Breath volatile organic compounds and inflammatory markers in adult asthma patients: negative results from the ALLIANCE cohort

To the Editor:

"Breathomics" in asthma is a rapidly growing area of significant scientific interest, as indicated by a recently published review, two research articles, and their accompanying editorials in high impact pneumology journals [1–5]. The repeatedly observed associations between breath volatile organic compounds (VOCs) and sputum or blood inflammatory cells [2, 3] suggest that breathomics are on the brink of introduction as a valuable clinically tool. However, there are also major concerns about unresolved methodological issues and a general paucity of high-quality data [1, 6]. In this letter we detail our concerns with breathomics based on data from a cohort of adult asthma patients with a broad spectrum of clinical phenotypes.

In the adult arm of the ALLIANCE asthma cohort we prospectively recruited patients with an established diagnosis of asthma [7] across different severity grades and inflammatory phenotypes, as identified by exhaled nitric oxide fraction (F_{ENO}), blood and sputum differential cell counts [8]. As recently recommended [5], the patients were clinically well characterised, the asthma diagnosis was thoroughly established, and breath VOCs were compared between different asthma phenotypes.

For the analysis we included 133 adult patients that attended their 12-month follow-up visit from 2015 to 2016 at LungenClinic Grosshansdorf. To examine patients under stable conditions, all visits were scheduled at least 4 weeks after an acute severe exacerbation (defined as oral steroid burst therapy for at least three consecutive days) or asthma-related hospitalisation. The study (NCT02419274) was approved (Medical School Luebeck ethics committee, Az.12-215) and all participants gave their written informed consent before inclusion.

The patients were grouped into five clinically established phenotypes according to disease severity (2014 European Respiratory Society/American Thoracic Society guidelines [9]), type 2 airway inflammation (blood eosinophils ⩾300 per µL), and smoking status (figure 1a). We performed spirometry, body-plethysmography, impulse oscillometry, and measured F_{ENO} [8]. Blood differential cell counts and induced sputum [10] were assessed by established protocols. The collection and analysis of breath VOCs has been described in detail previously [11]. Patients inhaled active-carbon filtered room air and exhaled into an aluminium reservoir tube to avoid the use of sampling bags. During 5 min collection, 2.5 L breaths were loaded onto each of two Tenax TA adsorption tubes, which were analysed by gas chromatography/mass spectrometry. 134 VOCs were assessed in total (listed in table 2 of [11]). 40 VOCs were excluded because in at least in 85% of the study participants the values were below the limit of detection. VOC data was log-transformed prior to analysis.

The validity and plausibility of the VOC data is supported by several aspects and observations. 1) The method used for collection and analysis was benchmarked in the peppermint oil trial [12, 13], in which we demonstrated washout kinetics of peppermint oil compounds after ingestion of a respective capsule. 2) The two simultaneously collected adsorption tubes in this study showed a very close agreement (median r>0.87). 3) As expected, acetone and isoprene were the most abundant VOCs in breath, while cleaning- and disinfectant-related VOCs such as propanol-1, propanol-2 and ethanol were predominantly

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Despite recent publications, we are not close to finding a clinically valuable breath VOC biomarker for asthma or asthma phenotypes https://bit.ly/3heTgtK

