N6-methyladenosine in RNA of atherosclerotic plaques: An epitranscriptomic signature of human carotid atherosclerosis

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ABSTRACT

Background: More than 170 post-transcriptional RNA modifications regulate the localization, processing and function of cellular RNAs, and aberrant RNA modifications have been linked to a range of human diseases. The RNA modification landscape in atherosclerosis, the main underlying cause of cardiovascular diseases, is still largely unknown.

Methods: We used mass spectrometry to analyse a selection of RNA-modifying enzymes and the N6-methyladenosine (m^{6}A) in carotid atherosclerotic lesion samples representing early and advanced stages of atherosclerosis as compared to non-atherosclerotic arteries from healthy controls.

Findings: (i) the detection of different levels of several enzymes involved in methylations occurring in rRNA and mRNA; (ii) these findings included changes in the levels of methyltransferases ('writers'), binding proteins ('readers') and demethylases ('erasers') during atherosclerosis as compared to non-atherosclerotic control arteries, with generally the most prominent differences in samples from early atherosclerotic lesions; and (iii) these changes were accompanied by a marked downregulation of m^{6}A in rRNA, the most abundant and well-studied modification in mRNA with a wide range of effects on cell biology.

Interpretation: We show for the first time that RNA-modifying enzymes and the well-studied RNA modification m^{6}A are differentially regulated in atherosclerotic lesions, which potentially could help creating new prognostic and treatment strategies.

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1. Introduction

Atherosclerosis is the major underlying cause of cardiovascular diseases (CVD) with its main complications, myocardial infarction (MI) and ischemic stroke, being the leading causes of death worldwide [1]. Most alarmingly, the incidence of atherosclerotic disorders is expected to increase over the next decade, mainly due to an ever-increasing aging population and the raising prevalence of obesity [2,3]. Atherosclerosis is now widely recognized as a chronic inflammatory disorder with the interaction between inflammation and lipids as a major hallmark [4]. Despite improvements in the understanding of the molecular mechanisms of atherosclerosis and the use of effective drugs e.g., statins, cardiovascular events in the population remain too high [5,6]. This prompts the design of more effective and supplementary therapies

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to enable improved individualized treatment. A prerequisite for such improvements is a better understanding of critical molecular mechanisms that drive the development of atherosclerosis.

During the last years, several studies have explored epigenetic modifications in CVD, reporting differential profiles of DNA methylation and histone methylation and acetylation in tissues and cells (e.g., in aortic lesions, vascular endothelium, monocytes, and vascular smooth muscle cells) from CVD patients [7,8]. More recently, the epitranscriptome, a set of RNA post-transcriptional modifications, has emerged as an additional mechanism of gene regulation, influencing fundamental aspects of cell function [9]. Thus, gene regulation relies not only on the translation of the messenger RNA sequence, but also on the folding process of different RNA species e.g., ribosomal RNA (rRNA) into more complex structures to form ribosomes, influenced by chemical modifications of the RNA bases [10]. In contrast to the limited number of DNA epigenetic modifications, more than 170 distinct types of RNA modifications have been found distributed along all classes of RNA molecules, including ribosomal RNA (rRNA), messenger RNA (mRNA), and transfer RNA (tRNA) [11−13]. Although RNA modifications have been found to affect localization, processing, structure and function of RNA molecules, the function of the majority of RNA modifications remains largely unknown. $N6$-metyladenosine (m^6A) is one of the most abundant and well-studied modifications in mRNA with a wide range of effects on cell biology [14]. m^6A has been previously related to cancers and brain disorders [15,16], but to this end, there are no data on m^6A in human atherosclerosis.

RNA-modifying enzymes fall into three main groups: 'writers' that establish the modifications; 'readers' that bind and interpret them; and 'erasers' that remove them. The dysregulation or mutations in approximately half of the presently known RNA-modifying enzymes has been linked to human diseases, particularly various forms of cancer and neurologic disorders [17,18]. However, except for reports on hypertrophic cardiomyopathy and myocardial ischemia [19,20], there are to the best of our knowledge no other data on the state of RNA modifying enzymes or post-transcriptional RNA modifications in CVD, specifically atherosclerotic disorders.

