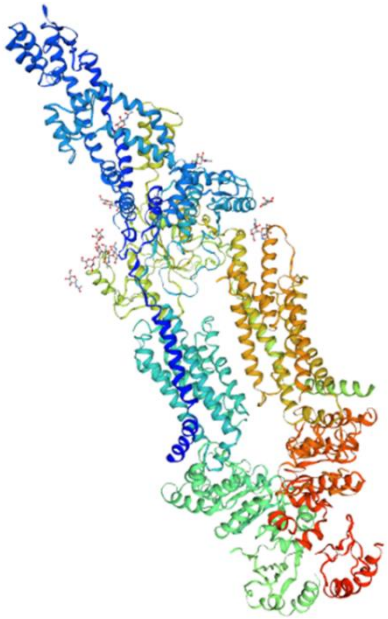
TITLE OF PROJECT: A Meta-Analysis Investigating the Impact of ABCA4 Polymorphisms on the STGD1 Phenotype.

PRINCIPAL CO-ORDINATOR:

Dr. Jan Bohringer OD, Ph.D.

SUPERVISORS: Dr. Shamjeet Singh BSc (General Agriculture). Ph.D. (Biochemistry) PgCert (University in Teaching and Learning), Mr. Niall Farnon MCOptom FIACLE

CO-INVESTIGATORS: Bevon Babwah (809002754), Karishma Ramnath (816002881)



AFFILIATION: Optometry Unit, Department of Clinical Surgical Sciences, Faculty of Medical Sciences, St. Augustine, The University of the West Indies

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ABSTRACT

Stargardt's disease (STGD1) is the most common inherited macular dystrophy, affecting 1 in every 10,000 persons. It is classified by compound heterozygous and homozygous polymorphisms of the ABCA4 gene, which is inherited in an autosomal recessive pattern. The ABCA4 gene is highly polymorphic and accounts for more than 500 disease-associated variants in relation to STGD1. Theoretically, there exists a strong relationship between ABCA4 mutations and the severity of clinical phenotypic variables. Therefore, the aim of this research to investigate the impact of specific ABCA4 polymorphisms on the STGD1 phenotype. The investigation of this relationship is important as it can provide significant insights in understanding the mechanism of

the disease, aide in patient counseling and possibly establish a foundation for future trials in treatment, as there is currently no cure for the disease.

A Meta-Analysis was performed on a curated database of 251 patients, having at least one classified polymorphism of the ABCA4 gene and at least one of the measurable phenotypic variables (STGD1): age of onset, BCVA, Fishman Phenotype Classification Grading. A total of 142 different polymorphisms of ABCA4 gene occurring at a frequency of 484 were identified. From this study, it was determined that the c.1622T>C polymorphism was the most prevalent, accounting for 11.20% of all mutations. This mutation was also found in a high frequency in European populations (27.0%) and was associated with significantly worst visual outcomes. This mutation existed in association with c.3113C>T as a complex allele [L514P; A1038V]. There was also evidence of haplotypic inheritance and a higher degree of pathogenicity in both instances. The c.6320G>A mutation was found to be prevalent (31.82%) in African populations and exhibited a milder phenotype when compared with all other polymorphisms considered. Missense functional change was found to be the most common molecular consequence for polymorphisms of ABCA4 and accounted for 86.06% of all functional changes. The phenotypic characteristics for Missense functional change varied from mild to severe dependant on the specific polymorphism. Nonsense functional changes or null mutations were found to have the most severe effect on the STGD1 phenotype and was associated with patients that had earlier age of onsets and more profound chorioretinal changes. In general, patients with earlier age of onsets had more severe phenotypical changes including worst visual outcomes than patients with a later disease onset. The number of independent mutations was shown to influence the average age of onset. The STGD1 phenotype produced by ABCA4 polymorphisms was shown to present in both males and females

approximately equally which is theoretically expected for an Autosomal Recessive inheritance pattern.

In conclusion, these findings suggest that individual polymorphisms of the ABCA4 gene can significantly impact the STGD1 phenotype, hence justifying the need for genotypic-phenotypic analysis of STGD patients. This is important as it can provide valuable insights into understanding the mechanism of the disease, provide accurate diagnosis of the disease, aide in patient counseling and most importantly, establish the foundation for future trials in treatment as there are currently no cures available for the disease.

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TERMS TO DEFINE

- **Genome**: The genome of an organism refers to the entire set of genes that a specific organism possesses. In the context of this research, the Human Genome was considered.
- **Gene:** Genes are the basic functional and physical unit of heredity and are passed from one generation to another. A gene is defined as a complete nucleic acid sequence which is essential for synthesis of a functional polypeptide. It has a coding

region which comprises of nucleotides that are responsible for encoding amino acid sequence of a single protein. Hence, mutations of a particular gene consequently alter the function of a single protein.

- **Genotype:** In the context of this study, the patient's genotype refers to the polymorphisms present at the ABCA4 genetic locus. Standardized NCBI notion for polymorphisms involves referencing a mutation by the specific location on exomic DNA in the form of numerals and letters. For e.g. c.2588GT>C represents a point mutation at the 2,588th nucleotide base where there is a substitution of Thymidine (T) for Cytosine (C).
- **Phenotype**: The STGD1 phenotype is produced by mutations in the ABCA4 gene. The phenotype of the patient refers to a specific set of measurements which characterizes a patient's physical characteristics which are related to a specific genotype. Guided by the data from the literature review, the following refers to how a patient's phenotype was measured: 1) Age of Onset, 2) Best Corrected Visual Acuity (BCVA) 3) Fishman Classification of Stargardt's Disease
- **STGD1 Phenotype:** The STGD1 phenotype in the context of this research refers to the physical observable and quantifiable characteristics of a patient diagnosed with Stargardt's disease, specifically Stargardt's Disease Type 1, due to polymorphisms in the ABCA4 gene.
- **Haplotype:** A haplotype refers to mutations that are frequently inherited together. They can display non-mendelin inheritance.

- **Alleles:** Alleles are variations of a specific gene which exists at the same locus of that gene. Alleles within this research were represented by the specific polymorphic change or nucleotide change.
- **Complex allele:** A complex allele is an arrangement of polymorphisms in a physical structure which are often closely associated together. They may exist in either cis or trans arrangements, the former being the more pathogenic form. Due to proximity, complex alleles may also be frequently inherited together.
- **Polymorphism:** Polymorphisms represent variants of a gene at a specific locus. It may refer to alleles or the specific nucleotide changes within an allele that is responsible for a trait or phenotypic outcome. Polymorphisms are naturally occurring phenomenon. They can be harmless variations for e.g. if they correspond to hair or eye color or they may pathogenic which is the case with disease-causing variants.
- **Functional change:** Mutations that change a protein coding gene may cause a change in the molecular structure of the eventual protein that is constituted of the amino acids code for by that gene. These mutations may change the way in which that protein may function, and this is referred to as a functional change.
- **Founder effect:** Mutations which are prevalent in a geographically isolated region are indicative of a common ancestor. This is referred to as the founder effect and indicates that the founding members of a population may contained the genetic polymorphism and passed it on too many generations of descendants.

- **Type 2 error:** This refers to a false negative or failure to reject a false null hypothesis ($p>0.05$). This can be as a result of large variability or small sample sizes.

1.0 INTRODUCTION

1.1 Background of study

Stargardt's Disease is the most prevalent inherited macular dystrophy which results from polymorphisms of multiple genes, with the most common being ABCA4, ELOVL4 and PROM1. The American Academy of Ophthalmology recognizes 3 classifications of the Stargardt's disease: Stargardt's 1 (STGD1), Stargardt's 3 (STGD3) and Stargardt's 4 (STGD4); depending on the genetic locus. STGD 1 is inherited in an autosomal recessive pattern and is classified by compound

heterozygous and homozygous mutations in the ABCA4 gene. STGD 3 and STGD 4 are inherited in autosomal dominant patterns and is due to mutations in the ELOVL4 and PROM1 genes respectively[1]. According to previous research, it was determined that mutations in the ABCA4 gene accounts for 95% of Stargardt's cases hence making STGD1 most prevalent[2]. As such, the general term Stargardt's Disease (STGD) usually implies STGD1 and is used interchangeably in the majority of books, journal and research papers.

ABCA4 is a large, highly polymorphic gene which consists of 50 exons and more than 900 disease-associated variants reported to date; 500 of which are associated with the Stargardt's Disease. As previously mentioned, the gene is inherited in an autosomal recessive fashion and is located on the short arm (p) at position 22, of chromosome 1[3]. ABCA4 encodes an adenosine triphosphate-binding cassette transporter which is vital for the function of the cones and rods in the retina. Biochemical studies indicate that the ABCA4 gene play a crucial role in transporting the compound, N-retinylidene-phosphatidylethanolamine, a retinal phospholipid across the photoreceptor outer segment disc membranes after the process of photoexcitation. This transportation process together with chemical isomerization is crucial as it allows for the removal of toxic retinal compounds from photoreceptor cells. However, defects in this gene results in the accumulation of cytotoxic derivatives in the RPE cells and photoreceptors eventually leading to their demise[4]. Furthermore, it has also been determined that mutations in the ABCA4 gene is also linked to a spectrum of diseases varying from STGD1 to Retinitis Pigmentosa, Cone and Rod Dystrophy (CORD) as well as severe early-onset retinal dystrophies. Therefore, due to the high complexity and multiple diseases associated with polymorphisms in the ABCA4 gene, it is suggested such STGD patients undergo genotypic and phenotypic analysis as previous research

indicates a strong association with polymorphisms of the ABCA4 gene and disease onset as well as phenotypic severity[5].

Genotypic analysis aims in identifying genetic variations or the changes within the DNA sequences; an essential factor in confirming a suspected genetic disease. The most common cause of genetic variation is Single Nucleotide Polymorphisms (SNP) occurring as a result of point mutations. Currently, SNPs account for the basis of genome wide association studies and provides a possible method to track ancestry since SNPs are inherited and are believed to be shared by individuals of common descent; which thus gives rise to the founders effect[6]. Multiple previous research indicates various specific mutations associated with a given population. A study conducted among European Patients with STGD1 found 19 novel mutations which included nonsense, missense, splice site mutations as well as deletions. It was determined that the 2588G>C mutation was prevalent in more than 1/3 of their population sample and was hence proposed that this mutation is a frequent founder mutation prevalent amongst patients of European descent[7].

