An investigation into possible causes of, and diagnostics for, Alpha Gal Syndrome

by

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Abstract

Alpha Gal Syndrome (AGS) is an allergic response to red meat, specifically to a glycan, galactose-α-1,3-galactose (αGal) in mammalian meats. AGS develops after tick bites, mainly from the lone star tick, *Amblyomma americanum*, which is commonly found in the Central and Southeastern United States. Diagnosing AGS is not straightforward and relies on a combination of symptoms, patient history, and a blood test. The only current diagnostic tool for AGS is measuring the amount of specific Immunoglobulin E anti-αGal (sIgE anti-αGal); AGS is diagnosed with the diagnostic threshold of ≥0.1 IU/mL. αGal is a sugar located on the terminal ends of glycans found in many glycolipids and glycoproteins of most mammals except for humans and Old world primates. However, healthy individuals without AGS are often found to have high levels of sIgE anti-αGal (false positive tests) and a substantial proportion of individuals diagnosed with AGS do not meet the diagnostic threshold at sIgE anti-αGal ≥0.1 IU/mL (false negative tests). As such, accurate diagnoses for AGS can be challenging and modifications to the current testing protocols are urgently needed. Although AGS is induced by tick bites, less than 10% of individuals bitten by the lone star tick will develop AGS. This variation in response to tick bites may arise from the differences in the potency of AGS sensitizers among tick populations, or from individual variation in innate sensitivity. We aim to understand this variation in responses to tick-mediated AGS sensitization, as well as to identify new potential targets for AGS diagnostic markers.

For chapter 1, we hypothesized that low levels of alpha-galactosidase activity were associated with AGS. Enzymatic degradation of αGal may protect immune systems during the sensitization and elicitation steps of AGS. We suspect that low levels of alpha-galactosidase activity may be part of the mechanism involved in the manifestation of AGS symptoms, which

may make it a good potential diagnostic marker for AGS. To test our hypothesis, we obtained 51 plasma samples from a population diagnosed with AGS and 102 control samples. We first measured the levels of plasma sIgE anti-αGal in AGS and control samples using an anti-αGal IgE detection ELISA (enzyme-linked immunosorbent assay). We confirmed that most of our AGS patient plasma samples had elevated levels of anti-αGal IgE with 80 % sensitivity and 80% specificity, which correlates with the general consensus seen in the literature. We also measured plasma alpha-galactosidase activity levels using a fluorescence substrate assay. The assay measured the amount of galactose removed from 4-Mu-Gal (4-Methylumbelliferyl-α-D-galactopyranoside). The total fluorescence of 4-Mu released from 4-Mu-Gal provides quantifiable alpha-galactosidase activity measurements over a given time period. We discovered that alpha-galactosidase activity is significantly lower in AGS samples. To confirm the validity of this result, given we utilized two different sample cohorts, we performed a series of freeze-thaw experiments to test our sample stability with different storage conditions. We found that alpha-galactosidase activity in plasma remains unaffected up to 6 freeze-thaw cycles, the maximum number we have tested so far. Additionally, we tested for correlation between the length of the AGS sample storage time and alpha-galactosidase activity and found no correlation. Therefore, we conclude that lower alpha-galactosidase activity is correlated with AGS, implying that the immune systems of AGS individuals may be exposed to greater levels of αGal. Conversely, we conclude that the higher alpha-galactosidase activity present in AGS-tolerant individuals may protect the immune system from developing an allergic response to αGal.

For chapter 2, we hypothesized that differences in the amounts of the proteins alpha-galactosidase A (GLA) and/or alpha-N-acetylgalactosaminidase (NAGA) were responsible for the significantly reduced alpha-galactosidase activity seen in AGS samples. In order to identify

the causal factor for the low alpha-galactosidase activity, we measured the amount of immunoreactive proteins (GLA or NAGA) by using sandwich ELISAs. We found that NAGA levels are significantly lower in AGS samples, but that there is no difference in GLA levels between AGS and control samples. We also found that NAGA level, but not GLA level, is correlated with alpha-galactosidase activity in plasma. Thus, it appears that NAGA is likely responsible for alpha-galactosidase activity observed in plasma.

Our results support the hypothesis that alpha-galactosidase is involved in the manifestation of, or tolerance to, AGS. We suspect that the breakdown of αGal may play an important role in the AGS sensitization and/or elicitation steps. In addition, we also demonstrated that NAGA levels in plasma are associated with alpha-galactosidase activity in plasma. This study suggests that alpha-galactosidase activity and NAGA level may be useful as diagnostic tools to add to the current AGS diagnostic method, the sIgE anti-αGal antibody test. The combination of all three tests provides a significantly improved specificity and sensitivity for AGS diagnostics.

Table of Contents

List of Figures

Chapter 1 - An Investigation into Possible Causes of, and Diagnostics for, Alpha Gal Syndrome

Introduction

Alpha Gal Syndrome (AGS) is a type 1 hypersensitivity response to red meat that can cause severe reactions in affected individuals (1). More specifically, AGS is an allergic response to alpha-gal (αGal), or galactose-α-1,3-galactose, which is a carbohydrate epitope found on non-human mammalian glycoproteins and glycolipids (2, 3, 4). AGS is induced through a sensitization of the human host to αGal through tick bites (5). Alpha-galactosidase A (GLA) and alpha-N-acetylgalactosaminidase (NAGA) are both lysosomal proteins with activity against αGal that, when mutated, lead to Fabry disease and Schindler's disease, respectively (6, 7). The inability, or reduced ability, of the proteins GLA and NAGA to metabolize αGal may increase the severity of AGS.

This thesis investigates the potential roles of alpha-galactosidases in the manifestation of AGS symptoms. This chapter reviews the current literature on several key topics: the development and symptoms of AGS, the biochemical characteristics of αGal, and the relationship between ticks and AGS. It also examines which tick species are linked to AGS onset and explores the roles of 2 alpha-galactosidases, GLA and NAGA. The ultimate aim of this research is to contribute to improved diagnostic methods for AGS and to offer new perspectives on potential therapeutic approaches.

Alpha Gal Syndrome (AGS): A Tick-Mediated Immune Response

AGS is an allergy to red meat that manifests with delayed and non-classical symptoms (5, 8). More specifically, AGS is an allergic response to galactose-α-1,3-galactose (αGal), a terminal glycan found in non-human and non-old world primate mammals (2, 9, 4). Unlike most food allergies, the development of AGS typically occurs later in life and is correlated with a history of tick bites (5, 2, 3).

Symptoms and Current Treatment Options

Symptoms of AGS include isolated abdominal reactions, urticaria, angioedema, and anaphylaxis in severe cases. Individuals with AGS have a wide range of symptoms that may be attributable to several factors: individual sensitivity, type of food ingested, form of allergen, and other co-factors. In general, more severe symptoms are associated with mammalian organ meat consumption and only highly sensitive individuals have reactions to meat-associated products such as milk (5). AGS is atypical among food allergies because its symptoms generally manifest 2 to 8 hours after allergen ingestion, in contrast to the classical rapid onset of symptoms occurring within 30 minutes (8, 10, 11, 12).

As with many food allergies, there is currently no treatment for AGS (5, 12). General medical interventions for food allergic responses include medications such as antihistamines for mild to moderate allergic responses and adrenaline for severe allergic responses (12). AGS is diagnosed through a combination of symptom observations and sIgE anti-αGal antibody titers unlike other food allergies diagnosed with skin prick tests or oral ingestion challenges (8). The diagnostic threshold for AGS diagnosis, as set by the CDC, is ≥0.1 kU/L (8). Patient treatment plans for AGS mirror the common food allergy plans; primarily avoiding products containing the

allergen, with secondary medical interventions post allergen exposure (12). As seen with other allergic responses, factors such as exercise, NSAIDS (non-steroidal anti-inflammatory drugs), and alcohol consumption can worsen AGS symptoms in some individuals (13). Avoidance of these potential symptom-worsening factors is recommended in individuals whose symptoms are exacerbated by these factors.

Prevalence of AGS

While AGS has been identified within many countries, it is not classified as a reportable disease in most regions. Therefore, it is difficult to obtain accurate counts of the number of individuals with AGS. For example, the CDC tracked the number of sIgE anti-αGal tests ordered between 2017 and 2022; there were 295,400 tests ordered and only 30.5% of those tests had a positive result (14). The CDC report extrapolated on their data and concluded that between 96,000 and 450,000 individuals may have been impacted by AGS in the United States since 2010 (3, 14). They explain the large disparity in predicted affected individuals by the lack of healthcare provider education on AGS in certain regions of the United States. It is likely that there are many individuals with AGS who have not received an AGS diagnosis (5, 3, 14).

There are several additional factors that may contribute to the expected diagnostic gap for AGS: geographic region, insurance constraints, and individual variance in reaction severity. AGS is more prevalent in certain regions of the United States, particularly in the Southeast (5, 2). AGS lacks diagnostic medical billing codes in a large portion of the United States (3). This means that many insurance companies may not cover the diagnostic test for AGS diagnosis. Finally, the delayed nature of AGS allergic reactions can lead to confounding allergy tests, which can also prevent an accurate diagnosis.

Mechanisms of Allergic Responses and AGS

There are 4 types of hypersensitivity reactions (allergic reactions) classified based on the types of immune cells involved in the allergic response (1). A type 1 hypersensitivity reaction, such as AGS, is a reaction that involves immunoglobulin E (IgE) (15, 1, 16). Type 1 hypersensitivities occur after exposure to an antigen. The first exposure, or sensitization, primes the immune system to respond to a particular antigen. In the sensitization stage, the host is not impacted by the allergen, as it is the first immune system-provoking exposure to an antigen. In this stage antigen-presenting cells will present the antigen to T-cells, which will then stimulate B-cells to produce IgE antibodies against the antigen (1). These IgE antibodies are then bound to the Fc receptors on mast cells and basophils, which sensitizes the immune system to a potential antigen re-exposure (1, 16, 17).

The elicitation step occurs when the antigen binds to the specific IgE antibodies on the surface of mast cells and basophils. This leads to an allergic reaction. Mast cells and basophils with activated antigen-IgE complexes will degranulate, releasing histamine, cytokines, and other immune signaling compounds (1, 16, 17). This results in a type 1 anaphylactic reaction or atopic immune response (1). Common symptoms of type 1 hypersensitivity reactions range from itching, sneezing, increased mucous secretions, and bronchospasm (1, 16). The severity of these symptoms varies widely between individuals. If the hypersensitivity reaction is severe enough, individuals may experience anaphylaxis (15, 1, 16).

Type 1 hypersensitivity reactions may also occur in 2 stages: the immediate reaction and a delayed reaction. The immediate reaction occurs suddenly within minutes of exposure to the antigen, while the delayed reaction may occur 4-12 hours after exposure. Delayed reactions are typically mediated by other immune cells such as eosinophils, neutrophils, and lymphocytes. The

symptoms of a delayed type 1 hypersensitivity reaction are the same as the immediate reaction (1).

Risk Factors for Development of AGS

Tick Bites and AGS Development

Several compounds containing αGal have been identified in tick saliva (5, 18). Bites from certain tick species, such as *Amblyomma americanum*, are correlated with the development of AGS (5). It is likely that the introduction of αGal into the bloodstream from tick saliva is the sensitization event necessary for the immune system to develop a response to αGal. Importantly, many individuals who are diagnosed with AGS describe a history of tick bite (or bites) prior to developing symptoms of AGS (5, 19, 20).

Several lifestyle factors have been linked to a higher risk of tick exposure and, consequently, an increased likelihood of developing AGS. Participating in outdoor activities, living in rural areas, and being male are all associated with developing AGS (3, 21). However, literature indicates that these risk factors may simply be risk modifiers for tick exposure in general, as males do not seem to develop AGS without a history of tick bites. It is important to note that males are, generally, more inclined to participate in lifestyles that could increase the number of chances for tick-human interactions (3).

B Antigen Theory

Interestingly, individuals with the B antigen blood group may have some protection against αGal sensitization (3, 13). The structure of αGal (Gal α-1,3-Gal-R) is different from the blood group B antigen by 1 fructose molecule (Gal α-1,3(Fuc α-1,2)Gal-R) (Figure 1.8) (22).

Brestoff et al. (2018) explored the potential protective role of blood group B in individuals at risk of developing red meat allergies, such as AGS (23). They compared blood groups in several red meat allergy patient cohorts from the United States, Japan, Sweden, and Spain. They also compared the frequency of blood group in red meat allergy patients to the general population blood group frequency in each country. They discovered that the frequency of individuals with red meat allergy and blood groups B or AB was lower than expected, given the expected frequency of blood groups A and AB occurring in a population (23). They also discovered that individuals expressing the blood group B antigen were five times less likely to be diagnosed with red meat allergy than individuals who do not express the blood group B antigen (23). Additionally, Brestoff et al. (2018) determined that red meat allergy patients with the B blood group antigen were less likely to produce sIgE anti-αGal or sIgE anti-beef (p-value = 0.023) (23). If there were production of sIgE anti-αGal in the B blood group, the patients appeared to produce less sIgE anti-αGal or sIgE anti-beef than the cohort without a B blood group antigen (23). Thus, they concluded that individuals with the B blood group antigen were less likely to develop allergic sensitization to αGal, which leads to the development of red meat allergy.

