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# Potential protective role of let-7d-5p in atherosclerosis progression reducing the inflammatory pathway regulated by NF-κB and vascular smooth muscle cells proliferation

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ABSTRACT

The prevalence of cardiovascular diseases (CVDs) is increasing in the last decades, even is the main cause of death in first world countries being atherosclerosis one of the principal triggers. Therefore, there is an urgent need to decipher the underlying mechanisms involved in atherosclerosis progression. In this respect, microRNAs dysregulation is frequently involved in the progression of multiple diseases including CVDs.

Our aim was to demonstrate that let-7d-5p unbalance could contribute to the pathophysiology of atherosclerosis and serve as a potential diagnostic biomarker. We evaluated let-7d-5p levels in vascular biopsies and exosome-enriched extracellular vesicles (EVs) from patients with carotid atherosclerosis and healthy donors. Moreover, we overexpressed let-7d-5p *in vitro* in vascular smooth muscle cells (VSMCs) to decipher the targets and the underlying mechanisms regulated by let-7d-5p in atherosclerosis.

Our results demonstrate that let-7d-5p was significantly upregulated in carotid plaques from overweight patients with carotid atherosclerosis. Moreover, in EVs isolated from plasma, we found that let-7d-5p levels were increased in carotid atherosclerosis patients compared to control subjects specially in overweight patients. Receiver Operating Characteristic (ROC) analyses confirmed its utility as a diagnostic biomarker for atherosclerosis. In VSMCs, we demonstrated that increased let-7d-5p levels impairs cell proliferation and could serve as a protective mechanism against inflammation by impairing NF-κB pathway without affecting insulin resistance.

In summary, our results highlight the role of let-7d-5p as a potential therapeutic target for atherosclerosis since its overexpression induce a decrease in inflammation and VSMCs proliferation, and also, as a novel non-invasive diagnostic biomarker for atherosclerosis in overweight patients.

*Abbreviations:* ACA, advanced carotid atherosclerosis; AKT, protein kinase B; CAD, coronary artery disease; CA, carotid atherosclerosis; CVDs, cardiovascular diseases; FAs, fibrolipidic plaque; IKK, inhibitor of nuclear factor-κB kinase; IκBα, inhibitor of nuclear factor-κB; miRNA, microRNA; NF-κB, nuclear factor kappa B; O.C.T., Tissue-Tek® O.C.T. Compound; ORO, oil-red O; TNF-α, tumor necrosis factor-α; VSMCs, vascular smooth muscle cells.

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## 1. Introduction

Cardiovascular diseases (CVDs) are one of the main causes of mortality and disability in the world [1]. The main underlying cause of most CVDs is the development of atherosclerosis in different locations of the vascular wall. In this sense, atherosclerosis is a chronic inflammatory disease that affects medium- and large-sized arteries of different vascular beds and is characterized by the thickening of the intima and media layer with loss of elasticity. Its basic lesion is the atheromatous plaque, which is mainly composed of lipids, fibrous tissue, and inflammatory cells, and goes through different stages [2].

The atherosclerotic process begins with endothelial dysfunction, resulting in the uncoupling of endothelial nitric oxide synthase due to several causes such as the loss of the glycocalyx or the shear stress generated by blood flow on the endothelium. These processes induce inflammation and oxidative stress of the endothelium and allow the entry of low-density lipoproteins (LDLs) or monocytes [3–9]. Once in the subendothelial space, monocytes differentiate into macrophages that capture modified LDLs, transforming into foam cells that release pro-inflammatory molecules [9,10]. In addition, the other resident cells in the arteries are the vascular smooth muscle cells (VSMCs), which also play a significant role in the progression of atherosclerosis. They could acquire different phenotypes, such as a synthetic with increased migration and proliferation capabilities, an osteoclastic-, fibroblastic-, or macrophage-like phenotype, allowing them to capture modified LDLs and give rise to foam cells [11,12]. In addition to cell type-specific effects and vascular layer-specific effects, there are altered pathways such as the insulin and insulin-like growth factor pathways [13–17] and the inflammatory nuclear factor-kappa B (NF-κB) pathway [18–23] in the atherosclerotic plaques.

MicroRNAs (miRNAs) are small non-coding RNAs of about 19 to 24 nucleotides that negatively regulate the expression of their target genes by binding to their messenger RNA (mRNA), serving as a signal for silencing or degradation of the mRNA [24]. Since miRNAs are negative regulators of the expression of proteins, their dysregulation is involved in the development and progression of different diseases. In fact, the expression of miRNAs has been altered in metabolic diseases such as obesity [25], non-alcoholic fatty liver disease (NAFLD) [26], diabetes [27] or CVDs like atherosclerosis. miRNA dysregulation has been observed in different stages of the atherosclerotic process. miR-217, a miRNA that is linked to aging is also involved in atherosclerosis progression, when miR-217 levels are increased in the endothelium, promotes endothelial dysfunction by decreasing the vascular endothelial growth factor receptor, an inhibitor of eNOS expression [28]. miR-345-3p expression decreases in HUVECs treated with oxidized-LDLs, increasing its target TRAF6, the NF-κB pathway, inflammation, and apoptosis [29]. miR-93, miR-145 and miR-128-3p modulate VSMCs switching from a healthy phenotype to a synthetic proliferative and migratory phenotype [30–32].

The let-7 family was one of the first-described miRNA families, with 12 highly conserved let-7 isoforms [33,34]. Mature let-7 family members contain identical sequences and suppress the expression of a common set of target mRNAs [35]. It has been described that let-7 miRNAs are dysregulated in clinical and experimental diabetes-associated atherosclerosis, and modulate VSMCs and endothelial cell activation and inflammation, *via* regulation of platelet-derived growth factor (PDGF) and TNF-α signalling [36]. Furthermore, restoration of let-7 levels can suppress mediators of vascular inflammation, including interleukin (IL)-6, IL-1β, and NF-κB. In this context, we studied the role of let-7d-5p in the development of human atherosclerosis, and whether let-7d-5p overexpression might have a protective role in the vascular insulin resistance and inflammation as well as in the proliferation of VSMCs. Finally, we tested whether circulating let-7d-5p might be a potential biomarker for advanced atherosclerosis diagnosis. To carry out the objectives of the current paper, we used vascular samples of subjects without atherosclerosis (Controls), subjects with initial aortic fibrolipidic plaques (FAs) and patients with advanced carotid atherosclerosis (ACA). Moreover, we also obtained the plasma from two groups, Controls and ACA, to analyze the levels of let-7d-5p as a potential non-invasive biomarker. Then, we confirmed the let-7d-5p levels in a cohort of 21 patients with Metabolic Syndrome (MeS) classified with carotid atherosclerosis (CA). Finally, we studied possible targets of this miRNA involved in the mechanisms of atherosclerosis development in a cell line of VSMCs previously generated in our laboratory [37].

## 2. Materials and methods

### 2.1. Human samples

Three cohorts of patients were analyzed in this study. In the first cohort, under the authorization of the French Biomedicine Agency (PFS 09-007) human aortas were collected from deceased organ donors from 2010 to 2013. After macroscopic evaluation, the aortas were classified following the Stary classification into two groups: control aortas (Controls, $n = 7$) in which there were no signs of atherosclerosis, and aortas with initial/early fibrolipidic atherosclerotic plaques [fibroatheromas (FAs), n = 7] as previously described [38]. The investigation was performed in accordance with the principles outlined in the Declaration of Helsinki.

The second cohort includes patients with ACA. Patients with carotid stenosis >70 % underwent carotid endarterectomy at IIS-Fundación Jiménez Díaz (Supplemental Table 1) and the atherosclerotic plaques ($n = 14$) were collected for further analysis. The plaques showed higher inflammatory cells infiltration (Stary stages V–VI), however, the adjacent areas showed mainly lipid depots and VSMCs (Stary stage III). In the same study, plasma was collected from subjects without atherosclerosis ($n = 7$) and ACA patients ($n = 19$) to obtain extracellular vesicles and analyze miRNAs levels (Supplemental Table 1). The study was approved by the Hospital's Ethics Committee (IIS-Fundación Jiménez Díaz) with the reference number PI1442016 according to the institutional and the Good Clinical Practice guidelines, which was performed in accordance with the Declaration of Helsinki. All participants gave written informed consent.

