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# Association of *IGF1* polymorphisms with exotropia in a Pakistani cohort

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**Purpose:** Strabismus (STBMS) is a multifactorial ocular disorder in children that leads to misalignment of the eyes. Insulin-like growth factor 1 (*IGF1*) has been shown to be involved in the development of extraocular muscles and myopia; however, data are limited on the genetic associations of *IGF1* with STBMS in Pakistan.
**Methods:** Two hundred seventy-four STBMS cases and 272 unaffected controls were recruited, and their DNA was extracted. Two *IGF1* single nucleotide polymorphisms, rs6214 and rs5742632, were genotyped using PCR–restriction fragment length polymorphism. Univariate logistic regression analysis was performed to determine the association of these single nucleotide polymorphisms with STBMS, and the results were adjusted for age and sex. In addition, 26 extraocular muscle tissues were collected from patients with STBMS undergoing squint correction surgery, along with 3 deceased control samples. *IGF1* mRNA expression was measured by quantitative PCR; the Mann–Whitney U test was applied, and fold change was calculated. Logistic regression analysis was applied to determine the association of RNA expression and fold change with genotype.
**Results:** Multivariate logistic regression analysis revealed that rs5742632 (odds ratio [95% confidence interval] = 1.05[1.01–1.06], p = 0.03) is associated with STBM. Moreover, rs6214 (1.03[1.01–1.05], p = 0.03) and rs5742632 (1.09[1.04–1.11], p = 0.04) were associated with exotropia. Statistically, no significant difference in *IGF1* mRNA expression in the extraocular muscles between the STBMS cases and the controls was observed.
**Conclusions:** *IGF1* polymorphisms rs5742632 (A>G) and rs6214 (C>T) are plausible risk factors for the development of exotropia. However, the physiologic mechanism requires further evaluation.

Strabismus (STBMS), also known as squint, is a common childhood disorder characterized by misalignment of the eyes [1]. It is conjectured to occur because of an abnormal development of vision during early childhood [2]. Anatomically, STBMS involves the absence of coordination between the extraocular muscles—which prevents both eyes from focusing together, thus hindering proper binocular vision [3]. Therefore, STBMS affects the ability of the eyes to perceive objects three-dimensionally [4]. STBMS can be classified into paralytic (incomitant) and nonparalytic (comitant) STBMS, which are further subclassified by the direction of misalignment. Horizontal misalignment is termed esotropia if the eye turns inwards and exotropia if it deviates outwards [3,4].

The prevalence of STBMS is about 1%–4% worldwide, of which more than 95% of cases are comitant STBMS (CS). However, these figures vary between different ethnic groups. The prevalence of STBMS in Europeans and Caucasians is approximately 2.5% and 2.7%, respectively, while lowest being in Africans (approximately 0.6%) [5]. In addition, the exotropic subtype is more common among Asians, while esotropia is more common among Caucasians [5]. Approximately 30% of individuals with STBMS have a positive family history [6], and even though CS accounts for more than 95% of the total STBMS cases, little is known about its pathological factors.

Insulin-like growth factor 1 (IGF1) is a somatic cell growth factor that facilitates the action of growth hormones and is involved in the regulation and development of various developmental processes, such as muscle differentiation [7]. The role of *IGF1* in the pathophysiology of STBMS and regulation of extraocular muscle function has been widely studied [8-11]. Single nucleotide polymorphisms (SNPs) in *IGF1* have been reported to be significantly associated with the development of myopia in Caucasian [12] and Egyptian populations [13], yet their role in STBMS is still inconclusive. Tanaka et al. [14] and Tang et al. [15] have shown that the prevalence of STBMS, especially exotropia, is relatively higher in patients with myopia; therefore, it is plausible that the same underlying components may contribute to the pathology of both conditions. Thus, our study aimed to determine whether

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Table 1. Demographic features of cohort.

| Subjects | Controls | STBMS | Esotropia | Exotropia | $p_1$ | $p_2$ | $p_3$ |
|---|---|---|---|---|---|---|---|
| **Total (N)** | 272 | 274 | 135 (49.3%) | 139 (50.7%) | | | |
| **Sex (Male)** | 129 (47.1%) | 147 (54.0%) | 72 (53.3%) | 71 (51.1%) | 0.17 | 0.19 | 0.38 |
| **Age (Years)** | 15.39 (2.21) | 16.82 (9.98) | 14.70 (9.43) | 18.90 (9.16) | 0.67 | 1.00 | 0.67 |
| **Diagnosed at Birth (Congenital)** | NA | 81 (29.6%) | 25 (18.5%) | 56 (40.3%) | NA | NA | NA |

