There are several limitations. One might think the changes in FeNO levels and oscillometry after 3 months were due to the regression toward the mean effect. However, these changes can be explained thoroughly by the mechanisms stated above. Since the patients of this study had well controlled asthma and good lung function, attention has to be paid for the generalizability. Further studies warrant the effect of high FeNO levels on asthma pathophysiology and the interpretation of R5-R20.

CONFLICT OF INTEREST

The authors have nothing to disclose.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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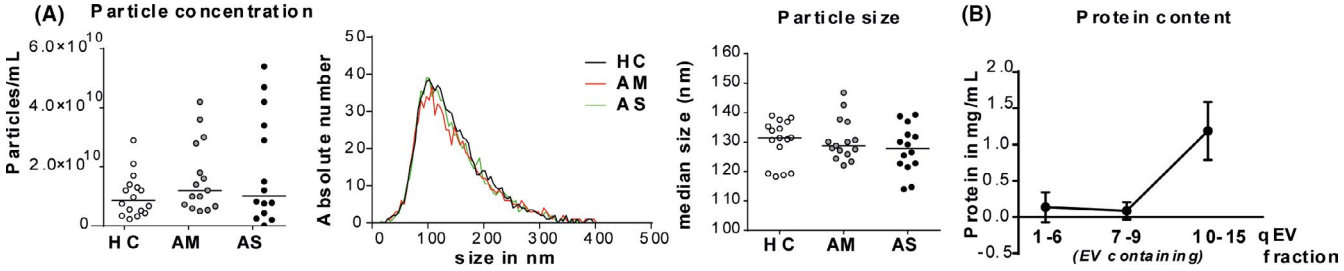
RNA-seq–based profiling of extracellular vesicles in plasma reveals a potential role of miR-122-5p in asthma

To the Editor,

Asthma is a heterogeneous disease encompassing several distinct sub-phenotypes with different etiologies and treatment responses,[1] but we are lacking markers to differentiate patient subgroups.

MicroRNAs (miRNAs) can regulate gene expression post-transcriptionally. Due to their high stability in body fluids, their easy detection, and their functional relevance in asthma, we and others have proposed extracellular miRNAs in noninvasive clinical samples as biomarkers for asthma.[2,3] Yet, extracellular miRNA profiles can be confounded by unspecific release of miRNAs from dying cells. Transfer of miRNAs by extracellular vesicles (EVs) is, however, supposedly a selective communication mechanism,[4] and EV-miRNA levels have been shown to be altered in bronchoalveolar lavage (BAL) fluid of patients with asthma.[5] In this pilot study, we profiled the

Thomas Bahmer and Susanne Krauss-Etschmann contributed equally to this study.



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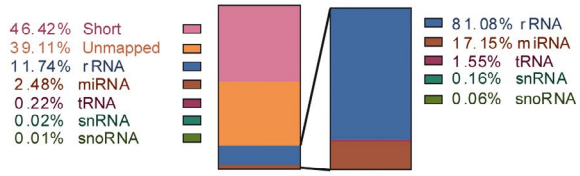

(D)

all Asthma vs HC

miRNA	baseMean	log2FoldChange	pvalue	FDR
hsa-miR-122-5p	1441.75	1.74	4.60E-05	0.03
hsa-miR-206	0.93	1.87	1.97E-02	0.99
hsa-miR-148a-3p	450.36	0.63	5.52E-03	0.99
hsa-miR-941	14.50	0.61	6.44E-03	0.99
hsa-miR-223-5p	8.59	0.53	2.23E-02	0.99
hsa-miR-191-5p	69.32	0.29	7.23E-03	0.99
hsa-miR-486-5p	1184.81	-0.39	1.89E-02	0.99
hsa-miR-3168	1327.95	-1.28	1.48E-04	0.05
hsa-miR-6842-3p	1.27	-1.38	2.36E-02	0.99

(E)