The third cohort included patients with MeS and CA (Supplemental Table 2). Plasma samples were obtained from January to December of 2021 at Hospital Universitario de La Princesa and belong to a collection managed by Dr. Pedro Landete Rodríguez (Registry Number: 3719). The biological samples were obtained after signing a specific informed consent approved by the Institutional Clinical Research Ethics Committee.

### 2.2. Cell culture

Primary VSMCs were obtained from thoracic aorta arteries of 3 male 8-week-old WT mice. Anesthetized mice (Avertin, 250 mg/kg, ip.) were saline-perfused, and thoracic aorta arteries were submitted to collagenase dispersion and primary culture as previously described [37]. Thus, primary culture of WT VSMCs was transfected by retroviral infection (viral particles containing pBabe retroviral vector encoding of SV40 large T antigen) and selected with 1 μg/mL puromycin for 3 weeks [37]. Cell lines were characterized with specific markers of vascular smooth muscle cells (α-SMA) and were used since 3 to 10 passages. Cell lines were cultured to subconfluence (70–80 %) with 10 % foetal bovine serum (FBS)-DMEM for *in vitro* experiments.

### 2.3. miRNA extraction from vascular cell lines and paraffin-embedded carotid tissue

The miRNA content from the cells were extracted by using the mirVana™ miRNA Isolation Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). The miRNA content from paraffin-embedded carotids was extracted using the RNeasy FFPE kit (Qiagen, Hilden,

Germany). All the extractions were made following the protocol recommended by the manufacturers. The mirVana™ kit allows the isolation of miRNAs and long RNAs in separate fractions by differential precipitation. The miRNA sample concentration was determined using a NanoDrop™ 2000 and the NanoDrop 2000/2000c Operating Software (Thermo Scientific, Waltham, MA, USA).

### 2.4. Cell transfection with miRNAs mimics

Approximately $5 \times 10^4$ cells were seeded in P60 culture plates (353002, Falcon™, Thermo Fisher Scientific, Waltham, MA, USA) and transfected with 10–20 nM of MISSION® miRNA mimic hsa-let-7d-5p (HMI0011, Sigma-Aldrich, Saint Louis, MO, USA) using Lipofectamine™RNAiMAX (13778500, Thermo Fisher Scientific, Waltham, USA) as specified in the manufacturer's protocol. The effect of the transfection in both, miRNA and protein levels, was assessed after 48 h transfection with Lipofectamine™RNAiMAX. Controls were cells transfected with miRNA mimic negative control oligonucleotide (scrambled control, D-001810-1005, Dharmacon). To evaluate the effect in NF-κB pathway after the transfection with mimic-let-7d-5p, the cells were deprived in 0 % FBS medium for 1 h and then stimulated with 10 ng/mL TNF-α (Sigma-Aldrich, Saint Louis, MO, USA) for 10 min. To evaluate let-7d-5p-induced insulin resistance, transfected cells with mimic-let-7d-5p (10 nM), anti-let-7d-5p (10 nM) or scrambled control (10 nM) were serum-starved in 0 % FBS medium for 1 h and stimulated with 10 nM insulin (Sigma-Aldrich, Saint Louis, MO, USA) for 10 min. Then, we analyzed whether there was a less phosphorylation of AKT, p70 S6 kinase (p70S6K) and p42/44MAPK.

In the proliferation studies, VSMCs were transfected with mimic-let-7d-5p for 48 h before serum starvation and stimulation with TNF-α (10 ng/mL), insulin (1 μM) or palmitic acid (PA) (0.4 mM) for 24 h. For the treatment with PA, we prepared a stock of PA dissolved in isopropanol at a concentration of 80 mM. The same day of the experiment, we conjugated PA with 0.25–1 % BSA medium. The final concentration of PA used to treat VSMCs was 0.4 mM.

### 2.5. miRNA retrotranscription and quantitative PCR (RT-qPCR)

Reverse transcription (RT) was performed on 10 ng of isolated miRNAs using the TaqMan™ Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Waltham, MA, USA). miRNA expression was determined by qPCR in a StepOnePlus™ Real-Time PCR System (Applied Biosystems) using the TaqMan Fast Advanced Master Mix (Applied Biosystems) and the corresponding TaqMan probes for hsa-let-7d-5p (mmu482962_mir), mmu-miR-16-5p (mmu482960_mir) and mmu-miR-191-5p (mmu481584_mir). miR-191-5p was used as an endogenous normalizer for vascular and cell samples, and both miR-191-5p and miR-16-5p for plasma samples. The relative abundance of miRNAs, normalized with the endogenous gene and relative to the control, was calculated as follows: Relative Quantification (RQ) $= 2^{-\Delta\Delta Ct}$; ΔCt (cycle threshold) = Ct (miRNA target) − Ct (miR-191-5p or miR-16-5p); ΔΔCt = [ΔCt (for any sample) − ΔCt (for the control)].

### 2.6. Western blot analysis

Proteins from cell lysates (20–40 μg), and tissue samples (60 μg) were separated on a 10 % or 10–20 % gradient polyacrylamide gel and then transferred to a 0.45 μM pore PVDF membrane (Merck, Darmstadt, Germany) as previously described [37]. The primary antibodies used were diluted in TTBS. Rabbit and mouse primary antibodies were immunodetected using horseradish peroxidase-conjugated anti-rabbit IgG (NA931V; 1:4000 in TTBS) or anti-mouse IgG secondary antibodies (NA934V; 1:5000 in TTBS) (GE Healthcare, Buckinghamshire, UK), respectively. When possible, phospho-proteins and their total expression were detected in the same gel, using Restore™ Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, USA) blocking the membrane again before the incubation with the next antibody. Loading was normalized by β-actin or α-tubulin. Protein bands were visualized using the SuperSignal™ West Pico PLUS Chemiluminescent Substrate (34,580, Thermo Fisher Scientific, Hercules, CA, USA). Band densitometry was analyzed using ImageJ Software (v1.52a, Wayne Rasband, National Institute of Health, Stapleton, USA).

### 2.7. Luciferase reporter assays

For reporter assays, a region of the wild-type 3′-untranslated region (3′UTR) from *INSR*, the wild-type 3′UTR from *IGF1R*, the wild-type 3′UTR from *AKT2*, as well as the corresponding mutated versions used as controls, were cloned into dual luciferase psiCHECK2 vector after the annealing the following primers:

3′UTR-*INSR* F: 5′-TCGAGACTGACTTTTTTCAGCACAGTCTACCTCATATTAAAAGGAG TATGC-3′ and 3′UTR-*INSR* R: 5′-GGCCGCATACTCC TTTTAATATGAGGTAGACTGTG CTGAAAAAAGTCAGTC-3′; 3′UTR-*INSR* mutated F: 5′-TCGAGACTGACTTTTTTCAGCA CAGTCGAACGCATATTA AAAGGAGTATGC-3′ and 3′UTR-*INSR* mutated R: 5′-GGCCGCA TACTCC TTTTAATATGCGTTCGACTGTGCTGAAAAAAGTCAGTC-3′; 3′UTR-*IGF1R* F: 5′-TCGAGACTGACCCCCCAAACATTTATCTACCTCATATTAAAAGGA GTATGC-3′ and 3′UTR-*IGF1R* R: 5′-GGCCGCATACTCCTTTTAATATGAG GTAGATAAATGTTTGGGGGGT CAGTC-3′; 3′UTR-*IGF1R* mutated F: 5′-TCGAGACTGACCCCCCAAACATTTATCGGAAC GCTATTAAAAGGAGTA TGC-3′ and 3′UTR-*IGF1R* mutated R: 5′-GGCCGCATACTCCTT TTAA-TAGCGTTCCGATAAATGTTTGGGGGGTCAGTC-3′; 3′UTR-*AKT2* F: 5′-TCGAGACT GACTGGGCACAGGCCTGGCTACCTCATATTAAAAGGAGTA TGC-3′ and 3′UTR-*AKT2* R: 5′-GGCCGCATACTCCTTTTAATATGAGGTA GCCAGGCCTGTGCCCAGTCAGTC-3′; 3′UTR-*AKT2* mutated F: 5′-TCGA-GACTGACTGGGCACAGGCCTGGCGGAACGCTATTAA AAGGAGTATGC-3′ and 3′UTR-*AKT2* mutated R: 5′-GGCCGCATACTCCTTTTAATAGCGT TCCGCCAGGCCTGTGCCCAGTCAGTC-3′.