Clinical phenotypic analysis includes determining age of onset, measuring BCVA and classifying the fundus appearance of the patient. An early age of onset is most prevalent in patients with STGD, however, there are also studies indicating noticeable peaks on early and late adulthood onset ranging from the 1st to 7th decade of life in association with BCVA ranging from Hand Movement to 0.3 logMAR. There are multiple methods which can be used to classify the fundus appearance of Stargardt's patients nevertheless, the Fishman Classification remains superior as it considers the progressive nature of the disease as well as the degree of retinal damage and extent of choroidal atrophy[8]. The Fishman Classification phenotypically characterizes the disease into 4 distinct stages based on the clinical examination findings such as fundus appearance and Visual Field Tests as well as ancillary testing including ERG, EOG tests (see Diagram 5 in appendix).

However, it is noted that the appearance of the fundus has no correlation with the progression of vision loss[2].

Additionally, it is determined that early onset STGD is associated with null variants including nonsense mutations with an accompanying severe phenotype as compared to early and late adulthood onset STGD which is associated with missense variants and accompanying milder phenotype and hence better visual prognosis[9,10]. Homozygous missense mutations such as c.5882G>A or c.6079C>T was found to be associated with milder, later onset of the disease. However, it is noted that not all missense mutations result in mild phenotype, as some are associated with very severe outcomes, similar to that of null mutations. Such missense mutations include complex alleles c.1622T>C, c.3113C>T and c.4918C>T. Additional research determined that patients having at least 2 mutations of the ABCA4 gene have more severe phenotypic outcomes and are more likely to present with an early onset of the disease[1]. Therefore, the range of various clinical findings can be due to variability in functional or structural effects of different alleles on the protein which they encode, differing biological effects of the altered proteins and effects in at least one modifying gene[11]. Hence, it is of great importance to examine the genotype-phenotype relationship in patients with STGD as it can provide important insights into the mechanism of the disease and aide in patient counseling, but most importantly, establish the foundation for future trials in treatment, as the availability of appropriate interventions increases since there are currently no cures available for the disease[12].

1.2 Rationale of Study

As previously mentioned, there exists a strong association with polymorphisms of the ABCA4 gene and corresponding phenotypic severity amongst STGD patients. Previous studies on the genotype-phenotype correlation in STGD patients is limited as the topic remains a challenge

for many researchers. This is as a result of patients presenting with compound heterozygous polymorphisms of the ABCA4 gene together with vast phenotypic variations observed among family members having the same mutations. Moreover, it has been established that the most prevalent occurring disease- associated ABCA4 mutations only affects approximately 10% of STGD patients. This low detection rate of identical mutations within the STGD population further contributes to difficulties in validating the pathogenicity of the disease and evaluating resultant clinical impacts and genotype–phenotype correlations [13]. As such, there remains a demand for more studies in this field hence justifying the aim of this research. This study is important as it can aide in predicting the severity and possible progression of the disease within various ethnicities and populations hence providing a better understanding in the mechanism of the disease on a large scale.

1.3 Aim of Study

To investigate the impact of specific ABCA4 polymorphisms on the STGD1 phenotype.

1.4 Objectives of Study

(i) To evaluate the prevalence of various ABCA4 polymorphisms on patients with the STGD1 phenotype.

(ii) To investigate the association of ABCA4 polymorphisms on the STGD1 phenotype by considering ethnicity and geographic location.

(iii) To assess the STGD1 phenotype based on the functional changes due to ABCA4 polymorphisms.

(iv) To analyze the impact of ABCA4 polymorphisms with respect to early or late age of onset amongst Stargardt’s patients.

1.5 Significance of Study

This research aims to compile multiple previous studies reporting frequent polymorphisms of the ABCA4 gene amongst STGD patients having phenotypic variables including age of onset, BCVA and phenotypic classification to identify any trends or relationships between the different phenotypic and genotypic variables via statistical analysis. This is of utmost importance as the information obtained can be utilized in the future to aide in predicting the severity and possible progression of the disease based on specific genotypic and phenotypic factors among various ethnicities. It can serve as baseline data in establishing the necessary foundation for prospective treatment trials since there is currently no cure for the disease.

2.0 LITERATURE REVIEW

In 1997, there was a major breakthrough which linked Stargardt's Disease to mutations of the ABCA4 gene which is now acknowledged as the most common cause of Mendelian retinal diseases [14]. STGD1 was determined to be caused by mutations of the large, highly polymorphic ABCA4 gene which consists of 50 exons and more than 900 disease-associated variants reported to date. Few researchers have made progression in aim of determining the exact protein or nucleic mutation responsible for the disease, however, as of today, there are no definite findings as, Stargardt's disease remains having a broad spectrum of possible linked variants. A study conducted by September et. al aimed to assess the mutation spectrum of ABCA4 found approximately 90% of the patients who had the defective ABCA4 gene, had at least 16 different sequence variants, of which two were novel in 40 patients [15]. Mutations c.4469G>A, c.6079C>T, and C.2588G>C mutations were the most prevalent variants identified in this study with the C1490Y allele being the most common disease-associated variant (present in 19 patients). Additionally, there were 10 ABCA4 disease-associated haplotypes identified, two of which were

carried by the c.4469G>A mutation. This study therefore identified mutations of ABCA4 gene as the major underlying cause of Stargardt's amongst persons in South Africa. Also, since at least two different origins of the common c.4469G>A mutation was identified, this suggested several founder effects for the Disease in South Africa. Another study conducted by Cremer et. al amongst 40 European patients revealed 19 mutations in the ABCA4 gene illustrating the high allelic heterogeneity of Stargardt's Disease[16]. One mutation, c.2588G>C, which was also present in South African patients (as previously mentioned), was present in 37.5% of the European patients in this study thereby portraying gene linkage disequilibrium with rare polymorphisms in exon 19 implying a founder effect. However, this research concluded that the c.2588G>C alteration is a mild mutation and only when combined with a severe ABCA4 mutation results in Stargardt's Disease. It was also determined that 1 in every 35 Western Europeans had the c.2588G>C mutation, which is a higher rate than that of the most prevalent autosomal recessive mutations.

An additional research paper conducted by Fritsche et al. investigating the ABCA4 genetic mutation in 25 patients noted the existence of complex alleles[17]. A complex allele refers to 2 or more variant mutation of a gene which may be present in either cis or trans configuration on the same chromosome. The study found 5/20 patients possessing the c.[1622T>C; 3113C>T] (p.[L541P;A1038V]) arranged in cis formation that had significantly worst visual outcomes than the other mutations investigated . Another study conducted by Audere et al. described a patient diagnosed with Retinitis Pigmentosa but possessing the same allelic complex with phenotypically characteristics to STGD1 [18]. The article found low visual acuities, central vision visual field loss and ERG reductions consistent with STGD1. Similar findings in other studies conclude that the allele complex L541P;A1038V is associated with varying phenotypes and is associated with an earlier age of onset. A study conducted by Tanna et. Al reported that the same complex occurred

with a high frequency in German populations which points to a founder effect [19]. It was found that the L541P;A1038V complex is associated with more severe versions of disease phenotypes bearing similarity to those found in patients with Null or Non-sense mutations. Another study conducted by Runhart et al. found a complex allele which contained 2 mutations in cis position, c.769-784C>T; 5603A>T compared against with the form of the disease with c.5603A>T to have had an additive effect on the observed pathogenicity[20]. Further research conducted by Schindler et al. discussed the possible additive effects on the phenotype of the alleles prevalent in STGD1[21]. The study purported that patients presenting with STGD1 phenotype have different severity coefficients depending on the number and sequence of the mutations in their genotype.

Finally, there was also another research conducted by Rajani B et. al determined that there were significant mutations in the ABCA4 gene of all of the patients analyzed[22]. Among these, 80% of the mutations were heterozygous whilst the remaining 20% were homozygous. There were numerous mutations ABCA4 mutations present amongst Indian patients with the disease which further confirmed the expansion of the existing spectrum of the disease-causing variants. This therefore fuels the importance of understanding both phenotypic as well as genotypic correlations thereby prompting the need for further research on this topic.

In Summary, it is evident that significant advances have been made within the past 20 years in an effort to better understand both the clinical and molecular features of the disease together with its underlying pathophysiology. However, the exact protein or nucleic mutation responsible for this disease is yet to be determined. It is evident that inconsistencies and high variabilities which exists amongst different populations give rise to genetic variations eventually leading to polymorphism. Thus, by conducting studies on these variations, the genetic differences which contribute to the development of the disease can be better understood.

3.0 METHODOLOGY

3.1 Research Design

As it pertains to the aim of this research, there is significant limitation in the availability of previous studies which were able to statistically establish relationships between genotypic and corresponding phenotypic variables of STGD patients. As a result, a Meta- Analysis was conducted. A Meta-Analysis is a specific subset of a Systematic Review which applies statistical techniques to integrate all of the results obtained from individual studies[23]. Meta- Analysis and Systematic Reviews are identified as having the strongest and highest quality of evidence as compared any other scientific based evidence reviews since it incorporates filtered information from multiple studies which greatly aides in mitigating bias; as this may be prevalent in smaller, individualized studies and is a factor of concern. As such, the conclusions established from Meta-Analysis Studies are deemed to be statistically stronger as compared to the analysis by a single study due to factors such as: increased number of subjects together with a larger diversity of accumulated results, hence, justifying the reason of choice[24].

3.2 Research Protocol

The PRISMA Protocol was utilized to provide the framework for the Method of data collection. This protocol was recommended for use when conducting Systematic Reviews and Meta- Analysis to reduce the risk of misinterpretation and bias in such studies. The protocol comprised of a 9-section checklist, and a flow chart (see Diagram 6 in Appendix). From the checklist, only the Method of data collection was used in this research. It was adapted accordingly into 6 subheadings ranging from 3.3 to 3.8. This, together with the four-phase flow chart was utilized in the process of data selection as it allowed us to effectively scan all of relevant data to

properly identify studies which were applicable to our research objectives by incorporating various inclusion and exclusion criteria.

3.3 Information Sources

Data was gathered from Electronic Databases including PubMed**,** Google Scholar and UWI linC (UWI Libraries Information Connexion).

Note: All nucleotide mutations identified in studies were subsequently verified through established bioinformatic databases particularly ClinVAR.

3.4 Search Method

An electronic database search was performed which contained keywords: "Stargardt's disease, polymorphisms, ABCA4, phenotype" with a custom date range of 2010-2020. Additional search filters included: Free full text availability, All article types and Human species.

