

Metabolomics applied to urine samples in childhood asthma; differentiation between asthma phenotypes and identification of relevant metabolites

Elia Mattarucchi^a, Eugenio Baraldi^b and Claude Guillou^{a*}

ABSTRACT: Asthma is a heterogeneous disorder and one of the most common chronic childhood diseases. An improved characterization of asthma phenotypes would be invaluable for the understanding of the pathogenic mechanisms and the correct treatment of this disease. The aim of this pilot study was to explore the potential of metabolomics applied to urine samples in characterizing asthma, and to identify the most representative metabolites. Urine samples of 41 atopic asthmatic children (further subdivided in sub-groups according to the symptoms) and 12 age-matched controls were analyzed. Untargeted metabolic profiles were collected by LC-MS, and studied by multivariate analysis. The group of the asthmatics was differentiated by a model that proved to be uncorrelated with the chronic assumption of controller drugs on the part of the patients. The distinct sub-groups were also appropriately modeled. Further investigations revealed a reduced excretion of urocanic acid, methyl-imidazoleacetic acid and a metabolite resembling the structure of an Ile-Pro fragment in the asthmatics. The meaning of these findings was discussed and mainly correlated with the modulation of immunity in asthma. Metabolic profiles from urines have revealed the potential to characterize asthma and enabled the identification of metabolites that may have a role in the underlying inflammation. Copyright © 2011 John Wiley & Sons, Ltd.

Supporting information can be found in the online version of this article.

Keywords: metabolomics; asthma phenotypes; urocanic acid; methyl-imidazoleacetic acid; isoleucyl-proline

Introduction

Asthma is a common heterogeneous disorder of the airways that presents physiological lung dysfunctions, chronic inflammation and tissue remodeling. Allergic asthma typically onsets early and it is emerging as the most common chronic disease in children (Bisgaard and Szefer, 2007). The diagnosis is usually based on medical history and manifestations, even if symptoms are frequently poorly recognized (Killian *et al.*, 2000). The measurement of lung function by spirometry greatly enhances diagnostic confidence, but the execution requires particular care and training (Enright, 2008). The aim of asthma treatment is limited to achieve and maintain control of the clinical manifestations, and is commonly assessed using standardized questionnaires (Nathan *et al.*, 2004). In the last few years there has been an increasing awareness of asthma heterogeneity in the underlying chronic inflammation. Different clinical manifestations and therapy responses (usually referred as 'asthma phenotypes') proved to be associated with the presence of different immune cells and inflammatory mediators (Bel, 2004). The recruitment of eosinophils in airway mucosal is common, and it can be assessed by sputum examination, measurement of the fraction of exhaled nitric oxide (FeNO), and the identification of eosinophil-derived proteins in blood or urine (Hoekstra *et al.*, 1996; Piacentini *et al.*, 1999). However, a portion of asthmatics has a prevalent neutrophilic infiltration (Murdoch and Lloyd, 2010). This occurrence can affect therapy (Pavord *et al.*, 1999), since inhaled corticosteroids (ICS), the most effective controller medications in allergic asthma (Frois *et al.*,

2009), act mainly on mast cells and eosinophils in the lungs (Djukanović *et al.*, 1992).

The continuous evolution of the concept of asthma has increased the need for new diagnostic markers (Louhelainen *et al.*, 2008). In this context, the collection and analysis of comprehensive nontarget metabolic profiles, generally referred to as metabolomics, has developed in recent years (Nordström and Lewensohn, 2010). Metabolomics can give a biochemical picture of the pathologic phenotype, as it results from the interaction of genetic, environmental and lifestyle makeup. Metabolic profiles can be collected by a variety of techniques, but ultra-high-performance liquid chromatography (UPLC)

* Correspondence to: C. Guillou, European Commission, Joint Research Centre, Institute for Health and Consumer Protection, Systems Toxicology Unit, Ispra (VA), Italy. E-mail: claud.guillou@jrc.ec.europa.eu

^a European Commission, Joint Research Centre, Institute for Health and Consumer Protection, Systems Toxicology Unit, Ispra (VA), Italy

^b University of Padova, Department of Pediatrics, Padova, Italy

Abbreviations used: EBC, exhaled breath condensate; ICS, inhaled corticosteroids; OPLS-DA, orthogonal projection to latent structures-discriminant analysis; PC, patients with poorly controlled symptoms using controller drugs; PLS-DA, partial least squares discriminant analysis; VIP, variable importance; WC, patients with well controlled symptoms without controller drugs; WCI, patients with well controlled symptoms using controller drugs.

coupled with electrospray mass spectrometry (ESI-MS) is emerging as one of the most used (Guillarme *et al.*, 2010). In a typical metabolomics workflow, chromatograms are aligned to extract those signals shared by the samples under investigation (Lange *et al.*, 2008). The resulting variables (extracted variables) are characterized by retention time, intensity and mass (m/z). Subsequently, multivariate data analysis is used to disclose differences between two or more groups of subjects (e.g. patients vs control; Trygg *et al.*, 2007). The major drawback of LC-ESI-MS is that the identification of chemical structures is still a challenging process (Kind and Fiehn, 2010). Existing libraries are still limited and computer-aided interpretation of mass spectra is hindered by the complex patterns of fragmentations or adducts (Holcapek *et al.*, 2010). For these reasons, many metabolomics studies only disclose a combination of variables that can discriminate different groups of samples under probabilistic assumptions, regardless of the biological nature of these variables. The number of metabolomics studies on asthma is still extremely limited; Saude *et al.* (2009) were able to statistically differentiate challenged animals in a murine model of asthma, analyzing urine samples. Carraro *et al.* (2007) claimed that metabolic profiles from exhaled breath condensate (EBC) could be effectively combined with spirometry and FeNO measurements to characterize asthmatic children. These results illustrated the potential for this approach, but further investigations are still necessary to translate metabolomics into clinical practice. In particular, the level of information brought by varied specimens remains unexplored: EBC is frequently used, but the concentration of metabolites in this matrix is frequently too low for a clear interpretation of the results (Louhelainen *et al.*, 2008). On the contrary, urine is one of the most informative, accessible and stable body fluids, but its utility in research on pulmonary diseases still lacks clear evidence.

