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Review

Up–to–date on the evidence linking miRNA-related epitranscriptomic modifications and disease settings. Can these modifications affect cross-kingdom regulation?

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ABSTRACT

The field of epitranscriptomics is rapidly developing. Several modifications (e.g. methylations) have been identified for different RNA types. Current evidence shows that chemical RNA modifications can influence the whole molecule's secondary structure, translatability, functionality, stability, and degradation, and some are dynamically and reversibly modulated. miRNAs, in particular, are not only post-transcriptional modulators of gene expression but are themselves submitted to regulatory mechanisms. Understanding how these modifications are regulated and the resulting pathological consequences when dysregulation occurs is essential for the development of new therapeutic targets. In humans and other mammals, dietary components have been shown to affect miRNA expression and may also induce chemical modifications in miRNAs. The identification of chemical modifications in miRNAs (endogenous and exogenous) that can impact host gene expression opens up an alternative way to select new specific therapeutic targets.

Hence, the aim of this review is to briefly address how RNA epitranscriptomic modifications can affect miRNA biogenesis and to summarize the existing evidence showing the connection between the (de) regulation of these processes and disease settings. In addition, we hypothesize on the potential effect certain chemical modifications could have on the potential cross-kingdom journey of dietary plant miRNAs.

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Introduction

Emerging evidence implicates a wide range of post-transcriptional RNA modifications (epitranscriptome) that play crucial roles in fundamental biological processes, including the regulation of gene expression. Collectively, these modifications are known as epitranscriptomics.

Most RNAs families possess several co- or post-transcriptional chemical modifications at a variety of locations [1,2]. Comparable to DNA and histone modifications, it is expected that many of these RNA modifications may be associated with regulatory functions. Current evidence shows that chemical RNA modifications can influence the whole molecule's secondary structure, translatability, functionality, stability and degradation, and some are dynamically and reversibly modulated [2–8]. These modifications have been studied in the past decades mostly in messenger RNA (mRNA), though, more recently, many began to be described also in traditionally considered 'functional' (i.e. transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs)) and 'non-functional' non-coding RNAs (ncRNAs) (i.e. microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs)) [9–16]. Understanding the biological consequences of such modifications is essential for the development of new therapeutic targets [17,18]. Although there are still many knowledge gaps, research in the field of epitranscriptomics is rapidly progressing and has been accelerated by the continuous advances in high throughput sequencing systems [19].

MiRNAs are a class of single-stranded small (19–25 nucleotides) ncRNAs involved in the regulation of gene expression at a post-transcriptional level via mRNA silencing and translational repression, among other mechanisms [20]. In humans and other mammals, dietary components have been shown to affect miRNA expression [21,22] and the likelihood that they may also induce chemical modifications in miRNAs, as was reported for mRNAs [23], deserves further research. Indeed, the regulatory effect of miRNAs can also be transmitted between different species and kingdoms as a means of communication between two organisms [24]. The identification of chemical modifications in miRNAs (endogenous and exogenous) – that can impact host gene expression – opens up an alternative way to select new specific therapeutic targets in different pathological settings.

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Hence, the aim of this review is to briefly address how RNA epitranscriptomic modifications can affect miRNA biogenesis and to summarize the existing evidence showing the connection between the (de)regulation of these processes and disease settings. In addition, we hypothesize on the potential effect certain chemical modifications could have on the potential cross-kingdom journey of dietary plant miRNAs.

Epitranscriptomic modifications

The chemical structure of RNA can be modified in cells to serve as an epigenetic mechanism for gene expression control. Around 170 different chemical modifications of RNAs have been reported so far, a great number of them consist of a methyl (CH_3) group being added to the nitrogenous base (e.g. N^6-methyladenosine (m^6A)), to the ribose sugar (e.g. 2′-*O*-methyladenosine) or to both (e.g. N^6,2′-*O*-dimethyladenosine (m^6Am)) [1]. Many of these covalent RNA additions are highly prevalent and can be dynamically added and removed through writer and eraser complexes, respectively. In addition, these modifications are interpreted by modification-specific binding proteins known as readers, providing a new layer of epitranscriptome-mediated post-transcriptional regulation [25]. The recent accumulation of knowledge has led to the development of RNA modification databases. For instance, MODOMICS database offers comprehensive evidence regarding the chemical structures of modified ribonucleosides, their biosynthetic pathways, the location of modified residues in RNA sequences, and RNA modifying enzymes [1].

Adenosine modifications: N6-methyladenosine (m^6A)

Adenosine methylation occurring at the sixth position of the nitrogenous base (N^6-methyladenosine; m^6A) is one of the most pervasive modifications found in eukaryotic RNA, including mRNA and ncRNA [6,26,27]. m^6A, which has been detected in animals, plants, single-cell organisms (archaea, bacteria and yeast) and viruses, has been linked to the regulation of multiple cellular processes, including RNA stability, translation, alternative splicing, secondary structure and nuclear export [17,28–36].

m^6A writers, erasers and readers

The reversible addition and removal of methyl in N^6 adenosine methylation is catalysed by methyltransferases (writers) and demethylases (erasers) proteins, respectively. In addition, methylated RNA binding proteins (readers) help regulate the downstream processes [26]. The positions, patterns and motifs of m^6A suggest that writers, readers and erasers might be conserved across kingdoms, though it has been suggested that individual members of m^6A writer complex achieved functional divergence in plants [37].

The association between methyltransferase-like (METTL) 3 and METTL14 with other regulator components, such as Wilm's tumour-associated protein (WTAP), METTL16, zinc finger CCCH-type containing 13 (ZC3H13), and RNA binding motif protein (RBM) 15/15B, among others, results in a functional complex. This complex uses an S-adenosyl methionine (SAM) binding domain on METTL3 to methylate specific mRNAs at a RRACH (R = A/G; H = A/C/U) m^6A consensus sequence, mainly in the 3′ untranslated regions (3′ UTRs) and near stop codons [26,38]. METTL3, the catalytic subunit, and METTL14, an allosteric activator, form a core heterodimer, whose localization in nuclear speckles is regulated by WTAP, which is also important for the methyl transferase activity of these enzymes [39,40]. WTAP-dependent binding between RBM15/15B and the methyltransferase complex is necessary for optimal methylation activity [41]. ZC3H13 plays a critical role in anchoring WTAP and other components in the nucleus to facilitate m^6A methylation [38]. METTL16, known for its role as an RNA m^6A methyltransferase, methylates mRNAs, including MAT2A, which encodes the SAM synthetase expressed in almost every cell [34]. Although METTL16 is usually found in the nucleus, it has been suggested that it is also a cytoplasmic methyltransferase with different RNA binding features according to its cellular location [42].

