

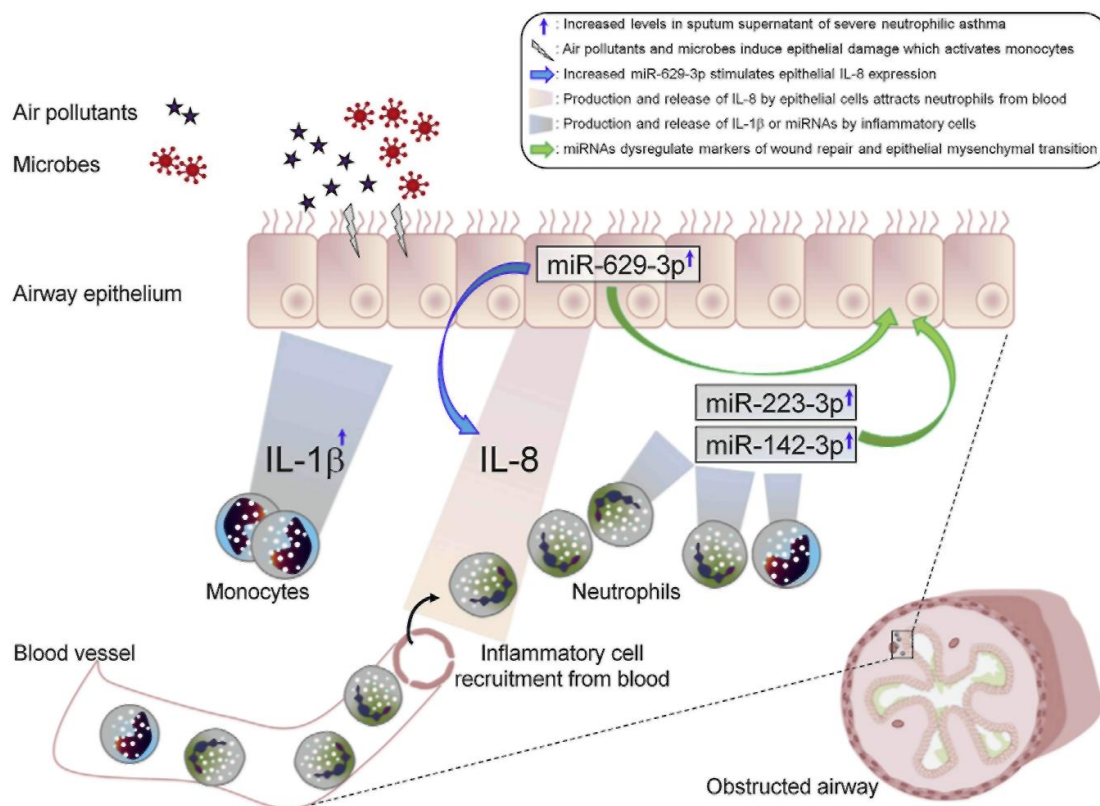
## Asthma inflammatory phenotypes show differential microRNA expression in sputum

Tania Maes, PhD,<sup>a</sup> Francisco Avila Cobos, MSc,<sup>b</sup> Florence Schleich, MD, PhD,<sup>c</sup> Valentina Sorbello, PhD,<sup>d</sup> Monique Henket, MLT,<sup>c</sup> Katleen De Preter, PhD,<sup>b</sup> Ken R. Bracke, PhD,<sup>a</sup> Griet Conicckx, Pharm,<sup>a</sup> Claire Mesnil, PhD,<sup>e</sup> Jo Vandesompele, PhD,<sup>b</sup> Lies Lahousse, PhD,<sup>a</sup> Fabrice Bureau, PhD,<sup>e</sup> Pieter Mestdagh, PhD,<sup>b</sup> Guy F. Joos, MD, PhD,<sup>a</sup> Fabio L. M. Ricciardolo, MD, PhD,<sup>d</sup> Guy G. Brusselle, MD, PhD,<sup>a</sup> and Renaud Louis, MD, PhD<sup>c</sup> *Ghent and Liege, Belgium, and Torino, Italy*

From <sup>a</sup>The Department of Respiratory Medicine, Ghent University Hospital; <sup>b</sup>the Center for Medical Genetics, Ghent University; <sup>c</sup>the Department of Respiratory Medicine, CHU Sart-TilmanB35, Liege; <sup>d</sup>the Department of Clinical and Biological Sciences, University of Torino; and <sup>e</sup>the Department of Cellular and Molecular Immunology, GIGA-Research Centre and Faculty of Veterinary Medicine, University of Liege.

### GRAPHICAL ABSTRACT

#### Role of microRNAs in severe neutrophilic asthma



### Abstract

**Background:** Asthma is classified according to severity and inflammatory phenotype and is likely to be distinguished by specific microRNA (miRNA) expression profiles. **Objective:** We sought to associate miRNA expression in sputum supernatants with the inflammatory cell profile and disease severity in asthmatic patients.

**Methods:** We investigated miRNA expression in sputum supernatants of 10 healthy subjects, 17 patients with mild-to-moderate asthma, and 9 patients with severe asthma using high-throughput, stem-loop, reverse transcriptase quantitative real-time PCR miRNA expression profiling (screening cohort, n = 36). Differentially expressed miRNAs were validated in an independent cohort (n = 60; 10 healthy subjects and 50 asthmatic patients). Cellular miRNA origin was examined by using *in situ* hybridization and reverse transcriptase quantitative real-time PCR. The functional role of miRNAs was assessed by using *in silico* analysis and *in vitro* transfecting miRNA mimics in human bronchial epithelial cells. Results: In 2 independent cohorts expression of miR-629-3p, miR-223-3p, and miR-142-3p was significantly upregulated in sputum of patients with severe asthma compared with that in healthy control subjects and was highest in patients with neutrophilic asthma. Expression of the 3 miRNAs was associated with sputum neutrophilia, and miR-223-3p and miR-142-3p expression was associated also with airway obstruction (FEV<sub>1</sub>/forced vital capacity). Expression of miR-629-3p was localized in the bronchial epithelium, whereas miR-223-3p and miR-142-3p were expressed in neutrophils, monocytes, and macrophages. Transfecting human bronchial epithelial cells with miR-629-3p mimic induced epithelial IL-8 mRNA and protein expression. IL-1 $\beta$  and IL-8 protein levels were significantly increased in sputum of patients with severe asthma and were positively associated with sputum neutrophilia. Conclusions: Expression of miR-223-3p, miR-142-3p, and miR-629-3p is increased in sputum of patients with severe asthma and is linked to neutrophilic airway inflammation, suggesting that these miRNAs contribute to this asthma inflammatory phenotype. (*J Allergy Clin Immunol* 2016;137:1433-46.)

**Key words:** *Asthma; microRNA; sputum; neutrophilic inflammation*

#### **Abbreviations used**

ACQ: Asthma Control Questionnaire

BMI: Body mass index

FVC: Forced vital capacity

GINA: Global Initiative for Asthma

HBEC: Human bronchial epithelial cell

ICS: Inhaled corticosteroid

IQR: Interquartile range

MAPK: Mitogen-activated protein kinase

miRNA: MicroRNA

NOD: Nucleotide-binding oligomerization domain

qPCR: Quantitative real-time PCR

SSC: Side scatter

VIM: Vimentin

Asthma is a chronic inflammatory airway disease characterized by airway hyperresponsiveness and reversible airway obstruction resulting in recurrent episodes of wheezing, shortness of breath, chest tightness, and cough. Asthma can be categorized into different phenotypes depending on the inflammatory profile (eosinophilic, neutrophilic, mixed granulocytic, and paucigranulo-cytic), presence of allergy (atopic vs nonatopic), and age of onset (childhood vs adult onset)<sup>1</sup>. This phenotyping has important therapeutic implications and can lead to more personalized medicine, in which identification of specific biomarkers or molecular mechanisms paves the way for specific (add-on) treatment. Such approaches can be especially relevant for patients with severe asthma, in whom current standard therapies do not offer sufficient benefit.

Both genetic and epigenetic alterations contribute to altered immune responses within the lungs of asthmatic patients. MicroRNAs (miRNAs) are highly conserved small, single-stranded, noncoding RNA molecules that control gene expression by targeting mRNAs for degradation or translational repression.<sup>2</sup> By targeting transcriptional activators or repressors, miRNAs can simultaneously regulate the expression of hundreds of genes, including other miRNAs. In this way miRNAs control various processes, such as cell proliferation, differentiation, and apoptosis. In patients with inflammatory conditions, miRNAs function in complex regulatory networks in which their expression can be controlled by either feed-forward or feed-backward mechanisms.<sup>3</sup> Although several studies addressing the role of specific miRNAs in asthmatic patients have been performed in mice, the number of studies in human airways, especially with regard to severe asthma, is very low.<sup>3</sup> In lung biopsy specimens of patients with mild asthma, no significant differences in miRNA expression could be demonstrated compared with healthy subjects.<sup>4</sup> In bronchial epithelial brushings of patients with mild-to-moderate asthma, changes in miRNA expression were reported, including downregulation of the miR-34/449 family, which could be mimicked by treatment with IL-13 and partly restored by corticosteroids.<sup>5</sup> In another miRNA profiling study with bronchial brushings of asthmatic patients, bioinformatics analysis revealed that the predicted miRNA targets belonged to a proinflammatory molecular network, which was confirmed by gene expression measurements.<sup>6</sup> Primary cell-culture studies on bronchial biopsy specimens of patients with severe asthma have revealed a role for miR-19a and miR-221 in epithelial cell and airway smooth muscle cell proliferation, respectively.<sup>7,8</sup> A T<sub>H</sub>2 cytokine-promoting role of miR-19a in patients with mild-to-moderate asthma was also demonstrated.<sup>9</sup> However, to our knowledge, no study has linked miRNA expression in the airways of patients with severe asthma to inflammatory phenotypes.

We have previously reported altered miRNA expression profiles in induced sputum supernatant of patients with chronic obstructive pulmonary disease. In the current study we performed miRNA gene expression analysis on induced sputum supernatants from 2 independent cohorts of well-characterized patients with mild-to-moderate or severe asthma and associated miRNA expression with inflammatory phenotypes. In both the screening and validation cohorts, we demonstrate that expression of miR-629-3p, miR-223-3p, and miR-142-3p is increased in patients with severe asthma and is associated with sputum neutrophilia, suggesting that these miRNAs contribute to disease mechanisms in patients with neutrophilic asthma.

