Diagnostic implications of genetic copy number variation in epilepsy plus

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Copy number variations (CNVs) represent a significant genetic risk for several neurodevelopmental disorders including epilepsy. As knowledge increases, reanalysis of existing data is essential. Reliable estimates of the contribution of CNVs to epilepsies from sizeable populations are not available.

Methods: We assembled a cohort of 1255 patients with preexisting array comparative genomic hybridization or single nucleotide polymorphism array based CNV data. All patients had “epilepsy plus,” defined as epilepsy with comorbid features, including intellectual disability, psychiatric symptoms, and other neurological and nonneurological features. CNV classification was conducted using a systematic filtering workflow adapted to epilepsy.

Results: Of 1097 patients remaining after genetic data quality control, 120 individuals (10.9%) carried at least one autosomal CNV classified as pathogenic; 19 individuals (1.7%) carried at least one autosomal CNV classified as possibly pathogenic. Eleven patients (1%) carried more than one (possibly) pathogenic CNV. We identified CNVs covering recently reported (HNRNPU) or emerging (RORB) epilepsy genes, and further delineated the phenotype associated with mutations of these genes. Additional novel epilepsy candidate genes emerge from our study. Comparing phenotypic features of pathogenic CNV carriers to those of noncarriers of pathogenic CNVs, we show that patients with nonneurological comorbidities, especially dysmorphism, were more likely to carry pathogenic CNVs (odds ratio = 4.09, confidence interval = 2.51-6.68; \( P = 2.34 \times 10^{-9} \)). Meta-analysis including data from published control groups showed that the presence or absence of epilepsy did not affect the detected frequency of CNVs.

Significance: The use of a specifically adapted workflow enabled identification of pathogenic autosomal CNVs in 10.9% of patients with epilepsy plus, which rose to 12.7% when we also considered possibly pathogenic CNVs. Our data indicate that epilepsy with comorbid features should be considered an indication for patients to be selected for a diagnostic algorithm including CNV detection. Collaborative large-scale CNV reanalysis leads to novel declaration of pathogenicity in unexplained cases and can promote discovery of promising candidate epilepsy genes.
**1 | INTRODUCTION**

Current estimates suggest that genetics contribute to causation in 50%-70% of the epilepsies.\(^1\) Copy number variations (CNVs) represent a prominent type of variant carrying risk for certain epilepsies.\(^2\) Whole genome oligonucleotide array CGH or SNP array is routinely included in evaluation of patients with complex phenotypes with a suspected genetic cause.\(^6\) CNVs, as a risk factor or cause, have been reported in ~5%-12% of patients with different types of epilepsies.\(^2\)\(^4\)\(^5\)\(^7\)-\(^9\) The risk of a pathogenic CNV is reportedly increased with concurrent intellectual disability (ID), dysmorphic features, autism spectrum disorder (ASD), drug resistance, or other comorbidities, from a study of 222 patients.\(^10\) Recurrent CNV “hotspots” predispose to different types of epilepsies.\(^5\)\(^11\) CNV detection has pointed to novel epilepsy genes.\(^12\)

Robust estimates of the frequencies and types of putatively relevant CNVs in epilepsy are needed to determine whether CNV detection should be included in genetic evaluation of patients with various epilepsy phenotypes. As knowledge of epilepsy genetics increases, systematic, iterative reevaluation of genetic data becomes essential. This process requires large numbers of individuals to be corralled, and because such data will inevitably come from different centers using different technologies, a robust means of joint reevaluation is essential.

Epilepsy is often a feature of neurodevelopmental disorders (NDDs). A recent study on individuals with NDDs and epilepsy reported similar results for rare variant frequency for individuals ascertained to have epileptic encephalopathy (EE) and for individuals ascertained for NDDs with unspecified epilepsy,\(^13\) suggesting that, genetically, epilepsy can be considered part of the spectrum of NDDs. Looking at this concept from the perspective of CNV, and to determine the frequency of CNVs in particular epilepsy phenotypes, we assembled a large international cohort of patients with the phenotype of “epilepsy plus,” which we define as the occurrence of epilepsy and comorbid features, including ID and psychiatric, neurological, and nonneurological features. Preexisting array data were systematically investigated using a workflow based on current knowledge of CNV classification. The workflow enabled combination of multicenter CNV data to provide a robust, up-to-date reevaluation of the contribution of CNVs to epilepsy plus and identified new candidate pathogenic autosomal CNVs. The method can be applied iteratively with additional cohorts at future time points, making optimal use of existing data.

**2 | MATERIALS AND METHODS**

**2.1 | Ethics**

This study was approved by the ethics committees of the participating centers. Written informed consent was provided by the patient, or the parent or the guardian of each patient as appropriate.

**2.2 | Data collection**

Preexisting CNV data, derived from array CGH or SNP array conducted for clinical or research purposes, were collected from eight specialist epilepsy and/or genetic centers (Table S1). All patients also had comorbid features including ID, autism, dysmorphic features, other neurological or nonneurological conditions, structural brain abnormalities, or multidrug resistance.\(^14\) Clinical information was collected through referring clinicians. Seizure and epilepsy/syndrome types were classified according to the International League Against Epilepsy criteria when available.\(^15\)

**2.3 | CNV analysis: Quality control and classification**

All CNV calls were provided by the contributing centers (Table S1). Figure 1 shows the workflow we used to classify CNVs (Data S1). We focused only on autosomal CNVs due to higher quality of CNV calls from nonsex chromosomes.\(^16\)
To ensure high reliability, we considered only CNVs with high calling confidence according to the following criteria: (1) size ≥ 150 kb, (2) coverage of ≥ 30 consecutive probes for SNP arrays and ≥ 3 probes for array CGH, and (3) microdeletion/microduplication frequency < 1% in the entire study sample. Samples with a total number of deletion or duplication (or both) calls > 2 SD from the mean number of any calls/sample across the entire dataset were excluded from the analysis. Further manual analysis used a bespoke workflow based on current understanding of classification, including the American College of Medical Genetics guidelines and additional literature. CNVs were classified into four groups: pathogenic, possibly pathogenic, benign, or of unknown significance. Briefly, the workflow was as follows: First, common CNVs, present in the healthy population, were classified as “benign.” All remaining CNVs were then classified as “pathogenic” if they met the following criteria: ≥ 80% overlap of the study CNV with any CNVs known to be associated with epilepsy; or, CNV with a size ≥ 3 Mb; or, CNV with a size < 3 Mb and > 1 Mb, and with de novo occurrence. The remaining CNVs were further classified according to their gene content. A CNV was classified as pathogenic when it involved a gene known to be associated with epilepsy (Table S2), the phenotype was concordant with that in the literature, and the type of CNV (deletion/duplication) matched current knowledge on the pathogenic mechanism of the gene change (gain or loss of function). If a CNV contained a gene associated with epilepsy but the other conditions were not fulfilled, the CNV was considered pathogenic only if proven to be de novo and was otherwise classified as “possibly pathogenic.” CNVs containing a brain-expressed gene, according to the published datasets and the database GTEx (http://www.gtexportal.org/home/), were classified as “possibly pathogenic” only if de novo. Analysis of recessive