Overall, there is good evidence supporting the theory that blood group B antigens provide a protective effect against the sensitization and allergy towards αGal. This is corroborated by the work of Gilstad et al. (2023) investigating a series of case studies from hospitals in the Washington D.C. area (24). Gilstad et al. (2023) investigated three case studies and demonstrated that individuals with AGS, or presenting with elevated IgE against αGal without AGS diagnosis, may develop anaphylactic reactions to certain blood products (24). In the case studies, all patients had blood group O blood and had received blood group B blood products. This is unusual because most blood transfusions aim to match the donor's blood type with that of the

recipient. Typically, individuals with blood group O only receive blood group O blood products, as they naturally have antibodies against blood groups A and B. However, there is a legitimate precedence for the situations detailed in the case studies; blood group O individuals needing urgent plasma or platelet transfusions may receive blood group B plasma or platelets when blood group O inventory is low (24). The blood group O patients from the case studies had severe allergic reactions attributable to a transfusion of blood group B blood products (24). The sIgE anti-αGal levels were measured in 2 of these case studies; the sIgE anti-αGal titers were measured at 0.72 kU/L and 3.04 kU/L, respectively (24).

Other Factors

There are a few other potential risk factors for the development of AGS, including poor healing rates for insect bites, previous allergic reactions, and genetics. In a study performed by Taylor et al. (2024) in the United States, they compared 82 AGS patients and 191 control patients in an effort to identify commonalities in AGS patients (13). They discovered that AGS patients were more likely to report large or unusually long-healing insect bites, childhood allergies, sIgE antibodies to a wide variety of allergens, and non-red meat food allergies (13). This finding is corroborated by a study performed in Sweeden (21). AGS patients were also more likely to have been hospitalized for a non-AGS allergic reaction. Additionally, AGS is often found in multiple members within closely-related familial groups (13). These factors support a correlation between genetic predisposition and the development of AGS; this is supported by the large proportion of individuals that have high titers for sIgE anti-αGal without showing symptoms of AGS (25).

It is important to note that genetic predisposition may not be the only risk factor leading to the development of AGS. While AGS is sometimes seen in familial groups, lifestyle and common household experiences (camping, hiking, gardening, and other outdoor activities) may contribute to increased risk of AGS development through increased tick exposure risk (13). Additionally, AGS diagnosis is primarily driven by symptom observation and a positive sIgE anti-αGal titer. As familial groups often share common tick exposure risks, it is possible that more than one individual in the group could develop AGS. If one member of the familial group received an AGS diagnosis, they would be more able to recognize the symptoms in another member of the group, which could lead to faster rates of diagnosis (13).

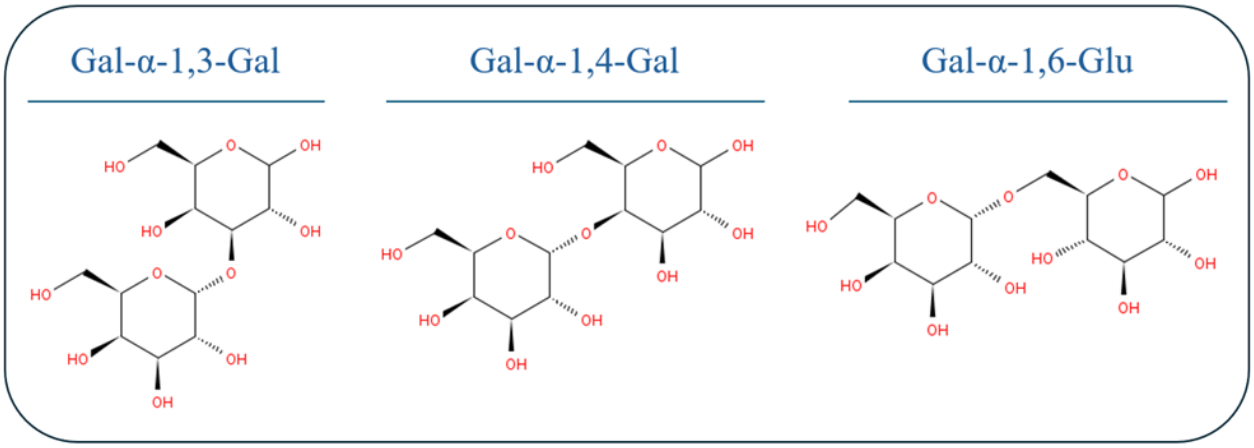
Glycans Containing Alpha-Galactose: A Human Health-Focused Perspective

AGS is a reaction to the disaccharide galactose-α-1,3-galactose (αGal), a carbohydrate epitope found on non-human (or old world primate) mammalian proteins and lipids. In some sources αGal is described in a trisaccharide form, Gal-α-1,3-Gal-β-1,4-GlcNAc, on the terminal ends of proteins (3). In addition, αGal is described as the terminal glycan on lipids as Gal-α-1,3-Gal-β-1,4-Glu (17).

Chemical Structure and Structural Isomers

There are several forms of galactose-a-1,3 linkages as terminal saccharides that are important to human health. As stated previously, αGal is the terminal disaccharide galactose-α-1,3-galactose (Figure 1.1) (3). AGS is an allergic response to this αGal epitope. However, compounds such as raffinose and globotriasylceramide (Gb3) possess terminal galactose residues linked via α-1,6 or α-1,4 glycosidic bonds, respectively, at the end of their glycans (6, 26).

Figure 1.1 The Structures of Alpha-Galactose



The terminal glycan structures of Alpha-Galactose. Structures built in Jmol (27) based on the structures described in sources (3, 6, 28, 29, 30).

Raffinose Family Oligosaccharides (RFOs) are found in legume seeds and cannot be digested by humans (26). This is because the lysosomal enzyme responsible for the first step of digestion, alpha-galactosidase, is not produced in the human intestinal lumen (31). The chemical structure of raffinose is galactose-α-1,6-glucose-α-1,3-fructose (Figure 1.1) (30). The terminal galactose of raffinose, and other RFOs, is removed by alpha-galactosidase produced by the gut microflora in humans. Some individuals lack the ability to comfortably consume legumes; they experience gastrointestinal distress symptoms such as bloating, abdominal pain, and flatulence. The common treatment for legume indigestibility includes supplementation with commercially available alpha-galactosidase capsules (26).

Globotriasylceramide (Gb3) is a glycosphingolipid that occurs naturally within humans (6, 26, 32). It is a chain of 2 galactose molecules and 1 glucose molecule attached to a ceramide. More specifically, Gb3 is galactose-α-1,4-galactose-β-1,4-glucose-1,1‘ceramide (6). The terminal galactose of Gb3 can also be removed by alpha-galactosidase. Individuals who lack the

ability to remove the terminal galactose from Gb3 will accumulate Gb3 in their tissues (6), leading to Fabry disease.

Correlation of Tick Bites with AGS Development

The development of AGS is correlated with bites from ticks introducing αGal as an allergen in affected individuals (5). Saliva samples from *Amblyomma americanum* has been shown to induce IgE responses to αGal in murine models. Red meat allergy can be induced in murine models when tick salivary gland extracts are injected intradermally (5). The αGal-containing glycoproteins and glycolipids in tick saliva have been identified as Gb3 and isoglobotriosylceramide (iGb3Cer) (33). iGbCer is a glycoform of the isoglobo GB3 series, with an αGal bound terminal glycosylation, which may play a role in the activation of natural killer T cells during immune responses (34).

Several αGal-containing N-glycosylated enzymes have also been identified in tick saliva (18). While the presence of αGal in tick saliva has been reported on many occasions, how αGal becomes a component of tick saliva is not fully understood. Previously, some groups favored the theory that ticks obtain αGal from prior blood meals from αGal-producing mammals; this has since been disproven (25). Other groups favored the theory that the ticks produce αGal-containing epitopes after an unknown trigger (25). There is a large variation in the amount of αGal present in the saliva of individual ticks (25). Levels of αGal in tick saliva appear to be influenced by the tick's habitat location, the source of its blood meals, and the volume of blood consumed. Maldonado-Tuiz et al (2023) showed that female ticks, especially ticks fed on human blood, were more likely to have elevated levels of αGal in their saliva (25). The cause of this

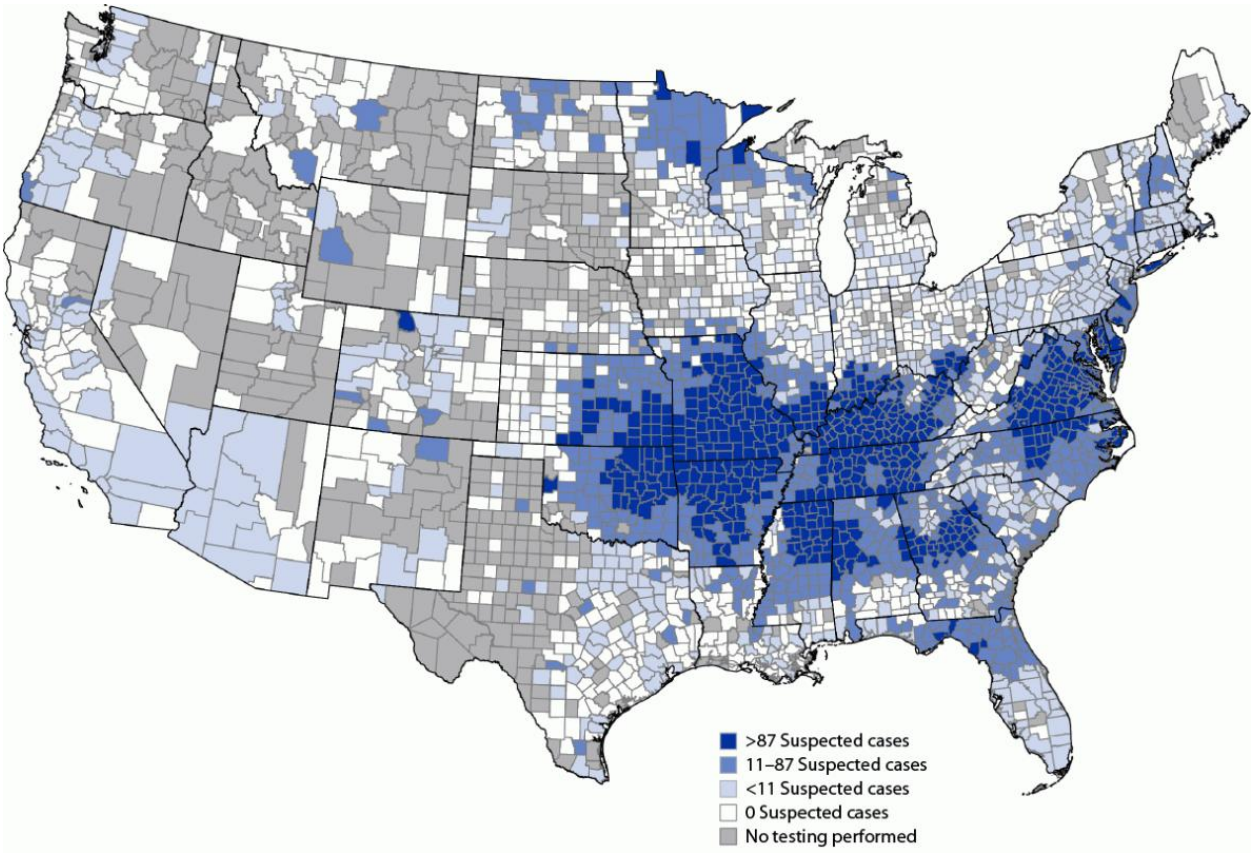
phenomenon is unknown. However, it is possible that other factors, such as the tick gut microbiome or tick genetics, may impact the amount of αGal found in specific ticks. (25).

It is known that the cutaneous exposure to food allergens in classical food allergies (such as IgE-mediated peanut allergy) can promote the initial sensitization of the immune system (12). This supports the hypothesis that αGal present in tick saliva may be enough to sensitize the immune system, priming it for an allergic response to αGal after the next exposure incident. However, it is important to note that the exact mechanism or trigger for inducing sensitivity to αGal is still unknown.

Tick Species Correlated with AGS Prevalence

Many tick species have been linked with the development of AGS. *Amblyomma americanum* is highly correlated with AGS development, while many other tick species are linked with AGS through case studies. These ticks usually, but not always, inhabit distinct geographic areas (35, 3, 36, 37, 38). In Figure 2, the number of suspected cases of AGS in the United States is shown for each county in the country (14). This has been used to correlate cases of AGS described in the literature with the ranges of tick species described in the reports (14).

Figure 1.2 Suspected Cases of AGS in the United States



Suspected cases of AGS in the United States based on number of sIgE anti-αGal antibody titer tests ordered by county (14). Source: Centers for Disease Control and Prevention (CDC). This image is available on the agency website free of charge [https://www.cdc.gov/mmwr/volumes/72/wr/mm7230a2.htm]. Reference to specific commercial products, manufacturers, companies, or trademarks does not constitute its endorsement or recommendation by the U.S. government, Department of Health and Human Services, or Centers for Disease Control and Prevention. (14).

