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Next-Generation Sequencing in Haematological Diagnostics: Innovations and Clinical Applications

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Abstract

Next-generation sequencing (NGS) technologies have transformed haematological diagnostics, offering unparalleled speed, accuracy, and depth in analysing genetic variations linked to blood disorders and malignancies [1]. This review highlights the advancements and clinical applications of NGS, including its use in identifying clonal architecture, detecting minimal residual disease, and enabling personalised treatment strategies [2]. The integration of NGS into routine diagnostics has allowed for the precise identification of driver mutations, inherited blood disorders, and pharmacogenomic insights. Despite challenges such as data interpretation, assay validation, and accessibility, NGS remains a pivotal innovation in molecular diagnostics. Emerging technologies, including single-cell sequencing [3] and AI integration, promise to further revolutionise the field, ensuring that genomic insights translate into improved patient outcomes. This work underscores NGS's transformative impact on haematology, from early disease detection to the realisation of precision medicine [4].

Keywords: Next-Generation Sequencing (NGS), Haematological Diagnostics, Molecular Residual Disease (MRD), Genetic Mutations, Personalised Medicine.

1. Introduction

Over the last few years, next-generation sequencing (NGS) technologies have revolutionised research in the fields of cancer genetics, diagnosis, and therapy. It performs massively parallel sequencing, thus increasing speed and reducing costs while providing deeper coverage compared to other sequencing methods [5]. The use of NGS has rapidly increased in both haematological malignancies and disease-related factors, such as the detection of minor variants, mutation tracking, clonal evolution, and assessment of homogeneity/heterogeneity [6]. The advent of NGS has also revealed a complex and often unexpected clonal architecture and will influence the molecular monitoring and identification of relapse in patients with haematological malignancies. In this review, we provide an overview of the clinical applications of NGS in haematological diagnostics, including both germline and somatic genetic studies, and the progress in early disease detection [7]. The recent development and implementation of a wide range of technologies focusing on selected areas or whole gene panels have significantly improved the capability to measure molecular residual disease in order to detect minimal residual disease (MRD) in leukaemia and other malignancies [8]. Indeed, MRD monitoring has strong clinical utility, and it is widely accepted as a valid biomarker for the prediction of relapse and outcome of treatment.

In blood cancers, NGS approaches have been utilised to detect driver lesions, clonal and subclonal heterogeneity, and the association between solid and haematological malignancies [9]. In addition, the creation of detailed mutational catalogues has been established as part of large international initiatives for the investigation of haematological cancer genomes. Overall, the knowledge produced has contributed significantly to the implementation of NGS-based molecular monitoring tests [10]. Indeed, protocols for NGS-based monitoring procedures have been applied not only in major clinical trials but also in routine diagnostic laboratories. With the increase in both the number of NGS requests and the strict requirements for faster and less costly methods, the diagnostic field dedicated to haematological disorders is undergoing rapid evolution. This review aims to provide advice about the current status, future perspectives, and the impact that NGS will have on the development of diagnostics, as well as the technological innovations that preclude translation from research to routine application [11].

1.1. Background and significance of haematological diagnostics

Haematological diagnostics substantially rely on traditional technologies, including peripheral blood smears, bone marrow smears, and cell cultures [12]. These methods have several limitations, including low positive rates for cloning culture of specific patient samples, inconsistencies in the manual interpretation of smears or confusion of sample quality interference factors, and higher detection thresholds for rare clones. With continuously updated molecular biology technologies and rapidly expanding next-generation sequencing (NGS) technologies, various haematological diseases can be detected at the gene level [13]. This helps further understand the various subclones and mutation information in the patient's disease state and assists in monitoring during the course of the disease. Because of the heterogeneity of various haematological diseases and the presence of minor clones, clinical applications face certain challenges. Furthermore, according to the needs of specific disease types and laboratory evaluation differences, targeted capture sequencing or contamination beyond the whole genome is often designed, including cytogenetically delineated leukaemia-specific prognostic factor development and configurations in suspension-based chromosome segregation studies [14]. Moreover, the development of multiple types of new targeted drugs for specific gene mutations has increased the need for rapid and accurate mutation diagnosis and for guiding targeted therapy. Currently, the clinical diagnostic platform for haematological diseases has been gradually updated from single-gene testing, but the clinical guidance of large-scale DNA sequencing is still not unified [15]. The haematologists' understanding of large data intelligence analysis cannot meet the needs of personalised medicine in the field of haematological diseases, and the purpose of this review is to introduce bioinformatics. These outstanding issues and problems are not the exploration and feasibility of novel technologies, platforms, analysis trends, and the possibility of early screening for targeted haematological diseases [16].

1.2. Evolution of sequencing technologies: From Sanger to NGS

In the past, the Sanger DNA sequencing method was the primary technology used to sequence both normal and tumour DNA and discover somatic mutations. Despite launching high-throughput platforms, genome-wide association studies that identified germline polymorphisms in age-related macular degeneration were based on the Sanger method [17]. Recently developed techniques have identified the first age-related macular degeneration risk gene. Although the method has bandwidth limitations compared to more advanced methods, the primary method for the first human genome sequenced under the Human Genome Project is the Sanger method. Subsequently, with the fast and large-scale genome sequencing projects, the rapidly increasing DNA database rates and falling DNA sequencing costs have rapidly increased the speed and reduced the cost of traditional germline mutation detection [18].

