Non-coding RNAs in disease: from mechanisms to therapeutics

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Abstract | Non-coding RNAs (ncRNAs) are a heterogeneous group of transcripts that, by definition, are not translated into proteins. Since their discovery, ncRNAs have emerged as important regulators of multiple biological functions across a range of cell types and tissues, and their dysregulation has been implicated in disease. Notably, much research has focused on the link between microRNAs (miRNAs) and human cancers, although other ncRNAs, such as long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs), are also emerging as relevant contributors to human disease. In this Review, we summarize our current understanding of the role of miRNAs, lncRNAs and circRNAs in cancer and other major human diseases, notably cardiovascular, neurological and infectious diseases. We further discuss the potential use of ncRNAs as biomarkers of disease and therapeutic targets.

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In this review, the authors describe our current knowledge of the role of miRNAs, lncRNAs and circRNAs in disease, with a focus on cardiovascular, neurological, infectious diseases, and cancer. They further discuss the potential use of ncRNAs as disease biomarkers and as therapeutic targets.

[H1] Introduction

The majority of the human genome (76–97%) encodes for RNAs that are not translated into proteins, termed non-coding RNAs (ncRNAs)[1-3]. Since their discovery, the biological importance of ncRNAs has become increasingly apparent, shifting the perspective of RNA as a simple intermediary of protein synthesis towards RNA as a functional molecule with essential roles in the regulation of gene expression and genome organization. The functional relevance of one class of ncRNAs in particular, microRNAs (miRNAs), has received much attention, with important roles in a myriad of cellular processes, including muscle differentiation and cardiac development[4,5], as well as neural stem cell differentiation and neurogenesis[6,7]. Compelling evidence further implicated dysregulated miRNAs in human diseases, particularly human cancers, such as by functioning as oncogenes and/or tumor suppressors[8]. miRNAs have also been found to be differentially expressed in a range of other human pathologies, including cardiovascular[9,10], neurological[6,7], and infectious diseases[11]. Most recently, their involvement in SARS-Cov-2 infection was demonstrated[12].

Over the years, high-throughput sequencing and other technologies have led to the identification of a wide range of ncRNAs of different types and sizes[4,5]. These include ribosomal RNAs (rRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), transfer RNAs (tRNAs) and more recently miRNAs, long ncRNAs (lncRNAs), circular RNAs (circRNAs), heterogeneous nuclear RNA (hnRNAs), PIWI-interacting RNAs (piRNAs).

Long ncRNAs and circular RNAs are recognized as essential regulators in a variety of biological processes. Similar to miRNAs, dysregulation of lncRNAs and circRNAs has

been associated with various human diseases[13-16]. One of the biggest challenges in the field today is to elucidate the diverse functions and mechanisms of action of ncRNAs, which is essential for defining their clinical relevance and exploiting their potential use as biomarkers or therapeutic targets.

Here, we review the role of ncRNAs in human diseases that account for the highest mortality worldwide, including cardiovascular diseases, cancer, neurodegenerative and infectious diseases, such as COVID-19. We place a focus on the disease-related ncRNAs that have received the most research focus: miRNAs, lncRNAs, and circRNAs. Readers are referred to other review articles for insights into additional classes of ncRNAs and their potential role in diseases[17-20]. We first provide a brief overview of the different ncRNA mechanisms and physiological roles, and then discuss the impact of ncRNA dysregulation in human disease. Finally, we review the use of ncRNAs as diagnostic and prognostic markers and targets of new therapeutic strategies.

[H1] Mechanisms of action and functions of ncRNAs

ncRNAs act through diverse mechanisms on target genes and interact with each other, creating a complex and dynamic regulatory RNA network[21]. Variations in the expression of a given ncRNA can affect the expression of other ncRNAs, altering many cellular processes including gene expression, RNA splicing, editing, intracellular transport, and translation[22].

[H2] microRNAs

miRNAs are short ncRNAs that were first identified thirty years ago in Caenorhabditis elegans (*C. elegans*) [23,24]. To date, more than 38,000 miRNAs from 271 species, including 2,654 human mature miRNAs, have been annotated in the miRNA archive miRBase (v22.1)[25]. Functionally, it is predicted that the majority of the human transcriptome is under miRNA regulation[26]. The complexity of such regulation is demonstrated by the fact that a single miRNA can target hundreds of different messenger RNAs (mRNAs) and that multiple miRNAs can target a single mRNA[27]. Overall, miRNAs have a function in every

fundamental biological process, including cell proliferation, differentiation, and embryonic development, and their tissue-specific functions have been demonstrated[28-30].

The classic function of miRNAs involves binding to the 3' untranslated region (UTR) of target mRNAs, leading to their degradation or translational repression[31]. This process requires miRNA association with an Argonaute (Ago) protein, which is the core component of the RNA-induced silencing complex (RISC). Once loaded onto an Ago protein, a miRNA can guide the RISC to a complementary target mRNA for translational repression or mRNA degradation. miRNAs also have the ability to inhibit protein expression by binding to coding regions (CDS) or the 5' UTR of mRNA molecules. For example, CDS-located miRNA interaction sites (miR-134, miR-296 and miR-470) in Nanog, Oct4 and Sox2, modulate embryonic stem cell differentiation[30].

Although miRNAs typically inhibit gene expression, there are instances in which they instead boost translation[32]. For example, human miR-369 has been shown to activate translation via a mechanism that involves direct binding to *TNF-α* and *FXR1*[32]. Moreover, let-7 miRNA has been shown to upregulate the translation of its target mRNAs during cell cycle arrest and to repress translation in actively proliferating cells, indicating that miRNA function alters between repression and activation during cell cycle[32]. Additional ways in which miRNAs activate genes include their attachment to the CDS or the 5' UTR of mRNAs[33,34]. Despite the fact that these alternative miRNA mechanisms of action are less well studied, there is increasing evidence of their cellular relevance (**Figure 1**).

In addition to regulating transcription within the cells in which they are produced, miRNAs can act as intercellular communication molecules through their secretion in extracellular vesicles or by acting as hormones[35,36]. Moreover, secreted miRNAs can directly target Toll-like receptor (TLR) proteins by acting as their ligands[37], a mechanism that activates TLR signaling transduction pathways and induces an immune response[38-41]. Recent studies have also revealed their interaction with non-Ago proteins [G], although the mechanism is poorly understood (**Figure 1**).

[H2] Long non-coding RNAs

lncRNAs are a large and highly diverse class of ncRNAs that are >200 nucleotides in length[42]. The first lncRNAs identified in eukaryotes, *H19* and *Xist,* were discovered long before the genomic era[43,44]; however, it took considerable time to recognize their broad biological functions. Although many lncRNAs have been identified to date, only a handful have been functionally characterized. The human GENCODE project estimated that there are 16,000 human lncRNAs, whereas the current version of the NONCODE database (v6.0) has annotated 96,411 human lncRNA genes, generating 173,112 lncRNA transcripts, an amount several times larger than the number of coding genes (estimated at around 20,000)[45,46].

lncRNAs can be transcribed in sense or antisense directions from various genomic regions, including introns or exons of overlapping protein-coding genes, intergenic regions (lincRNAs), pseudogenes (pseudogene-derived lncRNAs), transcribed ultraconserved elements (T-UCRs), telomeres (telomeric repeat-containing RNAs), centromeric repeats (centromeric lncRNAs), ribosomal DNA loci (promoter and pre-rRNA antisense, PAPAS), promoters (promoter-associated lncRNAs, PALRs), enhancers (eRNAs) and 3'-UTRs (UTR-associated RNAs)[47]. Similar to mRNAs, lncRNAs can be spliced; however, they usually contain fewer exons, are often retained in the nucleus and their abundance can be 10 times lower than mRNAs[48,49]. lncRNAs often show high tissue specificity and their expression alters dynamically during development[50].

The diversity of lncRNAs is also reflected in their function, which includes genomic, transcriptional, and translational regulation of neighboring and distant genes[22,51-53]. lncRNAs can directly interact with DNA, forming R-loops[54], and can associate with enhancers or promoters, activating or suppressing their function[55-58]. By forming a complex with proteins, lncRNAs can also bind to the DNA and regulate chromatin by recruiting chromatin modifiers to the promoter region of their target genes[59-61].

As well as associating with DNA, lncRNAs can interact with various other RNAs, including mRNAs, circRNAs, and miRNAs. They can further influence RNA splicing and act as miRNA sponges **[G]** and thereby inhibit the target-repressing function of miRNAs[62,63]. In addition, through their interaction with proteins, lncRNAs can serve as

scaffolds or guides to promote the colocalization of proteins or facilitate protein-protein interactions[64,65] (**Figure 2**).

[H2] Circular RNAs

CircRNAs are generated by back-splicing of linear transcripts and can be derived from exons, introns, exon-intron junctions, or intergenic regions of the genome[66-68]. Their circular structure makes circRNAs unsuitable for further processing, reducing susceptibility to exonuclease activity compared to linear RNAs, which results in a high degree of stability[69]. circRNA expression is often unrelated to the expression of their host genes[70], and due to their stability, they can be more abundant than their associated linear mRNA[71]. With regards to their localization, circRNAs usually accumulate in the cytoplasm[72]; however, they are also present in the nucleus, and similarly to lncRNAs, circRNAs can also bind to the DNA and form circR-loops {Conn, 2023 #337}. Although the turnover of circRNAs is largely unknown[70], it most likely involves secretion via exosomes[73].

CircRNAs can interact with miRNAs, mRNAs, or RNA-binding proteins (RBPs), activate or repress gene expression, or act as miRNA or protein sponges[74]. The complexity of the RNA network is well illustrated by the fact that circRNAs can sequester miRNAs and thereby indirectly influence the expression of their mRNA targets[74]. circRNAs can also function as protein 'enhancers', either by forming a circRNA–protein complex[75], acting as protein scaffolds[76], or recruiting proteins to a specific loci or subcellular compartment that facilitates their colocalization and thereby influencing protein-protein interactions[77,78]. (**Figure 3**).

[H2] ncRNA-encoded peptides

Despite their original non-coding classification, it was uncovered in the last decade that some ncRNAs contain short open reading frames **[G]** (sORFs) that encode small regulatory peptides (sPEPs) **[G]**, or micropeptides, consisting of less than 100 amino acids (AA)[79-81]. The first identified miRNA-encoded sPEPs (miPEP), miPEP171b (9 AA)

and miPEP165a (18 AA), were described in plants in 2015[82]. Both pri-miR-171b and pri-miR-165a miRNA precursors encode small proteins that enhance the accumulation of their corresponding mature miRNAs, leading to downregulation of their target mRNAs. After their discovery in plants, several studies have reported human sPEPs derived from ncRNAs and their potential roles in diseases[83-85].