Annealing was conducted by incubating both primers for 4 min at 95 °C and for 10 min at 70 °C in annealing buffer (100 mM potassium acetate, 30 mM HEPES, pH 7.4, and 2 mM magnesium acetate). Primers were then phosphorylated and cloned into the psiCHECK2 vector from Promega digested with *Xho*I and *Not*I.

Thirty thousand HEK293 cells were plated in DMEM containing 10 % FBS. Twenty-four hours later, cells were transfected with the psiCHECK2 vectors either with mimic-miR control, or mimic-let-7d-5p using lipofectamine (Invitrogen) following the manufacturer's instructions. The luciferase reporter assay was performed 72 h after transfection using the Dual-Glo luciferase kit (Promega, Madison, WI, USA).

The ratio between the firefly and the *Renilla* luciferase allows the normalization of luciferase values. Ratios were normalized against the ratio of the corresponding plasmid transfected with the miR-Control.

### 2.8. Extraction of exosomes of plasma from patients with ACA, CA and controls

To precipitate the exosomes from the plasma samples the total exosome RNA and protein isolation kit (from plasma) (4478545, Thermo Fisher Scientific, Waltham, MA, USA), were used following the protocol recommended by the manufacturer. First, the plasma was clarified with one centrifugation at 2000 xg for 20 min, and a second centrifugation at 10.000 xg for 20 min, in both cases the supernatant was collected, and the pellet was discharged. When the plasma was ready, we added 0.5 volumes of PBS 1× and 0.2 volumes of the Exosome Precipitation Reagent (from plasma) (4484451, Thermo Fisher Scientific, Waltham, MA, USA) mixing until the sample becomes cloudy, at this point the mix was incubated for 30 min on ice followed by a centrifugation at 10,000 xg for 30 min at room temperature. The pellet containing the exosomes was used to extract the miRNAs following the mirVana™ miRNA Isolation Kit.

### 2.9. Database search to find miRNAs and their potential targets

The miRNAs analyzed in this study were identified by a comprehensive review of literature in PubMed by using the terms (miRNAs, atherosclerosis, inflammation, insulin resistance) and GEO Database. Once the miRNA of interest was selected, to obtain the paired miRNA_mRNAs, miRMap [39], miRanda [40], miRDB [41], TargetScan [42], and miTarBase [43] were used to find potential mRNAs-miRNAs interactions. Subsequently, the overlapping predictions between the two or more databases were considered potential target pairs.

### 2.10. Proliferation assays

Cells were plated in 12-multiwell plates and cultured in 10 % FBS–DMEM until 40–50 % of confluence. Then cells were serum starved for 4 h and treated with TNF-α (10 ng/mL), insulin (1 μM) or PA (0.4 mM) for 24 h. Some cells have been previously transfected with mimic-let-7d-5p for 48 h. Finally, the cells were stained with violet crystal as described [37].

DNA synthesis were estimated by determining BrdU incorporation. Briefly, $10^4$ cells in 1 mL of complete medium were seeded into each well of an uncoated 96-well plate. The same day, the cells were transfected with vehicle or mimic-let-7d-5p for 48 h and after that serum deprived for 5 h and stimulated with TNF-α, insulin or PA for 24 h. After that, the rate of cellular proliferation was evaluated using a cell proliferation ELISA BrdU kit (Roche Applied Science). The incubation with BrdU labeling solution was for 24 h. [37].

### 2.11. Statistical analysis

The data were analyzed using the GraphPad Prism v8.2.1 software (GraphPad Software, San Diego, CA, USA). Normality and Lognormality tests were performed to confirm that the data followed a normal distribution. Statistical significance was assessed by Student's *t*-tests when comparing two groups, or with ANOVA tests followed by a Bonferroni post-hoc test when comparing more than two groups. In some of the analyses carried out in which we have subclassified ACA patients according to the BMI, when these groups did not follow a normal distribution, we applied the Kruskal-Wallis test. The exact *p* value is indicated in each figure when it reached statistically significance ($p < 0.05$). Receiver Operating Characteristic (ROC) analyses were performed to test the diagnostic accuracy of let-7d-5p in atherosclerosis.

