Genetics in Medicine (2022) 24, 681–693

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Genetics in Medicine

www.journals.elsevier.com/genetics-in-medicine

ARTICLE

Structural mapping of *GABRB3* variants reveals genotype–phenotype correlations

ARTICLE INFO

Article history:
Received 5 November 2021
Accepted 5 November 2021
Available online 7 December 2021

Keywords:
Epilepsy
GABA
GABRB3
Genetics
Mapping

ABSTRACT

Purpose: Pathogenic variants in *GABRB3* have been associated with a spectrum of phenotypes from severe developmental disorders and epileptic encephalopathies to milder epilepsy syndromes and mild intellectual disability (ID). In this study, we analyzed a large cohort of individuals with *GABRB3* variants to deepen the phenotypic understanding and investigate genotype–phenotype correlations.

Methods: Through an international collaboration, we analyzed electro-clinical data of unpublished individuals with variants in *GABRB3*, and we reviewed previously published cases. All missense variants were mapped onto the 3-dimensional structure of the *GABRB3* subunit, and clinical phenotypes associated with the different key structural domains were investigated.

Results: We characterized 71 individuals with *GABRB3* variants, including 22 novel subjects, expressing a wide spectrum of phenotypes. Interestingly, phenotypes correlated with structural locations of the variants. Generalized epilepsy, with a median age at onset of 12 months, and mild-to-moderate ID were associated with variants in the extracellular domain. Focal epilepsy with earlier onset (median: age 4 months) and severe ID were associated with variants in both the pore-lining helical transmembrane domain and the extracellular domain.

Conclusion: These genotype–phenotype correlations will aid the genetic counseling and treatment of individuals affected by *GABRB3*-related disorders. Future studies may reveal whether functional differences underlie the phenotypic differences.

Introduction

The *GABRB3* gene encodes the β3 subunit of the ligand-gated γ-aminobutyric acid type A receptor ($GABA_AR$). The β3 subunit is one of the most abundant subunits in the human central nervous system, particularly during early stages of life, and therefore has a crucial role in neurodevelopment during the embryonic stage.[1] Being abundantly expressed, the β3 subunit also plays an important part in regulating the number of receptors in the synapse during inhibitory synaptic plasticity and is critical for the pentameric receptor assembly.[2]

It has recently been reported that individuals with pathogenic *GABRB3* variants present a broad phenotypic spectrum from severe developmental and epileptic encephalopathy (de novo variants) to milder epilepsy syndromes, including generalized epilepsy with febrile seizures plus (GEFS+) (sometimes familial) and childhood absence epilepsy.[3] The current consensus dictates that pathogenic variants in *GABRB3* cause loss of $GABA_AR$ activity. When

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doi: https://doi.org/10.1016/j.gim.2021.11.004

$GABA_AR$s cannot effectively open, neuronal hyperactivity occurs that ultimately increases seizure susceptibility and leads to various neurological and behavioral abnormalities.[4-6] Several mechanisms have been suggested whereby variants in the β3 subunit cause structural changes in the protein, resulting in receptor malfunction or irregular expression levels in synapses.[2,7,8] Given the observed broad phenotypic spectrum in individuals with pathogenic variants in *GABRB3*, it is assumed that 1 or combinations of several of these mechanisms come into play to cause the phenotypic differences.[2,4,5,7] Intriguingly, a recent paper described 2 individuals who experienced severe adverse reactions to treatments with vigabatrin (VGB), and on the basis of functional analysis, it was speculated that these *GABRB3* variants were unique by causing gain of $GABA_AR$ activity.[9] This observation further adds layers of complexity to the genotypic and phenotypic spectrum of *GABRB3* variants.

Over the years, extensive analysis of the 3-dimensional (3D) structure of α1β3γ2 $GABA_AR$ has revealed important structure-function relationships, most of which was corroborated by recent cryogenic electron microscopy (Cryo-EM) structures of the pentameric receptor complex.[10] Each subunit has an extracellular domain (ECD) followed by helical M1 to M4 regions forming the transmembrane domain (TMD) with M2 segments lining the channel pore. $GABA_AR$ channels open upon binding of γ-aminobutyric acid (GABA) molecules to specific binding sites at the interfaces between β3/α1 subunits. This causes an influx of chloride ions into the cell and hence decreases neuronal excitability. Given that various structural regions in a subunit play specific roles in receptor activation, it can be speculated that pathogenic variants are predominantly located in these regions. Therefore, analyzing variants in the context of the 3D α1β3γ2 $GABA_AR$ structure will provide insight into their differential phenotypic outcome in *GABRB3*-related disorders.

To provide a comprehensive review of all available individuals with pathogenic *GABRB3* variants, in this study, we collated the knowledge of all known individuals and expanded this with a new cohort of 22 individuals. Furthermore, by correlating the 3D structural locations of the variants with clinical features, we investigated whether certain structural regions were more likely to be associated with more severe phenotypes.

Materials and Methods

Through an international collaboration including epilepsy centers in Europe and North America, we collected 22 unpublished individuals with *GABRB3* variants. The American College of Medical Genetics and Genomics/Association of Molecular Pathology guidelines were used to classify variant pathogenicity.[11] The *GABRB3* transcript NM_000814.5 was used for coding variant nomenclature.

Clinical information was collected by face-to-face interviews with the affected individuals and their caregivers and from clinical charts. The referring clinicians collected all data by a structured phenotype table, which included cognitive and motor milestones, details about epilepsy and electroencephalogram (EEG), and treatment response. The seizure types and epilepsy syndromes were classified according to the guidelines of the International League Against Epilepsy.[12] The classification of the epilepsies was based on a multilevel approach with the third level being the epilepsy syndrome. Epilepsy types were classified as generalized epilepsy (GE), focal/multifocal epilepsy (FE), or unknown on the basis of clinical grounds, supported by EEG and magnetic resonance imaging findings (epilepsydiagnosis.org).[13] After that, whenever possible, we further defined the epilepsy syndrome.