**TABLE I.** Demographic, functional, and inflammatory characteristics of the screening cohort according to disease severity

| Screening cohort                                | Healthy subjects | Patients with mild-to-moderate asthma | Patients with severe asthma |
|---|------------------|---------------------------------------|-----------------------------|
| No.   | 10               | 17                                    | 9                           |
| <b>Demographic characteristics</b>              |                  |                                       |                             |
| Age (y), median (IQR)                           | 40 (28-55)       | 58 (51-66)                            | 50 (46-63)                  |
| Sex (M/F)                                       | 3/7              | 5/12                                  | 2/7                         |
| BMI (kg/m <sup>2</sup> ), mean ± SD             | 24.5 ± 3.5       | 27.1 ± 5.5                            | 27.6 ± 6.5                  |
| Atopy,* no. (%)                                 | 4 (40)           | 10 (59)                               | 6 (67)                      |
| Smoking status (nonsmoker/ex-smoker)†           | 8/2              | 13/4                                  | 4/5                         |
| <b>Functional characteristics and therapy</b>   |                  |                                       |                             |
| FEV <sub>1</sub> (%), mean ± SD                 | 98.2 ± 17.8      | 97.7 ± 13.8                           | 53.8 ± 12.3  #              |
| FEV <sub>1</sub> /FVC ratio (%), mean ± SD      | 80.1 ± 6.3       | 74.1 ± 5.3 §                          | 61.1 ± 9.8  #               |
| Reversibility (%), mean ± SD                    | —                | 7.1 ± 7.6                             | 17.4 ± 28.1                 |
| ACQ score, median (IQR)                         | ND               | 0.7 (0.3-1.1)                         | 2.8 (1.9-3.9)#              |
| FENO (ppb), median (IQR)                        | 18.8 (15.9-22.8) | 32.3 (23.8-50.0)                      | 22.5 (16.5-72.9)            |
| ICS therapy (yes/no)                            | 0/10             | 8/9 §                                 | 9/0  ¶                      |
| ICS dose, median (IQR)                          | 0                | 0 (0-800)§                            | 2000 (1600-4000)  #         |
| <b>Sputum characteristics</b>                   |                  |                                       |                             |
| Inflammatory phenotype ‡ :                      | 0/0/10           | 11/4/2                                | 5/4/0                       |
| eosinophilic/<br>neutrophilic/paucigranulocytic |                  |                                       |                             |
| Macrophages (%)                                 | 62.7 (34.1-82.6) | 21.2 (16.6-57.8)                      | 12.0 (3.5-20.3)             |
| Neutrophils (%)                                 | 22.5 (1.3-62.8)  | 39.0 (15.5-71.0)                      | 72.0 (43.6-93.8)            |
| Eosinophils (%)                                 | 0.0 (0.0-0.0)    | 7.8 (0-26.9)§                         | 8.5 (1.2-19.7)§             |

Data are expressed as distributions (yes/no), means ± SDs, or medians with IQRs. Comparisons were done with Pearson  $\chi^2$  tests or the Kruskal-Wallis test, followed by the Mann-Whitney *U* test with the Bonferroni correction.

ACQ, Asthma Control Questionnaire; *F*, female; *FENO*, fraction of exhaled nitric oxide; *IQR*, interquartile range; *M*, male; *ND*, not determined.

\* Atopy is defined as positive test results for at least 1 specific IgE to common aeroallergens (see the Methods section for more information).

† Ex-smoker is defined as not smoking since at least 6 months.

‡ Sputum eosinophilic inflammation, ≥3% eosinophils and <76% neutrophils; neutrophilic inflammation, ≥76% neutrophils and <3% eosinophils; paucigranulocytic inflammation, eosinophils ≤3% and neutrophils ≤76% neutrophils.

§ *P* < .05 versus healthy subjects.

|| *P* < .001 versus healthy subjects.

¶ *P* < .05 versus patients with mild-to-moderate asthma.

# *P* < .001 versus patients with mild-to-moderate asthma.

## METHODS

### Subject characteristics and study design of the screening and validation cohorts

Two independent study populations were enrolled at the asthma clinic in CHU Liege (Belgium). Healthy subjects were recruited at the hospital and University of Liege, Belgium. All subjects from both cohorts were nonsmokers (never smokers or ex-smokers who quit smoking for at least 6 months).

The screening cohort consisted of healthy subjects (*n* = 10), patients with mild-to-moderate asthma (*n* = 17), and patients with severe asthma (*n* = 9). The validation cohort consisted of healthy subjects (*n* = 10), patients with mild-to-moderate asthma (*n* = 29), and patients with severe asthma (*n* = 21). Tables I and II provide an overview of the demographic, functional, and treatment characteristics of the 36

subjects from the screening cohort and 60 subjects from the validation cohort, according to disease severity and inflammatory phenotype, respectively. See this article's Online Repository at [www.jacionline.org](http://www.jacionline.org) for additional details.

Asthma severity was evaluated by a respiratory physician based on a combination of symptoms, lung function, and amount of medication to achieve control (Global Initiative for Asthma [GINA]<sup>11</sup> and European Respiratory Society/American Thoracic Society guidelines<sup>12</sup>). Severe asthma required GINA treatment steps 4 to 5 ( $\geq 1000$   $\mu\text{g}$  of beclomethasone/d and long-acting  $\beta_2$ -agonist), to prevent it to become uncontrolled or remained uncontrolled despite this therapy (uncontrolled asthma defined as poor symptom control [Asthma Control Questionnaire score  $>1.5$ ] and/or reduced lung function [ $\text{FEV}_1 < 80\%$  of predicted value]). Patients with mild-to-moderate asthma were defined as asthmatic patients treated with a low dose of inhaled corticosteroid (ICS;  $< 1000$   $\mu\text{g}$  of beclomethasone/d) or did not yet receive treatment and had an  $\text{FEV}_1$  of 80% or greater of predicted value.

Subjects were considered atopic if they had positive test results for at least 1 specific IgE ( $> 0.35$  kU/L; Phadia, Groot-Bijgaarden, Belgium) toward common aeroallergens (cat, dog, house dust mite, grass pollen, tree pollen, and a mixture of molds), had a positive skin prick test response, or both.

The study was approved by the local ethics committee, University of Liege, Belgium, (reference 2005/181; conforming to the declaration of Helsinki).

### **Sputum induction**

Sputum was induced and processed, as described previously.<sup>13,14</sup> See the Methods section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org) for more details. Whole sputum was weighted, and 3 volumes of PBS were added. After homogenizing by means of manual agitation for 30 seconds and centrifugation (800g) for 10 minutes at 4°C, the cell pellet and supernatant were separated. Sputum cell differentials were determined by counting 500 cells on cytopsin samples stained with Rapi-Diff II stain (Atomic Scientific, Manchester, United Kingdom) and used to define asthma inflammatory phenotypes. Eosinophilic asthma was defined as 3% or greater sputum eosinophils and less than 76% sputum neutrophils, neutrophilic asthma was defined as 76% or greater neutrophils and less than 3% eosinophils, and paucigranulocytic asthma was defined as less than 3% eosinophils and less than 76% neutrophils.<sup>15</sup> Patients with mixed granulocytic asthma ( $\geq 76\%$  neutrophils and  $\geq 3\%$  eosinophils) were excluded.

### **miRNA detection in sputum supernatants**

Total RNA from cell-free sputum supernatants (150  $\mu\text{L}$ ) was extracted by using miRNeasy Micro Kit without carrier RNA, according to the manufacturer's guidelines (Qiagen, Hilden, Germany). miRNA profiling on 80 ng of RNA was performed at Biogazelle (Zwijnaarde, Belgium) by using high-throughput, stem-loop RT quantitative real-time PCR (qPCR) miRNA expression profiling. See the Methods section in this article's Online Repository for more details and see the Online Repository for Cq values. The detection cutoff was put at a Cq value of 32. Data normalization was performed in qbase + software (Biogazelle, <http://www.qbaseplus.com>) by using the modified global mean normalization

procedure. Normalized expression data (calibrated normalized relative quantities) are logio-transformed.

In the validation study expression of miR-629-3p, miR-223-3p, and miR-142-3p was quantified with specific TaqMan Micro Assays (Life Technologies, Grand Island, NY) by using a custom RT and preamplification pool (12-cycle PCR, according to the manufacturer's guidelines). See the Methods section in this article's Online Repository for more details. Data were normalized in qbase + by using 2 stably expressed miRNAs (miR-20a and miR-30b) identified in the screening cohort. The normalized expression data are logio-transformed before data analysis.

TABLE II. Demographic, functional, and inflammatory characteristics of the validation cohort population according to inflammatory phenotype

| Validation cohort                             | Healthy subjects  | Patients with eosinophilic asthma‡ | Patients with neutrophilic asthma |
|---|-------------------|------------------------------------|-----------------------------------|
| No.   | 10                | 29                                 | 21                                |
| <b>Demographic characteristics</b>            |                   |                                    |                                   |
| Age (y), median (IQR)                         | 53 (31-58)        | 52 (41-64)                         | 61 (49-69)                        |
| Sex (M/F)                                     | 6/4               | 12/17                              | 5/16                              |
| BMI (kg/m <sup>2</sup> ), mean ± SD           | 23.9 ± 3.0        | 26.6 ± 4.5                         | 25.6 ± 4.3                        |
| Atopy,* no. (%)                               | 3 (30)            | 19 (66)                            | 10 (48)                           |
| Smoking status (nonsmoker/ex-smoker)†         | 7/3               | 19/10                              | 12/9                              |
| <b>Functional characteristics and therapy</b> |                   |                                    |                                   |
| Ratio mild/severe asthma                      | —                 | 18/11                              | 11/10                             |
| FEV <sub>1</sub> (%), mean ± SD               | 107.3 ± 12.8      | 82.3 ± 19.6                        | 78.7 ± 22.8§                      |
| FEV <sub>1</sub> /FVC ratio (%), mean ± SD    | 80 ± 6.1          | 69.9 ± 8.2§                        | 68.5 ± 11.0§                      |
| Reversibility (%), mean ± SD                  | 0.0 (-3.0 to 5.0) | 10.8 ± 10.4§                       | 12.5 ± 15.6                       |
| ACQ score, median (IQR)                       | ND                | 1.6 (1.2-2.4)                      | 2.1 (1.4-2.8)                     |
| FENO (ppb), median (IQR)                      | 24.0 (13.0-27.5)  | 34.5 (16.0-58.2)                   | 19.9 (15.0-37)                    |
| ICS therapy (yes/no)                          | 0/10              | 17/12§                             | 14/7                              |
| ICS dose, median (IQR)                        | 0                 | 400 (0-2000)§                      | 600 (0-2000)§                     |
| <b>Sputum characteristics</b>                 |                   |                                    |                                   |
| Macrophages (%)                               | 44.2 (32.6-64.8)  | 28.6 (22.6-38.8)§                  | 10.0 (4.0-15.0)  ¶                |
| Neutrophils (%)                               | 42.8 (19.9-63.1)  | 35.0 (19.2-51.0)                   | 85.0 (79.6-92.5)  ¶               |
| Eosinophils (%)                               | 0.0 (0.0-0.4)     | 21.0 (7.7-29.0)                    | 0.2 (0.0-0.8)1                    |

Data are expressed as distributions (yes/no), means ± SDs, or medians with IQRs. Comparisons were done with Pearson  $\chi^2$  tests or the Kruskal-Wallis test, followed by the Mann-Whitney *U* test plus Bonferroni correction.

ACQ, Asthma Control Questionnaire; *F*, female; *FENO*, fraction of exhaled nitric oxide; *IQR*, interquartile range; *M*, male; *ND*, not determined.

\* Atopy is defined as positive test results for at least 1 specific IgE to common aeroallergens (see the Methods section for more information).

†Ex-smoker is defined as not smoking since at least 6 months.

‡Sputum eosinophilic inflammation, ≥3% eosinophils and <76% neutrophils; neutrophilic inflammation, ≥76% neutrophils and <3% eosinophils.

§*P* < .05 versus healthy subjects.

|| *P* < .001 versus healthy subjects.

¶*P* < .001 versus patients with eosinophilic asthma (see the Methods section for the full table).

## Protein measurements

Levels of human IL-1 $\beta$ , IL-6, IL-8, and IL-5 protein in sputum supernatants were measured with the BD Cytometric Bead Array (Becton Dickinson, San Jose, Calif), a bead-based immunoassay. Detection limits for the proteins were 2.3 pg/mL for IL-1 $\beta$ , 1.2 pg/mL for IL-8, 1.6 pg/mL for IL-6, and 1.1 pg/mL for IL-5. Measurements were performed on a FACSCalibur flow cytometer, according to the Cytometric Bead Array

instruction manual.