## 3. Results

### 3.1. Let-7d-5p levels are increased in overweight patients with ACA

We aimed to delve into the role of let-7d-5p in human atherosclerosis

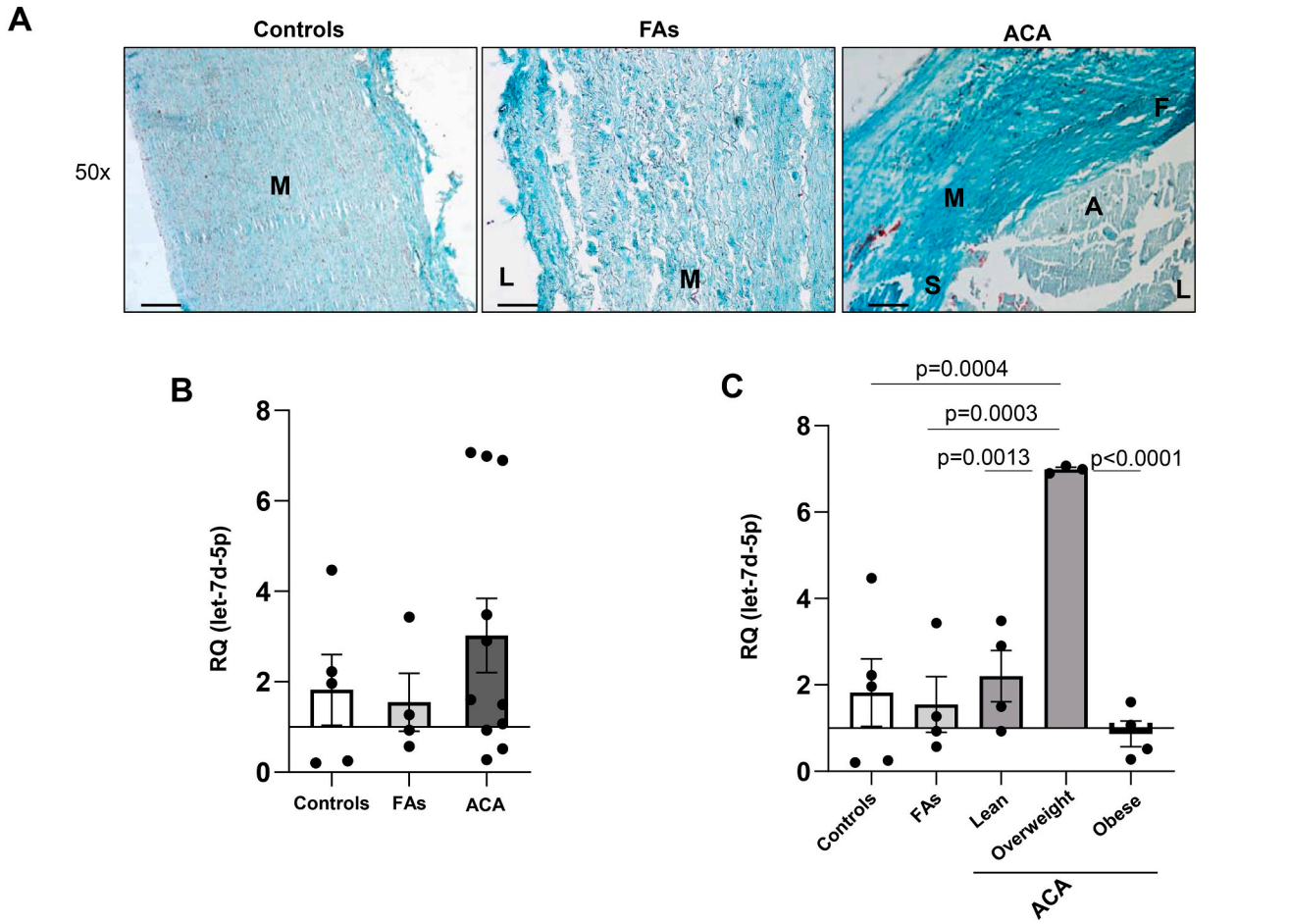


**Fig. 1.** Study of let-7d-5p levels in human atherosclerotic plaques. (A) Characterization of the human atherosclerotic plaques by Masson's Trichrome staining. Representative images of the groups at 50× (Scale bar = 100 μm) are shown. M = media; F = fibrous; S = shoulder; A = atheroma; L = lumen. (B) Relative expression of let-7d-5p in human vascular samples was measured by RT-qPCR. Amplification of miR-191-5p was used in the same reaction of all samples as an internal control. Controls = Control subjects; FAs = Fibrolipidic plaque subjects; ACA = Advanced carotid atherosclerotic plaque patients. RT-qPCR let-7d-5p: Controls ($n = 5$); FAs ($n = 4$); ACA ($n = 11$). (C) Analysis of let-7d-5p levels in ACA patients according to the BMI. RT-qPCR let-7d-5p: Controls (n = 5); FAs (n = 4); lean ACA (n = 4); overweight ACA ($n = 3$); obese ACA (n = 4).

progression. To this end, we analyzed vascular samples from control subjects (Controls), individuals with fibrolipidic plaques (FAs) and patients with advanced atherosclerosis undergoing carotid endarterectomy (ACA) (Fig. 1A). In those samples we performed Masson trichrome staining, and we could distinguish the media region in aortas from Controls and FAs and media, fibrous and shoulders in carotids from ACA (Fig. 1A). The histological analysis showed that complicated plaques from ACA contained an intraplaque hemorrhage with a higher percentage of inflammatory cells and/or a certain degree of calcification. The adjacent non-complicated regions showed a variable content of VSMCs and fibrous thickening (Fig. 1A).

In serial sections of vascular samples used for histological characterization, we isolated and analyzed let-7d-5p levels by RT-qPCR. Our results show that let-7d-5p was upregulated in ACA patients compared to samples from Controls and FAs (Fig. 1B). When we analyzed ACA samples regarding BMI, we observed that let-7d-5p levels were significantly increased in ACA overweight patients compared to ACA lean, ACA obese, FAs and Controls (Fig. 1C).

### 3.2. Let-7d-5p as potential circulating biomarker in patients with overweight and atherosclerosis

Next, to evaluate the role of let-7d-5p as possible non-invasive diagnostic biomarker for atherosclerosis, we isolated miRNAs from plasma EVs of individuals from the same study group as previously described [44].

Regarding circulating miRNA expression, we observed a significant increase in let-7d-5p levels in the ACA group compared to Controls (Fig. 2A). To further analyze our results, we separated the data obtained in ACA samples regarding the BMI. With this approach we obtained that let-7d-5p levels are only significantly increased in overweight ACA patients compared to Control subjects and ACA lean patients (Fig. 2B). To evaluate its putative role as a circulating diagnostic biomarker in atherosclerosis, we performed ROC analyses (Fig. 2C). For this miRNA, the area under the curve was 0.8045 ($p = 0.01$). The optimal cut-off value for atherosclerosis diagnosis was 1.275 or higher, with a sensitivity of 73.68 % and a specificity of 71.43 %.

To confirm these results, we used a third cohort of patients with MeS and CA ($n = 21$). We analyzed let-7d-5p levels in exosome-enriched EVs by RT-qPCR regarding the BMI. In this case, let-7d-5p levels were significantly increased in overweight patients compared to lean or obese patients (Supplemental Fig. 1).

### 3.3. Let-7d-5p regulates the expression of targets involved in insulin signalling

To determine whether the induction of let-7d-5p is involved in vascular insulin resistance during atherosclerosis, we transfected VSMCs with a mimic of let-7d-5p. A remarkable and significant increase in let-7d-5p expression was observed 48 h after transfection with the let-7d-5p mimic in VSMCs (Supplemental Fig. 2A and Fig. 3A). Next, we analyzed the expression of let-7d-5p targets involved in insulin signalling by Western Blot. Two doses of mimic-let-7d-5p, 5 and 10 nM, were analyzed and 10 nM was finally selected for the following experiments due to it induces a significant reduction of the insulin receptor (IR), IGF-I receptor (IGF-IR), AKT, mTOR and inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ) protein levels (Supplemental Fig. 2B and C).

Moreover, the cells were also transfected with an inhibitor of let-7d-5p, anti-let-7d-5p (10 nM), and we confirmed that the treatment did not alter let-7d-5p levels (Fig. 3A) and recovered the protein levels of its targets in relation to mimic-let-7d-5p treatment (Fig. 3B and C).

To demonstrate that let-7d-5p negatively regulates the expression of IR, IGF-IR and AKT by direct interaction with their mRNAs we performed experiments based on luciferase constructs containing the 3′UTRs of *INSR*, *IGF1R* and *AKT2* mRNAs (Fig. 3D-F). Our results demonstrate a direct and specific interaction of let-7d-5p with the 3′UTR of *INSR*, *IGF1R* and *AKT2* mRNAs (Fig. 3D-F).

Next, we analyzed the insulin signalling *in vitro* after transfection with mimic-let-7d-5p or anti-let-7d-5p in VSMCs. Our results show that increased levels of let-7d-5p did not alter insulin signalling in terms of AKT and p70S6K phosphorylation. However, we observed a significant decrease in p42/44MAPK phosphorylation after insulin stimulation in VSMCs with let-7d-5p overexpression (Fig. 4A and B). Moreover, anti-let-7d-5p treatment slightly increased AKT levels (Fig. 4D) but did not alter the phosphorylation of AKT and p70S6K whereas significantly diminished p42/44 phosphorylation (Fig. 4D).

### 3.4. Let-7d-5p overexpression protects against inflammation modulating proteins implicated in NF-κB pathway

Moreover, we also analyzed whether let-7d-5p could regulate the expression of targets involved in inflammation and we demonstrate that increased levels of let-7d-5p induced a statistically significant down-regulation of IKKβ (Fig. 5A). Next, we analyzed the effect of let-7d-5p overexpression on TNF-α signalling. In this case, our results show that increased levels of let-7d-5p induced a significant decrease in TNF-