EEG reports at seizure onset and at several points of follow-up were obtained for all novel individuals. All the available EEG reports were collected for every proband. Furthermore, a single epileptologist with EEG expertise reviewed raw EEG data of 13 affected individuals (including long-term monitoring video-EEGs) for background activity, interictal epileptiform abnormalities, ictal EEG discharges, and clinical manifestations.

Review of the literature

In parallel, we reviewed all available data on previously published individuals with *GABRB3* pathogenic variants. The literature search was performed using PubMed and Scopus, and the key words used included *GABRB3*, epilepsy, autism spectrum disorder, and psychiatric features. Papers in non-English language were excluded. Last search date was March 1, 2021.

Structure of $GABA_AR$ α1β3γ2

Three-dimensional crystallographic structure for human $GABA_AR$ β3 in a homopentameric form (Protein Data Bank [PDB] ID:4COF,[14] resolution = 2.97 Å, residue position coverage = 26-332 and 447-473) in complex with an agonist (benzamidine) was obtained from the PDB.[14] In addition, we performed our analysis on the biological assembly of $GABA_AR$ (PDB ID:6I53,[10] a Cryo-EM structure of the human α1β3γ2 $GABA_AR$ in a lipid bilayer, resolution = 3.20 Å, residue position coverage = 26-473). Mapping of Genome Aggregation Database (gnomAD) v2.1.1 missense variants and *GABRB3* variants on the structure was performed with PyMOL (Schrödinger, Inc.).

Results

We collected electro-clinical data on a total of 71 affected individuals with pathogenic or likely pathogenic *GABRB3*

variants,[3,8,9,15-30] including 22 previously unpublished individuals. Median age at follow-up was 6 years (range = 10 months-46 years). The main electro-clinical and genetic features are summarized in Supplemental Table 1. Complete information was not available for all individuals; denominators indicate the individuals for whom information on the clinical feature addressed was available.

Epilepsy

Of the 71 individuals, 99% (70/71) suffered from epilepsy. Of the 39 individuals, 38% (15/39) had GE and 62% (24/39) had FE. In 31 individuals, the epilepsy type was unknown. The median age of epilepsy onset was 12 months (range = 4 months-3 years) in individuals with GE and 4 months (range = 3 days-2 years) in individuals with FE (Figure 1A). The individuals with early onset GE had febrile seizures, which were later complicated by other seizure types. Photosensitivity was not reported in this cohort.

Epilepsy syndromes varied from individual to individual (Figure 1B). The most common syndromes in individuals with FE were early onset epileptic encephalopathy and developmental and epileptic encephalopathy, whereas those in individuals with GE were GEFS+ and myoclonic atonic epilepsy.

Each individual presented with up to 5 different seizure types, including focal seizures (29%, 20/70), bilateral tonic-clonic seizures (29%, 20/70), and myoclonic seizures (27%, 19/70) (Figure 1C). Status epilepticus was reported in 13% (9/70) of the individuals. Fever sensitivity was found in 29% (19/70) of the individuals.

Several antiseizure medications (ASMs) in various combinations were prescribed in each individual. At latest follow-up, 51% (26/51) of the individuals had ongoing daily to weekly seizures and 41% (21/51) were seizure free or had rare seizures. Of the individuals with GE, 37% (7/19) achieved seizure freedom, either with valproate (VPA) polytherapy (n = 3) or with lamotrigine and clobazam, barbiturates, clonazepam, or steroids (n = 1 each). Of the individuals with FE, 17% (4/23) achieved seizure freedom with VPA, clonazepam or stiripentol, levetiracetam (LEV), alone or in combination with VPA, or clobazam and VGB (n = 1 each).

EEG

The interictal EEG at epilepsy onset was normal in 32% (7/22) of the individuals. In the remaining individuals, the EEG showed background slowing (14%, 3/22) and irregular generalized spike and waves (14%, 3/22), multifocal (14%, 3/22) or focal spike/sharp-and-slow waves (14%, 3/22), hypsarrhythmia (14%, 3/22), or a burst suppression pattern (9%, 2/22).

At follow-up, the EEG deteriorated in most individuals, remaining normal in only 9% (2/22) of the individuals, and showed background slowing (18%, 4/22), focal spikes (36%, 8/22) or multifocal/generalized spike and waves (27%, 6/22), burst suppression (5%, 1/22), hypsarrhythmia (5%, 1/22), and migrating focal seizures of infancy (9%, 2/22).

In 5 individuals, ictal EEG recordings were available, including focal seizures (Figure 2A) in 3 individuals with FE and tonic seizures during sleep with ictal generalized fast activity (Figure 2B) in 2 individuals with GE.

Cognition, behavior, and additional features

At latest follow up, 39% (28/71) of the individuals had severe intellectual disability (ID) and 32% (23/71) had moderate ID, whereas 17% (12/71) had mild ID and 3% (2/71) had normal cognition. Cognitive stagnation or regression after epilepsy onset was described in 25% (18/71) of the individuals.

Individuals with FE had a higher prevalence of severe ID (75%, 18/24) than those with GE (16%, 3/19) (Figure 1D).

Autism spectrum disorder was diagnosed in 5% (4/71) of the individuals (1 GE, 4 unknown) and autistic features were seen in 15% (11/71) (1 GE, 4 FE, 6 unknown). Behavioral issues were present in 11% (8/71) of the individuals (4 GE, 1 FE), including aggression, impulsivity, anxious behaviors, or oppositional defiant disorders. Attention deficit hyperactivity disorder (ADHD) was reported in 10% (7/71) of the individuals and 8% (6/71) had stereotypic behavior.

Neurological disturbances were observed in 42 individuals (9 GE, 22 FE), consisting of axial hypotonia (40%, 17/42), ataxia (17%, 7/42), dystonia/dyskinesia (10%, 4/42), delayed speech and language development (55%, 23/42), microcephaly (12%, 5/42), and central visual impairment (14%, 6/42). Sleep disturbances were reported in 14% (6/42) of the individuals.

