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Metabolic disorders affecting the liver and heart: Therapeutic efficacy of miRNA-based therapies?

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ABSTRACT

Liver and heart disease are major causes of death worldwide. It is known that metabolic alteration causing type 2 diabetes (T2D) and Nonalcoholic fatty liver (NAFLD) coupled with a derangement in lipid homeostasis, may exacerbate hepatic and cardiovascular diseases. Some pharmacological treatments can mitigate organ dysfunctions but the important side effects limit their efficacy leading often to deterioration of the tissues. It needs to develop new personalized treatment approaches and recent progresses of engineered RNA molecules are becoming increasingly viable as alternative treatments. This review outlines the current use of antisense oligonucleotides (ASOs), RNA interference (RNAi) and RNA genome editing as treatment for rare metabolic disorders. However, the potential for small non-coding RNAs to serve as therapeutic agents for liver and heart diseases is yet to be fully explored. Although miRNAs are recognized as biomarkers for many diseases, they are also capable of serving as drugs for medical intervention; several clinical trials are testing miRNAs as therapeutics for type 2 diabetes, nonalcoholic fatty liver as well as cardiac diseases. Recent advances in RNA-based therapeutics may potentially facilitate a novel application of miRNAs as agents and as druggable targets. In this work, we sought to summarize the advancement and advantages of miRNA selective therapy when compared to conventional drugs. In particular, we sought to emphasise druggable miRNAs, over ASOs or other RNA therapeutics or conventional drugs. Finally, we sought to address research questions related to efficacy, side-effects, and range of use of RNA therapeutics. Additionally, we covered hurdles and examined recent advances in the use of miRNA-based RNA therapy in metabolic disorders such as diabetes, liver, and heart diseases.

1. Introduction

1.1. Metabolic disorders affecting liver and heart

Metabolic disorders are complex processes involving the disruption of whole or partial biochemical pathways, tissues and organs, and are often of genetic origin. Although the cause of errors in metabolic processes may be a disabled enzyme, the consequences can affect entire organs, such as worsening kidney function, increasing inflammation, and promoting the progression of liver and heart diseases.

A complicated cross-talk between liver and heart has long been recognized [1]: the liver and the heart are intimately related by various inflammatory and systemic or metabolic conditions that simultaneously affect them. The most prevalent metabolic disorder is represented by

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type 2 diabetes (T2D) whose complications on liver and heart are well known. Regarding the liver, Nonalcoholic fatty liver disease (NAFLD) represents the most common liver disease in western countries with a common link to metabolic syndrome (MetS) and T2D. Insulin, a key factor for diabetes, has a role in promoting liver dysfunction such as the activation of lipogenesis and inhibition of fatty acid oxidation (FAO), thus contributing to hepatic lipid accumulation [2,3]. NAFLD is typically silent and is the most frequent liver disease with a prevalence of approximately 25% in the general adult population [6]. Being closely linked to overweight and obesity, metabolic syndrome, and insulin resistance (IR), NAFLD is even more frequent in patients with T2D than in subjects without diabetes [4]. The relationship between these two metabolic diseases is bi-directional and complex. If NAFLD increases the risk of developing diabetes and its cardiovascular complications, diabetes increases the risk of progression of NAFLD towards more advanced forms of liver disease [5]. Growing evidence suggests that NAFLD potentially contributes to the increased risk of cardiovascular-related morbidity and mortality due to several pathogenic mechanisms that contribute to the accumulation of ectopic fat in the liver [6]. The resulting inflammatory response involves immune cells such as macrophages and T cells [7,8] exacerbating the abnormalities.

From the heart front, diabetes as metabolic disorder can cause a plethora of cardiovascular complications (resumed in [9]). Experimental and clinical evidence showed that impaired liver function per se aggravates cardiac function. The risk and prevalence of Coronary artery disease (CAD) are increasing concomitantly with the growing incidence of chronic liver disease-related hepatic dysfunction such as Nonalcoholic steatohepatitis (NASH). This disease, which represents the most severe histologic form of Nonalcoholic fatty liver disease (NAFLD) [7,10], is characterized by many metabolic abnormalities such as accumulation of fat in the liver, lobular inflammation, hepatocyte injury, and hepatic fibrosis [11,12].

Conventional drugs for either heart or liver may induce cross-side effects. Cardiovascular drug therapy may present serious side effects on the liver; hepato-biliary drug therapy may affect heart and vessels as well as post-transplantation immuno-suppressive drugs may show reciprocal cardio-hepatotoxicity. Given the frequency of these adverse effects and their association with intra- and extra-hepatic complications, the management of patients under treatment for liver or heart conditions requires a multidimensional approach.

Therefore, it is crucial to identify the mechanism linking them to provide the best treatment in clinical practice. Recently, engineered RNA molecules are emerging for a number of human diseases as novel therapeutic approach, in particular for Non-communicable Diseases (NCD) such as cardiovascular disease (CVD) and its complications whose incidence and mortality are dramatically increasing every year [13]. Among RNA-based technology, engineered microRNAs may be used as therapeutic agents as well as druggable targets. In fact, miRNAs inhibit messenger RNA (mRNA) target translation, thus repressing protein synthesis could even modify the disease phenotype. Similarly, modifying a single miRNA could produce changes in phenotype. On the other hand, the knowledge of miRNA targets and their functions is a challenge in network biology. Developing miRNA therapeutics requires a deep understanding of RNA targets as well as the pathways they regulate. In this work, we sought to explore the current FDA-approved RNA therapeutics, and the strategies for enhancing the efficacy of the novel RNA molecules used to treat metabolic, liver, and heart diseases. In particular, we sought to highlight the major advantages of using druggable non-coding RNA, such as miRNAs, concerning antisense oligonucleotides (ASOs) or other RNA therapeutics or conventional drugs.

2. RNA-based option therapy

2.1. Proof-of-concept in RNA world

The use of RNA therapeutics was initially proposed in the ‘90 s, but the concept was conceived after Craig and Venter sequenced the entire human genome during “The Human Genome Project". The direct consequence was a re-conceptualization of the central dogma of biology, in which "one gene codes one protein". About a quarter of the human genome is transcribed into mRNAs after the intron excision. In the past, the original concept of junk DNA, known as a repetitive portion of non-coding DNA sequence with undetermined properties, was replaced by the discovery of the "non-coding sequences" with specific roles in gene expression control. Therefore, it became clear that one gene could codify many protein-isoforms. The subsequent discovery of alternative splicing machinery, of introns and of Alu-based exonization processes [14] gave rise to the novel concept of a new gene expression modality. RNA therapeutics are attracting huge interest because of their wide range of potential uses as druggable molecules.

Only a few portions of the human genome have been drugged by the currently approved protein-targeted therapeutics (synthetic small-molecule and antibodies) because most human DNA sequences are transcribed into "non-coding" transcripts [15].

RNA molecules, namely antisense oligonucleotides (ASOs), and RNA-interference (RNAi), such as short interference RNAs (siRNAs) and miRNAs (or miRs), can directly target the mRNAs and non-coding RNAs (ncRNAs) through Watson–Crick base-pairing [16] (Fig. 1). Currently, RNA-targeting drug development programs, including mRNA-based vaccines or siRNAs, are at different stages: some are in pre-clinical or in early stage clinical trials, others are in phase III (primarily for genetically well-defined rare diseases). Conversely, some are awaiting regulatory decisions while others are not yet approved. A few examples may help illustrate this. The first successful FDA-approved RNA-targeting drug with extensive clinical use was the class of aminoglycoside antibiotics such as Neomycin, a small RNA-binding molecule that binds the 30 S subunit of bacterial ribosomal RNA, resulting in inhibition of mRNA translation [17]. Similarly, Fomivirsen [18], a 21-nucleotide guanosine-derived selective inhibitor of cytomegalovirus (CMV)-DNA polymerase, was the first small molecule approved by FDA in 1998 for CMV-induced chorioretinitis. Its properties trigger specific inhibition of human cytomegalovirus replication, by binding to complementary sequences on mRNA transcribed from the transcriptional unit of the virus [19]. More recently, a splicing modulator called Risdiplam, was approved for the treatment of spinal muscular atrophy (SMA) and has been successfully used as a therapeutic agent because of its ability to stabilize a selective splice-site in exon 7 of SMN2 (Survival of motor neuron 2) and promote the correct splicing event (https://www.fda.gov/news-events/press-announcements/fda-approves-oral-treatment-spinal-muscular-atrophy).

