Lung (2019) 197:523–531
https://doi.org/10.1007/s00408-019-00238-z

LUNG CANCER

Mass Spectrometry Analysis of the Exhaled Breath Condensate and Proposal of Dermcidin and S100A9 as Possible Markers for Lung Cancer Prognosis

Laura Núñez-Naveira[1,2] · Luis Antonio Mariñas-Pardo[1,2] · Carmen Montero-Martínez[1]

Received: 3 October 2018 / Accepted: 11 May 2019 / Published online: 21 May 2019

Abstract

Introduction New sampling techniques to analyse lung diseases, such as exhaled breath condensate (EBC), are a breakthrough in research field since they are less invasive and less traumatic for the patients compared to lung biopsies. Nevertheless, there is an increasing need to optimize not only the sampling protocols but the storage and processing of specimens to get accurate results.

Methods Exhaled breath condensate was sampled employing the ECoScreen device. Concentrated protein was obtained after ultracentrifugation, lyophilization and reversed-phase chromatography. MALDI-time of flight (TOF)/TOF mass spectrometry (MS) was applied to determine the protein profile in EBC. Commercially available ELISA kits were used to detect the selected biomarker in the EBC after MALDI-MS proteins identification.

Results The obtained EBC volume after two periods of 10 min doubled the amount obtained after 20 min. One hundred peptides were detected by MALDI-MS, and 18 proteins were identified after reversed-phase chromatography concentration. Dermcidin (P81605), S100A9 (P06702) and Cathepsin G (P08311) were selected to be analysed by ELISA. Dermcidin and S100A9 expression were statistically higher in lung cancer versus healthy volunteers. VEGF concentrations decreased, respectively, by 5.94 and 11.42-fold after 1 and 2 years of frozen EBC preservation in parallel with the declined number of proteins identified by MALDI-MS.

Conclusion Exhaled breath condensate analysis combined with MS technique may become a valuable method for lung cancer screening and Dermcidin and S100A9 may serve as biomarkers for lung cancer diagnosis or prognosis.

Keywords Biomarker · Exhaled breath condensate · Mass spectrometry · Protein profile

Abbreviations

EBC Exhaled breath condensate
VEGF Vascular endothelial growth factor

Introduction

Lung biopsy, bronchoalveolar lavage and pleural liquid are the most accepted and used samples to diagnose pulmonary diseases, but their sampling is time consuming, invasive and requires the use of sedation. The exhaled breath condensate (EBC) is a promising matrix due to the easiness of sampling and non-invasive characteristics. EBC was firstly described in the 1960s [1] and has been applied to measure pulmonary parameters [2–4] and also to quantify different proteins by using immune-specific assay kits (ELISA) [5, 6]. The final goal of these determinations is the discovery of early diagnosis markers to allow for the screening of large populations and to find out those individuals more susceptible to develop pathology [7].

Lung cancer accounts for a high percentage of population mortality [8] with an overall survival average of 1–3 years once the disease is diagnosed. At this timepoint, lung cancer is notoriously difficult to treat and ends with metastasis [9]. Many studies have focused on the research of a single protein, such as the use of VEGF for early diagnosis of disease and to discriminate patients with poor prognosis [7, 10]. However, the determination of the protein profile present in

✉ Luis Antonio Mariñas-Pardo
luismarinas@gmail.com

1 University Hospital Complex of A Coruña (CHUAC), As Xubias de Arriba, 84, 15006 A Coruña, Spain

2 Biomedical Research Institute of A Coruña (INIBIC), As Xubias de Arriba, 84, 15006 A Coruña, Spain

the EBC might be an excellent approach to investigate the differences between the healthy and disease statuses.

MALDI-MS is a common technique used to identify the protein profile of different samples. Nevertheless, the technique requires a minimum amount of protein concentration for a proper identification. EBC, due to its low protein concentration, which is quite far from the minimum amount needed (1 mg/ml) for proteomic analysis has yielded a modest number of protein identifications [11, 12]. Besides, contamination with keratins, which may come from sample handling or from the environmental air, is common [13] and may mask the true protein profile of the EBC.

