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Integrated Bioinformatic Approach for Precision Medicine: Prediction of Human GABRG2 Gene Pathogenic Variants, Characterized with Cellular Pathology and Epilepsy Phenotype Severity

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Abstract: Encoding for the γ2 subunit of inhibitory GABA (A) receptors, GABRG2 gene expression is widespread in the brain including cortex, hippocampus, cerebellum and nuclei of brainstem. Pathogenic variants of GABRG2 have been associated with epilepsy syndromes however, the difficulty in interpreting GABRG2 variants of unknown significance hinders the advancement of epilepsy precision medicine. Using computer algorithms, our study focused on 156 GABRG2 variants of unknown significance from ClinVar database, predicting 10 highly pathogenic variants within the γ2 (γ2S isoform) subunit. Integration with patient mutations and mutagenesis studies locates these variants within 'epileptogenic structural cassettes' of the γ2 subunit, aiding characterization of phenotype severity and cellular pathology. Our results predict milder phenotypes for N-terminus extracellular domain variants (S155F, C190F, M199T) and more severe phenotypes for transmembrane domain variants (Y280D, G308D, T310I, T314K, T317S, C342Y, Y460C), linked to cellular pathology with reduced cell surface expression and reduced cell current. Notably, 4 transmembrane domain variants (G308D, T310I, T314K, T317S in the receptor's pore-lining M2 region) may distort channel conductance. Our research aligns with ACMG/AMP criteria PP3.

Hassas Tıp için Bütünleşik Biyoinformatik Yaklaşım: İnsan GABRG2 Geninin Hücre Patolojisi ve Epilepsi Fenotip Şiddeti ile Karakterize Patojenik Varyantlarının Tahmini

Anahtar Kelimeler
GABA (A) reseptörü,
Gama-2 alt birimi,
Epilepsi
Hassas Tıp,
Patojenik varyant,
İn siliko

Öz: GABA(A) reseptörlerinin γ2 alt birimini kodlayan GABRG2 geninin ifadesi, korteks, hipokampus, serebellum ve beyin sapı çekirdekleri dahil olmak üzere beyinde geniş bir alana yayılmıştır. GABRG2 geninin patojenik varyantları epilepsi sendromları ile ilişkilendirilmiştir, ancak bilinmeyen öneme sahip GABRG2 varyantlarını yorumlamanın zorluğu, epilepsiye yönelik hassas tıbbın ilerlemesini engel teşkil etmektedir. Bilgisayar algoritmaları kullanılarak yapılan çalışmamızda, ClinVar veritabanındaki klinik önemi bilinmeyen 156 GABRG2 geni varyantına odaklanıldı ve γ2 (γ2S izoformu) alt biriminde bulunan 10 varyant patojenik olarak tahmin edildi. Hasta mutasyonları ve mutagenez çalışmalarıyla entegrasyon sonucunda varyantların γ2 alt biriminde 'epileptojenik yapısal kasetler' içinde konumlandırılmasıyla, fenotip şiddeti ve hücresel patoloji karakterize edildi. 3 tane N-terminus ekstrasellüler bölge varyantı (S155F, C190F, M199T), az şiddetli ve 7 tane transmembran bölge varyantı (Y280D, G308D, T310I, T314K, T317S, C342Y, Y460C) daha şiddetli epilepsi fenotipleri ile beraber azalmış hücre yüzeyi ifadesi ve azalmış hücresel akımla bağlantılı olarak öngörüldü. Özellikle, 4 transmembran bölge varyantının (Reseptörün kanal poruna katkıda bulunan M2 bölgesindeki G308D, T310I, T314K, T317S) kanal iletkenliğini bozabileceği belirlendi. Araştırmamız, ACMG/AMP kriterlerinden PP3 ile uyumludur.

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1. Introduction

Critical for the regulation of spike timing and modulation of neuronal rhythms, GABAergic interneurons appear to have an important role in brain information processing [1–4]. This depends on the precise matching of input signals from the diverse repertoire of GABAergic interneurons, with their molecular counterparts, the Gamma-Aminobutyric acid type A receptors ($GABA_ARs$)[5–9], located in distinct zones of the postsynaptic and extrasynaptic membrane [10–14]. This differential localization, along with other diverse features of receptor subtypes have specific physiological functions important during health and disease [15–20]. Thus, any alteration in the assembly, trafficking and cell surface expression of $GABA_ARs$ may cause the deterioration of the GABAergic process, posing a risk for a wide variety of psychiatric and neurological disorders [13,21–26], including epilepsy[27], a complex neurological condition characterized by recurrent unprovoked seizures [28].

Assembled from a large subunit pool (α1-6, β1-3, γ1-3, δ, ε, θ, π, ρ1-3), $GABA_ARs$ are GABA gated heteropentameric chloride channels and primary sites for brain inhibition[21]. They are composed of five subunits, with the prevalent subtypes typically consisting of two α subunits, two β subunits, and one γ2 subunit [21]. In recent years, advancements in cryo-electron microscopy (cryo-EM) have driven a series of breakthroughs in the structural biology of $GABA_ARs$ [16,29–35]. The receptor has a cylindirical shape, formed by the spatial arrangement of subunits such as β-α-β-α-γ in the counter-clockwise direction when observed from the extracellular space [22]. Of particular significance is the human γ2 subunit [37–39], which is encoded by the GABRG2 gene located on chromosome 5q34 [21]. The γ2 subunit containing receptors mediate fast phasic inhibition [15]. This subunit exhibits significant expression in both developing and mature brain and about 60% of all $GABA_ARs$ coassembly constitutes the γ2 subunit [40]. Studies of heterozygous γ2 knockout mice have shown a 25% reduction in γ2 subunits in the cerebral cortex, hippocampus, and thalamus, accompanied by decreased clustering of $GABA_ARs$ and increased anxiety [41]. In addition, these mice display absence-like spike-wave discharges, mild epilepsy, and altered biogenesis of the remaining wild-type γ2 subunits [42–44]. The γ2 subunit plays vital roles in various aspects of $GABA_AR$ function, such as clustering, synaptic maintenance, and current kinetics [37–39,45,46]. Indeed, via specific subunits $GABA_ARs$ have specific assembly rules, membrane localization, receptor clustering, pharmacology and plasticity [47–52,37,38,53–55,36,56]. These properties may be altered by numerous inherited or de novo mutations, which have been discovered in genes encoding the $GABA_AR$ subunits, including the GABRG2 gene, associated with a wide range of epilepsy conditions manifesting mild to severe phenotypic features [57–62,26,63]. By binding at the specific sites located in the $GABA_ARs$ subunit domains or subunit interfaces, many clinical central nervous system (CNS) drugs function by enhancing $GABA_AR$ mediated inhibition[56]. For instance, Phenobarbital has been used to treat epilepsy for more than 100 years[64,65]. Phenobarbital binds to the γ-β interface and α-β interface[66].

