Review

Current scenario of the genetic testing for rare neurological disorders exploiting next generation sequencing

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Abstract

Next generation sequencing is currently a cornerstone of genetic testing in routine diagnostics, allowing for the detection of sequence variants with so far unprecedented large scale, mainly in genetically heterogenous diseases, such as neurological disorders. It is a fast-moving field, where new wet enrichment protocols and bioinformatics tools are constantly being developed to overcome initial limitations. Despite the as yet undiscussed advantages, however, there are still some challenges in data analysis and the interpretation of variants. In this review, we address the current state of next generation sequencing diagnostic testing for inherited human disorders, particularly giving an overview of the available high-throughput sequencing approaches; including targeted, whole-exome and whole-genome sequencing; and discussing the main critical aspects of the bioinformatic process, from raw data analysis to molecular diagnosis.

Key Words: clinical practice; genetic testing; neurogenesis; next generation sequencing; sequencing approaches; variant interpretation

Introduction

So far, more than 7000 rare mendelian disorders are described and half of them affect the peripheral and central nervous system. The inherited neurological disorders are a group of heterogenous diseases from clinical and genetic point of view, often characterized by progressive and severe disability (Warman Chardon et al., 2015). The wide range of clinical manifestation includes ataxias, encephalopathies, genetic form of brain malformations, myopathies and muscular dystrophies, neuropathies and form of dementia. There is a significant phenotypic overlap between different forms of neurological diseases. For example, Emery-Dreyfuss muscular dystrophy can present similar proximal muscular weakness than the limb girdle muscular dystrophies, making sometimes difficult the precise diagnosis. From genetic point of view, the identification of the causative mutation can be very challenging, due to the heterogenous genetic nature of these disorders (Vgontzas and Renthal, 2019). For example, more than 300 genes are associated with ataxia or more than 50 genes are causative of the hereditary spastic paraplegia. On the other hand, there are rare neurological diseases for which the genetic basis is still unknown. Moreover this heterogenous picture is often characterized by reduced penetrance, variable onset and variable expressivity (Fogel, 2018). For that reasons, many patients affected by rare neurological disorders spend many years before receiving a molecular diagnosis or remain genetically undiagnosed (Adams and Eng, 2018).

For patients with rare neurological disorders, the rapid and correct diagnosis can reduce the time from onset of symptoms to medical treatment, reducing multiple specialists' visits, number of different clinical exams and avoiding ineffective medical treatments.

In this scenario, a precise molecular diagnosis may have several benefits on patient care. For example, the accurate genetic characterization can be useful for the prevention harmful immunosuppressant therapy in patients affected by progressive muscular dystrophies presenting like an inflammatory myopathy (Adams and Eng, 2018). Other example is the importance of the detection of causative mutations in the GAA gene associated with Pompe disease, that allow a timely enzyme replacement therapy that can significantly improve muscle strength and reduce mortality (Chan et al., 2017). Therefore, an early and accurate molecular diagnosis can be fundamental to initiating the timely and optimal treatment for patients affected by rare neurological disease.

Next generation sequencing (NGS) is a widely used approach for genetic testing in clinical laboratories. NGS presents the great potential to find causative mutation, *de novo* or inherited mutations, associated with genetic disorders characterized by variable phenotypic presentations and heterogeneous genetic background, such as in neurological disorders.

Prior to the advent of the high-throughput technologies, Sanger sequencing, referred to as a 'first-generation' sequencing, was the most-used method for the exon-by-exon analysis of a single or few genes in the diagnosis of inherited disorders (Sanger and Coulson, 1975; Yohe and Thyagarajan, 2017; Fernandez-Marmiesse et al., 2018). In recent years, while Sanger sequencing remains the gold standard, NGS has vastly changed genomics, allowing the fast generation of massive parallel sequencing reactions, overcoming the limitation of the single-gene analysis with the generation of a genome-scale data that was unthinkable in the previous Sanger era (Malentacchi et al., 2015; Yohe and Thyagarajan, 2017; Caspar et al., 2018). NGS opened a new scenario in the molecular diagnosis of Mendelian disorders, allowing for the detection of germline mutations exploiting different approaches, encompassing the targeted analysis of a panel of

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selected genes (targeted sequencing, TS), the sequencing of the entire genome (whole-genome sequencing, WGS) or of the entire coding region (whole-exome sequencing, WES; Di Resta and Ferrari, 2018).

