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43 **Abstract**

44 *Background*

45 Exhaled breath contains numerous volatile organic compounds (VOCs) known to be related to

46 lung disease like asthma. Its collection is non-invasive, simple to perform and therefore an

47 attractive method for the use even in young children. We analysed breath in children of the

48 multicenter All Age Asthma Cohort (ALLIANCE) to evaluate if "breathomics" have the potential

49 to phenotype patients with asthma and wheeze, and to identify extrinsic risk factors for

50 underlying disease mechanisms.

51 *Methods*

52 A breath sample was collected from 142 children (asthma: 51, pre-school wheezers: 55,

53 healthy controls: 36) and analysed using gas chromatography–mass spectrometry (GC/MS).

54 Children were diagnosed according to GINA guidelines and comprehensively examined each

55 year over up to seven years. Forty children repeated the breath collection after 24 or 48

56 months.

57 *Results*

58 Most breath VOCs differing between groups reflect the exposome of the children. We

59 observed lower levels of lifestyle-related VOCs and higher levels of the environmental

60 pollutants, especially naphthalene, in children with asthma or wheeze. Naphthalene was also

61 higher in symptomatic patients and in wheezers with recent inhaled corticosteroid use. No

62 relationships with lung function or TH2 inflammation were detected.

63 *Conclusion*

64 Increased levels of naphthalene in asthmatics and wheezers and the relationship to disease

65 severity could indicate a role of environmental or indoor air pollution for the development or

66 progress of asthma. Breath VOCs might help to elucidate the role of the exposome for the

67 development of asthma.

68 The study was registered at ClinicalTrials.gov (NCT02496468).

69

70 **Word count:** 235

71

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

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2

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

3

107 inflammatory phenotypes, clinical features such as lung function, asthma treatment and

108 exacerbation rate.

Methods

Study design

111 The ALLIANCE cohort of the German Center for Lung Research (DZL) is a prospective

112 multicenter asthma cohort [19]. For this study, we collected 182 breath samples from 142

113 children (51 children with asthma, 55 children with wheeze and 36 healthy controls) at two

114 pediatric specialist centers (Hannover, Munich) from October 2016 until spring 2020 (**figure**

115 **1, table S1**). In a subgroup of 40 patients, a second breath collection was performed after one

116 or two years (asthma n=20, wheeze n=20). The study was conducted in accordance with the

117 principles embodied in the Declaration of Helsinki and in accordance with local statutory

118 requirements. The study was registered at ClinicalTrials.gov (NCT02496468) and approved by

119 all local ethics committees. All parents of study participants <18 years as well as study

120 participants ≥8 years gave their written informed consent.

121 Children aged 6 months to 5 years were included if they had at least two episodes of wheeze

122 during the past 12 months ('pre-school wheezer') as indicated by the parents in the respective

123 questionnaire. Children ≥6 years were included based on doctor-diagnosed asthma according

124 to the Global Initiative for Asthma (GINA) guidelines and German guidelines [4, 20]. We also

125 recruited healthy control subjects who had never been diagnosed with asthma or pre-school

126 wheeze. Further inclusion and exclusion criteria are specified in the supplement or have been

127 published [19]. Laboratory tests included differential blood count, and specific

128 immunoglobulin E against 36 allergens measured by Euroline™ (Euroimmun, Germany). All

129 children performed exhaled nitric oxide (FeNO) measurements and spirometry from age 4

130 years onwards and VOC measurements from 3 years onwards. For the breath and near-subject

131 room air collection we used an in-house developed breath collection device [21]. Further

132 details regarding all procedures and study design are presented in the **online supplement**.

133

Analysis by gas chromatography-mass spectrometry (GC-MS)

135 GC-analysis of the samples was performed at Fraunhofer ITEM, Hannover, within eight days

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

4

139　Standards and Technology Mass Spectral Search Program Version 2.2 (NIST, USA)) [21, 22]. A

140　total of 158 VOCs were quantified using peak height of specific target ions which most

141　commonly matched with m/z signals of highest intensity in the respective VOC mass spectrum

142　(total Ion content (TIC)) [22]. Further details regarding measurement and analysis are available

143　in the **online supplement**.