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a)	Mild to moderate asthma		Severe asthma		Smoking asthmatics	p-value
	Eosinophilic	Non-eosinophilic	Eosinophilic	Non-eosinophilic		
Patients n	22	31	22	24	34	
Sex M/F	10/12	19/12	9/13	13/11	11/23	
Age years	46.0 (38.5–50.0)	47.0 (34.5–56.0)	54.0 (50.0–68.8)	62.0 (53.0–66.0)	56.5 (49.3–64.8)	0.002
Height cm	169.0 (165.2–176.8)	178.0 (169.0–182.0)	170.0 (165.2–176.2)	174.0 (165.5–178.0)	168.0 (161.0–175.0)	0.03
Body mass index kg·m^{-2}	26.3 (22.8–28.1)	26.3 (23.8–28.8)	27.7 (25.7–30.1)	26.1 (23.7–34.7)	26.7 (23.9–30.4)	NS
Smoking status	All patients were neversmokers or ex-smokers <10 pack-years				9 current/25 ex-smoker >10 pack-years	
Cumulative cigarette exposure pack-years	2.0 (2.0–5.8)	3.0 (2.0–6.0)	2.0 (2.0–4.0)	5.0 (3.0–5.0)	17.0 (12.3–40.0)	
Current passive smoke exposure at home n	2	1	5	2	4 (in ex-smokers)	
Exacerbation frequency never/1/>1 per year	15/4/2[¶]	28/2/1	6/4/12	5/5/14	15/7/11[¶]	
Asthma control test	22.0 (17.8–23.8)	23.0 (20.0–24.5)	17.0 (14.0–19.0)	17.0 (14.0–21.3)	20.0 (15.0–23.0)	<0.001
ICS use	17 (77%)	27 (87%)	22 (100%)	24 (100%)	31 (91%)	
ICS dose (in fluticasone equivalents) µg	250.0 (125.0–400.0)	250.0 (50.0–275.0)	700.0 (500.0–1000.0)	1000.0 (950.0–1100.0)	450.0 (200.0–500.0)	<0.001
Second asthma controller medication	17/77%	20 (65%)	22 (100%)	24 (100%)	28 (82%)	
OCS n	0	0	9	9	7	
OCS dose mg			10.0 (5.0–10.0)	10.0 (8.0–10.0)	5.0 (4.5–6.5)	
Biologicals[#] n	0	1	2	5	3	
Spirometry						
FEV_1 % pred	81.0 (75.9–92.8)	94.4 (84.8–104.6)	80.2 (67.7–87.5)	79.0 (53.0–93.0)	79.7 (65.0–93.0)	0.006
FVC % pred	108.2 (98.6–118.2)	109.5 (104.3–116.6)	106.6 (96.7–112.7)	97.1 (85.0–112.8)	104.6 (97.7–114.7)	NS
FEV_1/FVC	0.7 (0.6–0.7)	0.7 (0.7–0.8)	0.6 (0.5–0.7)	0.6 (0.5–0.7)	0.6 (0.5–0.7)	0.003
Bodyplethysmography						
RV/TLC	0.4 (0.3–0.4)	0.3 (0.3–0.4)	0.5 (0.4–0.5)	0.5 (0.4–0.5)	0.4 (0.4–0.5)	<0.001
sRtot kPa·s·L^{-1}	1.4 (1.0–1.6)	1.0 (0.8–1.1)	1.9 (1.1–2.8)	1.4 (1.1–2.7)	1.4 (1.2–2.3)	<0.001
sRtot % pred	136.5 (100.5–155.5)	89.0 (79.7–110.3)	158.5 (96.9–249.4)	145.8 (106.3–265.3)	135.9 (107.2–225.6)	<0.001
Impulse oscillometry						
R5Hz kPa·s·L^{-1}	0.5 (0.3–0.6)	0.4 (0.3–0.5)	0.5 (0.4–0.8)	0.5 (0.4–0.6)	0.5 (0.4–0.6)	0.004
FRDabs kPa·s·L^{-1}	0.1 (0.1–0.1)	0.1 (0.0–0.1)	0.2 (0.1–0.2)	0.1 (0.1–0.2)	0.1 (0.1–0.2)	0.003
***Fe*NO ppb**	26.0 (17.0–40.0)	19.0 (13.0–27.0)	29.0 (17.0–43.0)	24.0 (16.0–49.0)	16.0 (12.0–28.0)	0.02
Sputum						
Eosinophils %	3.8 (2.0–9.2)	0.4 (0.3–1.0)	5.4 (3.5–14.1)	0.9 (0.7–3.4)	3.5 (1.0–7.1)	<0.001
Neutrophils %	58.0 (35.1–82.8)	48.2 (36.3–66.7)	63.0 (36.8–69.3)	64.9 (53.1–80.9)	62.8 (48.2–75.5)	NS
Blood						
Eosinophils %	6.0 (5.0–7.8)	3.0 (2.0–4.0)	7.0 (4.0–9.0)	2.0 (1.0–2.0)	3.0 (2.0–6.0)	<0.001
Eosinophils ×10^6 per µL	475 (370–585)	170 (130–220)	500 (410–640)	135 (40–213)	255 (103–378)	<0.001
Neutrophils ×10^6 per µL	4455 (3558–5558)	3340 (3080–3945)	4300 (3400–5520)	6775 (3885–7850)	4100 (2920–5713)	0.001

