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REVIEW ARTICLE

NGS Technologies as a Turning Point in Rare Disease Research, Diagnosis and Treatment

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Abstract: Approximately 25-50 million Americans, 30 million Europeans, and 8% of the Australian population have a rare disease. Rare diseases are thus a common problem for clinicians and account for enormous healthcare costs worldwide due to the difficulty of establishing a specific diagnosis. In this article, we review the milestones achieved in our understanding of rare diseases since the emergence of next-generation sequencing (NGS) technologies and analyze how these advances have influenced research and diagnosis. The first half of this review describes how NGS has changed diagnostic workflows and provided an unprecedented, simple way of discovering novel disease-associated genes. We focus particularly on metabolic and neurodevelopmental disorders. NGS has enabled cheap and rapid genetic diagnosis, highlighted the relevance of mosaic and *de novo* mutations, brought to light the wide phenotypic spectrum of most genes, detected digenic inheritance or the presence of more than one rare disease in the same patient, and paved the way for promising new therapies. In the second part of the review, we look at the limitations and challenges of NGS, including determination of variant causality, the loss of variants in coding and non-coding regions, and the detection of somatic mosaicism variants and epigenetic mutations, and discuss how these can be overcome in the near future.

Keywords: Next generation sequencing, rare diseases, diagnosis, research, digenic inheritance, genome, exome.

1. INTRODUCTION: ADVANCES IN THE FIELD OF RARE DISEASES REACHED THANKS TO THE NGS TECHNOLOGY

There is no doubt that the emergence of NGS technologies constituted a turning point for the advancement of our understanding of rare diseases. Excellent reviews on the influence of NGS on rare diseases have been published [1-3]. In this first section, we will describe the main milestones that have been achieved thanks to the use of this technology.

1.1. Changes in Diagnostic Workflow

Until just several years ago, genetic analysis was considered the final stage of the diagnostic process in patients with a rare disease. After a process typically lasting years, involving the documenting of clinical manifestations and performance of imaging and biochemical tests, patients suspected to have a genetic disorder were referred for genetic analysis by Sanger sequencing, which is a highly manual and time-consuming chemical process. The success rates, however, were low. The emergence of NGS about 10 years ago radically changed this diagnostic workflow by providing a quick, powerful, and low-cost alternative for genetic analysis in the early stages of the process. In just a few weeks or even less, NGS-based tools can point to the implication of a single gene (or a small number of genes) and help to establish a rapid diagnosis in a considerable percentage of cases. This new workflow has drastically reduced waiting times and shortened the often endless quest that many patients had to embark on to find a prognosis. It therefore comes as no surprise that the best healthcare systems in

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the world now use these powerful tools as part of their routine diagnostic processes.

NGS has also led to a new process known as *reverse phenotyping*. On occasions the combined use of NGS and segregation analysis can identify a pathogenic mutation in a gene that is known to cause disease but that was previously linked to a different phenotype. In such cases, retrospective clinical investigation of the patient and family members can reveal additional, previously unrecognized features. In a review of over 300 studies that had investigated rare disease by WES, Boycott *et al.* [1] indicated that approximately 25% of reported mutations in known disease-causing genes were associated with a phenotype that, in retrospect, matched the clinical presentation of the patient being investigated. Many examples of reverse phenotyping can be found in recent literature [4-10]. Their findings show that the order and intensity in which symptoms appear in rare diseases vary greatly from patient to patient, explaining why it is so difficult to establish a diagnosis. Only a few years ago, physicians had no choice but to watch and wait as the different symptoms began to emerge over the course of years. The process could perhaps be likened to watching the grass grow. NGS, however, has provided clinicians with powerful molecular tools that they can use at the start of the diagnostic progress to unveil important clues that enable them to start investigating manifestations that are not yet fully expressed or may not even have yet appeared.

1.2. Easy and Rapid Isolation of Novel Disease-Causing Genes

The number of newly identified disease-associated genes has grown exponentially in all fields of medicine since the emergence of NGS technology. Figure (**1**) shows the increases in the number of entries for which the molecular basis of a particular phenotype is known in the Online Mendelian Inheritance in Man (OMIM) database over the past 10 years. This explosion of knowledge has taken place because NGS can be used to sequence any region of the human genome, ranging from several genes to the whole genome, in a fast and sensitive manner. Three main NGS-based tests are used in rare disease research. These tests can be ordered by cost, ease of analysis, and scope and include 1) parallel sequencing of coding sequences (exons) of groups of genes related by similar or overlapping phenotypes (gene panels); 2) whole-exome sequencing (WES), in which all known coding regions of the human genome are sequenced; and 3) whole-genome sequencing (WGS), which analyzes the entire human genome. WES has dominated rare disease research in recent years. It covers just the 1% (~30Mb) of the human genome that is translated into protein [11] but compared with WGS, it offers a significantly more cost- and time-effective method of collecting and analyzing genomic data. Gene panels, in turn, offer much faster turnaround times, fewer incidental findings, and higher coverage (thereby increasing the chances of detecting CNVs and somatic mosaic mutations) than WES. Un-

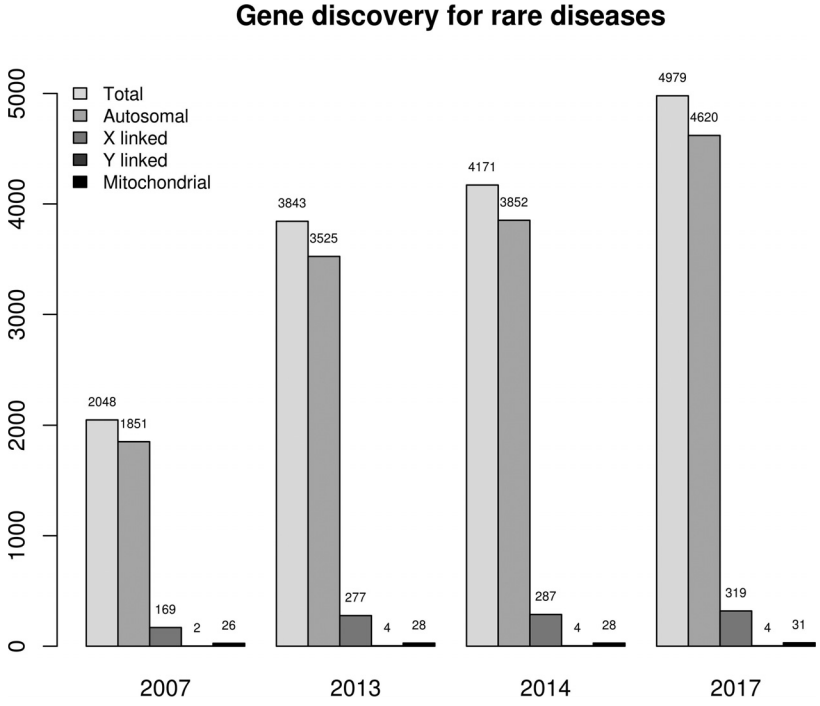

Fig. (1). Gene discovery for rare diseases.

fortunately, however, they are not capable of identifying novel disease-causing genes.

1.2.1. Strategies to Identify Disease-Causing Genes

The identification of new genes associated with human disease or known genes associated with new phenotypes contributes to our understanding of the biological bases of disease and is of great importance for patient management, and in some cases even for therapeutic intervention [12]. Two main approaches have been used to isolate disease-causing genes: 1) analysis of the exome (WES) or genome (WGS) of a group of patients with the same clinical characteristics and filtering of variants to be placed in a common gene for all or some of the members of the group; and 2) analysis of isolated patients in conjunction with parents and/or informative members of their family and filtering of variants by different modes of inheritance (autosomal dominant, recessive, X-linked or *de novo* hypothesis) to reduce the number of variants to a sufficiently small number to allow identification of the causal gene.