Dysmorphic features were reported in a subset of individuals (11%, 8/71) and included a mild prominence of the forehead, tented mouth appearance, and high-arched palate. One individual had cleft-palate.[30]

Affected family members

We also collected information about additional family members carrying pathogenic *GABRB3* variants. Of the family members with *GABRB3* variants, 54% (13/24) were asymptomatic and 46% (11/24) had epilepsy. All but 1 family member had normal intellect (data available for 73% (8/11) in the epilepsy group and for 92% (11/12) in the no-epilepsy group); 1 was deceased and thus was unavailable for follow-up. One family member had cognitive regression after seizure onset. No data about neurological or behavioral issues were available.

Four families carried a missense variant, whereas the remaining 8 families had truncating variants.

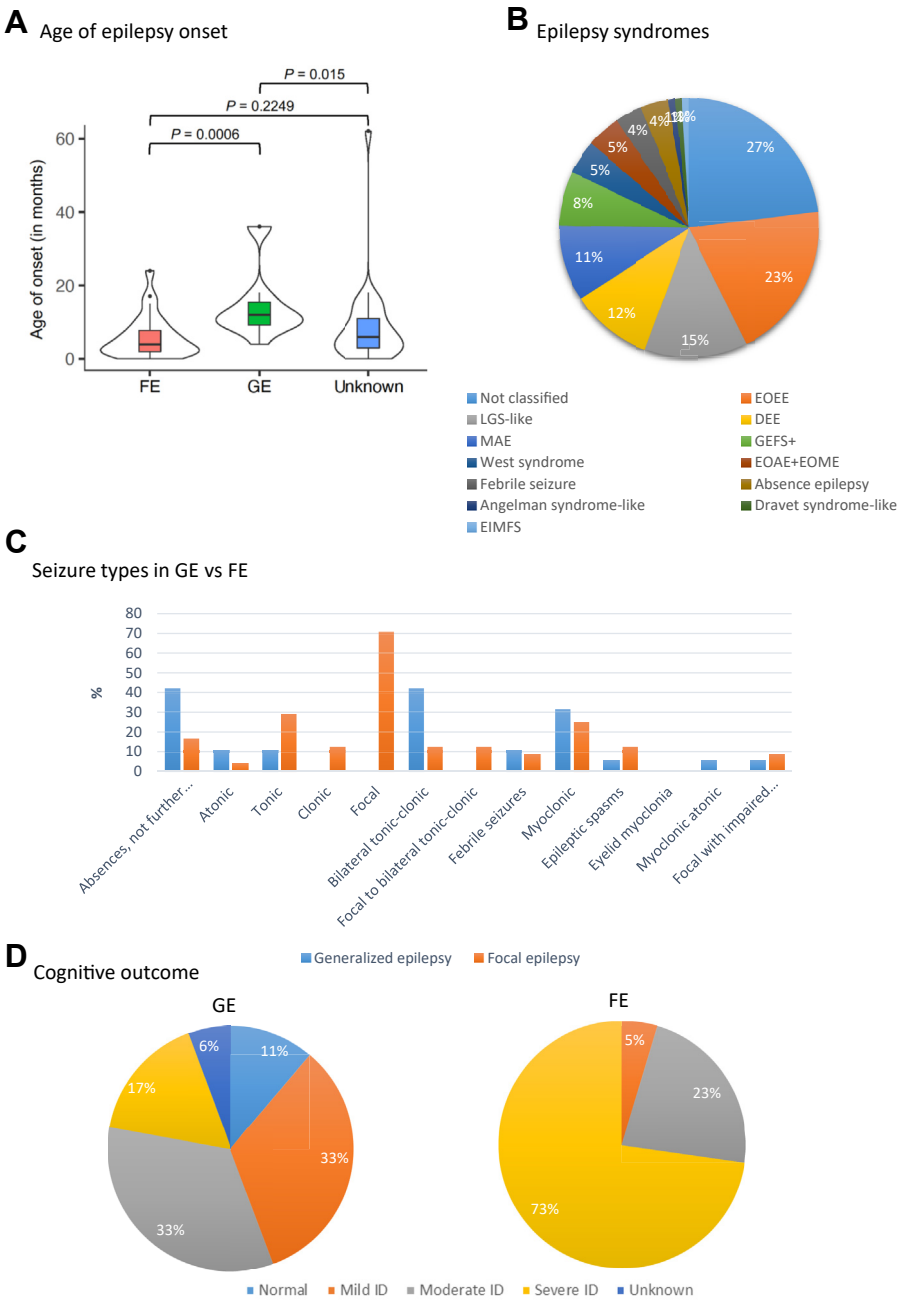

Figure 1 **Epilepsy features**. A. Seizure onset in individuals with GE vs FE. B. Epilepsy syndromes in the *GABRB3* cohort. C. Seizure types in individuals with GE vs FE. D. Cognition in individuals with GE and with FE. EOAE, early onset absence epilepsy; EOEE, early onset epileptic encephalopathy; EOME, early onset myoclonic epilepsy; EIMFS, epilepsy of infancy with migrating focal seizures; FE, focal epilepsy; GE, generalized epilepsy; GEFS+, generalized epilepsy with febrile seizures plus; ID, intellectual disability; LGS, Lennox Gastaut Syndrome; MAE, myoclonic atonic epilepsy.

Genetic landscape

A total of 53 different variants was observed in the 71 patients. Nine of them carried 8 different protein-truncating variants (PTVs) in the form of 6 stop-gained and 2 frameshift variants. One had an in-frame duplication (duplication of 1 amino acid), whereas 61 patients carried 44 different missense variants (single base substitution leading to a single amino acid change in the protein). *GABRB3* has a high probability of loss-of-function intolerance score for PTVs (ratio of expected vs observed variants) of 0.95 and only very few PTVs in gnomAD, indicating that the gene is intolerant for PTVs.

