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Critical Reviews and Perspectives

Toward the use of nanopore RNA sequencing technologies in the clinic: challenges and opportunities

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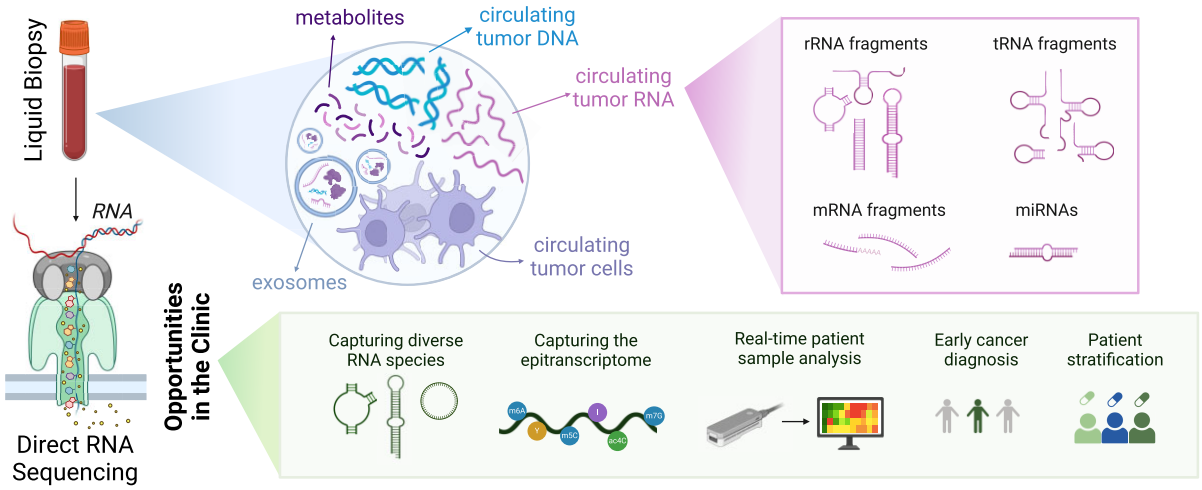
Abstract

RNA molecules have garnered increased attention as potential clinical biomarkers in recent years. While short-read sequencing and quantitative polymerase chain reaction have been the primary methods for quantifying RNA abundance, they typically fail to capture critical post-transcriptional regulatory elements, such as RNA modifications, which are often dysregulated in disease contexts. A promising cutting-edge technique sequencing method that addresses this gap is direct RNA sequencing, offered by Oxford Nanopore Technologies, which can simultaneously capture both RNA abundance and modification information. The rapid advancements in this platform, along with growing evidence of dysregulated RNA species in biofluids, presents a compelling clinical opportunity. In this review, we discuss the challenges and the emerging opportunities for the adoption of nanopore RNA sequencing technologies in the clinic, highlighting their potential to revolutionize personalized medicine and disease monitoring.

Graphical abstract



Introduction

RNA modifications and long-read sequencing technologies

RNA molecules have been the subject of extensive study over the past decades. While the function and regulation of messenger RNAs (mRNAs) have garnered the most attention in the past few decades, increasing evidence has highlighted the equal importance of noncoding RNAs (ncRNAs). Numerous studies have shown that, similarly to mRNAs, ncRNAs are also dynamically regulated across various cell types, throughout the cell cycle, in response to environmental stimuli, and in disease conditions [1]. Thus, it appears that both the coding and noncoding transcriptomes appear to convey more detailed insights about cellular states than was previously recognized.

In addition to exhibiting dynamic expression levels, RNA molecules can be extensively modified through various "epitranscriptomic" modifications, such as methylations,

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isomerizations, deaminations, and acetylations [2, 3], among others (Fig. 1A). These modifications expand the functional lexicon of RNA molecules, influencing the fate and activity of RNA molecules by fine-tuning their function, subcellular localization and/ half-life [4–6]. Furthermore, some RNA modifications can be dynamically regulated and even reversed, with enzymes such as FTO capable of removing N^6-methyladenosine (m^6A) and/or N^6-2′-O-dimethyladenosine (m^6Am) modifications from mRNAs [7, 8].

A substantial body of research has demonstrated that RNA modifications, along with the enzymatic machinery responsible for "writing", "reading", and "erasing" them, are frequently dysregulated or mutated in numerous human diseases, including diverse cancer types and a wide range of neurological diseases [9, 10] (Fig. 1A). This has brought RNA modifications to the forefront of drug development efforts, with both academic laboratories and pharmaceutical companies focusing on them as potential therapeutic targets [11–13]. These modifications not only hold potential as biomarkers for disease diagnosis but also as targets for novel treatments across a wide spectrum of diseases [14].

To validate the mechanisms of action of drugs targeting the RNA modification machinery, as well as to further understand the role of RNA modifications within cells, the scientific community has invested considerable effort in developing precise mapping techniques capable of detecting various epitranscriptomic marks across the entire transcriptome [15–19]. Notably, such readouts are critical to advance any therapeutic molecule targeting the epitranscriptomic machinery toward clinical use and eventual approval.