In this study, urine samples from 41 asthmatic children and 12 healthy controls were profiled by LC-MS to assess the amount of urine information that correlates with asthma, and possibly to disclose the biological origin of the prominent variables. Models explaining different asthma phenotypes are also discussed.

Experimental

Study Subjects

A total of 41 atopic asthmatic children (median age 11 years) were recruited at the hospital of Padua, Italy. Twelve age-matched healthy subjects with no history of atopy or respiratory diseases were used as controls. All these subjects were Caucasian; the ethics committee at the Padua hospital approved the study, and all parents gave their informed consent. Further information about these subjects and the correspondent pharmacological treatments is reported in Tables S1–S3 (Supporting Information). The diagnosis of asthma was confirmed by pediatric respiratory physicians according to international guidelines (GINA, 2009). Patients were further subdivided into three groups: WC (14 subjects), well controlled with beta2 short acting agonists as needed; WCI (16 subjects), well controlled with daily controller drugs; and PC (11 subjects), poorly controlled with daily controller drugs. Children's adherence to therapy was checked routinely and no significant allergen exposure or main co-morbidities, as well as no acute airway infections in the 3 weeks before the recruitment, were reported. None of the children was prescribed with a particular diet or food deprivation before sampling.

Chemicals

All the chemicals used in this study were purchased by Sigma (Sigma-Aldrich, St Louis, MO, USA). Chromatographic solvents and additives

were LC-MS grade, while the reference materials used to confirm metabolite identification and to produce the quality control sample were $\geq 99\%$ (HPLC) purity. Ultrapure water was produced through an Elix + Milli-Q Advantage A10 (Millipore, Billerica, MA, USA).

LC-MS Profiling of Urine

Metabolic profiles were acquired under UNI EN ISO 9001:2008 certification at the Institute for Health and Consumer Protection of the European Commission (registration no. 8203-A). In particular, standard operating procedures were adopted to avoid contamination according to the guidelines established by the manufacturer of the LC-MS apparatus used in this study (Waters, 2005).

Untimed spot urine samples were collected during working hours without the use of preservatives, centrifuged at 15000 *g* to remove debris, and diluted 1:4 in ultrapure water. For each sample, 10 μ L was analyzed on an Acquity UPLC system coupled to a Q-TOF Premier spectrometer (Waters Corporation, Milford, MA, USA). The analytical protocol was developed from the work of Wong *et al.* (2008) using an Acquity HSS T3 column (Waters) 100 $\times$ 2.1 mm, at 50°C. Mobile phases were: (A) water–0.1% formic acid; (B) methanol–0.1% formic acid. Chromatographic conditions were: phase A, 98% (for 0.5 min), subsequently reduced to 70% (at 5 min), 0% (at 9 min) and maintained at 0% until 10 min. Then, the starting conditions were restored and maintained until the end (11.5 min). The flow rate was 0.4 mL/min. Mass range was 50–1000 m/z , in W, centroid, positive and negative ionization modes (instrument settings are reported Table 1). For each ionization mode, all the samples were run in triplicate in a randomized order. Metabolic profiles of the three runs were merged in a single set and analyzed through MarkerLynx XS version 4.1 SCN639 (Waters), applying the parameters reported in Table 1. Extracted variables were normalized by the 'total useful MS signal' (i.e. the sum of the intensities of the variables shared by all the samples), and averaged over the three replicates (Warrack *et al.*, 2009). Finally, datasets were filtered against an exclusion list containing the contaminants observed in 'blank' samples and the main metabolites of the drugs taken by the subjects under investigation. In each run, a control

Table 1. Spectrometer settings and MarkerLynx extraction parameters

Parameter	+ Ionization	– Ionization
Capillary	3.5 kV	2.5 kV
Sampling cone	35	45
Extraction cone	3.0	3.0
Ion guide	2.0	2.0
Source temperature	100°C	80°C
Desolvation temperature	300°C	250°C
Cone gas flow	0.0 L/h	0.0 L/h
Desolvation gas flow	450.0 L/h	605.0 L/h
Collision energy	5 eV	5 eV
Collision gas flow	0.30 mL/min	0.45 mL/min
Ion guide gas flow	0.0 mL/min	0.0 mL/min
Initial/final retention time	0.3/9.5 min	0.3/9.5 min
Low/high mass	100/900 Da	100/950 Da
Mass tolerance	0.03 Da	0.03 Da
Peak with at 5% height	8.6 s	10 s
Peak to peak baseline noise	7	5
Smoothing	Yes	Yes
Intensity threshold	50 counts	55 counts
Mass window	0.03 Da	0.03 Da
Retention time window	0.5 min	1 min
Noise elimination level	3	3
Deisotope data	Yes	Yes

sample (a solution of sulfadimethoxine, Val-Tyr-Val and erythromycin, each at 2 ng/ μ L) was added at regular intervals and used to validate analytical uncertainty.