144　*Statistics*

145　We computed the mean value of the 158 target VOCs for all technical replicate samples. Data

146　was then log-transformed. We used the Mann-Whitney-U-Test (MWU) for univariate

147　comparisons between patients (asthma, wheeze) and healthy controls and the Pearson

148　correlation coefficient where appropriate. The false discovery rate was assessed by correcting

149　raw p-values with the *p.adjust* function in R (using the Benjamini and Hochberg (BH) option).

150　Corrected p-values are indicated as pBH. Site effects were accounted for by batch correction

151　using the *ComBat* function of the R package *sva* to our data. For some analyses that were

152　limited to data from one site we used both the original (uncorrected) and the batch corrected

153　"site-effect–free data" to test whether similar results were obtained.

154　Due to the exploratory nature of our analysis, we focused on the complete VOC dataset (n=158

155　VOCs). However, to avoid missing important observations by the multiplicity correction we

156　also ran the analysis with subsets of our 158 VOCs. A detailed description of these subsets is

157　provided in the **online supplement**. In addition we dimensionally reduced the dataset using

158　an unsupervised clustering approach called Cytomod [23]. Briefly, VOCs were clustered and

159　assigned to co-varying modules based on the pairwise Pearson-correlations between log-

160　transformed VOC values. For more details, please refer to the **online supplement**. Module

161　scores reflecting the expression of each module in each participant (see **online supplement**)

162　were compared between participant groups with different characteristics using MWU tests.

163　P-values were adjusted for the number of modules tested. A multivariable logistic regression

164　model was used to analyze the association between module 6 and disease status. We focused

165　our analysis on module 6, because most VOCs considered as air pollutants were found in

166　module 6.

167

168

169　**Results**

170　<u>Demographics</u>

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_1 and FEV_1/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**).
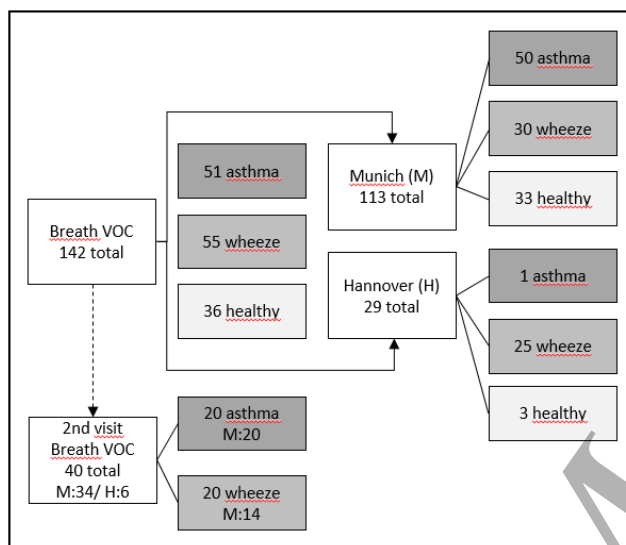
176

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

192

193　<u>Basic data description, quality, and plausibility of VOC data</u>

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

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 (pBH=0.068). No significant gender-specific differences for breath VOCs were

213   observed (pBH>0.05).

214

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   (pBH<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, pBH=0.27).

