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## Increased breath naphthalene in children with asthma and wheeze of the All Age Asthma Cohort (ALLIANCE)

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# 1 Increased breath naphthalene in children with asthma and wheeze of 2 the All Age Asthma Cohort (ALLIANCE)

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1  
2  
3 **43 Abstract**

4  
5 *44 Background*

6  
7 45 Exhaled breath contains numerous volatile organic compounds (VOCs) known to be related to  
8  
9 46 lung disease like asthma. Its collection is non-invasive, simple to perform and therefore an  
10  
11 47 attractive method for the use even in young children. We analysed breath in children of the  
12  
13 48 multicenter All Age Asthma Cohort (ALLIANCE) to evaluate if “breathomics” have the potential  
14  
15 49 to phenotype patients with asthma and wheeze, and to identify extrinsic risk factors for  
16  
17 50 underlying disease mechanisms.

18 *51 Methods*

19  
20 52 A breath sample was collected from 142 children (asthma: 51, pre-school wheezers: 55,  
21  
22 53 healthy controls: 36) and analysed using gas chromatography–mass spectrometry (GC/MS).  
23  
24 54 Children were diagnosed according to GINA guidelines and comprehensively examined each  
25  
26 55 year over up to seven years. Forty children repeated the breath collection after 24 or 48  
27  
28 56 months.

29 *57 Results*

30  
31 58 Most breath VOCs differing between groups reflect the exposome of the children. We  
32  
33 59 observed lower levels of lifestyle-related VOCs and higher levels of the environmental  
34  
35 60 pollutants, especially naphthalene, in children with asthma or wheeze. Naphthalene was also  
36  
37 61 higher in symptomatic patients and in wheezers with recent inhaled corticosteroid use. No  
38  
39 62 relationships with lung function or TH2 inflammation were detected.

40 *63 Conclusion*

41  
42 64 Increased levels of naphthalene in asthmatics and wheezers and the relationship to disease  
43  
44 65 severity could indicate a role of environmental or indoor air pollution for the development or  
45  
46 66 progress of asthma. Breath VOCs might help to elucidate the role of the exposome for the  
47  
48 67 development of asthma.

49 68 The study was registered at ClinicalTrials.gov (NCT02496468).  
50  
51 69

52 **70 Word count:** 235  
53  
54  
55 71

56 **72 Key words:** exhaled air, VOC, pediatric asthma, wheeze, ALLIANCE  
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60 74

## 75 Introduction

76 Asthma is one of the most prevalent pediatric chronic diseases worldwide and causes  
77 significant burden on patients, family, society and healthcare systems [1–3]. Characteristic  
78 clinical features are chronic airway inflammation and variable expiratory airflow obstruction  
79 presenting as cough, wheeze, chest tightness and dyspnea usually in response to specific  
80 triggers, e.g. viral infections and exposure to allergens [4].

81 Despite similar clinical manifestation, the pathobiology and course of the disease is very  
82 heterogeneous [5]. Many factors contribute to asthma development, among them genetics,  
83 epigenetics [6], prenatal influences as maternal smoking, viral respiratory tract infections in  
84 early life [7] and unfavorable environmental exposures to cigarette smoke, air pollution,  
85 allergens, or mold [8, 9]. There is particular need for non-invasive and simple to assess  
86 biomarkers for early disease detection and characterization, especially in children. While  
87 exhaled nitric oxide is considered as an established non-invasively accessible biomarker [10,  
88 11], it mainly reflects the level of eosinophilic airway inflammation. For a more comprehensive  
89 analysis, volatile organic compounds (VOC) in exhaled breath have gained considerable  
90 interest as biomarkers for lung diseases, especially in asthma [12–14].

91 Breath collection is non-invasive and only requires tidal breathing which makes it particularly  
92 attractive for the use in children even at a young age. A number of studies on breath VOCs in  
93 adult and pediatric asthma have been published in the last decade and are considered in  
94 recent reviews [12–14]. For example, Dallinga et al. showed that exhaled VOCs can distinguish  
95 asthmatic from non-asthmatic children [15], and there is evidence that breath VOCs identify  
96 distinct inflammation phenotypes [16] or predict exacerbations in asthmatic children [17, 18].  
97 Despite a large body of literature with a number of VOCs potentially discriminating between  
98 healthy controls and asthma patients or reflecting disease activity or treatment, there is  
99 currently no breath VOC biomarker or biomarker pattern that supports physicians in the  
100 diagnosis, treatment, and phenotyping of disease or in preventing exacerbations.

101 Here, we collected and analyzed breath VOCs from a subgroup of children of the All Age  
102 Asthma cohort (ALLIANCE), a multicenter prospective observational cohort recruiting children  
103 with pre-school wheeze and children and adults with asthma [19].

104 We hypothesized that VOCs or VOC patterns are distinct between children with asthma or pre-  
105 school wheeze compared to healthy children and contribute to identify extrinsic risk factors,  
106 and underlying disease mechanisms. Additionally, we investigated if VOCs were linked to

1  
2  
3 107 inflammatory phenotypes, clinical features such as lung function, asthma treatment and  
4  
5 108 exacerbation rate.

## 6 7 109 **Methods**

### 8 9 110 *Study design*

10  
11 111 The ALLIANCE cohort of the German Center for Lung Research (DZL) is a prospective  
12  
13 112 multicenter asthma cohort [19]. For this study, we collected 182 breath samples from 142  
14  
15 113 children (51 children with asthma, 55 children with wheeze and 36 healthy controls) at two  
16  
17 114 pediatric specialist centers (Hannover, Munich) from October 2016 until spring 2020 (**figure**  
18  
19 115 **1, table S1**). In a subgroup of 40 patients, a second breath collection was performed after one  
20  
21 116 or two years (asthma n=20, wheeze n=20). The study was conducted in accordance with the  
22  
23 117 principles embodied in the Declaration of Helsinki and in accordance with local statutory  
24  
25 118 requirements. The study was registered at ClinicalTrials.gov (NCT02496468) and approved by  
26  
27 119 all local ethics committees. All parents of study participants <18 years as well as study  
28  
29 120 participants ≥8 years gave their written informed consent.