The rapid development and data input of new methods, including the PGM Ion Torrent, Roche 454 GS FLX, and SOLiD platform, which provide low-cost sequencing solutions, have been able to identify the genome or exome of a patient with advanced cancer at a specifically defined clinically actionable timepoint. Subsequently, the rapid development and data input of next-generation sequencing (NGS), called second-generation sequencing technologies, including the HiSeq, NextSeq, and MiSeq instruments, has led to high sequence resolution and high read-throughput [19]. Currently, the majority of germline BRCA1 and BRCA2 genes and other somatic mutations detected in clinically relevant actionable cancer genes are identified by next-generation sequencing. The simplicity, speed, and scalable nature of the NGS method make it particularly well-suited for completing a significant amount of Titan studies. A number of other platforms, including those based on the Helicos single-molecule platform or the Pacific Biosciences single-molecule real-time (SMRT) technology, are suitable for investigating the tumour genome of specific genes or defining novel somatic mutations in patients with solid and haematological tumours using a comprehensive DNA mutational analysis approach and have been developed during the past four years using novel sequencing technologies [20].

2. Fundamentals of Next-Generation Sequencing (NGS)

High-throughput (HT)/Next-Generation Sequencing (NGS) technologies were developed as part of the genome projects in the last 20 years. Sequencing techniques emerged in 2005 with powerful and highly versatile tools. Despite the slightly altered implications of life and disease determining genotype characteristics, NGS is one of the most important influencing technologies in the 21st century. NGS technologies have developed with continuously increasing potential characteristics, namely reduced time, cost, and necessary quantity of starting material with a variety of detection methodologies [21]. Consequently, the field has erupted significantly, thereby offering wider applications, including the diagnostics of rare genetic diseases based on gene panel, whole-exome, whole-genome, transcriptome sequencing, as well as mtDNA, viral, microbial, and metagenome sequencing.

2.1. Principles and Workflow of NGS

Massively parallel sequencing represents the most recent, state-of-the-art technology for DNA sequencing. Although several platform-based experimental workflows are currently used, certain general considerations apply to all approaches [22]. The two initial steps of sample preparation and subsequent generation of sequence clusters are largely common to all protocols. The first step is the further fragmentation of DNA to increase the efficiency of sample preparation. All analytical approaches for second-generation sequencing rely on the simultaneous analysis of a large number of individual DNA fragments. The creation of a DNA fragment library is the next sample preparation step. To do that, the required DNA fragments are generated by fragmentation, followed by the end-repair of generated fragments. Specific sequencing adapters, comprising complementary sequences to adapters conjugated on the flow cell's surface and to sequencing primers used for cluster generation and subsequent sequencing, are ligated to the DNA fragments [23].

The next step of the library generation involves experimental cluster generation on a solid-phase surface. The two main approaches used by scientists when attempting to achieve dense immobilisation of DNA on flow cell surfaces are solid-phase amplification and bridge amplification [24]. Both methods enable DNA clusters to be generated by the surface binding of DNA fragments. However, differences exist between the two methods. Solid-phase amplification uses polonies in which some DNA fragments form a cluster containing up to a million copies of a single fragment. These clusters are mainly used for the

comparative analysis of the molecular or genetic diversity of DNA fragments. By contrast, contemporary massively parallel sequencing methods use templates. Each of these covers both DNA strands. The first bridge-amplified DNA cluster generation methods were developed for chemiluminescent detection. However, more recently, modified bridge amplification methods have been developed that are based on the direct sequencing of fluorescently labelled cluster-forming brushes. Since bridge amplification has several advantages associated with read length and density-related issues, it is treated as a gold-standard DNA cluster-forming method [25]. In this approach, specific primers are hybridised to the DNA fragments at the precise position of the sequencing adapters. These primers serve as a template for DNA polymerase, which synthesises a complementary DNA strand. The start of the amplification occurs prior to the very first sequencing reaction. After the first amplification cycle, one primer becomes free and can repeat the cycle. This series of continuous intermediate replication steps produces denser clusters on the solid surface [26].

2.2. Workflow of NGS in clinical diagnostics: From sample preparation to data interpretation

Routine use of NGS in clinical diagnostics has required the development of standardised workflows that ensure consistent results. Considering the plethora of possibilities between sample collection and the delivery of clinically meaningful insights to the treating physician, we provide an overview of the steps required to master NGS-based diagnostics. Nevertheless, as technology evolves, the different stages of a diagnostic NGS workflow in the field of haematology will change, resulting in better sensitivity, lower costs, and user compatibility [27]. The choice of the different workflows to be used will depend on the size of the project, the quality and/or amount of starting material, and the equipment available.

Here, we present a workflow common to all kinds of NGS-based tests with no initial quality profiling of the patient's sample, as it does not require a high percentage of neoplastic cells, which is often the case in the field of haematologic diseases. Pre-established quality criteria for the NGS diagnostic test dictate certain quality criteria, such as only samples for which the calculated signal-to-noise ratio is greater than two and that any imbalance in the coverage aerogrammes is less than twofold around the value one is eligible for downstream analysis [28]. We also state that, in the input planes specified by sample type, the average values of the super active base area and the bases with a negative q4/qc for a matched normal sample have to be above 80% and below 20%, respectively [29].