Values are N (%) or Mean (SD), $p_1$, comparison between STBM and controls; $p_2$, comparison between esotropia and controls; $p_3$, comparison of exotropia with controls, p values ($p_1$, $p_2$ and $p_3$) for sex (categorical variable) were calculated by Pearson's chi-square test while p values ($p_1$, $p_2$ and $p_3$) for age (continuous variable) were calculated by Mann–Whitney U test, respectively.

*IGF1* polymorphisms, which are known to be associated with myopia, are also associated with STBMS.

The *IGF1* polymorphism rs6214 (NC_000012.12: g.102399791C>T) has been widely reported to be associated with different conditions, including myopia. This SNP is a functional polymorphism located in the 3′-UTR of the gene that is a part of the regulatory motif, which is crucial for gene expression and mRNA stability [16,17]. Mak et al. [18] have also observed that an intronic SNP, rs5742632 (NC_000012.12: g.102462696A>G), is associated with myopia. The current study investigated the role of *IGF1* rs6214 and rs5742632 polymorphisms in STBMS among Pakistani patients. In addition, the relationship of these polymorphisms with *IGF1* expression levels in extraocular muscles was determined to better understand the pathogenesis of STBMS.

## METHODOLOGY

*Ethical declaration:* The current study conforms to the tenets of the Declaration of Helsinki. It was approved by the Ethical Review Board of the Department of Biosciences, COMSATS University Islamabad. Written informed consent was obtained from all participants before they participated in the study. Moreover, in the case of deceased donors, informed consent was obtained from accompanying family members.

*Panel recruitment:* In this study, STBMS cases were recruited from the Department of Pediatrics of Al-Shifa Trust Eye Hospital, Rawalpindi, Pakistan. Complete ophthalmological examinations were conducted for all the cases, including cover, prism cover, and Hirschberg tests.

*Inclusion and exclusion criteria:* All patients diagnosed with horizontal CS, including exotropia and esotropia, were enrolled in this study. Patients with any other ocular condition, including STBMS associated with any syndromic form, were excluded from the study. Patients with a history of any eye surgery other than squint repair were also excluded.

The control group included healthy individuals with no eye disorders. All subjects underwent a complete clinical assessment, and their medical histories were recorded before sample collection. First-degree relatives of cases and controls were excluded from the study. Demographic data, including age, sex, and age of diagnosis, were also recorded (Table 1).

*Blood sample collection and DNA extraction:* About 5 ml of blood was obtained from each participant in EDTA vacutainers (BD Vacutainer®, East Rutherford, NJ) for DNA extraction. The blood samples were refrigerated at 4 °C before genomic DNA extraction, which included an organic method (phenol/chloroform) as described by Sambrook and Russell [19].

*Selection of SNPs and genotyping of rs6214 and rs5742632:* Two *IGF1* SNPs, rs6214 [20] and rs5742632 [21], were selected for genotyping by PCR–restriction fragment length polymorphism using a previously described procedure (Appendix 1). These SNPs were selected because both have been studied in myopia. As a quality control measure, 10% of the total samples, including cases and controls, were analyzed by Sanger sequencing, and the results were 100% in concordance with the PCR–restriction fragment length polymorphism results.

*Association analyses:* The association analyses were performed with R v3.5.0, where the SNPs were additively coded. The univariate logistic regression analysis was applied to determine the association of both SNPs with i) STBMS (STBMS versus control), ii) esotropia (esotropia versus control), and iii) exotropia (exotropia versus control). These results were then adjusted for age and sex using a multivariate logistic regression analysis. The genotype data of both SNPs were also tested for Hardy–Weinberg equilibrium (HWE) by PLINK v1.9.0 [22].

