ACCEPTED MANUSCRIPT

Exhaled breath condensate as a potential biomarker tool for idiopathic pulmonary fibrosis - a pilot study

To cite this article before publication: Barbara Rindlisbacher *et al* 2017 *J. Breath Res.* in press <https://doi.org/10.1088/1752-7163/aa840a>

Manuscript version: Accepted Manuscript

Accepted Manuscript is "the version of the article accepted for publication including all changes made as a result of the peer review process, and which may also include the addition to the article by IOP Publishing of a header, an article ID, a cover sheet and/or an 'Accepted Manuscript' watermark, but excluding any other editing, typesetting or other changes made by IOP Publishing and/or its licensors"

This Accepted Manuscript is © 2017 IOP Publishing Ltd.

During the embargo period (the 12 month period from the publication of the Version of Record of this article), the Accepted Manuscript is fully protected by copyright and cannot be reused or reposted elsewhere.

As the Version of Record of this article is going to be / has been published on a subscription basis, this Accepted Manuscript is available for reuse under a CC BY-NC-ND 3.0 licence after the 12 month embargo period.

After the embargo period, everyone is permitted to use copy and redistribute this article for non-commercial purposes only, provided that they adhere to all the terms of the licence <https://creativecommons.org/licenses/by-nc-nd/3.0>

Although reasonable endeavours have been taken to obtain all necessary permissions from third parties to include their copyrighted content within this article, their full citation and copyright line may not be present in this Accepted Manuscript version. Before using any content from this article, please refer to the Version of Record on IOPscience once published for full citation and copyright details, as permissions will likely be required. All third party content is fully copyright protected, unless specifically stated otherwise in the figure caption in the Version of Record.

View the [article online](#) for updates and enhancements.

**Exhaled breath condensate as a potential biomarker tool for
idiopathic pulmonary fibrosis- a pilot study**

Barbara Rindlisbacher^{1*}, Carina Strebel^{2*}, Sabina Guler³, Attila Kollár⁴, Thomas Geiser³, Georg
Martin Fiedler¹, Alexander Benedikt Leichtle¹, Cédric Bovet¹ und Manuela Funke-Chambour³

¹University Institute of Clinical Chemistry, Inselspital, Bern University Hospital, University of Bern,
Switzerland

²Hospital of Zweisimmen, Zweisimmen, Switzerland

³Department of Pulmonary Medicine, Inselspital, Bern University Hospital, University of Bern, Bern,
Switzerland

⁴Department of Oncology, Inselspital, Bern University Hospital, Bern, Switzerland

*both authors contributed equally to this manuscript

§Corresponding author: Manuela Funke-Chambour, MD, Department of Pulmonary Medicine,
Inselspital, University Hospital Bern, CH-3010 Bern, E-mail: manuela.funke-chambour@insel.ch.
Phone: +41-31-632-2111, Fax: +41-31-632-1404

E-mail: carina.strebel@spitalstsag.ch, barbara.rindlisbacher@insel.ch, sabina.a.guler@gmail.com,
Attila.Kollar@insel.ch, martin.fiedler@insel.ch, alexander.leichtle@insel.ch,
Thomas.Geiser@insel.ch, cedric.bovet@insel.ch, Manuela.Funke-Chambour@insel.ch

Keywords

Idiopathic pulmonary fibrosis, exhaled breath condensate, mass spectrometry

Abstract

Idiopathic pulmonary fibrosis (IPF) is a devastating lung disease with poor survival. There is an urgent need to better diagnose and monitor IPF patients as new treatments which slow down disease progression are now available. Exhaled breath condensate (EBC) is easily and non-invasively collected, but analysis of potential biomarkers is difficult due to low concentrations and methodological limitations. We used a non-targeted metabolomics approach to identify discriminatory metabolic profiles that distinguish IPF patients from healthy controls.

For the pilot study set, we collected EBC from 10 stable IPF patients and 10 lung healthy controls. Samples were analyzed by ultra high-performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-HRMS) in positive and negative ion mode. After data processing and statistical analysis, 58 metabolites were found to be discriminative between IPF patients and controls in the positive ion mode. One metabolite candidate $m/z = 341.3514$ at a retention time of 9.94 min was 2.5-fold up-regulated in IPF patients compared to healthy controls and validated in a second set of eight IPF patients and healthy controls. The identity of this metabolic feature still remains elusive.