While next-generation sequencing (NGS)-based approaches initially revolutionized the field by enabling transcriptome-wide mapping of several RNA modifications (e.g. m^6A, Ψ, I, m^1A, m^7G, m^6Am, hm^5C, and 2′O-Me) [20–27], the field is gradually shifting toward nanopore sequencing technologies, such as those commercialized by Oxford Nanopore Technologies (ONT), which allow for sequencing of native RNA molecules, including their modifications [28]. The DRS platform allows researchers to simultaneously analyze both the transcriptome and the epitranscriptome in a single experiment, without needing to preselect specific RNA modification types that might be relevant to a particular condition or disease.

In nanopore sequencing, DNA or RNA molecules are translocated through protein nanopores embedded in a membrane connected to an ammeter. As each molecule passes through the nanopore, it causes characteristic disruptions in the electrical current, which are specific to the nucleotide sequence at that given moment. These current alterations are then translated back into nucleotide sequences using machine learning algorithms in a process known as "basecalling" [29]. RNA modifications can then be detected through three primary methods: (i) by measuring alterations in the current intensity signals during sequencing [30–32], (ii) by identifying systematic base-calling "errors" that are the result of RNA modifications at specific positions [33–36], or (iii) by using modification-aware base-calling models pretrained to detect specific types of RNA modifications [37] (Fig. 1B). For a comprehensive comparison of these methods, the reader is directed to several excellent detailed reviews [38–40], as this topic lies beyond the scope of this review.

The era of sequencing in liquid biopsies

The discovery of relatively stable extracellular RNAs in blood plasma has generated considerable interest in their potential use as noninvasive biomarkers. Unlike traditional tissue biopsies, liquid biopsies (LB) involve the collection and analysis of clinical fluids such as blood, saliva, urine, cerebrospinal fluid, pleural effusion, or bile for diagnostic, monitoring, and treatment purposes. The key advantage over tissue biopsy lies in their noninvasive or minimally invasive nature [41, 42]. Research in this field has primarily focused on identifying and detecting circulating biomarkers, including DNA, RNA, proteins, and metabolites, which may be found in circulating tumor cells [43], exosomes [42], or in free forms, such as cell-free DNA (cfDNA) [44, 45] or RNA (cfRNA) [46–48]. These biomarkers have been explored across diverse clinical contexts, including Alzheimer's disease [49], cardiovascular diseases [50], pregnancy complications [51], infectious diseases [44], and cancer [42, 47, 52–54], among others (Fig. 2).

Initial attempts to commercialize assays for cancer diagnosis using LB samples focused on detecting genetic alterations in circulating tumor DNA (ctDNA) from plasma samples. For instance, GRAIL's multi-cancer early detection tests, which use NGS to analyze cfDNA populations in peripheral blood, have shown promise in detecting pan-cancer signals [55]. RNA-based assays, while also commercially available, remain a relatively unexplored area [56]. Most efforts in this space have focused on the targeted capture of specific RNA populations, such as microRNAs (miRNAs) [57–59], mRNAs [46, 47, 60], long noncoding RNAs (lncRNAs) [61, 62], and tRNA-derived fragments (tRFs) [63–65]. One example is the company Exai Bio, which has developed assays for early-stage cancer detection based on orphan RNA signatures previously identified in patients with metastasis [66]. These assays have shown promising results in the early detection of invasive breast cancer and ductal carcinoma [67]. More recently, SLiPiR-seq, a specialized library preparation protocol developed to capture cfRNAs from plasma samples regardless of their 5′ ends, has proven successful at detecting early-stage tumors in a panel of diverse cancers [48]. Finally, long-read sequencing (LRS) methods have also been applied for early cancer diagnosis, in this case, by selectively capturing RNAs originated from transposable elements (TEs) and other repetitive regions. These repeat-originated RNAs, known as "oncRNAs", were found to exhibit significantly altered expression patterns in cancer patients, making them potential disease-specific diagnostic biomarkers [68].

In contrast, the use of RNA modifications as disease biomarkers remains largely underexplored. Pioneering studies have primarily relied on mass spectrometry-based methods to characterize RNA modifications in clinical samples [69], [70], with sequencing-based approaches being less common. Although nanopore native RNA sequencing has been available since 2017 and has demonstrated its ability to accurately identify and quantify native RNA modifications [30, 37, 39], its application in clinical settings remains rare. Given the well-established association between RNA modification dysregulation and various diseases, the question arises: what is preventing the adoption of DRS in clinical practice?