Ribosomal profiling **[G]** experiments have uncovered many unexpected associations between ncRNAs and ribosomes. Combined with the development of various computational methods, these experiments have led to the discovery of thousands of sORFs and many ncPEPs[86,87]. Several of these ncPEPs have been experimentally validated[88]. For example, a muscle-specific lncRNA is translated into the 35 AA protein DWORF, which was shown to regulate intracellular calcium signaling in heart tissue[87]. Moreover, the lncRNA *Linc00116* encodes a small peptide (56 AA), MTLN, that supports protein complex assembly in the mitochondria and inhibits the production of reactive oxygen species, thereby enhancing respiratory efficiency[89]. The identification of novel ncPEPs is ongoing, and their investigation can be facilitated by databases such as FuncPEP[90], which currently lists 112 functional sPEPs encoded by ncRNAs and provides details on the ncRNA 'host' transcripts. Another database, SPENCER, annotates cancer-associated sPEPs encoded by ncRNAs[91].

Recent studies showed that, similarly to miRNAs and lncRNAs, some circRNAs can also encode proteins[92,93]. Owing to the lack of the 5' end, translation initiation from circRNAs requires N^6-methyladenosine (m^6A) modification[94] or an IRES, which is usually rare in eukaryotic transcripts but has been identified in eukaryotes through systematic investigations[95,96]. Like the general function of many circRNAs, the function of their encoded peptides is largely unknown. Several hypotheses have been put forward about the role of translated circRNAs[97], including the generation of rapidly degraded peptides that regulate immune surveillance, acting analogous to lncRNA-encoded proteins and, therefore, participating in nonsense-mediated mRNA decay, or inhibiting the translation of other RNAs by sequestering ribosomes[97]. Regardless of the precise biological role, it has been hypothesized that translated circRNAs might have evolutionarily conserved functions, as their sequence is highly conserved across different species[93].

[H1] ncRNA dysregulation in human disease

Because their regulatory functions are crucial for normal cell activities, it is not surprising that dysregulation of ncRNAs leads to human disease[8,98]. Indeed, perturbations in ncRNA biology have been linked to a wide range of conditions, including cancer, cardiovascular diseases, neurological disorders, infectious diseases, and sepsis (**Figure 4**). Generally, in diseased tissues, ncRNAs are dysregulated as a consequence of genomic structural and copy number variations, epigenetic modifications, or transcription factor alterations [99,100]. Several databases, such as The Human MicroRNA Disease Database[101] for miRNAs, and LncRNADisease[102,103] for lncRNAs and circRNAs, can be useful resources for up-to-date information on disease-related ncRNAs. Ultimately, gaining a deeper understanding of the involvement of ncRNAs in disease could pave the way for the development of innovative diagnostic and therapeutic approaches.

[H2] Non-coding RNAs in cancer

The first evidence of ncRNA involvement in human cancer came in 2002 from genetic studies of patients with chronic lymphocytic leukemia (CLL)[104], the most common type of leukemia in the Western world[277]. Loss of chromosome region 13q14 is a common feature observed in CLL and is often the only genetic abnormality that is found in leukemic cells. Notably, the 13q14 region harbors genes encoding precursors of miR-15a and miR-16-1, which were later characterized as tumour suppressors through their targeting of *BCL-2*[104] and *MCL1*[105]. Soon after these discoveries, other miRNA-encoding loci were shown to be frequently located in the fragile regions of chromosomes[106,107] and lost or disrupted in various cancer types[108,109,52].

Due to the complexity of miRNA regulation, one of the biggest challenges is understanding whether miRNA dysregulation is the cause or consequence of the disease. Nonetheless, pan-cancer analyses have uncovered that certain miRNAs, such as the oncogenic miR-21 and miR-155, or the tumour suppressors miR-16 and miR-145, are commonly dysregulated in several types of cancer [110,111]. These studies have identified

miRNA signatures that are consistent across 15 different cancer types and indicate a major role in regulating the particular hallmarks of cancer. For example, miR-210, miR-21-3p, and let-7a-3p were associated with hypoxia gene signatures[110,111]. The miR-29 family regulates the DNA demethylation pathway members TET1 and TDG[110,111]. miR-21, which was shown to be overexpressed in a large variety of cancers[112,113], is involved in therapy resistance[114] and tested as a cancer biomarker[115]. miR-324 has an oncogenic role both in malignant cells and the surrounding tumor microenvironment (TME), specifically in neurons in mouse models of oral cancers[116], where miR-324 (in conjunction with miR-21 and opposition of miR-34a) promotes neuritogenesis.

Certain miRNAs can function as either an oncogene or a tumor suppressor, depending on tumor type, tumor stage, and the tumor microenvironment (TME), which further emphasizes that miRNA functions need to be investigated in a context-dependent manner[110,117]. For example, dysregulation of miR-324 was described in various cancer types including colorectal and gastric cancers, and its oncogene and tumor suppressor functions have been both demonstrated depending on the cellular context[118]. Meanwhile, several lncRNAs and circRNAs, including the lncRNA *MALAT1* can act as a sponge for miR-324[118]. miRNAs from the *let-7* family have been also shown to exhibit dual functionality, acting as tumor suppressors in cancer cells while concurrently exerting oncogenic effects within the TME[119]. Recently, an estrogen-driven mechanism was discovered in which estrogen receptor-positive breast cancer cells eliminate the tumor suppressor members of let-7 family via extracellular vesicles, and these have oncogenic effects through the immunostimulatory (M1) macrophage activation and polarization in the TME[36]. Similarly, miR-21 released inside extracellular vesicles by glioblastoma cells was demonstrated to act on microglial cells of the TME changing the levels of target genes, including *Btg2*, and consequently their phenotype[120].

Sequence conservation suggests positive selection during evolution and is therefore an important hint of potential functionality. Plenty of lncRNAs are transcribed from genomic regions that are perfectly conserved between humans, mice, and rats, termed ultraconserved elements (UCEs)[121]. However, newly-evolved, human, or primate-specific elements (pyknons) are also an interesting topic of research, and their non-coding transcripts were found to have a role in cancer progression[122].

After their discovery, miRNA-encoded peptides (miPEPs) gained intense research interest, with several studies reporting the involvement of human ncPEPs in growth and development, as well as disease. More recently, the pri-miR-34a-encoded miPEP133 (133 AA) has been demonstrated to positively regulate its own pri-miRNA in human cancer cell lines, in which it functions as a tumour suppressor[123]. In contrast, pri-miR-31-encoded miPEP31 (44 AA) decreases the expression of miR-31 by binding to its promoter region, inhibiting transcription initiation. miPEP31 is highly expressed in regulatory T cells (Treg), promotes Treg cell differentiation, and suppresses experimental autoimmune encephalomyelitis[124]. Pri-miR-155-encoded miPEP155 (17 AA) does not influence the expression of pri-miR-155 but increases the expression of the oncogenic *Rictor* and *EGFR* genes in HeLa cells[125]. Pri-miR-147b-encoded MOCCI micropeptide seems to have a role in the immune response to viral infection[126], whereas miPEP200a (187 AA) and miPEP200b (54 AA), encoded by pri-miR-200a and pri-miR-200b, respectively, inhibited the migration of prostate cancer cells in vitro[127].

A lncRNA-encoded peptide identified to have a role in cancer is HOXB-AS3 (53 AA), translated from the lncRNA *HOXB-AS3*, which was shown to suppress colon cancer growth; its loss is a critical oncogenic event in metabolic reprogramming[128]. Several lncRNA-encoded micropeptides have been further associated with cancer, such as the *LINC00665*-encoded CIP2A-BP (52 AA) and *LNC00908*-encoded ASRPS (60 AA), both of which inhibit breast cancer progression[129,130].

Through their roles in development, apoptosis, stress responses and cell cycle regulation, circRNAs-encoded peptides could be important in cancer initiation and progression[94]. Indeed, several circRNAs-encoded proteins, such as FBXW7-185aa, SHPRH-146aa, and PINT-87aa were shown to suppress glioma tumourigenesis[84,131,132]. Moreover, a recent study proposed a tumor suppressor role for the circRNA-encoded protein circFGFR1p (87 AA), which negatively regulates the FGFR1 oncoprotein[97]. Future research should focus extensively on the functions of ncRNA-encoded peptides and their coding mechanisms. Although there are still many challenges for such research, advances in coding prediction tools and genomics technologies should facilitate the progress.

[H2] ncRNAs in cardiovascular diseases

Cardiovascular diseases (CVDs), including myocardial infarction, atherosclerosis, heart failure, and cardiac hypertrophy, remain the leading cause of mortality and morbidity in the world[133]. ncRNAs are relevant for heart physiological activity and are involved in CVD processes through their functions in regulating apoptosis, proliferation, migration, cardiac remodeling, fibrotic responses and cardiac hypertrophy[134,135]. Emerging studies have revealed that miRNAs are involved in the pathogenesis of CVDs. A notable example is miR-21, which is upregulated in humans and mice with cardiac allograft vasculopathy (CAV), which is a complication of heart transplantation that limits long-term survival[136]. Moreover, miR-21 is also overexpressed in a mouse model of cardiac fibrosis caused by myocardial infarction, and correlated with attenuated *TGFβRIII* levels[137]. Silencing of miR-21 via antagomir-21 could disrupt CAV and prolong cardiac allograft survival[136] as well as reduce hypertrophy and fibrosis and restore impaired cardiac function[138]. It was also recently demonstrated that anti-miR-21 treatment successfully suppressed miR-21 and improved cardiac function in a pig model of ischemia–reperfusion injury with reduced cardiac fibrosis and hypertrophy[139].

miR-122 is abundantly expressed in various cardiovascular cell types. Upregulation of miR-122 was shown in patients with systolic dysfunction, cardiovascular fibrosis and cardiovascular remodeling[140]. Mechanistically, miR-122 was shown to directly inhibit the anti-apoptotic protein Xiap in a mouse model of cardiovascular disease, promoting endothelial cell apoptosis[141]. Moreover, circulating miR-122 level correlated negatively with cardiac function and has been shown to be an important indicator with both predictive and prognostic value for cardiac recovery[140]. In the same study, it was revealed that miR-122 inhibits the expression of the anti-apoptotic *BCL2* and thereby decrease the viability of cardiomyocytes[140].