3. Applications of NGS in Hematological Diseases

In recent years, numerous studies have demonstrated the potential of next-generation sequencing to revolutionise medicine, including haematological diagnostics. For that, NGS has been included in laboratory guidelines and in-patient work-up in the case of complex haematological diseases, such as acute myeloid leukaemia with normal karyotype alignment [30]. Regarding the higher mutation detection rate, NGS has already started to substitute first-tier analysis using techniques with limited throughput, such as Sanger sequence analyses. Among the overall advantages of NGS, its short turnaround time is of particular importance for the timely administration of specific therapy approaches [31]. In particular, methodologies for sequencing-based minimal residual disease monitoring have been described for leukaemia, such as acute myeloid leukaemia and chronic myeloid leukaemia, where rapid treatment decisions based on iterative genetic testing are crucial for the patient's outcome. In addition, the shift of treatment along with the primary goal mutation detection rate under target therapy modality provides a role for longitudinal NGS analysis in the context of polychemotherapy treatment protocols in the general population. Affected by the described clinical benefits, targeted NGS panels have been included in guideline rec-

ommendations, and the foundation of both National and European Medicines Agencies that regulate and provide trans-European access to NGS for diagnosing and treating children and young people allows insights into the utilisation of this technology in European Haematological and Oncological Departments. Aside from these and several other indications, few studies have undertaken a general diagnostic approach in larger cohort studies since, in most cases, specialised panels for a specific haematological disease are being utilised [32].

3.1. Diagnosis of inherited blood disorders (e.g., haemophilia, thalassemias)

Haematological diagnostics are among the medical fields that significantly profited from NGS sequencing. The vast majority of currently utilised inborn blood disease diagnostic and prenatal diagnostic sequencing assays are based on NGS sequencing [33]. Detection of causative variants or gene expression pattern testing, such as fusion gene detection, is straightforward by RNA sequencing of a prior transcriptome. Inherited bleeding disorders and thalassemia are two genetic conditions with an increased demand for diagnostics, both for the expected health gain and for a very high rate of structural changes, as the disease causative variants or expression regulator variants need to be distinguished [34]. This survey aims to highlight the main innovations in actual haematological diagnostics and related research in a wide approach from neonatology to molecular genetics [35]. In conclusion, NGS-based diagnostic panels commonly encompass numerous qualitative and quantitative disease causative variants and/or genes, providing ample gene testing data and achieving high diagnostic sensitivity and specificity in a single assay. This supports, reduces, or even abrogates the need for any labour-demanding and time-consuming routine laboratory techniques currently applied in the diagnostic practice of inherited blood disorders. One of the most significant disadvantages of the technique is the higher sensitivity of individual testing, as likely causative variants in several genes need to be considered whenever assigning the diagnosis to an individual, and these genes should be meticulously screened and evaluated at the same read depth for each gene [36]. As it is practically impossible to simultaneously amplify primer sets and/or the required DNA fragments at the same level with an acceptable workload, read depth coverage variation is typically inevitable even in a very well-designed and tested assay.

3.2. Characterization of hematological malignancies (e.g., leukemias, lymphomas, myelomas)

In recent years, the development of next-generation sequencing methods, also known as high-throughput sequencing technologies, has indeed provided a nearly complete view of the mutation landscape in various haematological malignancies [37]. These methods have been quickly adopted because there is an urgent need to discover clinically relevant mutations and, if possible, to develop personalised treatments for all of them. Researchers have found broad conservation of genetic alterations across cancers of a common tissue origin and have identified general principles of mutation categories and combinations that can define computations and tumour suppressor function. Despite an increase in the number of studies and the different haematological cancer samples analysed, only a few have focused on specific subtypes of cancer or compared the specific type to other common solid tumour types or tissues. These studies often described unique genetic alterations and gene mutations that are causative in the disease, as well as a myriad of non-causative, recurring, unclassified, uncharacterised alterations that represent competing candidates of disease causation [38]. Furthermore, they have significantly contributed to a better understanding of the mechanisms linking genetics with epigenetic modifications, transcriptomics, and protein-inflammatory pathways. These typing efforts were possible only because of the wide range of tumour-normal pairs of overlapping opening chromatin, DNA methylation, and matched RNA sequencing datasets for different haematological malignancies, with the generation of increasingly large

and cohesive datasets correlating comprehensive genomic and transcriptomic information with clinical outcome data [39].

3.3. Monitoring minimal residual disease (MRD)

Minimal residual disease (MRD) monitoring is a feature especially important in haematological malignancies and is usually related to the diagnosis or follow-up of chronic or acute leukaemia. In this case, PCR-based assays are currently serving as the gold standard due to their high level of accuracy and very high sensitivity in patient samples [40]. The underlying reason is quite obvious because neoplastic lymphoid cells generate clones with unique surface or cytoplasmic markers due to V(D)J recombination during development, and therefore, those cells can be solely distinguished from other cell types while healthy cells carry a non-functional V-region due to allelic exclusion, thereby using only one set of genes per cell for immunoglobulin light and heavy chains [41]. Consequently, the allele that is necessary for allelic exclusion can be taken as a unique biomarker. However, due to plenty of examples already reported, next-generation sequencing can at least be considered a possible option, especially for sorting different clonotypes to use specific PCR-based IMRD monitoring. Besides that, Sanger sequencing data is most likely accurate enough but not qualitatively appropriate for MRD monitoring.

