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REFERENCES

1. Russell R, Brightling C. Mepolizumab for the reduction of exacerbations in severe eosinophilic asthma. *Expert Rev Respir Med.* 2016;10(6):607-617.
2. Suzuki Y, Wakahara K, Nishio T, Ito S, Hasegawa Y. Airway basophils are increased and activated in eosinophilic asthma. *Allergy* 2017;72(10):1532-1539.
3. Leffler J, Jones AC, Hollams EM, et al. Basophil counts in PBMC populations during childhood acute wheeze/asthma are associated with future exacerbations. *J Allergy Clin Immunol.* 2018;142(5):1639-1641.
4. Mori Y, Iwasaki H, Kohno K, et al. Identification of the human eosinophil lineage-committed progenitor: revision of phenotypic definition of the human common myeloid progenitor. *J Exp Med.* 2009;206(1):183-193.

5. Buttner C, Lun A, Splettstoesser T, Kunkel G, Renz H. Monoclonal anti-interleukin-5 treatment suppresses eosinophil but not T-cell functions. *Eur Respir J.* 2003;21(5):799-803.
6. Flood-Page P, Menzies-Gow A, Phipps S, et al. Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. *J Clin Invest.* 2003;112(7):1029-1036.
7. Nair P, Pizzichini M, Kjarsgaard M, et al. Mepolizumab for prednisone-dependent asthma with sputum Eosinophilia. *N Engl J Med.* 2009;360(10):985-993.
8. Kelly EA, Esnault S, Liu LY, et al. Mepolizumab attenuates airway eosinophil numbers, but not their functional phenotype, in asthma. *Am J Respir Crit Care Med.* 2017;196(11):1385-1395.
9. Patil SP, Wisnivesky JP, Busse PJ, Halm EA, Li XM. Detection of immunological biomarkers correlated with asthma control and quality of life measurements in sera from chronic asthmatic patients. *Ann Allergy Asthma Immunol.* 2011;106(3):205-213.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Circulating miRNAs as diagnostic tool for discrimination of respiratory disease: Asthma, asthma-chronic obstructive pulmonary disease (COPD) overlap and COPD

To the Editor,

Asthma is a respiratory condition characterized by reversible airflow obstruction and airway inflammation usually provoked by eosinophils,¹ which is usually associated with atopy and early onset. Although asthma normally presents itself alone, it does not protect against chronic obstructive pulmonary disease (COPD), which is another lung pathology that differs from asthma in the no-reversible airflow obstruction, adulthood onset and the triggering by noxious substances.² This condition of having concomitant diagnosis of asthma and COPD has been identified as asthma-COPD overlap (ACO), and it is characterized by the features of both diseases.³

Nowadays, only spirometry and clinical manifestations can differentiate between both pathologies. Their differentiation is important because their treatment is different. The use of inhaled corticosteroids, the cornerstone of asthma management, could be harmful in COPD, since it has been associated with the occurrence of

pneumonia. For this reason, they are not indicated in COPD, unless some clinical characteristics are present, the presence of high blood eosinophil counts being the most important, which defines ACO. Thus, the use of disease biomarkers might help the clinical practice. MicroRNAs (miRNAs) are small noncoding RNAs associated with disease mechanisms and have been described as good biomarkers. We have previously described that differential miRNAs expression obtained from eosinophils of asthmatics compared with healthy individuals was able to cluster asthmatics and healthy subjects.⁴ Five of these miRNAs (miR-1246, miR-144-5p, miR-320a, miR-185-5p and miR-21-5p) measured in serum were able to differentiate asthmatics from healthy subjects by statistical models.⁴ We hypothesized that some of these miRNAs could be specific to asthma, and they may differentiate asthma from other respiratory diseases. Therefore, the aim of the study was to compare these miRNAs between asthmatics, COPD and ACO and to analyse whether they could be used as asthma biomarkers.

Abbreviations: ACO, asthma-COPD overlap; AMPK, AMP-activated protein kinase; AUC, area under the curve; COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in the first second; Fox, forkhead box; FVC, forced vital capacity; IL, interleukin; MAPK, mitogen-activated protein kinases; PI3K-AKT, phosphoinositide-3-kinase-protein kinase B; RNA, ribonucleic acid; ROC, receiver operating characteristic; sRAGE, soluble form of receptor for advanced glycation end products; TGF- β , transforming growth factor beta; YKL-40, Chitinase 3-like 1.

Rodrigo-Muñoz and Rial equally contributed to this publication.