The exact role of lncRNAs in CVDs has recently started to be elucidated. Notably, cardiac mesoderm enhancer-associated ncRNAs (CARMNs) are among the most thoroughly annotated lncRNAs. These lncRNAs are predominantly expressed in smooth muscle cells (SMCs) and are significantly upregulated in post-myocardial infarction[142,143].

Analysis of publicly available transcriptomic datasets has revealed a reduction in the expression of CARMNs in cerebral arteries with aneurysms and human atherosclerotic arteries[142]. Downregulation of CARMNs in human coronary artery SMCs resulted in enhanced cell proliferation and migration in vitro, and significantly attenuated the expression level of SMC-specific marker genes including *MYH11*, *ACTA2*, *CNN1*, and *TAGLN*[142]. Furthermore, RNA immunoprecipitation assays confirmed that *CARMN* could interact with *MYOCD* (Myocardin), an activator of SMC-specific genes and transcriptionally active in cardiomyocytes as well as in SMCs[142].

Adult mammalian hearts fail to regenerate after ischemic injury, primarily due to a decline in cardiomyocyte mitosis. Yet, the specific molecular mechanisms that account for the non-dividing nature of adult cardiomyocytes remain largely unknown[144]. Cardiac regeneration-related lncRNA (*CAREL*) is increased in expression in postnatal hearts and has been associated with regeneration during cardiac injury[144]. Indeed, the capacity for cardiomyocytes to proliferate, an important step in regeneration, was attenuated in transgenic mice that overexpress *CAREL*[144]. In contrast, knocking down of *CAREL* in cardiomyocytes by adenoviral shRNA increased the proliferation of cardiomyocytes and enhanced cardiac regeneration after injury. Further experiments using biotin-avidin pull-down and luciferase assays in human embryonic kidney cells (HEK293) and neonatal cardiomyocytes have revealed that *CAREL* acts as a competing endogenous RNA to miR-296, a positive regulator of cardiac replication and regeneration[144]. Other lncRNAs, such as *ZFAS1*[145], *SNHG3*[146], *ExACT1*[147], *MIAT*[148], *CPhar*[148], *Mhrt779*[148], *H19*[149] and *CPR*[150], have been implicated in myocardial ischemia-reperfusion injury, aortic valve calcification, pathological hypertrophy, and heart failure, atherosclerosis in carotid arteries, physiological cardiac hypertrophy, pulmonary arterial hypertension, and cardiomyocyte proliferation, respectively.

It has also been reported that several circRNAs have an important role in heart failure and myocardial infarction. Global circRNA profiling demonstrated that the ultraconserved *circ-INSR*, derived from the gene encoding the insulin receptor (*INSR*), regulates mitochondrial functions in cardiomyocytes under doxorubicin stress[151]. In human and mouse failing heart tissue, the expression of *circ-INSR* was diminished, inducing apoptosis of cardiomyocytes and impairing metabolic activity[151]. Consistent with this

observation, overexpression of *circ-INSR* successfully decreased doxorubicin-induced DNA damage and apoptosis of primary rat cardiomyocytes[151].

In a recent study, researchers identified and investigated the function of *circHIPK3*, derived from exon 2 of the *HIPK3* gene within mouse heart. *CircHIPK3* negatively regulates the RBP Hur at the post-transcriptional level, which leads to the destabilization of *p21* mRNA in a rat cardiomyocyte cell line (H9C2) and primary mouse cardiomyocytes[152]. In addition, *circMAP3K5* is known to be associated with SMC differentiation by sponging miR-22-3p and thereby inducing the expression of *TET2* [153].

A recent study demonstrated the protective effects of *circSlc8a1* (a circular antisense RNA) in the context of heart injury, highlighting the important role of *circSlc8a1* in preserving physiological heart function[154]. The experimental induction of cardiac-specific expression of *cA-circSlc8a1* in mice resulted in profound phenotypic alterations, notably characterized by a substantial increase in body weight, hepatic steatosis, and impaired cardiac function[154]. Another study demonstrated that the *circNlgn,* along with its translated product Nlgn173, is a mediator of doxorubicin-induced cardiofibrosis [155]. Silencing endogenous *circNlgn* has been found to reduce doxorubicin-induced cardiomyocyte apoptosis and enhance cardiomyocyte viability. Moreover, the silencing of *circNlgn* effectively inhibits collagen deposition and enhances the expression of fibrosis markers. These findings suggest that targeting *circNlgn* could potentially alleviate the adverse effects associated with doxorubicin, particularly its impact on fibrosis development[155].

In summary, ncRNAs have emerged as crucial regulators of cardiovascular diseases[156] [157], highlighting a promising research area that warrants further exploration. Future research should continue to elucidate the molecular mechanisms and potential therapeutic applications of ncRNAs, while large-scale, multicenter studies will have a crucial role in validating their translational feasibility from the laboratory to the clinic.

[H2] Non-coding RNAs in neurodegenerative disorders

Neurodegenerative disorders, such as Alzheimer disease, Parkinson disease, amyotrophic lateral sclerosis (ALS), and Huntington disease, are characterized by

degeneration and loss of neurons in specific areas of the brain and the spinal cord. Neurodegenerative disorders are currently irreversible and tend to worsen over time with no effective treatment available; they are thus associated with severe morbidity and are considered one of the leading causes of death by the World Health Organization (WHO).

Dysregulation of miRNA expression has been frequently observed in the central nervous system and is a powerful modulator of the onset of neurodegeneration[158]. It has been shown that increased expression of miR-29b-3p in striatal medium spiny neurons (MSN) is associated with age and contributes to the degeneration of MSNs in Huntington disease by directly targeting the 3'-UTR of *STAT3*[159]. Downregulation of STAT3 diminished autophagy and increased apoptosis in patient-derived MSNs. In Huntington disease-MSNs, administration of anti-miR-29b-3p reduced neural cell death, whereas the depletion of STAT3 counteracted the therapeutic effect of anti-miR-29b-3p treatment[159]. In other instances, miR-520f-3p, miR-135b-3p, miR-4317, miR-3928-5p, and miR-8082 have been found to be significantly differentially expressed in patients with Huntington disease compared to the control group[160]. Also, several miRNAs have been associated with Alzheimer disease through directly regulating disease-associated risk factors, including beta-site amyloid precursor protein cleaving enzyme 1 (BACE1), amyloid protein precursor (APP) cleavage, and presenilin-1 (PSEN1)[161]. Moreover, an elevated miR-543 level was found in the white matter tissue of patients with early-stage Parkinson disease, associated with a decreased level of SIRT1 protein, a potential target of mR-543. Subsequent in vitro experiments confirmed *SIRT1* as a direct target of miR-543 and the upregulation of miR-543 resulted in transcriptional downregulation of *SIRT1* in a neuroblastoma cell line and foetal astrocytes[162].

As many lncRNAs (~40%) are expressed in a brain-specific manner[163], experimentally altering their expression might lead to important insights into neuronal development and the pathogenesis of neurodegenerative disorders. However, only a small proportion of lncRNAs have been studied with regard to their role in neurodevelopment and brain function. For example, the lncRNA *RUS*, located upstream of the *Slitrk3* gene, is predominantly expressed in neural tissues, and its level increases during the differentiation of neural stem cells into neurons[164]. Depletion of *RUS* resulted in proliferation arrest and induced apoptosis in mouse embryonic cortical neural stem

cells[164]. Another example is the lncRNA *TUNA*, which has been implicated in the neural differentiation of mouse embryonic stem cells[165]. When *TUNA* is depleted, embryonic stem cell proliferation is compromised, although pluripotency is maintained. The *TUNA*-RNA binding proteins complex was detected at the promoters of important regulators of embryonic stem cell differentiation, including *Nanog*, *Sox2* and *Fgf4*. Single knockdown of each of these RBPs led to inhibition of neural differentiation of mouse ESCs, similar to the effect of *TUNA* knockdown[165].

The role of circRNAs in the molecular pathogenesis of neurodegenerative disorders and brain aging was recently reviewed[166]. Several circRNAs associated with neurodegenerative diseases were shown to act as miRNA sponges, such as *circHDAC9*, *circSAMD4A*, *circDLGAP4,* and *circSLC8A1*[166]. The mechanisms of action of these circRNAs during normal conditions remain unknown, and it is therefore difficult to determine their exact role in disease development. In rat spinal cord injury (SCI) models, the expression of *circRNA-2960* was found to be significantly enriched and it was suggested that *circRNA-2960* might exacerbate secondary damage to the spinal cord. Mechanistically, *circRNA-2960* inhibits its target miR-124, a molecule that prevents secondary injuries from SCI and promotes injury recovery. The regulation of miR-124 expression by *circRNA-2960* could therefore represent a crucial mechanism that influences the prognosis of SCI[167]. Overall, despite clear evidence of aberrant expression of circRNAs in neurological disorders[168-170], the functional significance of these alterations remains to be thoroughly investigated. Because each ncRNA could be involved in different pathways in neurons, further studies at the level of singular targets of specific ncRNAs are warranted to discover ncRNAs that could serve as biomarkers and therapeutic targets for neurodegenerative disorders.

[H1] ncRNAs in infectious diseases and sepsis

Many studies have assessed how ncRNAs are involved in immune defense against microbial infections[171,172]. When considered collectively, ncRNAs act as positive or negative regulators to encourage a balanced immune response for an effective defense

against pathogens[173]. In **Box 1** we highlight the example of miR-155, which is functionally involved in many types of diseases, and discuss its therapeutic use.

miRNAs have been functionally connected to the cellular response during microbial infections. For example, miR-718, which is encoded from the 5' UTR of *IRAK1*, was shown to have an anti-inflammatory function through targeting PTEN[174]. IRAK1 is an important component of the TLR signaling pathways and thereby has a role in innate immunity, whereas PTEN downregulation by miR-718 decreases proinflammatory cytokine production through its downstream target molecules[174]. Pre-miR-718 is highly conserved across mammals and decreased miR-718 expression was shown to be associated with *Neisseria gonorrhoeae* infection[174]. It was hypothesized that miR-718 can help to evade recurrent bacterial infections and lower the lipopolysaccharide (LPS)-induced mortality rate by establishing LPS-induced tolerance[174]. On the same TLR pathway, let-7i directly binds to and downregulates *TLR4*[175], participating in the immune response against *Cryptosporidium parvum*, a parasite that causes intestinal and biliary infections.