222

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

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

232   We found 24 significantly different VOCs (pBH<0.05) between healthy controls and asthma

233   patients and 23 significantly different (pBH<0.05) VOCs between healthy controls and children
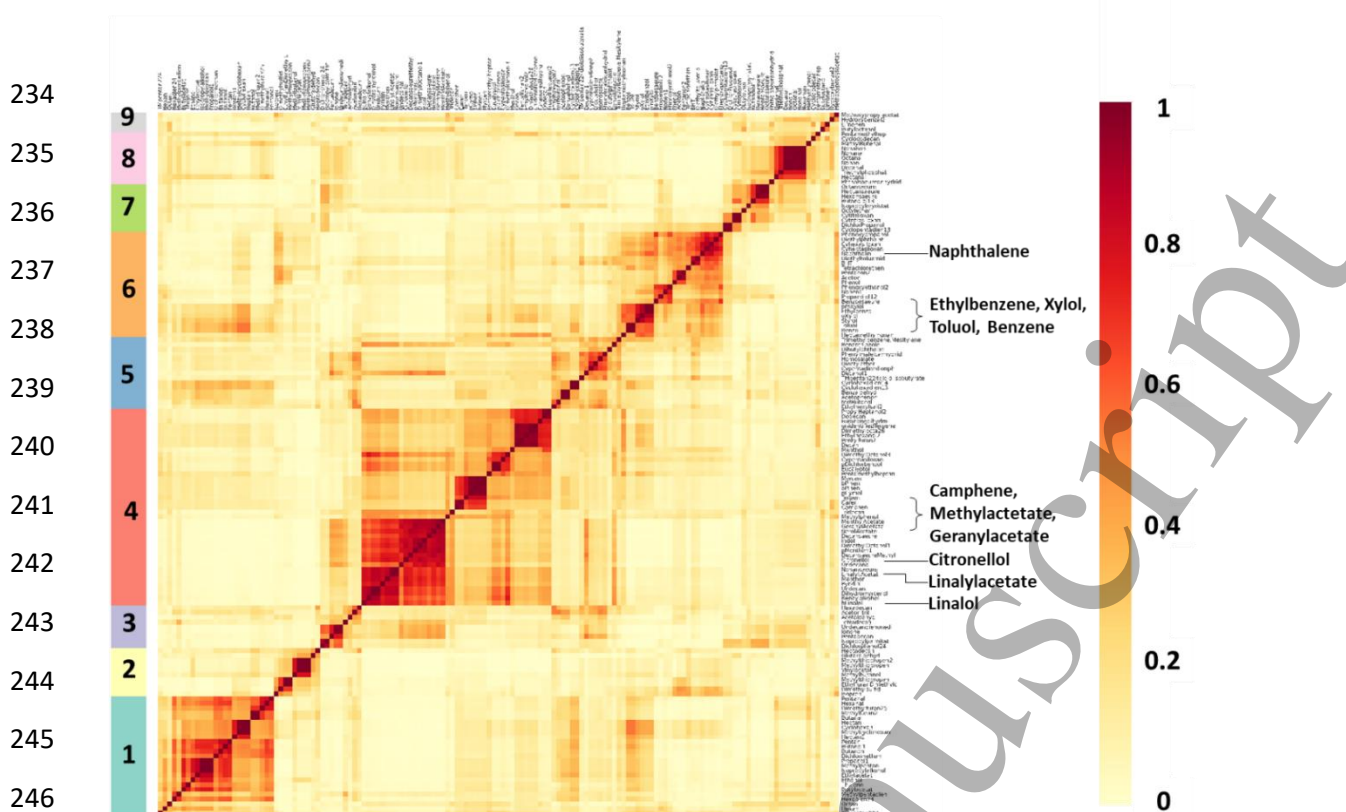
7

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 (pBH=0.006) and children with wheeze (pBH=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 (pBH>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 (pBH=0.07) and children with wheeze (pBH=0.03) compared to healthy controls (**figure S4**).

