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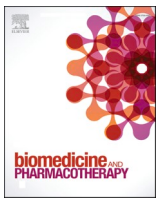

Review

Decoding viral and host microRNA signatures in airway-derived biosamples: Insights for biomarker discovery in viral respiratory infections

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ABSTRACT

The global public health crisis caused by the COVID-19 pandemic has intensified the global concern regarding viral respiratory tract infections. Despite their considerable impact on health, society and the economy, effective management of these conditions remains a significant challenge. Integrating high-throughput analyses is pivotal for early detection, prognostication of adverse outcomes, elucidating pathogenetic pathways and developing therapeutic approaches. In recent years, microRNAs (miRNAs), a subset of small noncoding RNAs (ncRNAs), have emerged as promising tools for molecular phenotyping. Current evidence suggests that miRNAs could serve as innovative biological markers, aiding in informed medical decision-making. The cost-effective quantification of miRNAs in standardized samples using techniques routinely employed in clinical laboratories has become feasible. In this context, samples obtained from the airways represent a valuable source of information due to their direct exposure to the infectious agent and host response within the respiratory tract. This review explores viral and host miRNA profiling in airway-derived biosamples as a source of molecular information to guide patient management, with a specific emphasis on SARS-CoV-2 infection.

1. Viral respiratory tract infections: a public health problem

Respiratory tract infections are a major threat to human health. Prior to the COVID-19 pandemic, there were approximately 489 million cases and 2.5 million deaths attributable to lower respiratory tract infections worldwide in 2019 [1]. Respiratory tract infections contribute to increased morbidity and mortality rates. This, in turn, places a considerable burden on both the economy and the public health infrastructure [2]. With 6.97 million deaths worldwide in January 2024 [3], the emergence of SARS-CoV-2 has put viral respiratory infections in the spotlight.

The most common viral respiratory infections are rhinoviruses, human respiratory syncytial virus (RSV), influenza viruses, parainfluenza viruses, coronaviruses and adenoviruses [4,5]. In general, the viral infection begins and persists in the upper respiratory tract, leading to diseases such as the common cold, sinusitis, pharyngitis or tonsillitis, among others [6]. Nevertheless, the infection could progress to the lower respiratory tract and cause bronchitis, bronchiolitis or pneumonia [7–9]. Viral infection in the lungs can lead to severe conditions such as acute respiratory distress syndrome (ARDS) and the need for admission to the intensive care unit (ICU) [10]. In fact, 28.8 % of ICU patients assisted by invasive mechanical ventilation (IMV) have viral respiratory infections [11].

In addition to vaccines for disease prevention, current therapeutic approaches for treating viral respiratory infections in the acute phase of the disease include administration of drugs to alleviate symptoms, antiviral drugs and ventilatory support. However, as underscored by the recent pandemic, exploration of novel therapeutic approaches is imperative for enhancing patient management. Furthermore, precise and timely diagnosis and prognosis play crucial roles in informed medical decision-making. In this scenario, biosamples derived from the airways (as outlined in Table 1) emerge as a valuable source of information owing to their direct exposure to the infectious agent and host response within the respiratory tract. Integration of airway-derived biosamples with cutting-edge omic approaches presents a promising and innovative framework for addressing these challenges.

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Table 1
Overview of airway-derived biosamples.

Respiratory tract compartment	Specimen	Collection location	Collection procedure
Upper respiratory tract	**Saliva**	Oral cavity	Fluid produced by the salivary glands, typically collected through passive drool.
	Nasopharyngeal swab (NPS), nasal swab, nasal airway secretions	Nasal cavity and nasopharynx	Obtained by swabbing with a swab or brush.
	Oropharyngeal swab (OPS), throat swab	Oral cavity and oropharynx	Obtained by swabbing with a swab or brush.
Lower respiratory tract	**Tracheal aspirate (TA)**	Trachea	Obtained via aspiration through an endotracheal tube or tracheostomy cannula.
	Sputum	Trachea, bronchi and lungs	Obtained by induced or spontaneous expectoration.
	Bronchial aspirate (BAS)	Bronchi and bronchiole	Obtained from the bronchial tree via flexible bronchoscopy.
	Bronchoalveolar lavage fluid (BALF), bronchoalveolar lavage (BAL)	Alveoli	Obtained from the alveolus via flexible bronchoscopy after instillation of variable volumes of physiological saline solution into a subsegment of the lung.

The COVID-19 pandemic has provided a unique opportunity for significant advancements in the field. Given the growing body of literature highlighting the clinical relevance of respiratory tract samples and the molecular insights they offer, microRNA (miRNA) profiling of these specimens has emerged as an interesting tool in the management of such infections. This review explores both SARS-CoV-2-encoded and host miRNAs as biomarkers in COVID-19 studies.

2. microRNAs: biogenesis, function and biomarkers

One of the most studied types of RNAs without coding potential, i.e., noncoding RNAs (ncRNAs), are miRNAs, which are single-stranded molecules of ~22 nucleotides in length. miRNAs are evolutionarily conserved molecules that posttranscriptionally regulate gene expression [12]. To date, 1917 human hairpin precursor miRNAs (pre-miRNAs) have been annotated in the miRBase database and are predicted to yield 2654 human mature miRNA sequences [13].

