

Differences in Serum miRNAs in Women Exposed to Biomass Smoke and Smokers with and without Severe to very Severe COPD

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Abstract

Background: Exposure to biomass smoke (BS) and smoking (TS) associated with the genetic environment are the main causes of Chronic Obstructive Pulmonary Disease (COPD). microRNAs (miRNAs) participate in the control of post-transcriptional regulation. The miRNAs involved in COPD-TS are well defined; however, those implicated in COPD-BS have not been identified.

Objective: It was analyzed which miRNAs participate in women exposed to biomass (COPD-BS) and smokers (COPD-TS), in a severe to very severe COPD (GOLD III-IV), comparing with women exposed to BS without COPD (H-BS), smokers without COPD (H-TS), and healthy control (C).

Material and methods: Clinical and physiological characteristics were analyzed. The serum miRNA expression profiles were analyzed using PCR arrays (n=3), followed by RT-qPCR (n=25).

Results: Women with COPD-BS were older, shorter, and weighed less related to the other groups. Four miRNAs were deregulated, miR-10a-5p that was overexpressed in COPD-TS versus H-TS; while three were underexpressed: miR-15b-5p comparing H-TS with C, miR-30d-5p in COPD-TS versus C, and miR-200c-5p in COPD-BS related to H-TS.

Conclusion: Our results suggest that women with COPD-SB have different serum expression of miRNA compared to those with COPD-TS; miR-200c could be relevant in the pathophysiology of COPD-SB, while miR-10a-5p, miR-15b-5p and miR-30d-5p in COPD-TS.

Keywords: Biomass smoke; COPD; miR-10-5p; miR-15b-5p; miR-30d; miR-200c-5p; Smoking

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Introduction

Chronic obstructive pulmonary disease (COPD) is defined as a preventable and treatable disease characterized by airflow limitation that is not fully reversible [1]. The main risk factor for the development of COPD is smoking (TS) [1]; however, in developing countries such as Mexico, women are mainly affected by domestic exposure to biomass smoke (BS) [2,3]. COPD from biomass smoke (COPD-BS) is considering a phenotype distinct from that of COPD from smoking (COPD-TS). The distinctive prognostic factors between both pathologies are widely described [2-4]. Thus, women with COPD-BS remain mostly in stages I-II of

COPD, evolving infrequently to stages III-IV, contrary to those with COPD-TS who most commonly evolve toward stages III-IV of COPD [2,3].

Several factors interact in the origin and development of the

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pathological process in COPD, including genetic, epigenetic, biochemical, physiological and environmental components [5]. Based on epigenetic factors, alterations in the expression of microRNAs (miRNAs or miRs) is essential [6]. miRNAs are small non-coding RNAs that silence and regulate posttranscriptional modification of mRNAs. Thus, certain miRNA expression profiles have been associated with the pathogenesis of various diseases [6,7]. miRNAs can be secreted by cell damage or lysis, and transported in body fluids in a stable manner, encapsulated or anchored in microvesicles, exosomes, lipoproteins or Argonaute-2 protein [7,8].

The profile analysis of extracellular miRNAs has been used as a tool to monitor the physiopathological state, by shown a good correlation with disease states and/or risk conditions [6-8]. Thus, for example, five circulating unregulated miRNAs were found in the serum of patients with COPD-TS, with four of them underexpressed: miR-20, miR-28-3p, miR-34c-5p, and miR-100; while miR-7 was overexpressed, when compared with healthy individuals [8]. Nevertheless, it is important to denote that those involved in COPD-BS have not been identified. In this sense, recently, our study group published a work analyzing which miRNAs participate in COPD-BS compared with COPD-TS in mild-to-moderate states (GOLD I-II) [9]. Nevertheless, the miRNAs that could be involved in COPD in severe to very severe stages (GOLD III-IV) have not been described. Accordingly, just we published a partial report from the same protocol performed in the present work, which showed that women with COPD-BS with a higher values of DLCOsb%P compared to COPD-TS in severe to very severe stages, based in the fact of that serum miR-22-3p was underexpressed, concomitantly to a higher concentration of serum the HDAC4, positively correlated with DLCOsb%P; considering that the miR-22-HDAC4-DLCO axis favored the development of chronic bronchitis over that emphysema in COPD-BS [10]. So that, in the present study we show more complete comparisons, considering 5 groups of women; women with COPD from smoking and biomass, women exposed to biomass or smoking without COPD, and healthy control women; focus in the comparative analysis in the expression of serum miRNAs. We hypothesize that COPD-BS as represents a different physio pathogenic phenotype with respect to COPD-TS, the profile of serum miRNAs present in women with COPD-BS, in severe to very severe COPD (GOLD stage III-IV), will be different from those with COPD-TS, and also with those healthy women exposed to BS (H-BS) or smoking (H-TS), and control women (C). Therefore, the objective in this study was to compare the serum expression of serum miRNAs in these five groups of women.