In the present study, we examine the protein levels of enzymes involved in post-transcriptional methylations of messenger and ribosomal RNA in patient samples representing both early- and advanced-stage atherosclerosis as compared to control arteries. We further detected reduced levels of m^6A, a widespread RNA modification associated with various diseases, in total RNA from the same human atherosclerotic samples.

2. Materials and methods

Ethics. The protocols were approved by the Regional Committee for Medical and Research Ethics, South-East Norway, ID 2009/613; no. 2769. The endarterectomy procedures were done according to routine medical indication. All participants gave signed informed consent. The study protocols were in agreement with the Declaration of Helsinki.

Study population. Atherosclerotic carotid plaques (n = 13) were retrieved from patients with moderate (50−69%) or severe (≥70%) stenosis in the internal carotid artery during carotid endarterectomy. From these plaques a central part of highly developed atherosclerosis ('advanced'), and the distal part with only early signs of atherosclerosis ('early') from the same plaque were processed for further analysis. All patients were recruited at the Department of Neurology, Østfold Hospital Trust Kalnes, Norway. Exclusion criteria were: concomitant infections, autoimmune disease, heart failure, malignancies, and kidney and liver disease. Non-atherosclerotic samples from the common iliac artery of deceased

organ donors (Ctrl; n = 9) were used as non-atherosclerotic controls.

Collection of tissue samples. Atherosclerotic carotid plaques and patient-matched 'early' atherosclerotic samples retrieved during carotid endarterectomy were divided into 'advanced' and 'early' plaques as described above, rapidly frozen in liquid nitrogen and stored at −80 °C until further analysis. The control arteries obtained from organ donors were processed and stored the same way as the atherosclerotic plaques. The histological analysis of the atherosclerotic plaques is included in the Supplementary File.

Targeted mass spectrometry. The targeted mass spectrometry, scan and protein quantification are based on a method previously described [21]. Sample preparation for mass spectrometry is described in the Supplementary File. First, peptide standards were analysed on a Thermo Scientific Q Exactive HF mass spectrometer functioning in parallel reaction monitoring mode (PRM). All methods used for targeted mass spectrometry were based on PRM, designed, analysed, and processed using the Skyline software 4.1.0.11714 [22]. The same software was used for *in silico* selection of proteotypic peptides, using the *Homo sapiens* reference proteome (www.uniprot.org/) to exclude non-unique peptides. Imported data into Skyline was used in the selection of the top ionizing peptides (charge states 2^+ and 3^+), as well as in making a scheduled method with retention times each 10 min. This method detected and quantified the corresponding peptides in the samples, while peptide identification occurred using the information on retention time and fragmentation pattern of the heavy labelled peptide. The same method was followed for the setup of PRM for standard peptides.

Targeted mass spectrometry scan. A Q Exactive HF mass spectrometer working in PRM mode attached to an EASY-nLC 1200 UHPLC system (Thermo Scientific) was used to analyse tryptic digests. Peptides were injected to an Acclaim PepMap C18 column (75 μm i.d. x 2 cm nanoviper, 3 μm particle size, 100 Å pore size; Thermo Scientific), and separated at 40 °C on an EASY Spray™ LC column (75 μm i.d. x 50 cm nanoviper, 2 μm particle size, 100 Å pore size; Thermo Scientific). A 120 min method was used at a 300 nl/min flow rate, as follows: 6% buffer B (80% Acetonitrile, 0·1% Formic acid), increasing to 31% buffer B at 105 min, and 100% increase in Buffer B over 6 min, where it was subsequently held for 9 min. Buffer A consisted of 0·1% Formic acid. The peptides eluting from the column were ionized by an Easy Spray™ Source (Thermo Scientific), and analysed on positive-ion mode using electrospray voltage 1·75 kV and HCD fragmentation. Each MS/MS scan was acquired with the following parameters: resolution of 60000 FWHM; normalized collision energy (NCE) 28; automatic gain control (AGC) target value of 2×10^5; maximum injection time (mIT) of 110 ms; and isolation window 1·4 m/z.