The use of high-throughput technologies allows an increased diagnostic rate than single-gene testing approach in the molecular diagnosis of diseases with an unknown genotype-phenotype correlation. Moreover, our limited knowledge on variable disease expressivity and penetrance in some neurological disorders can be expanded exploiting, for example, WES or WGS, that can allow the identification of novel disease genes. This would represent a key development toward a deeper understanding of disease variability in the future, with the potential for improved risk prediction in patients affected by neurological disorders. Indeed, so far, research use of high-throughput sequencing has successfully identified new pathogenic variants in new genes responsible for numerous rare genetic disorders (Dias et al., 2019; Maver et al., 2019; Royer-Bertrand et al., 2019).

In addition, for the effective use of the NGS approaches in diagnostic laboratories, geneticists and clinicians should take into account information on the relevant workflows including analysis and sequencing depth to understand the specific clinical application and diagnostic capabilities of these gene sequencing techniques. Due to the large amounts of data generated from NGS, even from small gene panels, bioinformatics pipelines are required to effectively process and evaluate the sequence information (Wong et al., 2019). Therefore, data analysis workflow and the choice of the most proper bioinformatic pipelines represent crucial issues in the application of NGS in clinical diagnostics, also determining sensitivity and accuracy in the detection of sequence variants. Furthermore, after prioritization, the interpretation, exploiting several available software tools, and the classification of detected variants are fundamental to reaching a molecular diagnosis (Mancini et al., 2015; Strande et al., 2018).

In the first section of this review, advantages of the three different approaches in the diagnostic practice are discussed, taking into account that NGS methods presents several pitfalls. Future trends of high-throughput technologies in genetic diagnostics are briefly discussed with a particular attention to the advent of third generation sequencing, characterized by long-read method, that is expected to further change also the clinical genome sequencing in the near future.

Then, in the second part we review the step-by-step process of data analysis, focusing on a selection of available tools and addressing the main critical issues related to the translation of NGS data to clinical application. The search of references has been done using PubMed. The cited references have been published in the last 5 years, with exception for few specific references in 2010. The bibliography were searched in the last 8 months.

Next Generation Sequencing Approaches

So far, NGS testing is used in clinical laboratories for the detection of germline mutations associated with different forms of inherited disorders, exploiting different approaches, including the analysis of selected genes panels (TS), WES or WGS (van Dijk et al., 2014; Caspar et al., 2018).

The main advantages and disadvantages are discussed below and summarized in **Figure 1**.

Targeted sequencing

The first approach, the TS, is used for screening of several disease-genes in the same run and it is useful for many neurological diseases characterized by an oligogenic inheritance. For example, many forms of ataxias, epilepsies and limb girdle muscular dystrophies are characterized by clinical overlap and different genes are associated with similar clinical presentation. For example, in limb girdle muscular dystrophies genetic diagnosis the TS approach allows a three times greater diagnostic rate than single-gene testing approach (Volk and Kubisch, 2017; Di Resta et al., 2018; Micaglio et al., 2019).

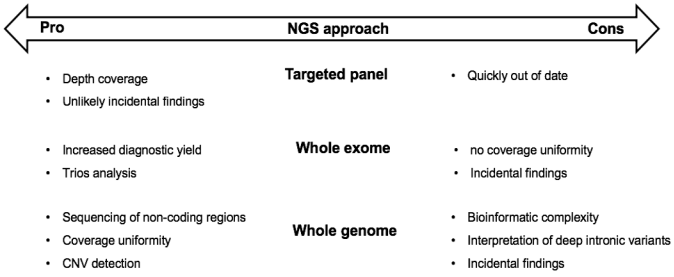

Figure 1 | **Comparison of pro and cons of different NGS approaches.**
The advent of NGS has opened a new era in molecular diagnosis. The massive parallel sequencing allows the screening of a panel of genes (targeted sequencing) or of the entire genome (whole-genome sequencing) or all of the coding regions (whole-exome sequencing). Each approach is characterized by advantages and still unsolved limitations, summarized in the figure. CNV: Copy number variation; NGS: next generation sequencing.

TS is a rapid diagnostic test characterized by a high read depth for the entire targeted region (200–1000×), allowing for the detection of low alternate allele frequencies, present in cases of germline mosaicism (Feliubadaló et al., 2017). Moreover, the analysis of disease-related genes minimizes the chance of detecting incidental findings, that are secondary findings not strictly related to the clinical reason for which the genetic test was requested. Indeed, according to the guidelines of the American College of Medical Genetics and Genomics, only causative genes should be included in the diagnostic testing (Rehm et al., 2013; Kalia et al., 2017).