All said, we aimed to optimize the processing of EBC as a prior step to MALDI-MS analysis by comparing three different methods of protein concentration: ultracentrifugation, lyophilization and reversed-phase chromatography. After protein identification in EBC samples from the healthy volunteers, some biomarkers would be selected and measured by ELISA in lung cancer EBC to investigate their possible role on lung cancer development.

Methods

Participants

The study was approved by the Research Ethics Committee of Galicia (Code 2009/283). Participants were recruited from the Pneumology Division at the University Hospital Complex of A Coruña (Spain) and signed a written informed consent. For EBC concentration optimization and mass spectrometry analysis, samples were collected from the healthy volunteers (smokers, $n=15$; non-smokers/ex-smokers, $n=15$). This group included subjects without lung cancer and no history of chronic obstructive pulmonary disease (COPD) or other respiratory conditions. Ex-smokers were defined as not having smoked for at least 1 year.

For biomarker determination by ELISA, patients with the newly diagnosed lung cancer ($n=9$) were recruited immediately before histological diagnosis. None of them had received any form of anti-cancer therapy, invasive diagnostic procedure or primary lung surgery. In addition, healthy volunteers were also recruited (smokers, $n=9$; non-smokers, $n=9$).

EBC Collection

Exhaled breath condensate was sampled employing the ECoScreen device (Viasys GmbH, Germany). Each subject was asked to breathe through the collection kit wearing a nose clip for 10 min, 20 min or two periods of 10 min separated by a short break. The volume of EBC recovered was measured, aliquoted in Lo-Bind Eppendorf tubes and stored at − 80 °C until analysis.

Protein Quantification and Concentration

Protein quantification was performed by BCA protein assay kit (Pierce, USA) following the datasheet protocol.

Protein concentration by ultracentrifugation was performed using Amicon Ultra-2 filters (Millipore Ibérica S.A.U, Spain) reaching a final volume of 100 µl. Lyophilization was done by centrifugation with negative pressure using a vacuum centrifugal evaporator. Protein pellet was resuspended in 100 µl of 1% urea buffer.

Samples were resolved in 12% polyacrylamide SDS-PAGE according to known protocols [14] in a BioRad Mini Protean Tetra Cell (BioRad Laboratories S.A., Spain) and stained with silver nitrate following standard protocols [15]. A protein ladder (PageRuler™ Prestained Protein Ladder, Fermentas Life Sciences) was used to monitor protein separation during SDS-PAGE development.

Mass Spectrometry Analysis

Reversed-phase chromatography concentration was performed with POROS R2 resin (MoBiTec GmbH, Germany). EBC was acidified with 100% trifluoroacetic acid (TFA) and mixed for 20 min at room temperature with POROS R2 resin. Afterwards it was centrifuged for 2 min at 2000 rpm in a column with a 10 µm diameter filter on the bottom and washed 5 times with 200 µl of 0.1% TFA in the same conditions. Sample elution was performed in 200 µl with 0.1% TFA/80% acetonitrile. Digestion was performed overnight with 12.5 ng/l of Sequencing Grade Modified Trypsin (Promega) at 37 °C. The samples were analysed using the MALDI-time of flight (TOF)/TOF mass spectrometer 4800 Proteomics Analyzer (ABSCIEX, Framingham, MA, USA) and 4000 Series Explorer™ Software (ABSCIEX). Data Explorer version 4.2 (ABSCIEX) was used for spectra analyses and generating peak-picking lists. Peak lists generated were analysed with Mascot search engine (http://www.matrixscience.com) using the following parameters: no taxonomy restriction; enzyme used: trypsin, 1 missed cleavage; fixed modifications: carbamidomethyl (C); variable modifications: oxidation (Met); peptide tolerance: 100 ppm; and fragment tolerance: 0.3 Da; peptide charge: +1. Searching was done against the SwissProt database (2019_02 release). All mass spectra were internally calibrated using autoproteolytic trypsin fragments and externally calibrated using a standard peptide mixture (Sigma-Aldrich). TOF/TOF fragmentation spectra were acquired by selecting the 10 most abundant ions of each MALDI-TOF peptide mass map (excluding trypsin autolytic peptides and other known background ions).