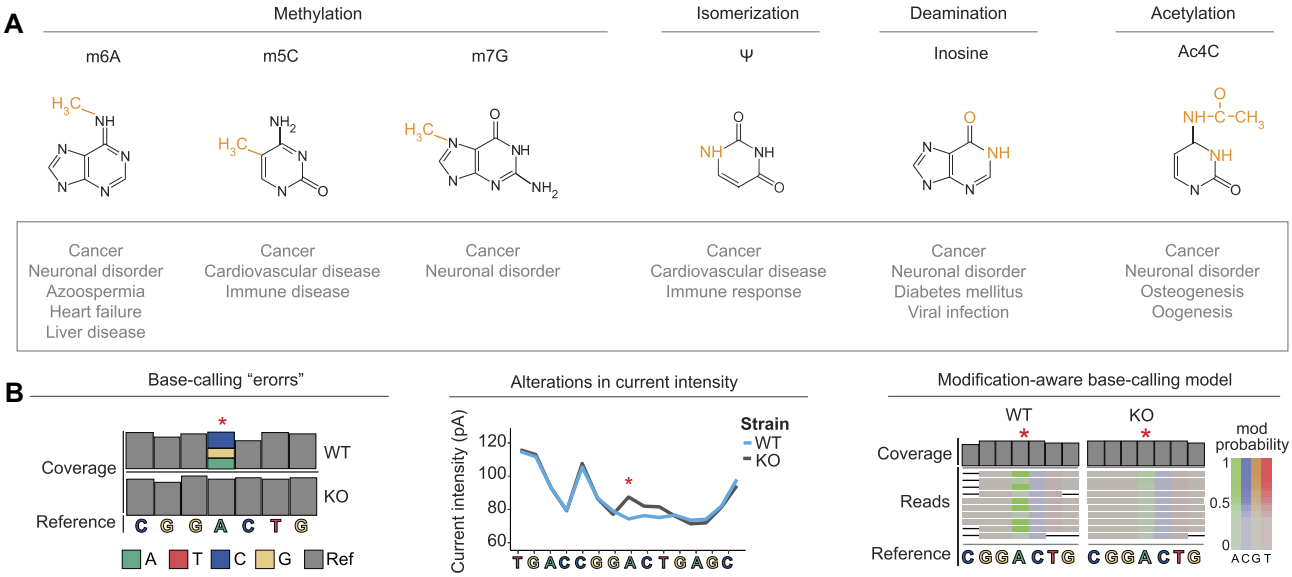

Figure 1. RNA modifications and their detection using direct RNA sequencing (DRS). (**A**) RNA modifications are chemical decorations to different parts of RNA molecules. Main types of RNA modifications are illustrated, with a different color highlighting where the alteration occurs. Examples of diseases associated with these modifications are listed below each modification. See also Supplementary Table S1. (**B**) RNA modifications in DRS datasets can be detected from base-calling "errors" (left), alterations in current features (middle), or using pretrained modification-aware base-calling models (right).

Challenges and potential solutions for DRS implementation into the clinic

The information-rich nature of RNA molecules, combined with the ability to capture them through native RNA sequencing methods, positions DRS as a promising approach for clinical applications. However, despite its potential, DRS is not yet routinely employed in clinical settings. Several challenges hinder its adoption, particularly when applied to liquid biopsy samples. Below, we summarize the key challenges that have so far prevented the adoption of DRS in clinical settings and outline potential solutions (Fig. 3).

High RNA input amounts are required

One of the main obstacles limiting DRS in clinical use is the substantial RNA input required to build DRS libraries. ONT's current native RNA kits (i.e. "RNA" flowcells and SQK-RNA004 kits) recommend starting with 300 ng of poly(A)-selected RNA or 1 ug of total RNA. However, liquid biopsy samples, such as plasma, yield much lower RNA amounts—typically ~10–35 ng of RNA from 9 ml of plasma [71]—making the implementation of DRS in such samples impracticable.

One possible solution to overcome this limitation is the use of multiplexing. Multiplexing involves pooling multiple samples into a single sequencing run, which could increase the total RNA input to the required levels. For example, pooling 100 samples into one flowcell could yield ~1 ug of total RNA, meeting the input requirements for DRS library preparation. Although this approach would reduce the sequencing yield per individual sample, the information-rich nature of RNA molecules may offset the loss of data by providing more detailed insights into the RNA modifications of each sample. While ONT does not yet offer commercial solutions for multiplexing DRS, community-driven efforts have demonstrated its feasibility [72, 73]. These studies show that DRS multiplexing is technically possible, and if further developed, could become a viable strategy for working with low RNA yields from LB samples.

Short reads are still not efficiently captured

Short reads have historically been challenging to capture efficiently using nanopore sequencing, which was initially developed and established as a LRS platform. Until recently, this technology was believed incapable of detecting RNA molecules shorter than ~100–150 nucleotides [74, 75]. However, recent studies have revealed that the *MinKNOW* software, which extracts FAST5/POD5 reads from raw current intensity data, was responsible for discarding short RNA reads by mistakenly identifying them as "adapters" [76]. Notably, manual adjustment of *MinKNOW*'s configuration parameters has been shown to increase the recovery of short RNAs from DRS by 12-fold [76], confirming that *MinKNOW* parameters were a primary factor in the loss of short RNA reads. It is important to note that in the last year, ONT has updated the default settings in the latest versions of *MinKNOW*, compatible with latest RNA chemistries (SQK-RNA004), enabling the detection of reads longer than 50 nt. However, this improvement remains insufficient for the efficient capture of RNA present in LB samples, where RNA populations are predominantly composed of shorter RNA molecules, i.e. 22–45 nt in circulating free RNA [71, 77] and ~100 nt in exosomal RNA [78].