1.2.2. Successful Application of WES in the Last 10 Years

The year 2010 witnessed the first two successful applications of WES. In the first case, a group from the University of Washington Center for Mendelian Genomics uncovered the genetic cause of Miller syndrome, characterized by severe micrognathia, cleft lip and/or palate, hypoplasia or aplasia of the postaxial elements of the limbs, coloboma of the eyelids, and supernumerary nipples [13]. They performed WES on four unrelated individuals with this syndrome in three independent kindreds. After filtering against public SNP databases and eight HapMap exomes, and looking for genes with two previously unknown variants in each of the four individuals, they were able to identify a single candidate gene, *DHODH*, which encodes a key enzyme in the pyrimidine *de novo* biosynthesis pathway. Sanger sequencing confirmed the presence of *DHODH* mutations in three additional families with Miller syndrome. In the same year, the same approach successfully identified the causative genes for Kabuki (*MLL2)* [14], Schinzel-Giedion (*SETBP1)* [15], and Sensenbrenner (*WDR35)* [16] syndromes**.**

Soon afterwards, publications of diagnostic applications of WES using a different strategy began to appear. One landmark study carried out by Goldstein and collaborators in 2012 tested trio-WES (sequencing of a patient and his/her biological parents) in 12 patients with different unexplained genetic conditions. They succeeded in confirming a genetic diagnosis in six of the patients, and were even able to propose a potential treatment based on the diagnosis in some of them [17]. In the same year, the NIH Undiagnosed Diseases Program (Bethesda) published its first large study describing the results of the first two years of a program for the application of genomic technology to establish rare disease diagnoses. They studied 160 patients (47% children and 53% with neurological disorders) and established a diagnosis in 39 of these (24%). Three disorders were diagnosed based on single nucleotide polymorphism (SNP) array analysis and an additional three were diagnosed using WES and variant filtering. Two new disorders were also discovered: arterial calcification due to deficiency of *CD73* and a familial distal myopathy caused by a mutation in *HINT3* [18]. In 2013, an almost identical diagnostic rate of 25% was reported by the Medical Genetics Laboratory from Baylor College (Houston, USA) in a study in which WES was applied to the clinical diagnosis of a cohort of 250 patients [19]. The diagnostic rate remained unchanged when the study population was later increased to 2000 patients [20]. Another study of 814 patients at the University of California reported a diagnostic rate of 26% [21]. In all the studies, diagnostic rates were higher when the trio-WES rather than a patient-only strategy was applied. The respective rates reported by Farwell and coworkers [20] were 37% versus 21%. Similar findings were reported by studies performed in Canada (FORGE: Finding of Rare Diseases Genes) [22] and by the Department of Pediatrics at Columbia University Medical Center [23]. The diagnostic yield of WES seems to be much higher than that of Sanger sequencing (which involves the sequential screening of many candidate genes), especially in the cases of highly heterogeneous disorders such as deafness, blindness, mitochondrial disease, and movement disorders [19]. According to one study from the exome sequencing center at St Radboud University Medical Center in Nijmegen, the Netherlands, the diagnostic yield of WES was at least 50% greater than that of Sanger sequencing [24]. In the next section, we will review, but by no means exhaustively, the most recent and/or relevant novel genes that have been identified in rare metabolic, neurodevelopmental, and neuromuscular diseases thanks to NGS.

1.2.2.1. Metabolic Disorders

Metabolic disorders are conditions that disrupt normal metabolism in human cells. Thousands of enzymes participating in numerous interdependent metabolic pathways are necessary for this process. Metabolic dis-

orders, however, are not restricted to diseases that show increased or decreased levels of biochemical parameters in blood, urine, or CSF samples. Many disorders do not have any biochemical markers and are associated with a wide variety of neurological phenotypes (movement, neuromuscular and myelin disorders), as we will see in the following sections.

Hyperphenylalaninemia is the paradigmatic example of a metabolic condition. It is an autosomal recessive inborn error of metabolism resulting from a deficiency of phenylalanine hydroxylase or one of four enzymes involved in the synthesis or regeneration of BH4, a cofactor for this enzyme. The first *HPA* mutation was identified 30 years ago [25]. Surprisingly, this year, a novel gene underlying this phenotype (*DNAJC12*) was found through WES [26]. This gene encodes a heat shock co-chaperone family member that interacts with phenylalanine, tyrosine, and tryptophan hydroxylases catalyzing the BH4-activated conversion of phenylalanine into tyrosine, tyrosine into L-dopa, and tryptophan into 5-hydroxytryptophan. This metabolic deficiency leads to dystonia and intellectual disability in addition to hyperphenylalaninemia.

Last year, two novel genes were added to the list of genes involved in complex molecule metabolism disorders, which are associated with all the enzymes involved in lysosomal, peroxisomal, and cholesterol metabolism. These were *ACER3*, which hydrolyzes the amide linkage of ceramides, leading to a leukodystrophy phenotype [27] and *NPL*, which codes for N-acetylneuraminate pyruvate lyase, which controls the cellular concentration of sialic acid by catalyzing the conversion of sialic acid into acylmannosamines and pyruvate. NPL mutations were found to be associated with dilated cardiomyopathy, sensorineural hearing loss, and unexplained sialic aciduria [28].

Mitochondrial disease deserves special mention within the category of metabolic disorders. Many genes associated with mitochondrial disease have been identified by WES since the explosion of NGS. The first findings in this field took place between 2010 and 2012 with the research of Haack, Calvo, and Taylor [29-32]. Their initial findings triggered the search for — and identification of — combined oxidative phosphorylation deficiency genes, with 16 new OMIM entries since 2013, and other genes associated with mitochondrial function or implicated in disorders resembling mitochondrial disease [28, 33-42]. One particularly relevant conclusion to be drawn from these studies is that many of the so-called mitochondrial diseases diagnosed on the basis of clinical or biochemical criteria are actually rare syndromes or other neurological or metabolic diseases with unusual presentations but a high level of phenotypic overlapping with mitochondrial disease [43-44]. There have even been reports of cases in which a supposed mitochondrial disease phenotype was in fact the result of two overlapping rare disease phenotypes. This overlapping probably explains why the diagnostic rate for mitochondrial disease using gene panels is so low (15-20%).

Using a combination of a panel targeting 135 genes associated with this group of disorders and WES, Legati *et al.* [45] revealed unexpected and conceptually new findings, including the possibility of marked variable penetrance of recessive mutations, the identification of large-scale DNA rearrangements explaining spuriously heterozygous cases, and the association of mutations in known genes encoding non-mitochondrial protein with unusual previously unreported clinical phenotypes.

1.2.2.2. Neurodevelopmental Disorders

Our molecular understanding of epileptic disorders has increased enormously in recent years. Many novel epilepsy-associated genes have been found through WES [46-58] since the publication of a large study from the EpiK consortium in 2013 that revealed several new loci potentially associated with early-onset epileptic encephalopathy [59]. Although *de novo* dominant mutations are frequently identified in association with epileptic disorders, somatic mosaicism and recessive disorders are also seen. Several genes can cause a single electro clinical syndrome, and, conversely, a single gene may be associated with phenotypic pleiotropy. Ion channels and proteins needed for synaptic, regulatory, and developmental functions have been found to underlie these disorders. Gene discovery provides the basis for neurobiological insights, and convergence of mechanistic pathways is frequently seen. These findings underpin the development of targeted therapies, which are essential for improving the outcome of these devastating disorders [60].

WES has also had an important role in the diagnosis of intellectual disability. WES studies performed with cohorts of patients with intellectual disability have provided strong experimental support for a *de novo* mutation paradigm for this condition. Together with *de novo* copy number variations (CNVs), *de novo* point mutations of large effect could explain the majority of all cases of intellectual disability in the population [61-63].

Very similar molecular findings have emerged from WES studies of autism spectrum disorder (ASD), a group of neurodevelopmental diseases that show strong heritability. As with epilepsy and intellectual disability disorders, the molecular architecture of ASD seems to be characterized by rare *de novo* events in many different genes [64-70]. Sporadic autism exomes reveal a highly interconnected protein network of *de novo* mutations [71]. A review by Reiner *et al.* (2016) connected ASD and aberrant neuronal migration during brain development and discusses recent advancements in the field connecting ASD with brain morphogenesis disorders [72-73].