AM vs HC

miRNA	baseMean	log2FoldChange	pvalue	FDR
hsa-miR-206	0.79	2.05	2.86E-02	1.00
hsa-miR-576-3p	0.80	1.51	6.64E-02	1.00
hsa-miR-3652	1.67	1.26	4.61E-02	1.00
hsa-miR-184	83.13	1.19	5.78E-02	1.00
hsa-miR-28-5p	2.52	1.04	1.77E-02	1.00
hsa-miR-941	14.04	0.82	3.30E-04	0.20
hsa-miR-122-5p	685.37	0.66	1.56E-02	1.00
hsa-miR-2110	2.66	-1.12	2.00E-02	1.00
hsa-miR-3168	1389.62	-1.37	6.69E-04	0.21
hsa-miR-6842-3p	1.28	-1.87	1.88E-02	1.00

AS vs HC

miRNA	baseMean	log2FoldChange	pvalue	FDR
hsa-miR-122-5p	1230.18	1.77	3.21E-05	0.02
hsa-miR-191-5p	71.15	0.39	3.81E-04	0.12
hsa-miR-223-5p	8.72	0.69	1.06E-02	0.73
hsa-miR-148a-3p	398.11	0.47	6.56E-03	0.73
hsa-miR-143-3p	60.94	0.45	1.04E-02	0.73
hsa-let-7e-5p	40.70	-0.37	7.32E-03	0.73
hsa-miR-409-3p	17.28	-0.88	1.03E-02	0.73
hsa-miR-3168	1626.92	-1.16	9.62E-03	0.73
hsa-miR-4433b-3p	4.63	-1.25	1.21E-02	0.75
hsa-miR-134-5p	1.50	-2.28	6.64E-03	0.73

(F)