Loss of 15 first nucleotides from each RNA molecule

In DRS, RNA molecules are translocated through a nanopore from their 3′ end (3′→5′), with translocation occurring at a relatively constant speed due to the helicase protein attached to each RNA molecule during the library preparation. However, the last ~15 nt at the 5′ end of each RNA molecule pass through the nanopore without the helicase assistance, causing these nucleotides to translocate much faster. As a result, the basecalling algorithm is unable to basecall these final ~15 bases accurately, leading to their effective loss [76, 79].

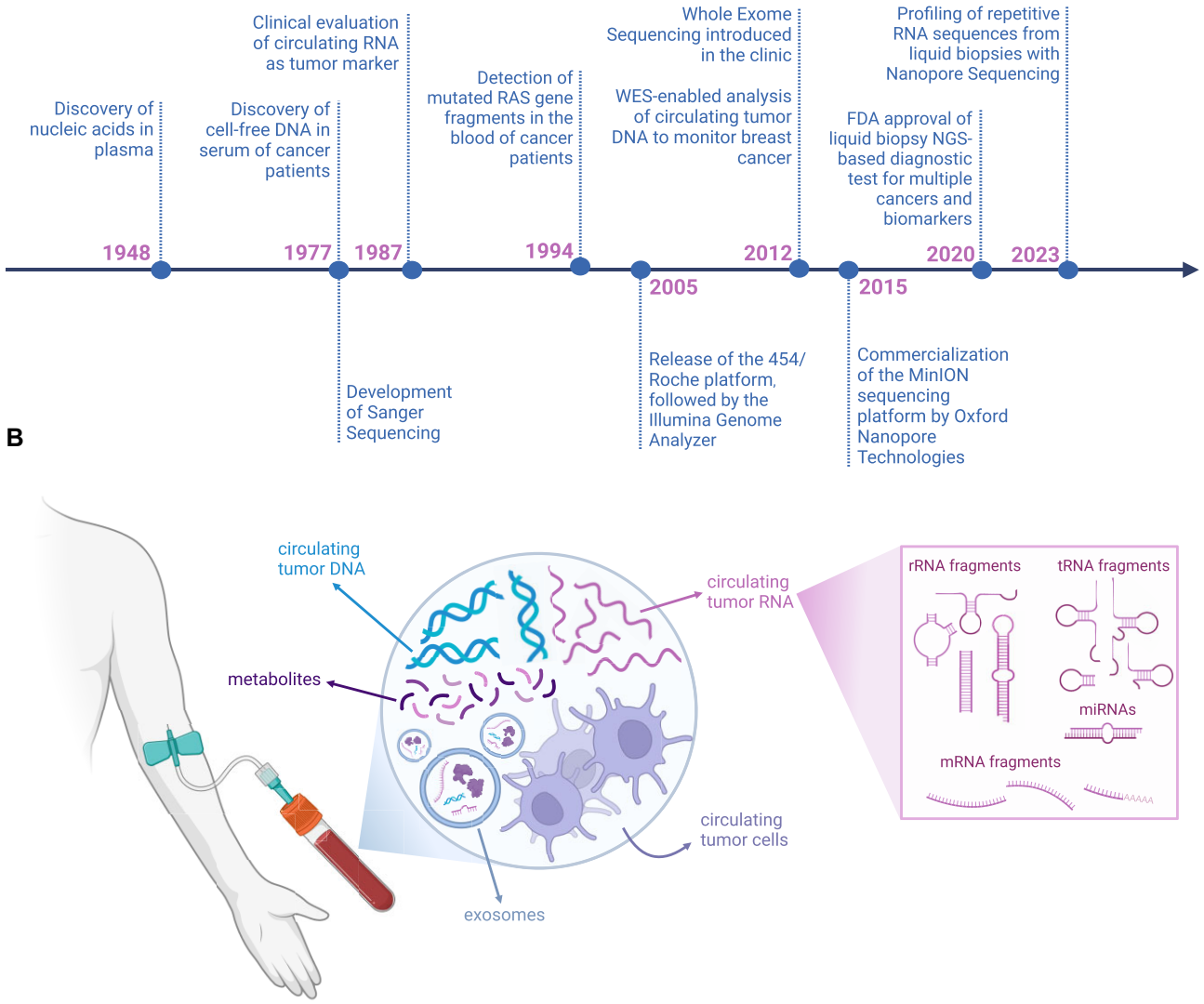

Figure 2. Clinical sequencing milestones and biomarkers in plasma. (**A**) Timeline of biological, technological, and clinical milestones for the utilization of nucleic acid biomarkers in the clinic [68, 132–138]. See also Supplementary Table S2. (**B**) Schematic overview of the sources of RNA molecules present in the blood, illustrating the RNA biotypes that are being currently used and/or studied as tumoral biomarkers, as well as the reservoirs in which they are studied (free, exosome, and tumor cells). Created in BioRender. Novoa, E. (2025). https://BioRender.com.

