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Cigarette Smoke Exposure and microRNA in Human Alveolar Macrophages: A Pilot Study

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Cigarette Smoke Exposure and microRNA in Human Alveolar Macrophages: A Pilot Study

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Abstract: Cigarette smoke (CS) is a major driver of many respiratory diseases including chronic obstructive pulmonary disease (COPD) and non-small cell lung cancer (NSCLC). Tobacco causes oxidative stress, impaired phagocytosis of alveolar macrophages (AMs), and alterations in gene expression in the lungs of smokers. MicroRNAs (miRNAs) are small non-coding RNAs that influence several biological processes and interfere with several regulatory pathways. The purpose of this study was to assess the effect of active CS on miRNAs expression in AMs obtained from bronchoalveolar lavage (BAL) of ever- or never-smoker subjects and patients with COPD or NSCLC. BAL specimens were collected from 43 sex-matched subjects to determine the expression of has-miR-34a-5p, 17-5p, 16-5p, 106a-5p, 223-5p, and 20a-5p before and after in vitro CS exposure by RT-PCR. In addition, bioinformatic analysis of miRNAs target genes linked to inflammation was performed. Distinct and common miRNA expression profiles were identified in response to CS, suggesting their possible role in smoking-related diseases. It is noteworthy that, following exposure to CS, the expression levels of hsa-miR-34a-5p and 17-5p in both ever- or never-smokers, 106a-5p in never-smokers and 20a-5p in ever-smokers, shifted towards those found in individuals with COPD, suggesting them like a risk factor in developing this lung condition. Moreover, we identified miRNA targets involved in the immune system or AMs property regulation using in silico analysis. In conclusion, our study identified miRNA signatures in AMs exposed to CS, indicating that CS is an important driver of epigenetic changes that contribute to the onset of various lung diseases.

Keywords: microRNAs; alveolar macrophages; cigarette smoke; COPD; lung cancer

1. Introduction

Cigarette smoke (CS) is the leading cause of preventable deaths worldwide and is commonly considered a major driver of many respiratory diseases [1]. Many epidemiological studies have established a high burden of diseases resulting from smoking, including non-small cells lung cancer (NSCLC) and chronic obstructive pulmonary disease (COPD) [2,3]. COPD is an heterogenous disease characterized by progressive deterioration of lung function over time and is generally associated with lung inflammation triggered by harmful particles or gases [4–10]. COPD and lung cancer, beyond a

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common etiology, are closely linked conditions, and patients with COPD have twice the risk of cancer diagnosis [12–16]. In the lungs of smokers, tobacco promotes oxidative stress and systemic /local inflammation, which is characterized by the upregulation of circulating inflammatory cells and release of inflammatory mediators [17–19]. In addition, CS has been shown to lead to genetic and molecular impairments, which may increase the chance of mutations and lung carcinogenesis [20]. Moreover, it is becoming increasingly evident that the development of COPD or NSCLC phenotypes in response to harmful agents is regulated by both the innate and adaptive immune systems. [20–22]. Alveolar macrophages (AMs) are essential effector cells that play a key role in the innate lung immune system by performing pathogen clearance, recruiting other immune cells, phagocyting and processing inhaled environmental particles, and producing pro-inflammatory mediators [23–27]. Smoking causes AMs impairment in phagocytosis and responses to pathogens, compromising their protection from noxious agents [28–30]. Importantly, AMs gene expression can be altered in response to environmental exposure, leading to epigenetic changes such as DNA methylation, covalent histone modifications, and microRNAs (miRNA) expression [31–33]. miRNAs are small non-coding endogenous RNA molecules capable of modulating gene expression by binding their target mRNAs at 3' end and, leading to gene silencing through mRNA cleavage or translational repression. miRNAs influence most biological processes and interfere with several regulatory networks, thereby coordinating gene expression under pathological conditions [34–36]. Indeed, aberrant miRNA expression appears to be a signature of human diseases, including tumors and inflammatory lung diseases [37,38]. In our previous study, we reported that has-miR-34a-5p, 17-5p, 16-5p, 106a-5p, 223-5p, and 20a-5p expression profiles in AMs are dysregulated in NSCLC, COPD and ever- or never-smoker controls, suggesting their potential role as an index of the smoking-related disease microenvironment [39]. Notably, all selected miRNAs have been shown to influence processes related to inflammation, carcinogenesis or immunity, which are closely linked to CS [40–46]. However, despite the known association between CS and lung diseases, little is known about the effect of active smoking on the expression levels of miRNAs in AMs, and how it affects the identification of potential candidate miRNAs as biomarkers of pulmonary conditions. Therefore, to further assess the role of CS in the regulation of AMs miRNA expression, we evaluated the levels of the above mentioned-miRNAs in AMs of bronchoalveolar lavage (BAL) from ever- or never-smoker controls and patients with COPD or NSCLC before and after CS exposure.

2. Materials and Methods

2.1. Ethics Statement

This study belongs to a cross-sectional nonpharmacological clinical study recorded at clinicaltrials.gov (NCT04654104) and all procedures and protocols described were approved by the local Ethics Committee "Calabria Centro". The criteria of the Institutional Review Board/Human Subjects Research Committee, the Declaration of Helsinki, and the Guidelines for Good Clinical Practice were followed and, all patients or legal guardians signed an informed consent form prior of the beginning of the study.

2.2. Study Population

We enrolled 43 individuals who were equally distributed in terms of age (≥18 years) and sex, at the "Mater Domini" Hospital in Catanzaro, Italy. All participants underwent spirometry in compliance to international guidelines as well as bronchoscopy and BAL for suspected pulmonary neoplasia [47]. Samples that were not employed for histopathological purposes or in our previous research were used in the current study 8 [39]. Based on the clinical data and the pathological diagnosis obtained after bronchoscopy, we divided the enrolled subjects into:1) healthy never-smoker control ("HNS"; n = 9); 2) healthy ever-smokers control ("HS"; n = 11); 3) smokers with Global Initiative for Obstructive Lung Diseases (GOLD) stage 1–4 ("COPD," n =11); 4) non-small cell lung cancer ("NSCLC"; n=12). The main clinical and pathological characteristics of the cohorts are reported in our previous study and are available in the online version, at

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https://doi.org/10.3390/biomedicines12051050. In summary, those who had lung infections, extrapulmonary tumors, airflow obstruction other than COPD, autoimmune disorders, or who did not sign the informed consent form were excluded. All enrolled subjects were smokers except for HNS group; specifically, HS were 8 current and 3 former smokers, COPD were 11 current smokers and NSCLC were equally distributed between current and ex-smokers. Within each group, the subjects were comparable in terms of age, sex, and lung cancer histology. Indeed, only those with NSCLC were enrolled among the subject's presenting cancer. The most frequent comorbidities were hypertension ($p < 0.05$), and the most used drugs were bronchodilators ($p < 0.0001$).

