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Epilepsia™

CRITICAL REVIEW

Solving the unsolved genetic epilepsies: Current and future perspectives

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Abstract

Many patients with epilepsy undergo exome or genome sequencing as part of a diagnostic workup; however, many remain genetically unsolved. There are various factors that account for negative results in exome/genome sequencing for patients with epilepsy: (1) the underlying cause is not genetic; (2) there is a complex polygenic explanation; (3) the illness is monogenic but the causative gene remains to be linked to a human disorder; (4) family segregation with reduced penetrance; (5) somatic mosaicism or the complexity of, for example, a structural rearrangement; or (6) limited knowledge or diagnostic tools that hinder the proper classification of a variant, resulting in its designation as a variant of unknown significance. The objective of this review is to outline some of the diagnostic options that lie beyond the exome/genome, and that might become clinically relevant within the foreseeable future. These options include: (1) re-analysis of older exome/genome data as knowledge increases or symptoms change; (2) looking for somatic mosaicism or long-read sequencing to detect low-complexity repeat variants or specific structural variants missed by traditional exome/genome sequencing; (3) exploration of the non-coding genome including disruption of topologically associated domains, long range non-coding RNA, or

other regulatory elements; and finally (4) transcriptomics, DNA methylation signatures, and metabolomics as complementary diagnostic methods that may be used in the assessment of variants of unknown significance. Some of these tools are currently not integrated into standard diagnostic workup. However, it is reasonable to expect that they will become increasingly available and improve current diagnostic capabilities, thereby enabling precision diagnosis in patients who are currently undiagnosed.

KEYWORDS

DNA methylation, epilepsy, epilepsy genetics, metabolomics, non-coding regions, re-analysis, somatic mosaicism, transcriptomics

1 | INTRODUCTION

Epilepsy is one of the most common neurological disorders worldwide and is a frequent feature of neurodevelopmental disorders (NDDs). Recognizing the cause of seizures is crucial for accurate diagnosis, treatment, and monitoring, and for providing prognostic guidance. Etiologies can be divided into a variety of categories such as genetic, metabolic, structural, infectious, and immune factors. These are not mutually exclusive categories, and many etiologies belong to more than one category; a metabolic disorder may fall into both the metabolic and genetic categories, whereas tuberous sclerosis may be classified as having both a genetic and a structural etiology. The intricate genetic architecture that underlies the epilepsies is estimated to contribute to ~60%–70% of all cases, indicating the significant impact of genetic factors.[1] Although our current understanding about the genetic similarities and differences between NDDs with and without epilepsy remains limited, it is clear that both groups will benefit from accurate genetic testing.[2]

Over the past decade, genetic testing in the form of next-generation sequencing (NGS) has become a less-expensive, faster, and thus more widely used diagnostic tool in the field of human genetics. With regard to epilepsy, genetic testing of affected individuals and their relatives has revealed numerous genes involved in monogenic epilepsies. Such monogenic epilepsies include familial focal epilepsies, including familial self-limited neonatal epilepsy, neonatal-infantile epilepsy, and infantile epilepsy, where rare variants in *PRRT2*, *KCNQ2*, and *SCN2A* are the underlying genetic cause in many families.[3–5] It also includes sporadic developmental and epileptic encephalopathies (DEEs), caused by ultra-rare and de novo variants that are often located in genes encoding ion channels.[6–8] In addition, ultra-rare variants in numerous other genes involved in diverse biological processes such as transcription, DNA repair, protein modulation, cell proliferation and differentiation, cellular trafficking, and extracellular matrix homeostasis have been identified as causative factors.[9–11] Indeed, according to a seminal paper by the Epi25Consortium,[12] individuals with any type of epilepsy carried an excess of ultra-rare, deleterious variants in constrained genes and in genes previously associated with epilepsy. Notably, the strongest enrichment of such variants was reported in individuals with DEEs.

Even in epilepsies due to malformations of cortical development, genetics seems to play a prominent role; a recent International League Against Epilepsy (ILAE) task force project advocates for integrating genetic testing in the diagnostic workup of focal cortical dysplasia.[13] Monogenic causes of generalized epilepsies exist but are less common.[14] Common genetic variation (polygenic risk) has been shown to contribute to epilepsy, in particular to generalized epilepsies, but also to focal epilepsies.[15] More recent work has shown that common genetic variation also contributes to DEE, even in cases where

Key Points

- Even if exome sequencing is negative in patients with epilepsy there are additional diagnostic options in the pipeline.
- Re-analysis of existing data and consideration of somatic mosaicism are the first steps to be taken when a genetic diagnosis is not reached by exome sequencing.
- Short- and long-read whole genome sequencing allow investigation of the non-coding regions of the genome.
- Analysis of the transcriptome, the methylome, and the metabolome helps to improve interpretation of genetic findings of unknown significance.

a presumed monogenic cause has been identified. This illustrates that although some DEEs are monogenic disorders, these disorders are also characterized by a more complex genetic architecture.[16]

An accurate genetic diagnosis can end the diagnostic odyssey. Moreover, a genetic diagnosis offers patients and relatives a better understanding of their condition and enables informed decision-making regarding reproductive choices for parents and other relatives. It also allows improved genotype-driven classification of epilepsy, thereby further facilitating homogenous cohort and natural history studies. Most excitingly, it may also directly influence treatment choices for patients and thus become the starting point of personalized medicine.[8]

