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Title page

Exhaled Volatile Organic Compounds as Markers for Medication Use in Asthma

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ABSTRACT

Rationale

Asthma is a heterogeneous condition, characterized by chronic inflammation of the airways, typically managed with inhaled bronchodilators and corticosteroids. In case of uncontrolled asthma, oral corticosteroids (OCSs) are often prescribed. Good adherence and inhalation technique are associated with improved outcomes, however it is difficult to monitor appropriate drug intake and effectiveness in individual patients. Exhaled breath contains thousands of volatile organic compounds (VOCs) that reflect changes in the body's chemistry, and may be useful for monitoring drug pharmacokinetics and pharmacodynamics. We aimed to investigate the association of exhaled VOCs in severe asthma patients from the U-BIOPRED cohort (by gas chromatography-time-of-flight-mass spectrometry) with urinary levels of salbutamol and oral corticosteroids (OCS) (by liquid chromatography-high resolution mass spectrometry).

Methods

Samples were collected at baseline and after 12-18 months of follow-up. Statistical analysis was based on univariate and multivariate modeling, followed by area under the receiver operating characteristics (AUROC) calculation. Results were verified through longitudinal replication and independent validation.

Results

Data were available for 78 patients (baseline: n=48; replication: n=30; validation: n=30). Baseline AUROCs (95% CI) were 82.1 (70.4-93.9) for salbutamol and 78.8 (65.8-91.8) for OCS. These outcomes could adequately be replicated and validated. Additional regression analysis between qualified exhaled VOCs and urinary concentrations of salbutamol and prednisone, showed statistically significant correlations ($p<0.01$).

Conclusion

In summary, we have linked exhaled VOCs to urinary detection of salbutamol and OCS. This merits further development of breathomics into a point of care tool for therapeutic drug monitoring.

INTRODUCTION

Asthma is a heterogeneous disease, characterized by variable airflow obstruction and chronic inflammation of the airways. Control of asthma in general is managed with inhaled bronchodilator therapy and inhaled corticosteroids (ICS). In uncontrolled asthma and acute exacerbations short courses of oral corticosteroids (OCS) are often prescribed, whilst in patients with severe disease maintenance therapy with OCS can be required [1]. Good adherence and adequate inhalation technique are associated with improved outcomes, however it is difficult for clinicians to monitor appropriate medication intake and drug effectiveness in individual patients [2, 3].

Besides pulmonologists, the World Anti-Doping Agency (WADA) and the International Olympic Committee (IOC) are interested in monitoring asthma treatments because of ergogenic effects. Although most bronchodilators and steroids are prohibited in-competition and require a Therapeutic Use Exemption (TUE), usage of both drugs is still under debate. An important element of this discussion is the significant variability among individuals in the metabolism of the drugs [4-6]. Therefore, techniques suitable for monitoring these drugs in both athletes and patients are needed.

Exhaled breath contains thousands of volatile organic compounds (VOCs) that reflect changes in the body's chemical metabolism, since they are by-products of metabolic and pathological processes at cellular and tissue levels. Metabolomic analysis of expired VOCs (breathomics) is commonly based on analytical methods such as gas chromatography and mass spectrometry (GC-MS) and/or cross-reactive gas-sensor electronic nose (eNose) technology and is suggested to be used for: 1) diagnosis of disease, 2) disease phenotyping,

3) prediction of exacerbations and 4) prediction of treatment response [7-11]. Furthermore it has been shown that some exhaled VOCs exhibit characteristic behavior shortly after the onset of exercise [12].

Since our lungs are in direct contact with the blood circulation, the analysis of exhaled volatiles may also be employed to monitor uptake and distribution of drugs [13, 14]. As breath sampling is non-invasive, (virtually) inexhaustible and, for the purposes of the Human Tissue Act, constitutes a “non-biological material”, it potentially is a preferable source to detect biological responses and metabolism kinetics of drugs than blood and urine. Despite all this, the analysis of exhaled VOCs for pharmacokinetic purposes has thus far received limited attention [13, 14]

Given that breathomics has real potential in the field of pharmacokinetics, we hypothesized that there is an association between markers of recent drug usage in urine, as current gold standard, and exhaled VOCs. We therefore aimed to detect asthma medications in urine of severe asthma patients from the U-BIOPRED project [15] by liquid chromatography–mass spectrometry (LC-MS) and to link those to exhaled VOCs or breath profiles measured by GC-MS.

METHODS

Participants. In this study, data from an unselected subset of adult subjects (aged ≥18 years) participating in the pan-European U-BIOPRED cohort study were included [15]. All participants were diagnosed with severe asthma according IMI-criteria [16]. In short, patients were prescribed high dose ICS [at least 1000 μg per day fluticasone propionate (FP) or equivalent] plus at least one other controller medication and were defined uncontrolled according to Global Initiative for Asthma (GINA) guidelines and/or had at least two severe exacerbations in the preceding year and/or required prescription of daily oral OCS (maintenance) to achieve asthma control. Potential participants were excluded if they had an exacerbation in the previous month prior to enrollment. The study was approved by all local medical ethics committees and all patients gave written informed consent. The study was registered at ClinicalTrials.gov identifier: NCT01976767.

