Epitranscriptomics of cardiovascular diseases (Review)

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Abstract. RNA modifications have recently become the focus of attention due to their extensive regulatory effects in a vast array of cellular networks and signaling pathways. Just as epigenetics is responsible for the imprinting of environmental conditions on a genetic level, epitranscriptomics follows the same principle at the RNA level, but in a more dynamic and sensitive manner. Nevertheless, its impact in the field of cardiovascular disease (CVD) remains largely unexplored. CVD and its associated pathologies remain the leading cause of death in Western populations due to the limited regenerative capacity of the heart. As such, maintenance of cardiac homeostasis is paramount for its physiological function and its capacity to respond to environmental stimuli. In this context, epitranscriptomic modifications offer a novel and promising therapeutic avenue, based on the fine-tuning of regulatory cascades, necessary for cardiac function. This review aimed to provide an overview of the most recent findings of key epitranscriptomic modifications in both coding and non-coding RNAs. Additionally, the methods used for their detection and important associations with genetic variations in the context of CVD were summarized. Current knowledge on cardiac epitranscriptomics, albeit limited still, indicates that the impact of epitranscriptomic editing in the heart, in both physiological and pathological conditions, holds untapped potential for the development of novel targeted therapeutic approaches in a dynamic manner.

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1. Introduction

In recent years, great technological leaps in sequencing technologies have enabled the in-depth investigation of the genetic basis of a multitude of human disorders, and they have paved the way for a new era of personalized medicine (1,2). Such breakthrough developments in sequencing technologies have substantiated the deep complexity of associations between genotype and phenotype and revealed unexpected cases, such as those of identical twins carrying the same disease mutations, but exhibiting different clinical features, such as balance problems and the development of blindness (3). Such discrepancies can be attributed to epigenetic and/or epitranscriptomic differences. The term epigenetics, first introduced

by C.H. Waddington in 1942, refers to the study of the mechanisms and molecules that can perpetuate variable gene activity states in the context of the same DNA sequence (4). Epigenetic mechanisms include DNA methylation, chromatin remodeling, histone modifications, gene activity regulation by non-coding RNA (ncRNA) molecules and protein-protein interactions (5). These mechanisms, which include a vast array of different molecules and pathways, regulate genomic structure and transcriptional activity in response to the ever-changing profiles of cell-intrinsic, cell-cell and cell-extrinsic signals (6).

Epigenetic regulation has been increasingly gaining interest due to its strong relationship with environmental adaptation. As new insights are gained, novel distinctions are also formed, leading to the emerging field of epitranscriptomics. Instead of encompassing all epigenetic regulation, epitranscriptomics focuses on modifications at the RNA level (7). Due to the vast array of effects that coding and ncRNAs exert in regulating the differential response of organisms to environmental stimuli, as well as homeostasis maintenance, epitranscriptomics has turned into an explosive field of research. Several different RNA modification databases have been established throughout the years in an effort to catalogue the plethora of RNA modifications that are continuously being detected. These include databases such as Modomics (https://iimcb. genesilico.pl/modomics/) (8-11), RMBase v2.0 (http://rna.sysu. edu.cn/rmbase/) (12), DARNED (https://darned.ucc.ie/) (13), the RNA Modification Database (https://mods.rna.albany. edu/) (14) and REDIportal v2.0 (http://srv00.recas.ba.infn. it/atlas/) (15), encompassing >172 RNA modifications to date.

Epitranscriptomic changes induced by such mechanisms have been implicated in various diseases and most of them display reversible chemistry, making epitranscriptomics a promising candidate for providing novel therapeutics (16). As such, a number of reviews have already been published discussing the ever-expanding field of epitranscriptomic modifications, with a limited number focusing on their effects under the prism of cardiovascular disease (CVD). Most notable reviews have focused on N6-methyladenosine (m⁶A) modifications, as the most prevalent epitranscriptomic modification and its role in CVD (17,18). Although, Kumari *et al* (17) featured a section about m⁶A readers, Chen *et al* (18) also discussed the potential for m⁶A modification to influence CVD risk factors. Focusing more on clinical trials investigating epigenetic-sensitive drugs for heart failure (HF), Napoli *et al* (19) also outlined the discovery of epigenetic biomarkers and signatures of cardiac remodeling. On the other hand, Fischer and Vondriska (20) focused their discussion on epigenetic changes occurring in CVD, but did not expand into RNA modifications, as was also the case for Schiano *et al* (21), who discussed epigenetic mechanisms underlying the various pathologies encompassed by the CVD umbrella-term. Although the authors mentioned CVD epitranscriptomics as an emerging layer of epigenetic regulation in CVD, they also highlighted the need for further research that covers this subject matter.

In the present review, the most prevalent epitranscriptomic modifications that have been shown to be involved in the field of CVD have been outlined (22), in an effort to extensively cover the area of RNA modifications, without focusing on a single one. This study also briefly discussed the mode of action of each modification and then explored their respective

effect on both coding and ncRNAs, including microRNAs (miRNAs/miRs) and long ncRNAs (lncRNAs), in the context of CVD. Furthermore, the current methods of RNA modification detection that have been on the forefront of epitranscriptomic research were also explored in brief. Finally, available data on genetic associations of RNA modifications, as well as therapeutic implications of epitranscriptomic approaches, in the heart were discussed.

2. Prevalence of epitranscriptomics in CVD and their activity

CVD is currently the leading cause of death worldwide, accounting for almost half the total number of deaths (23). CVD encompasses a wide array of heart and vessel-related pathologies, including, but not limited to HF, coronary heart disease, hypertension, hypertrophic and dilated cardiomyopathy, as well as congenital heart disease (24). Accumulating data have shown that cardiovascular risk factors may alter epigenomic patterns and that several cardiovascular biomarkers are associated with epigenetic modifications (25). DNA methylation appears to contribute to processes underlying CVDs, such as atherosclerosis, hypertension and inflammation (26-28). Moreover, epidemiological studies imply that methylation of repetitive sequences such as long-interspersed nucleotide repetitive elements-1 (LINE-1) and Alu elements are associated with CVD (26). Specifically, patients with prevalent ischemic heart disease (IHD) and stroke displayed lower blood LINE-1 methylation, while elevated methylation of Alu elements was associated with CVD and obesity in Chinese individuals (26). Histone modifications have also been implicated in processes, such as hypertension and atherosclerosis, while histone deacetylase 4 overexpression following myocardial infarction (MI) has been shown to increase myocardial fibrosis and cardiac hypertrophy, eventually leading to cardiac dysfunction (29). Although epigenetic regulation has been the focus of attention, RNA modifications have only recently started becoming the focus of CVD researchers.

Epitranscriptomic regulation manifests through the action of different enzymes. Enzymes that modify the RNA itself are called 'writers', while the ones that recognize and remove modifications are termed 'erasers'. Finally, 'readers' are the group of enzymes that bind the modifications themselves (30,31). These different modifications are classified into groups based on their different characteristics. These groups include classification into reversible and non-reversible (where erasers are lacking), substitutional and non-substitutional (32), cap (where the modifications happen to the 5'-end of the RNAs) or internal modifications [where the modifications occur within the 5'- or 3'-untranslated regions (UTRs) or within transcript introns] (33), and finally, modifications on coding or ncRNAs (34). NcRNAs have now been studied extensively and have been proven to have important regulatory effects in both physiological and pathological conditions. The term ncRNAs encompasses a large array of RNA molecules, including, but not limited to the major classes, such as miRNAs, lncRNAs and circular RNAs (circRNAs), as well as transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small nucleolar RNAs (snoRNAs), small nuclear RNAs (snRNAs) and others (35). ncRNA regulatory roles extend from interacting with RNA/DNA-binding

proteins, being part of complex structures, interacting with messenger RNA (mRNA) molecules to participating in translation and guiding chemical modifications (35). Through post-transcriptional modifications, ncRNAs display discrete temporal and spatial expression patterns, reflecting a precise regulation of their expression (36).

Epitranscriptomic editing of ncRNAs is quite prevalent during physiological, but also pathological conditions (37). miRNA editing is capable of creating alternative miRNAs, known as isomiRs (38). isomiR Bank, a database integrating >300,000 detected isomiRs (https://mcg.ustc.edu.cn/bsc/isomir/) (39), gives an estimate of the extent of additional layers of regulation that this editing process can generate. Although, isomiRs were first dismissed as artifacts, follow-up research has shown that almost half of miRNA transcripts are edited, and these edited transcripts can be loaded into the RNA-induced silencing complex and exert their regulatory activity (40). miRNA modifications happen either in the 3'-end or in the 5'-end sequences. Although, 3'-end editing is more prevalent (41), it mostly influences miRNA stability and activity. 5'-end editing, on the other hand, introduces modifications in the seed sequence, altering the target set of the miRNA and regulating new pathways (42-45). Moreover, circRNA efficiency and translation have been shown to be subject to regulation by distinct RNA modifications, such as m^6A, 5-methylcytosine (m^5C) and pseudouridylation (Ψ) modifications (46), as was also the case for numerous lncRNAs, which have been found to have roles in various CVD-related pathways, such as atherosclerosis and pulmonary hypertension (47).

