Chromatography **2014**, *1*, 108-119; doi:10.3390/chromatography1030108

OPEN ACCESS

chromatography

ISSN 2227-9075

www.mdpi.com/journal/chromatography

Review

Exhaled Breath Condensate for Proteomic Biomarker Discovery

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Received: 8 May 2014; in revised form: 20 June 2014 / Accepted: 24 June 2014 / Published: 2 July 2014

Abstract: Exhaled breath condensate (EBC) has been established as a potential source of respiratory biomarkers. Compared to the numerous small molecules identified, the protein content of EBC has remained relatively unstudied due to the methodological and technical difficulties surrounding EBC analysis. In this review, we discuss the proteins identified in EBC, by mass spectrometry, focusing on the significance of those proteins identified. We will also review the limitations surrounding mass spectral EBC protein analysis emphasizing recommendations to enhance EBC protein identifications by mass spectrometry. Finally, we will provide insight into the future directions of the EBC proteomics field.

Keywords: exhaled breath condensate; proteomics; mass spectrometry

1. Introduction

Exhaled breath condensate (EBC) has quickly become a subject of research interest due to the high potential for respiratory disease biomarker discovery. Considered an aqueous matrix featuring condensed volatile organic compounds (VOC) from breath and non-volatile droplets of liquid from the respiratory tract, EBC represents an expanded potential for respiratory biomarker discovery beyond

VOC breath analysis [1–5]. These properties have allowed EBC to come to the forefront of collection methods for biomarker discovery in respiratory diseases.

Historically, the biomarker discovery field has relied heavily on mass spectrometric approaches for characterizing the contents of complex biological samples and fluids. Exhaled breath condensate is no different. Both gas chromatography and liquid chromatography mass spectrometry (GC-MS and LC-MS/MS respectively) approaches have been applied to EBC to identify molecules of prognostic and predictive significance. This review will discuss the proteins identified using mass spectral methodologies for EBC biomarker discovery with a focus on the significance and limitations associated with EBC analysis.

2. Collection and Sampling

In contrast to invasive and minimally invasive collection methods, such as tissue biopsy, blood and bronchoalveolar lavage (BAL), exhaled breath condensate is obtained by a completely non-invasive method making it ideal for ailing individuals [6,7]. Furthermore, EBC collection is simplistic, requiring minimal technical skills, allowing for sampling from both children and adults alike [6,7]. These properties make EBC collection and analysis applicable to many respiratory diseases, such as asthma, cystic fibrosis and chronic obstructive pulmonary disease (COPD) [6,7].

Sampling of EBC can be conducted on commercially available equipment, most notably the Ecoscreen or RTube, or homemade devices such as that put forth by Schleiss *et al.* [4,8,9]. See Huttmann *et al*., for details regarding the Ecoscreen and RTube [10]. All these sampling devices strive to condense humid breath, without salivary contamination, into liquid or ice, based on the condensation temperature, for collection and analysis [11]. For review of collection, device variability, sampling pitfalls and recommendations for collection method standardization, in EBC sampling see Horvath *et al.*, Montuschi and Grob *et al.* [1,8,12]. Since EBC is >99% water, analytes are diluted to detection limits or lower [13]. Steps to concentrate analytes, such as centrifugal evaporation or lyophilization, have been employed to overcome this limitation [4,8]. Despite this analytical obstacle, it is these concentrated samples that hold the most promise for protein biomarker discovery.

3. Exhaled Breath Condensate Proteomics

In contrast to global bottom-up proteomics, EBC proteomic analysis has yielded only a modest number of protein identifications (Table 1). For example, shotgun proteomics of global lysates regularly identify several hundred proteins in a single study with significant protein scores [14]. Conversely, the largest number of significantly identified proteins from EBC using bottom-up approaches, in a single study, was accomplished by Fumagalli *et al*., with 44 total proteins [15]. This has been attributed to the low concentration, <1 μg/mL, of proteins contained in EBC [16]. While few in number, those already discovered have the potential for high physiologic relevance.