Our preliminary results identified a distinguished EBC profile of IPF patients compared to controls. Although these results need to be confirmed in additional individuals, EBC sampling for diagnosis and/or monitoring of IPF patients is a promising, new method, which should be further explored. The EBC samples have been obtained within the clinical trial NCT02173145 at baseline.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

44 **1. Introduction**

45 Idiopathic pulmonary fibrosis (IPF) is a progressive lung disease affecting mainly men over 60 years
46 [1]. Patients present with unspecific clinical symptoms like worsening dyspnea during exercise and
47 dry cough [1]. Clinical course and prognosis vary, ranging from slow progression to rapid disease
48 deterioration which ultimately leads to death, on average after two to three years [1, 2]. Diagnosis of
49 IPF requires a radiological and/or histological usual interstitial pneumonia (UIP) pattern and exclusion
50 of known causes for interstitial lung disease (ILD), which might impact treatment decisions [1].
51 Invasive diagnostic procedures, including bronchoscopy and surgical lung biopsy are often needed for
52 a confident diagnosis, but can be associated with high interventional risks, especially in patients
53 suffering from severe respiratory impairment and hypoxia [3]. In addition to diagnostic challenges, the
54 clinical course of the disease is unpredictable [2], treatment options are limited [4-6] and individual
55 treatment response is difficult to assess. Although new medications are in use, which slow down lung
56 functional decline, cure is still impossible and lung transplantation remains the ultimate option for few
57 selected patients [4, 5]. Non-invasive diagnostic and monitoring tools are urgently needed for a safer
58 and more efficient approach to IPF diagnosis, to predict the course of disease and monitor treatment
59 response. A promising solution might be the identification of new biomarkers by exhaled breath
60 analysis. [7].

61 Exhaled breath condensate (EBC) is a non - invasively collected biofluid of the respiratory tract. EBC
62 contains particles from the airway that enter EBC from the gas phase or from the alveolar lining fluid
63 [8, 9]. Similar to blood or urine, EBC represents a matrix from which biomarkers may be identified.
64 Breath monitoring by EBC sampling is simple and due to its non-invasiveness a particularly promising
65 option to collect biological samples from patients with respiratory impairment. Over the last years,
66 non-invasive methods to diagnose and monitor lung pathologies have gained increasing interest [10].

1
2
3 67 In clinical practice NO in exhaled breath of asthma patients is commonly measured to evaluate
4
5 68 treatment response [11]. Investigations of EBC in several lung diseases such as asthma [11], chronic
6
7
8 69 obstructive pulmonary disease [12-14], cystic fibrosis [15], lung cancer [16] and interstitial lung
9
10 70 diseases [11, 17] are ongoing. Different biomarker patterns in patients with smoking related lung
11
12 71 diseases and healthy smokers were identified by analyzing EBC samples [18-21] and an individual
13
14 72 metabolomic breath print is postulated [7].
15
16
17

18
19 73 The main advantage of EBC is its non-invasive collection. However, the use of EBC in clinical
20
21 74 practice is still limited by the difficulties in analysis of its composition [22]. As EBC contains highly
22
23 75 diluted molecules analytic biochemistry assays are difficult to perform. In this study, we investigated
24
25 76 EBC by ultra high-performance liquid chromatography coupled to high-resolution mass spectrometry
26
27 77 (UHPLC-HRMS), which has been recently suggested as a sensitive and selective technique to identify
28
29 78 biomarkers in EBC [23].
30
31
32
33

34 35 79 **2. Materials and Methods**

36 37 80 *2.1 Study participants*

38
39 81 All patients were recruited from the ILD clinic at the Department for Pulmonary Medicine, Inselspital,
40
41 82 Bern University Hospital, Bern, Switzerland. Main inclusion criterion for patients was definitive or
42
43 83 probable IPF diagnosis [1]. Definitive or possible UIP patterns were diagnosed on high resolution
44
45 84 chest CT scans and, if available, UIP was confirmed on lung biopsy. Secondary causes for lung
46
47 85 fibrosis were excluded and cases assessed by experienced ILD clinicians, radiologists, and
48
49 86 pathologists during multidisciplinary discussion according to the current diagnostic standards [1].
50
51 87 Mortality risk was estimated by the GAP index and staging system, which includes gender, age, forced
52
53 88 vital capacity (FVC) and diffusion capacity of the lung for carbon monoxide (DLCO) [24]. Healthy
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

89 volunteers without a history of chronic lung disease were recruited, age ranged between 61 and 86
90 years. EBC was collected from all patients and healthy controls after they signed informed consent.
91 The EBC samples from IPF patients have been obtained within the clinical trial NCT02173145 at
92 baseline. The study was performed according to human research law and approved by the local ethics
93 committee (KEK 002/14, 246/15).

94

95 *2.2 EBC Collection*

96 EBC was collected using the Ecoscreen EBC collecting device from Erich Jaeger, Hoechberg,
97 Germany, according to the manufacturer's instructions and the ATS/ERS methodological
98 recommendations for EBC collection [9]. All participants were advised not to eat at least one hour
99 before EBC collection. The cooling device was precooled (-15°C to -20°C). A common blotting paper
100 was placed inside the mouth piece just before the air enters the cooling chamber to reduce
101 contamination with saliva. Participants sat upright, wore a nose clip and performed tidal breathing
102 continuously for 10 minutes. Through cooling and condensation 3-5 ml of breath fluid was produced
103 on average. The samples were placed in aliquots and immediately stored on dry ice at -80 degrees
104 Celsius until analysis.