30  
31 121 Children aged 6 months to 5 years were included if they had at least two episodes of wheeze  
32  
33 122 during the past 12 months ('pre-school wheezer') as indicated by the parents in the respective  
34  
35 123 questionnaire. Children ≥6 years were included based on doctor-diagnosed asthma according  
36  
37 124 to the Global Initiative for Asthma (GINA) guidelines and German guidelines [4, 20]. We also  
38  
39 125 recruited healthy control subjects who had never been diagnosed with asthma or pre-school  
40  
41 126 wheeze. Further inclusion and exclusion criteria are specified in the supplement or have been  
42  
43 127 published [19]. Laboratory tests included differential blood count, and specific  
44  
45 128 immunoglobulin E against 36 allergens measured by Euroline™ (Euroimmun, Germany). All  
46  
47 129 children performed exhaled nitric oxide (FeNO) measurements and spirometry from age 4  
48  
49 130 years onwards and VOC measurements from 3 years onwards. For the breath and near-subject  
50  
51 131 room air collection we used an in-house developed breath collection device [21]. Further  
52  
53 132 details regarding all procedures and study design are presented in the **online supplement**.

54  
55 133

### 56 57 134 *Analysis by gas chromatography-mass spectrometry (GC-MS)*

58  
59 135 GC-analysis of the samples was performed at Fraunhofer ITEM, Hannover, within eight days  
60  
136 of initial breath collection. Turbo Mass Software 5.4 (PerkinElmer, USA) was used for  
137 automated identification of individual VOCs based on retention time of specific masses,  
138 comparing values with reference compounds and the NIST database (National Institute of

1  
2  
3 139 Standards and Technology Mass Spectral Search Program Version 2.2 (NIST, USA) [21, 22]. A  
4  
5 140 total of 158 VOCs were quantified using peak height of specific target ions which most  
6  
7 141 commonly matched with  $m/z$  signals of highest intensity in the respective VOC mass spectrum  
8  
9 142 (total ion content (TIC)) [22]. Further details regarding measurement and analysis are available  
10  
11 143 in the **online supplement**.

#### 12 144 *Statistics*

13  
14 145 We computed the mean value of the 158 target VOCs for all technical replicate samples. Data  
15  
16 146 was then log-transformed. We used the Mann-Whitney-U-Test (MWU) for univariate  
17  
18 147 comparisons between patients (asthma, wheeze) and healthy controls and the Pearson  
19  
20 148 correlation coefficient where appropriate. The false discovery rate was assessed by correcting  
21  
22 149 raw  $p$ -values with the *p.adjust* function in R (using the Benjamini and Hochberg (BH) option).  
23  
24 150 Corrected  $p$ -values are indicated as  $p_{BH}$ . Site effects were accounted for by batch correction  
25  
26 151 using the *ComBat* function of the R package *sva* to our data. For some analyses that were  
27  
28 152 limited to data from one site we used both the original (uncorrected) and the batch corrected  
29  
30 153 “site-effect-free data” to test whether similar results were obtained.

31 154 Due to the exploratory nature of our analysis, we focused on the complete VOC dataset ( $n=158$   
32  
33 155 VOCs). However, to avoid missing important observations by the multiplicity correction we  
34  
35 156 also ran the analysis with subsets of our 158 VOCs. A detailed description of these subsets is  
36  
37 157 provided in the **online supplement**. In addition we dimensionally reduced the dataset using  
38  
39 158 an unsupervised clustering approach called Cytomod [23]. Briefly, VOCs were clustered and  
40  
41 159 assigned to co-varying modules based on the pairwise Pearson-correlations between log-  
42  
43 160 transformed VOC values. For more details, please refer to the **online supplement**. Module  
44  
45 161 scores reflecting the expression of each module in each participant (see **online supplement**)  
46  
47 162 were compared between participant groups with different characteristics using MWU tests.  
48  
49 163  $P$ -values were adjusted for the number of modules tested. A multivariable logistic regression  
50  
51 164 model was used to analyze the association between module 6 and disease status. We focused  
52  
53 165 our analysis on module 6, because most VOCs considered as air pollutants were found in  
54  
55 166 module 6.

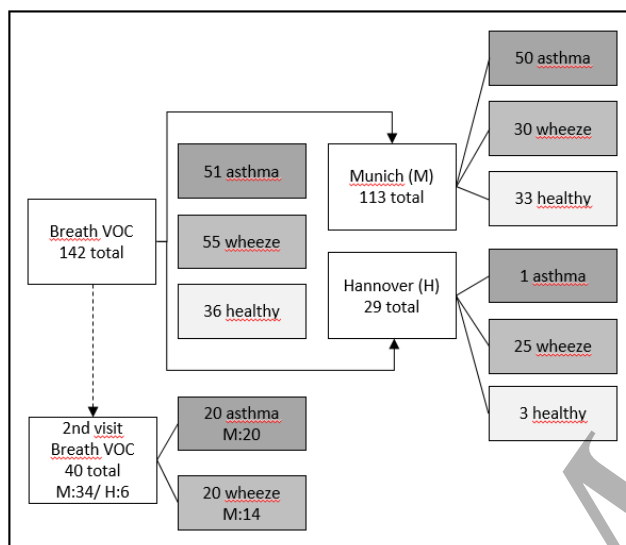
56 167

57 168

## 169 Results

### 170 Demographics

171 As shown in **figure 1**, we included 55 children with pre-school wheeze, 51 children with asthma  
 172 and 36 healthy controls (**table 1**). Total IgE and FeNO were higher ( $p < 0.01$ ) in asthmatics than  
 173 in healthy controls while FEV<sub>1</sub> and FEV<sub>1</sub>/FVC values were lower ( $p < 0.01$ ). Blood eosinophils  
 174 were higher in asthmatic children compared to controls ( $p = 0.01$ ), and atopy was more  
 175 prevalent in subjects with wheeze and asthma (**table 1**).