**FIGURE 1** Workflow used to classify the copy number variations (CNVs) in our cohort of patients with epilepsy plus. Stepwise procedures are shown for CNV classification into benign, pathogenic, possibly pathogenic, and unknown significance groups. CGH, comparative genomic hybridization; CNS, central nervous system; SNP, single nucleotide polymorphism.
inheritance of epilepsy genes was not considered due to limitations of most CNV platforms on calling homozygous deletions or duplications. The remaining CNVs were classified as “of unknown significance.”

2.4 | CNV confirmation

In each individual, the proposed possibly pathogenic CNVs were confirmed if DNA was available and inheritance status was confirmed using a second, locally developed technique for multiplex amplicon quantification (MAQ; Data S1; Agilent, https://www.agilent.com/en/products/next-generation-sequencing/amplicon-target-amplification-(multiplicom)/maq-overview).

2.5 | Phenotype enrichment analysis

Using Fisher's exact test, we investigated whether patients carrying a pathogenic autosomal CNV, compared to those not carrying a pathogenic CNV, had overrepresentation for specific phenotype components (nonneurological disorders, neurological or psychiatric disorder, ID, facial dysmorphism, brain abnormalities, epilepsy onset < 1 year of age, and EE). The analysis was conducted in two ways—(1) for any pathogenic CNV and (2) for only large (>1 Mb) pathogenic CNVs—and was corrected for multiple testing accordingly.

2.6 | Meta-analysis

To determine the impact of epilepsy on the probability of identifying pathogenic CNVs, we used the following strategy. First, we split our cohort into two subgroups including patients with (1) epilepsy and ID including autistic features and (2) epilepsy and other psychiatric/neurological disorders. We gathered two “historical control groups” through a systematic review of the literature and a meta-analysis and estimated the yield of pathogenic CNVs in patients with (1) ID including autistic features (without epilepsy) and (2) psychiatric/neurological disorders (without epilepsy). Then, we compared the yield of pathogenic CNVs between these groups with (1) epilepsy and ID including autistic features versus the historical-control group with only ID and autistic features and (2) epilepsy with other psychiatric/neurological disorders versus the corresponding control group from the literature manifesting only other psychiatric/neurological disorders. We used the Cochran Q test to assess heterogeneity across studies.

To evaluate whether epileptic encephalopathies might specifically contribute to the yield of pathogenic CNVs, we compared patients with epilepsy manifesting as EE (epilepsy-EE) from our cohort to those with epilepsy without EE (epilepsy-notEE) from a systematic review of the literature.

The full search strategy, inclusion criteria, and methods are available in Data S1.

3 | RESULTS

We assembled a cohort of 1255 patients. After quality control, 1097 patients were retained for analysis. Of these, 139 (12.7%) carried a total of 142 autosomal CNVs classified as either pathogenic (n = 122, 10.9%) or possibly pathogenic (n = 20, 1.7%). Eleven patients (1%) carried two pathogenic or possibly pathogenic CNVs (Table S3).

3.1 | Pathogenic CNVs

To simplify presentation, we further divided pathogenic CNVs into four subgroups: (1) recurrent CNVs with well-documented enrichment in epilepsy; (2) CNVs related to a genetic Online Mendelian Inheritance in Man database (OMIM) syndrome with neurological symptoms in which epilepsy can feature; (3) CNVs not known to be enriched in epilepsy and not associated with any other OMIM syndrome, but containing at least one gene that is already implicated in epilepsy; and (4) CNVs based on size combined with de novo occurrence.

3.1.1 | Recurrent CNVs with well-documented enrichment in epilepsy

Thirty-six individuals had a CNV known to be recurrent in people with epilepsy (36/120, 30%; Table 1).2,5,11 One individual had two recurrent pathogenic CNVs. The 16p13.11 deletion was the most frequent, occurring in 10 of 120 (8.3%) patients bearing pathogenic CNVs and 10 of 1097 (0.9%) of the studied individuals. Other frequently represented CNVs were 1p36 deletion (OMIM #607872, 5/120 patients, 4.2%), 15q11.2 duplication syndrome, distal (three patients), 16p11.2 duplication syndrome including PRRT2 (four patients), the Potocki-Lupski 17p11.2 duplication syndrome (two patients),
and 17p13.3 deletion syndrome, also known as Miller-Dieker lissencephaly deletion syndrome (three patients). We also identified de novo duplications at 2q24.3 and at 4p16.3-p13, for which regions both deletions and reciprocal duplications have been associated with epilepsy.22,23

### 3.1.3 CNVs including epilepsy-related genes

Nineteen individuals had a CNV (19/120, 15.8%) including epilepsy-related genes (Table 2). Five individuals had a CNV including HNRNPU (four de novo deletions and one duplication; two deletions and the duplication also contained the flanking AKT3 gene). The four probands carrying deletions presented with epilepsy classified as Lennox-Gastaut syndrome in one patient, genetic generalized epilepsy (GGE) in another, and early onset, drug-resistant epilepsy not otherwise classified in the remaining two. Moderate to severe ID was reported in four patients and one also had ASD. Three had microcephaly, congenital and severe (−4 SD) in one. Brain magnetic resonance imaging showed corpus callosum agenesis or hypoplasia in three of four patients. Facial dysmorphic features were observed in three patients. The patient carrying a large (>100 Mb) duplication involving, among many other genes, HNRNPU and AKT3, had a complex phenotype including neonatal seizure onset, polymicrogyria, and multiple cardiac defects. Three individuals had a 9q21.13 deletion, one de novo and two of unknown inheritance, including a gene with recently described association with epilepsy, RORB. All the patients presented with ID and generalized epilepsy with absences or atypical absences, with eyelid myoclonia in two cases and photosensitivity in one. Further clinical details of patients with CNVs including HNRNPU or RORB are provided in Tables S5a and S5b. Three deletions encompassed the ADGRV1 gene, two of which included MEF2C. Additional epilepsy genes that were found deleted or duplicated in single patients are listed in Table 2 and include GNAO1, NEDD4L, and SIK1.