According to the canonical pathway (Fig. 1), miRNA biogenesis begins with DNA transcription in the nucleus by RNA polymerase II and occasionally by RNA polymerase III [14]. First, a primary miRNA (pri-miRNA) hundreds to thousands of nucleotides in length is generated [15]. The pri-miRNA then becomes a pre-miRNA (70–100 nucleotides in length) through the processing complex formed by the RNase III ribonuclease Drosha and the cofactor DGCR8 [16,17]. The pre-miRNA is transported from the nucleus to the cytoplasm via exportin 5 and subsequently processed by the Dicer enzyme, cleaving the molecule into a miRNA duplex comprising the miRNA and its complementary strand [18]. The Argonaute-2 (AGO2) protein binds to the duplex and promotes its fusion to the RNA-induced silencing complex (RISC), which retains the guide strand that will form the mature miRNA [19]. The passenger

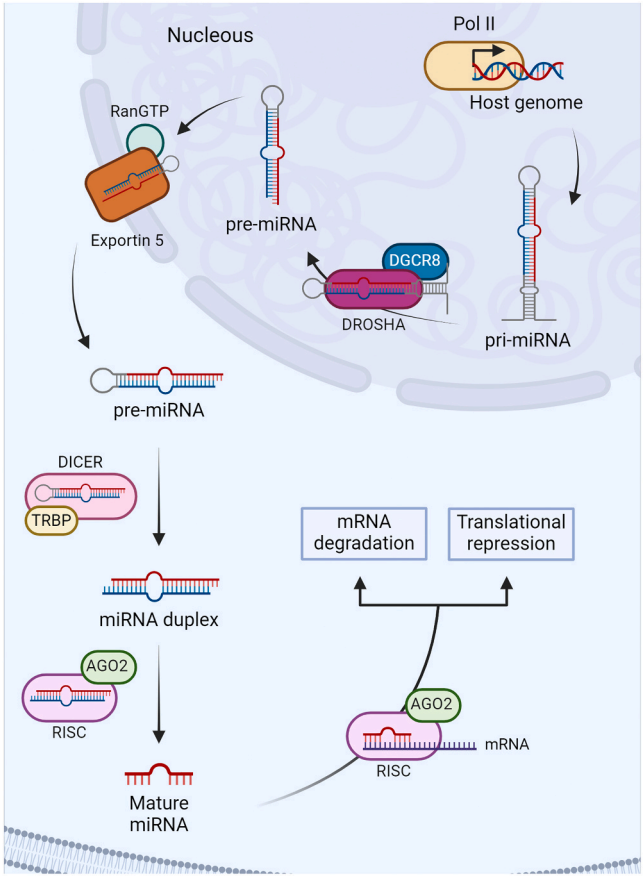

Fig. 1. Canonical microRNA biogenesis pathway. Biogenesis of microRNAs (miRNAs) is initiated by DNA transcription in the nucleus by RNA polymerase II and occasionally by RNA polymerase III, resulting in the formation of primary miRNAs (pri-miRNAs). Subsequently, the pri-miRNA is processed by the RNase III ribonuclease Drosha and its cofactor DGCR8, leading to the formation of precursor miRNAs (pre-miRNAs). The pre-miRNA is then transported via exportin 5 from the nucleus to the cytoplasm. In the cytoplasm, the Dicer enzyme processes the pre-miRNA, cleaving it into a duplex miRNA composed of the miRNA and its complementary strand. The Argonaute-2 (AGO2) protein binds to the duplex, facilitating its integration into the RNA-induced silencing complex (RISC). Within the RISC, the guide strand, which forms the mature miRNA, is retained, whereas the complementary strand is degraded. Created with BioRender.com.

strand may also form another mature miRNA and be loaded in AGO2 [20]. Multiple noncanonical pathways, such as the Drosha/DGCR8-independent pathway and the Dicer-independent pathway, result from modification at any of the above-described steps [21].

miRNAs recognize targeted mRNAs through base pairing. Binding occurs when the seed region of the miRNA, formed by a sequence between 6 and 8 nucleotides at the 5' end, binds to the complementary sequence of the 3' untranslated region (UTR) of the mRNA and, less frequently, with the 5' UTR interaction site and coding sequence (CDS) [22,23]. Full complementarity between the miRNA seed region and the target mRNA results in mRNA degradation. In mammals, where binding is generally not perfectly complementary, translational inhibition typically occurs [24]. Ultimately, the interaction between miRNAs and mRNAs leads to a reduction in protein synthesis, thereby modulating gene expression.

The results of computational prediction suggesting that miRNAs potentially govern around 60 % of human protein-coding genes underscore the pivotal role of miRNA-mediated gene regulation [25]. A single miRNA can interact with multiple mRNAs, and reciprocally, a single

mRNA can be subject to regulation by various miRNAs owing to partial sequence recognition. Extensive research has revealed that miRNAs regulate a myriad of biological mechanisms, with a prominent role in developmental processes, maintenance of homeostasis and responses to stress [26,27]. Consequently, dysregulated expression of miRNAs has been associated with initiation and progression of various diseases, including respiratory conditions [28].

In 2007, Valadi et al. [29] demonstrated for the first time that miRNAs can be detected in a stable form in the extracellular space. Subsequently, miRNAs have been identified in various body fluids, including serum, plasma and urine [30]. Extracellular miRNAs can originate passively, be released from necrotic cells, or be actively secreted by multiple cell types. They are found in the extracellular milieu encapsulated in extracellular vesicles (microvesicles, exosomes, or apoptotic cells) or bound to proteins such as AGO2 or lipoproteins [31–33]. Active export of miRNAs into the cell-free compartment suggests their role in intercellular communication, primarily in paracrine signaling but also as autocrine and endocrine signals [34].

The utilization of miRNA profiling offers several advantages in the field of biomarker development. Firstly, miRNAs are stable molecules that resist degradation, even under harsh conditions [35]. This inherent stability makes miRNAs well-suited candidates for routine clinical testing. miRNAs can be detected in various easily accessible specimens, including saliva, sputum and exhaled breath condensate [36]. The non-invasive nature of sample collection not only reduces patient discomfort but also enables longitudinal monitoring of disease progression and treatment efficacy. Furthermore, miRNA profiling allows for the simultaneous assessment of multiple biomarkers within a single experiment. The multiplexing capability enables a comprehensive evaluation of disease states and treatment responses, enhancing diagnostic, prognostic and predictive assessment. In some cases, miRNAs exhibit tissue-specific expression patterns [37], providing insights into the pathophysiological processes occurring within a specific tissue/organ. Additionally, quantification of miRNAs can be achieved using techniques that are widely adopted in most clinical laboratories, such as quantitative real-time polymerase chain reaction (RT-qPCR). The compatibility with standard laboratory protocols facilitates the integration into existing clinical workflows, minimizing the need for specialized equipment or expertise.