Protein quantification by targeted mass spectrometry. Heavy labelled peptides were spiked and used for chromatography quality control and peptide identification. Instead of using heavy/light ratios, peptide were quantified summing the integrated peak areas of the strongest fragments. Peptide areas for multiple peptides of the same protein were summed to assign relative level to that protein. To evaluate protein levels in different groups, relative level values of each protein in samples belonging to the same group (i.e., 'advanced' atherosclerotic carotid plaques, 'early' atherosclerotic samples, and controls) were averaged. A minimum of 2 peptides/protein were used for quantification. The Supplementary File and Supplementary Table 1 include the peptide standards and peptides used for quantification (marked in bold).

Quantification of m^6A using LC-MS/MS. RNA isolation is described in the Supplementary File, and quantification of m^6A was similarly done as in [23]. Briefly, RNA was hydrolysed to ribonucleosides using 20 U benzonase (Santa Cruz Biotech) and 0·2 U

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nuclease P1 (Sigma) in 10 mM ammonium acetate pH 6·0, and 1 mM magnesium chloride at 40 °C for 45 min. Next 50 mM of ammonium bicarbonate, 0·002 U phosphodiesterase I, and 0·1 U alkaline phosphatase (Sigma), were added and incubated at 37 °C for 45 min. The hydrolysates were added 3 vol of acetonitrile, and spun down at 4 °C, 16000×g for 30 min. For LC-MS/MS analysis of modified and unmodified ribonucleosides, dry supernatants were dissolved in 50 μl water. Chromatographic separation was performed by an Agilent 1290 Infinity II UHPLC system with a ZORBAX RRHD Eclipse Plus column (C18 150 × 2·1 mm ID; 1·8 μm) protected with a ZORBAX RRHD Eclipse Plus guard column (18 5 × 2·1 mm ID; 1·8 μm) from Agilent. The mobile phase consisted of water and methanol (both added 0·1% formic acid) run at rate of 0·23 ml/min, as follows: 5% methanol for 0·5 min, followed by a 3 min gradient of 5−15% methanol, a 3 min gradient of 15−90% methanol, and 4 min re-equilibration with 5% methanol. For the analysis of unmodified ribonucleosides, a portion of each sample was diluted and chromatographed isocratically with 20% methanol. Detection of m^{6}A was done using an Agilent 6495 Triple Quadrupole system operating in positive electrospray ionization mode, with monitory of transitions following specific parameters: molecular weight, 281·1 Da; Q1 molecular ion, 282·1 m/z, amu; Q3 product ion, 149·1 m/z, amu; and positive ionization polarity. For resolving 28S and 18S rRNA fractions, size-exclusion chromatography (SEC) was used as described elsewhere [24].

Statistical analyses are described in the Supplementary File.

3. Results

3.1. Ribosomal RNA methyltransferase levels are reduced in human atherosclerotic lesions

To investigate the role of post-transcriptional RNA modifications, we first quantified by use of mass spectrometry several methyltransferases ('writers') known to induce modifications in *rRNA*. First, protein levels of the *N*6-adenosine-methyltransferase ZCCHC4, the methyltransferase like 5 (METTL5), and the rRNA 2′-*O*-methyltransferase fibrillarin (FBRL), were significantly decreased in both early and advanced stage atherosclerosis compared to non-atherosclerotic controls (Fig. 1a−b). As for METTL5, the decrease was significantly more prominent in early atherosclerosic samples. Protein levels of the m^{6}A-methyltransferase dimethyladenosine transferase DIM1, and the m^{5}C-methyltransferases NSUN1 (NOP2) and NSUN5 were significantly decreased in early stage atherosclerosis, but not in advanced atherosclerosis, as compared to both non-atherosclerotic control arteries (Fig. 1d−f).