m^6A was identified as a novel regulator of miRNA processing where METTL3 methylates primary inter- and intragenic miRNAs, and this modification was proposed to allow the microprocessor complex to recognize its specific substrates and initiate miRNA biogenesis [43]. Moreover, METTL16 has been demonstrated to bind and methylate the U6 small nuclear RNA and the lncRNAs MALAT1 and XIST [14,34]. The fate of mRNAs containing m^6A is predominantly determined by different categories of m^6A-binding proteins, termed 'readers', which recognize the modified site and prompt downstream regulatory effects (e.g. tumorigenesis, viral replication, adipogenesis, haemopoiesis, immune regulation, etc.), by altering RNA metabolic processes [25,31–33,44–46]. Such proteins include the YT521-B homology (YTH) domain family, heterogeneous nuclear ribonucleoproteins (hnRNPs), and IGF 2 mRNA-binding proteins (IGFBPs). The YTH domain represents a family that recognizes the m^6A mark directly [40], interacting with m^6A present in RNA through a conserved aromatic (tryptophan) cage [47,48]. By recruiting different complexes to target m^6A sites, the YTH domain-containing proteins, as well as other potential m^6A-binding proteins, contribute to gene regulation post-transcriptionally in many aspects, such as splicing, translation, localization, and lifetime [28,29,31,33,49]. The YTH domain family consists of YTH domain family protein 1–3 (YTHDF1-3, DF family) and YTH domain containing protein 1–2 (YTHDC1-2, DC family) [49]. YTHDF1 protein can increment mRNA translation efficiency by way of interacting with the translation initiation factor eIF3 in a m^7G-cap-independent way [50]. The cytoplasmic m^6A reader YTHDF2 is necessary for mRNA degradation and contributes to reducing the stability of targeted transcripts using the CCR4-NOT deadenylase complex [28,51]. YTHDF3 works in cooperation with these proteins to improve RNA binding specificity and affinity [29,32]. YTHDC1 and the YTHDF families are primarily localized to the nucleus and cytoplasm, respectively, while YTHDC2 is found in both the nucleus and cytoplasm [31,52,53]. These proteins participate in the processes of gene splicing, exportation, degradation, and translation of m^6A-containing mRNAs [54–56]. Fragile-X mental retardation protein (FMRP) has been reported to be an indirect reader

via binding with YTHDF proteins to regulate m^{6}A-modified mRNA [57].

The structural alteration of mRNAs induced by m^{6}A methylation (m^{6}A-switch) enhances the binding of other reader proteins such as heterogeneous nuclear ribonucleoproteins (hnRNPs), including hnRNPA2B1, hnRNPC and hnRNPG, and insulin-like growth factor 2 mRNA binding proteins (IGF2BPs), including IGF2BP1, IGF2BP2, and IGF2BP3, to m^{6}A sites [35,36,40]. In the m^{6}A-switch mechanism, m^{6}A modulates protein binding by inducing an RNA structural change that alters the accessibility of a protein binding site. In this sense, HNRNPC and HNRNPG binding to m^{6}A-modified RNA depends on an m^{6}A-induced base-pairing disruption that exposes U-rich and purine-rich binding sites, respectively [35,36,58]. The former is involved in pre-mRNA processing, while HNRNPG plays a role in pre-mRNA alternative splicing. On the other hand, members -of the IGFBP family recognize m^{6}A-containing transcripts via common RNA binding domains, such as the KH domain. Although the exact molecular mechanisms have not been fully revealed, IGF2BPs have been reported to recognize m^{6}A-modified mRNAs and enhance their stability and translation. IGFBP proteins exert their functions by recruiting RNA stabilizers, such as HuR, to protect m^{6}A-containing mRNA from degradation [27,30].

m^{6}A modifications can be reversibly 'erased' in a dynamical process by RNA demethylases, such as fat mass and obesity-associated protein (FTO) and alkylation repair homolog protein 5 (ALKBH5) in mammals. The former is able to oxidize m^{6}A resulting in intermediates, which are then hydrolysed to adenine, whereas ALKBH5 can eliminate m^{6}A directly [3,27]. In plants, several ALKBH5 orthologs were detected, and it has been suggested that these proteins could have redundant functions in m^{6}A demethylation, whereas no FTO orthologs were found [59].

Other RNA modifications

Apart from m^{6}A, various other RNA modifications have been identified so far. One of the most well-known chemical modifications of RNA affects capping at the 5′-end (5′ cap), including N7-methylguanosine (m^{7}G) [60,61]. Other modifications, involving adenosine (e.g. N1-methyladenosine (m^{1}A), N6,2′-O-dimethyladenosine (m^{6}Am)), cytosine (e.g. 5-methylcytosine (m^{5}C)), and uridine (e.g. uridylation, pseudouridylation), have been identified, as well as N-glycan alterations, among others. However, their biological roles are still not fully disclosed due to the complexity of RNA structure and functions [27].

Adenosine modifications (other than m^{6}A)

Other modifications of adenosine found in the human epitranscriptome include a methylation at the N^1 position of adenosine, generating N1-methyladenosine (m^{1}A), which is widespread in tRNA, rRNA and also in mRNA [7,62,63]. Transcriptome-wide mapping of m^{1}A exposed highly conserved features in mouse and human cells. In particular, these modifications were seen to reside close to both canonical and alternative transcription start sites (TSS), to be present in highly structured regions around the start codon, and to correlate positively with protein production [62]. AlkB homolog 1, histone H2A dioxygenase (ALKBH1) was recently presented as an RNA demethylase that catalyzes the demethylation of m^{1}A in tRNA [64] and alkB homolog 3, alpha-ketoglutarate dependent dioxygenase (ALKBH3) has been suggested to be a potential demethylase of m^{1}A in mRNA [65].

In contrast to m^{6}A, which is an internal modification, in higher eukaryotes, N6,2′-O-dimethyladenosine (m^{6}Am) can be found in the first nucleotide after the 7-methylguanosine cap, near the TSS in certain mRNAs [9,65,66]. Recently, m^{6}Am was discovered to suppress cap-dependent translation, and PCIF1 was identified as mRNA m^{6}Am methyltransferase [67,68].