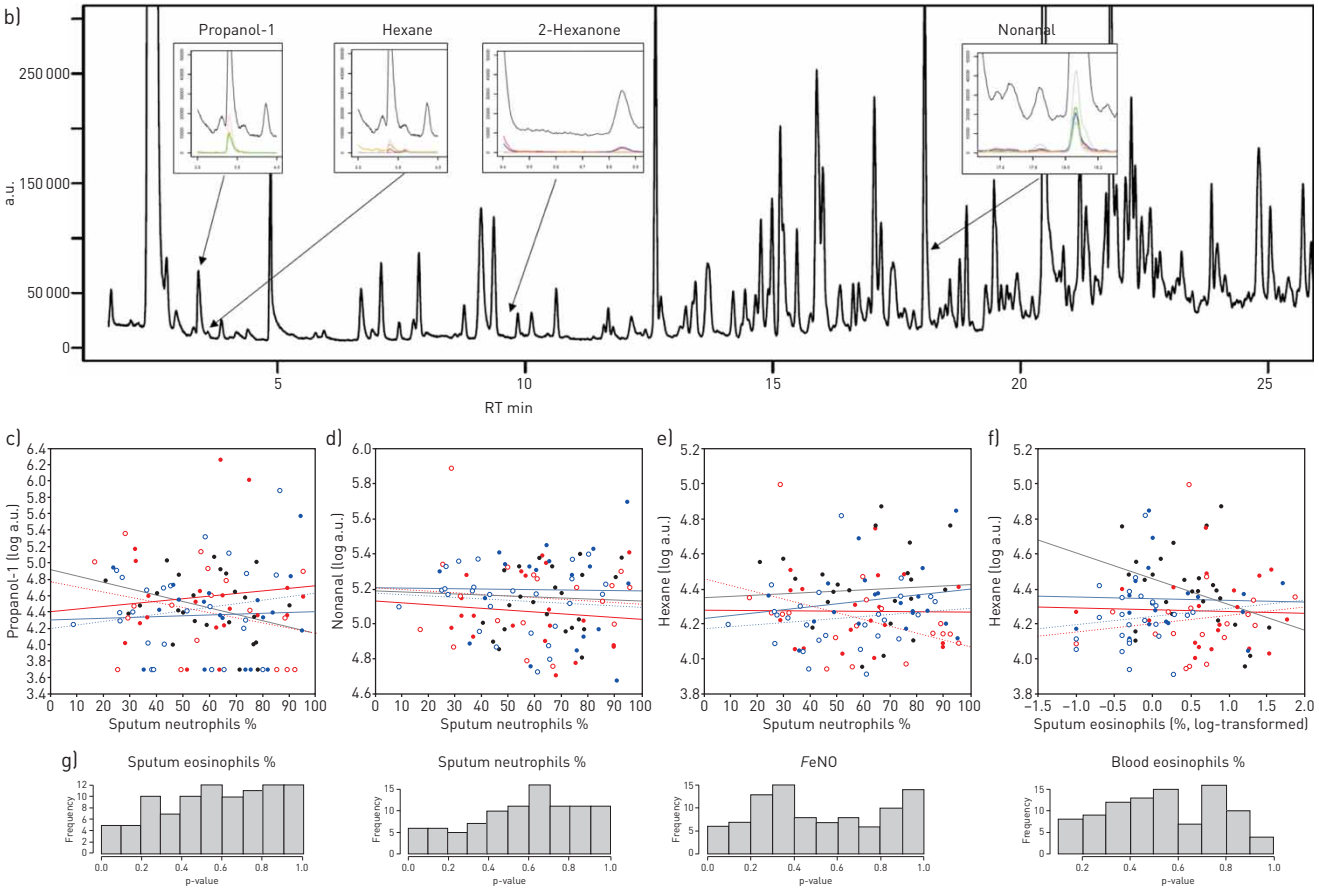

FIGURE 1 a) Patient demographics according to asthma phenotypes. Data are presented as median (interquartile range). Statistics: Kruskal–Wallis ANOVA. ICS: inhaled corticosteroids; OCS: oral corticosteroids; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity; RV: residual volume; TLC: total lung capacity; sRtot: specific total airway resistance; R5Hz: resistance at 5 Hz; FDRabs: frequency dependence of resistance (resistance at 5 Hz minus resistance at 20 Hz). [#]: omalizumab or mepolizumab; [¶]: missing values. b) Gas chromatography/mass

spectrometry chromatogram of one representative patient with inserts and arrows indicating the retention time (RT) for propanol-1, hexane, 2-hexanone and nonanal [3]. The black line indicates the total ion content (TIC), the insert coloured plots show specific masses of the respective volatile organic compounds (VOCs). c–e) Correlation between VOCs and sputum neutrophils (%). All patients and subgroup correlations not significant (unadjusted p-value >0.05). f) Correlation between hexane and sputum eosinophils (%). All patients and subgroup correlations not significant (unadjusted p-value >0.05), except for smokers (r=−0.45, unadjusted p-value 0.02, adjusted p-value 0.48). Selection of VOCs based on published data [3]. As shown in (b), 2-hexanone was not detectable in our samples. g) Histograms of unadjusted p-values for the correlation between all detected VOCs and sputum neutrophils and eosinophils (%), exhaled nitric oxide fraction (F_{ENO}) and blood eosinophils (%). a.u.: arbitrary units.

found in room air. 4) We found highly significant differences for smoking-related VOCs such as acetonitrile, benzene and cyclohexadiene ($p<0.001$, respectively) between active smokers and non-smokers (figure 1a). In addition, these compounds indicated that five patients potentially misjudged their smoking behaviour or experienced a substantial passive smoke exposure. 5) In line with others [14], we found higher levels of isoprene in male subjects ($p<0.001$).