Development disturbances that originate during fetal life are generally caused by changes in neuronal cell migration and/or proliferation that lead to brain morphogenesis disorders. The molecular story told by WES studies in this area has much to do with *de novo* and/or mosaic mutations in ectodermal tissues. The genes involved in brain morphogenesis disorders have also grown in recent years and many of the mutations implicated have been found in somatic mosaicism, as we will discuss later [74-81].

Psychiatric disorders had never been considered monogenetic diseases until recently, but the use of NGS in psychiatric patients has yielded findings that support a de novo mutational paradigm, including CNVs and single nucleotide variants (SNVs), for disorders of this kind [82-86].

1.2.2.3. Neuromuscular Disorders

Neuromuscular disorders are conditions that result from disorders or lesions of the peripheral nervous system. They include disorders of the motor unit or the sensory component and more specifically of motor neurons, dorsal roots ganglia, spinal roots, cranial nerves, peripheral nerves, neuromuscular junctions, and muscles. In this section we will look at some of the advancements in this field since the emergence of NGS.

Several novel genes have been found to underlie novel limb-girdle muscular dystrophies (LGMDs). These include *LIMS2,* which encodes a protein critical for muscle attachment (LGMD2W) [87], the *BVES* gene (LGMD2X), which encodes a membrane protein abundantly expressed in heart and muscle that may play an important role in development of these tissues [88], *TOR1AIP1,* involved in the regulation of torsin A ATPase (TOR1A) (LGMD2Y) [89], and the endoplasmic reticulum (ER) O-glucosyltransferase gene *POGLUT1* (LGMD2Z) [90].

Novel forms of myofibrillar myopathies have been found, such as myofibrillar myopathy 7, associated with the *KY* gene, which interacts with several sarcomeric cytoskeletal proteins including filamin C (*FLNC*) and the slow isoform of the myosin-binding protein C (*MYBPC1*) [91], and myofibrillar myopathy 8, associated with the *PYROXD1* gene, which belongs to a family of pyridine nucleotide-disulfide reductases [92]. Two new centronuclear myopathies have also been characterized molecularly: centronuclear myopathy 4, associated with the *CCDC78* gene [93], which encodes a protein important for skeletal muscle function; and centronuclear myopathy 5, associated with the *SPEG* gene [94], which encodes a protein that resembles members of the myosin light chain kinase family required for myocyte cytoskeletal development. *ALG2*, which codes for an alpha-1,3-mannosyltransferase that catalyzes the second and third mannosylation steps in the N-linked glycosylation pathway, and *ALG14,* which codes for a UDP-GlcNAc transferase, responsible for catalyzing a key step in endoplasmic reticulum N-linked glycosylation, have been linked to congenital myasthenic syndrome 14 and 15 [95]. Just last year, another novel congenital myasthenic syndrome was associated with mutations in the *MYO9A* gene [96], which encodes an unconventional myosin (containing the basic domains of conventional myosin but possessing distinctive tail domains) which functions as actin-based molecular motors.

Finally, biallelic mutations in the voltage-gated sodium channel SCN10A were recently implicated in both neuromuscular disease and epileptic encephalopathy [97].

Last year, mutations in two genes involved respectively in the metabolism of heparan sulfate and triglycerides were linked to dominant Charcot-Marie-Tooth (CMT) disease. A heterozygous mutation in the *NAGLU* gene, which in a recessive manner has long been associated with the well-known lysosomal storage disorder mucopolysaccharidosis type IIIB, was found to cause CMT type 2V in a dominant manner. The mutation, found by WES, segregated with the disorder in the family and patient leukocytes showed significantly decreased NAGLU enzyme activity (36-54% of controls), which is consistent with a detrimental effect of the mutation [98]. Mutations in *DGAT2*, a gene that codes for an enzyme responsible for the synthesis of triglycerides, was associated with another dominant form of early-onset axonal CMT [99]. Mutations in the *MORC2* gene, a DNA-dependent ATPase that relaxes chromatin to facilitate DNA double-strand break repair, have also

been linked to the dominant CMT [100]. Mutations in two aminoacyl-tRNA synthetase genes, *HARS* and *MARS,* have also been found to be responsible for dominant forms of CMT types 2U and 2W [101-102].

Finally, three novel hereditary sensory neuropathy disorders (1D, 1E, 1F) have been characterized through WES in the loci *ATL1* (whose protein product is potentially involved in axonal maintenance, as suggested by the spastic paraplegia type 3 caused by defects in this gene), *DNMT1* (the main enzyme responsible for maintaining methylation patterns following DNA replication), and *ATL3* (whose protein product is required for proper formation of the network of interconnected tubules of the endoplasmic reticulum), respectively [103-105].

1.2.2.4. Movement Disorders

NGS has also led to significant breakthroughs in three groups of neurodegenerative disorders: 1) spinocerebellar ataxia (SCA), characterized by coordination problems caused mainly by atrophy of the cerebellum; 2) dystonia, linked to basal ganglia dysfunction, although evidence is now pointing to involvement of the cerebellum and two major pathways (the synaptic transmission and neurodevelopment pathways); and 3) hereditary spastic paraplegia (HSP), whose main feature is progressive gait impairment. In this next part, we will summarize nine scientific findings related to novel genes associated with movement disorders identified by WES this year. The first case involves a family with a complicated form of HSP caused by a homozygous mutation in *SELENOI* [106], responsible for the final step in the Kennedy pathway forming phosphatidylethanolamine which, together with phosphatidylcholine, constitutes more than half of the total phospholipids in eukaryotic cell membranes. The association was supported by functional analyses. The second finding was the involvement of the *DOCK3* gene, which plays an important role in axonal outgrowth and cytoskeleton reorganization, in a disorder with hypotonia, ataxia, and intellectual disability. WES-trio analyses identified a maternally inherited nonsense variant and a paternally inherited 458- kb deletion in chromosomal region 3p21.2 disrupting the *DOCK3* gene [107]. A *de novo* point mutation in the *GRIK2* gene, a member of the ionotropic glutamate receptor gene family, was found to be the cause of disease in a 10-year-old patient with ataxia, motor and speech delay, and intellectual disability. Functional characterization of the mutated protein supported the association [108]. A homozygous mutation in *SLC30A9*, which is involved in intracellular zinc homeostasis, was found in a consanguineous Bedouin family with neurological deterioration progressing into severe intellectual disability, profound ataxia, camptocormia, and oculomotor apraxia [109]. For the first time ever, a report linked *PNPLA6* mutations with pure SCA without chorioretinal dystrophy or hypogonadotropic hypogonadism, constituting another example of phenotype expansion [110]. The next finding is an example of how dominant and recessive mutations in the same gene can lead to completely different phenotypes. Dominant mutations in *DSTYK* (dual serine-threonine and tyrosine protein kinase) had already been implicated in congenital urological developmental disorders. Nevertheless, a recent study showed that a homozygous large intragenic deletion in this gene was responsible for complicated HSP in a Palestinian-Jordanian pedigree. The authors supported the finding by isolating the same mutation in previously reported pedigrees with the same phenotype [111]. Another interesting association recently detected is that between an autosomal recessive SCA and a mutation in the non-coding RNA RNU12 that was detected by combining WGS and RNAseq analysis in a large consanguineous family. Homozygosity mapping, rare variant search, and comparison of the transcriptomes of affected and unaffected family members led to the detection of a homozygous point mutation in non-coding RNA RNU12 [112].