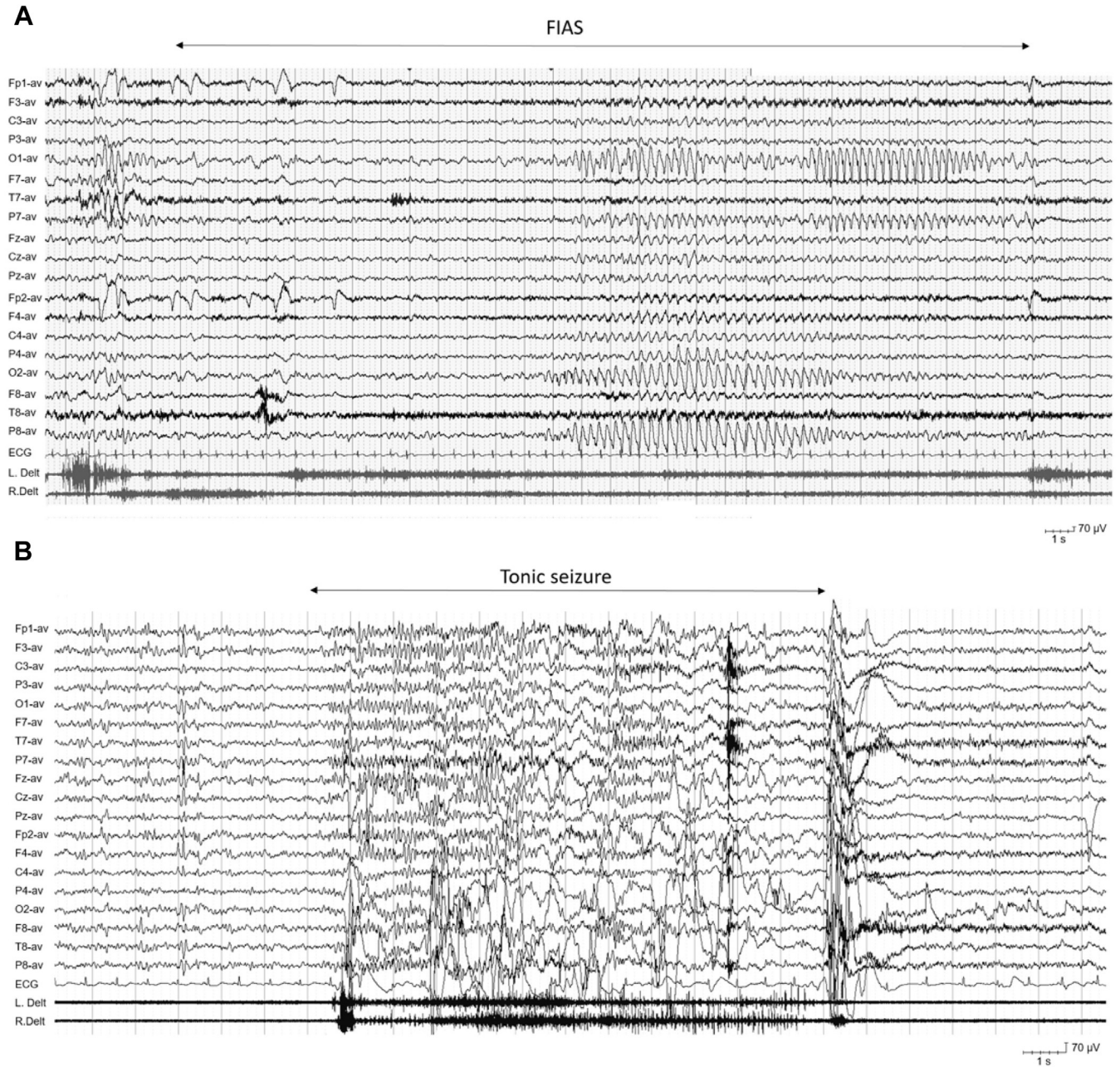

Figure 2 Ictal electroencephalogram (EEG) recordings in individuals with focal epilepsy (FE) or with generalized epilepsy (GE) related to *GABRB3* pathogenic variants. A. Individual 9 (FE; 5 years and 9 months at the time of EEG recordings): focal seizure during wakefulness with staring and impaired awareness (FIAS). The EEG shows an arrest of the background activity, followed by low amplitude rhythmic activity in the posterior regions, evolving to high amplitude 6 Hz activity with intermixed small spikes, in the occipital–post-temporal regions bilaterally. B. Individual 8 (GE; 45 years old at EEG recordings): tonic seizure during sleep. The surface electromyographic traces to the bottom show that the seizure starts with a spasm, followed by sustained tonic activation, with a superimposed vibratory component close to the seizures' end. The EEG correlate is a diffuse 10 Hz rhythmic activity with frontal predominance. FIAS was classified according to the guidelines of the International League Against Epilepsy.[12]

In the rest of the paper, all single amino acid changing variants are referred to as missense variants.

In case of missense variants, 76% (54/71) of the individuals had de novo variants and 17% (12/71) had variants inherited from a parent, either affected or unaffected, whereas for 5 individuals, segregation was unknown. All truncating variants were inherited from unaffected/affected parents. Thirteen variants were recurrent, displaying homogenous phenotypes for identical variants.

Missense variants were primarily located in the N-terminal ECD (22 variants in 34 individuals) and in the TMD (20 variants in 25 individuals) (Figure 3). A few missense