In studies on multiple myeloma, the numerous sub-clones usually extend the number of targets to become specifically amplified with patient sample-specific PCR primer designs [42]. Due to target saturation, specific amplification failure does not seem to be a critical rate-limiting factor. Therefore, the Genescan approach may be successfully tested in performing MRD monitoring of multiple myeloma using modified protocols of detection. Unique is the repeated insertion of random nucleotides by the error-prone reverse transcriptase, which is connected to specific priming and increased specificity of transcription in the presence of the enforced loop introduced by the specific primer-template. With high-throughput deep-set next-generation sequencing, such errors could indeed enable the identification of time-dependent extinction of specific sequences repeatedly occurring in patient RNA [43]. Moreover, the concept seems to be feasible for other gene composition analysis tasks covering specific multigene assays.

3.4. Pharmacogenomics and personalised treatment strategies

Pharmacogenetics examines the response to drugs and seeks to understand the contribution of specific genetic factors to this interaction. Genetic variations influence drug metabolism and response. Genetic polymorphisms result in variations to a great extent in the level of expression of specific genes or in the specificity or efficiency of the expressed proteins or enzymes, which can lead to differences in drug efficacy or toxicity [44]. These variations can occur through different elements and splicing of the transcribed genes. Next-generation sequencing provides a comprehensive approach to evaluating DNA variability, especially with regard to single-nucleotide base substitutions and small insertions or deletions. Furthermore, it permits the evaluation of extensive panels of pharmacogenes simultaneously, which is especially beneficial in combination with diagnostic panels for multimedication or multiple treatments in individual patients. The risk of experiencing side effects because of drug interactions or the absence of response to a specific treatment could be reduced to a great extent by such information [45].

There are numerous examples of the importance of NGS for pharmacogenetic diagnostics. The genotyping of CYP2C19 and CYP2C9 has been established, especially for pharmacogenomic-guided dose adjustment of warfarin, sulfonamides, nonsteroidal anti-inflammatory drugs, and platelet aggregation inhibitors [46]. Mutations in CYP2C19 should be identified before initiating clopidogrel therapy because reduced CYP2C19 activity affects the antithrombotic effect of clopidogrel [47]. Pretreatment identifica-

tion of TPMT and NUDT15 genotype status reduces the risk of developing life-threatening myelotoxic complications from standard treatment with thiopurines, which is still widely used for childhood cancer. The detection of the presence of HLA-B5701 is strongly recommended before initiating abacavir therapy in HIV-infected patients because HLA-B5701-positive patients have a predisposition to developing a hypersensitivity reaction to abacavir [48]. The genotype in VKORC1 and CYP2C9 is strongly associated with the individual dose requirements for acenocoumarol and warfarin-dependent patients. The list of important polymorphisms is not comprehensive. Other examples with increased relevance for haematological patients exist: CYP3A4/5 and IL28B influence the efficacy of tyrosine kinase inhibitors and direct antiviral agents for treating chronic hepatitis C patients [49]; STK11 predisposes to a combination treatment of metformin and an mTOR inhibitor in TSC syndrome [50]; GCLC and ABCB1 are primarily associated with antineoplastic acquired multidrug resistance syndrome in childhood ALL patients. These examples emphasise the clear benefits of NGS-based diagnostics for individualised pharmacotherapy in haematology patients because unsafe drug reactions can be highly reduced [51]. The latest recommendation for drug labels based on a preexistence of valid pharmacogenetic associations is one result of this.

4. Challenges and Limitations of NGS in Hematological Diagnostics

A key challenge is to optimise the diagnostic and treatment algorithms, which are still far from perfect. This ultimately requires not only a better understanding of haematological neoplasia at the molecular level but also a detailed knowledge of the mutations that occur and their evolutionary dynamics. NGS offers unrivalled opportunities not only for sequencing genomes of malignant samples but also for investigating genetic heterogeneity, clonal structure, and clonal evolution in haematological cancer patients. Relevant technological applications and recent work are reviewed, including whole-genome and whole-exome sequencing, chromosomal translocations and SNP analysis, DNA methylation and its implications, as well as single-cell and ultra-deep sequencing, and the genome mining of patient-derived xenograft models [52]. The advantages and complications of applying NGS in blood cancers are evaluated; unique cancer-associated gene mutations, gene fusions, and splice isoforms are highlighted, together with the prognostic and predictive mutation and biosignature analysis, which can be routine, thanks to next-generation blood diagnostics [53]. The clinical applications and comprehensive translational properties of NGS in hematologic cancer diagnosis, treatment, and prognosis are evaluated. Advances and severe limitations of the hypermutation phenomenon across the human B-cell and T-cell receptor repertoires are discussed [54]. We also point out novel potential utility for classical pathology specimens and identify several strategic research tenets for NGS applications in the haematological neoplasia of today. Finally, we summarise the field's most promising directions, which will significantly impact the healthcare of tomorrow by utilising both the evolutionary concept and the mutation knowledge of several cancers.