3. Role of microRNAs in virus—host crosstalk

Our understanding of the regulatory roles played by miRNAs during viral infections is evolving. These small RNA transcripts are not exclusively generated by host cells but also synthesized by the viruses themselves. The impact of miRNAs on host—pathogen dynamics is multifaceted, as their influence can either be advantageous for the host or favor the pathogen [38].

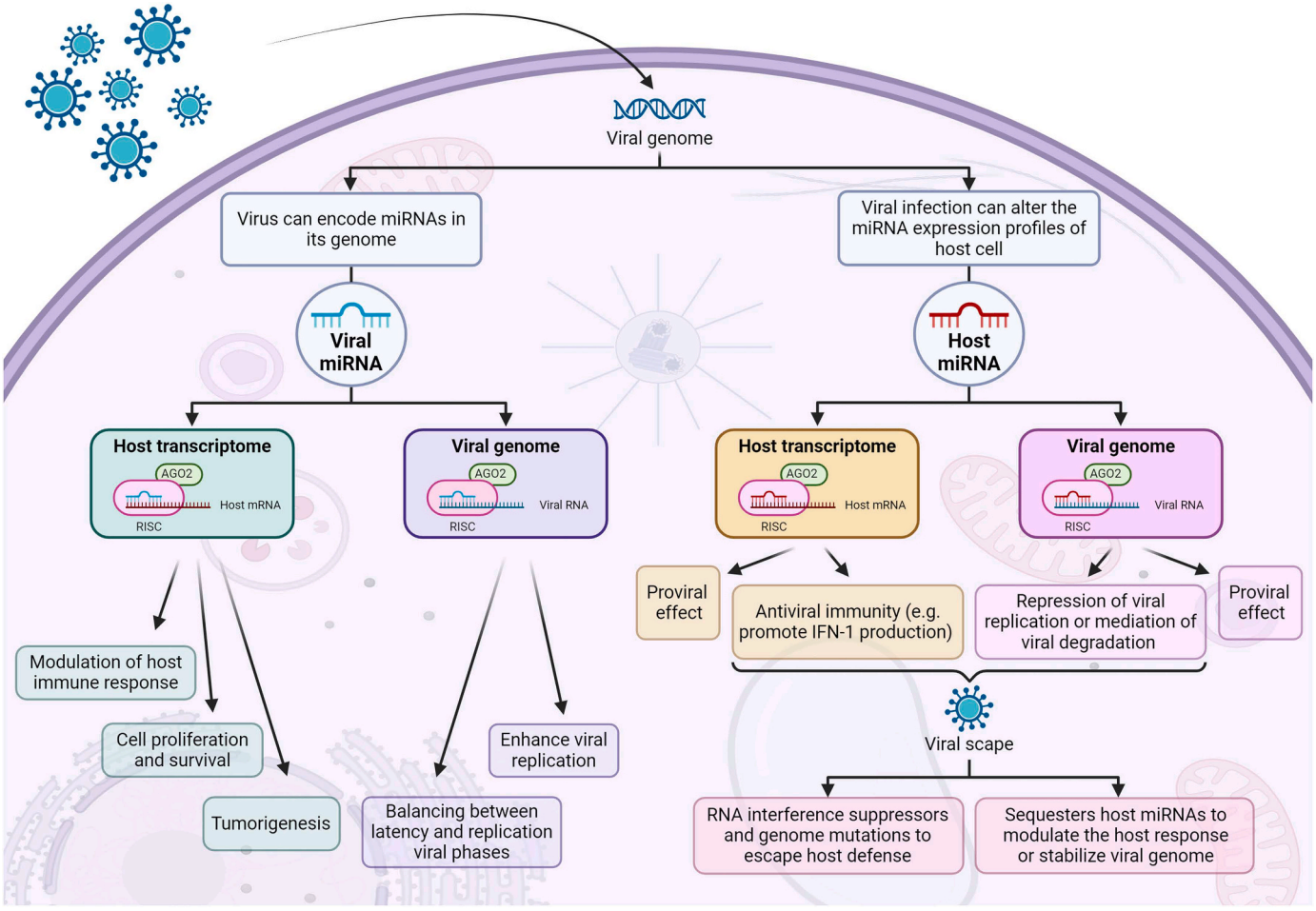

Fig. 2. Viral and host microRNAs in viral infection. Viral infections induce alterations in host cell microRNA (miRNA) expression profiles. Host miRNAs can exert a proviral effect or participate in antiviral immunity by binding to the host transcriptome. Simultaneously, miRNAs can interact with the viral genome, either by repressing viral replication or mediating viral degradation. The virus has developed various strategies to evade host defense mechanisms and can sequester host miRNAs to modulate the host response and facilitate its viral life cycle. Additionally, viruses can encode their own miRNAs, influencing processes such as cell proliferation, survival, modulation of the host immune response and tumorigenesis. Viral miRNAs also play a role in binding to the viral genome, orchestrating the phases of the viral cycle and enhancing viral replication. Created with BioRender.com.

Host miRNAs regulate both response to viruses and viral pathogenesis (Fig. 2). Host miRNAs participate in the antiviral response by promoting expression of interferon (IFN)-inducible antiviral genes and immune response regulators [39,40]. Host miRNAs also inhibit viral RNA translation or alter the stability of the viral RNA secondary structure by directly interacting with the viral genome through miRNA binding sites [41]. The interactions between viruses and hosts have evolved over time as both genomes undergo modifications. Viruses could undergo gain-of-function mutations to evade host defenses. miR-1307–3p suppresses replication of influenza A virus by interacting with the NS1 protein, but the H1N1 strain carries a mutation in the NS1 gene that allows the virus to escape miR-1307–3p inhibition [42]. Viral RNA can act as a 'sponge' sequestering host miRNAs from their cellular targets and creating an advantageous microenvironment [43,44]. Direct interaction with the viral genome may prompt host miRNAs to act as proviral factors, enhancing viral RNA stability, replication and infection. miR-122–5p interacts with the hepatitis C virus (HCV) genome, causing moderate stimulation of viral protein translation and protecting the viral genome from the host exoribonuclease XRN2 [45,46]. Increasing evidence supports host miRNAs playing a proviral role by inhibiting antiviral host factors. miR-146a-5p represses type I IFN production during vesicular stomatitis virus (VSV) infection and suppresses the NF-κB and antiviral Jak-STAT signaling pathways in dengue virus infection [47,48].

