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Review

Toward Clinical Implementation of Next-Generation Sequencing-Based Genetic Testing in Rare Diseases: Where Are We?

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Next-generation sequencing (NGS) technologies have changed the landscape of genetic testing in rare diseases. However, the rapid evolution of NGS technologies has outpaced its clinical adoption. Here, we re-evaluate the critical steps in the clinical application of NGS-based genetic testing from an informatics perspective. We suggest a 'fit-for-purpose' triage of current NGS technologies. We also point out potential shortcomings in the clinical management of genetic variants and offer ideas for potential improvement. We specifically emphasize the importance of ensuring the accuracy and reproducibility of NGS-based genetic testing in the context of rare disease diagnosis. We highlight the role of artificial intelligence (AI) in enhancing understanding and prioritization of variance in the clinical setting and propose deep learning frameworks for further investigation.

Introduction to Rare Diseases

Approximately 7000 rare diseases have been recognized, a substantial number of which are life-threatening or chronically debilitating [1]. Around 80% of rare diseases are genetic in origin. A single rare disease affects a small number of the population (defined as < 1/15 000 in the US and < 1/2000 in Europe) but on aggregate, an estimated 350 million people globally suffer from rare diseases. Most rare disease patients (50%–75%) show onset at birth or in childhood. As many as 30% of rare diseases patients die before the age of 5 years. Furthermore, each rare disease patient has been estimated to cost a total of 5 million dollars throughout their life span.

Incomplete knowledge of **natural history** (see Glossary) and lack of awareness confounds rare disease diagnosis. The average length of accurate diagnosis of a rare disease is 4.8 years and involves more than seven physicians or specialists who may be geographically distributed[i]. An often-protracted path to the diagnosis of rare diseases poses an immense burden and psychological distress to patients and their family and a strong challenge to the current healthcare system [2]. The rare disease patients and family may benefit from genetic diagnosis. The genetic diagnosis may not be directly associated with any treatment options, and physicians will continue to treat symptoms, albeit in a more informed way based on likely prognosis of the case. Therefore, genetic diagnosis could be of benefit beyond treatment management as it can offer information to families, many of who just want to know what is wrong with their family member, and can also inform fertility decisions.

Next-Generation Sequencing-Based Genetic Diagnosis: Challenge and Opportunities

Emerging genomics technologies, such as next-generation sequencing (NGS), have been intensively applied in a research setting but also offer great opportunities in the clinical setting [3–5]. Despite the remarkable progress of NGS-based genetic testing[ii] for improving the discovery of genetic variants in rare disease, the translational gap between NGS-based genetic testing and clinical implementation remains. Many factors contribute to the suboptimal translation of NGS technology into a rare disease diagnosis. The acceptability and uptake of NGS-based genetic testing depends upon a clear demonstration of patient benefit driven by providing physicians with the tools for enhanced decision making. In this context, real-world evidence in support of NGS-based genetic testing is often limited.

There are several challenges to overcome before NGS can deliver its potential for patients, clinicians, and society. The key debate is whether NGS-based genetic testing can produce accurate and reproducible results that would support clinical decision making for rare disease diagnosis. To address this,

Highlights

NGS-based genetic testing in the diagnosis of rare diseases holds great promise to serve as a first-tier genetic testing tool in the near future.

Advancement of NGS technologies provides many options for diagnosing rare disorders associated with different types of genetic variants. Factors must be balanced in a 'fit-for-purpose' implementation.

The accuracy and reproducibility of NGS should be evaluated in a clinical setting to deliver reliable genetic testing results in different clinical stages of rare diseases.

Artificial intelligence (AI) will play a central role in integrating diverse diagnosis information toward an enhanced diagnosis power for rare diseases.

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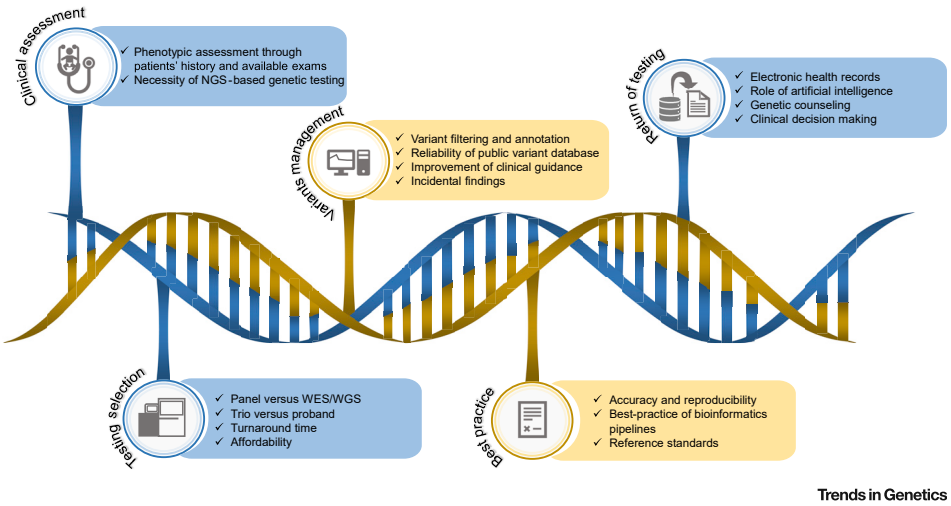

Figure 1. The Workflow of NGS-Based Genetic Testing in Rare Disease Diagnosis.
Some key steps and significant challenges are illustrated. Abbreviations: NGS, next-generation sequencing; WES, whole exome sequencing; WGS, whole genome sequencing.

the aim of genetic testing in rare disease diagnosis needs to be better defined. Secondly, there needs to be a seamless and harmonized relationship among genetic testing service providers, physicians, and patients. Key steps, parameters, and considerations are summarized in Figure 1.