Table 1. List of proteins identified in exhaled breath condensate by mass spectrometry in various diseases. Disease Abbreviations: NS = Non-Smoker, S = Smoker, ES = Ex-Smoker, LT = Lung Transplant, AATD = Pulmonary Emphysema Associated with α1 Antitrypsin Deficiency, COPD = Chronic Obstructive Pulmonary Disease, LC = Lung Cancer, ALI/ARDS = Acute Lung Injury/Acute Respiratory Distress Syndrome. Methodology Abbreviations: LC = Liquid Chromatography, MS/MS = Mass Spectrometry/ Mass Spectrometry, SELDI = Surface-Enhanced Laser Desorption/Ionization, MALDI = Matrix-Assisted Laser Desorption/Ionization, TOF = Time-of-Flight, 1DE & 2DE = Dimensional Gel Electrophoresis.

Protein Identifications from Exhaled Breath Condensate by Mass Spectrometry						
#	**Protein Name**	**Accession Number**	**Disease**	**Method of Detection**	**Reference**	**Reported as Contaminant**
1	Cytokeratin 1	P04264	Healthy NS, Healthy S, Healthy ES, LT, AATD, Asthma, LC	LC-MS/MS, SELDI-TOF, MALDI-TOF, 2DE-LC-MS/MS	[15–24]	[16,18,24]
2	Cytokeratin 9	P35527	Healthy NS, Healthy S, Healthy ES, LT, AATD, Asthma, LC, ALI/ARDS	LC-MS/MS, SELDI-TOF, MALDI-TOF, 2DE-LC-MS/MS, 1DE-LC-MS/MS	[15–23,25]	
3	Cytokeratin 2	P35908	Healthy NS, Healthy S, Healthy ES, LT, Asthma, LC, ALI/ARDS	LC-MS/MS, MALDI-TOF, 1DE-LC-MS/MS	[15,16,18–22,24,25]	[16,18,24]
4	Cytokeratin 10	P13645	Healthy NS, Healthy S, Healthy ES, LT, AATD, Asthma, LC, ALI/ARDS	LC-MS/MS, SELDI-TOF, MALDI-TOF, 2DE-LC-MS/MS, 1DE-LC-MS/MS	[15–22,25]	
5	Cytokeratin 14	P02533	Healthy NS, Healthy S, Healthy ES, LT, Asthma, LC	LC-MS/MS, SELDI-TOF, MALDI-TOF	[15,16,19,20,22,24]	[24]
6	Cytokeratin 5	P13647	Healthy NS, Healthy S, Healthy ES, LT, Asthma, LC	LC-MS/MS, SELDI-TOF, MALDI-TOF	[15,16,19,20,22]	
7	Cytokeratin 6B	P04259	Healthy NS, Healthy S, LT, Asthma	LC-MS/MS, MALDI-TOF	[15,16,18–20]	
8	Dermcidin	P81605	Healthy NS, Healthy S, Healthy ES, LT, Asthma, LC	LC-MS/MS, MALDI-TOF	[16,19,20,22,24]	[24]
9	Lysozyme C	P61626	Healthy NS, Healthy S, LT, AATD, Asthma, LC	LC-MS/MS, SELDI-TOF, MALDI-TOF	[15,19–22]	
10	Cytokeratin 6A	P02538	Healthy NS, Healthy S, Healthy ES, LT, Asthma, LC	LC-MS/MS, MALDI-TOF	[16,19,20,22]	