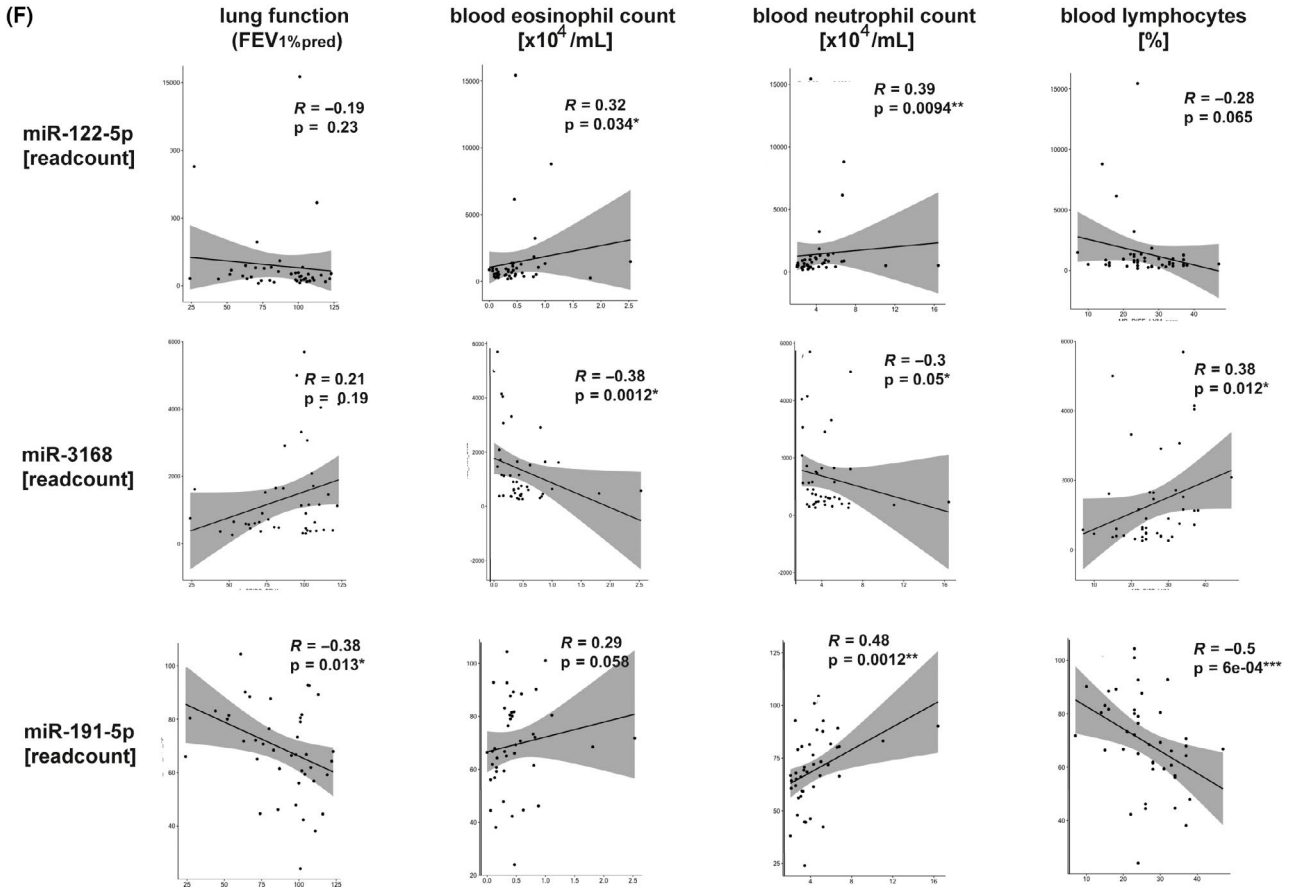

FIGURE 1 small RNA sequencing from plasma EVs. A, EV concentration (left panel), size distribution, and median size (right panels) in plasma EV isolations from healthy controls (HC, n = 16), mild-to-moderate (AM, n = 15), or severe asthma (AS, n = 14) assessed by nanoparticle tracking analysis (lines represent median). B, Protein content of qEV fractions in mg/mL, assessed by microBCA (n = 45 per fraction, mean ± SD). C, Sequencing distribution of small RNA classes in %, with relative distribution within mapped reads (right) (D and E) 10 top-regulated miRNAs (sorted by log2 fold change) in asthma patients vs HC (D) and AM vs HC (left) and AS vs HC (right) (E); FDR = false discovery rate. F, Spearman's correlations of miR-122-5p (upper panels), miR-3168 (middle panels), and miR-191-5p (lower panels) read counts with clinical characteristics of all included subjects (n = 45), and each graph depicts correlation co-efficient *R* and *P*-value

