

Analysis of the Genetic Variants Associated with Fabry Disease

by

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Abstract

Fabry Disease is a lysosomal storage disorder involving the dysfunction of the GLA (galactosidase alpha) gene, and follows an X-linked inheritance pattern with varying incidence rates in populations. The GLA gene is responsible for producing the enzyme alpha-galactosidase A, which breaks down a glycosphingolipid known as globotriaosylceramide, also known as Gb3. Thus, with a dysfunctional GLA gene, Gb3 builds up in the host's body and leads to complications in the heart, kidney, gastrointestinal tract, and ultimately death. As current treatments for Fabry Disease are extremely expensive and impractical in the long term, studies have been performed to look into the molecular mechanisms behind the disease to provide further insight on how to better treat the disease. For my study, I used a sample of 50 patients, provided by PerkinElmer's Lantern Project, a program designed to provide diagnostic testing for various lysosomal storage disorders and limb-girdle muscular dystrophies (LGMDs), to observe the types of variants produced, the enzyme and biomarker levels, and classification of pathogenicity. I found that there was one specific variant, c.679 C>T, that appeared the most often compared to other variants, that there was a weak negative association between enzyme levels and biomarker levels, and that those who had higher than normal biomarker levels were more likely to be labeled as likely pathogenic or pathogenic. My findings align with previous studies and conclude that there are specific variants associated with Fabry Disease that have certain implications, which may urge us to focus on specific genetic regions when looking into developing treatments.

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Preface

I came into the University of Pittsburgh's School of Public Health with the intention to learn about public health, whatever that may entail, move on to medical school, and that would be it. However, during my last year and a half in this program, I have come to discover that public health is something that I am truly passionate about and is not just a steppingstone towards higher education. The staff, the classes, the opportunities and the friends I have made have far beyond exceeded my expectations, and I am forever indebted to and grateful for this school. I did not think I would be where I am now today, but I would not have it any other way.

I would like to thank Andrew McCarty, who has served as an advisor, mentor, essay reader, and friend during my time in graduate school. He has undoubtedly been nothing but friendly, helpful, and has pushed me to grow as a person. I am very honored to have been able to work with him during my time at school as well as my time at PerkinElmer, and both this essay and I would not be where we are if it was not for his guidance. In addition, I would like to thank PerkinElmer for the opportunity to work with this data set and providing me with an interesting and wonderful first internship experience.

I would also like to thank Dr. Andrea Durst for serving as my counselor and professor, overseeing my journey of an atypical accelerated student and only supporting me. Over the last year and a half, she has made sure that I was on track to graduate in the time frame I desired, suggesting class schedules, checking up every so often, and making sure I was doing okay mentally. With her constantly encouraging me in all my decisions, I was confident in my ability to not only do well in my courses, but also explore what I wanted to do with my life.

Finally, I would like to thank the staff, faculty, and students of the University of Pittsburgh. I have spent the last five years of my life in this city I called home learning about the wonders of biology and genetics, and I will look back fondly at my time here. Every single person I have ever interacted with has only further justified why I decided to pursue my undergraduate and graduate career here. I am truly honored to be able to call myself a Pitt alum.

1.0 Introduction

Fabry Disease is a lysosomal storage disorder (LSD) regarding the galactosidase-alpha (*GLA*) gene and glycosphingolipid metabolism. Our lysosomes are responsible for degrading, recycling, and disposing macromolecules and cellular waste that builds up from cellular functions and aging, including dead cell components, carbohydrates, lipids, proteins, nucleic acids, and more. The *GLA* gene, located at Xq22.1 is responsible for producing an enzyme known as alpha-galactosidase A, located in the lysosome (Auray-Blais 2007). Alpha-galactosidase A is responsible for the catabolism of various glycosphingolipids, including globotriaosylceramide, also known as Gb3 (Scriver et al. 2000), through hydrolysis of the terminal alpha-galactosyl moieties of these molecules. When the *GLA* gene is defective, alpha-galactosidase A is no longer produced in sufficient quantities; thus, the body is unable to metabolize Gb3. This leads to Gb3 being built up in the lysosome as well as other organelles within the cell, including the endoplasmic reticulum, cell membrane, and nucleus (Germain 2010). Because of this, Gb3 has been used as a biomarker for the disease.

This results in irregularities and impairment of bodily function; symptoms can include extreme pain in the hands and feet, angiokeratomas in the skin, shortness of breath, chest pain, chest tightness, nerve pain, vision issues, and complications within the heart, kidney, and gastrointestinal (GI) tract (Germain 2010) (Rozenfeld 2009). Even though cases of Fabry Disease are rare among all ethnicities, and the estimated incidence rate is relatively low at 1 in 100,000 in the population (Germain 2010), with 1 in 50,000 males affected (Wilcox et al 2008), if left untreated, it can lead to multiple organ failure, which may result in death (Mehta 2002). This incidence rate can vary and is often emphasized to be underestimated.

There is still much unknown about Fabry Disease and much to improve upon. Currently, there is no known cure and treatment options are extremely expensive. The only current treatment is either Replagal and Fabrazyme, both which cost approximately \$300,000 per year. Replagal contains an enzyme known as agalsidase alfa, which has been shown to function similarly to alpha-galactosidase A, and is used to eliminate Gb3 buildup within endothelial cells (Pastores 2007). Likewise, Fabrazyme contains an enzyme known as agalsidase beta, which also functions similarly to alpha-galactosidase A, and is used for the exact same purposes. Both treatments are applied intravenously to patients (Germain 2015) and only Fabrazyme is currently approved by the FDA in the USA. However, because both options only serve as a temporary solution and are highly priced, it has provided incentive towards looking for alternative treatment solutions.

My project will be exploring the genetic makeup of Fabry Disease and determining if there are trends across variants in X chromosomal regions that are conserved across Fabry Disease patients, if there is a correlation between a patient's enzyme levels and their corresponding biomarker levels, and whether specific enzyme levels and biomarker levels are correlated with a classification of gene pathogenicity per ACMG (American College of Medical Genetics) guidelines. By expanding the underlying mechanisms of this disease, further insight can be gained towards more efficient and affordable treatment options and ultimately, farther into the future, a permanent cure.

1.1 The Lysosome

The lysosome is an essential organelle in the cell – located in the cytoplasm of the cell, its responsibilities are primarily the degradation, recycling, and disposal of all waste products and

materials of the cell, including carbohydrates, lipids, proteins, and nucleic acids (Ballabio 2019). Each human cell has a range of 50-1000 lysosomes scattered throughout the cytoplasm (Ballabio 2019). Considered a static organelle, meaning its position does not change in the cytoplasm, the lysosome consists of numerous proteins that are present on the membrane of the organelle as well as the interior, with each one having specific purposes such as signaling to other organelles, transporting macromolecules, or the notable enzymatic activity, hydrolyzing macro molecules (Ballabio 2019). Hydrolases, alongside other proteins located in the lysosome such as proteases and lipases (which degrade proteins and lipids, respectfully), function in a heavily acidic environment (pH 4.5 - 5.0), which is maintained by ion channels located along the membrane of the lysosome that regulate the concentration of various ions in the lysosome, such as calcium, hydrogen, sodium, potassium, chloride, iron, zinc, and copper (Trivedi 2020).