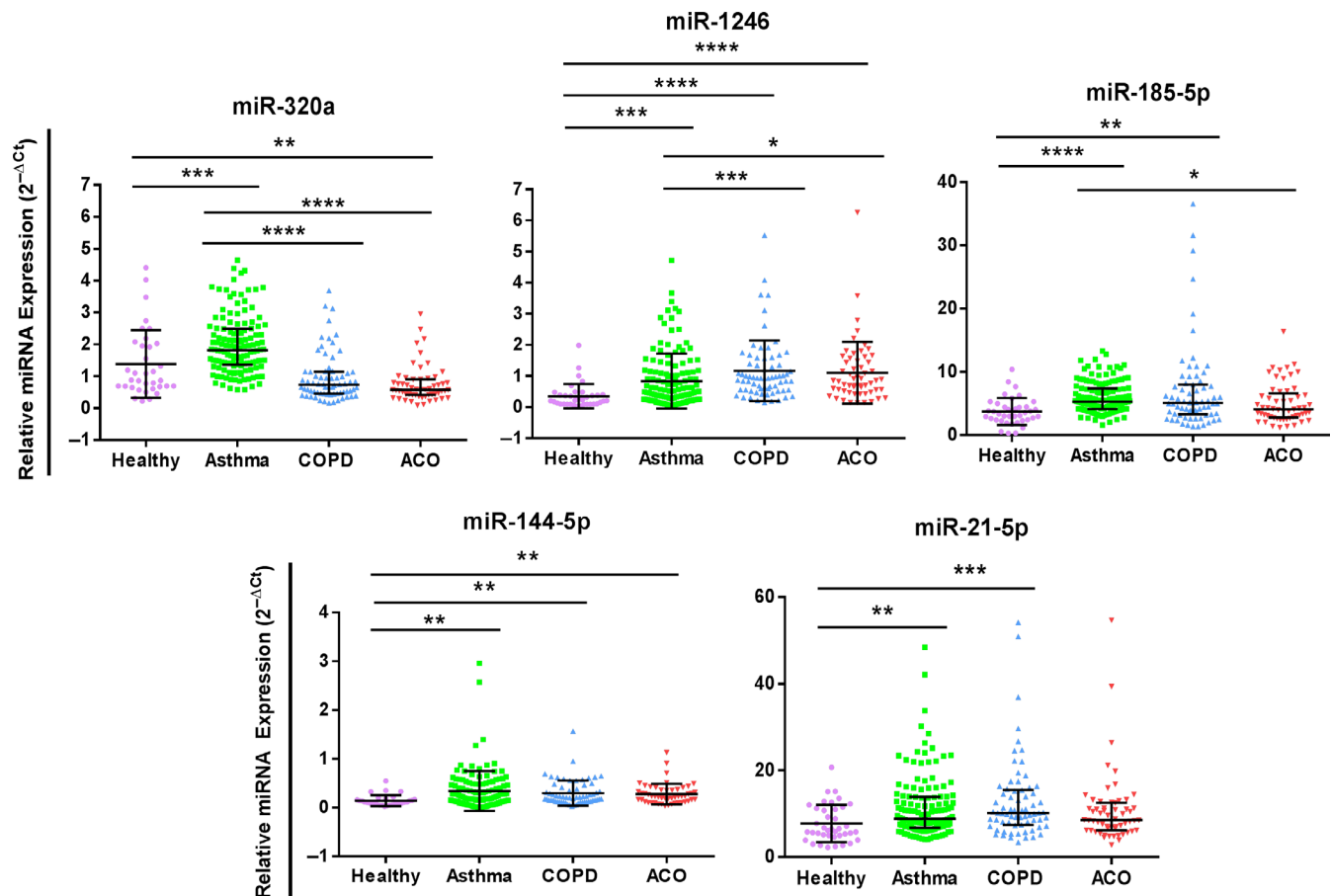


FIGURE 1 Relative miRNAs expression for healthy, asthma, chronic obstructive pulmonary disease (COPD) and asthma-COPD overlap individuals, represented as the $2^{-\Delta C_t}$. Comparisons between groups were made by Dunn multiple comparison test after nonparametric Kruskal-Wallis test (samples were nonparametric). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$

We extracted miRNAs from the serum of patients diagnosed with asthma, COPD or ACO recruited in Spanish hospitals for a cross-sectional, observational, multicentre study. COPD diagnosis was performed according to the GOLD initiative: postbronchodilator FEV1/FVC ≤ 0.70 , with smoking history > 10 packs-year, in the absence of asthma⁵ and ACO by the presence of COPD with ≥ 200 eosinophils/ μ L, or a previous asthma diagnosis with smoking history ≥ 20 packs-year.⁵ The asthmatic and healthy controls come from a different cohort previously published, the asthmatics being diagnosed according to the Spanish Asthma Guideline (GEMA) as described in Rodrigo-Muñoz et al⁴ (Table S1).

After extraction, RNA was retrotranscribed to cDNA, and afterwards, we evaluated miRNAs expression in serum from healthy individual, asthma, COPD and ACO patients by quantitative PCR. MiR-191-5p was used as endogenous control. Healthy and asthmatic miRNAs expression values are the same as described in Rodrigo-Muñoz et al⁴ (more details on the Data S1).

From the studied miRNAs, we found that miR-320a was differentially expressed in serum from asthmatics compared to patients with COPD and ACO; miR-1246 was also differentially expressed between asthma and COPD and ACO; and miR-185-5p was upregulated in asthma vs ACO (Figure 1). Nevertheless, only miR-320a expression

was able to differentiate asthmatics from COPD and ACO with a good area under the curve (AUC) value (over 0.8; Figure 2 and Table S2). MiR-1246 and miR-185-5p have AUCs lower than 0.7 (data not shown).

MiR-1246, miR-144-5p, miR-185-5p and miR-21-5p were differentially expressed between COPD and healthy subjects; besides, miR-1246 and miR-144-5p relative expression may be used to differentiate between them. This was similar for ACO where miR-320a, miR-1246 and miR-144-5p were differentially expressed and can differentiate ACO from healthy controls (Table S2). All these data seem to indicate the specificity of miR-320a for asthma.