Protein levels of IL-8 were measured in supernatants of human bronchial epithelial cells (HBECs) with a human CXCL8/IL-8 DuoSet ELISA (R&D Systems, Minneapolis, Minn).

### ***In situ* hybridization for miRNAs**

*In situ* hybridization was performed on bronchial biopsy specimens from patients with severe asthma and control subjects (obtained from Professor Ricciardolo; approved by the local ethic committee of the San Luigi Hospital, Turin, Italy: protocol 1759, 2008; conforming to the declaration of Helsinki); each subject provided written informed consent. Asthma diagnosis and severity were based on GINA and European Respiratory Society/American Thoracic Society guidelines.<sup>11,12</sup> Bronchoscopy was performed with a flexible fiberoptic bronchoscope (Pentax FB-18P; Asahi Optical, Tokyo, Japan), and bronchial (segmental/lobar airways) biopsy specimens were gently extracted, as previously described.<sup>18</sup>

More details on *in situ* hybridization are provided in the Methods section in this article's Online Repository. Fig E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org) shows *in situ* hybridization with scrambled negative controls.

For more details on neutrophil elastase staining, see the Methods section in this article's Online Repository.

### **Flow cytometry and miRNA analysis on sorted sputum cells**

Sputum cells (10,000/cell type) were sorted on a FACSAria II (BD) to greater than 92% purity and captured in QIAzol, followed by RNA extraction, cDNA synthesis, and RT-PCR with Qiagen reagents. Neutrophils were defined as CD45<sup>+</sup>CD14<sup>-</sup>CD16<sup>+</sup>, monocytes as CD45<sup>+</sup>CD14<sup>-</sup> side scatter (SSC)<sup>low</sup>, and macrophages as CD45<sup>+</sup>CD14<sup>-</sup>SSC<sup>high</sup> based on the method of Brooks et al.<sup>19</sup> More details are presented in the Methods section in this article's Online Repository, including the gating strategy.

For more information on *in silico* analysis into the functional role of miRNAs, see the Methods section in this article's Online Repository.

### **Transfection of HBECs with miRNA mimics**

Normal HBECs (CC-2540; Lonza, Basel, Switzerland) were cultured in BEGM medium (CC-3170, Lonza), according to the manufacturer's guidelines. At passage 2, 150,000 cells were transfected with mirVana 30 nmol/L miRNA mimics and controls (Ambion, Life Technologies) in 6-well plates by using lipofectamine (RNAiMAX; Invitrogen, Carlsbad, Calif; n = 4 for each condition). Forty-eight hours after transfection, culture supernatants was collected, and cells were lysed in QIAzol, followed by RNA extraction and RT-PCR. mRNA gene expression was normalized to 3 reference genes (*RPL13A*, *HPRT*, and *GAPDH*) with qbase+ software (Biogazelle, <http://www.qbaseplus.com>), followed by data analysis. More details are presented in the Methods section in this article's Online Repository.

### **Statistical analysis**

For continuous variables, results are expressed as means  $\pm$  SDs; medians and interquartile ranges were used for skewed distributions (verified by distribution graph and skewness factor  $>1$ ). For categorical variables, the number or percentage distribution of each category within the groups is shown. Comparisons between groups were performed either with nonparametric tests (Kruskal-Wallis, followed by the Mann-Whitney  $U$  test for pairwise comparisons or Fisher exact tests) or parametric tests (ANOVA followed by least significant difference tests, in case of normal distribution and similar variance among groups).  $P$  values of less than .05 were considered significant.

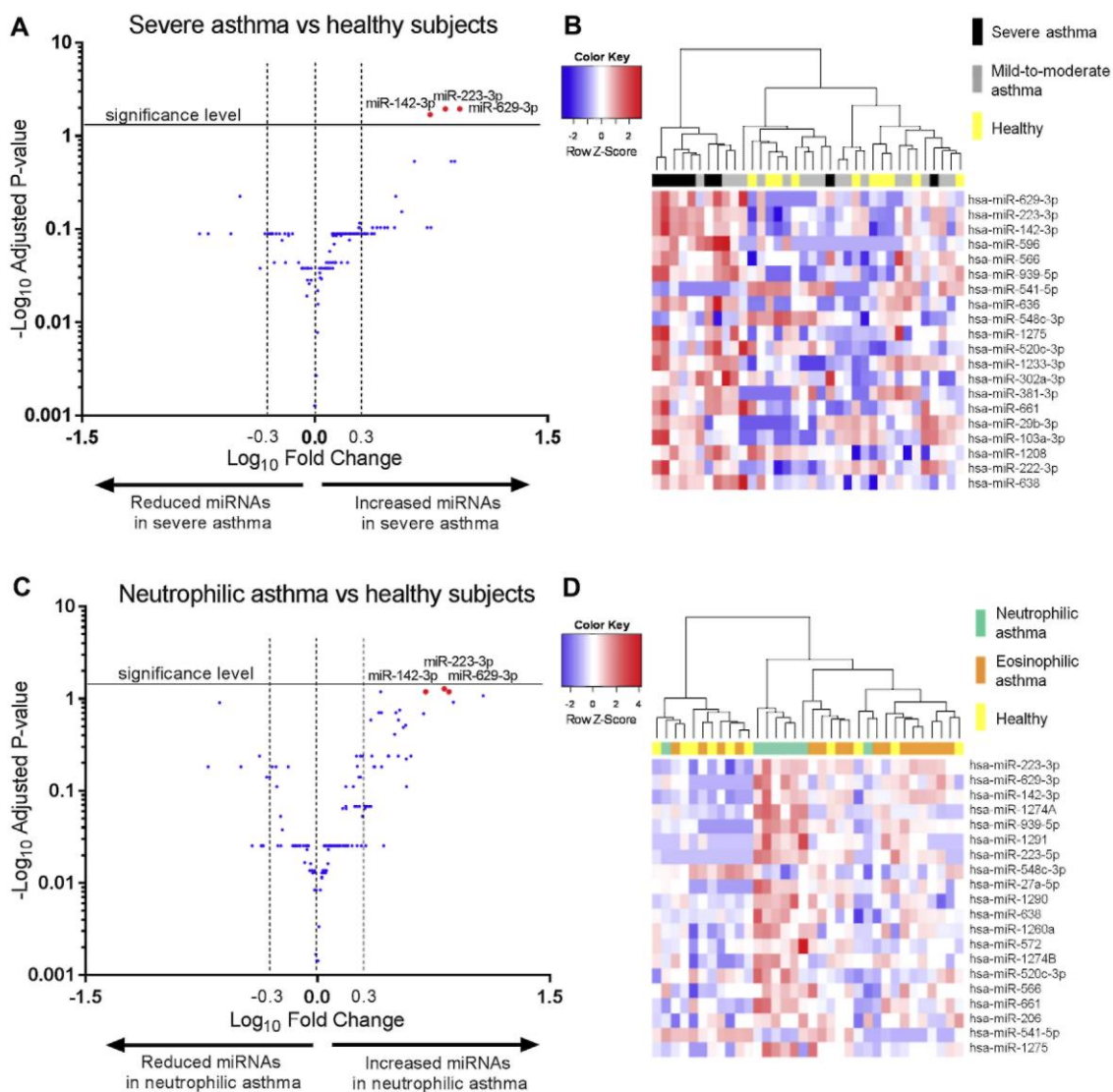
The association between differentially expressed miRNAs and clinical parameters, inflammatory cell counts, or cytokine levels was determined with the Spearman rank correlation coefficient. Multiple linear regression analysis was performed modeling all predictive factors (percentage neutrophils, FEV<sub>1</sub>/forced vital capacity [FVC], pack years, ICSs, age, atopy, sex, and body mass index [BMI]) in a first step, followed by stepwise selection of most contributing factors. Statistical analysis was performed with SPSS statistics software (Version 21; SPSS, Chicago, I11).

Only those miRNAs that were detectable (Cq  $<32$ ) in at least 80% of the samples of one of the groups were further analyzed to investigate differential miRNA expression between the groups of the screening cohort. The miRNAs with undetectable expression levels were imputed by subtracting one log<sub>2</sub> value from the minimum expression of this miRNA across samples of the cohort. Differential miRNA expression between 2 test groups was calculated in the data analysis software R3.1.2. with the limma package. An adjusted  $P$  value of less than .05 (false discovery rate method) was considered significant.

**FIG 1.** miRNA profiling in the screening cohort. **A** and **C**, Volcano plots of the  $-\log_{10}$ -adjusted  $P$  values versus the  $\log_{10}$  fold changes in miRNA expression between groups. The *horizontal line* represents the significance level of the false discovery rate-adjusted  $P$  values ( $P < .05$  was considered significant), indicating all miRNAs above this line are differentially expressed. A  $\log_{10}$  fold change of 0.3 indicates a 2-fold increase in miRNA expression. Fig 1, **A**, Healthy subjects ( $n = 10$ ) compared with patients with severe asthma ( $n = 9$ ); Fig 1, **C**, patients with neutrophilic asthma ( $n = 8$ ) compared with healthy subjects ( $n = 10$ ). **B** and **D**, Hierarchic clustering of the top 20 most differentially expressed miRNAs in

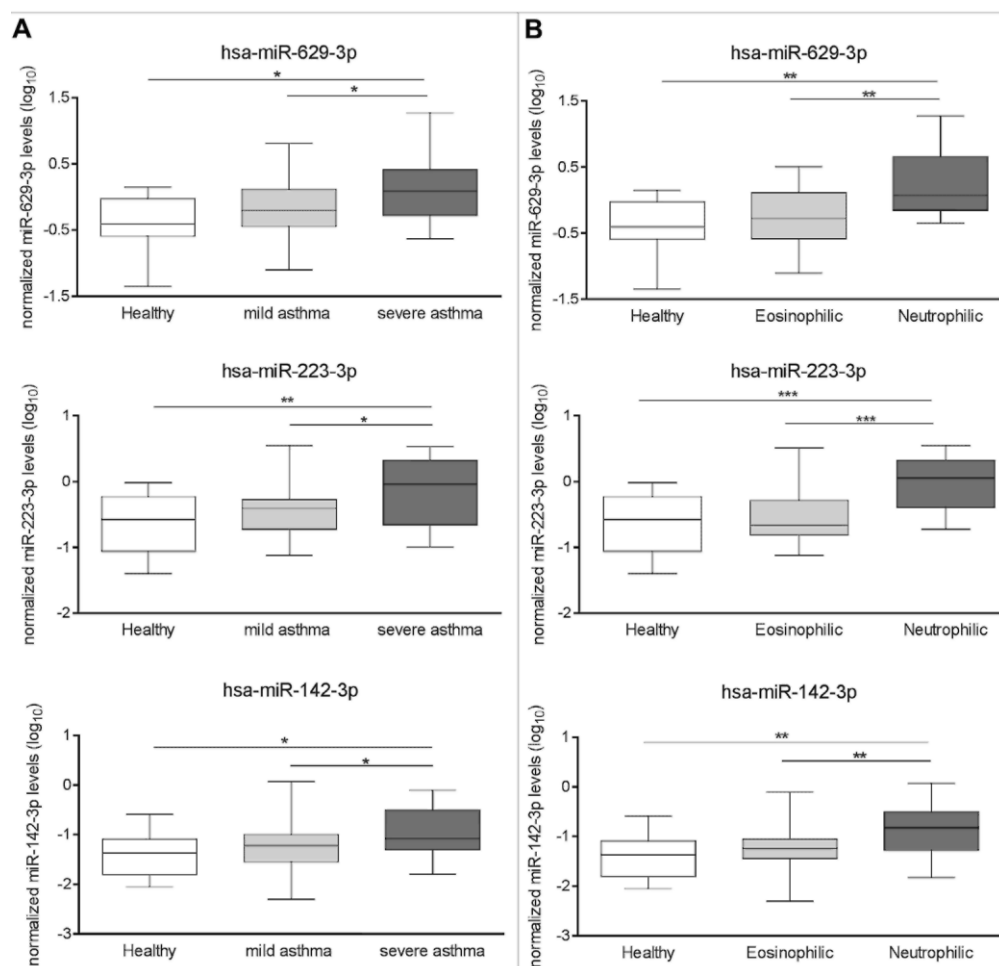


sputum supernatants. Samples are based on asthma severity (Fig 1, **B**) or the inflammatory phenotype (Fig 1, **D**). The heat map was built by using Manhattan distance and the Ward method. Red indicates an expression level greater than the mean across all subjects, and blue indicates an expression level lower than the mean.