2.1.1. Nucleoside-modified mRNA (modRNA) therapeutics

Another RNA-based approach involves the use of novel gene therapy characterized by the synthetic “nucleoside-modified mRNA” (modRNA) as a new approach to express protein in vitro and in vivo systems. The first observations on the usefulness of modRNA were as potential use in regenerative cardiology after myocardial infarction: Zangi et al. [20] transiently applied in vivo modRNA encoding human vascular endothelial growth factor-A (VEGF-A) results in the expansion and directed differentiation of endogenous heart progenitors in a mouse myocardial infarction model. The main characteristics of modRNAs are their transience, stability and safety to avoid genomic integration. They are non-immunogenic due to the specific design discussed above, RNAse resistance, and a controlled mRNA delivery to the tissues, making it possible to achieve the therapeutic effect in different organs. The design of modRNA-based constructs ensures the delivery of any gene to a tissue; modRNA is a modified nucleotide sequence with 100% replacement of uridine by N1-Methylpseudouridine-5’-Triphosphate (1- mψU) [21] and 5-methyl-cytosine [22], conferring a conformational change in secondary structure leading to the ability of modRNA to escape from the recognition by Toll-like receptors (TLRs) [23], allowing for more resistance to RNases, and inhibiting innate immune response [24]. Thus,

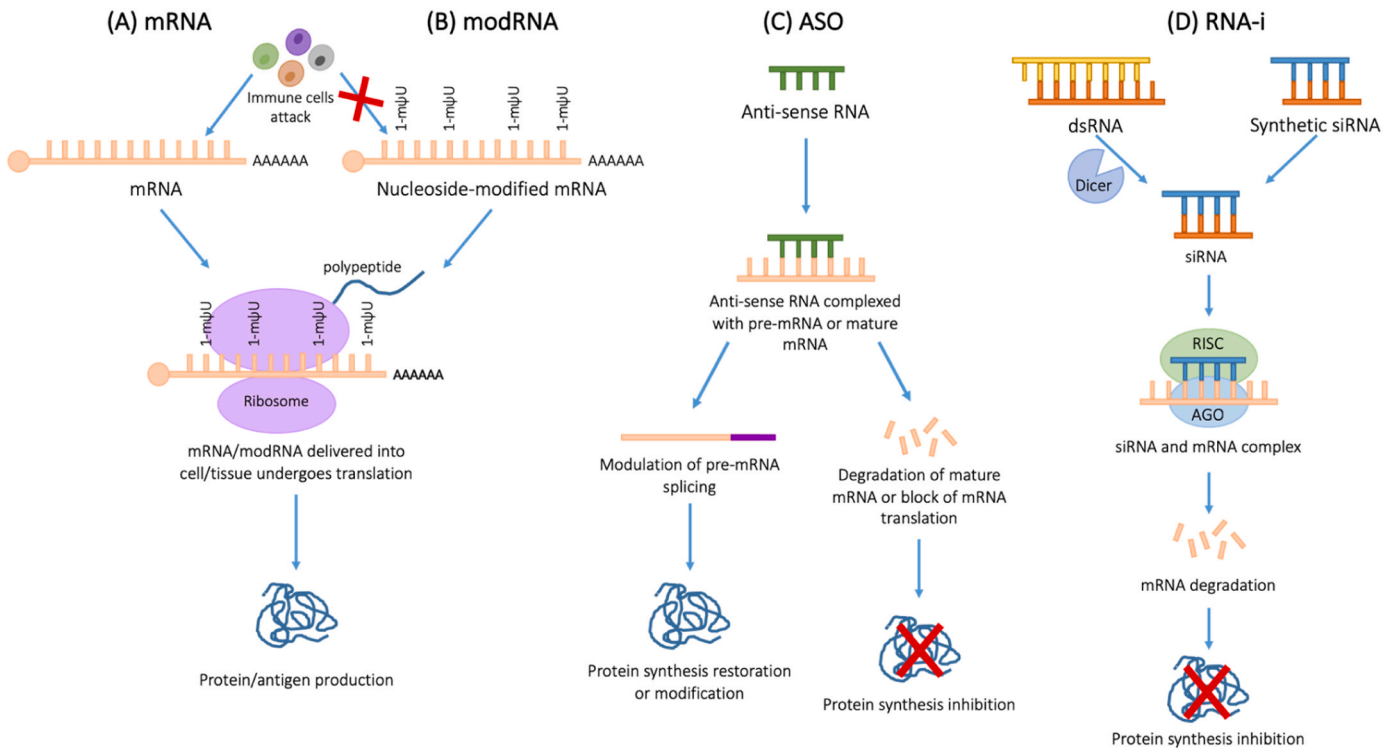

Fig. 1. Schematic representation of RNA-based therapeutics. Schematic representation of RNA-based therapeutics: (A) mature mRNA or (B) synthetic nucleoside-modified mRNA (modRNA), capable to immune escape and RNAse resistance, are introduced into cells, where recruit ribosomes and are translated to produce specific antigens or functional proteins. (C) ASO are small single-stranded oligonucleotides that bind to complementary target pre-mRNA or mature mRNA. They can alter pre-mRNA splicing pattern by targeting splicing regulatory factors, leading to restored or modified mRNA translation. They can also block mRNA translation by preventing ribosome recruitment or induce mRNA degradation, resulting in gene silencing. Post-transcriptional gene silencing can be triggered by (D) siRNAs, small double stranded RNA molecules produced by cleavage of longer double stranded RNAs by endonuclease Dicer and (E) synthetic siRNAs, small double-stranded RNA molecules that match with perfect complementarity to mRNA target. Once associated with the RISC complex, siRNAs and synthetic siRNAs cleave their target mRNA and induces gene silencing.

translation may start in a few minutes with a half-life of 12–120 h. modRNA has been extensively studied in the heart [20,25], whereas hepatic disease models used are for factor IX deficiency hemophilia B [12], porphyria [13], glycogen storage disease type 1 A [14], thrombotic thrombocytopenic purpura [15], alpha-1 antitrypsin deficiency [15], Crigler-Najjar syndrome type 1 (CN1) [16]. In ischemic heart disease, modRNA has been used for delivery of pyruvate kinase muscle isozyme M2 (PKM2), controlling the pentose phosphate cycle, reducing oxidative stress DNA damage, and providing building blocks for cell proliferation post-Pkm2 modRNA expression in the heart [26]. Sultana et al. [27] described a modRNA method to induce post-ischemic injury cardiac regeneration based on modulation of the 5'-untranslated regions (5'-UTR) of the fatty acid metabolism gene carboxylesterase 1D (Ces1d), resulting in enhanced translation efficiency in ischemic heart and liver. Among the hepatic dysfunctions, hereditary defects of bilirubin conjugation could be treated with modRNAs; preclinical data from Gunn rat studies - with known physiological differences between humans and Gunn rats - provided a model based on human uridine-diphosphate--glucuronosyltransferase (hUGT1A1)-modRNA useful for design human clinical trials [28]. The model consists of the uptake of modUGT1A1 mRNA-lipid nano-particles (LPNs) into liver hepatocytes, which release mRNA from the endosome into cytoplasm allowing for the transcription and production of UGT1A1 protein. The glucuronidation of bilirubin by UGT1A1 leads to increased clearance of bilirubin. With this approach, many hepatic hereditary diseases might be treated, such as CN1, type 2 (CN2) and Gilbert syndrome. Many other diseases could find many benefits from these applications. Preclinical studies on autoimmune diabetes used viable cellular therapies as alternative approaches to viral vectors (able to integrate into the genome). Remi et al. demonstrated the safe delivery of interleukine-4 (IL-4) modRNA in NOD mice, preventing glucose increase in 50% of treated diabetic mice [29,30]. Besides the available formulations, preferentially via injection, the combination of devices (pills) able to deliver mRNA is gaining popularity. Abramson et al. [31] developed a combo formulation to avoid enzymatic degradation and bypass the tissue barriers in the gastrointestinal tract (GI). This oral delivery enables local transfection of untargeted GI cells via parenteral administration. Although the authors have orally administered mRNAs encapsulated in branched polymer nanoparticles (BPN) into the stomach submucosa, they did not assess the response of mucosal immunity. These important considerations make modRNA extremely versatile in construct engineering; indeed, modRNA construct can contain also miR-recognised elements as "suppress the suppressor" approach, such as the already experienced cardio-specific miR1, miR-208 and miR-199 delivered in in-vivo cardiomyocytes [26].

2.1.2. *Antisense oligonucleotides (ASOs) therapeutics*

Antisense oligonucleotides (ASOs) are short single-stranded oligonucleotides (12–24 nucleotides) that function via alternative mechanisms, such as a changing splicing pattern targeting enhancers or silencers, to inhibit splicing or causing exon skipping or exon inclusion, respectively [32]. For example, ASO-mediated therapies are employed in the treatment of Duchenne muscular dystrophy by means of FDA-approved drugs like Eterlipsen, which modulates exon skipping [33], and Nusinersen, which induces exon inclusion [34]. ASOs can exert their function trough its feature to avoid degradation in serum [35] conferring a strong efficacy of the treatment. Beside their antisense binding with mRNA targets, ASOs bind also non-coding RNA associated with disease pathogenesis. They can inhibit miRNA-mediated down-regulation by directly binding to miRNAs (miRNA inhibitors or antagomirs) or combining with mRNAs at miRNA-binding sites to inhibit

miRNA interaction (miRNA competitors or block-mir) [36,37]. Along this category of antisense nucleotides, Miravirsen, used for the treatment of hepatitis C (HCV), functions to bind miR-122. Miravirsen, a locked nucleic acid (LNA) modified phosphorothioate oligonucleotide complementary to the 5' end which binds with high affinity to hepatic-specific miR-122, is a promising new HCV therapy. The inhibition of miR-122 has the advantage of reducing circulating viral titer and cholesterol in animal studies [38], and the long-lasting viremia without side effects. The highly abundant expression of miR-122 in the liver is a necessary cofactor for HCV accumulation in plasma. Its interaction with HCV by binding to two closely spaced target sites in the 5' non-coding region (NCR) of the HCV genome, increases circulating HCV viral titer, thus upregulating viral RNA levels [39]. Another effect of anti-miR-122 is the down-regulation of interferon (IFN)-regulated genes and the improvement of HCV-induced liver pathology. Regarding the side effects of ASO treatments, the administration of Miravirsen has been associated with a transient increase of transaminases without meaningful changes as well as minor renal effects.