3.2. Dysregulated m^{6}A mRNA modulators in human atherosclerotic lesions

We next examined enzymes known to catalyse m^{6}A modifications in *mRNA*. We quantified the level of the methyltransferases ('*writers*') forming the m^{6}A WMM complex [25]. Wilms' tumor 1-associating protein (WTAP) and *N*6-adenosine-methyltransferase catalytic subunit (METTL3) showed significantly higher protein levels in advanced-as compared to early-stage atherosclerosis lesions, but no differences as compared to control arteries (Fig. 2a−b). There were no significant changes in the protein level of the *N*6-adenosine-methyltransferase non-catalytic subunit (METTL14) in the three sample groups (Fig. 2c). When analysing levels of proteins binding to RNA ('*readers*'), we found a marked increase of the eukaryotic translation initiation factor 3 subunit A (eIF3a) in advanced atherosclerosis as compared to both control arteries and the early atherosclerosis samples. The YTH domain-containing family protein 2 (YTHDF2) showed significantly decreased levels

in early atherosclerosis as compared to both control arteries and samples from the advanced stage lesions. In contrast, we did not find significant differences between the three sample groups for YTHDF3 (Fig. 2d−f). Finally, the protein level of the demethylase ('*eraser*') fat and obesity-associated protein (FTO) was significantly decreased in early atherosclerosis as compared to both control arteries and advanced stage atherosclerosis, with no differences within the two latter groups. In addition, the '*eraser*' AlkB homologue 5 (ALKBH5) was not detected in our samples.

3.3. Decreased m^{6}A RNA levels in human atherosclerotic lesions

We finally performed analysis of m^{6}A in rRNA enriched samples from early and advanced stages of carotid atherosclerosis as well as non-atherosclerotic controls. m^{6}A represents the most common and extensively-studied RNA epitranscriptomic modification so far, previously associated with various diseases [26]. In our study, levels of m^{6}A were significantly lower in early and advanced atherosclerosis as compared to control arteries, with particularly low levels in samples representing early atherosclerosis (Fig. 3a). m^{6}A modification has been reported to be present in rRNA, mRNA, small nuclear RNA (snRNA), and microRNA (miRNA). Given the fact that 80−85% of total RNA is comprised of rRNA, we argue that most of the m^{6}A signal detected in total RNA comes from rRNA. Our results thus indicate that the rRNA m^{6}A levels were decreased in early and advanced atherosclerosis.

3.4. m^{6}A is decreased in 18S rRNA from human atherosclerotic lesions

Human ribosomes are comprised of four RNA molecules and 80 proteins assembled into two ribonucleoprotein subunits. The small (40S) subunit contains one ribosomal RNA (rRNA; 18S) and the large (60S) subunit contains three rRNAs (5S, 5.8S, and 28S) that together form the catalytic core for translation [27]. Ribosomes contain two m^{6}A modifications, one in 18S rRNA position 1832 established by METTL5, and one in 28S rRNA position 4220 (formerly 4190) established by ZCCHC4 [28,29]. To further investigate the m^{6}A affected in atherosclerosis, we applied preparative size-exclusion chromatography to separate 18S rRNA and 28S rRNA and quantified the m^{6}A levels in these rRNA species. Whereas m^{6}A in 28S rRNA fractions showed similar levels between atherosclerotic samples and healthy controls, m^{6}A in the 18S rRNA fraction was significantly lower in both early and advanced atherosclerosis as compared to healthy controls. The reduction was approximately 50% of control levels, indicating possible differential methylation of 18S rRNA A$_{1832}$ in early and advanced atherosclerosis (Fig. 3b).