The functional versatility and lack of immunogenicity of miRNAs make them attractive candidates as virally encoded regulators. In 2004, Pfeffer et al. [49] were the first to describe a virus with a genome encoding miRNAs, specifically the Epstein–Barr virus (EBV). To date, around 320 viral miRNAs have been annotated in miRbase (Release 22.1, May 2024, https://www.mirbase.org/). Most viral miRNAs are encoded by DNA viruses that utilize the host miRNA biogenesis machinery for their production via the canonical host miRNA processing pathway, which involves Drosha and Dicer machinery [50]. The origin and roles of viral miRNAs in RNA viruses remain a subject of debate [51]. First, the majority of RNA viruses lack access to nuclear miRNA processors, as these are located in the nucleus, whereas RNA virus genome replication predominantly occurs in the cytoplasm. Second, cleavage of pre-miRNAs poses a potential threat, as it can lead to degradation of RNA-based viral genomes. Finally, mature miRNAs have the capability to induce self-cleavage of the viral antigenome.

Viral miRNAs exert their effects via two main mechanisms of action (Fig. 2). First, viral miRNAs regulate viral gene expression. For instance, the human cytomegalovirus genome encodes a miRNA named miR-UL112–1 that regulates genes that contribute to viral replication [52]. Second, viral miRNAs can mimic host miRNAs, thereby influencing the host immune response and regulating processes such as tumorigenesis, cell proliferation and survival. This is the case for miR-BART5, a viral miRNA of EBV that modifies p53-upregulated modulator of apoptosis (PUMA) expression, promoting host cell survival and facilitating latent EBV infection [53].

4. Potential of microRNA profiling of airway-derived biosamples for management of COVID-19 patients

The presence of extracellular miRNAs in multiple body fluids, combined with their alterations in different pathophysiological conditions, offers optimal biological properties for their translation into biomarkers [54]. Quantifying viral and host miRNAs in airway-derived biosamples represents a promising approach for biomarker discovery in respiratory medicine. While relatively unexplored before the COVID-19 pandemic, the literature increasingly supports this concept [55–61]. In recent years, this field has experienced exponential growth, with numerous investigations assessing viral and host miRNA profiles in various respiratory tract samples from COVID-19 patients.

4.1. SARS-CoV-2-encoded microRNAs

Although the presence of viral miRNAs in RNA viruses is controversial, advances in high-throughput sequencing methods and computational predictions have enabled the identification of miRNAs encoded by SARS-CoV-2. The first investigation to identify a SARS-CoV-2-encoded miRNA in a respiratory sample was conducted by Meng et al. [62]. The authors transfected the Calu-3 and Vero E6 cell lines with two SARS-CoV-2 clones (HK-95 and HK-405) followed by deep sequencing analysis. Approximately 0.1 % of the small RNA sequences were mapped to the SARS-CoV-2 genome, revealing three viral miRNAs (v-miRNA-N-28612, v-miRNA-N-29094 and v-miRNA-N-29443) encoded by the N gene. Elevated copy numbers of these miRNAs were observed in nasopharyngeal aspirate samples from COVID-19 patients with high viral loads (Cq=14–20) compared to those with low viral loads (Cq=30–35). Computational analysis indicated that the target genes were associated with cellular metabolism and biosynthesis. Further investigation via RT-qPCR revealed decreased expression of host targets involved in metabolic processes (*ACO1*, *BCAS1*, *BNIP3L*, *CLDN10*, *DMBX1* and *SNCA*) following overexpression of v-miRNA-N-28612 using synthetic mimic transfection. Moreover, liposome transfection of v-miRNA-N-28612 in human peripheral blood mononuclear cells (PBMCs) led to altered expression of IL-1β, NLRP3 and caspase 1 protein, implying the involvement of SARS-CoV-2-derived miRNAs in modulating innate immune responses. More recent data support the role of SARS-CoV-2-encoded miRNAs in regulating innate immunity. By using mimic oligonucleotides, Meseguer et al. [63] demonstrated that the viral miRNAs svRNA 1 and svRNA 2, detected in saliva and nasopharyngeal swab (NPS) samples, may control the host expression of *SERINC5*, a factor that restricts the infectivity of certain viruses. Treatment with antiviral miRNAs before infection recovered *SERINC5* levels and reduced viral protein levels which point to the therapeutic potential of targeting viral miRNAs.

Using an analogous approach, a viral miRNA named CoV2-miR-O7a derived from the ORF7a region of SARS-CoV-2 was identified through small RNA-sequencing (smRNA-seq) after infection of three lung-derived human cell lines (Calu-3, PC-9 and A549-hACE2) [64]. Once more, only a minor proportion of small RNA reads mapped to the viral genome (ranging from 0.01 % to 1.63 %). The production of CoV2-miR-O7a was observed to rely on the cellular machinery but occurred independently of Drosha. Upon transfecting synthetic CoV2-miR-O7a into HEK293T cells, the authors evaluated the transcript levels of predicted target mRNAs. Consequently, two transcripts, namely *BATF2* (a mediator of IFN-gamma signaling) and *HSPG2* (a proteoglycan associated with the extracellular matrix), exhibited significant downregulation. Furthermore, CoV2-miR-O7a was detected in NPS samples obtained from SARS-CoV-2–infected individuals, with its abundance correlating positively with viral load. Remarkably, two viral miRNAs with a very similar sequence were identified by Singh et al. [65]. The authors transfected Caco-2 and A549-ACE2 cells and sequenced small RNAs at 24- and 48-hours post-infection. The findings indicated that CoV2-miR-O7a has two possible isoforms (CoV2-miR-O7a.1 and CoV2-miR-O7a.2) generated in a Dicer-dependent manner and loaded into AGOs, with efficiency comparable to that of host miRNAs such as miR-let-7a. A near-perfect antisense complementarity for the entire CoV2-miR-O7a.2 sequence to the 3′ UTR of *BATF2* was observed. Supporting this data, transfection of A549-ACE2 with CoV2-miR-O7a.2 caused the downregulation of *BATF2* mRNAs upon 8 hours of IFN-α treatment. Therefore, CoV2-miR-O7a.2 could regulate the expression of IFN-stimulated genes during SARS-CoV-2 infection. RT-qPCR assays in NPS samples revealed the presence of CoV2-miR-O7a.1 and CoV2-miR-O7a.2 exclusively in COVID-19 patients but not in seasonal human coronaviruses infected patients. The relative expression of CoV2-miR-O7a.1 and CoV2-miR-O7a.2 correlated with viral RNA levels indicating that the higher the abundance of the SARS-CoV-2 genome, the higher the level of both isoforms in the upper respiratory tract.