4.1. Current limitations in NGS technology for haematology

One of the challenges in diagnostics based on high-throughput sequencing is a very large volume of clinical data generated by even a single patient sample [55]. To detect all types of variants across the entire genome through targeted DNA enrichment, thousands of independent fragments need to be sequenced in sufficient depth. This means that a single complete myeloid panel can deliver a vast amount of data—about 3 Mb in total. This is because it must cover all mutated coding regions of genes and has to be associated with substantial sequencing coverage, i.e., enough depth of coverage to detect low-variant allele frequency events [56]. The constant evolution of panel composition due to the discovery of new driver

mutations and the development of new drugs targeting new mutations creates a potential challenge in comparing sequencing data from different patients tested at different times. It is fundamental to standardise different methodologies and to achieve a high level of agreement. Critical evaluation of sequence analysis and robust diagnostic pipelines to detect disease-related mutations at low frequency is crucial. It is important to follow the recommendations for each platform and methodology suggested in recent literature.

With regard to difficulties in aligning reads to the rather long repetitive sequence stretches, all strong NGS mutation detection methods are able to properly identify numerous repetitive regions due to unique flanking regions [57]. This is a limitation not only of NGS but of clinical diagnostics in general, although certainly an important aspect of the application of NGS in diagnostics. However, real problems might arise if attempts are made to detect deletions or duplications. Studies have shown high agreement between different methods for identifying structural variants in a pool of cancer cell lines and have reviewed various tools, which are some of the useful methods for this aim. A bioinformatics tool aimed at the identification of exon deletions in next-generation sequencing data has been developed [58]. This is capable of identifying single- or multiple-event indel positions in one or several genes, given a reference set of normal and cancer samples. We believe in the necessity of this complementary analysis both for diagnostic and prognostic reasons and to monitor the response to therapy, and we suggest that different tools should be applied in parallel to obtain the most reliable results [59].

4.2. Data Analysis and Interpretation

Data analysis and interpretation is the last step in a comprehensive NGS workflow for haematological diagnostics. Monogenic, oligogenic, and multigenic defects detected are interpreted in context with affected clinical phenotype, and the final diagnosis is discussed and correlated with clinicopathological findings. Sequencing read data is first quality-checked and converted to variant call format files to capture random errors. Annotation using large, manually curated variant databases and disease-specific mutation databases helps to quickly and accurately identify medically relevant genotypes. The filtering algorithm plays an important role as irrelevant variants are excluded first by applying common population allele frequency, as well as functional, genetic, and clinical guidelines [60].

Typical times for the development of sequence data, bioinformatics analysis, and generation of preliminary reports range from two to five weeks. Generally, clinical NGS services aim for a turnaround time of two to ten days. Therefore, standardised bioinformatics pipelines are pivotal to decreasing times from data generation to tablet [61]. While NGS and software development are excellent means to increase the depth and scope of genomic testing in haematology, we should not forget the consequences of technically based inadequate counselling and informed consent when dealing with 'uncertain findings'.

5. Integration of NGS into Clinical Practice

NGS platforms are capable of generating huge amounts of data from different biological samples in a very short period of time. Applying NGS in the diagnostics of acute leukaemias urgently raises the question of the clinical management of this rush in genomic and transcriptomic data. This chapter provides a brief overview of the stages from sample collection in a hospital to reporting results from an NGS-based report generation software. Most of the steps in sample processing are done according to the standard steps for NGS sample preparation. We outline unique specifics of using this technology for haematological diagnostics and provide a scheme and a general workflow for NGS sample collection until the final case report.

Patients and Sample Collection

Modern diagnostics of acute leukaemias require the collection of different types of samples from bone marrow, peripheral blood, and, in certain cases, lymph nodes or extramedullary infiltrate. Total RNA material is isolated from the primary sample, and then it is reverse transcribed to the first strand cDNA. For NGS library preparation, it is necessary to obtain double-strand cDNA to ensure the presence of the necessary adapters in the molecule [62]. In the case of DNA library preparation and certain cDNA NGS library preparation protocols, the sample processing may vary a bit.

Sample Preparation

When the NGS library preparation is finished, sequencing adapters are added to the cDNA in the process. These are necessary for the ligation of the sample to the flow cell and for the further primer annealing stages involved in template amplification and sequencing. The successful addition of the sequencing adapters is confirmed by the so-called library control qPCR assay, which is designed to confirm the presence of adapters at both ends of the molecule before the sequencing run starts [63]. Dreadful values are likely to fail the further sequencing.

5.1. Clinical Decision-Making

During the last decades, the development of antineoplastic treatment strategies for cancer patients was unrivalled in medicine. The most important tool for appropriate clinical decision-making was to prepare and analyse tumour and somatic tissues from bone marrow or peripheral blood. However, with the rise of NGS, the existing technologies and strategies for testing unfavourable genetic markers for the current therapy of cancer patients are extended in routine diagnostics, leading to early initiation and refinement of a drug selection strategy not only in patients with a highly increased risk of relapse but also in patients with a decreased neoplastic burden [64]. Additionally, recent developments, including multiplatform remote DNA biopsy tests and the introduction of molecular data and somatic illness data of tumour boards for data-based pharyngoscopy planning, might shift further toward the aspiration of individual precision pharyngoscopy for all patients with complex malignancies.

The diagnostic approach for FLT-DTKS is used as an important and paradigmatic example for demonstrating the power and perspectives of targeted mutant testing at diagnosis, disease monitoring during and after antineoplastic treatment, and serial targeting at progression and relapse of patients with complex haematological malignancies [65]. As illustrated with several case studies, all of these different testing phases feature specific usage of custom-designed NGS gene panels that allow robust and cost-effective mutant capture diagnostics with high diagnostic yield. Repeated FLT-DTKS studies not only identified relieving IR gene mutations occurring at specific time points but also revealed changes in mutant disposition and heterogeneity of gene mutation modes that are diagnostically relevant [66].