Several studies have attempted to address this issue by employing techniques such as splint adapter ligation [74, 76] or cap-dependent click chemistry ligation reactions [80] to extend the 5′ ends of the RNA molecules. However, this remains an unsolved problem for LB samples, where the composition of 5′ RNA ends is unknown, rendering these approaches impractical for use in such contexts.

Sequencing yield is significantly lower than other platforms

Unlike amplification-based sequencing methods such as Illumina and PacBio, DRS sequences native, unamplified RNA molecules, which avoids biases introduced by polymerase chain reaction (PCR), but results in substantially lower throughput. This limitation can be particularly problematic for low-input samples, such as plasma. However, certain improvements in the new sequencing chemistry have been shown to increase the sequencing throughput up to 10-fold, indicating potential progress in addressing this issue [81].

Lack of "ground truth" standards

A critical prerequisite for adopting a given sequencing technology into clinical practice is the availability of "standards" that can be used to calibrate, benchmark, and validate the instruments, methodologies, and clinical results [82]. However, the epitranscriptomics field lacks well-defined "ground truth" samples and reference datasets that can be uniformly employed across laboratories. Specifically, we lack mixtures of RNA molecules of known sequence, abundance, and modification content that can be introduced during the library preparation step to serve as intrinsic calibration standards. Analogous tools, such as the ERCC, SIRVs, or "sequins" [83], are commercially available for calibrating RNAseq libraries, but no equivalent exists for epitranscriptomic applications. Recent efforts to generate synthetic *in vitro* transcribed standards, such as the "curlcakes" [84], have been proposed. However, the need for independent production of these synthetic datasets by individual laboratories undermines their consistency and robustness, rendering them inadequate for clinical implementation.