ELISA Biomarker Measurement in EBC

Protein stability in EBC samples after long-term frozen storage was assessed as VEGF expression by using a commercially available sandwich enzyme linked immunosorbent assay (ELISA): Quantikine Human VEGF (R&D Systems Europe, Abingdon UK). This assay was specific with no cross activity and the lower limit of detection was 9 pg/ml for VEGF. VEGF is detectable in cancer and healthy controls EBC samples [6].

Commercially available ELISA kits for each biomarker selected after MALDI-MS proteins identification were used: ELISA Kit for Dermcidin (DCD), ELISA Kit for S100 Calcium Binding Protein A9 (S100A9) and ELISA Kit for Cathepsin G (CTSG) (Uscn Life Science Inc., China). The assays were specific for each biomarker and the lower limits of detection were 0.29 ng/ml for Dermcidin, 5.8 pg/ml for S100A9 and 0.058 ng/ml for Cathepsin G. All the assays were performed in duplicate as previously described [6].

Statistical Analysis

All data were analysed by GraphPad Prism® software Version 5.00 (GraphPad Software, Inc., La Joya, USA) and SPSS 10.0 for Windows (SPSS, Chicago, IL, USA). Box and Whisker plots were used to represent biomarker values. In the Tukey boxplot, the horizontal lines of the boxes represent 25%, 50% (median) and 75% percentiles (from bottom to top). Whiskers represent minimum and maximum values. Mann–Whitney test was conducted for the comparison of median values between the lung cancer group and both control groups: non-smokers and smokers. A p-value lower than 0.05 was considered statistically significant.

Results

EBC Collection and Concentration

Sampling in two periods of 10 min produced an EBC volume (mean ± SD; 1787.00 ± 141.70 µl, $n = 9$) that doubled the amount obtained in the 20 min period (918.90 ± 72.66 µl, $n = 9$).

EBC protein concentration in the raw sample was 28.71 ± 1.782 µg/ml. Ultracentrifugation and lyophilization increased the concentration to 58.16 ± 15.14 µg/ml and 68.13 ± 10.18 µg/ml, respectively (Fig. 1a). Raw and concentrated EBC samples (20 µl) were loaded in an SDS-PAGE gel for electrophoresis. The increase in protein concentration can be also observed in the band protein pattern (Fig. 1b). Reversed-phase chromatography was also applied for protein concentration ($n = 9$).

Mass Spectrometry Protein Identification

MALDI-MS protein identification was applied to EBC processed by (1) ultracentrifugation, (2) lyophilization and (3) reversed-phase chromatography (Fig. 2). One hundred peptides were detected and 18 proteins were identified after reversed-phase chromatography (Table 1). Keratins (P04264, P48668, P13645 and Q14533) or salivary and submucosal cells related proteins (P02788, P02768, P19961, P12273,