**FIG 2.** Validation cohort: normalized miRNA expression levels ( $\log_{10}$ -based) determined by using qRT-PCR. A, Expression of miRNAs according to asthma severity. The mild asthma category includes patients with mild-to-moderate asthma. B, Expression of miRNA according to inflammatory phenotype (eosinophilic asthma,  $\geq 3\%$  eosinophils and  $< 76\%$  neutrophils in sputum; neutrophilic asthma,  $\geq 76\%$  neutrophils and  $< 3\%$  eosinophils in sputum). \* $P < .05$ , \*\* $P < .01$ , and \*\*\* $P < .001$ .

## Validation Cohort



## RESULTS

### Characteristics of the screening cohort

Table I shows demographic, functional, and sputum characteristics of the 36 subjects (more details are provided in Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). The screening cohort consisted of 10 healthy subjects, 17 patients with mild-to-moderate asthma (ICS dose <1000 µg of beclomethasone/d or no ICS and FEV<sub>1</sub> ≥80% of predicted value), and 9 patients with severe asthma (ICS dose ≥1000 µg of beclomethasone/d plus long-acting β<sub>2</sub>-agonist and FEV<sub>1</sub> <80% of predicted value). There were no significant differences in age, sex, BMI, atopy, smoking status, and pack years among the 3 groups. Patients with severe asthma had significantly lower FEV<sub>1</sub>, FEV<sub>1</sub>/FVC ratio, and higher Asthma Control Questionnaire scores compared with healthy subjects and patients with mild-to-moderate asthma. Within the mild-to-moderate asthma group, 52.9% were ICS naive, and 47.1% were treated with low-to-moderate doses of ICSs. Based on differential cell counts in induced sputum, the inflammatory phenotype of patients with asthma was eosinophilic (16/26), neutrophilic (8/26), or paucigranulocytic (2/26). Compared with healthy subjects, the percentage of eosinophils was higher in both asthmatic groups, whereas the percentage of sputum neutrophils tended to be higher in patients with severe asthma (*P* = .066).

### **Differential expression analysis of miRNAs in healthy control subjects compared with patients with (severe) asthma in the screening cohort.**

Expression analysis was performed for 755 miRNAs, followed by differential expression analysis for 171 miRNAs that were detectable in at least 80% of the sputum samples from 1 group (healthy subjects, patients with mild-to-moderate asthma, or patients with severe asthma). Comparison of miRNA expression in healthy subjects versus patients with severe asthma revealed 3 miRNAs with expression that was significantly increased in patients with severe asthma: miR-629-3p, miR-223-3p, and miR-142-3p (false discovery rate, <0.05; Fig 1, A and B).

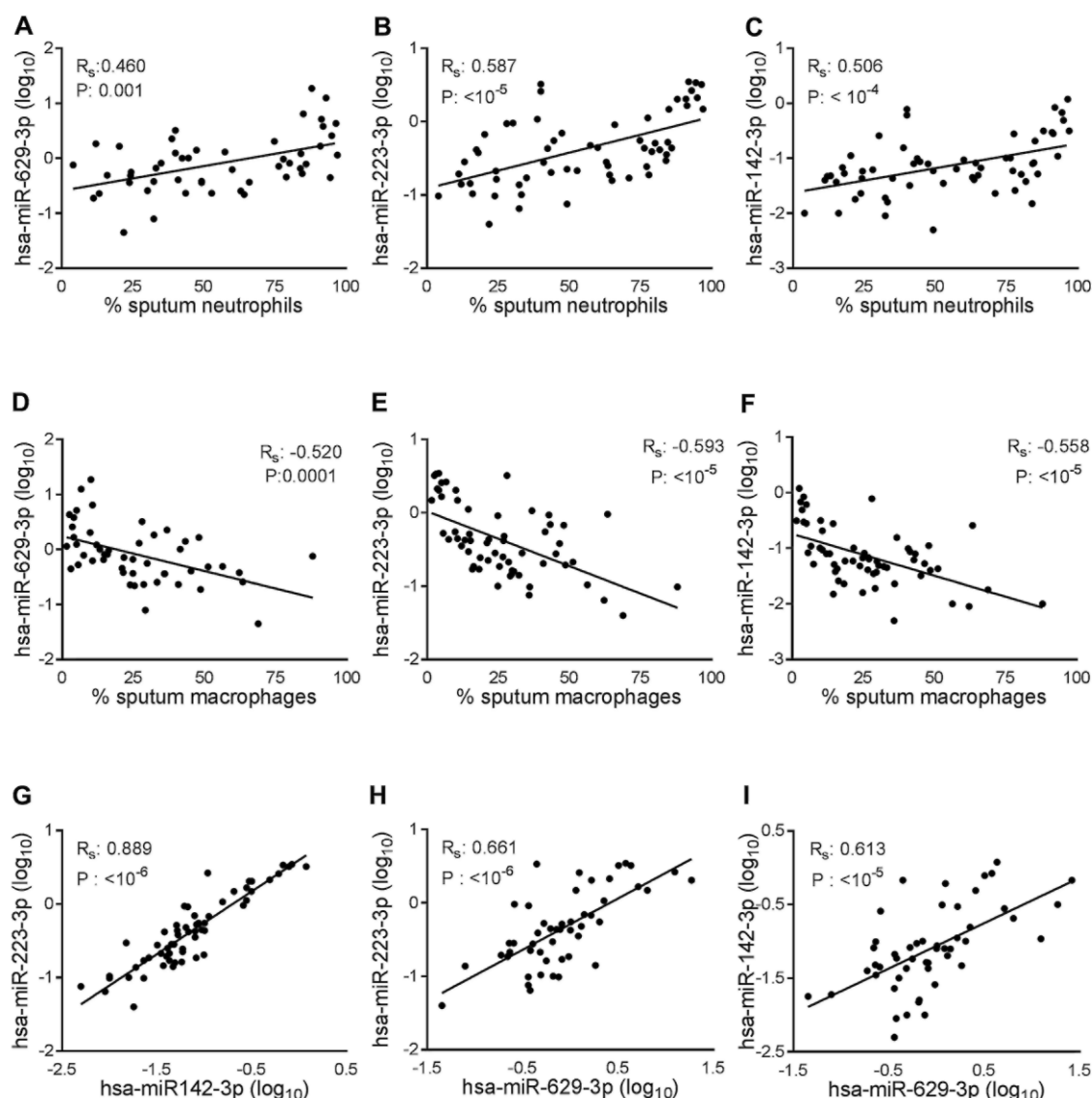
Comparison of patients with mild-to-moderate asthma with patients with severe asthma or comparison of healthy subjects with patients with mild-to-moderate asthma showed no differentially expressed miRNAs (see Table E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

### **Expression of miRNAs in healthy control subjects versus patients with eosinophilic or neutrophilic asthma in the screening cohort**

Considering the clinical relevance of inflammatory phenotypes in asthmatic patients, we investigated miRNA expression profiles according to the asthma inflammatory phenotype. The screening population consisted of 10 healthy control subjects, 16 patients with eosinophilic asthma, and 8 patients with neutrophilic asthma. See Table E3 and the Results section in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org) for details on demographic, functional, and sputum characteristics of the screening cohort based on inflammatory phenotype.

Expression of miR-629-3p, miR-223-3p, and miR-142-3p in sputum tended to increase in patients with neutrophilic asthma compared with that in healthy subjects (false discovery rate: 0.052, 0.065, and 0.065, respectively; Fig 1, C and D and Tables E4 and E5 for raw Cq values in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Comparing patients with eosinophilic asthma with healthy subjects and patients with eosinophilic asthma with patients with neutrophilic asthma did not reveal any differentially expressed miRNAs (see Table E4).

**FIG 3.** Validation cohort. **A-C**, Spearman correlation between the percentage of sputum neutrophils and expression of miR-629-3p (Fig 3, A), miR-223-3p (Fig 3, B), and miR-142-3p (Fig 3, C). **D-F**, Spearman correlation between the percentage of sputum macrophages and expression of miR-629-3p (Fig 3, D), miR-223-3p (Fig 3, E), and miR-142-3p (Fig 3, F). **G-I**, Spearman correlation between miRNA expression in sputum supernatants. Fig 3, A, miR-223-3p and miR-142-3p; Fig 3, B, miR-223-3p and miR-629-3p; and Fig 3, C, miR-142-3p and miR-629-3p.



## Characteristics of the validation cohort

Because the data from the screening cohort suggested that miR-629-3p, miR-223-3p, and miR-142-3p expression was not only increased in patients with severe asthma but also linked to neutrophilic asthma, we selected an independent validation cohort of 60 subjects, including 10 healthy control subjects and 50 asthmatic patients (29 patients with mild-to-moderate and 21 patients with severe asthma) with either an eosinophilic or neutrophilic phenotype. Characteristics of the validation cohort based on disease severity or inflammatory phenotype are summarized in Table II (more details are provided in Tables E6 and E7 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). There were no significant differences in sex, atopy, smoking status, and pack years when groups were compared according to either disease severity or inflammatory profile. Patients with severe asthma had significantly lower lung function and significantly higher Asthma Control Questionnaire scores compared with patients with mild-to-moderate asthma and healthy subjects (see Table E6). Within the asthmatic groups, 29 of 50 patients had an eosinophilic ( $\geq 3\%$  sputum eosinophils and  $< 76\%$  neutrophils) and 21 of 50 had a neutrophilic phenotype ( $\geq 76\%$  sputum neutrophils and  $< 3\%$  eosinophils).

### **Confirmation of miR-629-3p, miR-223-3p, and miR-142-3p expression in patients with severe asthma in the independent validation cohort**

Validation of the miRNA expression of the top 3 differentially expressed miRNAs (miR-629-3p, miR-223-3p, and miR-142-3p) was performed in the independent validation cohort by using RT-qPCR. Similar to the screening cohort, miR-629-3p, miR-223-3p, and miR-142-3p expression was significantly increased in patients with severe asthma versus healthy subjects. In addition, levels were significantly higher in patients with severe asthma compared with those in patients with mild-to-moderate asthma (Fig 2, A).