Clinical Assessment of Rare Diseases

Like the diagnosis of common diseases, diagnosis of rare diseases relies on physical characteristics at presentation, diagnostic testing, and clinical knowledge of physicians. However, the symptoms of rare diseases are often masked by more common conditions. Furthermore, rare diseases can be highly individual with intricate interplay between genetic background and environmental factors. Moreover, the pleiotropic effects of genes, where one gene does not equal one phenotype or disease, makes a clinical diagnosis increasingly complex. Also, the physician's awareness may point toward more pathological-based clinical tests for disease diagnosis, not just genetic testing. However, the phenotypic and locus heterogeneity makes rare diseases uncategorizable based on standard histological and pathological features. Lack of experience with rare diseases and an absence of case reports as references poses an obstacle for a physician to make a diagnosis. Consequently, rare disease patients experience a long referral loop from one physician/specialist to another, which could lead to inappropriate management and disease progression. Thus, it is timely to advocate for genetic testing strategies, particularly NGS, for diagnosing rare diseases (Box 1).

Selection of a Fit-for-Purpose NGS Assay

Research is ongoing regarding which NGS-based genetic approach would be most appropriate in the clinical setting. Rapid advancements in technology open up new possibilities but at the same time pose technical challenges as each innovation is evaluated and the optimum approach to NGS-based screening is repositioned. Clinical implementation of a fit-for-purpose NGS test in rare diseases is multifactorial but major contributing factors are knowledge of the rare disease, speed of delivery, and cost. Furthermore, an optimized experimental design is key in improving the diagnostic power and clinical utility of NGS-based genetic testing (Box 2).

Panel versus Whole Exome Sequencing/Whole Genome Sequencing

An incomplete knowledge of the natural history of each rare disease can make a substantial proportion (~60%) of rare diseases intractable and undiagnosable [6,7]. Panel-based NGS or targeted sequencing tests are designed to reveal causal mutations for genes known to be associated with a

Glossary

Allelic heterogeneity: the phenomenon in which different mutations at the same locus lead to the same or very similar phenotypes. These allelic variations can arise as a result of natural selection processes, as a result of exogenous mutagens, genetic drift, or genetic migration.
Artificial intelligence: a branch of computer science dealing with the simulation of human intelligence processes by computer systems.
Cell-free fetal DNA: fetal DNA which circulates freely in the maternal blood.
Extreme-phenotype sampling: a selective genotyping design for genetic association studies where only individuals with extreme values of a continuous trait are genotyped for a set of genetic variants.
FAIRsharing: a curated, informative, and educational resource on data and metadata standards, inter-related to databases and data policies.
GC content: the percentage of nitrogenous bases on a DNA or RNA molecule that are either guanine or cytosine (from a possibility of four different ones, also including adenine and thymine in DNA and adenine and uracil in RNA).
Genome editing: (genome engineering) a type of genetic engineering in which DNA is inserted, deleted, modified, or replaced in the genome of a living organism.
***In vitro* fertilization (IVF)**: a complex series of procedures used to treat fertility or genetic problems and assist with the conception of a child.
Induced pluripotent stem cells: a type of pluripotent stem cell that can be generated directly from adult cells.
Liquid biopsy: a simple and noninvasive alternative to surgical biopsies, which enables doctors to discover a range of information about diseases through a simple blood sample.
Locus heterogeneity: a single disorder, trait, or pattern of traits caused by mutations in genes at different chromosomal loci.
Natural history: the course a disease takes in individual people from its pathological onset until its eventual resolution through complete recovery or death.

Box 1. A Glimpse of Genetic Diagnostic Testing for Rare Diseases

Since the inception of NGS technology, various applications are emerging and have proven successful in diagnosing a proportion of rare diseases in both research and clinical arenas [3,18,19,44]. Numerous genetic testing services were established globally, and the market size is expected to be valued at USD 22 billion in 2024[ii]. Based on Orphanet data statistics (version 1.2.11, accredited by Apr. 12th, 2018) [86], there are a total of 11 548 diagnostic tests available provided by 522 institutions mainly from Europe, which primarily focus on the diagnosis of the known causative gene. The number could be doubled or tripled when combining the data from the US and PR China. The clinical purpose of these diagnostic tests varied (Figure S1A in the supplemental information online). Approximately 86% of 11 548 genetic diagnostic testings are for postnatal diagnosis. Somatic genetics for pediatric cancers and antenatal diagnosis occupied 5.24% and 5.05% of all the diagnostic measurements, respectively. It was not surprising that preimplantation diagnosis accounts for less than 1% of available diagnostic tests due to its technical immaturity.

Furthermore, Sanger sequencing, as a critical orthogonal verification technology, is still widely adopted by the community (55%). This panel-based sequencing has incrementally moved toward dominating the market (14%) (Figure S1B in the supplemental information online). The diagnostic tests utilized for detecting genetic mutation, including SNV, deletion, and duplication within the coding region occupied nearly 92% of all available genetic tests (Figure S1C in the supplemental information online). These diagnostic tests could be used for diagnosing 2321 genes that were associated with 3446 rare diseases. The 3446 rare diseases covered a broad spectrum of rare disease categories based on Orphanet rare disease classification (Figure S1D in the supplemental information online). The top five rare disease categories with available diagnostic tests were neurological diseases, developmental anomalies during embryogenesis, inborn errors of metabolism, eye diseases, and bone diseases.

Preimplantation genetic diagnosis (PGD): the genetic profiling of embryos prior to implantation and sometimes even of oocytes prior to fertilization. PGD is considered in a similar fashion to prenatal diagnosis.
Preimplantation genetic screening: similar to the definition of PGD but for screening purposes.

specific rare disease [8]. Since the NGS gene panel is predesigned or expert-selected, ultra-deep, uniform coverage allows for high sensitivity and also for specific variant calling for rare genetic variants. A panel-based NGS test has been successfully applied to rare diseases with genetic heterogeneity, including **allelic heterogeneity** [9] and **locus heterogeneity** [10], overlapping phenotypes [11], and with causal genes involved in the common disease-related pathways [12].

Clinical whole exome sequencing (WES)/whole genome sequencing (WGS) is usually applied to patients with negative results based on conventional genetic tests or panel-based NGS test. Compared with panel-based genetic testing approaches that are locus-specific or focus on variants for a few genes, WES/WGS can provide complete genetic information in support of rare disease diagnosis [2]. The comprehensive ability to assess diverse types of genetic variants across the genome enables more precise identification of pathogenic variants to further influence diagnosis and treatment.