4. Discussion

Post-transcriptional RNA modifications have a broad spectrum of functions in RNA metabolism regulating stability, localization, and translation of gene transcripts in response to environmental cues [30]. Herein, we report that carotid atherosclerotic plaques show differences in the levels of several enzymes involved in post-transcriptional modification of rRNA and mRNA. These findings include changes in the levels of methyltransferases ('writers'), binding proteins ('readers'), and demethylases ('erasers'), during atherosclerosis as compared to non-atherosclerotic control arteries, with generally the most prominent differences in samples from early atherosclerotic arteries (Table 1). Additionally, we found a marked reduction of m^{6}A in total RNA. m^{6}A is present in all classes of RNA, being one of the most abundant and well-studied modifications in mRNA with a wide range of effects on cell biology [26]. m^{6}A has been previously related to various human disorders like
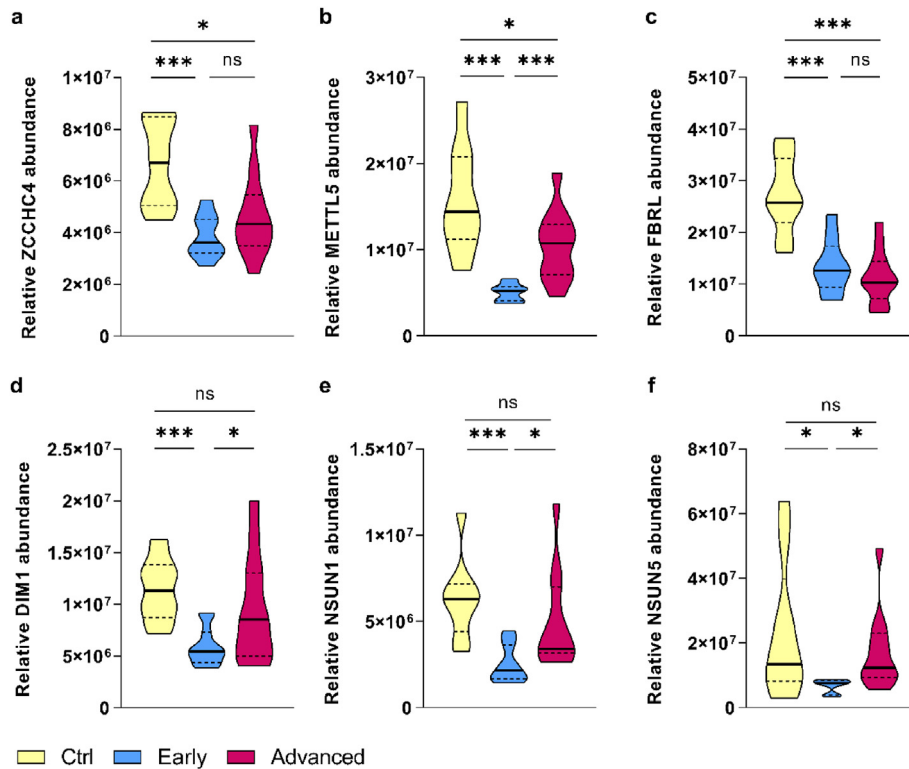
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Fig. 1. Ribosomal RNA methyltransferase levels are reduced in human atherosclerotic lesions. Relative protein level of the methyltransferases **(a)** ZCCHC4, **(b)** METTL5, **(c)** FBRL, **(d)** DIM1, **(e)** NSUN1, and **(f)** NSUN5. Data presented as violin plots with median and quartiles of samples from donor control arteries (Ctrl; n = 9), and 'early' (n = 11) and 'advanced' (n = 12) parts of the atherosclerotic lesions. *p < 0.05, **p < 0.01, ***p < 0.001; ns, not statistically significant.

cancers and brain disorders, but this is to the best of our knowledge the first report on a possible role of m^{6}A and regulation of its modulatory enzymes in atherosclerotic disorders.