Design. The U-BIOPRED study in adults comprised three visits as reported in detail elsewhere [15]. At the first visit participants were screened for eligibility to participate according to the inclusion and exclusion criteria. Of relevance to the present study several measurements were performed. In rank of order according to study protocol, the assessments during baseline visit (second visit) and at 12-18 months follow-up visit (third visit) were: urine sampling and completion of questionnaires, followed by pre- and post-bronchodilator spirometry testing. Next, fractional exhaled nitric oxide (FE_{NO}) was measured and exhaled breath collected, which was within 60 minutes from urine sampling. Finally, blood was drawn and allergy tests were carried out, all as outlined previously [15]. All baseline measurements were defined as training set, while 12-18 months follow-up samples were split into replication (revisits) and a validation (previously unsampled patients) sets.

Methods of measurement

Exhaled breath collection and analysis. Exhaled breath was collected at eight participating sites using a previously described method [9, 10]. In short, patients breathed for 5 min at tidal volume through a two-way non-rebreathing valve and an organic compound filter (A2, North Safety, Middelburg, Netherlands). Next, the subject exhaled a single vital capacity volume into a 10 L Tedlar bag (SKC Inc, Eighty Four, PA, USA). The exhaled VOCs were then trapped on sorbent tubes containing Tenax (Tenax GR SS 6mm x 7", Gerstel, Mülheim an der Ruhr, Germany), by pulling the air through the Tenax tube using a peristaltic pump at a flow rate of 250 ml/min. Such storage of VOCs preserves the exhaled marker signal [17].

At the Philips research lab (Philips research, Eindhoven, The Netherlands), Tenax tubes were thermally desorbed (Gerstel TDS, Mülheim an der Ruhr, Germany) using helium as carrier gas. The sample was transmitted to a packed liner, heated to 300°C for 3 min and subsequently transferred to a Tenax TA cold trap (at −150°C), which was heated after 2 min to 280°C at 20°C/s and splitless injected onto the chromatographic column. Compounds were separated using capillary gas-chromatography with helium as a carrier gas at 1.2 mL/min (7890 N GC, Agilent, Santa Clara, CA, USA) on a VF1-MS column (30 m × 0.25 mm, film thickness 1 µm, 100% dimethylpolysiloxane, Varian Chrompack, Middelburg, The Netherlands). The temperature of the gas chromatograph was adjusted in three steps: 40°C for 5 min, increased until 300°C with 10°C/min, and finally held isothermally for 5 min. A Time-of-Flight mass spectrometer (LECO Pegasus 4D, LECO, Sint Joseph, MI, USA), in electron ionization mode at 70eV, was used for the detection of product ions (ranging from 29 to 400 Da).

Urine collection and analysis. Before the visit, patients were asked not to take food and drinks that were diuretic. The urine sample was split into five 8 mL tubes (Sarstedt AG & Co, Nümbrecht, Germany). The capped tubes were immediately stored in vertical position in a freezer (-20˚C).

All urine samples were analyzed at the department of Medical Biochemistry and Biophysics of the Karolinska Institutet (Karolinska Institutet, Stockholm, Sweden). The sample preparation for determining fragments or metabolites of salbutamol and corticosteroids was performed on a robotic liquid-handling platform (Microlab STAR, Hamilton Robotics, Bonaduz, Switzerland). The corticosteroids and the salbutamol were prepared from an aliquot of urine fortified with internal standards and subsequently hydrolyzed using β-glucuronidase (E-coli). Purification was performed using mixed mode solid phase extraction (SPE) in 96 well plate format. The analysis of the extract was performed with reversed phase liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS, Thermo Q-Exactive, Thermo Fisher Scientific Inc, Waltham, USA). Acquisition of raw LC-HRMS data was performed in full scan mode at a resolution of 35000 with polarity switching.

As a next step two summarizing, dichotomous variables were established regarding the observation of salbutamol and corticosteroid residues in a sample. Presence of corticosteroids was determined based on the remains of prednisone and/or prednisolone and/or methylprednisolone and/or 16α-hydroxy-prednisolone and/or 20β-dihydro-prednisolone [18, 19]. These two outcomes were used to split the patients into groups (salbutamol detected: yes/no and corticosteroids detected: yes/no) for statistical analysis. The limit of detection (LoD) for all these compounds was 1 ng/mL [20], with an expected terminal half-life of 4-6 hours for salbutamol and 2-4 hours for prednisolone [21].

Lung function. Spirometry was executed before and 10 minutes after intake of 400 µg of salbutamol via a spacer according to ERS recommendations using daily-calibrated lung function equipment [22].

