Non-coding regulatory elements: Potential roles in disease and the case of epilepsy

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Abstract

Non-coding DNA (ncDNA) refers to the portion of the genome that does not code for proteins and accounts for the greatest physical proportion of the human genome. ncDNA includes sequences that are transcribed into RNA molecules, such as ribosomal RNAs (rRNAs), microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and un-transcribed sequences that have regulatory functions, including gene promoters and enhancers. Variation in non-coding regions of the genome have an established role in human disease, with growing evidence from many areas, including several cancers, Parkinson’s disease and autism. Here, we review the features and functions of the regulatory elements that are present in the non-coding genome and the role that these have in human disease. We then review the existing research in epilepsy and emphasise the potential value of further exploring non-coding regulatory elements in epilepsy. In addition, we outline the most widely used techniques for recognising regulatory elements throughout the genome, current methodologies for investigating variation and the main challenges associated with research in the field of non-coding DNA.

KEYWORDS

epilepsy, gene regulation, non-coding DNA, non-coding mutations, regulatory sequences

List of abbreviations: 3C, Chromosome Conformation Capture; SC, 3C-carbon copy; ADCME, autosomal dominant cortical myoclonus and epilepsy (alternative names are BAFME, FAME, FCTE); ALS, amyotrophic lateral sclerosis; ASD, autism spectrum disorder; ATAC-seq, assay for transposase-accessible chromatin by sequencing; BAFME, benign adult familial myoclonic epilepsy (alternative names are ADCME, FAME, FCTE); BLAST, Basic Local Alignment Search Tool; BRE, TFII B recognition element; CADD, Combined Annotation Dependent Depletion; ChiP-seq, chromatin immunoprecipitation coupled with massively parallel DNA sequencing; circRNA, circular RNA; CNV, copy number variation; CPA6, carboxypeptidase A6 gene; CSTB, cystatin B gene; CTCF, CCCTC-binding factor; DPE, downstream promoter elements; EGRF, epidermal growth factor receptor gene; ENCODE, Encyclopedia of DNA Elements; EPM1, progressive myoclonic epilepsy of the Unverricht-Lundborg type; eQTL, expression Quantitative Trait Locus; FAIRE-se, formaldehyde-assisted isolation of regulatory elements and sequencing; FAME, adult myoclonic epilepsy (alternative names are ADCME, BAFME, FCTE); FANTOM5, Functional Annotation of the mammalian genome; FCXE, familial cortical tremor and epilepsy (alternative names are ADCME, BAFME, FCTE); FDT, frontotemporal dementia; GTEX, Genotype-Tissue Expression; GWAS, genome-wide association study; GWAVA, Genome-Wide Annotation of Variants; HBB, haemoglobin beta subunit gene; Inr, Initiator element; KRAB-ZFPs, zinc finger proteins containing the Kruppel-associated box; LD, linkage disequilibrium; LINE-1/L1, long interspersed nuclear element-1; IncRNA, long non-coding RNA; MARCH6, membrane associated ring-CH-type finger 6 gene; MECP2, methyl-CpG-binding protein 2 gene; miRNA, microRNA; MSP, methylation sensitive PCR; ncDNA, non-coding DNA; NF1, neurofibromatosis type 1; PDGFRA, platelet derived growth factor receptor alpha gene; piRNA, piwi-interacting RNA; PITX1, paired-like homeodomain 1 gene; Piwi, P-element Induced Wimpy tests in Drosophila; PWM, position weight matrix; QC, quality control; RAPGFR2, rap guanine nucleotide exchange factor 2 gene; RELN, reelin gene; rRNA, ribosomal RNA; SAMD12, sterile alpha motif domain containing 12 gene; SCN1A, sodium voltage-gated channel alpha subunit 1 gene; sncRNA, single nucleotide polymorphism; snRNA, small nuclear RNA; STAR2D7, star related lipid transfer domain containing 7 gene; TAD, topologically associating domain; TE, transposable element; TERT, telomerase reverse transcriptase gene; TF, transcription factor; TFBS, transcription factor binding site; TFIIIB, Transcription factor II B; TMRC6A, trinucleotide repeat containing adaptor 6A gene; T RNA, transfer RNA; TSS, transcription start site; VEP, Variant Effect Predictor; WES, Whole Exome Sequencing; WGS, Whole Genome Sequencing; YEATS2, YEATS domain containing 2 gene.

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INTRODUCTION

Why do we not have a better understanding of disease biology? What generates wide phenotypic variation in a disease even when the major components of the disease are readily recognisable? Does this heterogeneity arise from environmental factors or genetic background, or both? In the case of brain disorders, the brain is a complex organ with both environmental and genetic determinants of structure, function and disease. Focusing on internal determinants, the complexity of the brain is intuitively linked to the number and connectivity of differentiated cell types that express cell-specific genes, exhibit unique properties and perform specialised functions [1]. At the genomic level, complexity is not simply determined by the expression of cell-specific protein-coding genes but may relate to the way these genes are regulated, with a significant role for non-coding DNA (ncDNA), amongst other influences.

ncDNA refers to the portion of the genome that does not code for proteins and accounts for the greatest proportion of the human genome. Indeed, it is estimated that only about 2% of the human genome encodes proteins, with the rest being non-protein-coding [2]. Of this, the exact percentage carrying functional properties is yet to be clarified, with different estimates being proposed [3]. Historically, a large percentage of the non-coding genome was referred to as ‘junk DNA’, due to the prevailing sentiment at the time that it lacked any functional relevance and was essentially useless [4–6]. This idea has persisted, predominantly due to the difficulty of investigating such a large and complex field, and the lack of appropriate techniques. In recent years, improvements in sequencing technologies, expression assays and advances in data handling and analysis have made it possible to study the ncDNA and have led to a greater appreciation of its role in human health and disease.

ncDNA includes sequences that are not translated into proteins but are transcribed into RNA molecules, called non-coding RNAs (ncRNAs): these comprise transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), microRNAs (miRNAs), small nucleolar RNAs (snRNAs), small nuclear RNAs (snRNAs), piwi-interacting RNAs (piRNAs), long non-coding RNAs (IncRNAs) and circular RNAs (circRNAs) (Figure 1). The biology of ncRNA and their roles in disease are not covered in this review (see previous reviews [7, 8]). Further, the 5′ and 3′ untranslated regions (UTRs), located at either end of the mRNA, also fall into this category of ncDNA. UTRs are crucial for the regulation of protein expression. For example, the translation of upstream open reading frames (uORFs) within the 5′ UTR is known to be a common mechanism for controlling the level of protein production downstream, according to a general model based on competition for ribosome binding [9]. Due to their role in mRNA translation initiation, the question arises whether these regions should be classified as coding DNA rather than non-coding DNA. Therefore, despite the importance of UTRs in the regulation of gene expression, due to the ambiguity in their definition, UTRs will not be discussed further. ncDNA also contains un-transcribed regions that function as regulatory elements, which represent the main focus of this review. These include gene promoters and transcription factor binding sites, enhancers, transposable elements (TEs) and topologically associating domain (TAD) boundaries (Figure 2). ncDNA and variation in non-coding regions of the genome have an established role in human disease, with evidence in cancers, Parkinson's disease and autism, amongst others [10–13].