Table 1. *Cont.*

Protein Identifications from Exhaled Breath Condensate by Mass Spectrometry						
#	**Protein Name**	**Accession Number**	**Disease**	**Method of Detection**	**Reference**	**Reported as Contaminant**
11	Cytokeratin 16	P08779	Healthy NS, Healthy S, Healthy ES, LT, Asthma, LC	LC-MS/MS, MALDI-TOF	[16,19,20,22]	
12	Serum Albumin	P02768	Healthy NS, Healthy S, Asthma, LC	LC-MS/MS, MALDI-TOF	[15,16,20,22]	
13	Cytokeratin 8	P05787	Healthy NS, LT, Asthma	LC-MS/MS, MALDI-TOF	[18–20]	
14	Ubiquitin	P62988	Healthy NS, Healthy S, LT	LC-MS/MS, SELDI-TOF	[15,16,19]	
15	Cystatin A	P01040	Healthy NS, Healthy S, LT, AATD, COPD	LC-MS/MS, SELDI-TOF	[15,16,19]	
16	Calgranulin B	P06702	Healthy NS, Healthy S, AATD, Asthma, COPD	LC-MS/MS, SELDI-TOF, MALDI-TOF	[15,20,21]	
17	Hemoglobin	P02042	Healthy NS, Healthy S, Asthma	MALDI-TOF	[20,21,24]	[24]
18	Cytokeratin 4	P19013	Healthy NS, LT	LC-MS/MS, MALDI-TOF	[18,19]	
19	Cytokeratin 17	Q04695	Healthy NS, Healthy S, Healthy ES, LC	LC-MS/MS	[16,22]	
20	α1-Microglobulin/Bikunin Precursor	P02760	Healthy NS, Healthy S	LC-MS/MS, SELDI-TOF	[15,16]	
21	Human Basement Membrane Heparan Sulfate Proteoglycan Core Protein	P98160	Healthy NS, Healthy S	LC-MS/MS	[15,16]	
22	Cerebroside Sulfate Activator	P07602	Healthy NS, Healthy S	LC-MS/MS	[15,16]	
23	Lyosomal-Associated Membrane Glycoprotein-2	P13473	Healthy NS, Healthy S	LC-MS/MS	[15,16]	
24	Kininogen 1	P01042	Healthy NS, Healthy S	LC-MS/MS, SELDI-TOF	[15,16]	
25	Calgranulin A	P05109	Healthy NS, Healthy S, AATD, COPD	LC-MS/MS, SELDI-TOF, MALDI-TOF	[15,21]	
26	Interferon ϒ	P01579	Healthy NS, Healthy S, AATD, COPD	LC-MS/MS, SELDI-TOF	[15,17]	
27	IL-2	P60568	Healthy NS, Healthy S, AATD, COPD	LC-MS/MS, SELDI-TOF	[15,17]	
28	IL-15	P04933	Healthy NS, Healthy S, AATD, COPD	LC-MS/MS, SELDI-TOF	[15,17]	
29	Heomglobin, Subunit β	P68871	Healthy NS, Healthy S	LC-MS/MS, SELDI-TOF	[15,16]	[16]

Table 1. *Cont.*

Protein Identifications from Exhaled Breath Condensate by Mass Spectrometry						
#	**Protein Name**	**Accession Number**	**Disease**	**Method of Detection**	**Reference**	**Reported as Contaminant**
30	Demoplakin	P15924	Healthy ES, LT	LC-MS/MS	[19,22]	
31	Filaggrin	Q5D862	Healthy ES, LT	LC-MS/MS	[19,22]	
32	Prolactin-Induced Protein	P12273	Healthy ES, LT, LC	LC-MS/MS	[19,22]	
33	Glyceraldehyde-3-Phosphate dehydrogenase	P04406	LT, LC	LC-MS/MS	[19,22]	
34	β Actin	P06709	Asthma, LC	LC-MS/MS, MALDI-TOF	[20,22]	
35	Cytokeratin 25	Q7Z3Z0	Healthy NS	LC-MS/MS	[16]	
36	Cytokeratin 26	Q7Z3Y9	Healthy NS, Healthy S	LC-MS/MS, SELDI-TOF	[15]	
37	Prostaglandin H2 D-Isomerase	P41222	Healthy NS	LC-MS/MS	[16]	
38	Leukocyte-Associated Immunoglobulin-Like Receptor 1	Q6GTX8	Healthy NS	LC-MS/MS	[16]	
39	α Actin, Cardiac Muscle 1	P68032	Healthy NS, Healthy S	LC-MS/MS	[15]	
40	Pulmonary Surfactant Associated Protein A1	Q8IWL1	Healthy NS, Healthy S, AATD, COPD	LC-MS/MS, SELDI-TOF	[15]	
41	Pulmonary Surfactant Associated Protein A2	Q8IWL2	Healthy NS, Healthy S, AATD, COPD	LC-MS/MS, SELDI-TOF	[15]	
42	Laminin β4	B4DX23	Healthy NS, Healthy S	LC-MS/MS	[15]	
43	Histone H1.5	P16401	Healthy NS, Healthy S, AATD, COPD	LC-MS/MS, SELDI-TOF	[15]	
44	α1-Antitrypsin	P01009	Healthy NS, Healthy S, COPD	LC-MS/MS, SELDI-TOF	[15]	
45	Erythropoietin	P01588	Healthy NS, Healthy S	LC-MS/MS, SELDI-TOF	[15]	
46	Complement C3	P01024	Healthy NS, Healthy S	LC-MS/MS	[15]	
47	Nucleolar Protein 4	O94818	Healthy NS, Healthy S	LC-MS/MS	[15]	
48	V-Set & Immunoglobulin Domain-Containing Protein 8	Q5VU13	Healthy NS, Healthy S, AATD, COPD	LC-MS/MS	[15]	