In current practice, gene panels or more recently exome sequencing (ES) and chromosome microarrays have been used to capture disease-causing single nucleotide variants (SNVs) and copy number variants (CNVs), respectively. It is important to note the limitations of these technologies, as certain genetic disorders, such as Angelman syndrome (an imprinting disorder) and Fragile X syndrome (a disorder with trinucleotide expansions), cannot be identified through ES or microarrays. Presently, separate diagnostic tools are still required for their detection. However, future advancements in whole genome sequencing (WGS), and especially long-read sequencing,[17] will eventually enable us to also capture these disorders in a one-fits-all test. ES and WGS offer a higher diagnostic yield compared to multigene panels or microarrays.[18,19] Therefore, ES and (when available) WGS should be considered as the first tier in the diagnostic workup for epilepsy and should be considered in patients with neonatal or infantile onset seizures, DEEs, familial epilepsies, or seizures accompanied by cognitive and psychiatric comorbidities.[14] Although ES captures mainly the protein-coding sequences, which is ~2% of the human genome, WGS assesses the complete genome, including non-coding sequences. WGS is also more performant than ES in detecting CNVs and structural variants (SNVs). Despite these advantages of WGS, it is important to emphasize that in most clinical applications, WGS analysis is still often only exome focused ("pseudo-exome/panel analysis"), as it remains difficult to interpret the effects of non-coding variants. Although NGS technologies have transcended our ability to detect new or known epilepsy genes, the diagnostic yield remains at ~45%–48% with ES or WGS.[14] Common explanations for negative exomes or genomes include that (1) the underlying cause is non-genetic; (2) there is a complex, non-mendelian genetic cause of illness (including oligogenic, polygenic, and multifactorial causes); (3) there is a monogenic cause that was not identified on ES or WGS, such as disease-causing variants that are located in a deep intronic or regulatory region, inherited causative variants with reduced penetrance that remained unnoticed upon variant filtering, a mosaic variant that is not detectable in all tissues, or a complex structural disease-causing variant that is often missed with exome sequencing; and (4) the disease-causing variant is present within the exome but remains a variant of unknown significance based on the tools and knowledge available at the time of the initial analysis.[20] This means that many patients with a genetic epilepsy still remain undiagnosed and that better diagnostic tools still need to be developed.

This review addresses the diagnostic strategies that could be utilized in patients that remain genetically unsolved despite extensive diagnostic testing (Figure 1). Some of these are diagnostic options that lie beyond the exome and genome but are not yet readily available in the clinical setting. However, we anticipate that they will become clinically relevant and integrated into routine diagnostics in the coming years, as technology continues to advance.

2 | FIRST STEP: RE-ANALYSIS OF EXOME/GENOME SEQUENCING DATA

Unlike many other diagnostic tests, a negative ES or WGS does not rule out a genetic disorder; instead, it only implies that a genetic diagnosis could not be identified, given the clinical and genetic information available at the time of analysis.

Regular re-analysis and re-interpretation of sequencing data is expected to increase the diagnostic yield based on new information from the literature, new bioinformatic tools, and the constantly updated disease and population databases. A key driver of new insights from re-analysis of exome/genome data is the discussion within a multidisciplinary team including laboratory and clinical geneticists, bioinformaticians and computational scientists, genetic counselors, disease specialists (in this case epileptologists), basic scientists, and the referring clinicians. Previous studies have shown that reanalyzing ES and WGS data can increase the diagnostic yield by 5%–26%.[20] Although the time frame between the initial analysis and re-analysis in these studies varies from 6 months to 7 years, most re-analyses were conducted at intervals of 1–2 years. Further studies should determine the optimal timing for re-analysis, considering the balance between the rate of new gene and variant discoveries and the costs and efforts required for re-analysis.

Re-analysis is currently considered to be an option to both the ordering health care provider and the clinical testing laboratory. In some instances, it may even