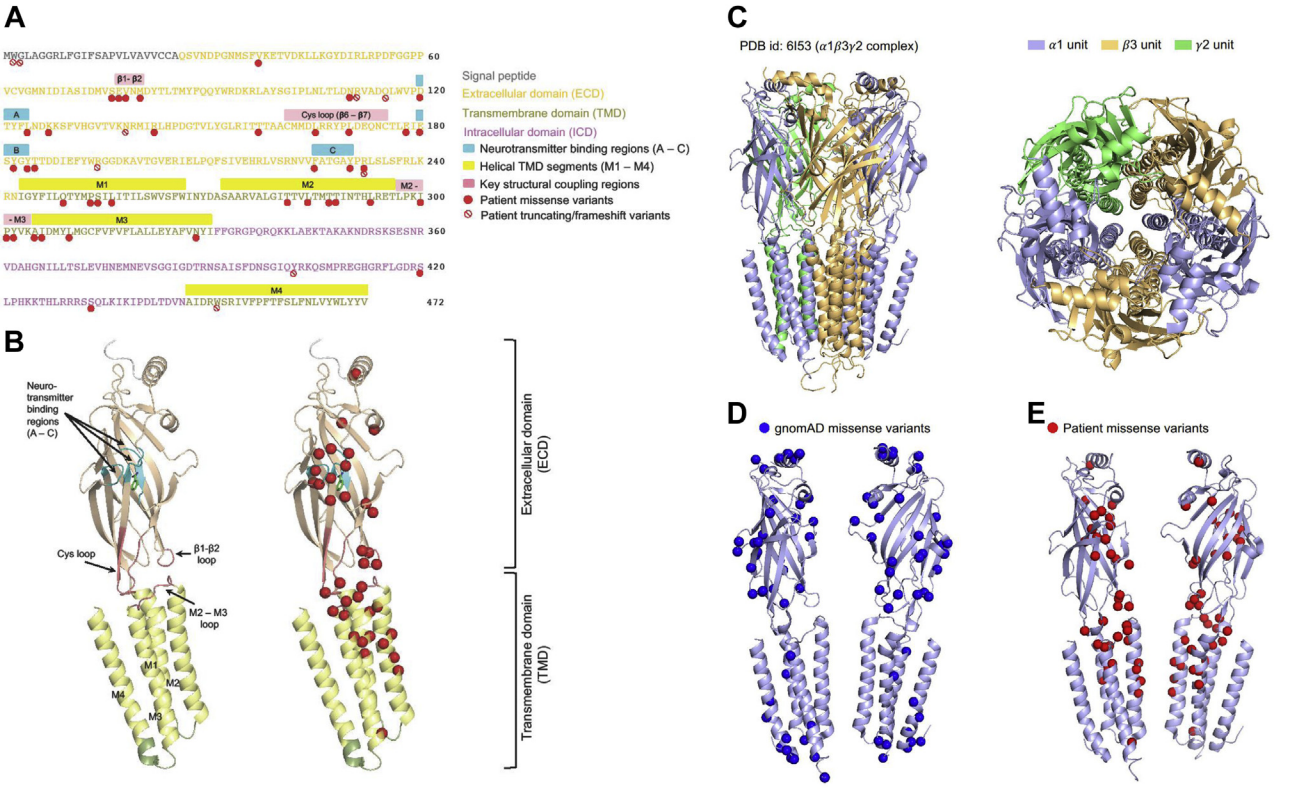

Figure 3 **Cognitive features.** A. Protein sequence encoded by *GABRB3*, annotated with protein domains, neurotransmitter binding regions and helical segments, structural coupling regions, and individual variants' positions. B. γ-Amino-butyric-acid type A receptor ($GABA_AR$) β3 structure (single chain, PDB ID: 4COF), annotated with protein domains, neurotransmitter binding regions and helical segments, structural coupling regions, and individual missense variants' positions (red spheres). C. Side (left) and top (right) view of the cryogenic electron microscopy structure of the human synaptic α1β3γ2 $GABA_AR$ complex (PDB ID: 6I53). Two α1 chains (encoded by *GABRA1*), 2 β3 chains (encoded by *GABRB3*), and 1 γ2 chain (encoded by *GABRG2*) are shown in light orange, purple, and green, respectively. D. *GABRB3* missense variants observed in the general population (gnomAD) mapped onto β3 chains (blue spheres). E. *GABRB3* missense variants observed in individuals (our cohort, Supplemental Table 1) mapped onto β3 chains (red spheres). gnomAD, Genome Aggregation Database; PDB, Protein Data Bank.

variants were located in the large intracellular loop (intracellular domain [ICD]) between M3 and M4 (2 variants in 2 patients).

For all variants in the TMD, these were mainly clustered in 2 regions: 3 helical segments (M1, M2, M3; 16 variants) and a key structural coupling region (small M2-M3 loop; 3 variants). All inherited variants were located in the ECD (11 individuals) except for 1 in the M4 (1 individual) helical segment of the TMD (Figure 3A).

Individuals with epilepsy that could not be classified as either FE or GE were evenly distributed between the ECD (21 individuals) and TMD (9 individuals) and included both missense variants (23 individuals) and PTVs (4 individuals).

Individual and population missense variants and structural locations

We mapped the location of the missense variants from our cohort and population missense variants from gnomAD onto the $GABA_AR$ β3 sequence (UniProtKB - P28472) and structure (PDB ID: 4COF, an X-ray structure, Figure 3B and PDB ID: 6I53, a Cryo-EM structure, Figure 4A). In the general population, 110 variants were observed that were located in 91 amino acid positions according to gnomAD.[31] Of these general population variants, 43% (44/110) were mappable on the transmembrane structure (Figure 3D), whereas the rest were located in the unstructured regions of the ECD and in the M3-M4 regions of the ICD.

In contrast, 95% (42/44) of the disease-associated missense variants, substituting 38 amino acid residues, were mappable to the structure (Figure 3B and E). Only 2 variants were located in the unstructured M3-M4 region of the ICD, which were not available in the crystal structure or in the Cryo-EM structure (Supplemental Table 1); these are de novo variants, p.(Ser420Ile) and p.(Ser433Leu).

Two amino acid positions were mutated in both affected individuals and in the general population (Asn110 and Arg142). Overall, it is notable that the disease-associated missense variants were located in distinct spatial regions of the structure (hotspots) compared with the general population variants (Figure 3D and E), preferentially on or in a spatial proximity to (1) the neurotransmitter binding regions