Research Methodology

Study population

This cross-sectional study was conducted in accordance with the Declaration of Helsinki, and was approved by the Science and Bioethics Committee at the Instituto Nacional de Enfermedades Respiratorias Ismael Cosío Villegas (INER) in Mexico City (protocol INER: B15-15), a referral center of respiratory diseases in Mexico. All participants answered a written questionnaire and received

and provided informed consent to participate in the study, agreeing to protocol approved by the Committees.

A total of 125 women divided into five groups of 25 were integrated in this study. Women were recruited at the COPD clinic at the INER, from a cohort that was followed regularly, from May 2015 to May 2017; all participants received complete information about the study and gave their informed consent for the study, and data were recollected by medical doctors previously trained on the questionnaire assessment. Women with COPD from biomass (COPD-BS), smokers with COPD (COPD-TS), all with COPD in severe to very severe COPD (GOLD stages III-IV), smokers without COPD (H-TS), exposed biomass without COPD (H-BS) and healthy control women (C).

Demographic, anthropometric, and clinical data were collected including TS history (>10 packs-years) and cumulative exposure to BS (h-year). No patient with COPD was exposed to both factors. Wood was the only fuel used by women with COPD-BS, who came from rural and suburban, low-income regions of Mexico. Were excluded women with a history of any other chronic pulmonary or non-pulmonary conditions, exacerbation 6 months, or lower respiratory tract infection 4 weeks prior to study enrollment.

Pulmonary function testing

COPD was classified according to the exposure history following the procedures recommended by the American Thoracic Society/European Respiratory Society [11], and the standard reference for Mexicans [10]. The pulmonary function test applied to all women to diagnose COPD was pre- and post-bronchodilator spirometry. Forced expiratory volume in the 1st sec % predicted ($FEV_1\%P$), forced vital capacity % predicted (FVC%P), and FEV_1/FVC ratio were measured. Tests were done using a SensorMedics dry seal spirometer (Yorba, Linda, CA, USA).

Blood samples

From each woman, blood samples were collected in anticoagulant-free tubes (5mL) (BD vacutainer; Becton, Franklin Lakes, NJ, USA), at morning with at least 8 fasting hours. The samples were centrifuged at 5,000g at room temperature for 15 min to obtain the serum and tested the same day. For continuous storage, the serum was aliquoted and frozen at -80°C until use.

Obtaining serum miRNAs

The extraction of the miRNAs was performed using the QIAGEN miRNeasy serum/plasma kit (Hilden, Germany) following the manufacturer's instructions. The miRNA was quantified, and integrity was assessed with the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA).

Differential expression of miRNAs in serum by PCR arrays

Serum miRNAs analysis was conducted in two stages: a screening stage to identify miRNAs differentially expressed in a samples from 3 women randomly selected from each study group. Once miRNAs differentially expressed were identified, we implemented the validation stage in 25 women of each group, using quantitative real-time PCR (RT-qPCR).