Besides its degradation enzymatic activity, lysosomes are important in cell signaling and creating more lysosomes – lysosome fusion and fission play a large role in maintaining the number of lysosomes in the cell, although primary lysosomes are synthesized from the Golgi apparatus (Trivedi 2020). Lysosomes can communicate with other organelles in the cell through membrane contact sites (MCS); for example, MCS between the lysosome and endoplasmic reticulum has been noted to be involved in calcium exchange, synthesis of new lysosomes, and maintaining cytoplasmic positioning (Trivedi 2020). Fusion and fission are also distinct characteristics of the lysosome – the lysosome has the ability to fuse with the plasma membrane, endosomes, and autophagosomes, allowing the lysosome to perform functions such as regulation of secretory vesicles, degradation of macromolecules, and monitoring of ion channels. (Trivedi 2020). Lysosomal fission is important in regulating both lysosomal numbers and size (Trivedi 2020).

1.2 Lysosomal Storage Disorders

Lysosomal storage disorders (LSDs) are diseases that are inherited from generation to generation and are recognized by a definitive quality of buildup of macromolecules in various organs and their cells due to a loss of or downregulated function of lysosomes. The loss of enzyme function makes up for about 70% of all LSD cases (Rajkumar 2021). There is a total of 70 known LSDs that involve 40 different hydrolases, making each LSD rare in occurrence individually but common overall. Each LSD can also fall under one of seven classifications, dependent on what substrates and defects are involved (Rajkumar 2021). LSDs commonly follow an autosomal recessive inheritance pattern with the exception of three diseases, including Fabry Disease.

Most LSDs are caused by dysfunctional proteins from expression of mutated genes, which creates a lack of mechanism needed to break down the toxic macromolecules (Rajkumar 2021). Those who inherit LSDs will have varying issues depending on which and where macromolecules are accumulated in the body – for example, Hurler Syndrome is an accumulation of mucopolysaccharides, and Pompe Disease is associated with glycogen buildup (Rajkumar 2021). Although the severity of symptoms and treatments vary depending on numerous factors, such as age or predisposing factors, all have devastating and lasting effects on the individual that prompt research efforts towards combating, preventing, and treating LSDs and their respective risks.

1.2.1 Fabry Disease

Fabry Disease is a specific type of LSD that is associated with the buildup of glycosphingolipids in the body due to dysfunctional or lack of alpha-galactosidase A, specifically a buildup of globotriaosylceramide, also known as Gb3 (Germain 2010). This leads to damage

across various organs of the host, including the skin, ears, heart, kidneys, eyes, lungs, and nervous system (Germain 2010). Fabry Disease follows an X-linked inheritance pattern, and because it is a rare and pan-ethnic disease, prevalence is difficult to determine (Germain 2010). Current treatments include enzyme replacement therapy (ERTs), where the host is infused with an enzyme with similar function to the enzyme that is missing or dysfunctional in the host's body (in the case of Fabry Disease, agalsidase alfa or agalsidase beta) (Germain 2010).

1.2.1.1 Prevalence/Incidence Rates

The incidence and prevalence rate of Fabry Disease are difficult to determine for a variety of reasons. Because the disease is so rare in occurrence, it is difficult to determine an accurate number of people who have the disease, and often times this number is vastly underestimated (Germain 2010). Regarding the general population, the incidence rate has been reported to range from 1 in 476,000 to 1 in 117,000. However, Fabry Disease has been shown to be a pan-ethnic disease and follows an X-linked inheritance pattern, and thus incidence and prevalence rates may vary based on the population's ethnicity and biological gender. Studies in both Italy and Taiwan found that the prevalence of the disease can reach up to 1 in 3,100 newborn males (Germain 2010). Thus, it is important to note that there are a multitude of factors that can affected the estimation of prevalence and incidence rate of Fabry Disease.

1.2.1.2 Inheritance Patterns

Fabry Disease is known as a X-linked disease, but it is unknown whether this disease has a dominant inheritance pattern, recessive inheritance pattern, or neither (Germain 2010). For males, diagnosis of an individual's Fabry Disease status is relatively straightforward; those who are hemizygous for a defective *GLA* gene will have deficient amounts of alpha-galactosidase A,

indicative of Fabry Disease (Germain 2010). However, females have shown varying inheritance patterns, with a penetrance of 70% in females with one copy of a defective GLA gene and with a spectrum of severity based on random X chromosome inactivation in the individual. Theoretically, female cases of Fabry Disease should be double the number of male cases; however, databases such as the NIH have shown that there are similar numbers of cases across biological sexes, indicating that the X-linked inheritance pattern in Fabry patients does not follow what is considered to be normal, or carriers who are asymptomatic have not been identified. Thus, the term “carrier” has been widely preferred over “heterozygote (Germain 2010).”

Random X chromosome inactivation is a natural phenomenon that balances the number of gene products produced in females, assumed to have two copies of an X chromosome, and males, assumed to have one copy of the X chromosome and one copy of the Y chromosome. X chromosome inactivation occurs through non-coding RNAs that silence transcription of a randomly chosen X chromosome; the silenced chromosome stays constant through all cells derived from the original (Panning 2008). It has been shown in the past that Random X-chromosome inactivation can play a part in various levels of enzymatic activity, which can range from no enzymatic activity to normal levels (Chamoles et al. 2001). Skewed X inactivation, which is when the expression of each X chromosome in a female is uneven due to cells not expressing an X chromosome evenly, also affects how Fabry disease can manifest in individuals (Echevarria et al. 2016).

1.2.1.3 Detection/Diagnosis

Fabry disease has an early age of onset in patients, which can be as early as birth (Laney et al. 2014). There are a large number of early symptoms of Fabry Disease, including acroparesthesia, nausea, vomiting, diarrhea, angiokeratomas on the skin, visual issues, hearing

loss, weight gain issues, unable to concentrate and many more (Germain 2010). Some of these symptoms may decrease in severity in adulthood, but this does not mean that Fabry Disease has stopped its manifestation in the patient.

Urine tests can be used to detect levels of Gb3 to screen for Fabry Disease (Auray-Blais 2007). Gb3 levels in urine in Fabry Disease patients have been shown to be at higher levels due to accumulation within epithelial cells in the distal tubules (Auray-Blais 2007). Through the use of liquid chromatography and tandem mass spectrometry, Gb3 levels in urine on filter paper can be observed. Dried blood spots on filter paper (DBFP) can also be used in similar fashion – rather than looking at levels of Gb3, levels of alpha-galactosidase A enzyme activity can be observed through fluorometric means to determine whether a person may have indications of Fabry Disease (Chamoles et al. 2001).

