

ORIGINAL ARTICLE

AIRWAY DISEASES

Asthma severity in childhood and metabolomic profiling of breath condensate

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To cite this article: S. Carraro, G. Giordano, F. Reniero, D. Carpi, M. Stocchero, P.J. Sterk, E. Baraldi. Asthma severity in childhood and metabolomic profiling of breath condensate. *Allergy* 2013; **68**: 110–117.

Keywords

asthma; exhaled breath condensate; mass spectrometry; metabolomics; pediatrics.

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Accepted for publication 27 September 2012

DOI:10.1111/all.12063

Edited by: Michael Wechsler

Abstract

Background: Asthma is a heterogeneous disease and its different phenotypes need to be better characterized from a biochemical-inflammatory standpoint. This study aimed to apply the metabolomic approach to exhaled breath condensate (breathomics) to discriminate different asthma phenotypes, with a particular focus on severe asthma in children.

Methods: In this cross-sectional study, we recruited 42 asthmatic children (age, 8–17 years): 31 with nonsevere asthma (treated with inhaled steroids or not) and 11 with severe asthma. Fifteen healthy children were enrolled as controls. Children performed exhaled nitric oxide measurement, spirometry, exhaled breath condensate (EBC) collection. Condensate samples were analyzed using a metabolomic approach based on mass spectrometry.

Results: A robust Bidirectional-Orthogonal Projections to Latent Structures-Discriminant Analysis (O2PLS-DA) model was found for discriminating both between severe asthma cases and healthy controls ($R^2 = 0.93$; $Q^2 = 0.75$) and between severe asthma and nonsevere asthma ($R^2 = 0.84$; $Q^2 = 0.47$). The metabolomic data analysis leads to a robust model also when the 3 groups of children were considered altogether ($K = 0.80$), indicating that each group is characterized by a specific metabolomic profile. Compounds related to retinoic acid, adenosine and vitamin D (Human Metabolome Database) were relevant for the discrimination between groups.

Conclusion: The metabolomic profiling of EBC could clearly distinguish different biochemical-metabolic profiles in asthmatic children and enabled the severe asthma phenotype to be fully discriminated. The breathomics approach may therefore be suitable for discriminating between different asthma metabolic phenotypes.

It is widely recognized that asthma has a heterogeneous nature, with both genetic and environmental components. Several asthma classifications have been proposed in the past, based mainly on the description of clinical characteristics, symptom triggers or lung function measurements (1). We presently know that asthma is a complex multifactorial disorder, and there is evidence that asthma symptoms can be sustained by different inflammatory patterns (2, 3). The characterization of the severe asthma phenotype is of particular interest because while mild to moderate asthma is usually well controlled by low-dose inhaled corticosteroids (ICS), severe asthma presents with chronic symptoms and/or recur-

rent exacerbations despite an appropriate anti-asthma treatment (4). Consequently, this latter form of asthma can benefit most from a better understanding of the underlying pathophysiological mechanisms (5) with a view to the potential development of targeted therapeutic strategies based on the knowledge of the molecular mechanisms involved.

Asthma and its phenotypes can hardly be investigated by concentrating on one specific biomarker because there are so many cellular and molecular mechanisms involved and they interact in complex networks, so new integrated systems approaches are needed (6, 7). Metabolomics, one of the core disciplines of system biology, is a high-dimensional biology

method that may allow hypothesis-free profiling of biomarkers, rather than a traditional hypothesis-driven approach. The metabolomic approach, in fact, is based on an unbiased approach that simultaneously considers a large number of metabolites in a given sample, and, with the aid of bioinformatic tools, it enables the identification of characteristic metabolite profiles capable of discriminating between different groups of individuals (8, 9). Interestingly, metabolome has been claimed as 'the best indicator of an organism's phenotype' (10).

A recent study demonstrated that the metabolomic analysis of urine can discriminate asthmatic from healthy children (11). Moreover, metabolomic analysis has lately been applied in subjects with respiratory diseases by studying exhaled breath condensate (EBC) (12, 13), an approach called breathomics (12). EBC is a biofluid collected noninvasively by cooling the air expired during tidal breathing, and it provides information on pathological processes involving the lung (14). Compared to other systemic biological matrix, as blood or urine, EBC, being collected directly at the lung, is less affected by the metabolism of the whole body.

We hypothesized that metabolomics in EBC allows the discrimination of children with severe asthma from those with nonsevere asthma and from healthy controls. Our aim was to apply breathomics to studying asthmatic children with different degrees of disease severity in an attempt to discriminate between their clinical phenotypes on the basis of their EBC metabolic profiles and to establish whether severe asthma can be characterized from a biochemical-metabolic fingerprint.