However, TS presents some limitations. At first, it is difficult to detect copy number variations (CNVs), due to a low coverage in GC-rich regions, such as the first exons, or by the absence of enrichment probes for a specific region (Meienberg et al., 2016; Caspar et al., 2018), depending on the adopted technology.

Moreover, since so far there is no an international consensus for the disease-related gene lists associated with a specific phenotype, the content of the clinical gene panels can be different among different diagnostic laboratories (Yohe and Thyagarajan, 2017; Courtney et al., 2018). Furthermore, a periodical update of the gene panels is needed, due to the identification of novel causative genes, mainly for inherited forms for which genetic basis is not completely understood. The clinical cases that remain undiagnosed after TS require further genetic analysis, such as the sequencing of a different gene panel or WES or WGS.

Whole-exome sequencing

WES are often performed in unsolved cases after TS approach (Worthey et al., 2011; Sawyer et al., 2016; Eldomery et al., 2017) or in cases affected by rare or unknown diseases, exploiting trios analysis, testing the proband and his/her parents (Di Resta and Ferrari, 2018; Splinter et al., 2018; Mazzarotto et al., 2020; Rossi et al., 2020).

Exome encompasses the entire coding regions of the genome, harboring the 85% of all known disease-causing variants (Abecasis et al., 2010). WES allows not only to analyze encoding regions already associated with human disorders, but also to identify new causative genes in diseases for which genetic basis is not completely characterized (Bick and Dimmock, 2011; Zhu et al., 2015). In particular, the trios analysis represents a cost-effective approach, which significantly increases the diagnostic yield, facilitating the

filtering and interpretation of the variants. In using the WES approach, the chance of detecting incidental findings in a diagnostic test can be reduced, limiting the bioinformatic analysis to a gene panel of interest with the possibility of subsequently expanding the computational analysis, if it is necessary (Bick et al., 2017).

However, WES suffers from some limitations. First, as discussed in TS, WES is not characterized by uniformity of target coverage, due to poor enrichment in GC-rich regions and the use of short reads covering the coding sequence (Yohe and Thyagarajan, 2017). It can create bias toward exonic variant identification in a subset of low covered genes or detection of large deletion, expansion, or structural rearrangement (Wells et al., 2019). In this regard, several studies showed that WGS approach can be more powerful than WES in detecting exonic variants, including also CNV (Belkadi et al., 2015; Meienberg et al., 2015, 2016), as further discussed. Second, it is estimated that 15% of variants with putative role on mendelian traits are localized in non-coding regions and all these variants would be missed exploiting WES (Mazzarotto et al., 2020).

Whole-genome sequencing

The limitations discussed above can be overcome by exploiting the WGS approach (Meienberg et al., 2016; Lionel et al., 2018).

WGS may solve the WES-negative cases in patients affected by a disorder with a strong familial segregation (Bick et al., 2017). WGS is characterized by a uniform coverage in coding and non-coding regions, leading to a low chance of losing disease-causing variants due to intrinsic technical errors.

For the first time WGS was successfully used in the neurogenetics field for the identification of a causative coding mutation in a family affected by a rare form of autosomal recessive Charcot-Marie Tooth disease or subsequently, for example, in molecular diagnosis of sensory and motor neuropathy with microcephaly or an early-onset epilepsy (Lupski et al., 2010; Gonzaga-Jauregui et al., 2013; Martin et al., 2014).

Moreover, WGS allows for the detection of CNVs, gross chromosomal abnormalities and deep intronic variants, leading to a higher diagnostic yield compared to WES or TS, reaching about 73% of genetically-solved cases (Clark et al., 2018; Scocchia et al., 2019).

However, it is important to keep in mind that approximately 3–4 million variants per individual are commonly identified through WGS and for sure, as in the WES approach, the analysis of the entire genome can lead to a high chance of detecting incidental findings. It again can be avoided by focusing the initial analysis only on a gene panel of interest (Barbitoff et al., 2020).

Moreover, the application of WGS in diagnostic routine may present some issues, such as the high costs for sequencing or for computational infrastructures suited to store and analyse terabytes of data; moreover, it is important taking into account the great complexity for data analysis and variant interpretation.