Cytidine modifications

As mentioned above, apart from adenosine, the epitranscriptome exhibits several, increasingly characterized, modifications in other bases, such as cytidine. Most modifications are present at levels 10- to 100-fold less than m^{6}A [69]. Methylcytidine (m^{5}C) modifications, identified in mRNAs and ncRNAs [70], are present in modest levels (measured by LC–MS/MS) in mRNAs and have been mapped primarily to 5′ UTRs, whereas CDS regions showed the lowest density [71,72]. In humans, m^{5}C in RNAs are catalysed by members 1–7 of the NOL1/NOP2/SUN domain (NSUN) family of proteins, together with the homologue of DNA methyltransferase (DNMT), DNMT2 [10,73]. NSUN6-methylation was recently associated with a role in regulating translation termination [74].

Uridine modifications

3′ RNA uridylation, i.e. the addition of non-templated uridine (s) to the RNA end, by Terminal Uridylyl Transferases (TUTases), is now considered to be functionally important for RNA processing [75]. Another important uridine-specific alteration is pseudouridylation, which consists in the creation of the 5-ribosyl isomer of uridine, pseudouridine (Ψ) [76]. Ψ modifications relative to U in the 3′-UTR were distinctly lower compared to the 5′-UTR and CDS region in mammals, including humans [77–79]. Pseudouridylation is catalysed by pseudouridine synthases (PUS), which have been linked to human diseases and important biological processes [7].

RNA capping modifications

N7-methylguanosine (m^{7}G) is a very-well described cap modification crucial for mRNA stability, translation and functional diversity [61,80,81]. Despite its relative abundance, it is challenging to study since it does not interfere with reverse transcription (as it is neutral to Watson–Crick base pairing) and cannot be detected by standard sequencing-based technologies [15].

N-glycan modifications

Glycans, which have been widely described in proteins and lipids, regulate a myriad of essential functions and are present across different kingdoms of life [82]. Very few RNAs have been previously described to have sugar modifications in the

side chain, including tRNA [83]. A recent study showed that small noncoding RNAs contain sialylated glycans ('glycoRNAs') and that the majority of cellular glycoRNAs are located on the cell surface [84]. These glycoRNAs seem to act as ligands of the sialic acid binding-immunoglobulin lectin-type (Siglec) receptors, which have been functionally associated with diseases, such as autoimmunity and cancer [84]. Although more research is needed on this topic, the existence of glycoRNAs expands the role of RNA modification in health and disease.

Epitranscriptomic modifications in plants

RNA modifications such as m^6A, m^5C and Ψ have been detected in transcriptome-wide studies in plants. m^6A sites are widespread in plants and exhibit the dominant m^6A consensus motif RRACH (R = A/G; H = A/C/U), as well as other lineage-specific m^6A motifs, such as URUAY [46,85,86]. Throughout the transcriptome, m^6A are more abundant near the stop codon and 3′ UTR in different developmental stages in *Arabidopsis* and other plants. Furthermore, several studies have shown m^5C throughout the *Arabidopsis* transcriptome [87,88]. Other mRNA modifications, such as m^1A, hm^5C, Nm, I and ac4C have vital functions in mammals, but need to be further depicted in plants [59].

Gene-expression levels of writers, erasers and readers vary across different plant tissues and developmental stages [87,89,90], suggesting the dynamic regulation of mRNA modifications in plants. Furthermore, the level of mRNA modifications in different plant species has revealed the dynamics of mRNA modifications during plant growth and development [91]. Even though a number of m^6A writers, erasers and readers have been identified in *Arabidopsis*, the exact molecular mechanism describing the dynamic m^6A generation and deletion in plants in response to environmental changes is still lacking.

The m^6A methyltransferase complex seems to be conserved between mammals and plants [92]. The core component with m^6A catalytic activity is METTL3, which belongs to the MT-A70 family. In plants, the MT-A70 family proteins can be grouped in MTA, MTB, and MTC, which are present in most plant species [93]. In *Arabidopsis*, the m^6A writer complex contains the METTL3 ortholog, MTA [89], and the METTL14 ortholog, MTB [94], among other components. Many functional sites are conserved between plants and humans, suggesting that plant MTA–MTB and human METTL3–METTL14 may have a similar mechanism of methylation. As mentioned earlier, the methylation of A to m^6A can be reversed by m^6A demethylases (erasers). In plants, even though no FTO orthologs are found, genomes encode multiple copies of ALKBH5 orthologs, including six orthologs (ALKBH9A/B/C and ALKBH10A/B/C) in *Arabidopsis*, suggesting these may have redundant functions in m^6A demethylation [95]. Moreover, m^6A mediates its biological function by recruiting reader proteins. Plant YTH proteins can be divided into two groups: group 1 includes YTHDF and the majority of YTH domain proteins, and group 2 contains YTHDC1, YTHDC2 and the remaining plant YTH proteins, which form two subgroups [45]. The residues involved in the cage and RNA binding are highly conserved between yeast and *Arabidopsis* YTH proteins, suggesting that the other YTH proteins in *Arabidopsis* could also be m^6A readers.

In mammals, m^6A is involved in almost all aspects of RNA metabolism, including transcript stability, translation initiation, mRNA export, polyadenylation, nuclear retention, and pre-mRNA splicing [2,3,5,6,54]. In plants, several studies have demonstrated that m^6A and m^5C affect RNA stability [46,85,87,90,95]. In contrast, a number of transcripts exhibited increased abundance after loss of m^6A in plants [85,86,90,95], raising the question of how m^6A regulates gene expression in different plant species and under specific circumstances.

In humans, m^5C RNA modifications are catalysed by NSUN2 [44,70], whereas in plants, its homolog tRNA-specific methyltransferase 4B (TRM4B), belonging to the RNA (C5-cytosine) methyltransferase (RCMT) family, functions as an m^5C mRNA methyltransferase in *Arabidopsis* [87,88]. In plants, m^5C seems to have an ancient origin and widespread distribution, since members of the RCMT family are present in a great number of plant species, ranging from green algae to flowering plants.

miRNAs biogenesis, target recognition and biological functions

As mentioned above, miRNAs are a class of single-stranded non-coding RNAs, highly conserved evolutionarily, which have been associated with key regulatory roles in a substantial number of cellular processes in eukaryotic cells [96].