In the present study, the epitranscriptomic modifications are classified into three major categories. The most prevalent form of epitranscriptomic modification, as in epigenetics, is RNA methylation (Fig. 1), which can affect adenosines in different positions [N^1-methyladenosine (m^1A), m^6A, 2'-O-methylation (Nm)], cytosines [m^5C, 5-hydroxymethylcytosine (hm^5C)] or guanosines [7-methylguanosine (m^7G)] (48). The second group encompasses substitutional modifications (Fig. 2), which include A-to-I and C-to-U RNA editing (49,50). Finally, the third group of modifications includes all epitranscriptomic changes that do not fall into any of the previous two categories [such as Ψ (51) and 8-oxoguanine (8-OxoG)], but nevertheless, have a proven or implied role in CVD (52) (Fig. 3).

3. RNA methylation modifications

m^6A. One of the most extensively studied RNA modifications in general is the m^6A modification. First discovered in 1974 (53,54), m^6A modification comprises the most common chemical addition to eukaryotic RNA. During m^6A modification, a methyl group (-CH3) is added to the N^6 site of the adenosine residue (m^6A) (55). This event can be detected in snoRNAs, tRNAs, rRNAs and other ncRNAs, and eventually represents 0.2-0.6% of all adenosine in mammalian mRNA (56). Specifically, 80% of the methylation that the total transcriptome undergoes is through the m^6A modification (57). In terms of localization, the distribution of m^6A follows a specific pattern in RNA, located mainly near the stop codons and the 3'-UTR (55,58). Several studies have shown
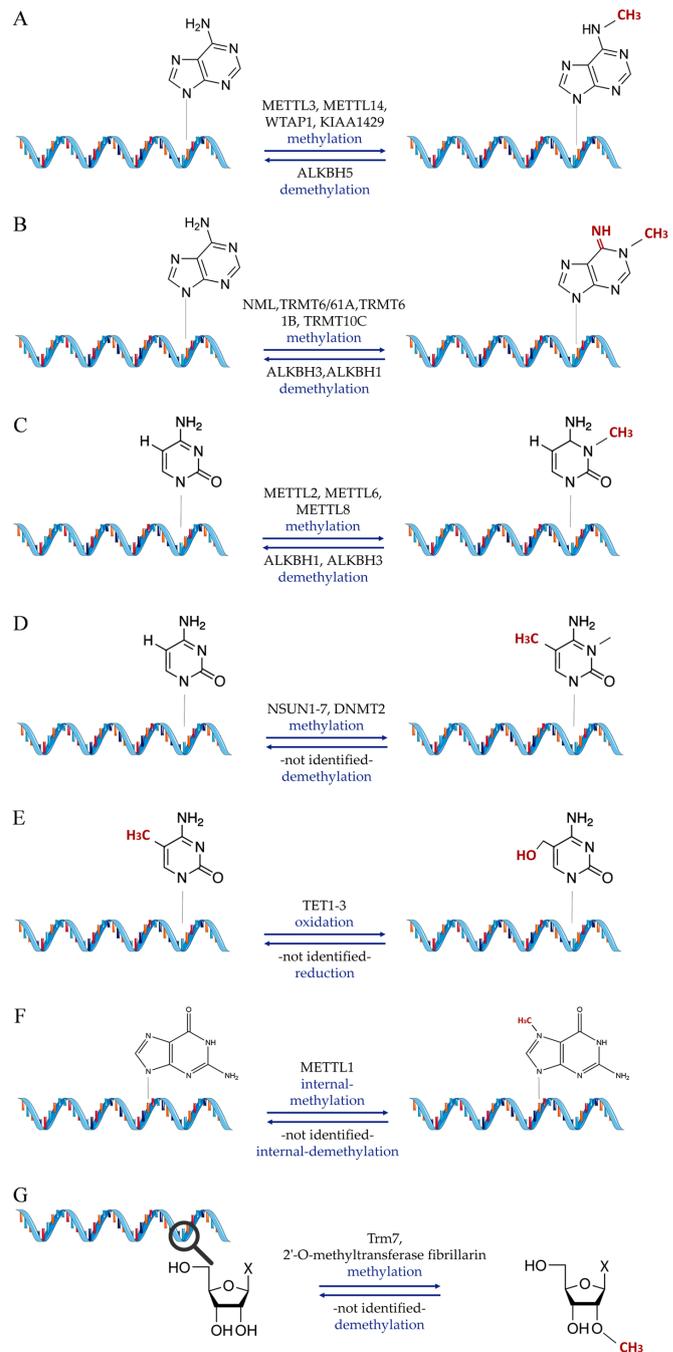
Figure 1. RNA methylation modifications along with their corresponding writers and erasers. (A) m^6A modification, (B) m^1A modification, (C) m^3C modification, (D m^5C modification, (E) hm^5C modification, (F) m7G modification and (G) Nm (where N stands for any nucleotide) modification. m^6A, N^6-methyladenosine; m^1A, N^1-methyladenosine; m^3C, 3-methylcytosine; m^5C, 5-methylcytosine; hm^5C, 5-hydroxymethylcytosine; m^7G, 7-methylguanosine; Nm, 2'-O-methylation; METTL1/2/6/8/14, methyltransferase 1/2/6/8/14; WTAP1, Wilms tumor-associated protein-1; KIAA1429, vir like m^6A methyltransferase associated; ALKBH5, RNA demethylase ALKBH5; NML, nucleomethylin; TRMT6, tRNA methyltransferase 6 non-catalytic subunit; TRMT10C, tRNA methyltransferase 10 homolog C; ALKBH3, α-ketoglutarate-dependent dioxygenase alkB homolog 3; ALKBH1, nucleic acid dioxygenase ALKBH1; NSUN, NOP2/SUN RNA methyltransferase; DNMT2, DNA methyltransferase-2; TET, methylcytosine dioxygenase; Trm7, tRNA [cytidine(32)/guanosine(34)-2'-O]-methyltransferase.

the importance of m^6A modifications in both mRNA and ncRNA stability (59), mRNA translation (60,61), in secondary
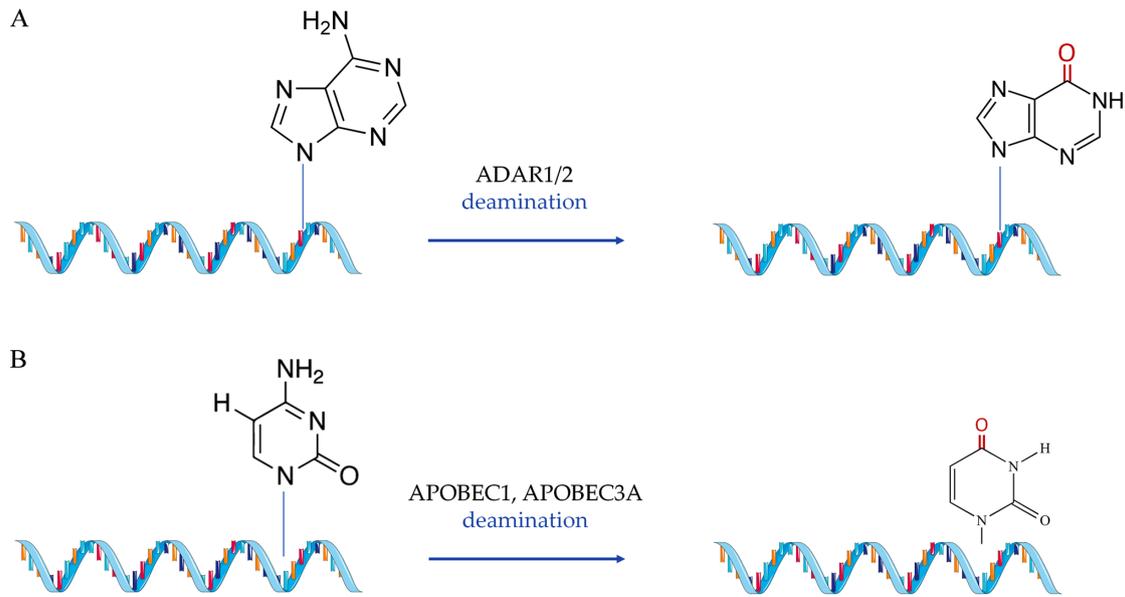
Figure 2. Substitutional RNA modifications and the two main mechanisms of RNA editing. (A) A-to-I editing and (B) C-to-U editing are presented. ADAR, adenosine deaminase acting on RNA; APOBEC1, apolipoprotein B mRNA editing enzyme catalytic subunit 1; APOBEC3A, apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3A.
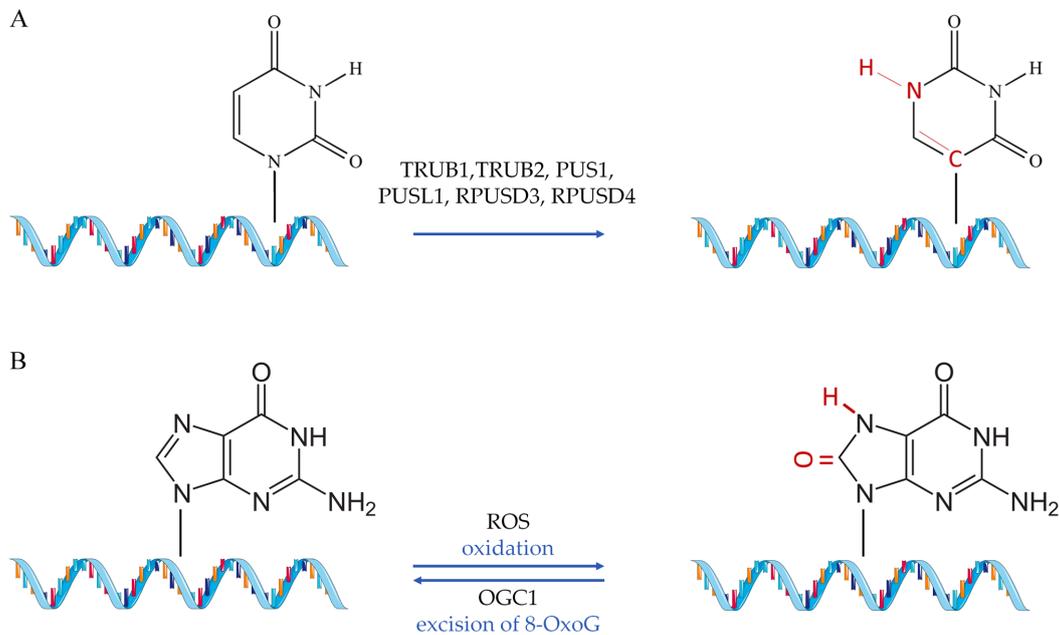
Figure 3. Other RNA modifications. Additional epitranscriptomic mechanisms that do not feature methylation or RNA editing, such as (A) pseudouridylation and (B) 8-OxoG modification. 8-OxoG, 8-oxoguanine; TRUB, TruB PUS family member; PUS1, pseudouridine synthase 1; PUSL1, pseudouridine synthase like 1; RPUSD, RNA pseudouridylate synthase domain; ROS, reactive oxygen species; OGG1, 8-oxoguanine glycosylase.