186 **Figure 1.** Study population. Breath VOCs were analyzed from 142 subjects. 40 subjects were  
 187 sampled on two visits, which were at least one year apart. The Hannover site had a focus on  
 188 wheezers and started the recruitment for VOC sampling later during the study. The one  
 189 patient with asthma from Hannover was planned to be included as wheezer but the visit could  
 190 not be scheduled before the 6<sup>th</sup> birthday, therefore based on the definition criteria, this  
 191 subject was considered as child with asthma.

### 193 Basic data description, quality, and plausibility of VOC data

194 As expected, acetone and isoprene were major VOCs in breath of children, while disinfectants  
 195 like 1-propanol, 2-propanol, ethanol, 2-phenoxyethanol, and 1-phenoxypropan-2-ol were the  
 196 major VOCs detected in the room air samples. In line with the “owncloud concept” [22] we  
 197 found the expected correlations between near-subject room air and breath for VOCs, that are  
 198 frequently used in lifestyle products (personal care, cosmetics, home care) or for  
 199 environmental pollutants. Among these were geranyl acetone, siloxanes, as well as benzene  
 200 (**figure S1**). Furthermore, patients exposed to second-hand smoke and in one patient to active  
 201 smoking showed increased levels of cigarette smoke related VOCs (**table S2**) [21, 24]. Isoprene

202 levels in exhaled breath significantly correlated with age ( $r=0.36$ ,  $p<0.0001$ ), as previously  
203 shown [25].

204

#### 205 Potential confounders: site, age, gender

206 The median levels of most VOCs were different between study sites. We consider technical  
207 reasons, like instrument drift, as unlikely for this observation (details are presented in the  
208 **online supplement**) and adjusted the data as outlined in material and methods. To evaluate  
209 the impact of age we checked the correlation between VOC values and age using the site  
210 effect-free data for all subjects. Besides the already mentioned positive isoprene correlation,  
211 we only found a negative correlation for 1,2-propandiol and age ( $r=-0.27$ ), with borderline  
212 significance ( $p_{BH}=0.068$ ). No significant gender-specific differences for breath VOCs were  
213 observed ( $p_{BH}>0.05$ ).

214

#### 215 Reproducibility between visits

216 For 40 children (asthma  $n=20$ , wheeze  $n=20$ ) we were able to collect a second sample in a  
217 follow-up visit one year after taking the initial sample. In three cases, the second sample was  
218 taken two years later. Initial and follow-up VOCs values correlated significantly for 13 VOCs  
219 ( $p_{BH}<0.05$ , **figure S2**, **table S3**). Most of these VOCs appear to be environment- and lifestyle-  
220 related. The correlation between the visits was not significant for naphthalene (**figure S3**,  
221  $p=0.06$ ,  $p_{BH}=0.27$ ).

222

#### 223 Cluster analysis

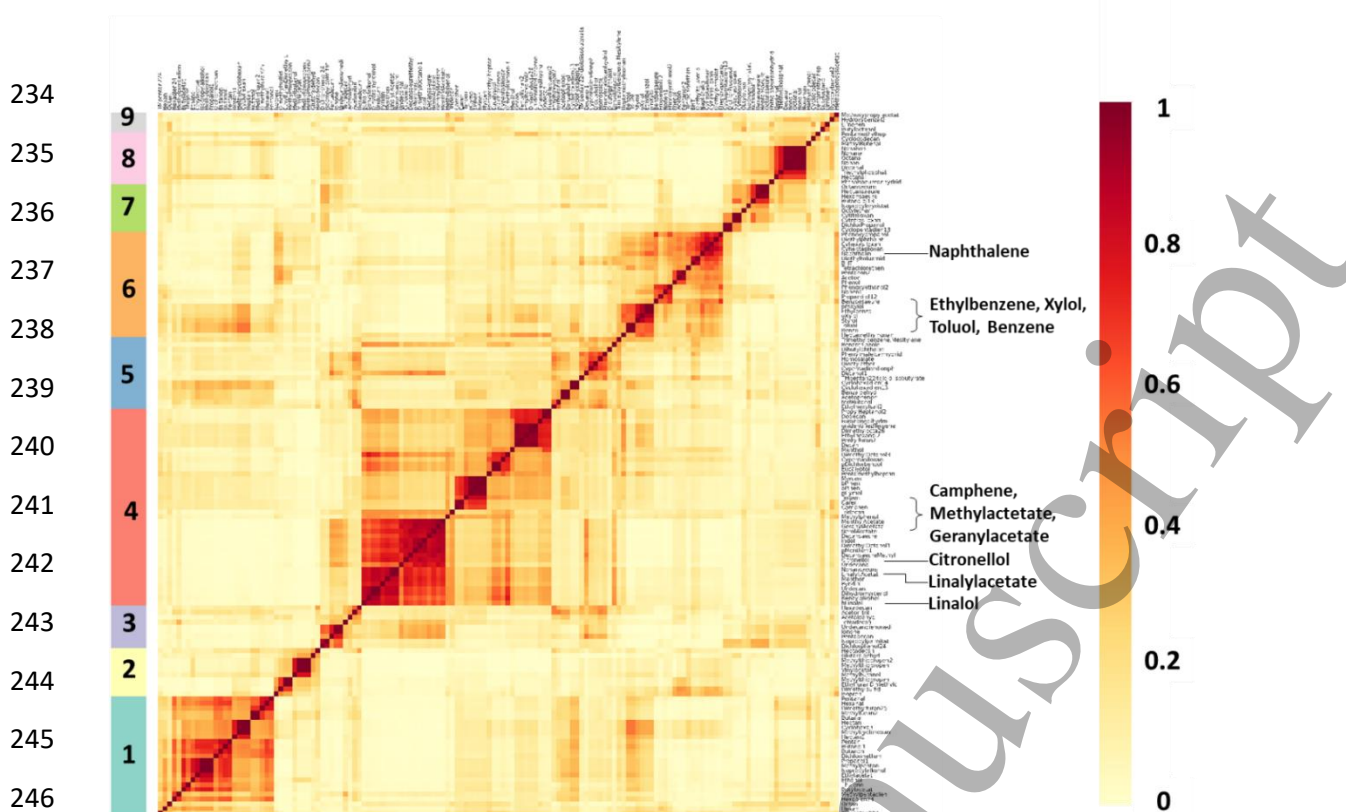
224 In the unsupervised cluster analysis, we identified a total of 9 modules (**figure 2**), consisting of  
225 3 to 39 VOCs. The VOCs contained in each module are listed in the supplement (**table S4**).  
226 Interestingly, all BTEX (benzene, toluene, ethylbenzene, xylenes) VOCs and other VOCs  
227 considered as air pollutants, like naphthalene, were found in module 6. A large number of  
228 VOCs in module 4 were substances used as fragrances and flavors typically found in many  
229 lifestyle and cleaning products.