Blood. Blood eosinophils and neutrophils percentages were obtained from standard complete blood counts.

Allergic status. Allergy testing was performed using total and specific serum IgE and skin prick test (SPT) to a panel of common aeroallergens. Atopy was defined as the presence of sensitization on SPT (wheal ≥ 3 mm) or serum specific IgE (≥ 0.35 kU/L)

$FE_{NO.}$ Fractional exhaled lower respiratory nitric oxide was measured with a portable device (NIOX Mino System; Aerocrine, Solna, Sweden) at a constant flow rate of 50 mL/s, according to ATS/ERS recommendations [23].

Analysis

Data cleaning and reduction. As a first step in preprocessing, raw GC-MS files were converted into netCDF files, subsequently peak alignment was realized using XCMS [24]. After ComBat batch correction [25] on the complete dataset of both baseline and 12-18 months follow-up samples, GC-MS data was divided into a training, replication and validation set. Subsequently, datasets were normalized by adjusting the average and standard deviation of each individual GC-MS fragment to respectively 0 and 1.

Univariate analysis. To select potentially relevant VOCs for the distinction between the drugs-detected *versus* no-drugs-detected groups, we applied a strategy reported by van Oort *et al* [26]. This applies Wilcoxon sum-rank testing combined with computation of area under

the receiver operating characteristics (AUROC) for each GC-MS fragment. Features with a p-value < 0.05 and an AUROC ≥ 0.7 were retained for further analysis. After filtering for multi-collinearity through correlation analysis (r ≥ 0.95), spectra of GC-MS features retained after univariate analysis were provisionally identified based on NIST–library (v.2014) matching. Compounds determined as instrument artefacts i.e. silanes and siloxanes [27], or Tedlar bag related substances [28] were considered as contaminants and removed for further analysis.

Multivariate analysis. A sparse partial least square (SPLS) analysis [29] was utilized on persevered GC-MS fragments in order to reveal most optimal set of features for discrimination between groups of interest. Optimal modeling settings for *eta* (thresholding parameter) and K (number of hidden components) were objectively defined by the algorithms with 10-fold cross validation. Next a composite score of the final set of selected variables was calculated via a leave-one-out linear discriminant analysis. The diagnostic performance of the (breath based) discriminant score *versus* urine LC-MS outcomes (drugs-detected or no-drugs-detected) was tested by calculation of the AUROC combined with 95% confidence interval (CI).

Validation. Baseline outcomes were validated by rerunning final two steps of analysis, linear discriminant score and AUROC calculation on two datasets: follow-up samples of baseline patients (*replication*) and an independent group of follow-up patients lacking paired (breath and urine) baseline samples (*validation*).

Correlation analysis. Finally the association between univariate preserved features and urinary concentrations of salbutamol, prednisone and prednisolone were determined on all samples (baseline + follow-up) together. After SPLS modeling, bootstrapped linear regression analysis (1000 replicates) was applied to test robustness of the models.

All analyses were performed in RStudio (v.1.0.136; RStudio Team (2016). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA; http://www.rstudio.com) using R (v.3.3.3; R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org) as engine and supported by R packages: XCMS, data.table, MASS, pROC, caret, spls, sva.

RESULTS

Complete data on both breath and urine were available for 48 baseline and 60 follow-up visits, occurring at eight research sites across five countries in Europe. Patient characteristics are summarized in Table 1. A flowchart on patient enrolment is shown in Figure 4

There was no complete overlap between the patients from who baseline and follow-up data was available. During the follow-up visits at 12-18 months, 30 patients were measured for the second time (called the "replication" set) and 30 patients for the first time (called the "validation" set). Of patients within the replication set, a few had changed medication regime after the baseline visit. Four of them were withdrawn from OCS, another four were prescribed an increased OCS dose and one patient was withdrawn from long-acting β2-agonists usage.

Univariate analysis. Analysis of the 48 baseline breath samples resulted in the detection of 7115 ion fragments. After preprocessing and combining outcomes of both Wilcoxon sum-rank (p<0.05) and AUROC (AUC>0.7) test, the number of discriminatory features for GC-MS breathomics in regard to traces of salbutamol and OCS in urine (yes/no) by LC-MS, were respectively 79 and 28. Out of these 107 fragments, 48 were excluded either due to multi-collinearity (n=26), or were considered to represent either contaminants (n=13) or internal standards (n=9), which resulted in 49 'salbutamol' and 10 'OCS' related fragments.

Multivariate analysis. After SPLS modeling, seven discriminatory compounds remained for salbutamol and four for OCS [Table 2].