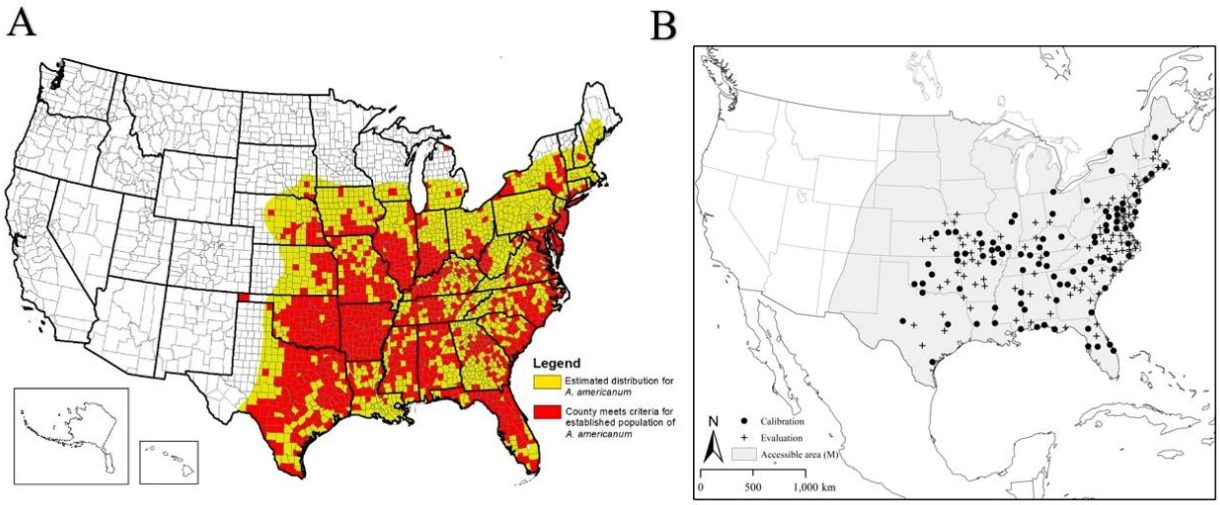
Ticks Correlated with AGS in the United States

Amblyomma americanum

Amblyomma americanum, the lone star tick, is the tick most commonly associated with AGS in the United States (Figure 1.3) (5). Lone star ticks are fairly aggressive and tend to be nonspecific feeders (39). In general, the larvae and nymphs tend to infest ground-feeding birds

and medium to large sized mammals (40). Adult *A. americanum* typically feed on medium to large sized mammals. The most common host for lone star ticks is the white-tailed deer (40).

Figure 1.3 The Current and Proposed Future Range of *A. americanum*

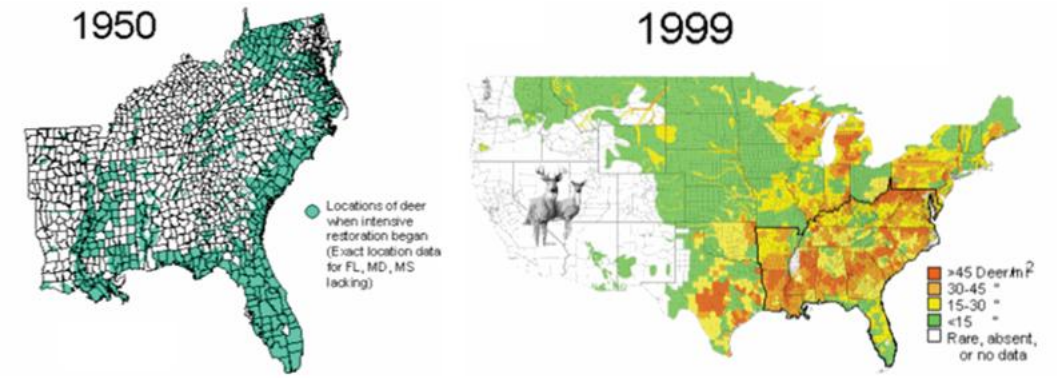


A) The range of *A. americanum* defined by the CDC. Red counties meet the criteria for established *A. americanum* populations. Yellow counties are projected to have suitable habitats for *A. americanum* and may indicate future areas where populations of *A. americanum* may become established. Source: Centers for Disease Control and Prevention (CDC). This image is available on the agency website free of charge [https://www.cdc.gov/ticks/data-research/facts-stats/lone-star-tick-surveillance.html]. Reference to specific commercial products, manufacturers, companies, or trademarks does not constitute its endorsement or recommendation by the U.S. government, Department of Health and Human Services, or Centers for Disease Control and Prevention. (41). B) The proposed range of *A. americanum* as defined by Raghavan et al (2019) (35). Image was made available under the Creative Commons CC0 public domain dedication and does not reflect the endorsement or recommendation of the authors (35).

The most common tick reported as a human parasite in the United States was *A. americanum* (40). In addition to inducing AGS, *A. americanum* is a known vector of several bacteria causing ehrlichiosis (*Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and "Panola Mountain" *Ehrlichia*) and tularemia (*Francisella tularensis*), as well as Bourbon virus and Heartland virus (42, 43).

The range of *A. americanum* (Figure 1.3) is currently under some debate. According to Rahgavan et al (2019), *A. americanum* has been routinely found further west than the CDC-defined lone star tick range, especially in Kansas, Oklahoma, and Nebraska (35). This is likely due to reforestation efforts and a marked increase in white-tailed deer populations (Figure 1.4) (40). However, it is important to note that a tick population is not considered established when a single tick is found in a location. The CDC defines an established tick population in an area where at least 6 ticks from 1 lifestage are found, or more than 1 lifestage is found, within 12 months (36).

Figure 1.4 Changes in White Tail Deer Population in the United States 1950-1999



The changes in Whitetail deer population spread between 1950 and 1999. Reprinted with permission from the Annual Review of Entomology, Volume 48, Issue 1, Childs, J.E. and Paddock, C.D., The ascendancy of *Amblyomma americanum* as a vector of pathogens affecting humans in the United States, Pages 307-337, Copyright (2002). Use of the image does not reflect the endorsement or recommendation of the authors (40).

Ixodes scapularis

Ixodes scapularis, the blacklegged tick, is primarily found in the Eastern United States (Figure 1.5) (37). It is associated with several human pathogens, including several also associated with *I. pacificus*. The pathogens transferred by *I. scapularis* bites include *B.*

burgdorferi, *B. miyamotoi*, *A. phagocytophilum*, *Babesia microti* (intraerythrocytic parasite), *Borrelia mayonii* (Lyme disease), *Ehrlichia muris eauclarensis* (Ehrlichiosis), and Powassan virus (37, 44).

Ixodes scapularis has also been associated with the development of AGS in a few case studies (19). One patient who developed AGS in Maine, where *A. americanum* is not known to have established populations, had a tick bite from *I. scapularis* prior to developing symptoms. The tick species was confirmed by entomologists at the Centers for Disease Control and Prevention (19). Although AGS is not a reportable disease in Maine, there were 57 cases of elevated sIgE anti-αGal identified by the Maine CDC between November 2014-October 2023. Twenty-three of these cases were confirmed as AGS, while the remaining 34 cases are categorized as suspected cases of AGS (19). They were unconfirmed because of the lack of patient interviews and insufficient data in medical charts. Additionally, the presence of galactosylated proteins containing αGal have been identified in the salivary glands of *I. scapularis* (45). This suggests that *I. scapularis* bites may contribute to the development of AGS in the United States (19).

Figure 1.5 The Ranges of *I. pacificus* and *I. scapularis* in the United States

A) Map of the range of *I. pacificus*. Green counties meet the criteria for established *I. pacificus* populations. Yellow counties are projected to have suitable habitats for *I. pacificus* and may indicate future areas where populations of *I. pacificus* may become established (36). Source: Centers for Disease Control and Prevention (CDC). This image is available on the agency website free of charge [https://www.cdc.gov/ticks/data-research/facts-stats/blacklegged-tick-surveillance.html]. Reference to specific commercial products, manufacturers, companies, or trademarks does not constitute its endorsement or recommendation by the U.S. government, Department of Health and Human Services, or Centers for Disease Control and Prevention (36).
B) Map of the range of *I. scapularis*. Red counties meet the criteria for established *I. scapularis* populations. Yellow counties are projected to have suitable habitats for *I. scapularis* and may indicate future areas where populations of *I. scapularis* may become established (37). Source: Centers for Disease Control and Prevention (CDC). This image is available on the agency website free of charge [https://www.cdc.gov/ticks/data-research/facts-stats/western-blacklegged-tick-surveillance.html]. Reference to specific commercial products, manufacturers, companies, or trademarks does not constitute its endorsement or recommendation by the U.S. government, Department of Health and Human Services, or Centers for Disease Control and Prevention (37).

Ixodes pacificus

Ixodes pacificus, the western blacklegged tick, is found along the west coast of the United States (Figure 1.5) (36). There are several pathogenic bacteria associated with *I. pacificus* bites: *Borrelia burgdorferi* (Lyme disease), *Anaplasma phagocytophilum* (anaplasmosis), and *Borrelia miyamotoi* (B. Miyamotoi disease) (46). In addition to *A. americanum*, *I. pacificus* has also been associated with the development of AGS. An article from Emerging Infectious Diseases described an AGS case study from Washington state (20). The patient had received a tick bite, species unconfirmed, prior to the first symptoms of AGS. Subsequent bites from *I. pacificus*,

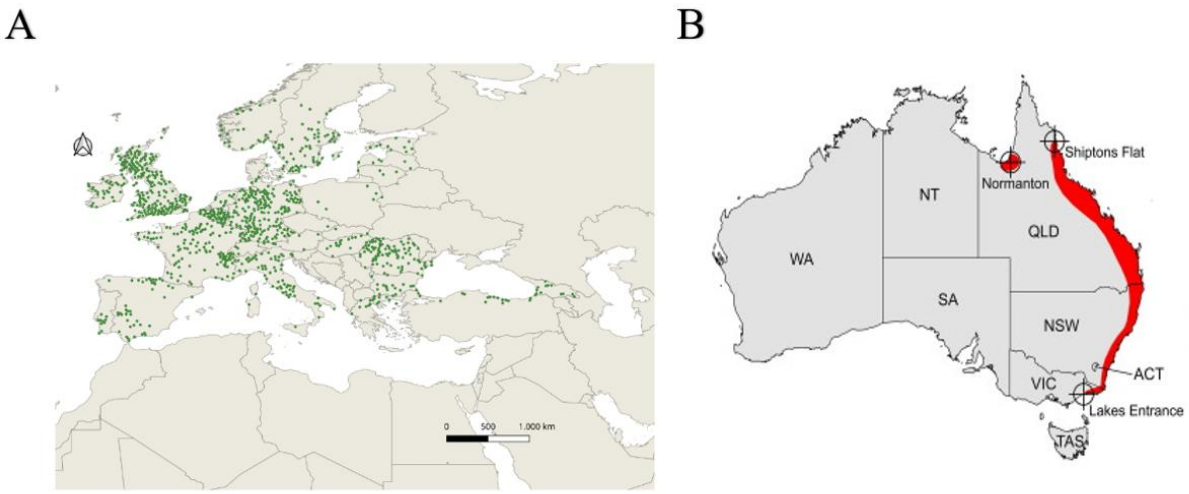
species confirmed by the Washington State Department of Health, preceded increased sIgE anti-αGal titers in the patient (20). A second case study out of California correlated larval *I. pacificus* bites with the development of AGS (22).

Ticks correlated with AGS in other regions

Ixodes ricinus

Ixodes ricinus, the castor bean tick, is mainly located in Europe (Figure 1.6) (38). It is associated with a wide range of pathogens including: *B. burgdorferi*, *B. microti*, *A. phagocytophilum*, *Babesia divergens* (intraerythrocytic parasite), *Rickettsia helvetica* (suspected spotted fever), *Rickettsia monacensis* (spotted fever), Louping ill virus, and Tribec virus (47, 38). In continental Europe, development of AGS is correlated with *I. ricinus* bites. The largest European AGS cohorts are from Germany and Sweden, but cases of AGS have been described from Portugal, Poland, and Turkey. In Germany, AGS is mainly identified in rural populations, which provides further support to the correlation between tick bites and AGS (8, 3).

Figure 1.6 The Ranges of *I. ricinus* and *I. holocyclus* in Europe and Australia



A) The confirmed locations of *I. ricinus* in Europe. Each data point represents a 1 km radius around a tick location confirmed by literature searches performed by Kuyucu and Hekimoglu (2024) (48). Image was made available under the Creative Commons CC BY license and does not reflect the endorsement or recommendation of the authors. B) The distribution of *I. holocyclus* in Australia (42). Reprinted from the International Journal for Parasitology, Volume 51, Issue 4, Teo, E. J. M., Vial, M. N., Hailu, S., Kelava, S., Zalucki, M. P., Furlong, M. J., Barker, D., & Barker, S. C., Climatic requirements of the eastern paralysis tick, *Ixodes holocyclus*, with a consideration of its possible geographic range up to 2090, Pages 241-249, Copyright (2021), with permission from Elsevier.

Ixodes holocyclus

Ixodes holocyclus, the eastern paralysis tick, is found on the east coast of Australia (Figure 1.6) (8, 3). *Ixodes holocyclus* is a known carrier of *Rickettsia australis*, which causes Australian scrub typhus. Unlike the other Ixodes species that are correlated with the development of AGS, *I. holocyclus* can cause disease directly through its bite. Most tick species spread disease through the spread of pathogens while biting a host. *I. holocyclus* injects holocyclotoxins (a neurotoxin) when feeding, which can lead to tick-induced paralysis in the host (42).

Other Regions with AGS Cases

Reports of AGS from Asia, specifically in Japan, Laos, and South Korea, are correlated with *Haemaphysalis longicornis* and *Amblyomma testudinarium* (3). African reports of AGS are rare, but there are some reports of high αGal IgE in rural areas. Additionally, *Amblyomma hebraum* and *Riphicephalus evertsi* have been correlated with AGS in South Africa (3). In South America, cases of AGS have been reported in Panama, Brazil, and French Guiana. These cases are correlated with *Amblyomma cajennense* (3).