The biogenesis of miRNA is classified into canonical and non-canonical pathways. Briefly, the canonical pathway is characterized by a preliminary cleavage of primary miRNA (pri-miRNA) to precursor miRNA (pre-miRNA) by the microprocessor complex consisting of Drosha and DGCR8 enzymes. Following its transfer to the cytoplasm by exportin 5, pre-miRNA is additionally cleaved by Dicer to generate double-strand RNAs, which are subsequently associated with Argonaute (AGO) proteins forming the RNA-induced silencing complex (RISC) [97]. Non-canonical pathways circumvent the steps of the canonical pathway and can be differentiated into Drosha/DGCR8-independent and Dicer-independent pathways [98].

It is acknowledged that miRNAs can directly mediate post-transcriptional gene silencing in the cytoplasm through a seed sequence, a run of six nucleotides spanning nucleotide 2–7 on the 5′ end of miRNAs, and the complementary sequences in the 3′-untranslated region (UTR) of target mRNA [99]. Nevertheless, the interaction between miRNAs and other regions, such as 5′ UTR, coding sequence, and gene promoters, have also been reported [100]. In addition, it seems that miRNAs can be shuttled between different subcellular compartments to control the rate of translation and transcription [101]. Recent studies have shed light on the dynamic nature of miRNA actions and further revealed the complexity of miRNA-mediated gene regulation [102]. miRNA actions are vital for animal development and viability, and the deregulation of their activity has often been associated with the development and progression of human diseases [103].

Table 1. Summary of studies reporting a crosstalk between RNA modifications and miRNAs in disease settings.

RNA modification	Disease/condition	ncRNA	Enzyme	Effects	Outcomes	Ref
m^6A	Acute Ischemic Stroke (AIS)	miR-335	METTL3	METTL3-mediated m6A modification upregulates mature miR-335 generation, which targets Erf1	METTL3/miR-335/Erf1 axis contributes to SG formation, which may reduce AIS injury in the early stage	[18]
	Liver fibrosis	miR-350	METTL3	ASIC1a upregulates the expression of mature miR-350, which targets SPRY2 and activates the PI3K/AKT and ERK pathways	Promotion of HSC activation and liver fibrosis	[97]
	Diabetic retinopathy	miR-25-3p	METTL3	regulation of miR-25-3p/PTEN/Akt signaling cascade in a DGCR8-dependent manner	Alleviated high-glucose induced RPE cell apoptosis and pyroptosis, and promoted cell proliferation	[98]
	Colistin-induced nephrotoxicity	miR-873-5p	METTL3	METTL3 positively modulates miR-873-5p mature process, thereby regulating the Keap1/Nrf2 pathway	Anti-oxidative stress and anti-apoptotic role	[99]
	Lung cancer	miR-143-3p	METTL3	METTL3 facilitates the biogenesis of miR-143-3p, which targets the 3'UTR of VASH1 to inhibit its expression	Overexpression of METTL3 is associated with increased invasion capability and angiogenesis of cancer cells	[100]
	Hepatocellular carcinoma	miR-126	METTL14	METTL14 interacts with DGCR8 and promotes the processing of pri-miR-126 in an m6 A-dependent manner m6A marks enhanced the recognition of pri-miR126 by DGCR8 and the subsequent processing to mature miRNA	METTL14 may be involved in HCC metastasis, acting as a negative regulator	[101]
	Pancreatic cancer	let-7a-5p, miR-17-5p	METTL3/	METTL14	1) Raised METTL3 /METTL14 levels are accompanied by increased methylation in cancer tissues and serum vs normal; 2) m6A modified miRNAs have a reduced ability to inhibit mRNAs	
		Methylated miRNAs could become cancer biomarkers	[102]			
	miR-25-3p	METTL3	METTL3	promotes the maturation of miR-25-3p, which suppresses PHLPP2, resulting in the activation of oncogenic AKT-p70S6K signaling	Overexpression of METTL3 associates with cancer development and progression	[103]
	Colorectal cancer	let-7a-5p, miR-17-5p	METTL3/	METTL14	1) Raised METTL3 /METTL14 levels are accompanied by increased methylation in cancer tissues and serum vs normal; 2) m6A modified miRNAs have a reduced ability to inhibit mRNAs	
		Methylated miRNAs could become cancer biomarkers	[102]			
miR-1246		METTL3	METTL3	promotes the maturation of pri-miR-1246, resulting in the downregulation of SPRED2 and inhibition of the MAPK pathway	Overexpression of METTL3 promotes cell migration and invasion	[104]
Bladder cancer	miR-221/222	METTL3	METTL3	promotes the maturation of pri-miR221/222, resulting in the reduction of PTEN	Overexpression of METTL3 promotes cancer proliferation	[105]
m^5C	Pancreatic cancer and colorectal cancer	miR-200c-3p, miR-21-3p	NSUN2	1) Raised NSUN2 levels were accompanied by increased methylation in cancer tissues and serum vs normal; 2) m5C modifications in miR-200c-3p did not affect its ability to inhibit mRNAs	Methylated miRNAs could become cancer biomarkers	[102]
	Glioblastoma multiforme	miR-16-5p, miR-181a-5p, miR-181b-5p, miR-181d-5p, miR-210-3p, miR-451a, miR-193a-5p	DNMT3A/ AGO4	m5C in miR-181a-5p and miRNA-451a abolishes their tumor suppressor functions	Poorer prognosis in GBM patients	[106]
2'-O-methylation	Lung cancer	miR-21-5p	HENMT1	1) HENMT1 was significantly increased in lung cancer tissues compared to non-cancerous lung tissues; 2) differential 3'-terminal 2'Ome was shown for miR-21-5p in non-small cell lung cancer (NSCLC)	Methylated miRNAs could become cancer biomarkers	[107]

(*Continued*)

Table 1. (Continued).