The subsequent linear discriminant score calculation resulted in final baseline AUROCs of 82.1 (95%CI: 70.4-93.9) % for GC-MS breath vs. LC-MS urine (salbutamol detected: yes/no) and 78.8 (95%CI: 65.8-91.8) % for GC-MS breath vs. LC-MS urine (OCS detected: yes/no). These outcomes were replicated and validated using the two longitudinal datasets, resulting in outcomes for salbutamol of: replication 67.0 (95%CI: 46.0-88.0) % and validation 90.9 (95%CI: 78.9-100.0) % and for OCS: replication 70.1 (95%CI: 50.8-89.5) % plus validation: 82.1 (95%CI: 67.2-97.1) % [Table 2 + Figure 1 + Figure 2].

Association with urinary levels of salbutamol and OCS. SPLS modeling followed by regression analysis among classifying GC-MS features and urinary concentrations of salbutamol, prednisone and prednisolone, resulted in three linear models with R^2 of respectively: 0.10 (95%CI: 0.00-0.23) , 0.63 (95%CI: 0.33-0.94) and 0.29 (95%CI: 0.05-0.48), all $p<0.01$. The most stable model (association with prednisone), was based on four compounds: lysine [retention time: 899.17 seconds], glycolic acid [retention time: 952.50 seconds], 4-carene [retention time: 1011.67 seconds] and octanal [retention time: 1046.48 seconds] [Table 3 & Figure 3].

DISCUSSION

The present study examined the link between exhaled VOCs by GC-MS and traces of asthma medications in urine of severe asthma patients as measured by LC-MS. The distinction between the urine based drugs-detected vs. no-drugs-detected groups by the exhaled marker models resulted in moderate to good AUROCs of approximately 80% for both OCs and salbutamol. Both outcomes could be replicated (revisits) and validated (previously unsampled patients) using samples obtained during 12-18 months follow-up visits. Our results indicate that profiles of exhaled VOCs might be useful for monitoring drug levels. These outcomes should be extended towards time series analysis, which argues for pharmacokinetic pathway and dose ranging studies on asthma medication. Additionally, expanding to other types of asthma medication like ICS and long-acting β2-agonists would strongly increase the added value of the proposed method of drug level monitoring.

To our knowledge, this is the first study examining exhaled VOCs in relationship with drug usage as detected by urinary metabolites in asthma. The novelty of the present study is represented by linking two sources of excretion, urine and exhaled VOCs regarding the metabolism of asthma medication. Previously breath profiles have been linked to the metabolic effects of inhaled salbutamol [30] and eucalyptol [31] during a controlled drug regimen. The present study takes an additional step by relating exhaled metabolomics to urinary traces of the drugs.

One of the strengths of this study is the availability of a longitudinal replication dataset and an independent validation cohort. Secondly, the analytical techniques for both urine and breath analysis were highly sophisticated and performed in experienced laboratories.

Thirdly, patient grouping regarding the usage of asthma medications was based on detection of urinary metabolites by LC-MS, which might be more accurate than patient self-reporting or prescriptions [32]. Finally this work arises from a pan-European study, with data originating from five different countries. Despite the multicenter and cross-border character of this study, which may have introduced variation within data, a link between urine and exhaled markers could be revealed. This may be a result of well documented standardized operating protocols and the application of a thoroughly tested breath collection technique [9, 10].

We realize that this study has several limitations. Firstly, due to the observational of this study the specific time and dose of medication intake, and levels of hydration and nutrition are uncertain. Which is emphasized by the relative poor agreement between prescription of maintenance oral corticosteroids and the urinary prednisolone positive patients within the training set. Additionally, there is a natural variability among individuals in the metabolism of the drugs [4, 5]. Despite those limitations, a link between systemic markers of drug uptake in urine and exhaled markers was revealed which could be replicated and validated. Furthermore, since adherence within this study was based on a direct urinary measurement, the time of dosing is less relevant. A second limitation is related to the correlation analysis between urine and breath, which could only include patients with traces of medication in their urine. As consequence of that, the number of samples were decreased in comparison to the first series of analyses of the study. This may have affected the robustness of the model, as represented by wide 95% confidence intervals. Therefore, the outcomes of our regression analysis should be cautiously interpreted. Finally, the results obtained within the replication group are lower than those of the training and validation set. Although we

couldn't find a direct cause for this, we assume that a minor (technical) variation, exhaled breath or urine related, among a few patients within the relatively small group (*n*=30) affected the results. The adequate outcomes of the validation set, which builds upon the models of the trainings set too, strengthens such assumption.