In both cohorts expression levels of miR-223-3p and miR-142-3p were negatively associated with the FEV<sub>1</sub>/FVC ratio and FEV<sub>1</sub> (see Table E8 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

### **Increased miR-629-3p, miR-223-3p, and miR-142-3p expression is associated with neutrophilic asthma.**

Expression levels of the 3 identified miRNAs in the validation cohort were also compared based on inflammatory phenotype. Expression of miR-629-3p, miR-223-3p, and miR-142-3p was significantly increased in patients with neutrophilic asthma compared with that in patients with eosinophilic asthma and healthy control subjects (Fig 2, B).

Multiple regression analysis with age, BMI, atopy, sex, FEV<sub>1</sub>/FVC ratio, ICS use, neutrophil percentage, and pack years as predictor variables was followed by stepwise multiple linear regression.

We further examined whether differences in miRNA expression could be detected within the mild-to-moderate or severe asthma groups with regard to inflammatory phenotype. For the 3 miRNAs, the expression was significantly increased in patients with mild-to-moderate neutrophilic asthma compared with those with mild-to-moderate eosinophilic asthma and healthy control subjects. In patients with severe asthma, miR-223-3p and miR-142-3p expression was significantly increased in patients with neutrophilic asthma compared with those with eosinophilic asthma, whereas miR-629-3p expression tended to increase (see Fig E2 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)).

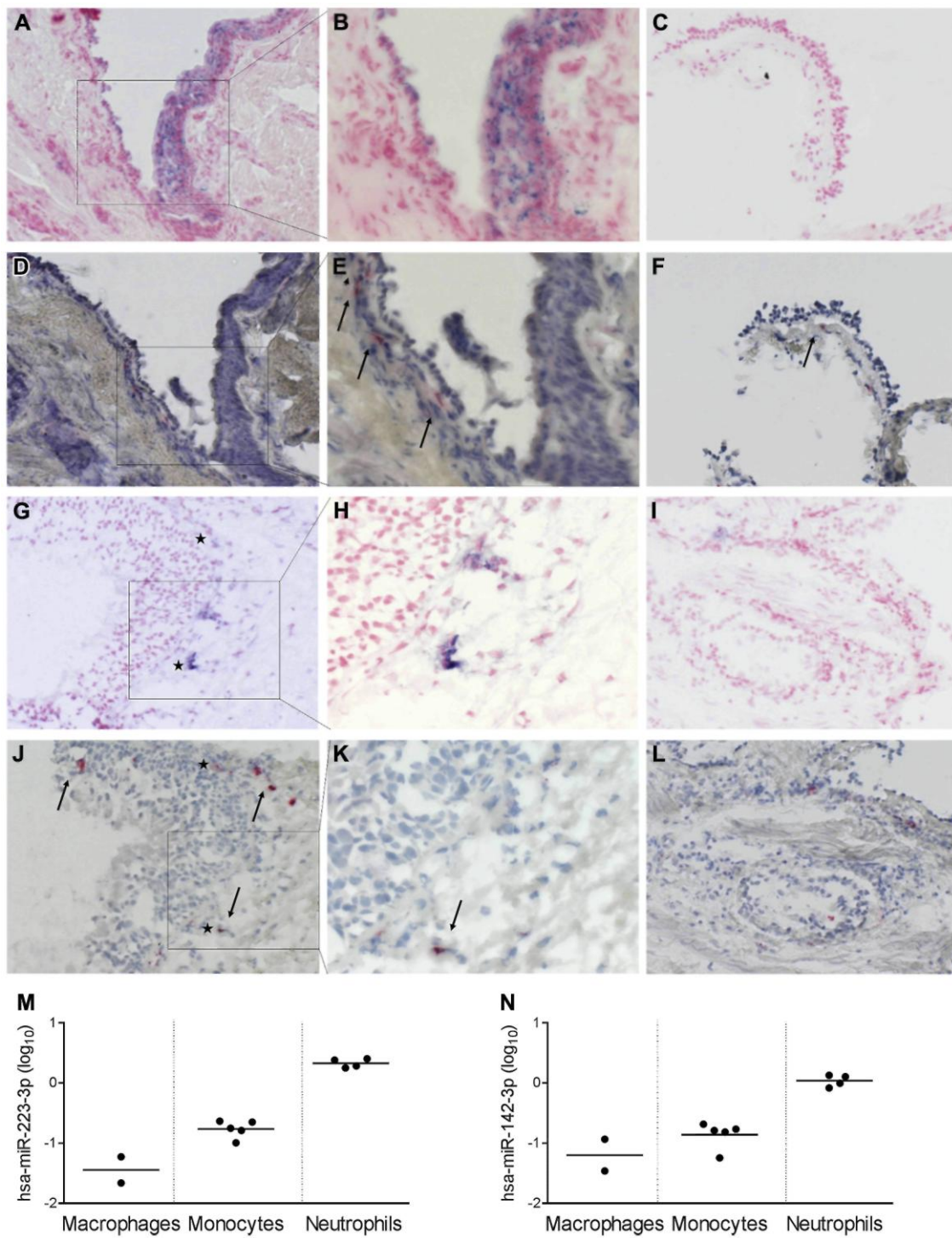
Correlation analysis between miRNA expression levels and sputum cell counts revealed that expression of miR-629-3p, miR-223-3p, and miR-142-3p was positively associated with the percentage of sputum neutrophils and negatively correlated with the percentage of macrophages in the 2 independent cohorts (Fig 3, A-F, and see Table E8). In addition, expression among miR-629-3p, miR-223-3p, and miR-142-3p was highly correlated (Spearman rho > 0.65,  $P < 10^{-6}$ ; Fig 3, G-I).

Multiple linear regression analysis demonstrated that miR-629-3p expression was significantly associated with neutrophil percentages, whereas expression of miR-223-3p and miR-142-3p was significantly associated with neutrophil percentages and FEV<sub>1</sub>/FVC ratio (or FEV<sub>1</sub>), even after adjusting for age, BMI, atopy, sex, ICS use, and pack years (Table III and data not shown).

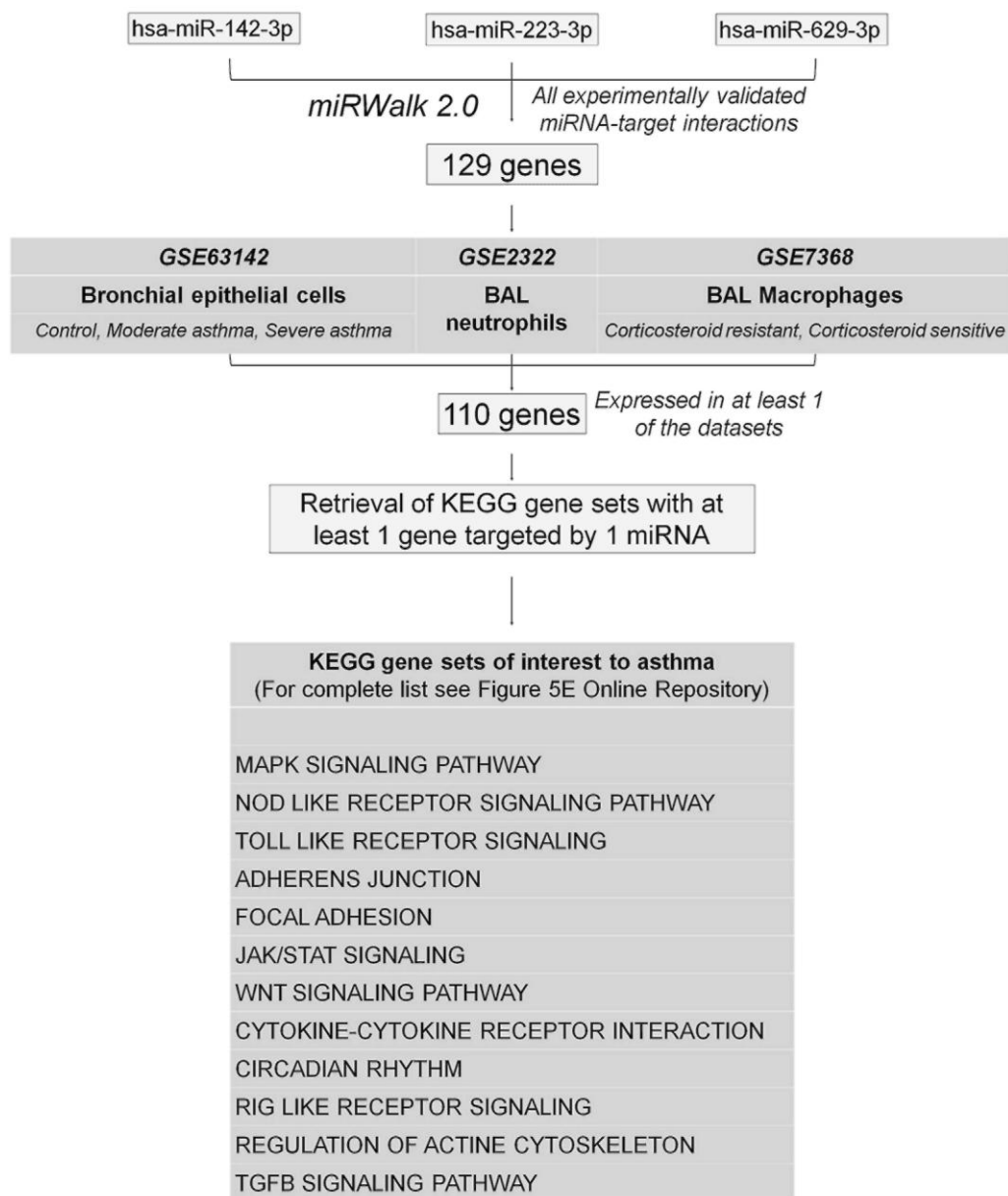
**TABLE III.** Multiple linear regression analysis to model which factors determine miRNA expression (validation cohort)

| miRNA ID  | Adjusted $R^2$ | $P$ value model                        | Predictor variable          | $\beta$ | $P$ value       |
|---|----------------|--|-----------------------------|---------|-----------------|
| <b>Multiple linear regression</b>                   |                |  |                             |         |                 |
| miR-629-3p  | 0.239          | <b>.011</b>                            | Age                         | 0.003   | .986            |
|   |                |  | BMI                         | -0.165  | .324            |
|   |                |  | Atopy                       | 0.146   | .290            |
|   |                |  | Sex                         | -0.209  | .144            |
|   |                |  | FEV <sub>1</sub> /FVC ratio | -0.255  | .126            |
|   |                |  | ICS (yes/no)                | 0.142   | .372            |
|   |                |  | Neutrophils (%)             | 0.494   | <b>.002</b>     |
| miR-223-3p  | 0.361          | <b>.0001</b>                           | Pack years                  | -0.197  | .236            |
|   |                |  | Age                         | -0.084  | .518            |
|   |                |  | BMI                         | -0.050  | .714            |
|   |                |  | Atopy                       | 0.002   | .989            |
|   |                |  | Sex                         | -0.170  | .154            |
|   |                |  | FEV <sub>1</sub> /FVC ratio | -0.329  | <b>.016</b>     |
|   |                |  | ICS (yes/no)                | 0.080   | .540            |
| miR-142-3p  | 0.319          | <b>.0005</b>                           | Neutrophils (%)             | 0.575   | <b>&lt;.001</b> |
|   |                |  | Pack years                  | -0.114  | .399            |
|   |                |  | Age                         | -0.014  | .918            |
|   |                |  | BMI                         | 0.060   | .669            |
|   |                |  | Atopy                       | 0.019   | .869            |
|   |                |  | Sex                         | -0.292  | <b>.021</b>     |
|   |                |  | FEV <sub>1</sub> /FVC ratio | -0.393  | <b>.006</b>     |
| <b>Multiple linear regression (stepwise method)</b> |                |  |                             |         |                 |
| miR-629-3p  | 0.228          | .0003                                  | Neutrophils %               | 0.493   | <b>&lt;.001</b> |
| miR-223-3p  | 0.382          | <b><math>4.1 \times 10^{-7}</math></b> | Neutrophils (%)             | 0.492   | <b>&lt;.001</b> |
| miR-142-3p  | 0.286          | <b>.00003</b>                          | FEV <sub>1</sub> /FVC ratio | -0.296  | <b>.007</b>     |
|   |                |  | Neutrophils (%)             | 0.413   | <b>.001</b>     |
|   |                |  | FEV <sub>1</sub> /FVC ratio | -0.284  | <b>.016</b>     |