Genetic testing is an integral component of epilepsy diagnosis [67,68]. However, in recent years, the genetic testing performed by next generation sequencing has led to the accumulation of variants of unknown significance (VUS), necessitating their interpretation [69]. Consequently, American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology (AMP) have established a framework for interpreting the clinical significance of genetic variants [70]. This framework categorizes the variants as “Pathogenic”, “Likely pathogenic”, “Uncertain significance” “Benign”, or “Likely benign” [70]. According to this framework, in silico methods, which use computational predictions to assess variant effects, are considered as supporting evidence for pathogenicity, corresponding to criterion PP3 [70]. Accumulating literature show successful integration of VUS, interpreted as damaging or deleterious by in silico methods [71,72], into the system of epilepsy diagnosis and management [68,72–74]. Given the importance of the GABRG2 gene variants described so far, this study specifically focuses on the comprehensive in silico analysis of the VUS detected in the coding region of the γ2 subunit. Through this analysis, the potential impact of these VUS on protein structure and function is assessed and elaborated through the integration with the data from the epilepsy patient mutations.

2. Material and Method

Methods are given in the **Appendix A.**

3. Results

3.1. Workflow

The structured analysis comprises several steps as detailed in **Figure 1**. Initially, GABRG2 variants were accessed from NCBI ClinVar database [75]. These variants underwent comprehensive assessment using a set of algorithms, namely SIFT[76], PANTHER [77], Polyphen-2 [78], PhD-SNP [79], and SNPs&GO [80]. These algorithms utilized a homology based analysis (expect for the PolyPhen2, which is based on both sequence and structural parameters)[71]. Following this, functional and stability analysis was performed with the help of MutPred2 [81] and I-Mutant 2.0 [82]. Evolutionary conservation scores were obtained via ConSurf algorithm [83–85] to gauge residue importance leading to the selection of 20 VUS, which

were then subjected to three-dimensional (3D) structural modeling for validation. The results (10 variants) were integrated with structural, functional data, in addition to epilepsy patient data from the literature.

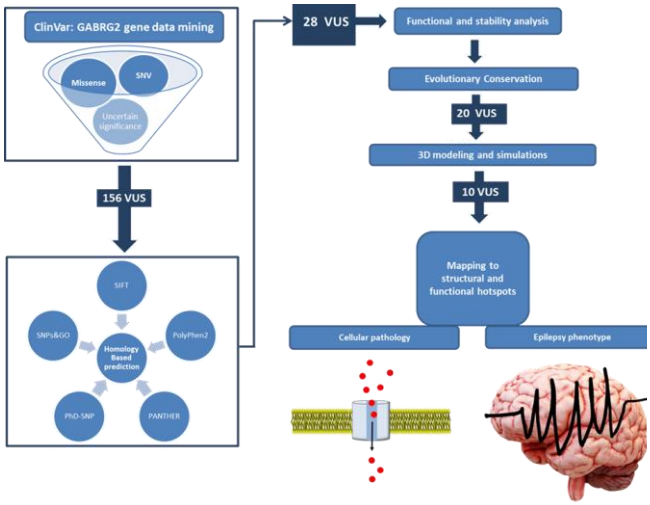

Figure 1. The general overview of the study.

3.2. Data mining

The variant data of GABRG2 transcript variant 2, encoding for the shorter isoform of the γ2 subunit (or γ2S) were classified according to classification criteria of ClinVar database [75]. This classification is based on the ACMG/AMP variant classification system[70] and other standards (https://www.ncbi.nlm.nih.gov/clinvar/docs/clinsig/#standard accessed 10.07.2024) . As seen in the **Figure 2.A** , a total of 588 GABRG2 variants are classified according to "Clinical significance", "Molecular consequence", and "Variation type". Among them, 494 are "Single Nucleotide Variants", and the rest include "Deletions" (64), "Duplications" (28), and "Insertions" (14). In terms of "Molecular consequence", there are 207 variants in untranslated regions or "UTRs", 24 are "Nonsense" variants, 18 are "Splice site" and 16 are "Frameshift". Almost half of the variants are missense (494) among the all variants (45 %, Figure 2.B). In the category of "Clinical significance", there are 156 "Missense" variants with "Uncertain significance" (VUS), as well as 14 variants, categorized as "Likely benign", 5 "Benign" variants, 12 "Pathogenic" variants, 16 "Likely pathogenic" variants, and 15 variants with "Conflicting interpretations" as shown in **Figure 2.B.**

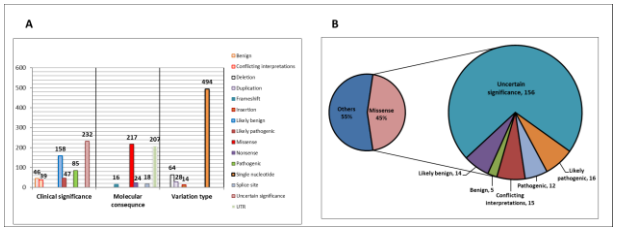

Figure 2. Profile of GABRG2 gene variants (A) Classification of GABRG2 variants according to three categories: "Clinical significance", "Molecular consequence" and "Variation type". (B) The categorization of missense variants, which constitute 45% of all GABRG2 variants, is based on their "Clinical significance", namely "Uncertain significance", "Likely benign", "Benign", "Likely pathogenic", "Pathogenic", and 'Conflicting interpretation'.