How can we explain our findings? Out of eleven GC-MS features adopted in the two distinction models, two compounds are associated with downstream metabolic pathways. This concerns methyl-acetate for salbutamol and octanal for OCS, which are reported in the KEGG database as products of carbohydrate pyruvate metabolism [33] and an aldehyde product of lipid peroxidation [33], respectively. These outcomes fit earlier findings regarding changes in metabolic energy processes after intake of both drugs [30, 34, 35]. Regarding the compounds fostered in the presented regression analysis, lysine and glycolic acid are of interest. Lysine, an essential amino acid, might have a link to the activation of genes by corticosteroids [36], while glycolic acid shows a remarkable similarity with the $COCH_2OH$-tail of prednisone. From the remaining set of compounds included in the final models, butyrolactone and 4-carene are described as potential biomarkers for respectively lung- and breast cancer [37, 38], however to our best knowledge no direct link to asthma nor medication usage has been reported yet. Closely related to 4-carene in their chemical structure are 3-carene and 3-carane, interestingly these two VOCs have been associated with lung infections [39]. Despite possible usage as flavor additive (levomenthol and carvone) or a compound emitted by a microorganism (chlorobenzene) [40], no direct nor indirect link was found yet for the other reported compounds.

What are the clinical implications of our data? The current study presents an association between excreted traces of asthma medication in urine and exhaled breath metabolomics. Therefore, breath might be a more favorable source to analyze biological responses and pharmacokinetics of asthma drugs than blood and urine, especially since it is non-invasive and practically inexhaustible. It suggests the advancement of current outcomes towards a breathomics based, quick and non-invasive point of care tool for pharmacokinetic monitoring purposes or adherence testing, as are currently undertaken for the diagnosis of asthma, COPD and lung cancer using gas-sensor driven electronic nose (eNose) technology. [11]. In addition, breathomics may qualify for rapid and frequent sampling of asthma drug use in sport science, for example testing misuse of drugs by athletes at real-time.

In conclusion, metabolomics of exhaled breath is associated with markers of recent drug usage in urine. This suggests that breathomics qualifies for development into a point-of-care tool for monitoring drug level changes in both patients and athletes.

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CONFLICT OF INTEREST/DISCLOSURE

Disclosure of potential conflict of interest: PB, CEW, SED, PM, PJS and SJF received grant support from Innovative Medicines Initiative. RGK consults through Knowles Consulting Ltd for a variety of companies and Universities, and particularly for Peptinnovate Ltd as CSO. AHM reports grants from Boehringer Ingelheim, AstraZeneca, GlaxoSmithKline and Novartis outside the submitted work. WMA, CG, HHK, HW, TJV, TMN and SJV have no relevant conflict no interest to disclose.

AUTHORS CONTRIBUTIONS

PB; Study design, dry lab analysis and wrote the manuscript. WMA; Dry lab analysis and amended the manuscript. CG; Wet and dry lab analysis (LC-MS). HHK; Wet lab analysis (GC-MS). HW; Dry lab analysis (GC-MS). TJV; Contributed to GC-MS analysis. TMN; Contributed to GC-MS analysis. CEW; Contributed to LC-MS analysis. SED; Contributed to LC-MS analysis. PM; Contributed to sample collection. RGK; Contributed to study design. SJV; Amended the

manuscript. AHM; Amended the manuscript. PJS; Study design and amended the manuscript. SJF; Study design and wrote the manuscript.

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TABLES

Table 1: Demographic data an baseline characteristics of study population

	Training	Replication	Validation	p-value
Subjects; *n*	48	30	30	
Age; years [mean ± SD]	52.3 ± 14.1	54.9 ± 11.9	56.2 ± 10.1	0.38
Gender; female [%]	52.1	50.0	60.0	0.71
Body Mass Index (kg/m2) [mean ± SD]	30.5 ± 5.8	30.7 ± 5.6	28.7 ± 6.3	0.32
Smoking status; current / ex- / non-smoker [%]	2.1 / 43.8 / 54.2	3.3 / 43.3 / 53.3	16.7 / 33.3 / 50.0	0.12
Pack years [median | IQR]	10.0 | 4.1 - 16.5	8.5 | 4.1 - 14.4	21.0 | 11.0 - 35.0	0.02
ICS(-LABA) prescription [%]	100	100	100	NA
OCS prescription [%]	64.6	56.7	56.7	0.71
Total daily OCS dose [median | IQR]	10.0 | 9.4 - 15.0	10.0 | 7.5 - 15.0	10.0 | 5.0 - 20.0	0.95
Short Acting β-Agonist prescription [%]	56.3	53.3	56.7	0.96
Long Acting β-Agonist prescription [%]	37.5	40.0	33.3	0.86
Leukotriene Modifiers prescription [%]	47.9	36.7	40.0	0.59
Anti-IgE therapy prescription [%]	20.8	23.3	16.7	0.81
Urinary prednisolone positive [%]	47.9	56.7	53.3	0.74
Urinary salbutamol positive [%]	29.2	43.3	20.0	0.14
Exacerbations/year [median | IQR]	1.00 | 1.00 – 2.25	1.50 | 0.00 - 2.75	1.00 | 0.00 - 2.75	0.60
Atopy positive [%]	66.7	63.3	66.7	0.95
ACQ; Juniper [median | IQR]	1.86 |1.43 - 3.00	1.64 |1.29 - 2.86	2.00 |1.18 - 3.18	0.85
$PbFEV_1$ % predicted [mean ± SD]	76.8 ± 23.7	80.8 ± 21.6	75.4 ± 24.7	0.65
$PbFEV_1$/FVC actual ratio [mean ± SD]	77.7 ± 18.9	76.0 ± 15.6	78.4 ± 19.8	0.87
$F_{E_{NO}}$; ppb [median | IQR]	33.5 | 20.0 – 53.0	37.8 | 19.6 – 55.3	18.5 | 13.5 – 27.5	<0.01
Blood eosinophils; 10^9/L [median | IQR]	0.17 | 0.10 - 0.37	0.18 | 0.60 - 0.43	0.20 | 0.09 - 0.40	0.99
Blood neutrophils; 10^9/L [median | IQR]	5.31 | 4.08 - 8.01	6.27 | 4.79 - 7.67	6.24 | 4.85 - 7.61	0.60