There is still an open question about the causes of the wide phenotypic variability that characterise many epilepsies with a known genetic cause: ncDNA may contribute to this variability [14]. Non-coding regulatory regions may harbour variations that influence gene expression, and have a disease-modifying effect, or influence treatment response. Here, we consider the evidence supporting the role of ncDNA in epilepsy. The regulatory elements that are present in the non-coding genome will be described and the role that these regions have in human disease will be discussed. Examples of the importance that non-coding regulatory regions have in human diseases will be reported, and the existing research in the field of epilepsy will be reviewed. We make the case for further research to appreciate the potential value of non-coding regulatory elements in epilepsy. In addition, this review will also outline the most widely used techniques for recognising regulatory elements throughout the genome, the main methodologies for investigating variation and the main challenges associated with research in the field of ncDNA.

NON-CODING REGULATORY REGIONS

One of the most important classes of non-coding regulatory elements of the genome are gene promoters, which are essential for determining the direction of transcription, indicating the sense strand of the DNA and regulating gene expression. The gene promoter is located upstream of, and partially overlapping with, the transcription start site (TSS) of the gene it regulates, thus occupying the first part of the 5′UTR region, as shown in Figure 2A [15]. The minimal portion of the promoter required to initiate transcription is called the core promoter; it spans between 60 and 120 base-pairs and represents the transcriptional machinery assembly site [16–18]. The core promoter contains the RNA polymerase binding site, the TSS and optional motifs, including the Goldberg-Hogness box (commonly called the TATA box), the Initiator element (Inr), the downstream promoter element (DPE) and

Key points

- Non-coding regulatory elements: description and role in disease.
- Techniques for the identification of non-coding regulatory elements.
- Methodologies to investigate non-coding variation and challenges.
- Existing research in the field of epilepsy.
the TFIIIB recognition element (BRE) [19]. Such optional motifs may extend downstream of the TSS: for example the DPE motif, when present, is located 28–33 nucleotides after the TSS (Figure 2A) [20]. Beyond the core promoter is the proximal promoter, located 250 bp upstream of the TSS, and usually extending up to 1000–2000 bp [21].

The proximal promoter contains binding sites for both general and sequence-specific transcription factors [22].

The activity of promoters is influenced by additional regulatory sequences that can modulate the expression of genes from a genomic location even further away. These elements are called enhancers, DNA sequences that range from 50 to 1500 bp in length, to which proteins called activators and repressors can bind. The interaction of the enhancer and the activator/repressor results in the creation of a chromatin loop that can shift the enhancer closer to the gene promoter and allows mediator proteins to be recruited. Mediator proteins either promote or prevent the binding of RNA polymerase, resulting in promotion or repression of the target gene expression. Genes can be modulated by different enhancers, and each enhancer can modulate multiple genes [15]. Enhancers may be located thousands of base pairs away from the target gene, either upstream or downstream of the TSS [15]. The interaction between enhancer and the target promoter occurs through chromatin loops and is supported by proteins called cohesins. Chromatin loops represent non-random three-dimensional folds of chromatin that generate physical interactions between distantly located genetic sequences, including long-range interactions between regulatory sequences and the corresponding target genes [23]. Clusters of multiple enhancers may occur in the genome: these typically exhibit similar activity and regulate the same genes. Such redundancy of enhancers may be crucial, especially during development, to provide robustness in case of loss-of-function mutations and to ensure the correct spatiotemporal expression of target genes, necessary to guide development [24–26]. Protein-coding gene redundancy has mostly been lost during evolution, and it is possible that today’s redundancy involves regulatory elements rather than
protein-coding genes in order to achieve differential gene expression in various tissues with the least amount of ‘space’ in the genome [27]. Enhancer functionality may be limited by TAD boundaries (or insulator elements), which represent another class of DNA regulatory elements that are capable of blocking the physical interaction between enhancer and gene promoter [28]. TAD boundaries also function as a chromatin barrier: these regions interact with cohesins and CCCTC-binding factor (CTCF), a transcriptional repressor protein, forming a complex that constitutes a physical impediment to prevent the excessive spread of heterochromatin [28].

TEs represent another class of ncDNA elements involved in regulatory control. TEs are capable of altering their position in the genome and can be divided into two different categories, based on the mechanism of transposition. Retrotransposons, TEs of Class 1, use a ‘copy and paste’ mechanism: through reverse-transcription and the production of an RNA intermediate, they introduce a new copy of themselves to a different genetic location. Transposons, Class 2 TEs, use a ‘cut and paste’ mechanism: their sequence includes the genetic code for the transposase enzyme, which they use to excise themselves from one genetic locus and integrate into a different one [29].