Statistical Analysis

The variables acquired using the positive and negative ionization modes were merged in a single dataset, which was subsequently used to generate three independent models: healthy controls vs (WC + WCI + PC), WC vs (WCI + PC) and WCI vs PC. Models were produced by partial least squares discriminant analysis (PLS-DA) considering only those variables shared by no less than 80% of the samples within at least one class (Trygg *et al.*, 2007). Variables were further reduced starting from those with the lowest values of the VIP parameter (variable importance for the projection), until a maximum goodness of prediction (Q^2) was reached, as proposed by Chong and Jun (2005). PLS-DA models were validated by permutation tests and then converted into correspondent orthogonal projection to latent structures-discriminant analysis (OPLS-DA) models. The advantage of 'two classes' OPLS-DA models is that the significant information is collected in a single discriminating component; thus prominent variables were pointed out by their correlation with this component (Wiklund *et al.*, 2008). Multivariate analysis was performed by SIMCA P+12 (Umetrics, Umeå, Sweden). To estimate of the overall effectiveness of the models, probabilistic rules for the classification of the samples were established by a naive Bayes analysis of the measured values of score (t) using WEKA 3.4.11 (University of Waikato, Hamilton, New Zealand). According to these rules, samples were classified through the score values calculated in cross validation (t_{cv}), reporting the percentage of correct predictions for each class.

Metabolite Identification

Variables extracted with MarkerLynx were further checked manually and mass spectra were investigated to disclose the presence of adducts and fragments (Kind and Fiehn, 2010; Holcapek *et al.*, 2010). Metabolites were identified through MS/MS fragmentation experiments under the same conditions as used for the profiling of the urine (except for the collision energy, which was ramped from 5 to 35 eV during the scan time). Elemental compositions were estimated by MassLynx version 4.1 (Waters) using the isotope abundance profile (i-FIT) with a mass error of 10 ppm (carbon prediction filter at ± 5). Lists of putative candidates were independently obtained querying the human metabolome database (Wishart *et al.*, 2009) and using the MetFrag software (Wolf *et al.*, 2010). Reference compounds were used for confirmation.

For the metabolites with a prominent difference between two classes of samples, the median of the intensities between the two classes and the relative spreads of distributions (from 25th to 75th percentile) were calculated and represented in box plots. The significance of the observed differences was assessed by the Mann-Whitney test.

Results and Discussion

LC-MS Profiling

A total of 53 samples of urine were analyzed in triplicate, in positive and negative ionization modes. Injections at regular intervals of a quality control sample revealed a root mean square error of 0.0031/0.0013 Da, an average retention time shift of $\pm 0.04/0.19$ min and an average intensity CV of 4/19% for the positive/negative ionization modes, respectively. The analysis of the control sample's chromatograms revealed that the performances of the chromatography gradually declined during the experiment, as expected due to the effect of urine on the stationary phase (Wong *et al.*, 2008). An increased analytical variability in the negative ionization mode was also observed. Nevertheless, all the performance parameters were within the expected range for the instrumentation used and adequate for metabolomics applications (Pandher *et al.*, 2009). A total of 4541 and 2203 variables were extracted from the positive and negative metabolic profiles of urines and merged into a single dataset used as input to produce multivariate models. The size of the initial dataset (6744 variables) is correlated with the biological complexity of the samples investigated. However, the majority of the extracted information proved to be orthogonal (structured noise), as demonstrated by the fact that the final datasets of the models were extremely reduced after the process of variable selection had been carried out (Table 2).

Multivariate Models and Metabolite Identification

In the first model, asthmatics (i.e. WC + WCI + PC) were compared with healthy controls, while in the second model patients taking controller drugs (WCI + PC) were compared with those who did not (WC). The two models proved to be very efficient; in the first model only one patient was wrongly classified as nonasthmatic, and even in the second model only one single misclassification was observed (Table 2). A graphical representation of the two models in cross validation is reported in Figures S1 and S2 (Supporting Information). The discrimination of the asthmatics proved to be uncorrelated with possible metabolic effects due to the chronic assumption of controller drugs (mainly corticosteroids) by some of the patients. In fact, no correspondences were observed between the final datasets of the two models (i.e. no common variables were identified). Special attention was given to the identification of the variables within the dataset of the first model, and in particular to

Table 2. Characteristics of the OPLS-DA models used to study the datasets of the extracted variables

	First model	Second model	Third model
Class 1	(WC + WCI + PC)	(WCI + PC)	WCI
Class 0	Controls	WC	PC
Final dataset	40 variables	72 variables	27 variables
Orthogonal comp.	1	1	1
R^2	0.85	0.81	0.88
Q^2	0.75	0.72	0.76
Percentage of correct predictions per class in cross validation	98% (WC + WCI + PC) 100% (controls)	96% (WCI + PC) 100% (WC)	100% (WCI) 91% (PC)
WC, patients with well controlled symptoms without controller drugs; WCI, patients with well controlled symptoms using controller drugs; PC, patients with poorly controlled symptoms using controller drugs.			

the variable with m/z of 121.0394 Da and retention time of 1.42 min, since it showed the most significant difference ($p=1.2\times 10^{-5}$) between asthmatics and healthy controls. Fragmentation studies and the use of reference compounds revealed that the observed signal corresponded to a fragment of urocanic acid after the loss of a molecule of water (Fig. 1). This compound is an intermediate in the catabolism of histidine and is produced by the enzyme histidase, which is expressed mainly in liver and skin, but not in lung (Sano *et al.*, 1997). Interestingly, *cis*-urocanic acid (produced after UV radiation) is well known to be an immune-suppressor (Gibbs *et al.*, 2008) and it has been considered for the topical treatment of inflammatory and autoimmune diseases of skin (Ong, 2009). A recent publication showed how *cis*-urocanic acid can also influence distal sites of inflammation, reducing the severity of colitis in a mouse model (Albert *et al.*, 2010). These considerations can provide a scientific explanation to many old anecdotes on the benefits of sun exposure in asthma treatment (Gorman *et al.*, 2010). In our study, the concentration of urocanic acid was found to be significantly ($p=1.2\times 10^{-5}$) reduced in patients' urines, leading to the intriguing hypothesis that this occurrence can have a negative influence on the resolution of the inflammation process in asthma. Fragmentation studies enabled also the identification of methyl-imidazoleacetic acid, which appears to be less concentrated in the urines of asthmatics, but with a more limited significance ($p=0.015$). Methyl-imidazoleacetic acid is the main metabolite and a specific marker of the histamine metabolism in the body. For this reason it has been monitored in asthmatics for many years, although the results of these studies appeared inconclusive (Mitchell *et al.*, 1974). This is probably because the alteration of the histamine metabolism is affected by the different pharmacological treatments and is mainly limited to the lungs. The interest in histamine further declined with the observation that asthmatics are quite unresponsive to common antihistamines