OCS prescription, require prescription of daily or alternate day oral corticosteroids; Total daily OCS dose, daily OCS dose normalized to prednisolone, amongst OCS users; Short Acting β-Agonist prescription, require prescription of daily short acting β-Agonists; Long Acting β-Agonist prescription, require prescription of daily long acting β-Agonists; Leukotriene modifiers prescription, require prescription of daily leukotriene modifiers; Anti-IgE therapy prescription, require prescription of anti-IgE therapy at weekly or monthly basis. Urinary prednisolone positive, prednisolone traces in urine detected by LC-HRMS; Urinary salbutamol positive, salbutamol traces in urine detected by LC-HRMS; Atopy, presence of sensitization on SPT (wheal ≥ 3mm) or serum specific IgE (≥ 0.35 kU/L); ACQ, Juniper - Asthma Control Questionnaire; PbFEV1, Post-bronchodilator Forced Expiratory Volume in one second; FVC, Forced Vital Capacity; $F_{E_{NO}}$, Fraction of Exhaled Nitric Oxide in parts per billion. Differences between groups were tested using ANOVA (parametric) or Kruskal-Wallis (non-parametric) testing for continous data and the Pearson's Chi-squared tests for categorical data.

Table 2: Adopted GC-TOF-MS features into the two (salbutamol detected: yes/no and corticosteroids detected: yes/no) distinction models after SPLS modeling.

Compound name	Ret.time (sec)	salbutamol	oral corticosteroids
Methyl acetate	498.90	X	
1-propanol	527.37	X	
Butanal	561.85	X	
Methyl propionate	611.23	X	
3-methyl-Butanal	639.07	X	
Chloro-benzene	896.80		X
Butyrolactone	929.10	X	
Glycolic acid	952.50		X
Octanal	1046.48		X
Levomenthol	1236.51		X
Carvone	1301.60	X	

Compound name, most probable compound based on NIST-library; Ret.time, retention time of fragment in seconds; salbutamol, fragments retaining after SPLS modeling of on traces of salbutamol in urine; oral corticosteroids, fragments retaining after SPLS modeling on traces of oral corticosteroids in urine.

Table 3: GC-TOF-MS features adopted into the SPLS based model used for the bootstrapped linear regression analysis between exhaled markers and concentrations of prednisone in urine.

Compound name	Ret.time
Lysine	899.17
Glycolic acid	952.50
4-Carene	1011.67
Octanal	1046.48

Compound name, most probable compound based on NIST-library; Ret.time, retention time of fragment in seconds.

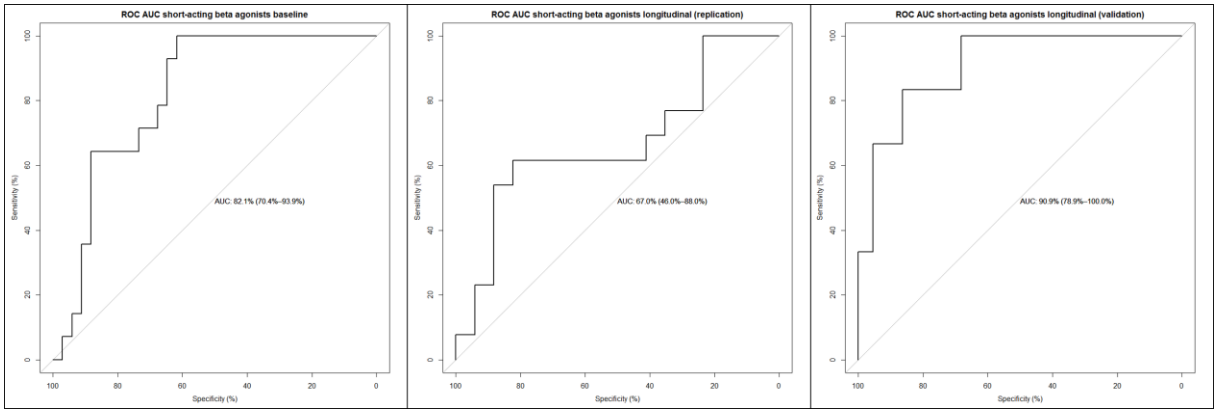


Figure 1: AUROC's: GC-MS breath profiles vs. LC-MS urine (salbutamol detected YES/NO), on baseline and validation dataset. Baseline: 82.1 (95%CI: 70.4-93.9), replication: 67.0 (95%CI: 46.0-88.0%) and validation: 90.9 (95%CI: 78.9-100.0).