However, because there are various manifestations of Fabry Disease, it is often difficult to diagnose. It can be easily confused with growing pains or other diseases such as rheumatic diseases, leading to multiple diagnoses by multiple physicians being necessary (Rozenfeld 2009). In addition, the tests mentioned previously can often fail to screen for Fabry Disease, and thus physicians should take caution when using these methods.

1.2.1.4 Treatment Options

Enzyme replacement therapy has been the primary means of treating Fabry Disease patients, with two options currently available to the consumer. Replegal, which contains agalsidase-alfa, is produced by Takeda Pharmaceutical Company through human fibroblasts, and Fabrazyme, which contains agalsidase-beta, is produced by Sanofi Genzyme through hamster ovary cells (van der Veen et al. 2020). Both treatments function by reducing the levels of Gb3 from endothelial cells, which provided short-term solutions, and has been shown to slow down the

development of cardiac and renal complications in the body, agalsidase-beta more so than agalsidase-alfa (van der Veen et al. 2020).

Although enzyme replacement therapy has been used as treatment for Fabry Disease over the last 17 years, it is still not optimal. It does not stop complications in the heart, kidney, or nervous system from developing, as reuptake of the recombinant enzyme is limited in the body and is not yet fully understood. The body can make antibodies specifically against agalsidase variations that may negatively affect their efficiencies (van der Veen et al. 2020). In addition, having to constantly apply the treatment is a burden – it is both time consuming to have to schedule appointments with physicians to inject the treatment and inefficient in the long term. This has led to the development of other treatment options, such as second generation ERTs that do not carry certain molecules on their protein surfaces that may make it difficult for the protein to enter certain pathways, and substrate reduction therapies, which attempts to stop the formation of the substrate, regardless if there's a presence of a defected enzyme.

Another point of discussion regarding treatment options is gene therapy, which is the transfection of normal genes into the host's cells to replace the dysfunctional genes. Fabry Disease is an optimal condition to look into for this treatment option, as the host only needs to restore a small amount of enzymatic activity to reduce Gb3 storage; this has been shown to be true in both in vitro and in vivo studies (Siatskas & Medin 2001). Khan et al. infused stem cells with functional GLA genes into five adult males with Fabry Disease and found that all patients were producing alpha-galactosidase A at normal levels in blood and bone marrow cells (Khan et al. 2021).

1.3 Perkin Elmer and the Lantern Project

Perkin Elmer introduced the Lantern Project as a collaboration project alongside Sanofi Genzyme in 2018; the goal of the initiative is to “offer complimentary genetic testing...and provide diagnostic testing for various lysosomal storage disorders and limb-girdle muscular dystrophies (LGMDs), including but not limited to: Fabry Disease, Pompe disease, Gaucher disease, Niemann – Pick Type A and B, and Mucopolysaccharidosis I (MPS I) (The Lantern Project).” The program was not designed as a replacement for a health care professional’s judgement, nor was it designed to interfere with the duties of one, but health care professionals can order testing kits (consisting of blood, saliva, and dried blood spot sample collections) for any of the diseases listed through a test requisition form (TRF). New York State regulations require samples that are collected from patients within New York State must be tested by clinical laboratories that present the appropriate licensing – the DOH requires that each test is cleared. Thus, testing for New York State residents may be skewed.

Testing for a disease only occurs should a patient present the given symptoms of the disease, which they can fill out manually on a page attached to the TRF. Should a patient fulfill all the requirements for testing, their sample is shipped via FedEx to a Perkin Elmer laboratory facility, where the sample is accessioned, extracted, concentrated, and sequenced. Should any issues arise during any part of this process, the appropriate hold is applied within the system indicating that a patient’s test cannot proceed. This hold is either resolved or the accession is cancelled; if the latter occurs, the patient and/or their ordering provider must request a new testing kit through a newly filled-out TRF.

Once a sample is sequenced, the data is reported back to the ordering provider and any other parties indicated on the TRF and is also stored within a Perkin Elmer database, which can be

used for reanalysis should the ordering provider see fit. The dataset I have acquired for this project is derived from this database, which includes patients that have tested for all the LSDs listed prior. Samples that were tested for Fabry Disease were filtered and removed from the dataset, leaving a sample size of 348 patients. This dataset includes de-identified patient test results, including any variants that were located (the specific nucleotide change), the corresponding protein change, alpha-galactosidase A enzyme levels, biomarker levels, zygosity, and classification of the gene based on ACMG guidelines, which falls under one of three categories: pathogenic, likely pathogenic, and uncertain significance.

2.0 The Three Aims of the Project

- Determine whether specific enzyme levels are correlated with biomarker levels of Fabry Disease.
- Analyze pathogenic variants across Fabry Disease patients to check for patterns, trends, and correlations that may suggest some conserved mutation responsible for Fabry Disease.
- Investigate whether specific enzyme levels (alpha-galactosidase A) and biomarker levels (Gb3) are correlated with a classification of gene pathogenicity per ACMG guidelines.

2.1 Description of Aims

Because of the vast descriptive statistics provided to me, there were various potential pairings that I wanted to analyze for correlations. From these analyses, I have three specific questions/aims I hope to accomplish: whether a patient's enzyme levels are correlated with their corresponding biomarker levels, whether these enzyme levels and biomarker levels are associated with the classification given to the patient, and potentially discover any correlations among a patient's specific nucleotide change and their other descriptors. Ultimately, I hope to provide some insight towards the underlying genetic mechanisms of Fabry Disease, pinpointing potential loci to focus on, suggesting appropriate treatment options, and contribute towards providing affordable and effective treatments of Fabry Disease.

The first specific question I'd like to investigate is whether a patient's enzyme levels are correlated with biomarker levels. As previously stated, the enzyme we wanted to focus on is alpha-

galactosidase A, as this is the primary gene affected in Fabry Disease patients, and the biomarker is either some intermediate of Gb3 or Gb3 itself. If a patient's enzyme levels are lower than 1.10 umol/L/hr, that patient is classified as having "below normal levels" of enzyme, which serves as an indicator of risk of having a pathogenic variant. Likewise, if a patient's biomarker levels are greater than 1.11 ng/mL, the patient is classified as having "above normal levels" of biomarker, which also serves as an indicator of risk. Because alpha-galactosidase A is supposed to reduce the amount of Gb3 present in a cell, it is assumed that if I were to observe an increased level of enzyme (1.10 umol/L/hr or higher) in a patient, then I would expect to observe a lower level of biomarker in the same patient (1.11 ng/mL or lower). Thus, our alternate hypothesis regarding this specific question would be, "Levels of enzyme greater than or equal to 1.10 umol/L/hr are negatively correlated with levels of biomarker less than or equal to 1.11 ng/mL."