Patient characteristics according to asthma phenotypes are shown in figure 1a. In contrast to others, we observed no statistically significant correlations between breath VOC levels and markers of inflammation such as sputum eosinophils, blood eosinophils, sputum neutrophils or exhaled nitric oxide, after adjusting the p-values for multiple testing using the Benjamini–Hochberg method [15]. The histograms of all unadjusted p-values of the correlations between VOCs and markers of inflammation are shown in figure 1g. We tested these correlations also within the subgroups of patients with comparable results. Figure 1c–f shows the correlations between sputum neutrophils and eosinophils for three markers suggested to discriminate between eosinophilc or neutrophilic asthma [3]. There were also no significant differences in breath VOCs between four different sputum inflammatory phenotypes (eosinophil cut-off 3% and neutrophil cut-off of 61% [16]). In a univariate analysis, we found nine VOCs with differences between severe and mild asthmatic subjects and only one VOC with a difference between high and normal blood eosinophils. After adjusting for multiple testing, all respective p-values were >0.11. Interestingly, the largest difference between moderate and severe asthma patients was found for an unidentified VOC (unadjusted $p<0.001$) that was suspected to be COPD-related in a previous study [11].

A recent paper suggests propanol-1 as a potential marker to discriminate between inflammatory phenotypes [3]. Although propanol-1 is known to occur in humans and to be associated with some diseases and metabolic disorders, propanol-1 is a major part of hand and surface disinfectant and detected in high concentrations in room air of hospital environments. We found no difference of propanol-1 between groups or sputum inflammatory phenotypes [16] in our study (figure 1c). The spectrum of compounds associated with asthma is very broad and diverse between studies. A certain overlap between studies appears to exist for alkanes in general, but not for individual alkanes. These as well as other markers or combination of markers suggested to be associated with asthma (reviewed in [1]) were either not among the VOCs that we detected [11] or showed no significant differences between groups. In an effort to find clinically relevant breath VOCs we used a comprehensively characterised asthma cohort and used a breath analysis method that has been benchmarked against others [13], but we were not able to reproduce the positive results of other asthma breath VOC trials.

There is an increasing interest in breath biomarkers [5] but it is important to keep in mind that still no validated VOC biomarker or biomarker pattern exists for any disease (Breath Summit 2019, Loughborough). Despite STARD guidelines, external validation is still rare in breath VOC studies [1] and importantly it is also heterogeneously defined. To evaluate the clinical value of a novel test system the discrimination model from the training patient cohort should be tested in independent patients. Showing that two discrimination models, derived from independent patient groups, result in a similar list of markers [3] or lead to similar clustering of data [2] is a major improvement with respect to independent data validation. However, a true external validation still is missing. The reason for the currently limited success in this field may be remaining methodological issues or the fact that readily detectable asthma VOC biomarkers do not exist. Despite our high quality standards we can also not exclude that methodological or sensitivity issues are responsible for the non-supportive findings presented here. However, breath biomarkers are not ready for clinical use until all standards are met.

Many valuable insights were gained from the numerous breath VOC studies, especially increasing our awareness for interfering environmental, lifestyle and metabolic factors and for the need of a more standardised methodological approach. Considering these interfering factors it currently appears crucial to identify breath VOCs to be able to assess their origin and biochemical meaning. Databases like PubChem, mVOC or HMDB provide detailed information for VOCs on endogenous production in different species, and relationship to human diseases, as well as occurrence in foods or products potentially playing a role for exogenous exposures. Available standardised collection methods (*e.g.* ReCIVA breath sampler, Owlstone,

UK) and efforts to make collection and analysis methods more comparable between research groups (Peppermint Oil Consortium) will strengthen research activities that involve multiple centres [3, 11] and thereby increase patient numbers and statistical power, which is desperately needed for a truly external validation.

However, at this point, we would like to add a word of caution to the ongoing discussion, as we did not find any significant correlations between VOCs and inflammatory markers in a well-characterised cohort of adult patients with asthma with a broad spectrum of clinical phenotypes.

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