**FIG 4.** Origin of miR-629-3p, miR-223-3p, and miR-142-3p in sputum. A-C and G-I, *In situ* localization of miRNA expression (blue color) in bronchial biopsy specimens. Fig 4, A and B, miR-629-3p expression in patients with severe asthma. Fig 4, C, No staining for miR-629-3p in healthy control subjects. Fig 4, G-H, miR-223-3p expression in patients with severe asthma. Fig 4, I, Faint staining for miR-223-3p in healthy control subjects. Tissue was counterstained with nuclear fast red. D-F and J-L, Localization of neutrophil elastase-positive cells (red color, indicated by black arrows) in bronchial biopsy specimens in corresponding regions of consecutive sections of Fig 4, A-C and G-I, respectively. Tissue was counterstained with hematoxylin (blue). Black stars represents regions where miR-223-3p and neutrophil elastase are colocalized. M and N, Expression of miR-223-3p (Fig 4, M) and miR-142-3p (Fig 4, N) in sorted cell subsets in sputum. Sputum cells (n = 2-5, depending on cell type) were sorted by means of flow cytometry. Macrophages were defined as CD45<sup>+</sup>CD14<sup>+</sup>SSC<sup>high</sup>, monocytes as CD45<sup>+</sup>CD14<sup>+</sup>SSC<sup>low</sup>, and neutrophils as CD45<sup>+</sup>CD14<sup>-</sup>CD16<sup>+</sup>; see Fig E3 for the gating strategy. miRNA expression was normalized to snord95, snord96A, and snord68. Log<sub>10</sub>-based values are shown.



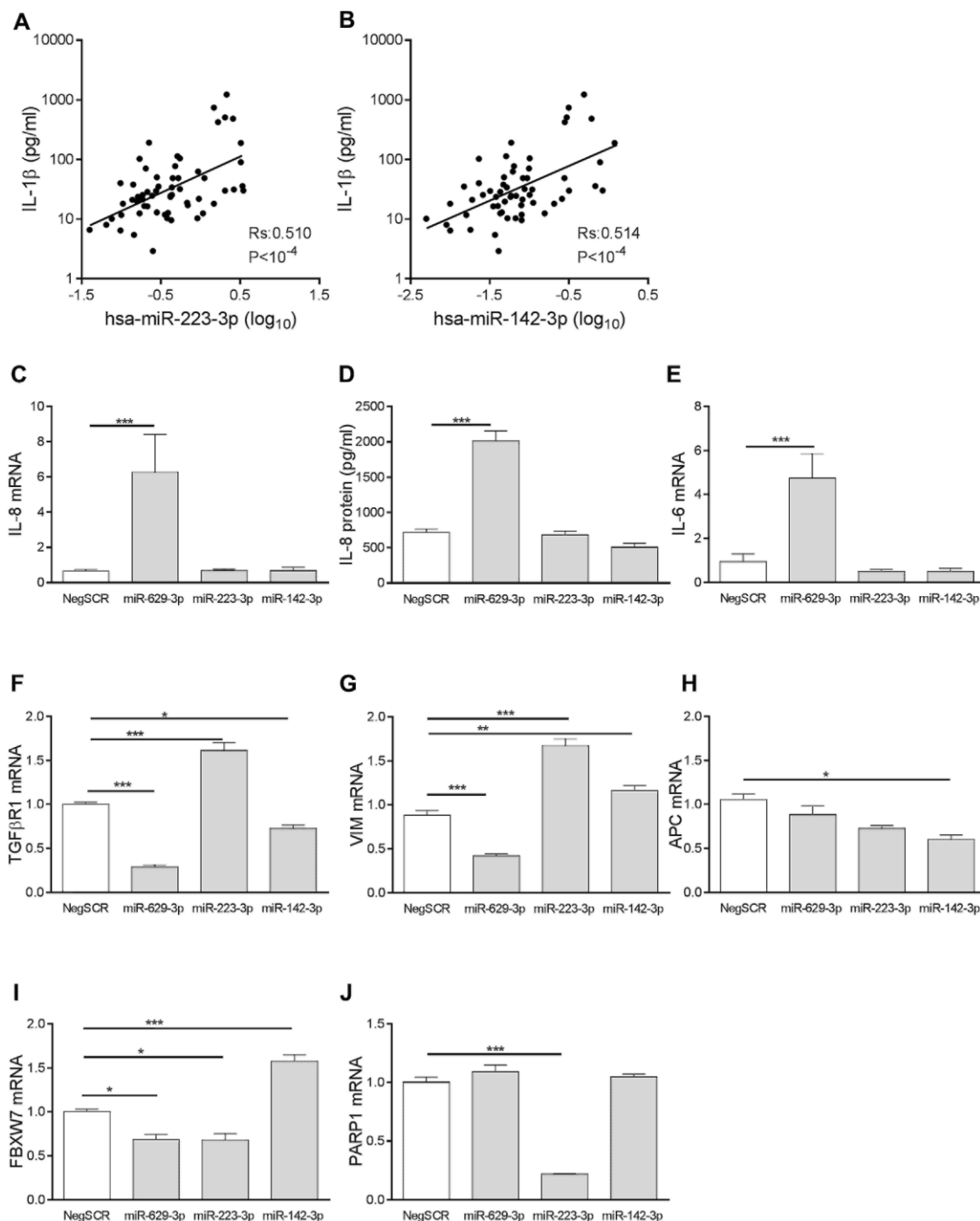
**FIG 5.** Bioinformatic workflow to discover the functional role of miR-629-3p, miR-223-3p, and miR-142-3p in asthmatic patients. *KEGG*, Kyoto Encyclopedia of Genes and Genomes.



**FIG 6.** A and B, Spearman correlations between IL-1  $\beta$  (in picograms per milliliter) and miR-223-3p (Fig 6, A) and IL-1 $\beta$  (in picograms per milliliter) and miR-142-3p (Fig 6, B) in sputum supernatants of the validation cohort. C and E-J, mRNA expression of genes relevant to asthma and/or direct miRNA



targets in HBECs transfected with mimics for miR-629-3p, miR-223-3p, and miR-142-3p or negative scrambled control (*NegSCR*; n = 4 per group). D, IL-8 protein levels in supernatants of HBECs transfected with mimics for miR-629-3p, miR-223-3p, and miR-142-3p (n = 4 per group). \* $P < .05$ , \*\* $P < .01$ , and \*\*\* $P < .001$ .



### Cellular origin of miR-629-3p, miR-223-3p, and miR-142-3p

Using *in situ* hybridization on bronchial biopsy specimens of patients with severe

asthma, we demonstrated that miR-629-3p expression was confined to epithelial cells (Fig 4, A and B). Neutrophil elastase-positive cells were present just below epithelial miR-629-3p expression (Fig 4, D and E). The miR-223-3p expression was mainly localized just below the epithelium in the lamina propria (Fig 4, G and H, respectively). Neutrophil elastase-positive cells colocalized with miR-223-3p expression (Fig 4, G, H, J, and K) but were also detectable in regions with faint or no miR-223-3p expression. In bronchial biopsy specimens of control subjects, expression of miR-629-3p was undetectable, whereas very faint expression of miR-223-3p was detectable (Fig 4, C and I, respectively).

We sorted sputum cell subsets using flow cytometry<sup>19</sup> to examine miRNA expression by using RT-PCR (Fig 4, M and N, and see Fig E3 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org) for the gating strategy). Expression of miR-223-3p and miR-142-3p was detectable in sputum macrophages, monocytes, and neutrophils and was highest in neutrophils (Fig 4, M and N). miR-629-3p was not detectable in these cell subsets (data not shown).

### ***In silico* analysis into the functional role of miRNAs**

All experimentally validated targets of the 3 miRNAs were retrieved by using miRWalk 2.0 (129 genes) to elucidate the potential role of the identified miRNAs in asthmatic patients.<sup>20</sup> Using gene expression data from bronchial epithelial cells (GSE63142),<sup>21</sup> bronchoalveolar lavage macrophages (GSE7368),<sup>22</sup> and bronchoalveolar lavage neutrophils (GSE2322),<sup>23</sup> we identified 110 genes from the 129 genes as being specifically expressed in these cell types (see Fig E4 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Within all Kyoto Encyclopedia of Genes and Genomes pathway<sup>24,25</sup> gene sets, we selected those containing at least 1 of the 110 potential target genes (see Fig E5 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org) for full list and Fig 5). Several of the identified pathways are of interest to asthma and inflammation, such as mitogen-activated protein kinase (MAPK) signaling, nucleotide-binding oligomerization domain (NOD)-like receptor signaling, Toll-like receptor signaling, Janus kinase-signal transducer and activator of transcription signaling, and the TGF- $\beta$  signaling pathways.

### **Sputum cytokine levels are increased in patients with severe asthma and correlate with expression of miR-223-3p and miR-142-3p**

Because several of the validated targets of miR-223-3p, miR-142-3p, and miR-629-3p are involved in inflammatory pathways (eg, NOD-like receptor, Toll-like receptor, and MAPK signaling), we determined protein levels of the proinflammatory mediators IL-1 $\beta$ , IL-6, and IL-8 in sputum supernatants. Patients with severe asthma showed significantly increased levels of IL-1 $\beta$  and IL-8 in induced sputum compared with healthy subjects in the 2 cohorts, whereas levels tended to be intermediate in patients with mild-to-moderate asthma (see Fig E6, A-F, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). IL-1 $\beta$  and IL-8 levels were negatively associated with FEV<sub>1</sub> and FEV<sub>1</sub>/FVC ratio, yet this reached significance only in the screening cohort (see Table E9 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Importantly, IL-1 $\beta$  and IL-8 levels correlated significantly with sputum neutrophilia, and expression of miR-223-3p and miR-142-3p correlated with IL-1 $\beta$  levels (Fig 6, A and B, and see Table E9). Levels of the type 2 cytokine IL-5 increased in patients

with mild-to-moderate and those with severe asthma and in patients with eosinophilic asthma but did not correlate with any of the identified miRNAs (see Fig E6, G and H, and Table E9).

### **Mimics of miR-629-3p, miR-223-3p, and miR-142-3p alter gene expression in HBECs**

The biological role of the identified miRNAs was investigated by transfecting primary HBECs with miRNA mimics. Expression of proinflammatory genes and direct or indirect miRNA targets related to asthma, wound repair, and inflammation (based on the literature<sup>26-30</sup> and miRWalk 2.0) was investigated. Expression of IL-8 (mRNA and protein) and *IL6* mRNA significantly increased after treatment with miR-629-3p mimic compared with the other groups (Fig 6, C-E). *TGFBR1*,<sup>26</sup> vimentin (*VIM*),<sup>27</sup> and adenomatous polyposis coli (*APC*)<sup>28</sup> mRNA levels were significantly modulated after treatment with the different miRNA mimics (Fig 6, F-H). *FBXW7* mRNA levels were downregulated by mimics of miR-223-3p and miR-629-3p and increased by miR-142-3p mimic. *PARP1* was downregulated by the miR-223-3p mimic (Fig 6, I and J).