structure formation (mRNA and lncRNA) (62,63), in polyadenylation (56,64-66), in circadian clock regulation (67), as well as in the response pathways to ultraviolet (UV)-induced DNA damage (68). The extent of m6A-modified RNA is both dynamic and reversible. m6A modification is carried out and reversed by effector proteins, called m6A writers and erasers (31). Writer proteins include methyltransferase 3 (METTL3)3 and methyltransferase 14 (METTL14) (69,70), Wilms tumor-associated protein-1 (71) and vir like m6A methyltransferase associated (72), which form a methyltransferase complex in mammals with its catalytic center located within METTL3 (72,73),

responsible for the deposition of m6A. Additional m6A readers include YTH domain-containing family (YTHDF) proteins, such as YTHDF1, YTHDF2, YTHDF3 and YTHDC1, as well as heterogeneous nuclear ribonucleoproteins (HNRNPs), including heterogeneous nuclear ribonucleoproteins C1/C2 (HNRNPC) (17). Conversely, for the reversal of m6A modification, RNA demethylase ALKBH5, an RNA demethylase, acts as an activation and deactivation regulator (74).

m6A modification is involved in various physiological and pathological aspects in the context of CVD, including cardiomyocyte remodeling, cardiac hypertrophy and regulation

of mitogen-activated protein kinases (MAPKs). Since its extensive role in CVD has been thoroughly reviewed elsewhere (17,18,75-77), we will only briefly mention some of the most prevalent findings, while updating information where necessary. Of heightened interest is the METTL3-mediated modification, which has been heavily implicated in cardiac function and homeostasis (78). METTL3 has been shown to be upregulated in mouse hearts following ischemia/reperfusion, acting in a negative feedback loop with transcription factor EB, which controls the expression of autophagy and lysosomal genes (79-82). In failing hearts, m^6A hypermethylation of mRNA encoding for myosin regulatory light chain 2 led to lower protein levels compared with healthy controls (83). Overexpression of METTL3 led to a hypertrophic response both *in vitro* and *in vivo*, while cardiac-specific deletion of METTL3 caused signs of HF accompanied by functional and morphological changes (78). All of the above, indicate a central role for METTL3-mediated modification in cardiac homeostasis as well as the development of HF. During the development of HF, m^6A methylation has also been implicated in the regulation of calmodulin-1 translation, a member of the calcium/calmodulin-dependent protein kinase II signaling pathway (84). Last, but not least, when examining obesity as a risk for the development of CVD, fat mass and obesity-associated protein (FTO), which was the first identified m^6A demethylase, appears to play a crucial role in obesity development, evidenced by the reduction effect on weight and adipose tissue after FTO knockdown (85). Aside from obesity, the FTO protein also appears to be decreased in the failing heart, leading to an increase in the content of m^6A, in a state of hypoxia (86). By contrast, the correlation between the FTO protein and changes in calcium dynamics have been documented, resulting in changes of cardiomyocyte contraction and arrhythmic events (87). This serves to show that CVD can be influenced by m^6A modification events from a variety of sources or comorbidities, directly or indirectly related to its development, while this modification has also been shown to play a pivotal role in CVD-related risk factors, such as inflammation, obesity, insulin resistance and adipogenesis, whose disruption increases the risk of atherosclerosis (18).

m^6A methylation is also capable of influencing miRNA biogenesis and activity. Specifically, heterogeneous nuclear ribonucleoproteins A2/B1, an m^6A-binding protein, reads m^6A modifications in primary miRNAs (pri-miRs) and promotes DICER-mediated processing. This process affects miRNA-mRNA binding strength by influencing non-canonical base pairing (88). In angiogenesis, m^6A modification of the two vasoactive pri-miRNAs, pri-miR-126 and -222 by METTL14 and METTL3, respectively, accelerated their maturation, while it correlated with poor prognosis in patients with bladder cancer (89,90). miRNA regulation has been extensively studied in the cardiovascular field and such miRNA epitranscriptomic events, further expand the complexity of the miRNA regulatory effects.

m^6A methylation has additionally been found to affect the stability of circRNAs and subsequently the circRNA-miRNA-mRNA network, activating the Wnt and FoxO pathways and promoting pulmonary hypertension (91,92). X-inactive specific transcript (XIST), a lncRNA that is important in X-chromosome inactivation during female development, has been shown to also act as a sponge for miR-101a-3p, promoting apoptosis during MI (93). Of note, XIST is highly methylated via two m^6A components, RNA binding motif protein 15 and RNA binding motif protein 15b (94). Metastasis associated lung adenocarcinoma transcript 1 (MALAT1), another lncRNA associated with cardiomyopathy and MI (95), was also found to contain a number of m^6A editing sites (96). Such modifications led to conformational changes and increased binding by a number of RNA binding proteins (RBPs) (97). In this context, a nuclear RBP that connects single-stranded poly-U tracts and has a role in RNA processing and maturation is HNRNPC (63). This RBP is an m^6A switching factor and its binding capacity depends on the methylation of METTL3 or METTL14. This modification favors single-stranded RNA binding sites that are necessary for the interaction of HNRNPC with RNA molecules such as MALAT1 (63). Finally, another MALAT1 interacting protein is METTL16, a methyltransferase that adds m^6A to single-stranded U6 snRNAs (98). METTL16 is essential for embryonic development (99) and regulates the splicing of S-adenosylmethionine synthase isoform type-2, thus controlling the levels of methyltransferase substrate S-adenosyl methionine, as well as interacting with the 3' triple helix region of MALAT1, however the underlying mechanism of this process is not yet known (98,100). In conclusion, it is evident that m^6A modifications have a vast array of effects in both coding and ncRNAs, while acting at multiple different levels of cardiac homeostasis and CVD development.

m^1A. The m^1A modification is one of the oldest detected RNA modifications, involving the methylation of the N^1 site of the adenosine residue (101). Alongside 3-methylcytosine (m^3C) and m^7G, it is the most common methylation modification encountered in physiological conditions (102). m^1A modifications are found in both mitochondrial and nuclear-encoded mRNAs, as well as rRNAs and tRNAs (103), catalyzed by a variety of enzymes, such as nucleomethylin in the case of rRNA methylation (104) or tRNA methyltransferase 6 non-catalytic subunit (TRMT6)/61A, TRMT61B and TRMT10C for tRNAs (105-107). It reversibly methylates adenosine at positions 9, 14 and 58 of tRNA, while multiple sites exist within coding RNA transcripts and lncRNAs (108). The main erasers of m^1A modification are nucleic acid dioxygenase ALKBH1 (ALKBH1) and α-ketoglutarate-dependent dioxygenase alkB homolog 3 (ALKBH3) (109). A study by Li *et al* (110) showed that the majority of the methylation sites in mitochondrial transcripts were found in either the 5'-UTR, where they increased transcription efficiency, or in the coding sequence region where they inhibited translation (110). Furthermore, a 3'-UTR site was also detected, leading to implications of potential miRNA specificity effects. Although no studies to date have directly correlated m^1A modifications with CVD, their impact on mitochondrial transcription cannot be overlooked. The heart is heavily reliant on mitochondria, due to its high energy demands. As such, dysregulated mitochondria have been associated with a number of cardiac diseases, including, among others, cardiac hypertrophy, HF and ischemia-reperfusion, as has already been reviewed elsewhere (111,112). Of note, inferred m^1A methylation levels of mitochondrial-tRNA (mt-tRNA), as well as mt-RNR2 and mt-ND5 transcripts, have been observed

in the cardiac left ventricle and the atrial appendage, as shown in a study by Ali *et al* (113). Nevertheless, further research is required to elucidate potential specific effects of m^1A editing events in the context of CVDs.