Enzymatic Degradation of Alpha-Galactose Terminal Glycans

Alpha-galactose is a common carbohydrate epitope present in a broad range of foods, including legumes (as raffinose) and red meat (as αGal) (26). Its widespread occurrence in the human diet necessitates the capacity to metabolize dietary alpha-galactose.

Potential Connection Between Alpha-Galactose Degradation and AGS

GLA, and NAGA to some degree, hydrolyze the alpha bond holding the terminal galactose of αGal to the rest of the glycoprotein/glycolipid. The removal of the terminal sugar may allow other enzymes to have access to their substrates further down the molecule. We hypothesize that the removal of the terminal galactose of αGal may protect an individual from developing an allergic response to glycoproteins and glycolipids containing αGal. If an individual has sufficient alpha-galactosidase activity, we theorize that there will not be sufficient αGal present to elicit an allergic reaction to αGal. This may partially explain how a significant proportion of the randomly selected control cohort from Taylor et al.'s study (2024) could have elevated sIgE anti-αGal titers without symptoms of AGS (13).

Furthermore, we hypothesize that protein disfunctions leading to reduced alpha-galactosidase activity may increase the chance of allergic reaction to αGal. If αGal is not broken down rapidly enough, sIgE anti-αGal can bind to the antigen in sufficient amounts to induce AGS allergic symptoms. Additionally, dysfunctional alpha-galactosidases may lead to increased concentrations of αGal in the bloodstream. This could lead to an increased chance of developing AGS, as well as an increased severity of AGS symptoms. Therefore, we suspect that GLA and NAGA, which are alpha-galactosidases with activity against the terminal galactose in alpha-gal glycolipids and glycoproteins, may be significant in the protection of immune systems from AGS sensitization and elicitation.

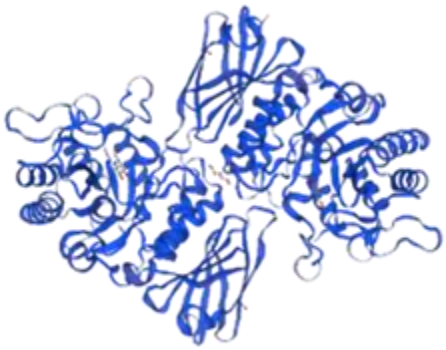
Alpha-Galactosidase A (GLA)

Protein Structure and Function

GLA (EC: 3.2.1.22) is a lysosomal protein that hydrolyzes the alpha bond between the two galactose molecules that make up αGal (49). More specifically, GLA catalyzes the removal of a terminal galactose from glycolipids, glycoproteins, and oligosaccharides during the catabolism of these molecules (6). GLA is also found in blood plasma (50, 51). The mature and active form of GLA functions in a homodimer (Figure 1.7) (6). The molecular weight of the GLA monomer is approximately 46 kDa (52) while the molecular weight of the homodimer is approximately 94 kDa (53). Molecular weight of GLA has some range, as 5-15 percent of the molecular weight is due to carbohydrates on the surface of the protein. The amount of glycosylation on GLA varies between different organisms and individuals (6). The gene encoding GLA is located on the X chromosome at position Xq21→→Xq22. It spans approximately 13000 base pairs and has 7 exons. (54, 53). The GLA monomer is 429 amino

acids, including a 31 amino acid signal peptide sequence (determines export location of enzyme) (53).

Figure 1.7 The Structure of the GLA Homodimer



The structure of the homodimer form of GLA. Structure built using Swiss-Model (55, 56, 57, 58, 59) using sequences obtained from UniProt (51). Sequence was made available under the Creative Commons CC BY 4.0 license and does not reflect the endorsement or recommendation of the authors (51).

The mature GLA active site involves 2 aspartic acid residues: Asp-170 and Asp-231. GLA hydrolyzes the terminal galactose of αGal through a double displacement reaction (6, 28). Asp-170 first acts as the nucleophile in the active site, donating electrons to carbon 1 of the trapped galactose. Then Asp-231 acts as an acid, donating a hydrogen ion to the newly freed oxygen molecule. Finally, Asp-231 acts as a base and removes a hydrogen from the oxygen molecule removed from the galactose. The result of this reaction is a free galactose molecule and a glycolipid or glycoprotein without its terminal galactose from αGal (28).

Fabry Disease: a Genetic Disorder Caused by Mutations in the GLA Gene

Mutations in lysosomal storage proteins like GLA can lead to the accumulation of specific substrates (αGal, in this case) in the tissues. In humans, pathogenic mutations in GLA

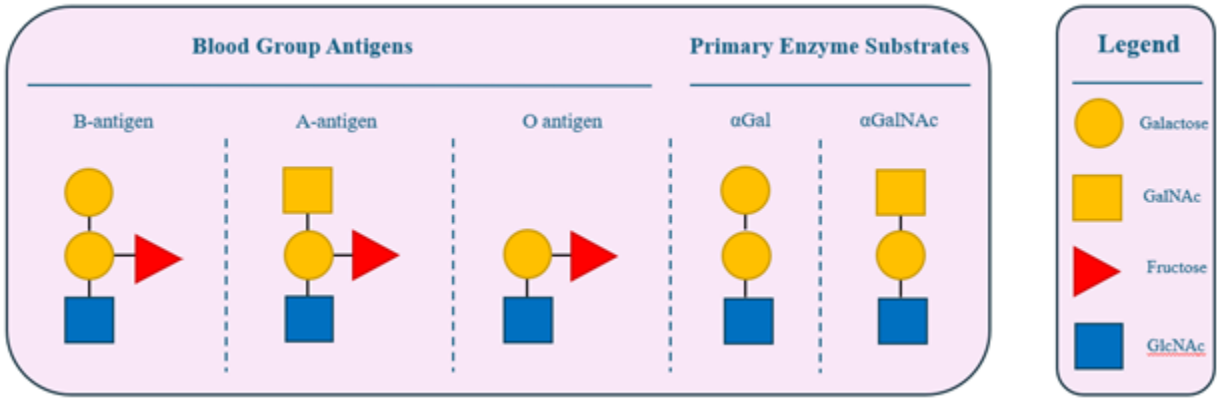
lead to the accumulation of galactosylated substrates, particularly Gb3 (6). Accumulation of galactosylated substrates leads to the development of Fabry disease. Fabry disease is a recessive X-linked disorder with an estimated prevalence ranging from 1 in 8,400 to 1 in 117,000 for severe phenotypes, and approximately 1 in 1,000 or 1 in 2,000 for all cases combined (49, 6, 7, 60). It is characterized by chronic pain, liver and kidney function impairment (6, 50). One of the best treatments for Fabry disease is enzyme replacement therapy using GLA. Treatment of Fabry disease with recombinant human GLA was approved by the FDA in 2003 (6).

There are two recombinant GLA products on the market for the treatment of Fabry disease, Replagal and Fabrazyme. Replagal is a recombinant GLA produced in a human cell line; it is different from Fabrazyme because it contains larger amounts of complex carbohydrates. Fabrazyme is a recombinant GLA produced in a Chinese hamster ovary (CHO) cell line and has higher amounts of sialylated and phosphorylated carbohydrate than Replagal (6, 50). The recommended dose for Fabrazyme infusion is 1 mg/kg body weight. The recommended dose for Replagal infusion is 0.2 mg/kg. The differences in carbohydrate composition between these two products are likely responsible for their differences in tissue distribution and dose requirements (50, 61). Lee et al. (2025) performed tissue distribution analyses in mice for Replagal and Fabrazyme (61). After a bolus dose of 3 mg/kg of each medication, approximately 2 times as much Fabrazyme was detected in the kidney, heart, and spleen when compared to Replagal. This is potentially due to increased binding efficiency for the mannose-6-phosphate receptor; this receptor is what allows for the uptake of the recombinant enzymes into Fabry disease fibroblasts (61).

Degradation of Blood Group B Antigen by GLA

The blood group B antigen present on type B red blood cells has a very similar structure to αGal (3, 22). The blood group O antigen, (Fuc α-1,2)Gal-R, is the base blood type antigen (Figure 1.8). Blood group B and A antigens are modifications of this antigen (26). The blood group B antigen (Gal α-1,3(Fuc α-1,2)Gal-R) is the base antigen with a terminal α-1,3-linked galactose (3, 26).

Figure 1.8 A Comparison between GLA and NAGA Enzyme Substrates and Human Blood Group Antigens.



The structures of human blood group antigens (A, B, and O) and enzyme substrates for GLA and NAGA (3, 26, 62, 63). Figure based on the structures described in sources (3, 26, 62, 63).

It has been proven that blood group B red blood cells can be converted into pseudo-blood group O through alpha-galactosidase activity (64, 26, 63). These pseudo-blood group O red blood cells have been successfully transfused in human volunteers in phase 1 and 2 clinical trials (26, 63). However, this process has been hampered by the low pH levels required for optimal GLA function, the amount of protein needed to fully cleave the terminal sugars from blood group B antigens, and the cost of the treatment. There is an ongoing search for cheaper and more

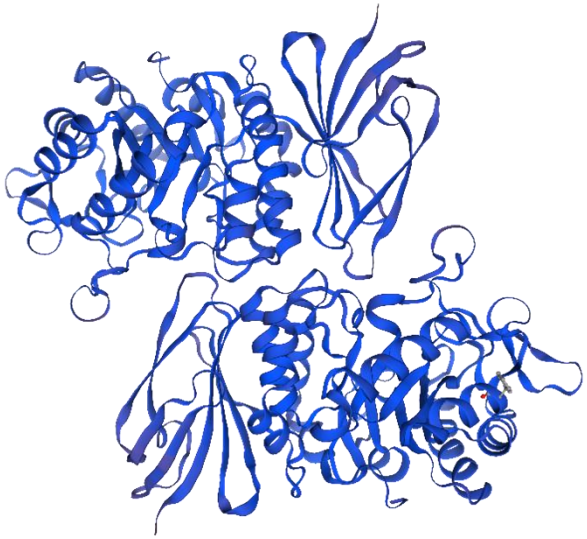
efficient methods of converting blood group B into pseudo-blood group O antigens using GLA from microorganisms (62, 63).

Alpha-N-acetylgalactosaminidase

Protein Structure and Function

NAGA (EC: 3.2.1.49) is a lysosomal protein that cleaves terminal α-N-acetylgalactosamine (α-GalNAc) from glycoproteins and glycolipids (65, 66). Previously, NAGA was known as alpha-galactosidase B, due to its activity as an alpha-galactosidase (67). NAGA functions as a homodimer (Figure 1.9). The NAGA gene is found on chromosome 22 (68, 7). The gene encoding NAGA is 1267 base pairs, has 10 exons, and generates a 411 amino acid sequence. After removal of the animo acid tail, each NAGA monomer contains 394 amino acids (65, 67, 66). This protein is negatively charged (charge is approximate to the pH of the lysosome) and functions optimally at pH 4.6 (65). NAGA is highly glycosylated and rich in disulfides (4 disulfide bonds in a single monomer) (65).

Figure 1.9 The Structure of the NAGA Homodimer



The structure of the homodimer form of NAGA. Structure built using Swiss-Model (55, 56, 57, 58, 59) using sequences obtained from UniProt (66). Sequence was made available under the Creative Commons CC BY 4.0 license and does not reflect the endorsement or recommendation of the authors (66).

NAGA is homologous to GLA in amino acid sequence and structure. Likewise, the active site and biochemical activity are known to be similar GLA. The active site of NAGA cleaves the terminal α-GalNAc from peptides using a double-displacement reaction. One carboxylate, D156, makes a nucleophilic attack on the substrate. D217 donates a proton to facilitate the breakage of the glycosidic linkage securing the terminal α-GalNAc. Then D217 deprotonates a water molecule to make a second nucleophilic attack on the bound glycosyl intermediate, which regenerates the initial active site conformation and releases the cleaved product from the active site (65). NAGA does have some hydrolytic activity against αGal. In fact, NAGA was originally known as GAL-B (GAL-A being alpha-galactosidase) (67). However, NAGA binds with αGal with much less efficiency than it does with α-GalNAc (65).

Schindler's Disease: a Genetic Disorder Caused by Mutations in the NAGA Gene

Schindler's disease is a lysosomal storage disorder caused by NAGA malfunction (7, 65). It is predicted to have a prevalence between 1:100,000-200,000 (7). There are 3 types of Schindler's disease: type I, type II, and type III (7). Type I Schindler's disease is the classic form of the disease that begins in infancy. The common symptoms are psychomotor retardation and developmental regression. This means that the infant will begin showing signs of delayed development in skills that require the coordination of both mind and body (psychomotor retardation), as well as loss of previous developmental milestones (developmental regression) (7, 65). Children with type I Schindler's disease may also display neurological symptoms, such as hypotonia, spastic involuntary muscle movements, rapid eye movements, and optic atrophy. Type II Schindler's disease begins in adulthood (7). Symptoms typically include angiokeratomas (small raised skin lesions), lymphedema, and mild intellectual impairments. Less common symptoms include distinctive facial features (thick lips, depressed nasal bridge, and enlarged nose tip), pain crises, vertigo, hearing loss, tinnitus, and muscle weakness (7, 65). Type III Schindler's disease is characterized by a spectrum of symptoms with varying severity, including seizures, autistic disorders, and cardiomyopathy (65). There is currently no treatment available for Schindler's disease.