Increasing evidence shows that NGS-based diagnostic testing is superior to the recommended first-line genomic testing tools from the perspective of both diagnostic rate and clinical utility [13]. Based on a meta-analysis of 37 rare genetic disorders studies with 20 068 children conducted using WGS, WES, and chromosomal microarray analysis (CMA), the diagnostic rate of WES/WGS (0.36 and 0.41, respectively) is significantly higher than that of CMA (0.10). Furthermore, clinical utility could be increased by approximately 50%–80% from CMA to WES/WGS [14]. The pace of rare disease-related causal genes discovered by WES/WGS has steadily increased to account for more than 85% of causal gene discovery [15,16]. Encouragingly, there have been increasing attempts to explore the opportunities for positioning WGS as a first-tier genetic test [17].

WGS, as a non-hypothesis driven approach, could be widely utilized to comprehensively assess the genetic variant picture of rare disease patients. However, validated and orthogonal technologies are strongly recommended to be applied to verify the detection results. Those validated genetic findings allow researchers to focus on and provide the diagnosis in a specific genetic region. Although WGS has been gradually adopted in the clinical setting for rare disease diagnosis, panel-based sequencing is still valuable for deeper deciphering the genetic complex in a specific area. Therefore, a composite of WGS and panel-based sequencing testing are suggested, which may augment the clinical utility.

Box 2. Experiment Design for NGS-Based Genetic Testing

Panel Design

The gene-panels designed for rare diseases fall mainly into three categories of disease-specific, organ-specific, or a universal panel. Gene panels typically include a mixture of causative genes located in genome regions with different GC contents, associated with differing technical difficulty in detecting genetic variants, with further impact on the diagnostic rate. As gene panels are being adopted for clinical diagnosis for rare diseases and are incrementally becoming the first-line option for causative variants identification, panel design should be optimized to permit better clinical adoption. First, gene panel design should take a comprehensive perspective on the genome properties of included genes (i.e., variant type and GC content), facilitating gene-capture tool selection, sequencing depth determination, and data interpretation. Second, consideration should be given to the likelihood that a universal panel may not yield a better diagnostic rate [87]. The diagnostic rates among the different panels ranged from 31.3% to ~57% [88,89] and were not correlated with the number of genes included in the panel. Finally, a dialogue should be established, aiming to optimize panel development for clinical diagnosis allowing different stakeholders to align and promote diagnosis success.

Trio versus Proband

The current diagnosis rate of NGS-based testing for rare diseases ranges from approximately 25% to 52%, depending on the rare disease type, availability of biological family members (i.e., trio-based or proband), and analytical strategies [90], where trio-based refers to affected child and unaffected parents and proband refers to the affected individual in a pedigree tree. The causative genetic variants of rare disease detection are mainly based on sequencing with population-based sampling (e.g., **extreme-phenotype sampling**) or trio-based sampling. WES/WGS with a trio strategy allows for more sensitive identification of *de novo* mutations (DNM) that are present only in the child and the establishment of the phase of variants in recessive or imprinted disorders by inheritance [91,92]. For example, WGS was employed for the clinical diagnosis of 217 individuals with a broad spectrum of diseases. In this work, it was suggested that the diagnostic rate could be significantly improved for family trios (57%) compared with proband cases (34%) [90]. Another example is WES for diagnosis of 278 infants in intensive care units within the first 100 days of life, yielding a 36.7% diagnostic rate and enabling effective medical management for 52% of patients with a diagnosis. A higher diagnostic rate at an earlier age could be achieved with critical trio samples offering a more likely benefit from subsequent medical procedures [3].

Time to Delivery

Turnaround time is one of the most critical factors for the clinical implementation of NGS diagnostics in rare diseases. For example, timely diagnosis of rare diseases in the neonatal intensive care unit could avoid further invasive, and at times misplaced, investigations for the neonate. The time to delivery of results ranged widely from 50 hours up to 58 weeks for NGS-based diagnosis of different rare diseases, depending on the teamwork of clinicians, bioinformaticians, and molecular geneticists and, more importantly, a close liaison among the team members [14]. Furthermore, sequencing depth as an essential parameter correlated with diagnostic accuracy. Ultra-depth sequencing also may improve diagnosis rate but at a higher cost, with demands for increased data storage and a possible slower data analysis.

Cost

NGS technologies continue to evolve, and the trajectory of NGS applications is tending toward use as a clinical tool, enabled by the continuing falling price [18]. Unlike Sanger sequencing, NGS allows us to sequence millions of fragments simultaneously per run and detect genetic variants for multiple genes in parallel. Two major paradigms exist in NGS technology. Short-read sequencing is a cost-effective and high accuracy tool for small variant detection. By contrast, long-read sequencing, including Pacific Biosciences, Oxford Nanopore Technology, and Ion Torrent, may be a good option for *de novo* genome assembly and complex **Structural** variants (SVs). However, the costs and error rates in variant detection for long-read sequencing limit its routine use in the clinical setting. Furthermore, the legacy conventional genetic testing approaches such as array-based genotyping, NanoString, and qPCR are less expensive [19] and may still be useful for less complicated variant detection [2,20,21].

Box 3. Variant Interpretation Guidelines

ACMG-AMP guidelines for interpreting sequencing variants have been widely adopted by clinical laboratories [23]. The key recommendation within the ACMG-AMP guidelines is the development and use of a systematic scoring system for prioritizing support evidence of variants coupled with a five-tier scheme for variant classification [22]. The current five tiers of variant classification in Mendelian genetics include pathogenic (P), likely pathogenic (LP), uncertain significance (VUS), likely benign (LB), and benign (B), but with more optional tiers. However, these tiers are currently not accepted by the public clinical variant databases such as ClinVar [93]. Based on a survey study on clinical adoption of ACMG-AMP guidelines, approximately 22% of surveyed laboratories defined variants in the VUS category with additional terms such as 'topline VUS' and 'uncertain clinical significance, possibly pathogenic', highlighting the need for further standardization and improvement of variant terminology for better clinical applications [23].

Furthermore, the thresholds for defining the minor allele frequency and multiple *de novo* occurrence are also disease-specific and data resource-related [94]. For example, the Clinical Genome RASopathy clinical domain working groups posted additional guidelines to compliment further the ACMG-AMP guidelines, in which the unpublished disease-specific clinical genetic testing data were employed to improve the accuracy of scoring of the strength of variants in different subcategories. Furthermore, some lab-based criteria still exist for variant interpretations. Based on the meta-analysis of concordance between lab-based standards and ACMG-AMP guidelines in nine laboratories, a high concordance (i.e., 79%) was established within each laboratory but only 34% concordance was obtained across the laboratories [95].