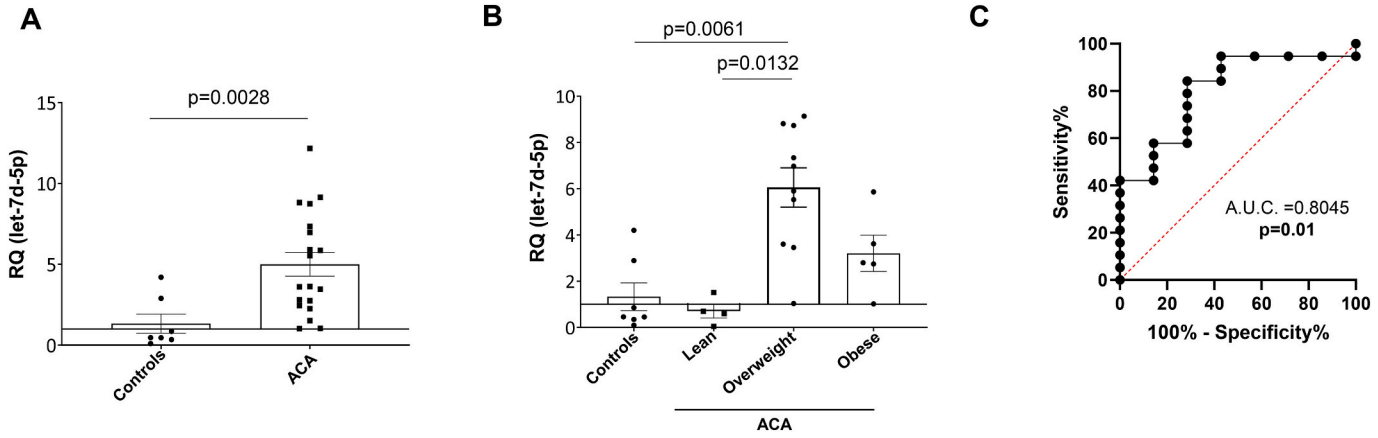


**Fig. 2.** Let-7d-5p is overexpressed in plasmatic EVs from advanced carotid atherosclerotic patients and may be a potential circulating biomarker of the disease. To test whether let-7d-5p could be a biomarker of advanced atherosclerosis, we precipitated EVs from the plasma of healthy donors and advanced carotid atherosclerosis patients. Afterwards the miRNAs were isolated from the exosomes and then the levels of let-7d-5p were analyzed by RT-qPCR, all ACA patients together (A) or separating ACA patients according to BMI (B). The validation of let-7d-5p as biomarker was confirmed by a ROC curve (C). Controls = Control subjects; ACA = Advanced carotid atherosclerosis patients; ROC = Receiver operating characteristic. RT-qPCR of let-7d-5p (A): Controls ($n = 7$), ACA ($n = 19$). RT-qPCR of let-7d-5p; (B): Controls (n = 7); lean ACA ($n = 4$); overweight ACA ($n = 10$); obese ACA ($n = 5$); (C) ROC curve let-7d-5p ($n = 26$).

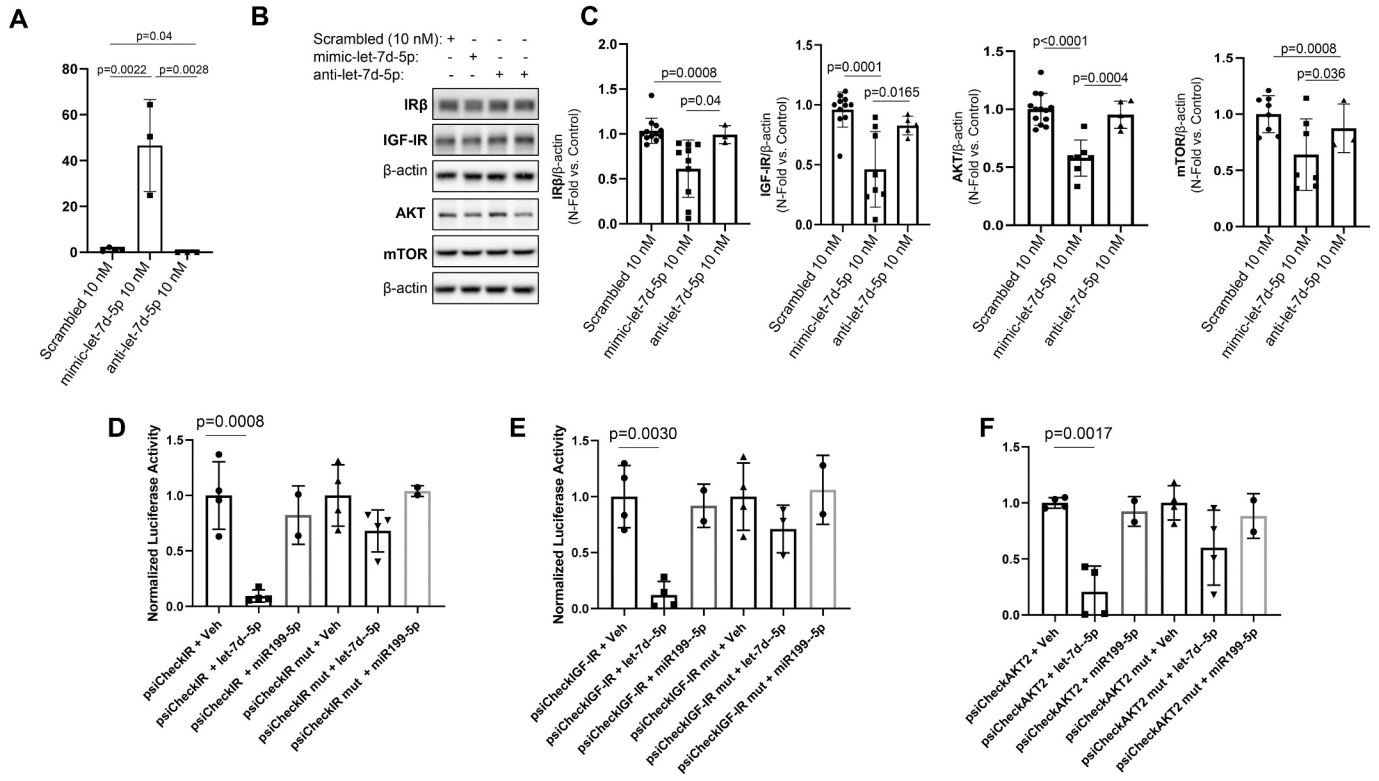


**Fig. 3.** Regulation of IR, IGF-IR and AKT by let-7d-5p. VSMCs were transfected with mimics of let-7d-5p and an inhibitor of let-7d-5p (A) for 48 h. The increase or decrease in miRNA levels was measured by RT-qPCR. The silencing effect of increased let-7d-5p on their targets IR, IGF-IR, AKT and mTOR, or the effect of a let-7d-5p inhibitor was analyzed by Western blot 48 h after transfection (B and C, images and quantifications, respectively). All the *in vitro* experiments were performed at least in triplicate. AKT = Protein kinase B; IR = Insulin Receptor; IGF-IR = Insulin-like growth factor-I receptor; mTOR = mammalian Target of Rapamycin; VSMCs = Vascular smooth muscle cells. RT-qPCR of let-7d-5p: Control ($n$ = 4); mimic-let-7d-5p (n = 4); anti-let-7d-5p ($n$ = 3). Measurement of let-7d-5p effect on IR: Control ($n$ = 11), mimic-let-7d-5p ($n$ = 10); anti-let-7d-5p (n = 3); effect on IGF-IR Control (n = 11), mimic-let-7d-5p ($n$ = 8); anti-let-7d-5p (n = 4); effect on AKT: Control (n = 10), mimic-let-7d-5p ($n$ = 5); anti-let-7d-5p (n = 5); effect on mTOR: Control (n = 8), mimic-let-7d-5p ($n$ = 7); anti-let-7d-5p (n = 3). Regulation of *INSR* (D), *IGF1R* (E) *and AKT* (F) expression by the interaction of let-7d-5p with their 3′UTR sequence. The graphs show the normalized Renilla luciferase activity in HEK293 cells transfected with the different constructs. (D) HEK293 cells were co-transfected with the psiCHECK INSR and psiCHECK INSR mut plasmids together with mimic-miR-199-5p (control) or mimic-let-7d-5p. (E) HEK293 cells were co-transfected with the psiCHECK IGF1R and psiCHECK IGF1R mut plasmids together with mimic-miR-199-5p (control) or mimic-let-7d-5p. (F) HEK293 cells were co-transfected with the psiCHECK AKT2 and psiCHECK AKT2 mut plasmids together with mimic-miR-199-5p (control) or mimic-miR-let-7d-5p. In all cases, Renilla luciferase activity was normalized to firefly luciferase activity. Bars show mean values ± SEM corresponding to 3–5 independent experiments.