It was shown that *MALAT1* lncRNA is a key player in controlling macrophage M1/M2 polarization[176]. Briefly, *MALAT1* expression is upregulated in LPS-treated macrophages, which differentiate towards a proinflammatory M1 phenotype, and it is downregulated in IL-4-treated cells, which differentiate in the M2 subtype. Notably, *MALAT1* knockdown decreases LPS-induced M1 macrophage activation, whereas IL-4–induced M2 differentiation and a macrophage profibrotic phenotype are increased by *MALAT1* knockdown[176]. Consistent with these observations, an independent study found that the expression of *Mirt2* lncRNA is elevated upon activation of the LPS-p38-Stat1 and LPS-IFN-α/β-Stat1 pathways in mouse macrophages[177]. Increased levels of *Mirt2* upon LPS treatment inhibited the K63-ubiquitination of TRAF6 and relieved inflammatory responses after TLR4 activation[177]. Other early experiments found that treating M2 microglia cells with IL-4 caused a dramatic decrease in the expression of lncRNA *GAS5* compared with resting microglia[178]. Mechanistically, *GAS5* negatively regulates the transcription of *IRF4* by binding PRC2 to inhibit M2 polarization[178]. Recently, it was reported that ablation of the mouse lncRNA *Malat1* activates the antioxidant pathway and alleviates sepsis[179].

[H2] Sepsis and the balance between human and viral miRNAs

Sepsis, the final stage of full-body disequilibrium to pathogenic bacterial, viral or fungal infections, remains a leading cause of human death and currently has no pathogenesis-specific therapy[180]. Since the initial discovery of downregulated miR-150 in peripheral blood cells and plasma from patients with septic shock[181], a substantial number of dysregulated cellular miRNAs in sepsis have been identified[182]. Cellular overexpression of miR-150 was sufficient to inhibit the pre-pro-B cell to pro-B cell transition by targeting MYB and FOXP1, respectively[183,184]. The miR-212 and miR-132 cluster was found to have a similar effect through negative regulation of FOXP1 and SOX4, respectively[185]. In another study, the loss of function of the miR-15 family, which comprises the miR-15a/miR-16-1, miR-15b/miR-16-2, and miR-497/miR-195 clusters, reduced normal pre-B differentiation by directly targeting cyclin E1 and D3[186]. Another recent study reported that miR-146a-5p is highly enriched in *Leishmania donovani*-infected bone marrow-derived macrophages (BMDMs), and positively correlates with dose and time of infection, which was further examined in an in vitro mouse model[187]. In infected BMDMs, downregulation of miR-146a-5p led to a decrease in *Arg1* expression and abundance of iNOS. It was observed that silencing BRD4 effectively restored miR-146a-5p expression and M2 polarization marker expressions in infected BMDMs[187]. Another therapeutically important sepsis-related miRNA, miR-93-5p, was uncovered by analyzing mouse and baboon models of sepsis, in addition to human peripheral blood mononuclear cells (PBMCs) obtained from patients with sepsis. In an in vivo mouse sepsis model, inhibition of miR-93-5p reduced inflammatory monocytes and increased circulating effector memory T cells, resulting in longer survival[188].

Although investigation of a single miRNA can yield insight into its biological function, it does not capture the complex, interconnected network of miRNAs that control cell biology and disease. In patients with sepsis, the miRNA network exhibits significantly less connection when compared to that of healthy controls. Perhaps explaining this observation, several miRNAs, including miR-16, miR-29a, miR-146, miR-155, and miR-

182, were reported to be 'sponged' by their protein coding targets in patients with sepsis[189].

Unexpectedly, it was discovered that Kaposi sarcoma virus (KSV)-produced miRNAs are differentially expressed in sepsis and may be used for diagnostic and therapeutic purposes. Specifically, elevated levels of miR-K-10b and miR-K12-12* play a functional role in sepsis as agonists of TLR8, leading to cytokine dysregulation characteristic of a cytokine storm[190]**.** Moreover, the viral Epstein Barr miR-BHRF-1 and the KSV miR-K12-12 were detected in plasma during the early systemic response to injury and were associated with unfavorable outcomes in polytrauma patients[191]. Starting from these observations, we suggest considering non-human (particularly viral) ncRNAs when using next-generation sequencing (NGS) methods to screen for ncRNAs involved in sepsis and other human diseases. Although virus-encoded-ncRNAs have largely been linked to immune evasion, virus life cycle regulation and virus-induced tumorigenesis[192], there is a considerable gap in understanding the specific mechanisms and processes underlying these functions.

[H2] SARS-CoV-2 infection and ncRNAs

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has led to hundreds of millions of confirmed cases and millions of deaths all over the world in the last few years[193]. The importance of ncRNAs in infectious diseases prompted scientists to swiftly investigate their potential role in COVID-19 soon after the beginning of the pandemic[194]. As a result, several studies published in the last three years demonstrate the involvement of miRNAs and lncRNAs in COVID-19[12,195-197].

A recent study reported that SARS-CoV-2 expresses a miRNA-like small RNA, termed CoV2-miR-O7a, which is derived from the coding region of the *ORF7a* transcript[195]. CoV2-miR-O7a is associated with Ago proteins, seems to influence interferon signaling pathways, and may contribute to SARS-CoV-2 pathogenesis[195]. It has also been shown that lncRNAs, such as *CHROMR,* are overexpressed in patients with SARS-CoV-2

infection[198]. Depletion of *CHROMR* resulted in attenuated interferon-stimulated gene expression and the sequestration of the nuclear repressor complex, IRF-2/IRF2BP2[198]. In addition, other lncRNAs, such as *PIRAT* and *LUCAT1* have been shown to be upregulated in patients with COVID-19 and were implicated in the progression of the disease. *PIRAT* appears to be preferentially expressed in myeloid cells and has been connected to tissue infiltration in infectious and inflammatory diseases[197]. Through the inhibition of alarmin expression, PIRAT creates a negative feedback loop with PU.1, located in the nucleus of human monocytes[**197**]. Consistent with this finding, negative feedback regulation was observed between LUCAT1 and Jack-STAT-dependent IFN immunity[**199**].

Coronavirus transcriptomes seem to contain additional components that contribute to the intensified inflammatory responses observed in patients with SARS-CoV-2, SARS, and Middle East Respiratory Syndrome (MERS) [200]. circRNAs encoded by coronavirus genomes have been identified and are implicated in viral pathogenesis[200]. For instance, two SARS-CoV-2 circRNAs contain IRES signals and have the potential for translation[200]. Moreover, in a study utilizing the human-pathogenic MERS-CoV as a model, the interactions between circRNAs and key components of the host cell competing endogenous RNA network were demonstrated, revealing several differentially expressed circRNAs during coronavirus replication[201]. Downregulation of circFNDC3B and circCNOT1 resulted in a substantial reduction in MERS-CoV viral load in human lung cancer cells (Calu-3) and fibroblast cells (HFL1), potentially related to the downregulation of their target genes, specifically *MAP3K9* and *USP15*[201]. Further research is needed to identify clinically applicable ncRNA signatures and explore the role of ncRNA in immunological and peripheral system regulation. However, a signature composed of 6 lncRNAs including *NRIR*, *BISPR*, *MIR155HG*, *FMR1-IT1*, *USP30-AS1*, and *U62317.2* has been shown to be associated with the regulation of SARS-CoV-2 infection[196]. Moreover, the importance of coronavirus infections as a source of interacting RNA and identifying novel drug targets for patients affected by SARS-CoV, SARS-CoV-2, and MERS patients remain important areas of investigation.

[H1] ncRNAs as disease biomarkers

Interest among the scientific community in the use of ncRNAs as disease biomarkers rely on several critical observations and considerations. First, relevant changes in ncRNA expression or activity have been detected in pathological tissues, rendering these molecules good indicators of underlying disease state or specific disease features[202]. Second, technologies are available for quantifying small and long ncRNAs in different tissue types and across different preservation methods (such as fresh frozen and fixed tissue samples). These span from PCR-based assays, through hybridization-based methods to NGS-based technologies. Finally, it is possible to quantify specific ncRNA molecules in cellular and subcellular compartments of diseased cells, as well as in extracellular compartments (such as extracellular vesicles, body fluids including urine, saliva, cerebrospinal fluid, synovial fluid, placenta, and breast milk)[203], which makes these molecules suitable for liquid biopsy applications. MicroRNAs were recently found to be selectively inserted in extracellular vesicles, that also display a moderate content of small ncRNAs, the distribution and composition of which depends on the size and isolation method. Comprehensive review articles have recently been published covering the main discoveries on biomarker ncRNAs and human diseases[8,204-210].

Several considerations should be made regarding studies on lncRNA in liquid biopsy samples. The levels of lncRNA in plasma and serum are particularly low compared to intracellular levels, their detection can be challenging, and their stability can be a concern[211-213]. Therefore, the preanalytical steps should be well-standardized, and the appropriate reference genes should be selected carefully[211-213]. Furthermore, the examination of genome-wide miRNA expression profiles in both healthy and diseased aging individuals has revealed that age has a significant impact on blood miRNA composition, potentially compounding the interpretation of results in the older population[214,215].

[H2] ncRNAs as biomarkers in cancer

Each year, thousands of papers are published on the differential expression of ncRNAs in various types of cancer. Although many of these studies suggest that the identified

dysregulated ncRNAs have potential as biomarkers and/or therapeutic targets, only a small fraction will reach in clinical trials. The NIH Early Detection Research Network (EDRN) and the ClinicalTrials.gov database are useful to track the hundreds of ongoing clinical trials of ncRNAs as potential biomarkers for cancer and other diseases. **Table 1** provides examples of clinical trials that examine the use of ncRNAs as biomarkers of disease; a comprehensive summary of clinical trials at ClinicalTrials.gov is provided in **Supplementary Table 1**.

[H3] ncRNAs as tissue biomarkers

Among the small ncRNA biomarkers most broadly tested in cancer tissues is miR-21-5p, which has been validated as a diagnostic or prognostic biomarker in most frequent cancers, such as lung[216], breast[217], colorectal[218,219], and CLL[220]. miR-21 is also released in circulation and detectable in extracellular vesicles, as described in the next section. miR-506-3p is another miRNA that has demonstrated significant prognostic potential across multiple cancer types, including ovarian, pancreatic, and gastric cancer[221-223]. Likewise, miR-31-3p has predictive potential and has been shown to have clinical relevance in metastatic colorectal cancer (CRC). Specifically, low levels of miR-31-3p have been identified as a predictor of response to anti-EGFR therapy in two clinical studies[224,225]. Currently, miR-10b-5p is the subject of investigation in an ongoing multi-site clinical trial on gliomas as a potential biomarker (NCT01849952) and has been identified as a potential therapeutic target in advanced glioblastoma[226].