(antagonists of the H_1 histamine receptor). However, the role of histamine was recently re-evaluated with the discovery of a new receptor (H_4) that is expressed by many inflammatory cells; low concentration of histamine seems to promote the recruitment of dendritic cells, eosinophils and mast cells through the H_4 receptors (Thurmond *et al.*, 2008). Thus, the slight reduction in the methyl-imidazoleacetic acid concentration observed in this study could be associated with an alteration of the histamine metabolism that is beyond the innate allergic response, while being part of the mechanisms that sustain the chronic inflammation in asthma. A third metabolite was found to have a significantly ($p=5.3\times 10^{-4}$) low concentration in asthmatics. MS/MS fragmentation experiments on this metabolite led to the hypothesis of an Ile-Pro dipeptide. However, only a rough confirmation of the structure was possible using an Ile-Pro-Ile standard, since the corresponding dipeptide was unavailable. The meaning of this finding is unclear, and it seems to contrast with the assumption that the accumulation of collagen in the lungs of the asthmatics is associated with a significant reduction in the prolidase activity (Cakmak *et al.*, 2009), since this occurrence would increase the urinary excretion of dipeptides containing proline. In the second model, many of the variables were associated with intermediates of the metabolism of C21-steroid hormones. In particular urocortisone and urocortisol were identified, and proved to have a reduced concentration in patients taking corticosteroids. This observation is in agreement with the corticosteroid related suppression of the adrenal function, and it confirms that the differentiation of the overall group of the asthmatics is not influenced by the metabolic effect of controller drugs. In fact, the overall group of the asthmatics was characterized by a set of metabolites (among which urocanic acid, methyl-imidazoleacetic acid, and an Ile-Pro fragment) that proved to be unrelated with the model in which patients taking controller drugs were compared with those who do not; only in this

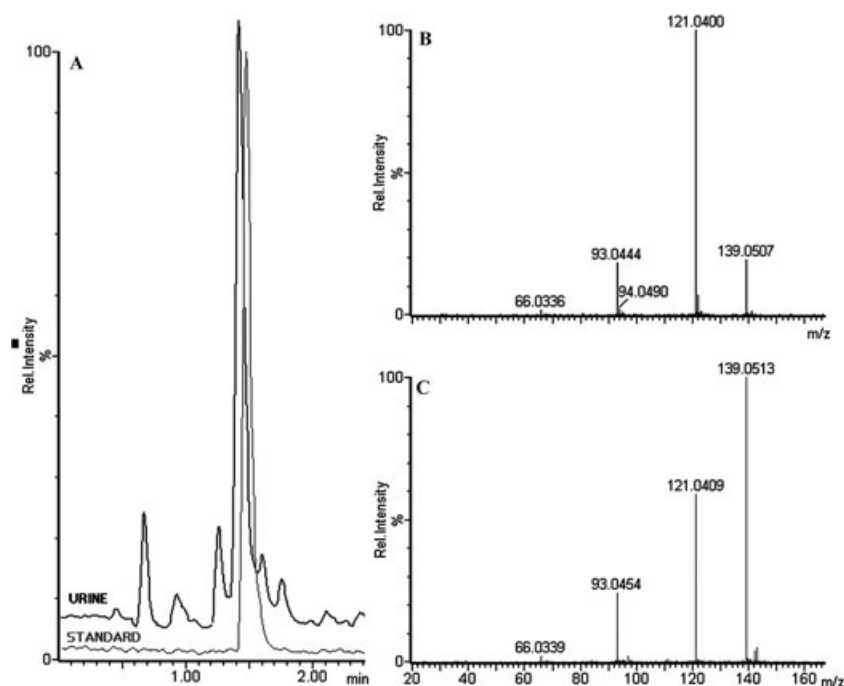


Figure 1. Chromatograms (A) and MS/MS mass spectra of a urine sample (B) and of a reference solution of urocanic acid at 1.6 ng/μl in ultrapure water (C).