Methods

Study subjects

We recruited 42 atopic asthmatic children among patients attending the Respiratory Medicine/Allergy outpatient's clinic at the Pediatrics Department in Padova. According to international guidelines (15), the diagnosis of asthma was based on clinical history and medical examination, spirometry and FEV1 reversibility $\geq 12\%$ demonstrated at least once in the previous 6 months. The children did not have symptoms of acute upper or lower airway infection during the last 3 weeks.

The following groups of children were recruited:

- 1 31 children (aged 8–17) with nonsevere asthma [14 steroid naive and 17 regularly treated with low-medium dose ICS, alone or in combination with long-acting beta2 agonists (LABA) ($n = 13$), montelukast ($n = 2$), theophylline ($n = 1$)].
- 2 11 children (aged 8–16) with severe asthma (asthma poorly controlled despite regular treatment with multiple controller medications); all these children were treated with high dose of ICS (4, 15) combined with LABA; 3 were also taking montelukast, and 1 was taking theophylline. This group's asthma was judged to be poorly controlled because the children had chronic symptoms and/or frequent severe exacerbations requiring multiple courses of oral steroids (all the children in group 3 had experi-

enced 3 or more exacerbations in the previous year) (16). Any significant allergen exposure in these children had been ruled out, and the main co-morbidities (e.g., gastroesophageal reflux and rhinosinusitis) had also been excluded or treated. These children's adherence to therapy was checked routinely;

- 3 as a control group, we recruited 15 healthy children (aged 9–17) with no history of atopy or respiratory diseases.

Design

The study had a cross-sectional design. At recruitment, all the children had a physical examination and underwent exhaled nitric oxide (FE_{NO}) measurement and spirometry. EBC was collected 15 min after spirometry and stored at -80°C for subsequent analysis using mass spectrometry (MS).

The Ethics Committee of our hospital reviewed and approved the protocol, and all parents gave their written informed consent.

EBC collection

EBC was collected and processed according to ATS/ERS recommendations (13). EBC was collected using the TURBO-DECCS (Medivac, Parma, Italy) as previously described (see the online supplement for additional information) (17).

Orbitrap LC-MS analysis of metabolites in EBC

Analyses were performed with an Ultimate 3000 Dionex HPLC system (Dionex, Softron GmbH, Germany) coupled to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with an Advion Triversa NanoMate source (Advion BioSciences, inc. Ithaca, NY, USA) (see the online supplement for additional information).

Data extraction was performed using MarkerLynx software after the transformation of the LTQ Orbitrap raw data in cdf file and then in masslynx file (Waters co., Milford, MA, USA).

Fractional exhaled nitric oxide (FE_{NO}) measurement and lung function test

FE_{NO} was measured with the NIOX system (Aerocrine, Stockholm, Sweden) using a single-breath online method according to the ERS/ATS guidelines (see the online supplement for additional information) (18).

Lung function parameters were measured with a 10-l bell spirometer (Biomedin, Padova, Italy).

Statistical analysis

Statistical analysis was performed using standardized methods to minimize false discoveries and obtain robust statistical models (19, 20). In particular, projection methods such as principal component analysis (PCA) (21) were used for pattern recognition, while Bidirectional-Orthogonal Projections

to Latent Structures-Discriminant Analysis (O2PLS-DA) (22, 23) was applied to distinguish between the different groups of children and to characterize them. O2PLS-DA is a supervised classification technique that produces parsimonious classification models where the information useful for discriminating between the different groups under investigation is summarized in a few predictive scores resulting from a suitable combination of the measured variables. The obtained model can often be interpreted in terms of single measured variables, the putative biomarkers, which characterize a specific group with respect to the others. The putative biomarkers can be highlighted by calculating the correlation loadings that describe the correlation structure existing between predictive scores and variables (23, 24).

Permutation test on the responses and procedures of *n*-fold cross-validation with different values of *n* was run for each model, to check its validity (19, 20).

The general model for the discrimination of the three groups of children (healthy controls, nonsevere and severe asthma cases) was obtained by combining the O2PLS-DA with the Naïve Bayes classification technique, using the O2PLS-DA predictive scores as attributes for building the Naïve Bayes classifier. The training set used to build the model and test set used to validate it were extracted from the candidate set of 57 subjects by a sampling procedure based on a combination of PCA and onion D-optimal design.

The measured variables were log-transformed, and Pareto scaling with mean centering was applied prior to perform the multivariate data analysis. The SIMCA P+ 12 software (Umetrics, Umea, Sweden) was used to apply the projection methods, while the Naïve Bayes classifier was calculated using the WEKA 3.4.11 software (University of Waikato, New Zealand).

The discriminating compounds were identified by comparing the mass spectra with data from the Human Metabolome Database (HMDB) (version 2.5) (25).

The clinical data (spirometric parameters and log-transformed FE_{NO} values) were analyzed by means of ANOVA, followed by Holm-Sidak test for between-group comparisons.