Other methodological approaches have been used to identify miRNAs encoded by SARS-CoV-2. For instance, computational prediction of the SARS-CoV-2 viral genome sequence, coupled with miRNA screening analysis of samples from COVID-19 patients, led to the identification of CvmiR-5–5p and its precursor pre-CvmiR-5, which has a hairpin secondary structure in the ORF1a region [66]. In line with works that have demonstrated the presence of SARS-CoV-2 derived miRNAs in the circulation [67,68], CvmiR-5–5p was detected in serum samples of asymptomatic patients, with higher levels observed in patients with severe COVID-19, suggesting its potential for early viral infection detection and its association with disease progression and severity. Furthermore, CvmiR-5–5p was detected in both NPS and sputum samples, with elevated expression observed in sputum, thus highlighting its utility for rapid detection in airway specimens. Subsequent analyses implicated CvmiR-5–5p in the regulation of COVID-19 pathogenesis, particularly the development of neurological disorders. Through bioinformatics analyses, a total of 8610 mRNAs were predicted as targets of CvmiR-5–5p in human host cells, with an enrichment in nervous system-related pathways such as neurogenesis, neuron development, and neuron differentiation. RNA-seq analysis performed on A549 cells overexpressing CvmiR-5–5p precursors further indicated its involvement in regulating various biological processes including the immune system, nervous/sensory system, viral infection and metabolic pathways. Additional investigations support the involvement of SARS-CoV-2-derived miRNAs in the onset of common symptoms in COVID-19 patients. For instance, CvmiR-2, identified in NPS samples, has been linked to fatigue, muscle pain and neurological disorders [68].

More recently, Tucker et al. [69] employed smRNA-seq analysis on NPS obtained from patients with COVID-19 to identify a distinct conserved sequence; CoV2-miR-O8 (SARS-CoV-2 miRNA-like ORF8--derived smRNA). CoV2-miR-O8 is not expressed in other coronaviruses but is consistently present across all SARS-CoV-2 variants. Detection of CoV2-miR-O8 was exclusive to COVID-19 positive patients, displaying a notable correlation with the expression levels of the envelope E gene. In a subset of patients, CoV2-miR-O8 exhibited substantially higher expression levels (up to 100-fold) compared to the viral miRNAs miR-CoV2-miR-O7a.1 and miR-CoV2-miR-O7a.2. Genes targeted by CoV2-miR-O8 were associated with the type I IFN signaling pathway, encompassing *RSAD2*, *OAS3*, *IFIT1* and *XAF1*. These findings point to a plausible role for CoV2-miR-O8 in modulating antiviral immune responses during the infection. Wu et al. [70] employed a similar methodological approach. By aligning RNA-seq data from NPS samples with the complete genome sequence of SARS-CoV-2 isolate Wuhan-Hu-1, they identified several SARS-CoV-2-derived miRNAs in positive samples. Among these, a small virus-derived non-coding RNA spanning from genomic site 346–382 (sv-CoV2–346) exhibited the highest expression levels. The presence of sv-CoV2–346 was further validated in SARS-CoV-2-infected A549-ACE2 cells.

SARS-CoV-2 shares high genetic identity (approximately 80 %) with SARS-CoV-1. Therefore, it is not surprising that these two viruses may share the same viral miRNAs. Given this high degree of similarity, Zhang et al. [71] conducted a comparative analysis of the ten most abundant SARS-CoV-1-encoded miRNAs with the genome of SARS-CoV-2, identifying six putative miRNAs that could be encoded by SARS-CoV-2. Subsequently, they validated the presence of two of these miRNAs (svRNA-5p and svRNA-3p) in NPS and lung tissue samples obtained from patients infected with SARS-CoV-2. Additionally, in a human bronchial epithelial cell line (16HBE cells), transfection with small viral RNA precursors induced a notable inflammatory response, primarily through the activation of CXCL8, CXCL11 and the type I IFN signaling pathway.