Both the enzyme level and biomarker level are candidates for indicators of pathogenic variants of the *GLA* gene, presuming that the alternate hypothesis of the previous question is supported. Lower levels of enzyme means that less Gb3 is degraded within the cell, allowing for buildup for Gb3, resulting in the increased biomarker levels we see. This leads to the symptoms we see within patients with Fabry Disease, allowing us to diagnosis a patient. Alternatively, a patient's variant is classified as pathogenic, likely pathogenic, or uncertain significance based on ACMG guidelines, which begs the question: do enzyme levels and biomarker levels have some correlation with what a patient is classified as due to its indirect effects on homeostasis?

The piece of information I find most key to the project is the specific variant detected within a patient tested – the variant discovered and the corresponding protein change. Given this information across 348 patients, the possibilities of pairings and questions become endless – are patients that have a specific variant associated with a range of enzyme levels, a range of biomarker

levels, or classifications? Is there a specific variant that is conserved among all 348 patients, and is there a specific type of variant, such as a substitution variant, that is most prominently present across all patients? It is this crucial piece of information that allows for relationships to be drawn across other factors related to Fabry Disease, and so I intend to test whether there is a specific variant that occurs more often than the others, which will allow me to determine if a specific variant is also associated with a certain enzyme level, biomarker level, ACMG classification, etc. It is also important to note that because my data exclusively comes from U.S. patients whose ordering providers are aware of the Lantern Project's existence, I will need to compare my results to those studies conducted not only in the U.S., but also internationally as well.

3.0 Materials and Methods

Patients were solely recruited through the Lantern Project, where their ordering provider would send a testing requisition form (TRF) to PerkinElmer staff. Ordering providers are required to fill out at least two identifiers of a patient, such as name, date of birth, or medical record number. The ordering provider must also indicate where they are ordering from, what test they would like to run, and how the test will be billed (institutional billing, commercial insurance, or out-of-pocket). Finally, the provider must sign the TRF to confirm multiple requirements - they are a licensed medical provider authorized to order genetic testing to patients; they have obtained the patient's consent for the testing order, which implies that the patient understands information regarding the test, sample and data retention, and that their sample's existence may be shared with other professionals for research purposes, in which the professionals will contact the patient; the testing is medically necessary and may impact how the patient is medically managed in the future; and patients would also send samples to PerkinElmer laboratories for DNA extraction and sequencing.

Agilent targeted sequence capture method is used to sequence patient DNA, and direct sequencing of capture regions are performed through 2X100bp reads on Illumina next generation sequencing (NGS) systems. These sequences are then aligned to the human reference genome (hg19) to locate variants in target regions. Sanger sequence analysis is used to validate single nucleotide variants (SNVs) and insertion/deletion variants (indels) that meet PerkinElmer guidelines. This assay does have its limitations, however, as it cannot detect variants in non-target regions in the exome, including deep intronic regions, transcription modification regions, and

anything related to mitochondrial DNA. In addition, because of the size of target regions, copy number variation (CNV) analysis may not be possible for detection of variants.

Primary data analysis is performed using Illumina DRAGEN Bio-IT Platform v.2.03. Secondary and tertiary data analysis is performed using PerkinElmer's internal ODIN v.1.01 software for SNVs and Biodiscovery's NxClinical v.5.1. Reports would then be created defining whether a patient exhibited the variant associated with the disease requested for testing, as well as enzyme levels, biomarker levels, and pathogenicity as defined by ACMG. This data would then be stored within PerkinElmer databases, ready for use. For the purposes of my essay, de-identified data from all patients who requested genetic testing under the Lantern Project were pulled and organized into an Excel spreadsheet, containing the report data listed previously. I then modified the data sheet to solely focus on those who requested testing for Fabry Disease, narrowing down the sample size to 348 patients. The data sheet was further narrowed down to those patients who were labeled as having "below normal enzyme" levels, resulting in the final data sheet containing 50 patients total. No comparisons were made between these patients and patients with "normal levels" of enzyme. The data was imported into R-Studio, where all graphs were created and statistical analyses were performed. Statistical tests include a chi-squared test and a linear regression model.

4.0 Results

4.1 Demographics of the dataset

A demographics chart displaying the age and gender of all 348 was produced (Figure 1). On the left is a bar graph depicting the age distribution of the data set. The majority of the subjects, at 54.6%, were between the ages of 0-18 years old with 190 out of the 348 patients being in this age range. There were 158 patients who exceeded the age of 18 included in this study. The average age of this data set is 24.59 years old, the median age is 17 years, the range spans 0 to 82 years and the IQR is 44 years. On the right is a bar chart depicting the counts of biological genders of subjects in the dataset. The majority of subjects were male, at 92.40% of the sample or 322 subjects out of 348. It is also worth noting that there is missing data for biological gender for some of the subjects.

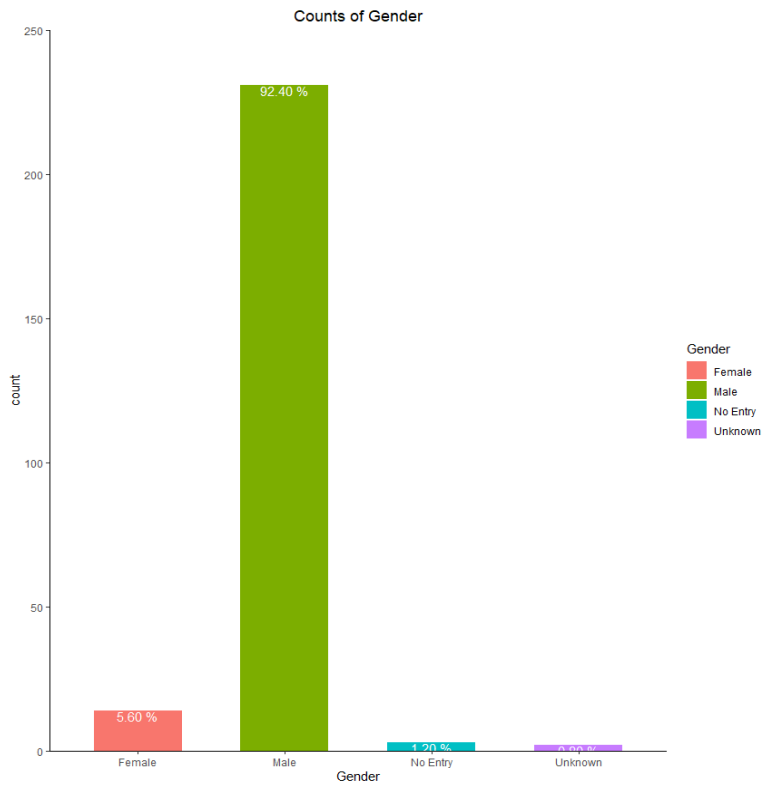
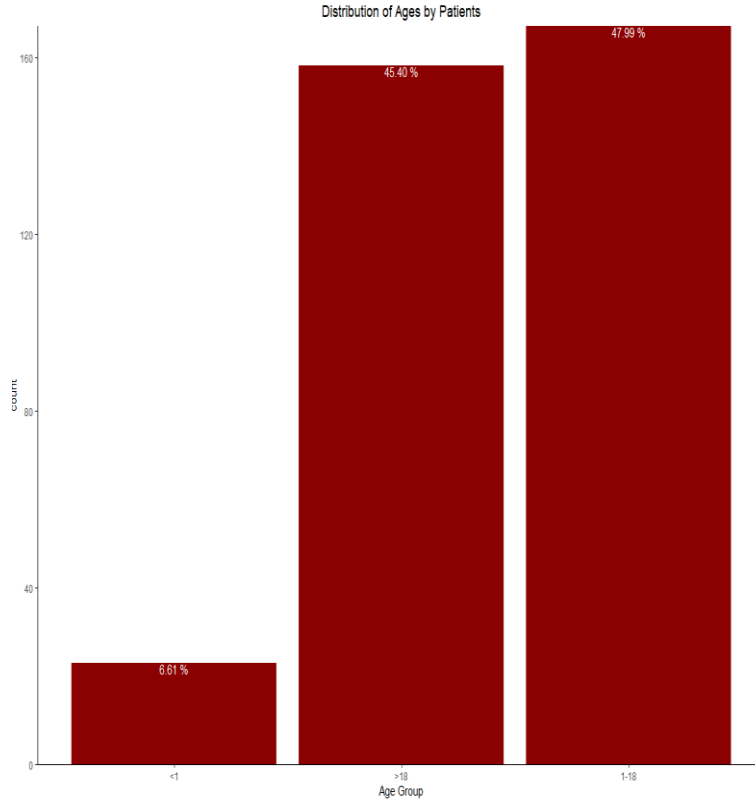