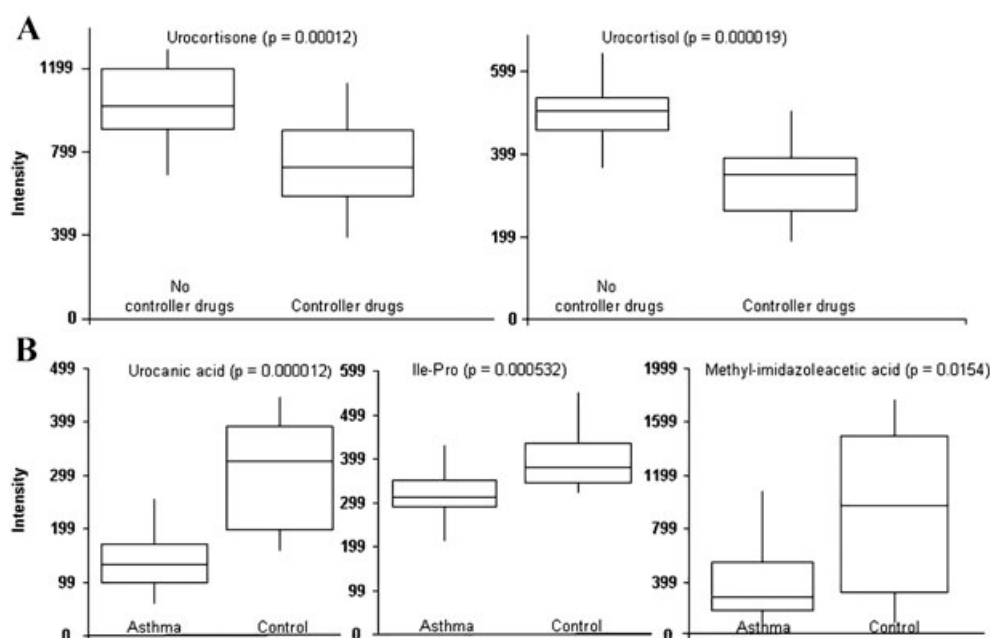


Figure 2. Box plots for the metabolites that showed the most relevant differences between patients taking, and not taking controller drugs (A), and between asthmatics and controls (B). The levels of significance were calculated using the Mann–Whitney test.

last model were the distinctive metabolites mainly ascribable to the assumption of corticosteroids. For the identified metabolites, the medians of the concentrations, the relative spreads of distribution and the levels of significance are reported in Fig. 2.

To further assess the level of information contained in urine, asthmatic patients were further subdivided according to the achieved level of control, and compared in the third model (i.e. WCI vs PC). Even in this case, class prediction was excellent (Table 2). A graphical representation of the model in cross validation is reported in Figure S3 (Supporting Information). However, in this case, the majority of the variables showing the highest correlations with the principal component of the model proved to have a level of significance lower than 95%. This revealed that the model is based on the whole dataset (i.e. the whole metabolic fingerprint) as opposed to on a restricted number of relevant variables which have the character of putative markers. This condition is probably due to the limited number of observations investigated (a subset of 27 patients), that hindered the further identification of relevant metabolites for this model.

These results highlighted the possible role of urine as a target for metabolomics studies on pulmonary diseases, even if this body fluid is generally considered a suitable target only for systemic disorders or with a direct involvement of the secretory apparatus.

Conclusion

The results of this study showed how the metabolic profiling of urine by LC-MS could be a promising approach for research on asthma and the detection of possible diagnostic markers. All the models reported had excellent prediction, highlighting possible biological interpretations for the observed differences. In particular, the finding of a reduction in the urocanic acid and methyl-imidazoleacetic acid contents may be correlated with the recently disclosed roles of these metabolites in inflammatory

diseases. However, further studies with more representative cohorts of patients will be needed to confirm these results, and to highlight possible markers for the discrimination of asthma phenotypes.

Supporting Information

Supporting information can be found in the online version of this article.

Acknowledgments

The authors would thank Dr M. V. Holland for her help for English revision, Dr N. Segebarth and Dr M. Stocchero for their valuable scientific advices and Dr D. Carpi and G. Giordano for the dispatching and preparation of the urine samples. The study was undertaken in the framework of the collaborative agreement no. CCR.IHCP.CA31220.X0 between the Joint Research Centre of the European Commission and the Department of Pediatrics of the University of Padova. The information contained in this article reflects the authors' views; the European Commission is not liable for any use of the information contained herein. The authors have no financial, professional or personal interests that might have influenced the outcome or presentation of the work described in this manuscript.

References

- Albert E, Walker J, Thiesen A, Churchill T and Madsen K. cis-Urocanic acid attenuates acute dextran sodium sulphate-induced intestinal inflammation. *PLoS One* 2010; **5**: 13676–13685.
- Bel EH. Clinical phenotypes of asthma. *Current Opinion in Pulmonary Medicine* 2004; **10**: 44–50.
- Bisgaard H and Szefer S. Prevalence of asthma-like symptoms in young children. *Pediatric Pulmonology* 2007; **42**: 723–728.
- Cakmak A, Zeyrek D, Atas A, Celik H, Aksoy N and Erel O. Serum prolidase activity and oxidative status in patients with bronchial asthma. *Journal of Clinical Laboratory Analysis* 2009; **23**: 132–138.