105

106 *2.3 Sample preparation*

107 Samples were thawed on ice and 80 µL of -20°C-cold acetonitrile:methanol (ACN:MeOH, 1:1, v/v)
108 containing chlorpropamide as internal standard (1 µg/mL) were added to 20 µL EBC. Samples were
109 shortly vortexed and stored at -80 °C for 30 minutes to precipitate proteins. Afterwards, the samples
110 were centrifuged in two consecutive steps (14'000 g at 4 °C for 20 min) to remove precipitates and
111 subsequently, the supernatants were transferred to total-recovery LC-MS glass vials (Waters, Milford,

MA, USA). A pooled quality control (QC) sample was prepared by mixing 25 μ L of individual EBC sample extracts.

114

115 *2.4 Non-targeted metabolomics analysis*

Non-targeted metabolomics analysis was performed on a 2D-UPLC I-Class system coupled to a quadrupole time-of-flight mass spectrometer (Synapt G2-S HDMS, Waters) operated in positive and negative electrospray ionization (ESI) mode. The mobile phases A and B consisted of 0.1% (v/v) formic acid in (A) 1% (v/v) MeOH in H₂O and (B) MeOH, respectively. The sample injection volume was 2 μ L and the chromatographic separation was performed on an ACQUITY UPLC HSS T3 column (1.0 mm x 100 mm, 1.8 μ m, Waters) by applying a solvent gradient from 100% A to 1% A over 11 min starting at 1 min. The solvent flow rate was set to 0.17 ml/min, the column temperature to 50°C, and the autosampler to 6°C. Standard mass spectrometric parameters were 0.5 kV and 50 V for capillary voltage and cone voltage, respectively. Desolvation and source temperature were kept at 450°C and 120°C, respectively. Cone and desolvation flows were set to 150 L/h and 800 L/h, respectively. Leucine-Enkephalin ([M+H]⁺ m/z = 556.2766, [M-H]⁻ m/z = 554.2620) was acquired every 20 s for lock mass correction. Mass spectra were acquired at a scan time of 0.2 s in the MSe resolution mode over a range of m/z 50-1200. The samples were analyzed with MSe continuum experiments in the positive and negative electrospray ionization (ESI) mode. The pooled QC sample was analyzed with a DDA experiment to obtain MS/MS spectrum information. A system suitability test (SST) containing standards was measured at the beginning and the end of the analytical run to ensure retention time stability, intensity stability and mass error $\leq$ 8 ppm. The system was equilibrated by injecting 10 times the pooled QC sample and at least 10 QC samples were analyzed in each

analytical batch. The instrument was controlled via MassLynx (version 4.1, Waters). Representative base peak ion (BPI) chromatograms of a control and a IPF patient EBC sample are presented in figure 1. The pilot study set was analyzed with one replicate per IPF patient and healthy control sample, whereas for the validation set, duplicate analysis was applied.

138

2.5 Data processing and statistical analysis

In step I, the raw mass spectrometric data were imported and processed with Progenesis QI (version 2.2, Nonlinear Dynamics, Newcastle, UK). After lock-mass correction and chromatographic alignment, ion patterns were deconvoluted between 0.45 – 11 min. As possible ions, $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$, $[M+2H]^{2+}$, $[M+3H]^{3+}$, $[2M+H]^+$, $[2M+Na]^+$ and $[2M+K]^+$ were defined in the positive mode, and $[M-H]^-$, $[M-H-H_2O]^-$, $[M-2H]^{2-}$, $[M-3H]^{3-}$, $[M+HCOOH-H]^-$ and $[2M-H]^-$ in the negative mode. Abundances of metabolic features were normalized to all compounds. Noise reduction was performed by excluding ion patterns from further analysis with a 2-fold higher abundance in blank versus QC samples, a peak width < 0.05 min, ion charge > 1 and a coefficient of variation (CV) of the peak area $> 30\%$ in the QC samples, as recommended by Dunn and colleagues [25]. Furthermore, the chromatographic window was specified between 0.5 – 10.5 min and low abundant metabolic features (normalized abundance < 100 , arbitrary threshold) were excluded. In step II, normalized abundances of the remaining features were subjected to multivariate analysis with SIMCA (version 14, Umetrics, Umeå, Sweden) for biomarker discovery. For the validation set, the features abundances of the duplicate analysis were averaged. After Pareto scaling, principle component analysis (PCA) was performed to visualize trends and detect outliers among observations in the scores plot. PCA was followed by orthogonal partial least square