m^3C. m^3C is a relatively under-investigated modification in CVD. Although it has been known as an RNA modification in yeast for some time, it was only recently detected in mammalian mRNA, in a study by Xu *et al* (114). Even though METTL2 and METTL6 are responsible for m^3C modification of tRNA in mice and human (114), these only account for ~50% of the total m^3C tRNA methylation levels, suggesting a role for yet undiscovered methyltransferases. As far as mRNA m^3C modification is concerned, METTL8 was shown to be the sole detected 'writer' enzyme (114). Some eraser enzymes that have been associated with this process are ALKBH1 and ALKBH3 (115,116). Even though these methyltransferases have not been implicated in CVD, METTL2 has been shown to form a complex with DALR anticodon binding domain containing 3 (DALRD3) in humans, while a patient with a mutated DALRD3 exhibited mild congenital heart disease, which resolved spontaneously (117). Since m^3C modifications are only now starting to garner attention, further research is required to identify their potential impact in CVD.

m^5C. Cytosine m^5C, another common type of RNA modification, occurs in both mRNA and ncRNAs, such as tRNA, rRNA and lncRNA (118,119). Research in both humans and mice has shown that the m^5C sites are ~100 nucleotides downstream of the translation initiation site, as well as present in the UTRs (120,121). Two main groups of m^5C protein writers have been found. The first group consists of seven members of the NOP2/SUN RNA methyltransferase (NSUN) family (122) and includes methylate tRNA (NSUN2, NSUN6), rRNAs (NSUN1, NSUN5), mRNAs (NSUN2), ncRNAs (NSUN2), mt-rRNAs (NSUN4) and mt-tRNA (NSUN3) (122-128). The second group comprises only DNA methyltransferase-2 (DNMT2) (129), previously speculated to be DNA specific, but has previously been found to also act on tRNA (130). Although, to date, eraser proteins of m^5C modifications have not been identified, a previous study suggested a role for m^5C in RNA transport (118). The Aly/REF export factor, an mRNA export adaptor protein identified as m^5C binding (reader) protein, is involved in this process (118).

Notably, mutations in both m^5C writer groups have been associated with adverse effects in various diseases, such as cancer and intellectual disability (131-134). According to an *in vitro* study, where the DNMT2 gene was examined, a total of >60 somatic mutations have been identified, with variable DNMT2 methylation activity (135). Mice lacking the NSUN4 gene, a mitochondrial m^5C methyltransferase, did not survive birth, while NSUN4 cardiac conditional deletion resulted in mitochondrial dysfunction and cardiomyopathy (126). DNMT2-deficient mice have also been shown to develop cardiac hypertrophy, possibly via the dysregulation of the interaction between positive transcription elongation factor b (P-Tefb), a transcription regulator, and RNA component of 7SK nuclear ribonucleoprotein (Rn7sk), a ncRNA (136). Decreased levels of m^5C methylation in DNMT2-deficient hearts suggested disruption of P-Tefb/Rn7sk association (136).

Luo *et al* (137) were able to show, both *in vitro* and *in vivo*, that NSUN2-mediated methylation of intercellular adhesion molecule 1 increased vascular inflammation, while its knockdown led to the development of atherosclerosis (137). In an atherosclerotic plaque mice study, increased IL-17A translation was observed as a result of m^5C methylation of the IL-17A mRNA coding region (138).

In regards to m^5C methylation of ncRNAs a study by Yuan *et al* (139) showed that m^5C editing was present during miR-125b biogenesis, a miRNA also heavily implicated in atherosclerosis (140,141). NSUN2-mediated methylation inhibited the function of miR125b, by repressing its processing in all maturation stages, including pri-miR, precursor miRNA (pre-miR) and mature miRNA both *in vitro* and *in vivo* (139). The previous findings regarding m^5C editing events of coding RNAs, imply extensive roles for m^5C methylation in multiple levels of the complex atherosclerosis network (137,142). Antisense ncRNA in the cyclin-dependent kinase inhibitors (INK4) locus (ANRIL), a lncRNA which has been previously identified in patients with MI as a poor prognostic marker for cardiac function, has also been shown to undergo m^5C modification, although the extent of this lncRNAs influence in cardiac function remains unknown (47,121). The pathophysiological conditions resulting from the deregulation of the system of m^5C modifications demonstrate the importance of these modifications in controlling the fate and function of RNAs (143).

hm^5C. Although the epigenetic importance of hm^5C DNA modification in mammals has long been established, the potential of such an editing event in RNA remains unclear. In a study by Fu *et al* (144), it was found that the same family of enzymes that catalyzes this modification in DNA, the ten-eleven translocation family, was able to catalyze the formation of 5-hydroxymethylcytosine (5-hmrC) in RNA *in vitro* and *in vivo* (144). They also determined that the ratio of conversion from 5-mrC to 5-hmrC was roughly 0.02%. Of note, out of all the tissue types tested, the highest levels of 5-hmrC were detected in the cardiac tissue, with 3.9 modifications per 10^6 ribonucleotides (144). Even though the relative levels of this modification in RNA are lower than in DNA, it is still unknown whether this is a stable or transient oxidation effect. Furthermore, in a study by Fang *et al* (145), it was observed that cardiac-specific deletion of methylcytosine dioxygenase TET2 and TET3 had adverse effects on DNA hydroxymethylation dynamics during embryonic heart development, while leading to a reduction in cardiomyocyte numbers and the emergence of non-compaction cardiomyopathy. In light of the aforementioned studies, it would be of interest to investigate the effects of cardiac deletion of TET family genes in RNA hydroxymethylation dynamics.

m^7G. m^7G, one of the most conserved modifications of nucleosides that make up eukaryotic mRNA, plays an essential role in regulating protein synthesis, pre-mRNA splicing and the export of mRNA (146). m^7G is additionally present at defined internal positions, within other classes of RNAs, such as tRNAs and rRNAs (147). One of the most extensively described enzymes that mediate internal m^7G methylation, METTL1, has been shown to mediate m^7G modification in miRNAs (148). More

specifically, it has been documented that METTL1-mediated methylation led to an increase in lethal-7 (let-7) miRNA processing (148). The family of let-7 miRNAs has been extensively studied in various diseases and participates in regulating cell differentiation (149,150). Members of this miRNA family have been implicated in CVD, as evidenced by a study in which the inhibition of let-7c miRNA was shown to prevent the deterioration of cardiac infarction, *in vitro* and *in vivo* (151). A study by Yang *et al* (152), which focused on angiotensin II-treated hearts and isolated cardiomyocytes, reported upregulation of several let-7 miRNA members, indicating their possible role in cardiac hypertrophy, while let-7i expression was downregulated in patients with dilated cardiomyopathy and correlated with poor clinical outcomes (153). The effects of m^7G modification in the context of CVD thus remain largely unexplored, but the implications of let-7-mediated effects hint to regulatory levels of potentially high importance.

Nm. Nm (N meaning any nucleotide in this case) is a modification of RNA occurring co-transcriptionally or post-transcriptionally, where a methyl group is added to the 2'-hydroxyl of the ribose moiety (154). This type of modification is recurrent and observed in numerous RNA classes, such as sncRNAs, mRNA and tRNA (154). This modification can be carried out by stand-alone methyltransferases (155), such as tRNA [cytidine(32)/guanosine(34)-2'-O]-methyltransferase or by the fibrillarin enzyme, which requires guiding by box C/D snoRNAs (156). Loss of snoRNA-guided Nm modifications on snRNAs reportedly leads to significant defects in the splicing of cardiac mRNA and the development of the heart (157,158). In cardiometabolic disease, small nucleolar RNA C/D Box 32A, a subtype of ncRNA from the Rpl13a locus, was found to target the mRNA of peroxidasin for Nm, indicating a role in the functional altering of peroxidase activity in the heart (159). As in the case of m^7G modifications, research in this modification area is still limited, but cardiovascular effect implications exist, capable of driving future research avenues.