- Carraro S, Rezzi S, Reniero F, Héberger K, Giordano G, Zanconato S, Guillou C and Baraldi E. Metabolomics applied to exhaled breath condensate in childhood asthma. *American Journal of Respiratory and Critical Care Medicine* 2007; **175**: 986–990.
- Chong IG and Jun CH. Performance of some variable selection methods when multicollinearity is present. *Chemometrics and Intelligent Laboratory Systems* 2005; **78**: 103–112.
- Djukanović R, Wilson JW, Britten KM, Wilson SJ, Walls AF, Roche WR, Howarth PH and Holgate ST. Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. *American Review of Respiratory Disease* 1992; **145**: 669–674.
- Enright P. The use and abuse of office spirometry. *Primary Care Respiratory Journal* 2008; **17**: 238–242.
- Frois C, Wu EQ, Ray S and Colice GL. Inhaled corticosteroids or long-acting beta-agonists alone or in fixed-dose combinations in asthma treatment: a systematic review of fluticasone/budesonide and formoterol/salmeterol. *Clinical Therapeutics* 2009; **31**: 2779–2803.
- Gibbs NK, Tye J and Norval M. Recent advances in urocanic acid photochemistry, photobiology and photoimmunology. *Photochemical and Photobiological Sciences* 2008; **7**: 655–667.
- GINA. Global strategy for asthma management and prevention, 2009. Available from <http://www.ginasthma.org/Guidelineitem.asp?l1=2&l2=1&intId=1561> (accessed 13 October 2009).
- Gorman S, McGlade JP, Lambert MJ, Strickland DH, Thomas JA and Hart PH. UV exposure and protection against allergic airways disease. *Photochemical and Photobiological Sciences* 2010; **9**: 571–577.
- Guillarme D, Schappler J, Rudaz S and Veuthey JL. Coupling ultra-high pressure liquid chromatography with mass spectrometry. *Trends in Analytical Chemistry* 2010; **29**: 15–27.
- Hoekstra MO, Hovenga H, Gerritsen J and Kauffman HF. Eosinophils and eosinophil-derived proteins in children with moderate asthma. *European Respiratory Journal* 1996; **9**: 2231–2235.
- Holcapek M, Jirásko R and Lisa M. Basic rules for the interpretation of atmospheric pressure ionization mass spectra of small molecules. *Journal of Chromatography A* 2010; **1217**: 3908–3921.
- Killian KJ, Watson R, Otis J, St Amand TA and O'Byrne PM. Symptom perception during acute bronchoconstriction. *American Journal of Respiratory and Critical Care Medicine* 2000; **162**: 490–496.
- Kind T and Fiehn O. Advances in structure elucidation of small molecules using mass spectrometry. *Bioanalytical Reviews* 2010; **2**: 23–60.
- Lange E, Tautenhahn R, Neumann S and Gröpl C. Critical assessment of alignment procedures for LC-MS proteomics and metabolomics measurements. *BMC Bioinformatics* 2008; **9**: 375–393.
- Louhelainen N, Myllärniemi M, Rahman I and Kinnula VL. Airway biomarkers of the oxidant burden in asthma and chronic obstructive pulmonary disease: current and future perspectives. *International Journal of Chronic Obstructive Pulmonary Disease* 2008; **3**: 585–603.
- Mitchell RG, Thom H and Richardson JE. Histamine metabolism in asthma. *Journal of Allergy and Clinical Immunology* 1974; **54**: 111–114.
- Murdoch JR and Lloyd CM. Chronic inflammation and asthma. *Mutation Research* 2010; **690**: 24–39.
- Nathan RA, Sorkness CA, Kosinski M, Schatz M, Li JT, Marcus P, Murray JJ and Pendergraft TB. Development of the asthma control test: a survey for assessing asthma control. *Journal of Allergy and Clinical Immunology* 2004; **113**: 59–65.
- Nordström A and Lewensohn R. Metabolomics: moving to the clinic. *Journal of Neuroimmune Pharmacology* 2010; **5**: 4–17.
- Ong PY. Emerging drugs for atopic dermatitis. *Expert Opinion on Emerging Drugs* 2009; **14**: 165–179.
- Pandher R, Ducruix C, Eccles SA and Raynaud FI. Cross-platform Q-TOF validation of global exo-metabolomic analysis: application to human glioblastoma cells treated with the standard PI 3-kinase inhibitor LY294002. *Journal of Chromatography B Analytical Technologies in the Biomedical and Life Science* 2009; **877**: 1352–1358.
- Pavord ID, Brightling CE, Woltmann G and Wardlaw AJ. Non-eosinophilic corticosteroid unresponsive asthma. *Lancet* 1999; **353**: 2213–2214.
- Piacentini GL, Bodini A, Costella S, Vicentini L, Mazzi P, Sperandio S and Boner AL. Exhaled nitric oxide and sputum eosinophil markers of inflammation in asthmatic children. *European Respiratory Journal* 1999; **13**: 1386–1390.
- Sano H, Tada T, Moriyama A, Ogawa H, Asai K, Kawai Y, Hodgson ME, Kato T, Wada Y and Suchi M. Isolation of a rat histidase cDNA sequence and expression in *Escherichia coli*; evidence of extra-hepatic/epidermal distribution. *European Journal of Biochemistry* 1997; **250**: 212–221.
- Saude EJ, Obiefuna IP, Somorjai RL, Ajamian F, Skappak C, Ahmad T, Dolenko BK, Sykes BD, Moqbel R and Adamko DJ. Metabolomic biomarkers in a model of asthma exacerbation: urine nuclear magnetic resonance. *American Journal of Respiratory and Critical Care Medicine* 2009; **179**: 25–34.
- Thurmond RL, Gelfand EW and Dunford PJ. The role of histamine H1 and H4 receptors in allergic inflammation: the search for new anti-histamines. *Nature Reviews Drug Discovery* 2008; **7**: 41–53.
- Trygg J, Holmes E and Lundstedt T. Chemometrics in metabolomics. *Journal of Proteome Research* 2007; **6**: 469–479.
- Warrack BM, Hnatyshyn S, Ott KH, Reilly MD, Sanders M, Zhang H and Drexler DM. Normalization strategies for metabolomic analysis of urine samples. *Journal of Chromatography B Analytical Technologies in the Biomedical and Life Science* 2009; **877**: 547–552.
- Waters. Controlling contamination in ultraperformance LC/MS systems, 2005. Available from http://www.waters.com/webassets/cms/support/docs/715001307d_cntrl_cntrl.pdf (accessed 12 May 2009).
- Wiklund S, Johansson E, Sjöström L, Mellerowicz EJ, Edlund U, Shockcor PJ, Gottfries J, Moritz T and Trygg J. Visualization of GC/TOF-MS-based metabolomics data for identification of biochemically interesting compounds using OPLS class models. *Analytical Chemistry* 2008; **80**: 115–122.
- Wishart DS, Knox C, Guo AC, Eisner R, Young N, Gautam B, Hau DD, Psychogios N, Dong E, Bouatra S, Mandal R, Sinelnikov I, Xia J, Jia L, Cruz JA, Lim E, Sobsey CA, Shrivastava S, Huang P, Liu P, Fang L, Peng J, Fradette R, Cheng D, Tzur D, Clements M, Lewis A, De Souza A, Zuniga A, Dawe M, Xiong Y, Clive D, Greiner R, Nazirova A, Shaykhtudinov R, Li L, Vogel HJ and Forsythe I. HMDB: a knowledge-base for the human metabolome. *Nucleic Acids Research* 2009; **37**: 603–610.
- Wolf S, Schmidt S, Müller-Hannemann M and Neumann S. In silico fragmentation for computer assisted identification of metabolite mass spectra. *BMC Bioinformatics* 2010; **11**: 148–159.
- Wong MC, Lee WT, Wong JS, Frost G and Lodge J. An approach towards method development for untargeted urinary metabolite profiling in metabolomic research using UPLC/QTOF MS. *Journal of Chromatography B Analytical Technologies in the Biomedical and Life Science* 2008; **871**: 341–348.