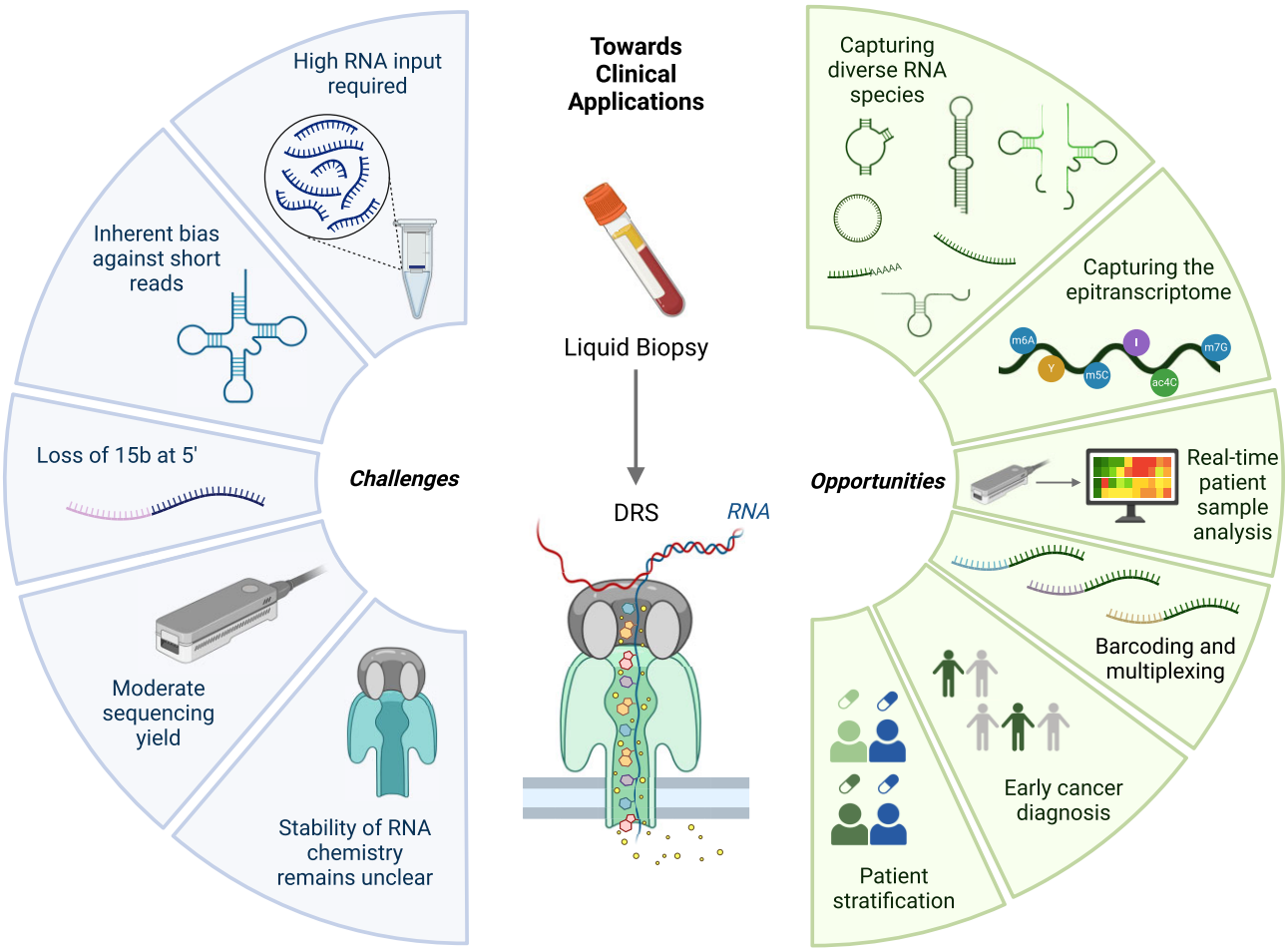

Figure 3. Challenges and opportunities of applying native RNA sequencing in the clinic. Ongoing challenges with utilizing native RNA sequencing include the requirement of increased RNA input for library preparation, the inherent bias against short reads that causes their inefficient capture, the loss of the first 15 bases of each molecule due to variable translocation speeds, the lower sequencing yields compared to short-read sequencing platforms, and the instability of the commercial DRS platform. Nonetheless, the utilization of DRS allows for the capture of a diverse set of RNA species and, in addition, for the capture of the epitranscriptomic information as well. When applied to the analysis of clinical samples and LB, it holds the potential of diagnostic applications through the identification of disease biomarkers. Created in BioRender. Novoa, E. (2025). https://BioRender.com.

Availability of up-to-date robust bioinformatic workflows for data analysis is limited

The relative novelty of DRS technology has led to frequent emergence of new methods for detecting RNA modifications. While this rapid innovation drives progress, it also complicates the systematic analysis of DRS datasets, increasing the risk of manual errors and inconsistencies. Efforts to mitigate these challenges have included the development of bioinformatic workflows such as MasterOfPores [85] and nf-core [86]. However, these workflows are susceptible to becoming rapidly outdated due to the continuous upgrades in RNA chemistry.

Robustness remains unclear

A critical requirement for any clinical test is its ability to produce consistent results when repeated on the same sample. However, simulations of *MinKNOW* software on bulk raw current intensity files [76] can lead to varying numbers of basecalled reads between simulations (data not shown). This variability could in turn lead to inconsistencies in results, as different subsets of reads may be recovered from the same sequencing run. Further investigation is needed to determine whether this issue persists with the latest versions of *MinKNOW*, which are compatible with the newest RNA chemistries.

Stability of the RNA chemistry remains unclear

A key requirement for bringing DRS into the clinic is the stability of the nanopore DRS chemistry. Since DRS was made available in 2017, four versions of the kit chemistry have been so far released (SQK-RNA001 to 004), from which three have been commercially released (all except SQK-RNA003). While each release has come with a significant increase in pore half-life, sequencing yield, and basecalling accuracy, the lack of stability in the DRS flowcell and kit chemistry is a major drawback to implementing this technology for clinical purposes. We should note that ONT has recently released a Q-line of products for which the company guarantees stable production for at least 3 years, however, the DRS chemistry is so far not included in this line of products.

The potential of DRS: leveraging RNAs as disease biomarkers

RNA molecules are starting to become more commonly used by various companies for early disease diagnosis and progno-

sis, demonstrating that RNA abundances alone can be sufficient for diagnosing certain conditions [87–89]. Although DRS has not yet been implemented as a technology for characterizing LB samples, significant advancements have been made in recent years by employing native RNA sequencing on cell lines [31, 90–92], model organisms [84, 93–96], blood samples [97], and even patient tissue biopsies [98]. The latter have resulted in the discovery of novel cancer signatures, thus constituting strong proof-of-concept for the potential of DRS in sample classification and/or patient stratification.

Various RNA biotypes show potential as biomarkers for human diseases using DRS. One such class is ribosomal RNAs (rRNAs), the most abundant RNA species in the cell, which are extensively modified [99–103]. Historically regarded as homogeneous molecular entities, ribosomes are now recognized for their dynamic nature and regulation, partly due to the changing modification repertoire of the rRNAs that they contain [104]. Research has demonstrated that the loss of key rRNA modifications is a hallmark of cancer [105, 106] and cancer-prone conditions, such as dyskeratosis congenita [107]. A recent study employing DRS revealed that rRNA modification patterns vary significantly across tissues, developmental stages, cell types, and disease states, including cancer. Importantly, it was shown that rRNA modification profiles alone were sufficient to accurately classify samples either as cancerous or normal in a cohort of matched tumor-normal lung samples [98], highlighting the potential of rRNA modifications as reliable biomarkers for disease classification.

Another class of promising biomarkers that can be effectively detected with DRS are transfer RNAs (tRNAs), which are both highly abundant and heavily modified. In addition to their crucial role in protein synthesis, tRNAs have been increasingly recognized for being dysregulated both in terms of abundances [108] and modification patterns in various diseases [108–111], including cancer [112], and in response to environmental stimuli [76, 113]. As a result, tRNAs are emerging as potential diagnostic and therapeutic targets [114]. Moreover, tRNAs hold potential as therapeutic agents themselves, as they could modulate the impact of mutations by facilitating readthrough of genetic mutations shared across multiple genes, positioning tRNAs as novel therapeutic tools [115].