Other important genes underlying movement disorders had been identified in previous years. Genes linked to different forms of SCA include genes involved in posttranslational modification, such as transglutaminase 6 (*TGM6*), metabotropic glutamate receptors (*GRM1* and *GRM6*), transporters (*ABCB7*, *ATP1A3*), ion channels (*KCND3*), members of the synaptotagmin family of genes that encode membrane-trafficking proteins (*SYT14*) and proteins associated with mitochondrial metabolism (*AFG3L2*) [113-121]. Dystonia, in turn, was associated with hippocalcin (*HPCA*), a member of a family of neuron-specific Ca (2+)-binding proteins found in the retina and brain [122], the stimulatory G-alpha subunit of the G protein receptor (*GNAL*) [123], a brain-specific member of the beta-tubulin family (*TUBB4A*) [124], and *COL6A3*, previously associated with Ullrich and Bethlem myopathy [125]. Finally, *VPS35*, a component of the retromer cargo-recognition complex critical for endosome-trans-Golgi trafficking and the recycling of membrane-associated proteins, has been associated with Parkinson disease [126-127].

Important conclusions can be drawn from the above NGS studies. These conclusions are well presented in

two recent reviews [128, 129]. The first explores the similarities between SCA and dystonia, and suggests the existence of potentially shared molecular pathways that use a gene co-expression network approach. The second states that, although autosomal dominant and recessive SCA and HSP have traditionally been included in separate clinicogenetic disease classifications, the advent of NGS has shown an overlapping phenotype generating a continuous phenotypic spectrum. Various genes traditionally rooted in either the SCA or HSP classification system (*e.g., SPG7*, *SYNE1*, *PNPLA6*) have now been shown to cause SCA at one end of the disease continuum and HSP at the other. Other genes, such as *GBA2* and *KIF1C*, were almost simultaneously implicated in both HSP and SCA. The variability and fluidity of phenotypes along the ataxia-spasticity spectrum warrants a rethinking of traditional classification systems. The concept of a continuous ataxia-spasticity disease spectrum is further supported by the existence of common cellular pathways and disease mechanisms, suggesting shared vulnerability of cerebellar and corticospinal neurons for common pathophysiological processes. This could in fact be the mechanistic overlap that drives their clinical overlap.

1.2.2.5. Demyelinating Disorders

A demyelinating disease is any disease of the nervous system in which the myelin sheath of neurons is damaged. The damage impairs the conduction of signals in the affected nerves, which in turn causes deficiency in sensation, movement, cognition, and other functions depending on which nerves are involved.

Many genes have been added to the list of myelin disorder genes since 2011, when *POLR3A* and *POLR3B*, which encode the two largest subunits of RNA polymerase III, were identified as the molecular cause of hypomyelinating leukodystrophy 7 and 8 [130]. One of these, the *TUBB4A* gene, which encodes a brain-specific member of the beta-tubulin family, was identified as the cause of hypomyelinating leukodystrophy 6 [131]. The two most recent findings involve *CNTNAP1* and *PYCR2*. *CNTNAP1*, which is required for high-velocity nerve conduction, had been previously associated with a recessive lethal congenital contracture syndrome 7, but recent investigations showed that recessive mutations also cause a severe CNS disorder with hypomyelinating leukodystrophy and peripheral neuropathy of sensory-motor type [132]. This is yet another illustrative example of phenotype expansion. *PYCR2*, in turn, which is a mitochondrial enzyme that catalyzes the final step of proline biosynthesis and reduces pyrroline-5-carboxylate (P5C) to L-proline, was found to be involved in the development of hypomyelinating leukodystrophy 10 [133].

1.3. Development of Gene Panels: Rapid, Low-cost NGS-based Tools for the Genetic Diagnosis of Rare Diseases

As genome and exome sequencing are usually overpowered for a diagnostic setting, analyses often focus (at least as an initial step prior to exome sequencing) on known disease-related genes. Only the coding regions of these particular genes are enriched by respective probes and subsequently sequenced within what are known as *gene panels*. While these genomic tools are not capable of isolating genes associated with novel diseases, they have important advantages with respect to WES in the field of clinical diagnosis. By reducing the number of regions to be examined, much deeper coverage can be achieved for each nucleotide interrogated. This reduces false positives and negatives and allows the detection of mosaic variants and much more powerful CNV detection. In addition, gene panel analyses do not reveal findings unrelated to the phenotype being investigated, avoiding incidental findings and ethical problems. Most important of all is the significant reduction in turnaround times, which have fueled the spread of these tools throughout rare disease reference centers.

Several studies in which gene panels have been used for clinical diagnosis have reported varying rates of diagnosis. Just some of the diseases studied to date are epilepsy [134, 135], metabolic disorders [31, 45, 136, 137], orodental diseases [138], neuromuscular disorders [139-142], hereditary immunodeficiencies [143-144], hereditary motor neuropathies [145-147], amyotrophic lateral sclerosis [148], intellectual disability [149], hematologic diseases [150, 151], skeletal dysplasias [152], cardiovascular disorders [153-155], hearing loss [156-157], retinal disorders [158-163], sex development [164-165], maturity onset diabetes of the young [166], ichthyosis [167], cystic kidney diseases [168], and aortopathies [169].

1.4. Uncovering the Wide Phenotypic Spectrum of Genes

As many of the cases mentioned above illustrate, one of the major revelations of NGS is that most genes have a broad phenotypic spectrum, correcting the classic assumption on which previous diagnoses had been based: that there was a “one-to-one” relationship between gene and phenotype. This assumption had guided (or perhaps misguided) diagnostic processes for

many years and has also filled medical textbooks. It has long been known that certain genes can cause not only different but completely opposing phenotypes depending on the mutation carried. Examples are *ABCC8*, *KCNJ11*, and *GCK*, which, according to the variant expressed, can lead to hyperglycemia (neonatal diabetes) or hypoglycemia (congenital hyperinsulinism), two life-threatening conditions. We now know, however, that this characteristic, which was previously believed to be restricted to specific genes, is a widespread phenomenon. A simple search of the OMIM database will show that many genes are associated with different disorders, and that some of them even have different modes of inheritance. Labeling a gene thus as *recessive* or *dominant* would also now appear to be inaccurate. Under this new knowledge, we can expect that known disease-causing genes will be increasingly linked to new phenotypes with distinct pathological mechanisms. We will illustrate this point with two examples. Missense mutations in the *NOTCH2* gene had been shown to cause a variant form of Alagille syndrome (paucity of intrahepatic bile ducts, cholestasis, cardiac disease, skeletal abnormalities, ocular abnormalities, and a characteristic facial phenotype) [170], but in 2011 it was discovered that carboxy-terminal truncating mutations in the same gene cause Hajdu–Cheney syndrome, an autosomal dominant skeletal disorder characterized by short stature, coarse, dysmorphic facies, bowing of the long bones, and vertebral anomalies [171-173]. The second example is the *KCNQ2* gene, in which missense activating mutations cause severe neonatal onset epileptic encephalopathy while loss of function truncating mutations cause the much milder phenotype of benign neonatal seizures.

It has been anticipated that a considerable percentage of novel phenotypes will be reassigned as atypical presentations of known disorders and that the list of genes in which distinct mutations cause separate rare diseases will continue to grow [1]. Caution, however, must be exerted, since the possible overlapping of two or more genetic disorders, a finding that has also been uncovered by NGS, could also be playing a role in the apparent phenotype expansion of genes, as we discuss in another section.

1.5. The Prominent Role of *De Novo* Mutations in Rare Diseases

The importance of *de novo* chromosomal abnormalities underlying neurodevelopmental disorders has been recognized by the cytogenetics community for many decades, and parental analysis is an important part of substantiating or excluding causality of rare chromosomal variants. The availability of high-resolution genomic microarrays in the past decade allowed the unbiased genome-wide analysis of *de novo* CNVs long before the same could be achieved for *de novo* SNVs and indels. Such analyses of CNVs have revealed the importance of genomic variations of this type in neurodevelopmental disorders such as intellectual disability, ASD, and schizophrenia [174, 175]. *De novo* CNVs larger than 100 kb are infrequent in the general population, occurring in approximately one in 50 individuals. A frequency, however, of approximately 10% is estimated for patients with sporadic intellectual disability [175, 176], ASD [177, 178], or schizophrenia [179]. The use of high-resolution genomic microarrays to analyze the genetics of these disorders has resulted in the identification of many new recurrent microdeletion syndromes, such as those caused by deletions affecting the chromosomal loci 1q21.1, 3q29, 15q13.3, 15q24, 17q12, and 17q21.31 [180].