3.5 Research Criteria

- Inclusion Criteria:
    1) Studies that only analyzed the **ABCA4 gene** and the alleles or polymorphisms at that locus.
    2) Studies must utilize internationally accepted genetic notation for nucleotide mutations that are verifiable through established bioinformatic databases; specifically, ClinVAR.
    3) Patients in these studies must have at least one classified polymorphism of the ABCA4 gene and at least one of the measurable phenotypic variables: age of onset, BCVA, Fishman Phenotype Classification Grading.

- Exclusion Criteria:
    1) Studies using phenotypic grading scales other than Fishman Phenotype Classification were excluded.
    2) Papers that were not written in English were excluded.
    3) Papers that included descriptive statistics without published raw data were excluded.
    4) Intronic DNA or non-coding DNA sequences were not investigated. (Only protein coding DNA or exomes were analyzed.)

3.6 Study Selection

Studies were sorted and selected by relevance as opposed to date. Additionally, there were no restrictions in selecting studies with respect to specific authors, demographics, age, sex, ethnicities, or population location. Furthermore, selection was not limited to Systematic Reviews and Meta- Analyses studies.

3.7 Data Collection

3.7.1 Data Extraction

Genetic data including Specific nucleotide mutations together with respective protein change and functional changes were extracted from all papers. The Specific nucleotide mutations extracted were then verified through the ClinVar Bioinformatic Database available from NCBI to verify their location at the ABCA4 genetic locus. Phenotypic data extracted comprised of at least one of the following: age of onset, BCVA OD and OS (logMAR), Fishman Classification Grading.

Additionally, if available, patient's current age at the time of the respective study and sex were extracted.

3.7.2 Synthesis of Results

The data extracted, as listed in 3.6.1, was compiled in an excel sheet alongside the patient's respective demographic data.

3.8 Data Analysis

As indicated in 3.6.2, the data compiled in the excel sheet was imported and analyzed accordingly in IBM SPSS Statistics Version 22 as outlined below. Prior to any statistical analysis, the distribution of data amongst all variables were determined as normal or skewed based on histogram plots, comparison of mean, median and mode values as well as skewness and kurtosis factors as computed by SPSS.

From the data extracted in 2.7, a database comprising of 251 patients with Stargardt's Disease (STGD1) from different populations, together with their mutations, respective functional changes and associated clinical variables was compiled in Microsoft Excel. The data was then evaluated through a series of segmented analyses. The following illustrates the steps in which the data was analyzed:

3.8.1 Whole Data Set Analysis

3.8.1.1 Cross-Population Analysis

Data compiled in Microsoft Excel as indicated in 3.7.2 was imported into SPSS for a cross-populational analysis. The average age of onset, BCVA (OD/OS) and Fishman Classification was determined for each population and tabulated alongside their respective study.

The distribution of these variables was diagrammatically illustrated. Central tendency[i] of all variables were determined and appropriate tests were conducted to determine statistically relationships[ii] for each population against respective clinical variable.

3.8.1.2 Evaluation of Frequently Occurring Mutations associated with STGD1 Phenotype

14 of the most frequently occurring ABCA4 polymorphisms (accounting for 10% of the total mutations), were evaluated against all clinical variables. The Prevalence of each mutation was calculated, and the corresponding Allele and Amino acid changes were identified. Central tendency[i] for all variables was determined and the data was then evaluated accordingly against all clinical variables to determine statistically significant relationships[iii]. A table was created to illustrate this data.

3.8.2 Analysis based on Functional Change

3.8.2.1 Prevalence of Functional Changes in the sample

The data was analyzed to determine the frequency of specific functional changes within the sample.

3.8.2.2 Comparison of Missense vs Nonsense

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[i] Data was deemed normally distributed if the mean, mode and median was approximately equal and skewness and kurtosis values were within the range of -1 to +1. Normally distributed data was analyzed using parametric tests (One-way ANOVA and Independent T test) while nonparametric tests (Kruskal Wallace and Mann Whitney-U test) were used to analyze data which were not normally distributed.

[ii] Statistically significant relationships were indicated by an " * " in-text on results tables.

The relationship between Missense and Nonsense functional changes was further categorically assessed. Central tendency[i] of all variables were determined and appropriate tests were conducted to determine statistically significant relationships[ii]. A table was created to represent this data.

3.8.2.3 Haplotypic Analysis of Missense functional changes

The most frequently occurring individual polymorphic pairs were compared against their mean clinical variables to quantify their individual impact on the STGD1 phenotype and to discern if there was evidence of haplotypic inheritance by equating prevalence rates. Central tendency[i] of all variables were determined and appropriate tests were conducted to determine statistically significant relationships[ii] A table was created to illustrate this data.

3.8.2.4 Complex Allelic Analysis

The most prevalent polymorphic pair from 2. c. above was compared against their respective clinical variables to establish their effect on the STGD1 phenotype through pairwise genetic analysis and to further determine whether these polymorphisms exist in a complex allelic arrangement. Central tendency[i] of all variables were determined and appropriate tests were conducted to determine appropriate statistically significant relationships[ii].

3.8.3 Analysis based on Age of Onset

Comparison of Early and Late Onset - Onset of the disease was evaluated against average presenting age, BCVA, and Fishman classification to determine whether there was a variation of the STGD1 phenotype based on Age of Onset. Patients were divided by Age of Onset and statistically analyzed under two categories, Early or Late. Early onset was classified as <20 years while Late onset is >20 years. Central tendency[i] for all variables were determined and age of

onset was evaluated accordingly against all clinical variables to determine statistically significant relationships[ii].

3.8.3.1 Early and Late Onset compared with Phenotypic Classification and Polymorphic Prevalence

Onset of the disease was evaluated against all clinical variables to determine whether there was a variation of the STGD1 phenotype based on Age of Onset. Patients were divided by Age of Onset and statistically analyzed under two categories, Early and Late. Early onset is classified as <20 years while Late onset is >20 years. The most frequent mutations which occurred in patients with Early Onset and Late Onset was then identified to determine the impact of that those individual polymorphisms had on the STGD1 phenotype with respect each to phenotypic classification.

3.8.3.2 Average age of onset compared with Fishman Classification

The data was further categorized by Phenotype Grade to determine whether there were significant differences in the Age of Onset for each of the individual phenotypic classes that was examined through comparison of means. Central tendency[i] for all variables was determined and the age of onset was then evaluated accordingly against Fishman Classification to establish statistically significant relationships[ii].

3.8.4 Analysis based on Number of Mutations

The number of mutations were analyzed against the respective clinical variables to determine if they affected the STGD1 phenotype. The ABCA4 gene contains complex alleles. However, patients with mutations in loci other than ABCA4 were excluded. Therefore, only patients that contained mutations in Alleles located within ABCA4 were analyzed. Up to 4

mutations were evaluated per patient. A non-Haplotypic analysis was then performed. The most prevalent mutation at each level (No. of Mutations 1-4) were recorded alongside their respective amino acid residue changes. Central tendency[i] for all variables was determined and the number of mutations were evaluated against all clinical variables to determine statistically significant relationships[ii].

3.8.5 Analysis based on Patient Sex

Sex was evaluated against respective clinical variables to determine whether there is a genetic basis for gender bias in STGD1. Central tendency[i] for all variables was determined and patient sex was evaluated against all clinical variables to determine statistically significant relationships[ii].

4.0 RESULTS

4.1 Whole data set and segmentation analysis

A. Results from Cross-Populational Analysis:

Table A below illustrates Analysis by Population Location of the entire database. The average values of considered clinical variables were tabulated and against respective geographic locations.

Author of Study	Location *[P]	Average age of onset ± SE *[NP]	Average BCVA OD ± SE *[P]	Average BCVA OS ± SE*[P]	Average Fishman Phenotype Classification ± SE *[P]

Yuan-Hu F, et. al	China N_T:4	29.25 ± 8.15 N: 4	0.78 ± 0.23 N: 4	0.80 ± 0.20 N: 4	N/A
Salles et. al	Brazil N_T:48	14.09 ± 1.29 N: 44	1.23 ± 0.08 N: 43	1.15 ± 0.10 N: 43	2.19 ± 0.12 N: 48
Paper1: Gemenetzi M, Lotery A Paper 2: Fujinami K et. al	Europe N_T:44	12.00 ± 1.19 N: 44	0.72 ± 0.06 N: 44	0.77 ± 0.05 N: 44	2.92 ± 0.06 N: 37
Tracewska A, et. al	Poland N_T:43	12.13 ± 1.22 N: 42	0.71 ± 0.08 N: 42	0.74 ± 0.08 N: 42	2.88 ± 0.10 N: 43
Joo K, et.al	Korea N_T:5	6.00 ± 1.68 N: 4	1.50 ± 0.16 N: 5	1.44 ± 0.17 N: 5	3.20 ± 0.20 N: 5
Zaneveld J, et al	Canada N_T:10	N/A	N/A	N/A	3.10 ± 0.31 N: 10
Smaragda K, et. al	Greece N_T:49	19.31 ± 1.60 N: 44	N/A	N/A	N/A
Zernant J, et. al	African/American N_T:39	34.62 ± 3.24 N: 39	0.69 ± 0.10 N: 38	0.72 ± 0.10 N: 38	1.75 ± 0.13 N: 36
Paper 1: Battu R et. al	India N_T: 9	11.00 ± 1.72 N: 7	0.90 ± 0.07 N: 5	0.96 ± 0.04 N: 5	2.44 ± 0.34 N: 9

Paper 2: Kadarkarai Raj R et. al					
	Total Average	17.87 ± 0.94 N: 228	0.86 ± 0.04 N: 181	0.87 ± 0.04 N: 181	2.49 ± 0.06 N: 188

Table A: Evaluation of clinical variables of patients in the 9 populations examined in this study. N/A: No data provided, N: Number of Patients. *Significantly different at $p<0.05$. NP- Nonparametric tests (Kruskal Wallace), P: Parametric test (One-Way ANOVA).

The results from Table A indicate that European populations or populations with significant European ancestry had worst clinical outcomes ($p<0.05$) when compared with non-European populations (with the exception of Korea). The most notable differences are that of Fishman Phenotype Classifications and Age of Onset as compared across different locations. Average age of onset was found to have statistically different means as compared to BCVA OD, OS and Fishman Phenotypes Classification Grades: 1&2, 1&3, 1&4, 2&3 and 2&4 ($p<0.05$). There was also a statistical difference between average Phenotype Classification and Specific Nucleotide Mutations recorded.