277
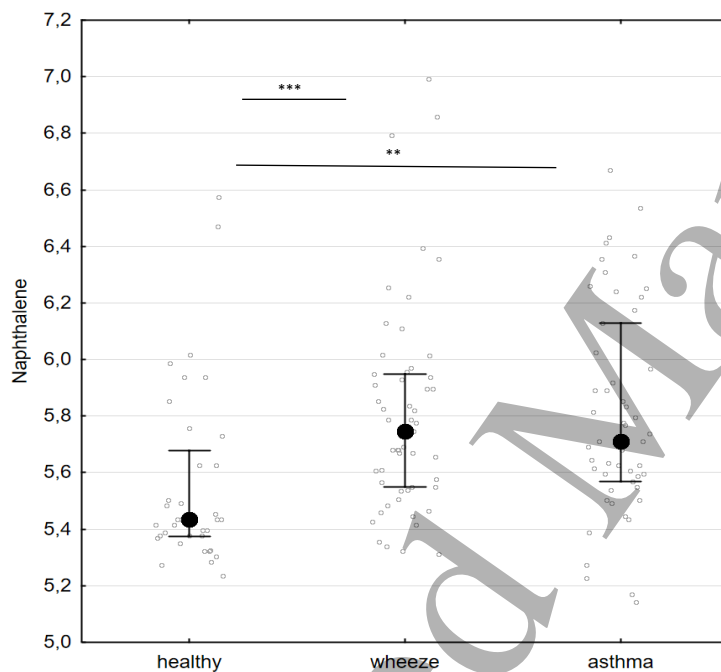
290 **Figure 3.** Differences in the levels of naphthalene between groups. Median and interquartile
291 ranges. Individual data is shown in grey open circles. *** p<0.001, ** p<0.01.

292

293

294 <u>VOC profiles associated with disease characteristics</u>

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_1 z-score,

299 neither for asthma patients, wheezers or both groups combined. There were also no

300 differences when comparing participants with FEV_1 z-scores <-1 to those with z-scores >-1. The

301   same results were found for the FEV_1/FVC data. For FEF_{25-75} the results were also similar,
302   except for asthma patients. Here we found higher levels for six VOCs (all in module 4), among
303   them capric-acid, undecane and undecanal and for module 4 values (pBH<0.05) in asthma
304   patients with a z-score <-1.

305

306   <u>VOC profiles associated with disease control</u>
307   No significantly different VOC levels were found between asthmatic children and wheezers
308   with controlled disease according to GINA guideline definition [4] (n=87 and those with
309   "partly-controlled" or "uncontrolled" asthma" (n=19) when testing the different VOC subsets
310   as defined in material and methods (pBH>0.05)). Testing only naphthalene showed
311   significantly higher levels in the partly- and uncontrolled group (p<0.008), which is compatible
312   with the observed trend to increased module 6 values (p=0.03, pBH 0.251). No difference was
313   observed for module 4.
314   VOCs or module 4 and 6 values did not differ between children with or without exacerbations
315   or wheeze episodes in the past 12 months. In wheezers with current use of inhalative
316   corticosteroids (ICS), we found significantly higher levels for 1-phenoxypropan-2-ol
317   (pBH=0.03) and for naphthalene (p=0.01). Both are found in module 6, which also showed
318   higher values in this group (pBH=0.06). In addition, higher levels for an unidentified terpene
319   (pBH≤0.008), and three alkanes (undecane, dodecane and decane) were observed (pBH=0.09)
320   Two of these VOCs were found in module 4, which showed a trend for higher module values
321   in this group (pBH=0.06).

322

323   <u>Association of cluster module 6 with asthma or pre-school wheeze</u>
324   As VOC module 6 was increased in children with asthma or pre-school wheeze, we were
325   interested in investigating this association while controlling for other known risk factors for
326   asthma development. In a multivariable analysis, increased values for VOC module 6 were
327   significantly associated with asthma and pre-school wheeze, also when adjusting for age,
328   parental asthma, gender, presence of atopy, elevated blood eosinophil counts, and secondary
329   cigarette smoke exposure (**table 3**). Only atopy was also significantly associated to asthma or
330   wheeze status in our multivariable regression model.

331

332

Discussion

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

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

364   described as a potential source of naphthalene [27], however, this contribution will be difficult

365   to control for in cohort studies.