4. Substitution modifications

A-to-I editing. A-to-I RNA editing is the most common form of substitutional RNA editing in mammals (160). During this process, two conserved mammalian enzymes, adenosine deaminase acting on RNA (ADAR)1 and 2, hydrolyze the adenosine residues into double-stranded RNA regions (mRNAs and ncRNAs) in order to convert them into inosines (161). Due to the similar chemical content inosines share with guanines, they are misread by the endogenous translational complex during reverse transcription and thus pair with cytosines (162). A-to-I editing has been shown to be indispensable both for physiological development and the emergence of pathological conditions in the heart, while an average of ~80,000 A-to-I editing sites have been identified in human cardiac tissue (163).

ADAR1 has been shown to edit cathepsin S (CTSS), a cysteine protease associated with atherosclerosis and angiogenesis (164-166). The editing event occurred in the 3'-UTR region of CTSS mRNA, enabling the recruitment of ELAV like RNA binding protein 1, which in turn regulated the mRNA expression and stability of CTSS. Of note, both ADAR1-mediated editing and CTSS mRNA expression were elevated in blood

samples from patients with coronary artery disease (167). In mice, ADAR1 was increased during oxidative stress in neonatal cardiomyocytes (168), while a knockout study in the developing heart showed that ADAR1 cardiac deletion is associated with embryonic lethality, establishing the importance of A-to-I RNA editing during cardiac embryonic development for both proliferation and survival (169). Additionally, El Azzouzi *et al* (170) were able to bypass embryonic lethality and knock out ADAR1 in adult cardiomyocytes by using an inducible knockout method under the control of the α-myosin heavy chain promoter, which is specifically expressed in cardiomyocytes. Their results showed increased lethality in Adar1-null mice, accompanied by a decrease in global miRNA expression, worsening of cardiac function and severe ventricular remodeling, via a pathway involving miR-199a-5p and the unfolded protein response (170). In a study by van der Kwast *et al* (44), an edited version of miR-478b-3p, a miRNA present in smooth muscle cells, fibroblasts and vascular endothelial cells, was responsible for neovascularization in response to ischemia. The A-to-I modification of miR-478b-3p was located in the seed sequence and modified its target set by enriching for proangiogenic pathways (44). Moreover, Filamin A (FLNA) mRNA has been previously shown to be one of the substrates for ADAR2 editing (171). In a study by Jain *et al* (172), mice with impaired FLNA editing developed left ventricular hypertrophy and cardiac remodeling, accompanied by elevated blood pressure. Additionally, FLNA mRNA editing in patients with CVD was found to be decreased by up to 50%, making ADAR2-mediated FLNA mRNA editing one of the first studies to highlight an editing event associated with cardiac disease in humans (172). In terms of occurrence, there is limited information about A-to-I modifications in lncRNAs. Nevertheless, ANRIL, a lncRNA acting as a regulator of coronary heart disease, was shown to undergo A-to-I editing at the site of its Alu motifs, potentially affecting its interaction with chromatin and its downstream effects (47).

C-to-U editing. C-to-U editing is another editing mechanism acting in mammals (173,174). It is regulated by a family of evolutionarily conserved cytidine deaminases called APOBEC, in a highly specific manner with a preference for cytidines in AU-rich regions (175). First observed regulating the expression of apolipoprotein-B48 in a tissue-dependent manner, C-to-U modification in the liver produces a truncated apolipoprotein, via the introduction of a stop codon in the gene transcript, resulting in the hepatic-specific apolipoprotein-B100 expression (176). The APOBEC1 complementation factor (ACF), an RNA-binding cofactor, integral in editing apolipoprotein B is one of the two minimal elements for a functional C-to-U editing complex (177,178). Of note, CUGBP Elav-like family member (CELF)2, another RBP that has been shown to also modulate C-to-U editing by recruiting ACF (179), is mainly expressed in the heart, alongside another member of the CELF family, CELF1 (180). The activity of CELF1 ranges from RNA editing to polyadenylation and alternative splicing, while CELF1 depletion can cause neonatal cardiac dysfunction with repression effects in ion-transport and circadian gene expression (181).

Although the activity of APOBEC1 is responsible for editing apolipoprotein B, it has been previously reported that

another member of the subfamily, APOBEC2, is exclusively expressed in the heart and skeletal muscle, and maintains low, but definite deaminase activity (182). Meanwhile, APOBEC3A is capable of C-to-U editing under hypoxic conditions (183), while its overexpression induced editing, among others, of primary pulmonary hypertension genes in an *in vitro* experiment (184). All of the above imply a yet undiscovered potential for RNA editing of cardiac-specific transcripts in a C-to-U editing manner, similar to the one observed for apolipoprotein modifications in the liver.

5. Other modifications

Ψ. 5-Ribosyluracil/Ψ, an isomer of the conventional RNA nucleoside uridine (185) is overall the most common RNA modification. It has been found in mRNA, as well as tRNA, rRNA, snRNA, snoRNA and ncRNA (186). This modification is observed in almost all tRNA molecules, as the TΨC loop is characteristic of tRNAs (187). One of the Ψ functions is to enhance the ability of RNA to stack bases by making the sugar-phosphate backbone more rigid (188,189). Additionally, it participates in the classic Watson-Crick base coupling with adenosine, such as unmodified uridine isomer, but presents a stronger pairing with the other four bases (190). A total of 13 pseudouridine synthases (PUS) have been identified in humans, acting both in an RNA dependent and independent manner (191).

In a previous study by Safra *et al* (192), a member of the TruB PUS family member 1 (TRUB1) family, also known as PUS4 and PUS7, was shown to combine ~60% of all reproducible Ψ detected sites in mRNA in three human cell datasets. These datasets consisted of fibroblasts and HEK293 cells, HeLa cells grown under wild-type or serum-starved conditions and HEK293 cells grown under a range of conditions/perturbations, respectively. A consensus motif (GUUCNANNC) for Ψ by TRUB1 has been identified, while its catalytic activity is mainly located in the nucleus (192). It is worth noting that several other PUS, such as PUS1, pseudouridine synthase-like 1, TRUB2, RNA pseudouridine synthase domain containing 3 (RPUSD3) and RPUSD4, have been predicted or found to be mitochondrial RNAs (mtRNAs), resulting in a number of mtRNAs being modified by PUS enzymes (193,194).

As in the previous modifications, the mutations in PUS are related to various diseases, such as cancer and mitochondrial myopathy (195,196). Of note, the absence of eraser proteins for the Ψ modifications, coupled with the inactivity of the C-C bond between the base and the sugar (Ψ), suggest that this is a potentially irreversible modification (188). Analysis of TRUB1 levels in human tissue revealed its high expression in the heart and skeletal muscle, with still unexplored modification potential mainly in tRNAs (197). Moreover, during both Ψ and Nm methylation modifications, snoRNAs have been found to act as guides for the modification process (159,198). A special class of guide RNAs concentrated in the Cajal body are responsible for guiding spliceosomal U modifications, these snRNAs are termed scaRNAs (199). In this regard, scaRNAs are responsible for regulated alternative splicing, with extensive implications for response to variable environmental conditions (158). Notably, in a study by Nagasawa *et al* (200), infants born with a common congenital cardiac defect

termed Tetralogy of Fallot, were shown to have decreased spliceosomal pseudouridylation levels in their right ventricle, which in turn depended on scaRNA1 levels, as exhibited in an *in vitro* experiment in primary cardiomyocytes (200). These findings imply that spliceosomal pseudouridylation depends on scaRNA levels in human tissue, revealing a novel potential regulatory mechanism for the alternative splicing of genes important in embryogenesis and cardiogenesis. KCNQ1 overlapping transcript 1, a lncRNA and a biomarker for MI, has also been shown to be able to be modified by Ψ (201). Establishing studies with a larger number of samples and the examination of additional RNA modifications and epigenetic factors is necessary for deeper investigation into the cardiovascular effects of pseudouridylation.

8-OxoG. Finally, 8-OxoG is conventionally formed through the interaction of the guanine base in DNA molecules with reactive oxygen species, under conditions of oxidative stress (202). Repair of this type of base lesion is executed by the enzyme 8-OxoG glycosylase (OGG1), which excises 8-OxoG (203). A study by Shah *et al* (204) documented the detrimental effects of 8-OxoG on the function of vascular smooth cells, reporting a reduction of human atherosclerotic plaque development when the activity of 8-OxoG glycosylase was restored. By sequencing oxidized miRNAs in rat models, 8-OxoG modifications at specific positions in miR-1 were found to promote cardiac hypertrophy (205). Additionally, 8-OxoG DNA glycosylase 1 overexpression was found to lower cardiac mitochondrial levels of DNA 7,8-dihydro-8-OxoG (8-oxo-dG) in mouse models (206). The same study evidenced the decrease in transverse aortic constriction-induced cardiac fibrosis in a state of OGG1 overexpression, suggesting that increased repair of 8-oxo-dG in mtDNA leads to decreased cardiac pathology (206). In a study by Noren Hooten *et al* (207), 8-oxo-dG levels were found to be associated with clinical cardiovascular risk factors, such as high sensitivity C-reactive protein, systolic blood pressure, IL-23 levels and body/mass index. Moreover, strong association between 8-oxo-dG and the levels of systolic blood pressure have been documented (207). Although there are implications for important regulatory effects mediated by 8-oxo-dG modification in the cardiac tissue, this field of research remains in its infancy.