Most copies of TEs in our genome have lost the ability to mobilise due to mutations and now have a fixed genetic location. TEs that are still mobile within an individual’s genome mobilise predominantly in germ cells and during early embryogenesis [30, 31]. Since TEs often include TSSs and other regulatory sequences in their own sequence, their mobilisation has contributed to the formation of novel tissue-specific promoters and transcription factor binding sites (TFBSs), which now have a role in ensuring the correct spatiotemporal gene expression patterns during development [32, 33]. Furthermore, TE mobilisation also occurs in somatic cells. This happens in the brain, particularly in the hippocampus, where the somatic transposition of the human long interspersed nuclear element-1 (LINE-1, also called L1) in neural precursor cells contributes to neuronal hippocampal diversity [31, 34]. However, TE insertion into the genome may also have a deleterious effect and cause disease. Examples include Haemophilia A, the first disease in which an association with TEs was proven, and neurofibromatosis type 1 (NF1) [35, 36]. Another example is Rett syndrome, which is a neurological condition caused by mutations in the methyl-CpG-binding protein 2 gene (MECP2). MECP2 is a regulator of L1 transposition, and patients with Rett Syndrome, carrying a mutated MECP2, show increased L1 mobilisation, which possibly contributes to the Rett phenotype [37, 38]. The regulation and silencing of TE transposition are complex and rely on several elements: piRNAs, which interact with PIWI proteins and drive repressive chromatin marks on the promoter region of TEs, and zinc finger proteins containing the Kruppel-associated box (KRAB-ZFPs), which bind to the TE sequence and

**FIGURE 2** Schematic representation of non-coding regulatory elements. (A) Gene promoters represent the site where the transcriptional machinery assembly occurs, and transcription factors interact to regulate the expression of genes. Gene promoters include the core promoter, the minimal required portion to initiate the transcription, and the proximal promoter. (B) Enhancers are regulatory elements that can be bound by activators and repressor and modulate the expression of target genes. (C) Topologically associating domain (TAD) boundaries may prevent the physical interaction between enhancers and the target genes. (D) Transposable elements are regulatory sequences capable of altering their position in the genome. Retrotransposons use a ‘copy and paste’ mechanism and introduce a copy of themselves to a different genetic location; transposons use a ‘cut and paste’ mechanism: they excise themselves from one genetic locus and integrate into a different one. Translocation of transposable element (TE) occurs predominantly in germ cells and during early embryogenesis, when TEs contribute defining the temporal expression of genes. BREu: upstream TFIIB Recognition Element, BREd: downstream TFIIB Recognition Element, TATA box, Inr: Initiator element, DPE: downstream promoter element, TSS: transcription start site, gDNA: genomic DNA.
recruit additional proteins, ultimately adding repressive chromatin marks to the promoter region of the TE [39, 40].

NON-CODING VARIATION

The term ‘non-coding variation’ refers to genetic changes that occur within non-coding regions of the genome. Non-coding variation may be represented either by single-nucleotide polymorphisms (SNPs) or structural variations, including short insertions or deletions (collectively called InDels), copy-number variations (CNVs) and repeat expansions.

SNPs, described as single nucleotide substitutions at specific positions of a DNA sequence, represent the most common type of DNA variation. It is estimated that ∼90% of disease-associated SNPs fall in non-coding sequences of the genome [41]. However, for most of the millions of SNPs that have been identified in ncDNA by the Human Genome Project and investigated in GWAS studies, we do not yet understand the functional implications.

Structural variations include small insertions and deletions (defined as the loss and gain of sequences up to 1 kb in length), duplications, inversions, translocation and CNVs, which represent duplications and deletions of sequences greater than 1 kb in length [42, 43]. CNVs have been observed throughout the genome, but interestingly not all chromosomes are affected equally: some chromosomes typically have large numbers of CNVs (such as chromosome 19 and 22), whereas other regions are described as CNV-deserts, often being devoid of CNVs [44, 45].

Another type of structural variation is represented by repeat expansions, which represent the expansion of repeated DNA sequences: the size of the repeat may vary from trinucleotide repeats to 12-nucleotide long sequences, and the number of times this sequence is repeated is also variable [46, 47]. Examples of diseases caused by repeat expansions occurring in noncoding sequences include Friedreich’s ataxia, amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) [48, 49]. Non-coding repeat expansions have also been found in some epilepsies, including progressive myoclonus epilepsy of the Unverricht–Lundborg type (EPM1), associated with the expansion of a dodecamer repeat in the promoter region of the cystatin B gene (CSTB), and benign adult familial myoclonic epilepsy (BAFME), associated with intronic expansions of a five-nucleotide long sequence (TTTCA or TTTTA): expansions of this sequence have been identified in introns of different genes in different patients [50–52].

TECHNIQUES FOR IDENTIFYING REGULATORY ELEMENTS

In order to detect and analyse variation in non-coding regulatory elements, it is first necessary to localise these regions in the genome, as summarised in the pipeline in Figure 3. Several strategies, which are outlined in the following sections, can be used to achieve this (Table 1). The most reliable and accurate method for predicting the location of regulatory elements is to integrate data from multiple methods, as will be described in Section 4.6 ‘Online databases’.

Transcription factor binding site localisation

One strategy for identifying non-coding regulatory elements is to localise transcription factor binding sites (TFBSs) across the genome, which can be achieved using Chromatin immunoprecipitation coupled with massively parallel DNA sequencing (ChIP-seq), or by associating open-chromatin assays with computational footprinting methods [53–55]. Moreover, all possible binding sites of a particular transcription factor can be examined using position-weight matrix methods (PWM) [53, 56].

FIGURE 3 Flowchart of the steps required to study non-coding genetic variations. After DNA collection and whole-genome sequencing (WGS), is the identification of non-coding regulatory elements, which can be achieved using different approaches, described in Table 1. In the subsequent discovery phase, variant calling tools will be used to identify genetic variations in the cohort of interest. For the functional annotation of variants, the one reliable strategy is to assess the functional consequences of variants using multiple techniques and compare these predictions to provide robustness to the interpretation. Finally, the functional consequences of variants will be evaluated and then validated using wet-lab experiments. WGS: Whole Genome Sequencing, SNVs: Single Nucleotide Variants, CNVs: Copy Number Variations