### 3.1.4 Pathogenic autosomal CNVs based on size combined with de novo occurrence

Thirty-two individuals (32/120, 26.6%) had CNVs that fell only into this category (one individual had two large pathogenic CNVs). We did not find overlapping CNVs in healthy individuals (Database of Genomic Variants; http://dgv.tcag.ca/dgv/app/home). Sixteen (16/32, 50%) of the CNVs ≥ 3 Mb showed an overlap or partial overlap with CNVs described in Decipher (https://decipher.sanger.ac.uk/) in patients exhibiting various clinical features including ID, seizures, and dysmorphisms (Table S4b). Interestingly, in a patient with an EE, we uncovered a de novo 13q33.1-q13.3 deletion including NBEA.

#### 3.2 Possibly pathogenic CNVs

Nineteen individuals (19/1097, 1.73%) had a total of 20 CNVs classified as possibly pathogenic (one individual had two possibly pathogenic CNVs); 10 were de novo (Table 3). For 17 of 19 individuals (18/20 CNVs), DNA was available to check the CNV and/or inheritance using MAQ analysis. Eleven of the 18 analyzed CNVs were confirmed; in seven cases, the test was inconclusive (Table 3). These CNVs were classified as possibly pathogenic because they included an epilepsy gene but were inherited or the direction of the change was not concordant with the known disease mechanism (loss or gain of function) or phenotype, or because they included a brain-expressed gene and were de novo. CNVs falling in the first category were a maternally inherited 10q23 deletion including LGI1, and a maternally inherited 20q13 duplication including KCNQ2, CHRNA4, and EEF1A2. Four other inherited CNVs included recessive genes: PLCB1, TBC1D24, ABAT, and CNTNAP2. A possible additional
single nucleotide variant (SNV) on the other allele cannot be excluded. Of note, the PLCB1 deletion was confirmed to be homozygous and would be considered pathogenic, but our flowchart was not developed for recessive analysis.

In the second category, we identified several interesting candidate genes including a de novo deletion including STAG1 and a de novo intragenic duplication in FGF12. In both genes, only recently have pathogenic SNVs been reported in patients with neurodevelopmental disorders including epilepsy.24,25 We further identified a deletion including SETBP1 associated with Shilton-Giedon syndrome and ID (OMIM 611060) and a duplication including HCN2, a gene in which SNVs exerting a gain-of-function effect have recently been suggested as a risk factor for genetic generalized epilepsies.26 HCN2 has also previously been associated with febrile epilepsy syndromes; interestingly, the patient carrying this CNV also had a history of febrile seizures.27 Other interesting candidate genes located in identified de novo deletions or possibly disrupted by intragenic breakpoints of identified duplications included FMN2 (also associated with AR mental retardation MIM616193), CHRM3, CSNK1G3, and NMT1, all of which are brain-expressed and predicted to be intolerant to loss of function (probability of loss-of-function intolerance ≥0.99) according to the latest gnomAD (http://gnomad.broadinstitute.org/about) constraint metrics (https://www.nature.com/articles/nature19057).

3.3 | Enrichment analysis

We collected phenotypic information on seven characteristics wherever this specific information was available: neurological or psychiatric disorder (528/956 patients, 55.2%), ID (727/944 patients, 77%), comorbidity with a nonneurological disorder (242/882 patients, 27.4%), facial dysmorphism (209/769 patients, 27.2%), brain abnormalities (288/613 patients, 47%), epilepsy onset before 1 year of age (175/340 patients, 51.5%), and diagnosis of known EE syndrome (238/487 patients, 49%). We compared carriers of pathogenic autosomal CNVs with those who did not carry a pathogenic autosomal CNV. Patients with pathogenic CNVs were significantly enriched for nonneurological disorders (2.68-fold) and for dysmorphism (4.09-fold; Figure 2). Beyond the overall pathogenic CNV enrichment, testing for large pathogenic CNVs (>1 Mb) separately showed a more profound and significant fold enrichment of 2.82 and 4.94 for comorbidity with nonneurological disorder and dysmorphism, respectively (Figure 2).

3.4 | Meta-analysis

The search identified 4806 citations, of which 59 papers met the inclusion criteria and were included in the systematic review. Overall meta-analysis showed that in patients with ID without epilepsy, the yield of pathogenic CNVs was 15% (95% confidence interval [CI] = 14-17), and in patients with psychiatric/neurological disorders without epilepsy, the yield was 8% (95% CI = 5-12; Figure S3, Table 4). These data were compared with the two subgroups from our cohort: (1) patients with epilepsy and intellectual disabilities, including autistic features, with a yield of 13.5% (95% CI = 9.2-18.9); and (2) patients with epilepsy and psychiatric/neurological comorbidities, with a yield of 10% (95% CI = 7.9-11.7). We did not find statistically significant differences for any of these comparisons (P values from heterogeneity test were >0.05).

The yield of pathogenic CNVs in patients with epilepsy-EE from our cohort (7.1%, 95% CI = 4.2-11.2) was lower, but not significantly different, than in patients with epilepsy-notEE from the meta-analysis (11%, 95% CI = 8-14; Table 4).

4 | DISCUSSION

Most epilepsies, especially when beginning in infancy and childhood, have a prominent genetic contribution. Numerous next generation sequencing, whole exome sequencing, and whole genome sequencing studies have been published in recent years uncovering single gene mutations in many epilepsies and epilepsy syndromes. Yet, the contribution of CNVs to the epilepsies, especially those complicated by comorbidities, has been less explored. Most published reports are single-center studies. The largest sample size was 2454 patients including a large cohort of 1366 patients with genetic generalized epilepsy in addition to 281 patients with rolandic epilepsy and 807 patients with adult focal epilepsy28,29; the biggest cohort specifically addressing the epilepsy plus phenotype studied 222 individuals.4 The maximum frequency of pathogenic CNVs reported in any of these series was 12%, with a range of 5%-12%. These studies tended to focus on individuals who were children at the time of testing.2,5,9 The importance of rare CNVs has been well recognized in patients with neuropsychiatric disorders including unexplained ID, congenital anomalies, and seizures. Thus, clinical geneticists, pediatric neurologists, and epileptologists commonly request chromosomal array CGH to obtain a genetic diagnosis for patients with such clinical features.