6. Tools of epitranscriptomic modification research

In the past decade, dramatic advances in the development of powerful sequencing technologies have facilitated transcriptomic investigation in a faster, more efficient and more in-depth manner than ever before. Such advances have also assisted greatly in the study of epigenetics and epitranscriptomics. The use of RNases constitutes one of the earliest methods of mapping mRNA modifications and still exhibits the highest sensitivity for m6A mapping (208). In the same manner, the more recent MAZTER-seq (209) and m6A-REF-seq (210) technologies exploit the discovery of methylation-blocked endoRNases. Another method, termed site-specific cleavage and radioactive-labeling followed by ligation-assisted extraction and thin-layer chromatography, also known as SCARLET, utilizes site-specific cleavage and splint ligation and has also been extensively used to detect m6A modifications in both

coding RNA and lncRNAs (211). Furthermore, antibody incorporating techniques have been established for the detection of RNA modifications. These include the m^6A-LAIC-seq or m^6A-level and isoform-characterization sequencing method, which uses immunoprecipitation in total RNA samples (56), as well as the widely used MeRIP-Seq technology, which maps m^6A-methylated RNA through the use of m^6A-specific antibodies (212). Combining the aforementioned technique with Ab cross-linking, allowed the enhancement of the resolution of the technique, giving rise to methylation individual-nucleotide resolution UV cross-linking and immunoprecipitation (124). Applications of the same principle of cross-linking include PA-m^6A-seq (213), but also m^1A-MAP (110) in the case of m^1A modification mapping.

Alternative methods for the detection of RNA modifications take advantage of chemical reactions that are limited to a certain type of RNA modification, combining them with short-read sequencing. RNA-BisSeq, aimed toward the mapping of m^5C, involves the chemical deamination of cytidines except for m^5C (118). Modern library preparation protocols, yield RNA fragments with nucleotide modifications at the 5'- or 3'-end, which can be used for the enrichment of the RNA fragments in RNA seq libraries (214,215). The Nm-Seq and RibOxi-Seq techniques used to map internal Nm modifications entail the treatment of RNA fragments with $NaIO_4$ oxidation, which along with additional steps, leads to the enrichment of 3'-Nm-containing fragments and improvement of the final transcriptome-wide RNA analysis (166,216). Using the same principle, RiboMethSeq is based on the protection of the phosphodiester bond in RNA when Nm occurs at the 5'-neighboring ribose (217). Following alkaline hydrolysis, library preparation and 5'- and 3'-extremity counting, the aforementioned protection is translated into a signal.

Mass spectrometry (MS) has also been an invaluable tool for RNA modification analysis. State-of-the-art MS methods are being employed for the detection and quantification of chemical modifications in RNA, yielding different types of information based on the type of MS analysis (218). Top-down MS analysis can identify and localize mass-altering RNA modifications in undigested RNA, while also allowing *de novo* sequencing to be performed. Nevertheless, non-altering mass modifications, such as m^1A, m^6A and mass-silent modifications, such as pseudouridine, remain a major challenge (219,220). Bottom-up MS is conducted for the mass mapping of partially hydrolyzed RNA, and MS approaches can generate oligonucleotides and sequencing ladders that can be subsequently interpreted into RNA sequences and localization of the modifications (221). Another MS-based method is nucleoside MS, which is performed on complete RNA hydrolysates, followed by liquid chromatography separation of the nucleoside mixtures. While highly accurate for the detection of chemical modifications, it cannot provide sequence information or localization of the modification (222). Still, each method's advantages can be combined to overcome limitations and drawbacks on high-throughput RNA modification mapping, while appropriate software for MS data processing should always be incorporated (223).

A-to-I modifications are either investigated via the traditional method of screening for A-to-G mismatches in reverse transcribed RNAs (224), by the use of the more recently developed inosine chemical erasing (ICE) methods, or by the use of transgenic mice where ADAR knockdown is followed by deep-sequencing. In the case of ICE, reverse transcription is blocked by the formation of N^1-cyanoethylinosine after acrylonitrile processing. This method combined with deep-sequencing gave rise to ICE-seq, for high-throughput investigation of A-to-I modifications (225). In the case of Ψ modification profiling, several high-throughput sequencing techniques are utilized, wherein treatment with N-cyclohe xyl-N'-(2-morpholinoethyl)-carbodiimide-metho-p-toluenesulfonate specifically modifies Ψ, G and U residues on RNA. Although the G and U modifications are later removed, the chemically induced modification on Ψ is stable and blocks reverse transcription (226). Such methods include Ψ-seq (227), PSI-seq (228), Pseudo-seq (229) and CeU-seq (230).

Novel sequencing approaches enable direct RNA sequencing without amplification or cDNA conversion. The rapidly developing technology of nanopore sequencers, such as the one created by Oxford Nanopore Technologies (ONT), includes the use of a synthetic membrane with embedded nanopores in an ionic solution (231). As an ionic current passes through the nanopore, an individual read is recorded by a sensor and the corresponding data is acquired by the sequencer's implemented software. Characteristic changes in the current reads during the movement of a nucleic acid strand, as it traverses the nanopore from one chamber to the other, enable the identification of the strand's nucleic acid sequence, in a process known as 'base-calling' (232). Nucleotide modifications in ONT reads can be determined with the use of specialized software, such as Nanopolish and the ONT integrated CpG-methylation calling software (233).

7. Epitranscriptomic genetic variation and CVD

High-throughput sequencing techniques have not only promoted the field of epitranscriptomic profiling, but have, through Genome-wide Association Studies (GWAS), facilitated the identification of single nucleotide polymorphisms (SNPs) in a variety of diseases, including CVD (234). These studies have led to the identification of >5,000 associations with CVD (https://www.ebi.ac.uk/gwas/) (235), exhibiting the importance of SNPs in CVD emergence. Several databases have also been developed in an effort to catalogue disease-associated polymorphisms that affect epitranscriptomic modifications. These databases include m^6Avar (236) and m^6ASNP (237), both of which catalogue m^6A-related polymorphisms, m^7GHub (238) focusing on m^7G-related SNPs, RMDisease encompassing >200,000 human SNPs that affect m^6A, m^1A, m^6Am, m^5U, m^7G, Ψ and Nm modifications (239) and the RNA Framework, which is a rounded toolkit for the analysis of post-transcriptional modifications (240).

In terms of epitranscriptomic genetic variation, research remains at an early stage. As expected due to the greater emphasis given so far on m^6A-related modifications, in the context of CVD, a number of m^6A-related SNPs have been recognized as genetic variants associated with CVD. Multiple GWAS studies by Mo *et al* (241) have paved the way in this field and associated m^6A-SNPs with a variety of CVD factors. More specifically, m^6A-SNPs were shown to be associated with coronary artery disease (241) and have a potential role

in the regulation of blood pressure (242), as well as in the regulation of lipid metabolism (243). Furthermore, several m⁶A-related SNPs were found to affect the expression of multiple disease-causing genes, with potential adverse effects for ischemic stroke in humans (244). In this context, the genetic variant rs12286, which is strongly associated with coronary artery disease, was shown to be able to affect ADAMTS7 expression, by regulating the upstream m⁶A methylation (241). Ali *et al* (113) analyzed the levels of m¹A/G methylation in mitochondrial-encoded RNA across multiple tissue types, followed by the identification of overlaps between peak associated nuclear variants and disease-associated variants with significance on a genome-wide level. Nuclear genetic variants (rs13874, rs1084535), which are associated with inferred methylation levels at mt-RNR2 and several mt-tRNA P9 sites, were in linkage disequilibrium (LD) with rs34080181, which has been linked to atrial fibrillation (245). Furthermore, the intronic variant in polyribonucleotide nucleotidyltransferase 1 mitochondrial (rs2627773) that is associated with inferred methylation levels of mt-RNR2, is in LD with rs1975487, which is associated with diastolic pressure (246). Franzén *et al* (247) mapped A-to-I RNA editing quantitative trait loci (edQTLs) in order to identify clinical features associated with RNA editing. Subsequently, they evaluated the disease relevance of RNA editing by intersecting the edQTLs with GWAS data (247). More specifically, the authors intersected edSNPs with lead SNPs from published GWAS data. Of note, the rs10847434 SNP, which is associated with coronary artery disease (248) had an edQTL with an editing site in the 3' exon of apolipoprotein C1 pseudogene 1, a locus that has been linked to coronary artery disease (249). Additionally, the SNP rs4739066, a polymorphism associated with MI (250), was also found to have two edQTLs with editing sites in the 3'-UTR of the α-tocopherol transfer protein gene, a gene associated with the level of severity of atherosclerotic lesions in the area of the proximal aorta (251). Taken together, the aforementioned studies suggest that there is still a large unexplored area of genetic variation related to CVD pathogenesis, especially in regards to epitranscriptomic modifications.