### **DISCUSSION**

In this study we identified miR-629-3p, miR-223-3p, and miR-142-3p expression as upregulated in sputum supernatants of patients with severe neutrophilic asthma. Linear regression analysis demonstrated that expression of the 3 miRNAs was associated with sputum neutrophilia. Moreover, miR-223-3p and miR-142-3p expression was significantly associated with airway obstruction (FEV<sub>1</sub>/FVC ratio). We demonstrated that miR-629-3p is expressed in the bronchial epithelium, whereas miR-223-3p and miR-142-3p are expressed in neutrophils, monocytes, and macrophages. *In silico* analysis and functional experiments on HBECs transfected with miRNA mimics indicate that the identified miRNAs are influencing pathways related to inflammation and wound response. Finally, sputum supernatants of patients with severe asthma contained increased IL-1 $\beta$  and IL-8 levels, which were positively associated with sputum neutrophilia.

To our knowledge, this is the first report describing a link between miR-629-3p, miR-223-3p, and miR-142-3p expression in sputum and in patients with (severe) neutrophilic asthma. Induced sputum, which enables monitoring of genetic, biochemical, molecular, and cellular markers in the proximal airways,<sup>15,31</sup> has been used to identify inflammatory phenotypes in asthma.<sup>32</sup> Treatment directed toward normalization of sputum eosinophilia reduces asthma exacerbations,<sup>33</sup> indicating that findings from induced sputum can have therapeutic potential.

We showed (1) increased miR-629-3p expression in sputum supernatants of patients with severe neutrophilic asthma, (2) positive correlation between miR-629-3p expression and percentage of sputum neutrophils, (3) miR-629-3p expression in the bronchial epithelium of patients with severe asthma, and (4) that a miR-629-3p mimic induces IL-8 and IL-6 expression in HBECs. These findings suggest that miR-629-3p influences the pathogenesis of neutrophilic asthma by inducing epithelial expression of the neutrophil chemoattractant IL-8. In patients with liver inflammation, IL-6/IL-6 receptor/signal transducer and activator of transcription 3 signaling promotes miR-629 expression through a complex positive feedback loop mechanism.<sup>34</sup> It is possible that IL-6 released during inflammation promotes epithelial miR-629-3p expression in

patients with severe asthma, further perpetuating IL-6 expression and signaling.

Because miRNAs are known as repressors of gene expression, the proinflammatory effects likely occur through an indirect mechanism. One possible candidate is *FBXW7*, a validated miRWalk 2.0 target of miR-223-3p, expression which was also downregulated in HBECs treated with the miR-629-3p mimic. *FBXW7* has been identified as a suppressor of Toll-like receptor 4-mediated inflammatory gene expression by downregulating C/EBP $\delta$ .<sup>29</sup> Further research is needed to unravel the method of action of miR-629-3p. Notably *PARP1*, a miR-223-3p target and coactivator of the nuclear factor kappa-light chain enhancer of activated B cells that facilitates IL-8 expression,<sup>30</sup> was downregulated by the miR-223-3p mimic in HBECs.

We demonstrated increased miR-223-3p expression in patients with severe and those with neutrophilic asthma. Previous studies with airway biopsy specimens and bronchial brushings revealed no differential expression<sup>4</sup> or upregulated miR-223-3p expression<sup>5</sup> in patients with mild asthma; however, no association with inflammatory cell profiles was made. Upregulated miR-223-3p expression was demonstrated both in patients with cystic fibrosis<sup>35</sup> and those with active tuberculosis<sup>36</sup> and was suggested to play an active role in disease pathogenesis. In smokers with chronic pulmonary disease, pulmonary miR-223-3p expression was increased compared with that in smokers without airflow obstruction,<sup>37</sup> whereas bronchial airway epithelium from smokers showed decreased miR-223 expression compared with nonsmokers. When ex-smokers were excluded from our cohort to avoid smoking effects, expression of the 3 miRNAs remained increased in patients with neutrophilic asthma (data not shown). Notably, in patients with different lung diseases characterized by neutrophilic inflammation and airway obstruction, increased miR-223-3p expression and increased IL-1 $\beta$  and IL-8 levels were reported.<sup>35,39-41</sup>

Because *in silico* analysis on validated miRNA targets expressed in bronchial epithelial cells, neutrophils, or macrophages suggested that the identified miRNAs are implicated in inflammatory pathways (Toll-like receptor, NOD-like receptor, and MAPK signaling, yielding in IL-1 $\beta$ , IL-6, and IL-8), we measured levels of proinflammatory cytokines in sputum supernatants. IL-1 $\beta$  and IL-8 levels were significantly increased in patients with severe asthma and correlated with neutrophil counts in sputum. These data are similar to recent findings of Simpson et al<sup>41</sup> and Baines et al,<sup>42</sup> who elegantly demonstrated increased IL-1 $\beta$  and IL-8 levels and NLRP3 expression in patients with neutrophilic asthma.

We demonstrated miR-223-3p and miR-142-3p expression in sputum neutrophils, monocytes, and macrophages, which corresponds with previous studies in human alveolar macrophages<sup>4</sup> and blood neutrophils and monocytes.<sup>43</sup> How miR-223-3p is implicated in inflammatory processes in neutrophilic asthma requires further investigation because miR-223-3p expression can downregulate IL-1 $\beta$  synthesis through NLRP3 repression on the one hand<sup>44,45</sup> but is also induced by IL-1 $\beta$  on the other hand.<sup>35</sup>

Notably, miR-223-3p and miR-142-3p expression in sputum supernatants was strongly correlated (Spearman Rho = 0.889,  $P < 10^{-6}$ ). This corresponds with literature because miR-223-3p can upregulate miR-142-3p expression through a miR-223-3p-CEBP- $\beta$ -LM02-miR-142-3p regulatory pathway<sup>46</sup>

In accordance with the literature, the miR-142-3p mimic reduced *TGFBR1* expression in HBECs.<sup>26</sup> The canonical TGF- $\beta$ -Smad3 pathway is activated in asthmatic patients and is associated with increased *VIM* expression during epithelial-mesenchymal

transition.<sup>47</sup> The miR-223-3p mimic induced *TGFBR1* and *VIM* expression and reduced *FBXW7* expression in HBECs. In the literature increased expression of miR-223-3p and *VIM* and reduced *FBXW7* expression was described in epithelial-mesenchymal transition in patients with pancreatic cancer.<sup>27</sup> However, the effects of the miRNA mimics on different genes suggest a complex regulatory network. In addition, miR-142-3p mimic also inhibited expression of *APC*, an inhibitor of WNT signaling.<sup>28</sup> We showed no epithelial miR-223-3p and miR-142-3p expression on tissue sections, yet a transfer of miRNAs (eg, miR-223-3p) from inflammatory cells to the epithelium is possible.<sup>48</sup>

Our findings for miR-629-3p, miR-223-3p, and miR-142-3p combined with the literature suggest that an imbalanced expression could determine the progression to uncontrolled inflammation and wound repair pathways in patients with severe asthma.

A limitation of our study is that the validation cohort included a significant number of patients with mild neutrophilic asthma (n = 11), a rather rare phenotype of mild asthma. Although this patient distribution does not reflect the distribution found in clinical practice, this enabled us to investigate whether miRNA expression was associated with disease severity, neutrophilic inflammation, or both. Remarkably, no published miRNAs related to eosinophilia or type 2 responses<sup>9</sup> were identified in the screening cohort. This is possibly because in sputum supernatants only a limited amount of miRNAs could be quantified. In addition, most published studies have analyzed more homogenous patient cohorts, with a typical mild-to-moderate T<sub>H</sub>2-type asthmatic phenotype. Finally, the dose of ICSs could have driven differential miRNA expression in patients with mild versus severe asthma. However, we found no correlation between ICS dose and miRNA expression (data not shown).

Despite these limitations, the strong associations between miRNA expression, inflammatory parameters, lung function, and cytokine levels in patients with severe neutrophilic asthma and the functional experiment in HBECs indicate that these miRNAs could contribute to the pathogenesis of neutrophilic asthma. Further research into the interactions between miR-629-3p, miR-223-3p, and miR-142-3p could provide potential therapeutic targets for lung diseases characterized by neutrophilia and airway obstruction.

In conclusion, we have identified expression of miR-629-3p, miR-223-3p, and miR-142-3p as increased in patients with (severe) neutrophilic asthma and suggest that these miRNAs actively contribute to the disease pathogenesis by modulating proinflammatory and wound-repair pathways.

### Key messages

- In 2 independent cohorts of asthmatic patients, expression of miR-629-3p, miR-223-3p, and miR-142-3p in induced sputum is increased in patients with severe asthma and positively associated with neutrophilic airway inflammation.
- A miR-629-3p mimic induces upregulation of IL-8 in bronchial epithelial cells,

## providing a mechanistic link between increased miR-629-3p expression and airway neutrophilia in patients with severe asthma.

### Acknowledgment

We thank Indra De Borle, Ann Neesen, Lien Coelembier, Marie-Rose Mouton, Greet Barbier, Anouck Goethals, Katleen De Saedeleer, and Kimberly Verniers for excellent technical assistance and Kelly Heyns for technical advice.

Supported by the Belgian Interuniversity Attraction Poles Program (IUAP) P7/30, Concerted Research Action of Ghent University BOF14-GOA-027, Belgium; Spearhead Immunology, Ghent University Hospital, Belgium; FWO Flanders (G.0A99.13 and G.0319.1 IN); and the Maro Fund of the King Baudouin Foundation.

**Disclosure of potential conflict of interest:** T. Maes has received grants from Belspo (Belgian Federal Government-Interuniversity Attraction Poles) and Ghent University Hospital (Concerted Research action and Spearhead Immunology); has received the GlaxoSmithKline Clinical Science Award; has received travel support from Glaxo-SmithKline and Boehringer Ingelheim; and is a shareholder in Oryzon Genomics. J. Vandesompele is cofounder, CSO, and member of the board of directors of Biogazelle and receives stock/stock options from Biogazelle. L. Lahousse has received travel support from the European Respiratory Society, the American Thoracic Society, and BVP G. F. Joos has received grants from the Belgian Federal Government, Belspo, Ghent University Hospital, Spearhead Immunology, the Maro Fund (King Baudouin Foundation), AstraZeneca, Chiesi, GlaxoSmithKline, and Novartis; has consultant arrangements with AstraZeneca, Boehringer Ingelheim, GlaxoSmithKline, Mundi-pharma, and Novartis; has received payment for lectures from AstraZeneca, Chiesi, GlaxoSmithKline, Novartis, and Sandoz Pharma; and has received travel support from

AstraZeneca and Novartis. F. L. M. Ricciardolo is a board member for Mundipharma; has consultant arrangements with Novartis, Chiesi, and AstraZeneca; has received grants from Boehringer Ingelheim; and has received payment for lectures from Novartis, Chiesi, and Mundipharma. G. G. Brusselle is a member of advisory boards for Astra Zeneca, Boehringer Ingelheim, GlaxoSmithKline, and Novartis and has received payment for lectures from AstraZeneca, Boehringer Ingelheim, Chiesi, GlaxoSmithKline, Novartis, Mundipharma, and Zambon. R. Louis has received grants from the Federal Office for Scientific Affairs (LAP P7/30), GlaxoSmithKline, Novartis, Chiesi, and AstraZeneca; is a board member for GlaxoSmithKline, AstraZeneca, Novartis, Mundipharma, and Pfizer; has received payment for lectures from Novartis and Pfizer; and has received travel support from Boehringer Ingelheim. The rest of the authors declare that they have no relevant conflicts of interest.