However, CNVs may be seen in healthy control individuals, and determination of the pathogenicity of newly identified CNVs can be challenging. To evaluate the role of pathogenic CNVs and identify possible candidate genes, we investigated the occurrence of CNVs in epilepsy plus, in a cohort among the largest reported to date.2-5,8 Data were collected from eight centers and included both adults and children. Autosomal CNV classification was conducted using a systematic filtering procedure specifically adapted
| Individual | CNV type | Chr region | Start (Mb) | Stop (Mb) | Size, Mb | Inheritance | Epilepsy genes | Epilepsy phenotype |
|------------|----------|------------|------------|-----------|----------|-------------|----------------|------------------|
| IT_FLO_041| Deletion | 1q42-q44   | 236852056  | 249212809 | 12.4     | De novo     | HNRNPu, AKT3    | Epilepsy NOS, DR  |
| BE_LEU_127| Duplication | 1q21.1-q44 | 144967252  | 249212666 | 104.2    | Unknown     | HNRNPu, AKT3    | Epilepsy NOS with infantile onset, DR |
| PO_W_031  | Deletion | 1q43-q44   | 241757184  | 250728885 | 3.3      | De novo     | HNRNPu, AKT3    | Focal of unknown origin |
| IT_FLO_062| Deletion | 1q44       | 244515959  | 247118959 | 2.6      | De novo     | HNRNPu          | Generalized epilepsy, DR |
| BE_LEU_009| Deletion | 1q44       | 244823848  | 248093878 | 3.3      | De novo     | HNRNPu          | Lennox-Gastaut syndrome |
| BE_ANT_005| Deletion | 2q24.3     | 163860225  | 172528095 | 8.7      | De novo     | SCN1A, SCN2A    | Generalized epilepsy of unknown origin |
| IT_FLO_020| Deletion | 5q14.3     | 88232244   | 90181244  | 1.9      | De novo     | ADGRV1          | Epilepsy and FS NOS |
| PO_W_027  | Deletion | 5q14.3-q15 | 87100153   | 92514871  | 5.4      | De novo     | ADGRV1, MEF2C   | Epilepsy NOS |
| IT_FLO_024| Deletion | 5q14q21    | 87770000   | 95780000  | 8        | Unknown     | ADGRV1, MEF2C   | Epilepsy NOS |
| BE_LEU_211| Deletion | 5q34       | 161059999  | 161446505 | 0.4      | Unknown     | GABRA1, GABRA6  | Epilepsy NOS |
| PO_W_019  | Deletion | 9q21.13    | 74741400   | 77306932  | 2.6      | De novo     | RORB            | Generalized photosensitive epilepsy (Jeavons syndrome) |
| BE_LEU_244| Deletion | 9q21.13    | 76474486   | 81651005  | 5.2      | Unknown     | RORB            | Generalized of unknown origin |
| US_267    | Deletion | 9q21.12-q21.13 | 72702925 | 77128468 | 4.4      | Unknown     | RORB            | Generalized epilepsy of unknown origin |
| Individual CNV type | Chr region | Start | Stop | Size, Mb | Inheritance | Epilepsy genes | Epilepsy phenotype | Other clinical features | Neuroimaging | Reported epilepsy phenotype associated with genes | Proposed disease mechanism (gain or loss of function) of reported epilepsy genes |
|---------------------|------------|-------|------|----------|-------------|----------------|---------------------|------------------------|--------------|-----------------------------------------------|---------------------------------------------------------------------|
| IT_FLO_041          | 1q42- q44  | 236852056 | 249212809 | 12.4     | De novo    | HNRNPU, AKT3   | Epilepsy NOS, DR    | ID, stereotypies, congenital microcephaly (-4 SD), facial dysmorphism | CC agenesis, holoprosencephaly | *HNRNPU*: epileptic encephalopathy, early infantile, 54 (MIM 617391)  
*AKT3*: megalencephaly, polymicrogyria, polydactyly, hydrocephalus syndrome 2 (MIM 615937) | Loss of function (Table S6a); loss and Gain of function (Table S6a for more details) |
| BE_LEU_127          | 1q21.1- q44 | 144967252 | 249212666 | 104.2     | Unknown    | HNRNPU, AKT3   | Epilepsy NOS, infantile onset, DR | ID, hypotonia, acquired microcephaly (-2 SD), facial dysmorphism, hypotonia | Frontal lobe atrophy and CC hypoplasia | *HNRNPU*: epileptic encephalopathy, early infantile, 54 (MIM 617391)  
*AKT3*: megalencephaly, polymicrogyria, polydactyly, hydrocephalus syndrome 2 (MIM 615937) | |
| PO_W_031            | 1q43- q44  | 241757184 | 245072885 | 3.3       | De novo    | HNRNPU, AKT3   | Focal of unknown origin | ID, hypotonia, acquired microcephaly (-2 SD), facial dysmorphism | Delayed myelination, atrophic septum pellucidum, aqueduct stenosis, hydrocephaly | *HNRNPU*: epileptic encephalopathy, early infantile, 54 (MIM 617391)  
*AKT3*: megalencephaly, polymicrogyria, polydactyly, hydrocephalus syndrome 2 (MIM 615937) | |
| IT_FLO_062          | 1q44       | 244515959 | 247118959 | 2.6       | De novo    | HNRNPU         | Generalized epilepsy, DR | ID, facial dysmorphism, hypotonia | CC agenesis, holoprosencephaly | *HNRNPU*: epileptic encephalopathy, early infantile, 54 (MIM 617391)  
*SCN1A*: epileptic encephalopathy, early infantile, 6 (Dravet syndrome; MIM 607208); epilepsy, generalized, with febrile seizures plus, type 2 (MIM 604403); febrile seizures, familial, 3A (MIM 604403);  
*SCN2A*: epileptic encephalopathy, early infantile, 11 (MIM 61372); seizures, benign familial infantile, 3 (MIM 6077451) | Loss of function; loss of function is associated with ASD, gain of function is associated with EE |
| BE_ANT_005          | 2q24.3     | 163860225 | 172528095 | 8.7       | De novo    | SCN1A, SCN2A   | Generalized epilepsy of unknown origin | ID, facial dysmorphism | Abnormal NOS |  
*SCN1A*: epileptic encephalopathy, early infantile, 6 (Dravet syndrome; MIM 607208); epilepsy, generalized, with febrile seizures plus, type 2 (MIM 604403); febrile seizures, familial, 3A (MIM 604403);  
*SCN2A*: epileptic encephalopathy, early infantile, 11 (MIM 61372); seizures, benign familial infantile, 3 (MIM 6077451) | Loss of function |
| IT_FLO_020          | 5q14.3     | 88232244  | 90181244  | 1.9       | De novo    | ADGRV1         | Epilepsy and FS NOS | ID, macrocephaly, facial dysmorphism | Abnormal NOS |  
*ADGRV1*: febrile seizures, familial, 4 (MIM 604352); myoclonic epilepsy (Table S2);  
*MEF2C*: mental retardation, stereotypic movements, epilepsy, and/or cerebral malformations (MIM 613443) | Loss of function |
| BE_LEU_211          | 5q34       | 161059999 | 161446505 | 0.4       | Unknown    | GABRA1, GABRA6 | Epilepsy NOS, ID | ID, scoliosis, gastroesophageal reflux, bilateral corneal opacity | Periventricular nodular heterotopia |  
*GABRA1*: epileptic encephalopathy, early infantile, 19 (MIM 615744); possible susceptibility allele; juvenile myoclonic epilepsy (MIM 611136) and childhood absence epilepsy (MIM 611136);  
*GABRA6*: possible susceptibility allele for childhood absence epilepsy (Table S2) | Loss of function |
| US_267              | 9q21.12- q21.13 | 72702925  | 77128468  | 4.4       | Unknown    | RORB           | Generalized epilepsy of unknown origin | ID, pyramidal sign, tremor, neurogenic bladder, psychotic episodes, severe macrocytic anemia, cold agglutinin disease, bilateral femuropatellar arthrosis, facial dysmorphisms | Corticosubcortical atrophy, supratentorial ventricular enlargement, periventricular vascular leukoencephalopathy, white matter lesions, lacunar infarcts in the basal ganglia and left thalamus |  
*GABRA1*: epileptic encephalopathy, early infantile, 19 (MIM 615744); possible susceptibility allele; juvenile myoclonic epilepsy (MIM 611136) and childhood absence epilepsy (MIM 611136);  
*GABRA6*: possible susceptibility allele for childhood absence epilepsy (Table S2) | Loss of function |
| BE_LEU_244          | 9q21.13    | 76474486  | 81651005  | 5.2       | Unknown    | RORB           | Generalized epilepsy of unknown origin | ID, episodic ataxia | Small nonspecific white matter lesions over right parietal hemisphere | Loss of function |
| PO_W_019            | 9q21.13    | 74741400  | 77306932  | 2.6       | De novo    | RORB           | Generalized epilepsy, ID | ID, autism, strabismus | Negative |  
*ADGRV1*: febrile seizures, familial, 4 (MIM 604352); myoclonic epilepsy (Table S2);  
*MEF2C*: mental retardation, stereotypic movements, epilepsy, and/or cerebral malformations (MIM 613443) | Generalized epilepsy, ID (Table S6b for more details) |