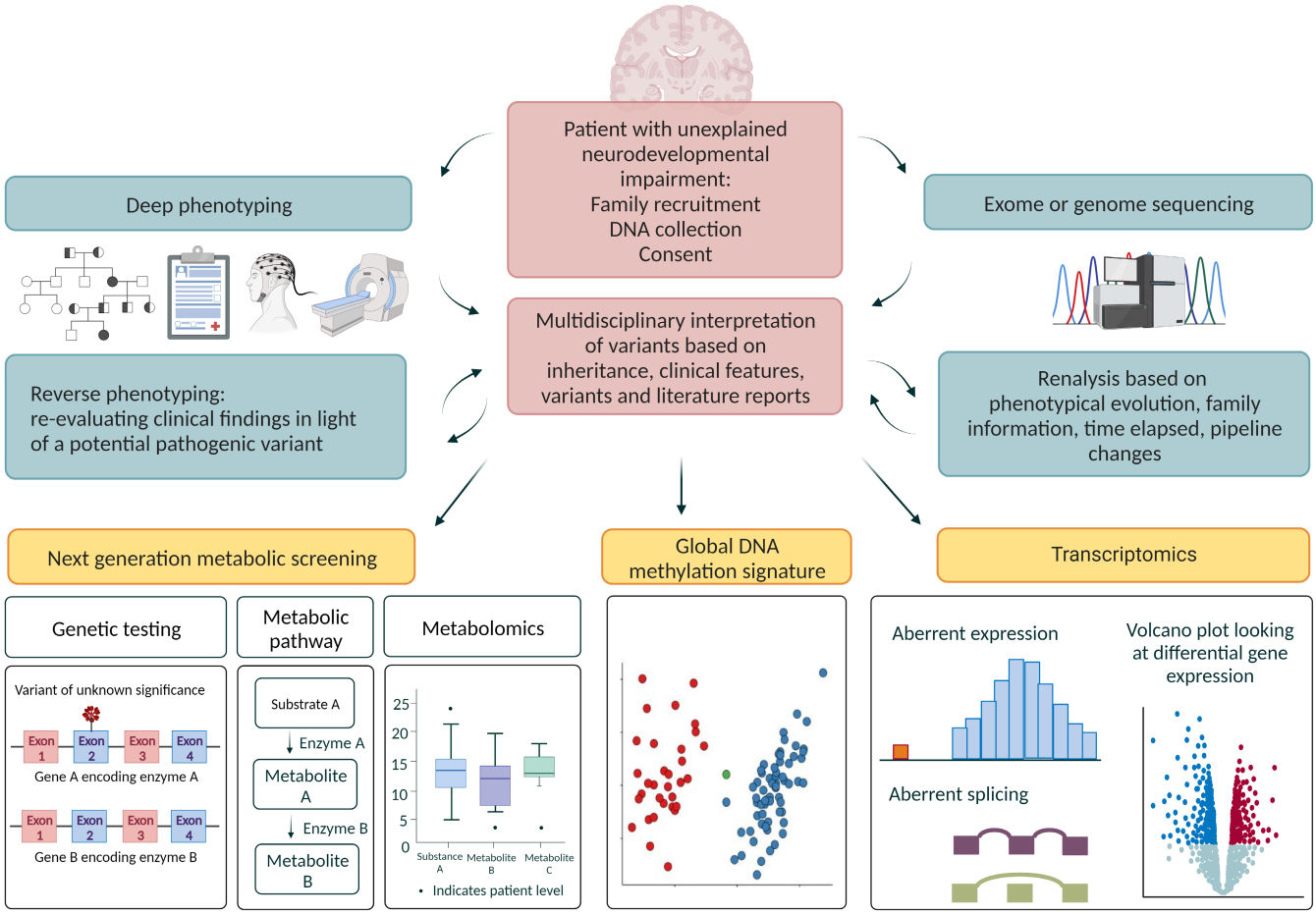

FIGURE 1 Beyond the exome or genome—the possibilities in the diagnostic pathway of genetically undiagnosed patients with epilepsy.

be requested by patients or caregivers themselves. Most commonly, re-analysis is ordered by the physician on a patient-by-patient basis, or by a clinical laboratory on a cohort level.[20] A health care provider may request re-examination of genetic test results for various reasons, such as changes in a patient's phenotype. A variant may have been excluded from further analysis or reporting if a commonly observed clinical feature of a disease was absent during the initial analysis. In addition, updates on the patient's family history are useful, as clinical assessment and genetic testing of family members can aid in identifying or ruling out variants. In addition, re-analysis may be performed if several years have passed since the initial test[21] or if a new gene has been identified that may be relevant to the patient's condition. The initial analysis may have detected one or more variants of unknown significance (VUS) or candidate genes with uncertain links to human disease; a re-analysis may be initiated to check for updates on new information regarding such variants. Finally, a health care provider may choose to request a re-analysis in the event of an updated bioinformatics pipeline, such as an improved capability in detecting CNVs.[20] There is also a great potential for automated data re-analysis tools to be integrated into the standard pipelines of routine diagnostic laboratories.

When reanalyzing ES/WGS data it is important to consider the quality and completeness of the data, such as the coverage of the entire coding region of clinically relevant genes and the read depth of the covered regions. This is particularly important for older ES data, as newer ES capture platforms have improved capabilities and performance; if ES failed to provide a diagnosis, it may be beneficial to consider resequencing the exome using a newer platform or to perform WGS. In clinical practice it is preferable that coverage of the targeted areas for ES is at least 98%, and the preferable read depth is 20x for both ES and WGS. WGS does not rely on the capture of selected genetic regions and covers all regions of the genome without the potential enrichment bias that ES capture platforms can introduce. This can help overcome the limitations of ES in sufficiently covering coding exons, especially GC-rich regions, as well as in characterizing structural variants that usually cannot be resolved using ES.[22,23] Despite all these options, ultimately, the decision of which sequencing method to be used is often determined based on the available local resources.

3 | THE HIDDEN GENETICS OF THE EPILEPSIES: VARIANTS TYPICALLY MISSED BY CURRENT DIAGNOSTIC SEQUENCING STRATEGIES

3.1 | Somatic mosaicism

Somatic mosaicism refers to the presence of genetically distinct cell populations within an individual, which can arise due to variants occurring during embryonic development or later in life. Depending on the timing and location of the variant, mosaic variants will be present in most or only very specific tissues. As such, they can still be detectable in a sizable fraction of blood-derived DNA used in conventional diagnostic testing or might be identifiable only in specific cell types such as neurons. Even low-level mosaicism in blood may not be detectable in routine genetic testing, and its identification requires specialized techniques such as deep sequencing. In recent years, somatic mosaicism has been shown to play an important role in genetic epilepsies.