2.1.3. RNA-interference (RNAi) therapeutics

RNA-interference technologies based on synthetic siRNAs (double-stranded RNA) and, similarly, on endogenous miRNAs, are promising therapies for many diseases. These novel approaches could be able to overcome the limits of disorders caused by alterations of multiple pathways, such as diabetes, in which targeting a single gene may be unsuitable, or insufficient, for therapeutic action. Both miRNAs and siRNAs multitargeting actions offer the unique advantages of treating entire pathways. RNAi is now known as precise, efficient, stable, and better than antisense therapy, such as ASO, for gene suppression [40]. The gene knockdown technique used by synthetic siRNAs shares the same mechanism of action of the endogenous miRNAs (see paragraph 2.1) with the only difference that siRNA can perfectly match its mRNA target, cleaving it [41]. This particular feature of siRNA can determine specific mRNA knockdown thus avoiding the production of proteins. MiRNAs even with the same mechanism of siRNA molecules exert different knockdowns due to the conformation in a secondary structure which can modulate miRNA-mRNA interactions. This characteristic could explain the different degrees of genetic regulation of the specific miRNAs involved in the regulation process [42]. The first clinical application of RNAi was for the treatment of age-related macular degeneration by means of vascular endothelial growth factor (VEGF)-A-targeting Bevasiranib and VEGFR1-targeting AGN211745. However, these siRNAs triggered significant induction of Toll-like receptor 3, Interleukin-12 (IL-12), and Interferon-gamma (IFNg) [43–46], rousing immune response. The main medications approved by regulatory authorities use siRNA-based technology (Table 1) for pathologies that predominantly originate in the liver. Some recent approved drugs based on these technologies are given below.

Patisiran (2018), a drug based on siRNA, is used in the treatment of hereditary transthyretin-mediated (hATTR) amyloidosis, a rare disease caused by mutations in the gene encoding the transthyretin (TTR) protein produced by the liver [47]. The disease is primarily characterized by sensory, motor, and autonomic neuropathy and/or cardiomyopathy. Mutations result in the accumulation of misfolded TTR protein as amyloid fibrils in multiple sites including nerves, heart, gastrointestinal tract, eye, and central nervous system [48] in which case liver transplantation represents the only therapeutic option [49]. The delivery of Patisiran is ensured by lipid nanoparticle (LNP) formulation for delivery to the liver [50]. Long-term treatment with Patisiran (phase II and III clinical trials) showed reverse neuropathy progression due to the reduction of TTR levels, and tolerability [51,52]. However, hepatic delivery was improved by modifying the patisiran-based structure. Thus, the siRNA molecule was conjugated with a trivalent N-acetylgalactosamine (GalNAc) ligand to ensure better hepatic delivery, resulting in Vutrusiran. This molecule demonstrate comparable efficacy as Patisiran which both target the key pathogenic protein TTR and have similar pharmacodynamic effects in terms of TTR lowering. Finally, Vutrusiran is being investigated for potential application on cardiomyopathy [53].

Givosiran (2019), another drug based on a siRNA molecule, is reported to be able to silence aminolevulinic acid synthase 1 (ALAS1) mRNA in the liver. Givosiran was approved by the FDA and EMA for the treatment of adults with acute hepatic porphyria (AHP) [54]. This drug prevents the accumulation of aminolevulinic acid (ALA) and porphobilinogen (PBG) [55], responsible for neurovisceral attacks [56] triggered by AHP. Its mechanism is based on selective targeting of hepatocytes due to its linkage to GalNac leading to its selective uptake into hepatocytes via asialoglycoprotein receptors (ASGPR) [57]. Givosiran was further modified to protect it from nuclease digestion [58] and also to escape from cellular trafficking [59].

Inclisiran (2021), a promising siRNA able to silence hepatic Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9), is prescribed for adult subjects with heterozygous familial hypercholesterolemia (HeFH) or clinical atherosclerotic cardiovascular disease (ASCVD). The principal action mechanism is focused on the ability of siRNA to specifically bind PCSK9, whose binding to the Low-Density Lipoproteins (LDL) receptors on the surface of hepatocytes increases LDL-cholesterol (LDL-C) in circulation [60]. Synthetic monoclonal antibodies (mAbs), such as Evolocumab and Alirocumab, can bind circulating PCSK9 and inhibit its effect. Similarly to the abovementioned hepatic siRNA, Inclisiran delivery is ensured by ligation to GalNAc. Inclisiran has not revealed pleiotropic effects that, instead, occurred with the use of statins such as proinflammatory markers, interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) [61], or reduction of C-reactive protein (CRP) [62]. The only siRNA, FDA-approved drug advised for rare kidney disease is Lumasiran (2020). Lumasiran is used in pediatric and adult subjects with primary hyperoxaluria type 1 (PH1) that reduces urinary oxalate levels. The drug acts in the liver by silencing hydroxy acid oxidase 1 (HAO1) mRNA. The hepatocyte uptake is due to conjugation with the GalNAc ligand. Another siRNA molecule under investigation targeting PH1 is Nedosiran. However, Nedosiran acts on the hepatic enzyme lactate dehydrogenase (LDH), whereas Lumasiran acts on the hepatic enzyme glyoxylate oxidase (GOX).

Table 1
RNA-therapy FDA-approved.

Drug name	Disease	Tissue	Molecule
Fomivirsen	Cytomegalovirus retinitis	eye	ASO
Eteplirsen	Duchenne	muscle	ASO
Nusinersen	spinal muscular atrophy	muscle	ASO
Risdiplam	spinal muscular atrophy	muscle	RNA
Inclisiran	PCSK9-i	hepatic	siRNA
Givosiran	Acute phorphiria	hepatic	siRNA
Miravirsen	HepaticC-Virus (HCV)	hepatic[1]	anti-miRNA
Patisiran[52]	Amiloidosis hATTR	hepatic	siRNA
Lumasiran	Hyperoxaluria type 1	kidney	siRNA

[1] eradication for HCV (hepatic C virus).

3. miRNA power in therapeutic intervention

3.1. miRNA biogenesis and mechanism of action

MiRNAs represent a broad class of endogenous small, non-coding single-stranded RNA molecules of about 22 nucleotides in length, They have attracted increasing interest for their critical role in post-transcriptional regulation of gene expression through the promotion of mRNA degradation and the suppression of protein translation [63,64]. Ambros et al. first discovered lin-4 miRNA in Caenorhabditis elegans in 1993. [64,65] Over the last decades, miRNAs have been found abundantly in virtually all animal organisms and their number is constantly increasing [66].

To date, the miRBase database (http://www.mirbase.org) accounts for 2000 miRNA precursor sequences encoding 3000 different mature

miRNAs in the human genome, of which a great number still has an unknown function [67]. miRNA coding sequences in the human genome are located mostly in intronic regions, often in clusters, to a lesser extent in exons of transcripts [68], and more rarely in intergenic regions [69]. The biogenesis of miRNA is a multi-step process that implies a nuclear and a cytosolic phase. In the nucleus, miRNA genes are transcribed by RNA polymerase II in polycistronic long primary transcripts (pri--miRNAs) ranging from 200 nucleotides to kilobases in length, with a stem-loop structure and having a stem-loop region and two single-stranded ends at 5' 7-methylguanosine cap and at 3' poly-adenylated tail [64,70]. pri-miRNAs are cleaved into hairpin-shaped molecules of 60–70 nucleotides (pre-miRNAs) by the Microprocessor complex consisting of the RNase III endonuclease Drosha and the cofactor double stranded-RNA binding protein DiGeorge syndrome critical region 8 (DGCR8), [71,72]. pre-miRNAs, are exported from the nucleus to the cytoplasm [73,74] through a complex with the carrier protein Exportin 5 (EXP5) and its cofactor GTP-binding nuclear protein RAN-GTP. Once in cytoplasm, pre-miRNAs are further processed by RNase III endonuclease Dicer into small double-stranded (ds-miRNAs) 22 nt long [75,76]. They are then incorporated into a ribonucleoprotein complex, the RNA-induced silencing complex (RISC), comprising Dicer, its cofactor trans-activation response RNA-binding protein (TRBP) and Argonaute (Ago2) [77,78]. miRNA duplex intermediates are unwinded and produce two single-stranded mature miRNAs: the guide strand is loaded on Ago2 and directs RISC towards complementary sequences on target mRNAs whereas the passenger strand is released and addressed to degradation [79] (Fig. 2).

The fine regulation of miRNA levels can occur at each step of the biogenesis process, including transcription, processing, loading on RISC complex, RNA editing, RNA post-transcriptional modification, and RNA decay, and can involve multiple participating proteins, such as transcription factors, RNA-binding proteins, protein-modifying enzymes, RNA-modifying enzymes, exoribonucleases and endoribonucleases [64]. These small molecules are highly conserved across species, suggesting the importance of their regulatory role [80–84]. Moreover, the number and expression of miRNA genes as well as the diversity of miRNA target interactions seem to be directly correlated with the complexity of the organisms [84,85]. It has been estimated that in humans up to 60% of total protein-coding genes can be modulated by miRNAs and a single miRNA can silence several target genes [86]. Due to their ubiquitous and pivotal role in controlling the transcription of most protein-coding genes, miRNAs are implicated in almost all biological processes in mammals, ranging from embryo development, cell cycle regulation, proliferation, and differentiation to apoptosis, angiogenesis, cell