Table 1. *Cont.*

Protein Identifications from Exhaled Breath Condensate by Mass Spectrometry						
#	Protein Name	Accession Number	Disease	Method of Detection	Reference	Reported as Contaminant
49	Thyroid Hormone Receptor-Associated Protein 3	Q9Y2W1	Healthy NS, Healthy S	LC-MS/MS	[15]	
50	Chromodomain-Helicase-DNA-Binding Protein 1	O14646	Healthy NS, Healthy S	LC-MS/MS	[15]	
51	Zinc Finger CCCH Domain-Containing Protein 4	Q9UPT8	Healthy NS, Healthy S	LC-MS/MS	[15]	
52	Monocyte Chemoattractant Protein 1	P13500	Healthy NS, Healthy S, AATD, COPD	LC-MS/MS, SELDI-TOF	[15]	
53	Interferon α 1/13	P01562	Healthy NS, Healthy S, COPD	LC-MS/MS, SELDI-TOF	[15]	
54	Tumor Necrosis Factor	P01375	Healthy NS, Healthy S, AATD, COPD	LC-MS/MS, SELDI-TOF	[15]	
55	Growth-Regulated Oncogene α Protein	P09341	Healthy NS, Healthy S, AATD, COPD	LC-MS/MS, SELDI-TOF	[15]	
56	IL-1 α	P01583	Healthy NS, Healthy S, AATD	LC-MS/MS	[15]	
57	IL-1 β	P01584	Healthy NS, Healthy S, COPD	LC-MS/MS, SELDI-TOF	[15]	
58	IL-12, Subunit α	P29459	Healthy NS, Healthy S, COPD	LC-MS/MS, SELDI-TOF	[15]	
59	IL-12, Subunit β	P29460	Healthy NS, Healthy S	LC-MS/MS	[15]	
60	Lipocalin	-	LT	LC-MS/MS	[19]	
61	Cytochrome C	P99999	LT	LC-MS/MS	[19]	
62	Desmoglein	-	LT	LC-MS/MS	[19]	
63	Hornerin	Q86YZ3	LT	LC-MS/MS	[19]	
64	Annexin A1	P04083	LT	LC-MS/MS	[19]	
65	Serine Protease Inhibitor	-	LT	LC-MS/MS	[19]	
66	Bleomycine Hydrolase	Q13867	LT	LC-MS/MS	[19]	
67	Arginase 1	P05089	LT	LC-MS/MS	[19]	
68	Peroxiredoxin	-	LT	LC-MS/MS	[19]	
69	Caspase 14	P31944	Healthy S, Healthy ES, LC	LC-MS/MS	[22]	