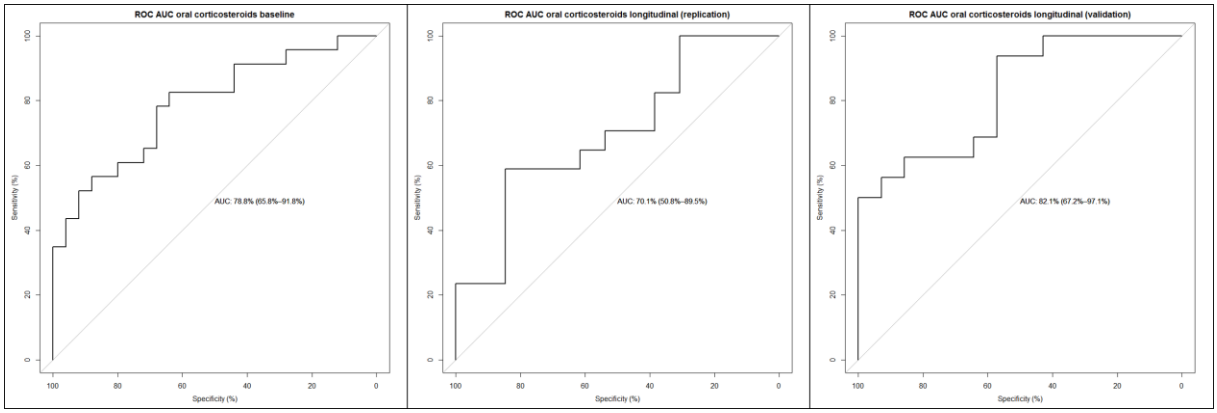


Figure 2: AUROC's: GC-MS breath profiles vs. LC-MS urine (OCS detected YES/NO), on baseline and validation dataset. Baseline: 78.8 (95%CI: 65.8-91.8), replication: 70.1 (95%CI 50.8-89.5) and validation: 82.1 (95%CI: 67.2-97.1).