As previously discussed, NGS applications have also brought to the fore the prominent role of *de novo* point mutations in the development of rare diseases, and in particular neurodevelopmental disorders such as epilepsy, brain morphogenesis defects, intellectual disability, ASD, and schizophrenia. *De novo* mutations represent the most extreme form of rare genetic variation: they are more deleterious, on average, than inherited variations because they have been subjected to less stringent evolutionary selection. *De novo* mutations provide a mechanism by which early-onset reproductively lethal diseases remain frequent in the population. They are thus prime candidates for causing severe genetic diseases that occur sporadically. Furthermore, they can occur in the germline, during embryo genesis, or somatically. Because *de novo* mutations are not rare events collectively, they might be responsible for an important fraction of more commonly occurring diseases through disruption of any one of a large number of genes. An excellent review of the role of *de novo* mutations in the genetic architecture of human diseases can be found in the paper by Veltman and Brunner [181].

Several of the studies mentioned in section 1.2 revealed that, together, *de novo* mutations affecting many different genes in different individuals might explain most rare neurodevelopmental diseases such as epilepsy [46, 59], intellectual disability [61], ASD [64, 67-69, 71], brain morphogenesis defects [73, 74], and psychiatric disorders [82, 86]. This *de novo* model for

complex neurodevelopmental genetic diseases essentially points to a monogenic basis of disease, with the mutation representing a single event of large effect. This contrasts with the multifactorial model, which invokes the interplay of many genetic and non-genetic factors of small effect in any individual patient. Thus, although it needs to be acknowledged that the phenotypic effect of any single mutation depends on the genetic background in which it occurs, the current overall picture is decidedly more monogenic than that envisaged just a few years ago [182]. The application of large-scale NGS studies demonstrates that many previously enigmatic sporadic syndromes, malformations, and diseases are due to *de novo* germline gene mutations, whereas others reflect somatic mosaicism for gene mutations.

1.6. Mosaic Mutations Accounting for a Significant Portion of Mutational Load in Rare Diseases

Until recently, genetic research was based on the premise that the mutations associated with human disease were present in all the cells of a patient. Increasingly however, evidence suggests that post-zygotic mutations occur much more frequently than previously thought, and may account for a significant portion of the mutational load causing human disease [183-193]. In fact, a subset of human diseases is obligatorily somatic. This is because constitutive mutations in certain genes are incompatible with embryonic development, whereas mosaicism allows development but causes very serious diseases, the etiology of which is hard to discern. Examples are McCune-Albright syndrome, Maffucci syndrome, and various chromosomal aneuploidy syndromes.

NGS has highlighted the role of somatic mosaicism in the development of rare diseases and neurodevelopmental diseases in particular [194]. Mosaicism refers to the existence within an individual of two genetically distinct populations of cells resulting from a postzygotic mutation. It has been shown that during embryonic development, both point mutations and large deletions or insertions continuously accumulate in dividing cells. Somatic mutations may be overlooked or they may cause normal human variation or a rare genetic disease. They can also be transmitted to the next generation if their distribution pattern includes gonadal tissue. These mutations range from SNVs to whole chromosomes and have been implicated in numerous diseases, in particular cancer. The phenotypic consequences of somatic mosaicism depend on many factors, including the stage of development in which the mutation occurs, the areas of the body affected, and the pathophysiological effect of the mutation. To date, mosaicism has been described in a range of rare diseases (primary immunodeficiencies, rheumatologic, hematologic disorders, *etc.*), but especially in neurodevelopmental disorders. This is because the probability of somatic mutation in neurons is greater than in other tissues due to the high rate of neuronal proliferation between weeks 4 and 24 of gestation (estimated at 105 divisions/minute) [195].

Somatic mosaic mutations in genes associated with severe diseases can result in highly enigmatic diseases that are very difficult to diagnose even with high-throughput sequencing. The difficulty lies not in the throughput but in the tissue from which the DNA is extracted. Recent technical advances have identified mosaic variants that cause many clinical phenotypes, underscoring the omnipresence of mosaicism. The influence of these variants on phenotype depends not only on their presence, but also on the specific tissues in which they are expressed. These observations represent the starting point of a research avenue that will be essential to better understand the molecular architecture of rare disease. Recently, Acuna-Hidalgo and coworkers reported that 6.5% of so-called *de novo* mutations were in fact mosaic mutations that had occurred post-zygotically [183]. Several research groups have demonstrated the high incidence of mosaicism in patients with brain morphogenesis disorders, particularly in the *LIS1*, *DCX*, *mTOR*, *PIK3R2*, *PIK3CA*, *AKT3*, *AKT1*, *FLNA*, and *TUBB2B* genes [75, 196-200], and in some cases restricted to brain tissue. Mosaic variants has also been found in epilepsy patients, especially those with Dravet (*SCN1A*) and Rett (*MECP2*, *CDKL5*) syndromes [201-204], *KCNQ2*-related epilepsy [205], X-linked early onset-epileptic encephalopathy caused by mutations in the *PCDH19* gene [206], and *SCN2A*-related epilepsy [207]. Cases of adult neurodegenerative diseases caused or modulated by somatic mutations have also been described, and include Alzheimer's disease [208, 209].

1.7. Digenic Inheritance

The power of NGS to elucidate the whole spectrum of variants in a given individual will also stimulate the discovery of digenic or polygenic disease causes as, after identifying what initially appears to be a disease-causing mutation, additional analyses can be carried out because the data are already available. A remarkable example includes the detection of heterozygous mutations in the two functionally related genes

GUCY1A3 and *CCT7* in an extended family with myocardial infarction [210]. Digenic inheritance is a phenomenon also seen in neurological diseases such as Parkinson disease with, for example, mutations in *PINK1* and *DJ-1* [211]. However, it remains to be proven that the combination of two mutations is, indeed, the cause of disease rather than the simple co-occurrence of two mutations by chance [212].

1.8. Co-occurrence of Two or More Genetic Diseases in the Same Patient

Before the explosion of NGS, it had never been postulated that a patient could have two or more rare diseases simultaneously. This year, however, Posey and colleagues carried out a retrospective analysis of data from 7374 consecutive unrelated patients who had been referred for molecular diagnostic by WES [213]. A molecular diagnosis was achieved for 2076 of these patients (28.2%). Surprisingly, 101 (4.9%) had diagnoses that involved two or more disease loci, and of these, 97 had two molecular diagnoses, three had three diagnoses, and one had four. According to the authors, this finding challenges the notion that a genetic investigation is complete once a single diagnosis is established. They also applied two models to investigate whether such a finding should be theoretically expected. The first model, a Poisson model, assumed that pathogenic variants arose independently at different loci within each patient's genome, and the second model, an independence model, used the observed rate of singleton diagnosis. Under both models, the observed proportion of patients with multiple molecular diagnoses (4.9%) was significantly lower than would be expected (14.0% according to the Poisson model and 26.4% according to the independence model). It could therefore be speculated that in at least a small proportion of rare diseases, the severe end of the clinical spectrum may be the consequence of this mechanism and that some such combinations may be lethal and thus escape detection. In other words, phenotype expansion of a rare disease might be influenced by such co-occurrences. The findings reported by Posey and coauthors suggest that any finding in a well-recognized syndrome that is reported only once or twice should be viewed with caution. Another study that supports co-occurrence of genetic disease is the one carried out in the FORGE and Care4Rare Canada WES initiatives, which retrospectively analyzed WES results for 802 undiagnosed probands referred for testing over the previous 5 years [214]. Of the 802 probands, 226 (28.2%) were diagnosed based on mutations in known disease genes. Eight (3.5%) had two or more genetic diagnoses that explained their clinical phenotype, and seven of these had family members with one or more of the molecularly diagnosed diseases. Consanguinity and multisystem disease appeared to increase the likelihood of multiple genetic diagnoses within a family.