In this study, we did not find any differentially expressed miRNA between COPD and ACO patients, not even when differentiating subjects by smoking habit for each disease (Figure S1), showing that ACO and COPD share the expression of these miRNAs. When we studied correlations between blood eosinophils and miRNAs expression, no correlation was found, with the exception of a low correlation for miR-144-5p in ACO ($r = -0.35$) that probably had no clinical implication.

Next, we performed qualitative logistic regression models to evaluate whether the miRNAs combination was able to better distinguish asthmatics from ACO/COPD. Combined expression of miR-1246, miR-320a and miR-185-5p improves the differentiation of asthma from COPD because AUC values reached 0.90. We can conclude

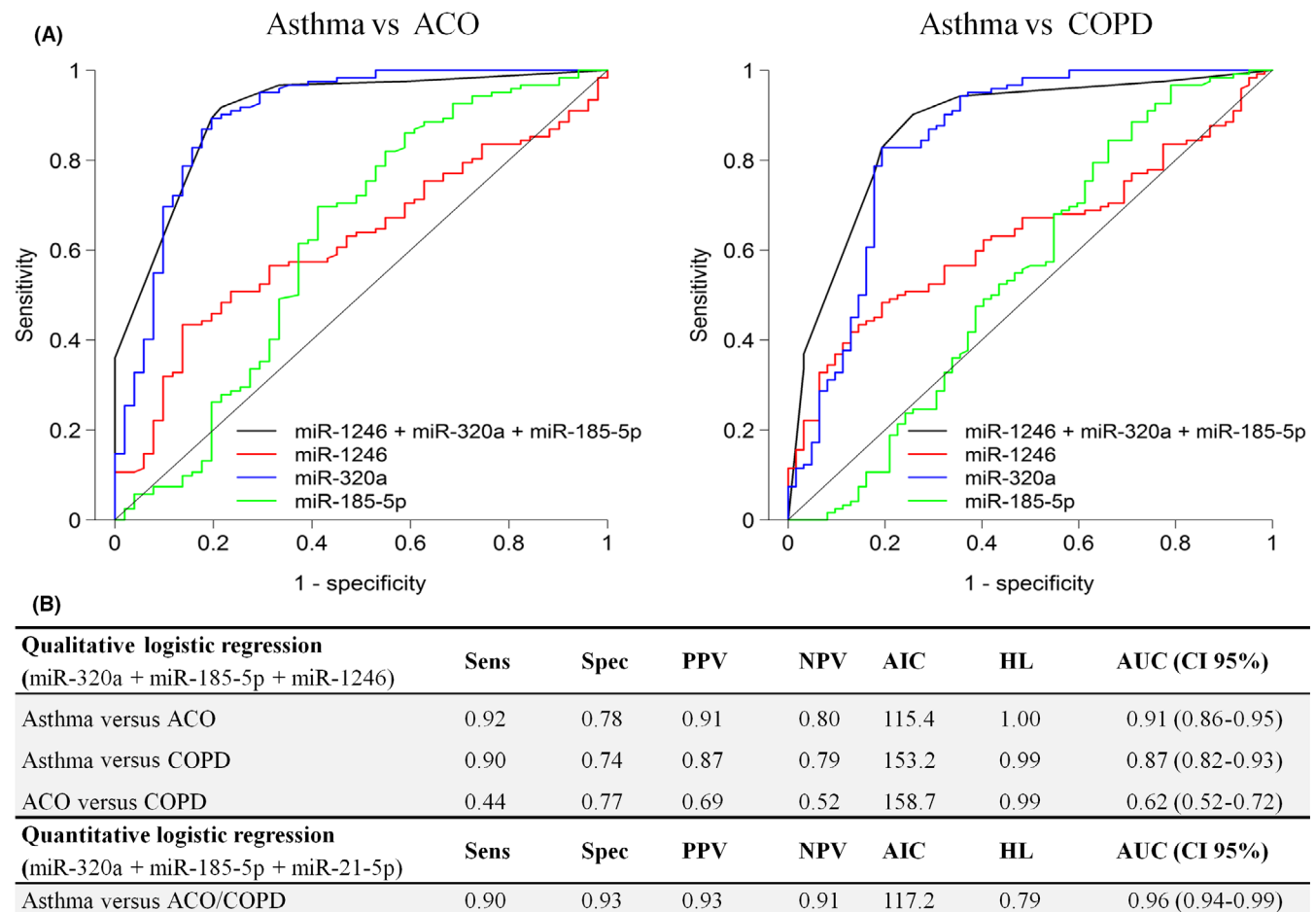


FIGURE 2 A, Graphical representation of the area under the curve (AUC) values obtained for each comparison receiver operating characteristic (ROC) curve, and representation of the AUC for the qualitative logistic regression models for asthma vs asthma-chronic obstructive pulmonary disease (COPD) overlap and COPD. B, Table shows the individual values of miRNAs expression cut-off (Δ Ct), sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and AUC. For the logistic regression models, table shows the same data as for the ROC curves plus the Akaike information criteria and the *P* value for the Hosmer-Lemeshow test

that the miRNAs combination is better to differentiate asthma from COPD, whereas miR-320a alone is the most appropriate to differentiate asthma and ACO. In our study, miRNAs expression is even better for asthma discrimination than blood eosinophils, which has an AUC for ACO-asthma of 0.56 and an AUC of COPD-asthma of 0.86.

Similar to what happened with the miRNAs individually, the expression of combined miRNAs was not useful enough to differentiate COPD from ACO (AUC = 0.62), showing that the expression of studied miRNAs is similar in ACO and COPD (Figure 1).