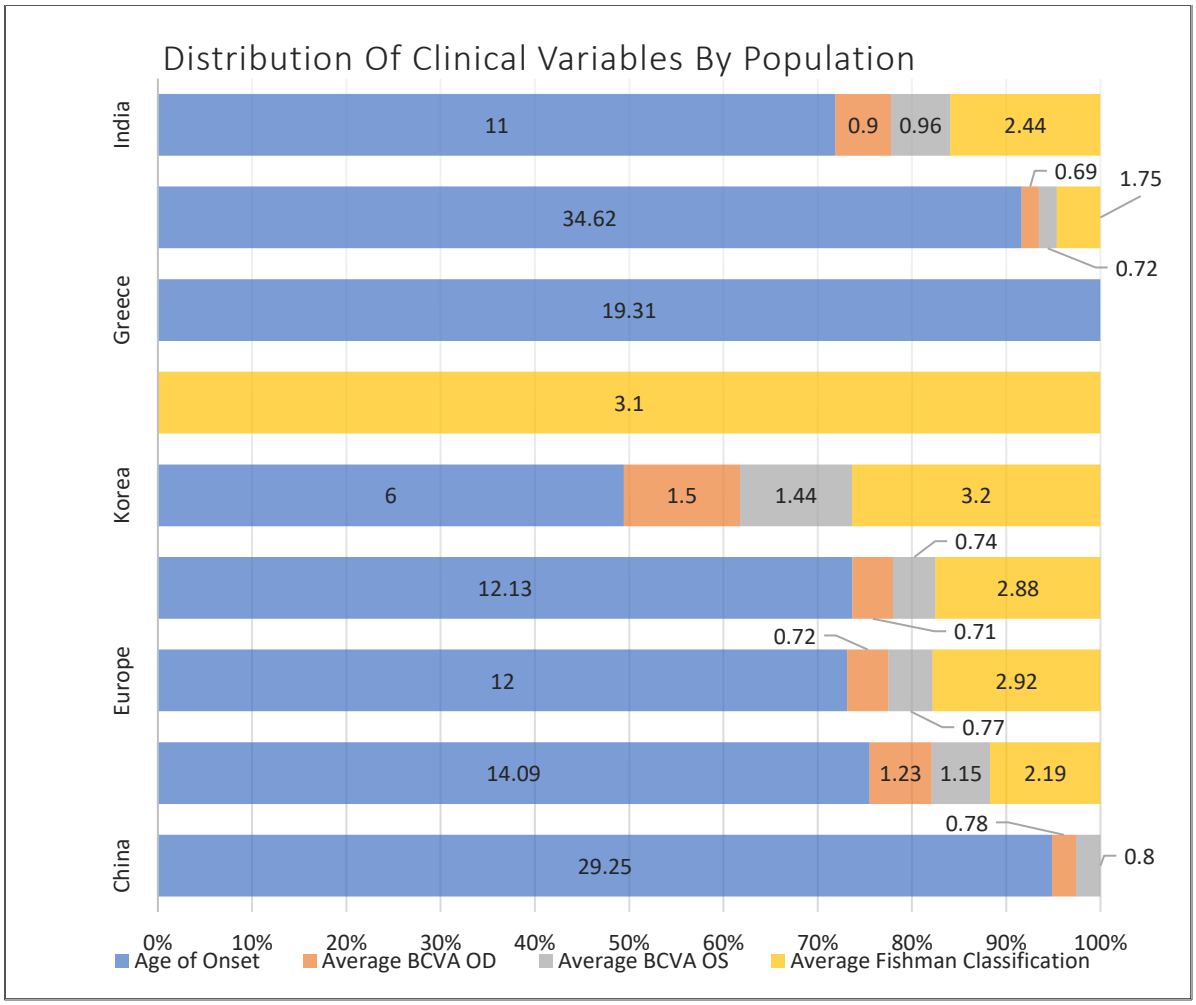

Diagram 1: Bar Chart of the distribution of measured clinical variables amongst different populations examined. Unavailable data is not represented.

From the data illustrated in Diagram 1, African populations show the lowest average disease severity with the lowest Average Fishman Phenotype Grade (1.75), BCVAs OD/OS (0.69/0.72) and highest Age of Onset (34.62) when compared with other populations.

B. Evaluation of Frequently Occurring Mutations associated with STGD1 Phenotype:

Upon further analysis of the data, a total of 142 different mutations occurring at a frequency of 484 were identified. 10% of the most frequent mutations, accounting for 14 specific nucleotide mutations, were retained for further analysis as indicated by Diagram 2 below.

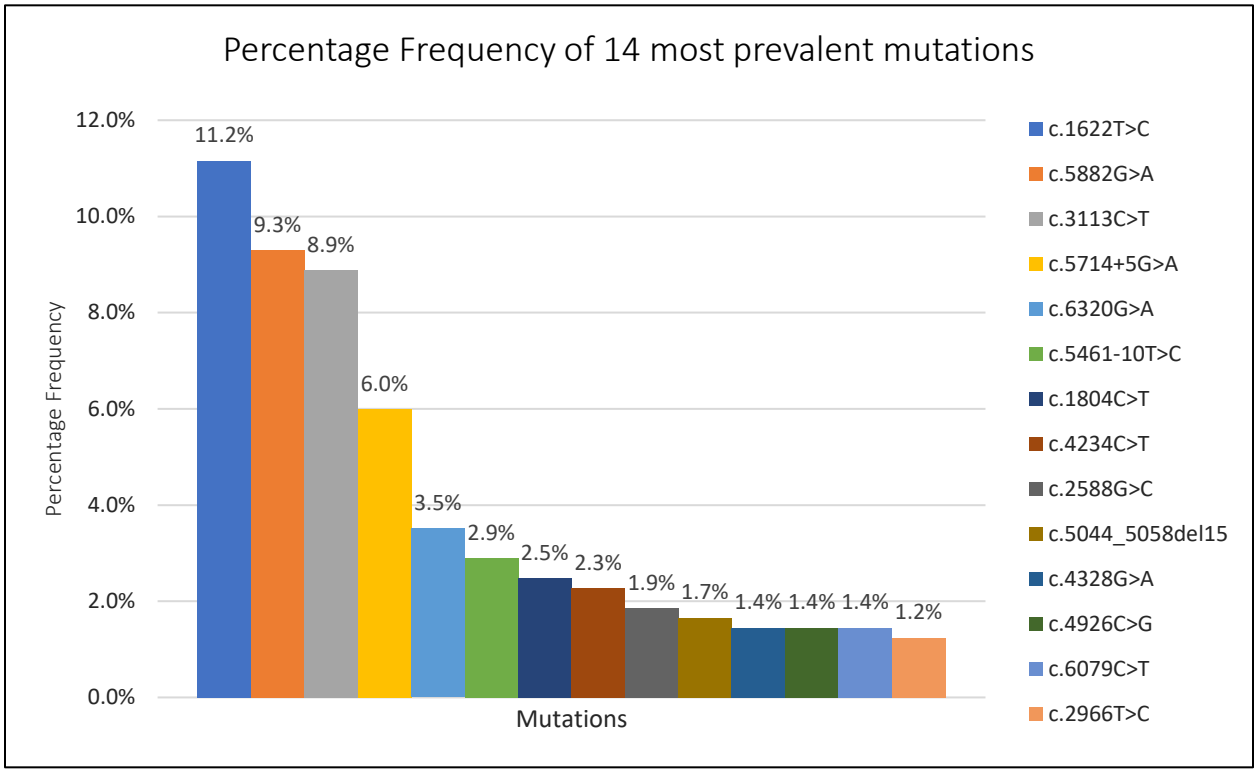

Diagram 2: Bar chart representation of the top 10% of the most frequent occurring mutations in the populations examined.

Mutation	Prevalence in entire population / %	Allele and Amino Acid change	Average Age of Onset ± SE	Average BCVA OD ± SE	Average BCVA OS ± SE	Average Fishman Phenotype Classification ± SE
c.5714+5G>A $N_{T:}$29	6.00%	N/A	19.96 ± 2.51 N:23	0.90 ± 0.19 N:8	0.88 ± 0.19 N:8	2.89 ± 0.20 N:9
c.1622T>C N_T: 54	11.20%	L541P, p.Leu541Pro	12.98 ± 1.34 *NP	0.98 ± 0.09	0.94 ± 0.09	2.86 ±0.13 *P N:35

			N:44	N:34	N:34	
c.3113C>T N_T: 43	8.90%	A1038V, p.Ala1038Val	14.81 ± 1.64 N:37	0.91 ± 0.11 N:28	0.84 ± 0.12 N:28	2.97 ± 0.14 N:29
c.5882G>A N_T: 45	9.50%	G1961E, p.Gly1961Glu	18.28 ± 1.61 N:40	0.67 ±0.08 N:25	0.68 ± 0.07 N:25	2.33 ± 0.18 N:27
c.6320G>A N_T : 17	3.50%	R2107H, p.Arg2107His	38.25± 6.03 *NP N :16	0.68± 0.17 N :16	0.78± 0.16 N :16	1.56± 0.20 *P N :16
c.5461-10T>C N_T : 14	3.30%	N/A	12.93± 4.13 *NP N :14	0.85± 0.09 N:14	0.81± 0.10 N :14	2.77± 0.17 N :13
c.1804C>T N_T : 12	2.50%	R602W, p.Arg602Trp	10.50± 1.25 *NP N :12	1.25± 0.21 N :10	1.26± 0.20 N :10	2.25± 0.22 N:12
c.4234C>T N_T : 11	2.30%	Q1412, p.Gln1412Ter	12.64± 1.88 N :11	0.45± 0.17 N :11	0.45± 0.17 N :11	2.91± 0.25 N:11
c.2588G>C N_T : 9	1.90%	G863A, p.Gly863Ala	15.11± 3.30	0.84± 0.11	0.90± 0.10	2.63± 0.18 N :8

			N :9	N :9	N :9	
c.5044_5058del15 N_T : 8	1.70%	N/A	8.71± 1.19 *NP N :7	1.20± 0.31 N :6	1.43± 0.19 N :6	2.50± 0.27 N :8
c.4328G>A N_T : 7	1.40%	R1443H, p.Arg1443His	7.25± 0.25 *NP N :4	1.27± 0.16 N :6	1.27± 0.16 N :6	2.43± 0.30 N :7
c.4926C>G N_T : 7	1.40%	S1642R, p.Ser1642Arg	9.00± 1.37 N :6	1.20± 0.31 N :6	1.43± 0.19 N :6	2.43± 0.30 N :7
c.6079C>A N_T : 7	1.40%	P2009T, p.Pro2009Thr	16.33± 3.02 N :6	0.80± 0.23 N :5	0.72± 0.18 N :5	2.60± 0.51 N :5
c.2966T>C N_T : 6	1.20%	V989A, p.Val989Ala	30.00± 8.80 N :5	0.80± 0.32 N :4	0.75± 0.29 N :4	1.50± 0.29 N :4

Table B: Comparison of frequently occurring mutations with phenotypic clinical variables. * Significantly different at $p<0.05$, N: Number of Patients, N_T: Total number of patients with specified mutation, NP: Nonparametric test – Kruskal Wallace, P: Parametric test (One-Way ANOVA). N/A: Not Applicable

As indicated by table B, there was a statistical difference in the average phenotype classification amongst patients with c.4328G>A and c.6320G>A mutations; it was noted that the latter has lower Fishman classification grading. There was also a statistical difference in average age of onset amongst pairs c.5044_5058del15 and c.6320G>A, c.5461-10T>C and c.6320G>A, c.1804C>T and c.6320G>A, c.1622T>C and c.6320G>A.

