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Molecular characterization of β-Thalassemia in Mehsud and Wazir tribes of inbreed Pashtun Population showed different patterns, indicating genetic heterogeneity for β-Thalassemia

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Abstract: β-Thalassemia (β-thal) is a monogenic disorder characterized by mutations in the HBB gene, which affect globin production, leading to hypochromic and microcytic anemia. This hereditary blood condition is marked by a decreased or absent synthesis of the β-globin chain of hemoglobin. It is notably prevalent in regions with a high prevalence of consanguineous marriages, such as the Pashtun population. The present study aimed to assess and compare the incidence of the most commonly occurring mutations of β-thalassemia in the Mehsud and Wazir tribes of Pashtun ethnicity. Additionally, the study examined the inheritance patterns of these mutations in patients and explored the level of consanguinity among parents. The findings hold potential significance for prenatal diagnosis (PND), genetic counseling, and carrier screening, which could help manage and reduce the occurrence of affected births within these tribes and the broader Pashtun population. This study involved the collection of 230 peripheral blood samples from affected patients, their parents, and siblings from families with at least one transfusion-dependent child, as well as sporadic patients from the Mehsud tribe. These samples were collected from various areas of the South Waziristan tribal district in the Mehsud region of Khyber Pakhtunkhwa (KP), Pakistan. A similar approach was followed for collecting 230 blood samples from the Wazir tribe, residing in different areas of the Frontier Region (FR) Bannu, KP, Pakistan. All samples were analyzed using the Amplification Refractory Mutation System Polymerase Chain Reaction (ARMS-PCR) technique to detect the six most common β-thalassemia mutations prevalent among Pashtun populations. The findings revealed some unique characteristics, particularly within the Mehsud tribe. The most prevalent mutation in this tribe was codon 41/42 (–TTCT), followed by frameshift codons FSC 8/9 (+G) (HBB: c.27_28insG), IVS-I-5 (G>C), and FSC-5 (–CT). Interestingly, the mutation spectrum in the geographically adjacent Wazir tribe differed. In this tribe, IVS-I-5 (G>C) was the most frequently detected mutation, followed by FSC-5 (–CT), among the six reported mutations. The findings of this study reveal distinct heterogeneity in the mutation patterns of β-thalassemia between the geographically adjacent Mehsud and Wazir tribes of the Pashtun ethnicity. These results highlight the importance of tailored parental counseling to address disease recurrence effectively. Furthermore, they emphasize the need for large-scale mutation screening and comprehensive prenatal diagnostic efforts within the Mehsud and Wazir tribes, as well as the broader Pashtun population.

Keywords: Hemoglobin (Hb), Prenatal Diagnosis (PND), beta-thalassemia (β-thal).