Moreover, when using a quantitative logistic regression model grouping ACO and COPD patients due to their similar miRNAs expression, the combination of miR-185-5p, miR-320a and miR-21-5p was able to differentiate asthmatics against ACO/COPD with an AUC of 0.96 (Figure 2).

Trying to get an insight into the pathophysiological mechanisms that may differ between asthma and COPD/ACO consequence of these differences in miRNAs expression, we performed target-miRNA analysis by miRSystem. Afterwards, we performed an enrichment analysis within the STRING database with the targeted genes to obtain altered pathways (more details on the Data S1).

Gene targets for the deregulated miRNAs between COPD/ACO and asthma belong to MAPK, AMPK, PI3K-AKT and Erb2 pathways that may regulate cell functions as proliferation or muscle contraction. Other genes targeted by these miRNAs belong to pathways related to immune regulation as T-cell receptor, FoxO or TGF- β (Table S3). Results show that the differences found between ACO/COPD and asthma may be due to the targeting effect of these miRNAs.

Until now, several biomarkers have been investigated in asthma, COPD and ACO such as sRAGE, periostin, YKL-40 or IL-5, in different biofluids.⁶⁻⁸ Nevertheless, the field of miRNAs as biomarkers for these diseases is still scarce, since it has only been described that COPD, ACO and asthma show higher sputum levels of miR-145 and miR-338 compared with healthy subjects.⁹

Here, we show that serum miRNAs are promising biomarkers of respiratory diseases, with high values of sensitivity and specificity. We previously described a specific miRNAs profile that was upregulated in asthma compared with healthy controls. Interestingly, miR-320a is only upregulated in asthma, so it may be asthma-specific, and it could be used for differentiating asthma from other pulmonary diseases such as COPD and ACO. Besides,

some of these miRNAs, such as miR-1246 and miR-144-5p, are also upregulated in COPD/ACO, which could be explained as related to a broad spectrum of airway diseases. Altogether, our results show the emerging importance of miRNAs as diagnostic tools in respiratory diseases.

CONFLICTS OF INTEREST

JS reports having served as a consultant to Thermo Fisher, Novartis, Sanofi, Leti, FAES FARMA, Mundipharma and GSK; having been paid lecture fees by Novartis, GSK, Stallergenes, LETI and FAES FARMA; and received grant support for research from Thermo Fisher and ALK. VDP reports having served as a speaker/consultant to Astra-Zeneca. BGC reports personal fees and/or grants from AstraZeneca, Boehringer, Novartis, Chiesi, Rovi, Menarini, Esteve, outside the submitted work. The rest of the authors declare no conflicts of interest.

FUNDING INFORMATION

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REFERENCES

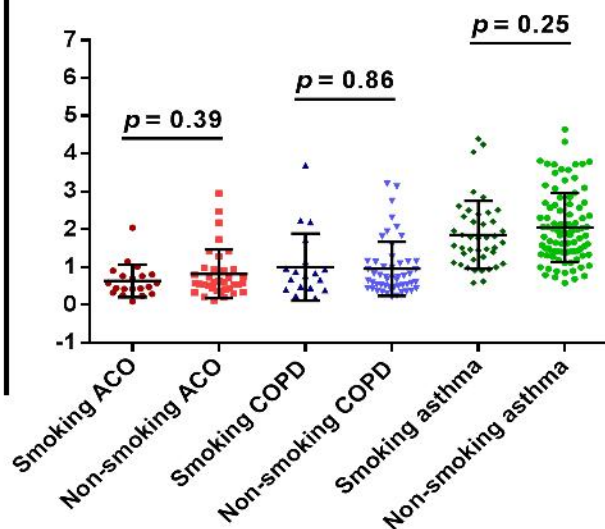
1. Sastre B, Rodrigo-Muñoz JM, Garcia-Sanchez DA, Cañas JA, Del Pozo V. Eosinophils: old players in a new game. *J Invest Allergol Clin Immunol*. 2018;28:289-304.
2. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol*. 2008;8:183-192.
3. *Global Strategy for Asthma Management and Prevention*, Global Initiative for Asthma (GINA) [Internet]. 2015. <http://www.ginasthma.org/>
4. Rodrigo-Muñoz JM, Cañas JA, Sastre B, et al. Asthma diagnosis using integrated analysis of eosinophil microRNAs. *Allergy*. 2019;74:507-517.
5. Cosío BG, Pérez de Llano L, Lopez Viña A, et al. Th-2 signature in chronic airway diseases: towards the extinction of asthma-COPD overlap syndrome? *Eur Respir J*. 2017;1:49.
6. Iwamoto H, Gao J, Koskela J, et al. Differences in plasma and sputum biomarkers between COPD and COPD-asthma overlap. *Eur Respir J*. 2014;43:421-429.
7. Shirai T, Hirai K, Gon Y, et al. Assessment of serum periostin and YKL-40 may identify asthma-COPD overlap. *J Allergy Clin Immunol*. 2019;7:134-145.
8. Pérez de Llano L, Cosío BG, Iglesias A, et al. Th2 and non-Th2 inflammatory pattern in the asthma-COPD overlap: a network approach. *Int J Chron Obstruct Pulmon Dis*. 2018;13:591-601.
9. Lacedonia D, Palladino GP, Foschino-Barbaro MP, Scioscia G, Carpagnano GE. Expression profiling of miRNA-145 and miRNA-338 in serum and sputum of patients with COPD, asthma, and asthma-COPD overlap syndrome phenotype. *Int J Chron Obstruct Pulmon Dis*. 2017;12:1811-1817.