Schindler's disease is caused by very low to nonexistent NAGA activity. This leads to an accumulation of glycosphingolipids, glycoproteins and glycolipids in tissues and urine of patients (7). The neurological characteristics of Schindler's disease are associated with dystrophic axonal swellings (swelling of the end of axons). This severe alteration of nerve cell structure was first seen in type I Schindler's patients (69). The causes for symptoms of type II and type III Schindler's disease are uncertain.

Degradation of Blood Group A Antigen by NAGA

As seen with blood group B antigens and αGal, the blood group A antigen present on type A red blood cells has a similar structure to αGalNAc (Figure 1.8) (3, 62, 64). The blood group A antigen (GalNAc α-1,3(Fuc α-1,2)Gal-R) has a terminal αGalNAc. NAGA has activity against the terminal αGalNAc on the blood group A antigen (26, 62).

It has been suggested since the 1980s that, through the removal of the αGalNAc and αGal terminal groups on blood group A and B antigens, a universal supply of red blood cells could be created (62, 63). Several groups have tested NAGA derived from microorganisms for the best activity against the blood group A terminal αGalNAc, because, as a lysosomal protein, human-derived NAGA functions best in an acidic environment (62, 63). When Gao et al. (2012) combined GLA and NAGA from bacterial sources with type AB red blood cells, they demonstrated that the conversion to type O red blood cells was successful (26, 63). As with GLA and blood group B conversion into pseudo-blood group O, there is ongoing research into cheaper and more efficient methods of converting blood group A into pseudo-blood group O using microbial-derived NAGA (62, 63).

NAGA as "Nagalase": A Controversial Immunosuppressant and its' Relation to Cancer

When describing NAGA found in human serum, Korbelik, Naraparaju, & Yamamoto (1998) described the enzyme as 'nagalase' (70, 71). Nagalase is involved in several processes, including immune system modulation. This function is thought to occur through the inactivation of Gc protein-derived macrophage activating factors (GcMAFs) produced by B and T cells (71, 72). There are several isoforms of nagalase found in healthy human serum and they function at

different optimal pH conditions, which range from pH 4 to pH 8. The range of optimal pH conditions is thought to be due to changes in N-glycosylation patterns. Lysosomal nagalase (Naga4) has an optimum pH of ~4.5 and is less active in serum, while the isoform Naga6 has an optimum pH of 5.8 and has been shown to be active in serum (72).

A standard NAGA detection ELISA cannot fully detect Naga6 in plasma and serum samples, due to a reduction in antibody binding efficiency to Naga6. This reduction is potentially attributable to highly charged, low molecular weight substances in serum. To detect Naga6 in plasma or serum, Yamamoto et al. used an unorthodox approach (71, 72). They extracted the inhibitor with ammonium sulfate fractionation with final precipitate saturation concentrations ranging from 30% to 70%. Then the precipitate was dialyzed and tested using the NAGA detection ELISA (71, 72). Naga6 is of particular interest because it is associated with cancer cell proximity (71). It is secreted in larger quantities by cancerous cells, hypothetically allowing for immune evasion through the inactivation of GcMAF (71, 72). It has also been shown that nagalase levels in serum drop within 24 hours of tumor removal and increases a 'precursor activity', which is thought to be GcMAF previously inactivated by the elevated levels of excreted nagalase in and around the tumor (71).

While this information sounds like an interesting avenue for cancer therapies, it is important to note that Yamamoto, the primary author of papers discussing nagalase and GcMAF, is a controversial figure in oncological research. Ugarte et al. published a letter to the editor in 2014 that led to the retraction of 3 key Yamamoto papers that were published in 2008-2009, by citing several inconsistencies (71, 72, 73). These inconsistencies include the inability of the Ugarte et al. to find co-authors of papers and IRB members that supposedly approved Yamamoto's human studies (73). However, some of their charges could be explained by

language and location barriers; many of the coauthors and IRB members that could not be located were listed as members of Japanese institutions. On the other hand, while Albracht disagreed with several of Yamamoto's methodological approaches, he also disagreed with the letter written by Ugarte et al. (72). He was suspicious of the motives behind the letter to the editor, stating that several major discoveries in science have been rejected because they did not fit into popular ideas held by the scientific community at the time. Additionally, Ugarte et al. did not mention any of Yamamoto's earlier papers or patents describing nagalase and GcMAF in their letter to the editor, nor have any of these materials been retracted (72). If Yamamoto's discoveries are treated with caution, and repeatable confirmatory experiments are performed, the correlation between nagalase, GcMAF, and immunosuppression could be useful for oncological research in the future.

Conclusion

In conclusion, AGS is an allergic response to αGal found as a terminal carbohydrate epitope on most (non-human or old world primate) mammalian glycolipids and glycoproteins. It is induced through tick bites from multiple species of ticks throughout the world. More investigation is required on AGS and how symptoms of AGS develop. More specifically, information on the potential ways that some individuals may be protected from the development of AGS, while others are susceptible to developing AGS, is needed.

The aim of this thesis is to explore the variation in response to sIgE anti-αGal levels and the development of AGS. We suspect that the removal of terminal galactose molecules from αGal may have a protective effect for the immune systems of AGS-resistant individuals. Conversely, we suspect that individuals susceptible to developing AGS may have lower rates of alpha-galactosidase activity. This would expose the immune system to greater levels of αGal,

allowing for an allergic reaction. Furthermore, we suggest that proteins with alpha-galactosidase activity, predominantly GLA and NAGA, are expressed in different levels in individuals susceptible to AGS.

Chapter 2 - Alpha-Galactosidase: Potential Roles and Implications in Alpha Gal Syndrome Tolerance

Introduction

Alpha Gal Syndrome (AGS) is an allergy to red meat with an unusual presentation (8). More specifically, AGS is an allergy to a carbohydrate epitope, galactose-α-1,3-galactose (αGal). αGal is found in non-human and non-old-world primate mammalian products on the terminal ends of glycans found on glycolipids and glycoproteins (9, 3, 22). AGS symptoms are delayed after exposure to αGal, which is unusual compared to general food allergy symptoms. Most typical food allergy symptoms develop rapidly, within 30 minutes, post ingestion of allergens. AGS symptoms typically develop between 2 and 8 hours after ingestion of mammalian meat (1). Many individuals with AGS also have allergic responses to products such as dairy and gelatin. In severe cases of AGS, individuals cannot take medications with gelatin capsules or other animal-derived products, such as anti-venoms, some vaccines, and the cancer drug Cetuximab. All these products may contain mammalian-derived allergens, αGal or immunologically similar compounds, in the final product (5).

Typical AGS symptoms include gastrointestinal distress, urticaria, and itching. More severe cases of AGS could include symptoms of anaphylaxis such as trouble breathing, drops in blood pressure, and swelling (5). AGS is currently diagnosed through a combination of reported symptoms and testing for the presence of specific IgE against αGal (sIgE anti-αGal). The CDC defines the minimum threshold of IgE anti-αGal for AGS diagnosis at 0.1 IU/mL (11). There are atypical cases of AGS where the patient will present with the symptoms of AGS without a IgE anti-αGal titer of ≥0.1 IU/mL. There are also studies demonstrating that approximately 30% (13, 74) of individuals tested in some regions will have elevated titers of IgE anti-αGal without

displaying AGS symptoms. This suggests that a second mechanism may contribute to the presence or absence of AGS symptoms.

AGS is classified as a type 1 hypersensitivity reaction (1). This is a type of allergic reaction that is an exaggerated immunoglobulin E (IgE) controlled response to allergens. Type 1 hypersensitivities involve a sensitization step prior to the elicitation, or immune response, step. The body is first sensitized for a future allergic reaction to αGal through tick bites. After αGal from tick salivary glands is introduced into the bloodstream, the sensitization steps may involve the typical antibody-mediated responses: The antigen-presenting cells will take the antigen (αGal) to T-cells. T-cells will then stimulate B-cells, which will produce IgE antibodies against αGal (1). The IgE anti-αGal antibodies are then bound to basophils and mast cells. After this step, the immune system is primed to respond to αGal during the second exposure. The elicitation, or allergic response step, occurs after the next exposure to αGal (1, 16). αGal will be bound by the IgE anti-αGal, leading to the release of histamine, cytokines, and immune signaling compounds by basophils and mast cells. The compounds released by basophils and mast cells lead to the onset of allergic symptoms (1, 16).

There are several risk factors associated with the development of AGS. First, there are lifestyle components (participating in outdoor activities such as hunting and farming or living in rural areas) and non-lifestyle components (having childhood allergies, having non-meat food allergies, and having a wide variety of IgE antibodies) (3, 21, 13). The lifestyle-associated risk factors typically allow for more human-tick interactions. Other non-lifestyle factors that are associated with more severe allergic responses are not exclusive to AGS. These include the use of NSAIDS (non-steroidal anti-inflammatory drugs), consumption of alcohol, and exercise (13). The non-lifestyle-associated risk factors are closely tied to how an individual's immune system

may be impacted by the introduction of new antigens. There are also likely some genetic factors involved in the development of AGS, as AGS seems to be found in family groups (13).

There are several tick species that are currently correlated with the development of AGS. In the United States, the most common tick associated with AGS is *A. americanum* (5). The range of *A. americanum* is currently being updated by the CDC, but previous estimates of established *A. americanum* populations very closely align with the suspected cases of AGS in the United States (39, 14). Species of the *Ixodes* genus are also commonly correlated with the development of AGS (8). Case reports from the United States demonstrate that bites from *Ixodes pacificus*, found along the west coast, and *I. scapularis*, whose range closely resembles the range of *A. americanum*, are correlated with AGS development (19, 20). Global cases of AGS are correlated with *Ixodes* species as well. *I. ricinus*, found in continental Europe, and *I. holocyclus*, found on the eastern coast of Australia, are linked to cases of AGS in those locations (48, 42).

The exact mechanism for how tick bites are capable of inducing AGS is still under investigation. It has been shown that saliva samples from *Amblyomma* species can induce IgE anti-αGal responses in murine models. Red meat allergy can be induced in murine models when mice are injected intradermally with tick salivary gland extracts (5). The method by which tick saliva can contain αGal is currently under some debate. There are several prevailing theories as to how αGal is found in tick saliva. One theory is that the ticks may obtain αGal through previous blood feedings on an αGal-producing mammal prior to biting a human host. Another theory is that the ticks produce αGal in their saliva after some currently unknown trigger (25). It is also theorized that other factors, such as genetic or endosymbiont variation within ticks, could impact the amount of αGal found in an individual tick (25).

αGal is removed from terminal glycan structures of glycoproteins and glycolipids by enzymes with alpha-galactosidase activity in the lysosomes (6, 32). In human lysosomes, the primary alpha-galactosidase is alpha-Galactosidase A (GLA) (28). The secondary alpha-galactosidase is alpha-N-acetylgalactosaminidase (NAGA), formerly known as alpha-galactosidase B (69). The primary substrate of NAGA is terminal α-N-acetylgalactosamine (α-GalNAc), however, it does have activity against terminal alpha-galactose (69). Functional alpha-galactosidase activity is essential for maintaining human health. Mutations in proteins responsible for alpha-galactosidase functions can result in the accumulation of substrate in tissues (6). This can contribute to the onset of lysosomal storage disorders such as Fabry disease and Schindler's disease, which are caused by mutations in GLA and NAGA, respectively (6, 69).

We are interested in the variety of responses to tick bites, by which some individuals will develop AGS after tick bites, while other individuals remain AGS symptom free, even with elevated sIgE anti-αGal levels. Our aim is to understand this variation in response to tick-mediated AGS sensitization. Given our current understanding of how AGS symptoms may arise, we hypothesize that immune system of AGS-susceptible individuals is not protected by the degradation of αGal through alpha-galactosidase activity. We suspect that enzymatic degradation of αGal may protect immune systems during the sensitization and elicitation steps of AGS, which suggests that plasma alpha-galactosidase activity may serve as a diagnostic marker for AGS. In this study we have compared alpha-galactosidase activity levels in both AGS and control plasma samples.

Methods

Samples

A total of 51 plasma samples collected from AGS patients and 80 plasma samples collected as CDC controls were provided by Dr. Scott Commins (UNC School of Medicine). An additional 23 plasma samples purchased from Innovative Research were used as Random controls.

sIgE anti-αGal Detection ELISA

First, the plate (Corning Costar Assay Plate, ref:9018:) was coated with a 5µg/well solution of rabbit anti-human IgE (Fortis Life Sciences, ref: A80-109A) in bicarbonate buffer (0.0125 M sodium bicarbonate, 0.0875 M anhydrous sodium carbonate, pH adjusted to 9.2) overnight at room temperature. Then standards (sIgE anti-αGal (InBio, ref: E-16D9)) and 20 µL plasma with 80 µL SuperBlock blocking buffer (Thermo Scientific, ref: 37515) were added to the appropriate wells and incubated at 37ºC for 1 hour. After 2 washes with Tris-Buffered Saline with 0.05% Tween 20 (TBST), a third last wash was left on the wells for one hour at room temperature. Then, 100 µL of a 1 µg/mL solution of αGal conjugated to a polyacrylamide backbone with a biotin tag was added (GlycoNZ, ref: GNZ-0070-BP) and incubated at 37ºC for 1 hour. Another set of washes as described above was performed, followed by the addition of 100 µL of a 1 µg/mL solution of Streptavidin conjugated to HRP (horseradish protein) (Thermo Scientific, ref: 21130). The Streptavidin-HRP was incubated in a dark area at 37ºC for 1 hr. The plate was then washed 3 times with TBST and 100 µL TMB (3,3',5,5'-Tetramethylbenzidine) (Thermo Scientific, ref: 34022) was added to the plate and incubated in a dark area at room temperature for 30 minutes. Finally, the reaction was stopped with a 1M Phosphoric acid

solution. The plate was read at OD 450nm using a microplate reader (Thermo Scientific Varioskan lux).