RNA modification	Disease/condition	ncRNA	Enzyme	Effects	Outcomes	Ref
Uridylation	Osteosarcoma	miR-24, miR-29a	TUT1	Upregulation of miR-24 and miR-29a by TUT1 reduced PPARgamma and SREBP-1c expression	Reduced lipogenesis and tumor progression	[108]
	Breast cancer	let-7a, let-7f	LIN28	Wnt–b-catenin pathway enhances HRAS and HMGA2 protein expression through Lin28 activation and let-7 repression	Proliferation of cancer stem cells	[109]
	Head and Neck cancer	let-7 family	LIN28B	Down-regulation of the let-7 family by Lin28b results in enhanced HMGA2, CCND2, IGF1R, and IGF2BP2 levels	Enhanced tumor progression	[110].
m^7G	Lung and colon cancer	let-7-5p, miR-125a-5p, miR-92b-3p	METTL1	METTL1-dependent m7G promotes the processing of tumor-suppressive miRNAs, including the let-7 family reducing the levels of HMGA2	Reduced cell migration	[15]

AGO4, Argonaute RISC catalytic component 4; CCND2, cyclin D2; DNMT3A, DNA (cytosine-5)-methyltransferase 3A; Erf1, eukaryotic translation termination factor; GBM, Glioblastoma multiforme; HMGA2, High mobility group protein HMGI-C; HRAS, GTPase HRas; IGF1R, insulin like growth factor 1 receptor; IGF2BP2, insulin like growth factor 2 mRNA binding protein 2; LIN28B, lin-28 homolog B; m^5C, 5-methylcytosine; m^6A, N6-Methyladenosine; m^7G, 7-Methylguanosine; METTL1, (guanine-N(7)-)-methyltransferase 1; METTL3, N6-adenosine-methyltransferase catalytic subunit; METTL14, N(6)-adenine-specific methyltransferase; NSUN2, Myc-induced SUN domain-containing protein; PHLPP2, PH domain leucine-rich repeat protein phosphatase 2; PPARgamma, peroxisome proliferator-activated receptor gamma; SREBP-1c, Sterol regulatory element-binding protein 1; TUT, terminal uridylyl transferase 1; VASH1, vasohibin-1.

Crosstalk between RNA modifications and miRNA in disease settings

Evidence of the crosstalk between RNA modifications and miRNA biogenesis in pathophysiological contexts is rapidly growing. Examples of these associations are summarized in Table 1.

Adenosine modifications, miRNAs and disease

Significant overlap between m^6A and miRNA-binding sites has been reported in 3′ UTRs of mRNAs, and this co-localization generally shows an inverse allocation pattern, where m^6A peaks abound near the stop codon, usually preceding miRNA-binding sites, which prosper near the 3′ end of 3′ UTRs [104]. The inverse distribution pattern seems to indicate that the interaction between m^6A and downstream-bound miRNA requires a particular partition. Furthermore, raised expression of miRNAs was frequently associated with larger amounts of m^6A-modified target transcripts, suggesting that miRNAs may regulate m^6A modifications on their target transcripts [104]. In addition, it has been reported that m^6A marks the initiation of pri-miRNAs processing [5]. HNRNPA2B1 was categorized as m^6A-reader protein in the nucleus that interacts with the Microprocessor protein DGCR8, enhances binding of DGCR8 to pri-miRNA transcripts, and positively regulates pri-miRNA processing in a similar manner as METTL3 [5,43]. Moreover, HNRNPA2B1 modulates the alternative splicing of mRNA transcripts [43]. Recent studies have provided evidence on the link between m^6A modification, miRNAs processing and tumour progression scenarios. As previously described, METTL3, in an m^6A-dependent manner, interacts with DGCR8 to support the maturation of pri-miR221/222, which then targets PTEN, leading to accelerated cell proliferation in bladder cancer [105]. In colorectal cancer, METTL3 can methylate pri-miR-1246, promoting the maturation of pri-miR-1246, which targets the anti-oncogene SPRED2 [106]. Moreover, METTL3 was shown to prompt the splicing of pre-miR-143-3p into its mature form, which is involved in the brain metastasis of lung cancer cells via down regulation of VASH1 [107]. In a recent investigation, cigarette smoke-induced overexpression of METTL3 resulted in increased m^6A modification, with the involvement of NF-κB associated protein (NKAP). In turn, this generated excessive maturation of pri-miR-25, which impairs PH domain leucine-rich repeat protein phosphatase 2 (PHLPP2), contributing to the activation of AKT-p70S6K signalling, a pathway linked to the development and progression of pancreatic cancer [108]. In another study, METTL14 has been shown to interact with DGCR8 enhancing the maturation of pri-miR-126, which then counteracts the suppressing impact of this m^6A writer on the metastasis in hepatocellular carcinoma (HCC) [109]. Evidence, concerning other pathological scenarios associated with irregular m^6A-dependent miRNA processing, is beginning to accumulate. For example, it has been reported that METTL3 interacts with DGCR8 and stimulates the maturation of miR-873-5p, which could regulate Keap1-Nrf2 pathway against oxidative stress and apoptosis in colistin-evoked nephrotoxicity [110]. In addition, METTL3-catalyzed m^6A resulted in a negative regulation of pre-miR-320

and miR-320, which targets RUNX2, a key transcription factor for osteoblast differentiation and bone formation [111]. In gastrointestinal cancer, Konno and colleagues reported a rise in the levels of METTL3 and METTL14 together with a higher number of RNA methyl marks, including m^6A, in miR-200 c, miR-17-5p, let-7a-5p, among other miRNAs [112]. Lastly, METTL3-dependent m^6A was shown to regulate miR-7212-5p maturation via DGCR8, a microRNA that targets FGFR3 to inhibit osteoblast differentiation in mouse osteoblast precursor cells [113].

Accumulating evidence supports the concept that m^6A influences miRNAs processing, yet it does not appear to play a unidirectional regulation as the role of miRNAs in regulating m6A formation of mRNAs has been revealed [13]. Whereas the involvement of AGO proteins in regulating m^6A formation was discarded, this was not the case for Dicer, whose induction raised the abundance of m^6A without altering the protein levels of METTL3, FTO and ALKBH5. In addition, Dicer seems to influence the nuclear speckle localization of METTL3 [13]. Overexpression and knockdown of specific miRNAs increased and decreased, respectively, m^6A abundance in mouse neural crest stem cells (NSCs) and in HeLa cells. Finally, miRNAs were shown to regulate the m^6A methyltransferase activity of METTL3 by modulating its binding to mRNAs [13]. The above is supported by a study performed in HCC cells, where miR-145 was reported to regulate m^6A level through the inhibition of YTHDF2 expression [114]. In another study, focused on hepatoblastoma progression, miR-186 was proposed to control METTL3 expression via Wnt/β-catenin signalling [115]. Moreover, the existence of a feedback loop was proposed, wherein HBXIP suppresses let-7 g inducing METTL3, which in turn increases HBXIP expression, leading to the acceleration of proliferation in breast cancer [116]. Regarding the RISC complex structural changes influenced by methylated miRNAs, *in vitro* and *in silico* analyses suggest that m^6A modifications in miR-17-5p and let-7a-5p, located away from the RNA-binding site (at positions 13 and 19, respectively) affect target-RNA recognition efficiency [112]. These results demonstrate that m^6A modifications may reduce the ability of miRNAs to suppress target mRNA translation. Nevertheless, m^6A-modified miR-200 c-3p did not repress target gene expression, contrary to non-methylated and m^5C-modified miR-200 c-3p in HCT116, a low-level endogenous miRNA expressing cell line [112].