4.2. Host microRNAs

Numerous studies have utilized upper respiratory tract samples to investigate changes in the host miRNA profile during SARS-CoV-2 infection. In 2021, Pimenta et al. [72] assessed the expression of miR-200c-3p in saliva samples obtained from COVID-19 patients. The study population was subdivided into four groups: i) 39 individuals testing negative for SARS-CoV-2; ii) 37 positive and symptomatic patients with no indication of hospitalization (absence of respiratory dysfunction); iii) 21 hospitalized patients exhibiting respiratory disorders; and iv) 14 patients with severe COVID-19 requiring oxygen therapy. miR-200c-3p level exhibited a progressive increase correlating with disease severity. Intriguingly, individuals over 42 years of age demonstrated elevated levels of this miRNA, without considering the evolution of the disease and regardless of sex. Multivariable analysis identified miR-200c-3p expression and systemic arterial hypertension as independent factors associated with severe COVID-19 cases. Of note, both *Ace2* mRNA and ACE2 protein levels are inhibited by miR-200c in rat primary cardiomyocytes and in human induced pluripotent stem cells (iPSC)-derived cardiomyocytes [73], suggesting that this miRNA could be used as a preventive strategy to treat cardiovascular complications of COVID-19. Alteration of miRNA levels due to the infection in saliva samples is supported by more recent findings. Hicks et al. [74] analyzed saliva samples from individuals (<18 years) with nonsevere COVID-19 (n=152) and severe COVID-19 (n=45) employing RNA-seq methodology. Among the 1606 human miRNAs quantified in the saliva, the study identified 43 differentially expressed miRNAs (fold difference ≥ 2 and adjusted p-value < 0.05), 72.1 % exhibiting downregulation in severe cases. The miRNAs showing greatest intergroup disparities were miR-296–5p, miR-548ao-3p, miR-1273c and miR-4495. These four miRNAs demonstrated enrichment in gene ontology pathways such as viral processes, the transforming growth factor beta receptor signaling pathway and Fc-gamma receptor signaling involved in phagocytosis. In a related study involving both mild (n=10) and severe COVID-19 (n=10) patients, an altered miRNA profile was observed in saliva samples [75]. Specifically, members of the let-7 antiviral family (let-7a-5p, let-7b-5p and let-7c-5p) exhibited downregulation, while miR-23a-3p and miR-29c-3p showed significant upregulation. Notably, different therapeutic approaches have been proposed to enhance the expression of let-7 family members in the context of SARS-CoV-2 infection [76,77]. Moreover, several miRNAs with immunomodulatory functions, such as miR-34a-5p, miR-146–5p and miR-181d-5p, were upregulated in patients with severe disease. The modulation of most miRNAs by infection was consistent in both saliva and plasma samples.

The mucosal immune response of the upper respiratory tract assumes a critical role in viral entry, replication and infection dissemination. Farr et al. [78] conducted smRNA-seq analysis to investigate miRNA profiles in NPS obtained from eight uninfected controls and 12 COVID-19 patients. Among COVID-19 patients, the most up-regulated transcripts were miR-142–3p, miR-486–5p and miR-451a, while the most down-regulated were miR-3065–3p and miR-3065–5p. Particularly, miR-142–3p, a negative regulator of IL-6 production, demonstrated the most substantial alteration, which may be associated in the role of this cytokine in mitigating the viral cytokine storm and inflammation observed in severe COVID-19 cases [79]. Comparison of differentially expressed miRNAs in NPS and plasma revealed alterations in miR-142–3p and miR-3065–3p across both sample types. Through a supervised machine learning technique, the authors developed a COVID-19 classifier (comprising miR-30c-2–3p, miR-93–5p and miR-628–3p), non-validated, capable of independently identifying COVID-19 cases with accuracy, precision and recall rates of 100 %, along with an area under the curve (AUC) of 1.0. Using smRNA-seq, another investigation compared NPS from SARS-CoV-2-infected (n=10) and non-infected patients (n=10) [80]. The results revealed 943 conserved miRNAs, with statistically significant differences observed in let-7i, miR-29a, miR-34b, miR-34c, miR-100, miR-200a and miR-342 between the two groups. RT-qPCR quantification demonstrated upregulation of miR-21, miR-34b and miR-342 in infected patients. Further analysis demonstrated a correlation between the expression levels of miR-21 and miR-342 and the viral relative quantity, suggesting their association with the presence of SARS-CoV-2 in nasopharyngeal tissues.

Garnier et al. [81] also analyzed the miRNA expression in NPS of patients with severe COVID-19 (n=20), patients with nonsevere COVID-19 (n=21) and non-COVID-19 patients (n=20). Consistent with other studies [82], their investigation revealed a significant reduction in global miRNA expression levels among patients with severe COVID-19 compared to the other groups. Univariate analyses showed the downregulation of 14 miRNAs in patients with severe COVID-19 and non-COVID-19 patients compared to controls, including miR-27a-3p, miR-30c-5p, miR-30d-5p, miR-125a-5p, miR-200b-3p, miR-200c-3p, miR-218–5p, miR-340–5p, miR-375, miR-378a-3p, miR-422a, miR-455–5p, miR-491–5p and miR-532–5p. Five miRNAs; miR-125a-5p, miR-200b-3p, miR-340–5p, miR-455–5p and miR-491–5p, exhibited downregulation in severe versus nonsevere COVID-19 cases. Given that miRNA depletion enhances proinflammatory cytokine production [83], it was hypothesized that the widespread suppression of miRNA expression in severe COVID-19 may contribute to the hyperinflammatory state characteristic of this condition. Employing multivariable analysis with sparse partial least squares-discriminant analysis (sPLS-DA), the authors identified eight miRNAs; miR-15b-5p, miR-103a-2–5p, miR-125a-5p, miR-200b-3p, miR-491–5p, miR-532–3p, miR-629–5p and miR-1290, that accurately predicted severe disease in 85.37 % of the study sample. miR-125a-5p, miR-200b-3p and miR-491–5p were common between the univariate and multivariate analyses and demonstrated discriminatory ability between severe and nonsevere cases, with AUC values ranging from 0.76 to 0.79. *In silico* analyses of miRNA targets showed enrichment in mechanisms related to viral infection, immune response and inflammation, which suggests that these miRNAs could serve as targets for antiviral or anti-inflammatory therapeutic interventions. In the same year, Latini et al. [84] proposed that let-7b-5p may be involved in the pathogenesis of infection by targeting and regulating the *ACE2* and *DPP4* genes. Initially, *in silico* prediction analyses indicated binding sites within both genes. Subsequent *in vitro* functional assays utilizing let-7b-5p mimics and inhibitors in HeLa cells verified the capacity of the miRNA to modulate both *ACE2* and *DPP4* levels, which play crucial roles in viral infections. This result points to the involvement of let-7b-5p in regulating genes implicated in SARS-CoV-2 infection. Furthermore, the expression level of let-7b-5p in NPS from 35 COVID-19 patients and 25 non-COVID-19 individuals was examined. Differential expression analysis revealed the downregulation of let-7b-5p, accompanied by an overexpression of *ACE2* and *DPP4* genes, in COVID-19 patients compared to non-infected subjects. Other approaches have also provided results on the association of host miRNAs with the viral infection. Devadoss et al. [85] identified a long noncoding RNA (lncRNA), named LASI, as a mediator of SARS-CoV-2 infection and the consequent airway mucoinflammatory response. A smRNA-seq analysis in conjunction with a SARS-CoV-2-infected 3D-airway model identified miRNAs regulated in a LASI-dependent manner. The expression of these miRNAs was also assessed in NPS from patients with COVID-19 (n=20). let-7b-5p, miR-150–5p and miR-200a-5p exhibited increased expression levels dependent on viral load. It was hypothesized that these miRNAs may facilitate SARS-CoV-2-induced airway epithelial mucoinflammatory responses in a LASI-dependent manner, with LASI functioning as a molecular sponge for these inflammatory miRNAs.