Results

The clinical characteristics of recruited children, including exhaled nitric oxide levels and spirometric values, are reported in Table 1. As expected, we found significantly

reduced spirometric parameters in children with severe asthma ($P < 0.001$ for FVC, FEV₁, FEF_{25–75} and $P < 0.01$ for FEV₁/FVC), while no significant difference was found between nonsevere asthma cases and healthy controls (Table 1). FE_{NO} was higher ($P < 0.01$) in asthmatic than in healthy subjects, but there was no significant difference between nonsevere and severe asthmatics (Table 1).

The mass spectrometry analysis of the EBC samples generated spectra in which the metabolites are represented according to their mass to charge ratio (the spectra of some representative metabolites are reported in Fig. 1).

The information contained in the spectra was investigated using multivariate statistical analysis tools.

Preliminary data analysis was performed by principal component analysis (PCA). No outliers within the different groups of children were highlighted, so all the subjects were included in the following steps of our data analysis strategy.

Severe asthma vs healthy subjects

A robust O2PLS-DA model was found for discriminating between severe asthma cases and healthy controls ($R^2 = 0.93$ and $Q^2_{\text{sevenfold cross-validation}} = 0.75$). The ROC curve analysis of the predictive score calculated during cross-validation with sevenfolds confirmed the robustness of the obtained model (AUC = 1.0; Fig. 2A).

Measured variables that might play a key role in group discrimination were highlighted by analyzing the distribution of the correlation loadings arising from the model (23, 24).

By searching the HMDB (25), among the discriminating variables, we could characterize from a chemical point of view the putative biomarkers, potentially having a role in asthma pathophysiology, as follows.

Variable 225 [327.204 m/z (M+H⁺); retention time 9.40 min] and variable 127 [252.182 m/z (M+H⁺); retention time 11.05 min] emerged as variables characteristic of severe asthma subjects. Variable 225 is a compound chemically related to retinoic acid, while variable 127 is related to deoxyadenosine.

On the other hand, among the variables characterizing the healthy controls, we identified the variable 412 [429.286 m/z (M+H⁺), retention time of 11.18 min] that, according to the HMDB (25), might be ercalcitriol, the active metabolite of vitamin D2. In Table 2, we report the statistical parameters describing the power in discrimination of the selected

Table 1 Spirometric parameters and FE_{NO} levels in children recruited

	Nr (male)	Age (range)	FVC* (%pred)	FEV ₁ * (%pred)	FEV ₁ /FVC* (%)	FEF _{25–75} * %pred	FE _{NO} [#] (ppb)
Healthy	15 (7)	12.6 (9–17)	101 (1.9)	98 (1.9)	90 (1.5)	106 (4.9)	11.3 [9.7–16.1]
Nonsevere asthma	31 (21)	11.3 (8–17)	102 (1.9)	98 (2.0)	87 (1.2)	96 (4.4)	26.3 [17.6–46.2] [§]
Severe asthma	11 (5)	10.4 (8–16)	86 (4.2) [†]	73 (3.3) [†]	78 (1.9) [‡]	55 (4.2) [†]	41.5 [19.5–90.0] [§]

*Data are expressed as mean ± SEM.

[#]Data are expressed as median and IQR.

[†] $P < 0.001$ vs healthy and nonsevere asthma.

[‡] $P < 0.01$ vs healthy and nonsevere asthma.

[§] $P < 0.01$ vs healthy.