Table 1. *Cont.*

Protein Identifications from Exhaled Breath Condensate by Mass Spectrometry						
#	Protein Name	Accession Number	Disease	Method of Detection	Reference	Reported as Contaminant
70	Cystatin SN	P01037	Healthy ES	LC-MS/MS	[22]	
71	Growth Hormone Regulated TBC Protein 1	Q5TC63	LC	LC-MS/MS	[22]	
72	Apolipoprotein D	P05090	Healthy S	LC-MS/MS	[22]	
73	Junction Plakoglobin	P14923	Healthy ES	LC-MS/MS	[22]	
74	Submaxillary Gland Androgen-Regulated Protein 3B	P02814	Healthy ES	LC-MS/MS	[22]	
75	Zymogen Granule Protein 16B	Q96DA0	Healthy ES	LC-MS/MS	[22]	
76	Polyubiquitin-B	P0CG47	Healthy ES	LC-MS/MS	[22]	
77	ϒ-Glutamylcyclotransferase	O75223	Healthy S, Healthy ES, LC	LC-MS/MS	[22]	
78	Zinc α-2-Glycoprotein 1	P25311	Healthy ES	LC-MS/MS	[22]	
79	α Actin	P68133	Healthy S, LC	LC-MS/MS	[22]	
80	Pulmonary Surfactant Associated Protein D	P35247	Healthy NS	MALDI-TOF	[18]	

The dominant proteins found in EBC are cytokeratin proteins. Most often Cytokeratins 1, 2, 9 and 10 are more prevalent while 4, 5, 6A, 6B, 8, 14, 16, 17, 25, and 26 are also present at a lower frequency [15–25]. Several of these cytokeratins, specifically 5, 6, 8, 14 and 17, are expressed in alveolae and bronchii of the lung suggesting EBC is a representative sample of the lower respiratory tract environment [18]. Similarly, Gianazza *et al*., have reported a 3× increase in the keratin content of EBC from smokers when compared to non-smokers [23,26]. However, many of these cytokeratins and other proteins have been reported as contaminants, associated with either the sampling environment or patient derived, dust and skin debris in ambient air [18,24]. Cytokeratins 1, 2 and 14, dermcidin and hemoglobin fall into this category (Table 1) [16,18,24]. Potentially more important than cytokeratin identification, a recent study has found EBC derived inflammatory mediators, e.g., IL-1α, IL-1β, IL-2, IL-12α, IL-12β, IL-15, IFN-α, IFN-ϒ, and TNF-α, suggesting the proteomic community is progressing toward the lower end of the dynamic range of EBC [15].

Cytokines in exhaled breath condensate have been reported in the low pg/mL range often bordering the lower limits of detection and/or quantitation by common immunological methodology [27]. For example, tumor necrosis factor alpha (TNF-α) was shown by Garey *et al*., by ELISA to range from 7.4 ± 17.5 to 3.9 ± 8.5 pg/mL between smokers and non-smokers which is near their reported 2 pg/mL detection limit for the assay [28]. Mass spectral detection of these potentially low abundant and highly localized proteins by Fumagalli *et al*., in EBC suggests the methodological and instrumental limitations of EBC proteomics are slowly being overcome [15]. Such results hold promise for using MS to characterize the EBC protein content, across many diseases, expanding our understanding of EBC as a source of protein biomarkers.

The identification of cytokines in exhaled breath condensate also holds diagnostic significance in determining lung inflammation. For example, IL-2 was detected in the EBC of children with asthma and CF while remaining undetectable in control children [29]. Furthermore, protein array data provided by Matsunaga *et al*., showed increases in IL-4, IL-8, IL-17, TNF-α, RANTES, IP-10, TGF-β, MIP-1α and MIP-1β in asthmatics when compared to healthy individuals [30]. Matsunaga *et al*., also correlated values of EBC RANTES, TNF-α, and TGF-β with physiological parameters of airway disease [30]. Additionally, Colombo *et al*., demonstrated IL-8 correlated with clinical biomarkers of cystic fibrosis by biochip array [31]. However, more sensitive assays, such as multiple reaction monitoring (MRM), are needed as these cytokines are often near immunological reagent dependent assay's detection limits [29]. These examples emphasize the potential for using EBC as a medium to monitor lung inflammatory mediators. Additionally, these data stress the need for novel highly sensitive methods for EBC analysis.