Developmental syndromes, cancer, obesity, and type 2-diabetes, have been associated with mutations in the rRNA methylation machinery [31]. Moreover, recent studies revealed ribosomes being much more diverse than previously anticipated, with heterogeneity arising from both differential rRNA modifications and bound proteins [32,33]. A recent study has described more than 228 sites with 14 distinct post-transcriptional modifications in rRNA [34]. Moreover, rRNA modification patterns have been observed to change in response to environmental fluctuations, during embryonal development, and in diseased states [35–37]. Here, we show a significant decrease in the methylation mark m^{6}A measured in total RNA, mainly composed of rRNA. m^{6}A is regulated by several highly specialised enzymes and, importantly, the decrease in m^{6}A modification levels in samples from carotid atherosclerosis was associated with decreased levels of the m^{6}A-'writers' enzymes in rRNA, i.e., ZCCHC4 and METTL5, in samples from early atherosclerotic lesions. Moreover, post-transcriptional modifications in mRNA are of outmost importance in gene regulatory processes with m^{6}A as the most abundant and best studied mRNA modification. m^{6}A mRNA is regulated by a complex interplay between methyltransferases ('writers') and demethylases ('erasers'), as well as binding proteins ('readers') that recognize the modified RNA bases, which affect mRNA metabolism, including translation initiation and mRNA stability [38]. Herein we show that the 'reader' YTHDF2 and the 'eraser' FTO were reduced in early atherosclerotic lesions as compared to control samples. Although we have no data on m^{6}A levels in pure mRNA, these finding may suggest that the differences in m^{6}A levels in atherosclerotic as compared to control arteries are not restricted to rRNA.

Because m^{6}A plays a critical role in mRNA splicing, degradation and translation, it is conceivable that it may also play an important role in immunity. Indeed, it has been suggested that m^{6}A mRNA and differential changes in its regulatory enzymes can play an important role in immunological processes, including the interaction with Toll-like receptors and activation of the transcriptional factor NF-kB, both major regulator of immune responses and inflammation [39]. However, to this end, the functional consequences of the present findings with reduced levels of m^{6}A RNA and its regulatory enzymes are unclear. It is noteworthy, however, that the regulation of these molecules were at least to some degree different between samples from early- and more advanced-carotid lesions. Thus, the m^{6}A rRNA 'writers' ZCCHC4 and METTL5 were increased in advanced-as compared to early-atherosclerotic lesions. Even more strikingly, the m^{6}A mRNA 'writers' WTAP and METTL3, the 'readers' eIF3a and YTHDF2, and the 'eraser' FTO were significantly increased in advanced-as compared to early-atherosclerotic lesions. Although it could be hypothesized that such a pattern could be associated to enhanced immunological processes, cell proliferation and cell composition, the consequences for these differences remain to be solved.