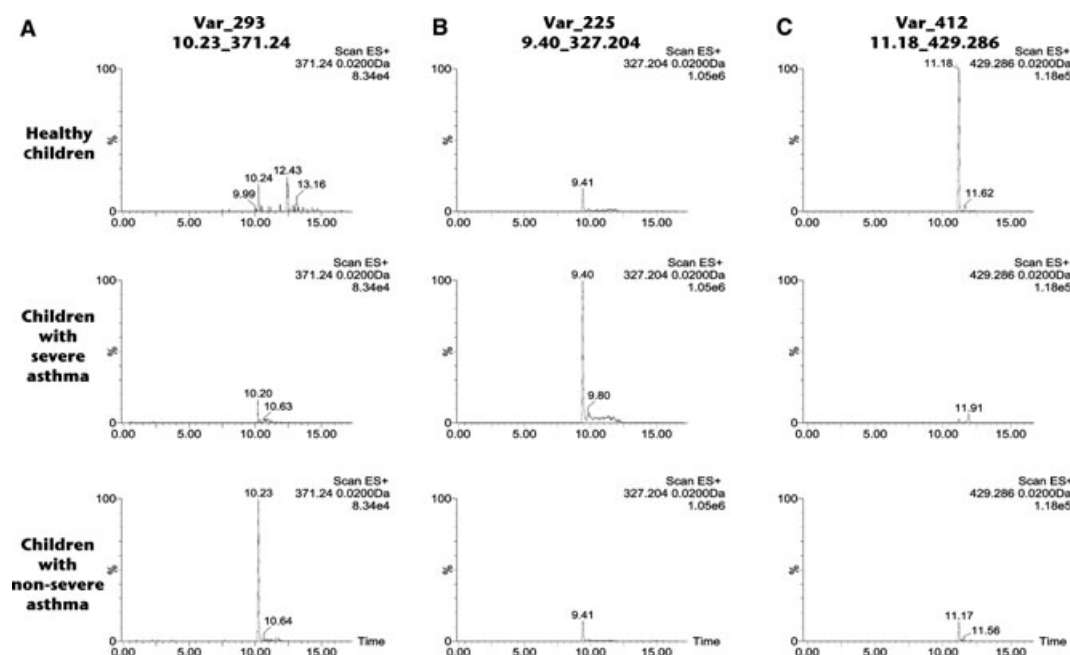


Figure 1 Representative ion-extracted chromatograms (in positive ions) for the three variables that allow a qualitative separation of the

samples in three principal clusters (i.e., panel A: variable 293; panel B: variable 225; panel C: variable: 412).

variables and the compounds chemically related to such variables.

Severe asthma vs nonsevere asthma

A reliable O2PLS-DA model was found also for discriminating between severe asthma and nonsevere asthma ($R^2 = 0.84$ and $Q^2_{\text{sevenfold cross-validation}} = 0.47$). The ROC curve analysis of the predictive score calculated during cross-validation with sevenfolds (AUC = 0.83; Fig. 2B), and the permutation test for the response (20) confirmed the validity and robustness of the obtained model.

In the characterization of the nonsevere asthma group compared to the severe asthma group emerged the role of the variable 293 [371.24 m/z ($M+H^+$); retention time 10.23 min]. For this variable, the most likely related metabolites reported in the HMDB (25) are 20-Hydroxy-PGF2a, Thromboxane B2, and 6-Keto-prostaglandin F1a. Table 2 shows the statistical parameters for the selected variable.

Within the nonsevere asthma group, it was impossible to obtain a reliable O2PLS-DA model for discriminating between asthmatic children regularly treated with controller medications and those with well-controlled asthma steroid naïve.

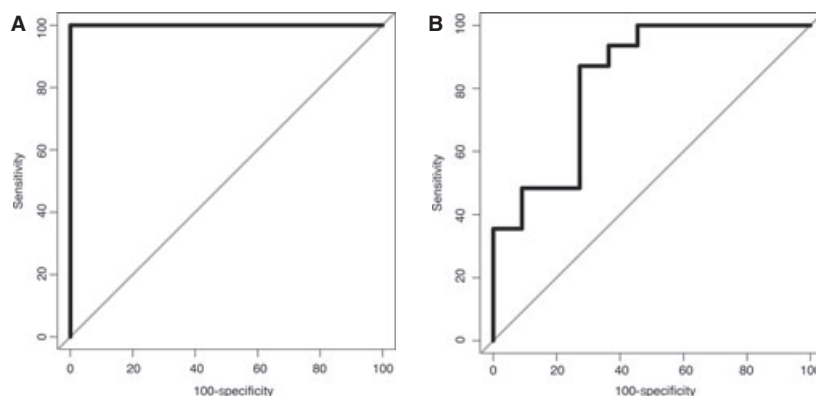


Figure 2 ROC curve for the predictive score of the O2PLS-DA models calculated during sevenfold cross-validation. (A) Severe asthma vs healthy subjects; the curve represents the performances in prediction estimated for the model (AUC = 1.00, $\sigma_{\text{AUC}} < 0.01$).

(B) Severe asthma vs nonsevere asthma subjects; the curve represents the performances in prediction estimated for the model (AUC = 0.83, $\sigma_{\text{AUC}} = 0.08$).

Table 2 Statistical parameters characterizing the discriminant properties of the selected putative biomarkers (log-transformed variables) (the hypothesis tested were $H_0: \mu_1 = \mu_2$ and $H_1: \mu_1 \neq \mu_2$). Analysis performed accordingly to the suggestions of Broadhurst et al. (19)

Variable	<i>P</i> -value <i>t</i> -test	β ($\alpha = 0.05$)	<i>P</i> -value Mann–Whitney test	ROC curve analysis AUC	σ_{AUC}	Chemically related compounds
Var_225	0.01	0.28	0.02	0.77	0.11	Retinoic acid
Var_127	0.04	0.48	0.11	0.77	0.09	Deoxyadenosine
Var_412	0.03	0.43	0.13	0.73	0.09	Ercalcitriol
Var_293	0.006	0.19	0.01	0.76	0.09	20-Hydroxy-PGF2a Thromboxane B2 6-Keto-prostaglandin F1a

Severe asthma vs nonsevere asthma vs healthy children

When the breathomics data were analyzed all together for the three sets of children – that is, healthy controls, nonsevere and severe asthma cases – the O2PLS-DA was combined with the Naïve Bayes classification technique, using the O2PLS-DA predictive scores as attributes for building the Naïve Bayes classifier. As a result, a robust classification model was obtained. We report the performance in prediction of the model in the ‘model validation’ section.