Alpha-Galactosidase Activity Assay

A 100 μM and a 20 μM stock of 4-MU (4-methylumbelliferone) (Cayman Chemical Company, ref: 29603) were prepared as the 4-MU standards. The alpha-galactosidase substrate was prepared by making a 15 μM solution of 4-MU-Gal (4-methylumbelliferone-α-D-galactopyranoside) (Cayman Chemical Company, ref: 16551). Assay Buffer was made with a final concentration of 20 mM citric acid, 30 mM sodium phosphate, and 0.1% bovine serum albumin (BSA). 30 μL assay buffer and 20 μL substrate was added to each sample well to create a final substrate concentration of 5 μM/well. Standards and 10 μL plasma samples were added to respective wells of a black Costar plate (Corning, ref: 3915). The plate was incubated in the dark at 37ºC for 2 hours. A 1M glycine and 1M sodium hydroxide stop solution was added and the results were read on a microplate reader at excitation range 360 nm and emission range 445 nm.

Freeze-Thaw

One aliquot of human plasma was pulled from -80ºC stocks was prepared to test for the impact of multiple freeze-thaws on alpha-galactosidase activity. The aliquot was thawed completely on ice. The aliquot was then separated into 3 different stocks. Stocks were frozen at -20ºC and thawed completely on ice for each freeze-thaw cycle. Samples with 1, 3, and 6 daily freeze-thaw cycles were prepared as samples.

Statistical Analysis

The Shapiro-Wilk test was used to determine whether or not data was normally distributed. Normal and non-normal data need different types of later analysis to be performed. A p-value ≤0.05 indicated that the data was not normally distributed. The Mann Whitney U test was used to compare non-parametric datasets when the data was not normally distributed. The purpose of the test was to identify whether the datasets came from the same population or if they came from different populations. A p-value ≤0.05 indicated that one dataset was statistically significantly different from the other dataset. A Pearson correlation, which fitted a linear model to each set of data and tested the significance of the slope for each group, was used on our data. An ANOVA test determined whether there was significance between 3 or more groups by comparing the variation between groups and the variation within groups. The Kruskal-Wallis test was a non-parametric alternative to ANOVA tests. It was used to determine whether there was a difference between the medians of at least 3 independent groups. A p-value ≤0.05 indicated that there was a difference between at least 2 of the independent groups. The Bonferroni correction was used to help correct family-wise error rates when we performed multiple statistical comparisons.

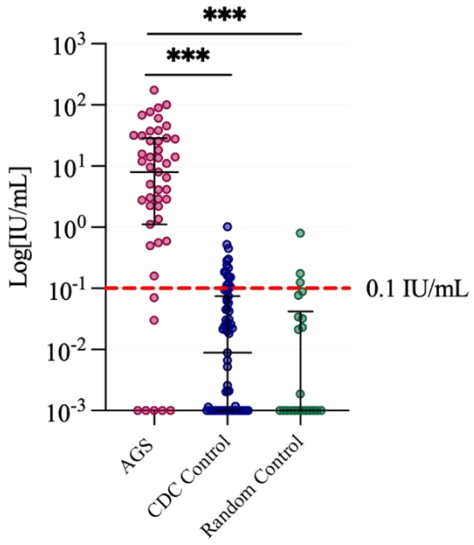
Results

sIgE Anti-αGal Levels

We found that sIgE anti-αGal levels were significantly elevated in AGS patients when compared to controls (Figure 2.1). Using the Kruskal-Wallis test with Bonferroni correction, we determined that the CDC Control group was lower than the AGS group (p-value = 8.9×10^{-12}) and that the Random control group was lower than the AGS group (p-value = 7.3×10^{-7}). The CDC Control group and the Random control group had no difference between them (p-value = 0.77).

The false positive rate, or number of control individuals who meet the CDC threshold for AGS diagnosis (sIgE anti-αGal ≥ 0.1 IU/mL), was 19.6%. The false negative rate, or number of AGS patients that did not meet the CDC diagnosis threshold, was 21.6%.

Figure 2.1 Log Transformed sIgE Anti-αGal Levels from AGS, CDC Control, and Random Control Samples

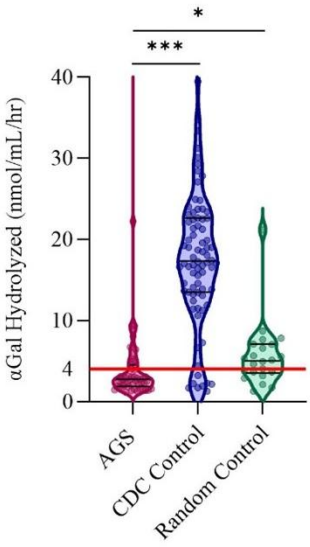


AGS samples display significantly higher sIgE anti-αGal levels ($p<0.001$) than either control group. AGS diagnostic threshold is marked at 0.1 IU.mL. All values lower than 10^{-3} were transformed to 10^{-3} for graph legibility.

Alpha-Galactosidase Activity

We found that alpha-galactosidase activity is significantly lower in AGS patient plasma when compared to controls (Figure 2.2). We assigned an arbitrary protein activity value of 4 nmol/μL/hr, as it was similar to the false positive and false negative rates of the sIgE anti-αGal test. The false positive rate was 15.7% and the false negative rate was 29.4%. When comparing the datasets with the Kruskal-Wallis test with Bonferroni correction, the AGS group and CDC control group comparison had a p-value of 9.1×10^{-14}. The AGS group and Random control group comparison had a p-value of 0.015.

Figure 2.2 Measurement of Alpha-galactosidase Activity (nmol/mL/hr)

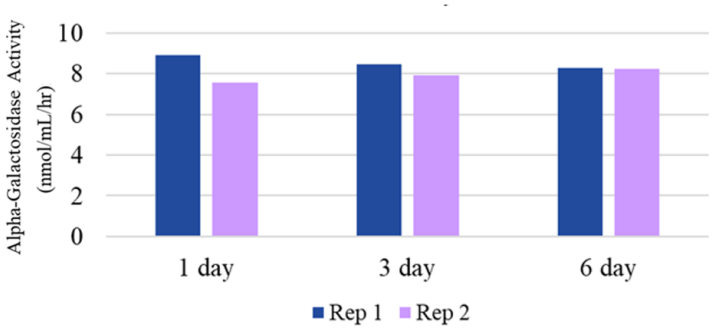


AGS had significantly lower alpha-galactosidase activity when compared to CDC Control ($p<0.001$) and Random Control ($p<0.05$).

Alpha-Galactosidase Activity in Freeze-Thaw Sample Set

We discovered that, up to 6 freeze-thaw cycles, there is no significant difference in plasma alpha-galactosidase activity (p-value = 0.993) (Figure 2.3). This suggests that, up to 6 freeze-thaw cycles, there is no statistically significant difference in alpha-galactosidase activity.

Figure 2.3 Comparison Between Number of Thaws and Alpha-galactosidase Activity.

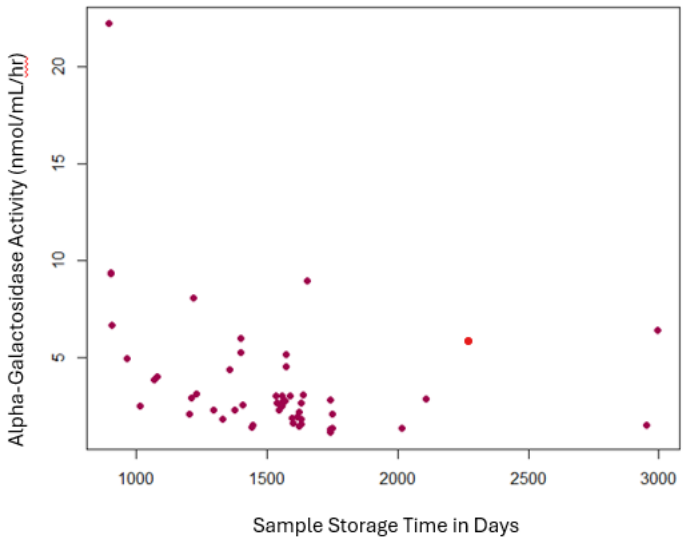


There is no significant difference between plasma alpha-galactosidase activity up to 6 freeze-thaws.

Comparison between Length of Sample Storage and Alpha-Galactosidase Activity

Outliers were removed from our dataset (n = 1) before the correlation analysis was performed. The outlier was excluded, as the alpha-galactosidase activity value was 18-fold higher than the second-highest value. We discovered that there is no statistical significance between the length of sample storage and the levels of alpha-galactosidase activity in AGS patient plasma (p-value = 0.0119, r-squared value = 0.1246) (Figure 2.4). While the p-value alone would indicate relevance, the r-squared value indicates that less than 13% of the alpha-galactosidase activity can be attributed to the length of sample storage. This indicates that there is no relationship between the storage time in our plasma samples and the levels of plasma alpha-galactosidase activity.

Figure 2.4 Correlation Between Sample Storage Time and Alpha-Galactosidase Activity



There is no correlation between length of sample storage and alpha-galactosidase activity in plasma.

Discussion

We found that alpha-galactosidase activity is significantly lower in AGS samples when compared to control samples and that neither the length of sample storage time nor number of freeze-thaw cycles, up to 6 cycles, impacts plasma alpha-galactosidase activity. We also confirmed that our AGS samples had higher sIgE anti-αGal levels when compared to control samples. This supports our hypothesis that individuals susceptible to developing AGS were more likely to do so based on lowered alpha-galactosidase activity. This confirmation indicates support for our further hypothesis that an increased amount of αGal in the bloodstream, caused by a lowered ability to hydrolyze the terminal galactose from glycoproteins and glycolipids, could lead to a sIgE anti-αGal-mediated allergic response in AGS-susceptible individuals.

As previously mentioned, sIgE anti-αGal is not a perfect diagnostic tool. In literature, it is shown that a subset of healthy individuals (or, at the very least, AGS-symptom-free individuals) have sIgE anti-αGal levels elevated above the 0.1 IU/mL threshold for AGS diagnosis. In our control sample sets, we found this 'false positive' result in 19.6% of samples. It is possible that these individuals with elevated sIgE anti-αGal and a history of tick bites may be protected from developing AGS through sufficient alpha-galactosidase activity. There are also individuals diagnosed with AGS, with symptoms and tick bite histories, who have sIgE anti-αGal levels below the 0.1 IU/mL diagnostic threshold. In our AGS sample set, we found this 'false negative' result in 21.6% of samples. While a portion of these individuals likely have low total IgE counts, and therefore cannot meet the unit measurement, they may reach the 1% total IgE proportion that the 0.1 IU/mL threshold represents with average total IgE counts. There will be other patients with AGS symptoms who do not meet either definition of sIgE anti-αGal threshold.

Using our alpha-galactosidase activity threshold at 4 nmol/mL/hr, we identified the number of AGS samples and control samples that did not fall into the expected values. If we follow that AGS samples should fall below the activity threshold and control samples should fall above the activity threshold, we have a 'false negative' rate of 29.4% in our AGS sample set and a 'false positive' rate of 15.7% in our control sample sets. We compared sample identities of the 'false negative' and 'false positive' samples in both the sIgE anti-αGal levels and alpha-galactosidase activity levels. We found that, if alpha-galactosidase activity testing was performed after the sIgE anti-αGal antibody titer test, 27.3% of our AGS sample set with sIgE anti-αGal antibody titers below the threshold would have received an AGS diagnosis. Additionally, we found that 15% of our control sample sets with an elevated sIgE anti-αGal antibody titer would not have received an AGS diagnosis after alpha-galactosidase activity testing. This requires further testing, however we suggest that testing for alpha-galactosidase activity, along with sIgE anti-αGal levels, may provide a more accurate diagnostic measure than measuring sIgE anti-αGal alone.

Alpha-galactosidase activity is a measure of how much αGal is removed from the terminal end of a structure, 4-MU in our assay, over time. In humans, the enzymes with alpha-galactosidase activity are typically active in the lysosome, which has a considerably lower pH than blood plasma. However, given our finding that alpha-galactosidase activity is significantly lower in AGS patient plasma samples, we suggest that there may be a correlation between the patient's ability to break down αGal and the development of AGS symptoms.

Several important variables remain uncharacterized in our current datasets, presenting opportunities for future research to enhance the resolution and interpretability of our findings. First, the donor metadata (sex, age, race, lifestyle, and medical comorbidities) is currently

unavailable. Incorporating these demographic and clinical variables in future studies would allow for further investigation into other potential influences on alpha-galactosidase activity. Second, detailed tick exposure histories, including tick bite incidence, are unknown. Such data could enable correlation analyses between exposure events, tick bite occurrence, and enzymatic activity. Third, although we investigated the relationship between length of sample storage time and alpha-galactosidase activity within the AGS group, full sample history data remains incomplete for our control dataset. Future work that includes well-documented sample handling protocols, including sample collection and storage conditions, would allow for a more thorough evaluation of potential pre-analytical variables. Together, these variables represent promising directions for future investigation into the impact of alpha-galactosidase activity on the sensitization and elicitation of AGS.