2.3. Bronchoalveolar Lavage and AMs Extraction

After obtaining informed consent, the subjects underwent standard flexible bronchoscopy for clinical indications [47]. Premedication and local anesthesia were followed by BAL with 200 ml of sterile isotonic saline solution (37 °C) in the right middle lobe. Specifically, BAL was obtained by instilling 50 ml up to four times, as previously reported [39]. The samples were filtered through sterile gauze and centrifuged at 400 g for 10 min at 4 C° to pellet cellular material. The cells were washed, resuspended in buffer phosphate saline (PBS), and counted in a Bürker chamber. The cell yield was determined as the total cells /total volume obtained for each saline installation. Then, cell viability was determined by Trypan blue exclusion assay, and differential cell count was performed with QUICK-DIFF staining; at least 100 cells were counted.

2.4. Preparation of CS Extract

CS extract was prepared as previously described bubbling ten Red Marlboro cigarettes (Phillip Morris; Cracow, Poland) without a filter through 250 ml of serum-free RPMI with a customized vacuum pump apparatus. The obtained suspension was adjusted to pH 7.4 and filtered through a 0.20 μm pore filter to remove bacteria and large particles [19].

2.5. TH-P1 Culture and Cytotoxicity Assay

Macrophages from acute monocytic leukemia (THP-1) ware used as a pilot model to establish the exact dose (2%, 5%, or 10%) of CS that was able to affect cell viability at 24h using the Thiazolyl Blue Tetrazolium Bromide solution (MTT) assay. THP-1 cells (ATCC® TIB-202™), purchased from the American Type Culture Collection (Manassas, Virginia, USA), were maintained at 2×10^5 cells/ml in RPMI-1640 medium containing 10% FBS and 2 mM L-glutamine, 200 U/ml penicillin, and 200 mg/ml streptomycin. To obtain a macrophages-like phenotype, THP-1 cells were treated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for two days. The cells were then incubated with fresh medium for one day to allow cell recovery and exposed to 2%, 5%, or 10% CS medium for 24h. Following 24 h MTT was added and incubated for 4 h to perform the proliferation assay. MTT is a colorimetric method that allow to assess the mitochondrial reductive function as an indicator of growth inhibition. After 4 h, DMSO was added to measure the absorbance at 595 nm using a microplate reader.

2.6. AMs Culture and CS Exposure

BAL cell pellets were suspended in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 200 U/ml penicillin, and 200 mg/ml streptomycin. The cell suspension was added at 0.5×10^6 cells/mL to a 75 tissue culture flask and maintained at 37 °C in a 5% CO2 humidified milieu for 2 h to allow AMs adherence. Lymphocytes, red blood cells and other non-adherent cells were removed by washing several times with PBS. AMs purity, as determined by morphology, was greater than 95%. The AMs were then exposed to 10% CS for 24 h, based on THP-1 treatments results.

2.7. Biochemistry Assays and Real Time PCR (RT-PCR)

The extraction of miRNAs in AMs obtained from BAL was carried out through the miRNeasy mini kit and RNA was eluted at a volume of 15 μL, as previously described 37). RNA degradation

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was assessed using a qubit RNA Integrity and Quality (IQ) assay (catalog number Q33222) with a Qubit 4 fluorometer (serial number 2322618032114). The expression levels of has-miR-34a-5p, 17-5p, 16-5p, 106a-5p 223-5p and 20a-5p were determined using TaqMan™ Advanced miRNA Assay RT-PCR, following Thermo Fisher Scientific procedures (Waltham, MA, USA), with U6 snRNA as the housekeeping miRNA as previously described [39]. Nine biological replicates for the HNS group, eleven for HS, eleven for COPD, and twelve for NSCLC were analyzed, and all samples were run in triplicate; after the achievement of the RT-PCR, the cycle threshold (Ct) of the reactions was determined. Data from all RT–PCR experiments and miRNA expression was analyzed applying the comparative and normalizing to the endogenous miRNA control 2^{-DDCt} method, where $DCt = Ct_{miRNA} - Ct_{housekeeping\ miRNA}$, whereas the relative differences in expression was determined with $DDCt = DCt_{HS/COPD/NSCLC\ (with\ or\ without\ CS)} - DCt_{HNS}$.

2.8. In Silico Prediction of hsa-miRs Target Genes

mRNA targets of has-miR-34a-5p, 17-5p, 16-5p, 106a-5p 223-5p and 20a-5p that are linked to inflammation or AMs properties were analyzed in silico using DIANA Tools (http://diana.imis.athena-innovation.gr/DianaTools/index.php) and miRTargetLink 2.0 (https://ccb-compute.cs.uni-saarland.de/mirtargetlink2) databases. Only genes that were already validated experimentally were selected and potential biochemical pathways were checked using the GeneCard database (https://www.genecards.org/).

2.9. Statistical Analysis

Unless specified, all data are expressed as mean ± standard deviation (SD). The ordinary one-way ANOVA test followed by Dunnet Multiple Comparison Test (for MTT assay) and Tukey Multiple comparison test (for miRNAs analysis) with a single pooled variance, was used to assess the differences between the groups. Nominal (sex, age, comorbidity, or treatment) and categorical variables were considered and the correlation between clinical data was calculated using one-way ANOVA followed by Tukey Multiple Comparison Test. GraphPad software (version 9.1.0) was used for statistical analyses (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. CS Effect on TH-P1 Viability

To determine the dose of CS for subsequent analysis, we used THP-1 macrophage cell line. Treatment of THP-1 cells with CS for 24h significantly affect cell viability, which peaked at 10% CS ($p < 0.0001$) (Figure 1). Therefore, 24h 10% CS was used to perform AMs exposure.

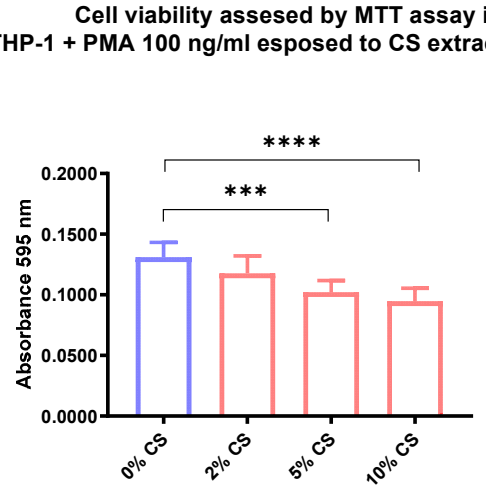

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Figure 1. THP-1 cells viability after CS exposure. THP-1 cells were treated with CS at 24h for the indicated concentration, and the cell viability was assessed by MTT assay. Cell viability is shown as absorbance at 595 nm. All samples were run in triplicate, and results are shown as means ± SD. The statistical tests used in these analyses were one-way analysis of variance followed by Dunnet Multiple Comparison Test. *** $p < 0.001$, **** $p < 0.0001$.