discriminant analysis (OPLS-DA) including model cross-validation to compare discriminant features between healthy controls and IPF patients. Results were further integrated if the 10-fold cross-validated correlation $R^2(\text{cum})$ and $Q^2(\text{cum})$ were ≥ 0.5 , if the cross validatory ANOVA p-value was ≤ 0.05 and if the permutation test was passed (20 permutations, $R^2 > R^2_{\text{permutation}}$ and $Q^2_{\text{permutation}} < 0$) [26] [27]. The VIP-plot was used to find discriminative metabolite candidates having a loading vector $p(\text{corr}) < -0.5$ or > 0.5 and a VIP score of > 1.5 . All possible ion features were removed if they were not significant (i.e., confidence interval crossed zero) within model cross-validation. In step III, univariate statistical analysis was performed to consistently evaluate the filtered metabolic features. For the pilot study, pairwise comparisons of continuous variables were carried out with arc-sinh transformed normalized abundances in the Progenesis Q1 software. For the validation study, log10 transformed normalized abundances were used in the SIMCA software. Nominal two-sided p-values < 0.05 were considered statistically significant and corrections for multiple testing were done using the false discovery rate (FDR) method by Benjamini and Hochberg [28].

In step IV, each potential metabolite candidate was reviewed for its ion alignment and chromatographic peak shape. In step V, the selected metabolites were searched against the Human Metabolome Database (HMDB, version 3.6) with a mass accuracy of 8 ppm to obtain potential structure and formula of the metabolite features. Structure elucidation was performed by comparing MS/MS spectra from DDA experiments with data from the open source platforms MetFrag [29] and CSI:FingerID [30] [31].

3. Results

3.1 Study participants

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

178 In total 10 controls and 10 IPF patients were included in the pilot study set. The baseline
179 characteristics of the patients are described in table 1. Mean age of controls and IPF patients was 69
180 and 68 years with a male predominance (9 out of 10). IPF diagnosis was confirmed histologically in 6
181 out of 10 patients. Pulmonary function tests showed reduced FVC (mean FVC 67% predicted) and
182 DLCO (mean uncorrected DLCO 43% predicted) in IPF patients. Calculated GAP index (0 to 8
183 possible score points) ranged from 1 to 6 points (mean 4.2), reflecting GAP stage I to III and a
184 consecutively increased risk of mortality (predicted 3-year mortality 16% for stage I and 76% for stage
185 III) [24].

186 All except one patient were treated with antifibrotic drugs (Pirfenidone or Nintedanib) for at least three
187 months before study inclusion. All patients and controls were currently non-smokers, most of them
188 with a history of cigarette smoking. Basic physical exam and pulmonary functional tests in controls
189 were normal.

190 In the validation set, eight IPF patients and healthy controls were included. Healthy control samples
191 were from additional eight individuals. From IPF patients a new sample was taken two weeks after the
192 sample of the pilot study.

193

194 *3.2 Non-targeted metabolomics analysis*

195 The workflow for measurement, data processing and statistics is illustrated in Figure 2. After data
196 filtration, 1671 and 981 metabolic features were detected by UHPLC-HRMS in the positive and
197 negative ionization mode in the pilot study, respectively. The OPLS-DA models only resulted in
198 acceptable model characteristics in the positive ion mode (table 2). Based on the multivariate analysis,

26 metabolic features were found to be differentially regulated between healthy controls and IPF patients. With this approach, only metabolic features with strong model contribution and high reliability were selected. Univariate statistical analysis was then performed to consistently screen for additional metabolites. By univariate statistics, 39 metabolic features were significantly regulated in the positive ion mode. By combination of multivariate and univariate approaches, 58 metabolic features were found differentially regulated in the pilot study. The profile of the metabolic features isolated by multivariate and/or univariate statistical analysis revealed a clear discrimination between the healthy and IPF patients (figure 3, list of regulated metabolic features with the corresponding fold changes presented in supplementary file 1).

To validate our results, a new set of samples was profiled by UHPLC-HRMS. Similar to the pilot study, satisfactory multivariate model parameters were only obtained in the positive ionization mode (table 2). 48 discriminative features were selected by multivariate or univariate analysis (list of regulated metabolic features with the corresponding fold changes presented in supplementary file 2).

Among them, two metabolic features (m/z 410.3731 and m/z 341.3514 at retention time 10.06 and 9.94 min, respectively) of the pilot study were confirmed. The feature m/z 410.3731 was however regulated in different direction in both study sets, thus only feature m/z 341.3514 with a retention time of 9.94 min remained consistently more than two-fold up-regulated in IPF patients compared to healthy controls (figure 4, fold change IPF/healthy of 2.5 in the pilot study and of 2.0 in the validation study, FDR adjusted p -value ≤ 0.01). The corresponding extracted ion chromatograms of the validated metabolic feature in the pilot and validation study sets are presented in figure 5.