Clinical Management of Genetic Variants

Although NGS provides a complete genetic makeup of rare disease patients, it also poses a significant challenge to pinpoint a subset of clinically relevant genetic variants. The filtration and annotation of genetic variants includes multiple components, and the diagnostic decision on inclusion or exclusion of variants depends heavily on data resources and the functional annotation algorithms employed [2]. More importantly, the validation of candidate genetic variants mainly relies on whether there is sufficient supporting evidence of their clinical relevance and functional impact. Moreover, the strategy adopted for decision making is also profound. The hard cut-off strategy for variant selection is mainly based on allele frequency or read depth, which could omit rare genetic variants below the predefined threshold representing pathogenic meaning. Thus, the variant prioritization strategy could be utilized for ranking in order the variants regarding their potential clinical impact. Furthermore, WES/WGS has a high probability of incidental or secondary findings (ISFs), adding another layer of complexity for clinical management of genetic variants. Standards and recommendations have been developed by the professional communities, such as the American College of Medical Genetics and American College of Pathologists (ACMG-AMP), as educational resources and guidelines to facilitate clinical laboratories managing sequencing variants [22]. However, further refinement and update of these guidelines is needed to continually improve the accuracy and reliability of variant annotation, expand the standard terms for clinical variant classification, to promote spontaneous genetic information submission, and to call for redundancy standards [23] (Box 3).

Public Variant Databases

Variant databases support the curation, accumulation, and clinical interpretations of disease-associated variants, a crucial adjunct to clinical variant management. Every individual has >20 double-null genes and >150 heterozygous null genes, so even a complete and perfect WGS does not declare the disease-causing variant. Therefore, the public variant databases are imperfect, however, they are still a good resource for facilitating decision making of variant selection [24]. The current public human variant databases are mainly divided into two categories: population-based resources and disease-specific variant atlases. Population-based databases provide information on the variant frequency in large populations. Although efforts have been made to expand the number of healthy individuals and to drive population stratification, certain ethnic populations, age groups, and genders remain under-represented. For example, the minor allelic frequency of minority groups such as African Americans is significantly under-represented compared with populations with European ancestry [25]. Furthermore, the population-based genetic variant database may include some samples with a disease or carry some phenotypic correlates of the disease. For example, the Exome

Aggregation Consortium [26] studied 60 706 individuals of diverse ancestries and found 3230 genes with near-complete depletion of predicted protein-truncating variants. However, 72% of these genes have not been reported in public variant databases such as Online Mendelian Inheritance in Man and ClinVar databases. This information could be valuable as criteria (e.g., the hard cutoff filtering) to facilitate identification of candidate rare disease-causing variants.

Moreover, few variant databases incorporate family-based trio, quartet, or pedigree samples, all of which are of great importance for distinguishing *de novo* variants [27]. The disease-based variant database is an excellent resource to curate the knowledge of rare disease-related variants in the patient level. Disease-based variant databases suffer a relatively high error rate and have insufficient statistical power for specific diseases or phenotypes (e.g., pairs of genes that cause disease), leading to an unreliable assignment of pathogenicity. Furthermore, more detailed information on clinical site description, submission agreement, and data governance should be a mandatory requirement for data sharing and curation to enhance the utility of public variant databases [28]. Some community health initiative efforts are currently underway to sequence patient populations and incorporate various lifestyle choices and patient information to make the data available for diagnostic purposes. For example, MyCode Initiative is promoting disease diagnosis by analyzing the DNA of patient-participants for various diseases to advance precision medicine [29]. Another example is GeneMatcher, which aims to collect rare disease patient samples sharing the variants from the same candidate disease genes. These efforts could greatly improve the quality and representation of genetic variants curation for specific rare diseases [30].

Bioinformatics Analysis and Statistical Methods

Bioinformatics approaches have been widely utilized to filter out variants without functional impact and annotate variants regarding their causative effect with specific phenotypes [31]. The pros and cons of those *in silico* filters and annotation tools have been intensively discussed elsewhere [32]. Concerns regarding inconsistency among these methodologies have arisen, mainly attributed to parameters such as inconsistency of input and output variant format, lack of a unified measure on predictive power, and frequency of annotation database update. Consensus and cloud-based variant functional prediction platform have been introduced, including Combined Annotation-Dependent Depletion (CADD) [33] and Bystro [31], which aim to provide more consistent and reproducible variant annotation results. Moreover, clinical guidelines were also developed by ACMG/AMP for evaluation of variant interpretation tools in diagnostic laboratories, which also suggested the combinations of *in silico* algorithms with an increased concordance for variant annotation in a clinical setting [34]. However, the computational-based variant prediction tools are not capable of providing definitive proof regarding the clinical relevance of the candidate variants. Some limitation of variant prediction tools, such as inaccurate function prediction of gain/loss of function, need further improvement. Besides, the fast-moving pace of NGS technologies causes rapid movement of scientific evidence. The question raised is how to uptake the novel technologies and findings to revisit and reannotate the existing knowledge, which is of great importance for better deciphering the clinical relevance of candidate variants [35,36].

Incidental Findings

Management of ISFs derived from WES/WGS is the subject of fierce debate in the clinical setting, where ISFs refer to genetic results that are unrelated to the initial purpose of testing. The capture of ISFs depends on the physician and the willingness of patients and their families to share information, which limits the consensus reporting system implemented [37]. Recommendations on diagnosing ISFs have been proposed and updated by ACMG, resulting in a list of 59 medically actionable genes recommended for return in clinical genomic sequencing [38]. However, some actionable genes included in the ACMG recommendations are adult-onset specific, and other actionable gene-related conditions are not aging specific, which poses additional challenges when applying ISFs to pediatric patients [39].