3.3. Homology-based prediction of pathogenicity for GABRG2 variants

The 156 GABRG2 VUS **(Appendix B, ClinVar)** were evaluated using SIFT [76], PolyPhen-2 [86], PANTHER [77], PhD-SNP [79], and SNPs&GO [80]. (SIFT found 80 deleterious variants with a score ≤ 0.05, while PolyPhen-2 marked 66 as probably damaging (scores close to 1). PANTHER [77] identified 126 as Likely damaging based on evolutionary conservation. PhD-SNPs [79] predicted 84 as Disease, and SNPs&GO labeled 57 as Disease. SNPs&GO had the highest number of Benign predictions (98), followed by SIFT (76), PhD-SNP (72), and PolyPhen2 (60). Additionally, PANTHER [77] and PolyPhen2 [86] identified 30 and 29 Likely pathogenic variants, respectively. It is important to note that the variant classification terms used in the prediction tools are dissimilar and do not correspond to the terms of 5-tier ACMG system [70]. For instance "Pathogenic" which is the classification category in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/docs/clinsig/#standard accessed 12.07.2024) is based on ACMG system[70]. It represents clinically validated category with comprehensive evidence and may be used for direct medical decision-making. For clarity, this and other ClinVar variant classifcation categories are shown with double quotation marks ("…") in the entire manuscript. However when a prediction tool classifies a variant as 'Pathogenic', 'Disease', 'Damaging', or 'Deletereious', it is an algorithm-based prediction suggesting potential harm, requires further validation by additional evidence and clinical correlation to be used in decision-making. Therefore, in this manuscript, only ClinVar categories were written with double quotation ("…") marks. In contrast, other terms, specifically in silico prediction terms, were capitalized as proper nouns or, if they were not terms but adjectives, they were written in lowercase, such as the adjective 'pathogenic'. The algorithmic prediction results presented in the **Figure 3** summarizes the classification under three categories: Deletereious, Possibly damaging, and Neutral, which represent classifications of in silico assessments (SIFT [76], PolyPhen-2 [86], PANTHER [77], PhD-SNP [79], and SNPs&GO [80]). To identify the most pathogenic variants, a filter was applied: variants had to meet specific criteria, including being predicted as damaging or intolerant by at least four tools, with a SIFT score of 0 and a PolyPhen2 score of 1. This filter identified 28 pathogenic variants (L81F, P83T, R125H, D149H, F152S, S155F, M199T, C190F,

E217G, Y220C, Y280D, V292G, G308D, I309T, I309M, T310I, I313M, T314K, T317S, V329F, Y331N, D336G, C342Y, R446C, F453L, N457Y, Y460C, and W461R). These variants are given in the **Appendix C Table 1**, together with ClinVar accession and version numbers. These variants scored 0 in SIFT, 1 in PolyPhen2, and were predicted as Probably damaging and Disease by PANTHER [77]. PhD-SNP [79] also categorized them as Disease while SNPs&GO [80] classified all as Disease, except for L81F, I309T, I309M, and L313M, which were considered Neutral.

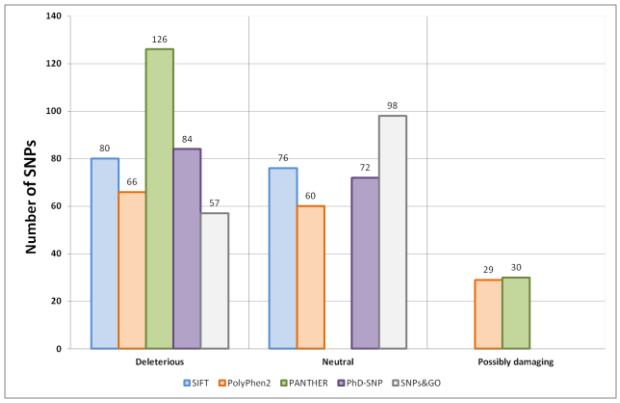

Figure 3. Deletereious, Neutral and Possibly damaging variants of GABRG2 predicted by five in silico tools (SIFT [76], PolyPhen2 , PANTHER [77], PhD-SNP and SNPs&GO [80]).

The identified 28 deleterious or pathogenic GABRG2 gene variants are mapped to the corresponding protein domains[87] of the γ2 subunit. In the NCBI database (RefSeq: NP_000807.2), this 467 amino acid long subunit is characterized with a signal peptide between the residues 1-39, N terminus extracellular domain (ECD) between the residues 90 and 271, first transmembrane domain (M1) between the amino acids 274-296, second transmembrane domain (M2) between the residues 300-322, third transmembrane domain (M3) between the residues 334 and 356, fourth transmembrane domain (M4) spanning from the amino acid residue from 444 until the 466. In addition, it has an intracellular domain (ICD) in between the residues 357-443, where it interacts with GABA(A)-receptor-associated protein (GABARAP)[88] in the region between the residues 425 and 442. The 10 of the variants, predicted as pathogenic, are located within the ECD (L81F, P83T, R125H, D149H, F152S, S155F, M199T, C190F, E217G and Y220C), two in the M1 (Y280D & V292G), 7 in the M2 (G308D, I309T, I309M, T310I, I313M, T314K, T317S), 2 variants in the linker between the M2-M3 (V329F & Y331N), 2 in the M3 (D336G & C342Y), and 5 in the M4 region (R446C, F453L, N457Y, Y460C, W461R). Specificities of these variants are summarized in **Appendix C Table 2.**

3.4. Functional and stability analysis

3.4.1. Analysis of variant effects: Molecular mechanisms

The MutPred2 [81] server is used to investigate variant impact on molecular mechanisms of γ2 subunit. To predict the relevant molecular mechanisms, the identified 28 variants were studied by submitting the amino acid sequence of GABRG2 Isoform 2 (RefSeq NM_000816.3 and NP_000807.2, NCBI database [89]) to Mutpred2 server [81]. MutPred2 is based on the machine learning approach that combines genetic and molecular data to assess the likelihood of amino acid substitutions being pathogenic. It works by offering two key features: a general prediction of pathogenicity and a ranked list of specific molecular changes that could potentially impact the phenotype. The method is trained on a dataset comprising 53,180 pathogenic variants and 206,946 unlabeled variants (assumed to be neutral) sourced from the Human Gene Mutation Database (HGMD) [90], SwissVar [91], dbSNP [89], and inter-species pairwise alignment. MutPred2 follows a series of steps to assess the impact of a substitution on protein structure and function. Mutpred2 data sets were developed for the training of various property predictors that rely on public data with different dates. For instance, protein-protein interaction datasets date back to 2012 [81]. On the other hand, the $GABA_AR$ structural data, which also present the subunit interaction interfaces for instance, have been published only recently [30,32]**.** Thus, for supporting evidence, the Mutpred2 results were integrated with the recent evidence from the literature (NCBI [89] and PDBe [92,93]) and described in the Supplementary File for MutPred Analysis (**Appendix B, MutPred)** that involves the recent structural data of $GABA_AR$ subunits [30]. This approach does not only allow a comprehensive analysis but also an opportunity to test the accuracy of the MutPred2 results.