$C_{21}H_{44}N_2O$ was obtained as potential molecular formula for the regulated metabolic feature. The measured isotope ratio of m/z 341.3514 was in excellent agreement with the simulated one of

221 $C_{21}H_{44}N_2O$ (98.4% similarity). The major fragment detected by MS/MS experiment (DDA
222 experiment, $m/z = 128.1076$, supplementary figure 1) could be assigned to $C_7H_{14}NO^+$ (with 0.8 ppm
223 error), which could fit with the suggested formula of the parent ion. Based on the accurate m/z ratio,
224 possible identities were searched against HMDB and METLIN databases for the validated metabolic
225 feature m/z 341.3514. No reasonable metabolite was found against these databases by assuming a
226 protonated $[M+H]^+$ ion. Possible identifications against the databases ChemSpider including
227 PubChem and MassBank are shown in the supplementary file 3. None of these identifications reflect a
228 known biologically relevant molecule.

229

230 4. Discussion

231 In this study, we found 58 metabolic features that discriminated EBC of IPF patients from EBC of
232 healthy controls in a pilot study set. These discriminative features were isolated by UHPLC-HRMS in
233 the positive ionization mode. Among them, one metabolic feature (m/z 341.3514 at a retention time of
234 9.94 min) was up-regulated in IPF patients (FC 2.5) and was validated in an independent set of
235 samples. It is tenable that this metabolic feature is not related to a drug or a drug metabolite, because it
236 was also detected in the healthy controls. $C_{21}H_{44}N_2O$ was found as a potential molecular formula and
237 might represent a potential biomarker for IPF from non-invasively collected biofluids for diagnosis.
238 Further work will be required to identify its structure.

239 Previous studies on EBC from IPF patients showed promising results. Docosatetraenoyl
240 lysophosphatidic acid (LPA 22:4), isoprostane and H_2O_2 were elevated in IPF compared to healthy
241 controls [32, 33]. In our metabolic study, LPA 22:4 was not detected. This may be due to the lower
242 sensitivity of our methodology compared to the targeted LC-MS/MS assay used by Montesi et al. [32].

1
2
3 243 EBC has many advantages over other biofluid sampling methods. Collection is simple, non-invasive,
4
5 244 cheap and can be repeated as often as desired. Opposed to induced sputum sampling or BAL it is
6
7
8 245 nearly always safe even in severely respiratory limited patients. These advantages make EBC a
9
10 246 promising new tool to investigate lung diseases. Given the possibility of sampling at any point of
11
12
13 247 disease state, EBC can provide a real-time assessment of pulmonary pathologies. Nevertheless, this
14
15 248 technique has several pitfalls: collecting and measuring EBC needs to be standardized in order to
16
17
18 249 reduce the effect of confounders on data collection and interpretation and to ensure reproducibility.
19
20 250 Recent reviews show high variation in the sampling of EBC due to limited standardization [8, 9].
21
22 251 Some aspects of collecting EBC can be easily standardized for example the material of the collecting
23
24 252 tube, the temperature of the collecting device and the duration of collection. However, conditions like
25
26 253 ventilation parameters and the degree of dilution vary between subjects and are impossible to
27
28 254 standardize but must be taken into account when interpreting and comparing EBC results [10]. We
29
30 255 recognize this limitation and further investigations are needed to define the influence of ventilation
31
32 256 parameters and the degree of dilution on the molecular profile of EBC. Sampling methods and storage
33
34 257 after collection need to be standardized to improve sample reliability [10]. Recently, after submission
35
36 258 for this study, a proposed standardization for EBC collection has been published and hopefully will
37
38 259 help to improve data about EBC [34]. Overall, poor reproducibility, a lack of large representative
39
40 260 studies and the absence of large norm reference cohorts hinder EBC to enter clinical practice [22].
41
42
43
44
45
46
47
48
49 261 EBC has already been investigated by liquid chromatography mass spectrometry (LC-MS) in several
50
51 262 lung diseases, for example in chronic obstructive pulmonary disease (COPD) [13, 21]. Although
52
53 263 smaller clinical trials must be confirmed by larger studies, LC-MS seems to be a promising strategy in
54
55
56 264 investigating EBC of other lung diseases [13, 23] [35] and our results contribute to the current body of
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

evidence. By UHPLC-HRMS a large amount of data are being generated. Artefact peaks can result in false positives and over-interpretation can bias the results especially in limited sample sets. By validating a single metabolic feature over the 58 regulated features isolated in the pilot study, we demonstrated the importance of a validation set for such an analysis. The discovery of new candidate biomarkers might eventually lead to clinical tools for diagnosis and monitoring of lung diseases. Nevertheless, molecular biomarkers should reflect relevant pathobiological processes and mechanisms of a disease. Using screening methods as we did, carries a risk of detecting markers of physiological processes that have questionable pathobiological importance, and the relevance of many biomarkers in IPF is not yet clearly defined [36]. However, new non-invasive biomarkers are urgently needed.

5. Conclusions

Our study showed that metabolic profiling by UHPLC-HRMS can be applied to investigate EBC as a novel matrix for IPF biomarker discovery. We showed distinctive differences in the molecular profile of EBC from IPF patients compared to healthy controls. Our results should still be confirmed in a larger cohort and further studies might be able to confirm a profile of biomarkers, that could contribute to IPF diagnosis, disease state assessment, prediction of disease progression and response to therapy.