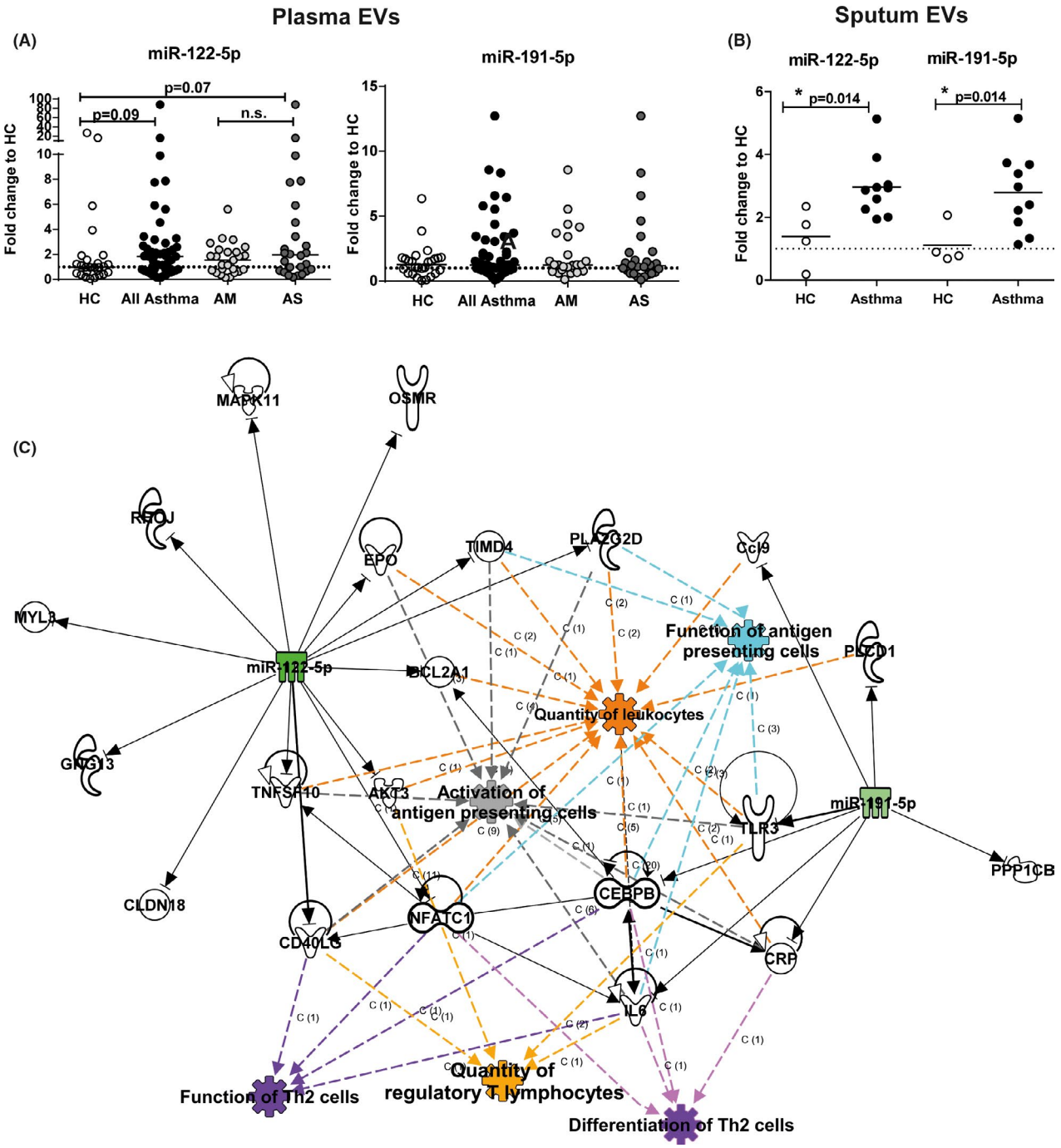

FIGURE 2 EV-miRNA from plasma correlates with clinical characteristics of asthma. A and B, RT-qPCR for miR-122-5p and miR-191-5p normalized to miR-21 levels (reference) in (A) plasma EVs from healthy controls (HC, n = 26), all asthma (n = 51), mild-to-moderate (AM, n = 25) or severe asthma (AS, n = 26), or (B) sputum supernatant EVs from healthy controls (HC, n = 4) and patients with asthma (n = 10). All Mann–Whitney U to HC or AM. **P* ≤ .05, ***P* ≤ .01, ****P* ≤ .001. Graphs depict fold changes to healthy controls with lines indicating the median. (C) Ingenuity pathway analysis showing association of miR-122-5p and miR-191-5p with *quantity of leukocytes*, *function of antigen-presenting cells*, *activation of antigen-presenting cells*, *function of Th2 cells*, *quantity of regulatory T lymphocytes*, and *differentiation of Th2 cells*

EV-miRNA signature in plasma of patients with mild-to-moderate (AM) or severe eosinophilic asthma (AS) (as defined by ERS/ATS guidelines[6]) and healthy control (HC) subjects (Tables S1 and S2).