### REFERENCES

1. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 2012;18:716-25.
2. Lodish HF, Zhou B, Liu G, Chen CZ. Micromanagement of the immune system by microRNAs. *Nat Rev Immunol* 2008;8:120-30.
3. Rebane A, Akdis CA. MicroRNAs : essential players in the regulation of inflammation. *J Allergy Clin*

Immunol 2013;132:15-26.

4. Williams AE, Lamer-Svensson H, Perry MM, Campbell GA, Herrick SE, Adcock IM, et al. MicroRNA expression profiling in mild asthmatic human airways and effect of corticosteroid therapy. *PLoS One* 2009;4:e5889.
5. Solberg OD, Ostrin EJ, Love MI, Peng JC, Bhakta NR, Hou L, et al. Airway epithelial miRNA expression is altered in asthma. *Am J Respir Crit Care Med* 2012;186:965-74.
6. Jardim MJ, Dailey L, Silbajoris R, az-Sanchez D. Distinct microRNA expression in human airway cells of asthmatic donors identifies a novel asthma-associated gene. *Am J Respir Cell Mol Biol* 2012;47:536-42.
7. Haj-Salem I, Fakhfakh R, Berube JC, Jacques E, Plante S, Simard MJ, et al. MicroRNA-19a enhances proliferation of bronchial epithelial cells by targeting TGFbetaR2 gene in severe asthma. *Allergy* 2015;70:212-9.
8. Perry MM, Baker JE, Gibeon DS, Adcock IM, Chung KF. Airway smooth muscle hyperproliferation is regulated by microRNA-221 in severe asthma. *Am J Respir Cell Mol Biol* 2014;50:7-17.
9. Simpson LJ, Patel S, Bhakta NR, Choy DF, Brightbill HD, Ren X, et al. A microRNA upregulated in asthma airway T cells promotes TH2 cytokine production. *Nat Immunol* 2014;15:1162-70.
10. Van Pottelberge GR, Mestdagh P, Bracke KR, Thas O, van Durme YM, Joos GF, et al. MicroRNA expression in induced sputum of smokers and patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2011; 183: 898-906.
11. Global Initiative for Asthma. Global strategy for asthma management and prevention: updated 2014. Available at: [www.ginasthma.org](http://www.ginasthma.org). Accessed March 17, 2015.
12. Chung KF, Wenzel SE, Brozek JL, Bush A, Castro M, Sterk PJ, et al. International ERS/ATS guidelines on definition, evaluation and treatment of severe asthma. *Eur Respir J* 2014;43:343-73.
13. Delvaux M, Henket M, Lau L, Kange P, Bartsch P, Djukanovic R, et al. Nebulised salbutamol administered during sputum induction improves bronchoprotection in patients with asthma. *Thorax* 2004;59:1111-5.
14. Manise M, Holtappels G, Van CK, Schleich F, Bachert C, Louis R. Sputum IgE and cytokines in asthma: relationship with sputum cellular profile. *PLoS One* 2013;8: e58388.
15. Schleich FN, Manise M, Sele J, Henket M, Seidel L, Louis R. Distribution of sputum cellular phenotype in a large asthma cohort: predicting factors for eosinophilic vs neutrophilic inflammation. *BMC Pulm Med* 2013;13:11.
16. Mestdagh P, Feys T, Bernard N, Guenther S, Chen C, Speleman F, et al. High-throughput stem-loop RT-qPCR miRNA expression profiling using minute amounts of input RNA. *Nucleic Acids Res* 2008;36:e143.
17. D'haene B, Mestdagh P, Hellemans J, Vandesompele J. miRNA expression profiling: from reference genes to global mean normalization. *Methods Mol Biol* 2012;822:261-72.
18. Ricciardolo FL, Sabatini F, Sorbello V, Benedetto S, Defilippi I, Petecchia L, et al. Expression of vascular remodelling markers in relation to bradykinin receptors in asthma and COPD. *Thorax* 2013;68:803-11.
19. Brooks CR, Van Dalen CJ, Hermans IF, Douwes J. Identifying leukocyte populations in fresh and cryopreserved sputum using flow cytometry. *Cytometry B Clin Cytom* 2013;84:104-13.
20. Dweep H, Gretz N. miRWalk2.0: a comprehensive atlas of microRNA-target interactions. *Nat Methods* 2015;12:697.
21. Modena BD, Tedrow JR, Milosevic J, Bleecker ER, Meyers DA, Wu W, et al. Gene expression in relation to exhaled nitric oxide identifies novel asthma phenotypes with unique biomolecular pathways. *Am J Respir Crit Care Med* 2014;190:1363-72.
22. Goleva E, Hauk PJ, Hall CF, Liu AH, Riches DW, Martin RJ, et al. Corticosteroid-resistant asthma is associated with classical antimicrobial activation of airway macrophages. *J Allergy Clin Immunol* 2008;122:550-9.
23. Coldren CD, Nick JA, Poch KR, Woolum MD, Fouty BW, O'Brien JM, et al. Functional and

genomic changes induced by alveolar transmigration in human neutrophils. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L1267-76.

24. Kanehisa M, Goto S, Sato Y, Kawashima M, Furumichi M, Tanabe M. Data, information, knowledge and principle: back to metabolism in KEGG. *Nucleic Acids Res* 2014;42(Database issue):D199-205.

25. Kanehisa M, Goto S. KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res* 2000;28:27-30.

26. Lei Z, Xu G, Wang L, Yang H, Liu X, Zhao J, et al. MiR-142-3p represses TGF-beta-induced growth inhibition through repression of TGFbetaR1 in non-small cell lung cancer. *FASEB J* 2014;28:2696-704.

27. Ma J, Fang B, Zeng F, Ma C, Pang H, Cheng L, et al. Down-regulation of miR-223 reverses epithelial-mesenchymal transition in gemcitabine-resistant pancreatic cancer cells. *Oncotarget* 2015;6:1740-9.

28. Carraro G, Shrestha A, Rostkovich J, Contreras A, Chao CM, E1 Agha E, et al. miR-142-3p balances proliferation and differentiation of mesenchymal cells during lung development. *Development* 2014;141:1272-81.

29. Balamurugan K, Sharan S, Klarmann KD, Zhang Y, Coppola V, Summers GH, et al. FBXW7 $\alpha$  attenuates inflammatory signalling by downregulating C/EBP $\delta$  and its target gene Tlr4. *Nat Commun* 2013;4:1662.

30. Hassa PO, Haenni SS, Buerki C, Meier NI, Lane WS, Owen H, et al. Acetylation of poly(ADP-ribose) polymerase-1 by p300/CREB-binding protein regulates coactivation of NF-kappaB-dependent transcription. *J Biol Chem* 2005;280: 40450-64.

31. Alexis NE. Biomarker sampling of the airways in asthma. *Curr Opin Pulm Med* 2014;20:46-52.

32. Simpson JL, Scott R, Boyle MJ, Gibson PG. Inflammatory subtypes in asthma: assessment and identification using induced sputum. *Respirology* 2006; 11 : 54-61.

33. Green RH, Brightling CE, McKenna S, Hargadon B, Parker D, Bradding P, et al. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* 2002;360:1715-21.

34. Hatziapostolou M, Polytarchou C, Aggelidou E, Drakaki A, Poultsides GA, Jaeger SA, et al. An HNF4 $\alpha$ -miRNA inflammatory feedback circuit regulates hepatocellular oncogenesis. *Cell* 2011;147:1233-47.

35. Oglesby IK, Chotirmall SH, McElvaney NG, Greene CM. Regulation of cystic fibrosis transmembrane conductance regulator by microRNA-145, -223, and -494 is altered in DeltaF508 cystic fibrosis airway epithelium. *J Immunol* 2013;190: 3354-62.

36. Dorhoi A, Iannaccone M, Farinacci M, Fae KC, Schreiber J, Moura-Alves P, et al. MicroRNA-223 controls susceptibility to tuberculosis by regulating lung neutrophil recruitment. *J Clin Invest* 2013;123:4836-48.

37. Ezzie ME, Crawford M, Cho JH, Orellana R, Zhang S, Gelinas R, et al. Gene expression networks in COPD: microRNA and mRNA regulation. *Thorax* 2012: 67:122-31.

38. Schembri F, Sridhar S, Perdomo C, Gustafson AM, Zhang X, Ergun A, et al. MicroRNAs as modulators of smoking-induced gene expression changes in human airway epithelium. *Proc Natl Acad Sci U S A* 2009;106:2319-24.

39. Seiscento M, Vargas FS, Acencio MM, Teixeira LR, Capelozzi VL, Sales RK, et al. Pleural fluid cytokines correlate with tissue inflammatory expression in tuberculosis. *Int J Tuberc Lung Dis* 2010;14:1153-8.

40. Eickmeier O, Boom L, Schreiner F, Lentze MJ, NGampolo D, Schubert R, et al. Transforming growth factor beta1 genotypes in relation to TGFbeta1, interleukin-8, and tumor necrosis factor alpha in induced sputum and blood in cystic fibrosis. *Mediators Inflamm* 2013;2013:913135.

41. Simpson JL, Phipps S, Baines KJ, Oreo KM, Gunawardhana L, Gibson PG. Elevated expression of the NLRP3 inflammasome in neutrophilic asthma. *Eur Respir J* 2014;43:1067-76.

42. Baines KJ, Simpson JL, Wood LG, Scott RJ, Fibbens NL, Powell H, et al. Sputum gene



expression signature of 6 biomarkers discriminates asthma inflammatory phenotypes. *J Allergy Clin Immunol* 2014;133:997-1007.

43. Allantaz F, Cheng DT, Bergauer T, Ravindran P, Rossier MF, Ebeling M, et al. Expression profiling of human immune cell subsets identifies miRNA-mRNA regulatory relationships correlated with cell type specific expression. *PLoS One* 2012;7:e29979.

44. Haneklaus M, Gerlic M, O'Neill LA, Masters SL. miR-223: infection, inflammation and cancer. *J Intern Med* 2013;274:215-26.

45. Bauernfeind F, Rieger A, Schildberg FA, Knolle PA, Schmid-Burgk JL, Hornung V NLRP3 inflammasome activity is negatively controlled by miR-223. *J Immunol* 2012;189:4175-81.

46. Sun W, Shen W, Yang S, Hu F, Li H, Zhu TH. miR-223 and miR-142 attenuate hematopoietic cell proliferation, and miR-223 positively regulates miR-142 through LM02 isoforms and CEBP-beta. *Cell Res* 2010;20:1158-69.

47. Hackett TL, Warner SM, Stefanowicz D, Shaheen F, Pechkovsky DV, Murray LA, et al. Induction of epithelial-mesenchymal transition in primary airway epithelial cells from patients with asthma by transforming growth factor-beta1. *Am J Respir Crit Care Med* 2009;180:122-33.

48. Ismail N, Wang Y, Dakhallah D, Moldovan L, Agarwal K, Batte K, et al. Macrophage microvesicles induce macrophage differentiation and miR-223 transfer. *Blood* 2013;121:984-95.