Somatic mosaicism is emerging as a significant contributor to the hidden genetics of epilepsy. Mosaicism can be restricted to brain tissue, as evidenced by studies of individuals with malformations of cortical development (MCDs) such as focal cortical dysplasias (FCDs), disorders that often lead to co-morbid focal epilepsy and often require epilepsy surgery, which sometimes allows the study of brain-derived DNA. In patients with MCDs, somatic mutations have been identified in genes involved in the regulation of neuronal proliferation, migration, and differentiation, affecting key signaling pathways such as the PI3K–AKT–mTOR pathway.[24,25] The mosaic distribution of mutant cells in affected brain regions leads to abnormal cortical architecture and connectivity, as well as increased neuronal excitability and seizures. Although somatic variant detection in resected brain tissue is not yet routinely applied in most epilepsy centers, the ILAE Diagnostic Methods Commission advocates integrating genetic findings as an objective measure for the diagnosis of FCD after surgery, and recommends the use of appropriate protocols for reliable detection of low-level brain mosaicism in FCDs including high-depth NGS of >1000× reading depth.[13]

Recent evidence indicates that somatic mutations restricted to the brain are also involved in focal epilepsy without obvious brain abnormalities on MRI. Brain somatic variants in the X-linked gene *SLC35A2* have been identified in individuals with the newly identified clinical entity of mild malformation of cortical development with oligodendroglial hyperplasia in epilepsy (MOGHE), associated with pediatric drug-resistant focal epilepsy.[26] Although MRI is most often suggestive of an MCD, structural imaging can be reportedly normal. Germline variants *SLC35A2* are known to lead to a rare congenital disorder of glycosylation, leading to a severe neurodevelopmental disorder including seizures, again highlighting the relationship between phenotypic severity and cellular distribution of the culprit gene variant.

The inaccessibility of brain tissue in most epilepsy patients is obviously a major hurdle for the identification of somatic variants. To overcome this, several studies have attempted to uncover recurrent mutations in a variety of brain disorders, including brain malformations and tumors, by analyzing cell-free DNA (cfDNA) in cerebrospinal fluid (CSF), also called CSF liquid biopsy. This cfDNA is the product of fragmented genomes released from necrotic or apoptotic cells. Targeted amplicon sequencing and droplet digital polymerase chain reaction (PCR) of cfDNA in CSF have been successful in confirming known somatic mutations in these disorders.[27,28] To date, it is not yet feasible to identify somatic variants in CSF cfDNA when no prior knowledge of the mosaic variant is available. Once such a technique is optimized and given the potential treatment implications associated with a diagnosis of an mTOR pathway defect (e.g., mTOR inhibitors such as rapamycin) or *SLC35A2*-related glycosylation defects (oral galactose supplementation), the minimal risk of a lumbar puncture may be a decent trade-off for the potential of precision therapy. The identification of somatic mosaicism in MCD further provides a proof of concept for the role of somatic mutations in epilepsy and highlights the need for more sensitive and comprehensive genetic testing approaches to uncover the hidden genetics of unexplained, presumed genetic, sporadic epilepsies.

3.2 | Structural variation and repetitive regions

The typical approach for performing ES or WGS involves generating numerous "short" sequencing reads, often in the form of paired-end 150 bp reads. Subsequently these reads are aligned to the human reference assembly and variations from the reference are detected. However, this process comes with several challenges, such as accurately aligning variant reads to a specific genomic location in a confident manner or bridging highly repetitive regions. Examples of variations that are typically overlooked with short-read sequencing include low-complexity repeat variants (short tandem repeats and repeat expansions), structural variants such as inversions, translocations and segmental duplications, and mobile element insertions.[29] Recently a number of methodologies facilitating longer read lengths up to tens of kilobases of length, such as nanopore-based sequencing and single-molecule

real-time sequencing, have emerged.[30–32] Because of their ability to directly sequence individual DNA molecules, long-read sequencing technologies can also directly detect base modifications, such as DNA methylation, enabling the profiling of epigenetic marks at single-base resolution. This enables the simultaneous analysis of the genome and epigenome. There is an increasing amount of evidence supporting the superior variant detection ability of long reads compared to short reads, including in the field of epilepsy and neurodevelopmental disorders.[29,33–36] However, the technique remains very expensive and the diagnostic utility remains to be reported in larger cohorts of patients with epilepsy.