To the best of our knowledge, solid evidence on the impact of m^1A on the processing of a specific miRNA has not yet been reported in a pathological context. Nevertheless, m^1A modification sites have been identified in lncRNA, such as MALAT1 (m^1A8398), which was found to be upregulated in various forms of cancer [117], and in a group of nuclear-encoded lncRNAs [118].

Cytidine modifications, miRNAs and disease

In 2019, Konno et al. showed that miRNAs could be cytosine-methylated and reported that adenosine and cytosine methylated miRNA-17-5p could be used as a biomarker of early-stage pancreatic cancer [112]. In gastric cancer cell lines, small RNA methyl marks, including 5mC, 3-methylcytosine, m^1A and m^6A, were found in 1–8% of total adenines and cytosines. Interestingly, the fraction of methylated miRNAs increased upon stimulation with epidermal growth factor, suggesting a regulatory mechanism for RNA modification [112]. Cheray et al. (2020) confirmed the existence of cytosine methylation in mature miRNAs and show it is associated with poor prognosis in glioblastoma multiforme [119]. In addition, this study provided evidence that miRNAs can be cytosine-methylated by DNMT3A/AGO4-including complexes and demonstrated that 5mC in miRNAs suppresses their gene expression repressive capacity [119]. Very recently, Carissimi and colleagues confirmed the presence of m^5C in human miRNAs and reported the existence of (hydroxy)-methyl-5 cytosine (hm^5C) modifications [16]. In human cell lines and PBMCs, these authors found that several miRNAs (i.e. miR-125a-5p, miR-191-5p, miR-25-3p and many others) harbour hm^5C and that this is not restricted to CG sequence context. These findings suggest that, in addition to DNMT3A, other RNA methyltransferases might be involved, although the functional consequences of these modifications are unknown [16]. Another RNA cytosine modification, ac^4C, is regulated by *N*-acetyltransferase 10 (NAT10). Ac4C is enriched within the 5′ regions of the coding sequence and is associated with substrate mRNA stability [8].

Uridine modifications, miRNAs and disease

It is known that uridylation plays a role in the regulation of canonical microRNA biogenesis in the tumour suppressor let-7 microRNA family. In addition, uridulation involves two different non-canonical microRNA biogenesis pathways contrasting on their dependence on enzymes Drosha and Dicer. Furthermore, uridylation of mature miRNAs can generate isomiRs, sequence variant microRNAs, with modified action [75]. TUT1 has been suggested to positively influence, through 3′ nucleotide additions, the levels of miR-24 and miR-29a, which, in turn, negatively modulate lipogenesis regulators PPAR-gamma and SREBP-1 c. As lipogenesis is considered to be a cancer hallmark, TUT1 might act as a tumour suppressor [120]. miRNAs play important roles in disease progression, mainly through the regulation of their target genes; however, miRNAs can themselves be post-transcriptionally regulated by other genes, which contributes to the overall complexity of the regulation process. A clear example occurs with the regulation of the Let-7 miRNA family biogenesis through pre-miRNA uridylation, where TUTases 4 and 7 interacts with Lin28, which, in turn, is regulated via Let-7 miRNA, generating a negative feedback loop [12,121]. In head and neck cancer [122], and also in breast cancer [123], a suppression mediated by LIN28 was observed in the processing of pre-let-7. Notably, in the former study, this was accompanied by the up-regulation of genes in the IGF pathway in Lin28b-expressing cells [122], namely IGF2BP2, while in the latter study the influence of Lin28 on let-7 maturation was associated to the Wnt–b-catenin pathway [123]. DIS3-like exonuclease 2 (DIS3L2) was proposed to be the reader for uridylated pre-let-7 in vivo, forming a LIN28-TUT4/7-DIS3L2 pathway [124]. However, it was recently reported that DIS3L2 loss had no effect on mature let-7 levels [125].

Most studies of PUSs and Ψ have focused on abundant ncRNAs, and evidence regarding whether PUSs are able to influence metabolic pathways of other types of RNA is scarce. Recently, Song et al. (2020) reported that the depletion of PUS10 caused a noticeable decrease in the expression of many mature miRNAs together with the build-up of unprocessed pri-miRNAs in several human cells [126]. This study also showed that PUS10 directly binds to pri-miRNAs and interacts with the microprocessor to promote miRNA biogenesis; however, authors concluded that the catalytic activity of PUS10 does not play a relevant role in this process [126].

RNA capping modifications, miRNAs and disease

Recently, two complementary high-throughput sequencing strategies were used to demonstrate that a subset of miRNAs harbours internal m^7G modification, including the let-7 family. In particular, it was shown that m^7G methylation by METTL1, a methyltransferase that regulates cell migration and promotes the processing of let-7e-5p miRNA precursor [15]. Apart from m^7G, other somewhat less characterized RNA caps exist in eukaryotes (e.g. yeasts, plants and humans) [4,127–129]. The recognition of the 5′ mono-phosphate of pre-miRNAs by Dicer is important for the subsequent processing of miRNAs. In this sense, a study by Xhemalce et al. (2012) showed that the BCDIN3 domain containing RNA methyltransferase (BCDIN3D) was shown to O-methylate the 5′ mono-phosphate end of pre-miR-145 and pre-miR-23b, inhibiting their processing by Dicer [127].

3′ end modifications affect the regulation of miRNA stability and target recognition

As mentioned above, miRNA are important regulators of many vital cellular processes; hence, turnover and degradation of these regulators needs to be tightly and dynamically controlled. This can be achieved through the manipulation of miRNA stability, which involves 3′ end modifications (i.e. 3′ methylation and 3′-to-5′ truncation), AGO association, and miRNA-target RNA interaction. Extended information on these mechanisms can be found elsewhere [130]. Regarding 3′ end modifications, the HEN1-catalysed 2′-*O*-methylation modification at the 3′ end (3′-terminal 2′Ome) of various small RNAs (including miRNAs) in plants and piRNAs in animals is crucial for their stability (Figure. 1). Loss of function mutations involving *hen1* cause miRNA deterioration and 3′ end heterogeneity due to combined actions of 3′-to-5′ truncation and 3′ tailing, mainly by uridylation [11,131,132]. The addition of non-uridine nucleotides to miRNAs was also shown to occur at very low frequencies through other non-identified terminal nucleotidyl transferases. However, their biological relevance (if any) remains to be ascertained [132].