366   In children with pre-school wheeze, increased breath naphthalene levels have been observed

367   before [35], but its potential role was not further discussed. Increased naphthalene levels have

368   also been reported in blood and urine of children with asthma. In a study comparing PAH levels

369   in 195 children, naphthalene serum levels showed one of the strongest associations with

370   asthma [36] , while increased 2-naphthol levels, a naphthalene metabolite, could be found in

371   urine of asthmatic children [37]. Increased PAH metabolite levels in urine of children with

372   asthma were associated with higher disease-specific symptoms and consequently decreased

373   lung function parameters [38]. All substances mentioned above were part of module 6 with

374   considerable correlation among each other, which suggests regular exposure to a potential

375   common source. Little is known so far regarding the potential pathomechanisms linking

376   naphthalene and asthma. Murine studies showed that naphthalene could have local effects

377   on the respiratory epithelium as well as systemic affects due to high plasma concentrations

378   after inhalation [30, 39, 40], however, the exact role in asthma development is still uncertain.

379

380   In contrast to the strong pollution signature in children with asthma and wheeze, we found

381   increased levels of flavor and lifestyle related VOCs in healthy subjects. Although speculative,

382   one possible explanation could be a higher awareness of parents of asthmatic or wheezing

383   children limiting exposure to strong flavorings, cosmetics, or cleaning products while parents

384   of healthy children might be less restrictive.

385

386   Eleven of the compounds found to be different between healthy children and patients (**table**

387   **2)** have been related to asthma in previous studies [13, 14], however the overlap with our

388   results is quite small. Recent reviews show that this is not uncommon in studies with asthma

389   or wheezing patients [12, 13] and very likely due to differences in patient populations and in

390   sampling and analysis methods. Furthermore, as we used a predefined target VOC dataset

391   comprising of 158 VOCs, we might not have detected some of the previously reported VOCs

392   in comparable trials with asthma patients.

393

394   We found no relationships of VOC profiles with clinical characteristics such as asthma control

395   or risk for exacerbations [17, 18]. Similarly, VOCs were not associated with inflammatory

396   phenotypes, i.e. type 2 inflammation, confirming previous negative results from the adult arm

397   of the ALLIANCE cohort [41]. In this respect it is important to note that about 73 % of the

398   asthmatic children were under anti-inflammatory treatment when the breath collection was

399   performed. We cannot exclude that this has reduced our chance of finding VOC patterns

400   associated with inflammation, however we still observed significantly higher FeNO levels in

401   the asthmatic children, indicating ongoing airway inflammation.

402   Naphthalene and two other VOCs of module 6 were increased in pre-school wheezers treated

403   with ICS, supporting the proposed link between air pollution as a disease driving factor, as ICS

404   treatment in this age group identifies children with a more severe phenotype. Additionally,

405   children with pre-school wheeze and regular ICS treatment showed higher levels of three

406   different long chain alkanes, which have previously been identified in COPD patients with

407   respiratory tract infections caused by Rhinovirus [42]. This could potentially point towards an

408   inflammatory phenotype driven by recurrent viral infections. However, it needs to be

409   mentioned that one of these alkanes (undecane) was also part of module 4 and increased in

410   healthy controls thus illustrating the complexity of the data and the difficulties in

411   distinguishing exogenous and endogenous sources of VOCs.

412

413   A strength of our study is the choice of method for breath collection and VOC analysis which

414   has been benchmarked in a previous trial [43]. Furthermore, our results were in concordance

415   with several expected findings. For example, we were able to identify cigarette smoke related

416   VOCs in children exposed to environmental tobacco smoke, and we saw significant

417   correlations for lifestyle related VOCs between repeated measurements over at least one

418   year. Correlations between near-subject room air and breath for VOCs related to hygiene

419   product and detergents are in line with our "owncloud" concept [22].

420   It's well known that the VOC composition of breath is influenced by many factors [44], among

421   them demographic, diet, life style and recent activity. Therefore, we interpret the observed

422   correlations of VOCs between the repeated measurements as remarkable, despite rather low

423   correlation coefficients (r≤ 0.6) and consider these as an indication for stable living and

424   lifestyle conditions.

425   However, we also have to report some limitations. Our data showed a marked center effect

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

13

428 material) and the comparable results indicate that batch correction did not introduce an

429 unwanted bias to our data.