3.2. miRNA Expression Levels

hsa-miR-34a-5p, 17-5p and 16-5p

We assessed miRNA signatures in each pathological condition (NSCLC and COPD), smoking habit (HS), and control group (HNS) before and after exposure to 10% CS for 24h. The effects of CS in vitro stimulation on hsa-miR-34a-5p, 17-5p, and 16-5p expression in BAL AMs in vitro are shown in Figures 2–4. Following stimulation with 10% CS for 24 h, we observed a significant increase in hsa-miR-34a-5p ($p < 0.01$), 17-5p ($p < 0.001$), and 16-5p ($p < 0.001$) expression in AMs obtained from HNS group (Figures 2A–4A). Interestingly, acute in vitro CS stimulation also led to significant positive modulation of hsa-miR-34a-5p ($p < 0.001$), 17-5p ($p < 0.001$), and 16-5p ($p < 0.001$) expression in AMs from HS (Figures 2A–4B). In contrast, CS stimulation of COPD and NSCLC AMs did not affect hsa-miR expression (Figures 2C,D–4C,D). This could suggest that acute CS stimulation is sufficient to affect hsa-miR expression exclusively in AMs from subjects without preexisting lung diseases.

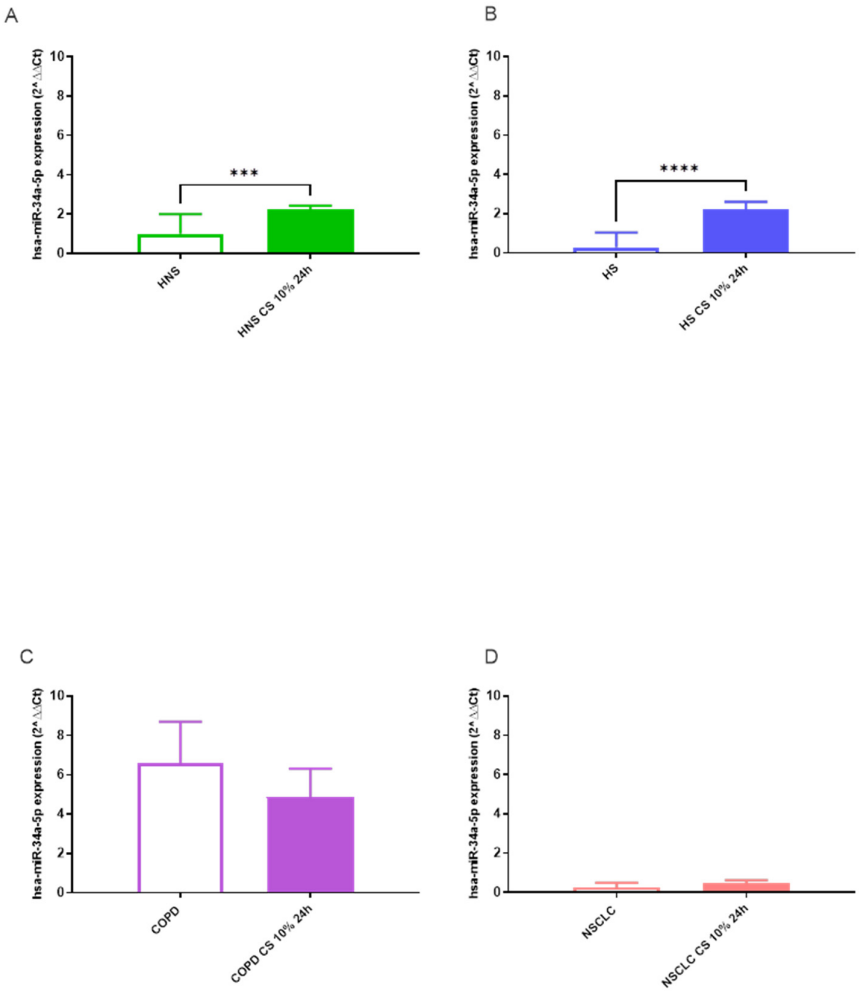

Figure 2. Analysis of hsa-miR-34a-5p AMs expression levels in HNS (2A; biological replicates n = 9), HS (2B; biological replicates n = 11), COPD (2C; biological replicates n = 11) and NSCLC (2D; biological

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replicates n = 12) before and after 10% CS at 24 h. AMs from BAL all groups were treated with CS at 10% for 24 h and the expression of hsa-miR-34a-5p was assessed by real-time RT-PCR. All samples were run in triplicate, and results are shown as means ± SD. The statistical tests used in these analyses were one-way analysis of variance followed by Tukey Multiple Comparison Test. ** $p < 0.01$, **** $p < 0.0001$.