The differential expression analysis was performed for 96 miRNAs using MiScript miRNA PCR Array (MIHS-106Z; Qiagen, Valencia, CA, USA), isolated from serum. After obtaining all raw results, the data was analyzed in the Qiagen software (Data analysis file for miScript miRNA PCR Array All miRNA QC; Qiagen, Valencia, CA, USA) (available at <https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overviewpage/>). The software uses the 2- Δ Ct method, which analyzed and compared two specific groups using Student's t-test. The data is expressed as fold change = relative quantification of miRNA.

Validation of samples by RT-qPCR

The validation was performed by RT-qPCR, obtaining the cDNA of the miRNAs extracted with the RT kit and amplified with TaqMan Universal Master Mix II with the UNG kit, all from Applied Biosystems by Thermo Fisher Scientific (USA). Pre-designed commercial assays for each miRNA were obtained from Thermo Fisher Scientific: hsa-miR-10a-5p (ID 002288), hsa-miR-15b-5p (ID 000390), hsa-miR-30d-5p (ID 000420), and hsa-miR-200c-5p (ID 002300). The expression level of each miRNA was evaluated using the comparative threshold cycle method (2- $\Delta\Delta$ Ct) and normalized with a corresponding miRNA sequence from *C. elegans* as an exogenous normalizer in gene expression (spike-in cel-miR-39). The relative concentration of each miRNA was described by the equation Δ Ct = (Ct miRNA - Ct spike). The cut-off value was set as the cycle \leq 40 and it was considered that a gene was not detectable when the Ct was $>$ 40 and the signal was under established limits [12].

Statistical analysis

To obtain the sample size, the free software G Power (version 3.1.9.2; Heinrich-Heine-Universität, Düsseldorf, Germany) was

used. The change in gene expression was reported in multiples (fold-change). The statistical analysis for qPCR array was performed with the Qiagen software (available at <https://www.qiagen.com/us/shop/genes-and-pathways/data-analysis-center-overviewpage/>). RT-qPCR was analyzed by relative quantification ($\Delta\Delta$ Ct method). The analyses were performed using the statistical package GraphPad (Graph-Pad v6.01 Software, Inc., La Jolla, CA, USA). $P < 0.05$ were considered significant.

Results

Characteristics of women in the study

The demographic, anthropometric, and clinical data of the study groups are shown in **Table 1**. The women with COPD-BS were older and shorter, showing differences with the other groups ($P < 0.01$). Women with COPD-BS were weighed less, and showed differences with COPD-TS and H-BS; H-TS also showed difference with COPD-TS ($P < 0.01$); although the BMI did not show difference between the groups. Tobacco and biomass index did not show difference between groups.

FEV₁%P and FEV₁/FVC ratio in both groups of women with COPD showed differences among the H-BS, H-TS, and C groups ($P < 0.01$); likewise, women from the H-BS and H-TS groups showed differences compared with both groups with COPD ($p < 0.01$). Additionally, with no difference between the groups with COPD.

Differential expression of miRNAs in serum by PCR array

The differential expression analysis of the miRNAs was performed in three patients chosen randomly from each study group. The results obtained are shown in **Table 2**. Only one miRNA was

Table 1 Anthropometric, clinical, and physiological characteristics of the study in women (n=25).

Variables	Control	COPD-TS	COPD-BS	H-TS	H-BS
Characteristics					
Age (years)	66.59 ± 7.11	66.57 ± 6.37	73.27 ± 8.69**/**/#	63.12 ± 8.21	66.58 ± 7.1
Height (cm)	158.26 ± 8.10	155.31 ± 7.57	146.52 ± 5.69*/**/#	158 ± 0.07	153 ± 0.1 */*//##
Weight (kg)	68.55 ± 11.59	59.52 ± 12.13	56.25 ± 10.82**//##	70.58 ± 13.46/**	65.87 ± 12.57
BMI (kg/m ²)	27.33 ± 3.91	24.79 ± 5.78	26.33 ± 5.04	28.16 ± 4.86	26.35 ± 9.01
Exposure characteristics					
Tobacco index (pack-years)	0	36.62 ± 23.1	0	30.25 ± 19.26	0
Biomass smoke Index (h-years)	0	0	366.88 ± 219.3	0	325.87 ± 186.58
Lung function characteristics					
FEV ₁ %P	96.25 ± 3.1	39.85 ± 5.36**	39.92 ± 6.57**	83.14 ± 2.3*/**	84.32 ± 4.2*/**
FEV ₁ /FVC ratio	80.02 ± 2.4	45.63 ± 5.36*	45.23 ± 10.08*	74.12 ± 2.3*/**	73.5 ± 2.2*/**
Case numbers (%)					
GOLD grades					
III	0	16 (64)	20 (80)	0	0
IV	0	9 (36)	5 (20)	0	0