Figure 1: Demographics of the Dataset

4.2 c.679 C>T variant is the most frequent variant

After the datasheet was narrowed down from 348 patients to 50 patients based on classification of enzyme levels (all 50 are classified as “below normal levels”), a count of specific nucleotide variants was produced (Figure 2). Overall, there is a relatively even distribution of variants across all 50 patients with the exception of a couple. c.679C>T has, by far, the largest count out of any variant at 8 counts, with others at 3 or lower. Running a chi-squared test of homogeneity produces a p-value of 0.238, which at a 0.05 significance level means there is no difference between the observed and expected counts between any of the variants, meaning that despite having 8 counts, c.679 C>T is not statistically significantly higher than the other variants.

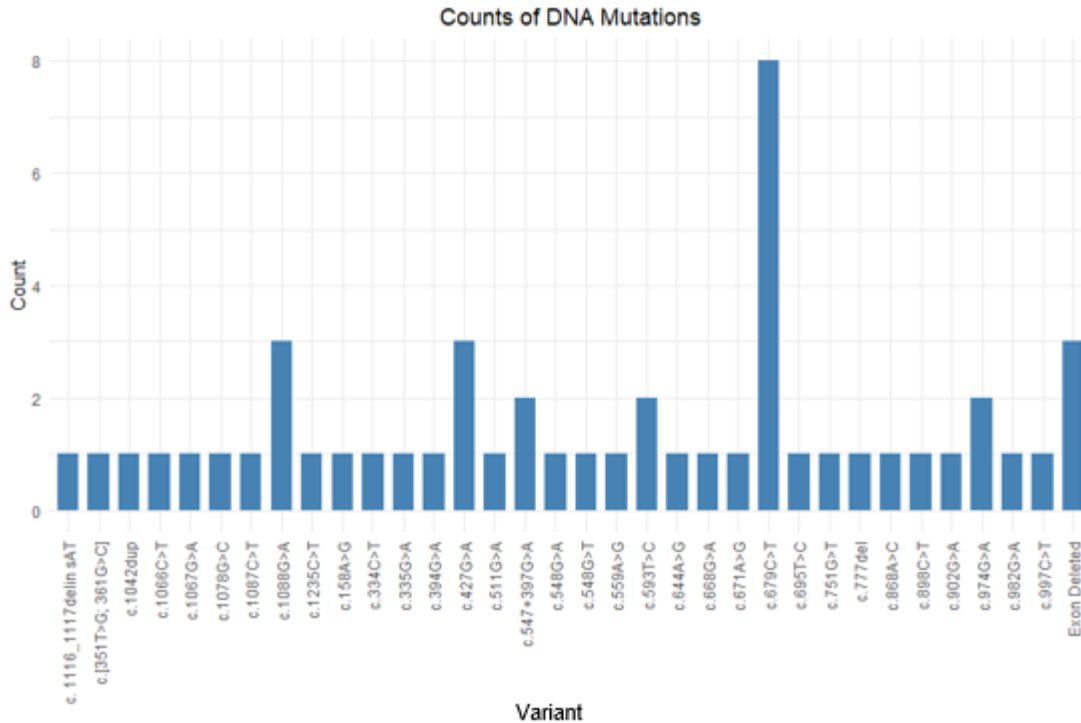


Figure 2: Counts of Variants

4.3 There is a negative association between enzyme levels and biomarker levels

A linear regression model was used to analyze the relationship between enzyme (GLA) levels and biomarker (Gb3 and related intermediate) levels (Figure 3). As indicated by the red best-fit-line, there is a weak linear inverse relationship between the two variables. An r-squared value (coefficient of determination) of 0.1277 and a p-value of 0.0108 was obtained; since the p-value is less than a significance level of 0.05, it can be argued that the relationship between the two variables is statistically significant. However, two outliers were identified (circled in red) by multiplying the interquartile range by 1.5 and subtracting and adding that number from the first and third quartile, respectively. Removal of outliers changes the r-squared value to be 0.1158 and

the p-value to be 0.018, which still falls under the significance level of 0.05. Given the shape of the scatterplot, it should be noted that a non-linear model may be more suited towards this data (i.e. quadratic, logistic) that may produce a higher r-squared value and a lower p-value. In addition, the point circled in green did not fall under the definition of an outlier for this calculation, but because of how isolated it is from the rest of the points, it may also need to be removed to better represent the population.