The present study has several limitations such as the lack of data on m^{6}A levels in purified mRNA and tRNA, as well as the screening of other RNA modifications. We also lack mechanistic data pointing to the functional consequences of our findings. It is also conceivable that the differences between controls and samples from carotid atherosclerosis are influenced by differences in cell composition, and forthcoming studies should also analyse epitranscriptomic modifications at the single cell level. Nonetheless, these novel data on the regulation of rRNA- and mRNA-modifying enzymes, as well as m^{6}A presence in total RNA from carotid atherosclerosis, could potentially represent the start of a new era in the treatment of
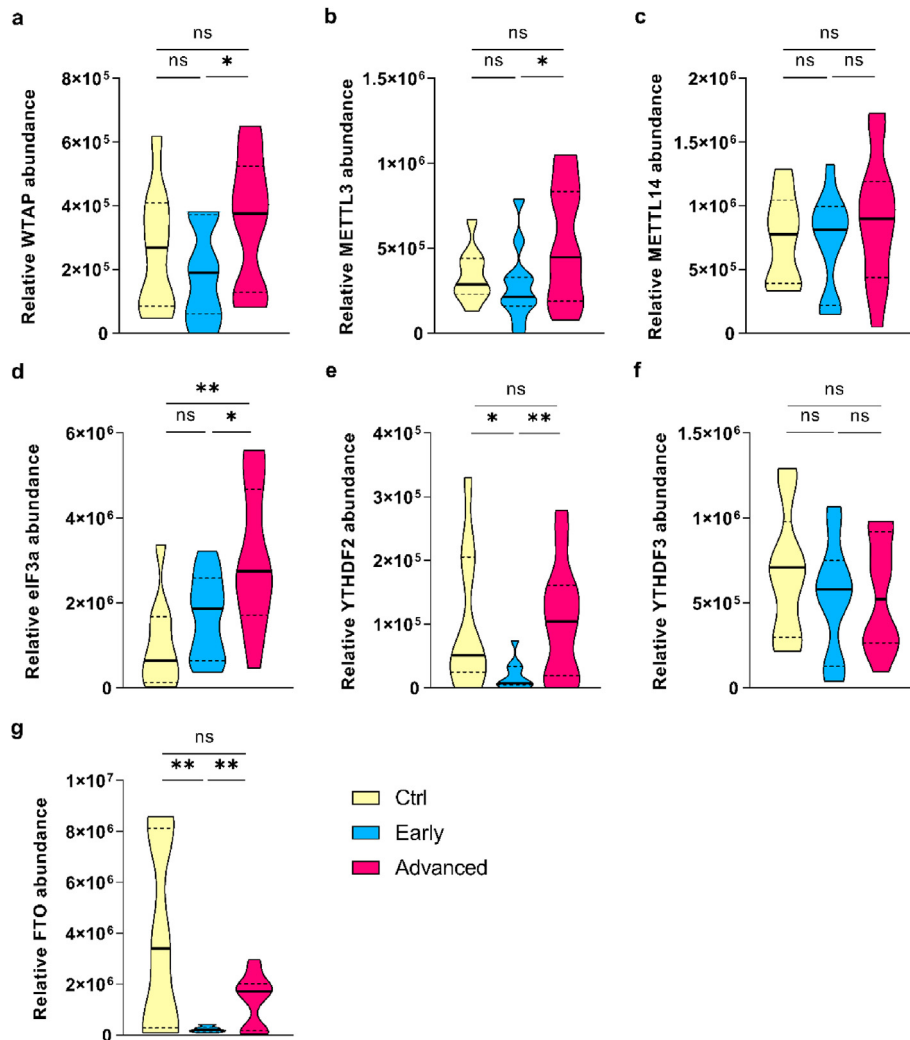
Fig. 2. Dysregulated m⁶A mRNA modulators in human atherosclerotic lesions. Relative protein level of the 'writers' **(a)** WTAP, **(b)** METTL3, and **(c)** METTL14; the 'readers' **(d)** eIF3a, **(e)** YTHDF2, **(f)** YTHDF3; and **(g)** the 'eraser' FTO. Data presented as violin plots with median and quartiles of samples from donor control arteries (Ctrl; n = 9), and 'early' (n = 11) and 'advanced' (n = 12) parts of the atherosclerotic lesions. *p < 0.05, **p < 0.01; ns, not statistically significant.
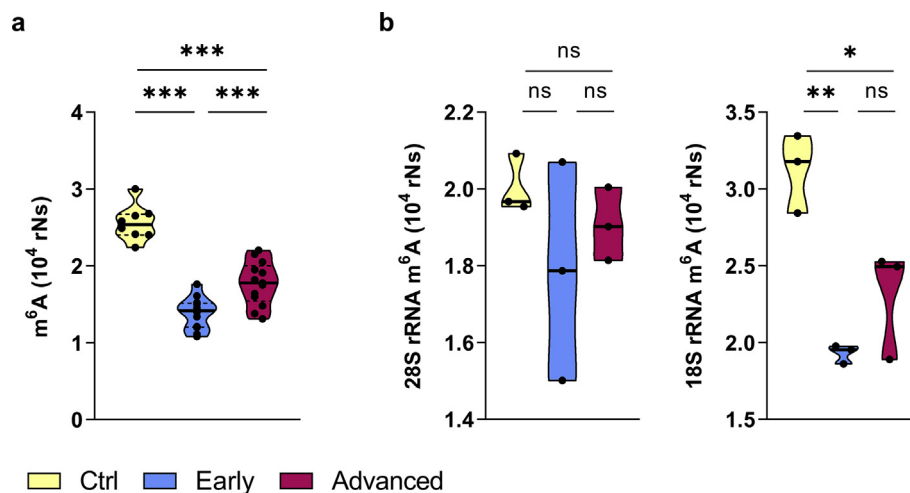