Conclusion

Our results show that sIgE anti-αGal levels are significantly elevated and alpha-galactosidase activity levels are significantly lowered in the plasma of individuals with AGS. This supports our hypothesis that lowered alpha-galactosidase activity may allow for the immune systems of AGS-susceptible individuals to have more interactions with αGal, leading to the development of allergic responses. This also provides evidence that individuals with elevated sIgE anti-αGal levels and no AGS symptoms may be protected from developing an allergic response to αGal through sufficient levels of alpha-galactosidase activity. In conclusion, our findings indicate that measuring alpha-galactosidase activity has potential as a valuable supplemental measure in AGS diagnostics.

Chapter 3 - Proteins Involved in Alpha-Galactosidase Activity in AGS Plasma

Introduction

Alpha Gal Syndrome (AGS) is an allergic response to red meat. More specifically, AGS is an allergy to galactose-α-1,3-galactose (αGal), a carbohydrate epitope found on the terminal ends of glycans from non-human and non-old world primate mammals (8). Common symptoms of AGS include urticaria, gastrointestinal distress, angioedema, and anaphylaxis in severe cases (13). This wide range of symptoms and severity may be attributable to several factors, such as individual sensitivity, the form of the allergen, and what form of mammalian product was consumed. In general, the consumption of mammalian organ meat seems to induce more severe symptoms in AGS patients than other forms of mammalian products (5). As with many food allergies and intolerances, there is no current treatment for AGS, other than avoidance of the allergen. Medical interventions for allergic symptoms, such as antihistamines for mild symptoms, or adrenaline for anaphylaxis, are recommended (12).

AGS is a somewhat unusual food allergy, as symptoms are typically delayed for 2-8 hours after ingestion (8, 11, 11, 12). AGS is also not diagnosed with either skin prick tests or oral ingestion challenges. AGS is diagnosed by measuring the amount of specific IgE against αGal (sIgE anti-αGal) (8). The diagnostic threshold for AGS, as set by the CDC, is ≥ 0.1 IU/mL (8). This test has approximately 80% sensitivity and 80% specificity, which is not ideal for a diagnostic test (Chapter 2). Approximately 20% of non-AGS patients will be measured as false-positive where they exhibit sIgE anti-αGal titers outside the specificity range (≥ 0.1 IU/mL), despite lacking AGS symptoms (13).

As we discussed in Chapter 2, the false-positive individuals may be explained by high alpha-galactosidase activity, in which the enzymatic removal of αGal could protect the immune system from developing a reaction to αGal. This could create a high tolerance for αGal, even when the individual has elevated levels of sIgE anti-αGal. Likewise, we found that alpha-galactosidase activity is significantly lower in AGS patients, which provides opportunities to improve testing procedures and explore the ways in which AGS symptoms occur.

There are two enzymes that have been described as having alpha-galactosidase activity in humans: alpha-galactosidase A (GLA) and alpha-N-acetylgalactosaminidase (NAGA). GLA is a lysosomal enzyme that hydrolyzes the alpha bond of αGal, removing the terminal galactose (49). Each GLA monomer is 429 amino acids, including a 31 amino acid signal peptide sequence that determines the export location of the enzyme (53, 54). The molecular weight of the monomer is approximately 46 kDa; approximately 5-15% of this weight is attributable to glycosylation of GLA, which can vary widely depending on the organism, or individual, from which the GLA originated (6, 52). The gene for GLA is located on the X chromosome and is approximately 13000 base pairs with 7 exons (53, 54). The mature and active form of GLA acts as a homodimer (6). NAGA is also a lysosomal enzyme that hydrolyzes the alpha bond of α-N-acetylgalactosamine (α-GalNAc), cleaving the terminal α-GalNAc from glycoproteins and glycolipids (51, 66). NAGA also displays some hydrolytic activity against αGal, but with far less activity than it does with α-GalNAc (66, 67). Each NAGA monomer is 411 amino acids, including a 17 amino acid tail that is cleaved during dimerization; NAGA functions as a homodimer (51, 66, 67). The gene for NAGA is located on chromosome 22 and is approximately 1270 base pairs (66).

Overall, we were interested in which enzyme was responsible for the significant differences in alpha-galactosidase activity we have previously observed between AGS and control plasma samples. We investigated the levels of GLA and NAGA enzymes in the plasma of AGS patients and control individuals. We sought correlations between the levels of these proteins and alpha-galactosidase activity in the plasma samples. From these observations, we aimed to identify potential targets for future research into therapeutic treatments and improved diagnostics for AGS through the identification of proteins potentially involved in the lowered alpha-galactosidase activity seen in AGS patients

Methods

Alpha-Galactosidase A Detection ELISA

We used the RayBiotech Human Alpha-Galactosidase A/GLA ELISA Kit (catalog number: ELH-aGLA) to quantify the amount of GLA in plasma samples. This kit utilized a sandwich immunoassay method to detect GLA at a threshold between 41 and 10000 pg/mL. We performed the assay as detailed by the kit manual, with 1 exception. We used a 10-fold dilution of plasma per well, 10 µL plasma in total 100µL, rather than the 2-fold dilution as recommended by the kit. Any sample that did not show activity above the minimum detection threshold was retested with a larger volume of plasma up to 20 µL.

NAGA Detection ELISA

We used the antibodies.com Human NAGA ELISA Kit (catalog number: A78503) to quantify the amount of NAGA in plasma samples. The kit detected NAGA at a threshold between 0.156 and 10 ng/mL by using a sandwich immunoassay method. The assay was

performed according to the kit manual. Any sample that had activity above the detection threshold was tested again with an appropriate dilution factor. The plasma volume used in the 100 µL reaction volume was in the range of 1 to 5 µL.

Statistical Analysis

The data was not normally distributed, as calculated with the Shapiro-Wilk test (at p-value ≤0.05), therefore for correlation analyses between different experimental tests Spearman's rank correlation coefficient was used. All of our datasets had at least 1 non-normally distributed set of results. Spearman's rank correlation was used on non-parametric data to quantify the monotonic relationship between 2 variables. This coefficient was calculated by applying the Pearson correlation formula to ranked data, allowing for it to be used to compare non-parametric datasets. The Mann Whiteny U test was used to test the rank differences between each pair of non-parametric variables. The Kruskal-Wallis test was utilized as a non-parametric alternative to a one-way ANOVA. This non-parametric test was utilized to determine if there was a statistically significant difference between the medians of at least 3 independent groups. A p-value ≤0.05 indicated that there was a significant difference between at least 2 of the groups. The Bonferroni correction was used to correct family-wise error rates that may occur when performing multiple simultaneous statistical comparisons.

Chapter 2 Experiments Relevant to This Chapter

The following assays were performed and described in Chapter 2: sIgE anti-αGal detection ELISA, alpha-galactosidase activity assay, and the freeze-thaw sample treatments. The sIgE anti-αGal detection ELISA was used to quantify the amount of sIgE anti-αGal in blood

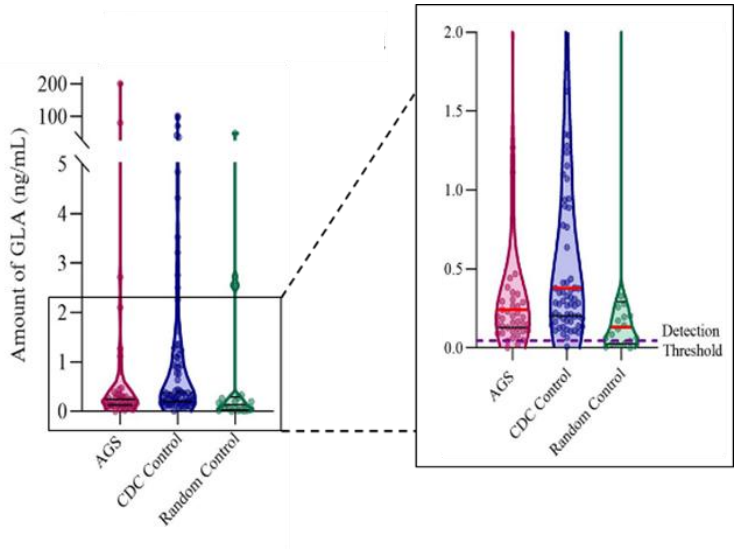
plasma. The alpha-galactosidase activity assay was used to quantify the levels of alpha-galactosidase activity potential in plasma. Samples from the freeze-thaw treatments were used to check the stability of GLA levels in plasma using the RayBiotech Human Alpha-Galactosidase A/GLA ELISA kit (catalog number: ELH-aGLA).

Results

GLA Enzyme Level

There was no significant difference between AGS, CDC control, and Random control groups when comparing amounts of GLA present in plasma (Figure 3.1) in a Kruskal-Wallis test with Bonferroni correction. We confirmed p-values using pairwise Mann-Whitney U tests. The p-value between the AGS group and the CDC control group was 0.15 and the p-value between the AGS group and the Random control group was 0.55. The median values are 0.242 for the AGS group, 0.379 for the CDC control group, and 0.132 for the Random control group. The distribution range is 0.0018-200.85 ng/mL for the AGS group, 0.0074-101.19ng/mL for the CDC control group, and 0.0063-47.39 for the Random control group.

Figure 3.1 Levels of GLA Enzyme in Plasma



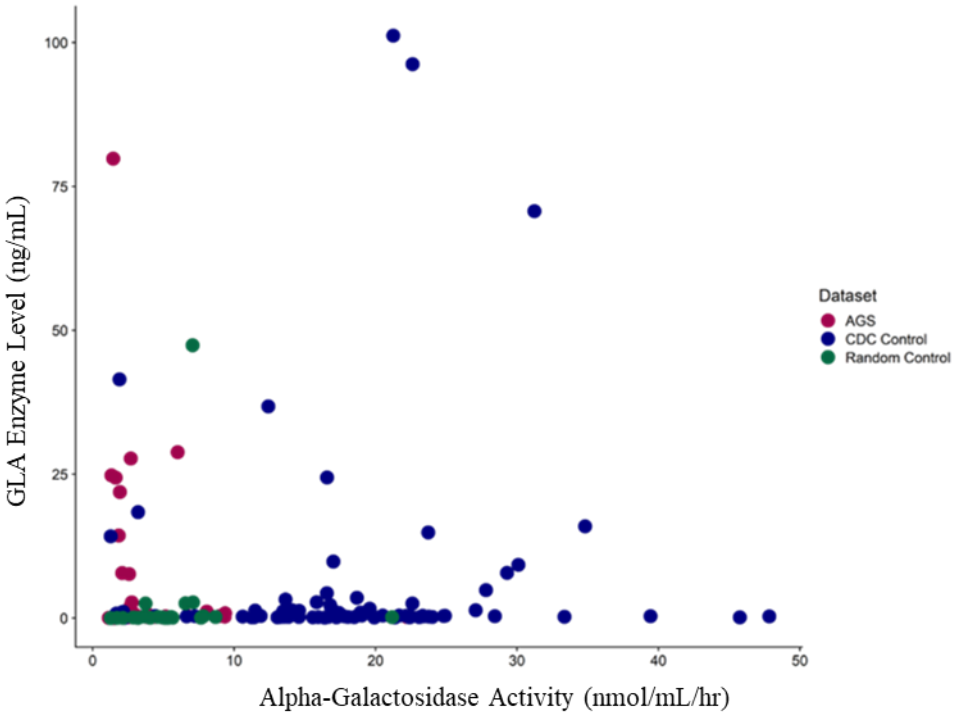
The upper and lower quartiles are marked in black and the median is marked in red. The 'Detection Threshold' line marks the bottom threshold of detection for the Human Alpha-Galactosidase A/GLA ELISA Kit from RayBiotech.

We also measured the amount of GLA in samples that underwent 1, 3, or 6 daily freeze-thaw cycles. There was no difference in the amount of GLA detected in any of the freeze-thaw samples (data not shown).

Correlation Between Alpha-Galactosidase Activity and GLA Enzyme Level

We observed no clear relationship between alpha-galactosidase activity and GLA enzyme levels in plasma (Figure 3.2). We performed a Spearman correlation analysis to test the monotonic relationship, which gave a p-value of 0.79 and an R-squared value is 0.00046. Thus, there was no evidence for a monotonic relationship between GLA enzyme level and alpha-galactosidase activity.