Supporting Information

Metabolomics applied to urine samples in childhood asthma; differentiation between asthma phenotypes and identification of relevant metabolites

Elia Mattarucchi^a, Eugenio Baraldi^b and Claude Guillou^a

^aEuropean Commission, Joint Research Center, Institute for Health and Consumer Protection, Systems Toxicology Unit, Ispra (VA), Italy; ^bUniversity of Padova, Department of Pediatrics, Padova, Italy.

Tables

Table S1. Features of the healthy subjects (controls) and the children with well controlled symptoms without controller drugs. FVC, Forced vital capacity; FEV₁, Forced expiratory volume in one second. Lung function parameters were measured at the best of three attempts as a percentage of the reference values.

ID	Sex	Age	FVC (%)	FEV ₁ (%)
55	F	9	106	112
56	M	14	92	100
57	F	10	96	91
58	F	9	97	93
59	M	14	98	92
60	M	14	99	92
61	M	17	118	114
62	M	13	109	94
63	M	9	96	92
64	F	15	108	100
65	M	12	105	100
66	F	13	93	92
15	F	14	104	85
21	M	13	98	101
24	F	14	108	107
30	F	13	109	106
33	M	13	101	103
36	M	10	112	110
42	M	17	117	103
43	M	11	117	103
45	M	11	92	94
47	M	9	125	119
48	M	8	124	120
50	F	16	121	125
52	M	9	89	84
53	M	14	94	95

Table S2. Features of the patients with well controlled symptoms under controller drugs. FVC, Forced vital capacity; FEV₁, Forced expiratory volume in one second. Lung function parameters were measured at the best of three attempts as a percentage of the reference values.

ID	Sex	Age	Drug per day	FVC (%)	FEV ₁ (%)
13	M	10	Budesonide + Formoterol (160 + 9 µg)	101	85
16	M	12	Budesonide + Formoterol (160 + 4.5 µg)	105	85
22	M	12	Budesonide + Formoterol (160 + 9 µg)	111	84
			Theophylline (400 mg)		
23	M	8	Fluticasone + Salmeterol (200 + 100 µg)	97	95
25	F	12	Budesonide + Formoterol (160 + 9 µg)	98	98
27	M	8	Fluticasone + Salmeterol (200 + 100 µg)	99	102
28	F	9	Fluticasone (200 µg)	90	84
29	F	9	Fluticasone (200 µg)	99	99
31	M	10	Budesonide + Formoterol (160 + 9 µg)	101	104
32	M	10	Budesonide + Formoterol (160 + 9 µg)	97	98
			Montelukast (5mg)		
34	M	15	Budesonide + Formoterol (160 + 4.5 µg)	106	101
35	F	11	Fluticasone (125 µg)	88	89
			Montelukast (5mg)		
37	M	11	Budesonide + Formoterol (160 + 9 µg)	97	96
39	M	8	Budesonide + Formoterol (160 + 9 µg)	102	103
44	F	10	Fluticasone (250 µg)	93	92
51	M	11	Fluticasone + Salmeterol (200 + 100 µg)	90	84

Table S3. Features of the patients with poorly controlled symptoms. Exa, Exacerbations requiring oral corticosteroids in the last year; FVC, Forced vital capacity; FEV₁, Forced expiratory volume in one second. Lung function parameters were measured at the best of three attempts as a percentage of the reference values.

ID	Sex	Age	Exa.	Drug per day	FVC (%)	FEV ₁ (%)
12	F	9	3	Budesonide + Formoterol (480 + 18 µg)	70	62
14	M	16	2	Budesonide + Formoterol (640 + 18 µg)	95	75
17	F	8	3	Fluticasone + Salmeterol (200 + 100 µg)	91	76
18	M	13	5	Budesonide + Formoterol (640 + 18 µg)	79	71
				Montelukast (5mg)		
19	F	10	3	Budesonide + Formoterol (320 + 18 µg)	90	73
20	M	12	7	Budesonide + Formoterol (480 + 13.5 µg)	93	83
				Theophylline + Lansox (500 + 15 mg)		
38	F	11	>8	Fluticasone (200 µg)	60	52
				Montelukast (5 mg)		
40	F	7	4	Fluticasone + Salmeterol (200 + 100 µg)	112	92
41	M	10	3	Budesonide + Formoterol (160 + 9 µg)	89	80
46	M	7	5	Fluticasone + Salmeterol (200 + 100 µg)	82	64
				Montelukast (5mg)		
54	F	11	8	Fluticasone + Salmeterol (500 + 100 µg)	91	73

Figures

Figure S1. Score scatter plot in cross validation of the OPLS-DA model based on the subdivision of 53 samples between asthmatics (triangle) and healthy controls (dots). In sequence, each sample was excluded from the training set of the model and it was used as test sample estimating its score value in cross validation (t_{cv}).