Therefore, we isolated small EVs (EVs) (<200 nm) by size exclusion chromatography (SEC) (qEV, Izon Bioscience) from 1 mL plasma of 45 adult subjects with AM (n = 15) or AS (n = 14), and HC (n = 16) (Table S1). All study participants were enrolled in the all age asthma (ALLIANCE) cohort, a multi-center longitudinal asthma patient cohort of the German Center for Lung Research (DZL).[7]

SEC-isolated particles were confirmed to be <200 nm by nanoparticle tracking analysis (NTA) (ZetaView PMX 110, Particle Metrix) and were equal in concentration and size distribution across groups (Figure 1A). The median particle concentration isolated from 1 mL of plasma was 8.6×10^9 particles/mL (interquartile range [IQR]: 4.5×10^9-1.4×10^{10}) for HC, 1.2×10^{10} particles/mL (IQR: 6.6×10^9-2.8×10^{10}) for AM, and 1.0×10^{10} particles/mL (IQR: 3.9×10^9-3.6×10^{10}) for AS. Furthermore, SEC fractions 7-9 used for sequencing were without protein contamination (Figure 1B) and expressed different amounts of typical small EV markers CD63, CD81, and/or CD9 (Figure S1).**FIGURE 1** small RNA sequencing from plasma EVs. A, EV concentration (left panel), size distribution, and median size (right panels) in plasma EV isolations from healthy controls (HC, n = 16), mild-to-moderate (AM, n = 15), or severe asthma (AS, n = 14) assessed by nanoparticle tracking analysis (lines represent median). B, Protein content of qEV fractions in mg/mL, assessed by microBCA (n = 45 per fraction, mean ± SD). C, Sequencing distribution of small RNA classes in %, with relative distribution within mapped reads (right) (D and E) 10 top-regulated miRNAs (sorted by log2 fold change) in asthma patients vs HC (D) and AM vs HC (left) and AS vs HC (right) (E); FDR = false discovery rate. F, Spearman's correlations of miR-122-5p (upper panels), miR-3168 (middle panels), and miR-191-5p (lower panels) read counts with clinical characteristics of all included subjects (n = 45), and each graph depicts correlation co-efficient *R* and *P*-value

EV-RNA was isolated (miRNeasy Micro Kit; Qiagen) and subjected to RNA sequencing (RNA-seq) (HiSeq 2500, Illumina) of small RNAs (below 35 nt) according to Ref.8 While all samples achieved high Phred scores (>30) indicating excellent sequencing quality (Figure S2A), 46.42% of reads were short or unmapped (39.11%) (Figure 1C). From all mapped reads, 17.15% were classified as miRNAs, while 81.08% were ribosomal RNAs. In total, we detected 139 distinct EV miRNAs with ≥10 reads (AM: 115; AS: 114; HC: 128) (Figure S2B, Tables S3-S5), and 35 of which had read counts ≥ 50. Unsupervised clustering and principal component analysis did not separate the different groups (Figure S2C, D), indicating that there is no difference between asthma patients and healthy controls based on total EV-miRNA expression. However, miR-122-5p was significantly increased in all patients with asthma (log2 fold change (log2FC) = 1.74, false discovery rate (FDR) = 0.03) and in AS (log2FC = 1.77, FDR = 0.02) compared with healthy controls, while miR-3168 was decreased (log2FC = −1.28; FDR = 0.05) (Figure 1D). In AS, we found similar trend for miR-191-5p (log2FC = 0.39, FDR = 0.12) (Figure 1E).