4.2 Results of Functional Change Analysis

Comparison of Functional changes detected: 80.95% of mutations detected in the ABCA4 gene that produces STGD1 were found to be Single Nucleotide Polymorphisms (SNPs). SNPs commonly resulted in a Missense functional change (86.65%). Diagram C below illustrates the prevalence of Functional Change Occurrences.

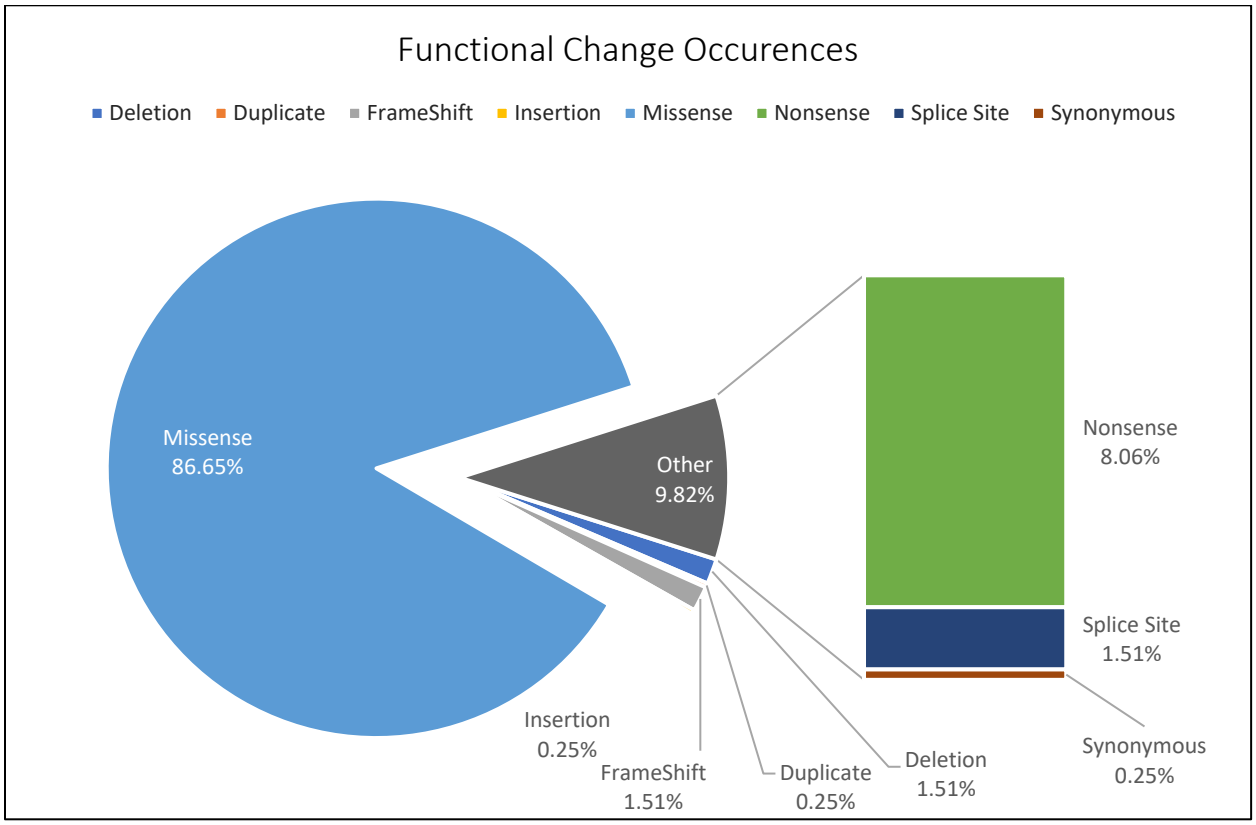

Diagram 3: Pie Chart representation of functional changes of mutations in sample population.

A. Comparison of Missense vs Nonsense Functional Change:

Functional Change	Prevalence in entire population /%	Average Age of Onset ± SE*^{NP}	Average BCVA OD ± SE	Average BCVA OS ± SE	Average Fishman Phenotype Classification ± SE*^{P}
Missense N_{MO}: 203	80.88	19.49 ± 1.10 N: 183	0.91± 0.05 N:132	0.90± 0.05 N:132	2.37± 0.07 N:143
Nonsense N_{NO}: 15	5.98	8.77± 1.17 N:15	0.93± 0.17 N:13	0.85± 0.15 N:13	3.13± 0.17 N:15
Total Average	86.86	18.68± 1.04	0.91± 0.04	0.89± 0.04	2.44± 0.69

Table C: Comparison in means of phenotypic variables between patients with Missense and Nonsense Functional Changes. N_{MO} – Total number of patients with Missense only. N_{NO} – Total number of patients with nonsense only. N: Number of Patients. * Statistically significant at $p < 0.05$. NP: Nonparametric test – Mann-Whitney U test, P: Parametric test (One-Way ANOVA)

Table C indicates statistical significance in means between age of onset and phenotype classification (groups 1&4, 2&4, 3&4) with respect to Functional Change. It is evident that patients with nonsense mutations had significantly lower average age of onset together with a higher Fishman phenotype classification as compared to patients with missense mutations.

B. Haplotypic Analysis of Missense functional change – Missense functional changes accounted for the majority of all mutations (86.06%). The most frequently occurring individual variants were the C.1662T>C and c.3113C>T SNPs. These mutations were first evaluated independently to quantify their individual impact on STGD1 phenotype. Table

D illustrates the individual impact each of these specific polymorphisms had against clinical variables.

Missense Mutation	Prevalence in entire population/ %	Average Age of Onset ± SE*NP	Average BCVA OD ± SE	Average BCVA OS ± SE	Average Fishman Phenotype Classification ± SE*P
c.1622T>C N: 49	19.52	12.98 ± 1.34 N:44	0.98 ± 0.09 N:34	0.94 ± 0.09 N:34	2.86 ± 0.13 N:35
c.3113C>T N: 39	15.54	14.81 ± 1.64 N:37	0.91 ± 0.11 N:28	0.84 ± 0.12 N:28	2.97 ± 0.14 N:29

Table D: Comparison of c.1622T>C and c.3113C>T individual effect against clinical variables. N: Number of Patients. * Statistically significant at $p < 0.05$. NP: Nonparametric test- (Kruskal Wallace), P: Parametric test- (One-way ANOVA)

There is a statistical difference in means between the age of onset and Fishman phenotype classification amongst patients with c.1622T>C and c.3113C>T mutations. Patients with c.1622T>C mutation had an average lower age of onset together with lower grade classification as compared to patients with c.3113C>T mutation. Through comparison of their individual prevalence rates, it is evident that they occurred independently at a similar rate.

C. Complex Allelic Analysis – Through comparative analysis of the data obtained in Table B, it was noted that some nucleotides had similar prevalence prevalent rates. Both the c.1622T>C and c.3113C>T SNPs frequently occurred alongside each other in 14.3% of patients with Missense functional changes. It was determined that 59% of all the

c.1622T>C mutations occurred together with c.3113C>T. Table E shows this nucleotide pair and the impact on the associated phenotypic variables.

Alleles		Average presenting Age ± SE	Average Age of Onset ± SE *NP	Average BCVA OD ± SE	Average BCVA OS ± SE	Average Fishman Phenotype Classification ± SE *P	Percent Occurrence in Pairs
Allele 1	Allele 2						
L541P (c.1622T>C, p. Leu541Pro) N: 29	A1038V (c.3113C>T, p. Ala1038Val) N:29	11.95 ± 1.88 N: 24	12.40 ± 1.44 N: 24	0.91 ± 0.09 N:20	0.88 ± 0.10 N:20	3.00 ± 0.15 N: 24	14.3%
Other Missense Mutations N: 174		22.14 ± 1.70 N:93	20.61 ± 1.22 N:160	0.87 ± 0.05 N:110	0.87 ± 0.05 N:110	2.28 ± 0.08 N:118	85.7%

Table E: Comparison of clinical variables among patients who had c.1622T>C and c.3113C>T occurring together. N: Number of Patients. * Statistically significant at $p < 0.05$, NP: Nonparametric test- Mann-Whitney U test, P: Parametric test- (One-way ANOVA).

The results from Table E indicate statistical differences in means for the age of onset and Fishman phenotype classification (1&4, 2&4, 3&4) for this polymorphic pair on the L541P and A1038V alleles as compared to patients with other Missense functional changes.

4.3 Results of Age of Onset Analysis

A. Comparison of Early and Late Onset: The results shown in the Table F below compare the average presenting age, BCVA and Fishman classifications between two segments; Early and Late Onset in patients with the STGD1 phenotype.

Classification of Onset	Average Presenting Age ± SE[a NP]	Average BCVA OD ± SE[a P]	Average BCVA OS ± SE[a P]	Average Fishman Phenotype Classification ± SE[a P]
Early N_T: 161	18.30 ± 1.09 N: 124	0.96 ± 0.05 N: 123	0.94 ± 0.05 N:123	2.70 ± 0.07 N:129
Late N_T: 67	39.33 ± 2.43 N: 21	0.74 ± 0.07 N:44	0.72 ± 0.07 N:44	1.78 ± 0.124 N:41
Total Average	21.35 ± 1.17	0.90 ± 0.04	0.88 ± 0.04	2.48 ± 0.07

Table F: Relationship between classification of onset and clinical variables. N: Number of Patients. [a] Statistically significant at $p<0.05$. NP: Nonparametric test – Mann- Whitney U test, P: Parametric test (ANOVA test)

As illustrated by Table F, there were statistical differences in means amongst all 4 variables analyzed with respect to classification of onset. It was noted that patients with an earlier age of onset (less than 20 years) have significantly worse averages of BCVA OD, BCVA OS and Severity of Disease (Fishman Classification) as compared to patients having a late onset of the disease ($p<0.05$).