SUPPORTING INFORMATION

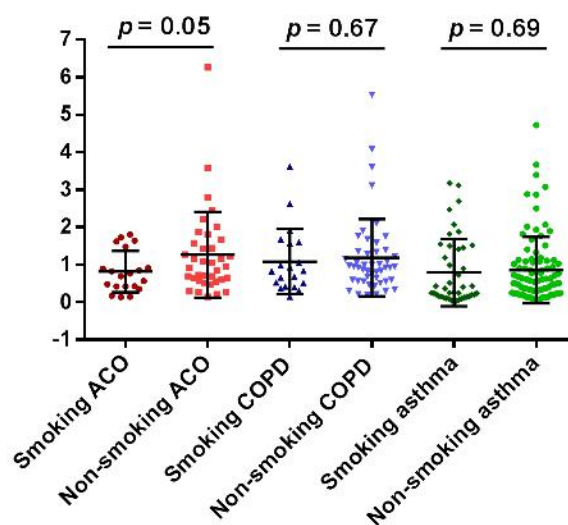
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Relative miRNA Expression ($2^{-\Delta Ct}$)

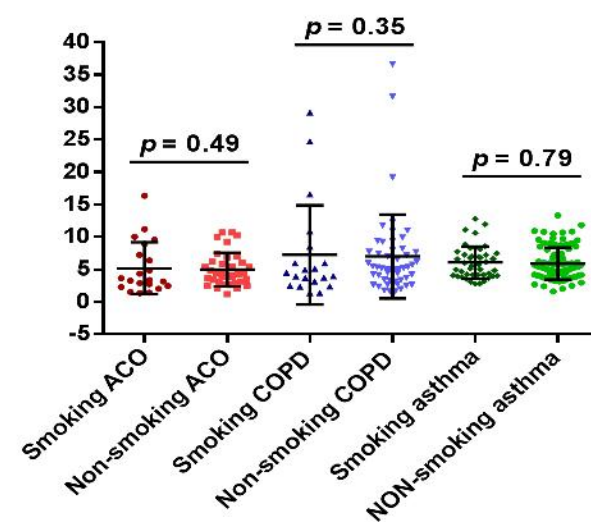
miR-320a



miR-1246

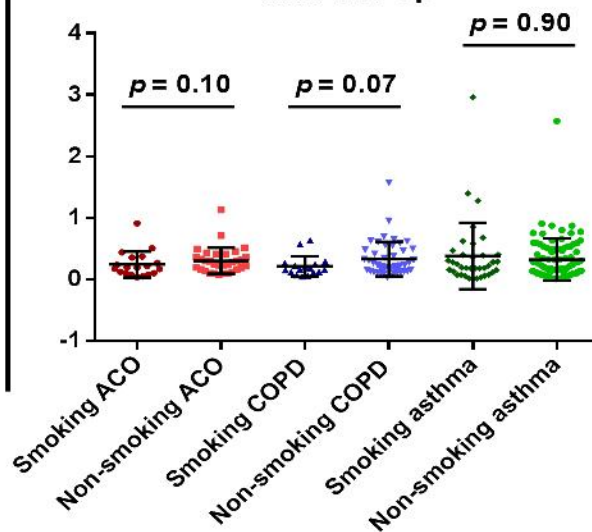


miR-185-5p



Relative miRNA Expression ($2^{-\Delta Ct}$)

miR-144-5p



miR-21-5p

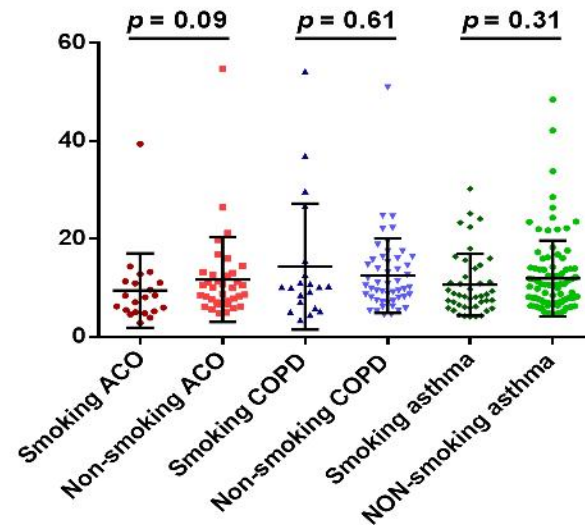


Table S1. Clinical characteristics of the subjects of study.

		Healthy (n = 39)	Asthmatics (n = 138)	ACO (n = 63)	COPD (n = 75)
Age (years)*		40.92 (21-79)	54.20 (16-88)	65.71	67.95
Atopy (%)		3 (7.69)	103 (75.63)	13 (20.63)	8 (10.67)
Smoking history	Current (%)	2 (5.13)	13 (9.42)	22 (34.92)	21 (28.0)
	Ex smoker (%)	0 (0.00)	30 (21.73)	41 (65.08)	54 (72.00)
	Non-smoker (%)	16 (41.02)	94 (68.11)	0 (0.00)	0 (0.00)
FVC (%)†		NA	87.00 (35-140)	108.00 (62-150)	110.00 (59-150)
FEV₁ (%)†		NA	84.00 (32-147)	54.00 (8-91)	55.00 (23-110)
Total serum IgE (kU/L)†		NA	172.00 (4-2000)	70.50 (5-788)	82.49 (4-478)
Peripheral blood eosinophils (cells/μl)*		<350.00	365.20 (3-1140)	375.29 (210-940)	113.47 (0-200)
Bronchodilators	Long action (%)	0 (0.00)	105 (76.08)	59 (93.65)	70 (93.33)
Corticoid daily dose (μg)†		0.00	720.00 (0-4000)	400.00 (92-640)	320.00 (92-960)
Leukotriene receptor agonist (%)		0 (0.00)	51 (36.95)	0 (0.00)	1 (1.33)

Results are shown as *Mean (range), †Median (range). FEV₁: forced expiratory volume in first second. FVC: forced vital capacity.