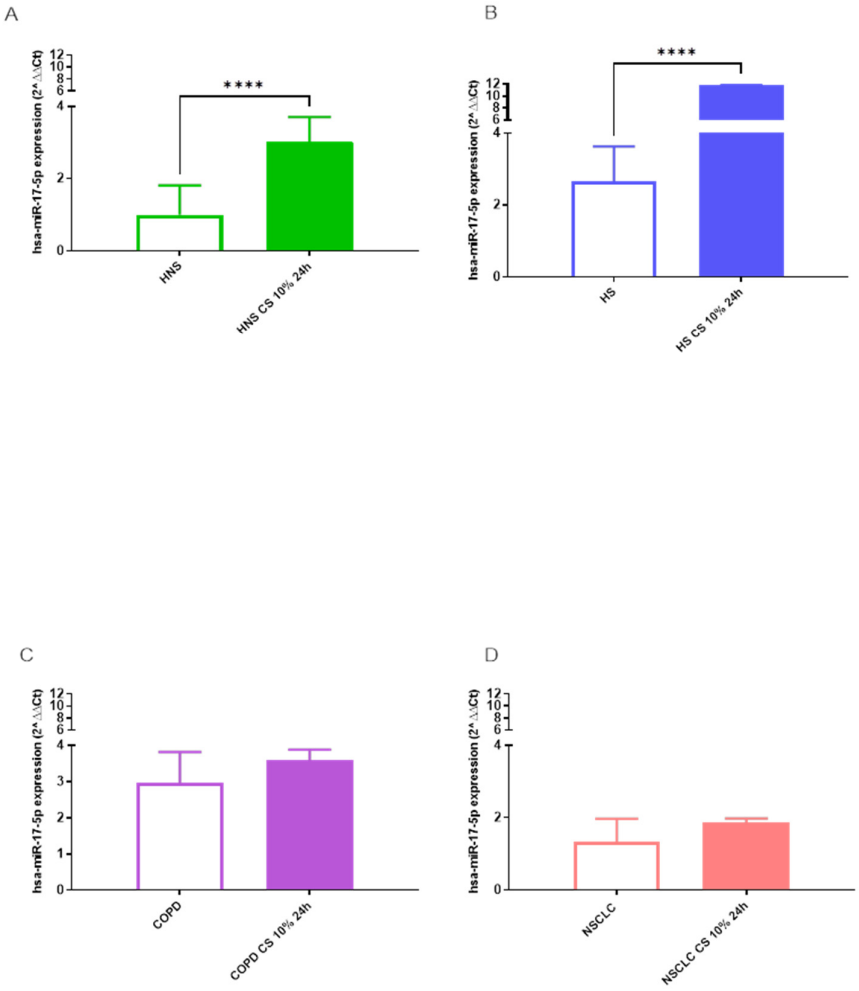

Figure 3. Analysis of hsa-miR-17-5p AMs expression levels in HNS (2A; biological replicates n = 9), HS (2B; biological replicates n = 11), COPD (2C; biological replicates n = 11) and NSCLC (2D; biological replicates n = 12) before and after 10% CS at 24 h. AMs from BAL all groups were treated with CS at 10% for 24 h and the expression of hsa-miR-17-5p was assessed by real-time RT-PCR. All samples were run in triplicate, and results are shown as means ± SD. The statistical tests used in these analyses were one-way analysis of variance followed by Tukey Multiple Comparison Test. *** $p < 0.001$, **** $p < 0.0001$.

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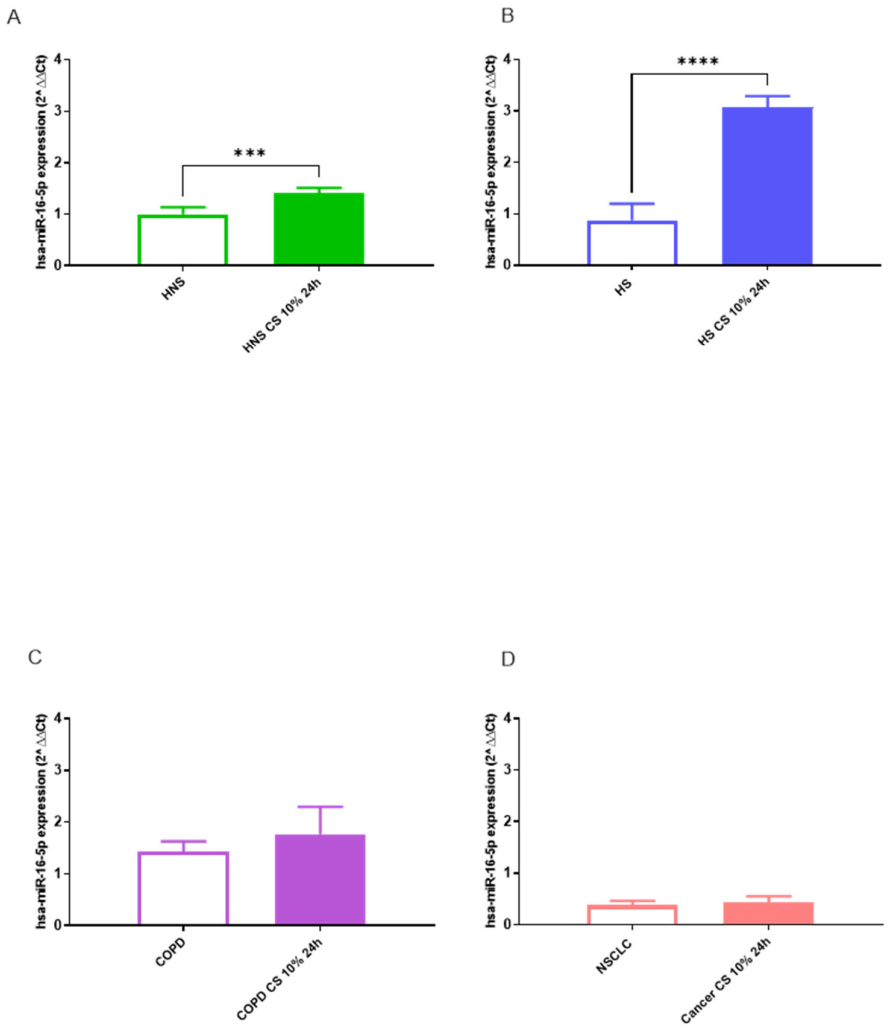

Figure 4. Analysis of hsa-miR-16-5p AMs expression levels in HNS (2A; biological replicates n = 9), HS (2B; biological replicates n = 11), COPD (2C; biological replicates n = 11) and NSCLC (2D; biological replicates n = 12) before and after 10% CS at 24 h. AMs from BAL all groups were treated with CS at 10% for 24 h and the expression of hsa-miR-16-5p was assessed by real-time RT-PCR. All samples were run in triplicate, and results are shown as means ± SD. The statistical tests used in these analyses were one-way analysis of variance followed by Tukey Multiple Comparison Test. *** $p < 0.001$, **** $p < 0.0001$.

hsa-miR-106a-5p

Following in vitro exposure to 10% CS for 24 h, we observed a significant increase in hsa-miR-106a-5p expression in HNS AMs ($p < 0.01$) (Figure 5A) and in AMs from the COPD group ($p < 0.05$) (Figure 5C). Interestingly, CS led to the opposite trend in AMs from HS ($p < 0.01$) (Figure 5B) while it did not affect hsa-miR-106a-5p expression in AMs obtained from patients with NSCLC (Figure 5D).