230

#### 231 VOCs differ between children with asthma or pre-school wheeze and healthy controls

232 We found 24 significantly different VOCs ( $p_{BH}<0.05$ ) between healthy controls and asthma  
233 patients and 23 significantly different ( $p_{BH}<0.05$ ) VOCs between healthy controls and children



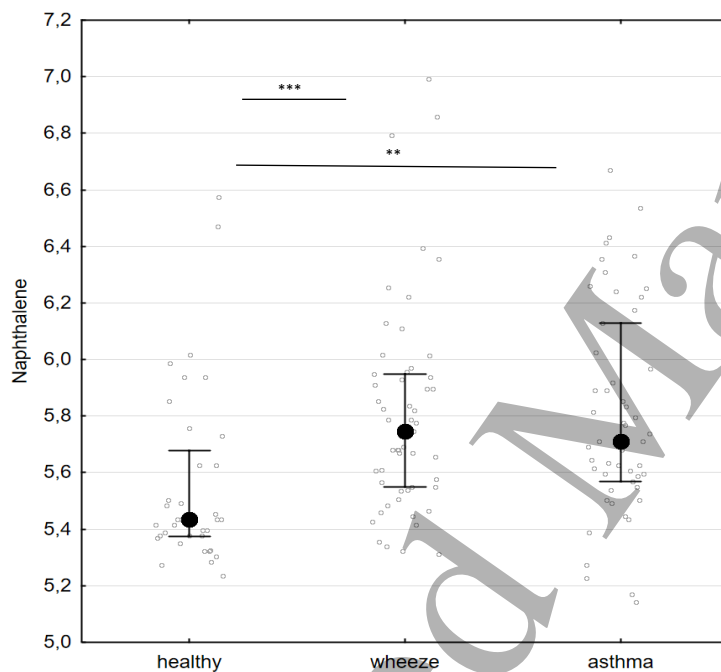


**Figure 2:** Heatmap of VOC modules: VOCs are first clustered together based on pairwise Pearson correlations. A reliability score (i.e., the fraction of times that a given VOC is assigned to the same cluster) is calculated over 1,000 permutations of participants and is used to assign VOCs to modules. The color bars on the left side depict the module membership for each VOC (a full list is given in supplement **table S4**), and the coloring in the heatmap represents the reliability score of each VOC.

with wheeze (**table 2**). There was a large overlap, with 13 VOCs having significantly different levels both in asthma patients and wheezers compared to healthy controls (**table 2**). As clinical characteristics differed between healthy controls and patients (**table 1**), we performed additional subgroup analyses which are described in detail in the **online supplement**. We did not find any evidence that these differences influenced the results.

Most of the VOCs with significantly higher levels in breath of healthy controls are used as fragrances or flavors and can therefore be found in lifestyle products and foods (**table 2**). Some of these VOCs with suspected fragrance or flavor origin correlated among each other, which could indicate common sources (**table S5**). This result was also mirrored in the cluster analysis data, as most of the VOCs, which were increased in healthy controls, were found in module 4 as indicated in **table 2** and **figure 2**. Module 4 values were also significantly higher in healthy controls compared to asthmatic children ( $p_{BH}=0.006$ ) and children with wheeze ( $p_{BH}=0.03$ ), respectively (**figure S4**).

268 Breath naphthalene levels showed the largest difference between children with asthma or  
 269 wheeze and healthy controls (**table 2, figure 3**). Furthermore, other VOCs considered as air  
 270 pollutants, such as 1,2-propanediol and ethylhexanol were also increased in asthma or  
 271 wheeze. Although not statistically significant ( $p_{BH} > 0.05$ ), we additionally observed higher  
 272 breath levels for other aromatic air pollutants (**table 2**). These were significantly correlated  
 273 (all  $p < 0.01$ ) among each other and with naphthalene (**figure S5**), again suggesting a common  
 274 source. All these environmental pollutants were found in module 6 as indicated in **table 2**.  
 275 Consistent with this result, overall module 6 values were higher in asthmatic children  
 276 ( $p_{BH} = 0.07$ ) and children with wheeze ( $p_{BH} = 0.03$ ) compared to healthy controls (**figure S4**).



290 **Figure 3.** Differences in the levels of naphthalene between groups. Median and interquartile  
 291 ranges. Individual data is shown in grey open circles. \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$ .