B. Early and Late Onset compared with Fishman Phenotypic Classification and Polymorphic Prevalence: The following Table illustrates the results of the prevalence of specific polymorphisms between early and late onset found at each Stage of Fishman classification.

Fishman Phenotype Classification	Early Onset		Late Onset	
	Most Prevalent Mutation	% Frequency	Most Prevalent Mutation	% Frequency
1	c.5882G>A N:4; N_T: 24	16.78%	c.6320G>A N:7; N_T: 22	31.82%
2	c.1622T>C c. 1804C>T c.5882G>A N: 15; N_T: 58	25.86%	c.1622T>C c.6320G>A c.5882G>A N:6; N_T: 22	27.27%
3	c.1622T>C N:18; N_T: 123	14.63%	c.5882G>A N:2; N_T: 14	14.29%
4	c.1622T>C N:6; N_T: 25	24.00%	N/A	N/A

Table G: The most frequently occurring mutations for early and late onset according to Fishman phenotype classification. N: Number of patients with specified mutation for a given phenotypic grade, N_T Total number of patients presenting with specific phenotypic grade

As shown by Table G, the most prevalent mutation associated with a Fishman phenotype classification of grade 1 amongst patients with early onset was c.5882G>A >C (16.78%) and c.6320G>A (31.82%) in late onset. It is noted that both mutations c.1622T>C and c.5882G>A occurred together in patients with both early and late onset with approximately equal frequency of 25.86% and 27.27% respectively. Mutation c.1622T>C was the most prevalent mutation in patients with early onset for both phenotype grades 3 and 4.

C. Average age of onset compared with Fishman Classification

Fishman Phenotype Classification	Average age of onset ± SE * NP
1	32.66 ± 3.44 N: 29
2	20.05 ± 2.66 N:42
3	11.18 ± 0.78 N:88
4	8.00 ± 0.76 N:11
Total Average	16.83 ± 1.15

Table H: Average age of onset for each Fishman Phenotypic grade. N: Number of Patients. * Statistical significance at $p < 0.05$. NP: Nonparametric test – Kruskal Wallace.

As shown by Table H, there is a statistical significance in the average age of onset as compared to phenotypic grade; particularly among classes 1 &2, 1&3, 1&4, 2&3 and 2&4. This implies that the Age of Onset and severity of the STGD1 phenotype could be linked. The average

age of onset for Phenotype grade 1 is 32.66 years (Corresponding to Late Onset) which is significantly different from that of grade 4, which is 16.83 years (Corresponding to Early Onset).

4.4 Results on Analysis of Number of Mutations

A. Table I below shows the results of the evaluation of the number of mutations a patient contained in their genotype against all clinical variables considered.

Number of Mutations	Average Presenting Age ± SE	Average Age of Onset ± SE*[p]	Average BCVA OD ± SE	Average BCVA OS ± SE	Average Fishman Phenotype Classification ± SE	Most frequent Mutations
1 N: 76	24.86 ± 2.61 N: 39	21.60 ± 2.22 N:62	0.85 ± 0.08 N:45	0.86 ± 0.08 N:45	2.49 ± 0.13 N:43	c.5882G>A
2 N: 116	21.00 ± 1.55 N:76	16.02 ± 1.20 N:104	0.90 ± 0.06 N:81	0.86 ± 0.06 N:81	2.56 ± 0.09 N:89	c.5882G>A c.5174+5G>A
3 N: 52	21.08 ± 2.21 N:39	14.98 ± 1.57 N:50	0.94 ± 0.08 N:42	0.96 ± 0.08 N:42	2.52 ± 0.14 N:42	c.1622T>C c.3113C>T c.5882G>A
4 N: 7	22.33 ± 4.22 N:6	9.50 ± 1.32 N:4	0.60 ± 0.37 N:5	0.60 ± 0.37 N:5	2.83 ± 0.40 N:6	c.1622T>C c. 4328G>A c.3113C>T c.508+2T>C
Total N:251	22.01 ± 1.12	17.24 ± 0.93	0.89 ± 0.04	0.88 ± 0.04	2.54 ± 0.06	c.1622T>C

Table I: Analysis of number of mutations with respect to phenotypical variables N: Number of Patients. * Statistical significance between number of mutations and average age of onset. P: Parametric test (ANOVA test).

4.5 Results of Analysis based on Patient Sex

Sex	Average Age of Onset ± SE	Average BCVA OD ± SE	Average BCVA OS ± SE	Average Fishman Phenotype Classification ± SE
Male N:81	15.68 ± 1.23 N: 81	0.64 ± 0.07 N: 81	0.66 ± 0.07 N: 81	2.45 ± 0.11 N: 81
Female N:83	13.35 ± 1.10 N:83	0.59 ± 0.08 N:83	0.59 ± 0.07 N:83	2.65 ± 0.12 N:83
Total Average	14.50 ± 0.83	0.62 ± 0.05	0.62 ± 0.05	2.55 ± 0.08

Table J: Comparison of means of different clinical variables against sex. N: Number of Patients.

As shown by Table J, clinical measurements for STGD1 did not exhibit bias based on sex. The clinical variables all show approximately equal phenotypic characteristics for both males and females based on Age of Onset, BCVA and Severity (Fishman Phenotype Classification). There were no statistical differences observed in the means among the various clinical variables measured with respect to sex ($p>0.05$).

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

A Meta-Analysis conducted on a database of 251 patients across 9 different populations and ethnicities including: Africa, Asia, South Asia, South East Asia, Europe, North and South America revealed the characterization of 484 mutations which included 142 different polymorphisms in the ABCA4 gene. This finding is consistent with the highly polymorphic nature of the ABCA4 gene as reported in other studies. As a result of such large genetic variation, it difficult to establish specific relationships between genotype and phenotype since there is also a large phenotypic spectrum[13]. Hence studies have attempted to describe the STGD1 phenotype by utilizing phenotypic characteristics which include measurable clinical variables like BCVA, Age of Onset or the physical appearance of the fundus according to the Fishman Classification system. The following averages for various clinical phenotypes in the database are as stated: age of onset to be 17.87 ± 0.94 years, BCVA (logMAR) to be 0.86± 0.04, 0.87± 0.04 (OD/OS) and Fishman Classification to be 2.49 ± 0.06. Furthermore, as evidenced in the literature review, it is expected to have variation in presentation of phenotypes and genotype among different populations; which was also reflected in this study. It was noted that there were variations in each of these characteristics in relation to both patient demographics and the individual polymorphisms which constitute their genotype; which will be further discussed.

The top 14 frequently occurring mutations identified in this study were independently analyzed to determine genotypic-phenotypic relationships. Among these mutations, it was noted that mutation c.1622T>C was the most frequent occurring (11.20%) while mutation c.2966T>C occurred with least frequency (1.20%). Prevalent polymorphisms are indicative of a common origin for a specific genetic mutation. This is referred to as the founder effect[25]. As previously

mentioned, the most prevalent polymorphism found in the sample was the c.1622T>C on the L541P allele which causes the p. Leu541Pro amino acid substitution resulting in a Missense functional change. This mutation accounted for 11.20% of all mutations in this study and was also one of the most frequently occurring mutation amongst European populations, accounting for 27.07% of all mutations in that cohort. This finding is consistent with a study conducted by Maria-Lopes which investigated 21 Portuguese families in Europe. The study also found that this polymorphism was frequently found in Spanish families as well. This correlation indicates a possible European origin for this mutation and suggests of a founder effect. Within this study, other European populations analyzed included the countries of Greece and Poland as well as otherwise unspecified European nationalities. Mutations evaluated in this group accounted for 159 or 32.85% of all 484 mutations in the sample. The data showed the most frequently occurring polymorphisms within this group to be the c.1622T>C at 27.04% followed by the c.3113C>T at 23.27% and then the c.5882G>A at 20.13%. Together, these mutations also represent the most prevalent 3 of the Top 10% of all mutations within this study as indicated by Table B. This finding is consistent with other studies on European populations. The c.1622T>C mutation is the most frequently occurring mutation found in this study and amongst all European populations as well. It occurs within the L541P Allele responsible for the p. Leu641Pro amino acid substitution. Furthermore, the average age of onset for this group was found to be 12.98 ± 1.34 Years and mean phenotype classification to be 2.86 ±0.13. When compared to the 14 mutations which comprise the top 10% of prevalent mutations, both these characteristics were found to be statistically significant in their variation. This implies that a patient possessing the c.1622T>C polymorphism, may present with different measurements of clinical variables (phenotype) when compared with other polymorphisms. This was found to be the case through comparison of the phenotypic

classification with the c.5882G>A polymorphism. The c.5882G>A mutation is responsible for the p.Gly1961Glu amino acid substitution and the average age of onset was found to be 18.28 Years ± 1.61 and Phenotypic classification of 2.33 ± 0.18 which when taken together are less negative when compared with the findings for the c.1622T>C mutation as stated before. A study conducted by Zernat et. al classified the c.1622T>C mutation as prevalent in German populations[26] whereas the c.5882G>A was the most prevalent mutation in a Russian cohort as indicated by study conducted by Shurygina et. al[27]. This illustrates the heterogeneity of the phenotypes through comparison with geographically and ethnically similar populations but also evidences the variation of the phenotypic spectrum by a single polymorphism.