1. Introduction

β-Thalassemia is an inherited blood disorder characterized by diminished or absent synthesis of the β-globin chain within hemoglobin. Its prevalence is notably high in regions with a tradition of consanguineous marriages, as seen within Pashtoon populations. This research paper aims to investigate and compare the molecular characteristics of β-thalassemia in two Pashtun tribes, Mehsud and Wazir, to assess whether genetic heterogeneity exists within the affected population. Beta-thalassemia is an autosomal recessive genetic disorder affecting the red blood cells, characterized by the mutation in the beta-globin present on the short arm (p) of the 11th chromosome represented by 11p15.4-11p15.5 [1]. The history of β-thalassemia dates to ancient times, although the condition was not formally recognized and understood until more recent centuries. While the precise etiology of β-thalassemia was not known, historical records suggest that the disease was prevalent in various populations around the Mediterranean region and parts of Asia. Particularly in areas where malaria was endemic, individuals carrying β-thalassemia mutations had a survival advantage against malaria, contributing to the disease's persistence in certain populations. The term "thalassemia" was first introduced by the American hematologist Dr. Thomas B. Cooley in 1925. He described a severe form of chronic anemia that affected individuals of Mediterranean descent, particularly those of Greek and Italian origin. Cooley coined the term Cooley's Anemia to refer to this inherited blood disorder, which is now known as β-thalassemia major. The duplication of the β-globin gene consists of three coding segments (exons) positioned between codons 30 and 31, spanning a length of 1,600 base pairs. Any alteration in the β-globulin gene causes a reduction (β+) or absence (β0) of a β-globulin chain within the hemoglobin tetramer, leading to a disturbance in the transport of oxygen throughout the body[2]. The precise global prevalence of β-gene mutations remains uncertain; however, the Itha Genes database reports approximately 400 distinct forms of β-thalassemia mutations documented worldwide, with 35 of them being more prevalent in Pakistan [3-5]. Studies in molecular genetics of various thalassemia populations indicate that each group tends to exhibit its unique assortment of mutations. The mutations most frequently identified within Pakistani populations are IVS-I-5(G-C), FSC 8-9(+G), CD 41/42(–TTCT), IVS-I-1(G-T), FSC-5(–CT), and CD 15(G-A). Roughly 3% of the global populace carries the β-thalassemia gene, while in Pakistan, approximately 9,000 thalassemia patients (5-8%) are born in each ethnic group annually [6]. In the mid-20th century, different research-based studies have been conducted to distinguish between alpha-thalassemia and beta-thalassemia based on their specific genetic and clinical characteristics. It was concluded from the results of the research studies that thalassemia encompassed a group of disorders characterized by a deficiency in either the alpha-globin or beta-globin chains of hemoglobin. It was further investigated that beta-thalassemia was particularly prevalent in regions where consanguineous marriages were common. The genetic inheritance pattern of beta-thalassemia made it more likely for individuals to be affected if both parents carried a beta-thalassemia mutation, a situation that is more likely to occur in populations with a history of intermarriages. The precise frequency of β-thalassemia mutations within the human population is essential for implementing effective health control programs targeting the disease. Through initiatives such as population screening and informative sessions with parents to raise awareness about the potential risk of genetic disorders as well as pre-birth testing, numerous countries around the world have successfully decreased the incidence of homozygous β-thalassemia [7]. This research-based study aims to investigate and compare the molecular characteristics of β-thalassemia in two Pashtun tribes, to assess whether genetic heterogeneity exists within the affected population. The current study aims to evaluate and compare the incidence of the most frequently occurring beta-thalassemia mutations in the geographically adjacent Mehsud and Wazir tribes of Pashtun Ethnicity. Additionally, the current study also aims to analyze the inheritance pattern of these mutations in patients and

explore the consanguinity among the parents in these tribes. Moreover, the current study holds the potential valve for various aspects such as prenatal diagnosis (PND), genetic counseling, and carrier screening. These applications can contribute to the reduction of affected births within tribes and the whole Pashtun Population. Furthermore, understanding the genetic diversity of β-thalassemia in Pashtun tribes can provide insights into disease management and inform targeted prevention strategies. The analysis of thalassemia blood samples involves a combination of hematological, electrophoretic, and molecular techniques to ensure accurate diagnosis and identification of specific mutations. Genetic counseling and prenatal diagnosis play a vital role in managing thalassemia in affected families. Additionally, carrier screening programs are instrumental in identifying individuals at risk and facilitating the implementation of preventive measures.