#### 294 VOC profiles associated with disease characteristics

295 VOCs or module 4 and 6 values did not differ depending on presence of atopy, blood  
 296 eosinophilia, or increased FeNO (see **online supplement** for definition and further details).  
 297 Similarly, we did not find any correlations between VOCs and FeNO or absolute blood  
 298 eosinophil levels. No correlations were found for VOCs or module values and FEV<sub>1</sub> z-score,  
 299 neither for asthma patients, wheezers or both groups combined. There were also no  
 300 differences when comparing participants with FEV<sub>1</sub> z-scores  $< -1$  to those with z-scores  $> -1$ . The

1  
2  
3 301 same results were found for the FEV<sub>1</sub>/FVC data. For FEF<sub>25-75</sub> the results were also similar,  
4  
5 302 except for asthma patients. Here we found higher levels for six VOCs (all in module 4), among  
6  
7 303 them capric-acid, undecane and undecanal and for module 4 values (pBH<0.05) in asthma  
8  
9 304 patients with a z-score <-1.

10  
11 305

#### 12 306 VOC profiles associated with disease control

13  
14 307 No significantly different VOC levels were found between asthmatic children and wheezers  
15  
16 308 with controlled disease according to GINA guideline definition [4] (n=87 and those with  
17  
18 309 “partly-controlled” or “uncontrolled” asthma” (n=19) when testing the different VOC subsets  
19  
20 310 as defined in material and methods (pBH>0.05)). Testing only naphthalene showed  
21  
22 311 significantly higher levels in the partly- and uncontrolled group (p<0.008), which is compatible  
23  
24 312 with the observed trend to increased module 6 values (p=0.03, pBH 0.251). No difference was  
25  
26 313 observed for module 4.

27 314 VOCs or module 4 and 6 values did not differ between children with or without exacerbations  
28  
29 315 or wheeze episodes in the past 12 months. In wheezers with current use of inhalative  
30  
31 316 corticosteroids (ICS), we found significantly higher levels for 1-phenoxypropan-2-ol  
32  
33 317 (pBH=0.03) and for naphthalene (p=0.01). Both are found in module 6, which also showed  
34  
35 318 higher values in this group (pBH=0.06). In addition, higher levels for an unidentified terpene  
36  
37 319 (pBH≤0.008), and three alkanes (undecane, dodecane and decane) were observed (pBH=0.09)  
38  
39 320 Two of these VOCs were found in module 4, which showed a trend for higher module values  
40  
41 321 in this group (pBH=0.06).

42 322

#### 43 323 Association of cluster module 6 with asthma or pre-school wheeze

44  
45 324 As VOC module 6 was increased in children with asthma or pre-school wheeze, we were  
46  
47 325 interested in investigating this association while controlling for other known risk factors for  
48  
49 326 asthma development. In a multivariable analysis, increased values for VOC module 6 were  
50  
51 327 significantly associated with asthma and pre-school wheeze, also when adjusting for age,  
52  
53 328 parental asthma, gender, presence of atopy, elevated blood eosinophil counts, and secondary  
54  
55 329 cigarette smoke exposure (**table 3**). Only atopy was also significantly associated to asthma or  
56  
57 330 wheeze status in our multivariable regression model.

58 331

59  
60 332

## 333 Discussion

334 In this study, we showed that breath VOCs differ between healthy children and patients with  
335 asthma or pre-school wheeze. Most of the differential VOCs were related to the exposure of  
336 the patients. Lifestyle-related VOCs were generally lower in children with wheeze or asthma  
337 compared to healthy children. More importantly, several air pollutants, especially  
338 naphthalene, showed higher breath levels in children with wheeze or asthma. The association  
339 between pollutant-related VOCs and asthma or wheeze held true in a multivariable model  
340 with adjustment for other known disease risk factors. The most prominent VOC linked to  
341 asthma or wheeze was naphthalene, however cluster analysis also identified several other  
342 pollutants like benzene, toluene, ethylbenzene, and xylene (BTEX), which were linked to  
343 asthma or wheeze. Benzene and ethylbenzene originate mainly from traffic emissions and  
344 incomplete combustion, while other BTEX are related to indoor exposure caused by proximity  
345 to road traffic, cigarette smoking or cooking methods [26, 27]. In agreement with our results,  
346 other authors have also shown increased levels of these VOCs in patients with asthma [28,  
347 29].

348  
349 Naphthalene is a very volatile polycyclic aromatic hydrocarbon (PAH) predominantly present  
350 in the atmosphere in its vapor form [27]. It's a natural constituent in coal tar and oil, and a  
351 ubiquitous pollutant in the environment [27]. Large amounts are produced worldwide [30],  
352 primarily as an intermediate for other chemicals and airborne emissions originate from  
353 incomplete combustion, e.g. in traffic exhaust, during burning of biomass, or tobacco smoke  
354 [27]. Inhalation is being considered as the most significant route of exposure [31]. We did not  
355 find differences in breath naphthalene levels for children who lived in the proximity of larger  
356 roads or who were exposed to environmental tobacco smoke, but this analysis was only based  
357 on questionnaire data which is only a crude estimate of the real life exposure. Naphthalene  
358 concentrations can also be increased indoors [27], especially in buildings with attached  
359 garages or integrated fireplaces [27, 32]. A range of indoor building materials has been  
360 reported to emit naphthalene, e.g. caulking, carpeting, rubber or vinyl flooring [33]. Cabins of  
361 diesel- and gasoline-fuel cars have also been reported as relevant sources [34]. In our study,  
362 we did not have information about these potential exposures. Therefore, it would be  
363 interesting for future studies to include personal sampling devices. Diet has also been

1  
2  
3 364 described as a potential source of naphthalene [27], however, this contribution will be difficult  
4  
5 365 to control for in cohort studies.