African populations showed the lowest degree of severity amongst all the clinical variables assessed and across every population. The mean age of onset was shown to be 34.62 ± 3.24, BCVA (logMAR) to be 0.69 ± 0.10, 0.72 ± 0.10 (OD/OS) and a Phenotypic classification of 1.75 ± 0.13. In comparison, European populations were found to be worst in each assessment with the age of onset to be 12.00 ± 1.19, BCVA (logMAR) 0.72 ± 0.06, 0.77 ± 0.05 (OD/OS) and a Phenotypic classification of 2.92 ± 0.06. The differences in average age of onset between European and African populations were found to be statistically significant which suggests higher age of onset of STGD1 in African populations when compared with European populations ($p<0.05$). This research identified the most prevalent mutation found in African populations to be the c.6320G>A polymorphism. This accounted for 20% of all disease-causing alleles (R2107H) in this cohort. This specific polymorphism was also found to be the most prevalent mutation amongst all patients with an early onset of (<20 Years) and the mildest phenotypic severity (Grade 1), accounting for 31.82% of all mutations in that category. A lower age of onset is associated with worst visual outcomes and more debilitating disease consequence[28]. This finding was also replicated when comparing

African populations against non-homogenous populations with high European ancestry for e.g. Brazil where the mean age of onset also differed significantly from African populations $p<0.05$. Therefore, these comparisons support the theory that the severity of the STGD1 phenotype may affect different ethnicities differently. Particularly, the comparisons show that African populations are less susceptible to more severe forms of STGD1 than European populations or those with European ancestry. These results coincided with a study conducted by Zernant et al. on a cohort of 44 patients of West African descent which also found a significant difference in average age of onset and phenotype severity between Europeans and Africans. It was concluded that those of the former experience a later onset and "milder" disease symptoms[28].

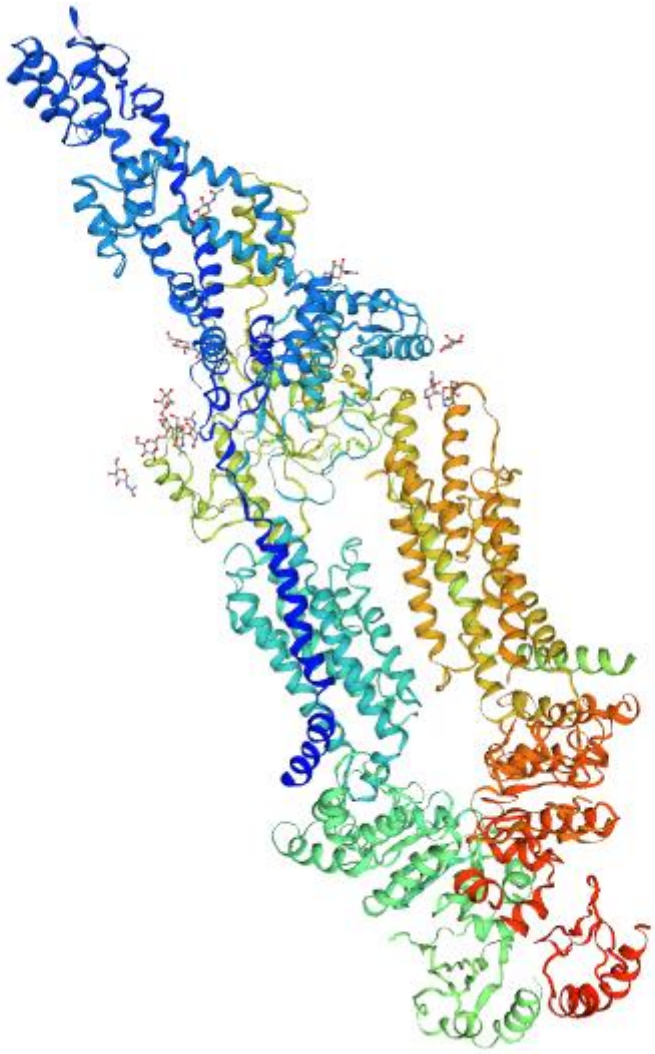

Diagram 4: Molecular Representation of ABCA4 Transporter [29]

The ABCA4 gene codes for the ABC ATP-Binding cassette superfamily A, group 4. It is a transmembrane complex protein transporter only expressed in retinal photoreceptors (See Diagram 4). Therefore, mutations within this gene directly affects the expression of the amino acids which constitute this oligomer. This ultimately affects the conformation of the tertiary and quaternary structure of the transporter. The most common functional change detected in our study was Missense which accounted for 86.6% due to Single Nucleotide Polymorphisms (SNPs). This is consistent with findings in several other studies that report similar findings for the STGD1

phenotype[30, 31]. There is some debate that some Missense mutations by themselves carry a greater pathogenicity than other Missense mutations. The mean age of onset for c.6320G>A missense mutation was found to be 38.25± 6.03 with patients having an average BCVA (logMAR) of 0.68± 0.17, 0.78± 0.16 (OD/OS) and a Fishman phenotypic classification of 1.56± 0.20. When compared to another Missense mutation, the c.1804C>T mutation on the R602W allele responsible for the p. Arg2107His amino acid substitution there was statistically significant difference with respect to average age of onset and Fishman phenotypic classification. The mean age of onset for c.1804C>T was determined to be 10.50± 1.25. A study conducted on an 8-year old pediatric patient of Hispanic descent, described this polymorphism as extremely rapidly progressing and resembling an inflammatory disease[32]. From the time of presentation, the child became legally blind within 2 years. Our studies corroborate these findings for early age of onset with this mutation $p<0.05$. These findings for age of onset and Fishman phenotypic classification together confirm that the Missense functional change is the most prevalent molecular consequence underlying the STGD1 phenotype and further exemplifies the relationship between specific polymorphisms and their impact on the resultant phenotype.

A study conducted on a German cohort of 50 patients by Maugeri et al in 2000, evidenced the existence of a complex allele in patients with the STGD1 phenotype. The study described the existence of a complex genetic structure which comprised of the [L514P; A1038V] alleles. This complex is characterized by 2 polymorphisms, the c.1622T>C and the c.3113C>C, which result in Missense functional changes[26]. These individual mutations were identified in our sample occurring frequently within a Polish population. Individually, the c.1622T>C was identified in 49 patients and the c.3113C>T in 39. It was found that patients with the former polymorphisms had an earlier average age of onset at 12.98 Years ± 1.34 and an average phenotype classification of

2.86 ± 0.13 $p<0.05$. The latter had an average age of onset of 14.81 Years ± 1.64 and a phenotypic classification of 2.97 ± 0.14. These polymorphisms were further investigated for signs of haplotypic inheritance. Polymorphic haplotypes are a collection of nucleotide mutations which are frequently inherited together. This was found to be the case with the [L514P; A1038V] complex allele. Through pair-wise analysis, it was found that these alleles occurred together in 14.3% of all patients with Missense functional change. Patients who inherited this complex allele were found to develop disease symptoms almost 2x earlier as compared with patients with other Missense functional changes $p<0.05$. It was also noted that these patients had a more severe version of disease with an average Fishman phenotypic classification of 3.00 ± 0.15 when compared to the same $p<0.05$. These results indicate that there is a complex relationship between these specific polymorphisms and the resultant STGD1 phenotype[33]. It illustrates their individual contribution in patients that possessed at least one copy of the mutation and the effect they have when combined. This demonstrates the direct and combined impact of these polymorphisms on the STGD1 phenotype and the possible existence of complex alleles within these patients.

The second largest functional change following Missense, was found to be Nonsense which accounted for 36 mutations or 8.06% of all changes. The most prevalent nonsense polymorphism was c.4234C>T which is located on the Q1412 allele responsible for the p.Gln1412Ter amino acid change and subsequent aberration of the sequence. These loss-of-function or null mutations and are often associated with more severe disease consequences[31]. It was determined that patients with Nonsense mutations had an average age of onset of 8.77 Years ± 1.17 and Fishman phenotypic classifications of 3.13 ± 0.17 whereas patients with Missense functional had an average age of onsets at 19.49 Years ± 1.10 and mean Fishman phenotypic classification of 2.37± 0.07. It was hence suggested nonsense functional changes had a significantly earlier age of onset and a more

severe phenotypic classification as compared to those of missense functional change in this study, $p<0.05$. These findings are consistent with previous studies which indicate that patients with nonsense mutations generally have earlier age of onsets and more severe phenotypic changes[34].

As previously stated, and evidenced by the aforementioned, age of onset is strongly associated with the STGD1 disease prognosis and severity. The earlier a patient develops the disease, the worst the eventual visual outcomes. This research compared early age of onset (<20Y) with late age of onset (>20Y). Within these parameters, it was found that the average BCVA (logMAR) for early onset patients were 0.96 ± 0.05, 0.94 ± 0.05 (OD/OS) with an average phenotypic classification of 2.70 ± 0.07. Patients with early age of onset showed significantly worst outcomes with all clinical variables assessed when compared with patients that had Late onset. This included an earlier age of presentation, higher BCVAs and more severe Fishman phenotype classification $p<0.05$. A study conducted by Lambertus et. al on 51 STGD patients with early age of onset found that patients in the study had a rapid decline in visual acuity along with profound chorioretinal atrophy[34]. The study concluded that early-onset STGD lies at the severe end of the ABCA4 phenotypic spectrum which was consistent with the previous stated findings.

The most prevalent mutations found between patients with early onset differed at each phenotypic classification when compared to patients with late onset. Fishman Classification Stage 1, characterized by the mildest changes to the fundus, was associated the c.5882G>A mutation being prevalent at that stage. This mutation occurred with the highest frequency, of 16.87%, in all patients with early onset. The c.1622T>C was the most prevalent mutation in those with more severe changes (Fishman Stage 2-4). It also accounted for 24% of all patients with early onset and the most severe Fishman phenotypic classification, Stage 4. The average age of onset for patients with Stage 1 Fishman phenotypic classification was 32.66 Years ± 3.44. In comparison, patients

with the Stage 4 Fishman phenotypic classification had an average age of onset of 8.00 Years ± 0.76. Therefore, patients with the mildest classification (Fishman Stage 1) of the disease had a later average age of onset when compared with those who had more profound changes (Fishman Stage 4) to the fundus $p<0.05$. These findings are consistent with those found in other studies as well[35].

The average Fishman phenotypic classification for patients with late onset was found to be 1.78 ± 0.124 with BCVA (logMAR) 0.74 ± 0.07, 0.72 ± 0.07 (OD/OS). These visual outcomes were significantly better when compared with to patients with an earlier disease onset $p<0.05$. 59.09% of all patients with late onset had a milder version of physical changes (Fishman Stage 1-2) to the fundus. The most prevalent mutation found in patients with late onset was the c.6320G>A mutations which accounted for 31.82%. As previously discussed, this is also the most prevalent mutation found in patients with African ancestry and coincidentally accounted for the majority of patients with the mildest observable changes to the fundus[28] (Fishman Stage 1). It is also notable that there was no recorded patient which had both a late onset of STGD1 and severe physical changes to the fundus (Fishman Stage 4). A study by Lee et al. described similar findings in a cohort of patients with late onset STGD1 and identified the c.6320G>A polymorphism as having a milder pathogenicity hence coinciding with our findings[36].