Figure 3.2 Scatterplot Relationship Between GLA Enzyme Level and Alpha-Galactosidase Activity

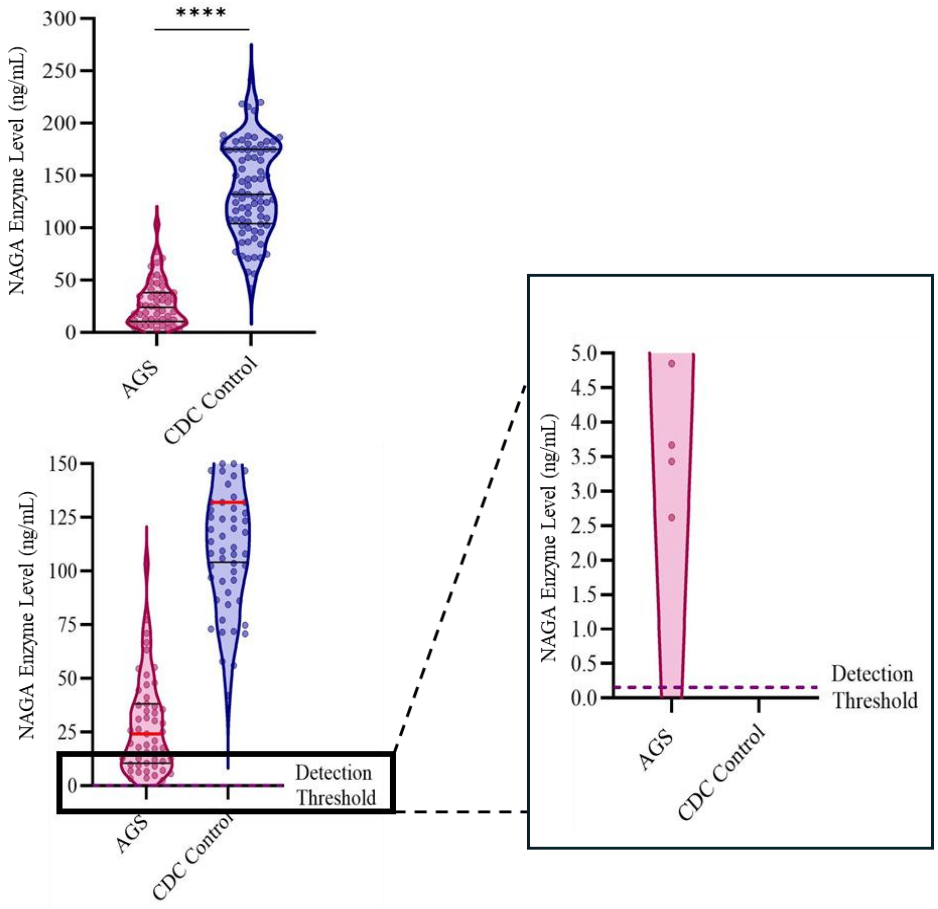


AGS samples are marked in pink, CDC control samples are marked in blue, and Random control samples are marked in green.

NAGA Enzyme Level

We observed clear distributional differences suggestive of a mean difference between NAGA enzyme levels in AGS samples when compared to the CDC control group (Figure 3.3). We validated observed differences with a Mann-Whitney U test ($p < 0.0001$), which indicated evidence for systematic differences between the rank distributions of NAGA enzyme level in CDC control plasma when compared to AGS plasma. The median values are 24.15 for the AGS group and 132.12 for the CDC control group, while the mean values are 28.27 for the AGS group and 137.45 for the CDC control group. The distribution range is 2.62-103.35 ng/mL for the AGS group and 42.10-241.51ng/mL for the CDC control group.

Figure 3.3 The Amount of NAGA Present in Sample Plasma

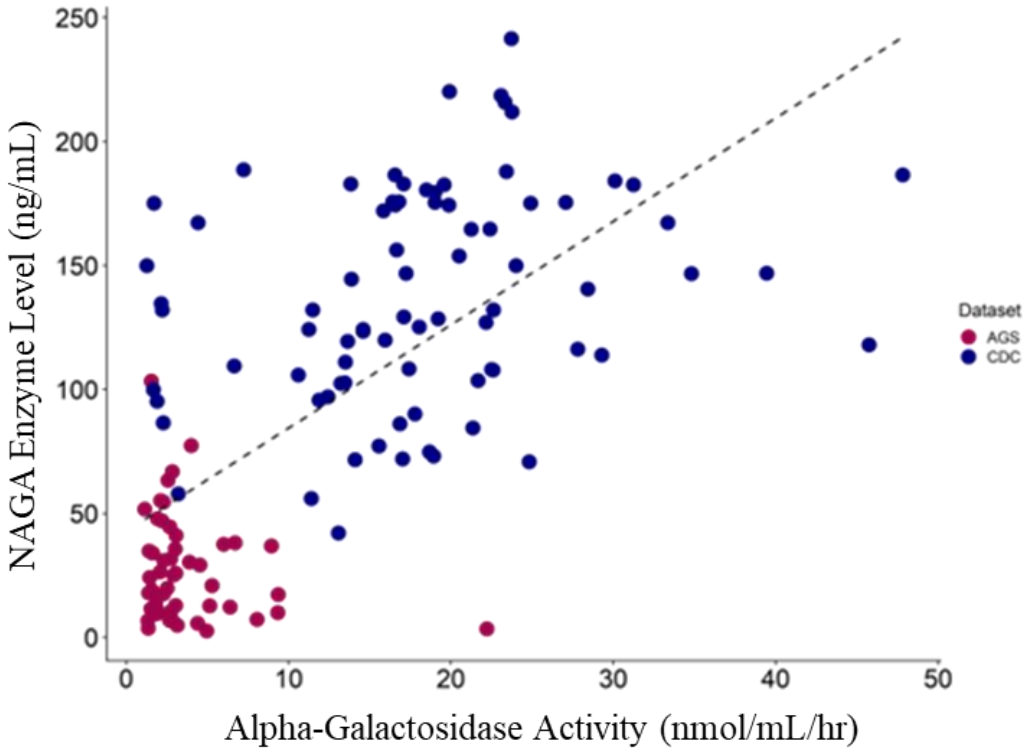


The outer quartiles are marked in black and the median is marked in red. The 'Detection Threshold' marks the bottom detection threshold of the antbodies.com Human NAGA ELISA Kit.

Correlation Between Alpha-Galactosidase Activity and NAGA Enzyme Level

We performed a Spearman correlation analysis to compare alpha-galactosidase activity and NAGA enzyme level in plasma. There appeared to be a clear relationship between alpha-galactosidase activity and NAGA concentration (Figure 3.4) and there was evidence of a monotonic relationship between alpha-galactosidase activity and NAGA enzyme level (p-value of 0.001; Figure 3.4). One outlier was excluded from this comparison, as the alpha-galactosidase activity value was 18-fold higher than the second-highest value.

Figure 3.4 Scatterplot Relationship Between NAGA Enzyme Level and Alpha-Galactosidase Activity in Plasma

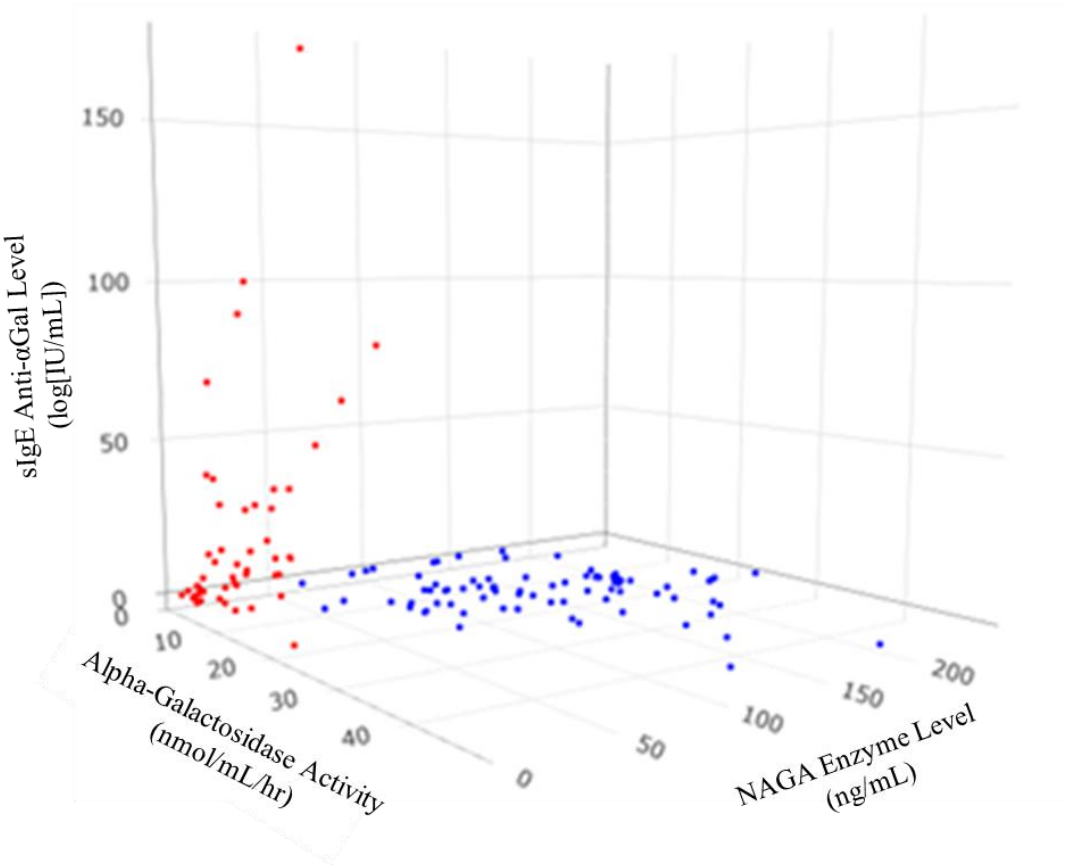


The AGS samples are marked in pink and the CDC control samples are marked in blue. The regression line is marked in black.

Improved AGS Diagnostics when Alpha-Galactosidase Activity, NAGA Enzyme Level, and sIgE anti-αGal Level are Combined

We plotted AGS and CDC control data in a 3D plot using the data from our alpha-galactosidase enzymatic activity test, NAGA detection ELISA, and sIgE anti-αGal detection ELISA from Chapter 2 (Figure 3.5). We observed that the 3D graph visually separates the AGS and CDC control groups into 2 distinct populations. These distinct groups had very little observable overlap, which indicated that a combination of these 3 tests can be used to improve the specificity and sensitivity of future AGS diagnostics.

Figure 3.5 A Comparison Between NAGA Level, Alpha-Galactosidase Activity, and sIgE Anti-αGal Level



AGS samples are pictured in red and CDC control samples are pictured in blue. 1 outlier (alpha-galactosidase activity level of 866 nmol/mL/hr) was removed for graph legibility.

Discussion

We found a significant difference in the amount of NAGA present in AGS plasma samples when compared to CDC controls. Additionally, we found a strong correlation between the amount of NAGA in plasma and alpha-galactosidase activity in plasma. On the other hand, we found that the amount of GLA in plasma is not statistically different between AGS patient samples and control samples and there is no correlation between alpha-galactosidase activity and

GLA enzyme level in plasma. We confirmed that this finding was not an artifact by performing a set of daily freeze-thaw cycles on a single plasma sample and testing for changes in alpha-galactosidase activity and GLA enzyme level; there was no difference in the amount of GLA detected up to 6 daily freeze-thaw cycles in plasma. As we discovered in Chapter 2, alpha-galactosidase activity is significantly lowered in our AGS samples.

Kytidou et al. (2017) used enzymes produced by HEK293 cells, a human cell line, to identify how much alpha-galactosidase activity could normally be attributed to either GLA or NAGA (75). They found that GLA was responsible for approximately 90% of alpha-galactosidase activity in an alpha-galactosidase activity test and we hypothesized that GLA concentration would correlate with alpha-galactosidase activity in our plasma samples (75). We were surprised by the lack of correlation between GLA enzyme level and alpha-galactosidase activity in our samples. We were further surprised by our NAGA enzyme level findings, as NAGA has been shown to be responsible for approximately 10% of alpha-galactosidase activity in an enzyme activity assay (75).

It is important to note that both GLA and NAGA are lysosomal proteins and their optimal pH is approximately 4.6 (66). The pH of blood is not ideal for either GLA or NAGA functionality. We are not certain that alpha-galactosidase activity is happening natively in plasma; it is possible that the proteins have been excreted into the blood for some other purpose and measuring plasma concentrations of these enzymes may not be indicative of biological activity. However, we are using protein concentrations from plasma samples to test the amount of possible alpha-galactosidase activity in an ideal environment.

Given that NAGA expression is, on average, 3-fold higher than GLA in AGS plasma and shows a 21-fold increase in CDC control plasma, we suspect that NAGA contributes to the

modulation of alpha-galactosidase activity in plasma samples. Only 4 AGS samples, and none of the CDC control samples, had NAGA levels that fell within the majority of GLA levels (0-5 ng/mL). However, this may be somewhat unlikely, as GLA has been demonstrated to have a much higher catalytic activity for αGal than NAGA (75)

Additional factors may contribute to the correlation between alpha-galactosidase activity and NAGA, which is not seen with GLA. First, it is possible that GLA found in AGS patients is less efficient at binding to αGal than GLA present in control individuals, while NAGA's binding potential towards αGal remains unaffected. Second, due to its higher plasma concentration, NAGA may interact more frequently with αGal in plasma, potentially interfering with GLA's access to αGal and thereby modulating downstream enzymatic activity. The increased interaction rate may be able to compete with α-GalNAc for NAGA's active site. Finally, it is possible that other proteins with alpha-galactosidase activity are present in the plasma, which could be acting on αGal in an as-yet-undiscovered mechanism.

NAGA enzyme levels and alpha-galactosidase activity are significantly correlated, irrespective of the responsible mechanism. If we take this correlation and plot both variables along with sIgE anti-αGal levels from Chapter 2 (Figure 3.5), we see two distinct populations form: AGS and CDC controls. We suggest that combining the sIgE anti-αGal, alpha-galactosidase activity, and NAGA enzyme level tests may provide a more precise AGS diagnostic profile than using the sIgE anti-αGal test alone.

Conclusion

In conclusion, we discovered that NAGA, not GLA, is correlated with alpha-galactosidase activity from plasma samples. We have further identified that a combination of sIgE anti-αGal, NAGA enzyme level, and alpha-galactosidase activity may make for a more

accurate diagnostic panel for AGS. This is an important discovery, as these findings implicate both alpha-galactosidase activity and NAGA enzyme levels as potential targets for future research into therapeutic treatments and improved diagnostics for AGS.

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