6  
7 366 In children with pre-school wheeze, increased breath naphthalene levels have been observed  
8  
9 367 before [35], but its potential role was not further discussed. Increased naphthalene levels have  
10  
11 368 also been reported in blood and urine of children with asthma. In a study comparing PAH levels  
12  
13 369 in 195 children, naphthalene serum levels showed one of the strongest associations with  
14  
15 370 asthma [36], while increased 2-naphthol levels, a naphthalene metabolite, could be found in  
16  
17 371 urine of asthmatic children [37]. Increased PAH metabolite levels in urine of children with  
18  
19 372 asthma were associated with higher disease-specific symptoms and consequently decreased  
20  
21 373 lung function parameters [38]. All substances mentioned above were part of module 6 with  
22  
23 374 considerable correlation among each other, which suggests regular exposure to a potential  
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25 375 common source. Little is known so far regarding the potential pathomechanisms linking  
26  
27 376 naphthalene and asthma. Murine studies showed that naphthalene could have local effects  
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29 377 on the respiratory epithelium as well as systemic effects due to high plasma concentrations  
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31 378 after inhalation [30, 39, 40], however, the exact role in asthma development is still uncertain.

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33 379  
34  
35 380 In contrast to the strong pollution signature in children with asthma and wheeze, we found  
36  
37 381 increased levels of flavor and lifestyle related VOCs in healthy subjects. Although speculative,  
38  
39 382 one possible explanation could be a higher awareness of parents of asthmatic or wheezing  
40  
41 383 children limiting exposure to strong flavorings, cosmetics, or cleaning products while parents  
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43 384 of healthy children might be less restrictive.

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45 385  
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47 386 Eleven of the compounds found to be different between healthy children and patients (**table**  
48  
49 387 **2**) have been related to asthma in previous studies [13, 14], however the overlap with our  
50  
51 388 results is quite small. Recent reviews show that this is not uncommon in studies with asthma  
52  
53 389 or wheezing patients [12, 13] and very likely due to differences in patient populations and in  
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55 390 sampling and analysis methods. Furthermore, as we used a predefined target VOC dataset  
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57 391 comprising of 158 VOCs, we might not have detected some of the previously reported VOCs  
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59 392 in comparable trials with asthma patients.

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61 393  
62  
63 394 We found no relationships of VOC profiles with clinical characteristics such as asthma control  
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65 395 or risk for exacerbations [17, 18]. Similarly, VOCs were not associated with inflammatory

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3 396 phenotypes, i.e. type 2 inflammation, confirming previous negative results from the adult arm  
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5 397 of the ALLIANCE cohort [41]. In this respect it is important to note that about 73 % of the  
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7 398 asthmatic children were under anti-inflammatory treatment when the breath collection was  
8  
9 399 performed. We cannot exclude that this has reduced our chance of finding VOC patterns  
10  
11 400 associated with inflammation, however we still observed significantly higher FeNO levels in  
12  
13 401 the asthmatic children, indicating ongoing airway inflammation.

14 402 Naphthalene and two other VOCs of module 6 were increased in pre-school wheezers treated  
15  
16 403 with ICS, supporting the proposed link between air pollution as a disease driving factor, as ICS  
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18 404 treatment in this age group identifies children with a more severe phenotype. Additionally,  
19  
20 405 children with pre-school wheeze and regular ICS treatment showed higher levels of three  
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22 406 different long chain alkanes, which have previously been identified in COPD patients with  
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24 407 respiratory tract infections caused by Rhinovirus [42]. This could potentially point towards an  
25  
26 408 inflammatory phenotype driven by recurrent viral infections. However, it needs to be  
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28 409 mentioned that one of these alkanes (undecane) was also part of module 4 and increased in  
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30 410 healthy controls thus illustrating the complexity of the data and the difficulties in  
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32 411 distinguishing exogenous and endogenous sources of VOCs.

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34 412  
35 413 A strength of our study is the choice of method for breath collection and VOC analysis which  
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37 414 has been benchmarked in a previous trial [43]. Furthermore, our results were in concordance  
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39 415 with several expected findings. For example, we were able to identify cigarette smoke related  
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41 416 VOCs in children exposed to environmental tobacco smoke, and we saw significant  
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43 417 correlations for lifestyle related VOCs between repeated measurements over at least one  
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45 418 year. Correlations between near-subject room air and breath for VOCs related to hygiene  
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47 419 product and detergents are in line with our “owncloud” concept [22].

48  
49 420 It's well known that the VOC composition of breath is influenced by many factors [44], among  
50  
51 421 them demographic, diet, life style and recent activity. Therefore, we interpret the observed  
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53 422 correlations of VOCs between the repeated measurements as remarkable, despite rather low  
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55 423 correlation coefficients ( $r \leq 0.6$ ) and consider these as an indication for stable living and  
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57 424 lifestyle conditions.

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59 425 However, we also have to report some limitations. Our data showed a marked center effect  
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426 which we had observed in a previous multicenter study as well [24]. All analyses were  
427 performed using batch-corrected data and original data whenever possible (supplementary

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3 428 material) and the comparable results indicate that batch correction did not introduce an  
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5 429 unwanted bias to our data.

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7 430 In line with our previous studies [21, 22, 24] we limited our GC-MS analysis to preselected 158  
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9 431 VOCs instead of performing a broad analysis of all potential peaks in a chromatogram. While  
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11 432 the latter is likely to include more features for comparison, there is a risk for false identification  
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13 433 of VOCs, which coelute at the same retention time. On the downside, by including only  
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15 434 preselected VOCs, we cannot exclude to have missed VOCs which could have been relevant  
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17 435 for the comparison between groups. Unfortunately, healthy control children were not age-  
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19 436 matched to children with asthma or wheeze. However, only two VOCs out of 158 VOCs were  
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21 437 actually age dependent, which makes a significant influence of age on the result unlikely.  
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23 438 Overall, we focused our analysis on single VOC comparisons as well as the two modules 4 and  
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25 439 6, which comprised most of the significantly different VOCs found in the single VOC analysis.  
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27 440 Some other VOCs modules were also significantly different between the study groups,  
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29 441 however, further detailed analysis of all modules was beyond the scope of this manuscript.