Risks in reporting ISFs include spurious courses of action, such as parents pursuing IVF with a PGD for a variant that may not lead to disease [39]. Consequently, it will be important to develop

Box 4. Technical Barriers to NGS-Based Genetic Testing in Rare Diseases

Accuracy

One of the significant difficulties for NGS technology is to detect rare variants with extremely low allele frequency accurately. For example, causative genetic variants, such as *de novo* mutations, were discovered in various rare diseases with a shallow frequency and a low penetrance rate [96]. The mutation rate of *de novo* mutations (DNM) is estimated at around $1–3 \times 10^{-8}$ per base pair per generation, although the rate may vary across different genome locations, families, and patient ages [97]. The error rate of NGS is in the range of ~1% to 0.1% or even lower in optimal scenarios [98], which is much higher than the mutation rate of DNM. It has been a notable challenge for accurate and reliable DNM identification.

Detection of complex variants such as SVs and large CNVs is another hurdle for accurate NGS genetic testing [99–101]. The SVs detected by short-read sequencing approaches suffer low sensitivity (~10% to 70%), and high false positive rates (up to 89%), resulting in insufficient detection power for complex or nested SVs with breakpoints in repetitive regions [62]. The long-read single molecule sequencing technologies have the potential to increase the resolution and sensitivity of SV detection. However, relatively high error rates in Pacific Biosciences (PacBio) with ~10%–15% and Oxford Nanopore with ~5%–20% still exist [18].

Reproducibility

Reproducibility is vital for a successful clinical diagnosis. NGS-based genetic testing is no exception. Complexities in NGS-based genetic testing include samples and library preparations, gene capture, bioinformatics pipelines, and results interpretations, all of which confound the generation of repeatable and reproducible results. Each step is subject to some uncertainty. Therefore, a rigorous examination of accuracy and reproducibility is imperative to underpin clinical implementation in rare diseases.

recommendations and standards for managing the return of information from genomic sequencing in children, especially in early life, to ensure the best interests of the family. For example, the BabySeq Project is a randomized trial for exploring the medical, behavioral, and economic impacts of sequencing-based genetic testing on pediatric patients and the further improvement of ISF management [40].

Standardizing Practices for NGS-Based Genetic Testing

Accurate and reproducible NGS data are a prerequisite for reliable clinical diagnosis. NGS technologies have reshaped the potential for genetic-based diagnosis at an astounding pace and the pros and cons of different sequencing platforms have been intensively discussed [4,18]. Intrinsic sequencing errors and artifacts introduced during sample and library preparation cause inaccurate and irreproducible variant detection, especially for low-frequency and complex structural variants (SVs) (Box 4). Feasible and effective procedures should be developed for enhancing clinical adoption of NGS-based genetic testing for rare disease diagnosis (Figure 2).

Biological Certified Reference Materials

Systematic errors could be addressed using a reference standard approach to evaluate diagnostic performance and reproducibility of diagnostic testing, and to establish best practices for variant calling [41]. A wide range of professional organizations has recommended utilizing reference standards to calibrate NGS measurements routinely [16,17].

Efforts have been made to develop reference materials, including well-characterized cell-based genetic materials [42–44] and synthetic spike-in controls [45,46]. Many laboratories have moved to apply the Genome in a Bottle Consortium (GIAB) reference samples (e.g., NA12878) as process controls to estimate detection limits, ensure repeatability and reproducibility, and calibrate their NGS workflow [47]. However, there are concerns regarding how well these reference samples, derived from cells of healthy donors, represent the complex variant distribution of rare diseases patients in a clinical setting.

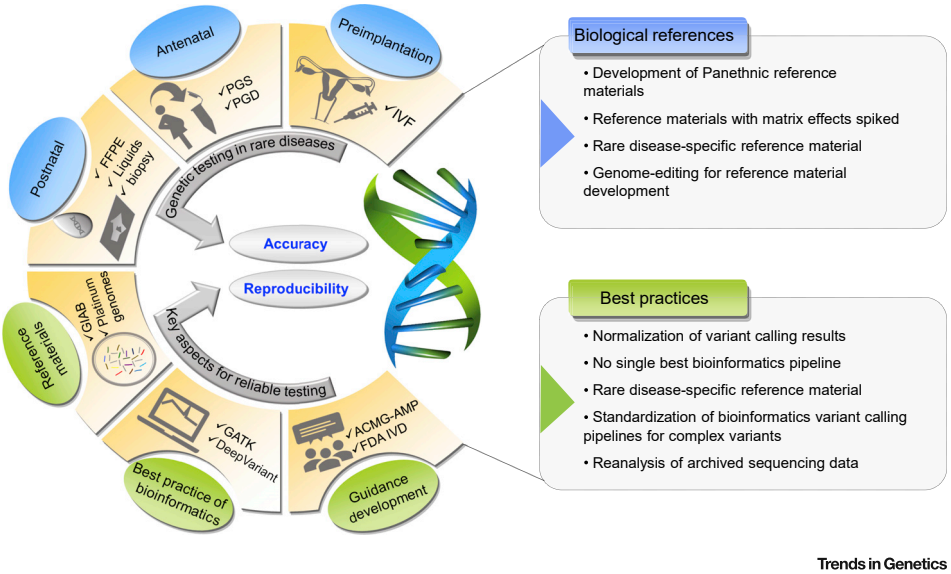

Figure 2. The Outstanding Challenges and Potential Solutions for Enhancing Accurate and Reproducible. Next-Generation Sequencing (NGS)-Based Genetic Testing. NGS-based genetic testing has great potential for application to different clinical stages of rare diseases, including IVF, PDS, and PDG, and different tissues such as FFPE and liquid biopsies. Detailed solutions for improving the accuracy and reproducibility of NGS-based testing are suggested: development of biological certificated references and standardization of best practice of NGS data analysis. Abbreviations: ACMG-AMP, American College of Medical Genetics and American College of Pathologists; FFPE, formalin-fixed, paraffin-embedded; GIAB, Genome in a Bottle Consortium; IVD, *in vitro* diagnostic; IVF, *in vitro* fertilization; NGS, next-generation sequencing; PGD, preimplantation genetic diagnosis; PGS, preimplantation genetic screening.