Regarding lncRNA biomarkers, the most studied lncRNA in cancer tissues is *HOTAIR*. HOTAIR is involved in chromatin reprogramming, and its expression has been tested as a diagnostic or prognostic and predictive biomarker in ovarian[227], colorectal[228], breast[229], esophageal[230], and pancreatic[231] cancer. *MALAT1* has been identified as an early prognostic marker for metastasis development in surgically removed lung cancer[232], while *CCAT1* and *CCAT2* have well-characterized mechanistic roles and serve as prognostic biomarkers for CRC[233,234]. The detection of *PCA3* in urine is an FDA-approved diagnostic biomarker for prostate cancer. Furthermore, the combined measurement of *PCA3* and

619 the *TMPRSS2:ERG* gene fusion in prostate biopsy can improve the detection of prostate
620 cancer[235].

621

622 *[H3] ncRNA biomarkers in liquid biopsies and extracellular vesicles*

623 Circulating ncRNA biomarkers gained much attention in biomedical research when it was
624 discovered that several tumour-associated miRNAs, including miR-155 and miR-21, were
625 elevated in the serum of patients with diffuse large B cell lymphoma (DLBCL)[236].
626 Additionally, serum levels of miR-141 were found to distinguish patients with prostate
627 cancer from healthy controls. It subsequently became clear that miRNAs are stable in a
628 large variety of biological fluids[203].

629 As aberrant miR-21-5p expression is associated with inflammatory events, increased
630 levels of this miRNA in serum or plasma have been reported in many human conditions,
631 spanning from cancer to cardiac disorders. In cancer, there is now convincing data on the
632 value of miR-21-5p as a biomarker for gastric, esophageal and prostate cancer[218,237-239].
633 Among the circulating miRNAs with the confirmed diagnostic value, miR-371-3p is highly
634 promising; indeed, a prospective multicentric study on testicular germ cell tumours[240]
635 (teratoma excluded), revealed that miR-371-3p had a detection accuracy greater than
636 90%, higher than standard-of-care biomarkers, including alpha-fetoprotein in surgically
637 resected tumours[241]. ncRNA biomarkers can be particularly useful in diseases that are
638 difficult to diagnose, and where no coding gene-based biomarkers have been
639 identified[242].

640 A frequently explored opportunity in biomarker studies of circulating miRNAs is the
641 use of miRNA signatures, composed of miRNAs that are expressed concordantly. Two
642 studies have proposed miRNA signatures consisting of 13 miRNAs for early detection of
643 lung cancer, to be used alone[243,244] or in combination with low-dose Computer
644 Tomography (CT) scan[245], with promising results in clinical trials. Another group validated
645 an eight miRNA signature for the early diagnosis of esophageal squamous cell
646 carcinoma[239] in different prospective cohorts. A circulating miRNA signature composed
647 of five miRNAs can distinguish more aggressive prostate cancers[246].

The levels of miR-25-3p and miR-92a-3p were tested as prognostic biomarkers in patients with liposarcoma since they are secreted by liposarcoma cells through extracellular vesicles. The secreted miRNAs indeed act as proinflammatory signals for tumour-associated macrophages[247].

Detection of unmethylated fragments of the lncRNA *XIST* in plasma has been associated with testicular cancer presence[248] and proposed as a testicular germ-cell tumour biomarker[249]. Moreover, the lncRNA *ANRIL* was found to be upregulated in bone marrow mononuclear cells of patients with acute myeloid leukemia (AML) compared to healthy donors and suggested to serve as a valuable prognostic biomarker for AML[250].

[H2] ncRNAs biomarkers in cardiovascular diseases

ncRNAs with a diagnostic role in cardiovascular disease have been extensively investigated[206]. Cardiac tissue is not easily accessible; therefore, biomarkers of cardiac disorders are commonly tested in the blood or blood derivatives (plasma and serum)[251]. A prospective study tested the association between extracellular miRNA levels and heart failure risk in approximately 2,400 individuals[252]. Three plasmatic miRNAs (miR-17, miR-20a, and miR-106b) were associated with heart failure, such that individuals with higher levels of these miRNAs had a 15% reduction in long-term incident heart failure, after adjustments for other risk factors.

Cardiac tissue-specific miR-1, miR-133, and miR-208 (collectively known as myomirs) have been tested as circulating biomarkers in different settings. These miRNAs are collectively released to the circulation upon heart failure[253] and were proposed as diagnostic biomarkers to distinguish coronary artery disease (CAD, the primary cause of mortality in the United States), acute coronary syndrome, and heart failure[254]. As prognostic biomarkers, circulating miR-132, miR-140-3p, and miR-210 were validated as survival predictors in CAD on 1112 individuals using a multivariate model[255].

The detection of various lncRNAs in the bloodstream suggests that they are either protected from RNase-mediated degradation, similar to miRNAs or that they originate from a plentiful source with a continuous release. Two circulating lncRNAs, *ZFAS1* and

ICDR1AS, have recently been identified as independent predictors of myocardial infarction[256]. However, the precise origins and mechanisms linking these lncRNAs to myocardial infarction remain uncertain, as they were derived from whole blood[256]. Conversely, decreased levels of the lncRNA *HOTAIR* were observed in the plasma of myocardial infarction patients and were described to be cardioprotective through interaction with miR-1[257].

CircRNAs are present in abundance in bodily fluids such as blood, urine, and extracellular vesicles. These molecules have high stability and exhibit differential expression patterns in response to stress stimuli[258]. The circRNA *MICRA*, also known as *circ-ZNF609*, was found to be significantly reduced in the blood of individuals who have experienced myocardial infarction and serves as a promising prognostic biomarker for left ventricular dysfunction following myocardial infarction[259,260]. Furthermore, circRNA microarray analysis of PBMCs from patients with CAD has revealed *hsa_circRNA_0001879* and *hsa_circRNA_0004104* as potential diagnostic biomarkers for this condition[261]. Besides the lncRNAs and circRNAs mentioned above, additional transcripts have been suggested as potential circulating biomarkers in CVD[262,263]. The testing of lncRNAs and circRNAs as biomarkers in CVD is still at an early stage, although their above-mentioned dysregulation points toward potential usefulness in the near future, as it was recently reviewed[264,265]. A large proportion of the findings that suggest lncRNAs and circRNAs may serve as biomarkers for CVD are based on studies that involve limited numbers of participants and therefore require independent validation in larger studies.

[H2] ncRNAs biomarkers in neurodegenerative diseases

As obtaining nervous tissue from living individuals can be challenging, body fluids are often used as the most reliable source of ncRNAs. Several studies investigated the use of circulating small RNAs as Alzheimer disease biomarkers, tested either in cerebrospinal fluid (CSF), serum, or plasma, and derived extracellular vesicles. Serum miRNA signatures to differentiate frontotemporal lobar degeneration[266] or other forms of dementia[267] and Alzheimer disease were proposed. The neuronal-released miR-181a-5p was proposed as a circulating prognostic biomarker for ALS[268]. In this study, the authors

performed longitudinal monitoring of miR-181 in 252 patients subdivided into discovery and validation cohorts, and the potential of plasma miR-181 in predicting patients' death risk was assessed.

A panel of lncRNAs, quantified in PBMCs, was proposed as a diagnostic biomarker for multiple sclerosis[269]. Two of these lncRNAs, *NRON* and *TUG1*, were validated in an independent cohort. As for discrimination of multiple sclerosis subtypes, both CSF- and blood-circulating miRNAs have been investigated. Specifically, high levels of CSF miR-181c were associated with conversion from clinically isolated syndrome to relapsing-remitting (recovering) multiple sclerosis[270]. Serum miR-191-5p and miR-128-3p were associated with progressive forms (no recovery) of multiple sclerosis[271].

A total of 4,060 circRNAs with differential expression levels were identified in PBMCs derived from patients diagnosed with Alzheimer disease[272]. In silico analysis showed that the top 10 dysregulated circRNAs were strongly associated with various risk factors of AD, including inflammation, metabolism, and immune responses. These findings suggest that these circRNAs might play a potential role in the diagnosis of AD[272]. As another example, high expression levels of three circRNAs, *circFUNDC1*, *circPDS5B,* and *circCDC14A*, were found in patients with acute ischemic stroke (AIS) compared with healthy controls[273]. The elevated expression levels of these circRNAs were found to be positively correlated with infarct volume. These findings suggest that the three circRNAs may serve as potential biomarkers for the diagnosis of AIS[273].

[H1] Non-coding RNA therapeutics

The use of RNA-based therapies has emerged as a promising treatment approach for human diseases and vaccine development. Certain endogenous ncRNAs can regulate the expression of genes involved in human diseases, and their dysregulated expression can contribute to the onset of disease, highlighting the potential of these ncRNAs as targets for drug development. ncRNAs play a dual role in cancer as either oncogenes or tumour suppressors, leading to the abnormal inhibition or degradation of their target mRNAs, and as a result, they serve as both direct therapeutic targets and potential therapeutic candidates for cancer treatment. In this respect, the utilization of miRNA-

based therapeutics offers dual advantages. Firstly, as natural molecules found within human cells, miRNAs possess pre-existing mechanisms for their processing and downstream target selection, in contrast to artificial chemotherapy compounds or Antisense Oligonucleotides (ASOs). Secondly, miRNAs target multiple genes within a single pathway, leading to a more comprehensive and specific response. Several recent reviews have extensively discussed a range of approaches to modulate the therapeutic potential of ncRNAs, including the use of siRNAs, ASOs, shRNAs, anti-miRNAs, miRNA mimics, miRNA sponges, therapeutic circRNAs, and CRISPR-Cas9-based gene editing[52,274-278]. Currently, several ongoing clinical trials investigate the specific targeting of miRNAs for therapeutic purposes (**Table 2**); it is expected that similar clinical trials for lncRNAs and circRNAs will begin in the near future.

[H2] Approaches of non-coding RNA therapeutics

Currently, two main approaches exist for ncRNA-based therapeutic interventions, depending on the desired molecular outcome. The first is ncRNA antagonism, which involves inhibiting or repressing the expression or function of target ncRNA transcripts, often achieved by antisense RNAs. The second approach, known as ncRNA replacement therapy, aims to restore the expression or function of the target ncRNA and mostly involves the introduction of small RNAs.