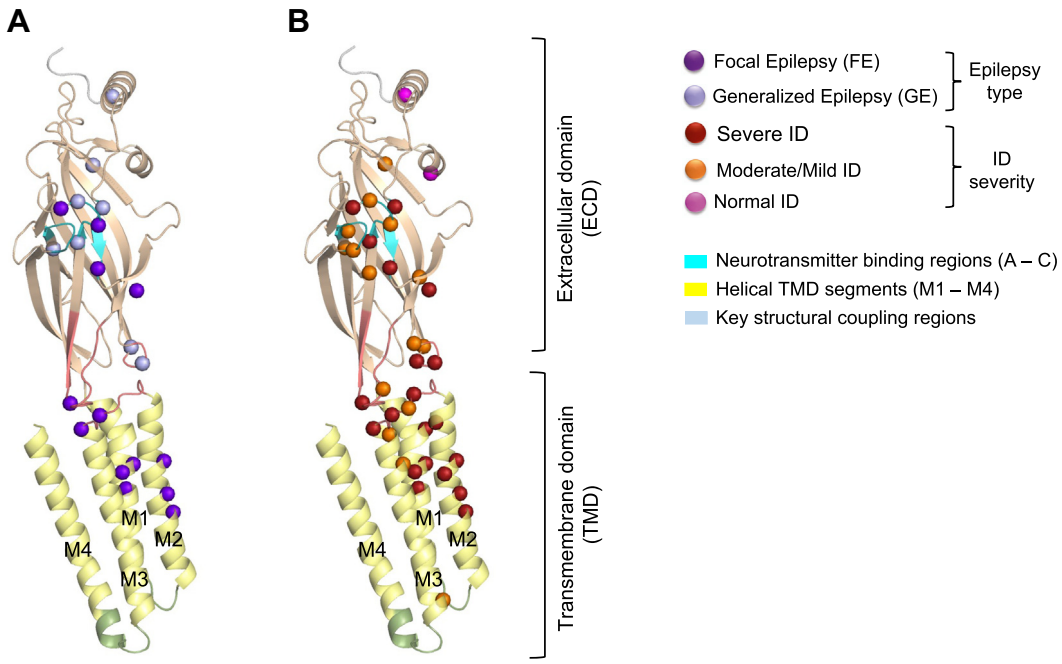

Figure 4 Investigation of individual missense variant positions by different phenotypes on the $GABA_AR$ β3 structure (Protein Data Bank ID: 4COF, single chain). A. Protein residue positions affected by missense variants in individuals with different epilepsy types: focal epilepsy and generalized epilepsy are indicated by dark purple and light purple, respectively. B. Protein residue positions affected by missense variants in individuals with different severity of ID: severe ID, moderate/mild ID and normal ID are indicated by dark red, orange and magenta spheres, respectively. ID, intellectual disability.

A, B, and C in the ECD, which are essential for the neurotransmitter recognition and binding (Figure 3B); (2) the β1-β2, Cys, and M2-M3 loops, which are key structural regions for coupling and signaling between the ECD and TMD; and (3) the channel forming helical segments M1, M2, and M3 (Figure 3B).

Are individual phenotypes correlated with the structural location of the variant?

Given the clustering of variants in key regions of the GABRB3 protein (Figure 3), it is likely that the structural position of a variant is correlated with the individual phenotype. To investigate this, we grouped the individuals with a missense variant ($n = 61$) on the basis of their ID severity and epilepsy type (Table 1) and mapped the affected amino acids on the structure (Figure 4). All 13 individuals with GE in our cohort had missense variants located in the ECD of the protein (Figure 4A), except p.(Leu284Pro) in the transmembrane domaine 2 and p.(Ser433Leu) located in the ICD between M3 and M4 helical regions. Conversely, FE associated missense variants are located in both the ECD and TMD. All individuals, except 1 (p.(Leu284Pro), with a variant in the M2 helical segment of TMD had FE (Figure 4A) and severe ID (Figure 4B). Hence, it appears that at least certain clinical phenotypes are indeed associated with the structural location of the missense variant.

Genotype–phenotype correlations and protein position of missense variants

We analyzed the individual phenotypes of missense variants (61 individuals) by separating them into 3 groups on the basis of key structural locations, namely ECD, TMD, and ICD (Table 1).

In the ECD group, all individuals ($n = 34$) suffered from epilepsy with a median age at epilepsy onset of 8.5 months (range = 1.5 months-7 years). Of the 34 cases, 38% (13/34) had GE, with fever sensitivity in 29% (10/34) of the cases. Seizure freedom was achieved in 35% (12/34) of individuals at a median age of 1.5 years, after trying 1 to 3 ASMs. The most effective medications included VPA (15% [5/34] of the individuals) in combination with either topiramate, LEV, zonisamide, or steroids.

Cognition before epilepsy onset was normal in 71% (12/17) of subjects, whereas 24% (4/17) had mild to moderate ID; cognition was unknown in 17 subjects. At latest follow-up, cognition deteriorated in the subjects; 3% (1/32) had normal cognition, 72% (23/32) had mild ID to moderate ID, and 28% (9/32) had severe ID. In 2 individuals, cognition was unknown.

Table 1 Clinical characteristics of the *GABRB3* cohort

	Missense Variants[a]		
	ECD	TMD	Truncating Variants
Number of individuals/variants	34/22	25/20	10/9
Epilepsy onset (range)	8.5 mo (1.5 mo-7 y)	4 mo (birth – 5 y)	1 y (birth – 5 y, 2 mo)
Seizure types (top 3)	Myoclonic (31%) Atonic (29%) Bilateral TC (29%)	Focal (50%) Tonic (33%) Myoclonic (21%)	Absences (40%) Bilateral TC (20%) Myoclonic (10%)
Epilepsy type (number of individuals)	13 GE/7 FE	1 GE/14 FE	2 GE/1 FE
Fever sensitivity	29%	13%	50%
Seizure freedom (mean age at offset)	34% (1.5 y)	17% (12 mo)	40% (5 y)
Most effective ASMs	VPA in combinations	None specific	VPA in combinations
Cognition	Normal 3% Mild ID 30% Moderate ID 39% Severe ID 28%	Normal 0% Mild ID 9% Moderate ID 18% Severe ID 72%	Normal 10% Mild ID 0% Moderate ID 60% Severe ID 30%
Neurological deficits	Language delay (63%) Hypotonia (26%) Ataxia (16%)	Hypotonia (52%) Language delay (29%) Ataxia (19%) Visual impairment (19%)	Language delay (40%)
Additional features	ADHD (50%) Stereotypies (33%) Autistic features (23%)	Autistic features (13%) Aggression (8%) Microcephaly (13%) Digestive issues (13%)	Autistic features (40%) Aggression (20%) Stereotypies (20%)
Recurrent variants	p.(Asp120Asn), p.(Leu124Phe), p.(Lys127Arg), p.(Thr157Met), p.(Thr185Ile), p.(Arg232Gln)	p.(Ser254Phe), p.(Leu256Gln), p.(Thr281Ala), p.(Pro301Leu), p.(Tyr302Cys)	p.(Trp2[a])

ADHD, attention deficit hyperactivity disorder; *ASMs*, anti-seizure medications; *ECD*, extracellular domain; *FE*, focal epilepsy; *GE*, generalized epilepsy; *TC*, tonic-clonic seizures; *TSM*, transmembrane domain.