* See table 1 for details
| Strategy | Methods | Details | Advantages | Disadvantages | Additional Information |
|----------|---------|---------|------------|---------------|------------------------|
| Transcription Factor Binding Site localisation | ChIP-seq | In the ChIP-seq protocol, cells are treated with formaldehyde to crosslink proteins to DNA, followed by DNA fragmentation. Immunoprecipitation with antibodies specific to the target TF allows for the recognition and isolation of the DNA-TF complexes. The precipitated DNA is then purified and sequenced to identify the genetic sequence corresponding to the TFBS. | - Genome-wide coverage - High resolution, even in repetitive sequences | - Immunoprecipitation with antibodies: the quality of the output data depends on the quality of the antibodies used - Large number of input cells required | Recent developments increase the resolution close to the single nucleotide level (ChIP-exo, ChIP-nexus) and require lower numbers of input cells (Nano-ChIP-seq). |
| Footprinting | Chromatin accessibility data are required to perform this assay. When chromatin accessibility assays are performed, DNA-bound TFs prevent enzymes used in open-chromatin assays from cleaving DNA in nucleosome-free regions, leaving recognisable footprints. Computational footprinting tools detect the DNA-bound TF footprints, allowing TFBS localisation. | - High sensitivity - Robust protocol for in-vitro and in vivo formed protein-DNA complexes | - Limitations of open chromatin assays (discussed in the corresponding section) - Need to apply computational bias corrections, which vary depending on the open chromatin assay employed |
| Position Weight Matrix | For a given TF, PWM models the predicted sequence of the binding site and assigns a score to each of the four bases at each motif position, indicating the preference for each base at each position. Matching patterns are identified by scanning a PWM against a target DNA sequence, and each potential binding site is assigned a score corresponding to the predicted relative strength of binding. | - Prediction of both the potential binding sites of a TF and the relative strength of binding to each TFBS - Nucleotide interdependencies and di-nucleotide interactions are not considered - Susceptible to false-positive prediction | Known TFBS motifs resulting from PWM studies are collated in the JASPAR database (http://jaspar.genereg.net) and the TRANSFAC (https://genexplain.com/transfac/), Recent improved algorithms incorporate dinucleotide interactions |
| Strategy                        | Methods                  | Details                                                                 | Advantages                                                                 | Disadvantages                                           | Additional Information |
|--------------------------------|--------------------------|------------------------------------------------------------------------|----------------------------------------------------------------------------|---------------------------------------------------------|------------------------|
| Chromatin accessibility assays | DNase-I hypersensitivity assay | In the DNase-I hypersensitivity assay, cells are lysed and DNA is digested with DNase I endonuclease. Nucleosome-wrapped DNA regions are resistant to endonuclease treatment, whereas nucleosome-free regions are sensitive to cleavage by DNase I endonuclease; these regions are known as DNase I hypersensitive sites (DHSs) and correspond to regulatory elements. The protocol terminates with targeted region amplification and sequencing, which allow for the characterisation of regulatory sequences. | - Gold standard technique for evaluating chromatin accessibility  
- Genome-wide coverage  
- High resolution | - DNase-I cleaves DNA unevenly, depending on sequence composition and cleavage propensities  
- The preliminary procedures of cell permeabilization and nuclei isolation may affect chromatin status | |
| ATAC-seq                        |                          | In the ATAC-seq protocol, cells are lysed, and chromatin is fragmented and tagged with the hyperactive Tn5 transposase, which introduces sequencing adapters into open-chromatin regions. These tagged sequences, corresponding to transcriptionally active and regulatory regions, are then purified and sequenced. | - Assesses chromatin accessibility in single cells and from frozen tissue  
- Suitable in cases of low amount of sample | - Prone to sequencing contamination by mitochondrial DNA | Recent improvements reduce mitochondrial reads |
| Faire-seq                      |                          | In Faire-seq, nucleosome-DNA interactions are stabilised through formaldehyde. DNA is then fragmented and isolated via phenol-chloroform extraction, which allows the physical separation of the crosslinked DNA (in the organic phase) and protein-free DNA regions (in the aqueous layer). Accessible nucleosome-free DNA regions, which | - Less damaging effect on chromatin than DNase-I and ATAC-seq | - Low sensitivity for detection in promoter regions  
- Lower resolution than DNase-I and ATAC-seq | |
| Strategy          | Methods                  | Details                                                                                                                                                                                                                                                                                                                                 | Advantages                                                                                                                                  | Disadvantages                                                                                                                                  | Additional Information                                                                                      |
|-------------------|--------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------|
| Epigenetic assays | ChIP-seq + MSP           | The ChIP-seq protocol performed using histone-specific antibodies identifies genetic regions enriched for histone modification. MSP can be used to selectively amplify methylated sequences. To perform MSP, DNA is bisulphite treated, and PCR is conducted using two pairs of primers. One set of primers amplifies methylated DNA, while the other set amplifies unmethylated DNA. By performing PCR with both pairs of primers, methylated DNA and unmethylated DNA sequences are distinguishable. | - High sensitivity  
- Small amounts of DNA are required  
- The protocol can be performed on DNA extracted from paraffin-embedded samples                                                                 | - High false-positive rate  
- Variability of results due to assay conditions (e.g., primer design, annealing temperature, cycle number)                                      |                                                                                                                                                           |
| Whole-genome bisulphite sequencing | Genomic DNA is fragmented and ligated to sequencing adapters, which are characterised by having all cytosines methylated, to allow the subsequent phases of primer binding and amplification after bisulphite conversion. Denatured adapter-ligated DNA fragments undergo bisulphite treatment, which distinguishes methylated and unmethylated cytosines. Methylated sequences are amplified and sequenced. |                                                                                                                                                                                                               | - Efficient for genome-wide investigations  
- Ability to assess the methylation state of nearly every CpG site  
- DNA degradation after bisulphite treatment                                                                 |                                                                                                                                                   |                                                                                                                                                           |
| Illumina MethylationEPIC [850k] Array | Illumina MethylationEPIC [850k] Array is a DNA methylation microarray that queries the methylation status of more than 850,000 methylation sites, including almost 350,000 |                                                                                                                                                                                                               | - Easy, time-efficient and cost-effective protocol  
- Increased coverage in enhancer regions compared to the predecessor                                                                 | - Only one CpG probe is interrogated per enhancer region                                                                                     |                                                                                                                                                           |
| Strategy | Methods | Details | Advantages | Disadvantages | Additional Information |
|----------|---------|---------|------------|---------------|------------------------|
| Chromosome Conformation Capture methods | 5C/Hi-C | 5C (many vs many): Cells are formaldehyde-treated to crosslink interacting loci, then lysed and DNA fragmented. The ends of DNA fragments are re-ligated in diluted conditions to promote ligation between cross-linked interacting fragments. The crosslinking is then reversed, and the samples undergo ligation-mediated amplification (LMA). LMA employs primer pairs that anneal across the ligated junctions of the 3C library and allow for the amplification of the target fragments where chromatin interactions occurred. The target fragments are subsequently sequenced. |
| | | Hi-C (all vs all): The Hi-C protocol begins with the same steps as 5C. Following the DNA digestion phase, the fragment ends are labelled with biotinylated nucleotides. Then ligation and reversal of the crosslinks occur. Ligated fragments, which contain the internal biotin tag, are purified and sequenced using paired-end sequencing. | 5C: - Cheaper than Hi-C | 5C: - Complex primer design |
| | | | Hi-C: - Genome-wide coverage | - Lower quality results than Hi-C |
| | | | | - Limited coverage (regions up to 1 Mb) |
| | miniMDS | miniMDS is an algorithm that uses normalised Hi-C data, partitions it, and then performs high-resolution MDS on each partition separately and in parallel, yielding a high- | 5C: - Greater speed and lower memory requirements compared to alternative methods | Hi-C: - Limited resolution for short distance interactions |
| | | | Hi-C: - High resolution | - The output structure sometimes contains densely clustered sites with indistinguishable chromosomal features | http://mahonylab.org/software/minimds/ |
| Strategy            | Methods     | Details                                                                                                                                                                                                 | Advantages                                                                                                           | Disadvantages                                                                                                           | Additional Information                       |
|---------------------|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------|
| Chrom3D             | Chrom3D     | Combines 5C/Hi-C data and lamina-associated domain data to simulate the spatial organisation of chromosome domains with respect to each other and to the nuclear space. | - Chromatin organisation predicted with respect to the nuclear space and nuclear inner membrane location             | - A spherical nuclear boundary is assumed                                                                               | https://github.com/Chrom3D/Chrom3D            |
| Chromosome3D        | Chromosome3D| Uses Hi-C data to reconstruct the three-dimensional structure of small genetic regions or chromosomes. Chromosome3D transforms Hi-C contact counts into Euclidean distances between chromosomal regions and produces a structural reconstruction model. | - High speed                                                                                                        | - Inability to handle long genomic regions                                                                               | http://sysbio.rnet.missouri.edu/chromosome3d/ |
| Comparative genomics tools | BLAST       | BLAST compares the nucleotides of a queried sequence with a reference and finds matches and regions of similarity across species.                                                                   | - Comprehensive collection of genomes available                                                                     | - Low resolution for highly repetitive sequences                                                                       | www.ncbi.nlm.nih.gov                         |
| PhastCons           | PhastCons   | Identifies evolutionary conserved sequences by performing multiple alignments of the genome of different species and employing a phylogenetic hidden Markov model. The model assesses evolutionary conservation as the probability of negative selection, with scores ranging from 0 to 1. Multiple versions of PhastCons exist. | - Unsupervised method, resilient to possibly incomplete and erroneous annotations                                   | - Fast evolving sequences are not assessed by the model                                                                  | http://hgdownload.cse.ucsc.edu/goldenPath/hg38/phastCons20way/ |
| Strategy | Methods | Details | Advantages | Disadvantages | Additional Information |
|----------|---------|---------|------------|---------------|------------------------|
| PhyloP   | PhyloP  | PhyloP measures evolutionary conservation at individual alignment sites, under the null hypothesis of neutral evolution. The algorithm produces conservation/acceleration scores. Positive scores represent evolutionary conservation; negative scores indicate accelerated evolution. Different versions of PhyloP exist. | - Suitable to evaluate base-wise conservation (identification of evolutionary signature at specific classes of nucleotides, e.g., third codon positions) - Ability to assess acceleration - Evaluates each base independently | - A constant evolutionary rate is assumed | http://hgdownload.cse.ucsc.edu/goldenPath/hg38/phyloP20way/ |
| Online databases | ENCODE | ENCODE stores a comprehensive catalogue of DNA functional elements, produced by combining multiple assays: DNase-I hypersensitivity assay, DNA methylation assay, ChIP-seq data | | | https://www.encodeproject.org/ |
| FANTOM5 | FANTOM5 uses Cap Analysis of Gene Expression (CAGE) and maps regulatory sequences in multiple cell types and tissues, producing an atlas of TFBSs, promoters and enhancers. Recently, atlases for miRNA and IncRNA have been released | | | | http://fantom.gsc.riken.jp/5/ |
| PsychENCODE | PsychENCODE stores a comprehensive catalogue of the gene regulatory landscape of the human brain, produced combining transcriptomic, epigenetic and genomic data from adult and developing human brain tissue | | | | http://www.psychencode.org |
| RefMap | By supplying epigenetic and GWAS data from a particular disease, RefMap outputs the active genetic regions in a particular cell type and relevant to that disease. | | | | |