Table S2. Individual miRNAs ROC curves for diagnosis.

miRNAs individual ROC curve	Cutoff ($2^{-\Delta C_t}$)	Sens	Spec	PPV	NPV	AUC (CI 95%)
miR-1246 healthy vs. ACO	0.41	0.84	0.78	0.86	0.76	0.85 (0.77 - 0.93)
miR-144-5p healthy vs. ACO	0.16	0.73	0.74	0.82	0.62	0.75 (0.64 - 0.86)
miR-320a healthy vs. ACO	0.64	0.58	0.80	0.83	0.55	0.72 (0.61 - 0.82)
miR-1246 healthy vs. COPD	0.51	0.81	0.87	0.92	0.71	0.87 (0.80 - 0.95)
miR-144-5p healthy vs. COPD	0.15	0.69	0.71	0.83	0.54	0.75 (0.65 - 0.86)
miR-320a asthma vs. ACO	0.99	0.82	0.89	0.77	0.92	0.90 (0.85 - 0.96)
miR-320a asthma vs. COPD	1.17	0.80	0.82	0.70	0.89	0.84 (0.78 - 0.91)

Table S3. KEGG pathways and genes for each pathway targeted by the deregulated miRNAs between ACO/COPD and asthma, studied by STRING.

KEGG PATHWAY	FDR	TARGETED GENES FOR EACH PATHWAY
MicroRNAs in cancer	3.24e-07	<i>ABCC1, BCL2L2, BMF, BMI1, CCND2, CCNE1, CDK6, CDKN1A, CRKL, DNMT1, E2F3, FZD3, GRB2, HDAC4, HMGA2, KIF23, MAPK1, MCL1, MMP16, PTEN, RHOA, SOX4, STAT3, STMN1, TP53, TPM3, VEGFA, VIM</i>
Focal adhesion	0.000322	<i>ACTN4, AKT1, AKT3, ARHGAP5, CAV1, CCND2, CDC42, COL24A1, CRKL, CTNNB1, DIAPH1, ELK1, FLNC, GRB2, IGF1R, MAPK1, PAK1, PAK6, PAK7, PPP1R12A, PPP1R12B, PTEN, RAC1, RAP1A, RHOA, VEGFA, XIAP</i>
AMPK signaling pathway	0.00114	<i>AKT1, AKT3, CAB39, CPT1B, CREB3L2, CREB5, FOXO1, HMGCR, IGF1R, PFKM, PPARGC1A, PPP2R2C, PPP2R5B, PPP2R5D, PRKAG2, RAB14, TSC1, ULK1</i>
MAPK signaling pathway	0.00489	<i>AKT1, AKT3, CACNA1E, CACNB4, CACNG4, CDC42, CRKL, DUSP4, ELK1, FGF13, FLNC, GRB2, MAPK1, MAPK14, MAPK8IP3, MAX, NFATC3, NTRK2, PAK1, PPM1B, PPP3R1, RAC1, RAP1A, RASA1, SRF, STMN1, TP53</i>
PI3K-Akt signaling pathway	0.00533	<i>AKT1, AKT3, CCND2, CCNE1, CDK6, CDKN1A, COL24A1, CREB3L2, CREB5, CSF1, EIF4B, FGF13, GRB2, IGF1R, IL6R, KITLG, MAPK1, MCL1, PHLPP2, PPP2R2C, PPP2R5B, PPP2R5D, PTEN, RAC1, RXRA, TP53, TSC1, VEGFA, YWHAH, YWHAQ, YWHAZ</i>
Adherens junction	0.0112	<i>ACTN4, CDC42, CTNNB1, IGF1R, IQGAP1, MAPK1, RAC1, RHOA, SMAD4, TJP1, YES1</i>
ErbB signaling pathway	0.0137	<i>AKT1, AKT3, CAMK2D, CDKN1A, CRKL, ELK1, EREG, GRB2, MAPK1, PAK1, PAK6, PAK7</i>
cGMP-PKG signaling pathway	0.0153	<i>ADCY6, AKT1, AKT3, ATP1A3, ATP1B2, ATP2B2, CREB3L2, CREB5, MAPK1, MEF2D, NFATC3, PDE3A, PLCB1, PPP1R12A, PPP3R1, PRKG1, RHOA, SRF</i>
FoxO signaling pathway	0.0153	<i>AKT1, AKT3, CCND2, CDKN1A, FOXO1, GRB2, IGF1R, MAPK1, MAPK14, PLK2, PLK3, PRKAG2, PTEN, SMAD4, STAT3</i>
Cell cycle	0.0157	<i>CCND2, CCNE1, CDK6, CDKN1A, E2F3, RAD21, SKP1, SMAD4, STAG2, TP53, YWHAH, YWHAQ, YWHAZ</i>
Hippo signaling pathway	0.0157	<i>BMP6, BMPR1A, CCND2, CTNNB1, DLG2, FZD3, MOB1B, PARD6B, PPP2R2C, SMAD4, TEAD1, WNT11, YWHAH, YWHAQ, YWHAZ</i>
T cell receptor signaling pathway	0.0163	<i>AKT1, AKT3, CDC42, GRB2, ICOS, MAPK1, MAPK14, NFATC3, PAK1, PAK6, PAK7, PPP3R1, RHOA</i>
p53 signaling pathway	0.0188	<i>BAI1, CCND2, CCNE1, CDK6, CDKN1A, PTEN, RFW2, SESN2, TP53, ZMAT3</i>
TGF-beta signaling pathway	0.0188	<i>ACVR1B, ACVR2A, BMP6, BMPR1A, FST, MAPK1, RHOA, SKP1, SMAD4, SMAD5, SP1</i>
Rap1 signaling pathway	0.0201	<i>ADCY6, AKT1, AKT3, CDC42, CRKL, CSF1, CTNNB1, FGF13, GRIN2A, IGF1R, KITLG, MAPK1, MAPK14, PARD6B, PFN1, PLCB1, RAC1, RALA, RAP1A, RHOA, VEGFA</i>
mTOR signaling pathway	0.0262	<i>AKT1, AKT3, CAB39, EIF4B, MAPK1, PTEN, TSC1, ULK1, VEGFA</i>
Ras signaling pathway	0.0401	<i>AKT1, AKT3, CDC42, CSF1, ELK1, FGF13, GRB2, GRIN2A, IGF1R, KITLG, MAPK1, PAK1, PAK6, PAK7, RAC1, RALA, RAP1A, RASA1, RHOA, SYNGAP1, VEGFA</i>