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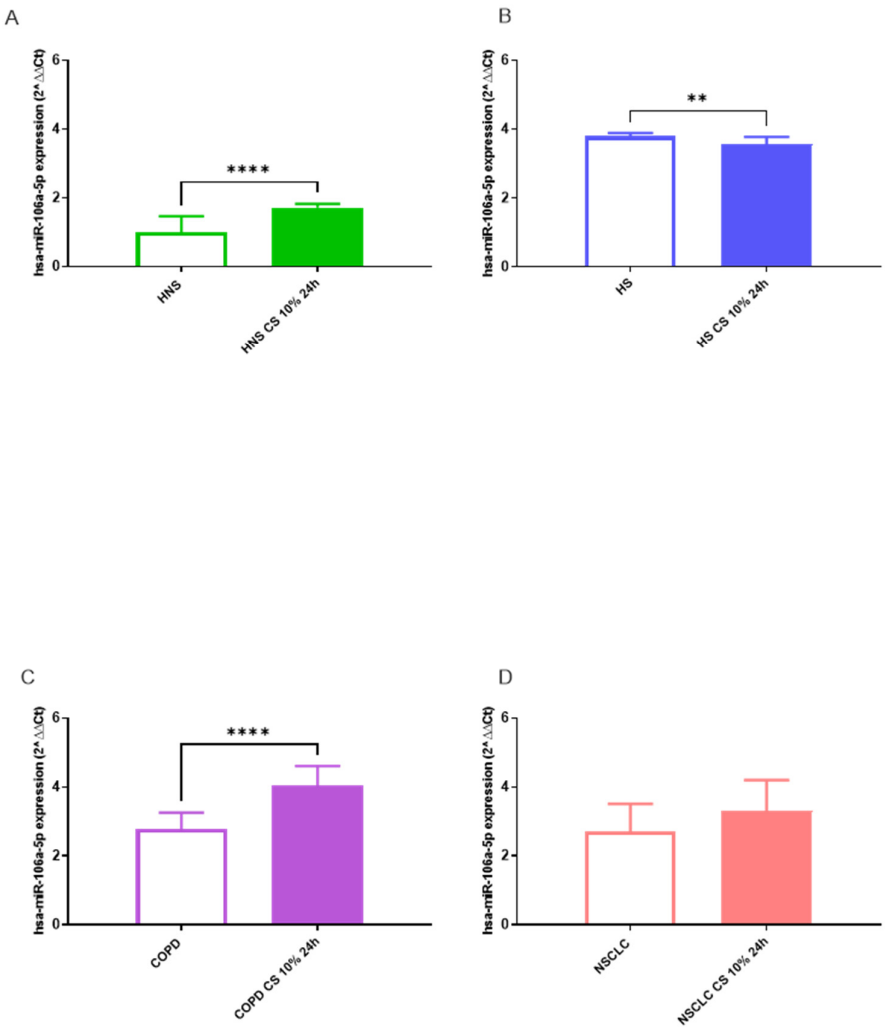

Figure 5. Analysis of hsa-miR-106a-5p AMs expression levels in HNS (2A; biological replicates n = 9), HS (2B; biological replicates n = 11), COPD (2C; biological replicates n = 11) and NSCLC (2D; biological replicates n = 12) and after 10% CS at 24 h. AMs from BAL all groups were treated with CS at 10% for 24 h and the expression of hsa-miR-106a-5p was assessed by real-time RT-PCR. All samples were run in triplicate, and results are shown as means ± SD. The statistical tests used in these analyses were one-way analysis of variance followed by Tukey Multiple Comparison Test. * $p < 0.05$, ** $p < 0.01$.

hsa-miR-223-5p and 20a-5p

RT-PCR results showed that CS significantly decreased hsa-miR-223-5p ($p < 0.001$) and 20a-5p ($p < 0.01$) expression in HNS AMs (Figures 6A and 7A). In contrast, in vitro CS stimulation in COPD AMs induced both hsa-miR-223-5p ($p < 0.01$) and 20a-5p ($p < 0.05$) upregulation (Figure 6C and 7C). Moreover, CS affected only hsa-miR-20a-5p expression in AMs from HS, leading to a significant positive modulation ($p < 0.0001$) (Figure 7B), while did not further impact hsa-miR expression in AMs from individuals with NSCLC (Figure 6D and 7D).

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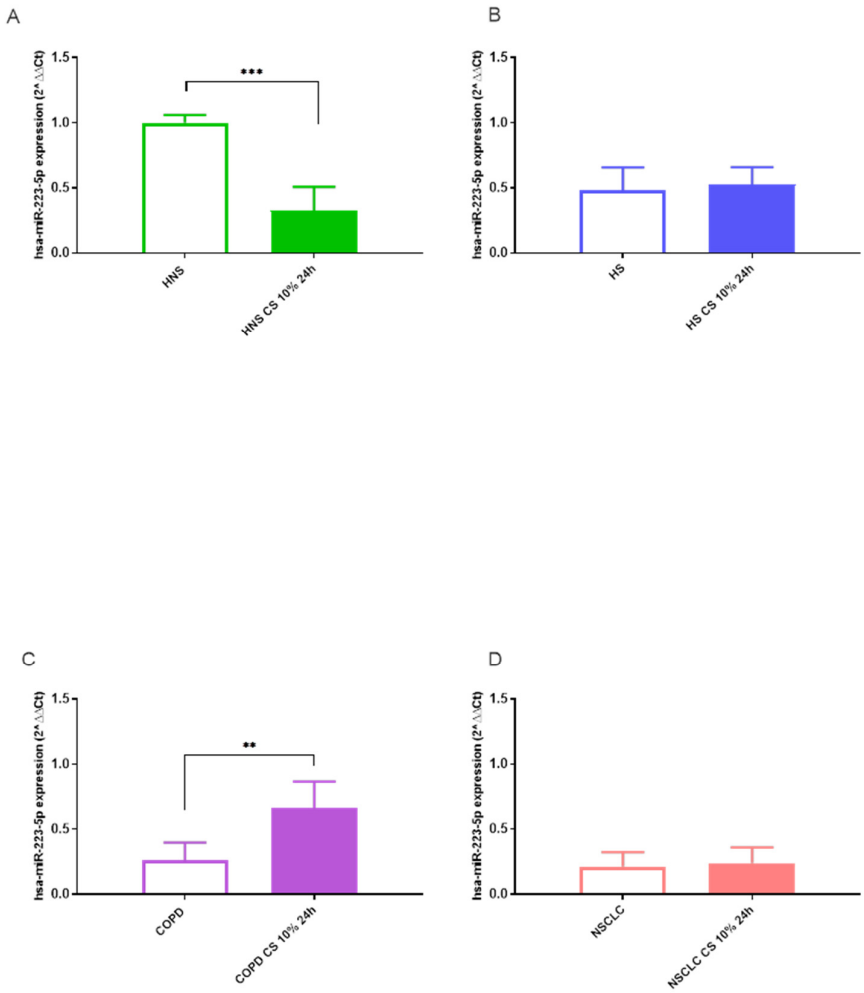

Figure 6. Analysis of hsa-miR-223-5p AMs expression levels in HNS (2A; biological replicates n = 9), HS (2B; biological replicates n = 11), COPD (2C; biological replicates n = 11) and NSCLC (2D; biological replicates n = 12) before and after 10% CS at 24 h. AMs from BAL all groups were treated with CS at 10% for 24 h and the expression of hsa-miR-223-5p was assessed by real-time RT-PCR. All samples were run in triplicate, and results are shown as means ± SD. The statistical tests used in these analyses were one-way analysis of variance followed by Tukey Multiple Comparison Test. ** $p < 0.01$, *** $p < 0.001$.