[a]Two individuals with missense variants located in the intracellular domain are not included here.

Behavioral issues were common (35%, 12/34), including ADHD (6/12), stereotypic behavior (4/12), and aggression (2/12). Autism or autistic features were reported in 24% (8/34).

Neurological deficits were reported in 56% (19/34). Language delay was the most common issue (63%, 12/19), followed by hypotonia (26%, 5/19) and ataxia (16%, 3/19). One individual, carrying a p.(Tyr182Phe) variant, died at 35 months of sudden unexpected death in epilepsy.[20]

In the TMD group, 14 individuals (58%, 14/24) had an FE and 1 had a GE. The median age at onset was 4 months (range = first day of life to 5 years). Fever sensitivity was not prominent (13%, 3/24). Seizure freedom was achieved in 17% (4/24) of the individuals at a median age of 12 months, both with monotherapy and polytherapy with LEV, lamotrigine, phenobarbital, stiripentol, VGB, and VPA, and no ASM was preferred over others.

Cognition at onset was known only for 4 individuals, being normal in 2, whereas 2 had mild ID. All individuals were intellectually disabled at latest follow-up: 9% (2/22) had mild ID, 18% (4/22) had moderate ID, and 72% (16/22) had severe ID. The level of cognition was unknown in 3 individuals. A few individuals had autistic features ($n = 3$) or behavioral issues ($n = 2$).

Neurological deficits were reported in 84% (21/25) of the individuals, mainly consisting of hypotonia (11/21), language delay (6/21), ataxia (4/21), and visual impairment (4/21). Two individuals were deceased: 1 (p.[Thr288Asn]) died at 18 months in the context of severe neurological deterioration[8] and 1 (p.[Leu284Arg]) died at 54 months of unknown causes.[21]

In the ICD group, only 2 individuals carried missense variants in the ICD, not permitting any comparison with the other subgroups.

Individuals with truncating variants

All individuals ($n = 10$) had epilepsy with a median age at onset of 1 year and with prominent fever sensitivity (50%). Seizure freedom was achieved in 40% (4/10) of individuals, with VPA polytherapy in 2 individuals. Cognition before seizure onset was normal in half of the individuals (2/4). At latest follow-up, most individuals had moderate ($n = 6$) or severe ($n = 3$) ID. Language delay was the only

neurological deficit reported ($n = 4$). Autistic features ($n = 4$) and behavioral issues ($n = 4$) were prominent.

Discussion

In this study, we analyzed the phenotypic and genetic features of a large cohort of 71 individuals with a *GABRB3*-related disorder, including 22 previously unpublished individuals. We delineated a spectrum of developmental diseases associated with *GABRB3* variants, including various epilepsy phenotypes. Furthermore, we found a possible correlation between the location of the variants within the protein structure and the clinical features, including age of epilepsy onset, epilepsy type, and degree of ID.

Clinical and genetic landscape

Similar to previous studies, when analyzing the whole cohort, we were unable to delineate a unique *GABRB3* phenotype. We found that phenotypes were distributed across a large clinical spectrum, encompassing a wide variety of epilepsy syndromes both generalized and focal, as well as different degrees of ID.

Interestingly, by clustering affected individuals on the basis of the location of their variants within the protein, we observed more homogeneous clinical subgroups with similar phenotypic features and severity. Individuals with variants located in the ECD primarily had onset of myoclonic, atonic, or absence seizures at a median age of 12 months. Furthermore, 28% of these individuals were fever sensitive. Epilepsy was both generalized and focal. Behavioral issues, including ADHD, and autistic features were reported in half of the individuals (52.8%). The clinical features correlated with the findings in the *Gabrb3*$^{+/D120N}$ (extracellular variant) mouse that shows atypical absence seizures, myoclonic seizures, tonic seizures, and bilateral tonic-clonic seizures, as well as rare atonic seizures and epileptic spasms in the *Gabrb3*$^{+/D120N}$ pups.[5] Behavioral issues have also been described for the *Gabrb3*$^{+/D120N}$ mouse that have learning and memory deficits, hyperactivity, reduced socialization, and anxiety, all of which increase with age.[5] In addition, a phenotype of early onset febrile seizures with subsequent additional seizure types and/or developmental impairment was seen in a subset of patients with variants in the ECD. These patients were classified with GEFS+ or a Dravet-like phenotype, confirming *GABRB3* as one of the genes that should be investigated in individuals with these phenotypes.[27]

The few individuals with PTVs displayed a phenotype resembling that found in individuals with variants located in ECD that included GEs, moderate ID, and relatively common behavioral issues or autistic features. Interestingly, most PTVs were inherited from presumed unaffected parents, suggesting reduced penetrance as previously discussed.[3] Why reduced penetrance is seen more commonly in PTVs is puzzling but is also seen for other genes such as *GABRG2*.[32]

In contrast, individuals with variants in the TMD displayed a more severe phenotype with early onset FE, often refractory to ASMs, and they were usually not fever sensitive. Most of these individuals had severe ID and prominent neurological deficits, whereas behavioral issues and autistic features were rarely described.

Clinical phenotypes in key structural regions

When analyzed in a structural context, it was clear that variants were clustered in 3 regions of the β3 subunit that are key to its function.

GABA binding region

A hotspot for variants was identified in the neurotransmitter binding pocket formed by neurotransmitter binding regions A, B, and C.[14] Neurotransmitter (GABA) binding activates the $GABA_AR$ channel, and therefore, variants in this pocket should have considerable effect on the activation and/or deactivation of the channel. In total, 7 variants (11 individuals) were observed in this pocket in our cohort. Six of these variants led to moderate to mild ID with or without GE. Interestingly, this implies that individuals with variants in the GABA binding pocket are less severely affected than those with variants in other structural motifs. One notable exception to this is the p.(Leu124Phe) variant that was found in 3 individuals with severe ID and FE, suggesting that this specific location is somewhat crucial for the function of the protein.