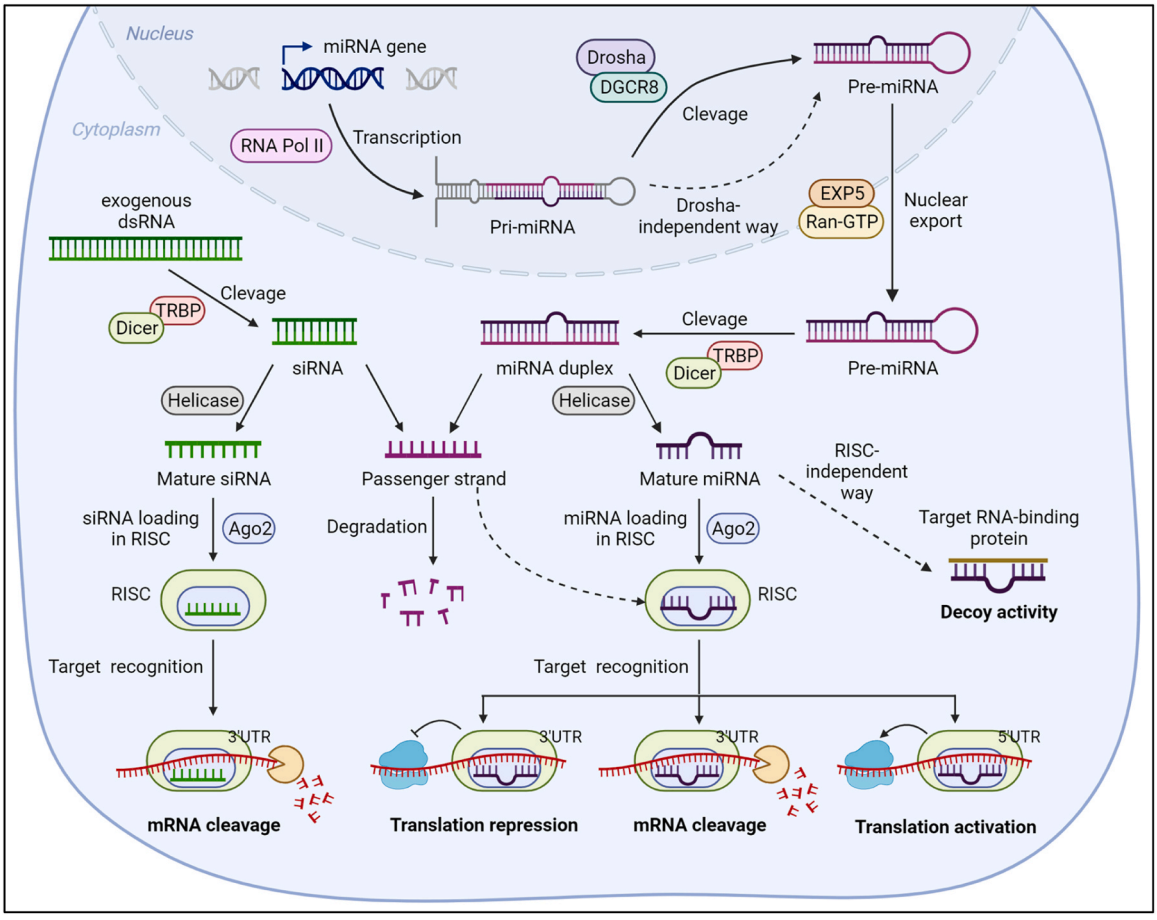

Fig. 2. Differences between miRNA and siRNA biogenesis, and their mechanism of action. Differences between miRNA and siRNA biogenesis, and their mechanism of action. miRNAs (violet strands) are transcribed by RNA Polymerase II in the nucleus as long pri-mRNA, which is then cleaved by Drosha into pre-miRNA. The pre-miRNA hairpin is exported by Exportin 5 (EXP5) to the cytoplasm and further is processed by the Dicer to form miRNA duplex. Mature miRNA (blue strand) can recognize regions at 3' UTR of target mRNA with partial or full complementarity, leading to translational inhibition or mRNA degradation respectively. It can also bind to specific sequence at 5' UTR of target mRNA, inducing protein translation or function as molecular decoys for RNA-binding proteins. The passenger strand of miRNA duplex is degraded, while the guide strand is incorporated into RISC and guides the active complex to the target mRNA. Long dsRNA (in green strands) introduced exogenously are processed by Dicer to form siRNA duplex. The passenger strand of siRNA/miRNA duplex is degraded, while the guide strand is incorporated into RISC and guides the active complex to the target mRNA. Mature siRNA binds target mRNAs with a perfect complementary sequence, causing mRNA degradation.

signaling, metabolism, innate and adaptive immune responses [69, 87–89] (Table 3). Since miRNAs are critical contributors to the maintenance of physiological homeostasis, their biogenesis and function are maintained under tight temporal and spatial regulation [64]. MiRNAs expression, or function, is significantly altered in numerous diseases - including different cancer types [90], cardiovascular and liver diseases [91,92], as well as metabolic inflammatory and autoimmune disorders [90,93,94], neurodegenerative and neurodevelopmental diseases [95, 96], diabetes [97,98] and prediabetes [96,99–101], psoriasis [102] and muscular dystrophy [103]. Recently, miRNAs have been considered as risk factors for cardiovascular disease (CVD) due to their correlation with cardiovascular events. Many miRNAs have been associated with conventional cardiovascular risk factors (CRFs) such as hypertension, dyslipidemia, obesity, and diabetes (which substantially increase the CV risk), and thus, miRNAs may be targeted to reduce CV risk [104].

3.2. miRNA linked with therapeutic areas

Until now, miRNAs' utility is limited to serve as biomarkers because they are stable in biological fluids such as blood [97], saliva [105] urine, and cerebrospinal fluid [106], thus providing relevant biological information among cells and tissues. This cross-talk allows a cell-to-cell communication in physiological and pathological situations thanks to miRNA association with RNA-binding proteins (Argonaute2 [Ago2]), lipoprotein complexes (high-density lipoprotein [HDL]) [107], and with membrane-bound extracellular vesicles (EVs).

Therefore, the fascinating role of miRNAs as endocrine and paracrine messengers holds a promise for a new source of molecular biomarkers able to identify a plethora of human diseases. Recent discoveries have revealed that several miRNAs are shared with many pathological processes such as liver lipid metabolism, atherosclerosis, CVD, NAFLD, metabolic syndrome, and T2D. The multitude of therapeutic areas targeted by miRNAs may control a wide spectrum of different diseases. Currently, several miRNAs are being implicated in factors of development and progression of the liver and heart failure, such as cardiac hypertrophy, fibrosis, and apoptosis (Table 3). Studies in miRNAs can increase the understanding of pathologies such as heart and liver failure at a deeper pathophysiology level and may provide new means of clinical diagnosis, prognosis, and even targeted treatments. Along the process of cardiac metabolism, executors such as peroxisome proliferator-activated receptors (PPARs) family regulate the switch from fatty acids (FA) to glucose (Table 3) in substrate utilization [108]. PPARα and PPARγ, and the PPARγ-coactivator-1 (PGC1) that regulates FA oxidation (FAO), are modulated by several miRNAs including miR-199a, miR181c, miR-214, miR-378, miR-378 * , miR-24, miR-126, miR-21, and miR-29.

Temporal changes in the levels of different circulating miRNAs can have an accurate diagnostic and prognostic value in patients with stable and advanced heart failure (HF) [109]. The muscle-specific miRNAs (miR-1 and miR-133a) orchestrate skeletal muscle and cardiac development by influencing myoblast and cardiomyocyte growth, differentiation, proliferation, and apoptosis, whereas miR-208, miR-208b, and miR-499 control cardiac remodeling, contractility, and response to stress and hormonal signaling [110]. Levels of miR-1, miR-133, miR-208b, and miR-499 in the circulation are found rapidly and significantly dysregulated in various CVDs such as acute myocardial infarction (AMI), HF, hypertrophy and arrhythmias, and are associated with adverse cardiac remodeling and clinical outcome [111,112]. miR-208 is significantly upregulated while miR-1 and miR-133a are significantly downregulated in AMI patients [112]. Increasing expression levels of miR-133 or miR-1 as well as suppression of miR-208 level might be useful to diminish cardiac hypertrophy and improve cardiac function [110]. In particular, Rac and Cdc42, regulators of the pro-hypertrophic mitogen-activated protein (MAP)-kinase pathways, have been identified as miR-133 targets [113,114]. Enhanced circulating levels of miR-29 and miR-21, and reduced levels of miR-133 and miR-30, which are important modulators of the extracellular matrix, have been correlated with increased structural remodeling and fibrosis [112]. On the contrary, downregulation of miR-134 inhibits ischemic injury both *in vitro* and in vivo by targeting HSP-A12B [115]. Moreover, the knockdown of miR-181b exerts a neuroprotective role against ischemic injury through repression of HSPA5 and ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1) protein expression [116]. In addition, downregulation of miR-30a attenuates ischemic injury by upregulating HSPA5 level and decreasing ER stress-induced apoptosis [117]. Recent clinical trial data indicate that the anti-miR compounds, specific inhibitors for miRNAs, are a potentially new class of drugs. In light of these findings, miRNA-based therapies can have beneficial effects in ameliorating cardiac hypertrophy, fibrosis, and function.

3.3. Are miRNA druggable targets or therapeutic agents?

Since miRNA-inhibitors bind to mature miRNAs forming inactive complexes and targeting multiple pathways of disease, miRNA-based drugs should represent a novel and potentially powerful therapeutic approach. There are about 2600 predicted mature human miRNAs 600 of which are considered to be well-validated and provide a wide range of novel target opportunities (miRBase v22 [67], https://www.mirbase.org). The combination of miRNA expression and functional delivery of chemically modified miRNA-modulators enable the scientific community to consider novel therapeutic opportunities.

The inhibiting feature of anti-miRNAs makes miRNAs specific druggable targets. Several druggable miRNAs are being considered for clinical trials. In-vitro studies have shown the inhibiting ability of Cobomarsen, an LNA-ligated anti-miR-155, that simultaneously inhibits multiple parallel survival pathways (including JAK/STAT, MAPK/ERK, and PI3K/AKT) [118], thus demonstrating that miR-155 is a potential druggable target. Similarly, LNA-ligated anti-miR-92 up-regulates its target, integrin alpha 5 (ITGA5) mRNA, produces "on-target" effects by accelerating angiogenesis, wound healing in human vascular endothelial cells, primary human skin fibroblasts, and mouse skin [119].