References

1. Raghu, G., et al., *An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management*. Am J Respir Crit Care Med, 2011. **183**(6): p. 788-824.

2. Ley, B., H.R. Collard, and T.E. King, Jr., *Clinical course and prediction of survival in idiopathic pulmonary fibrosis*. Am J Respir Crit Care Med, 2011. **183**(4): p. 431-40.

3. Kolb, M. and Y. Shargall, *Lung surgery in interstitial lung disease-a safe and useful procedure?* J Thorac Dis, 2013. **5**(4): p. 375-7.

- 292 4. Raghu, G., et al., *An Official ATS/ERS/JRS/ALAT Clinical Practice Guideline: Treatment of*
293 *Idiopathic Pulmonary Fibrosis. An Update of the 2011 Clinical Practice Guideline.* Am J
294 Respir Crit Care Med, 2015. **192**(2): p. e3-19.
- 295
- 296 5. Funke, M. and T. Geiser, *Idiopathic pulmonary fibrosis: the turning point is now!* Swiss Med
297 Wkly, 2015. **145**: p. w14139.
- 298
- 299 6. Myllarniemi, M. and R. Kaarteenaho, *Pharmacological treatment of idiopathic pulmonary*
300 *fibrosis - preclinical and clinical studies of pirfenidone, nintedanib, and N-acetylcysteine.* Eur
301 Clin Respir J, 2015. **2**.
- 302
- 303 7. Martinez-Lozano Sinues, P., R. Zenobi, and M. Kohler, *Analysis of the exhalome: a*
304 *diagnostic tool of the future.* Chest, 2013. **144**(3): p. 746-9.
- 305
- 306 8. Grob, N.M., M. Aytakin, and R.A. Dweik, *Biomarkers in exhaled breath condensate: a review*
307 *of collection, processing and analysis.* J Breath Res, 2008. **2**(3): p. 037004.
- 308
- 309 9. Horvath, I., et al., *Exhaled breath condensate: methodological recommendations and*
310 *unresolved questions.* Eur Respir J, 2005. **26**(3): p. 523-48.
- 311
- 312 10. Kuban, P. and F. Foret, *Exhaled breath condensate: determination of non-volatile compounds*
313 *and their potential for clinical diagnosis and monitoring. A review.* Anal Chim Acta, 2013.
314 **805**: p. 1-18.
- 315
- 316 11. Rihak, V., Zatloukal, P., Chladkova, J., Zimulova, A., Havlinova, Z., Chladek, J., *Nitrite in*
317 *exhaled breath condensate as a marker of nitrooxidative stress in the airways of patients with*
318 *asthma, COPD, and idiopathic pulmonary fibrosis.* J Clin Lab Anal, 2010. **24**(5): p. 317-22.
- 319
- 320 12. Santini, G., et al., *Exhaled and non-exhaled non-invasive markers for assessment of*
321 *respiratory inflammation in patients with stable COPD and healthy smokers.* J Breath Res,
322 2016. **10**(1): p. 017102.
- 323
- 324 13. Borrill, Z.L., K. Roy, and D. Singh, *Exhaled breath condensate biomarkers in COPD.* Eur
325 Respir J, 2008. **32**(2): p. 472-86.
- 326
- 327 14. Vestbo, J., et al., *Evaluation of COPD Longitudinally to Identify Predictive Surrogate End-*
328 *points (ECLIPSE).* Eur Respir J, 2008. **31**(4): p. 869-73.
- 329
- 330 15. Antus, B., Barta, I., Csiszer, E., Kelemen, K., *Exhaled breath condensate pH in patients with*
331 *cystic fibrosis.* Inflamm Res, 2012. **61**(10): p. 1141-7.
- 332
- 333 16. Dent, A.G., T.G. Sutedja, and P.V. Zimmerman, *Exhaled breath analysis for lung cancer.* J
334 Thorac Dis, 2013. **5 Suppl 5**: p. S540-50.
- 335
- 336 17. Psathakis, K., Mermigkis, D., Papatheodorou, G., Loukides, S., Panagou, P.,
337 Polychronopoulos, V., Siafakas, N. M., Bouros, D., *Exhaled markers of oxidative stress in*
338 *idiopathic pulmonary fibrosis.* Eur J Clin Invest, 2006. **36**(5): p. 362-7.
- 339
- 340 18. de Laurentiis, G., Paris, D., Melck, D., Montuschi, P., Maniscalco, M., Bianco, A., Sofia, M.,
341 Motta, A., *Separating smoking-related diseases using NMR-based metabolomics of exhaled*
342 *breath condensate.* J Proteome Res, 2013. **12**(3): p. 1502-11.
- 343
- 344 19. Santini, G., et al., *Electronic Nose and Exhaled Breath NMR-based Metabolomics*
345 *Applications in Airways Disease.* Curr Top Med Chem, 2016. **16**(14): p. 1610-30.
- 346