4. Exhaled Breath Condensate Proteomic Limitations

In contrast to the numerous small molecule biomarkers identified, such as leukotrienes (LTB_4, CysLT) and nitric oxide (NO), proteomic analysis of EBC has had a limited number of potential protein biomarkers identified due to methodological and instrumental hurdles [4,8,32,33]. The analytical difficulties of EBC proteomics can be attributed to several factors including, low protein concentration, inconsistent sample preparation and sample loss and instrumental sensitivities [34–36]. Although present, the limitations surrounding EBC proteomics can be potentially overcome.

Low protein content, <1 μg/mL, in EBC requires measures to concentrate the proteins prior to analysis [16]. Traditionally accomplished through centrifugal evaporation or lyophilization, these steps are reported to concentrate samples up to 20% [4]. Although beneficial, lyophilization has been shown to reduce peptide identifications 50%–90% suggesting other methods or combination of methods such as gel electrophoresis, ultrafiltration, protein precipitation and solid-phase extraction, for sample concentration could improve sample quality and protein ID quantity [15–25]. Additionally, lyophilizing instruments are not readily available at most institutions limiting their potential use in EBC methodology [1]. These results suggest lyophilization for sample concentration alone is insufficient to probe low abundant proteins in EBC.

The most common sampling procedure for EBC collection involves normal tidal breathing through a collection device for 10 minutes, ultimately yielding approximately 1 mL of condensate [1,2]. Although variations exist, *i.e*., condensing temperature, collection device, *etc.*, this procedure has been used routinely for many applications including proteomics [15–25]. A 1 mL sample of EBC for proteomics is insufficient due to the low overall protein abundance. While increasing the sampling time is ideal, it may provide added stress to an already sick individual [16]. To circumvent this limitation, Fumagalli *et al*., pooled samples from healthy smokers and non-smokers to increase sample protein concentration and obtain MS spectra from the low end of the dynamic range [15]. While they could not characterize distinct individuals in this manner, sample pooling did allow for the MS detection of the highest number of proteins from EBC to date [15].

Many proteomic sample preparation techniques have been applied to exhaled breath condensate with varying success, such as solid-phase extraction, protein precipitation, delipidation and ultrafiltration [15–25]. The most thorough attempt to characterize the differences in sample preparation was conducted by Kurova *et al*., [16]. They concluded lyophilization followed by standard tryptic digestion yielded the best MS results by reducing sample loss [16]. These results suggest sample handling has a profound effect on MS detection.

The proteomic analysis of EBC has been conducted on many different instruments, such as MALDI-TOF, SELDI-TOF, Ion-trap LC-MS/MS and LC-FT-ICR, each with variable sensitivities (Table 1) [15–25]. As newer more sensitive instruments with high MS^N scan rates become available, the field of EBC proteomics will quickly progress. Additionally as the interest in exhaled breath as a source of protein biomarkers grows, more researchers will become involved in EBC protein analysis further advancing the field.

5. Future Directions

Although significant strides have been made in qualitatively analyzing EBC, *i.e*., small molecule, proteomics, pH, *etc.*, the clinical significance and application of these discoveries currently remains unclear. Further development of MS techniques and instrumentation, sample collection and preparation are required to advance the proteomic characterization to the low end of the dynamic range. Such research will allow for the identification of low abundant disease biomarkers facilitating the development of highly sensitive quantitative MS assays, *i.e*., multiple reaction monitoring, supporting large-scale clinical biomarker studies. It is these low abundant proteins in conjunction with high

sensitivity quantitative assays that could hold significant clinically relevant diagnostic and therapeutic information.

Acknowledgment

This work was supported by a subcontract from UES Inc. under Air Force contract FA8650-14-D-6516. Opinions, interpretations, conclusions and recommendations are those of the authors and not necessarily endorsed by the United States Government. The authors would like to thank Jae Kwak, Catherine A. Powell and Grant M. Slusher for their scholarly input for this manuscript.

Author Contributions

Sean W. Harshman prepared and wrote the review. Claude C. Grigsby and Darrin K. Ott provided guidance and direction for the written review.

Conflicts of Interest

The authors declare no conflict of interest.

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