α-induced NF-κB activation in terms of IKKα/β and p65 phosphorylation. Moreover, TNF-α stimulation did not induce IκB-α degradation in VSMCs overexpressing let-7d-5p (Fig. 5B). In contrast, when VSMCs were transfected with 10 nM anti-let-7d-5p for 48 h, deprived of FBS and stimulated with TNFα, p65 protein levels and its phosphorylation together with IκBα degradation was significantly increased (Fig. 5D). Finally, we confirmed some results in PC WT VSMCs, TNF-α induced a significant decrease in IκBα protein levels whereas let-7d-5p overexpression avoided its degradation and let-7d-5p inhibition did not avoid it (Fig. 5E).

### 3.5. Let-7d-5p reduces VSMCs proliferation

Finally, we analyzed whether let-7d-5p was involved in the proliferation induced by TNF-α, insulin and PA in VSMCs by performing two different approaches, violet crystal staining and BrdU incorporation. Our results obtained by violet crystal staining demonstrate that let-7d-5p overexpression was able to inhibit the proliferation induced by TNF-α and PA. However, we did not observe any change in insulin-stimulated cells (Fig. 6A). The BrdU incorporation assays revealed the same behavior in VSMCs stimulated with TNF-α and PA in which increased levels of let-7d-5p were able to inhibit such proliferation. In the case of insulin-induced VSMCs proliferation, increased let-7d-5p levels did not exert any effect (Fig. 6B and C).

## 4. Discussion

CVDs are one of the major clinical problems worldwide, with atherosclerosis being one of the main causes [1–3]. New biomarkers are necessary to help to identify the presence of vulnerable atherosclerotic plaques and avoid the progression of the disease and the development of acute events. In this context, miRNAs might be implicated in the progression of atherosclerosis due to each miRNA is able to regulate multiple targets involved in several processes related to plaque instability. The current study focused on let-7d-5p, a member of the let-7 family within the let-7a-1/let-7f-1/let-7d cluster located on chromosome 9q22.3 in the human genome (GenBank, NCBI database). In this sense, we propose that let-7d-5p might have a protective role in the inflammation and stenosis of atherosclerotic plaque, mainly in overweight patients.

Firstly, we analyzed the levels of let-7d-5p in patients with atherosclerosis and healthy subjects. We observed an increase of let-7d-5p levels in carotid plaques of ACA patients in relation to subjects without atherosclerosis or with fibrolipidic plaques. In line with these results, it had previously been published that in an experimental model of atherosclerosis in mice there was a significant increase in let-7d-5p levels, identified as a key node along with miR-378a-5p and miR-30c [44].

Secondly, miRNAs can also be found circulating in plasma, inside

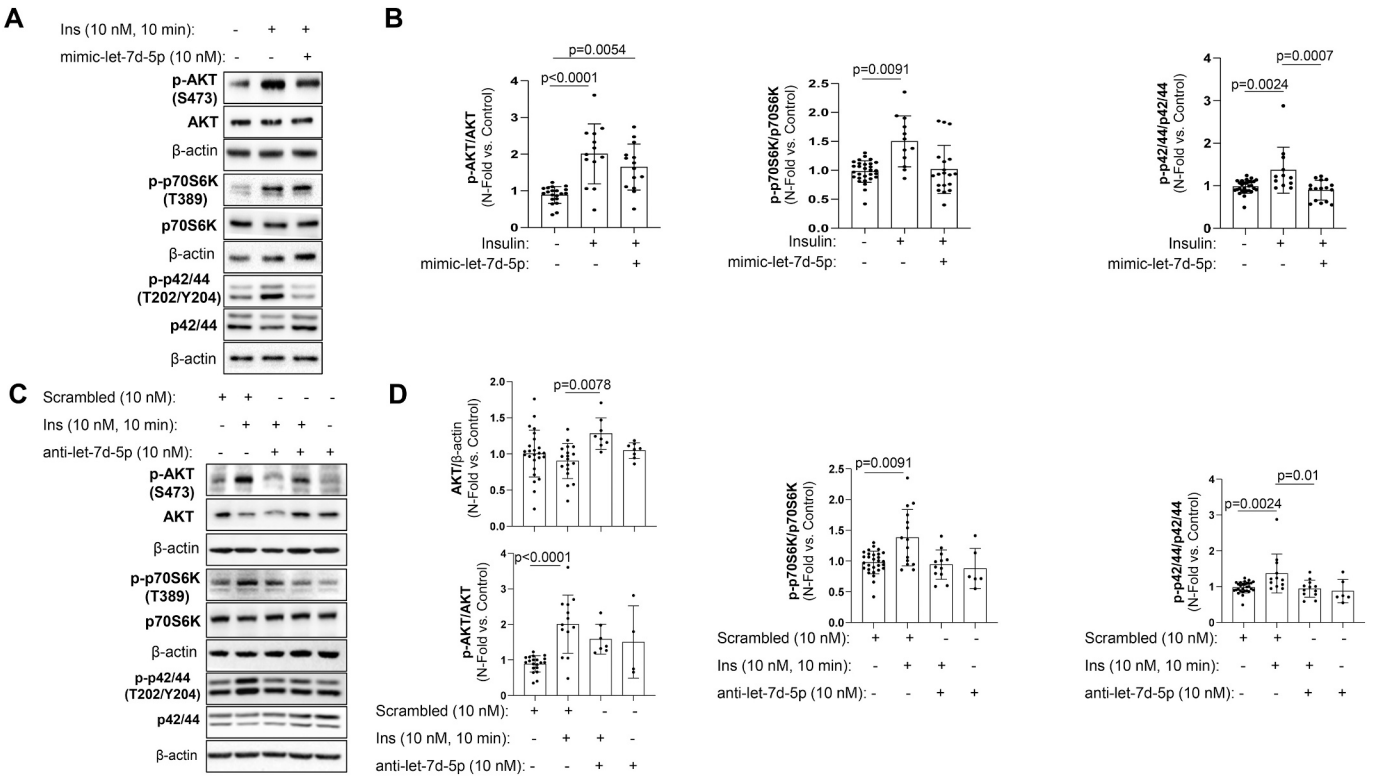


**Fig. 4.** Effect of let-7d-5p on insulin signalling in VSMCs. To analyze the role of let-7d-5p in insulin signalling of VSMCs, the cells were transfected with 10 nM mimic-let-7d-5p or 10 nM anti-let-7d-5p for 48 h and then they were deprived of FBS for 1 h and finally, the cells were stimulated with 10 nM insulin for 10 min. Representative images of Western blot (A and C) and their respective quantifications (B and D) of p-AKT/AKT, p-p70S6K/p70S6K and p-p42/44MAPK/p42/44MAPK. Bars show mean values ± SEM corresponding to 4–16 independent experiments.

EVs [45,46]. Thus, we wanted to isolate EVs from healthy subjects and ACA patients in order to show that let-7d-5p was also found in EVs and whether their levels fluctuated in atherosclerosis. The data obtained allow us to infer a significant increase in let-7d-5p levels in ACA patients compared to healthy subjects, as well as overweight ACA patients compared to obese and lean ACA patients, and healthy subjects. Moreover, we have confirmed in another cohort of patients an increase in let-7d-5p in EVs from overweight patients with MeS and *CA*. Our group had previously demonstrated increased levels of circulating let-7d-5p in patients with NAFLD [47]. In the same manner, it was recently published that let-7d-5p along with miR-503-5p, miR-106b-3p and miR-93-5p levels were significantly upregulated in plasma from patients with type 1 diabetes (T1D) with carotid calcified plaque compared with T1D patients with carotid fibrous plaque [48]. Therefore, we decided to carry out a ROC study to check if let-7d-5p could be postulated as a non-invasive biomarker of atherosclerosis, and the results obtained in the curve indicate that it may be a potential diagnostic biomarker of the disease. These results, although obtained in two different cohorts of patients, have the limitation of the small sample size and would have to be confirmed in other cohorts with a higher number of subjects per group.