A study conducted by Utz et al on 112 patients with STGD1 found that patients having at least two polymorphisms of the ABCA4 gene were associated with an earlier age of onset and reduced BCVA (logMAR). This was similar to what was found in this research[37]. The average age of onset and BCVA for patients with 1 polymorphism was 21.60 Years ± 2.22 and 0.85 ± 0.08, 0.86± 0.08 (OD/OS). In comparison, the average age of onset and BCVA for those with 4 polymorphisms was found to be 9.50 Years ± 1.32 and 0.60 ± 0.37, 0.60 ± 0.37 (OD/OS). Through

a non-haplotypic analysis, it was determined that the number polymorphic variants a patient possessed was associated with the average age of onset $p<0.05$. However, even though there was a difference in the means for both variables, there was no statistical difference between the specific number of mutations and BCVA $p>0.05$. This could be attributed to the limited availability of data for this category which resulted in a possible type 2 error.

STGD1 is a genetic disease and as such there are complex patterns that govern inheritance which can be influenced by the sex of a patient. OMIM recognizes the STGD1 disease to exhibit an autosomal recessive inheritance pattern[38]. Barring epigenetic influence, it is expected to equally affect both males and females in terms of incidence and severity[39]. This was found to be the case in this research. The average age of onset, BCVA (logMAR) and phenotypic classification for males was found to be 15.68 Years ± 1.23, 0.64 ± 0.07, 0.66 ± 0.07 (OD/OS) and 2.45 ± 0.11. For females, it was determined to be 13.35 Years ± 1.10, 0.59 ± 0.08, 0.59 ± 0.07 (OD/OS) and 2.65 ± 0.12. By comparison, the means for all clinical variables are approximately equal and there were no statistically significant relationships amongst any $p>0.05$.

5.2 Conclusion

Stargardt's disease (STGD1) is classified by compound heterozygous and homozygous polymorphisms of the ABCA4 gene and is inherited in an autosomal recessive pattern. Due to the highly polymorphic nature of the ABCA4 gene, currently, researchers have been unable to determine the exact protein or nucleic mutation responsible for the disease. There exists a theoretical relationship between the genotypic and phenotypic classifications amongst Stargardt's patients. However, there is significant limitation in the availability of studies which were able to statistically establish relationships between genotypic and corresponding phenotypic variables of STGD1 patients. As such, a Meta-Analysis was conducted to investigate the impact of specific

ABCA4 polymorphisms on the STGD1 phenotype. A database comprising of 251 patients obtained from 11 research studies was compiled based on the specific requirements as outlined in the Methodology 3.0 and analysed accordingly.

A total of 142 different polymorphisms of ABCA4 gene occurring at a frequency of 484 were identified. The most prevalent mutation found in this study was the c.1622T>C polymorphism which accounted for 11.20% of all mutations in sample. This mutation was found frequently occurring in European populations which suggests a possible common ancestor originating in Europe and is indicative of a founder effect. Additionally, it was established that European populations contained the following prevalent polymorphisms: c.1622T>C (27.04%), c.3113C>T (23.27%), c.5882G>A (20.13%). These polymorphisms had a relative higher pathogenicity than those prevalent in African populations with significantly worst visual outcomes. The most prevalent polymorphism in African populations was the c.6320G>A (31.82%) mutation which was found to have milder pathogenicity and better visual outcomes. The most prevalent functional change was found to be Missense which accounting for 86.06% of all functional changes, was found to have a varying impact on the STGD1 phenotype dependant on the specific polymorphism and nature of the allele. The existence of the complex allele [L514P; A1038V] in 14.3% of patients with Missense functional changes, exhibited haplotypic inheritance and impacted the STGD1 phenotype more severely than all other mutations in that category $p<0.05$. Patients with null mutations for e.g. c.4234C>T had worst visual outcomes than all other functional changes $p<0.05$. Additionally, on average, patients with earlier age of onsets had more profound changes to the fundus when compared to patients with a later age of onset $p<0.05$. The c.1622T>C was found to be prevalent in patients with early onset (24%) while the c.6320G>A, was prevalent (31.82%) in those with late age of onset. A relationship in average age of onset and

number of mutations in the ABCA4 gene was also established ($p<0.05$). ABCA4 polymorphisms affect both males and females approximately equally with no significant variation in any of the clinical variables assessed.

Hence, these findings altogether suggest that polymorphisms of the ABCA4 gene can significantly impact the STGD1 phenotype as defined by age of onset, BCVA and fundus appearance as classified by Fishman Phenotypic Grading.

5.3 Limitation of the study

- There was limited amount of data and relevant information which was available hence this contributed to small sample sizes when analyzing certain genotype-phenotype relationships.

5.4 Recommendations

- It is recommended that future studies investigate cis and trans configurations when studying complex alleles since the location of the mutation can influence the phenotypic variables.

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7.0 APPENDIX

Fishman Classification of Stargardt Disease[5]	
Stage 1	• Pigmentary changes in macula (ranging from faint/irregular pigment mottling to beaten-bronze appearance to atrophy) • Pisciform ring of flecks within 1 DD on all sides of the fovea • Normal ERG and EOG
Stage 2	• Pisciform flecks present beyond 1 DD from the margin of the fovea, often extending beyond the arcades and nasal to the optic disc • ERG and EOG normal, but cone/rod response may be subnormal • Prolonged period for dark adaptation
Stage 3	• Fundus exam shows diffusely resorbed flecks and choriocapillaris atrophy in the macula • EOG testing reveals subnormal ratios • ERG shows subnormal cone or cone and rod amplitudes • Central field defects as well as peripheral/midperipheral field impairment can be seen
Stage 4	• Fundus exam shows diffusely resorbed flecks and extensive choriocapillaris/RPE atrophy throughout entire fundus • ERG testing shows reduced cone and rod amplitudes • Peripheral fields show moderate to extensive restriction

ABBREVIATIONS: DD, disc diameter; **EOG,** electro-oculogram; **ERG,** electroretinogram.

Diagram 5: Fishman Classification of Stargardt's Disease[2].

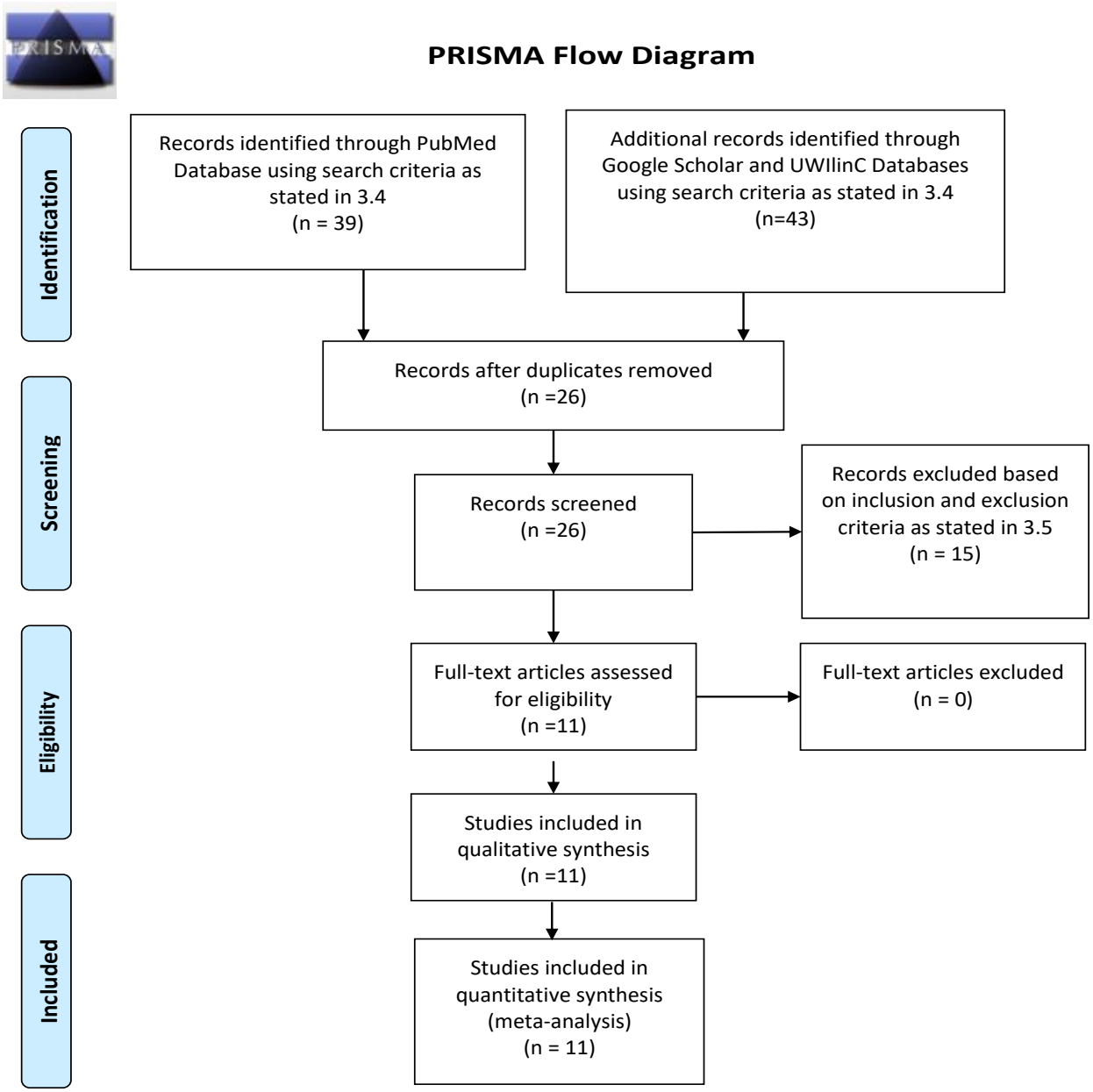

Diagram 6: PRISMA 4-Step flow chart illustrating the method of selection of research papers. Retrieved from http://prisma-statement.org/PRISMAStatement/FlowDiagram

Note: The same studies were used for both qualitative and quantitative synthesis. It is also important to note that not all data presented within each of the 11 studies was utilized. Each ABCA4 nucleotide mutations identified in these studies were subsequently verified through

ClinVAR Bioinformatic Database to verify their location at the ABCA4 genetic locus. Patients who had mutations on additional genes in association with the ABCA4 was excluded from the study.