The data are expressed as means ± SD. The data were analyzed by one-way ANOVA and Tukey's the post hoc test. * $P < 0.05$, ** $P < 0.01$, * vs. control; /** $P < 0.05$, * $P < 0.01$, vs. COPD-TS or COPD-BS groups; //# $P < 0.05$; //## $P < 0.01$, vs. H-TS or H-BS groups, respectively.

Abbreviations: BMI: Body Mass Index; C: Control Healthy Women; COPD-BS: COPD Secondary to Biomass Smoke Exposure; COPD-TS: COPD Secondary to Tobacco Smoking; FEV₁% Pred: Forced Expiratory Volume in the 1st Second (% Predicted); FVC: Forced Vital Capacity; FEV₁/FVC Ratio: Forced Expiratory Volume in the 1st Second (% Predicted)/ Forced Vital Capacity Ratio; H-BS: Exposed to BS Without COPD; H-TS: Smokers Without COPD.

Table 2 Differentially expressed miRNAs the serum of women in women with COPD from biomass exposure or smoking (GOLD stages III–IV), women exposed to biomass or smokers without COPD, and healthy control women (n=3).

Compared groups	miRNA	Regulation level	Fold change	P-value
COPD-TS vs. H-TS	miR-10a-5p	underexpressed	-4.10	0.0177
H-TS vs. C	miR-15b-5p	overexpressed	15.38	0.0236
COPD-TS vs. C	miR-30d-5p	underexpressed	-5.93	0.0060
COPD-BS vs. H-BS	miR-200c-3p	underexpressed	-4.15	0.0088

Samples were analyzed by PCR arrays. The data are presented as the change in regulation (fold-change) and the obtained p-value.

Abbreviations: C: Control Healthy Women; COPD-BS: COPD Secondary To Biomass Smoke Exposure; COPD-TS: COPD Secondary To Tobacco Smoking; H-BS: Exposed To BS Without COPD; H-TS: Smokers Without COPD.

overexpressed, miR-15b-5p, in women with H-TS group with the C group. The remaining three miRNAs were underexpressed: miR-10a-5p in women with H-TS compared with COPD-TS, hsa-miR-30d-5p in women with COPD-TS compared with the C group, miR-200c-5p in women with COPD-BS related to those with H-BS.

Validation of miRNAs by RT-qPCR

The four miRNAs obtained from the PCR array were subsequently validated by RT-qPCR to corroborate the differences found between the study groups. miR-10a-5p that was underexpressed in the PCR array analysis (Table 2), was overexpressed by RT-qPCR in women of the COPD-TS group compared with those of the H-TS group (Figure 1; $P < 0.0001$); similarly, miR-15b-5p that was overexpressed in the PCR array and was inversely underexpressed in the RT-qPCR among the women of the H-TS group compared with the C group (Figure 2; $P < 0.0001$).

The two remaining miRNAs showed matching results with the PCR array and RT-qPCR and were found to be underexpressed: miR-30d-5p in the COPD-TS group compared with that in the C group (Figure 3; $P < 0.0001$); miR-200c-5p in women with COPD-BS compared with those with H-BS (Figure 4; $P < 0.0001$).