(Continues)
to epilepsy. The workflow was an essential tool to identify, reanalyze, and reinterpret CNVs in this retrospectively collected cohort, in which CNV testing had been performed using different platforms in different laboratories. About 11% of patients with epilepsy plus harbored a pathogenic autosomal CNV. This number reaches 12.7% when we also consider the possibly pathogenic CNVs. Previous similar studies report a diagnostic yield ranging from ~5% to 12%.\(^2\)\(^-\)\(^5\)\(^8\)\(^9\) Thus, our result fits at the upper limit of this range, probably mainly due to the “epilepsy plus” phenotype of our cohort and to the application of a standardized workflow. Previously published studies\(^3\)\(^4\)\(^8\) that reported similar yields of pathogenic CNVs (9.3%, 8.1%, and 12%, respectively) also examined patients with complex epilepsy including ID. Overall, results from both our and similar previous studies indicate that within the complex phenotype of neurodevelopmental disorders, when seizures are associated with ID or with other neurological and nonneurological comorbidities, there is a higher probability of identifying a pathogenic CNV than in epilepsy alone.

We checked the original classification, where available (138/142), of pathogenic and possibly pathogenic CNVs before and after applying the workflow method we propose here. We found that 7.2% (10/138) of cases were discrepant. The main direction of change was from CNVs (8/10 CNVs) originally classified as of “unknown significance” to “pathogenic” and “possibly pathogenic” (Table S7). This is expected as information about brain-expressed genes or gene regions associated with epilepsy increases. We have confirmed that reanalysis of existing data over time is essential.

Our study confirms the importance of specific CNVs in epilepsy and broadens some of the associated phenotypic spectra.

Recurrent microdeletions at 1q21.1, 15q11.2, 15q13.3, 16p13.11, and 22q11.21 have been reported as risk factors for GGEs and focal epilepsies.\(^2\)\(^8\)\(^9\) The most frequent CNV identified in our cohort was the 16p13.11 deletion, which accounts for 8.3% of the pathogenic CNVs, supporting a critical region have been associated with ID, dysmorphism, hypotonia, dyspraxia, clumsiness, ataxia, spasticity, kyphoscoliosis, and aortic valve deficiency; \(15q11.23\) is known to be associated with autism spectrum disorder (ASD), atrial septal defects; \(15q13.3\) with cerebral palsy, lissencephaly, and congenital heart defects; and \(16p13.11\) with intellectual disability. The most frequent CNV identified in our cohort was the 16p13.11 deletion, which accounts for 8.3% of the pathogenic CNVs, supporting a critical region have been associated with ID, dysmorphism, hypotonia, dyspraxia, clumsiness, ataxia, spasticity, kyphoscoliosis, and aortic valve deficiency. The most frequent CNV identified in our cohort was the 16p13.11 deletion, which accounts for 8.3% of the pathogenic CNVs, supporting a critical region have been associated with ID, dysmorphism, hypotonia, dyspraxia, clumsiness, ataxia, spasticity, kyphoscoliosis, and aortic valve deficiency. The most frequent CNV identified in our cohort was the 16p13.11 deletion, which accounts for 8.3% of the pathogenic CNVs, supporting a critical region have been associated with ID, dysmorphism, hypotonia, dyspraxia, clumsiness, ataxia, spasticity, kyphoscoliosis, and aortic valve deficiency.
Online Mendelian Inheritance in Man database.