The general score of MutPred2 [81] prediction for the 28 variants of the γ2 subunit is presented in the **Table 3**, which shows that all the 28 variants (L81F, P83T, R125H, D149H, F152S, S155F, C190F, M199T, E217G, Y220C, Y280D, V292G, G308D, I309T, I309M, T310I, L313M, T314K, T317S, V329F, Y331N, D336G, C342Y, R446Q, F453L, N457Y, Y460C, W461R) have a high probability of pathogenicity with a score higher than 0.75. Additional scores including Property score (Probability) and P value as well as the mechanisms for the pathogenicity can be found in **Appendix B, MutPred**. The property score, which ranges from 0 to 1, represents the probability of loss or gain of the specific property due to the substitution. A higher property score suggests that the alteration of that property is more likely to be involved in the molecular mechanism of the associated disease. Reflecting on these, the variant L81F has a MutPred2 [81] score 0.818. Among the candidate molecular mechanisms with a p value < 0.05, 'Altered ordered

interface' has the highest probability (0.31) (P value = 0.01). Additionally, mechanism of 'Altered transmembrane protein' (Probability score is 0.27, P value is 0.00082) was predicted. This is well reflected by structural studies: when we identified variants predicted to be pathogenic, we primarily observed that they corresponded to critical amino acid positions, and predominantly involved interface interactions. **Appendix C, Table 4** lists these residues found in the receptor subunit interaction interfaces. Interestingly, L81 is in the ECD interface of the γ2 subunit interacting with β2 or β3 subunits as described in Cryogenic electron microscopy (cryoEM) structures of human $GABA_AR$ [30,32](**Appendix C, Table 4**). Thus, the impact of L81F will likely be the distortion of interaction of the γ2 subunit with β subunits leading to the alteration of receptor oligomerization. The significance of ECD was demonstrated by a point mutation (R43Q) found in the γ2 subunit, which is linked to childhood absence epilepsy and febrile seizure[94]. The ECD consists of an N-terminal α-helix followed by a core β sandwich composed of 10 β strands, with the GABA-binding site located near the middle of the ECD. Studies show that receptor assembly process, which starts in the endoplasmic reticulum (ER), is primarily regulated by the N-terminal ECD of the subunits[95]. N-terminal deletions in the γ2 subunit impaired its incorporation into receptors [96]. Consequently, when interpreting the MutPred2 prediction, we will examine the ECD variants from this perspective. In addition to L81F, there are 9 more variants in the ECD: P83T, R125H, D149H, F152S, S155F, C190F, M199T, E217G, Y220C. Among these P83T, which has MutPred2 score of 0.895 was predicted as 'Altered ordered interface' (probability score 0.29, p value 0.03) and 'Altered transmembrane protein' (probability score 0.28, p value 0.00067) (**Appendix B, MutPred)**.

Like L81, the P83 is in the ECD interface of the γ2 subunit interacting with β2 or β3 subunits as shown in cryoEM structures of human $GABA_ARs$ [30,32] (**Appendix C, Table 4**). Thus, the variant P83T, which is located in the α-β1 loop of the γ2 subunit at γ2+/β2− subunit interface, will pose the risk of altered interaction of the γ2 subunit with β subunits leading to the alteration of receptor oligomerization. Indeed, another variant in the same position (GABRG2, P83S variant) was previously reported in a family with idiopathic generalized epilepsy, where it was observed to be associated with the seizure phenotype [97]. The mutant receptors with this variant had reduced cell surface expression owing to the altered receptor assembly and ER retention, decreased whole-cell current amplitudes and increased sensitivity to Zinc ions (Zn^{2+}), despite some inconsistent findings in the literature [62]. These likely confirm that the P83T identified in this study, may cause pathogenic effect. Similarly, the rest of the variants (R125H, D149H, F152S, S155F, E217G) and their localization in the molecular interaction interfaces verify the prediction of 'Altered ordered interface' and 'Altered transmembrane protein' although these mechanisms are not necessarily have the highest MuPred2 [81] prediction probability (**Appendix B, MutPred)**. For instance, D149H is described as 'Altered metal binding' (Probability score: 0.42) according to MupPred2 candidate mechanism for pathogenicity (MutPred2 score: 0.942, **Appendix B**). Zn^{2+}, the divalent metal cation, acts as non-competitive inhibitor of both αβ and αβγ $GABA_ARs$ [98,99] but the Zn^{2+} binding site is primarily found in the β subunit. According to structural analysis at 3.0 Å resolution, this site is formed by a group of three histidine residues positioned at amino acid position 267 within the pore lining M2 helices of β3 subunits [100]. So 'Altered metal binding' effect of D149H in the γ2 subunit is an unlikely mechanism although it has highest probability score among the candidate mechanisms **(Appendix B, MutPred).** Indeed, the distortion of the Zn^{2+} binding site, would not be expected as a mechanism relevant to epilepsy, since Zn^{2+} is an inhibitor of $GABA_ARs$[3].

On the other hand, 'Altered ordered interface' and 'Altered transmembrane protein" appears to be the possible mechanisms for pathogenic effect since D149 is a residue at the interaction interface with β2 & β3 subunits (**Appendix C, Table 4**). Same conclusions can be made for the other variants (C190F, M199T, E217G) in the ECD that "altered ordered interface" and 'Altered transmembrane protein" appears to be the possible MutPred2 predicted mechanisms (**Appendix B**) instead of 'Altered metal binding' since these ECD variants correspond to residues at the subunit interfaces (**Appendix C, Table 4**).

Taken together the mechanism of pathogenic effect of the seven ECD variants (L81F, P83T, R125H, D149H, F152S, S155F, E217G) is predicted as 'Altered ordered interface' and 'Altered transmembrane protein'. The remaining ECD variants, C190F, M199T and Y220C, will be examined separately since they do not correspond to subunit interface sites. The variant C190F is predicted as 'Altered metal binding site' (MutPred2 probability=0.6, **Appendix B, MutPred**). Similarly, M199T and Y220C are predicted as 'Altered metal binding sites' (MutPred2 probability scores are 0.29 and 0.57 respectively (**Appendix B, MutPred**). The mechanism of pathogenicity for these variants are unclear since there is not a significant metal (such as Zn^{2+}) binding site in the γ2 subunit and supporting data are required for the proposition of molecular mechanism of pathogenicity in later sections of this study (protein modeling).