4 | THE HIDDEN GENETICS OF THE EPILEPSIES: THE DARK MATTER OF THE GENOME

4.1 | Introducing the non-coding genome

The focus of current-day diagnostics for most genetic disorders including epilepsy, is on the part of the human genome that contains protein-coding genes. The exome consists of ~2% of all human DNA nucleotides, with the remaining 98% being referred to as the non-coding genome. Whereas in the past considered "junk DNA," the non-coding genome has now been recognized as instrumental to ensure correct spatiotemporal regulation of gene expression. Non-coding regulatory elements such as enhancers and gene promoters engage together in long distance three-dimensional (3D) interactions, ensuring that genes are switched on and off at the right moment, in the right cell type, and to the right extent. The playfield of these 3D interactions is set up by a tightly controlled genome organization, facilitated by structural proteins, histones, and their post-translational modifications, dividing our genome into transcriptionally silenced and closed heterochromatin and transcriptionally active euchromatin compartments, of which the latter harbors an open chromatin configuration. It is in these accessible regions of the non-coding genome that enhancers and promoters of active genes are located, being accessible to the transcriptional machinery. The level of this accessibility can be influenced by methylation of CpG-dinucleotides (see subsequent text) and by histone modifications such as acetylation of lysine 27 of histone 3 (H3K27ac) and changes in nucleosome spacing, mediated by chromatin-modifying enzymes. At the sub-compartment level, euchromatin is further divided by insulators into so-called topologically associating domains (TADs) (Figure 2). These TADs are in most instances <1 Mb in size and delineate those regions of our genome in which sequences preferentially interact with each other in the 3D genome space (Figure 2). Within these TADs, DNA loops are formed between enhancers and gene promoters and these enhancer-promoter (E-P) interactions are finally involved in the regulation of gene expression.

Enhancers are short DNA sequences (usually <1 kb) that can act as positive regulators of gene transcription, by recruiting transcription factors and RNA polymerase II while interacting with gene promoters. These E-P interactions can occur at long distances (e.g., the *ZRS* enhancer important for limb development is located >1 Mb away from its target gene *SHH*), enhancers can function at an orientation-independent manner relative to the transcriptional start site of a gene, and multiple enhancers can regulate one gene, but also multiple genes can be regulated by one enhancer. Non-coding DNA further also gives rise to long non-coding RNAs, which are RNA molecules that are not translated into proteins. lncRNAs participate in diverse cellular processes, including chromatin remodeling, transcriptional regulation, RNA splicing, and post-transcriptional regulation. Understandably, disentangling the gene regulatory networks underlying such complex gene expression regulatory mechanisms can be cumbersome and challenging. Still, in the last decade, multiple examples have accumulated where alterations of the non-coding genome, including enhancers or disruption of 3D chromatin organization have resulted in developmental disorders.[37–41] This includes (1) SNVs, insertions and deletions (indels), CNVs, and structural variants (SVs) disturbing the regulatory landscape of protein-coding genes; (2) alterations affecting the expression and function of long non-coding RNAs (lncRNAs) that can be either directly implicated in disease or indirectly affect regulation of disease implicated genes[42,43]; (3) deep intronic variants affecting mRNA splicing; and (4) epigenetic alterations such as aberrant DNA-methylation leading to gene expression perturbation. However, none of these non-coding mechanisms of genetic disease are currently routinely assessed in epilepsy diagnostics, and this may explain at least part of the missing heritability that is observed in the epilepsy field.

4.2 | How non-coding variants in the regulatory landscape may cause epilepsy

As complex as the gene regulatory mechanisms are, so too are the mechanisms that lead to their dysfunction. Although few studies have addressed these mechanisms on a large scale,[44,45] it is clear from the many published examples that virtually any conceivable mechanism for perturbing gene regulation is likely to be associated with disease.[46] To identify these mechanisms, WGS analysis