For these applications, it is crucial to be able to study tRNA populations in a simple and cost-effective manner. However, this has so far proven challenging with NGS-based methodologies due to the need for complex library preparation protocols, some of which require demethylation and/or pretreatment steps [116, 117], and their limited ability to detect RNA modifications [118]. Recently, several alternatives have been proposed to capture both tRNA abundance and modification information in a single experiment through the use of native RNA sequencing [74, 76], making the study of tRNAs using DRS a promising avenue for future clinical applications.

In contrast to rRNAs and tRNAs, which have only recently emerged as promising biomarkers, mRNAs have long dominated the field of RNA research and biomarker discovery, primarily studied through their reverse-transcribed cDNA products. Beyond differential mRNA expression analysis, there is increasing capturing differential mRNA modification information, particularly in disease contexts [119], and specifically cancer [120–122]. Although the use of DRS to study the mRNA (epi)transcriptome is still in its infancy, pioneering studies have already demonstrated the feasibility of using DRS to chart the transcriptome and epitranscriptome in peripheral blood samples, indicating its potential for clinical applications [97].

Future outlook of DRS: arising opportunities

Increased sequencing yields of the SQK-RNA004 chemistry

After much anticipation, ONT finally released the SQK-RNA004 kit in 2023, which required a new "RNA" flowcell, bypassing the commercial release of SQK-RNA003. This new chemistry introduced several significant improvements, most notably a substantial increase in sequencing yields, with an average 5- to 10-fold increase. This boost in throughput was partly due to the introduction of a faster helicase protein in the kit, which translocated RNA molecules through the nanopore at 150 nt/s, compared to the previous 70 nt/s, effectively doubling the sequencing output per flowcell. Additionally, the "RNA" pores appeared to have a longer half-life compared to the R9.4 generation, further enhancing the sequencing yield per run. The ability to achieve higher sequencing yields from the same RNA input enables the capture of RNA molecules expressed at lower levels, such as lncRNAs, which have been minimally studied using DRS up to this point. This improvement opens new possibilities for deeper transcriptome and epitranscriptome studies using DRS, especially for low-abundance RNA species.

New basecallers with improved basecalling accuracy

The availability of the new DRS chemistry has been accompanied by the release of novel models and basecalling algorithms, such as Dorado's high-accuracy (hac) and super-accuracy (sup) models, which can achieve basecalling accuracies >97% (Table 1). It is important to note, however, that the improved basecalling accuracy is not exclusively tied to the new chemistry or kits. Recent works have shown that similar basecalling accuracies (~97%) can be achieved with the previous R9.4/SQK-RNA002 chemistry when using community-trained sup models [29]. We should note, however, that these new basecalling algorithms and models have not been systematically benchmarked or validated by the scientific community. Future efforts should assess whether this improved accuracy is consistently observed across species, RNA biotypes, and samples.

New generation of modification-aware basecalling models

Since its introduction, the promise of nanopore DRS has been its potential to detect native RNA modifications at single molecule resolution. While considerable efforts demonstrated that RNA modifications can be identified using DRS, it is only recently that true *de novo* detection of RNA modifications—during the basecalling step itself—has been achieved [37]. These efforts have shown that basecalling models can be trained to predict not only the four canonical RNA nucleosides (A, C, G, and U), but also a fifth, representing a non-canonical nucleotide, such as m^6A. More recently, ONT has expanded these capabilities, releasing basecalling models for additional RNA modifications, including N^5-methylcytosine (m^5C), inosine (I), and pseudouridine (Ψ), with modification accuracies ranging 92.7%–99.5%, depending on the modification type (https://nanoporetech.com/platform/accuracy/). A notable advantage of these *de novo* basecallings is that they do not require more than one read to make accurate modifi-

Table 1. Comparison of short-read and long-read sequencing technologies and their potential use in clinical applications

Technology	Short-read sequencing Illumina	Long-read sequencing PacBio	ONT-cDNA	ONT-dRNA
Accuracy	~99.9%	~99.9%	99.75% (v5 sup) 99.25% (v5 hac)	98.66% (v5 sup) 97.54 (v5 hac)
Cost	High	High	Medium	Medium
Turnaround time	Days to weeks	Days to weeks	Real-time to days	Real-time to days
Main clinical applications	SNPs, differential expression, alternative splicing, gene fusion, *de novo* transcriptome assembly	SNPs, differential expression, isoform discovery, gene fusion, *de novo* transcriptome assembly, repetitive elements	SNPs, differential expression, isoform discovery, gene fusion, *de novo* transcriptome assembly, repetitive elements	Differential expression, isoform discovery, repetitive elements, RNA modifications
Strengths	High-throughput, high accuracy, well-established	High accuracy, long-read	Long-read, minimal assembly required	Long-read direct sequencing, No PCR bias
Weaknesses	Limited ability to capture long-read related information	High cost, long run time	Relatively lower accuracy than other cDNA-based methods, lower throughput	Higher error rate, lower throughput high RNA input required not well-established for short RNAs

cation predictions. This enables the study of RNA modifications regardless of transcript coverage, thereby broadening the range of molecules that can potentially be used as biomarkers. However, it is important to note that the accuracy and false positive rates of these modification-aware models have not been independently tested or systematically validated in peer-reviewed studies. As such, conclusions drawn from these models should be interpreted with caution until further validation is conducted.