The other variants analyzed by MutPred2 are located in M1 domain (V292G) in addition to those in the M2

domain (I309T & I309M, T310I, L313M, T314K, T317S), D336G and C342Y in the M3, R446Q in M4 as well as the variants in the M2-M3 linker (V329F, Y331N). The Mutpred2 score of M1 variant V292G is 0.82. Among the candidate mechanisms for pathogenic effects of the G variant in the position of 292, are "Altered transmembrane protein", "Altered ordered interface" and "Altered stability" (**Appendix B, MutPred**). Since these mechanisms are complementary each other and the variant V292G is located at the interaction interface with α1 & β3 subunit (**Appendix C Table 4**), we conclude that pathogenic effect of this variant is predicted as 'Altered ordered interface' and 'Altered transmembrane protein". The M2 domain is the region for the pore lining of the receptor channel for chloride ion to pass through [21]. The M2 region plays a role in forming the ion channel pore of the receptor, allowing the passage of ions. On the other hand, the intracellular domain (ICD) located between M3 and M4 domains, contains sites where phosphorylation occurs and interacts with other proteins, thereby influencing the function and trafficking of the channel [3, 60, 61]. For the variants predicted as pathogenic in this region, 'Altered stability" or 'Altered transmembrane protein" has the highest probability for the mechanism of pathogenicity according to MupPred2 [81] (**Appendix B, MutPred**). In the M2 domain, the variants I309T and I309M are found in the subunit interaction interface between the γ2 subunit and β2 or β3 subunits. The variant T314K in is located at the subunit interaction interface of γ2 subunit with α1 or β3 subunits (**Appendix C, Table 4**). These residues do not correspond to any variants in the M2 region identified in this study. CryoEM studies determined structural coordinates of $GABA_A$Rs that the chloride ion interacts with the residues V104, L237, Y238, Q239, F240 [34].

Interestingly, D336G in the M3 is predicted as loss of helix (P value ≤ 0.05) according to MutPred2 [81] analysis. This effect will likely cause an effect in the receptor integrity since it is located in the interaction interface with β2 & β3 subunits (**Appendix C, Table 4**). Also, the variants in the M2-M3 linker (V329F, Y331N) have the following properties: Val329 is located at the interaction interface with β2 & β3 subunits and Tyr331 is located at the interaction interface with β2 subunits (**Appendix C, Table 4**). These results are well correlated with the Mutpred2 analysis that represents "Altered ordered interface" (P value ≤ 0.05) (**Appendix B, MutPred**). Thus, these residues will likely cause a molecular mechanism that will presumably impact on receptor assembly.

M4 of the γ2 subunit is known to be critical for the postsynaptic targeting of the γ2 subunit containing $GABA_A$Rs. Initially, it was believed that the ICD of the γ2 subunit played a crucial role in postsynaptic targeting[53]. However, research has revealed that the localization γ2 subunit containing $GABA_A$Rs to postsynaptic sites primarily occurs through a mechanism that is mostly unrelated to the ICD of the γ2 subunit [14,51,53]. Instead, it relies on the presence of the γ2 subunit's C-terminal sequence, which includes the M4 [51]. Thus, the transmembrane domain (M4) —not the ICD as previously thought[14]—of the γ2 subunit appears to be important for the membrane targeting of receptor subtypes[14,51]. We have identified R446Q, F453L, N457Y, Y460C, W461R as pathogenic in the this domain. According to MutPred2 [81] results, R446Q variant causes "altered ordered interface" (P value < 0.05). Indeed, structural data show that among the variants identified in this domain only R446Q is located at the subunit interaction interface of the receptor (**Appendix C, Table 4).** Thus, this molecular mechanism of this variant effect predicted by MutPred2 seems to be reasonable. For the remaining variants, Mutpred2 results suggest 'Altered ordered interface' and 'Altered transmembrane protein' as most probable mechanisms for the variants N457Y (MutPred2 score: 0.909, P value < 0.05), Y460C (MutPred2 score: 0.942, P value < 0.05) and W461R (MutPred2 score: 0.946, P value < 0.05) and the "altered transmembrane protein" for the F453L (MutPred2 score: 0.911, P value < 0.05) **(Appendix B and Appendix C Table 3).**

3.4.2. Stability prediction

The impact of the 28 variants on GABRG2 protein stability was predicted by I-Mutant2.0[82] web server. According to I-Mutant2.0, the 28 variants considered to decrease the stability of γ2 subunit (except the S155F which predicted to increase the stability). The prediction conditions were 25 ℃ and PH=7, and the resulted DDG, which stands for the change in Gibbs free energy (ΔΔG) due to the variant. This, and the reliability index (RI), a score that indicates the confidence level of the predicted DDG value, are presented in the **Figure 4.** DDG > 0 is associated with stabilizing effect while DDG < 0 is associated with the destabilizing mutation. The variants V929G (RI= 9, DDG= -3.98), V329F (RI=9, DDG= -3.59), D336G (RI=7, DDG= -2.16), F152S (RI= 9, DDG= -2.05) and E217G (RI=7, DDG= -2.04) show the highest destabilizing effect. Furthermore, the variants R125H (RI= 9, DDG= -1.71), F453L (RI= 8, DDG= -1.56), Y331N (RI= 4, DDG= -1.51), W461R (RI= 8, DDG= -1.48), M199T (RI= 7, DDG= -1.42), P83T (RI= 8, DDG= -1.33), D149H (RI= 7, DDG= -1.13), R446Q (RI= 8, DDG= -1.08), G308D (RI= 3, DDG= -0.87), L81F (RI= 7, DDG= -0.76), C342Y (RI= 4, DDG= -0.75), Y460C (RI= 6, DDG= -0.71), L313M (RI= 5, DDG= -0.66), I309T (RI= 2, DDG= -0.66), I309M (RI= 5, DDG= -0.64), Y280D (RI= 2, DDG= -0.24), N457Y (RI= 1, DDG= -0.01) are also predicted to decrease the stability. S155F and T314K were predicted to decrease the stability with values of RI=3, DDG=-0.63 and RI=0, DDG=-0.56, respectively. On the other

hand, the variants C190F (RI= 3, DDG= 0.11), Y220C (RI= 2, DDG= 0.57), T310I (RI= 0, DDG= 0.37), T317S (RI= 2, DDG= 0.12) are predicted to increase the protein stability. However, their DDG values are near to zero and RI values are relatively low suggesting that these variants may not lead to the predicted effects. Thus, in the following steps we will include these variants in addition to other destabilizing variants.