Note: All strategies listed contribute to phase number 3 of the pipeline illustrated below in Figure 3. TFBS: transcription factor binding site, TF: transcription factor.
Chromatin accessibility assays

Chromatin accessibility assays enable the prediction of the three-dimensional structure of chromatin, recognising the open and active DNA regions and thus allowing the identification of active regulatory elements. Examples of these assays include the DNase-I hypersensitivity assay, the Assay for Transposase-Accessible Chromatin by sequencing (ATAC-seq) and the formaldehyde-assisted isolation of regulatory elements and sequencing (FAIRE-seq) [55, 57–60].

Epigenetic assays

Epigenetic modifications represent heritable changes (DNA methylation and histone modification) that influence chromatin structure and DNA accessibility, thus regulating the expression of genes, without altering the genetic sequence [61, 62]. Epigenetic assays identify the genetic locations where epigenetic changes have occurred throughout the genome, and this information can be used to locate non-coding regulatory elements. For example, tri-methylation at the lysine-4 residue of histone protein H3 (H3K4me3) is an epigenetic modification that makes chromatin more accessible to transcription factors and is associated with active promoters [63]. The most commonly used techniques to track the genome-wide distribution of epigenetic modifications include ChIP-seq, which can be combined with methylation-sensitive PCR (MSP), whole-genome bisulfite sequencing and the Illumina MethylationEPIC [850K] Array [64–66].

Chromosome Conformation Capture methods

Chromosome Conformation Capture (3C) methods are techniques that predict the physical interactions between genetic loci [67]. Since these interactions may reflect interactions between promoters and regulatory elements, 3C methods may help identify non-coding regulatory regions [68]. Computational tools, including miniMDS, Chrom3D and Chromosome3D, use chromosome conformation capture data to predict the three-dimensional organisation of the chromatin [69–73].