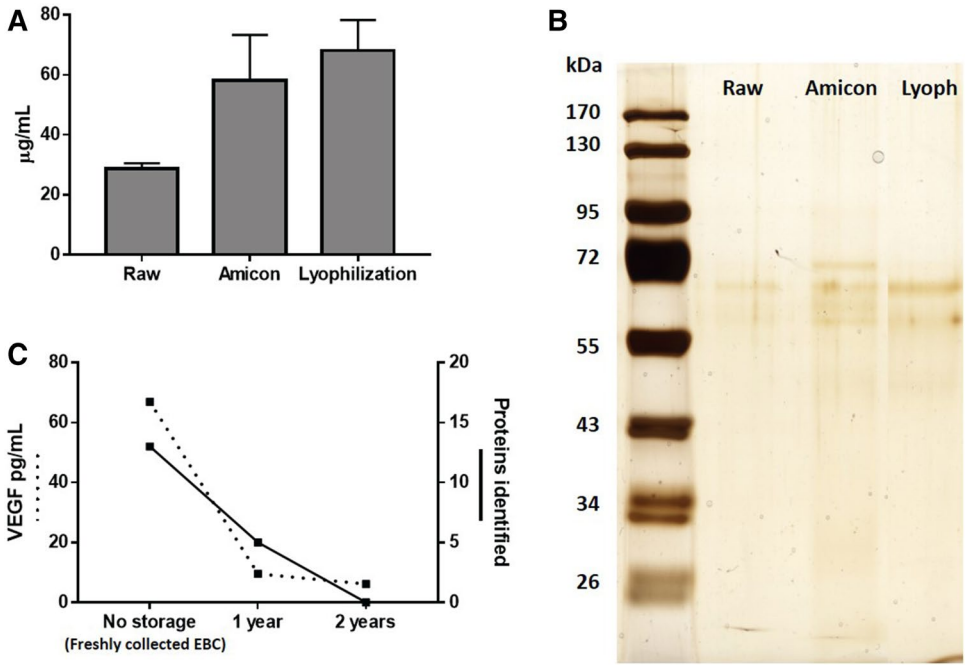

Fig. 1 **a** Protein concentration (µg/ml) in EBC (Raw) and after concentration using Amicon tubes or lyophilization. **b** SDS-Page electrophoresis with the three EBC samples: without concentration (Raw) and after processing by centrifugation using Amicon tubes or lyophilization (Lyoph). **c** Protein stability and number of proteins identified in EBC by MALDI-MS after long-term frozen storage

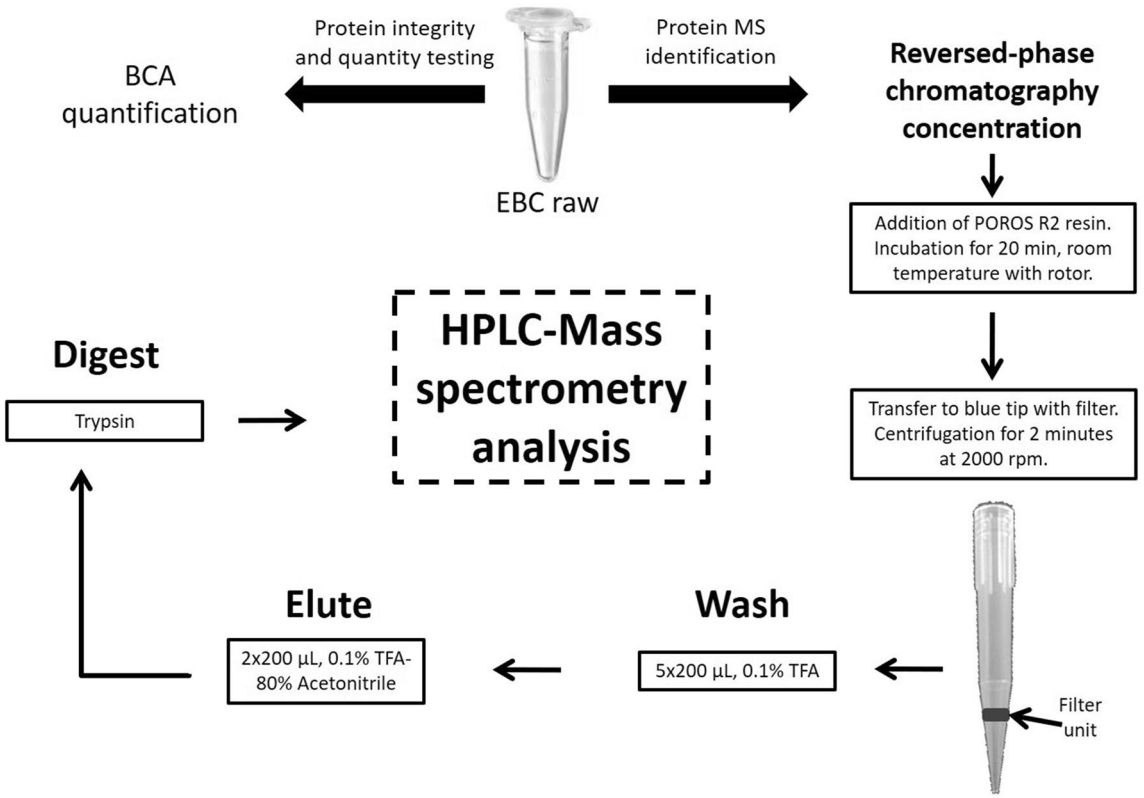

Fig. 2 Schematic workflow for the protocol of EBC processing by reversed-phase chromatography and MALDI-MS analysis