Bronchoalveolar lavage fluid (BALF) samples are frequently employed for molecular profiling in respiratory diseases [86–88]. In one of the earliest studies utilizing this biospecimen in SARS-CoV-2 infection, publicly available BALF RNA-seq data and upstream regulator analysis identified miR-2392 as an upregulated miRNA in COVID-19 patients [89]. Computational and experimental analyses indicated a role for this miRNA in regulating mitochondrial gene expression, glycolysis and inflammation. Subsequent analyses in human samples, including serum, urine and NPS, reported elevated levels of miR-2392 in both COVID-19 positive from serum and urine samples, with a notable potential for discrimination (AUC 0.95 for serum) in ROC curve analysis in serum samples. NPS samples exhibited differences close to significance. Another study conducted smRNA-seq analysis on BALF and blood samples from severe COVID-19 patients (n=5) and non-COVID-19 patients (n=3) [90]. The comparison of COVID-19 and non-COVID-19 BALF samples revealed 8223 differentially expressed miRNAs, with 825 miRNAs significantly upregulated and 432 miRNAs downregulated. miR-155–5p and miR-4284 emerged as the most upregulated miRNAs in infected BALF samples compared to uninfected samples. Validation via RT-qPCR confirmed significant upregulation of miR-155–5p and miR-4284, alongside downregulation of their respective target genes *HDAC* and *SMAD-7*.

Supporting these findings, miR-155–5p has previously been linked with viral respiratory infections. Elevated levels of miR-155–5p have been detected in nasal samples from young children ($\leq$2 years) infected with rhinovirus, RSV and other respiratory viruses [61]. The increased miRNA levels were correlated with reduced severity of respiratory illness among individuals exhibiting robust Th1 antiviral responses in the airways. Indeed, miR-155–5p, known for its multifunctionality, plays a pivotal role in the response to viral infections by regulating various dimensions of the immune system: innate, humoral and cellular [91]. Papadopoulos et al. [92] proposed that the elevated levels of miR-155–5p during SARS-CoV-2 infection serve as a preparatory mechanism for the SARS-CoV-2-S-ACE2-induced Renin-Angiotensin-Aldosterone System (RAAS) hyperactivity. The upregulation of miR-155–5p leads to the repression of *AGTR1*, *ARG2* and *ETS1*, thereby reshaping the RAAS towards a balanced and protective cardiovascular phenotype mediated by Angiotensin II type 2 receptors (AT2R). This modulation enhances erythropoietin (EPO) secretion, which has a protective role against infections, activates eNOS, increases NO availability and mitigates the proinflammatory effects of Angiotensin II. Moreover, miR-155–5p represses *BACH1* and *SOCS1*, creating an anti-inflammatory and cytoprotective environment that enhances the antiviral responses. Dysregulation of miR-155–5p homeostasis, particularly observed in elderly individuals and those with comorbidities, exacerbates RAAS activation, contributing to a more severe course of COVID-19 with detrimental immune and pathophysiological consequences. Additionally, the same authors [93] suggested that miR-155–5p influences Anexelekto (AXL) homeostasis, a receptor tyrosine kinase implicated in various cellular processes, including cancer progression and susceptibility to viral infections like SARS-CoV-2. AXL overexpression is associated with epithelial to mesenchymal transition, tumor angiogenesis, compromised antitumor immune response and resistance to therapeutic agents. Therefore, miR-155–5p, through its regulatory effects on AXL and RAAS, emerges as a crucial component of the host defense mechanism against viral entry and replication. Targeted modulation of miR-155–5p holds promising therapeutic potential, with repurposed drugs like metformin enhancing its regulatory effects. Future research should focus on elucidating the tissue-specific roles and temporal dynamics of miR-155–5p to develop precise therapeutic strategies that could mitigate severe outcomes in COVID-19 and other diseases involving immune and cellular dysregulation.

In addition to BALF, other lower respiratory tract samples have been evaluated in viral respiratory infections [94]. Molinero et al. [95] were the first group to underscore the potential of host miRNAs as biomarkers in bronchial aspirate (BAS) samples. In the univariate analysis, the study identified five dysregulated miRNA ratios (miR-122–5p/miR-199a-5p, miR-125a-5p/miR-133a-3p, miR-155–5p/miR-486–5p, miR-214–3p /miR-222–3p and miR-221–3p/miR-27a-3p) when comparing critically ill COVID-19 patients and non-COVID-19 patients. Additionally, another set of five ratios (miR-1–3p/miR-124–3p, miR-125b-5p/miR-34a-5p, miR-126–3p/miR-16–5p, miR-199a-5p/miR-9–5p and miR-221–3p /miR-491–5p) exhibited dysregulation in ICU nonsurvivors compared to survivors. The authors developed a miRNA ratio-based prediction model for ICU mortality by integrating three miRNA ratios (miR-125b-5p/-miR-34a-5p, miR-199a-5p/miR-9–5p, and miR-221–3p/miR-491–5p) with high discriminatory power (AUC of 0.85 to distinguish between survivors and nonsurvivors). This model outperformed the best clinical

predictor for ICU mortality (days from first symptoms to IMV initiation, AUC 0.73). *In silico* analysis was performed to identify the molecular pathways and biological processes most closely linked to the miRNA profile from nonsurvivors. Twenty-five KEGG pathways and 122 GO terms were enriched with the verified targets of miRNAs that comprised the ratios. Pathways and GO terms related to COVID-19 pathophysiology were identified, including the mechanisms associated with immune response, fibrosis and cell death.