One example of the former approach is Remlarsen (MRG-201), a molecule designed to mimic the activity of miR-29, which was shown to reduce the expression of proteins involved in skin fibrosis[279]. MRG-201 was investigated in a phase 2, double-blind, placebo-controlled study (NCT03601052) to explore the efficacy, safety and tolerability of the drug following intradermal injection in individuals with a history of scar fibrosis (keloids). Furthermore, based on the reports that MRG-201 reduced fibrosis in animal models, a peptide-conjugated MRG-229 mimic was developed as a potential therapy in humans with idiopathic pulmonary fibrosis[280]. After detailed anti-fibrotic activity tested in multiple models, including TGF-β1-treated human lung fibroblasts (NHLFs) and human precision-cut lung slices (hPCLS), in vivo bleomycin studies and toxicology in rats and non-human primates, the outcomes supports further clinical development[280].

A recent example of directly targeting ncRNA transcripts is a phase 1 randomized, double-blind, placebo-controlled study to assess the safety, pharmacokinetics, and pharmacodynamic properties of CDR132L, an antisense oligonucleotide-based inhibitor of miR-132, in patients with stable heart failure of ischaemic origin (NCT04045405). This trial described the linear plasma pharmacokinetics of miR-132, with no signs of accumulation, and was associated with cardiac functional improvements[281].

Additional developments in ncRNA-related therapeutics are of particular interest. A new therapeutic option is to target the downstream pathways of master regulator ncRNAs, including their target coding genes. As an example, the miR-15a/16-1 cluster is an essential player in CLL pathogenesis by targeting key anti-apoptotic proteins, BCL2 and MCL1[282]. When this cluster is downregulated or deleted, as in CLL, the downstream coding genes are upregulated, and the malignant cells lose anti-apoptotic potential and survive for longer periods. This mechanism had made CLL a deadly disease, until the development of Venetoclax (ABT199), a BCL-2 homology 3 (BH3) mimetic that specifically inhibits Bcl-2. Its use has fundamentally changed the natural history of the disease. Today, the therapeutic combination of Venetoclax and inhibitors of Bruton tyrosine kinase (such as Ibrutinib) increase progression-free survival and overall survival rates at 24 months to 95% and 98%, respectively[283]. As the downregulation of miR-15a/16 cluster is frequent also in other human cancers[284], the identification of patients with genomic deletions or mutations and/or with reduced expression of miR-15a/16 can identify patients who might respond well to Venetoclax or other novel BH3 mimetics.

Due to different mechanisms of action, a specific microRNA can be considered either a drug or a drug target in different pathologic conditions. For example, miR-16 was discovered as a tumour suppressor miRNA and therefore restoration by a miR-16 mimetic constitutes a suitable therapeutic strategy in cancers where this miRNA has reduced expression. For example, the safety and activity of miR-16-loaded minicells in patients with recurrent malignant pleural mesothelioma showed an acceptable safety profile and early signs of activity[285]. By contrast, recent studies showed that endothelium-targeted deletion of the miR-15a/16-1 cluster ameliorates blood-brain barrier dysfunction in ischemic stroke[286] and poststroke angiogenesis and improves long-term neurological recovery[287]. In this setting, the use of anti-miR-16 agents can result in adequate

798 therapeutic progress. Another promising therapeutic target is miR-21, which, besides its
799 relevance to other diseases, has recently been demonstrated to be upregulated in
800 pulmonary macrophages of both patients with COVID-19 and mice exhibiting acute
801 inflammatory lung injury. The inhibition of miR-21 (using RCS-21) reversed the
802 pathological activation of the macrophages and prevented pulmonary dysfunction and
803 fibrosis after acute lung damage in the mouse model[288]. The development of small-
804 molecule inhibitors (SMIs) of miRNAs, which directly bind and inhibit the activity of an
805 oncogenic or disease-causing miRNA (**Box 1**), has important advantages over the use of
806 oligonucleotides, such as superior metabolic stability, solubility and bioavailability[276,289-
807 291]. By use of small molecule inhibitors **[G]**, effective inhibition of oncomiRs (such as miR-
808 10b), as well as the upregulation of some important tumour suppressor targets (such as
809 PTEN) has been achieved in pre-clinical studies[292]. In addition, two small molecules
810 targeting specifically the triple-helical element for nuclear expression in *Malat1* RNA, but
811 not other similar structures present in the lncRNA *Neat1*, were identified by high-
812 throughput screening. The compounds significantly reduced *Malat1* levels and activation
813 of its downstream genes and induced the phenotypic attenuation of mammary gland
814 organoids branching[293]. This approach has great therapeutic potential as it can be
815 developed against small RNAs with very similar structures (as miR-21 and miR-10b), as
816 well as any large RNA that has a known secondary structure and that is involved in any
817 non-cancer disease including infectious diseases. An example of a potentially targetable
818 RNA is the genome of SARS-CoV-2, which is the largest single-stranded RNA virus
819 known to infect humans[294].

820

821 ***[H2] Challenges of non-coding RNA therapeutics***

822 Although ncRNAs have demonstrated therapeutic potential in vitro and in vivo, their
823 limited bioavailability in vivo presents a major challenge to their clinical translation[275]. To
824 overcome this obstacle, advanced drug delivery strategies are urgently required. To
825 address the problems of a short half-life, off-target effects, and low transfection efficiency
826 associated with RNA delivery, various ncRNA carriers and systems have been proposed
827 and extensively investigated, including several types of nanoparticles, ncRNA

828 modification, and the oncolytic adenovirus strategy. These strategies represent promising
829 approaches to enhance the delivery and efficacy of ncRNA-based therapies in vivo[275].

830 In parallel to improving delivery systems, challenges regarding the safety of ncRNA
831 therapies need to be addressed. Despite ongoing clinical trials evaluating miRNA
832 therapeutics for the treatment of human diseases, immune-related side effects still
833 present a significant challenge[295]. To illustrate, a phase I clinical study involving MRX34,
834 a liposomal miR-34a mimic, was prematurely terminated due to severe immune-related
835 side effects that resulted in the unfortunate deaths of four patients[296]. However, there is
836 still uncertainty regarding the specific cause of the clinical effects (including both toxicity
837 and anti-tumor activity) observed in MRX34. Serious adverse events (SAEs) attributed to
838 the treatment were predominantly observed later in the treatment cycle, occurring after
839 the completion of daily MRX34 infusions. These SAEs included sepsis, hypoxia, cytokine
840 release syndrome, and hepatic failure, which collectively suggest a pattern indicative of
841 immune-mediated toxicity[296]. It is unclear whether these effects are attributable to the
842 targeted gene-suppressing activity of the miR-34a nucleotide, a non-specific
843 inflammatory response triggered by the dsRNA present in the MRX34 formulation, or
844 possibly another underlying mechanism[296]. Considering the administration of
845 dexamethasone pre-medication and the absence of similar SAEs associated with the
846 same liposomal carrier used for a different investigational oligonucleotide drug, it is not
847 likely that the severe toxicities observed in MRX34 were caused by the liposome
848 carrier[297,298]. Moreover, the immune-related toxicities observed, along with the
849 unconventional response patterns occasionally seen with other immune-activating agents
850 such as CTLA-4 and PD-1/L1 immune checkpoint inhibitors, indicate an immune-
851 mediated mechanism underlying the clinical effects of MRX34[299,300].

852

853 **[H1] Conclusions and outlook**

854 Recent advancements in comprehensive functional genomics have improved our
855 understanding of the mechanism of action of ncRNAs on fundamental pathways related
856 to human diseases, thereby enhancing our knowledge of the clinical manifestations and
857 natural history of human diseases including cancer. However, understanding the full

range of ncRNA functions in human diseases still necessitates extensive investigation and clarification. For example, the amount of ncRNA exploration in infectious diseases could be widely expanded and such knowledge be used to prevent (through innovative biomarkers or vaccines) or control (through new therapeutics) future pandemics. This review has exclusively focused on the ncRNAs that have garnered the most attention in the literature. It is important to recognize that many other ncRNA types were not addressed here and therefore the impact of the entire ncRNA landscape in human diseases is even broader. Conducting large-scale expression screens and clinically evaluating ncRNAs can aid in identifying new non-coding transcripts that have a role in human diseases, potentially serving as therapeutic targets or biomarkers.

An essential question is: where is the field heading in the near future? We anticipate at least three major areas of development. First, we need to catalog and annotate all ncRNAs from each human cell type and body fluid in populations of millions of individuals from different races and ages, and possibly at the single-cell level. We are witnessing the start of such a huge and beneficial effort[301]. From a technological point of view, ncRNA quantification methods and data analysis methods are already available, so the bottleneck for this goal is more related to coordination and funding rather than technology availability. Such a catalog will be essential for understanding new mechanisms of diseases and even more so for biomarker development. Second, there is a need to advance proteomics methodologies capable of accurately and consistently identifying micropeptides ranging from 10–20 amino acids in length. These extremely short peptides originated from ncRNAs could be in a far greater number than what is currently known thus holding a more extensive biological relevance than previously assumed. If this is the case, then we will witness another major transformation in the ncRNA paradigm that will offer the scientific community an additional large category of ‘micro’ molecules to examine in-depth, carrying relevant translational implications. Another pivotal potential advancement will be the development of secure and effective ncRNA therapeutics. A revolutionary therapeutic breakthrough on par with the targeted efficacy of Gleevec or the immunotherapeutic impact of anti-PD1/PD-L1 drugs could substantially propel the field forward. One promising avenue in this domain involves

harnessing artificial intelligence to facilitate high-throughput development and investigation of small molecules that bind to coding and non-coding RNAs.

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Commentato [MA1]: Copy editor and MPS: Please leave names written out in full in dedication.

Author contributions

The authors contributed equally to all aspects of the article.

Competing interest's statement

G.A.C. is one of the scientific founders of Ithax Pharmaceuticals. The other authors declare no competing interests.

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Supplementary information

Supplementary information is available for this paper at https://doi.org/10.1038/s415XX-XXX-XXXX-X

Table 1. Representative examples of clinical trials investigating ncRNA biomarkers in cancer, cardiovascular disease, infectious diseases and neurogenerative disorders. For each miRNA, example trials were chosen based on a high number of study participants. COPD: Chronic Obstructive Pulmonary Disease.