In a bivariate analysis, miR-191-5p normalized read counts correlated negatively with $FEV_{1\%pred}$ (Spearman's $R = -.38$; $P = .013$) and lymphocyte percentage in blood ($R = -.5$; $P = .0006$) (Figure 1F). We found a positive correlation with blood neutrophil ($R = .48$; $P = .0012$) counts and a similar trend for blood eosinophil counts ($R = .29$; $P = .058$). miR-3168 read counts correlated significantly with blood eosinophils ($R = -.28$, $P = .012$), neutrophils ($R = -.3$, $P = .05$), and lymphocytes ($R = .38$, $P = .012$). Read counts of miR-122-5p positively correlated with both eosinophil ($R = .32$; $P = .034$) and neutrophil ($R = .39$; $P = .0094$) counts in blood, and trendwise with blood lymphocytes ($R = -.28$; $P = .065$) (Figure 1F). We do acknowledge that some correlation coefficients are quite low ($R < .3$) which could be due to the small sample size and needs to be confirmed in larger future studies.

Next, we isolated EVs from additional plasma samples of the same subjects and 10 new subjects per group (Figure 2A), and found a strong trend for an increase of miR-122-5p (FC = 1.84; $P = .07$) in severe asthma, and in all asthma subjects (FC = 1.96; $P = .09$) (Figure 2A) by RT-qPCR, confirming the sequencing results. miR-191-5p did not differ significantly among the groups, and miR-3168 was not detectable by RT-qPCR. Of note, in a pilot approach both miRNAs could also be detected in EVs isolated from sputum supernatant samples of four healthy control and ten asthma subjects, and we have a first hint that they are increased in asthma compared with healthy controls (miR-122-5p: FC = 2.90; $P = .014$; miR-191-5p: FC = 2.69, $P = .014$) (Figure 2B).**FIGURE 2** EV-miRNA from plasma correlates with clinical characteristics of asthma. A and B, RT-qPCR for miR-122-5p and miR-191-5p normalized to miR-21 levels (reference) in (A) plasma EVs from healthy controls (HC, n = 26), all asthma (n = 51), mild-to-moderate (AM, n = 25) or severe asthma (AS, n = 26), or (B) sputum supernatant EVs from healthy controls (HC, n = 4) and patients with asthma (n = 10). All Mann–Whitney U to HC or AM. $^*P \le .05$, $^{**}P \le .01$, $^{***}P \le .001$. Graphs depict fold changes to healthy controls with lines indicating the median. (C) Ingenuity pathway analysis showing association of miR-122-5p and miR-191-5p with *quantity of leukocytes*, *function of antigen-presenting cells*, *activation of antigen-presenting cells*, *function of Th2 cells*, *quantity of regulatory T lymphocytes*, and *differentiation of Th2 cells*

Ingenuity Pathway Analysis (IPA) of predicted targets of miR-122-5p and miR-191-5p revealed a concise network, containing the biological functions *quantity of leukocytes*, *function of antigen-presenting cells*, *activation of antigen-presenting cells*, *function of Th2 cells*, *quantity of regulatory T lymphocytes*, and *differentiation of Th2 cells* (Figure 2C). This is a first hint that miR-122-5p (and potentially miR-191-5p) could influence immune cell function upon uptake. This could systemically perpetuate the asthmatic phenotype and should be confirmed in larger studies, where also the specificity for asthma vs a general immune reaction should be assessed.

The relatively low number of significantly altered miRNAs here might be due to the isolation of highly pure EVs by SEC. This is in line with previous reports,[8] but SEC isolation is superior to other methods in separating EVs from contaminating proteins.[9] Further, we have here focused on eosinophilic asthma of different severities, hampering comparisons of different asthma sub-phenotypes. This will require considerably larger patient numbers and was thus beyond the scope of this study.

In summary, in relatively well-controlled asthma the total miRNA expression in plasma EVs is not different to controls. miR-122-5p is increased in plasma and sputum supernatant EVs derived from patients with (severe) asthma, and this miRNA correlated with immune cell types in the blood. Combined with the IPA-predicted role in lymphocyte differentiation and function, it is intriguing to speculate that this miRNA can sub-differentiate different forms of asthma, such as neutrophilic from eosinophilic asthma. This should be investigated in larger asthma cohort studies with a broad spectrum of clinically well-defined phenotypes and different treatment regimen also including steroid-naïve patients.