Commutability, defined as the ability of reference materials to mimic patient samples, is the most critical parameter in qualifying reference materials [41]. Here, we recommend developing biological certified reference materials for evaluating the accuracy and reproducibility of NGS testing systems in a clinical setting as follows:

1. Development of pan-ethnic reference materials. It has been demonstrated that the distribution of genetic variations across the global populations varies, resulting in difficult regions, such as extreme **GC content**, low complexity, or repetitive sequences differences [48]. Single ethnic population-based reference material may not be appropriate for covering genetic variants associated with different rare diseases with broad geographical distribution and diverse epidemiology. Therefore, a drive toward coordinated, local implementation on the development of reference materials from different ancestries is highly encouraged to improve their relevance to the local populations [47].
2. Reference materials spiked to take account of matrix effects. The variant distribution within formalin-fixed, paraffin-embedded or **liquid biopsy**-based samples from rare disease patients may be very different to that seen in normal tissues [49]. For example, NGS-based genetic testing has been applied to tissues with complex matrix effects, including ***in vitro* fertilization (IVF)**, **preimplantation genetic diagnosis (PGD)**, and **preimplantation genetic screening** to help patients to select embryos free of rare diseases [50–54]. Furthermore, liquid biopsies such as **cell-free fetal DNA** in maternal plasma also holds promise for the development of noninvasive PGD testing in rare disease diagnosis [55,56]. Therefore, the reference materials with different degrees of matrix effects should be developed to better understand the pros and cons of NGS-based genetic testing in real-world applications.

3. Rare disease-specific reference material. Healthy donor-based reference materials may only cover a tiny proportion of causative variants of rare diseases. Cells from patient samples such as human **induced pluripotent stem cells** harboring pathologic variants may be a feasible solution to refine the development of reference material. Efforts such as the Genetic Testing Reference Materials Coordination Program (GeT-RM) aim to coordinate a self-sustaining community process to improve the availability of appropriate and characterized reference materials for inherited diseases and pharmacogenetics [57].
4. **Genome editing** for reference material development. Advances in bioengineering, including genome editing, can be used to introduce specific genetic variants into cells, offering the opportunity to develop a synthetic reference material that includes various rare disease-related causative variants [58]. However, careful verification of the engineered cell line to account for unexpected off-target variants is a requirement [59]. Furthermore, confidentiality concerns for maintaining the anonymity of de-identified patients arose and need a better consideration.

Best Practice for NGS Data Analysis

Bioinformatics is an essential component of NGS-based genetic testing. The NGS bioinformatics pipeline consists of multiple steps, including sequencing alignment, variant calling, variant filtering, variant annotation, and prioritization for performing appropriate analyses. With developed reference materials, GIAB, along with Global Alliance for Genomics and Health (GA4GH), is working on generating benchmarking data to serve as the baseline to establish best practices in NGS data analysis [42,60].

Lessons learned from the GIAB project are beneficial to understand the pros and cons of sequencing technologies better and to standardize NGS data analysis further. First, normalization of the variant calling format from different bioinformatics pipelines is a prerequisite for further comparison of performance. GA4GH developed a standard procedure to standardize variant representation and suggested cloud-based bioinformatics pipeline management. Second, best practice is not solely dependent upon the utilization of the best bioinformatics pipeline. Based on the results of precisionFDA challenges, the performance (defined as recall and precision) of NGS bioinformatics pipelines are variation type and genome content dependent. For example, the top-ranked two bioinformatics pipelines including DeepVariant [61] and GATK-best practice in precisionFDA challenges show higher concordance (99.7% versus 76.5%) in and outside high-confidence regions, indicating that the big challenge for performance improvement resides in difficult genome regions. Third, the standardization of bioinformatics variant calling pipelines for complex SVs, large copy number variants (CNVs), and *de novo* mutation is imperative. The primary focus of GIAB is small variants, such as SNVs and small indels/deletions. Although intensive research has been conducted to provide bioinformatics solutions to improve the accuracy of variant calling for SVs [62,63] and *de novo* genetic variants [64,65], a comprehensive assessment and evaluation of these methods was not routinely carried out. Finally, a reanalysis of NGS data should be routinely conducted, along with the update of tools, reference genomes, and annotation databases [35,36].

Good Laboratory Practice for Sequencing-Based Genetic Testing

NGS data analysis requires extensive data storage, data transfer, and computational resources. The development of a consistent framework for managing the standards, repositories, and policies is imperative to underpin a reproducible and reusable NGS-based workflow. Community efforts have been made to standardize the bioinformatics pipelines for establishing good laboratory practice (GLP) for clinical NGS application. The Next-Generation Sequencing: Standardization of Clinical Testing II informatics workgroup have presented their recommendations for the design, optimization, and implementation of an informatics pipeline for germline mutation detection in the clinical NGS field to comply with the existing quality standards and regulations [66].

Furthermore, the Association of Molecular Pathology, with organizational representation from the College of American Pathologists and the American Medical Informatics Association, has developed

a set of 17 best practice consensus recommendations for the validation of clinical NGS bioinformatics pipelines, with emphasis on the training and qualifications of the molecular professional for improved NGS testing [67]. Moreover, the US FDA finalized two guidances to accelerate the development of reliable, beneficial NGS-based tests for making innovative and accurate testing technologies available to patients as efficiently as possible[iii]. Efforts such as **FAIRsharing** to maximize the community efforts toward a better adoption and visibility of standards could be an effective way to promote GLP [68].

Artificial Intelligence for Enhancing NGS-Based diagnosis

The impact of **artificial intelligence (AI)** is global and multidisciplinary. Today, AI, and deep learning in particular, has been widely applied in different biomedical frameworks [69,70] and has been revolutionizing the healthcare system [71] as well as other fields outside of the scope of this paper [69–73]. NGS-based diagnosis consists of various steps, where AI has begun to show its merits in improving variant calling accuracy, augmenting variant prediction, and enhancing the physician-friendliness of electronic health record (EHR) systems (Table 1).

Variant Calling

Variant calling is the process to identify different types of genetic variants from NGS data. Although various variant calling algorithms have been developed, the performance of most variant callers is still suboptimal, especially in a clinical setting. Deep learning-based algorithms provide an alternative scenario for variant calling. For example, DeepVariant developed by Google transforms the variant calling problem into an image recognition task by imagizing the BAM file and modeling with a convolutional neural network (CNN). The performance of DeepVariant is superior to most of the conventional variant calling algorithms for identifying SNV and small indels [61]. Furthermore, A SpliceAI, a 32-layer deep neural network (DNN) was developed for predicting *de novo* mutations (DNM) with predicted splice-altering consequence in patients with autism and intellectual disability, which paves the way for the application of AI on complex genetic variant prediction [74].