2. Methodology

This study involved a comprehensive molecular analysis of β-thalassemia in individuals from the Mehsud and Wazir tribes. Blood samples were collected from affected individuals, and genomic DNA was extracted for genetic analysis. Thalassemia blood sample analysis requires strict quality control measures to ensure accurate and reliable results. Consistent calibration of instruments and participation in external proficiency testing programs are essential to maintain the quality of the analysis. In this study, Polymerase Chain Reaction (PCR) was performed to amplify the β-globin gene, and DNA sequencing was carried out to identify mutations responsible for β-thalassemia. A total of 460 peripheral blood samples comprised 230 samples each from the Mehsud and Wazir tribes. The specimens were collected from individuals who were impacted by the condition, along with their parents and siblings. These samples were gathered from families that had at least one child dependent on transfusions, as well as sporadic cases. The population of the study comprised 265 (57.5 %) males and 195 (42.5 %) females. The samples were preserved in a 5 ml anticoagulant vacuum-sealed EDTA (Ethylenediamine tetra acetic acid) tube. The samples were acquired from the Thalassemia Center for Women and Children in Bannu, KP, Pakistan. Relevant clinical and demographic information, such as the frequency of blood transfusions, symptoms and signs of thalassemia, age, gender, and family history, was collected directly from patients through in-person interactions at the facility. A consent form was signed by the patient and their family member before the data collection [6]. Ethical approval was obtained from the Bio-Ethics committee of the University of Science and Technology Bannu, KPK, Pakistan [8]. The DNA extraction was performed in the laboratory of Human genetics, situated in the biotechnology department at the University of Science and Technology Bannu, KP, Pakistan. The extraction of genomic DNA was carried out using the organic (phenol/chloroform) method. [9].The Amplification Refractory Mutation System Polymerase Chain Reactions (ARMS-PCR) technique was utilized. This involved the use of six common allele-specific ARMS primers, along with control A, B, and C primers (serving as internal control forward and reverse primers), to detect β-thalassemia mutations (refer to Table 1). Initially, a pair of internal control primers, referred to as Control A and Control B (forward and reverse), along with Control C (forward), were used. In the context of this method, either a mutant (Mt) or a normal (N) allele-specific sequence would serve as the reverse primer. A PCR reaction mixture of 20 μL was prepared, comprising the following components: 0.4 μL (2.5 units) of Taq DNA polymerase, 0.5 μL of deoxynucleotide triphosphates (0.2 mM), 1 μL (100 ng) of genomic DNA, 2 μL of 1X Taq polymerase buffer (containing 10 mM Tris-HCl at pH 9.0, 1.2 μL (1.5 mM) of MgCl2, 50 mM KCL, and 0.1% Triton X-100), and 0.4 μL (10 pmol) of each of the four primers (Controls A, Control B, Common C, and either Mt or N). The remaining volume was filled with PCR water, and the mixture was gently mixed. A total of 27 cycles were performed, beginning with initial denaturation at 94°C for 1 minute, followed by

annealing at 65°C for one minute, and then extension at 72°C for one and a half minutes. The first cycle consisted of denaturation at 95°C for six minutes and the final extension at 72°C for 10 minutes. The amplified PCR products were subsequently subjected to electrophoresis on a 2% agarose gel, which was run for 35 minutes at 110 volts.

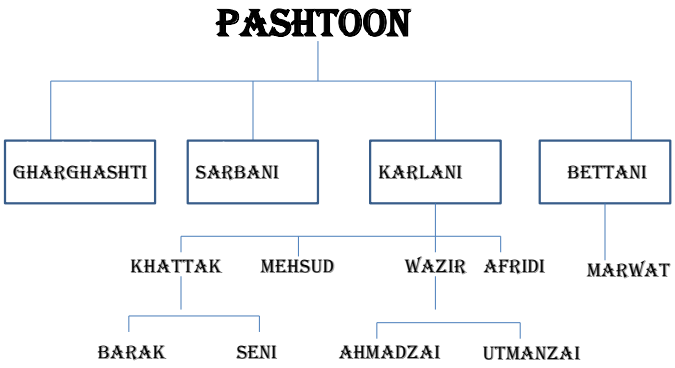

Figure 1. A family tree for the Pashtoon Ethnic group.

Table 1. Allele-specific primers for amplification refractory mutation system, polymerase chain reaction, and allele were used in this study.

Primers	Sequences (5' > 3')	Product Size (bp)
Control A	CAATGTATCATGCCTCTTTGCACC	861
Control B	GACTCAAGGCTGAGAGATGCAGGA	861
Common C	TCACTTAGACCTCACCCTGTGGAGCCAC	-
Codons 41/42 (Mt)	GAGTGGACAGATCCCCAAAGGCCTTGTTAG	439
Codons 41/42 (N)	GAGTGGACAGATCCCCAAAGGACTCAAAGA	-
IVS-1-5 (Mt)	CTCCTTAAACCTGTCTTGTAACCTTGTTAG	285
IVS-1-5 (N)	CTCCTTAAACCTGTCTTGTAACCTGATACGAAA	-
IVS-1-1 (Mt)	TTAAACCTGTCTTGTAACCTTGATACGAAA	280
IVS-1-1 (N)	GATGAAGTTGGT GACGCCCRG GGTAGG	-
FSC 8/9 (Mt)	CCTTGCCCCACAGGGCAGTAACGGCACACC	215
FSC 8/9 (N)	CCTTGCCCCACAGGGCAGTAACGGCACACT	-
Codon 15 (Mt)	CACCAACTTCATCCACG5TCACCTTGGCCT	500
Codon 15 (N)	CACCAACTTCATCCACGTTCACCTTGGCCC	-
FSC-5 (Mt)	ACAGGGCAGTAACGGCAGACTTCTACTCG	170
FSC-5 (N)	ACAGGGCAGTAACGGCAGACTTCTCATCAG	-