Fig. 3. Decreased m⁶A RNA levels in human atherosclerotic lesions. Modification levels of the RNA modification m⁶A in **(a)** total RNA (mainly rRNA), and **(b)** 28S and 18S rRNA fractions, expressed as 10⁴ unmodified ribonucleotides. Data presented as violin plots with median and quartiles of samples from donor control arteries (Ctrl; n = 3–9), and 'early' (n = 3–13) and 'advanced' (n = 3–13) segments of atherosclerotic lesions. Unpaired t-test performed for statistical significance. *p < 0.05, **p < 0.01, and ***p < 0.001; ns, not statistically significant.

Table 1
Ribosomal and messenger RNA modification-regulatory enzymes in human atherosclerotic lesions.

Protein	Uniprot Accession Number	Function; RNA target	RNA modification regulated	'Early' vs Ctrl	'Advanced' vs 'Early'	'Advanced' vs Ctrl
ZCCHC4	Q9H5U6	Writer; rRNA	m^6A	↓***	ns	↑*
METTL5	Q9NRN9	Writer; rRNA	m^6A	↓***	↑***	↑*
FBRL	P22087	Writer; rRNA	Nm	↓***	ns	↓***
DIM1	Q9UNQ2	Writer; rRNA	$m^6_{,6}A$	↓***	↑*	ns
NSUN1	P46087	Writer; rRNA	m^5C	↓***	↑*	ns
NSUN5	Q96P11	Writer; rRNA	m^5C	↓*	↑*	ns
WTAP	Q15007	Writer; mRNA	m^6A	ns	↑*	ns
METTL3	Q86U44	Writer; mRNA	m^6A	ns	↑*	ns
METTL14	Q9HCE5	Writer; mRNA	m^6A	ns	ns	ns
eIF3a	Q14152	Reader; mRNA	m^6A	ns	↑*	↑**
YTHDF2	Q9Y5A9	Reader; mRNA	m^6A	↓*	↑**	ns
YTHDF3	Q7Z739	Reader; mRNA	m^6A	ns	ns	ns
FTO	Q9C0B1	Eraser; mRNA, tRNA, snRNA	m^6Am; m^6A; m^1A	↓**	↑**	ns

Overview of the RNA-modifying enzymes analysed, with their Uniprot database accession number (https://www.uniprot.org/), known function, RNA target, and RNA modification they regulate, as well as the statistical comparisons between sample groups in the present study. Unpaired t-test performed for statistical significance. *$p < 0\cdot05$, **$p < 0\cdot01$, ***$p < 0\cdot001$; ns, not statistically significant.

atherosclerosis by targeting the enzymes responsible for the regulation of RNA modifications.

Declaration of competing interest

None of the authors declare any conflict of interest.

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None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.09.057.

Author contributions

AQJ, IG, MMLS, PA, MB, and BH contributed to the conception and design of the study. AQJ, MMLS, AA, XYK, SH, KS, MS, PA, MB, and BH contributed to the acquisition of the samples and data. AQJ, IG, MMLS, XYK, IA, SH, TBD, PA, MB, and BH contributed to the analysis and interpretation of the data. AQJ, PA, MB, and BH contributed to the original drafting of the article. All authors contributed to the review and editing of the article, and gave final approval of the document to be submitted.

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