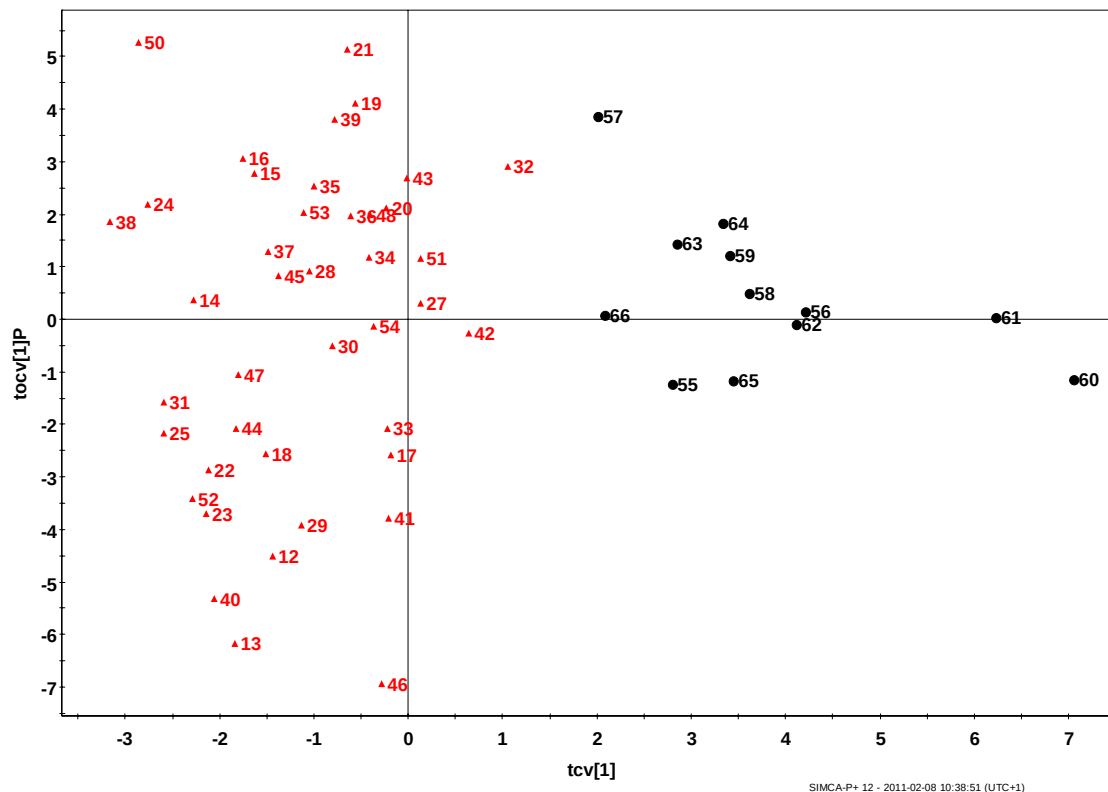


Figure S2. Score scatter plot in cross validation of the OPLS-DA model based on the subdivision of 41 samples between patients that does not take controller drugs (triangle) and patients under controller drugs (dots). In sequence, each sample was excluded from the training set of the model and it was used as test sample estimating its score value in cross validation (t_{cv}).

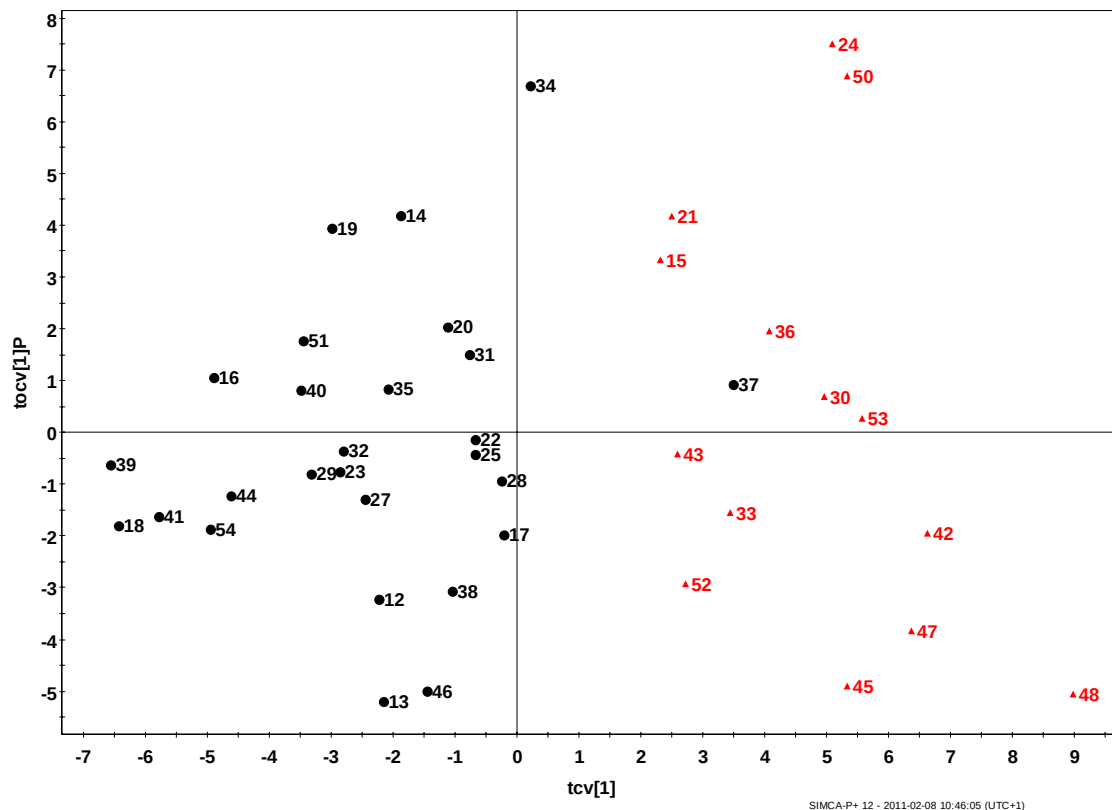


Figure S3. Score scatter plot in cross validation of the OPLS-DA model based on the subdivision of 27 samples between patients well controlled under controller drugs (triangle) and patients poorly controlled under controller drugs (dots). In sequence, each sample was excluded from the training set of the model and it was used as test sample estimating its score value in cross validation (t_{cv}).