Discussion

The PCR array study showed that 4 miRNAs were differentially expressed a finding that was subsequently validated in RT-qPCR assays. Importantly, two of the five miRNAs that were differentially expressed in the PCR array study did not coincide with the results of the RT-qPCR, miR-10a-5p and miR-15b-5p, that could be attributed, in part, to the genetic and epigenetic susceptibility of each individual, socioeconomic and hereditary family heterogeneity of the different women recruited, as well as to the randomness in sample selection that was three women for each group [13]. On the other hand, miR-30d-5p and miR-200c-3p were coincident in both PCR array and RT-qPCR.

Based on the results of the RT-qPCR, miR-10a was overexpressed in the COPD-TS compared with the H-TS group, miR-15b-5p was underexpressed in H-TS related C group, miR-30d-5p was underexpressed in the COPD-TS group compared with C, and miR-200c-3p was underexpressed in the COPD-BS with H-BS group. The deregulation of these serum miRNAs has not been documented thus far, but especially with respect to women with COPD-BS.

It is relevant to note that a search in various academic databases

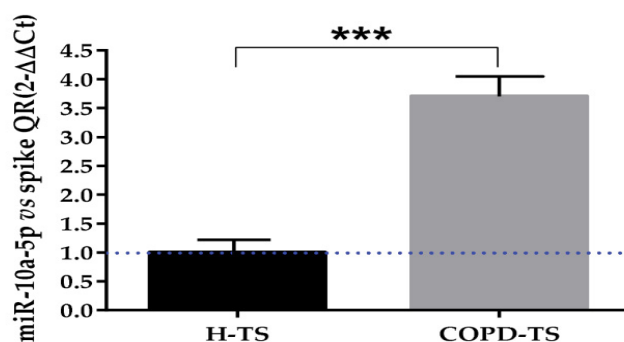


Figure 1 miR-10a-5p is overexpressed in COPD-TS women compared with that in H-TS women (n=25). RT-qPCR analysis of serum miR-10a-5p. The data are presented as ΔCt values from three independent experiments. Student's independent samples t-test was used. (Abbreviations: COPD-TS: COPD secondary to tobacco smoking; H-BS: exposed to BS without COPD; H-TS: * $P < 0.0001$.)

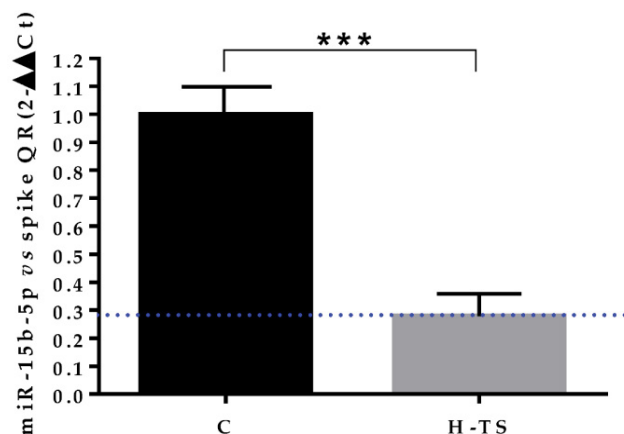


Figure 2 miR-15b-5p is underexpressed in the H-TS group compared with that in the C group (n=25). RT-qPCR analysis of serum. The data are presented as ΔCt values from three independent experiments. Student's independent samples t-test was used. (Abbreviations: C: control healthy women; H-TS: exposed to TS without COPD; *** $P < 0.0001$.)

shows heterogeneity in the analysis performed in COPD, especially since miRNAs have been examined in different body fluids such as serum, plasma, BAL and sputum.