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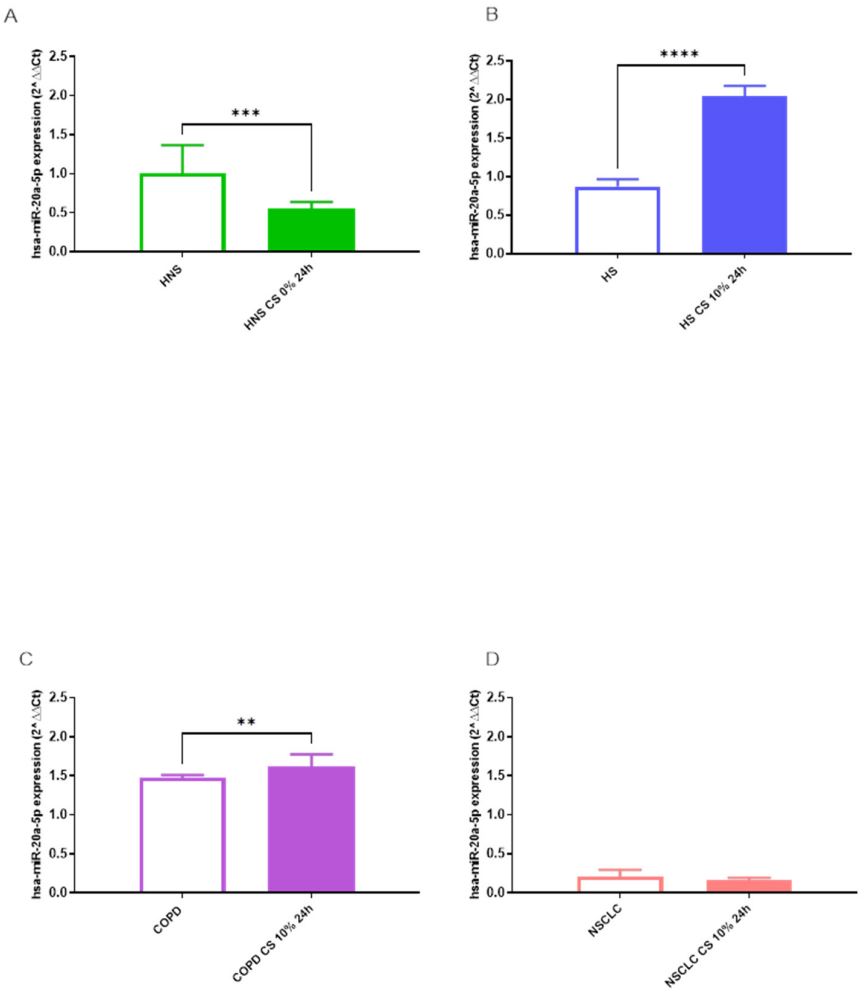

Figure 7. Analysis of hsa-miR-20a-5p AMs expression levels in HNS (2A; biological replicates n = 9), HS (2B; biological replicates n = 11), COPD (2C; biological replicates n = 11) and NSCLC (2D; biological replicates n = 12) before and after 10% CS at 24 h. AMs from BAL all groups were treated with CS at 10% for 24 h and the expression of hsa-miR-20a-5p was assessed by real-time RT-PCR. All samples were run in triplicate, and results are shown as means ± SD. The statistical tests used in these analyses were one-way analysis of variance followed by Tukey Multiple Comparison Test. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

3.3. In Silico Identification of Target mRNAs

The relationship between miRNAs and lung response to CS, was assessed by in silico analysis of sequence similarity between miRNAs and different mRNAs. Experimentally validated target genes were compared in two different databases of which miR target Link 2.0 was chosen as it had a higher number of validated genes for each miRNA. We analyzed all selected miRNA targets, focusing on those that are common to several miRNA and implicated in the cellular pathways regulating inflammation or AMs properties. Abbreviations, names of the selected genes, methods, and validation tissues are listed in Table 2. The results showed that the miRNA may be involved in regulation of several inflammation driver genes, such as BCL2, LAMTOR, MCL1, SOCS5 or VEGFA, among others. However, other target genes linked to apoptosis or cytokines production were found, as shown in Table 3. Given that several authors have experimentally confirmed all genes, miRNA might modulate these targets in a coordinated or individual manner, affecting several hallmarks of lung response to CS.

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Table 1. Bioinformatics tools for in silico analysis. Number of validated genes for each miRNA analyzed in miR Target Link 2.0 and Diana Tools databases.

	Number of Target Genes	
miRNA	**miR Target Link 2.0**	**DIANA Tools**
hsa-miR-223-5p	551	10
hsa-miR-16-5p	2.279	455
hsa-miR-20a-5p	1.659	611
hsa-miR-17-5p	1.817	136
hsa-miR-34a-5p	968	324
hsa-miR-106a-5p	1.166	435

Table 2. Abbreviations, gene names, methods, and tissue on which the miRNA selected were validated targets from miR Target Link 2.0.

Abbreviation	Gene Name	Methods	Tissues	References (PMID)
ATG14	Autophagy Related 14	Sequencing, HITS-CLIP	Embryonic kidney cells, B cells	20371350 22473208
BCL2	BCL2 Apoptosis Regulator	Luciferase reporter assay, qRT-PCR, Western blot, Proteomics analysis, Immunohistochemistry, Microarray, Sequencing, HITS-CLIP, Immunoblot, Immunoprecipitaion	Cervix cells, gastric cells, bone cells, marrow cells, spleen, liver, kidney, lymph node, tracheal/bronchial epithelial cells, breast cells, ovary cells, human embryonic kidney cells, B cells, mesothelial cell, glioma cells	17877811 18449891 18362358 17351108 17707831 20643754 20876285 19269153 16166262 19903841 20371350 23907579 22473208 24148817 25435430 26397135 26722459
CPT1A	Carnitine Palmitoyltransferase 1A	Proteomics HITS-CLIP	Cervix cells, neuronal cells	18668040 23313552
FOXC1	Forkhead Box C1	PAR-CLIP	Human embryonic kidney cells	21572407
HAS2		HITS-CLIP	B-cell	22927820

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	Hyaluronan Synthase 2			
HSPA1A	Heat Shock Protein Family A (Hsp70) Member 1A	Microarray pSILAC, Proteomics, PAR-CLIP	Leukemic cells, cervix cells, human embryonic stem cells	18362358 18668040 22012620
LAMTOR1	Late Endosomal/Lysosomal Adaptor, MAPK And MTOR Activator 1	Proteomics, PAR-CLIP	Cervix cells, brain tissue human embryonic kidney cells, B cells	18668040 24398324 23446348 20371350
MCL1	MCL1 Apoptosis Regulator, BCL2 Family Member	HITS-CLIP, microarray, Immunohistochemistry, Luciferase reporter assay, qRT-PCR, Western blot, PCR array	Human embryonic kidney cells, leukemic cells, liver	22473208 18362358 23594563 28097098
MFN2	Mitofusin 2	Proteomics, luciferase reporter assay, western blot, CLASH	Breast cells, lungs, human embryonic kidney cells	18668040 27640178 23622248
SCAMP5	Secretory Carrier Membrane Protein 5	HITS-CLIP	B cells	22473208 22473208
SENP1	SUMO Specific Peptidase 1	HITS-CLIP, PAR-CLIP	Human embryonic kidney cells	22473208 20371350 21572407
SOCS5	Suppressor Of Cytokine Signaling 5	CLASH, PAR-CLIP	Human embryonic kidney cells, peripheral blood mononuclear cells, macrophages, brain tissue	23622248 23592263 23446348