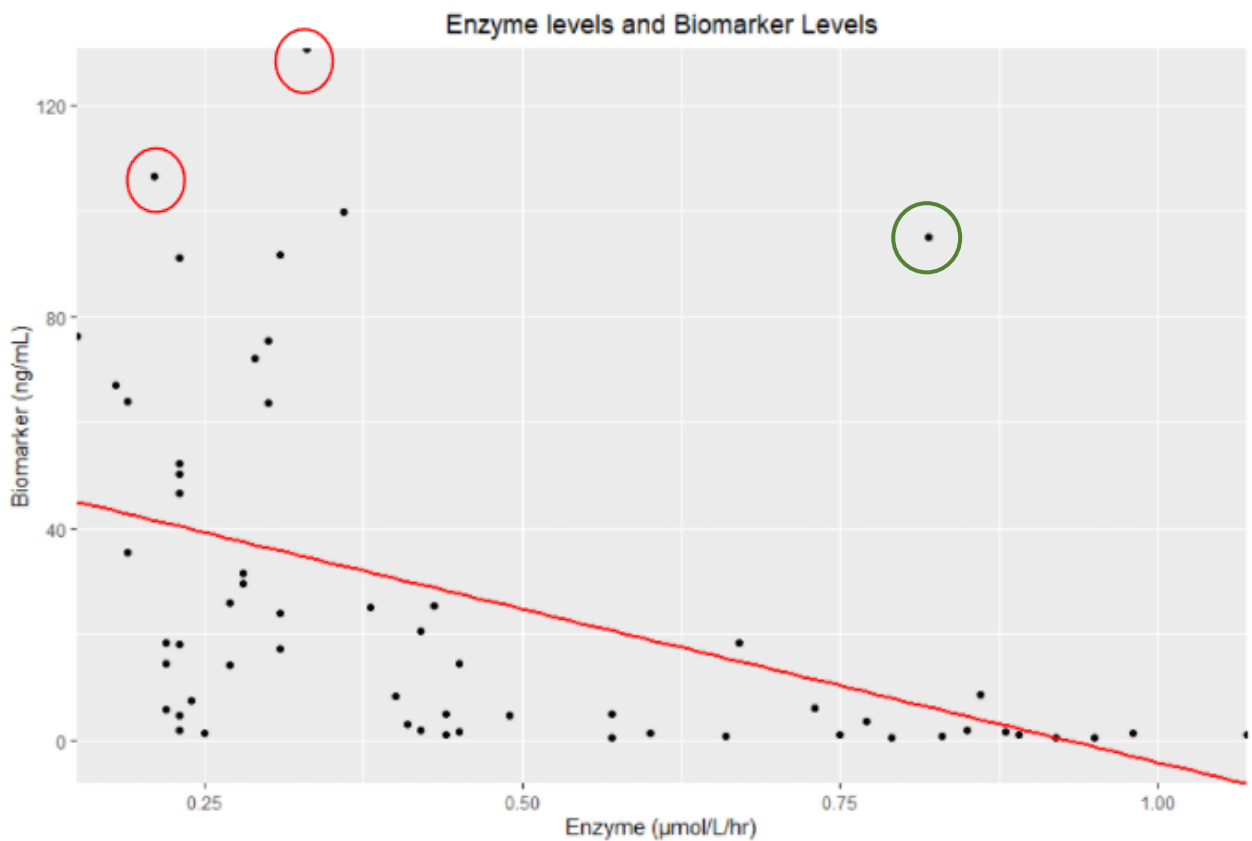


Figure 3: Enzyme Levels and Biomarker Levels

4.4 Substitution mutations were the most common

Although specific variants were listed in Figure 2, the data was organized and labeled to show what type of variant each patient had, and was made into a bar chart (Figure 4). Note that the majority of variants are under substitution, and follow the nomenclature c.###X>Y, where ### is the position of the variant and X>Y is the specific variant. Other variants that are present in the data set include deletions, deletion and insertion, double substitution, duplication, exon 2 deletion, and splice site substitution. However, all of these other variants have extremely low counts compared to the substitution variant.

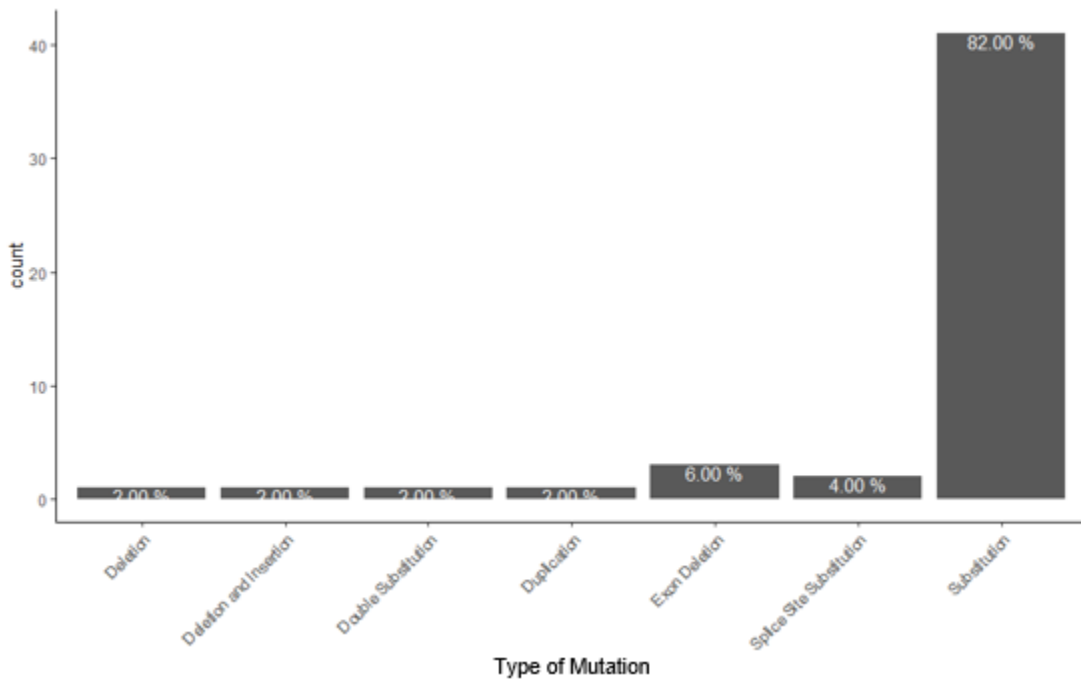


Figure 4: Counts of Types of Mutations

4.5 Types of mutation and classification

Three bar charts were created to determine whether there was a relationship between the type of mutation and its pathogenic classification (Figure 5). Bar height is based on the percentage of that variant that is part of that classification (i.e. 13 counts out of the 41 substitutions, or 31.7% of substitutions, are classified as likely pathogenic). It can be seen that all types of mutations fall underneath one classification except for substitutions, which over half fall under pathogenic, followed by likely pathogenic, and finally uncertain significance. All patients who had a deletion, duplication, or exon deletion variant were classified as having a pathogenic variant, all patients who had an indel or double substitution variant were classified as having a likely pathogenic variant, and all patients who had a splice site substitution were classified as having a variant of uncertain significance.