In Arabidopsis, HEN1 Suppressor 1 (HESO1) is an important TUT that catalyzes the addition of uracils to the 3′ end of unmethylated miRNAs [131]. UTP:RNA uridylyltransferase 1 (URT1) is another TUT reported to be a functional paralog of HESO1 [132,133]. URT1 and HESO1 act cooperatively on AGO1-bound miRNAs and the tailed miRNAs stay bound by AGO1. Moreover, miRNA tailing affects the activity of miRNAs, in addition to causing miRNA degradation [133].

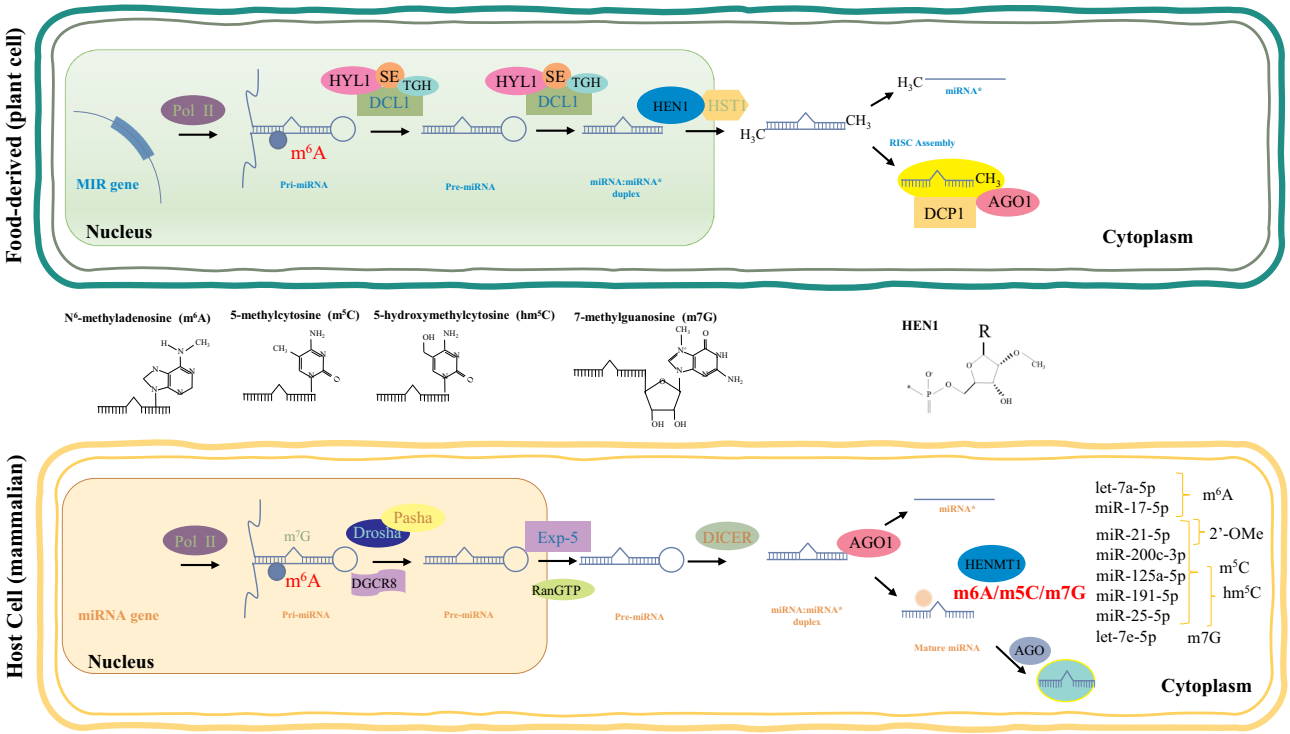

Figure 1. Epitranscriptomic modifications in miRNAs. Schematic representation of miRNA biogenesis in mammals and plants. HEN-1 is a methyltransferase that adds a methyl group to the ribose of the last nucleotide (represented as R) of small RNAs (sRNAs). This methylation protects the 3′-end of sRNAs from uridylation activity and subsequent degradation. Not all miRNA biogenesis mechanisms are depicted. Frequently reported miRNA modifications, along with representative examples, are shown on the right bottom corner.

In addition, small RNA Degrading Nucleases (SDNs), which are 3′ to 5′ end exoribonucleases, contribute to miRNA degradation by 3′ truncation of AGO1-bound and methylated miRNAs to result in AGO1-bound, 3′ truncated-and-unmethylated miRNAs. These are then uridylated by HESO1 and/or URT1, and further degradation is catalysed by other enzymes [134,135]. Small RNA degradation associated with uridylation seems to be a conserved mechanism in a variety of species, as is the case of the green algae *Chlamydomonas reinhardtii*, where MUT68, a terminal nucleotidyl transferase homologous to HESO1, is involved in the untemplated uridylation of the 3′ end of miRNAs and siRNAs [136].

Recently, HEMT1, an HEN1 homolog, was identified as the methyltransferase responsible for 3′-terminal 2′Ome of mammalian miRNAs [137]. Moreover, HENMT1 was significantly increased in lung cancer tissues compared to non-cancerous lung tissues, and differential 3′-terminal 2′Ome was shown for miR-21-5p in non-small cell lung cancer (NSCLC) and miR-26-5p in non-cancerous lung tissues. Furthermore, in vitro cleavage assays indicated that 3′-terminal 2′Ome protected miRNA 3′→5′ degradation by PNPT1. In addition, methylated miR-21-5p exhibited a higher affinity to AGO2 than the unmethylated form. miRNA target recognition should not be affected by 3′ uridylation since alterations at the 3′ end of miRNAs do not alter its seed sequence. However, it was recently shown that TUT4/7-mediated uridylation of miR-27a can modulate its pairing, enabling it to repress a distinct set of targets, despite the absence of a seed pairing match [138].

Despite recent advances, further research is needed to understand how the (de)regulation of 3′-terminal methylations in mammalian miRNAs is associated with disease settings.

Epitranscriptomic modifications potential contribution to cross-kingdom regulation

In recent decades, research has found that miRNA signals can be transmitted between different species and kingdoms as a means of communication between two, often unrelated, interacting organisms, such as a host and a pathogen, pest, parasite, or symbiont [24,139]. The identification of food-derived miRNAs from animal [140] and plant kingdoms [141] brought attention to the possibility that dietary miRNAs could influence host gene expression [142,143]. Nevertheless, even though several studies have been reported since the first paper by Zhang et al. (2012), kingdom crossing of food-derived miRNAs is far from being a consensual matter [144,145].