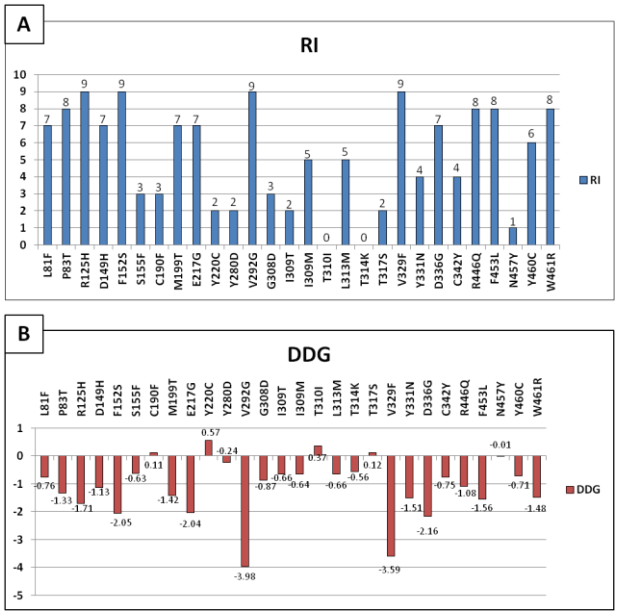

Figure 4. Stability prediction by I-Mutant server[82]. Bar charts showing the RI (A) and DDG values (B) of the 28 nsSNPs. All predicted to decrease stability except one of them (S155F). (RI= reliability index, DDG= Free energy change value, DDG>0 increase in stability, DDG<0 decrease in stability)

3.5. Evolutionary conservation profile of GABRG2 gene variants

Comparing amino acid sequences of homologous proteins can reveal crucial residues, that have likely undergone purifying natural selection, indicating their functional importance and conservation. ConSurf server, which performs a search for closely related homologous sequences [101], was used to identify the highly conserved residues. As presented in the **Figure 5** (and **Appendix C Table 5**), the variants P83T, R125H, D149H, C190F, Y280D, G308D, T310I, L313M, T314K, T317S, Y331N, D336G, C342Y, R446Q, N457Y, Y460C and W461R all share the ConSurf [101] score of 9, that considered to be the most conserved. Furthermore, it was also identified if the residue is a structural or functional residue (**Appendix C Table 5**).

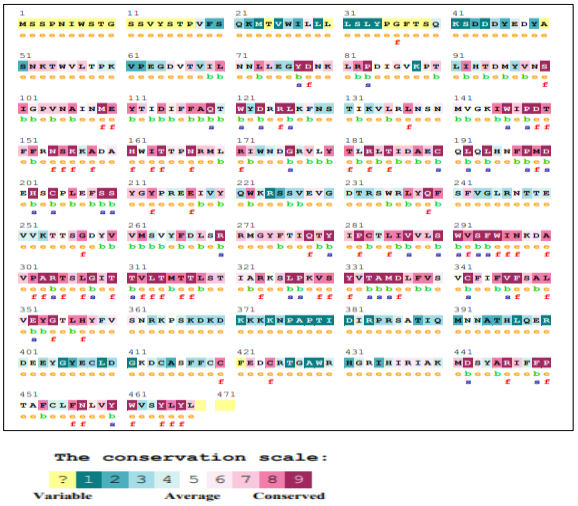

Figure 5. Conservation profile of GABRG2 residues.

Among the most deleterious 28 variants, 20 of them (P83T, R125H, D149H, S155F, C190F, M199T, E217G, Y280D, G308D, T310I, L313M, T314K, T317S, Y331N, D336G, C342Y, R446Q, N457Y, Y460C and W461R) were predicted as highly conserved structural/functional residues (**Appendix C, Table 5**). Among these 20 variants, 3 variants (S155F, M199T and E217G) scored 8 by Consurf [101] which also indicated them as highly conserved residues. These 20 variants were chosen for further investigation in this study.

3.6. Three-dimensional modeling

The utilization of 3D structural analysis of proteins has important clinical implications [72]. In our research, we identified a notable number of missense variants that were predicted to completely impair protein function. To visualize the effect of these 20 variants on GABRG2 protein structure, the wild-type GABRG2 and its mutant 3D structures were generated using Phyre2 web server [102]. The wild-type sequence and each mutation were run separately to the server. The generated structures were then submitted to the TM-align server [103] to calculate the TM-scores (template modeling scores) and RMSD (root-mean-square deviation) values for each mutant structure in alignment with the wild-type structure **(Appendix C Table 6).** TM-score [104] evaluates topological similarity between wild-type and mutant structures, while RMSD value measure the root-mean-square distance between corresponding atom pairs of the two protein models, to assess the degree of similarity of two protein 3D structures [105]. A higher RMSD value suggests a greater structural difference between the wild-type and mutant forms[105]. TM-score assigns a numeric value ranging from 0 to 1, with 1 signifying an exact match between the two structures. Only structures with highest RMSD (cut off > 3.0) among the lowest TM-scores (cut off < 0.7) were chosen leading to the identification of 10 variants (S155F, C190F, M199T, Y280D, G308D, T310I, T314K, T317S, C342Y, Y460C)

selected to be the most deleterious. The summary of the results identified so far are shown in the **Figure 6**, where 10 the most deleterious variants are highlighted with yellow box.