*Tissue sample collection:* A total of 26 extraocular muscle (EOM) tissue biopsies were obtained from patients undergoing squint correction surgery. Of these, 17 were medial rectus tissue samples obtained from patients with exotropia,

Table 2. Genotype counts and Hardy–Weinberg (HWE) Analysis for rs6214 and rs5742632.

| | Genotype counts | | | | | HWE |
|---|---|---|---|---|---|---|
| SNP | Controls (aa/aA/AA) | STBM (aa/aA/AA) | Esotropia (aa/aA/AA) | Exotropia (aa/aA/AA) | MAF | [1]p |
| rs6214 | 21/120/131 | 34/ 120/120 | 12/58/ 64 | 22/62/56 | 0.30 | 0.47 |
| rs5742632 | 8/108/156 | 22/ 119/133 | 9/54/71 | 13/65/62 | 0.29 | 0.04 |

[1]HWE p value, Minor allele frequency (MAF)

and 9 were lateral rectus tissue from esotropia patients. Moreover, three control muscle tissues were obtained from deceased donors.

Of these 26 patients, only 12 subjects consented to blood collection in addition to the tissue samples—these included 4 and 8 patients with esotropia and exotropia, respectively. Their DNA samples were extracted and genotyped for both *IGF1* SNPs. Resected EOMs were collected in RNA later (Applied Biosystems, Carlsbad, CA), and the samples were stored at −20 °C until further use.

*Expression profiling of IGF1:* Expression profile analysis was performed on total RNA, which was extracted from EOM samples using an all-in-one purification kit (Norgen Biotek Corp, Thorold, Canada). The extracted RNA was quantified using a spectrophotometer (IMPLEN, München, Germany) and reverse-transcribed using the RevertAid First Strand cDNA synthesis kit (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. Real-time quantitative PCR (RT-qPCR) analysis was performed using the Maxima SYBRGreen/ROX qPCR kit (Thermo Scientific, Waltham, MA). *GAPDH* was used as an internal control for the normalization of data. Primer sequences for *IGF1* (Uniprot ID: P05019) and *GAPDH* (Uniprot ID: P04406) are provided in Appendix 1. The relative expression level of *IGF1* was computed using the Mann–Whitney U test, and fold change (FC) was calculated using the $2^{-\Delta\Delta CT}$ method as described previously by Livak and Schmittgen, 2005 [23].

*Power calculations:* Power estimation was done in R v4.3.1 using package tidyverse v1.3.1 [24]. We generated power curves and estimated the power of our analysis at $\alpha = 0.05$. In addition, we estimated the sample size at $\alpha = 0.05$ with our observed effect size to achieve a power of 0.80.

## RESULTS

*Cohort description:* In total, 546 subjects were recruited for this study, including 274 STBMS cases and 272 healthy controls. The STBMS cases included 135 (49.3%) esotropia and 139 (50.7%) exotropia STBMS cases. Of the total STBMS cases, 54.0% were males, while 47.1% of the controls were males. The average ages of the STBMS and control cohorts were 16.82 and 15.39 years, respectively. It was also noted that 29.6% of the patients (n = 81) in the STBMS cohort were diagnosed with the disease at birth (Table 1).

*Association of SNPs with STBMS and its subclasses:* Among the two genotyped SNPs, rs6214 was in HWE ($\chi^2$ p value = 0.47), but rs5742632 was not in HWE ($\chi^2$ p value = 0.04; Table 2). The SNP rs5742632 was found to be associated with a higher risk of STBMS (A>G, odds ratio [OR]; 95% confidence interval [CI]) = 1.09 [1.02–1.17]; p = 0.01). In addition, both SNPs rs6214 and rs5742632 were associated with increased risk of exotropia (OR [95% CI] = 1.09 [1.01–1.16], p = 0.01 and 1.13 [1.05–1.20], p = 0.001, respectively) (Table 3).

Table 3. Univariate and multivariate logistic regression analyses of rs6214 and rs5742632.

| | Univariable logistic regression Analysis (Additive model) | | | | | | Multivariable logistic regression Analysis (Additive model) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| SNP | [1]OR (95%CI) | [1]p | [2]OR (95%CI) | [2]p | [3]OR (95%CI) | [3]p | [1]OR (95%CI) | [1]p | [2]OR (95%CI) | [2]p | [3]OR (95%CI) | [3]p |
| rs6214 | 1.05 (0.99- 1.12) | 0.10 | 1.09 (1.01- 1.16) | 0.01 | 1.01 (0.94-1.09) | 0.81 | 1.11 (0.91–1.36) | 0.29 | 1.03 (1.01–1.05) | 0.03 | 1.01 (0.97–1.05) | 0.54 |
| rs5742632 | 1.09 (1.02- 1.17) | 0.01 | 1.13 (1.05–1.20) | 0.001 | 1.06 (0.98–1.14) | 0.18 | 1.05 (1.01–1.06) | 0.03 | 1.09 (1.04–1.11) | 0.04 | 1.00 (0.96–1.05 | 0.91 |