P61626, P01037, P01834 and P0DOY3) were discarded. Three candidates were selected based on their possible role in lung cancer according to the literature: Dermcidin (P81605), 25.5% protein sequence coverage and five peptides identified; S100A9 (P06702), 23.1% protein sequence coverage and three peptides identified; and Cathepsin G (P08311), 29.5% protein sequence coverage and three peptides identified.

Biomarkers' Detection by ELISA

Dermcidin, S100A9 and Cathepsin G were quantified by ELISA in EBC samples obtained from lung cancer and healthy volunteers (Table 2).

Dermcidin expression was statistically higher in lung cancer EBC samples when compared to the levels obtained in healthy EBC measurements, smokers ($p = 0.0002$) and non-smokers ($p = 0.0012$) (Table 3 and Fig. 3a). Same observations were obtained for S100A9, with statistically significant higher protein expression in lung cancer when compared to healthy volunteers ($p < 0.0001$) (Table 3 and Fig. 3b). Cathepsin G was hardly detectable and no statistically significant differences were observed in the three groups analysed (Table 3 and Fig. 3c).

Protein Stability After Frozen Storage

Frozen storage affected the number and stability of proteins present in EBC. The number of proteins identified decreased with larger periods of frozen EBC storage falling from 13 proteins in fresh collected EBC (Fig. 1c) to six proteins after 1 year of storage and zero proteins after 2 years of frozen conservation.

After 1 and 2 years of frozen preservation, VEGF concentration decreased by 5.94 and 11.42-fold, respectively, and in parallel with the declined number of proteins identified by MALDI-TOF–TOF (Fig. 1c).

Discussion

Exhaled breath condensate is a non-invasive sampling technique, very promising for protein identification [16] which could help to understand how a tumour develops from carcinogenesis to metastasis. This information would consequently improve the patients' treatments [17]. Although EBC collection is easy and reproducible [6, 10], some difficulties were described along with some considerations for storage and manipulation of the samples [6]. Following our collection protocol, the volumes fell within the amounts published

Table 1 Proteins identified by MALDI-MS in EBC samples

#	Human proteins identified ($n=18$)	Gene	Protein accession number (UniProt)	Molecular weight (Da)	Role
1	Myeloperoxidase	MPO	P05164	83,869	Part of the host defence system of polymorphonuclear leucocytes. It is responsible for microbicidal activity against a wide range of organisms. It was found increased in sputum supernatant of smokers compared with non-smokers, while no differences were observed between smokers and COPD stage 0 [26]
2	Lactotransferrin	LTF	P02788	78,182	Lactoferrin is a natural forming iron-binding glycoprotein with antibacterial, antioxidant and anti-carcinogenic effects. It is described as expressed in the respiratory tract but also may come from the salivary gland [20]. No differences in expression between lung cancer and control tissues were found [27]
3	Serum albumin	ALB	P02768	69,367	It is described as expressed in the respiratory tract but also may come from the salivary gland [20]
4	Keratin, type II cytoskeletal 1	KRT1	P04264	66,039	It is identified as contaminant [20].
5	Keratin, type II cytoskeletal 6C	KRT6C	P48668	60,025	It is identified as contaminant [20]
6	Keratin, type I cytoskeletal 10	KRT10	P13645	58,827	It is identified as contaminant [20]
7	Alpha-amylase 2B	AMY2B	P19961	57,710	It is identified as salivary origin [20]
8	Keratin, type II cuticular Hb1	KRT81	Q14533	54,928	It is identified as contaminant [20]
9	Cathepsin G	CTSG	P08311	28,837	Cathepsins are highly expressed in various human cancers, associated with tumour metastasis [22]. Cathepsin G activity was suggested to be related to the grade of tumour differentiation and particular clinical stages of disease [38]
10	Azurocidin	AZU1	P20160	26,886	Found to be differentially expressed in lung cancer tissues compared to controls [27] but no previously detected in EBC samples
11	Prolactin-inducible protein	PIP	P12273	16,572	It is identified as salivary origin [20]
12	Lysozyme C	LYZ	P61626	16,537	Previously detected in EBC from asthmatic patients [11], it is described as expressed in the respiratory tract but also may come from the salivary gland [20]
13	Cystatin-SN	C13ST1	P01037	16,388	It is identified as salivary origin [20]
14	Transthyretin	TTR	P02766	15,887	Found to be expressed in lung carcinoma biopsies with the exception of adenocarcinoma [29]
15	Protein S100-A9	S100A9	P06702	13,242	It expression is increased in monocytes from patients with non-small cell lung cancer (NSCLC) [33] and in lung biopsies it was described to be correlated with tumour differentiation degree [34]
16	Immunoglobulin kappa constant	IGKC	P01834	11,765	It is described as expressed in the respiratory tract but also may come from the salivary gland [20]
17	Dermcidin	DCD	P81605	11,284	Previously detected in EBC [11, 12], high number of Dermcidin peptides were found in the EBC of patients with lung cancer [21, 30]
18	Immunoglobulin lambda constant 3	IGLC3	P0DOY3	11,266	It is described as expressed in the respiratory tract but also may come from the salivary gland [20]