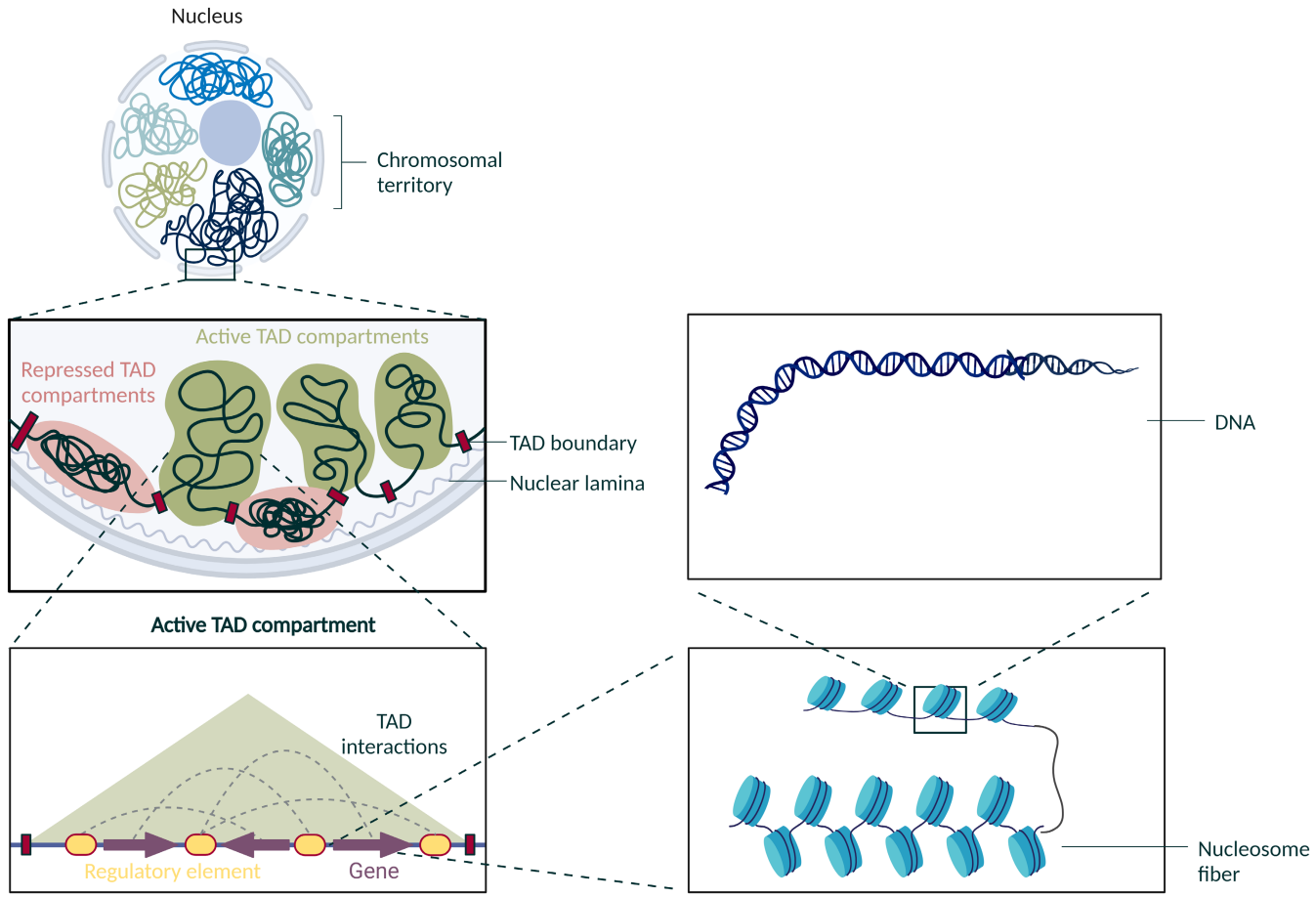

FIGURE 2 Overview of the genome organization in a eukaryotic nucleus (TAD = topologically associated domains). The DNA string is highly organized by structural proteins, histones, and their post-translational modifications, working not only linearly but also in a three-dimensional manner, such that specific areas of the genome are regulated by the same enhancers or inhibitors. This enables spatiotemporal regulation of gene expression in selected cells. Pathogenic variants in such enhancers or inhibitors might be able to cause disorders, such as epilepsy.

needs to be combined with knowledge of where regulatory elements are located, for example, obtained from large-scale computational studies that integrate multiple layers of omics data to predict the location of regulatory elements.[39] A 15 base pair deletion in a non-coding regulatory element upstream of an alternative transcript of *GPR56* was found to cause polymicrogyria restricted to the Sylvian fissure, leading to speech delay, intellectual disability, and refractory seizures in five individuals from three families.[47] Similar mechanisms have been described to play a role in epilepsy. In 28.8% of 198 investigated individuals with epilepsy, CNVs in proximity to known epilepsy genes were identified, which might indicate that disruption of the gene regulatory landscape of these genes might contribute to the development of epilepsy. Four individuals in this cohort carried a large deletion in proximity to the *GABRD* gene, a known epilepsy/ID gene.[48,49] Similar CNVs affecting regulatory sequences have been found in the proximity to the *ARX* gene in individuals with intellectual disability.[50] Some deep intronic SNVs in *SLC2A1* were hypothesized to disrupt enhancer element interactions and lead to *GLUT1* deficiency,[51] another cause of seizures.

Next to directly affecting the sequence of the enhancer by point mutations or deletions, CNVs and SVs can also indirectly affect the enhancer function by altering the 3D regulatory landscape. Copy number–neutral SVs, such as inversions and translocations, can disrupt or even create new regulatory landscapes, resulting in loss or gain of regulatory function. Clinical examples of such events include structural variants that delete TAD boundaries. In the case of the *LMNB1* locus, such deletion of a TAD boundary resulted in the misregulation of a gene by an enhancer by which it is normally not regulated, and this so-called enhancer adoption subsequently caused an adult-onset demyelinating leukodystrophy, a progressive neurological disorder affecting the myelination of the central nervous system.[52] Similar examples affecting TAD boundaries and subsequent misregulation of gene expression have been identified

using Hi-C, a chromatin conformation capture technology that allows the study of 3D genome interactions, and which is about to enter clinical practice; Hi-C maps of cells derived from nine patients with developmental disorders revealed structural variants altering TADs and regulatory elements.[53] Among those was a patient with disruption of the TAD of *FOXG1*, a gene in which several non-coding SVs disrupting downstream enhancers had been identified in individuals with Rett(-like) syndrome previously.[54] Similar TAD disruptions have been found in multiple individuals with SVs disrupting the regulation of the *MEF2C* locus, leading to ID, epilepsy, and cerebral malformations.[41,55] We expect that the widespread implementation of technologies and data analysis tools that allow the assessment of the impact of non-coding variants on the regulatory landscape will help to further diagnose a substantial number of individuals with currently unexplained causes of epilepsy.