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31 442  
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33 443 In this study, we were able to identify VOCs associated with asthma and pre-school wheeze  
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35 444 indicating an increased exposure of these children to environmental pollutants. Due to the  
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37 445 observational nature of our study, we cannot deduce a causal relationship of these  
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39 446 environmental exposures to the development of asthma. Still, the strong association  
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41 447 confirmed in a multivariable analysis as well as the fact that the result was already seen in pre-  
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43 448 school wheezers and in the case of naphthalene particularly in those more severely affected  
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45 449 supports previous findings that exposure to pollutants might be an early life factor driving  
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47 450 asthma pathology [7]. Although we could not find associations between VOCs and clinically  
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49 451 relevant features as inflammatory phenotypes or risk for exacerbations, we consider non-  
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51 452 invasive breath analysis an interesting tool to assess the exposome of children and to decipher  
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53 453 the role of environmental exposures in the development of airway diseases. The increased  
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55 454 levels of breath naphthalene and other PAH in asthmatic and wheezing children would be a  
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57 455 strong argument to include breath sampling and potentially also personal sampling devices  
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59 456 into future studies to better understand the role of environmental and especially indoor air  
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457 pollutants.

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464

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3 583 **Legends to figures**  
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5 584 **Figure 1.** Study population. Breath VOCs were analyzed from 142 subjects. 40 subjects were  
6 585 sampled on two visits, which were at least one year apart. The Hannover site had a focus on  
7 586 wheezers and started the recruitment for VOC sampling later during the study. The one  
8 587 patient with asthma from Hannover was planned to be included as wheezer but the visit could  
9 588 not be scheduled before the 6<sup>th</sup> birthday, therefore based on the definition criteria, this  
10 589 subject was considered as child with asthma.  
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13 590  
14 591 **Figure 2.** Heatmap of VOC modules: VOCs are first clustered together based on pairwise  
15 592 Pearson correlations. A reliability score (i.e., the fraction of times that a given VOC is  
16 593 assigned to the same cluster) is calculated over 1,000 permutations of participants and is  
17 594 used to assign VOCs to modules. The color bars on the left side depict the module  
18 595 membership for each VOC (a full list is given in supplement **table S4**), and the coloring in the  
19 596 heatmap represents the reliability score of each VOC.  
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22 597 **Figure 3.** Differences in the levels of naphthalene between groups. Median and interquartile  
23 598 ranges. Individual data is shown in grey open circles. \*\*\* p<0.001, \*\* p<0.01.  
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604 **Table 1.** Demographics

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		n	asthma	n	wheeze	n	healthy
Number of subjects	M/H	51	50 / 1	55	30 / 25	36	33 / 3
Gender	female/male	51	16 / 35	55	20 / 35	36	12 / 24
Age	years	51	12.9 ± 3.5***	55	5.7 ± 1.3***	36	9.7 ± 3.3
Age at inclusion	years	51	10.8 ± 3.1	55	2.7 ± 1.5***	36	9.7 ± 3.3
Atopy <sup>§</sup>	% pos.	46	85	43	58	35	37
FeNO	ppb	44	24.7 (11.1;36.9)**	23	9.6 (5.1;19.9)	24	9.1 (5.6;20.2)
FEV1	z-score	49	-0.53 (-1.09;0.23)***	48	-0.39 (-1.09;0.55)***	36	0.24 (-0.24;1.26)
FEV1/FVC	z-score	49	-0.57 (-1.34;0.15)***	47	-0.28 (-1.18;0.87)	36	0.16 (-0.67;1.12)
Blood leucocytes	cells/ $\mu$ L	46	7350 (6100;8300)	42	7500 (6400;9200)**	34	6675 (5500;7730)
Blood eosinophils	cells/ $\mu$ L	46	358 (198;570)*	42	381 (260;582)**	34	201 (139;395)
Blood neutrophils	cells/ $\mu$ L	46	3327 (2654;4300)	42	3430 (2603;4304)	34	3046 (2452;3696)
Total IgE	kU/L	45	232 (162;562)**	41	111 (35;302)	34	110 (41;240)
Asthma medication:							
SABA	n (%)	51	19 (37 %)	55	15 (27 %)		
Montelukast	n (%)	51	2 (4 %)	55	4 (7 %)		
ICS	n (%)	51	10 (20 %)	55	13 (24 %)		
LABA/ICS	n (%)	51	26 (51 %)	55	8 (15 %)		
wheezing episode (yes) <sup>§</sup>	n (%)	51	13 (26 %)	55	25 (45 %)		
≥ 1 exacerbation (yes) <sup>§</sup>	n (%)	51	2 (4 %)	55	5 (9 %)		

M=Munich, H=Hannover

Mean ± SD or median (25%;75% quartile) are presented. §: specific IgE  $\geq$  0,7 against at least 1/36 food- and aeroallergens.