[1]Logistic regression analysis STBMS versus controls,[2] Logistic regression analysis Exotropia versus controls, [3] Logistic regression analysis Esotropia versus controls, Odd's Ratio (OR)

When the results were adjusted for age and gender in multivariate analysis, rs5742632 showed association with STBMS (A>G, OR [95% CI] = 1.05 [1.01–1.06], p = 0.03]. Further, both SNPs rs6214 and rs5742632 were associated with increased risk of exotropia (OR [95% CI] = 1.03 [1.01–1.05], p = 0.03 and 1.09 [1.04–1.11], p = 0.04, respectively) (Table 3). However, none of these SNPs showed an association with esotropia when compared with the controls.

*Expression analysis of IGF1 in EOMs: IGF1* expression in EOMs was determined by calculating the FC value. *IGF1* was downregulated (FC = 0.65) in EOMs obtained from patients with STBMS compared to the controls. However, no statistically significant difference was observed in mRNA expression between the cases and the controls (z-score = 1.3494; p value = 0.18).

Correlation of the tissue expression with the studied SNPs was also determined, and logistic regression analysis showed no association with gene expression (β estimate [standard error (std. error)] = -0.08 [0.10], p = 0.39). We further checked the association of both SNPs via linear regression for minor alleles but found no association for either of the SNPs (rs6214: β estimate [std. error] = -0.59 [4.82], p = 0.15); rs5742632: β estimate [std. error] = -0.83 [3.43], p = 0.82). Moreover, we found no association between FC and the genotypes (rs6214: β estimate [std. error] = 11.00 [18.85], p = 0.57); rs5742632: β estimate [std. error] = 24.33 [26.50], p = 0.38) (Table 4).

*Power analysis:* The ORs of rs6214 and rs5742632 from their beta estimates in Table 4 were 0.55 and 0.44, respectively. The power curves are presented in Figure 1, which for our sample size (n = 29) showed that our tissue sample analyses were underpowered at α = 0.05 (power of rs6214 analysis = 0.55 and power of rs5742632 = 0.44; Figure 1A). The estimates of the needed sample size to achieve the desired power (0.80) at α = 0.05 with a similar effect size as obtained in our current analysis showed that we needed a minimum of 53 samples for rs6214 and 82 samples for rs5742632 (Figure 1B,C).

## DISCUSSION

In the current study, we observed an association of *IGF1* rs6214 and rs5742632 with STBMS, which is a multifactorial disorder. Our results showed that both SNPs are genetic risk factors for the development of exotropia; however, none of the SNPs showed an association with esotropia. Moreover, no significant difference in *IGF1* mRNA expression in the extraocular muscles was observed between the STBMS cases and the controls.

STBMS is an oculomotor disorder, and any abnormality in the regulatory factors involved in the development of the skeletal muscle may play a vital role in the pathogenesis of the disease [8]. *IGF1* is one of several growth factors that have been implicated in the development of extraocular muscles and binocular vision [8]. Endogenous and exogenous *IGF1* has been shown in animal models to regulate contractile force [8,9], as well as the kinetics (contraction time) of extraocular muscles [10]. Asymmetric application of exogenous *IGF1* to developing EOMs can induce STBMS in primates [11]. Studies have found that the mRNA levels of *IGF1* were reduced in strabismic EOMs, thereby implicating *IGF1* as one of the important components involved in the disease pathogenesis [25]. However, contradictory findings have reported no significant difference between the levels of *IGF1* mRNA in strabismic and control EOMs [26].

Until now, *IGF1* SNP-based genetic association studies for STBMS are limited; however, the genetic role of *IGF1* has been studied extensively in patients with myopia (short-sightedness) worldwide, which has overlapping clinical features with STBMS. It has been reported that *IGF1* regulates scleral protein production, which interferes with scleral remodeling and causes the development of myopia [27]. According to our clinical experience, myopia is often observed in patients with STBMS. Common clinical features of myopia and STBMS also overlap and include refractive error, blurry vision, and defects in EOMs.