A therapeutic agent for the treatment of cholestatic diseases is under development. The candidate is an unconjugated anti-miR-27 whose mechanism most likely depends on the inhibition of the c-Myc circuit. In animal models of chronic cholestatic liver injury, induced by lithocholic acid (LCA), anti-miR-27 (or knockdown of c-Myc) attenuates the suppression of prohibitin 1 (PHB1) and increases glutamate-cysteine ligase (GCL) subunit expression [120]. miR-27 seems to function as negative regulator of adipocyte differentiation by inhibition of proliferator-activated receptor-γ (PPAR-γ) [121]. This has a key role in hepatic fibrosis and endothelial-mesenchymal transition (End-MET) transforming growth factor β (TGF-β)-induced [122]. Reduction of miR-27 levels was shown to inhibit the activated hepatic stellate cell (HSC) proliferation.

However, in addition to their function of druggable targets, miRNAs may also play the role of therapeutic agents. MiRNAs that belong to miRNA-29 family could be therapeutic agents thanks to their peculiarity of extracellular matrix (ECM) proteins modulators. They are largely homologous in sequence with only a few mismatches among the different members in the 3' regions of the mature miRNA. Upon treatment, the miR-29 mimic reduces the expression of the ECM gene, or the activity for collagen synthesis. Several studies have demonstrated miR-29 reduced levels in different types of fibrotic tissues. It has therefore been suggested that increasing miR-29 levels could be a therapeutic benefit in aortic dilatation [123], systemic sclerosis [124], cardiac [125], renal [126,127], liver [128], and pulmonary fibrosis [128]. Therapeutic effects of encapsulated mimic miRNAs are already being tested in phase I and II clinical trials (32238921). Pre-clinical studies on white blood cells (WBC) identified the mRNA targets of miR-34 as BCL2, DNAJB1, CTNNB1, FOXP1, HDAC1 by NGS (RNA-Seq) and qRT-PCR analysis. miRNA-34a mimic appears to have a promising anti-tumor activity as tumor suppressor p21CIP1/WAF1 and, therefore, it is

eligible for the treatment of advanced and refractory solid tumors or hematologic malignancies. Unfortunately, miRNA-34a mimic did not meet expectations and the trial was interrupted because of its immune-related serious adverse events [129]. miRNA-1 replacement therapy has recently shown to revert cardiac hypertrophy and fibrosis in diabetic cardiomyopathy by targeting Fibulin-1, a secreted protein implicated in extracellular matrix remodeling [130].

3.4. Delivery system for miRNA therapeutics

Other applications of miR-based therapeutics regard engineered delivery vesicles, such as EVs, loaded with specific (or patterns) of miRNAs (or anti-miRNAs) to counteract/activate specific pathways. Also, is possible to specifically target miR pathways: the mature miR sequences, could be druggable e.g. by modified ASOs that mitigate the abnormally expressed miR, or with anti-miRs (Table 2). Moreover, miR sponges, which reduce miR levels by trapping, and or erasers, which inhibit miR activity, can be used as modulators in the most favorable gene activation or suppression.

The efficacy of miRNA therapeutics depends on their delivery system. The technology able to carry the miRNA oligonucleotides already exists, but many challenges need to be faced to develop reliable clinical applications. The main issue relates to systemic delivery to allow-miRNAs to reach their targets and to avoid degradation by nucleases. Engineering of miRNA takes into account the cellular uptake since miRNA has a hydrophilic peculiarity,a negative charge, and a high molecular weight which can block the penetration through the cell membrane. Therefore, cationic-lipid delivery systems are the best option. In order to facilitate tissue delivery and cellular uptake, modified miRNAs are entrapped into cationic lipid nanoparticles (LNPs) such as polymers and liposomes. Modified miRNAs are engineered for efficient and endosomal escape as the binding with a lipid such as cholesterol-conjugated miRNAs, exosomes [131], or viral vectors such as adenoviruses [132,133], or aptamers or antibodies conjugated on the particles result in targeting via recognition of specific receptors [134–136].

Chemical miRNA (mimics and/or anti-miRs) modifications on the phosphodiester backbone and the 2' of the ribose [2'-O-2-Methoxyethyl (2'-MOE), 2'-O-Methyl (2'-O-Me), 2'-locked nucleic acid (LNA) and 2'-Fluoro] protect the miRNA from degradation, improve stability and affinity, promote the long-lasting potency of the miRNA [137], and diminish the activation of Toll-like receptors [138] and innate immune response [139,140].

Endosomal entrapment is another major challenge for efficient miRNA delivery since the intracellular trafficking of miRNA often begins in the early endosome compartment. Endosomes are composed of a lipid

Table 2
Druggable-miRNA ongoing clinical trials.

Target	Disease	Tissue	Molecule	Clinical trial number
miR-103/ miR-107	T2D with NASH/ NAFLD	Phase II	Anti-miR	NCT02826525 NCT02612662
miR-21	Alport syndrome	Phase I/ Phase II	Anti-miR	NCT03373786
miR-29	Fibrosis	Phase I	Mimic-miRNA	NCT02603224
miR-34a	Solid tumor suppressor [129]	Phase II	Mimic-miRNA	NCT01829971
miR-124	Crohn's disease[219] and ulcerative colitis [220] and rheumatoid arthritis[221]	Phase II	inducer-miRNA	NCT03813199
miR-92	CVD and wound healing[119]	ongoing	Anti-miR	NCT03603431
miR-155	Lymphoma (CTCL)	ongoing	Anti-miR	NCT03713320
miR-132	Heart Failure	ongoing	Anti-miR	NCT04045405

Table 3
miRNAs selected as circulating biomarkers.

miRs	Tissue	Role in physiology	Therapeutic intervention
miR-122	Liver	Liver homeostasis and function	Liver fibrosis, HCV [39][38], NAFLD [174]
miR-34a	Liver	Mediator of p53 function	NAFLD[195] CHC[171–173]
miR-103 and miR-107	Liver	Glucose homeostasis and insulin sensitivity	T2D[144]; NAFLD; NASH
miR-21	Liver	Liver stellate cells activation and liver fibrosis[183], bile acid homeostasis	steatohepatitis[186] HCV, NAFLD[196]
miR-33[222, 223]; miR-33 * , − 223, − 30c, − 144, − 148a, − 23b, − 24, − 29, − 122 [224]	Liver	Lipid metabolism, insulin signaling and glucose homeostasis, inflammation	Cardiometabolic diseases; liver diseases
miR-128-3p [225]; miR-192[189, 190]	Heart, liver	Cardiomyocyte proliferation, ischemia/ reperfusion and oxidative stress mediated injury	Cardiac hypertrophy [225]; liver fibrosis, NAFLD[189,190]
miR-192[120] [192]; miR-27a/b [121]	Hepatocytes-derived exosomes	Insulin response, hepatic lipid metabolism	T2D[192], NAFLD [120]; Hepatic steatosis [121]
miR-21-5p, miR-151a-3p, miR-192-5p, and miR-4449 [196]	Hepatocytes-derived exosomes	Hepatic metabolism, inflammation, and lipid metabolism	NASH[196]
miR-21	Renal, urine	Renal fibrosis	Alport Syndrome [226], cardio-renal syndrome[227]
miR-21	Myocardia	Myocardial fibrosis	Atrial fibrillation [228]
miR-29[123] [125]; miR-21 and miR-133[111, 112], miR-30 [112]	Circulating	Myocardial matrix remodeling	cardiac fibrosis and dilated cardiomyopathy
miR-181b[116]	Heart	Cardiomyocytes apoptosis and myocardial damage	Ischemic injury
miR-23, miR-24, miR-199a, miR-378, miR- 378 * , miR-126[108]	Heart	Cardiac function and matrix remodeling, cardiomyocyte proliferation	Cardiac hypertrophy and fibrosis
miR-214[108]	Cardiac and skeletal muscle	Cardio-protection against cardiomyocyte apoptosis, cardiac remodeling, modulation of inflammation and angiogenesis	Ischemic cardiac diseases, coronary artery diseases, cardiac hypertrophy and fibrosis, dilated cardiomyopathy, myocarditis
miR-30a[117]	Brain, heart	Neurons and cardiomyocytes cell death, myocardial remodeling	Neuronal and Cardiac ischemic injury, myocardial fibrosis
miR-134[115]	Brain	Ischemia/ reperfusion injury-induced neuronal cell death	Cerebral ischemic injury
miR-216a[201]	Brain	Neuroprotection against ischemic	Cerebral ischemic injury

(continued on next page)

Table 3 (*continued*)

miRs	Tissue	Role in physiology	Therapeutic intervention
miR-216a-5p [201]	Exosomes	injury-induced apoptosis and inflammation Modulation of microglia polarization under hypoxia	Spinal cord injury

bilayer barrier that prevents the vast majority (~99%) of RNA therapeutics from entering the cytoplasm. RNA therapeutics escaping from endosomes results in a long duration of responses (3 to 6 months or more). The increase of endosomal escape efficiency can be conceived of as two generalized approaches: (i) endosomal rupture, or (ii) enhanced endosomal escape [141]. The conjugation to specific macromolecules, such as cholesterol and tri-antennary N-acetyl galactosamine (GalNAc), can favor endosomal escape [142] by binding to the Asialoglycoprotein receptor (ASGPR), which is predominantly expressed in the liver hepatocytes [57,143]. Upon binding, sialyl-GalNAc molecules are rapidly engulfed into hepatocytes by endocytosis. Due to a pH drop, GalNAc-miRNA conjugates can be released from ASGPR into the lumen of the endosome, and ASGPR recycles back to the hepatocyte surface. GalNAc and the linkers are rapidly degraded off of the miRNA conjugate and by a currently unknown mechanism, a small fraction of free miRNA, likely < 1%, escapes across the endosomal lipid bilayer membrane into the cytoplasm of the hepatocyte. Once in the cytoplasm, miRNAs could be rapidly loaded by transactivation-responsive RNA-binding protein into Ago to induce robust and sustained RNAi responses.