On the other hand, we analyzed the levels of let-7d-5p both in vascular and plasma samples and observed that the most significant increase was found in patients with overweight in relation to lean patients and those who were obese. In this context, it would make sense that an initial situation of low-grade inflammation and an increase in the lipid profile, triglycerides and oxidized-LDLs, as occurs in overweight ACA patients, could induce an increase in let-7d-5p levels as a protective compensatory mechanism and it would fail in a situation of obesity. In this sense, it has been described that the induction of let-7d-5p, and repression of its target HMGA2 in macrophages, is a protective response to the challenge of increased cholesterol influx into these cells; dysregulation of this response as it happens in obesity may contribute to atherosclerosis [49]. In addition, other study has also demonstrated a significant increase of let-7d-5p in overweight patients with obstructive sleep apnea-hypopnea syndrome and arterial hypertension [50].

Regarding VSMCs, there is evidence of their importance during the atherosclerotic process due to the change in phenotype they undergo, losing contractile capacity and capturing oxidized-LDL in a similar way to macrophages, transforming into foam cells. This change is induced by the accumulation of lipoproteins in the plaque and the inflammatory environment, which leads to changes in signalling pathways involved in insulin signalling, inflammation [51], death [52,53] or cell proliferation [52]. In relation to insulin signalling in VSMCs, we analyzed different targets of let-7d-5p: IR, IGF-IR, AKT and mTOR. Firstly, we confirmed that let-7d-5p directly regulates IR, IGF-IR and AKT. However, let-7d-5p overexpression only affected to p42/44MAPK phosphorylation whereas AKT and p70S6K activation was not modified. In this sense, a significant increase of let-7d-5p maintains partially insulin signalling and globally fails to reduce insulin-induced proliferation in VSMCs. This result could be due to insulin needs to activate both, the PI3K/AKT and p42/44MAPK signalling pathways, to up-regulate proliferation of VSMCs [54]. Finally, IGF-IR activation also induces the proliferation of VSMCs, in fact, it has been described that the decrease in their levels mediated by

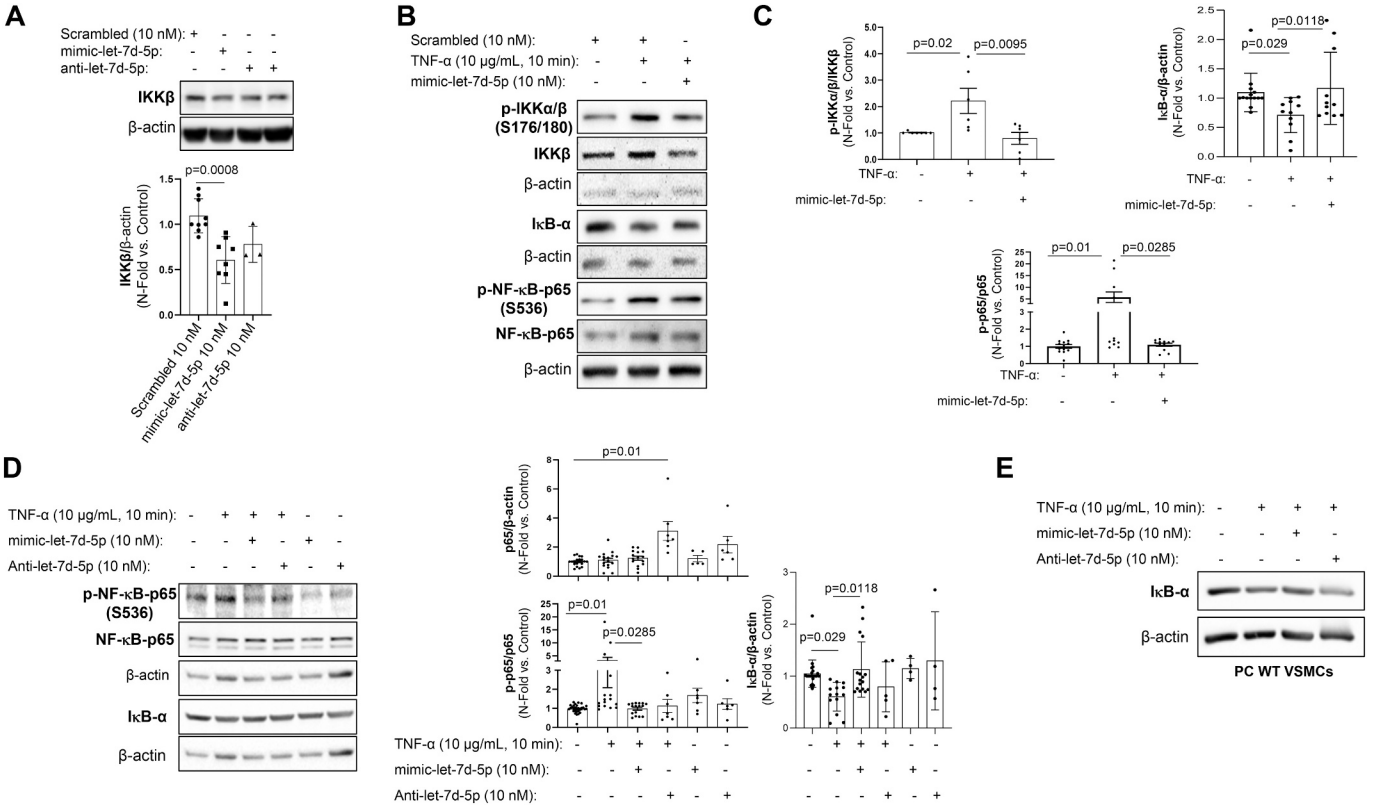


**Fig. 5.** Effect of let-7d-5p on NF-κB pathway in VSMCs. (A) The effect of mimic-let-7d-5p and anti-let-7d-5p on IKKβ was analyzed by Western blot. Bars show mean values ± SEM corresponding to 4–6 independent experiments. (B - D) The activation of NF-κB pathway was analyzed measuring the activation of IKKβ and p65. For it, VSMCs were transfected by mimic-let-7d-5p or anti-let-7d-5p for 48 h, then they were deprived of FBS for 1 h and finally, they were treated with 10 ng/mL TNF-α for 10 min. We analyzed both proteins on their phosphorylated forms, and the expression of IκBα by Western blot. (E) PC WT VSMCs were transfected with 10 nM mimic-let-7d-5p or 10 nM anti-let-7d-5p for 48 h, then they were deprived of FBS for 1 h and, finally were treated with 10 ng/mL TNF-α for 10 min. We analyzed the expression of IκBα by Western blot. Bars show mean values ± SEM corresponding to 4–6 independent experiments.

miR-1 inhibits the proliferation of these cells [55].

Another of the studied potential targets of let-7d-5p has been IKKβ for its role in the activation of NF-κB and in atherosclerosis progression [38,56]. Phosphorylation and activation of IKKβ induces phosphorylation and degradation of the inhibitor IκBα. Consequently, it triggers the release and activation of NF-κB in VSMCs, increasing inflammatory damage and inducing the worsening of the atherosclerotic lesion [38,56]. So, decreased phosphorylation of p65 and IKKβ as well as increased IκBα levels decreases NF-κB activation [56], which has been shown *in vitro* to mitigate the inflammatory damage in VSMCs [57]. In addition, exosomes derived from mesenchymal stem cells (enriched in let-7d family) ameliorated atherosclerosis in ApoE$^{-/-}$ mice and promoted M2 macrophage polarization in the plaque through miR-let7/HMGA2/NF-κB pathway [58]. According to these results, in this work we have shown that let-7d-5p overexpression significantly reduced IKKβ expression and phosphorylation as well as p65 phosphorylation leading to lower NF-κB activation in VSMCs. Therefore, we postulate let-7d-5p as an anti-inflammatory and atheroprotective miRNA.