Several genetic studies have been conducted to evaluate *IGF1* association with myopia in different populations [12,13,20,28]. Rydzanicz et al. [20] examined multiple *IGF1* SNPs, including rs6214 for myopia in a Polish population,

Table 4. Association of delta CT (gene expression) and fold change with rs6214 and rs5742632.

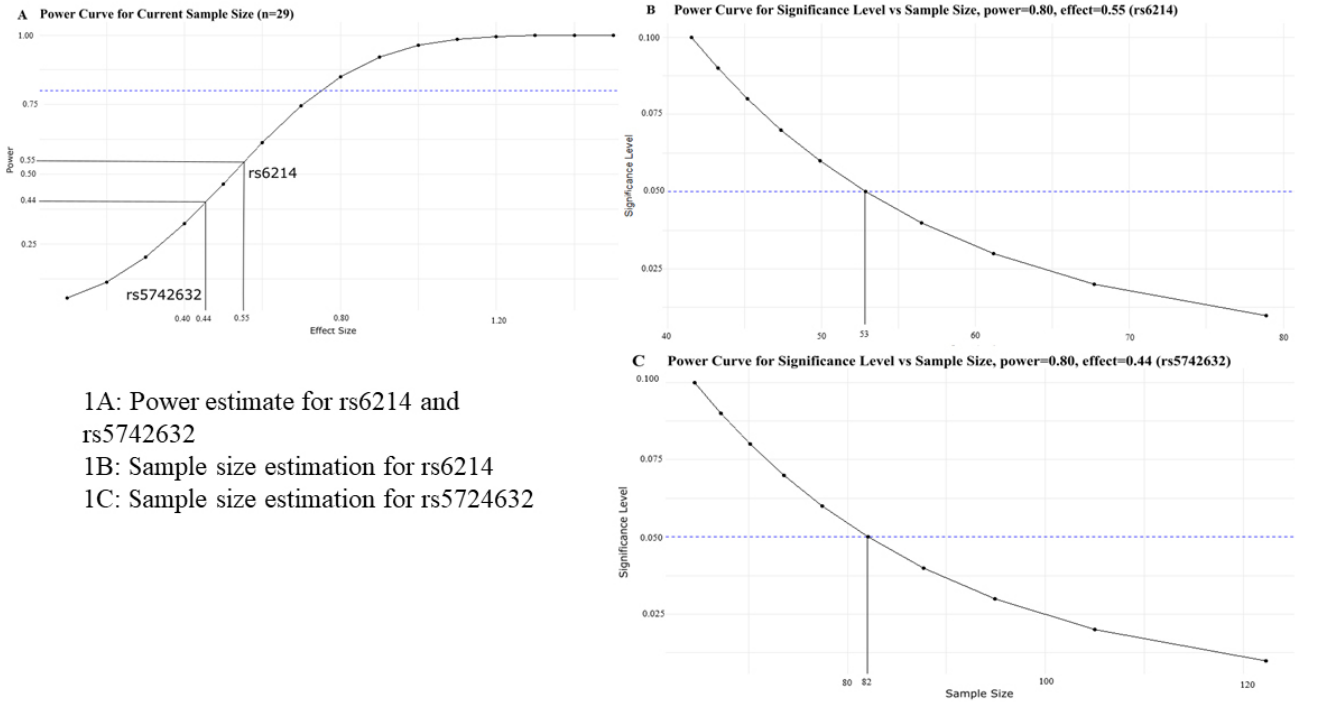
| | Regression Analysis | | | |
|---|---|---|---|---|
| **SNP** | **β estimate (SE)[1]** | **P value[1]** | **β estimate (SE)[2]** | **P value[2]** |
| rs6214 | −0.59 (4.82) | 0.15 | 11.00 (18.85) | 0.57 |
| rs5742632 | −0.83 (3.43) | 0.82 | 24.33 (26.50), | 0.38 |