Coupling region

Another important region of the $GABA_AR$ β3 subunit is the ECD–TMD interface. For a neurotransmitter binding event to transmit to the channel gate, the signal must be transduced across the ECD–TMD interface. Given the importance of this interface for intersubunit structural coupling and signal transmission, variants in this interface are likely to cause attenuation of the signaling events and thus lead to disease. In our cohort, 10 variants (in 14 individuals) in the ECD–TMD interface showed different phenotypes. This suggests that even if these structural motifs are important for neurotransmitter binding, not all variants in these locations will lead to a severe phenotype.

TMD:

All but 1 of the 8 individuals with variants in the M2 had FE and either severe ID (7/9) or moderate ID (2/9). Because the M2 segment lines the channel pore, it is reasonable to hypothesize that variants in the M2 will lead to significant perturbation of the channel structure, affecting the passage of chloride through the pore. Similarly, individuals with variants in the M1 and M3 mainly had focal seizure types

with early onset and severe ID, confirming the crucial function of these domains.

Treatment implications

Treatment was only effective in a limited number of individuals. These were treated according to their seizure types because currently there is no precision medicine available for $GABA_AR$s. Even if benzodiazepines target the receptor, the sedative effects of these medications render them less useful for long-term treatment. In addition, it was recently shown that VGB should be avoided in certain individuals because it can aggravate symptoms.[9] Seizure freedom was more common in individuals with variants in the ECD and in individuals with truncating variants, most likely because of their milder phenotype. However, only 36% and 40% of the individuals, respectively, achieved seizure freedom, highlighting the severity of disease and limited treatment options.

A novel drug targeting the $GABA_AR$ is showing promising results in individuals with *PCDH19*-related epilepsy (ClinicalTrials.gov Identifier: NCT02358538). The neurosteroid ganaxolone is a positive allosteric modulator of the $GABA_AR$ and as such it would theoretically be a relevant ASM in individuals with *GABRB3* variants. Future studies are needed to investigate these theories further.

Perspectives

It is widely accepted that pathogenic variants in the GABA-related genes cause loss-of-function of the resulting receptors. This leads to disinhibition in the GABAergic neurons and thus increased neuronal excitability. Numerous studies have shown that variants in both the ECD and TMD cause loss-of-function and lead to severe phenotypes in individuals.[3,33] In contrast to this, 2 variants in *GABRB3* were recently shown to lead to gain-of-function receptors.[9] Interestingly, the affected individuals showed hypersensitivity to VGB, a GABA enhancer, adding treatment response to the list of implications associated with functional effects of *GABRB3* variants. In this study, we observed clear differences in the phenotypic severity depending on the structural location of the variants. This suggests that the functional effects of variants in, eg, the TMD, could differ from those in the ECD and that equating the impaired function caused by missense variants to the loss-of-function caused by PTVs is an oversimplification. However, the previous report on *GABRB3* gain of function variants included both a variant in the ECD and a variant in the TMD, indicating that a correlation is not straight forward.

More patients are expected to be found and aggregated as genetic test availability is expanding. Considering that the estimated incidence for pathogenic de novo variants within *GABRB3* is 2.1 per 100.000 live births, we can expect just in the United States at least 81.9 new cases per year.[34] Future functional characterization of variants is needed to investigate the hypothesis presented in this study and to determine whether the associations between genetic location and phenotype found in this study also correlates with functional effects. Such studies could potentially also elucidate the relationship between variant location, phenotype, and treatment response, paving the way for a personalized medicine approach.

Data Availability

De-identified data will be made available to those eligible per request to the corresponding authors.

Acknowledgments

We thank the individuals and their families for participating in this study.

A.M. and M.A.K. are partly funded by the NIHR Great Ormond Street Hospital Biomedical Research Centre. A.M. is funded by UK Regenerative Medicine Platform (MRC) and the Rosetrees Trust. The views expressed are those of the author(s) and not necessarily those of the The National Institute for Health Research (NHS), the National Institute for Health Research (NIHR), or the Department of Health. The Cambridge NIHR Bioresource provided support in this study. The Deciphering Developmental Disorders (DDD) study presents independent research commissioned by the Health Innovation Challenge Fund (grant number HICF-1009-003), a parallel funding partnership between Wellcome and the Department of Health, and the Wellcome Sanger Institute (grant number WT098051). The views expressed in this publication are those of the author(s) and not necessarily those of Wellcome or the Department of Health. The study has UK Research Ethics Committee (REC) approval (10/H0305/83, granted by the Cambridge South REC, and GEN/284/12 granted by the Republic of Ireland REC). The research team acknowledges the support of the NIHR, through the Comprehensive Clinical Research Network.

This work was supported by Novo Nordisk Foundation (NNF19OC0058749 to R.S.M.), Lundbeck Foundation (R324-2019-1083 to R.S.M.), the Agencia Nacional de Investigación y Desarrollo (ANID, PAI77200124 to E.P.) of Chile, and the FamilieSCN2A Foundation 2020 Action Potential Grant (to E.P.). P.S. worked within the framework of the Dipartimento di Neuroscienze, riabilitazione, oftalmologia, genetica e scienze materno-infantili (DINOGMI) Department of Excellence of Ministero dell'Istruzione Ministero dell'Università e della Ricerca (MIUR) 2018-2022 (legge 232 del 2016).

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Ethics Declaration

All institutions involved in human participant research received local Institutional Review Board approval (main IRB: The ethics committee of Region Zealand, Denmark). Written informed consent, including authorization for reproduction of video images, was obtained for all individuals (or legal guardians) and family members where necessary. Individual data were collected according to local ethics committee guidelines.

Conflicts of Interest

The authors of declare no conflicts of interest.

Additional Information

The online version of this article (https://doi.org/10.1016/j.gim.2021.11.004) contains supplementary material, which is available to authorized users.

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