The analysis of the correlation structure existing between the predictive scores of the O2PLS-DA part of the classification model and the measured variables allowed us to interpret the model in terms of the single measured variables. This confirmed the role of the variables emerging from the

previous analysis and highlights how variable 412, related to vitamin D, distinguishes not only healthy children but also nonsevere asthma cases from children with severe asthma. In the 3D scatter plot (Fig. 3), we reported the distribution of the samples with respect to the variables 412, 225, and 293. The three variables allow a qualitative separation of the samples in three principal clusters. With respect to such variables, a representative ion-extracted chromatogram for each group has been reported in Fig. 1.

For the analysis of the metabolomic data together with the clinical data, please see the online supplement.

Model validation

Here, we report the performance in prediction of the model comparing severe asthma vs nonsevere asthma vs healthy children.

Balanced training (39 patients) and test (18 patients) sets were extracted from the data set by applying onion D-optimal design on the PCA scores calculated by considering each individual group of patients. All patients of the training set were correctly classified while the confusion matrix for the test set showed a kappa coefficient equal to 0.80 (Table 3). The robustness of the obtained classifier was confirmed by permutation test on the responses.

Analytical repeatability

35 quality control (QC) samples were interposed in the sequence analysis of the 171 study samples (i.e., the 57 collected samples, each analyzed in triplicate).

The principal component analysis (PCA) of the QC samples indicated that the system was stable.

Analytical repeatability was assessed by checking the ion intensity on the QC (difference of retention time,

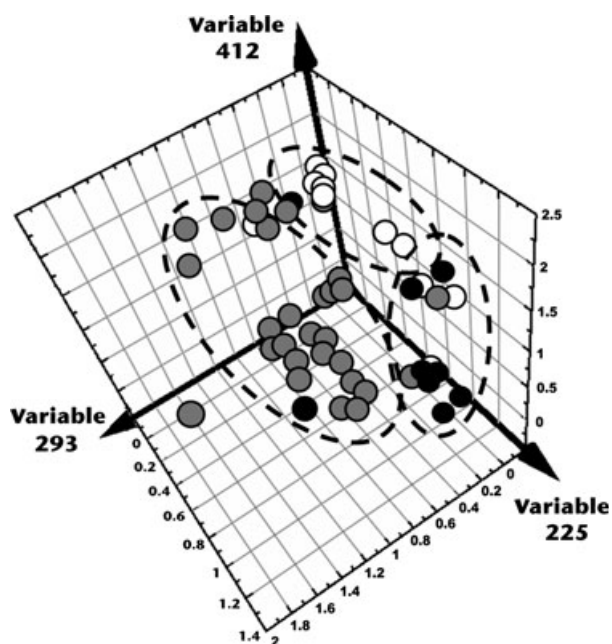


Figure 3 Scatter 3D-plot showing the three groups of children on the basis of the three selected putative biomarkers [variables are expressed as $\log(X+1)$ where X is the normalized intensity of the measured MS signals and $\log$ is the base-10 logarithm]. Healthy children are represented with white dots, children with nonsevere asthma with gray dots, and children with severe asthma with black dots.

Table 3 Analysis of the breathomics data using O2PLS-DA combined with a Naive Bayes classifier. The confusion matrix was obtained for the test set (Cohen's kappa coefficient $K = 0.80$). H, healthy subjects; SA, severe asthma; NSA, nonsevere asthma

	Predicted H	Predicted SA	Predicted NSA	Total
H	4	1	0	5
SA	0	2	1	3
NSA	0	0	10	10

0.34 ± 0.05 min (mean $\pm$ SD); ppm error -3.8 ± 2.9 ; CV% ion intensity extraction between 20% and 30%).

Discussion

We report here on the application of an innovative approach, breathomics (the metabolomic analysis of exhaled breath condensate) (12), to the characterization of pediatric asthma phenotypes, focusing particularly on severe asthma. Metabolomic analysis clearly distinguished between asthmatic children with different degrees of severity of the disease and a specific metabolite fingerprint emerged in the characterization of severe asthma.

There is a recognized need for a better comprehension of the heterogeneity of asthma through the characterization of the different phenotypes not only from a clinical standpoint but also from a biochemical-inflammatory one (16).