The use of miRNA mimetics and anti-miRNA oligonucleotides have been proposed in pre- and clinical trials, and many of them have been developed and completed in phase I or phase II. A novel insulin sensitizer, GalNAc-conjugated anti-miR targeting miR-103/107 (RG-125/AZD4076), could be useful in the treatment of complicated metabolic diseases such as (NAFLD), and (NASH) in patients with T2D/prediabetes. Anti-miR-103/107 completed phase I (http://www.regulusrx.com) clinical trials and it is progressing to phase II trials for the treatment of T2D, or prediabetes (http://clinicaltrials.gov – NCT02826525 and NCT02612662). By evaluating hyperinsulinemic-euglycemic clamp, it has been observed that anti-miR-103/107 functions as unique insulin sensitizer, by targeting insulin signaling, the liver decreases adipocyte size, liver triglycerides, and steatosis. In mouse models of diabetes, anti-miR-103/107 leads to a sustained reduction in fasting glucose and fasting insulin levels [144]. The molecular mechanism seems to be focused on the high miR-103 and miR-107 levels in obese micein which targeting caveolin-1 (CAV1) might regulate glucose homeostasis [59]. As approximately one third of the population is estimated to have NAFLD globally [145], the treatment with anti-miR-103/107 might be promising. Currently, glucagon-like peptide-1 receptor agonists (GLP-1 RA) could be recommended for the treatment of hepatic steatosis, liver function, lipid, glucose, inflammatory and insulin sensitivity markers, which indicates a synergistic treatment approach for patients with T2DM and NAFLD, as suggested by a recent metanalysis [146]. Among the many patients with T2DM and NAFLD, GLP-1 RA treatment improves body composition, glycemic control, insulin sensitivity, biomarkers of inflammation [147–149], and hepatic steatosis. The beneficial effects of GLP-1RA on NAFLD are likely to derive indirectly from weight loss whereas they may be directly linked to its effect on liver hepatocytes and hepatic lipid metabolism.

4. Putative druggable miRNAs in the control of inflammation as a strategy to target metabolic, liver, and heart dysfunction

Several studies have revealed specific alterations in miRNA expression profiles in mostly acute and chronic diseases, including liver disorders, atherosclerosis and cardiovascular diseases, metabolic syndrome, and T2D. The "common soil" of the pathogenesis of these chronic diseases could be attributable more generically to inflammation, and their response processes could share common mechanisms. Many miRNAs are well known as "inflamma-miR" and trigger inflammatory signaling pathways, most commonly the nuclear factor-kB (NF-κB), MAPK, and JAK-STAT pathways.

4.1. targeting NF-kB pathways

It is well acknowledged that NF-κB induces transcription of a variety of target genes mediating inflammatory responses for liver, heart, and metabolic diseases, including innate and adaptive immunity, stress responses, B-cell development, and lymphoid organogenesis. NF-kB/Rel complex includes NF-κB2 p52/p100, NF-κB1 p50/p105, c-Rel, RelA/p65, and RelB. This complex seems to promote inflammation through the canonical pathway that is induced by aproinflammatory stimulus such as cytokines, LPS, growth factors, and antigen receptors, that activates IKK complex (IKKβ, IKKα, and NEMO), which in turn phosphorylates IκB proteins. As a consequence, ubiquitination and proteasomal degradation occur, thus freeing NF-κB/Rel complexes. Active NF-κB/Rel complexes are further activated by post-translational modifications (phosphorylation, acetylation, glycosylation) and translocate to the nucleus where, either alone or in combination with other transcription factors including AP-1, ETS, and STAT, they induce target gene expression. In the non-canonical NF-κB pathway, mostly related to immune cell differentiation and maturation, NF-κB2-p100/RelB complexes are inactive in the cytoplasm. Serial phosphorylation from a subset of receptors, including LTβR, CD40, and BR3, activates the kinase NIK, which in turn activates IKKα complexes that phosphorylate C-terminal residues in NF-κB2 p100. Phosphorylation of NF-κB2 p100 leads to its ubiquitination and proteasomal processing to NF-κB2 p52. Many conventional drugs have been designed to exert the inhibitory role of this pathway. For example, aspirin and salicylate specifically inhibit IKK; lactacystin, by inhibiting 26 S proteasome complex, can prevent IκBα degradation. Finally, at the nuclear level, tacrolimus specifically prevents NF-κB subunits RelA, p50, c-Rel and other members from entering the nucleus [150].

4.1.1. NF-kB-related "inflamma-miR"

Many miRNAs could play a significant role in these processes, thus establishing the potential use of miRNA-based therapeutic interventions to treat the disease upstream. Bioinformatic prediction analysis [151] showed a plethora of miRNAs as regulatory elements of the NF-κB pathway. One of the first miRNAs to be characterized as NF-κB-dependent genes was miR-146a-5p because of its ability to regulate NF-κB promoter sequences and TLR and cytokine signaling [152], exerting its potential negative inflammatory regulation [153]. miR-146a-5p involved in the IL-1 receptor cascade negatively regulates the adapter proteins TRAF6/IRAK1, thereby controlling IKK levels [154] (Fig. 3). miR-126, miR-21, and miR-375 are potential targets NF-κB inhibitor alpha targeting IκBα, IKBA or NFKBIA [155]. Inflammasome can also be regulated by NF-kB signaling through induction of NLRP3 gene expression and activation of IL-1b and IL-18, inhibited by miR-574-3p and miR-92–5p, respectively. Alternatively, miR-379/miR-411 mimics may silence IL-1b and IL-18. Therefore, targeting NF-κB signaling with a single or many miRNAs could prevents the NF-κB activity and the entire signaling pathway, from the phosphorylation of IκBα to the inhibition of protease activity (Fig. 3).

In vascular endothelial cells (ECs), the pleiotropic NF-kB is activated during hyperglycemia, and it might be dependent on the activation of the family of guanosine triphosphate (GTP)-ases, which leads in turn to several inflammatory mediators activation (tumor necrosis factor-alpha (TNF-a), IL-1b, IL-6, protein kinase C (PKC), including adhesion molecules (cellular adhesion molecule (CAM)) that facilitate monocyte and T-cells adhesion to ECs. Hyperglycemia and IR are hallmarks of T2D.