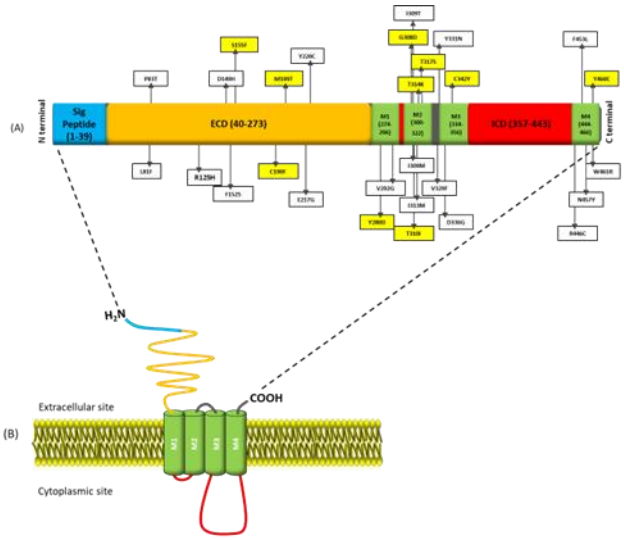

Figure 6. Graphical representation of the pathogenic variants located in the γ2**.** The diagram **(A)** showing the distribution and peptide sequence position of the initially identified 28 variants along the γ2 subunit in the cell membrane in diagram **(B).** The variants written in the yellow box in the diagram **(A)** highlights 10 variants (S155F, C190F, M199T, Y280D, G308D, T310I, T314K, T317S, C342Y, Y460C) representing the most pathogenic. (ECD: N-terminus extracellular domain, M1: First transmembrane domain, M2: Second transmembrane domain, M3: Third transmembrane domain, M4: Fourth transmembrane domain. ICD: Intracellular domain between the third and fourth transmembrane domains, Sig peptide: Signal peptide)

The 3D structures for these 10 variants were regenerated using the I-TASSER [106,107] server, which provides 5 different structures for each entry, with a C-score that ranged from -5 to 2, where a C-score of higher value signifies a model with a high confidence. Then these structures were validated by calculating their overall quality factors using ERRAT server [108] **(Appendix C Table 7).** The structures with highest C-score and highest quality factors variants (>70%, **Appendix C Table 7)** were chosen and visualized by UCSF Chimera 1.17 [109] The structure of these variants (S155F, C190F, M199T, Y280D, G308D, T310I, T314K, T317S, C342Y, Y460C) were superimposed over the wild-type structure to assess the similarity between the two models. The superimposed models are shown in the **Figure 7**. The superimposition of structures indicates whether these models share the same structure and the extent of the differences between them. As shown in **Figure 7,** the wild-type GABRG2 protein structure (represented by the yellow structure) was superimposed with the mutated GABRG2 structures for each mutation separately (shown as colored structures) and all of them resulted in RMSD values (**Figure 7**), greater than 0.5, indicating significant variations from the wild-type structure. The superimpositions, along with the RMSD values demonstrate that these mutations might significantly affect the structure of the γ2 subunit.

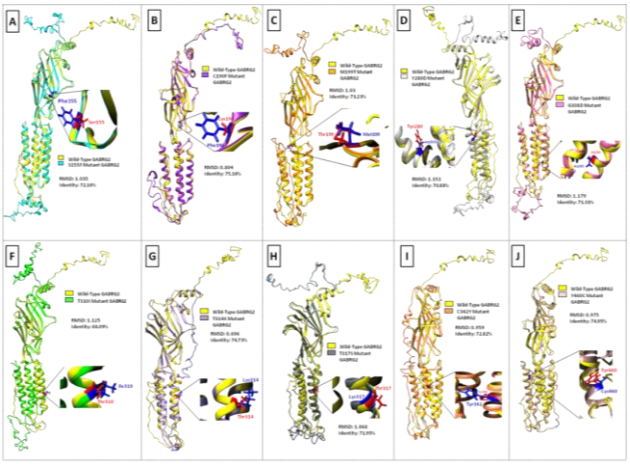

Figure 7. γ2 subunit three dimensional structures generated by I-TASSER[106,107] and visualized by Chimera [109]. The structures A-J present the variant amino acid superimposed over the wild-type amino acid (yellow).

3.7. Mapping the variants on to the structural/functional hotspots

As epilepsy research is continuously evolving, new findings shed light on the specific structural and functional alterations in the γ2 subunit that is associated with the specific cellular pathology and other characteristics of epilepsy syndromes [27,59,110–115]. Thus we also studied our results from this emerging perspective.

3.7.1. Cellular pathology

Frequent presence of mutants in key structural domains of $GABA_AR$ subunits with shared functional characteristics indicates a link between structure and function[115]. Thus, there exist "epileptogenic structural cassettes" within the $GABA_AR$ subunits [115]. $GABA_AR$ mutations contribute to epilepsy through affecting receptor assembly, trafficking, GABA binding, chloride channel function, and receptor kinetics [116]. These alterations are linked to the mutations in the specific structural domains causing the cellular pathology[115]. For instance, epilepsy mutations in the ECD of the γ2 subunit affect receptor assembly, leading to ER retention and decreased surface expression[27]. As a result, the trafficking of γ2-$GABA_ARs$ has emerged as critical in epilepsy, primarily due to frequent alterations in this process, such as inadequate subunit incorporation during assembly in the ER, which poses a limitation to forward trafficking [27,95]. Thus, we predict that pathogenic variants like S155F, C190F, M199T in the ECD will alter receptor assembly, increase ER

retention, and reduce trafficking, surface expression, and GABAergic current. Especially, the variant S155F, being at the interaction interface of γ2 subunit with β2 & β3 subunit (**Appendix C, Table 4**), has the highest possibility for this incidence. Nevertheless, for the γ2 subunit, this mechanism appears as a general pathological mechanism for many of the GABRG2 subunit mutations in differen domains [27]. As a result, the proposed pathology of all variants i.e., variants in the ECD (S155F, C190F, M199T), variant (Y280D) in the M1, variants (G308D, T310I, T314K, T317S) in the M2, variant (C342Y) in the M3 and the variant Y460C in the M4 would be reduced cell surface expression mostly via the reduced trafficking, increased ER retention, leading the reduced cell current. Notably, our previous results (**Supplementary File 3, Table 3**) support the findings for the variants T310I, T310I, T314K, T317S in the M2 since these variants are located at the subunit interaction interfaces **(Appendix C, Table 4).** Thus, for the variants (G308D, T310I, T314K, T317S) in the M2, forming the ion channel pore of the receptor[21], alteration in trafficking is expected, in addition to disturbance in the ion channel conductance. These altogether will likely manifest higher degree of alteration.