5.2. Standards for NGS in clinical settings (e.g., CAP, CLIA guidelines)

With these advances, NGS has raised the potential to improve both clinical care and translational research studies across different haematological diseases, including leukaemias, lymphomas, myeloproliferative neoplasms, and myelodysplastic syndromes. Leading multicenter trials in malignant and non-malignant haematology meeting federal standards suggest that this technology is ready for introduction. Standards for NGS in some areas of clinical diagnostics are available now, with the most widespread guidelines already in place for those meant for tumour profiling. However, no uniform guidelines are available across all diagnostic applications or from a worldwide perspective in the various fields of medicine [67]. In the field of clinical laboratory tests, accreditation standards are available only on a national basis in some cases. The absence of standards in some fields represents a significant hindrance to the de-

velopment of NGS.
These and other concerns have led to conflicting reports from investigators and sometimes delays in the implementation of NGS into routine clinical practice. International and inter-professional panels should be coordinated in the development of clinical practice guidelines, elaborating every critical clinical-interpretative aspect of NGS to define the scientific basis and clinical application of NGS based on methodological treatments [68]. This standardisation would instead offer a huge advantage for many patients and the clinical community as well. The final objective is a new environment of international professional guidelines that offer a set of international standards, which can be easily implemented through recommendations from the national guidelines of individual countries to develop uniform quality measurements in this rapidly evolving set of methodologies [69].

5.3. Challenges in assay validation and reproducibility

Clinical laboratory genetic testing requires commitment in resources and time to develop, validate, and implement. Despite the commercialisation of many gene panels, the fundamental problem is that comprehensive panels are unique, needed only at a rare frequency, or customised to a familial sequence variant required for relative testing. Although robust guidelines exist to develop panel-based NGS research assays, these guidelines do not cover a clinical setting where the assay is utilised in service in investigative studies for the identification of therapeutic targets or advancing scientific research. The complexity of some genes and the number of reference library sequence mutations create the possibility of primer matching with data inaccuracies, inconsistencies in the mapping of sequence reads, mapping of duplicated reads, or the risk of incidental sequencing of a homologous target gene. An important, common error is the failure to read small insertion/deletion sequences, resulting in the consistent misassignment of genotype. Batch analysis of any reads at the time can be validated with positive and negative controls, certified calibrators, and routine performance of commercially available reference sequence control assays. Accurate pre-PCR sample qualification is essential for the control of error and NGS utility.

5.4. Cost-effectiveness and accessibility in diverse healthcare systems

While the price of NGS has considerably decreased, the benefits of using NGS technologies cannot be accessible to all patients in routine haematological diagnostics. The most recent cost-effectiveness analysis of studies using NGS examining five different conditions found that the use of NGS in haematological diagnostics was only marginally cost-effective at the diagnosis step and not cost-effective at the monitoring step in most EU member states [70]. The only three exceptions to this were priced at very moderate estimates for establishing the minimum utility and time cost in the EU. However, they emphasise that their results rely on parameters that are not reliably available. According to the cost-effectiveness model, NGS testing was cost-effective in specific situations [71].
Moreover, diverse healthcare systems globally, including uninsured and underinsured patients, could face barriers to the use of NGS. The reduction in both the sequencing costs and the price of bioinformatic tools paves the way for NGS application in a variety of medical disciplines [72]. There are four challenges regarding the cost and price of an NGS test: the sequencing cost, the validation phase, the maintenance cost of the bioinformatic tools, and the fact that NGS tests may provoke a cascade of expensive reflex tests while the need for supporting legal, ethical, and social issues are reported. The widespread use of NGS-related corollary tests, from extended versions of well-established classical techniques to full NGS, may reduce costs and improve overall access to specific medical services.

6. Future Directions and Emerging Technologies in NGS

Target NGS, which involves resequencing only known clinically relevant loci, is an alternative to WES. It enables screening for a larger number of disease-associated genes than panels based on other methods. Importantly, resources can be focused on the known regions and avoid trapping thousands of non-informative intronic regions. Because the technology is designed to only sequence areas of known relevance, it offers a very high accuracy level, easily meeting the high coverage and very high accuracy that are generally required for clinical diagnostics [73].

For some diseases, such as HLA typing, targeted sequencing of certain genes or coding exons may be insufficient to identify patients who would benefit from clinical RNA interference, antisense oligonucleotide therapy, or other genetically based drug response modifier therapies. Consequently, researchers are also exploring transcriptome and whole-genome sequencing of patients to assess RNA splicing abnormalities. Screen-reading software platforms are required for pathogenic coding changes to be identified, and in general, the sheer volume of data generated by NGS necessitates bioinformatics expertise. Genomic data interpretation, annotation, and storage are also major challenges and opportunities for the field of medical genomics [74].