The reported phenotype associated with each known epilepsy gene refers to the phenotype reported in the OMIM or, if not available, the citation indicated in the supplementary material (Table S2).

| Other clinical features | Neuroimaging | Reported epilepsy phenotype associated with genes | Proposed disease mechanism (gain or loss of function) of reported epilepsy genes |
|-------------------------|-------------|-------------------------------------------------|--------------------------------------------------------------------------------|
| ID, facial dysmorphism  | Negative    | Epileptic encephalopathy, early infantile, 27 (MIM 616139) | Loss and gain of function                                                      |
| ID                      | NA          | Rett syndrome, congenital variant (MIM 613454)    | Loss of function                                                              |
| ID, language delay, facial dysmorphophism, microcephaly, cryptorchidism | Polymicrogyria | GNAO1: epileptic encephalopathy, early infantile, 17 (MIM 615473); neurodevelopmental disorder with involuntary movements (MIM 617493); movement disorder with or without EE; GPR56: polymicrogyria (MIM 606854, 615752) | Loss of function; gain of function (recessive, loss of function) |
| ID, hypotonia, dyspraxia, clumsiness, convergent strabismus | Vermis hypoplasia | OMIM: periventricular nodular heterotopia (MIM 617201); Lennox-Gastaut syndrome–infantile spasms | Loss of function                                                              |
| Bilateral deafness, facial dysmorphism, lumbar kyphosis, sacral dimple, bilateral clinodactyly, small hands and fingers, hypoplastic flexion creases, atrial and ventricular septal defects, left renal agenesis defects, left renal agenesis | Periventricular nodular heterotopia | KCNQ2: epileptic encephalopathy, early infantile, 7 (MIM 613720); myokymia (MIM 121200); seizures, benign neonatal, 1 (MIM 121200); CHRNA4: epilepsy, nocturnal frontal lobe, 1 (MIM 600513) | Gain and loss of function; loss of function |
| ID, ataxia, spasticity, kyphoscoliosis, aortic valve deficiency | Enlarged lateral ventricles with pronunciation of occipital horns (colpocephaly) | Epileptic encephalopathy, early infantile, 30 (MIM 616341) | Loss of function                                                              |

Abnormalities of the corpus callosum, and seizures. This critical region includes HNRNP as the most relevant candidate epilepsy gene. Around 30 point mutations, mainly including truncating, splice-site, and a few missense variants, in HNRNP have recently been identified in individuals with ID and seizures (Table S6a). In our cohort, five patients carried CNVs mapping to the 1q43q44 critical region, and in addition to the HNRNP gene, in two duplications and one deletion, the chromosomal rearrangement included also the AKT3 gene, which might contribute to brain abnormalities observed in these patients. Patients with deletions showed dysmorphic features, early onset psychomotor delay, and early onset epilepsy. These data confirm the role of HNRNP in neurodevelopment and epileptogenesis.

Mutations in RORB were first reported in a patient with mild ID and partial epilepsy. More recently, other mutations were identified in patients with neurodevelopmental disorders and mostly GGE, including absence seizures (Table S6b). In our cohort, three patients carried deletions including RORB and exhibited ID and generalized epilepsy, including absence seizures with eyelid myoclonia, and autistic features in one patient, supporting a role for RORB in GGE and, more broadly, in several neurodevelopmental disorders.

Among the syndromic pathogenic autosomal CNVs, we identified three patients with duplications mapping to the 17p11.2 Potocki-Lupski syndromic region, which is reciprocal to the Smith-Magenis deletion syndrome in which epilepsy is often seen. These three patients had a phenotype consistent with Potocki-Lupski syndrome; the occurrence of epilepsy supports previous evidence of its presence as a rare feature of 17p11.2 duplications. Interestingly, we identified five patients with a 7q11.23 deletion containing the Williams-Beuren region; four of these individuals had Lennox-Gastaut syndrome, and the fifth (previously reported by Ramocki et al) had a generalized drug-resistant epilepsy.

CNVs classified as pathogenic only because of large size (Table S4b) represented 27% (33/122) of all the pathogenic CNVs. These CNVs included a large number of genes, but the phenotype of affected individuals was complex and we were unable to identify an association with known genetic syndromes or with candidate epilepsy genes. However, for
| Individual   | CNV type | Chr region | Start       | Stop        | Size, Mb | Inheritance       |
|--------------|----------|------------|-------------|-------------|----------|-------------------|
| BE_LEU_009  | Duplication | 1q43      | 239842929   | 240356854   | 0.5      | De novo           |
| US_184      | Duplication | 3q28      | 191886383   | 192432844   | 0.5      | De novo           |
| BE_LEU_141  | Deletion  | 3q22.3     | 136035522   | 136412948   | 0.4      | De novo           |
| IT_FLO_036b | Duplication | 4q21.22-q21.23 | 84035965   | 84813544   | 0.8      | De novo           |
| IT_FLO_127  | Deletion  | 5q23.2     | 122481284   | 122987185   | 0.5      | De novo           |
| PO_W_039    | Duplication | 7q35-q36.1 | 146934489   | 148471787   | 1.5      | Inherited (M)     |
| IT_FLO_131  | Deletion  | 6q26       | 161725639   | 16187527    | 0.2      | De novo           |
| IT_FLO_109  | Duplication | 8p23.3-p23.2 | 161272     | 801514     | 0.6      | Unbalanced segregation of a balanced translocation (M) |
| IT_FLO_134  | Deletion  | 8p23.3-23.2 | 221611     | 801373     | 0.6      | Unbalanced segregation of a balanced translocation (M) |
| BE_LEU_236  | Duplication | 9q22.31    | 95208377    | 95590171    | 0.4      | De novo           |
| IT_FLO_144  | Deletion  | 10q23.33   | 95490322    | 95791986    | 0.3      | Inherited (M)     |
| BE_LEU_012  | Duplication | 15q13.2    | 32509932    | 32509932    | 1.6      | Inherited (P)     |
| UK_L_056    | Duplication | 16p13.3    | 2481289     | 2888632     | 0.4      | Unknown           |
| US_175      | Deletion  | 16p13.2    | 8368145     | 8860296     | 0.5      | Inherited (M)     |
| IT_FLO_023  | Deletion  | 17q21.31   | 43160474    | 43922220    | 0.8      | De novo           |
| IT_FLO_083  | Deletion  | 18q12.3    | 42605437    | 42784321    | 0.2      | De novo           |
| BE_LEU_116  | Duplication | 19p13.3    | 538568      | 2268870     | 1.7      | Unknown           |
| US_124      | Deletion  | 20p12.3    | 8314301     | 8688028     | 0.4      | Inherited (M+P)   |
| PO_W_030    | Duplication | 20q13.33   | 61925286    | 62724437    | 0.8      | Inherited (M)     |

ADHD = attention-deficit/hyperactivity disorder; BP = breakpoint; CC = corpus callosum; CNV = copy number variation; CT = computed tomography; DR = drug resistant; FS = febrile seizures; ID = intellectual disability; M = maternal; MAQ = multiplex amplicon quantification; NA = not available; NOS = not otherwise specified; P = paternal.

*Column with candidate genes also includes CNVs including known epilepsy genes that have not been considered pathogenic for various reasons; for example, the direction of the change or the phenotype did not fit to what is reported in the literature, or the CNV was inherited from a parent with unknown affectedness status.