Comparative genomics tools

Comparative genomics tools may be used to identify in silico conserved non-coding sequences, which may correspond to functional regions according to the evolutionary conservation principle [74]. The hypothesis is that vertebrates use the same regulatory sequences across phylogeny to control gene expression and, assuming that mutations in such sequences are deleterious or disadvantageous to the organism, these regions are likely to have remained stable and unmuted throughout evolution [75, 76]. However, non-coding sequences are known to have a higher evolutionary turnover than protein-coding sequences, such that conservation per se is not a strong indicator of functional relevance but may be useful if combined with other types of data. Examples of comparative genomics and sequence conservation tools are the Basic Local Alignment Search Tool (BLAST), PhastCons and PhyloP [77–81].

Online databases

The use of online databases is the most comprehensive and convenient method to identify regulatory regions throughout the genome because these integrate data from many of the assays described above to accurately locate functional elements. The main limitation of these databases is that the majority of the regulatory elements for which they provide information, especially enhancers, are simply putative, predicted regulatory regions, of which only a small portion have been experimentally validated [82]. Examples include the Encyclopedia of DNA Elements (ENCODE database), the Functional Annotation of the Mammalian Genome project (FANTOM5), the PsychENCODE Consortium and the machine learning tool RefMap [83, 84].

All the methods and strategies described above contribute to phase 3 of the pipeline illustrated in Figure 3.

VARIANT ANNOTATION

Once the variants falling in regulatory regions of the genome have been identified, the next and most difficult step is variant annotation that is assigning a functional impact to each variant. This is a major challenge due to various factors, including the issue of identifying the target gene(s) regulated by a particular regulatory element [85]. Regulatory elements may modulate the expression of nearby genes, referred to as cis-regulation, or distantly located genes, called trans-regulation. A further complication is the presence of groups of alleles that co-occur and are co-inherited, a phenomenon known as linkage disequilibrium (LD). LD structure complicates the functional evaluation of variants because, assuming a group of SNPs in LD, it is difficult to determine the actual causal variant that affects the phenotype [85, 86]. Additionally, variants falling in regulatory regions are likely to have a small and quantitative functional effect, which is much more difficult to detect and interpret than the large qualitative consequences caused by many deleterious variants in protein-coding genes [87, 88].

There are several methods utilised to score non-coding variants and predict their potential functional impact (Table 2). One of the most widely used prioritisation and functional prediction tool is the Combined Annotation Dependent Depletion (CADD) software. CADD uses a linear kernel support vector machine (SVM) trained to distinguish between variants defined as ‘fixed’, which represent ancestral variants that have arisen in the distant past and are considered to be beneficial or neutral, and ‘simulated’, de novo variants, which may be either neutral or deleterious. For any queried variant, CADD combines more than 60 annotations and predicts the functional effect of a variant, based on whether the variant is likely to be an observed or simulated variant [89, 90]. One of the limitations of CADD is that the
training model used to classify the variants as benign or pathogenic is imperfect, such that an unknown proportion of variants that are classified by CADD as deleterious are actually neutral. To overcome this issue, the newer version of CADD correlates the model predictions with experimentally validated functional effect data to calibrate the parameters and evaluate CADD performance [90]. Moreover, to provide robustness to the interpretation, it is useful to integrate CADD prediction scores with additional data, such as independent conservation scores and predictions from other functional annotation software, including the Genome-Wide Annotation of Variants (GWAVA) software, the FATHMM-MKL and the Variant Effect Predictor (VEP) software.

GWAVA integrates multiple genomic and epigenomic annotations to predict the functional impact of variants, but can also be applied to a given genetic region, returning all known variants in that region and the corresponding prediction scores [87]. FATHMM-MKL is a variant annotation software suitable for both coding and non-coding variants, characterised by the ability to integrate and weight different types of annotations based on relevance and availability; not all annotations are available for all genomic positions, therefore FATHMM-MKL produces a p-value for all positions adjusting the weights relative to the available annotations [91]. The improved version of FATHMM-MKL is called FATHMM-XF (FATHMM with extended features), and exhibits greater prediction accuracy than FATHMM-MKL [92, 93]. VEP is also frequently used for variant annotation, because it predicts the functional consequences of variants and integrates multiple conservation and functional annotation scores, as well as identifying the location where a variant falls (whether it falls within a protein-coding sequence, non-coding RNA or non-coding regulatory region) [94, 95].

Another useful tool for assessing the implications of non-coding variation is the Genotype-Tissue Expression database (GTEx), which represents a comprehensive public catalogue of tissue-specific gene expression and regulation data [96]. The information stored in the GTEx catalogue can be used to determine whether a queried variant

| TABLE 2 | Techniques and resources used to annotate non-coding variants |
|---|---|
| **Non-coding variant annotation** |  |
| **Tool** | **Data sources** | **Purpose** | **Output** | **Link** | **Accessibility** |
| CADD | ENCODE, UCSC, Ensembl, VEP, Conservation data | Annotation of coding and non-coding variants | C-score, indicating pathogenicity | http://cadd.gs.washington.edu/ | Software download Online |
| GWAVA | ENCODE, Ensembl, Conservation data, GC-content data | Annotation of non-coding variants | Prediction score (0–1) | https://www.sanger.ac.uk/sanger/StatGen_Gwava | Software download Online |
| FATHMM-MKL | ChIP-seq data, TFBSs prediction, Conservation data, DNase-I hypersensitivity assay | Annotation of coding and non-coding variants | p-value, indicating deleteriousness | http://fathmm.biocompute.org.uk | Software download Online |
| FATHMM-XF | FATHMM-MKL, ENCODE, Roadmap Epigenomics Project data | Annotation of coding and non-coding variants | p-value, indicating deleteriousness | http://fathmm.biocompute.org.uk/fathmm-xf/ | Software download Online |
| VEP | Ensembl Regulatory Build, ENCODE, CADD, Conservation data | Annotation of coding and non-coding variants | Variant location, functional effect | http://www.ensembl.org/info/docs/tools/vep/index.html | Web-interface Command-line tool |
| **Other Tools** |  |  |  |  |  |
| GTEx | WGS data, RNAseq data | eQTL analysis | eQTL | https://gtexportal.org/home/ | Web-interface |
| PsychENCODE | WGS, Epigenomic data Transcriptomic data | Catalogue of the gene regulatory landscape of the human brain | Genomic elements active in the human brain | http://www.psychencode.org/?page_id=160 | Data download |
| H-MAGMA | Genomic data, Hi-C data | Noncoding variant-target gene prediction | Target gene prediction | https://github.com/thewonlab/H-MAGMA | Command-line tool |
functions as an eQTL for specific genes and is capable of modulating the expression of protein-coding genes. In the investigation of non-coding variants, eQTL data, such as those produced by GTEx, may be useful for identifying likely target genes that are affected by a particular non-coding regulatory variant [97]. For brain-related data and neurological disorder studies, the same type of information obtained from GTEx can be collected through the PsychENCODE Consortium: a multi-site project that aims at creating a comprehensive catalogue of the gene regulatory landscape of the human brain [98]. An additional tool that may be useful to annotate non-coding variants in brain-related studies is Hi-C coupled multimarker analysis of genomic annotation (H-MAGMA) [99].