IVS: Intervening sequence; FSC: frameshift codon; Mt: mutant; N: normal

3. Results

3.1 Molecular Characterization of β-Thalassemia in Mehsud and Wazir tribes

The molecular characterization of β-thalassemia in the Mehsud and Wazir tribes has revealed distinct patterns of mutations. Several known mutations were detected in both tribes, including point mutations and small deletions in the β-globin gene. However, the prevalence of specific mutations exhibited differences between the two tribes, indicating genetic heterogeneity in β-thalassemia. A total of 920 alleles, with 460 alleles from both Mehsud and Wazir tribes each, were investigated for the presence of six β-thalassemia mutations. Among these six beta-thalassemia mutations, four mutations were found to be most prevalent in the Mehsud tribe. As indicated in Table 2, among the 230 samples, 82

individuals were identified as Homozygous for four β-thalassemia mutations while 126 were reported heterozygous for the same mutations. The data shown in Table 2 also revealed that ARMS-PCR could not characterize 13 individuals (9.59%) while 9 individuals (3.42%) did not exhibit any mutation on the β-globulin gene. The HBB: c.126_129delCTTT mutation was observed to be significantly prevalent in both heterozygous and homozygous states. The results shown in Table 3 revealed that the occurrence of HBB: c.126_129delCTTT mutation was the highest (36.9 %) followed by HBB: c.27_28insG mutation (32 %), HBB: c.92+5G>C (21.8 %), and HBB: c.17_18delCT (9.3 %)within the studied area.

Table 2. Homozygous, Heterozygous, β-thal mutations, uncharacterized mutation & normal alleles were reported in the studied individuals from the Mehsud region, KP province Pakistan by the present study.

S. No	HbVar Nomenclature	Mutations	Number of homozygous individuals	Number of heterozygous individuals	Uncharacterized Patients	Number of homozygous normal individuals
1	HBB: c.126_129delCTTT	Codons 41/42(–TTCT)	31	45	-	-
2	HBB: c.27_28insG	FSC 8/9(+G)	27	39	-	-
3	HBB: c.92+5G>C	IVS-I-5(G>C)	17	29	-	-
4	HBB: c.17_18delCT	FSC-5(–CT)	7	13	-	-
5	-	Uncharacterized patients	-	-	13	-
6	-	homozygous normal individuals	-	-	-	9
Tota	-	-	82	126	13	9

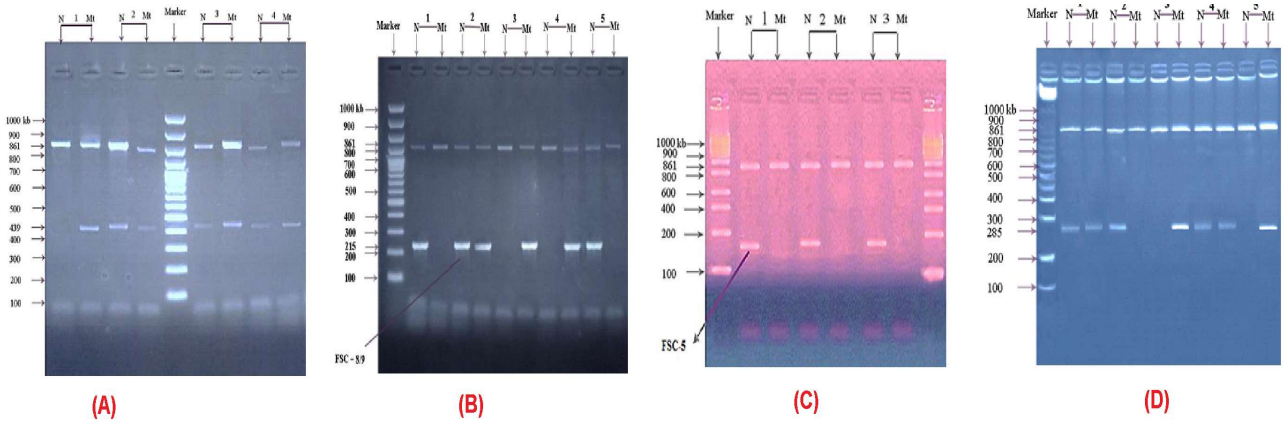

Figure 1: illustrates the ARMS PCR process and the subsequent electrophoresis analysis employed for detecting (A) Codons 41/42 mutation (B) FSC 8/9 mutation (C) FSC-5 mutation (D) IVS-I-5 mutation

Table 3. The frequency of mutant alleles in the studied individuals from the Mehsud tribe.