430 In line with our previous studies [21, 22, 24] we limited our GC-MS analysis to preselected 158

431 VOCs instead of performing a broad analysis of all potential peaks in a chromatogram. While

432 the latter is likely to include more features for comparison, there is a risk for false identification

433 of VOCs, which coelute at the same retention time. On the downside, by including only

434 preselected VOCs, we cannot exclude to have missed VOCs which could have been relevant

435 for the comparison between groups. Unfortunately, healthy control children were not age-

436 matched to children with asthma or wheeze. However, only two VOCs out of 158 VOCs were

437 actually age dependent, which makes a significant influence of age on the result unlikely.

438 Overall, we focused our analysis on single VOC comparisons as well as the two modules 4 and

439 6, which comprised most of the significantly different VOCs found in the single VOC analysis.

440 Some other VOCs modules were also significantly different between the study groups,

441 however, further detailed analysis of all modules was beyond the scope of this manuscript.

442

443 In this study, we were able to identify VOCs associated with asthma and pre-school wheeze

444 indicating an increased exposure of these children to environmental pollutants. Due to the

445 observational nature of our study, we cannot deduce a causal relationship of these

446 environmental exposures to the development of asthma. Still, the strong association

447 confirmed in a multivariable analysis as well as the fact that the result was already seen in pre-

448 school wheezers and in the case of naphthalene particularly in those more severely affected

449 supports previous findings that exposure to pollutants might be an early life factor driving

450 asthma pathology [7]. Although we could not find associations between VOCs and clinically

451 relevant features as inflammatory phenotypes or risk for exacerbations, we consider non-

452 invasive breath analysis an interesting tool to assess the exposome of children and to decipher

453 the role of environmental exposures in the development of airway diseases. The increased

454 levels of breath naphthalene and other PAH in asthmatic and wheezing children would be a

455 strong argument to include breath sampling and potentially also personal sampling devices

456 into future studies to better understand the role of environmental and especially indoor air

457 pollutants.

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583 **Legends to figures**

584 **Figure 1**. Study population. Breath VOCs were analyzed from 142 subjects. 40 subjects were
585 sampled on two visits, which were at least one year apart. The Hannover site had a focus on
586 wheezers and started the recruitment for VOC sampling later during the study. The one
587 patient with asthma from Hannover was planned to be included as wheezer but the visit could
588 not be scheduled before the 6th birthday, therefore based on the definition criteria, this
589 subject was considered as child with asthma.
590

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

597 **Figure 3.** Differences in the levels of naphthalene between groups. Median and interquartile
598 ranges. Individual data is shown in grey open circles. *** $p < 0.001$, ** $p < 0.01$.
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604   **Table 1.** Demographics

605

		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§	% 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/µL	46	7350 (6100;8300)	42	7500 (6400;9200)**	34	6675 (5500;7730)
Blood eosinophils	cells/µL	46	358 (198;570)*	42	381 (260;582)**	34	201 (139;395)
Blood neutrophils	cells/µL	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)$	n (%)	51	13 (26 %)	55	25 (45 %)		
≥ 1 exacerbation (yes)$	n (%)	51	2 (4 %)	55	5 (9 %)		

M=Munich, H=Hannover

Mean ± SD or median (25%;75% quartile) are presented. §: specific IgE >= 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