- 1
2
3 347 20. Malerba, M. and P. Montuschi, *Non-invasive biomarkers of lung inflammation in smoking*
4 348 *subjects*. Curr Med Chem, 2012. **19**(2): p. 187-96.
5 349
6 350 21. Martinez-Lozano Sinues, P., Meier, L., Berchtold, C., Ivanov, M., Sievi, N., Camen, G.,
7 351 Kohler, M., Zenobi, R., *Breath analysis in real time by mass spectrometry in chronic*
8 352 *obstructive pulmonary disease*. Respiration, 2014. **87**(4): p. 301-10.
9 353
10 354 22. Konstantinidi, E.M., Lappas, A. S., Tzortzi, A. S., Behrakis, P. K., *Exhaled Breath*
11 355 *Condensate: Technical and Diagnostic Aspects*. ScientificWorldJournal, 2015. **2015**: p.
12 356 435160.
13 357
14 358 23. Beck, O., A.C. Olin, and E. Mirgorodskaya, *Potential of Mass Spectrometry in Developing*
15 359 *Clinical Laboratory Biomarkers of Nonvolatiles in Exhaled Breath*. Clin Chem, 2016. **62**(1):
16 360 p. 84-91.
17 361
18 362 24. Ley, B., et al., *A multidimensional index and staging system for idiopathic pulmonary fibrosis*.
19 363 Ann Intern Med, 2012. **156**(10): p. 684-91.
20 364
21 365 25. Dunn, W.B., et al., *Procedures for large-scale metabolic profiling of serum and plasma using*
22 366 *gas chromatography and liquid chromatography coupled to mass spectrometry*. Nat Protoc,
23 367 2011. **6**(7): p. 1060-83.
24 368
25 369 26. Triba, M.N., Le Moyec, L., Amathieu, R., Goossens, C., Bouchemal, N., Nahon, P., Rutledge,
26 370 D. N., Savarin, P., *PLS/OPLS models in metabolomics: the impact of permutation of dataset*
27 371 *rows on the K-fold cross-validation quality parameters*. Mol Biosyst, 2015. **11**(1): p. 13-9.
28 372
29 373 27. Szymanska, E., Saccenti, E., Smilde, A. K., Westerhuis, J. A., *Double-check: validation of*
30 374 *diagnostic statistics for PLS-DA models in metabolomics studies*. Metabolomics, 2012.
31 375 **8**(Suppl 1): p. 3-16.
32 376
33 377 28. Benjamini, Y. and Hochberg, Y., *Controlling the False Discovery Rate: A practical and*
34 378 *powerful approach to multiple testing*. Journal of the Royal Statistical Society, 1995. **B 57**
35 379 ((1)): p. 289-300.
36 380
37 381 29. Ruttkies, C., Schymanski, E. L., Wolf, S., Hollender, J., Neumann, S., *MetFrag relaunched:*
38 382 *incorporating strategies beyond in silico fragmentation*. J Cheminform, 2016. **8**: p. 3.
39 383
40 384 30. Duhrkop, K., Shen, H., Meusel, M., Rousu, J., Bocker, S., *Searching molecular structure*
41 385 *databases with tandem mass spectra using CSI:FingerID*. Proc Natl Acad Sci U S A, 2015.
42 386 **112**(41): p. 12580-5.
43 387
44 388 31. Shen, H., Duhrkop, K., Bocker, S., Rousu, J., *Metabolite identification through multiple kernel*
45 389 *learning on fragmentation trees*. Bioinformatics, 2014. **30**(12): p. i157-64.
46 390
47 391 32. Montesi, S.B., Mathai, S. K., Brenner, L. N., Gorshkova, I. A., Berdyshev, E. V., Tager, A.
48 392 M., Shea, B. S., *Docosatetraenoyl LPA is elevated in exhaled breath condensate in idiopathic*
49 393 *pulmonary fibrosis*. BMC Pulm Med, 2014. **14**: p. 5.
50 394
51 395 33. Chow, S., Thomas, P. S., Malouf, M., Yates, D. H., *Exhaled breath condensate (EBC)*
52 396 *biomarkers in pulmonary fibrosis*. J Breath Res, 2012. **6**(1): p. 016004.
53 397
54 398 34. Horvath, I., et al., *A European Respiratory Society technical standard: exhaled biomarkers in*
55 399 *lung disease*. Eur Respir J, 2017. **49**(4).
56 400
57 401 35. Montuschi, P., Santini, G., Valente, S., Mondino, C., Macagno, F., Cattani, P., Zini, G., Mores,
58 402 N., *Liquid chromatography-mass spectrometry measurement of leukotrienes in asthma and*

other respiratory diseases. J Chromatogr B Analyt Technol Biomed Life Sci, 2014. **964**: p. 12-25.