The first issue can be overcome in the near future, considering the continuous decreasing of sequencing costs and the improvement of the new high-throughput platforms (van Dijk et al., 2014; Meienberg et al., 2016).

On the second point, based on our current knowledge, it is certainly more difficult to predict the possible pathogenic effect of a intergenic, regulatory or deep intronic variant (Kremer et al., 2017; Lionel et al., 2018). However, in the near future, WGS will be exploited by a larger number of laboratories, enabling much research on such variants, allowing a better understanding of their pathogenic role and increasing the yield of genetic testing (Meienberg et al., 2016). Moreover, knowledge of the variants in non-coding regions is also important to better understand their clinical implications, that is important for their further classification in the near future and for new insight into disease pathophysiology (Vgontzas and Renthal, 2019).

Next Generation Sequencing Data Analysis

The analysis of the sequencing data requires an important computational effort. Due to the complexity of the bioinformatic process, so far dedicated and skilled bioinformaticians have an essential role in the genetic laboratory, in order to continuously implement the computational pipeline in the clinical NGS service (de Leng et al., 2016).

The entire workflow of the NGS data analysis can be distinguished in primary, secondary and tertiary analysis; starting from the mapping of reads, to the reference genome, and on to variant calling and interpretation (**Figure 2**). Below, we give an overview of the main steps of the entire process.

Primary and secondary analysis: from the raw data conversion to the variant calling

The first step of NGS analysis includes the conversion of the output signal generated by sequencing platforms to short sequences of nucleotides, or reads, and the assignment of the base quality scores and the generation of the FastQ file, the raw data file (Wong et al., 2019).

Secondary analysis includes the alignment of shorts reads to the reference genome, generating SAM/BAM file, and variant calling, obtaining the vcf file. So far, several tools are available for this process (**Table 1**).

The BWA/GATK pipeline is recognized as the current standard for the alignment of short reads and calling of single nucleotide variants or indels in TS, WES or WGS analysis. It is based on a robust read mapping algorithm and it is continuously implemented by its development team at the Broad Institute, as the technologies improve, in order to formulate and share the best practices recommendations (Wong et al., 2019).

In order to improve the specificity of variant calling process, the alignment of sequence reads against the full human reference assembly is recommended, even if a TS has been performed, reducing a possible mismapping due to, for example, homologous regions (Gargis et al., 2015).

Most algorithms used for single nucleotide variants and indels calling are not suited to the CNVs detection and dedicated tools have been developed, such as Manta, CNVnator, BreakDancer or Pindel (Chen et al., 2009, 2016; Ye et al., 2009; Abyzov et al., 2011). As previously discussed, the WGS is the most favourable approach for the identification of CNVs, using short-read sequencing. However, new tools have been developed for the CNV detection also in TS or WES, such as CNVkit, taking into account the intrinsic enrichment bias, although they achieve a lower accuracy than the WGS data (Talevich et al., 2016).

Therefore, so far several bioinformatics tools are available and the use of several variant callers is recommended to optimize the accuracy of the variant calling step in a clinical assay (false negative results; Wong et al., 2019).

Tertiary analysis: filtering, interpretation and classification of next generation sequencing data

The tertiary analysis is the last step of the data analysis workflow. It consists in the prioritization and interpretation of the identified variants, assessing their functional impact and

Table 1 | **List of examples of bioinformatic tools used in the alignment and variant calling process, or interpretation of next generation sequencing data**

	Tool	Function
Primary and secondary analysis	BWA, GATK	Analysis, alignment, variant calling
	Manta, CNVnator, BreakDancer, PINDEL, CNVkit	Indels and CNV calling
Tertiary analysis	ANNOVAR, VEP, VAAR	Prediction of the effect of genetic variants on genes, transcripts, and protein sequences
	PhyloP, GERP	Analysis of evolutionary conservation
	PolyPhen2, SIFT, MutationTaster2	Prediction of the effect of the amino acid substitution
	MaxEntScan, NNSplice	Analysis of effect of CNV
	DECIPHER, DGV	Clinical interpretation of CNV

CNV: Copy number variation.