Medication: number of children with respective treatment in the month before the VOC visit

Wheeze episode: symptoms of wheeze which required treatment with salbutamol for > 2 / 7 days

Exacerbation: wheeze episode which required treatment with systemic steroids or admission to hospital

§: refers to 12 month prior to the VOC collection visit

\*\*\*p<0.001,

\*\*p<0.01, \*p<0.05

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**Table 2.** Differences between healthy controls, asthmatic children, and children with wheeze

VOC	Module 4	Asthma vs. Healthy		Wheeze vs. Healthy		Fragrance / Flavor	Food	Personal Care	Cleaning	Disinfectant	Air pollutant	VOC	Module 6	Asthma vs. Healthy		Wheeze vs. Healthy		Fragrance / Flavor	Food	Personal Care	Cleaning	Disinfectant	Air pollutant	
		pMWU	pBH	pMWU	pBH									pMWU	pBH	pMWU	pBH							
Lower in Diseased Patients												Increased in Diseased Patients												
unidentified C10H18 (a)	x	<0,001	<b>&lt;0,001</b>	<0,001	<b>0,007</b>							Isoprene (b)		<0,001	<b>&lt;0,001</b>	0,318	0,507							
Geranyl acetate	x	<0,001	<b>0,003</b>	0,003	<b>0,035</b>	x	x	x	x															
Pentadecane		<0,001	<b>0,003</b>	0,006	<b>0,042</b>	x	x	x				1-Phenoxypropan-2-ol	x	0,005	<b>0,034</b>	0,039	0,144			x	x	x		
Menthyl acetate	x	<0,001	<b>0,005</b>	0,001	<b>0,028</b>	x	x	x	x	x		Cycloheptasiloxane	x	0,021	0,093	0,004	<b>0,037</b>			x	x			
Decanoic acid methyl ester	x	0,001	<b>0,009</b>	0,002	<b>0,033</b>	x			x			Benzoic acid	x	0,022	0,093	<0,001	<b>0,008</b>	x	x	x	x			
Decanoic acid	x	<0,001	<b>0,009</b>	0,008	<b>0,054</b>	x	x	x	x			1-Nonene	x	0,068	0,179	0,004	<b>0,037</b>							
unidentified VOC (a)		<0,001	<b>0,009</b>	0,064	0,213							1,2-Propanediol	x	0,23	0,40	0,001	<b>0,028</b>	x	x	x	x	x	x	
Neryl acetate	x	0,002	<b>0,019</b>	0,038	0,144	x	x	x	x			2-Ethylhexanol		0,008	<b>0,047</b>	0,005	<b>0,038</b>	x	x				x	
2-Propanol		0,002	<b>0,018</b>	0,128	0,302	x	x	x	x	x		Limonene		0,051	0,146	0,001	<b>0,028</b>	x	x	x	x			
1-Decanol		0,002	<b>0,020</b>	<0,001	<b>0,007</b>	x	x	x	x	x		Naphthalene	x	<0,001	<b>0,005</b>	<0,001	<b>0,007</b>							x
Ionone		0,002	<b>0,017</b>	<0,001	<b>0,007</b>	x	x	x	x															
Citronellol	x	0,001	<b>0,015</b>	0,002	<b>0,033</b>	x	x	x	x			Ethylbenzene	x	0,070	0,179	0,159	0,328							x
b-Linalool	x	0,001	<b>0,017</b>	0,129	0,302	x	x	x	x	x		Benzene	x	0,046	0,138	0,344	0,537							x
Decanal		0,003	<b>0,023</b>	0,025	0,120	x	x	x	x	x		p-/m-Xylene	x	0,144	0,297	0,151	0,328							x
Linalyl acetate	x	0,005	<b>0,031</b>	0,158	0,328	x	x	x	x	x		o-Xylene	x	0,238	0,411	0,114	0,280							x
Diocetyl ether		0,003	<b>0,025</b>	0,078	0,222	x		x	x			Toluene	x	0,113	0,260	0,036	0,144							x
Camphene	x	0,002	<b>0,018</b>	0,057	0,201	x	x	x	x	x														
Undecane	x	0,003	<b>0,025</b>	0,005	<b>0,038</b>	x	x	x				Hexanoic acid				0,002	<b>0,034</b>	x	x	x	x			
2,5-Dimethylfuran		0,251	0,425	0,004	<b>0,037</b>	x	x					Myrcene				0,005	<b>0,038</b>	x	x	x	x			
t-Butyl alcohol		0,115	0,261	0,003	<b>0,034</b>	x	x	x	x			The table contains all compounds which were different between healthy and asthma or healthy and wheeze												
unidentified Terpene	x	0,030	0,107	0,004	<b>0,038</b>							Additional information was provided for other airpollutants irrespective of the level of significance												
3,7 - Dimethyloctan-1-ol	x	<0,001	<b>0,005</b>	0,003	<b>0,034</b>	x		x	x			(a) sign. correlation with e.g. Geranyacetate and other lifestyle VOCs suggests common source												
Octanoic acid		0,007	<b>0,043</b>	0,385	0,570	x	x	x	x			(b) endogenous VOC, difference between groups mainly due to age dependency												
Nonane		0,007	<b>0,043</b>	0,033	0,141							Occurrence and main use information based mainly on European Chemical Agency (ECHA)												
												pMWU: p-value uncorrected using non-parametric testing												
Hexanoic acid		0,401	0,554			x	x	x	x			pBH: p-value corrected for multiple testing (Benjamini-Hochberg)												
Myrcene	x	0,029	0,107			x	x	x	x			For more details on clusters refer to supplement table S4												

**Table 3.** Multivariable regression model investigating the association between module 6 and asthma or pre-school wheeze while controlling for known risk factors for asthma development

	<b>Estimate</b>	<b>SE</b>	<b>Odds ratio</b>	<b>98% CI</b>	<b>p-value</b>
<b>Module 6</b>	0.83	0.29	2.28	1.34 - 4.17	0.004
<b>Atopy</b>	1.96	0.53	7.08	2.62 - 21.0	<0.001
<b>Age</b>	-0.02	0.06	0.98	0.87 - 1.10	0.7
<b>Gender (male)</b>	-0.11	0.51	0.9	0.32 - 2.42	0.8
<b>parental asthma</b>	0.1	0.56	1.10	0.37 - 3.42	0.9
<b>Eosinophils <math>\geq 470/\mu\text{l}</math></b>	-0.87	0.59	2.39	0.79 - 8.05	0.14
<b>Cigarette smoke exposure</b>	-0.001	0.59	1.00	0.32 - 3.34	>0.9