S. No.	HbVar Nomenclature	Mutations	Homozygous	Heterozygous	Total No. of mutant alleles (n)	Frequency (%)
1	HBB: c.126_129delCTTT	Codons 41/42 (–TTCT)	31	45	107	36.9
2	HBB: c.27_28insG	FSC 8/9(+G)	27	39	93	32
3	HBB: c.92+5G>C	IVS-I-5(G>C)	17	29	63	21.8
4	HBB: c.17_18delCT	FSC-5(–CT)	7	13	27	9.3
Total			82	126	290	100.00%

Molecular analysis for the Wazir tribe was interestingly different from the closely situated Mehsud tribe and reported IVS-I-5 (G>C) to be the most recurrent followed by FSC-5(–CT) among the six reported mutations. It is evident from Table 4 that out of 230 samples, 60 individuals were found Homozygous for two β-thalassemia mutations i.e., IVS-I-5(G>C) and FSC-5(–CT) while for the remaining two mutations only 16 individuals were found homozygous. The data in Table 4 also revealed that 14 individuals (6%) were un-characterized by ARMS-PCR while 13 (5.7%) did not show any mutation on the β-globulin gene. The percentage of HBB: c.92+5G>C mutation was found to be higher in both hetero and homozygosity. The results shown in Table 5 revealed that the frequency of HBB: c.92+5G>C mutation was seen higher (39.8 %) followed by HBB: c.17_18delCT mutation (35 %), HBB: c.126_129delCTTT (16.8 %) and HBB: c.27_28insG (8.4 %) in the studied area.

Table 4. Homozygous, Heterozygous, β-thal mutations, uncharacterized mutation & normal alleles were reported in the studied individuals from FR Bannu region, KP province Pakistan

S. No	HbVar	Mutations	homozygous individuals	heterozygous	Uncharacterized Patients	homozygous normal
1	HBB: c.92+5G>C	IVS-I-5(G>C)	32	45	-	-
2	HBB: c.17_18delCT	FSC-5 (–CT)	28	40	-	-
3	HBB: c.126_129delCTTT	Codons 41/42 (–TTCT)	11	24	-	-
4	HBB: c.27_28insG	FSC 8/9 (+G)	5	13	-	-
5	-	Uncharacterized patients	-	-	14	-
6	-	homozygous normal individuals	-	-	-	13
Total	-	-	80	123	14	13

Table 5. The frequency of mutant alleles in the studied individuals from the Wazir tribe

S. No.	HbVar Nomenclature	Mutations	Homozygous	Heterozygous	Total	Frequency (%)
1	HBB: c.92+5G>C	IVS-I-5(G>C)	32	45	109	39.8
2	HBB: c.17_18delCT	FSC-5(–CT)	28	40	96	35
3	HBB: c.126_129delCTTT	Codons 41/42(TTCT)	11	24	46	16.8
4	HBB: c.27_28insG	FSC 8/9 (+G)	5	13	23	8.4
Total	-	-	76	122	274	100

Codons 41/42 mutation

4. Discussion

β-thalassemia is one of the most significant and extensively studied genetic disorders worldwide. Approximately 80 million individuals with beta-thalassemia have been recorded globally, with 23,000 major β-thalassemia patients being born every year [10]. β-thalassemia is highly prevalent in various groups of Pakistan. Roughly, five thousand (5-6 %) thalassemic patients are born annually in Pakistan [11]. There are several types of thalassemia gene mutations, each associated with different clinical presentations and levels of severity. The major categories of thalassemia gene mutations include β-Thalassemia Mutations, α-Thalassemia, and other globin chain mutations. Some of these mutations include the β-Thalassemia trait, point mutation, deletion mutation, silent carrier state mutation, α-Thalassemia trait, and non-deletion α-mutation. Almost 400 β-thalassemia alleles have been identified, with about 40 mutations accounting for 90% of the global β-thalassemia mutations [12]. Geographically, the distribution of β–thalassemia gene mutations is highly diverse among different ethnic groups. Each ethnic group and family tends to have a specific common set of mutations [13]. The gene mutation for β–thalassemia in the Asia subcontinent is very unusual and over 40 different mutations have been