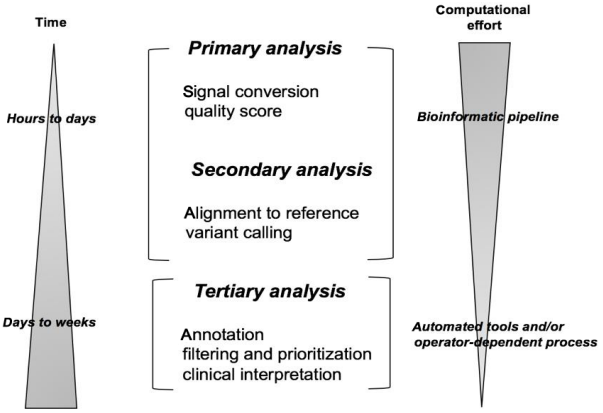

Figure 2 | **Schematic representation of the workflow of next generation sequencing data analysis.**
The entire process can be divided into three different stages: primary, secondary and tertiary analysis. Time and computational efforts are inversely related with each single step of the entire process.

likely pathogenic role, exploiting several tools and algorithms that have been developed for this process.

Tertiary analysis is the critical step and the real bottleneck for the clinical application of NGS.

The first process of the tertiary analysis is the variant annotation, in which functional information, such as conservation, population-specific allele frequency, effect on DNA sequence, are assigned to each detected variations (Krier et al., 2016; Eilbeck et al., 2017).

Several tools are available (**Table 1**) for determining the variant location and their effect on transcripts, such as ANNOVAR (Wang et al., 2010) or Variant Effect Predictor (McLaren et al., 2016), based on Ensemble transcripts, or The Variant Annotation Analysis and Search Tool, a suite, freely available for academic research, combining information on amino acid substitution and allele frequency for probabilistic disease-gene discovery (Yandell et al., 2011).

The assessment of the most likely causative variant in a plethora of detected variants of unknown significance is the most difficult process and a series of appropriate filtering can be useful in the operator-dependent evaluation, in order to filter out common and nonpathogenic variations.

Moreover, as discussed above, in order to prioritize the large numbers of detected genetic variations for diagnostic purpose, a gene list should be defined in the initial analysis, filtering and analyzing only variants localized on those known genes with a clinical utility and associated with the clinical suspicion (Fahrioğlu, 2018).

At first, a prioritization based on the population frequency can be applied, interrogating several databases, such as gnomAD (Scheps et al., 2020) or 1000 Genomes (Birney and Soranzo, 2015), and filtering out the common variations.

Subsequently, the known disease genotype-phenotype association can be evaluated in databases, such as Leiden Open Variation Database (Fokkema et al., 2011), Human Gene Mutation Database (Stenson et al., 2017), ClinVar (Landrum et al., 2014) or Online Mendelian Inheritance in Man (Amberger and Hamosh, 2017).

The inheritance, penetrance and expressivity of the variants should also be included in the interpretation criteria (Di Resta et al., 2014; Roy et al., 2018).

Other possible criteria for the variant prioritization is based on the evolutionary conservation (e,g,. PhyloP or GERP) (Davydov et al., 2010; Pollard et al., 2010) and on the *in silico* prediction of the potential effect on the protein structure (**Table 1**). In particular, tools such as PolyPhen2, SIFT or MutationTaster2 allow for the prediction of the possible impact of an amino acid substitution on the structure and function of a human protein (Adzhubei et al., 2010; Tosetti et al., 2017), while other tools (e.g., MaxEntScan, NNSplice) are specifically dedicated to evaluating the potential impact on splicing (Ng et al., 2010). The clinical interpretation of the CNVs can also be based on their population frequency and size, aided by databases such as DGV (Eilbeck et al., 2017) or DECIPHER, that enables the sharing and comparison of phenotypic and genotypic data in the scientific community worldwide (Firth et al., 2009).

Moreover, in recent years, collaborative efforts in global projects have led to the data sharing of the genotype-phenotype associations, in order to facilitate the clinical interpretation of rare or novel variants identified in similar clinical cases (Moorthie et al., 2013). It is important to cite for example the Genomics England 100,000 Genome Project, the NHLBI Trans-Omics for Precision Medicine the NHGRI Centers for Common Disease Genomics, which comprise genome sequencing data of tens of thousands of individuals (Siva, 2015; Kowalski et al., 2019).

Finally, in order to classify the inherited variants, the American College of Medical Genetics and Genomics and the Association for Molecular Pathology have developed guidelines, that remain a cornerstone in medical genetics (Richards et al., 2015). These guidelines define a framework for variant classification criteria, in order to have a standardized process for the clinical evaluation of genetic information, establishing the criteria necessary for classifying a genetic variant into five possible categories (pathogenic (class V), likely pathogenic (class IV), uncertain significance (class III), likely benign (class II), or benign (class I)) (Richards et al., 2015).