Barcoding strategies for DRS multiplexing

Currently, ONT does not offer commercial barcoding kits specifically designed for DRS, which would enable the pooling of multiple samples into a single flowcell. As a result, barcoding initiatives remain largely been driven by community efforts, including the development of tools such as DeePlexiCon [72], WarpDemuX [73], supporting 12 barcodes, and SeqTagger [123], which accommodates up to 96 barcodes. The ability to multiplex up to 96 samples within a single flowcell has the potential to significantly reduce sequencing costs per sample and mitigate batch effects, and represents a critical advancement in transitioning the DRS technology toward clinical applications.

Development of toolkits and workflows for live nanopore data analysis

In addition to identifying reliable biomarkers, the use of rapid and cost-effective approaches to diagnose and/or monitor diseases is crucial in the clinical context. Nanopore sequencing stands out as a promising technology in this regard, as it enables real-time processing of the sequencing data [124, 125]. For instance, real-time nanopore sequencing has enabled rapid brain tumor classification by leveraging genomic and epigenomic markers [126–128]. Building on this, the ROBIN framework, which integrates methylome and variant information, allows for real-time intraoperative tumor classification and single-day molecular profiling, significantly reducing turnaround times from several days to just 2 h for intraoperative classification and up to 24 h for detailed molecular profiling [129]. In addition, real-time processing of DRS data combined with RNA modification information, has shown promise when applied to the sequencing of blood cells [97]. The simplicity of the DRS library preparation, combined with the portability of nanopore sequencing and the capability for real-time analysis positions DRS a promising tool for viral epidemiology studies, including monitoring virus mutagenesis and controlling pandemics [130].

Conclusions and future perspectives

Every year, 20 million people are diagnosed with cancer, and 10 million people die from this disease [131]. Early detection and timely interventions can significantly improve patient outcomes and have a major impact on public health. Thus, there is an urgent need for population-based screening assays that can facilitate early cancer detection, particularly for those cancer types such as lung or pancreatic cancer, which are often diagnosed at advanced stages and have very poor patient prognosis and survival rates. Implementing population-based cancer screening programmes could substantially reduce the burden on healthcare systems, lower healthcare costs, and contribute to a healthier society.

RNA modifications have been extensively studied over the past decades, however, they are currently not routinely used as biomarkers for disease prediction or monitoring. While recent efforts using mass spectrometry demonstrate that these can be used as biomarkers, for example, in glioma [70], population-based studies would require a more rapid and cost-effective approach. In this regard, several efforts directed toward developing population-based cancer screening have primarily focused on the use of NGS technologies to study DNA or RNA (in the form of amplified cDNA) molecules present in plasma, exosomes, and circulating tumor cells, offering promising results [47, 50, 68]. However, these methods are still blind to RNA modifications.

In addition to DRS, tailored NGS-based methods and mass spectrometry (LC-MS/MS) have also demonstrated their potential for detecting RNA modifications in clinical applications. NGS technologies offer quantitative measurements with high accuracy and sequencing yields, however, the detection of RNA modifications relies on enzymatic reactions and/or antibodies, and thus is only applicable to a restricted RNA modification repertoire, often focusing on one RNA modification type at a time. Conversely, LC-MS/MS offers highly accurate

quantitative information for a wide range of modifications, but lacks sequence information.

The possibility to chart RNA modifications transcriptome-wide in a rapid and cost-effective manner, while preserving the sequence context information [30], comes with the hand of direct RNA nanopore sequencing. While DRS offers considerable potential for clinical applications, particularly due to its capacity to capture RNA abundances and their modifications, both of which are dysregulated in diseases, several challenges have so far hindered its implementation in clinical practice. These include high RNA input requirements, platform complexity, the lack of robust production lines for RNA chemistry, and the complexity of data analysis. Despite the challenges, solutions such as multiplexing, cost reduction strategies, standardized workflows, stable RNA chemistry product lines, and enhanced bioinformatics tools provide a path forward. With continued advancements in DRS technology and collaborative efforts to address these challenges, DRS has the potential to become a valuable tool in clinical diagnostics, especially for liquid biopsy applications.

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Author contributions: Conceptualization: X.L.K, O.B. and E.M.N. ; writing-original draft: X.L.K, O.B. and E.M.N.; supervision: E.M.N.; visualization: X.L.K. and O.B.

Supplementary data

Supplementary data is available at NAR online.

Conflict of interest

E.M.N. is a member of the Scientific Advisory Board of IMMAGINA Biotech. E.M.N. holds patents on demultiplexing methods (European Patent Application EP24382340.8 and EP24383144.3) and on tRNA sequencing methods (PCT/IB2023/059599/ WO2024/069464A1). X.L.K., O.B., and E.M.N. have received travel bursaries from ONT to present their work at conferences.

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Data availability

No new data were generated or analyzed in support of this research.

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