[1]Logistic regression showing association of minor alleles with gene expression[2] logistic regression analysis showing association of fold change with genotypes

and reported nonsignificant findings. Metlapally et al. [12] studied the genetic association of SNPs in several populations of Caucasian origin and reported a statistically significant association of rs6214 with myopia in these populations. In addition, Zidan et al. [13] reported a significant association of rs6214 with myopia in Egyptians, while no association was observed for rs5742632. Such differences between studies could be due to differences in the cohort size of the populations or inherent ethnic differences in these groups. The *IGF1* SNPs have also been found to be associated with the risk of different diseases; for example, rs6214 is associated with glaucoma and colorectal cancer [29,30]. Both rs6214 and rs5742632 were chosen because their clinical significance has been observed in several previous studies. The SNP rs6214 is present in the 3′-UTR of *IGF1*, which is crucial for gene expression and mRNA stability and its cellular location, where it can play an important role in the regulation and expression of *IGF1* [16,17]. Whereas, rs5742632 is an intronic SNP, which may affect mRNA splicing and thereby gene expression (Homo_sapiens/Info/Index).

We observed that rs6214 was in HWE; however, rs5742632 was not in equilibrium. Although our sample size was reasonable, our results can possibly improve if the sample size is further increased. Khan et al. (2020) found rs5742632 to be in HWE [21] even though their study included older control subjects (mean [SD] = 47.05 [11.29] years) due to the nature of the study. Thus, the sample size may be the main limiting factor in achieving HWE in our current controls. Therefore, we suggest replication studies to achieve better insights into HWE.

In the current study, we observed an association of the GG genotype of rs5742632 not only with STBMS but also with its subclinical class exotropia, while for rs6214, the




1A: Power estimate for rs6214 and rs5742632
1B: Sample size estimation for rs6214
1C: Sample size estimation for rs5724632

Figure 1. Line plots for power calculations and estimation of power based on effect size(s) obtained in the current study for EOM analysis. **A:** The power curve for the sample size of 29 showed that the test had a power of 0.55 and 0.44 for rs6214 and rs5742632, respectively. The blue dotted line indicates the required power (i.e., 0.80). It is obvious from the plot that with our current sample size of 29 and at α = 0.05, the effect size must be close to 0.80 to attain the desired power. **B:** The power curve estimated a minimum sample size of 53 to achieve the desired 80% power for an effect size of 0.55 for rs6214 association analysis, and the current sample size of 29 EOMs made the analysis underpowered. The blue dotted line indicates the level of significance (i.e., 0.05). **C:** The power curve estimated the minimum sample size (82) to achieve the desired 80% power for effect size of 0.44 for rs5742632 association analysis, and the current sample size of 29 EOMs made the analysis underpowered. The blue dotted line indicates the level of significance (i.e., 0.05). Both Figure 1B,C also indicate that sample size might also be increased while changing the level of significance, and more samples may even be needed to estimate true estimation with the current effect sizes.

association was found only with the exotropic subgroup. To the best of our knowledge, no other study has reported an association of rs5742632 and rs6215 with STBMS. As exotropia typically develops several years earlier than myopia [31,32], it is unlikely that myopia causes exotropia, but exotropia may be a risk factor for myopia. Expression profile studies of *IGF1* in humans have reported interesting results using different techniques, such as qPCR and expression array. Altick et al. [26] reported downregulation of *IGF1*, while Zhu et al. [25] showed reduced levels of *IGF1* mRNA in most STBMS tissues using expression array and qPCR analysis. Consistent with previous studies, our results also indicated downregulation of *IGF1*, even though it was not statistically significant. Variability in the expression levels of *IGF1* in strabismic muscles likely reflects variability in the causes of STBMS [33] or a mismatch between gene expression and protein levels [34,35].

*IGF1* regulates many downstream signaling pathways by binding to the *IGF1* receptor, a membrane-bound receptor tyrosine kinase, which then results in a conformational change in the intracellular component of the *IGF1* receptor, thereby activating it [36]. Once activated, it phosphorylates many substrates, including insulin receptor substrates (IRSs) and Src homology collagen [36]. Phosphorylated insulin receptor substrate 1 binds to PI3kinase, triggering the PI3kinase-Akt signaling pathway [37]. Activated Akt protein interacts with numerous molecules to not only promote cell growth and survival in the early developmental stages but also promote regeneration and maintenance of muscles and the central nervous system [37].