Variant Prediction

One of the hurdles for clinical implementation of NGS-based diagnosis is the difficulty of distinguishing pathogenic mutations from benign genetic variants. Although a lot of variant effect prediction tools have been developed to fill the gap, it is still a limiting factor that needs to be further established within the decision-making process [75]. Deep learning has been showing some promise in this field to augment the variant prediction accuracy. One example is the deleterious annotation of genetic variants using neural networks (DANN), which modified the CADD tool with a DNN model for variant prediction. The DANN outperformed the original CADD with support vector machine (SVM) classifier with a 14% relative increase in the area under the curve (AUC) metric [76]. Another interesting study led by Sundaram *et al.* developed a DNN model combining common variants derived from human and other non-human primate species to identify pathogenic mutations in rare disease patients. The proposed model achieved an 88% accuracy and found some unreported genes associating with intellectual disability [77].

EHR

Connecting genetic testing with EHR systems is a key step in bringing genomics into clinical care [78]. Meanwhile, the EHR system has served as a hub to incorporate diverse digitized health information, enabling better clinical decision making and precision medicine. The question is how to fuse data profiles of differing complexity in the EHR system to augment diagnosis in a clinical setting. Advances in AI may provide a solution.

Free-Text and Genetic Variants

The physician-friendliness of EHR data is the key to boost its utility. For example, physician notes, also known as patient narratives, were used to summarize the diagnosis for patients based on different testing results. However, the language used in the patient narrative is free-text in nature and

Models	Algorithms	Notes	Refs
Variant calling			
DeepVariant	Deep convolutional neural network (CNN)	Variant calling from short-read sequencing by reconstructing DNA alignments as an image.	[61]
Clairvoyante	Deep CNN	A multitask five-layer convolutional neural network model for predicting variant type (SNP or indel), zygosity, alternative allele, and indel length from aligned reads. It could be applied in long-read sequencing data such as PacBio and Oxford Nanopore	[102]
DeepNano	Deep RNN	A deep recurrent neural network for base calling in MinION nanopore reads, which achieves a comparable performance to Nanonet base caller provided by Oxford Nanopore	[103]
Variant prioritization and annotation			
Skyhawk	Deep neural network (DNN)	An artificial neural network-based discriminator that mimics the process of expert review for clinically significant genomics variants identification	[104]
DANN	DNN	A DNN algorithm for predicting deleterious annotation of genetic variants, which is superior to the state-of-art algorithms such as support vector machine	[76]
DeepSEA	Deep CNN	A deep CNN model for prediction of the noncoding-variant effects *de novo* from sequence and further applied to autism spectrum disorder-related variant functional prediction.	[105]
Phenotype–genotype association			
DeepGestalt	Deep CNN	A deep CNN model to distinguish more than 200 rare diseases based on patient face images, which could also separate different genetic subtypes in Noonan syndrome	[80]
DeepPVP	DNN	A deep DNN model for variant prioritization by integrating patients' phenotype information.	[106]

Table 1. Examples of Deep Learning for Genetic Variants

challenging to employ for systematic analysis of hidden knowledge tied to genetic testing results. AI-based text-mining strategies have been widely applied for different tasks, including information retrieval, text summarization, question and answering, and sentiment analysis. Some deep word embedding algorithms, such as word2vec and GloVe, have been developed to map the words or phrases to the vectors of numeric values, which provides an opportunity to integrate text-based information with image and genetic information (Figure 3A). For example, Wang *et al.* proposed a novel multilevel attention model named Text-Image Embedding Network (TieNet) to combine clinical image and free-text radiological reports by using deep hybrid CNN and recurrent neural network (RNN) models. The proposed TieNet has been successfully applied for auto-annotation of chest X-ray with the associated common thorax disease subtypes, with a high AUC value of 0.9 [79].

Phenotypes and Genetic Testing Association

Deep learning has been widely applied to medical image diagnostic systems, yielding better diagnostic performance than radiologists and pathologists [71]. Very recently, Gurovich *et al.* [80] proposed a DeepGestalt framework to identify facial phenotypes of rare genetic disorders with a CNN embedded. DeepGestalt comprised over 17 000 images for more than 200 rare diseases and achieved 91% accuracy. More importantly, the model could separate different genetic subtypes in Noonan syndrome, shedding light on the correlation of phenotypes with genotypic information for variant prioritization, and also demonstrated the deep CNN framework for the association analysis between phenotypic data and genetic variants (Figure 3B).

Omics Data Fusion

Efforts have been made to integrate multiple layers of omics data for prioritizing causative variants in rare diseases [81]. Furthermore, the concept of digital medicine continues to emerge, and wearable