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TGFBR2	Transforming Growth Factor Beta Receptor 2	Immunoblot, Luciferase reporter assay, Microarray, qRT-PCR, Western blot HITS-CLIP PAR-CLIP Immunohistochemistry, In situ hybridization	Colorectal cancer cells, umbilical cord blood cell, B cells, human embryonic stem cells, human embryonic kidney cells, B cells, epithelial cells of the small and large intestines, esophageal cells	20940405 19435428 22473208 22012620 21572407 20371350 27080303 27508097 26729221
VEGFA	Vascular Endothelial Growth Factor A	ELISA, Luciferase reporter assay	Kidney cells	18320040

Table 3. miRNA gene interaction and possible biochemical pathways involved.

Biochemical Pathways	miRNA	Validated target genes
Autophagy- adaptive immune response regulation	hsa-miR-16-5p hsa-miR-20a-5p hsa-miR-17-5p	ATG14
Apoptosis- ROS production	hsa-miR-16-5p hsa-miR-17-5p hsa-miR-20a-5p hsa-miR-34a-5p	BCL2
Apoptosis- inflammatory response regulation	hsa-miR-16-5p hsa-miR-20a-5p hsa-miR-17-5p hsa-miR-106a-5p	CPT1A
Oxidative stress-inflammation responses- cell apoptosis	hsa-miR-20a-5p hsa-miR-17-5p hsa-miR-223-5p	FOXC1

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Cytokines, chemokines, and matrix metalloproteinase production	hsa-miR-20a-5p hsa-miR-17-5p hsa-miR-106a-5p	HAS2
Protein folding- prevention of protein aggregation - apoptosis	hsa-miR-16-5p hsa-miR-34a-5p hsa-miR-223-5p	HSPA1A
Macrophages polarization-innate immune response regulation	hsa-miR-16-5p hsa-miR-20a-5p hsa-miR-17-5p	LAMTOR1
Apoptosis- bacterial clearance	hsa-miR-16-5p hsa-miR-17-5p hsa-miR-20a-5p hsa-miR-34a-5p	MCL1
Mitochondrial fusion-mitochondrial membranes regulation	hsa-miR-16-5p hsa-miR-17-5p hsa-miR-34a-5p hsa-miR-106a-5p	MFN2
TNF secretory pathway	hsa-miR-16-5p hsa-miR-20a-5p hsa-miR-17-5p	SCAMP5
Cytokines secretion- NF-κB pathway	hsa-miR-20a-5p hsa-miR-16-5p hsa-miR-34a-5p hsa-miR-223-5p	SENP1
EGFR signaling pathway	hsa-miR-16-5p hsa-miR-20a-5p hsa-miR-17-5p	SOCS5
TGF-β signaling pathway	hsa-miR-20a-5p hsa-miR-17-5p hsa-miR-34a-5p	TGFBR2
VEGF pathway- ROS generation-Akt/eNOS/NO pathway	hsa-miR-16-5p hsa-miR-20a-5p hsa-miR-17-5p	VEGFA

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4. Discussion

In this study, we assessed the effect of in vitro CS exposure on the regulation of miRNA expression in AMs, the main participants in the development of smoking-related conditions such as COPD and NSCLC. Tobacco promotes oxidative stress, systemic inflammation, and local inflammation in the lungs of smokers, leading to innate and adaptive immune system impairments [17–19]. In addition, CS leads to genetic and molecular impairments, which may increase the chance of mutations and lung carcinogenesis [20]. Given the high prevalence of smoking-related diseases, the search for new biomarkers has prompted in-depth epidemiological studies [48,49]. Several authors attempted to identify miRNA signature of CS and evidence for their causal role in smoking-related inflammation. Willinger et al. profiled 283 miRNAs and found six associated with serum levels of C-reactive protein, interleukin-6 and pulmonary function [50]. In our previous study, we reported that has-miR-34a-5p, 17-5p, 16-5p, 223-5p, 20a-5p, and 106a-5p expression profiles in AMs were dysregulated in NSCLC, COPD and ever- or never-smoker controls, suggesting their possible role as an index of smoking-associated conditions [39]. To further investigate the effect of active smoking on the expression levels of these miRNAs, we analyzed the changes in their expression before and after in vitro CS exposure in the above mentioned groups. This profiling was carried out in AMs recovered from BAL, a precious biological sample that is highly representative of the pulmonary microenvironment [51]. First, we identified that never-smokers AMs in vitro stimulated with 10% CS for 24h results in two specific trends, leading to hsa-miR-34a-5p, 17-5p, 16-5p, and 106a-5p upregulation and negative modulation of hsa-miR-223-5p and 20a-5p levels. In contrast, in vitro CS exposure in AMs obtained from individuals chronically exposed in vivo to CS such as ever smokers or with pre-existing lung conditions such as COPD or NSCLC variably affects miRNAs. In the parenchyma of COPD patients, a key hallmark of alveolar epithelial and endothelial cells is apoptosis and, as one of the most important risk factors for COPD, CS can initiate apoptosis in several cell types, including macrophages [52]. In this context, Long et al. reported that Notch-1 receptor protein, a transmembrane receptor which has been implicated in cell proliferation and apoptosis control, is lower expressed in primary lung microvascular endothelial cells (HPMECs) treated with CS, with the upregulation of hsa-miR-34a-5p [53]. In addition, Zeng et al. showed that exposure of BEAS-2B cells to CS increased the expression of hsa-miR-34a-5p and senescence-associated pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α) in a dose-dependent manner [54]. Furthermore, in our previous study, we reported a significant positive modulation of hsa-miR-34a-5p in the tissue and AMs of COPD subjects compared to healthy never-smoker controls [55]. Consistent with these data, we observed after in vitro exposure to CS a significant positive modulation of hsa-miR-34a-5p in the AMs of healthy individuals towards COPD level, providing evidence of the role of CS in COPD-like dysregulation. Interestingly, in vitro CS exposure equally affected hsa-miR-34a-5p expression in AMs obtained from HS suggesting the potent effect of acute CS stimulation. CS can directly damage epithelial cells, the first barrier for the respiratory tract, and cause infiltration of immune cells in the lungs, including AMs [56]. Leukocyte signal-regulatory protein-α (SIRPα), a member of the immunoglobulin superfamily, modulates many aspects of the inflammatory response to noxious agents, including immune cell activation, chemotaxis, and phagocytosis [57]. In this regard, Zhu et al. showed that upregulation of hsa-miR-17-5p by lipopolysaccharide (LPS) in macrophages is the mechanism underlying LPS-induced SIRPα reduction and AMs activation [58]. This was consistent with our finding of higher hsa-miR-17-5p levels in AMs of never-smokers and ever-smokers following acute in vitro CS treatment, supporting the importance of CS in the mechanisms underlying AMs impairment in lung diseases. The innate immune system represents the first line of host defense against harmful particles or bacterial infections through phagocytosis by resident macrophages [59]. One of the most important features of this process is the activation of TLRs immune receptors and the release of a variety of toxic products, including reactive oxygen species (ROS) such as NO, hydrogen peroxide, and superoxide anions [60]. Moon et al. reported that bacterial LPS enhanced the level of has-miR-16-5p in bone marrow–derived macrophages, resulting in decreased phagocytosis and the generation of mitochondrial ROS [61]. Accordingly, our findings showed a positive modulation of has-miR-16-5p in never-smokers and