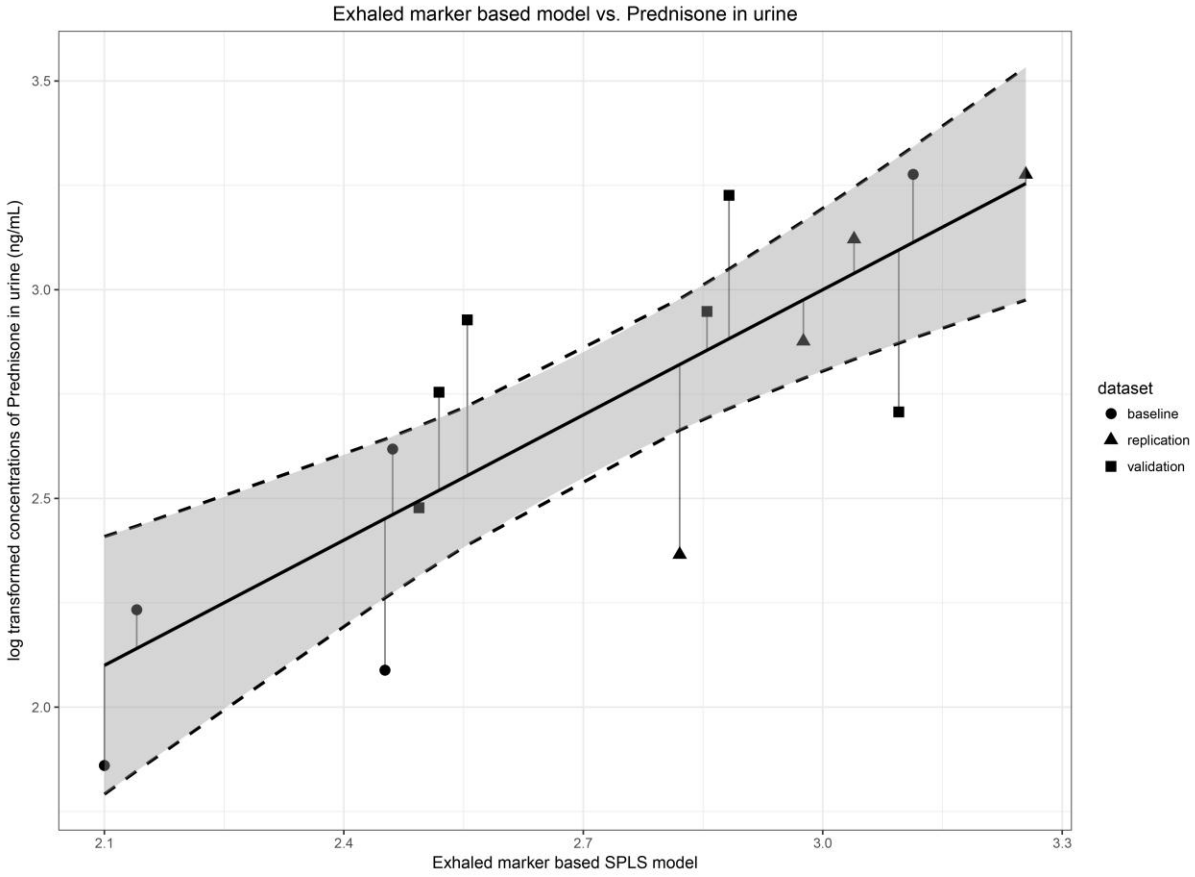
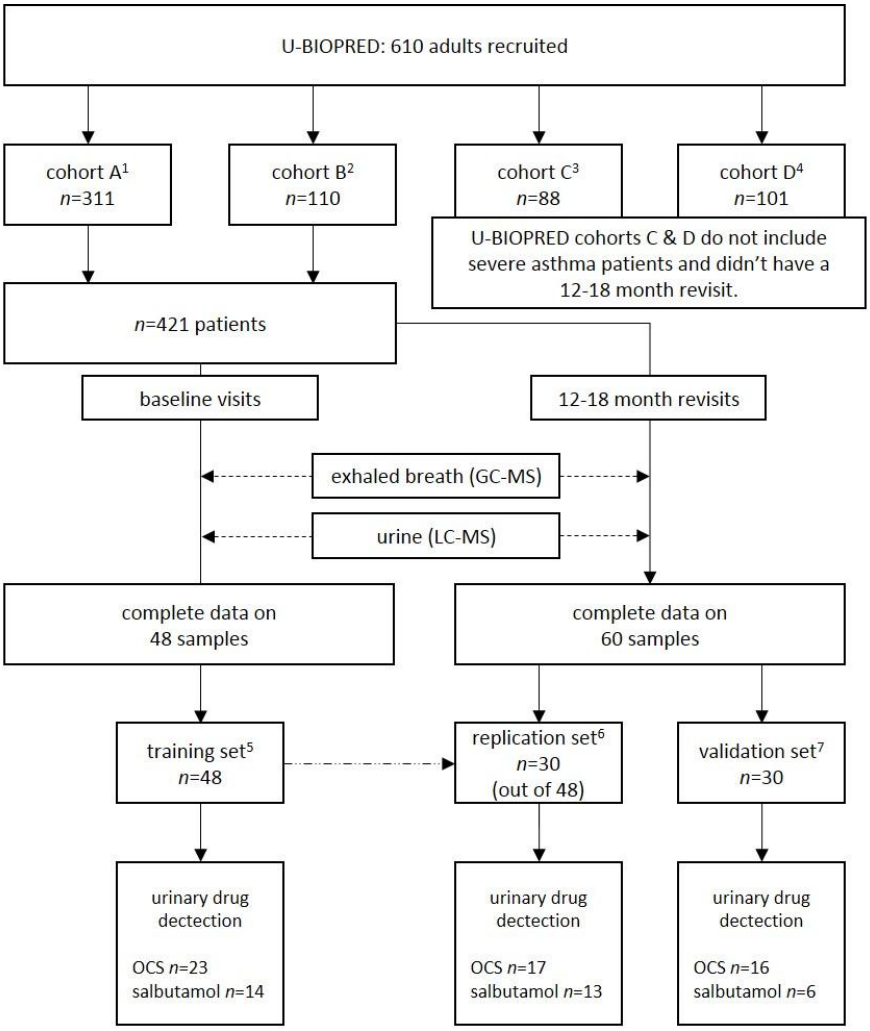


Figure 3: Associations between exhaled marker based SPLS model vs. log transformed concentrations of prednisone in urine (ng/mL) by linear regression analysis (R2=0.63, 95%CI(0.33-0.94) p<0.01), n=15.



[1]cohort A: severe nonsmoking asthma (<5 pack years)
[2]cohort B: smokers and ex-smokers with severe asthma (>5 pack years)
[3]cohort C: mild/moderate nonsmoking asthmatics
[4]cohort D: healthy nonsmoking controls
[5]training set: all baseline samples of patients from cohort A & B
[6]replication set: all available 12-18 month revisit samples of patients included in the training set
[7]validation set: all available 12-18 month revisit samples of patients without a baseline sample

Figure 4: A flowchart of the patients enrolled in the study.

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