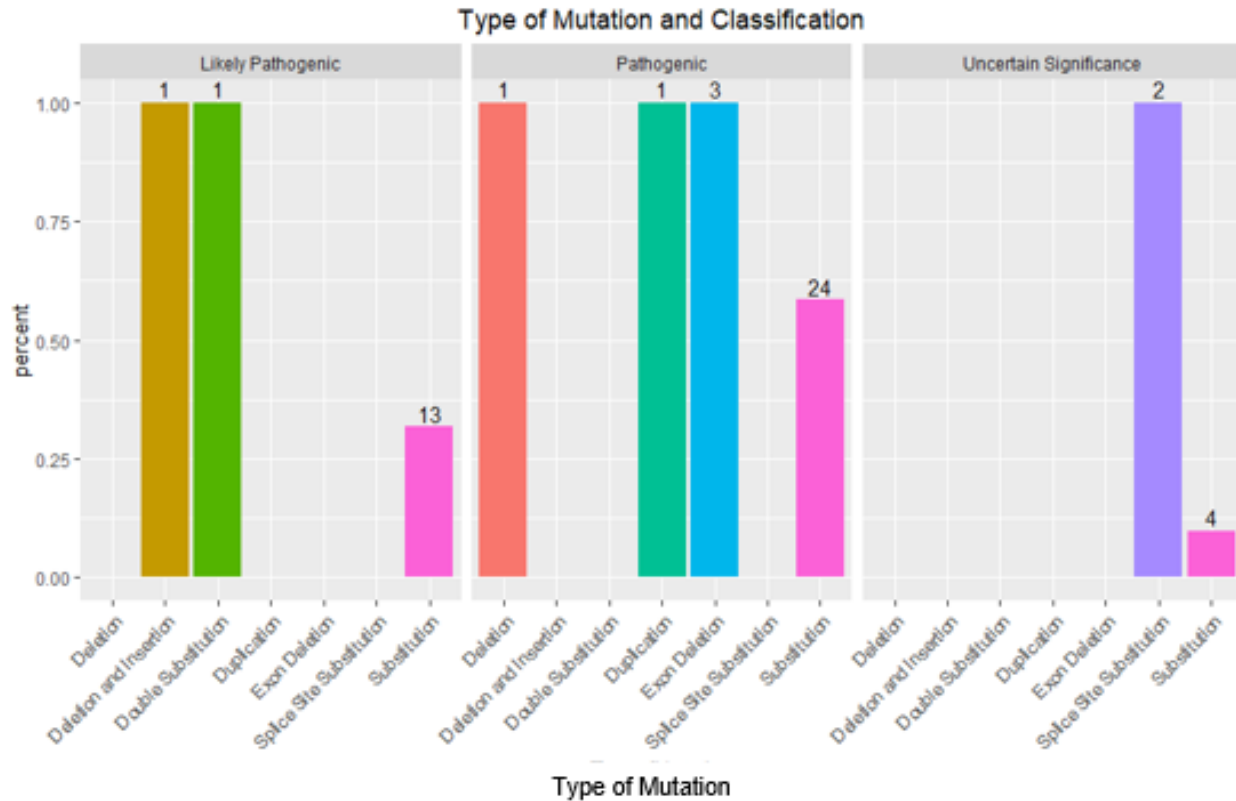


Figure 5: Types of Mutation and Classification

4.6 Biomarker levels and pathogenicity classification

Because the entire data sheet was organized based on the enzyme levels, the relationship between the biomarker level of a patient and their pathogenicity classification was observed. Table 1 shows the distribution of patients, in proportions, on whether their biomarker levels are above or below the standard level of 1.11 ng/mL. It can be seen that across all patients, the proportion of patients that had a biomarker level above the standard to those patients that had a biomarker level below the standard was the highest in those labeled to have pathogenic variants and the lowest in those with uncertain significance.

Table 1

	Likely Pathogenic	Pathogenic	Uncertain Significance
Biomarker Above	0.7333	0.9655	0.6667
Biomarker Below	0.2667	0.0345	0.3333

5.0 Discussion

Some of the specific aims that have been raised in this paper have supporting evidence from the analysis. With the creation of Figures 2 and 4, we can see that although there isn't any specific nucleotide variant that is statistically significant across all subjects, we do see that substitution mutations seem to be the most conserved type of mutation. In addition, with Figure 5, 37 out of the 41 patients, or approximately 90.2% of the patients, who had a substitution mutation had some form of pathogenic variant, suggesting that a substitution mutation, more than a deletion/insertion mutation, in the GLA gene could be cause for risk of inheriting Fabry Disease. Overall, these three figures help to answer the first specific aim.

For the second specific aim, we turn our focus primarily to Figure 3. Figure 3 is a scatterplot of all 50 subjects' enzyme levels and biomarker levels, and we can clearly see from the figure's regression line and p-value that there is some association between the two variables, which perfectly answers the second specific aim. For the third specific aim, the biomarker levels of patients were compared to their classification of pathogenicity in Table 1, and it was found that a patient was more likely to have at least a likely pathogenic variant. However, there was no comparison between the enzyme levels of patients to the patient's classification of pathogenicity.

A comparison was made to determine whether the nucleotide changes that were observed in this study were similar to those observed in other studies, but the results I found were surprising. Both Pastores and Lien, 2002 and Gal et al., 2006 found that most of their variants do not exceed position 400 in the GLA gene (Pastores and Lien 2002) (Gal et al. 2006). However, my results found that most variants occurred on the X chromosome and never went below position 400. This might have occurred simply because there are multiple variants on the X chromosome that have

associations with Fabry Disease, or it may be in regards to my limitations, which I will discuss later. However, both Gal and Pastores found that missense substitution mutations were the most prevalent among their subjects, which is similar to the dataset that I have, further supporting that substitution mutations on the X chromosome are associated with inheritance of Fabry Disease.

There were studies, however, that support my findings of specific variants. From Figure 2, I found that the variant c.679C>T was the most prominent among the dataset, which parallels findings from other studies. In Sakuraba et al., the study found a number of different variants in Japanese patients with deficiencies in the GLA gene, including c.679 C>T (Sakuraba et al. 2018). Further studies explored other ethnic populations, including Finnish, Brazilian, and France, and found patients with the same variant as well (Germain et al. 2002) (Turaça et al. 2002) (Pietilä-Effati et al. 2019). As this variant is prominent in my findings, as well as its prevalence in other studies, future directions should consider its value in discovering more about Fabry Disease. It is also important to note that not only are my findings consistent with others, but also c.679C>T is not population-specific either, which gives us insight on how to better combat Fabry Disease.

5.1 Public Health Significance

Fabry Disease has a large incidence rate range among populations, ranging from as low as 1 in 476,000 in the general population to as high as 1 in 1818, and as such it poses many health threats and challenges (Del Pinto and Ferri 2020). Those susceptible to Fabry disease develop a wide variety of symptoms and conditions, including complications in the heart, skin, eyes, ears, and nervous system, and failure to treat these may result in death. In addition to physical debilitations, Fabry Disease patients tend to develop psychiatric complications as well (Laney et

al. 2010). Life expectancy for males with Fabry Disease is 58.2 years, over 15 years less than that of someone unaffected at 74.7 years, and life expectancy for females with Fabry Disease is 75.4 years, over 5 years less than that of someone affected at 80.0 years (Waldek et al. 2009). Because of the wide range of prevalence of Fabry Disease across various populations, as well as expensive and inefficient treatment options and no available cure, it must be determined whether Fabry Disease can be deemed a public health issue. To do so, emphasis on the negative physical and mental detriments to patient quality of life will be observed and described.