The metabolomic profiling enabled a clear separation between the following 3 groups of subjects: (1) healthy children, (2) children with nonsevere asthma, and (3) children with severe asthma.

The nonsevere asthma group included both asthmatic children with well-controlled asthma regularly treated with controller medications and asthmatic children with well-controlled asthma steroid naïve. These two subgroups of children could not be discriminated by the metabolomic analysis, suggesting that, irrespective of any need for regular treatment, children with nonsevere asthma have similar EBC metabolic profiles, indicative of similar metabolic-inflammatory processes. Conversely, the fact that the metabolomic analysis clearly separated the severe asthma phenotype suggests that an overall EBC metabolite fingerprint specifically characterizes the severe asthma group. The identification of different biochemical-metabolic profiles in severe and nonsevere asthma may play an important role in clinical practice providing an objective tool to establish the severity of the disease and potentially enabling the identification of factitious diseases.

It is worth pointing out that the separation between the 3 groups of children (i.e., healthy, nonsevere asthma and severe asthma) was allowed by a combination of metabolites, in other words the discrimination was enabled by the overall metabolic profile. Metabolomic analysis, in fact, does not aim at the identification of each variable within the profile, but it is based on the integrated readout of the overall metabolic profile.

Nonetheless, some variables, within the profiles, turned out to have a key role for group characterization. By searching the HMDB (25) we were able to characterize from a chemical point of view the putative biomarkers potentially having a role in asthma pathophysiology.

Variable 225, characteristic of severe asthma subjects compared to healthy controls, is a compound chemically related to retinoic acid. This is consistent with recent data suggesting a role for retinoic acid and its metabolites in both inflammation (26) and airway remodeling in asthma (27), particularly in the more severe forms (27).

Variable 127, which was again characteristic of severe asthma subjects compared to healthy controls, is coherent with the deoxyadenosine chemical structure. Adenosine is a purinergic mediator with several pro-inflammatory effects (28), with a role in asthma that has been demonstrated by previous studies showing higher levels in bronchoalveolar lavage (29) and EBC of asthmatic subjects, and especially in those with worsening symptoms (30).

More interestingly, among the variables characterizing the healthy controls and the nonsevere asthma cases and lacking in the severe asthma group, we identified the variable 412, which, according to the HMDB (25), might be ercalcitriol, the active metabolite of vitamin D2. As far as we know, this is the first report of vitamin D being recovered specifically in the lung, the target organ in asthma.

There has recently been an increasing interest in the possible link between serum vitamin D deficiency and the onset of asthma, and there are reports of an inverse correlation between serum vitamin D levels and asthma severity (31–34). Moreover, a recent study suggests that oral supplementation of vitamin D facilitates asthma control in children (35). It is worth pointing out that the EBC analysis in our study was not targeted to detect vitamin D or its metabolites. The metabolomic approach, in fact, is not guided by any a priori hypothesis on the components that might be associated with a given condition; instead, it considers a vast number of metabolites and tries to identify which of them can discriminate between groups. It is therefore interesting that such an untargeted approach suggests the lack of a vitamin D metabolite in the EBC of children with severe asthma. In keeping with the results presented by other authors (31–34), we speculate that children with severe asthma and treated with high-dose ICS may have insufficient vitamin D in the airways.

Comparing the nonsevere asthma, phenotype with cases of severe asthma brought to light the role of variable 293, for which the related metabolites reported in the HMDB are 20-hydroxy-PGF2 α , thromboxane B2, and 6-keto-prostaglandin F1 α . Among these candidate metabolites, thromboxane B(2) is a stable metabolite of thromboxane A(2) and a potent bronchoconstrictor (36), and increased levels of this mediator have been demonstrated in the EBC of subjects with mild asthma (37).

Our data indicate that metabolomic analysis can be complementary to conventional clinical assessment in asthma. Compared to the other measurements taken in this study (spirometry and FE_{NO}), the metabolomic analysis appears to be much more informative. In fact, lung function could not distinguish between nonsevere and healthy children, and FE_{NO} could not distinguish between nonsevere and severe asthma (Table 1).

Nonetheless, it is noteworthy that when the clinical data (FE_{NO} and spirometric values) are considered together with the metabolomic data, the robustness of the model that discriminates the three sets of children – that is, healthy controls, nonsevere and severe asthma cases – improves (Table 1 in the online supplement). The comparison of the information in the set of clinical data with the information in the breatho-

mics data set demonstrates that the two representations contain different information on the system being investigated and combining them afforded a better understanding of the system itself. This observation underlines the need for combining metabolomics and clinical data as previously advocated by other authors (6).