The most important conclusion to be drawn from these studies is the relevance of comprehensive clinical phenotyping of family members to ultimately provide accurate genetic counseling. Another important conclusion is that rare diseases that are thought to be novel (*i.e.,* unlike any other previously described conditions) may in fact be two rare diseases segregating in the same family. One example is the case of a Newfoundland family in which two siblings were thought to have a novel, variably penetrant syndrome characterized by ocular and skin hypopigmentation, congenital neutropenia, immune dysregulation, and Crohn's disease. Using WES, both siblings were shown to have severe congenital neutropenia type 4 but one of them also had oculocutaneous albinism type 4 [215]. It is likely that other "unique" phenotypes represent a conflation of two known phenotypes in this way [1]. Isolated examples of co-occurrence of two diseases have been reported. One of them, a boy presenting with severe muscular hypotonia, multiple fractures, and joint hyperflexibility, features that are compatible with mild osteogenesis imperfecta and hypermobility type Ehlers-Danlos syndrome [216] were found to carry a dominant *COL1A1* mutation associated with osteogenesis imperfecta and two recessive mutations in *TNXB* associated with Ehlers-Danlos syndrome.

Another case was uncovered when investigating the cause of disease in a male patient with typical features of mitochondrial disease, including infantile cataracts, chronic progressive external ophthalmoplegia, ptosis, progressive distal muscle weakness, and ataxia. WES showed a homozygous splice site mutation in *SETX*, which is known to cause SCA, autosomal recessive 1 (SCAR1). Additionally, a missense mutation was identified in a highly conserved position of the X-linked *OCRL* gene, which causes Lowe syndrome and Dent disease 2. Therefore, the patient did not have mitochondrial disease but rather overlapping symptoms of two different rare diseases mimicking this disease [217]. This finding raises an important question. Mitochondrial disease is one of the most common groups of inherited genetic diseases, with an incidence of 1/5000, but ~90% of cases do not have a DNA-based diagnosis. Could it be then that a significant percentage of what clinicians consider to be mitochondrial disease

is actually a combination of two or more different phenotypes?

Another illustrative example of co-occurrence is the case of a consanguineous family with 2 rare diseases: hereditary hypophosphatemic rickets and congenital myopathy, segregating independently. Phenotypic manifestations, linkage analysis, and homozygosity mapping yielded elusive results in this family because physicians assumed that it was a single rare disease. The mystery, however, was unravelled when WES identified two homozygous mutations in two genes, *SLC34A3* and *SEPN1*, which segregated in this family [218].

Combinations of diseases in a single patient can be replicated over time, as in Fitzsimmons' syndrome, a condition characterized by progressive spastic paraplegia, brachydactyly with cone-shaped epiphyses, short stature, dysarthria, and low-normal intelligence. This syndrome was recognized as a single entity until WES recently showed that it was two different diseases [219].

1.9. The Promise of Effective Treatments

Effective treatments are lacking for most rare diseases, largely because of the failure to identify a molecular cause. NGS, however, has opened the door not only to molecular diagnosis, but also to the search for treatments for diseases that in the past were a death sentence. Sometimes, the detection of a molecular cause can pinpoint an effective treatment. This is the case of many metabolic disorders in which diet can be used to maintain a controlled metabolic state. A more recent example is the discovery of *SLC18A2* as the causative gene for an infantile-onset movement disorder characterized by severe parkinsonism, non-ambulation, mood disturbance, autonomic instability, and developmental delay [220]. This gene encodes a translocator of dopamine and serotonin into synaptic vesicles, so the use of dopamine agonists resulted in a marked improvement in symptoms and the resumption of development. A similar case arose from the identification of the riboflavin transporter genes *SLC52A3* and *SLC52A2* as the cause of Brown–Vialetto–Van Laere syndrome (progressive sensorineural deafness in combination with childhood amyotrophic lateral sclerosis). Subsequent treatment with riboflavin produced encouraging results in terms of both biomarker response and clinical endpoints [221]. Unfortunately, however, in most cases treatment cannot be inferred directly from a molecular defect.

Occasionally, a rare disease pathogenic pathway can overlap with one that is being targeted in common disorders. This phenomenon will occur with increasing frequency as the molecular 'atlas' of human pathology is completed and the modern pharmacopoeia grows, and is best exemplified by Marfan syndrome and the beneficial TGFβ pathway modulation [222]. Another example is the study of the effect of kinase inhibitors originally formulated for cancer treatment on cardiac function in Noonan syndrome, an inherited kinasopathy caused by activation of the RAS signaling pathway [223].

When the origin of a rare disease lies in protein dosage problems (excess or defect), one effective approach could be to influence mRNA or protein levels. The identification of drugs that restore inadequate levels of mRNA or protein to a clinically significant level is a promising research avenue [224]. A second approach involves the use of increasingly detailed transcription factor binding-site maps that incorporate the majority of annotated genes. For a number of transcription factors, there are well-characterized, clinically approved agents that are expected to upregulate genes containing the transcription factor-binding site; the use of this information has led to potential therapeutics for spinal muscular atrophy [225, 226].

The future of rare disease treatment, however, undoubtedly lies in gene therapy. For many years, gene transfer trials have been associated with very few success stories, producing largely disappointing results and hit by major setbacks. However, the successful results achieved in ocular diseases and primary immune deficiencies have brought gene therapy back to the therapeutic front line [227, 228], and effective, long-lasting treatments from gene therapy trials are now being reported at an increasing pace. Positive results have been documented for a wide range of genetic diseases (including hematological, immunological, ocular, and neurodegenerative and metabolic disorders) and several types of cancer. To date, about 2000 clinical trials for various diseases have been completed or are ongoing, and many more are in the pipeline [229].

A promising form of gene therapy that has emerged forcefully in recent times is genome-editing technology, designed to either correct endogenous disease-causing genes or specifically target the integration of a therapeutic gene into a defined genetic locus. Such tools include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)–associated systems (CRISPR–Cas). These

engineered endonucleases can be programmed to specifically target and alter a DNA sequence by introducing a double-strand break. They can therefore be employed to correct a disease-causing mutation with great efficiency and represent a sophisticated tool for precision medicine. The CRISPR-Cas system, for instance, has been employed to study the effects of gene modifications in postmitotic neurons in the mouse brain and to correct a hereditary disease, tyrosinemia, in a mouse model [230, 231]. However, the current efficiency of gene editing may be subtherapeutic for certain diseases where edited cells have no proliferative or survival advantage, and off-target double strand breaks may induce genotoxicity. These gene-editing tools therefore need further refinement before they can be safely and effectively used in the clinic.

1.10. Implementation of NGS in Neonatal Intensive Care Units

Rare diseases often present in the first days or weeks of life and may require complex management in the setting of a neonatal intensive care unit (NICU). Both diagnostic yield and speed are important considerations in neonatal care. In this context, the impact of WES/WGS can perhaps be compared with what happened when Guthrie cards were replaced by mass spectrometry. The fundamental premise is that application of NGS as early as possible can have high clinical impact.

Some experiences of genomic implementation in NICUs have been reported. The Children's Mercy Hospital Kansas City, for example, applied WGS to a select population of ill infants in a level IV NICU [232, 233]. They reported a diagnostic rate of 57%, which contrasted sharply with the rate 9% achieved with standard genetic testing (p=0·0002). Most of the diagnoses altered the management of patients, and 65% were associated with *de novo* mutations. Acute clinical usefulness was noted in 65% of patients, 20% had diagnoses with strongly favorable effects on management, and 30% were started on palliative care. Diagnoses like these may lead to clinical management changes ranging from transition to palliative care for uniformly lethal conditions to modification or initiation of medical or surgical therapy to improve outcomes in others.