36. Ley, B., K.K. Brown, and H.R. Collard, *Molecular biomarkers in idiopathic pulmonary fibrosis*. Am J Physiol Lung Cell Mol Physiol, 2014. **307**(9): p. L681-91.
37. Xia, J. and D.S. Wishart, *Using MetaboAnalyst 3.0 for Comprehensive Metabolomics Data Analysis*. Curr Protoc Bioinformatics, 2016. **55**: p. 14 10 1-14 10 91.

Acknowledgments

Liselotte McEvoy, Susanne Krebsner, Jeannette Frey (sample collection), volunteers and patients (participation), UHPLC-HRMS analysis was performed at the Clinical Metabolomics Facility, Center of Laboratory Medicine from the Bern University Hospital (Inselspital Bern). Samples from IPF patients were collected within a clinical trial sponsored by the Research fund of the Swiss Lung Association, Berne.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

425 **Figure legends**

426 **Figure 1:**

427 Representative base-peak ion (BPI) chromatogram in positive ion mode of an EBC sample from
428 control (upper panel) and IPF patient (lower panel). *Individual differences (Single detection)
429 **Chromatographic region with accumulation of similar as well as differentially regulated metabolites
430 between IPF patients and healthy controls.

431 **Figure 2:**

432 Workflow for sample measurement, data processing and statistical analysis.

433 **Figure 3:**

434 Heat map representing the log10-transformed abundance profile of the regulated metabolic features
435 isolated by multivariate and/or univariate statistical analysis in the pilot study sample set. Identity
436 (summarized by the retention time followed by the corresponding m/z ratio or neutral mass n) of the
437 metabolic features are shown on the right side, individual samples in rows, respectively. Cells colored
438 in red represent up-regulated, colored in blue down-regulated abundances. The analysis was done with
439 the MetaboAnalyst online platform [37].

440 **Figure 4:**

441 Distribution of the validated metabolic feature m/z 341.3514 eluting at 9.94 min in the pilot (left) and
442 validation (right) study sets (standardized abundance, FDR adjusted p-value < 0.05) between controls
443 and IPF patients in positive ion mode.

444

1
2
3 445 **Figure 5:**
4

5
6 446 Extracted ion chromatograms of the metabolic feature m/z 341.3514 eluting at 9.94 min detected in the
7
8
9 447 healthy (pink) and IPF (dark blue) patients in the pilot (left) and validation (right) study sets.
10

11
12 448
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

449 **Table 1.** Baseline characteristics of controls and IPF patients. Showing either mean $\pm$ standard
450 deviation or number and percentage of total.

	Controls	n=10	IPF patients	n=10
Age (years)	68.70 $\pm$ 7.91	10	68.44 $\pm$ 9.08	10
Men n (%)	9 (90)	10	8 (88.9)	9 (90)
UIP pattern				
HRCT scan	-	-	8	10
Pathology	-	-	6	10
Pulmonary functional tests				
FVC, L	4.14 $\pm$ 0.40	10	2.43 $\pm$ 0.49	10
FVC (% predicted)	114.60 $\pm$ 8.49	10	65.1 $\pm$ 11.27	10
DLCO uncorr. (% predicted)	129.60 $\pm$ 31.10	10	43 $\pm$ 15.02	10
GAP Index score (0-8)	-	-	4.2 $\pm$ 1.4	10
Smoking habits				
Current Smokers n (%)	0	10	0	10
Former Smokers n (%)	8 (80)	10	7 (77.8)	9
Pack years	5.50 $\pm$ 5.72	8	25.37 $\pm$ 15.18	8
Antifibrotic therapy				
Pirfenidon n (%)	-	-	6 (66.7)	10
Nintedanib n (%)	-	-	2 (22.2)	10
No antifibrotic therapy n (%)	-	-	1 (11.1)	10

451

Table 2. Characteristics of the OPLS-DA models for the pilot and validation study sets analysed in positive (ESI+) and negative (ESI-) ion mode.

Sample Set	Amount of features	$R^2(\text{cum})$	$Q^2(\text{cum})^a$	Permutation test (n=20)	CV-ANOVA p-value
Pilot ESI+	1671	0.896	0.626	$R^2 = 0.535$ $Q^2 = -0.78$	0.03
Pilot ESI-	981	0.605	0.254	$R^2 = 0.357$ $Q^2 = -0.577$	0.32
Validation ESI+	906	0.693	0.544	$R^2 = 0.301$ $Q^2 = -0.479$	0.05
Validation ESI-	220	0.646	-0.112	$R^2 = 0.537$ $Q^2 = -0.41$	1

R^2 (cum), cumulative sum of squares explained by all extracted components; Q^2 (cum), cumulative fraction of the predicted variation;

^a cross validation groups n = 10

Figure 1:

468 **Figure 2:**

469

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

470 **Figure 3**

471

472

473

474 **Figure 4**

475

476 **Figure 5**

477