5. Perspectives

Based on the existing literature, the exploration of miRNA profiling in airway specimens emerges as a promising tool for biomarker development in viral respiratory infections. Nevertheless, it is imperative to acknowledge several inherent limitations.

Additional large-scale population studies and independent cohort validations are for effectively translating research findings into clinical practice. Current research predominantly focuses on identifying dysregulated SARS-CoV-2-encoded or host miRNAs in respiratory samples associated with infection and/or its clinical outcomes. In-depth investigations into the association between miRNAs and specific outcomes are fundamental, alongside comprehensive evaluations of biomarker value and cost-effectiveness analyses. The role of miRNAs as predictors of post-viral syndrome should also be addressed [96]. Detailed information on biomarker performance, e.g., the area under the ROC curve, reclassification or accuracy, should be provided. In addition, the combination of circulating miRNA data with sociodemographic and clinical data has provided intriguing results [97]; however, this strategy has been poorly explored in this field.

The confounding factors such as comorbidities, should also be accounted in the analysis. Considering the influence of factors such as age, obesity and hypertension on the disease and miRNA levels [98], studies on naive groups, such as children and young adolescents in health and disease/infection, are warranted to establish a reference line and better understand the impact of these factors on host and viral miRNA expression profiles. Studies elucidating whether changes in miRNA profiles in airway specimens are attributable to differences in patient management are limited. It is plausible to hypothesize that variations in therapeutic approaches, such as different pharmacological interventions or supportive care measures, may influence viral and host miRNA expression profiles in respiratory samples similar to observations made with circulating miRNAs [99,100]. For example, the administration of specific antiviral agents may modulate SARS-CoV-2-encoded miRNA expression, affecting the host response to viral infection and influencing disease progression. To address this issue, future studies should evaluate changes in miRNA profiles in patients receiving different management strategies. By correlating miRNA expression patterns with clinical outcomes and treatment modalities, researchers can elucidate the impact of patient management on miRNA dynamics and their role as biomarkers for monitoring disease progression and treatment response.

In terms of biomarker development, it is imperative to compare viral and host miRNA profiling in airway specimens with other traditional and emerging biomarkers to elucidate their relative utility and complementarity in the management of viral respiratory infections. Additionally, combining information from viral and host miRNAs could provide a comprehensive understanding of the pathophysiology of viral respiratory infections and its impact on the host response. Given the intricate nature of viral respiratory infection and the myriad contributors to its onset and development, it is imperative to adopt a systems biology approach. Diverse omic technologies, including genomics, epigenetics, transcriptomics, proteomics or metabolomics, offer a source of extensive information encompassing diverse pathophysiological aspects of a given disease [101]. Consequently, implementing a panomic strategy that integrates miRNA data with other omic information emerges as a key step. The experimental designs should take advantage of artificial intelligence and machine learning strategies, not only for analyzing molecular information [102] but also for integrating data with clinical datasets [97].

Substantial disparities exist in the candidates identified across independent investigations. Reproducibility of miRNA testing depends on factors related to sample collection and processing, quantification and data analysis, which can introduce wide variations in results [further discussed in [103]]. Additionally, the lack of consensus on collection approaches and nomenclature, along with the heterogeneity of respiratory samples, complicates the comparability of findings between groups [104]. Further steps towards standardizing miRNA quantification and airways sample collection are required.

Identifying and quantifying viral miRNAs in respiratory samples presents significant methodological challenges, particularly in biomarker discovery. One primary obstacle is the low abundance of viral miRNAs compared to host miRNAs, complicating their detection and quantification. Developing robust bioinformatics pipelines is critical for accurate identification. In the context of viral biology, it is imperative to investigate the capacity of RNA viruses to encode miRNAs. To achieve this, additional research focused on the biogenesis of viral miRNAs is essential for decipher the intricate interplay between the virus and the host. This knowledge will shed light on whether viral miRNAs arise from regulated and patterned expression or are merely generated as random fragments or degradation resulting fragments. Moreover, most studies on viral miRNAs have only focused on their expression in the upper respiratory tract, primarily using NPS samples. Including lower respiratory tract samples such as BAS and BALF is vital for fully understanding the role of SARS-CoV-2-derived miRNAs in infection and their value as biomarkers.

Exploring the mechanistic insights into how miRNAs contribute to the symptomatology and outcomes of COVID-19 is essential. Understanding the role of SARS-CoV-2-encoded and host miRNAs in the pathogenesis of COVID-19 provide valuable insights into disease mechanisms and therapeutic targets. Despite their potential as therapeutic targets due to their regulatory roles in gene expression, their application in therapeutic interventions remains relatively unexplored, also in COVID-19 [105]. Gain- and loss-of-function studies, conducted both *in vivo* and *in vitro*, are indispensable to thoroughly delineate the functional roles and underlying mechanisms of action of the viral and host miRNAs under investigation. In the same line, exploring the correlation between miRNA profiles of respiratory epithelial cells and respiratory samples from the upper or lower respiratory tract constitutes a useful approach for the development of more precise biomarkers and the identification of novel therapeutic targets [106].

6. Conclusions

In summary, extensive research has been conducted on miRNA profiling of respiratory samples since the outbreak of the COVID-19 pandemic, aiming to identify specific biomarkers and extract molecular information. While this approach holds tremendous potential, addressing the aforementioned limitations in future studies is fundamental for facilitating the integration of miRNA-based biomarkers into clinical practice.

CRediT authorship contribution statement

Marta Molinero: Writing – original draft, Conceptualization. **Manel Perez-Pons:** Writing – review & editing, Conceptualization. **Jessica González:** Writing – review & editing, Conceptualization. **Ferran Barbé:** Writing – review & editing, Conceptualization. **David de Gonzalo-Calvo:** Writing – review & editing, Writing – original draft, Supervision, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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