5 | BEYOND DNA: COMPLEMENTARY ANALYSIS METHODS TO DETECT CAUSES OF UNSOLVED EPILEPSIES

Although the sequence of DNA of our genome is classically the center of genetic investigations, recent years have witnessed the rise of a complementary set of diagnostic modalities that can help to identify the cause of genetic disease, despite not directly focusing on sequencing DNA sequences. In the next sections, we discuss the utility of new testing options such as transcriptomics, DNA methylation signatures, and metabolomics to help diagnosing unsolved patients.

5.1 | Transcriptomics

In recent years, assessment of the transcriptome of patient cells by so-called RNA sequencing (RNA-seq) has made its entry into clinical genetics diagnostics. RNA-seq allows the detection of aberrant gene expression, mono-allelic gene expression, and aberrant mRNA splicing, all of which are mechanisms that have been found to cause or contribute to human Mendelian disease. Since its first clinical application in 2017,[56,57] a variety of studies have applied RNA-seq to help diagnosing genetically unsolved patients with a variety of phenotypes, ranging from metabolic disorders to neurodevelopmental disorders presenting with epilepsies, yielding in most instances a diagnostic yield of up to 15%.[58–64] At some centers, clinical RNA-seq can now be diagnostically requested even in routine clinical care.[64] Benefits of RNA-seq include detection of gene expression changes thereby pinpointing to disease relevant genes and variants, as well as offering improved functional interpretation of deep-intronic variants affecting mRNA splicing. These types of variants are either not identified (ES) or are often classified as variants of unknown significance due to the lack of good in silico prediction models for the effects of such variants. Additional benefit lies in the interpretation of synonymous variants within coding exons that can influence mRNA splicing, and the increased notice that intronic variants creating altered splice acceptor and donor sites (often far away from the nearest exon) can result in the increased inclusion of poison exons with a premature termination codon in the mature mRNA transcript of an expressed gene, targeting the transcript for nonsense-mediated decay and thus leading to gene dysfunction. Such poison exons have been found to affect the expression of *SCN1A*, *SCN2A*, and *SCN8A*, causing epilepsy in affected individuals.[65,66]

Whereas in most current studies, RNA derived from peripheral blood was investigated, sampling other clinically accessible tissues such as fibroblasts might be further beneficial, given that these tissues express up to 70% of all OMIM morbid genes (including the majority of known epilepsy-related genes) at sufficient coverage, and not all genes are expressed in blood cells.[63,64] In the future, innovations from the field of cell reprogramming such as fibroblast to induced-neuron transdifferentiation might further advance the utility of RNA-seq to specifically analyze genes with a neuronal restricted gene expression pattern that can only be analyzed in neurons but not in patient derived fibroblasts. In addition, integration of RNA-seq data with WGS analysis promises benefits for the interpretation of the effects of variants in non-coding regulatory elements. Furthermore, although currently not investigated in routine clinical investigations, RNA-seq studies also have the capacity to address the expression of long non-coding RNAs (lncRNAs). This might help to address previously unanticipated explanations of missing heritability, as several lncRNAs have been implicated in human disease. A particularly relevant example for the epilepsy field is the *CHASERR* lncRNA, which is located upstream of the gene *CHD2*, a gene in which haploinsufficiency leads to a developmental and epileptic encephalopathy. *CHASERR* was identified as a negative regulator of *CHD2* expression,[67] and it was hypothesized that interfering with this negative regulation could result in therapies aiming to restore *CHD2* expression in haploinsufficient individuals. In addition, two individuals were recently described as harboring de novo variants in *CHASERR*, presenting with neurodevelopmental delay and showing concomitant *CHD2* upregulation.[62] This further highlights that variants and expression changes of lncRNAs can cause phenotypes presenting with epilepsy, and RNA-seq-based

studies will be crucial to further unravel such causes of missing heritability.

5.2 | DNA methylation analysis

Epigenomics refers to the study of epigenetic modifications to the DNA and DNA-associated proteins that regulate gene expression through chromatin remodeling and changing the accessibility of the chromatin.