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ever-smokers AMs following CS treatment suggesting the ability of CS exposure to modulate the lung inflammatory response. Although a few studies have investigated the hsa-miR-106a-5p expression patterns associated with CS and chronic lung diseases, Liu et al. reported that it dramatically inhibited the activation of autophagy induced by M. tuberculosis in human THP-1 macrophages [62]. Indeed, CS impairs AMs autophagy playing an important role in COPD [63]. Moreover, Sharma et al. reported that hsa-miR-106a-5p negatively regulates IL-10 expression with an increase in proinflammatory cytokines in in vitro and in vivo model of airway inflammation [64]. This was in line with our findings of increased has-miR-106a-5p in AMs of both never-smokers and COPD subjects following in vitro CS exposure. However, the regulatory effects of has-miR-106a-5p in CS-related diseases are not fully understood, making its role controversial, as suggested by its reduction in AMs from ever-smokers after in vitro CS treatment. Our findings highlight that in vitro CS stimulation of AMs obtained from never-smokers results in negative modulation of hsa-miR-223-5p and 20a-5p levels. Several authors have described hsa-miR-223-5p role in macrophage differentiation, neutrophil recruitment, and pro-inflammatory responses, which are key features of lung inflammation and remodeling [65]. Interestingly, in never-smokers AMs, CS resulted in the modulation of hsa-miR-223-5p to levels comparable to those observed in individuals with smoking-related conditions. Consistent with our data, Schembri et al. reported lower has-miR-223-5p levels in bronchial epithelial cells from current smokers than in those from never-smokers [66]. Furthermore, it is important to point out that in COPD AMs, the expression of this miRNA is increased following exposure to CS, indicating a unique function for acute CS in COPD microenvironment. In fact, acute CS exposure can induce chemotactic factors in the lungs, stimulate AMs, and lead to neutrophil influx, which can require at least six months to normalize completely [67]. In this context, Roffel et al. detected higher levels of has-miR-223-5p in the lung tissue of COPD patients, assuming that it could be associated with impaired lung function and higher neutrophil counts [68]. As for hsa-miR-223-5p, CS led to hsa-miR-20a-5p downregulation in AMs obtained from never-smokers. Importantly, our data showed that ever-smokers and patients with COPD shared increased levels of this miRNA after exposure to CS. Specifically, exposure to CS in ever-smokers increases levels towards those reported in COPD, highlighting the close link between CS and the development of a COPD-like phenotype. hsa-miR-20a-5p has been shown to regulate AMs inflammatory responses by targeting SIRPα [58]. Moreover, Liu et al. reported higher hsa-miR-20a-5p levels in children with pneumonia and in lung cells exposed to LPS, highlighting its role in inflammation through activation of the NF-κB signaling pathway [69]. However, given its role in controlling cellular networks, such as the PI3K/Akt axis, the regulatory effects of CS on its expression cannot be generalized, making a more in-depth analysis necessary to explain our results in AMs from never-smokers [44]. Finally, in vitro CS exposure did not influence the expression of any miRNAs in AMs from subjects with NSCLC. In our previous study the same trend was seen for the Programmed death-ligand 1 (PD-L1) mRNA expression. Indeed, we reported that after CS exposure, PD-L1 mRNA expression was increased in AMs derived from never-smoker subjects but not in NSCLC patients, suggesting an overwhelm effect of cancer on acute CS exposure [70]. It is important to note that the intensity of the reaction against immunogenic antigens produced in response to CS varies across a wide range of disease manifestations highlighting the crucial role of immune responses in regulating the development of distinct phenotypes (such as COPD or NSCLC) in response to CS [71,72]. In this context, dysregulation of miRNAs could reflect phenotype switching or the onset of different lung manifestations, underlining the prominent, but not exclusive, role of CS. Indeed, miRNA expression profiles can be influenced by other environmental factors, which can further modulate the correlation between the expression of miRNAs and mRNA targets in response to CS [73]. Finally, to assess the potential interconnection of miRNAs in lung response to CS, we performed in silico prediction of hsa-miR target genes. Bioinformatic results revealed that the miRNAs analyzed may potentially be involved in the regulation of several inflammation driver genes, such as ATG14, BCL2, CPT1A, FOXC1, HAS2, HSPA1A, LAMTOR1, MCL1, MFN2, SCAMP5, SENP1, SOCS5, TGFBR2 and VEGFA, which are involved in immune system regulatory pathways [74–87]. Since several authors have experimentally

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confirmed all miRNA-regulated genes, miRNAs may modulate these targets combined or individually, affecting different hallmarks of lung response to CS.

5. Conclusions

This study, even if preliminary, indicates that CS is an important driver of epigenetic changes that contribute to the onset of various lung diseases. Moreover, we demonstrated that the effects of acute CS on miRNA expression levels could differ between never-smokers and subjects who were already chronically exposed to CS, such as HS, or had pre-existing lung diseases, such as COPD. It is noteworthy that, following exposure to CS, the expression levels of hsa-miR-34a-5p and 17-5p in both ever- or never-smokers, 106a-5p in never-smokers and 20a-5p in ever-smokers, shifted towards those found in individuals with COPD, suggesting them like a risk factor in developing this lung condition. A potential limitation of our study was the small sample size used for miRNA analysis, which did not allow subanalysis according to COPD severity. However, our data could be of clinical relevance and lead to future studies involving larger populations, allowing to better understand the networks involved in the pathogenesis of smoking-related diseases.

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