KEGG: Kyoto Encyclopedia of Genes and Genomes; FDR: False Discovery Rate

Online Supplement

Material and methods

Patient selection

For the purpose of studying miRNA expression in COPD and ACO patients, subjects diagnosed with COPD (n=75) or ACO (n=63) were recruited in several Hospitals around Spain. Patients were included in a cross-sectional observational, multicenter study. COPD diagnosis was performed according to the GOLD initiative: post bronchodilator FEV1/FVC $\leq$ 0.70, with smoking history $>$ 10 packs-year, in the absence of asthma¹, and ACO by the presence of COPD with $\geq$ 200 eosinophils/ μ L, or a previous asthma diagnosis with smoking history $\geq$ 20 pack-years². The study followed the guidelines set by the Helsinki declaration and was approved by Research Ethics Committee of the Balearic Islands. Subjects included signed a declaration of consent.

We also included an asthmatic and healthy patient's cohort that is described in our previous article³. Briefly 138 asthmatics and 39 healthy donors were recruited in the Allergy Department of IIS-Fundación Jiménez Díaz and Hospital La Paz-Institute for Health Research (IdiPAZ) in Madrid. Asthma clinical diagnosis was confirmed by a greater than 12% improvement in FEV1 after ten minutes of administration of inhaled terbutaline or a positive methacholine airway hyperresponsiveness test defined by PC20 methacholine $<$ 16 mg/mL.

Serum miRNA extraction

Serum from studied subjects was obtained by centrifugation at 3000 rpm for 10 minutes at 4°C in anticoagulant free tubes and stored at -80°C until used. For microRNA extraction 200 μ L of serum was used as main input for the isolation kit (miRNeasy serum/plasma advanced kit, Qiagen, Hilden, Germany), as provider described. Three synthetic miRNA Spike-ins (Sp2, Sp4 and Sp5) were included to ensure optimal RNA

extraction (spike-in mix, miRCURY LNA™ Universal RT microRNA PCR, RNA spike-in kit, Exiqon, Qiagen, Copenhagen, Denmark).

cDNA retrotranscription

MiRNAs extracted from serum were retrotranscribed to cDNA by using the kit Universal cDNA Synthesis kit II (Exiqon, Denmark), following the manufacturer instructions. Briefly, 2 µL of total RNA are mixed with reverse transcription enzyme, and with another synthetic miRNA Spike-in (Sp6), which accounts for control of a correct retrotranscription to cDNA; yielding a total volume of 10 µL. The reaction was performed during 60 minutes at 42°C, then 5 minutes at 95°C, and immediately at 4°C; in a Veriti Thermal Cycler (Applied Biosystems, Warrington, UK).

miRNA expression analysis by Real Time-PCR (qPCR)

After cDNA retrotranscription miRNAs expression was evaluated by quantitative PCR (qPCR) which were performed using ExiLent SYBR® Green master mix (Exiqon, Denmark) following manufacturer's instructions. Summarized, for evaluation of miRNA expression, cDNA produced in the previous step was diluted 1:40 in RNase free water (Exiqon, Denmark), and 4 µL of this diluted cDNA was mixed with the SYBR Green and the different probes. The evaluated miRNAs were selected by being deregulated between asthmatic patients compared to healthy controls in both eosinophils and serum, miRNAs miR-1246, miR-144-5p, miR-320a, miR-185-5p and miR-21-5p as previously described³. Besides, the miRNAs listed before other miRNAs were evaluated; like miR-191-5p and miR-103a-3p as endogenous controls; Sp2, Sp4, Sp5 and Sp6 as controls for correct RNA extraction and retrotranscription and miR-451a and miR-23a-3p for hemolysis control. Each sample was run in triplicate. All reactions were performed in a Light Cycler® 96 thermocycler (Roche, Basilea,