*Reported in Ottaviani et al.43
| Proposed candidate genes<sup>a</sup> | Epilepsy phenotype | Other clinical features | Neuroimaging | MAQ validation |
|-------------------------------------|--------------------|------------------------|--------------|----------------|
| *FMN2* (start BP within gene), *CHRM3* (stop BP within gene) | Epilepsy NOS | ID, scoliosis, gastroesophageal reflux, bilateral corneal opacity | Delayed myelination, atrophic septum pellucidum, aqueduct stenosis, hydrocephaly | De novo |
| *FGF12* (intragenic duplication) | Epilepsy NOS | Learning disabilities, attention deficit | Malrotation anterior and central part left hippocampus | NA |
| *STAG1, PCCB* (start BP within gene) | Epilepsy NOS | ID, autism, hypertonia, scoliosis, | NA | Confirmed in patient, absent in mother |
| *COQ2* (stop BP within gene) | Myoclonic-atomic epilepsy | ID | Negative | De novo |
| *CEP120, CSNK1G3* (stop BP within gene) | Myoclonic epilepsy | ID, hypotonia | Negative | De novo |
| *CNTNAP2* (start BPs within gene) | Epilepsy NOS | ID | CC hypoplasia | Inconclusive |
| *PARK2* (stop BP within gene) | Epilepsy NOS, FS | ID, hypotonia, obesity crowding of the fingers in both hands and feet, onychodystrophy | NA | Inconclusive |
| *FBXO25* | Generalized epilepsy of unknown origin, DR | Language disorder | Negative | Inconclusive |
| *FBXO25* | Myoclonic epilepsy, FS | No | NA | Inconclusive |
| *BICD2* | Epilepsy NOS | ID | NA | De novo |
| *LGI1* | Epileptic encephalopathy NOS | Severe ID, quadriplegia, congenital cardiomyopathy (implanted pacemaker) | Cerebral atrophy microcephaly | Maternally inherited |
| *CHRNA7* | Focal of unknown origin | ID | No | Inconclusive |
| *TBC1D24* | Myoclonic-atomic epilepsy | ID | Negative | Confirmed in proband, parents NA |
| *ABAT* (stop BP within gene) | Epileptic encephalopathy NOS | ID, apraxia, dyskinesia, generalized hypotonia | Negative | Maternally inherited |
| *NMT1* (start BP within gene), *PLCD3* (start BP within gene) | Focal epilepsy of unknown origin, FS | ID, macrocephaly, facial dysmorphism, cardiac defect, skin dyschromia | CC hypoplasia | Inconclusive |
| *SETBP1* (start BP within gene) | Generalized epilepsy of unknown origin, DR | ID | Negative | De novo |
| *HCN2* | Epilepsy NOS and FS | Learning disabilities, ADHD, facial dysmorphism | Negative | Confirmed in proband, parents NA |
| *PLCB1* (start BP within gene) | Epileptic encephalopathy NOS | Profound ID, microcephaly, hypertonia, hyperreflexia more prominent on the left side, squint in left eye | Atrophy on CT brain | NA |
| *KCNQ2, CHRNA4, EEF1A2* | Focal epilepsy of unknown origin | ID, facial dysmorphism | NA | Confirmed in proband, parents NA |

<sup>a</sup>Column with candidate genes also includes CNVs including known epilepsy genes that have not been considered pathogenic for various reasons; for example, the direction of the change or the phenotype did not fit to what is reported in the literature, or the CNV was inherited from a parent with unknown affectedness status.

<sup>b</sup>Reported in Ottaviani et al.43
one individual with EE and a large de novo 13q13.1-q13.3 deletion, we can suggest that a key gene is \textit{NBEA}, which was reported as a possible EE gene through an in silico prioritization approach\textsuperscript{35} and was recently associated with neurodevelopmental disease with epilepsy.\textsuperscript{36}

Four of the CNVs we classified as large and pathogenic were inherited. Interestingly, a duplication on 12q21.31 was inherited from a mother with a family history of autism. Autism has been reported in Decipher in a patient carrying an overlapping duplication (Table S4b). Following our algorithm, we consider these CNVs pathogenic, noting the incomplete penetrance often characterizing neurological and epileptic disorders and because we could not exclude related neurological traits in the transmitting parent.

A possibly pathogenic autosomal CNV was identified in 1.7\% of the patients. As well as some known epilepsy genes, discussed in the results section, we propose other genes in these regions that can be considered potential candidates for causing epilepsy, but need further validation. We found a de novo 18q12.3 deletion, which only encompassed the gene \textit{SETBP1}. Heterozygous missense mutations in \textit{SETBP1} cause Schinzel-Giedion syndrome (OMIM #269150), characterized by severe ID and specific craniofacial features,\textsuperscript{37} wherein seizures also occur.\textsuperscript{38,39} Mutations leading to haploinsufficiency, such as the deletion in our patient, have been reported in association with a distinct neurological syndrome, which includes mild to moderate ID without the typical syndromic craniofacial features.\textsuperscript{17,40,41} The patient in this study only showed severe epilepsy and ID, suggesting that the \textit{SETBP1}-mutation phenotype may be broader than previously described. One patient had a microdeletion, classified here as possibly pathogenic, which includes \textit{STAG1}, now linked with epilepsy as a cohesinopathy,\textsuperscript{24} and one patient carried a de novo intragenic duplication in \textit{FGF12} in which SNVs have recently been reported in patients with epileptic encephalopathies.\textsuperscript{25}

Other interesting candidate genes are highlighted in Table 3 and include \textit{HCN2}, \textit{FMN2}, \textit{CHRM3}, \textit{CSNK1G3}, and \textit{NMT1}.

Eleven patients (1\%) in our study cohort had a double hit (including pathogenic and possibly pathogenic CNVs). Here, the CNV burden alone could contribute to the neurodevelopmental phenotype; as shown by Girirajan and colleagues,\textsuperscript{42} children with two or more rare and large CNVs of unknown significance were eight times more likely to have developmental delay compared to controls, possibly by disruption of dosage-sensitive genes.\textsuperscript{42} We note, however, that our analysis focused only on CNVs with a certain pathogenic meaning and as such gives no insight into the general burden of CNVs per patient.
The enrichment analysis showed a significant association of pathogenic autosomal CNVs with non-neurological disorders and dysmorphism (for both large pathogenic and any pathogenic CNV); large pathogenic CNVs showed a more profound and significant association with dysmorphism and non-neurological disorders only. An enrichment of CNVs in patients with dysmorphism has been observed in previous studies, underscoring the importance of testing for CNVs in patients with epilepsy and associated comorbidities. Likewise, results from our data compared to historical controls, from a systematic literature review and meta-analysis, confirm that the percentages of pathogenic CNVs, when the phenotype includes or excludes epilepsy, do not vary significantly. Thus, although a search for CNV is undoubtedly worthwhile in people with epilepsy plus, it may not be that such CNVs drive only epilepsy, but for patients ascertained through their epilepsy, the presence of additional features points to an elevated likelihood of finding an underlying pathogenic CNV. We hypothesize that although epilepsy as a phenotype does not add a quantitative contribution to the diagnostic yield, its presence could be related to the type, location, and gene content of an underlying pathogenic CNV. Results from our data, comparing patients with epilepsy-EE versus historical controls with epilepsy-notEE, showed a nonsignificantly lower yield of pathogenic CNVs in patients with EE, raising a possible hypothesis that when epilepsy manifests as EE, the likelihood of finding a pathogenic CNV decreases and that EE is more often the consequence of single gene mutations.