The NICU at the Children's Hospital of Eastern Ontario used a gene panel with clinically relevant genes to test 20 newborns, 8 of whom received a diagnosis [234]. The authors stated that such an approach alters health care delivery in these units and that even a 10-20% success rate is invaluable if it allows for an intervention window that would otherwise be nonexistent. Because disease progression is extremely rapid, albeit heterogeneous, in newborns, molecular diagnoses must occur quickly to be relevant for clinical decision-making. Saunders *et al.* [235] describe 50-hour differential diagnosis of genetic disorders by WGS that features automated bioinformatic analysis and is intended to be a prototype for use in NICUs. Retrospective 50-hour WGS identified known molecular diagnoses in two children, while prospective WGS identified a potential molecular diagnosis of a severe *GJB2*-related skin disease in one neonate and *BRAT1*-related lethal neonatal rigidity and multifocal seizure syndrome in another. It also identified *BCL9L* as a novel, recessive visceral heterotaxy gene (*HTX6*) in a pedigree and ruled out known candidate genes in one infant. Sequencing of parents or affected siblings expedited the identification of disease genes in prospective cases. Thus, rapid WGS can potentially broaden and foreshorten differential diagnosis, resulting in fewer empirical treatments and faster progression to genetic and prognostic counseling [235].

2. LIMITATIONS AND CHALLENGES OF NGS TECHNOLOGIES

Despite its enormous strengths and potential, NGS also has limitations and challenges. First and foremost, NGS provides horizontal coverage and accuracy rates <100%, resulting thus in missing variants and false-positive results. Another challenge is related to the filtering and interpretation of data; as more than one candidate variant is usually found. Indeed, the greater the number of kilobases sequenced, the greater the chance of finding more candidates. There are also ethical challenges related to incidental findings and how to guide unaffected probands seeking direct-to-customer testing. There is thus an urgent need to define standards for run quality, variant interpretation, and quality control.

2.1. Demonstration of Causality of a Variant

When geneticists are looking for a molecular diagnosis and find a likely pathogenic variant(s) in a known gene that is associated with a similar phenotype to that of the patient being studied and has an appropriate mode of inheritance based on the family history (*de novo* for a dominant disorder with healthy parents, co-segregation of variant for a dominant familial disorder or in trans couple of variants in a recessive gene), they can be confident that they are close to a diagnosis. However, when the variant found is in a gene that has never been associated with disease or in a gene previously associated with a different phenotype, then they

are faced with a problem. It is incredibly difficult to prove causality for any mutation in a novel gene when there is only one affected individual. While finding rare variants that may be damaging in a compound heterozygous or *de novo* state can be a good first step for linking a gene to a disease, ultimately, functional studies, additional families, co-segregation and other genetic analysis are essential to support the link. It is accepted by the international scientific community that a prerequisite for new disease gene identification is the detection of more than one mutation in a gene in more than one pedigree. One obstacle in this regard, however, is the fragmented distribution of patients across institutions. One of the problems associated with NGS is the inability to share sequencing data quickly and universally. Standards and bioinformatic tools are needed to create international repositories of clinical and NGS results provided by families and scientists for comparison purposes. This problem can be circumvented by tools already created for and by the internet and social media. This point is well illustrated in several studies [17, 236-238].

Another handicap that has to be taken into account is the fact that some genes are dispensable, meaning that variants truncating in even one of these genes can lead to erroneous categorization of variant pathogenicity.

2.2. Demonstration of Causality of a Variant

There are many known neurogenetic disorders involving trinucleotide repeat expansion, such as Huntington's disease, fragile X syndrome, and Friedreich's ataxia. Expanded repeats range from small expansions of 20-100 copies to larger expansions of up to several thousand units. Most NGS technologies rely on reading signals from bulk DNA populations, and they are limited by the loss of sequence phase coherence—a particular problem for GC-rich sequences—as well as decreasing size resolution with increasing DNA length [239]. Due to the short length of reads NGS technology is, at least for the moment, unable to detect dynamic trinucleotide expansions. However, third-generation sequencing technology may overcome this limitation. SMRT, single-molecule, real-time sequencing, uses a zero-mode waveguide (single molecule of DNA) as a template [240] and sequencing reads are only limited by loss of activity of individual polymerase molecules. Read lengths can be as high as 15 kb (average of 3 kb) [241]. In a study of SMRT technology, Loomis and colleagues determined that it was possible to generate sequence data for *FMR1* (the human fragile X mental retardation 1 gene) alleles in excess of 750 CGG repeats, which translates to over 2.25 kb of 100% CGG-repeat DNA [239].

2.3. Missed Coding Variants

2.3.1. GC-rich Exons

In terms of capture efficiency, an important subset of GC-rich exons of the coding genes is missed in NGS studies. Accordingly, causative disease mutations present in these regions will be missed, producing a false-negative result, and the second-best gene, which may not be the gene of interest, may be falsely implicated. Because there is frequently more than one candidate gene, missing the actual gene may result in both a false-negative and a false-positive result.

2.3.2. Highly Homologous Regions

Coverage shortfalls generated by the presence of highly homologous regions are another limitation of NGS technology. Although these regions are captured and covered by multiple reads, QC filters discard them because the same read can be aligned in multiple different regions. Therefore, coverage drops and variants present in those regions may be missed. Alignment without QC filtering can be used in order not to eliminate these reads and detect variants, although in such a case it will not be possible to determine the specific region to which a variation belongs. This problem could be resolved as read length progressively increases. At present, duplications can be solved only by amplifying each specific region using specific primers placed in a non-homologous site at the edge of the region, and then, sequencing each amplification by Sanger using internal primers or by NGS using a purpose-prepared library with the specific PCR without any additional capturing.

2.3.3. Bioinformatic Limitations

Computational algorithms used at all stages, be it alignment, variant calling, or annotation, are still subject to final optimization. Different software packages may result in different final interpretations, and different thresholds for statistical significance and variant calling would produce a different final list of putative genes.

2.3.4. Lack of International and Reliable Databases

Prediction strength in NGS is dependent on the comprehensiveness of the databases containing both the filtering negative genes and the annotating positive genes. Large numbers of variants are identified when

large panels, such as exome panels, are used. Even after filtering, it is still very challenging to differentiate mutations from rare polymorphisms. However, as more and more exomes and genomes are sequenced, population-based filtering will become increasingly helpful. Newer algorithms based on hundreds or even thousands of samples at a time will help to reduce error rates.

2.4. Missed Variants in Non-Coding Regions

NGS assays available in diagnostic units (*i.e.,* genes panels and WES rather than WGS tools) are limited to known coding regions of the human genome. Exome sequences account for just 1% of the genome and it is therefore only logical that many disease-causing mutations will be missed. According to data from the ENCODE project, about 80% of the human genome may have a regulatory role [242]. Thus, with usual assays one can lose potential protein-coding regions that have not yet been annotated as genes, as well as regulatory regions, such as non-coding RNAs (or transcription factor binding sites). Many splicing mutations are missed in clinical settings due to the limitations of in silico prediction algorithms or because they are located in non-coding regions. Subtle changes in splice site variations, 3' untranslated regulatory regions, non-coding RNAs, and direct interaction of transcription factors may have significant effects on gene expression patterns that can only be assessed by transcriptome interrogation.

When applying NGS to DNA and RNA simultaneously, we can scan for potential disease-causing variations in non-coding DNA, and at the same time learn about the functional implications of genomic changes with the additional benefit of learning about transmission of alleles and potential imbalance in chromosome X expression due to skewed X chromosome inactivation. Such an approach can reveal genotype-phenotype correlations, highlight gene expression profiles associated with the genetic condition being studied, and allow immediate evaluation by in silico prediction algorithms of the effect genomic variants have on gene expression, alternative splicing, exon usage, and gene fusions [243]. Transcriptome assessment has led to the identification of small, single-stranded RNA molecules, such as microRNA (miRNA) and short, interfering RNA (siRNA) [244]. Small RNAs are short non-coding RNAs that regulate translation of their target messenger RNAs (mRNA) through mRNA degradation or suppression of translation. Advantages of miRNAs are their relative chemical stability, which facilitates miRNA isolation and analyses from clinical samples. Some miRNAs are actively secreted in exosomes and can be measured in blood specimens, rendering miRNAs targets for biomarker discovery [245]. Specifically designed siRNAs block miRNA action and may have therapeutic potential [246].