Switzerland). The incubation program was carried out for 45 cycles of 95°C during 10 seconds and 60°C during 1 min. DNA melting was performed by heating at 95°C for 5 seconds, then 65°C during 1 min, and finally at 97°C for 1 second. Samples were cooled for 10 seconds at 40°C. Cycle threshold (Ct) data was taken in the last step of the melting cycle in a continuous way. Results were analyzed with LightCycler® 96 SW 1.1 (Roche) software. Hsa-miR-191-5p was selected as endogenous control. Healthy and asthmatics miRNAs expression values data are the same as described in Rodrigo-Muñoz *et al*³. Each miRNA expression was calculated by using the $2^{-\Delta Ct}$ method⁴, where: $\Delta Ct = Ct_{miRNA} - Ct_{hsa-miR-191-5p}$. Fold expression of miRNAs between COPD/ACO and asthma were calculated by the $2^{-\Delta\Delta Ct}$ method where $\Delta\Delta Ct = \Delta Ct_{COPD/ACO} (Ct_{miRNA} - Ct_{miR-191-5p}) - \Delta Ct_{Asthma} (Ct_{miRNA} - Ct_{miR-191-5p})$.

Analysis of gene targets and pathway enrichment

Determination of gene targets for the deregulated miRNAs was studied by miRSystem miRNA targets database⁵. We included the miRNAs deregulated between COPD/ACO and asthma. This software allows to introduce the miRNAs of interest alongside with their expression rates (also known as fold) between two conditions, to obtain the targeted genes for those miRNAs. We calculated the fold differences between asthma and COPD/ACO by the $2^{-\Delta\Delta Ct}$ method⁴ for measuring relative miRNAs expression, and results showed a 1.8-fold for miR-1246; a 0.39 fold for miR-320a and a 0.9 fold for miR-185-5p. We did not include miR-144-5p as it does not have validated targets in miRSystem database, neither miR-21, which presented with a fold increase equal to one. After targets were defined an enrichment analysis was performed with the targeted genes. The aim is to extract the specific pathways and biological functions in which the set of miRNAs is involved as a signature. We used the enrichment analysis facility implemented in the STRING database⁶. We report KEGG pathways with an FDR <0.05.

Statistical Analysis

Unpaired comparisons were made with Student *t* test for Gaussian samples and Mann-Whitney U-test for non-Gaussian samples. For more than two groups comparisons ANOVA with Bonferroni *post-hoc* for Gaussian samples and Kruskal-Wallis with Dunn *post-hoc* test for non-Gaussian ones were applied. *P* value less than 0.05 was considered as significant.

Receiver Operating Characteristic (ROC) curve was performed to determine the capacity of miRNAs expression to be a disease biomarker, setting an Area Under the Curve (AUC) over 0.7 as a good biomarker. A Logistic Regression Analysis was elaborated for enhancing the capacity of individual miRNAs expression to be utilized for diagnosis, by combining expression of various miRNAs. Akaike Information Criteria (AIC) and the *p* value for the Hosmer-Lemeshow (HL) test were also used for determination of the best logistic regression model, setting as the best model the one that met the following requirements: higher AUC value, lower AIC value, and HL higher than 0.1

All statistical calculations and graphs were performed with GraphPad Prism 6 (GraphPad Software Inc., San Diego, USA). ROC analysis and multivariate logistic regression model with quantitative predictors were performed using R software.

Online Supplement References:

1. Global Initiative for Chronic Obstructive Lung Disease (GOLD). Global strategy for the diagnosis, management, and prevention of Chronic Obstructive Pulmonary Disease - 2019 Report. Glob Initiat Chronic Obstr Lung Dis Inc .

2019;

2. Cosío BG, Pérez de Llano L, Lopez Viña A, Torrego A, Lopez-Campos JL, Soriano JB et al. Th-2 signature in chronic airway diseases: towards the extinction of asthma-COPD overlap syndrome?. *Eur Respir J*. 2017; 1;49.
3. Rodrigo-Muñoz JM, Cañas JA, Sastre B, Rego N, Greif G, Rial M, et al. Asthma diagnosis using integrated analysis of eosinophil microRNAs. *Allergy: European Journal of Allergy and Clinical Immunology*. 2019;74:507-517
4. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods*. 2001; 25:402-8.
5. Lu T-P, Lee C-Y, Tsai M-H, Chiu Y-C, Hsiao CK, Lai L-C, et al. miRSystem: An Integrated System for Characterizing Enriched Functions and Pathways of MicroRNA Targets. Watson M, editor. *PLoS One*. 2012;7.
6. Szklarczyk D, Morris JH, Cook H, Kuhn M, Wyder S, Simonovic M, et al. The STRING database in 2017: Quality-controlled protein-protein association networks, made broadly accessible. *Nucleic Acids Res*. 2017; 45:362-368.

Supplementary Figure Legends.

Figure S1. Relative miRNAs expression depending on smoking habit for each studied disease condition: healthy, asthma, COPD, and ACO represented as the $2^{-\Delta Ct}$. Comparisons between groups were made by Student's *t* test for Gaussian samples or

Mann-Whitney test for non-Gaussian ones. P values are shown above each pair of comparisons.