NCT Number	Conditions	ncRNA	n=	Scope of the study
Cancer				
NCT02618538	Breast cancer	miRNAs	26,600	Investigating whether circulating miRNAs are significantly altered in the plasma of patients with cancer compared to matched healthy controls.
NCT05633342	Various cancer types	miRNAs	15,000	Characterizing intra-cellular multi-omic profiles of cancer and adjacent healthy tissues to aid the selection of circulating cancer biomarkers.
NCT02338167	Breast cancer	miRNAs	13,500	Discovery of biomarkers that predict progression free survival of patients with breast cancer. Biomarkers include gene expression profiling of the primary tumor and the corresponding metastases, somatic mutations, germline genetic variation, epigenetic changes and miRNA variation.
NCT03830619	Lung cancer	lncRNAs	1,000	Analysis of the sensitivity and specificity of serum exosome ncRNA as a biomarker for the diagnosis of lung cancer.
NCT05397548	Gastric cancer	lncRNA-*GC1*	700	Investigating whether circulating, exosomal lncRNA-GC1 can be used to monitor gastric cancer.
NCT05647941	Gastric cancer	lncRNA-*GC1*	700	Investigating whether lncRNA-GC1 can serve as a non-invasive biomarker for monitoring the neo-adjuvant chemotherapy response to personalized medicine for gastric cancer.
NCT04584996	Pancreatic cancer, biliary tract cancer	circRNAs	186	Defining the circRNA expression profile of pancreatic ductal adenocarcinoma (PDAC) tissues compared to controls, in an attempt to identify circRNA PDAC biomarkers.
NCT04464122	Neuroendocrine tumors	circRNAs	60	Identifying new circRNA biomarkers from tumor-educated platelets (TEPs) for the diagnosis and evaluation of treatment response in pulmonary and gastro-entero-pancreatic neuroendocrine neoplasms.
NCT05771337	Breast cancer	Circ-*ELP3*	80	Investigating the diagnostic value of *hsa_circ_0001785* (Circ-*ELP3*) and *hsa_circ_100219* (Circ-*FAF1*) in serum samples of patients with breast cancer .
Cardiovascular Diseases				
NCT05766046	Lung cancer, Cardiovascular diseases, COPD	miRNAs	7,324	Developing a diagnostic test analyzing miRNAs from blood of patients with cardiovascular diseases and lung cancer.
NCT03049254	Various cardiovascular conditions	miRNAs	6,000	Investigating blood-based biomarkers that predict disease onset, disease progression, and the likelihood of arrhythmia.
NCT04189029	Heart failure	miRNAs, lncRNAs	2,620	A prospective multicenter study to decipher phenotypic variability within patients with heart failure and preserved left ventricular ejection fraction.
NCT03170830	Acute Myocardial Infarction	circRNA-*Uck2*	178	Evaluating the diagnostic value of circRNA-Uck2 in acute myocardial infarction.
NCT02297776	Cardiac Arrest	miRNA, circRNA	160	Evaluating circRNA and miRNA plasma biomarkers for their ability to estimate the extent of brain injury after cardiac arrest.
NCT03076580	Cardiomyopathies	miRNAs, lncRNAs	2,000	A multi-omics study of cardiomyopathies patients, aiming to determine genetic risk factors and serial biomarkers of cardiomyopathies in diagnosis and prognosis.
NCT03225183	Cardiovascular Disease, Hypertension	lncRNAs	1,700	Characterizing the relationship between of lncRNAs and cardiovascular diseases and risk factors.
Neurological Diseases				
NCT05418023	Autism spectrum disorder, developmental delay	miRNAs	6,604	Validating a salivary miRNA diagnostic test for autism spectrum disorder.
NCT04961450	Amyotrophic Lateral Sclerosis, Frontotemporal Dementia, Motor Neuron Disease	miRNAs	2,500	Investigating miRNA biomarkers in blood, saliva, feces, cerebrospinal fluid, muscle tissue and nerve tissue of patients with motor neuron disease and frontotemporal dementia.
NCT04509271	Alzheimer disease	miRNAs	1,300	Investigating miRNA biomarkers for the diagnosis of mild cognitive impairment due to Alzheimer disease.
NCT03152630	Dementia	lncRNA	600	Investigating early and prognosis diagnosis of vascular dementia.
NCT04807738	Multiple sclerosis	lncRNA	110	Studying the effect of virtual reality on upper limb function and postural stability in people with multiple sclerosis; lncRNA biomarkers were analyzed to assess the biological effect of rehabilitation intervention.
NCT05341453	Spinal muscular atrophy	lncRNA	16	~~A randomized controlled trial is aimed to discover~~ Studying the effect of physiotherapy and hippotherapy ~~effect and efficacy~~ on children with SMA, with efficacy assessed in part using measurement of lncRNA in blood.
NCT05098340	Acute Ischemic Stroke	circRNAs	500	Analyzing the expression pattern of circRNAs in patients with acute ischemic stroke and healthy controls, to identify detection and prognosis biomarkers.
NCT04175691	Acute Stroke, Ischemic Stroke	miRNAs, lncRNAs, circRNAs	500	Analyzing the expression pattern of ncRNAs in patients with acute ischemic stroke and healthy controls, to identify detection and prognosis biomarkers.

Commentato [MA2]: **[Au: Please clarify how lncRNAs were studied in thie clinical trial.]**

Commentato [FN3R2]: **MFerracin:**
George I don't know here- The lncRNA is just the target of the disease, I don't think it is correct to cite the study among the biomarkers

Commentato [FN4R2]: In this trial the efficacy of physiotherapy and hippotherapy is analyzed on children with SMA by biomedical measures, by molecular **biological markers (lncRNA) in blood** and by surface electromyography (EMG). The primary goal of this study is to compare two physiotherapeutic approaches - the recommended form of classical physiotherapy and the method on a neurophysiological basis - hippotherapy.

I think this is a good example of how ncRNA biomarkers can be used to monitor therapy, however, we can change this to another example for lncRNAs if you prefer so.

Commentato [MA5R2]: OK – how is this for a description?

Commentato [MOU6R2]: George: this is fine for me

NCT04230785	Acute and Ischemic Stroke, Endovascular Treatment	ncRNAs	300	Analyzing the expression pattern of ncRNAs in patients with acute ischemic stroke before and/or after endovascular treatment.
Infectious Diseases and Sepsis				
NCT01780298	COPD	miRNAs	739	Investigating biomarkers for the differentiation of participants with COPD and three matched control groups: one of non-smoking subjects (never smoked), one of ex-smokers and one of current smokers.
NCT03280576	Sepsis	miRNAs	556	Analyzing the expression levels of miRNAs isolated from plasma, circulating exosomes and blood cells by next-generation sequencing to characterize epigenetic influences on progranulin plasma levels.
NCT05398952	Post viral fatigue, viral myocarditis	miRNAs, lncRNAs, circRNAs	2,000	Examining circulating ncRNA biomarkers in patients with post-COVID-19 persisting symptoms to identify new diagnostic and prognostic biomarkers.

1893 **Table 2. Examples of clinical trials investigating miRNA-targeting therapies.**

Related disease	Study ID	Treatment	Target(s)	Scope of the study	Phase
Cutaneous T-cell Lymphoma, Mycosis Fungoides (CTCL, MF)	NCT03837457	A synthetic locked nucleic acid-modified oligonucleotide inhibitor of miR-155 (MRG-106)	miR-155	Evaluation of MRG-106 impact on skin lesion severity, disease symptoms, quality of life, and the duration of stable or improved disease status, while ensuring no evidence of disease progression.	2
Cutaneous T-cell Lymphoma, Mycosis Fungoides (CTCL, MF)	NCT03713320	A synthetic locked nucleic acid-modified oligonucleotide inhibitor of miR-155 (MRG-106)	miR-155	Comparison of the effects of MRG-106 to vorinostat, a drug that has been approved for the treatment of CTCL.	2
Liver Cancer, Small Cell Lung Cancer, Lymphoma, Melanoma, Multiple Myeloma, Renal Cell Carcinoma, Non-Small Cell Lung Cancer	NCT01829971	A liposomal miR-34a mimic (MRX34)	Multiple oncogenic genes (such as, MEK1, MYC, PDGFR-α, CDK4/6, BCL2, WNT 1/3, NOTCH1, CD44)	Evaluation of MRX34 safety on patients with primary liver cancer, selected solid tumors, and hematologic malignancies.	1
Malignant Pleural Mesothelioma, Non-Small Cell Lung Cancer	NCT02369198	Targeted minicells containing a miR-16 mimic (TargomiRs)	EGFR-expressing cancer cells with an anti-EGFR bispecific antibody	Evaluating the safety, optimal dosing, and activity of TargomiRs in patients with malignant pleural mesothelioma	1
Colorectal Cancer	NCT03362684	Cetuximab, FOLFOX	miR-31-3p and miR-31-5p	Identifying the prognostic role of miR-31-3p and miR-31-5p in stage III colon cancer, specifically their potential as indicators of patient outcomes in the context of anti-EGFR therapy.	3
Coronary Heart Disease, Acute Myocardial Infarction	NCT02850627	Tongguan capsule	Global regulation of miRNA levels	Assessing the impact of Tongguan capsule on miRNA profiles in patients.	4
Preclinical Alzheimer Disease	NCT02045056	Gemfibrozil	miR-107	Examining the safety and efficacy of Gemfibrozil in regulating miR-107 levels as a potential strategy for	Early 1

				Alzheimer Disease prevention.	
Organ Protection	NCT05503043	Lidocaine	MiR-135a, Rock2, Add1	Investigating the impact of intravenous lidocaine on serum miR-135a levels and its downstream proteins (Rock2 and Add1) in patients.	NA
Alport Syndrome	NCT03373786	RG-012 (lademirsen)	miR-21	Assessing the impact of RG-012 on renal miR-21.	1
Keloid	NCT03601052	An oligonucleotide mimic of miR-29b (MRG-201/Remlarsen)	Multiple multiple factors involved in the fibrotic response (eg, collagen)	Assessing the efficacy of Remlarsen in preventing or reducing Keloid formation	2
Endometriosis	NCT05331053	Atorvastatin	Global regulation of miRNA levels	Investigating the role of miRNAs (let7-a, let7-b, let7-g, miR-98, miR-590) in driving elevated LOX-1 receptor expression and function in endometriosis.	4

Figure legends

Figure 1. The classic and non-classic functions of miRNAs. | a) The classic function of miRNAs is to target the 3' UTR of sequence-specific mRNAs, causing mRNA degradation or translational repression, as illustrated for miR34a targeting *PDL1* transcripts. **b)** Certain miRNAs can target the 5' UTR or coding sequence (CDS) of their target mRNA, resulting in either mRNA degradation, translational repression, or even increased translation of their target. For example, miR-24 can bind both to the 3' and 5' UTR of *Jab1* mRNA causing its posttranscriptional inhibition, whereas miR-10a binds to the 5' UTR of ribosomal-encoding mRNAs, such as *Rps16,* and enhances their translation. **c)** miRNAs can act as mediators of intracellular communication by being secreted via extracellular vesicles (EVs) and acting as hormones[39]. On immune cells, miRNAs can directly target Toll-like receptor (TLR) proteins by acting as their ligands, in turn activating TLR signaling pathways and inducing an immune response[38,40 41]. For example, let-7i can target TLR4, whereas miR-21 and miR-29a can target TLR8. **d)** Some miRNAs can also interact with non-Ago proteins, so-called miRNA-binding proteins (miRBPs), which can work in cooperation or competition with Ago, thereby enhancing or silencing miRNA function on its target molecule. Examples include miR-1 and the TNRC6B miRBP, and miR-21 and PDCD4 miRBP, respectively. miRNA can also be transported between Ago2 and miRBP; however this mechanism is less studied and not currently well understood. **e)** Some pri-miRNAs encode regulatory peptides that can influence the expression of the mature miRNA.