8. Therapeutic implications of epitranscriptomics in CVD

Although the field of epitranscriptomics is still in its infancy, there are already efforts being made to utilize such new regulatory knowledge for the development of novel therapeutic approaches, both for epigenetic and epitranscriptomic modifications in the context of CVD (19). As previously discussed, most epitranscriptomic research in CVD has so far been focused on m⁶A modifications and, as such, methods have focused on identifying ways to manipulate m⁶A methylation levels in the context of various therapeutic approaches. In a seminal study by Lu *et al* (252), it was established that curcumin was able to attenuate the effects of lipid metabolism disorder and increase total cholesterol in the liver, via the increase of m⁶A methylation, suggesting a protective role for this modification against hyperlipidemia. Recently, a large scale epitranscriptomic study has been established to identify IHD biomarkers in circulation, termed the IHD-EPITRAN study. This study is expected to include 200 patients, split into two cohorts of IHD and non-IHD patients, focusing on the identification of m⁶A and

A-to-I modification biomarkers (253). Additionally, limited approaches have also been taken in an effort to modulate m⁶A demethylation. Inhibition of demethylation was selectively blocked by the use of meclofenamic acid (MA) *in vitro* (254), while using the recently developed CRISPR-Cas13b technology, Li *et al* (255) attempted to manipulate m⁶A modified transcripts and specifically demethylate m⁶A marks. In a similar manner, Cox *et al* (256) developed the RNA Editing for Programmable A to I Replacement system, also utilizing CRISPR-Cas13b technology, to address disease-causing mutations. Although such tools are still outside the reach of clinical practice, as multiple technical and ethical concerns remain unaddressed, they pave the way for the development of future personalized CVD therapeutics.

9. Conclusions

The field of epitranscriptomics has been rapidly emerging, as the focus regarding disease development, environmental adaptation and homeostasis maintenance, shifts from the rigid genomic structure to the much more dynamic transcriptomic landscape. Although there have been major advances in transcriptomic profiling, understanding the mechanisms in which the transcriptome itself is differentially regulated through modifications, will allow for the development of novel and precise pharmacological interventions. The additional level of regulatory sensitivity that epitranscriptomic modifications are shown to offer, corresponds to the increased level of specificity required for any successful therapeutic intervention. To date, epitranscriptomic modifications are nearing 200, but not all of them have been thoroughly evaluated, nor do they all appear with equal frequency. Although epitranscriptomic research progresses rapidly in the fields of cancer and neurodegenerative disorders (257,258), in the context of CVD the number of modifications that have a significant impact are just beginning to be elucidated (Table I). However, their biological and clinical significance cannot be denied, as shown by the plethora of studies published in the past couple of years, showing the effect of RNA modifications in CVDs (Fig. 4).

Regarding RNA methylations, undoubtedly m⁶A has garnered the most attention. Although coding RNA modifications have been the focus of most m⁶A studies in the heart, a number of publications have emerged, pinpointing the importance of various RNA methylations in multiple levels of non-coding regulation in the heart. These events occur in multiple miRNA maturation stages, including primary-miRNA, pre-miR and mature miRNA levels. Coupled with recently emerging implications regarding methylation modifications in circRNAs and lncRNAs, such as XIST, in the context of CVD, it is becoming evident that this type of epitranscriptomic modification is paramount for physiological non-coding regulation and offers an additional regulatory level of gene expression, sensitive to environmental factors. Nevertheless, methylations are only one of the available modifications in the RNA modification toolset. Substitutional modifications have also been gaining attention in the cardiovascular field, especially with the emergence of recent studies exhibiting the importance of ADAR1 for cardiac development, homeostasis, as well as physiological cardiac function in adult mice (169,170,259). What is noteworthy is that the ADAR1-mediated modification,

RNA methylation modifications

m⁶A modification

METTL3 overexpression — Hypertrophic response

FTO decrease — Changes in cardiomyocyte contraction/arrhythmic events Observed in failing hearts

m⁶A hypermethylation of Myl₂ mRNA — Observed in failing hearts

m⁶A of circRNAs — Pulmonary hypertension promotion

m¹A modification

m¹A methylation of mitochondrial RNAs — Cardiac hypertrophy / Heart failure / Ischemia-reperfusion

hm⁵C modification

Cardiac-specific deletion of TET2 and TET3 — NCC Cardiomyocyte numbers reduction

m⁵C modification

Lack of NSUN4 gene — Stillborn

NSUN4 cardiac conditional deletion — Mitochondrial dysfunction Cardiomyopathy

DNMT2-deficiency — Cardiac hypertrophy

NSUN2-mediated methylation of miR-125b — Atherosclerosis

m⁷G modification

METTL1-mediated methylation of let-7 miRNA — CVD Cardiac hypertrophy Dilated cardiomyopathy

Nm modification

Loss of snoRNA-guided Nm modifications on snRNAs — Defects in cardiac mRNA splicing and heart development

Snord32A-guided methylation of peroxidasin — Peroxidase activity alteration in the heart

Substitutional RNA modifications

A-to-I editing

ADAR1 editing of CTSS — Atherosclerosis Disruptions in angiogenesis

ADAR1 cardiac deletion — Lethal in mouse embryos

Impaired FLNA mRNA editing — Left ventricular hypertrophy Increased blood pressure

C-to-U editing

CELF1 depletion — Neonatal cardiac dysfunction with repression effects on ion-transport and circadian gene expression

Other RNA modifications

Pseudouridylation

Infants born with tetralogy of Fallot — Decreased spliceosomal pseudouridilation levels in right ventricle

8-OxoG modification

OGG1 malfunction — Atherosclerosis

8-oxoG modifications at specific positions in miR-1 — Cardiac hypertrophy

Figure 4. Schematic representation of RNA modifications and their role in CVDs. CVD, cardiovascular disease; METTL3, methyltransferase 3; FTO, fat mass and obesity-associated protein; m⁶A, N6-methyladenosine; Myl2, myosin regulatory light chain 2 ventricular/cardiac muscle isoform; circRNAs, circular RNAs; m¹A, N¹-methyladenosine; TET, methylcytosine dioxygenase; NCC, non-compaction cardiomyopathy; m⁵C, 5-methylcytosine; NSUN4, NOP2/SUN RNA methyltransferase 4; DNMT2, DNA methyltransferase-2; NSUN2, NOP2/SUN RNA methyltransferase 2; miR/miRNA, microRNA; METTL1, methyltransferase 1; let-7, lethal-7; snoRNA, small nucleolar RNA; Nm, 2'-O-methylation; snRNA, small nuclear RNA; Snord32A, small nucleolar RNA C/D Box 32A; ADAR1, adenosine deaminase acting on RNA 1; CELF1, CUGBP Elav-like family member 1; OGG1, 8-oxoguanine glycosylase; OxoG, 8-oxoguanine.

Table I. Epitranscriptomic modifications in CVDs.

A, Methylation

Modification	Coding RNAs influenced	Pathologies associated with coding RNAs	ncRNAs influenced	Pathologies associated with ncRNAs
m^6A	6PGD, MAP3K6, MAP4K5, MAPK14, p53, TFEB, CCNA2, CDK2, PNPLA2, p65, p38, SERCA2a, KLOTHO, SIRT1, p16, FBLN5, TNC	Cardiac remodeling, atherosclerosis, heart failure, congenital heart disease, CVD-related risk factors, cardiac fibrosis, myocardial infraction, myocardial ischemia, stroke, aortic dissections, cardiac hypertrophy, dilated cardiomyopathy, diabetic cardiomyopathy, chemotherapy induced cardiotoxicity	miR-126, miR-221/222, XIST	H/R-induced injury, atherosclerosis, myocardial infraction
m^1A	N/A	N/A	mt-tRNAs, rRNAs	Coronary artery disease, cardiac remodeling, hypertrophic, cardiomyopathy dilated cardiomyopathy, diabetic cardiomyopathy, atherosclerosis, chemotherapy-induced cardiotoxicity
m^3C	DALRD3	Mild congenital heart disease, developmental delay and early-onset epileptic encephalopathy	N/A	N/A
m^5C	P-Tefb, ICAM-1, IL-17A	Cardiac hypertrophy, vascular inflammation, atherosclerosis	Rn7sk, miR-125b	Cardiac hypertrophy, atherosclerosis
hm^5C	TET genes[a]	Non-compaction cardiomyopathy	N/A	N/A
m^7G	N/A	N/A	let-7	Myocardial infraction, cardiac hypertrophy, dilated cardiomyopathy
Nm	Peroxidasin	Altered peroxidase activity in the heart	N/A	N/A

B, Substitutions

Modification	Coding RNAs influenced	Pathologies associated with coding RNAs	ncRNAs influenced	Pathologies associated with ncRNAs
A-to-I	CTSS, FLNA	Atherosclerosis, severe ventricular remodeling, cardiac remodeling, left ventricular hypertrophy	miR-199a-5p, miR-478b-3p	Severe ventricular remodeling, ischemia response
C-to-U	CELF1, APOBEC3A	Neonatal cardiac dysfunction, editing of primary pulmonary hypertension genes		

Table I. Continued.