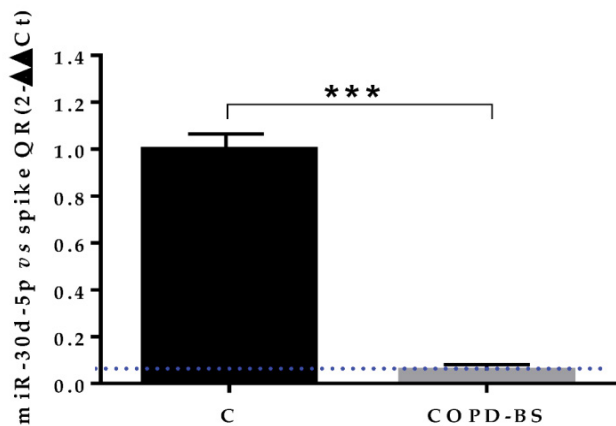


Figure 3 miR-30d-5p is underexpressed in the COPD-TS group compared with that in the C group (n=25). RT-qPCR analysis of serum. The data are presented as ΔCt values from three independent experiments. Student's independent samples t-test was used. (**Abbreviations:** C: control healthy women; COPD-TS: COPD secondary to tobacco smoking; *** $P < 0.0001$).

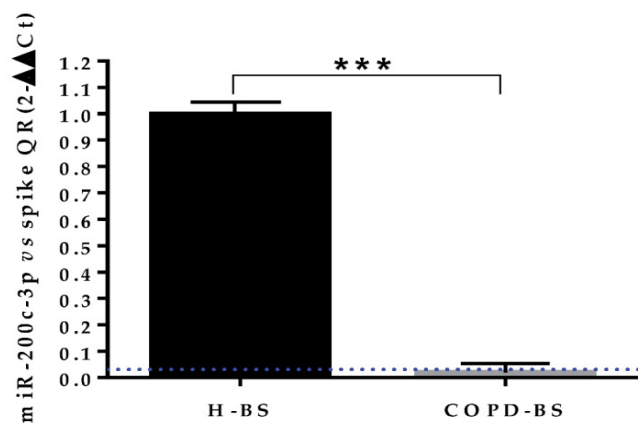


Figure 4 miR-200c-3p is underexpressed in the COPD-TS group compared with that in the H-BS group (n=25). RT-qPCR analysis of serum. The data are presented as ΔCt values from three independent experiments. Student's independent samples t-test was used. (**Abbreviations:** H-BS: exposed to BS without COPD; COPD-TS: COPD secondary to tobacco smoking; *** $P < 0.0001$).

Concerning miR-10a-5p, has been shown that angiogenic processes are inhibited when it miRNA is underexpressed in rats exposed to TS [14]. However, in our validation between the COPD-TS group regarding the H-TS, it was overexpressed. This overexpression was observed in primary cultures of smooth muscle cells of airways, where it inhibits the synthesis of DNA and proliferation [15], that could be related to the pathogenic effects that occur with the musculature in COPD, such is the case of cachexia [1,2,5]. However, to corroborate this fact, it is necessary to carry out focused studies to determine whether this miRNA exerts this effect in women with COPD-TS.

miR-15b-5p was reported overexpressed in the lung tissue from COPD-TS compared with healthy smokers, being its expression different among patients with in different stages of COPD. The expression of SMAD7 was validated as a target for miR-15b-5p, being reduced in bronchial epithelial cells in COPD; instead, when miR-15b-5p is underexpressed, there is an increase in SMAD7 protein inhibit apoptosis. However, we found that miR-15b-5p was underexpressed in H-TS related to C group, supporting the fact that miR-15b-5p could conferred a potential for the development apoptosis in COPD-TS. However, this aspect needs further clinical and experimental analysis [6].

miR-30d-5p that was underexpressed in women with COPD-TS compared with the C group. It was analyzed mainly in the serum of patients with nonsmall cell lung cancer (NSCLC), showing a significant association between underexpression and long-term patient survival in stages I-III; conversely, when it was overexpressed, there was a lower survival [16,17], could serve as a prognostic predictor of survival in NSCLC. In the case of women with COPD-TS where it was underexpressed, it is necessary to carry out further studies to determine whether it could have diagnostic and prognostic values.

Regarding miR-200c-3p, is a regulators/inhibitors of the epithelial-mesenchymal transition, that maintenance the epithelial phenotype in various cancers [18]. The downregulation of miR-200c-3p induced by TS extract activates the nuclear factor NF- κB pathway through the production of IL-6, contributing to the mechanism of carcinogenesis [19]. miR-200c-3p also acts as an inducer in the reduction of oxidative stress induced by H_2O_2 in lung cell cultures [20]. Nevertheless, it is important to continue studying this microRNA because, in the case of women exposed to BS, it would seem to promote the development of COPD by decreasing the antioxidant response, which requires a deeper analysis.