The best-studied epigenetic modification is methylation of the cytosine of the CpG-dinucleotides, and it plays an important role in mammalian development and differentiation. In humans, there are ~30 000 CpG islands, and two thirds of these are located within the promoter regions of several housekeeping or developmentally regulated genes. CpG islands are DNA segments rich in CpG dinucleotides. The promoter-associated CpG islands are generally unmethylated, and they are involved in *cis* regulation of gene expression through binding of specific proteins such as transcription factors. Methylation of these CpG islands results in gene silencing. DNA methylation shows dynamic changes during development, where DNA methylation is globally erased during proliferation and migration of the primordial germ cells and a re-methylation occurs in the germ cell precursors following sex determination. The second wave of DNA demethylation takes place after fertilization but it does not include the imprinted germline differentially methylated regions, resulting in parental allele–specific expression of imprinted genes. Methylation status is stably maintained in somatic tissues and inherited with high fidelity through cell lineages. Aberrant DNA methylation may directly or indirectly lead to disease development. In early development, a genetic event may cause DNA methylation changes in specific locations in the genome, and these changes are in principle maintained through mitotic divisions. Recently, machine learning–based approaches have been used to take advantage of these stable DNA methylation patterns in a given somatic tissue to identify global DNA methylation (DNAm) signatures (also called episignatures) across multiple loci associated with a given genetic disorder.[68,69] The most widely used method to detect global DNA methylation changes is bisulfite conversion of DNA followed by sequencing or hybridization to methylation arrays. To establish a DNAm signature for a given disorder, DNA from patients and control cohorts are typically investigated with methylation arrays and a minimum set of independent CpG sites that are differentially methylated in patients compared to controls are selected. These sites (or probes) define the DNAm signatures. Subsequently, a classifier is constructed using statistical and machine learning methods such that it can distinguish patients from controls using these selected probes.[68,69] The DNAm signature method can then be used, for example, to assess the clinical significance of a variant in a given disease gene. Currently, more than 120 disease-specific DNAm signatures have been characterized, mostly associated with neurodevelopmental disorders. Of these, at least 15 signatures are linked to gene defects involved in epilepsy: *ANKRD11*, *ARID1B*, *ATRX*, *CHD2*, *CREBBP*, *EHMT1*, *NSD1*, *SETD1B*, *SETD5*, *SMARCA2*, *SMC1A*, *SMS*, *UBE2A*, *FAM50A*, and *TET3*. For successful identification of disease-specific methylation patterns, a minimum number of methylation data sets from individuals with a given disorder and of age-/sex-matched controls are required. Given the rarity of several neurodevelopmental disorders, this hindrance may be overcome through data sharing. It should be emphasized that the majority of known signatures have been investigated predominantly in the context of neurodevelopmental disorders, rather than epileptic disorders.

Genetic disorders can also result from methylation deficits at a single locus. Barbosa et al. studied 489 individuals with neurodevelopmental disorders and congenital anomalies, of which 16% exhibited epilepsy, and observed an enrichment of de novo rare differential methylation regions (DMRs) in cases compared to controls.[69,70] A substantial subset of these methylation changes were secondary events caused by underlying regulatory sequence mutation encompassing CNVs, sequence variants in regulatory elements, and repeat expansions.[70] A separate study involving a large cohort of undiagnosed neurodevelopmental disorders looked both at episignatures and DMRs and confirmed that DNA methylation analysis can be used to assign a diagnosis to a significant subset of individuals who may remain undiagnosed using conventional approaches.[69]

5.3 | Metabolomics

Metabolomics is the study of small molecules produced by the metabolism of a biological system. The Human Metabolome Database[71] lists more than 74 000 unique metabolites and targeted metabolic screenings of specific class of metabolites such as amino acids and organic acids that have been used for decades in the diagnostics of metabolic disorders, such as phenylketonuria and maple syrup urine disease.[72] Untargeted metabolomics, also known as next-generation metabolic screening, is a newer approach to metabolic screening that is becoming increasingly popular.[73] It involves analyzing a wide range of metabolites simultaneously and provides a more comprehensive view of the metabolic state of an affected patient.

Approximately 2000 inborn metabolic disorders (IMDs) have been reported, of which more than 600 are associated

with epilepsy.[74] Tumiene et al. suggested that the number of metabolic epilepsies is likely to increase in the future, as more than 3200 genes are involved in human metabolic pathways.[74] The diagnosis of metabolic epilepsies relies on metabolic examinations (including metabolomics) as well as genetic testing. Each approach has its advantages and disadvantages and international guidelines for either testing modality are missing. Metabolomics is typically conducted in specialized laboratories, and its availability varies across different countries and regions.[74] On the other hand, genetic testing is performed in both clinical and commercial laboratories and is generally more widely accessible. In certain cases, when ES/WGS detect VUSs in a gene associated with IMDs, targeted metabolic testing can be employed to properly classify the genetic variant(s). In other situations, metabolomics exhibits greater specificity and sensitivity and provides quicker results. However, for many IMDs it often identifies only non-specific metabolic biomarkers, necessitating further confirmation through genetic testing.[74]

Although IMDs are not a common cause of seizures, early detection is crucial, as many of these disorders require treatment beyond traditional anti-seizure medication that aims to restore the alteration in metabolism caused by the metabolic defect. This may include measures to manage seizures or prevent neurodegeneration. Some patients with rare neurological disease may harbor a VUS found through NGS in genes that are involved in a metabolic pathway. In such instances, metabolomics has the potential to provide evidence supporting disease causality of the identified VUS and in some cases indication for potential treatment strategies. A recent study with 170 patients presenting predominantly with neurological symptoms showed that untargeted metabolomics contributed to the variant interpretation in 74 patients (43.5%) in over 73 different genes involved in a metabolic pathway.[75] In addition, untargeted metabolomics is a valuable tool for uncovering new metabolic causes of neurological disorders and for identifying diagnostic markers for inherited metabolic disorders where none exist so far.

6 | CONCLUSION

Epilepsy genetics has been rapidly incorporated into clinical practice, and the techniques used and understanding of the genetic mechanisms involved continue to evolve. Here, we have presented some of the more recent technologies that may be added to the genetic toolbox of epilepsy genetics when ES/WGS have failed to provide a diagnosis. Although we are still awaiting their implementation in routine diagnostics, ultimately all of these tools will improve our diagnostic capabilities and allow us to move forward on the path toward precision diagnosis and precision medicine for patients with genetic epilepsies.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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