C, Other

Modification	Coding RNAs influenced	Pathologies associated with coding RNAs	ncRNAs influenced	Pathologies associated with ncRNAs
Ψ	N/A	N/A	KCNQ1OT1	Myocardial infraction
8-OxoG	Differences in OGG1 levels	CVD-related risk factors, cardiac fibrosis, blood pressure levels	miR-1	Cardiac hypertrophy

[a]Deletion of TET genes. CVD, cardiovascular disease; m6A, N6-methyladenosine; 6PGD, 6-phosphogluconate dehydrogenase; MAP3K6, mitogen-activated protein kinase kinase kinase 6; MAP4K5, mitogen-activated protein kinase kinase kinase kinase 5; MAPK14, mitogen-activated protein kinase 14; TFEB, transcription factor EB; CCNA2, cyclin A2; PNPLA2, patatin-like phospholipase domain containing 2; SERCA2a, sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a; FBLN5, fibulin; 5KLOTHO, klotho β-glucuronidase; TNC, tenascin C; H/R, hypoxia/reoxygenation; miR, microRNA; ncRNA, non-coding RNA; XIST, X-inactive specific transcript; m1A, N1-methyladenosine; tRNA, transfer RNA; rRNA, ribosomal RNA; m3C, 3-methylcytosine; m5C, 5-methylcytosine; hm5C, 5-hydroxymethylcytosine; m7G, 7-methylguanosine; Nm, 2'-O-methylation; DALRD3, DALR anticodon binding domain containing 3; P-Tefb, positive transcription elongation factor b; ICAM-1, intercellular adhesion molecule 1; Rn7sk, RNA component of 7SK nuclear ribonucleoprotein; TET, methylcytosine dioxygenase; let-7, lethal-7; CTSS, cathepsin S; FLNA, Filamin A; CELF1, CUGBP Elav-like family member 1; APOBEC3A, apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3A; Ψ, pseudouridylation; KCNQ1OT1, KCNQ1 opposite strand/antisense transcript 1; OGG1, 8-oxoguanine glycosylase; OxoG, 8-oxoguanine.

in this case, involved miR-199a-5p, exhibiting the intricate and dynamic regulation that such modifications offer in tandem with ncRNA regulation. This fact is further highlighted by studies by van der Kwast et al (44,260), where A-to-I editing of miR-478b-3p created an isomiR with a completely different targetome and extensive angiogenic pathway effects, further establishing the relationship between epitranscriptomic modification and miRNA-mediated regulation. Alongside A-to-U editing events, C-to-U editing offers a much more delicate regulation network, with increased specificity. Although cardiac-specific effects in vivo have yet to be reported in relation to C-to-U editing, the expression of the tissue-specific APOBEC-2 deaminase in the heart, coupled with the capacity of APOBEC3A to edit hypertension genes under hypoxic conditions in vitro, point to still unexplored events, similar to the editing of apolipoprotein in the liver, in a tissue-specific manner.

In this context, cardiac aging has recently emerged as an exciting new field, exploring among others, the possible connection between RNA modifications and the various morphological and biomolecular changes that take place during the cardiac aging process. Increased cardiac fibrosis, left ventricular hypertrophy and valvular degeneration are just some of the main physiological changes that occur during human cardiac aging (261). Of note, cardiac fibrosis emergence has been linked to changes in RNA modifications, such as m6A and inosine (262), while cardiomyocyte aging has been found to be affected by RNA methylation (263). Cardiomyocyte hypertrophy, responsible for the thickening of the left ventricle walls during cardiac aging, has also been linked to m6A methylation. More specifically, in vitro and in vivo experiments have shown that m6A RNA methylase METTL3 could promote cardiomyocyte hypertrophy, whereas METTL3 inhibition inhibited the hypertrophic potential of cardiomyocytes (78). All of the above provide just a glimpse of the still unexplored effects that epitranscriptomic regulation may potentially have during cardiac aging.

Finally, a large number of modifications have also been detected in mitochondria. Due to the importance of mitochondria for physiological cardiac function, these editing events can have severe implications for both homeostasis and disease emergence. HF, despite its various complications, has historically been studied as a left ventricular disease. As such, m1A modifications in mitochondrial 16s rRNA, as well as tRNAs, but also components of the mitochondrial complex I (such as mt-ND5) in the left ventricle, can have severe implications for both HF and various other CVDs. Additionally, NSUN4-mediated mitochondrial m5C methylation is required for physiological function, as shown by the emergence of cardiomyopathy, after NSUN4 cardiac-specific deletion. While examining mitochondrial RNA methylation, Van Haute et al (264) demonstrated NSUN3 as a novel human m5C RNA methyltransferase, specializing in mitochondrial tRNA^{Met}. Mutations of NSUN3 caused reduced methylation and absence of formulation of cytosine residues at position 34 of the mitochondrial tRNA^{Met}, leading to reduced mitochondrial translation and the development of mitochondrial disease. Thus, mitochondrial RNA methylation seems to affect mitochondrial function as well as the translation of mitochondrial proteins, leading to the emergence of pathology (264). Even though other mitochondrial RNA modifications have not been implicated in CVD, it is safe to assume that we are only starting to scratch the surface, as various more mitochondrial modifications have been described, such as Ψ modifications. As already reviewed by Bohnsack and Sloan (265), the mitochondrial epitranscriptome is rapidly gaining interest as a key regulator of dynamic, efficient and accurate responses to metabolic needs. Mutations in mitochondrial RNase P protein 2, a mitochondrial RNase P subcomplex cofactor, participating in m1A and m1G mt-tRNA modifications, were shown to cause cardiomyopathy (106,266). Last, but not least, a few RNA modifications, such as C-to-U editing by APOBEC3A or m6A modification of the FTO protein were shown to be manifesting

during hypoxic conditions. Taking into account the extensive role of hypoxia in metabolic regulation, mitochondrial biogenesis and cardiac remodeling, such modifications further cement the role of epitranscriptomic regulation in the adaptation to ever-changing environmental stimuli both in physiological, but also in pathological conditions.

This new knowledge is now paving the way towards a new chapter in personalized medicine (267), where an in-depth understanding of epitranscriptomic modifications could not only enable more accurate patient classification based on epitranscriptomic 'profiles' or specific epitranscriptomic biomarkers, but, more importantly, allow for early predictions of response to treatment. Early evidence in this direction stems from the area of oncology, where specific RNA modifications (e.g. m⁶A) appear to be associated with therapeutic response and/or resistance (268). Significant promise also lies in the development of novel targeted epitranscriptomic therapies against CVD. The fine mapping of the role of epitranscriptomic changes in different aspects of CVD development, is likely to unveil a multitude of promising therapeutic targets, that could subsequently be modulated by targeted approaches, such as small molecule inhibitors. A number of such approaches are currently being pursued against FTO in the milieu of cancer (269). For example, the US Food and Drug Administration-approved nonsteroidal anti-inflammatory drug ethyl ester form of MA, MA2, was found to be an FTO inhibitor, which led to elevated levels of m⁶A modification in mRNAs in glioblastoma cells, suppressing tumor progression and prolonging the lifespan of glioblastoma stem cell-grafted mice (270). MO-I-500 was developed to selectively inhibit the m⁶A demethylase activity of FTO and was found to successfully inhibit the survival and/or colony formation of a triple-negative inflammatory breast cancer cells (271,272). R-2HG was found to bind directly to FTO and inhibit m⁶A demethylase activity leading to the inhibition of leukemic cell growth/survival and leukemia progression (273). FB23-2 was also effective in inhibiting the progression of human AML in xenotransplantation mice, by achieving the potent inhibition of FTO (274). In an effort to expedite the discovery of such inhibitors different predictive *in silico* approaches are also employed (275-279). The majority of the aforementioned approaches are based on conventional pipelines on databases' data management (280,281). However, at present in the post-genomic era, state-of-the-art approaches based on artificial intelligence are being employed, thus providing novel and radical solutions for the management and analysis of high amounts of data, where algorithms and convolutional networks not only decipher information by removing noise and reducing dimensionality, but also produce new knowledge and associations (282). The tremendous clinical potential of these advancements is supported by the early establishment of multiple companies focusing on epitranscriptomics, such as Accent, Gotham and Storm Therapeutics. It is only a matter of time before similar avenues are explored in the field of CVD, as evidenced by recent studies on epitranscriptomic modification-based therapy solutions.

All of the above serve to show we currently stand at the shore of cardiac epitranscriptomic research, where a vast ocean of information still remains unexplored. We still have to understand the relationship between the number of modifications that each coding or ncRNA carries, to their respective

effect, or the methods of action for tissue-specific RNA modifications in response to physiological or pathological stimuli. As we delve deeper into CVD epitranscriptomics, we are sure to come closer to the 'holy grail' of personalized medicine and targeted therapeutics.

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Authors' contributions

SL, EP, KID, KP, TM, KD, FB, DS, GPC and DV contributed to conceptualization, designing, writing, drafting, revising, editing and reviewing of the manuscript. Data authentication is not applicable. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

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Competing interests

The authors declare that they have no competing interests.

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