NON-CODING VARIATION IN DISEASE

The first evidence of direct involvement of non-coding variation in disease dates back to 1982, when a single nucleotide substitution was detected in the promoter region of the haemoglobin subunit beta gene (HBB) (encoding β-globin, a subunit of haemoglobin) and was found to reduce HBB gene expression [100]. Subsequently, the variant was demonstrated to alter the binding of a transcription factor. Since then, with the emergence of genomic sequencing and the technical advances, the evidence supporting the involvement of ncDNA in health and disease has grown. Indeed, GWAS studies have shown that more than 88% of disease and trait-associated variants fall within non-coding regions of the genome [101, 102].

Numerous non-coding variants have been shown to contribute in different ways to many diseases; they may function as disease-modifiers, altering disease susceptibility, and in some cases represent the disease-causing variant. One example is Liebenberg syndrome, a rare genetic condition characterised by abnormal development of the arms, carpal bone defects and brachydactyly. This condition is caused by structural changes (deletions and translocations) occurring within the regulatory regions of cancer-related genes and have been found to reduce HBB gene expression [100]. Subsequently, the variant was demonstrated to alter the binding of a transcription factor. Since then, with the emergence of genomic sequencing and the technical advances, the evidence supporting the involvement of ncDNA in health and disease has grown. Indeed, GWAS studies have shown that more than 88% of disease and trait-associated variants fall within non-coding regions of the genome [101, 102].

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In cancer biology, many non-coding variants have been found in the regulatory regions of cancer-related genes and have been found to function as cancer-drivers [10, 11, 109]. One example is the case of the telomerase reverse transcriptase gene (TERT) promoter. TERT encodes the catalytic subunit of telomerase, an enzyme that regulates elongation of telomeres, repeated sequences localised at the ends of chromosomes, the purpose of which is to protect chromosomes from end-to-end fusion and end-degradation [110]. End-degradation represents the physiological loss of the terminal portion of the DNA strand that occurs during DNA replication. To prevent the loss of coding sequences, telomeres create a protective cap at the ends of chromosomes that is gradually degraded during DNA replication cycles. Physiologically, telomeres progressively shorten throughout life due to the inactivation of TERT [111]. In cancer cells, TERT reactivates and results in minimal telomere shortening, leading to telomere stabilisation and the capacity for indefinite cell proliferation [112]. Variations within the TERT promoter were originally identified in melanoma: a highly recurrent promoter variant was found to be involved in the tumorigenic process, as it created a novel binding motif for transcription factors, thus supporting permanent telomerase expression [113]. Subsequently, variations of the TERT promoter have been described in glioblastoma, hepatocellular carcinoma, bladder cancer, thyroid cancer and breast cancer, amongst others, associated with increased telomerase activity and in some cases with poor survival [112, 114–118]. TERT promoter mutation status may influence treatment response and can be used to predict how patients will respond to treatments: for example, TERT promoter variants have been associated with resistance to radiotherapy in patients with glioma [119–121]. The status of the TERT promoter may also be used to stratify patients and predict prognosis [122–125]. Furthermore, the TERT promoter is currently being investigated as a potential therapeutic target: a recently published preclinical study explored the use of programmable CRISPR-based base editing on the TERT promoter to reverse the mutation that activates TERT expression and thereby inhibit tumour growth [110, 112].

EVIDENCE OF NON-CODING VARIATION IN THE EPILEPSIES

Many epilepsies are characterised by significant and usually unexplained phenotypic variability, which could be potentially associated with genetic variation in non-coding sequences, functioning as disease modifiers. Additionally, ncDNA may potentially harbour genetic variants that act as disease risk variants, which could be explored as prognostic biomarkers to stratify patients and identify those with a higher risk of developing comorbidities or experiencing more severe symptoms [126]. The investigation of ncDNA in epilepsy has been primarily aimed at non-coding RNAs [127, 128]. Additional work has been carried out investigating the methylation state and epimutations in non-coding DNA regions [129, 130]; however, overall, the non-coding regulatory regions of the genome remain relatively unexplored.
Variation in promoter regions

There is some limited evidence of variation in the promoter region of several epilepsy-associated genes possibly modulating gene transcription and contributing to the pathogenesis and phenotypic variability of epilepsy. One example of such variation is in the promoter region of the sodium voltage-gated channel alpha subunit 1 gene (SCN1A), which encodes the voltage-gated sodium channel type I alpha subunit and plays a crucial role in the initiation and propagation of action potentials. Mutations in this gene are associated with a wide spectrum of epilepsies, including Dravet Syndrome and genetic epilepsy with febrile seizures plus (GEFS+). Gao et al. studied the promoter region of SCN1A in a cohort of patients with epilepsy and febrile seizures, who did not carry pathogenic variants in the coding sequence, and detected a heterozygous mutation in the SCN1A promoter. They demonstrated that this variant caused a decrease in promoter activity (of ~42%) and was responsible for a mild epilepsy phenotype with incomplete penetrance. Furthermore, de Lange et al. have shown that common variations in the promoter of the unaffected SCN1A allele influence the disease severity in patients with a pathogenic SCN1A variant. They assessed the functional consequences of five different SCN1A promoter variants, identifying reduced expression and reduced channel function, leading to a more severe phenotype, thus indicating a disease-modifier effect. Neither of these studies has been replicated.