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CONFLICT OF INTEREST

This study was supported by an unrestricted grant (Next Generation Award) from Bencard Allergie GmbH to SB, which did not influence the content of the manuscript or the conclusions drawn. The authors declare no further conflict of interest relating to this manuscript.

AUTHOR CONTRIBUTIONS

TB, HW, A-MK, FP, BW, OF E.vM., KFR, GH, and MVK designed and conducted the clinical study including patient recruitment and processing of plasma samples. DB, MWP, J.B, S.K-E., and SB performed experiments and critically analyzed the data. IRK performed the bivariate analysis with clinical data. SB had primary responsibility for the experimental study design and writing of the manuscript. All authors have contributed to discussion of the data and writing of the manuscript, and approved the final version.

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ETHICAL APPROVAL

The study was approved by the local ethics committee of the Medical School Luebeck, Schleswig-Holstein (Germany, Az. 12-215), and is registered at clinicaltrials.gov (Identifier: NCT02419274). All participants gave their written informed consent.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

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Comparison of diagnostic accuracy of acoustic rhinometry and symptoms score for nasal allergen challenge monitoring

To the Editor,

The nasal allergen challenge (NAC) is a safe and reproducible method to identify the allergic triggers of rhinitis[1] in both atopic and non-atopic patients; besides, it can guide the composition and monitor the effect of allergen immunotherapy.[2,3] Nevertheless, NAC is scarcely implemented in the clinic due to the lack of standardized protocols, among other factors. In this regard, the European Academy of Allergy and Clinical Immunology (EAACI) published in 2018 a consensus document on NAC methodology.[3] National guidelines already existed in Spain[4] and Germany.[5] All documents advise to monitor the provocation by both subjective (symptoms score) and objective (nasal patency) parameters. Nonetheless, the guidelines differ on recommended symptom score (Lebel score, visual analogue scale [VAS], etc.), method to assess the nasal patency (acoustic rhinometry [AcRh], active anterior rhinomanometry, etc.), and cutoff points for both parameters. EAACI guidelines consider NAC positive if either a moderate change in both parameters (increase >23 mm^{-3} points for VAS/symptom score AND decrease >20-27% for nasal patency) or a clear change in at least one of them (increase >55/5 points for VAS/symptom score AND/OR decrease >40% for nasal patency) occurs.[3] However, these recommendations are mainly based on expert consensus, as there is a scarcity of data coming from ad hoc studies. Selection of cutoff points relies on the intended use of the test under evaluation. While screening methods need a high sensitivity, confirmatory tests require high specificity. Importantly, sensitivity and specificity inform on the proportion of diseased and disease-free subjects who are correctly diagnosed, but cannot predict the probability of a condition to be present in an individual patient referred for evaluation. Conversely, positive (PPV) and negative (NPV) predictive values inform directly about the probability of a subject who displayed a positive or negative result to have been or not correctly diagnosed. Nevertheless, predictive values are influenced by disease prevalence, implying that their diagnostic performance can vary in different populations.[6] On the other hand, positive (LR+) and negative (LR-) likelihood ratios display a direct mathematical relationship between pretest and posttest probability, and are independent of disease prevalence.[7] Therefore, LR+ and LR- are the most helpful indicators for the clinician to take decisions on individual patients. In this study, we investigated the discriminative power, and the pretest and posttest predictive power of NAC, together with optimal cutoff points for positivity, as measured by Lebel nasal-ocular symptom score (NOSS) and AcRh. We prospectively recruited 1165 and 369 adults with allergic rhinitis (AR) and nonallergic rhinitis (NAR), respectively, based on the results of skin prick test, serum allergen-specific IgE, and NAC (Table S1 and Table S2). We also enrolled 361 healthy nonatopic control (HC) subjects. All study