These guidelines clearly aim to increase the consistency in variant interpretation process between different laboratories, since it still presents limitations due to the subjective nature of variant classification (Kleinberger et al., 2016).

Indeed, published data highlight that after the classification of the same list of genetic variants between different centers, the obtained classification consensus is 71% (Amendola et al., 2016; Nykamp et al., 2017). In order to overcome this issue, several automated tools have recently been developed to facilitate and support the classification process in increasing the inter-laboratory consistency in the clinical interpretation of the identified variants (Nykamp et al., 2017).

Conclusions and Future Perspective

In recent years, the advent of NGS has opened a new era in

neurogenetics and certainly, so far, the second-generation sequencing is the cornerstone for molecular diagnosis for patients affected by heterogenous genetic diseases. For example, its adoption in the clinical molecular laboratories significantly increased our ability to identify the causative variants in rare neurological diseases.

In the previous Sanger sequencing era, single-gene tests were time-consuming and relatively expensive. Genetic tests were requested after the clinical evaluation and other general examinations, which suggested a specific gene as the likely disease cause. However, as previously mentioned, many neurological phenotypes are characterized by heterogenous genetic basis and testing all known disease genes was not feasible (Fogel, 2018). As a result, many patients remained genetically undiagnosed.

So far, thanks to the reduction of cost of DNA sequencing, NGS approach become widely used in clinical laboratories. The massive parallel sequencing allows the screening of a large number of genes or of the entire genome or all of the coding regions, as described in this review. The advances can allow a rapid identification of causative genes and a more precise genetic diagnosis of many neurological disorders. Thus, genetic testing should be considered earlier in the diagnostic procedure.

Moreover, the characterization of new causative genes responsible for many neurologic diseases has provided new insight into their pathogenic mechanisms, leading advances in gene therapy and identification of new treatments for previously incurable diseases (Žitnik et al., 2018; Vgontzas and Renthal, 2019).

However, despite the undoubted advantages, some issues should be solved from technical point of view, as discussed in this review. For example, one limitation is that NGS relies on PCR, that doesn't allow an efficient amplification of GC rich genomic regions. Moreover, while single nucleotide variants or small indel can be detected using short reads, the identification of CNV is more challenging exploiting NGS, leaving some cases genetically unsolved (Ebbert et al., 2019).

Now TGS are being developed in order to overcome these issues (Ståhl et al., 2016). Their main technical feature is the ability to sequence the single molecule, avoiding the intrinsic amplification bias of the first and second generation sequencing (Ståhl et al., 2016). Two commercial platforms such as the PacBio SMRT (Pacific Biosciences, CA, USA) and the Oxford Nanopore Technologies (Oxford, UK) have been developed. These instruments differ in their chemistry. The former is fluorescent detection-based while the Oxford Nanopore records a current change, as DNA molecule flows through a membrane pore. Both instruments produce long reads (up to 40,000 for the PacBio SMRT and up to 100,000 for the Nanopore). The third generation platforms could overcome issues for pseudogene, repeat regions sequencing or CNV detection and they can produce genome assemblies of unprecedented quality, allowing also the detection of epigenetic modifications or whole-transcriptome analysis (van Dijk et al., 2018). So far, third-generation sequencers are developed mainly for research purpose and they are expected to further change also the clinical genome sequencing. However, even if these instruments are a great promise for the genomic sequencing, it is necessary to overcome their intrinsic limitations before transfer to clinical practice may be possible. For example, nanopore sequencing still present a quite a high error rate (~15%) while SMRT sequencing have a maximum read length limited by polymerase processivity (~80 kb). Moreover, the cost of sequencing is relatively high per Gb and a large amount of starting material is needed (van Dijk et al., 2018).

Finally, so far, other challenges remain unsolved in the use of NGS for diagnostic testing, regarding the bioinformatic analysis and data interpretation, for which there is no clear guidelines. For example, as discussed in this review, open questions are the management and report of incidental findings or the subjectivity of variant classification and interpretation, mainly for the effect of variants of unknown significance (cl.III) or non-exonic variants (Meienberg et al., 2016).

In conclusion, although there are still open challenges, NGS provides an expanding approach to identify rare mutation in genetically heterogenous diseases and it is expected to lead advances in identification of better treatment and gene therapy in a new era of precision medicine for genetic neurological disorders.

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