Additionally, and to relevant, miR-22a-3p is of great interest because it is underexpressed in women with COPD-TS comparatively with those with COPD-BS, as was published elsewhere [10]. miR-22a-3p has been use a biomarker in pulmonary tuberculosis, NSCLC, and lung adenocarcinoma [21-23]; which, it could also be a prognostic marker for the development of COPD-BS, but this warrants further study. Additionally, serum HDAC4, a target of miR-22a-3p, was incremented in COPD-BS compared with COPD-TS [10,24].

Concerning miRNAs and HDAC4 in COPD-TS, these participate in muscle dysfunction with and without mass loss, with impairment and dysfunction of muscle strength in diaphragm and quadriceps [25]. Furthermore, miR-22, when overexpression, induces a strong response to Th17 cells and participates in the development of pulmonary emphysema in smokers [24].

To contextualize the potential relevance of the four deregulated miRNAs among the five groups of women in this study, also considering miR-22-3b under-expressed in COPD-BS compared to TS-COPD [22], we can comment that the overexpression of miR-15-3b could help prevent COPD in smokers, keeping them as healthy smokers; however, if miR-10a-5p overexpression occurs

in these women, it could favor the development of COPD; on the other hand, the under-expression of miR-30d-5p may contribute to the development of emphysema more directly in female smokers. In relation to the exposure to BS, the underexpression of miR-200c-3p could contribute to the prevention of COPD, maintaining the health of these women; while miR-22a-3p, which was under-expressed compared to female smokers [10], would participate more directly in the development of COPD. Additionally, and taking in consideration that COPD by smoking is considered a phenotype different to COPD by BS exposure, these miRNAs, would be part of the molecular and pathophysiologic mechanisms that operate specifically in each one of these phenotypes.

Finally it is pertinent comment that this is the first comparative study carried out in women with COPD-BS and COPD-TS (in severe to very severe stages), healthy smokers, exposed to BS without COPD, and healthy controls, demonstrating the genotypic difference based on serum miRNA expression, which a priori supports the fact that COPD from biomass has a different phenotype from that of COPD from smoking, which could have important implications for diagnosis, prognosis and treatment of COPD [25].

Limitations

The limitations of this study are due, in the first place, to the heterogeneity between the women of the different study groups, which is relevant in terms of genetic susceptibility, ethnicity and socioeconomic status, habitat and severity of exposure to BS

or TS, which makes it difficult to determine the contribution of each factor. Related to the validation of RT-qPCR, two miRNAs overexpressed in the PCR matrix, contrary to the RT-qPCR, were under-expressed, miR-10a-5b and miR-15b-5p, which would be related to the small number in the sample ($n = 3$) and with the randomness in the selection process. Regarding the size of the sample used in the validation ($n = 25$), it is consistent with the fact that the majority of women with COPD-TS are in GOLD III-IV status, which is not a limitation to select, while those with COPD-BS commonly have GOLD I-II status, and rarely GOLD III-IV status, making it difficult to select a larger sample of COPD-BS. Additionally, it will be necessary in future protocols to validate some of the target miRNAs from the mRNAs that were deregulated between groups, to fully understand their possible involvement in the of prevention or contribution in COPD.

Conclusion

Our findings suggest that women with severe to very severe BS-COPD have a different serum miRNA expression profile compared to those with TS-COPD, and with respect to healthy women exposed to BS or TS; deregulated miRNAs could be potentially relevant, miR-200c-3p, which was under-expressed in COPD-BS, while miR-10a-5p, which was overexpressed, and miR-15b-5p and miR-30d-5p under-expressed in COPD-TS; nevertheless, more studies are needed to corroborate the specific role of each miRNA in the group of women that were compared, for which the expression of some target mRNAs and their proteins might be analyzed.

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