VSMCs proliferation provokes plaque growth as a key factor in vessel stenosis. In the current work, we assess VSMCs proliferation in response to different stimuli such as hyperinsulinemia, a proinflammatory cytokine such as TNF-α and a saturated fatty acid such as PA. Our group had previously shown that all three stimuli induced VSMCs proliferation [37,59]. Our results clearly demonstrate that let-7d-5p overexpression significantly reduced the VSMCs proliferation induced by PA possibly by NF-κB modulation. In according to our results, it had previously published that let-7d overexpression decreased KRAS expression and the inhibition of VSMC proliferation [60] as well as E2F5 inhibited the progression of Gallbladder cancer and activated JAK2/STAT3 signalling [61].

## 5. Conclusions

In conclusion, this study postulates let-7d-5p as a possible atheroprotective miRNA since, the raising of its levels in overweight patients may be an attempt to compensate for the inflammation that occurs in the initial and intermediate stages of the disease [55]. However, in a situation of obesity where there is a very exacerbated inflammatory process, the levels of let-7d-5p decrease and this protective effect is lost. In this sense, the existence of other athero- and cardioprotective miRNAs within the let-7 miRNA family has been previously demonstrated [36,59].

The anti-inflammatory role of let-7d-5p can therefore be proposed as a cellular defense mechanism for the inhibition of phenotypic change of VSMCs, since the inflammatory environment promotes this change and the further advance of atherosclerotic damage [50]. Therefore, let-7d-5p exerts a protective role by modulating the inflammation and proliferation of VSMCs.

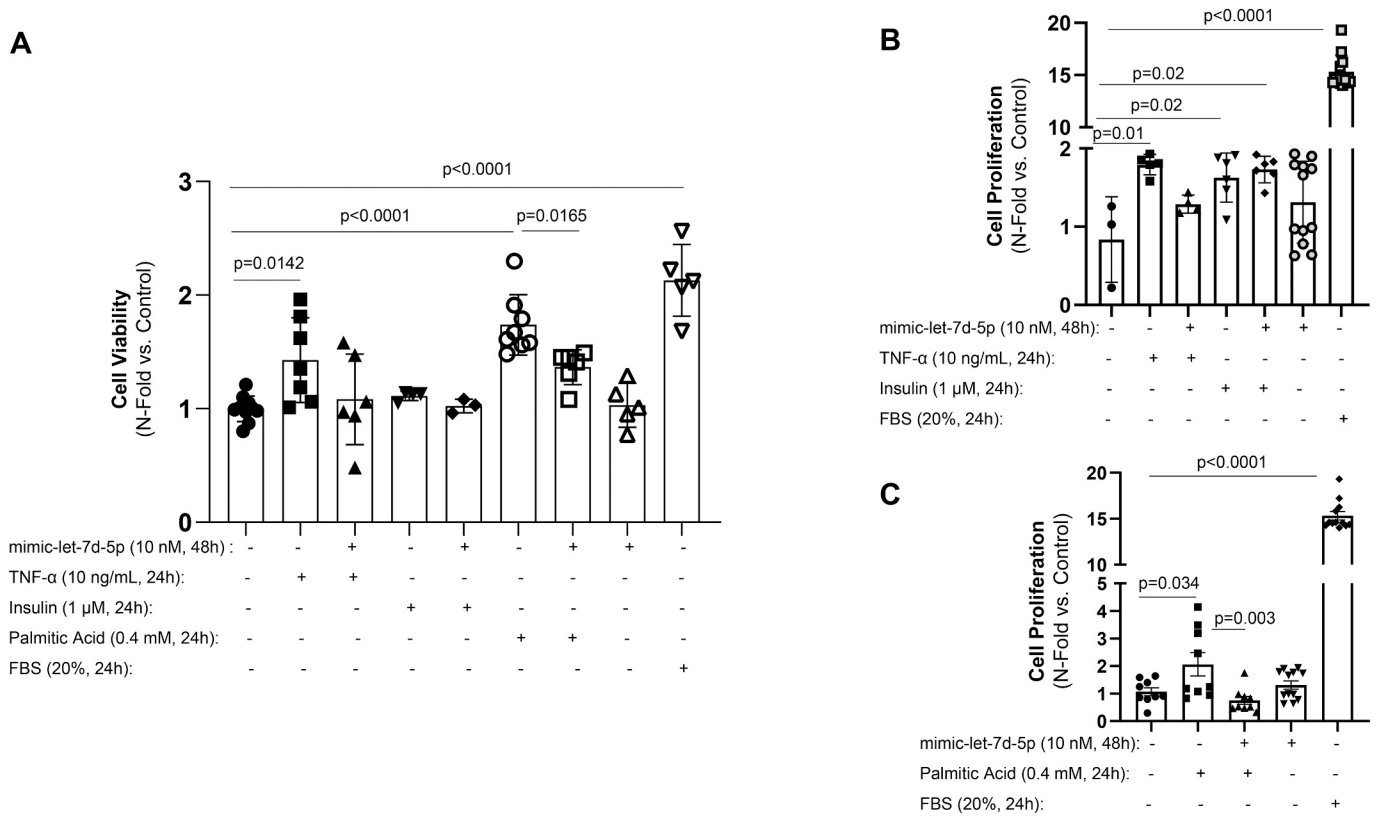


**Fig. 6.** Effect of let-7d-5p overexpression in VSMCs proliferation. (A) Cell viability and cell proliferation studies were performed in VSMCs. The cells were cultured to 50 % confluence in 10 % FBS–DMEM overnight, then, cells were transfected with mimic-let-7d-5p for 48 h and after serum starved for 5 h and after that, 1000 nM insulin, 10 ng/mL TNF-α or 0.4 mM PA were added to the wells in serum-starved 5 mmol/L glucose DMEM. After 24 h, the medium was withdrawn, and the cell lines were stained with violet crystal as described in Section 2. Rates of proliferation measured by BrdU incorporation in response to insulin (B), TNF-α (B), or PA (C) in presence or absence of let-7d-5p overexpression. Results are expressed as mean ± SEM from five independent experiments performed in duplicate.

Finally, our results propose let-7d-5p as a diagnostic biomarker of advanced atherosclerosis and in overweight patients with MeS and carotid atherosclerosis.

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## CRediT authorship contribution statement

**Javier Aroca-Esteban:** Methodology, Investigation, Data curation, Writing – review & editing. **Francisco V. Souza-Neto:** Data curation, Investigation, Methodology, Writing – review & editing. **Carlota Aguilar-Latorre:** Data curation, Investigation, Methodology, Writing – review & editing. **Alba Tribaldo-Torralbo:** Data curation, Investigation, Methodology, Writing – review & editing. **Paula González-López:** Methodology, Investigation, Data curation, Writing – review & editing. **Rubén Ruiz-Simón:** Methodology, Investigation, Data curation, Writing – review & editing. **Marta Álvarez-Villareal:** Methodology, Investigation, Data curation. **Sandra Ballesteros:** Methodology, Investigation, Data curation. **Melina Vega de Ceniga:** Investigation, Conceptualization, Resources. **Pedro Landete:** Investigation, Conceptualization, Resources. **Águeda González-Rodríguez:** Investigation, Conceptualization, Resources. **José L. Martín-Ventura:** Writing – review & editing, Methodology, Investigation. **Natalia de las Heras:** Writing – review & editing, Methodology, Investigation. **Óscar Escribano:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Almudena Gómez-Hernández:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

## Declaration of competing interest

The authors declare no conflict of interest.

## Data availability

The datasets used and/or analyzed during the current study are available from the corresponding authors (algomezh@ucm.es or oescriba@ucm.es) on reasonable request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2024.167327.

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