recognized [14]. About 35 beta-thalassemia alterations have been defined in Pakistan five of them are very common and make 90 % of the total mutated genes (i.e., HBB: c. 27_28 ins G, HBB: c. 92+5G > C, HBB: c. 126_129delCTTT, HBB: c.92+1 G > T & HBB: c. 17_18 del CT) [14]. The current treatment of β-thalassemia such as blood transfusions, chelation therapy, bone marrow transplant, and gene therapy is not only costly but not sufficient to eliminate the disease. Prenatal diagnosis in Pakistan has been available since 1994 enabling the identification of fetal or mutated genes in pregnant mother fetuses [15]. If β-thalassemia mutations (homozygous or compound heterozygous) are detected, the parents make decisions regarding whether to continue with the pregnancy or terminate [16]. The ARMS-PCR procedure using allele-specific primers has been used extensively to facilitate prenatal diagnosis. The high prevalence of β-thalassemia patients and carriers in developing countries can be attributed to factors such as limited public awareness and inadequate facilities, including thalassemia centers for molecular diagnostics, premarital screening, and prenatal screening [7, 15]. The current study presents four common mutations (as shown in Table 2) within the Mehsud tribe, revealing deviations from previous findings [17][19]. According to the current research, the HBB: c.126_129delCTTT gene mutation exhibits the highest prevalence (38.35%) among the Mehsud tribe which contrasts the previous findings in the Charsada and Kohat regions of KPK, Pakistan [8][20]. Results obtained for the Wazir tribe were different from above, and interestingly IVS-I-5 (G > C) mutation was reported as the most frequent, followed by FSC-5 (–CT), among the six mutations reported. Mehsud and Wazir are subtribes of the Pashtun group situated geographically and sharing Pashtun ethnicity. Both tribes originated from Afghanistan and exhibit a high degree of consanguinity. While they share several social and cultural attributes and have interactions, the intriguing divergence in the molecular patterns of beta thalassemia highlights a clear distinction in molecular heterogeneity [20]. The observed genetic heterogeneity in β-thalassemia within the Mehsud and Wazir tribes can be attributed to the practice of consanguineous marriages which increases the likelihood of inheriting identical recessive alleles. This results in a higher prevalence of specific mutations within distinct subgroups. The study findings suggest that the Mehsud and Wazir tribes have unique genetic backgrounds that influence the spectrum of β-thalassemia mutations present in each group. Understanding the molecular characteristics of β-thalassemia in specific Pashtun tribes is crucial for implementing effective disease management strategies. Population-based screening programs can be tailored to the unique mutation profiles within each tribe, enabling early detection and intervention. Additionally, prenatal genetic counseling can play a vital role in reducing the incidence of affected births. These findings need serious consideration in implementing parental meetings about disease recurrence in the future, large-scale mutation screening, and PND for the Population of Mehsud and Wazir tribes and the whole Pashtun ethnicity as well.

5. Conclusions

This research-based study will provide valuable insights into the molecular characterization of β-thalassemia in the Mehsud and Wazir tribes of the inbred Pashtun population. The observed genetic heterogeneity underscores the importance of conducting tribe-specific studies to tailor prevention and management approaches effectively. The findings of this study contribute to the broader understanding of β-thalassemia genetics and its implications for public health initiatives in consanguineous populations. Furthermore, this study explains the demarcation of molecular heterogeneity of Beta Thalassemia and elaborates that the distribution of mutations is not the same in adjacently situated tribes of inbreed Pashtun Population. While implementing programs like parental meetings about disease recurrence in the future, large-scale mutation screening, and PND in Pashtun Population, these findings must be considered carefully.

Ethics approval and consent to participate

Consent was taken from all patients for blood collection. All the patients agreed to publish their data. All the patients expressed their willingness to take part and share their data. Following the assurance of privacy and addressing social considerations, the institutional ethical committee provided approval from an ethical committee.

Conflicts of Interest

The authors declare no conflicts of interest.

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