Lower in Diseased Patients

VOC	Module 4	Asthma vs. Healthy pMWU	Asthma vs. Healthy pBH	Wheeze vs. Healthy pMWU	Wheeze vs. Healthy pBH	Fragrance / Flavor	Food	Personal Care	Cleaning	Disinfectant	Air pollutant
unidentified C10H18 (a)	x	<0,001	**<0,001**	<0,001	**0,007**						
Geranyl acetate	x	<0,001	**0,003**	0,003	**0,035**	x	x	x	x		
Pentadecane		<0,001	**0,003**	0,006	**0,042**	x	x	x			
Menthyl acetate	x	<0,001	**0,005**	0,001	**0,028**	x	x	x	x	x	
Decanoic acid methyl ester	x	0,001	**0,009**	0,002	**0,033**	x			x		
Decanoic acid	x	<0,001	**0,009**	0,008	0,054	x	x	x	x		
unidentified VOC (a)		<0,001	**0,009**	0,064	0,213						
Neryl acetate	x	0,002	**0,019**	0,038	0,144	x	x	x	x		
2-Propanol		0,002	**0,018**	0,128	0,302	x	x	x	x	x	
1-Decanol		0,002	**0,020**	<0,001	**0,007**	x	x	x	x	x	
Ionone		0,002	**0,017**	<0,001	**0,007**	x	x	x	x		
Citronellol	x	0,001	**0,015**	0,002	**0,033**	x	x	x	x		
b-Linalool	x	0,001	**0,017**	0,129	0,302	x	x	x	x	x	
Decanal		0,003	**0,023**	0,025	0,120	x	x	x	x	x	
Linalyl acetate	x	0,005	**0,031**	0,158	0,328	x	x	x	x	x	
Dioctyl ether		0,003	**0,025**	0,078	0,222	x		x	x		
Camphene	x	0,002	**0,018**	0,057	0,201	x	x	x	x	x	
Undecane	x	0,003	**0,025**	0,005	**0,038**	x	x	x			
2,5-Dimethylfuran		0,251	0,425	0,004	**0,037**	x	x				
t-Butyl alcohol		0,115	0,261	0,003	**0,034**	x	x	x	x		
unidentified Terpene	x	0,030	0,107	0,004	**0,038**						
3,7 - Dimethyloctan-1-ol	x	<0,001	**0,005**	0,003	**0,034**	x		x	x		
Octanoic acid		0,007	**0,043**	0,385	0,570	x	x	x	x		
Nonane		0,007	**0,043**	0,033	0,141						
Hexanoic acid		0,401	0,554				x	x	x	x	
Myrcene	x	0,029	0,107				x	x	x	x	

Increased in Diseased Patients

VOC	Module 6	Asthma vs. Healthy pMWU	Asthma vs. Healthy pBH	Wheeze vs. Healthy pMWU	Wheeze vs. Healthy pBH	Fragrance / Flavor	Food	Personal Care	Cleaning	Disinfectant	Air pollutant
Isoprene (b)		<0,001	**<0,001**	0,318	0,507						
1-Phenoxypropan-2-ol	x	0,005	**0,034**	0,039	0,144			x	x	x	
Cycloheptasiloxane	x	0,021	0,093	0,004	**0,037**			x	x		
Benzoic acid	x	0,022	0,093	<0,001	**0,008**	x	x	x	x		
1-Nonene	x	0,068	0,179	0,004	**0,037**						
1,2-Propanediol	x	0,23	0,40	0,001	**0,028**	x	x	x	x	x	x
2-Ethylhexanol		0,008	**0,047**	0,005	**0,038**	x	x				x
Limonene		0,051	0,146	0,001	**0,028**	x	x	x	x		
Naphthalene	x	<0,001	**0,005**	<0,001	**0,007**						x
Ethylbenzene	x	0,070	0,179	0,159	0,328						x
Benzene	x	0,046	0,138	0,344	0,537						x
p-/m-Xylene	x	0,144	0,297	0,151	0,328						x
o-Xylene	x	0,238	0,411	0,114	0,280						x
Toluene	x	0,113	0,260	0,036	0,144						x
Hexanoic acid				0,002	**0,034**	x	x	x	x		
Myrcen				0,005	**0,038**	x	x	x	x		

The table contains all compounds which were different between healthy and asthma or healthy and wheeze

Additional information was provided for other airpollutants irrespective of the level of significance

(a) sign. correlation with e.g. Geranyacetate and other lifestyle VOCs suggests common source

(b) endogenous VOC, difference between groups mainly due to age dependency

Occurence and main use information based mainly on European Chemical Agency (ECHA)

pMWU: p-value uncorrected using non-parametric testing

pBH: p-value corrected for multiple testing (Benjamini-Hochberg)

For more details on clusters refer to supplement table S4

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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

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

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