6.1. Single-Cell Sequencing

The authors would like to acknowledge high-dimensional single-cell analysis in rare malignant hematopoietic cells. Single-cell sequencing technology provides a comprehensive understanding of the progression and fate of cancer cells and their microenvironment. This technology reveals the complexity of individual immune cell types, the spatial distribution of immune cells, the transcriptional activities of immune cells, lymphocyte receptor repertoire, phylogenetic distance group, reconstruction of cell clonal architecture, lineage tracing, construction of tumour pseudotime, and so on [75]. Moreover, at present, time-dependent single-cell RNA sequencing and TCR/BCR sequencing are widely used to trace cell differentiation processes and cell fate changes in haematology and malignant neoplastic research. By using advanced medical genomics technology, haematological malignancies have made certain developments in the spatial distribution and fate lineage of hematopoietic tumours, which helps to clarify confusion in related haematology and neoplastic problems and expands our understanding of hematopoietic neoplastic diseases.

The analysis of complex cell populations, such as in the tumour microenvironment, is facilitated by single-cell RNA sequencing. This allows for capturing the complete landscape of cellular compositions, their proliferation states, and corresponding exogenous signalling cascades within the microenvironment. In addition, single-cell technologies have also made feasible the assessment of modules of immune receptor profiling or rugged cellular barcoding to allow for cellular lineage tracing. Further, emerging technologies such as prime editing, single-time guide RNAs, or CRISPR trace integration that are refining gene-editing capabilities are expected to provide applicable functional studies of single-cell populations. Such studies are expected to be carried out for individual lineages or in their evolutionary context during treatment response patterns, alterations of cellular dynamics, clonal evolution, or treatment resistance properties [76].

6.2. Emerging trends: AI integration, multi-omics approaches

The integration of AI methods with NGS data remains at the cutting edge of bioinformatics research. Through its integration with machine learning and deep learning algorithms, NGS data preprocessing, QAQC, biomarker identification, and thus the clinical utility of NGS applications is certain to greatly benefit [77]. As NGS is, at its core, high-throughput, multi-dimensional data processing, it can be re-

garded as a multi-omics technology. It is not an inherent feature of NGS to generate only transcriptomic, miRNA, epigenetic, or chromatin conformation data [78]. The development of comprehensive single-cell NGS has enabled the generation of multimodal gene and protein data, which pushes towards single-cell multi-omics. Such single-cell multi-omics data not only empower but also call for the development of new statistical and computational methodologies, in their alignment and integration, for accurate and informative concordance in the complementary features present.

Systematic data collection discussing the massively multimodal nature of single-cell multi-omics—the generation of multimodal features of tens of thousands of cells—all hitherto appear manageable, reporting total data numbers that are relatively modest from the machine learning perspective. Single-cell NGS may scale up, so mass analysis will become a real burden. An AI-powered clustering and annotation tool can assist in data analysis, making it both fast and accurate. Whether innovations of this kind are already being developed and tested in the single-cell multi-omics community, an integration that will surely promise scientists and clinicians who use NGS methodologies optimally informative and deeper insight into biological processes is not yet an actively explored area [79].

7. Ethical and Legal Considerations in NGS

Next-generation sequencing technologies are revolutionising medicine and healthcare today [80]. Despite the enormous potential of these platforms, a number of challenges still restrict the implementation or routine application to patient care [81]. There are substantial ethical and legal implications associated with the generation and interpretation of sequencing data in a clinical setting [82]. Patients need clear pretest information about the aim, significance and limitations, risks related to obtaining, testing and storing their DNA, and the potential for incidental findings [83]. A particular challenge to ensure the safe and beneficial application of NGS technologies is to make sure to harmonise the introduction of advanced technologies with ethical, psychological, clinical, legal, and societal implications in line with accelerating new tools in sequence and information processing [84].

With the installation of NGS methods in clinical routine diagnostics and the increasing number of novel NGS-based diagnostic tests, periodic updates of general and test-specific guidelines and laws, particularly related to genetic counselling and informed consent procedures, are essential for the protection of patient rights [85]. Thus, a bi-directional communication between technology developers, health service providers, government and patients must be implemented [86]. The worldwide increasing efforts on guidelines for the use of NGS technologies represent a major advancement but still do not cover all the different NGS field applications [87]. With the ongoing affordable price of NGS, we thus expect an increasing complexity of the ethical and legislative debate in the near future [88].

7.1. Patient Consent and Data Privacy

The increase in next-generation sequencing analysis also raises questions about the handling of the data and the associated ethical implications and data privacy [89]. The increasing use of NGS for scientific or medical purposes also confronts geneticists and physicians with the duty to provide information and counselling for patients, relatives, and the public based on best clinical practice and scientific evidence. Legal repercussions may arise from errors or incomplete interpretations of sequencing data, as well as the misuse of this data [90]. While considering data and intellectual property rights, it has to be ensured that medical data are primarily used in the best interest of any patient. The optimal and safe use of genome and exome data for clinical and research groups requires several measures that exceed the actual generation, data analysis, and interpretation of NGS datasets. Highly reliable and well-maintained data

repositories, as well as adequate data security standards, including software and hardware, will be of utmost importance in the future when considering the possibility of performing standardised analyses in different applications as well as international collaborations [91]. In addition to these technical requirements, enhanced international collaborations in patient cohorts, standardised experimental designs, and optimal use of biological resources are essential and can be facilitated by optimal preparation and commercial or medical exploitation of molecular data [92]. Thus, the ethical implications of genome and especially exome sequencing remain challenging and require continuous consideration in science, diagnostics, and clinical trials involving NGS methods within the research community, the medical community, and governmental bodies [93]. Several initiatives with respect to the stabilisation of legislative regulations have already been initiated and have to be continued.