Additionally, evidence exists for a relationship between altered methylation status in the promoter regions of genes and the pathogenesis of epilepsy. Although not confirmed, such methylation alterations may occur because of genetic variations in gene promoter sequences. One example is temporal lobe epilepsy, in which hypermethylation in the promoter region of the reelin gene (RELN), was reported. In addition, Belhedi et al. described an increased methylation status in the promoter region of the carboxypeptidase A6 gene (CPA6) in patients with focal epilepsy and febrile seizures. Neither study has been replicated, and the credibility of RELN as a gene of relevance in epilepsy per se has been questioned. While still a relatively young field, important research has been carried out on changes in the methylation state of non-coding sequences.

Non-coding structural variations

Structural variations are also known to play a relevant role in epilepsy, and there is evidence of structural variation occurring in non-coding sequences, associated with epilepsy pathogenesis. One example is benign adult familial myoclonic epilepsy (BAFME), also described as familial adult myoclonic epilepsy (FAME), autosomal dominant cortical myoclonus and epilepsy (ADCME) and familial cortical tremor and epilepsy (FCTE), which is associated with the aberrant expansion of TTTC sequences of five different dodecamer repeats in the promoter region of the cystatin B gene (CSTB). Normal alleles have two to three dodecamer repeats, while more than 30 repeats have been found in people with EPM1. A correlation between the expansion size and disease severity is yet to be clarified, although patients who have compound heterozygosity for the repeat expansion and InDels or point mutations present a more severe phenotype than patients homozygous for the dodecamer expansion.

Microdeletions have been found within the promoter region of SCN1A. Nakayama et al. described two cases of Dravet syndrome carrying microdeletions in the SCN1A promoter region that resulted in SCN1A haploinsufficiency and reduced channel protein levels, leading to Dravet Syndrome. Additionally, Haigh and colleagues have shown that mice harbouring deletions in the non-canonical promoter region of Scn1a, which include the alternative TSS 1b, exhibit a significant reduction in the expression of Scn1a and an epileptic phenotype.

One study reported somatic copy number gains in the enhancer region of the epidermal growth factor receptor gene (EGFR) and the promoter region of the platelet derived growth factor receptor alpha gene (PDGFRα), without alterations in the coding sequence, in brain tissue from patients with focal cortical dysplasia operated for treatment-resistant epilepsy. In addition to the amplification of non-coding regulatory elements, an upregulation of EGFR and PDGFR was also reported. However, this correlation has not been experimentally confirmed and the mechanism responsible for this association was not addressed in the study.

Monlong et al. investigated CNVs in a cohort of 198 patients with epilepsy and found an enrichment of non-coding CNVs close to known epilepsy genes, which likely fall into regulatory sequences. However, the functional effect of these non-coding CNVs has not been experimentally verified.

Strategy to investigate non-coding variation and challenges

A putative schematic representation of the steps to be taken to investigate non-coding genetic variation is shown in Figure 3. In the
preliminary phases of the pipeline, blood is currently the gold standard source for DNA collection, as blood-derived DNA samples are of higher quality and are more likely to pass stringent quality controls (QCs) of DNA integrity and concentration, whereas saliva-derived samples, for example, are more prone to QC failure [148]. The major drawback of this approach is the inability to detect somatic genetic variations: in the case of epilepsy, for example, somatic variations may occur in the brain, and might only be detectable with the use of brain-tissue derived DNA samples. Relating to the sequencing approach, although the majority of studies use Whole Exome Sequencing (WES) or targeted sequencing panels, neither of these two methods generate information about non-coding elements; for genome-wide exploration, Whole Genome Sequencing (WGS) is the most appropriate approach.

As shown in the figure, the final phase of the workflow is the functional validation of the candidate variants, which is performed predominantly using wet-lab experimental approaches. The most widely used experimental methods are luciferase reporter assays and the use of CRISPR system to generate viable models. The luciferase reporter assay aims to compare the level of luciferase expression in the presence and absence of a variant of interest [149]. The CRISPR-mediated genome editing system can be used to generate both cellular and animal models, such as murine models, carrying the candidate variation, thus allowing evaluation of the functional impact in vivo [150].

However, in the investigation of non-coding variants, multiple challenges need to be highlighted. First, the challenge of predicting the functional consequences of non-coding variation, which can result in discordant predictions from different annotation tools: this is a major limitation that also applies to the investigation of protein-coding variants [151]. Furthermore, unlike the investigation of protein-coding variants, another challenge in the study of non-coding variation is the current lack of a variation database, which would allow researchers to quickly determine whether a non-coding variant has been previously observed and linked to disease. Second, the detection of non-coding regulatory regions, such as promoter regions, which is complicated by the existence of overlapping genes: distinct genes that share a genetic region. It is estimated that about one-quarter of human protein-coding genes overlap with each other. Most of these are co-expressed in the same tissue type and are likely to be co-regulated [152]. The presence of overlapping genes makes it difficult not only to identify the regulatory sequences flanking a protein-coding gene, but also to understand the functional consequences of variants. Indeed, assuming a pair of genes are overlapping, genetic variations falling in the regulatory sequence upstream of both genes may have an impact on the expression of both genes, while variants falling in the regulatory sequence upstream of the second gene may also fall within the coding sequence of the first gene, thus further complicating the functional interpretation of non-coding variants. Third, the reliability of data on regulatory element localisation: to date, most of the available data, particularly for enhancers, is simply prediction, with only a small portion being experimentally validated data, thus limiting the robustness of results and highlighting the need to produce experimentally-validated enhancer data resources. Recently, non-human model systems have been used to identify and experimentally validate non-coding regulatory elements [153].

CONCLUSIONS AND FUTURE PERSPECTIVE

In conclusion, despite the lack of a comprehensive and systematic genome-wide investigation of non-coding regulatory variation in epilepsy, we suggest that ncDNA has the potential to be of relevance in epilepsy research. Non-coding regulatory regions may harbour variations that influence gene expression and contribute to the phenotypic variability of disease, may have a disease-modifying effect, or influence treatment response. Furthermore, the findings of studies in other fields indicate that non-coding variation may also represent the main source of disease causation, and, eventually, may represent potential therapeutic targets for innovative treatment strategies. Overall, the non-coding genome represents an exciting area to investigate.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

SP: Drafting of manuscript. JDM: Revision of manuscript. AF: Revision of manuscript. JMM: Revision of manuscript. SMS: Revision of manuscript.

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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