We believe that the strength of the present study is represented by the careful clinical characterization of the patients, the analytical repeatability of the data, and the cross-validation of the results. Particularly, the latter minimize the risk of false discoveries (19). Our study has also some limitations. First of all, we recognize that we can only speculate at this stage on the metabolic nature of the discriminating compounds. Further studies are needed to characterize, by applying also other spectroscopic techniques (e.g., high-field NMR with cryo-probe), the biochemical structure of the metabolites that emerged. It is worth pointing out that although, as reported above, the separation between groups is based on the overall metabolic fingerprint, this does not mean that in each subject, all the group's metabolic characteristics cluster together. This is particularly true for heterogeneous groups as the severe asthma group. The full identification of the most important metabolites within each group's profile will provide the rationale for a targeted analysis that might guide the therapeutic choices by establishing the metabolic pathways involved in each specific patient.

Another limitation relates to the low number of subjects of the severe asthma group, which prevents us from investigating the existence of multiple sub-phenotypes of severe asthma. To be properly addressed, this issue requires multicenter studies, as the ongoing pan-European project U-BIOPRED that aims to phenotype adult and pediatric severe asthma through a system biology approach (38). Finally, there was an inevitable treatment bias between severe and nonsevere asthmatic children that cannot be avoided because of the cross-sectional design of our study. Further studies with a prospective design are required to definitively assess the treatment effect on the EBC metabolic profile.

In conclusion, the metabolomic approach applied to exhaled breath condensate enabled the characterization of

different biochemical-metabolic profiles in asthmatic children, with a specific metabolic fingerprint emerging in the severe asthma group.

Our data suggest that breathomics is a promising approach for characterizing different asthma metabolic phenotypes, with the potential for discovering unknown biological pathways and developing new targeted therapies.

Authors' contribution statements

SC contributed to study design, collected clinical data and wrote the manuscript draft; GG performed the MS analysis and contributed to manuscript revision; FR and DC contributed to MS analysis; MS performed the statistical analysis and contributed to manuscript revision; PJS contributed to result interpretation and manuscript preparation; EB contributed to study design, result interpretation and manuscript preparation.

Conflict of interest statement

SC, GG, FR, DC, MS, EB have no conflict of interest to disclose. PJS's institute is recipient of a grant from the Innovative Medicines Initiative (IMI) on systems medicine of severe asthma, including exhaled breath metabolomics.

Funding

The research project has been supported by Grant Giovani Ricercatori – 2009 (GR 2009 – 1604820) funded by the Italian Ministry of Health.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1: Analysis of the breathomics data considered together with the spirometric parameters and exhaled nitric oxide measurements.

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ASTHMA SEVERITY IN CHILHOOD AND METABOLOMIC PROFILING OF BREATH CONDENSATE

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ONLINE SUPPLEMENTAL MATERIAL

METHODS

EBC collection

EBC was collected and processed according to ATS/ERS recommendations¹. EBC was collected using the TURBO-DECCS (transportable unit for research on biomarkers obtained from disposable exhaled condensate collection systems) (Medivac, Parma, Italy) as previously described².

TURBO is a refrigerating system that relies on a thermo electrical module giving rise to a Peltier effect. The cold side of the Peltier module is connected to an aluminium support shaped to house the test tube. TURBO is supplied with DECCS, a disposable respiratory system that consists of a mouthpiece equipped with a one-way valve and a reliable saliva trap, connected to a collecting vial (50 ml) by means of a tube. The children breathed tidally through the mouth for 20 minutes, while sitting comfortably and wearing a nose clip. They kept their mouth dry during EBC collection by periodically swallowing excess saliva. EBC samples were immediately stored at -80°C in polypropylene tubes until assay.

Orbitrap LC–MS analysis of metabolites in EBC

Analyses were performed with an Ultimate 3000 Dionex HPLC system (Dionex, Softron GmbH, Germany) coupled to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with an Advion Triversa NanoMate source (Advion BioSciences, inc. Ithaca, NY, USA).

Twenty μL of EBC were injected into an Atlantis T3 (Waters co., Milford, MA, USA) trap column $3\mu\text{m}$ particle size, $10\text{mm} \times 2.1\text{mm}$ inner diameter, and were diverted, starting after a time period of 1.25 min and continuing up to 7 min, onto an analytical Atlantis T3 (Waters co., Milford, MA, USA) column, $3\mu\text{m}$ particle size, $150\text{mm} \times 1\text{mm}$ inner diameter, at 40°C . For the trap column solvent A was H_2O with 0.1% formic acid and solvent B was

acetonitrile with 0.1% formic acid. Solvent B varied as follows: 0 min 2%, 1.25 min 100%, 8 min 2%; the flow rate was 40 μ L/min.

For the analytical column solvent A was H₂O with 0.1% formic acid, solvent B was acetonitrile with 0.1% formic acid, solvent C was methanol with 0.1% formic acid; the flow rate was kept at 40 μ L/min. Solvent B and C varied as follows: 0 min B 0% C 5%, 1.5 min B 0% C 5%; 10-11 min B 48% C 50%; 11.1-12 min B 98% C 0%; 12.1 min B 0% C 5%. The column was then equilibrated for 5 min at the initial conditions before analyzing the next sample.