3.7.2. Epilepsy phenotype

Studies indicate that the relationship between genotype and epilepsy phenotype, especially in relation to genes that encode ion channels and receptors, is complex [74]. Despite this,

These mutations may be linked to the severity of epilepsy phenotypes [111]. Mutations in the N-terminus extracellular domain (ECD) of the receptor subunits are thought to be linked with milder phenotypes (generalized epilepsy associated with mild to moderate intellectual disability) while mutations in the transmembrane regions (M1-M4) are considered for a more severe early-onset epilepsy, with severe intellectual disability [111]. For instance, patient mutations in the pore-lining M2 region exhibited notably severe phenotypes[91]. Thus, we anticipate that among the predicted pathogenic variants, those in the ECD will manifest milder epilepsy, and transmembrane variants will result in more severe phenotypes. Specifically, we propose that based on the patient mutations described in the literature [115], the variants in the ECD (S155F, C190F, M199T, **Figure 6A**) are predicted to be associated with milder epilepsy phenotypes. The variants in the transmembrane domains (variants in the M2: Y280D, variants in the M2: G308D, T310I, T314K, T317S, variant in the M3: C342Y and variant in the M4: Y460C, **Figure 6A**) are expected to manifest severe epilepsy phenotypes. In our previous paper[117] we described the utility of HPO (The Human Phenotype Ontology)[118] for a discussion of epilepsy phenotype and genetic variation. This perspective can be used for an extended evaluation of phenotypic severity in relation to variants prediced as pathogenic.

4. Discussion and Conclusion

Our study focused on the prediction of variant impact for a set of non-synonymous single-nucleotide polymorphisms (nsSNPs) with unknown molecular consequence within the coding region of the γ2 subunit of $GABA_AR$. These variants or VUS were subjected to predictive algorithms and the variants identified with highest probability of pathogenicity were validated by protein modeling. We have predicted 10 variants as the most pathogenic. These variants are S155F, C190F, M199T Y280D, G308D, T310I, T314K, T317S, C342Y Y460C. These resultant variants were integrated with the data of epilepsy patient mutations mapping the predicted variants on the 'epileptogenic structural cassettes'[115]. Thus, these ten variants are anticipated to contribute to cellular pathology characterized by reduced trafficking, increased endoplasmic reticulum retention and reduced cell current. Further integration of epilepsy patient mutations[111] have led to the presumed phenotype severity as a consequence of variant effect. Among these, the variants in the ECD (S155F, C190F, M199T) are predicted to be associated with milder epilepsy phenotypes. The variants in the transmembrane domains (variants in the M1: Y280D, variants in the M2: G308D, T310I, T314K, T317S, variant in the M3: C342Y and variant in the M4: Y460C) are expected to manifest severe epilepsy phenotypes. Consequently, our integrative approach implies that the specific position of GABRG2 variants might potentially forecast the intensity of clinical features, as also discussed in our previous papers[117].

Our results suggest the powerful utilization of accumulating data to support our comprehensive analysis which has the potential to guide wet lab experimentation and help decision making for differential diagnosis. Differential diagnosis may benefit from neuropathological examination, but neuropathological examination for late onset epilepsy, for instance, does not have definitive guidlines [119]. Genetic testing is another way which might support differential diagnosis but prevalence of VUS is a major challange . As utilization of genetic testing represents a shift towards a more tailored and individualized approach in managing epilepsy [68], determining whether the VUS are benign or disease-causing requires a thorough assessment of their effects. As a result, the use of computer-based tools to predict the consequences of these variants, known as in silico tools, has become indispensable. The ACMG/AMP provide guidelines for variant interpretation, categorizing variants as "Pathogenic", "Likely pathogenic, "Benign", or "Likely benign". In silico methods, such as predictive algorithms, are

considered as supporting evidence for pathogenicity, as outlined in criterion PP3 [70]. Thus, our results corresponding to PP3, have implications for the clinical management of epilepsy since it aligns with this framework for the categorization the pathogenic variants. Regarding this, accumulating examples from literature provide evidence for successful integration of the PP3 criterion into the clinical decision making [68] suggesting that our in silico results presenting the presumed pathogenic GABRG2 variants have potential for aiding epilepsy diagnosis and management, especially with the available examples for integrating in silico variant prediction into the diagnostic pathway [120].

There are some limitations of this study. The use of in silico tools and reference sequences may affect prediction accuracy, warranting cautious interpretation. Generalizing from single studies should be avoided. The focus here was on nonsynonymous point mutations in the γ2 subunit coding region. However, other variants, like those in splice sites or untranslated regions (UTRs), may play vital roles in epilepsy syndromes or $GABA_AR$-related channelopathies. Computational findings indicate transcription factor recognition sites in the 5' UTR of $GABA_AR$ subunit genes, suggesting potential impact on subunit expression. Further research should explore rare noncoding region variants in the GABRG2 gene using novel tools and frameworks. Utilizing functional annotation databases and machine learning for transcriptomic profiling can enhance precision medicine. Additionally, in the present study we specifically focused on the identification of pathogenic variants however, variants that might impact the on the ligand binding sites may have consequences for drug response. For instance, there are variants such as Phe343Leu (ClinVar Accession and version number: VCV000205551.2) identified in our study (Appendix B), located at the binding site (PDB 6X3W) of the Phenobarbital[66], an antiseizure medication[64]. Also, this variant overlaps with Diazepam binding site (PDB 6X3X)[34]. Although Phe343Leu was not predicted as pathogenic in our study, it may impact on the Phenobarbital and Diazepam response of the epileptic patients. Similarly, variant Asp336Gly (ClinVar Accession number: VCV000408214.7) overlaps with Diazepam binding site[34]. Although Asp336Gly was not predicted as pathogenic in our study, it may impact on the Diazepam response of the patients medicated by this drug. Thus, conducting docking simulations to study the impact of these variants on ligand binding (e.g., Phenobarbital, Diazepam) is crucial for future research.

Appendices

Appendix A. Material and Method
Appendix B. The summary of ClinVar data for GABRG2, The MutPred2 Analysis Results
Appendix C. Table 1-7 summarizing the results in the relevant sections in the main text.

Acknowledgment

Not applicable

Declaration of Ethical Code

In this study, we undertake that all the rules required to be followed within the scope of the "Higher Education Institutions Scientific Research and Publication Ethics Directive" are complied with, and that none of the actions stated under the heading "Actions Against Scientific Research and Publication Ethics" are not carried out.

Ethical approval

Not applicable

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