This year provided us with two examples of the use of transcriptome to solve rare disease diagnosis. The first was that of the large consanguineous family with an autosomal recessive SCA discussed in the movement disorders section [93]. Two months later, there was a report of an individual with sporadic atypical spinal muscular atrophy in whom clinical DNA sequencing identified one pathogenic *ASAH1* missense mutation. Transcriptome sequencing of patient leukocytes identified a highly significant and atypical ASAH1 isoform not explained by the mutation found. Subsequent Sanger sequencing identified the mutation responsible for the alternative splicing (c.504A>C; p. Lys168Asn) and provided a molecular diagnosis of autosomal-recessive spinal muscular atrophy with progressive myoclonic epilepsy [247].

2.5. Missed Variants in Somatic Mosaicism

We mentioned earlier on that one of the milestones achieved with the use of NGS was the discovery of the important role that mosaic mutations play in the development of rare diseases. NGS, however, only can detect mosaic mutations if: 1) the test achieves high coverage and 2) if the somatic mutation is present in blood cells, since genetic analysis is usually performed with DNA extracted from these cells. However, even mosaic mutations present in blood tissue can be difficult to detect because, unfortunately, the resulting genetic material is unstable due to the multiple rounds of self-renewal to which it is subjected during hematopoiesis. In addition, the diversity of clonal lines that give rise to circulating blood cells decreases with age. Thus, the selective effects of certain mutations, whether positive or negative, can bias the frequency of a mutation in the blood compared with in the rest of the body. One example is the iso-chromosome 12p, which is undetectable in blood, but detectable in samples of ectodermal origin.

In neurodevelopment diseases, where mutations may only be present in tissues of ectodermal origin, the largest challenge for detecting mosaicism lies in testing the actual tissue in which the mutation is present. Levels of mosaicism in different tissues and locations can also vary considerably, even within the same germline. These variations are probably a result of a combination

of factors, such as the moment at which the mutation occurs, cell migration and determination during development, and selective effects that occur in specific tissue types. To overcome these limitations, we must investigate the use of other sources of DNA for genetic analyses (*e.g.* buccal cells, hair roots, skin fibroblasts), and implement and optimize these approaches as key tools in routine genetic analyses.

2.6. Missed Epigenetic Mutations

Epigenetic modifications provide a system of gene regulation that allows clonally heritable yet reversible alterations in gene transcription. Errors in this complex system can give rise to abnormal gene silencing, known as *epimutation*; importantly, this can occur in the absence of an underlying genetic defect. Epimutations are commonly somatic events, and are particularly prevalent in tumors, but Suter and others [248, 249] have shown that they can also arise in the germline, giving rise to soma-wide transcriptional silencing of a gene. A germline epimutation can mimic the effect of an inactivating mutation, and in doing so, phenocopy a genetic disease. Evidence that this mechanism occurs in humans was recently provided by Suter *et al.* [248], who identified individuals in whom one allele of the gene encoding the DNA mismatch-repair protein MLH1 was epigenetically silenced throughout the soma (implying a germline event). These individuals had hereditary nonpolyposis colorectal cancer but did not have any identifiable mutation in *MLH1*, but it is silenced, which demonstrates that an epimutation can phenocopy a genetic disease.

WGS, WES, and gene panels cannot capture epigenetic phenomena. Other orthogonal technologies such as RNA-seq or ChIP-seq can detect gene fusions, expression differences, or changes in regulatory regions that are missed by exome sequencing alone. A small number of studies have performed detailed analyses of epigenetic events and their role in neurological disease. Most of these studies have examined methylation patterns and their role in gene expression and ultimately phenotype. For example, different levels of methylation of the ataxin 2 (*ATXN2*) gene promoter were found to be associated with disease development in a family with SCA type 2 [250]. Another example can be found in the *RYR1* gene, associated with myopathy, which had never been thought to be affected by epigenetic regulation. In 2008, during the mutation analysis of a cohort of patients, Zhou *et al.* discovered that RYR1 was transcribed from just one allele (*i.e.,* monoallelic expression) in skeletal muscle in a proportion of the patients. The transcribed allele in skeletal muscle carried a recessive mutation. Surprisingly, transcription analysis of patient fibroblasts and lymphoblastoid cell lines indicated biallelic transcription, suggesting tissue-specific allele silencing [251, 252]. These results suggest that recessive *RYR1* mutations in a proportion of patients with core myopathies may be unveiled by lack of expression of the other, apparently wild-type, allele. Various lines of experimental evidence suggest that this is due to genomic imprinting in these patients, although other epigenetic modification of allele expression cannot be definitively excluded. These data indicate that *RYR1* undergoes polymorphic, tissue-specific, and developmentally regulated allele silencing and that this unveils recessive mutations. The question that arises is: how many patients with genetic recessive diseases in which only one mutation has been found might have an epimutation that leads to the silencing of the healthy allele in a critical tissue? Only transcriptomic analysis of the affected tissue can reveal this kind of genetic mutation.

2.7. Ethical Challenges

Exome and genome sequencing has raised numerous ethical issues that have not yet been fully resolved. One major area of debate concerns the question of which results should be returned to a patient and in what context. Genetic sequencing may confirm the presence of the pathogenic variant being investigated, but it may also return incidental findings that may be relevant to the patient's current or future health yet unrelated to the initial line of questioning. Often, these incidental findings may be of unclear significance. Other concerns include how to best obtain adequate informed consent from the individuals submitting testing samples, and how to allow for data sharing among researchers while protecting individuals' privacy. These concerns are further complicated by the fact that exome sequencing may be undertaken in a clinical setting or as part of a research study. The ethics of data return to patients are less clear cut in the second case.

CONCLUSION

There is no doubt that NGS technologies have constituted a turning point in rare disease research, diagnosis, and treatment. Findings to date have provided a glimpse into the age of genomic medicine and into how our understanding of human disease will change. Although many rare diseases are still without treatment, a diagnosis itself can mean great relief for patients and families. By understanding what is happening to their

child and why, for example, parents can stop going from doctor to doctor in search of an answer, contact other parents with children in the same circumstances, form an idea of their child's prognosis, and access genetic counselling for family planning. Diagnosis is a necessary step for gaining control of one's situation and learning to accept it. The above reasons alone are more than sufficient to illustrate the impact that NGS technologies have had in the rare disease community.

Nevertheless, the journey has only begun and much work remains to overcome current challenges and increase the diagnostic power of NGS technologies. Full-coverage genomic studies accompanied by other modalities, such as methylome, transcriptome, and proteome and functional studies will help in this respect. The creation of reliable, accessible databases of genotypic and genomic information linked to clinical information is also essential for advancing knowledge. This is an enormous endeavor that will require global cooperation involving the sharing of vast amounts of data between many countries. Nonetheless, we have already embarked on this path of exponential growth and endless possibilities and we must not let this path become blocked by our failure to resolve the important ethical questions posed by the systematic use of new genomic tools.

Another important conclusion to be drawn from this work is that genomic science is cross-sectional, i.e., it cannot be understood as a single service provided to a patient with a suspected genetic disorder at the end of the diagnostic process. The main clinical departments should have their own head of genomics who, in conjunction with clinical, biochemical, and pathology specialists, can interpret genomic data at the beginning of the diagnostic process.

In sum, the implementation of NGS tools is imperative in the best health systems worldwide. There is no longer any excuse for diagnostic odysseys.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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