As *IGF1* is known to have a role in muscle growth during development [38] and regeneration [39], any change in its expression during the critical developmental period could result in severe abnormalities, such as aberrant orbital development and EOM organization [40]. *IGF1* has been reported to regulate muscle tone in the EOM in association with other myogenic factors, such as myogenic differentiation 1, myogenin, retinoblastoma 1, cyclin-dependent kinase inhibitor 1A, cyclin-dependent kinase inhibitor 1C, and muscle creatine kinase [25].

In addition, in contrast to other skeletal muscles, EOM requires highly precise force regulation to maintain appropriate movements and alignment of the eyes [41]. It must be noted that *IGF1* is also involved in the prenatal development and maturation of the central nervous system [42]. In adults, its persistent expression has been linked to increased neurogenesis [37], while in vitro studies have shown an increase in neural progenitor cell proliferation and maintenance in neural stem cell cultures after *IGF1* treatment [43]. Other studies investigating the effect of external stimuli on promoting neurodevelopment have shown that the accelerated development of the visual cortex and, in turn, enhanced visual acuity is inhibited when treated with *IGF1* antagonists and mimicked by *IGF1* treatment in the absence of external stimuli [44]. In adults, administration of *IGF1* is reported to restore the susceptibility of cortical neurons to monocular deprivation and promote the recovery of normal visual functions by reducing the intracortical GABA levels [45]. *IGF1* also promotes motor neuron survival in developing oculomotor neurons [46]. It has also been shown that *IGF1* injection into the eye muscles increases motor neuron survival by 30% in chicks [46]. Also, *IGF1* treatment in nonhuman primates has been previously reported to result in improved eye alignment by increasing innervation density [47] and force generation in EOM [9]. The observed relative downregulation of *IGF1* in the current study could result in the disruption of the regeneration capacity of EOM. This, combined with the fact that *IGF1* also protects against muscle degeneration [48], means its downregulation could cause more degeneration, which is commonly observed in EOM in patients with STBMS.

One major limitation of this study was the smaller sample size of the control tissues, which greatly impacted the power of expression results. Our power calculations estimated the desired sample sizes of 53 and 82 muscle tissues for rs6214 and rs5742632, respectively, as shown in Figure 1B,C. Due to societal and religious barriers in the area where the study was conducted, we were unable to collect more normal EOM tissues.

Our samples were collected from the twin cities of Islamabad and Rawalpindi, both metropolitan cities host to people from various ethnic backgrounds. Whether the population in this region of Pakistan is genetically homogeneous requires extensive genetic evaluation of DNA samples. Moreover, we cannot make a claim about the homogeneity and/or heterogeneity of the samples based on only two SNPs. In an ongoing project of our research group, the authors compared genotype calls and allele frequencies of 50 SNPs spanning the different genes [data not shown]. The results showed significant differences in genotype calls and allele frequencies when compared to the reference population of Punjabi in Lahore (PJL) from 1000 Genomes data [49]. These differences might suggest that the twin cities' population is probably heterogeneous as compared to the reference PJL population. Our comparison of the genotype calls and allele frequencies with PJL data for rs6214 and rs5742632 showed a significant difference between the genotype and allelic frequencies for both SNPs (Appendix 2). This could be explained by the fact that both cities are approximately 400 km geographically apart. Moreover, due

to its geographical location, Islamabad receives a high influx of patients from other cities. Currently, the collection of ethnic background information was beyond the scope of this study; therefore, future studies with this information might indicate ethnicity-based associations among patients.

STBMS is a multifactorial disease thought to be caused by an interplay of genetic and environmental risk factors. Therefore, studying genetic predisposition based on polymorphisms in different genes related to the development of STBMS can help in understanding the genetic causes of STBMS. It is, therefore, necessary to perform replication studies of these SNPs in a larger cohort and in other populations to validate their true association. In addition, other *IGF1* SNPs residing in the coding sequence should also be analyzed to establish the role of *IGF1* in the development of STBMS, specifically its comitant form. Elucidating the genetic mechanisms of STBMS can potentially lead to the development of new therapeutic strategies to reduce the disease burden.

## APPENDIX 1. PRIMER DETAILS FOR QUANTITATIVE PCR (QPCR).

To access the data, click or select the words "Appendix 1."

## APPENDIX 2. COMPARISON OF STUDY COHORT WITH 1000GENOMES PJL DATA.

To access the data, click or select the words "Appendix 2."

## ACKNOWLEDGMENTS

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