Before any biologically meaningful effects may eventually occur, dietary miRNAs must surpass many obstacles before (food processing-related) and after (bioavailability, reaching and entering target cells, etc.) entering host organisms [143]. First, miRNAs stability must be maintained throughout the harsh food processing chain (e.g. ripening, storage, cooking and/or other technological practices). Preliminary studies suggest that the stability of miRNAs depends on their specific sequence (i.e. GC content) [146] and secondary structure [147]. Specific dietary miRNAs have been reported to remain highly stable during different handling, storage and/or cooking conditions [141,148] or to resist degradation even under harsh conditions, such as acidic pH and substantial heat exposure (e.g. boiling) [149,150]. For example, MIR2911, a plant 26S ribosomal RNA-derived small RNA, exhibited high stability against RNases and strong stability in circulation[150]. However, many miRNAs do not survive food processing intact and certain conditions, such as high temperature and pressure, starch dextrinization or protein denaturation, reduce their contents [151,152]. For example, in animal-derived products, such as milk and its derivatives, processing (raw milk pasteurization and homogenization) leads to significant miRNA loss [153]. In plant-based foods, such as olive oil or beer, miRNAs are barely detected, most likely as a consequence of extensive degradation due to cellular compartment rupture and long-term contact between miRNAs and RNases [154]. Regarding the harsh biological factors miRNAs face after entering the host organism, sequence [146] and secondary structure [147] also influence their stability. The unfavourable environment of the gastrointestinal tract, including RNases and lytic enzymes of the oral cavity, extreme stomach acidity, flow of gastroenteric fluid and peristaltic activity, nucleases and degradative enzymes, mucus composition and gut microbiota, are conditions that dietary miRNAs must surpass before they can be taken up, biodistributed to target tissues and, eventually, exert biological effects [142,143]. In this sense, some studies failed to detect the transfer of plant or exosomal miRNAs 155,156 .

Although it is yet to be demonstrated *in vivo*, dietary miRNAs may cross the intestinal epithelium using the available routes of absorption (i.e. paracellular or transcellular). Some stable plant miRNAs can be absorbed via clathrin- and caveolin-mediated endocytosis [147]. *In vitro*, miRNA uptake was observed to be sequence-dependent and facilitated by NACh and TLR9 cell membrane receptors [147]. The absorption of single-stranded dietary miR168a seems to be facilitated compared to other types of miRNA structure [141], while the uptake of single-stranded mature miRNA was significantly lower than that of double-stranded miRNA mimics at pH 7.4 in cultured cells [157]. However, the absorption of dietary miRNAs by gastric pit cells in the stomach has been reported to be pH-dependent, as a near 30-fold rise was seen in single-stranded miR2911 absorption in pH conditions of 3.5 compared to 7.4 [157]. Although it is not clear whether the epitranscriptomic modifications of miRNAs influence their dietary absorption, the stability increasing effects (described above) may increase survival chances. Increased absorption of dietary small RNAs due to alterations in intestinal permeability have been reported [146]. miRNA packaging in extracellular vesicles (EVs), followed by their release in the circulatory system, enhances nuclease resistance and facilitates their uptake compared to the unpackaged form [141]. Why and how dietary miRNAs are packaged into mammalian EVs remains to be determined. Once taken up by the target cells/tissues, the biological functions potentially exerted by dietary miRNAs will depend on factors, such as subcellular localization [158], number of target

transcripts [159], and/or concentration reached within the cell [160].

There have been very few studies where the mechanisms of molecular interaction between human AGO proteins and plant miRNAs within the RISC complex, where miRNA functions have been explored. It has been shown that 3′-terminal 2′Ome (but not at the 5′ termini) of miRNAs largely protects them from nuclease degradation, suggesting that miRNAs are likely to be destabilized at their 3′ ends upon target binding [161]. It is also well known that 3′-end methylation is a common step in miRNA and siRNA metabolism and expectedly protects the 3′ ends of small RNAs from the uridylation activity and trimming in plants [11]. Moreover, HENMT1-mediated 3′-terminal 2′Ome of miRNAs in humans seems to increase their higher affinity with the AGO2 complex and provide some protection against degradation [137]. In this sense, it cannot be discarded that certain chemical modifications of dietary plant miRNAs provide them with enhanced protection against degradation by host organisms, and that these miRs could, eventually, reach target cells in sufficient numbers for biological effects to occur.

The hypothesis of cross-kingdom regulation of gene expression by dietary miRNAs represents a novel approach for future dietary therapy and deserves further research [142,145]. However, the theory of cross-kingdom regulation leading to relevant biological effects has been refuted by many researchers, and there are still many loopholes to fulfill and risks to consider before the use of dietary or plant miRNAs as effective therapeutic tools can be a reality.

Future Perspectives

Aberrant expression of ncRNAs, particularly miRNAs, is found in pathological contexts. In recent times, target selection in the development of miRNA-based therapeutics is mainly based on quantitative criteria (expression studies) and less on qualitative criteria (e.g. presence/absence of specific chemical modifications) [18,162,163]. RNA modifications offer a novel layer of complexity to the intricate mechanisms of disease development and therapy. Synchronously to their functioning as post-transcriptional mediators, miRNA biogenesis and target recognition can be regulated through chemical modifications. New specific therapeutic targets can be selected by studying chemically modified miRNAs. For example, uridylation of miR-26a prevents the miRNA from repressing its mRNA target, without affecting its abundance [164]. The growing knowledge in the epitranscriptomics field needs to continue in order to achieve the ultimate goal of providing the bases for the implementation of dependable, minimally invasive, diagnostic and therapeutic tools in the clinical setting.

On the other hand, it is important to determine if miRNA modifications can have an impact on cross-kingdom regulation (e.g. by enhancing miRNA stability) and contribute to potentiate the development of therapeutic miRNAs within edible plants. In this context, potent practical applications based on RNA oligonucleotides heavily rely on the inclusion of chemical modifications to exert enhanced biological effects [165]. Moreover, the development of genetically modified crops containing miRNAs is guaranteed [146,166,167,168].

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Author contribution

AD, JT-C and MCLH designed the manuscript and contents. JT-C, AD and MCLH wrote and revised the first version of the manuscript. HB, YB, AC and MMG revised and edited the second version of the manuscript. All authors revised and approved the final version.

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