The LC (liquid chromatography) flow was split (1/100) on chip-based electrospray ionization technology from Advion and directed to the mass spectrometer. MS analysis was done in positive FTMS (Fourier Transform Mass Spectrometry) mode at a resolution of 30,000 (at m/z 400) with a 50 – 1000 scan range and using the following source parameters: tube lens 150 V, capillary voltage 45 V, capillary temperature 200°C, and ion spray voltage 1.7 kV.

All chemicals were purchased from Sigma (St. Louis, MO, USA). The acetonitrile, formic acid, and methanol used for HPLC solvents were of LC–MS degree.

All samples were injected three times in three random sequence to avoid any effect of the analytical conditions on the classification.

A standard test solution (Sulfadimethoxine, Val-Tyr-Val, Leucine-Enkephalin, Erythromycin) was used as quality control (QC) and to test conditioning and stability. Data extraction was performed by MarkerLynx software after the transformation of the LTQ Orbitrap raw data in cdf file and then in masslynx file (Waters co., Milford, MA, USA).

35 quality control (QC) samples were interposed in the sequence analysis of the 171 study samples (i.e. the 57 collected samples each analysed in triplicate). The principal component analysis (PCA) of the QC samples indicated that the system was stable.

Analytical reproducibility was assessed by checking the ion intensity on the QC (difference of retention time 0.34 ± 0.05 min (mean $\pm$ SD); ppm error -3.8 ± 2.9 ; CV% ion intensity extraction between 20 and 30%).

Fractional exhaled nitric oxide (FE_{NO}) measurement

FE_{NO} was measured with the NIOX system (Aerocrine, Stockholm, Sweden) using a single-breath on-line method according to the ERS/ATS guidelines for measuring FE_{NO} in children³. Children inhaled NO-free air to total lung capacity and exhaled through a dynamic flow restrictor with a target flow of 50 ml/sec for at least 6-7 seconds. No nose clip was used. The NIOX system was calibrated using a 200 ppb NO tank (Lindegas Hoek Loos Speciality gases, Amsterdam, Netherlands) according to the manufacturer's instructions.

RESULTS

When the breathomics data were analysed all together for the three sets of children - i.e. healthy controls, non-severe and severe asthma cases - the O2PLS-DA was combined with the Naïve Bayes classification technique, by using the O2PLS-DA predictive scores as attributes for building the Naïve Bayes classifier.

The proposed approach allowed a satisfactory classification model to be built. The confusion matrix for the prediction of the extracted test set is shown in table 1, panel A (kappa coefficient equal to 0.80). During cross-validation with 7 folds the kappa coefficient was $K=0.59$.

When the metadata (FE_{NO} and spirometric values) were considered altogether with the metabolomic data, the robustness of the built model improves (kappa coefficient for 7-fold cross-validation was $K=0.72$) and only one subject of the test set was incorrectly predicted (table 1, panel B, kappa coefficient equal to 0.90), whereas the O2PLS-DA model obtainable using the metadata alone was much weaker. Indeed, when we used the O2PLS to directly compare the information contained into the set of metadata with the information in the breathomics dataset, we found that the two blocks of data have only a weak joint co-variation structure (only the 4% of the total variance of the breathomics dataset is joint-correlated with the 20% of the total variance of the metadata block), while the unique information characterizing each block is dominant. In other words, the information contained separately into each set of data is proper to that dataset and the combination of the two sets of data can improve the whole knowledge of the system under investigation. By using the information contained in both the sets of data we were able to improve the performance of the classification model as confirmed by the observed rise of the kappa coefficient during both cross-validation and test set prediction.

A.

	Predicted H	Predicted SA	Predicted NSA	Total
H	4	1	0	5
SA	0	2	1	3
NSA	0	0	10	10

B.

	Predicted H	Predicted SA	Predicted NSA	Total
H	5	0	0	5
SA	0	2	1	3
NSA	0	0	10	10

Table 1. Panel a. Analysis of the breathomics data using O2PLS-DA combined with a Naive Bayes classifier. The confusion matrix was obtained applying the classification model to the extracted test set (Cohen's kappa coefficient $K=0.80$). H: healthy subjects; SA: severe asthma; NSA: non-severe asthma. **Panel b.** Analysis of the breathomics data considered together with the metadata (spirometric parameters and FE_{NO} measurements) using O2PLS-DA and a Naive Bayes classifier. The confusion matrix was obtained applying the classification model to the extracted test set (Cohen's kappa coefficient $K=0.90$). H: healthy subjects; SA: severe asthma; NSA: non-severe asthma

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