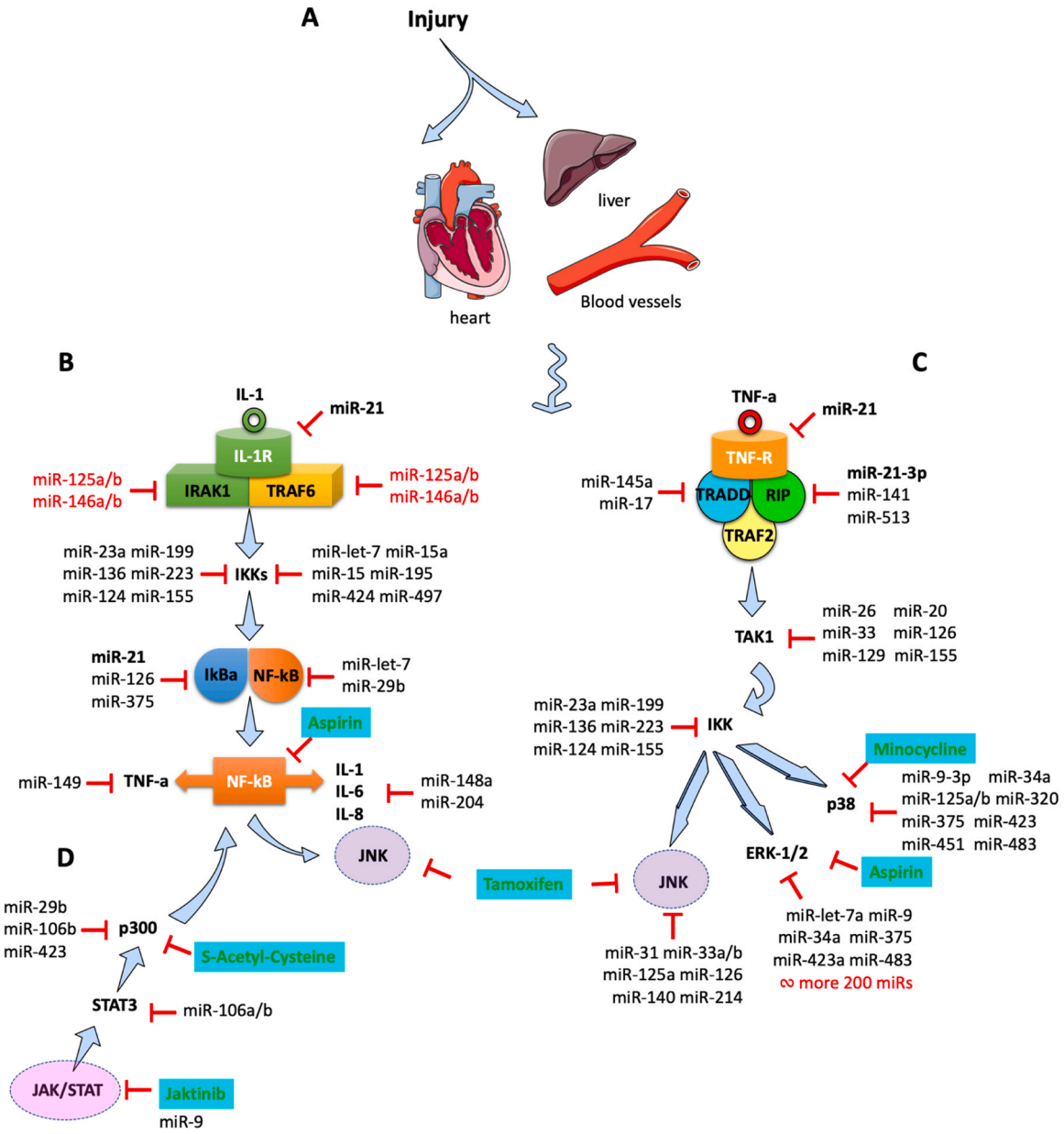

Fig. 3. Depiction of druggable miRNA in inflammation pathways. Depiction of druggable miRNA in inflammation pathways: (A) The major organs affected by inflammatory injury are heart and liver which determine many derangements on metabolic pathways. (B) In a classical pathway of NF-kB, IL-1 binds to its receptor IL-1R which in turn activates binding to the mediators IRAK1/TRAF6 and activates subsequent transcriptional responses via NF-kB and MAPK pathways. TRAF6 ubiquitously participates in many protective responses. Particularly, TRAF6 has an essential role in the primary and secondary inflammatory responses via driving type 17 (IL-17). Inflammation could be modulated by the use of miRNA-based drugs, in which miR-125a and -b, and miR-146a and -b inhibiting upstream signals of NF-kB could repress the inflammation cascade; also, for miR-21, − 126 and − 375, inhibiting the dissociation of IkBa from NF-kB, and repress the activation of NF-kB and subsequent translocation to nucleus for transcription of inflammatory genes. Targeting NF-κB-miR-146 and NF-κB-miR-155 networks could fine-tune the activity, intensity, and duration of inflammation. (C) Stimulus as TNF, and other molecules, exerts a signalling cascade with the activation of TRADD, TRAF2 and RIP (Receptor (TNFRSF)-Interacting Serine-Threonine Kinase 1) in order to activate TAK1, a MAP kinase able in turn to active NF-kB and MAPK signalling in different ways [229]. Drugs as minocycline, aspirin and tamoxifen, inhibit the pathway. However, miR-21–3p, − 141 and − 513 could repress upstream NF-kB and MAPK signalling and the formation of cytokines. (D) miRNAs involved in the JAK/STAT signalling were extrapolated by DIANA tools (https://dianalab.e-ce.uth.gr/microt) which collected experimental validated miRNA targets. Together with miRNAs, Jaktinib and s-acetyl-cysteine, targeting Jak/Stat and p300 [230] respectively (https://go.drugbank.com), could potentiate therapeutics effects.

Dangerous manifestations of T2D translate into activation of inflammatory patterns [156] and Oxidative stress (Ox-S). Several studies demonstrated that intensive management of hyperglycemia reduces the incidence of deep sternal wound infection in diabetic patients undergoing cardiac surgery [36]. Experimental and clinical studies highlighted the concept that during hyperglycemia a deficit in the ability of the cells to counteract the damage stimuli might occur, from reactive oxygen species (ROS) to cytokines production. Indeed, it seems that the response to fight hyperglycemic damage is attributable to a deficit in antioxidant responses [98–101,104,157–161]. Recent in vitro studies have demonstrated that miR-21 acts on both glucose and ROS homeostasis [99]. Similarly, it has been demonstrated that in prediabetic people with high BMI high levels of circulating miR-21 are associated with high levels of ROS and defective antioxidant responses [100]. In particular, anti-miR-21 seems to inhibit KRIT1 by generating the defective activation of antioxidant responses. Yet, this is not the only

mechanism. Several in vitro studies show that miR-21 targets several genes related to adipogenic differentiation [162]. Seeger et al. observed miR-21 positive regulation of lipid metabolism in db/db mice, with a reduction of obesity and adipocyte size of the white adipose tissues, whereas no change occurred in the HbA1c level [163]. Comparable results were found in white adipose tissues of obese people compared to lean controls, whereas in vitro studies have implied an association of miR-21 with adipogenesis [164].

4.1.2. NF-kB-related miR-122 and the link with liver, heart and metabolic disorders

In addition, these inflammatory pathways could be mediated by a specific miRNA such as miR-122 [165]. This miRNA is considered the most abundant and unique miR to be liver-specific showing a high reproducibility in serum, platelet-poor (PPP) and platelet-rich plasma (PRP); it regulates several genes associated to cholesterol and FA metabolism [166], hepatocytes proliferation and differentiation [128]. Its relevance is associated with thereplication of hepatitis C virus, making it a promising druggable candidate biomarker. Recently, miR-122 has been suggested to play an anti-inflammatory role in the liver. This hypothesis is corroborated by an animal study in which the miR-122 knock-out-mice showed an up-regulation of Ccl2 with intrahepatic recruitment of CD11bhiGr1 + inflammatory cells, which are a major source of cytokines such as IL-6 and TNF during liver inflammation [167]. It has been acknowledged that miR-122–5p has been used in phase II clinical trials as a drug target in the treatment-naïve subjects with chronic hepatitis C infection. Due to these pleiotropic features, miR-122 might represent "the linking-miR" able to treat heart, liver and dysmetabolism. Growing evidences make of miR-122 an actor in cholesterol biosynthesis, silencing of miR-122 and increases HMGCR and SREBP-2. *In vivo*, depletion of miR-122 causes iron deficiency due to increased mRNA expression of the peptide hormone hepcidin, which is the key factor of duodenal iron absorption and release [168]. In addition, it seems that miR-122–5p, modulating the ECM gene expression through the binding to MMP2–3'UTR, functions as substrate for its potential therapeutic role in CVD [169].

A role of miR-122 as circulating biomarker has been evidenced yet. Although its role in the eradication of HCV is clear, miR-122 seems to regulate lipid homeostasis (cholesterol, HDL and LDL) in a cross-sectional study [170]. Serum levels of miR-122 and miR-34a are upregulated both in patients with NAFLD and chronic hepatitis C infection (CHC) and are strongly correlated with histopathological disease severity [171–173]. However, the results are contraddictory because the data point to the role of miR-122 in NAFLD as a protective effect and others highlighting as an anti-steatotic [174]. NAFLD is also a risk factor for atherosclerotic CVD, which is the principal cause of death. It is noteworthy that Willeit et al. [166] identified miR-122 as a circulating marker linked to liver and the risk of heart disease. The association of miR-122 with the development of metabolic syndrome and T2D in the general population, could explain the cardiometabolic risk associated with miR-122.

To strengthen the role in NF-κB signaling, anti-miR-122 injected in serum of HFD-induced NAFLD rats [175] silences of TLR4/MyD88/NF-κBp65 [176] that result in lipid accumulation and inflammation.

Beside these considerable evidences candidate miR-122 as druggable molecule, an effect as therapeutic agent has been tested. Thereby, upregulation of miR-122 protects against ischemic neuronal cell death and brain damage by targeting FOXO3 via heat shock protein (HSP) 70-dependent NF-κB pathway [177].

With reference to liver inflammation, NF-κB regulates multiple functions in hepatocytes, Kupffer cells, and hepatic stellate cells (HSCs) making it the major factor activated in chronic liver disease, including alcoholic liver disease, NAFLD, viral hepatitis, and biliary liver disease. Therefore, the inhibition of different NFκB signaling components could lead to fibrosis and carcinogenesis suggesting that NF-κB makes an essential contribution to liver homeostasis and wound-healing processes. Once again, growing evidence makes miR-21 a powerful druggable target for the treatment of T2D [178], in particular for its link with beta-cell death [179].

miRNAs may be able to promote NF-κB-mediated inflammatory response in NAFLD as in the case of miR-125b directly targeting tumor necrosis factor-alpha inducing protein 3 (TNF-alpha IP3) and that mechanism might be a target for treating NAFLD [180]. In animal studies, miR-378 plays a key role in the development of hepatic inflammation and fibrosis by positively regulating the NF-κB-TNFα axis [181].

On the other hand, overexpressing miR-27b in the RAW264.7 murine macrophage cell line suppressed lipopolysaccharide (LPS)-induced activation of NF-κB [182].

Recent findings have shown that numerous miRNAs could provide potential therapeutic targets in contributing to brain injury in ischemic stroke.