Figure 2. The main functions of lncRNAs. | a) DNA interaction. lncRNAs can directly bind to DNA, forming R-loops, or can have a role in chromatin regulation in a complex with DNA-binding proteins. For instance, GADD45A can bind to the R loop formed by the lncRNA *TARID* at the TCF21 promoter, triggering local DNA demethylation by recruiting TET1 to the DNA[59]. *SWINGN* lncRNA, which is transcribed from an enhancer, modulates the activation of GAS6 oncogene by binding to SWI/SNF tumor suppressor complex and influences its ability to drive epigenetic activation of specific promoters[60]. *Xist* lncRNA,

which is responsible for X chromosomal inactivation, binds to SAF-A chromatin-interacting protein and is thereby able to localize to sites on the X chromosome. *Xist* directly binds to SHARP and the resulting complex recruits SMRT to these DNA regions and recruits HDAC3 to the X chromosome or induces HDAC3 enzymatic activity, which results in chromatin compaction and transcription silencing[61]. **b)** Various RNA interactions. lncRNAs can interact with mRNAs and affect translation, RNA stability or block miRNA binding sites and thereby inhibit the effect of miRNAs. For example, the lncRNA *GAS5* interacts with the translation initiation complex eIF4F, by directly binding to eIF4E and decreasing the translation of c-Myc[302]. The *TINCR*–STAU1 complex seems to mediate the stabilization of different mRNAs, such as *KRT80*[303]. *PTB-AS* substantially increases *PTBP1* mRNA levels by directly binding to its 3' UTR and blocking miRNA binding sites[304]. **c)** Sponge activity by miRNA interaction. *MALAT1* can act as a miRNA sponge for miR-34c and thereby upregulate *SATB2* expression and alleviate the symptoms of osteoporosis in mice[62]. *HOTAIR* can sponge the tumor-suppressor miR-222-3p and thereby contribute to ovarian cancer progression[63]. **d)** Protein interactions. lncRNAs can interact with proteins and act as their scaffolds or guides. For example, NFAT1 kinases are scaffolded by the lncRNA *NRON*[64], whereas *HOTAIR* specifically binds to YBX1, and promotes YBX1 nuclear translocation[64,65]. **e)** Some lncRNAs can harbor 'coding' activity and produce micropeptides. For example, *LINC0065* lncRNA can be translated to the CIP2a-BP micropeptide.

Figure 3. The main functions of circRNAs. | a) CircRNAs have the potential to serve as sponges for miRNAs, as demonstrated by *circTDRD3*, which harbors target sites for miR-1231. **b**) CircRNAs can interact with specific mRNAs and regulate their stability and/or translation. An example is *circZNF609* interaction with *CKAP5* mRNA. **c**) CircRNAs can undergo translation and produce small peptides, as demonstrated by *circCDYL2* translation into Cdyl2-60aa small peptide, which is approximately 7 kDa. **d**) CircRNAs that contain motifs capable of binding to RNA-binding proteins possess the capacity to act as decoys or sponges for proteins, consequently modulating their activity. CircRNAs harboring motifs that facilitate binding between an enzyme and its substrate can act as scaffolds, enabling the co-localization of the two molecules and optimizing

reaction kinetics. CircRNAs can interact with gene promoters, recruit TET1 demethylase, and initiate significant demethylation of CpG islands within the DNA. Additionally, circRNAs can bind to U1 snRNP and subsequently engage with the RNA polymerase II transcription complex, enhancing protein expression.

Figure 4. Examples of the different mechanisms of ncRNAs in human diseases. | a) *H19* lncRNA acts as a miRNA sponge in breast cancer tissue and thereby reduces the level of miR200a and miR200b in tissues and circulation, resulting in ARF protein accumulation that facilitates epithelial-mesenchymal transition (EMT). **b)** *Dworf* lncRNA has a heart-specific expression and is down-regulated in ischemic failing human hearts. Expression changes in DWORF small peptide, which is encoded by *Dworf* lncRNA and acts by regulating the Sarcoendoplasmic Reticulum Calcium ATPase (SERCA) calcium pump in myocytes, have a potential role in heart failure. **c)** *Cdr1as* circRNA contains 73 binding sites for let-7 and thereby acts as a miRNA sponge. As a consequence, reduced let-7 levels cause decreased UBE2A and increased SNCA protein levels that contribute to amyloid beta plaque accumulation and thereby to Alzheimer Disease. **d)** SARS-CoV-2 integrates and increases miR-2392 expression, promoting COVID-19 disease progression.

Figure 5. NcRNAs are important biomarkers and therapeutic targets.

A. Various ncRNA species can be detected and analyzed in standard biopsy samples and liquid biopsy specimens through various techniques including quantitative PCR (qPCR), droplet digital PCR (ddPCR), RNA Sequencing or in situ Hybridization. All types of ncRNA species can be isolated from blood cells, serum, plasma, extracellular vesicles, urine, saliva, breast milk, and cerebrospinal fluid among others. They have the potential to serve as diagnostic and prognostic biomarkers as well as to help monitor the diseases treatment and outcomes. **B**. Representative examples of the two main types of ncRNA therapies ~~e~~ investigated in pre-clinical and clinical stages. In response to cardiomyocyte stress, miR-132 is upregulated in the cardiac tissue of patients with cardiac stress or injury. Intravenous infusion of CDR132L containing antisense miR-132 is under clinical

investigation to improve cardiac function. MesomiR 1 is a miR-16 mimic encapsulated in minicells that aim to restore the level of miR-16 tumour suppressor in cancer cells and is under clinical investigation to treat malignant pleural mesothelioma.

Box 1. miR-155 regulation of the immune system and therapeutic use.

The involvement of ncRNAs in the various facets of immune function is an extensively studied area[305-307]. Various well-characterized miRNAs, such as miR-146, miR-150 and miR-155, have been reported to be involved in regulating lymphocyte, monocyte and macrophage phenotypes, respectively[308]. From a clinical perspective, the most advanced therapeutic applications are related to miR-155, which has important roles in T and B cell proliferation and cytokine production[309-312]. Overexpression of miR-155 in activated T helper (Th) cells can participate in Th2-mediated airway inflammation through targeting of sphingosine receptor S1PR1[313]. In addition, miR-155 is required for optimal proliferation of regulatory T cells (Treg) in vitro[314], which suggests an important role in regulating T-cell expansion. In vivo studies also showed that miR-155 acts in regulating interferon (IFN) responsiveness and the $CD8^+$ T cell response against pathogens[311]. Furthermore, overexpression of miR-155 has been shown to enhance cytokine responsiveness, engraftment, cytokine production and anti-tumour function[310]. Consistent with these findings, overexpression of miR-155 directly suppresses SHIP1 levels, while enhancing Polycomb repressor complex 2 (PRC2) activity by promoting the expression of the PRC2-associated factor PHF19[310].

miR-155 has a central role in regulating serine/threonine kinase (Akt)-dependent M1/M2 activation of macrophages[315]. In addition, miR-155 overexpression enabled successful reprogramming of tumour-associated macrophages (TAMs) into pro-inflammatory M1 macrophages[316]. Notably, miR-155 may contribute to the development of resistance to chemotherapy, as evidenced by its role in the cross-talk between neuroblastoma cells and human monocytes in chemoresistance, involving cell-to-cell communications with malignant cells via an exosomic miR-21/TLR8-NF-κB/exosomic miR-155/TERF1 signaling pathway[317]**.** Clinical applications related to suppressing miR-155 for cancer therapy targeted to the TME were developed for DLBCL**[318]** using the attachment of nucleic acid antimiRs to a peptide with a low pH-induced transmembrane structure (pHLIP). Further combinatorial therapeutics of anti-miR-155 with chemotherapy for the treatment of lung cancers was reported using the non-toxic DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) Food and Drug Administration (FDA)-approved

nanoliposomes[319]**.** Cobomarsen (also known as MRG-106) is a locked nuclei acid (LNA)-based inhibitor of miR-155, used for the treatment of cutaneous T-cell lymphoma (CTCL), mycosis fungoides (MF) subtype. Promisingly, it is likely that this drug could be repurposed for any other non-cancer type of disease in which miR-155 has abnormally high expression, and is pathogenetically involved, such as autoimmune inflammatory disorders[320]. A phase I trial demonstrated that Cobomarsen was well-tolerated, had clinical activity and had the potential to improve patients' quality of life[301].

Glossary terms

Small peptides (sPEP)

Small peptides, also called micropeptides, are polypeptides that are encoded by small open reading frames (sORFs) and consist of less than 100-150 amino acids, sometimes translated from ncRNAs.

Non-Ago proteins

Argonaute (Ago) proteins are interactor partners of small ncRNAs, such as miRNAs and siRNAs, which facilitate their target binding and thereby their effector mechanisms. It was recently uncovered that miRNAs can interact with other, non-Ago proteins as well.

Sponge

RNA molecules such as circRNAs that can bind and sequester RNAs or proteins and thereby inhibit their effects.

Ribosome profiling

A deep-sequencing-based method that reveals ribosome-associated mRNAs, thereby predicting regions subjected to translation.

Small-molecule inhibitor (SMI)

Compounds smaller than 500 Da developed to target any portion of a target molecule and to cause its inhibition.

Short open reading frame (sORF)

Also known as small ORF, these are 100 nucleotide-long putative protein-coding sites, which were previously overlooked as non-relevant regions.