7.2. Implications of incidental findings in haematological contexts

The focus of NGS-based haematological diagnostics is the assessment of a specific genetic disorder. Nonetheless, next-generation studies also include disease-causing gene mutations as well as a variety of incidental findings. Such findings vary greatly and might not necessarily be expected to influence health, especially in light of the variability of penetrance and expressivity of various disease-causing mutations. These questions are more than valid, particularly for stratified analysis in children. On a more general scale, the frequent detection of pathogenic cancer-related gene mutations in healthy individuals and the detection of disease-causing gene mutations in a percentage of unaffected siblings provide further evidence for limited or age-dependent penetrance with unknown implications on gene mutation discrimination. However, given their rate of detection and the potential implications for the health of analysed patients, such findings should still be reported according to good practice guidelines, especially to prevent unnecessary investigations or suspected health risks.

7.3. Regulatory hurdles for NGS-based diagnostics

To unlock the potential of NGS-based precision medicine for haemato-oncological patients, various stakeholders may need to address regulatory hurdles. These comprise certification and accreditation of laboratory-developed tests in which the vast majority of clinical NGS testing is performed, and there are marked variations in usability, performance, and quality among the assays offered by individual laboratories [94]. Enhanced standardisation and clinical validation of the assays, which are pivotal to defining decisive requirements for independent quality control, proficiency testing, benchmarking, and biobanking, are necessary. Ensuring that information regarding NGS test specifications and clinical validity can be made public is essential. These requirements are not necessarily met by strict approval processes. In practice, however, NGS-category clinical studies can fall under supplementary regulatory restraints and expenses. The nature of the situation is encapsulated by comparing information on which genes and variants should be analysed as an initial genetic test for individuals with a relevant personal history to several diverse national NGS guidelines [95]. Given the rapidly increasing pressure from payers, European consortia are focused today on quality performance improvement in clinical NGS testing.

8. Conclusion and Summary

Next-generation sequencing has revolutionised the practice of medicine in many fields, and it has been an essential tool for advancing our understanding of the pathogenesis of haematological malignancies and achieving more accurate, high-throughput diagnoses in clinical haematological laboratories. Unlike single gene assays, NGS allows testing for a comprehensive set of target genes simultaneously, with a single DNA or RNA input in different sample types. Although there are some challenges in incorporat-

ing NGS into clinical reports, such as measurement error, artefacts, variant interpretation or reporting, and compliance with the accreditation standards for clinical laboratories, NGS remains a unique and essential platform to use for the genomic profiling of haematological patients. The healthcare service system and social environment also need to be prepared for this new rapid, innovative, and knowledge-based diagnostic approach. We anticipate that the improvements in the above issues can be achieved through continuous innovation and development. The diagnostics and treatment of haematological disorders are being transformed by excellent, high-throughput, comprehensive technologies. Unravelling the molecular architecture also affects patient management. The application of NGS technology to clinical medicine has been implemented faster in academic research centres as a result of the rapid enhancements and decrease in the cost of technology. Some traditional community healthcare systems are not well-prepared for the arising issues, such as international collaboration or data sharing and patient informed consent for genomic analysis. Data security should also be a concern. All of these, taken together, indicate that next-generation sequencing technology can work as a powerful driving force for successfully managing and diagnosing patients with haematological malignancies.

8.1. Summary of NGS innovations and their clinical relevance

Next-generation sequencing is set to revolutionise DNA sequencing in human complex disorders, including pathogenesis studies and personalised treatment strategies. Here, we aim to discuss state-of-the-art NGS innovations and their use in the setting of haematological disorders, i.e., ascertaining clonal architecture in complex as well as small malignancies, the status of individual biomarkers in association with treatment and minimal measurable residual disease, germline mutations to build the hereditary background to the disease, and the host tissue microenvironment. Moreover, it is currently evolving and resulting in further insight and expansion of the mutational landscape of haematological disorders, leading to the identification of novel subgroups. This does not only pertain to rare diseases but can also help identify patient versus healthy samples, as well as subgroups in common complex diseases, for which it has become clear that a fair portion of so-called somatic mutations are actually derived from persistently expanded immune clones of the peripheral immune system, which rests upon healthy genotype clusters. Further, our evolving understanding of specific clonal biomarkers, whether they are derived from direct functional insight into clinically applicable nucleotide innovations or through overall treatment guidelines using shorthand NGS-based molecular evaluation, needs to be described.

8.2. Potential to transform haematological diagnostics

Mankind has been infatuated by the diagnosis, treatment, and cure of limited repertoires of blood diseases since early antiquity. The wide array of blood diseases, therefore, has earned attractive appellations such as "the royal diseases" and "the princes of haematology." "Hereditary," "congenital," "inherited," and "acquired" blood disorders are used to reflect the aetiology of blood diseases, and leaders in this area were mostly wealthy or leaders in other areas prone to consanguineous marriages. It is our great fortune to have next-generation sequencing technology and to have had that vast knowledge of human genomes. The influx of insights over the last two decades is transforming clinical applications with respect to whole genome sequencing and whole exome sequencing. These advances can be expected to profoundly change the future of haematological diagnostics, not only for diagnosis but also for predicting, preventing, and treating for generations to come.

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