Fabry Disease can appear in childhood, where classic symptoms such as pain in the limbs, angiokeratomas, hypohidrosis, and cardiac complications can begin to form. For cardiac variants of Fabry Disease, hypertrophy is generally the first indication of onset, specifically left ventricular hypertrophy, although cases of asymmetric septal hypertrophy have been reported before; if left untreated, this leads to heart failure or ventricular arrhythmias, which results in death (Kubo 2017). Cardiac complications do not affect just the cardiovascular system; areas of the body that develop Fabry Disease-associated complications, including the eyes, ears, kidneys, and brain, all show some connection to vascular complications.

In addition to its involvement in the cardiovascular system, Fabry Disease has also shown its manifestations in the central nervous system (CNS) (Lelieveld et al. 2015). White matter lesions, cerebral micro- and macro- angiopathy, hippocampal atrophy, and strain on intercranial arteries can result in strokes and decreases in processing speed, memory, attention, and motor functions at an early age (Lelieveld et al. 2015). Because of its severe damage on the CNS, patients with Fabry Disease may develop further neurological disorders, such as Alzheimer's Disease and clinical depression. (Lelieveld et al. 2015). Despite these neuropsychiatric symptoms,

however, cognitive decline has been shown to be limited, although further evidence and research is necessary for this argument (Lelieveld et al. 2015).

Ocular and auditory complications are also a sign of the manifestation of Fabry Disease, with the most common ocular symptom being corneal verticillata at a prevalence rate between 44% and 94% and the most common auditory symptom being hearing loss at a prevalence rate between 16.7% and 46.7% (Pitz et al. 2015) (Yazdanfard et al. 2019). Both hearing loss and ocular symptoms can develop early in childhood, with cornea verticillata appearing as early as 22-weeks in a fetus (Pitz et al. 2015) (Hajioff et al. 2003). There is no histopathological explanation as to why hearing loss develops in Fabry Disease patients, where as ocular symptoms have been shown to be a result of vascular abnormalities, which circles back to Fabry Disease manifestation in the cardiovascular system (Pitz et al. 2015) (Yazdanfard et al. 2019).

The psychiatric effects Fabry Disease can have on a patient can also severely negatively impact their quality of life. Fabry Disease has been shown to be correlated with generalized anxiety, depression, and panic attacks (Laney et al. 2010). Furthermore, due to the physical and mental complications, Fabry Disease has been shown to lead to a decrease in quality of life, especially in males ages 18-25 and females ages 25-35 (Laney et al. 2010). Fabry Disease patients report having difficulty performing everyday tasks, maintaining employment, and seeking help (Laney et al. 2010).

My data has shown that there are certain variants that occur more often in Fabry Disease patients than other variants, that substitution mutations occur the most often in Fabry Disease patients, and has reinforced the idea that a decrease in enzyme, or alpha-galactosidase A, level increases the level of biomarker, or Gb3. Although these points have been discussed in previous literature, not only does it support evidence that already exists and provides more insight into how

Fabry Disease may develop in a person, but also furthers the argument that Fabry Disease needs to be looked into to better the quality of life of those people with Fabry Disease. Using the data that I have produced and analyzed, alongside previous literature's findings, all symptoms, complications, and negative effects of Fabry Disease can be minimized or even be eradicated, thus ensuring that those members of the public with Fabry Disease no longer have to suffer from these conditions.

5.2 Limitations

With any dataset comes its limitations, and the data set I used for analysis is without exception. The severest and more glaring limitation is that I only used 50 out of the 348 available data points given to me, which is a relatively small sample size considering what I could've used. This explains why there were only one or two counts of a type of mutation, or why certain variants only appeared once in the dataset. For a more accurate and detailed analysis, I would need to include all 348 samples, regardless of their enzyme levels, biomarker levels, types of mutations, etc. In addition, many of the samples in the 348 data points have missing information, such as the nucleotide change, the classification, and more. While this is not as detrimental, it still may not be representative of the population, and must be taken into consideration when discussing any result produced.

The dataset is also almost exclusively male; as this is a X-linked disease, it comes as no surprise that the majority of those diagnosed with a risk of Fabry Disease are all biologically male, as the risk of inheriting an X-linked disease for males is higher. Therefore, any results or conclusions made in this analysis would be difficult to apply to a female population. Finally, those

in the dataset were only chosen to be included based on Perkin Elmer's judgment on whether a subject should be included or not, which was decided through a symptoms page on the TRF as well as the interpretation of genetic counselors and geneticists. Those who are asymptomatic but still carry some variant associated with Fabry Disease, as well as reports that came back with benign or likely benign variants, were excluded from the dataset and may not be representative of the population.

5.3 Future Directions

For future directions, I would take into account all 348 subjects and perform the same data analyses I have for the 50-sample subset, and review whether this changes any trends or conclusions I have already made. In addition, I would include a bin statistical test to determine whether enzyme level and biomarker level affect the classification of the variant, in addition to other statistical tests regarding associations between other variables, such as nucleotide variant and enzyme level. Furthermore, if possible, I would like to obtain data related to blood relatives of any subject who was tested for Fabry Disease; it would be interesting to see if there are any generation inheritance patterns between Fabry Disease subjects and their family members. Ultimately, I believe that cross checking all these associations may provide some insight into how Fabry Disease is inherited and what treatments and cures may be available in the far future.

6.0 Conclusions

While Fabry Disease may have much still unknown about it, from its inheritance patterns to its prevalence, the impact it can have on people's lives warrant enough reason to look into the molecular basis of its functions. The analysis is far from solving any problem related to Fabry Disease, but it has provided a solid foundation towards discovering the underlying genetic basis. We may have not found any associations other than enzyme level to biomarker level and biomarker level to classification of pathogenicity, but they have nonetheless provided us a step forward in solving the mystery behind Fabry Disease at the molecular level. I am unbelievably excited for the future, as it holds much potential and answers towards the enigma that is Fabry Disease.

Appendix A – Letter of Exemption



EXEMPT DETERMINATION

Date:	April 28, 2022
IRB:	2204007
PI:	Aoxue Chenqi
Title:	Analysis of the Genetic Variants Associated with Fabry Disease

The Institutional Review Board reviewed and determined the above referenced study meets the regulatory requirements for exempt research under 45 CFR 46.104.

Determination Documentation

Determination Date:	4/28/22
Exempt Category:	(4) Secondary research on data or specimens (no consent required)

If you have any questions, please contact the University of Pittsburgh IRB Coordinator, Jean Barone at 412-383-1480.

Please take a moment to complete our [Satisfaction Survey](#) as we appreciate your feedback.

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