Besides a plethora of regulated pathways, miR-21 is also involved in the control of liver dysfunctions. Nowadays, miR-21 represents a novel and attractive target for therapeutic intervention in cholestatic liver disease (CLD); In animal models of CLD, such as bile duct ligation (BDL), miR-21 was able to activate liver stellate cells and liver fibrosis [183]; Thus the use of anti-miR-21 via Cyclin Dependent Kinase 2 Associated Protein 1 (CDK2AP1) might constitute a novel approach to prevent necroptosis-associated liver injury, fibrosis and improve the adaptive responses of bile acid homeostasis gene expression [184]. It is noteworthy that miR-21 is upregulated in the liver of patients with cholangiocarcinoma [185] and non-alcoholic steatohepatitis [186]. Moreover, some studies demonstrated the role of miR-21 on hepatic glucose metabolism [187]. The inhibitory role of miRNAs on the NF-kB pathway is also evident in diabetes and obesity as well as in liver fibrosis. In chronic diseases, such as NAFLD, activation of hepatic stellate cells (HSCs) induced fibrosis mediated by the internalization of some miRNAs (e.g. miR-128–3p which mechanism is associated with inhibition of NF-kB axis [188], miR-122 and miR-192, contained in EVs released by lipid-induced HepG2 cells, or hepatocytes) [189,190]. Indeed, increased levels of circulating miR-122 in human subjects are associated with IR, obesity, and diabetes [170,191]. The detection of hepatic fibrosis by non-canonical markers remains a challenge in clinical practice. Therefore, many miRNAs can help to increase the understanding of hepatic phenotypes for clinical utility. In a high-fat diet (HFD) animal study has been demonstrated that increased miR-192 and exo-miR-122 were linked with IR [192], miR-27a-3p, and miR-27b-3p whereas overexpression of hepatic miR-146a improved IR and reduced inflammation in a mouse model of NASH [193,194]. On the other hand, in a meta-analysis study, Shengliang et al. determined that miRNA-34a can be used as a biomarker to differentiate between NASH and NAFLD [195]. Conversely, Kim et al. have demonstrated that the expression profile of four circulating miRNAs (miR-21–5p, miR-151a-3p, miR-192–5p, and miR-4449) in sera reveal a significant accuracy in diagnosing NASH in NAFLD [196]. Transfection of exosomes with mimic miR-192, miR-122, and miR-27a/b, induced central obesity and hepatic steatosis in recipient mice [192] through alteration of adipocyte-PPAR/RXR pathway.

4.2. targeting JAK/STAT pathways

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway is regarded as one of the central communication nodes in the cell function and are critical component of many cytokine receptor systems regulating growth, survival, and differentiation [197]. JAK/STAT-mediated downstream events include hematopoiesis, immune fitness, tissue repair, inflammation, apoptosis, and adipogenesis. Thus, the inhibition of the JAK/STAT pathway is promising for treating various diseases. Jaktinib is known as a JAK-1, JAK-2, and JAK-3 inhibitor used for the treatment of myelofibrosis

currently in phase Iof clinical trial NCT04866056 [198] in Chinese people. Recently, Whang et al. demonstrated that miR-9 could inhibit activation of the NLRP3 inflammasome and attenuate atherosclerosis-related inflammation, likely through the JAK1/STAT1 signaling pathway [199]. In addition, JAK-1 and matrix metalloproteinase 13 (MMP-13) were identified as the target genes of miR-9. In oxLDL-stimulated human THP-1 derived macrophages, knockdown of JAK-1 by siRNA blocked the phosphorylation of signal transducer and activator of transcription 1 (STAT1) and mimicked the effects of miR-9 [200]. Also, targeting JAK2/STAT3 by mimic miR-216a, it could be possible to exert neuroprotective effects by its inhibitory role in inflammation [201].

4.3. targeting MAPK pathways

MAPKs with their sub-families, c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinases (p38), and extracellular signal-regulated kinase (ERK)− 1/2, are involved in the regulation of various biological processes such as cell proliferation, differentiation, migration, and apoptosis [202]. JNK and p38 are primarily activated under the conditions of inflammation,and stress, and stimulated by growth factors, but the activation of ERK-1/2 is dependent on growth factors and cytokines [202]. Recently, De Nigris et al. demonstrated the role of p38 in the activation of insulin signaling in vasculature [203], thereby enabling us to hypothesize that many disorders could share common pathways.

In the MAPK signaling has been demonstrated that a stimulus, such as TGF-beta, could activate phosphorylation of p38 inducing Myocyte-specific enhancer factor 2 C (MEF2C), a transcription factor that role in cardiovascular disease [204], in the pathogenesis of T2D [205] and liver fibrogenesis in vivo [206]. Importantly, several miRNAs such as miR-21–5p and miR-155 might target MEF2C to reduce the burden of inflammation that MEF2C might generate (https://dianalab.e-ce.uth.gr/home).

Moreover, it has been suggested that p38 and ERK phosphorylation are modulated by miR-22 in cells and mice. The inhibition of miR-22 resulted in increased levels of pro-inflammatory cytokines regulated by this signaling, such as TNF-α, IL-1β, p38 phosphorylation and overexpression of inducible nitric oxide synthase (iNOS), NF-κB, macrophage inflammatory protein (MIP), prostaglandin E2 (PGE2), and cyclooxygenase-2 (COX-2) in ischemic models. A miR-22 therapy would be a potential candidate for protection against the neurotoxic effects caused by inflammation through the p-38 MAPK pathway inhibition [207].

miR-21 could target the MAPK pathway by negative modulation of Mitogen-Activated Protein Kinase Kinase 3 (MAP2K3). Treatment with miR-21 mimic and SB203580 (MAPK inhibitor) induced overexpression of miR-21 which consequently reduced the levels of MAP2K3, p38, iNOS, and metalloproteinase (MMP)− 9, markers of inflammation that were increased in the ischemic conditions [208].

Using bioinformatic approaches (https://diana-lab.e-ce.uth.gr/app/miRPathv4) four microRNAs such as miR-125b-5p, − 145–5p, − 155, − 21–5p, targeted 90 genes in the MAPK pathway (p-value <0.0001 and FDR<0.01), most of them linked to inflammation and organ damage.

5. Advantages of RNA therapy versus conventional drugs

RNA therapy could be a good alternative to conventional drugs (CD) because of the advantage of selectively binding many targets of the same pathway that CDs do not cover. For example, despite the success of the well-known LDL-C-lowering drugs in reducing adverse cardiovascular disease (CVD) events, growing limitations suggested to consider the screening for Lipoprotein-a (Lp-a), a pro-atherogenic molecule usually employed as a sensitive marker of residual risk (RR) in people at moderate to high risk of CVD. No CDs currently are available for specifically targeting Lp-a alone. Recently, the European Atherosclerosis Society (EAS) Consensus Panel recommended reaching a treatment goal of < 50 mg/dL (125 nmol/L), after therapeutic management of LDL-C [209]. LDL-C-lowering CDs are limited in targeting Lp-a, and this limitation is extended also to currently used monoclonal antibodies (mAB) treatments targeting PCSK9. RNA-therapy (Inclisiran in this specific case) that determin a higher adherence to therapy and lower adverse cardiovascular outcomes [210], gain ground in the treatment for hypercholesterolemia. While a recent meta-analysis highlighted the reduction rate of 25% of (Lp-a) concentration (< 50 mg/dl, threshold for risk of heart disease) with the use of two mAbs [211], on the other side a phase 2 clinical trial showed a reduction of Lp-a levels by up to 80% with the use of a novel ASO (AKCEA-APO(a)-LRx) able to target the hepatic biosynthesis of Lp-a binding apo-a (that avoid the link with apoB100) [212]. Thereby, Inclisiran peculiarities regard the ability to be complementary to statin medication, and most importantly, can successfully reduce Lp-a levels.

6. Limitations

Although miRNA therapeutics appear to be an innovative treatment for diseases, our literature review aims to highlight several limitations. The first problem is the “miR-drug discovery”: the high degree of redundancy and the multiple miRNA-targets, hampered the development of specific pharmacological treatments and diagnostic biomarkers. Once identified, miRNA targets and developed miRNA-agent (such as mimic or anti-miR), miRNA efficacy for therapeutic purposes could be engineered and optimized for avoiding a) easy degradation by RNases, b) stimulation of the innate immune system through activating Toll-like receptors, c) high dose that may determine miRNA aggregation in the liver, limiting their applications, and subsequent degradation in acidic compartments of the endo/lysosomal pathway. In addition, another relevant problem is d) the inability to escape from cellular trafficking such as endosome recruitment, due to unknown mechanism: only 1% of miRNA agent reach the target. To overcome this obstacle, one approach is to use low doses of combined miRNAs that synergistically regulate the expression of the same target gene [213,214]. Some evidences supported this hypothesis to decrease the dosage of mimics to achieve effective gene modulation and minimize unwanted toxicity: Orellana et al. [215] by a construct of folate (for endosomal recruitment) and Nigerin (able to open bilayer of endosome), facilitates the escape of RNA cargo from their entrapping endosomes, helping the small RNAs to become available in the cytoplasm, engage the RISC, and improve their RNAi activity. The strength of binding with the target (weak "on-target") and the occurrence of off-target events (due to specific chemical modifications that could alter the physical characteristics and activities of naturally synthesized, modified, and folded RNA [216,217]), f) toxicity and side effects (some studies evidenced decreased lymphocyte counts or cardiac events [218] or death [129]), raise their limitations.These inquiries are not completely solved, but chemical modifications responsible for toxicity could be replaced by bio-engineering miRNA sequences which could improve tolerability and stability in different animal models, increase binding affinity, and help loading into the miRNA-induced silencing complex. Noteworthy, novel biologically engineered RNAs have been conjugated with aptamers or cholesterol molecules. Even if engineered RNAs are biological tolerated, this form of encapsulation delivery includes cytotoxicity, poor transfection efficiency, and nonspecific biodistribution.

7. Conclusion

Taken together, the latest pre-clinical findings and emerging clinical data on the metabolic liver and heart diseases increasingly suggest that RNA-based therapy constitutes a very promising approach with the potential to complement the deficiencies of conventional therapeutic methods. The engineering of nucleic acids is enabling a new generation of therapeutics and vaccines to prevent or treat a wide range of diseases. Based on gain- or loss-of-function data collected in animal disease

models through the use of genetics or pharmacological modulation of miRNAs it is now well-accepted that miRNAs are important players during disease. The many advantages of RNA drugs include their potential to target a wide range of genetic molecules, fast and efficient production, long-term effects, and reduced risk of genotoxicity. Targeting miRNAs with anti-miRs, chemically modified, single-stranded oligonucleotides, or mimicking miRNAs offers a unique approach to treat liver and heart disease through the modulation of entire biological pathways and may become a new and major class of drugs with broad therapeutic applications.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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