Our study has limitations beyond its retrospective structure. The filtering workflow used allowed us to obtain a systematic classification of the large number of CNVs examined, but we recognize it is not perfect and might not accurately classify CNV mapping to hypervariable chromosomal regions. Pathogenic CNVs could be missed due to filtering out of small CNVs, misclassification of abnormalities, or an incomplete list of genes associated with epilepsy (new epilepsy-related genes continue to be reported). We excluded the sex chromosomes from the CNV calling and subsequent analysis, because copy number calling from these chromosomes is prone to false-positive calls and might inflate the reported frequencies of diagnostically relevant CNVs as the X chromosome in particular has been associated with neurodevelopmental disorders. Also, recessive disease cannot be ruled out with this type of analysis unless the second allele is studied with another approach.

In conclusion, we highlight the pathogenic causative role of autosomal CNVs in almost 11% of patients (and up to 12.7% when also considering the possibly pathogenic) with unexplained epilepsy with comorbidities and reiterate the concept that CNVs should be sought in patients with seizures especially when associated with other neurological and non-neurological conditions.

This study opens new perspectives for a better understanding and evaluation of CNVs identified in patients with epilepsy plus. We show that the reinterpretation of preexisting data using an adapted workflow can highlight new findings, and we recommend periodic systematic review of preacquired genetic data, as new methods and data become available. The workflow used here, specifically designed for epilepsy, can be used to homogenize data from different cohorts often collected at different times. Establishing the causative role of some CNVs can be challenging, especially when the CNV is not associated with a known syndrome, or similar CNVs may not be of the same size, might include different genes, and not have familial segregation data available to help interpretation. Bespoke, disease-specific algorithms may assist in assignment of CNVs to diagnostic categories that are more definitive than either “possibly pathogenic” or “of unknown significance.” There remain CNVs whose role will only be clarified by increasing the number of cases studied, functional studies, and continued exchange between clinicians and laboratory scientists. This study represents the first project of a newly formed and growing international consortium for CNVs in epilepsy (EpiCNV), in which large-scale data aggregation and sharing will be utilized as a new tool for CNV and gene identification in the epilepsies.

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DISCLOSURE OF CONFLICT OF INTEREST

A.C. has received honoraria from Eisai for participating in on an advisory board and as a speaker. B.C. has received honoraria for consulting and advisory board services from Brabant Pharma, Zogenix, and Novartis. J.H.C. is involved in clinical trials for GW Pharma and Zogenix; has received research grants from Vitaflo, NIHR, Action Medical Research, SPARKS, and the European Union; has been part of advisory boards for Takeda, Shire, UCB, and Eisai; and has given lectures for Shire and Zogenix; all honoraria and funds were given to the department. L.L. has received honoraria from Zogenix, Livanova, Shire, Takeda, UCB, and Novartis. P.S. has received speaker honoraria and travel grants from Eisai, Kolfarma, FB Health, and Zogenix. C.M. is an associate editor of Epileptic Disorders and has received speaker honoraria from SOBI (Swedish, Orphan Biovitrum). None of the other authors has any conflict of interest to disclose. We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design of the study, acquisition of data, or
analysis and interpretation of data; drafting of the article or revising it critically for important intellectual content; or appropriate investigation of accuracy and integrity of some part of the manuscript, in addition to giving final approval of the submitted version. The individual contribution for the manuscript of each author is as follows. A.C.: analysis and interpretation of data, manuscript preparation, revising manuscript critically for important intellectual content; E.C.: analysis and interpretation of data, manuscript preparation, revising manuscript critically for important intellectual content; H.S.: analysis and interpretation of data, manuscript preparation, revising manuscript critically for important intellectual content; V.C.: analysis and interpretation of data, manuscript preparation, revising manuscript critically for important intellectual content; E.Sa.: analysis and interpretation of data, manuscript preparation, revising manuscript critically for important intellectual content; H.V.E.: acquisition of data, revising manuscript critically for important intellectual content; V.C.: analysis and interpretation of data, manuscript preparation, revising manuscript critically for important intellectual content; E.Sz.: acquisition of data, revising the manuscript; A.P.: analysis and interpretation of data, revising the manuscript; J.H.C.: acquisition of data, revising the manuscript; D.L.: analysis and interpretation of data, revising the manuscript; M.B.-G.: acquisition of data, revising the manuscript; B.C.: acquisition of data, revising the manuscript; J.H.C.: acquisition of data, revising the manuscript; S.D.M.: analysis and interpretation of data; T.D.: acquisition of data, revising the manuscript; N.L.: acquisition of data, revising the manuscript; L.L.: acquisition of data, revising the manuscript; R.G.: study concept, revising manuscript critically for important intellectual content; D.H.-Z.: acquisition of data, revising the manuscript; F.K.: acquisition of data, revising the manuscript; L.L.: acquisition of data, revising the manuscript; R.G.: study concept, revising manuscript critically for important intellectual content; D.H.-Z.: acquisition of data, revising the manuscript; N.L.: acquisition of data, revising the manuscript; A.P.: analysis and interpretation of data, revising the manuscript; A.T.: acquisition of data, revising the manuscript; J.R.V.: acquisition of data, revising the manuscript; H.V.E.: acquisition of data, revising the manuscript; W.V.P.: acquisition of data, revising the manuscript; J.J.W.: acquisition of data, revising the manuscript; F.Z.: acquisition of data, revising the manuscript; P.D.J.: study concept and supervision, manuscript preparation, revising manuscript critically for important intellectual content; S.M.S.: study concept and supervision, manuscript preparation, revising manuscript critically for important intellectual content; C.M.: study concept and supervision, interpretation of data, manuscript preparation, revising manuscript critically for important intellectual content.

ETHICS

This study was approved by the ethics committees of the participating centers. Written informed consent was provided by each patient, or the parent or guardian of each patient, as appropriate.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.
APPENDIX: COLLABORATORS

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