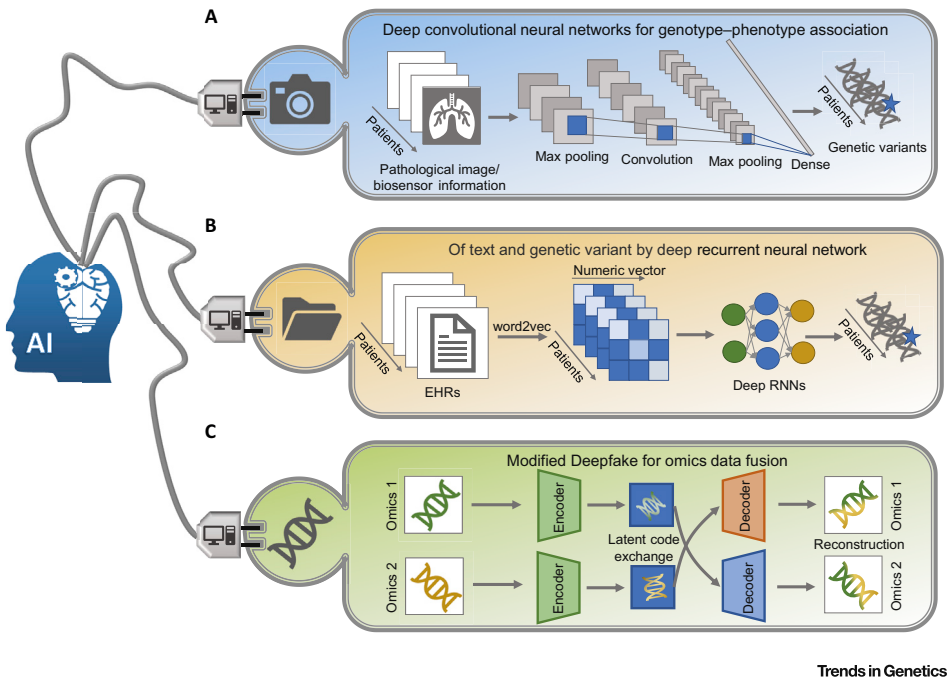

Figure 3. Potential Deep Learning Strategies for Improving the Variant Prioritization in Clinical Rare.
Disease Diagnosis. Three deep learning models are proposed, including (A) a deep convolutional neural network model to associate phenotypic testing results such as pathological images and digital wearable sensor signals with genetic variants from next-generation sequencing (NGS); (B) a data fusion model that employs a deep autoencoder by exchanging high representation code between omics data profiles to augment the prediction power; and (C) a deep recurrent neural network (RNN) model for linking electronic health records (EHRs) processed by word2vec and genetic variants from NGS for prioritization.

biosensors have begun to show their potential to provide continuous, dynamic, and real-time physiological information for clinical diagnosis [82]. Deep learning has a great advantage in linking high-dimensional data of a different nature and with different levels of complexity to gain maximal predictive power. For example, a deep Autoencoder model was proposed by integrating gene expression and CNV data from high-risk neuroblastoma patients with different subtypes showing significant survival differences, which yielded an improved survival curve compared with other state-of-the-art classifiers [83]. One of the interesting deep learning frameworks, Deepfake, is a technique for human image synthesis based on generative adversarial networks and is capable of combining and superimposing existing images to give a new representation[iv]. Inspired by the Deepfake framework, we envision a conceptual deep learning framework for data fusion of multiple layers of information, which may potentially improve variant prioritization, although further investigation is a 'must' (Figure 3C).

Database Storage and Management

AI and NGS are a golden combination since AI requires the big data and NGS generates big data. Along with the large amount of NGS data, other diagnosis-related testing data is being amassed, bringing up the issue of proper data storage. Clinical diagnosis laboratories are not capable of handling increasingly accumulated data. Additionally, data storage requires a sophisticated informatics infrastructure to store the data securely. More and more efforts have been made to bring cloud-based services in compliance with health privacy laws for NGS data storage, along with harmonizing data privacy across different stakeholders [84].

Despite the potential for AI to enhance clinical diagnosis in rare diseases, the complexity and diversity of clinical data profiles may pose many challenges for AI. That is due to the fact that developing an AI

model for rare disease diagnosis requires a training set of many patients with known clinical outcomes (i.e., labeled data).

However, the curation of labeled data needs manual efforts from the domain expert to delineate disease-specific information. Furthermore, although AI is capable of integrating diverse types of clinical data for augmenting prediction power, careful data annotation and standardization is still the key to warrant the establishment of a high quality model. AI is accelerating the transformation in clinical diagnosis from rule-based strategies to more individual-based or data-driven recommendation. However, AI-based approaches should be positioned as a 'last resort' and not as an assistance tool until the clinical utility is well-proven and the regulation and standardization on the clinical application of AI are well-established.

Outstanding Questions

How do we increase the visibility and adoption of developed standards and guidance for clinical implementation of NGS-based genetic testing?

How do we adapt best practice to promote the NGS-based genetic testing in a clinical setting?

How do we develop a variant selection framework to agilely implement the hard cut-off and prioritization?

How do we effectively combine genetic testing with omics and phenotypic information to understand the etiology of rare diseases, better to improve diagnosis rates?

How do we work with different stakeholders toward harmonization and adoption of NGS-based genetic testing?

Concluding Remarks

NGS technologies have tremendous potential to serve as the first step in genetic testing in rare disease diagnosis, although a lot of concerns and challenges remain (see Outstanding Questions). As described in this review, clinical implementation of NGS-based genetic testing depends on many factors but also on some important considerations not covered here, such as genetic counseling for harmonizing and coordinating the patient–physician relationship, ethnic issues for adopting and delivering genetic testing, and educational efforts for promoting the acceptance of genetic testing in a clinical setting [85]. Today, genetic tests are becoming more mainstream and more accessible to individuals and to physicians. Accordingly, there is much debate on how to deal with direct-to-consumer genetic testing (DCGT) in a clinical setting. Difficulty in the interpretation of genetic testing results, and issues relating to privacy and confidentiality, suggest that DCGT should be discouraged in the diagnosis of rare diseases at present.

The clinical application of NGS is approaching an upward trajectory as more and more experiences and confidence are accumulated through real-world applications. The high-potential application of NGS-based diagnosis in noninvasive prenatal testing, complex genetic variants detection, and preventive genetic screening has promoted and improved in a clinical setting. Furthermore, the application of NGS technologies will be expanded to different liquid biopsies for early diagnosis. Moreover, the consummation of NGS clinical practice will be gradually improved and established through efforts of different clinical communities, as well as regulatory bodies.

The modernization of diagnosis is progressively intertwined with emerging technologies and with AI. NGS-based genetic testing is one of the most promising diagnostic options and, as such, should sit alongside well-established clinical testing and other innovative diagnostic tools such as wearable biosensors. AI is starting to realize its potential in augmenting phenome-wide and genome-wide data profiles to improve clinical utility and diagnostic power. Alongside guidance efforts from the professional community, the regulatory standardization of NGS-based testing and AI application have been advocated and initialized by the government agencies[v]. We hope our paper will trigger debate among stakeholders to promote further NGS-based genetic testing, toward a more effective clinical implementation for millions of rare disease patients.

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Supplemental Information

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Resources

[i]https://globalgenes.org/rare-diseases-facts-statistics/.

[ii]www.gminsights.com/industry-analysis/genetic-testing-market

[iii]www.fda.gov/news-events/press-announcements/fda-finalizes-guidances-accelerate-development-reliable-beneficial-next-generation-sequencing-based

[iv]https://en.wikipedia.org/wiki/Deepfake

[v]www.fda.gov/medical-devices/software-medical-device-samd/artificial-intelligence-and-machine-learning-software-medical-device

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