in the literature [18, 19], but two periods of 10 min of collection produced more volume than 20 min of continued breathing.

Exhaled breath condensate protein concentration is insufficient for proteomics analysis [11, 12], and some authors have pooled a high number of EBC samples to overcome this issue [20, 21]. Another approach to increase the protein concentration consists in processing the EBC previous to the analysis by mass spectrometry. The band protein pattern observed for raw, lyophilized and ultracentrifugation concentrated EBC in an electrophoresis gel reflected the increase in concentration in a pattern similar to that previously published [13].

Table 2 Demographic, smoking and clinical data in the three groups analysed

	Cancer ($n=9$)	Smoker ($n=9$)	Non-smoker ($n=9$)
Sex (n (%))			
Male	7 (77.8)	5 (55.6)	1 (11.1)
Female	2 (22.2)	4 (44.4)	8 (88.9)
Age (mean ± SD)	59.78 ± 10.75	43.67 ± 8.02	54.11 ± 8.16
Smoking status (n (%))			
Smoker	4 (44.4)	9 (100.0)	–
Non-smoker	1 (11.2)	–	9 (100.0)
Ex-smoker	4 (44.4)	–	–
Cancer histology (n (%))			
Adenocarcinoma	3 (33.34)	–	–
Squamous cell carcinoma	6 (66.66)	–	–
Cancer stage (n (%))			
II	1 (11.11)	–	–
III	1 (11.11)	–	–
IV	7 (77.78)	–	–

Table 3 Dermcidin, S100A9 and Cathepsin G expression differences between cancer and control (smoker and non-smoker) groups

	Group	*N*	Median (ng/ml)	Minimum–maximum	Mean rank	Sum of ranks	Mann–Whitney *U*	Asymp. sig. (2-tailed)
Dermcidin	Cancer	7	0.22240	0.09244–0.49130	13.00	91	0	0.0002
	Smoker	9	0.05782	0.04981–0.08132	5.00	45		
Dermcidin	Cancer	7	0.22240	0.09244–0.49130	12.57	88	3	0.0012
	Non-smoker	9	0.08973	0.06120–0.11470	5.33	48		
S100-A9	Cancer	9	2.84300	2.39300–5.27600	14.00	126	0	< 0.0001
	Smoker	9	1.40000	1.36400–1.85200	5.00	45		
S100-A9	Cancer	9	2.84300	2.39300–5.27600	14.00	126	0	< 0.0001
	Non-smoker	9	1.69900	1.03000–1.88600	5.00	45		
Cathepsin G	Cancer	9	0.11690	0.01589–1.20700	11.78	106	20	0.0770
	Smoker	9	0.04646	0.01314–0.13970	7.22	65		
Cathepsin G	Cancer	9	0.11690	0.01589–1.2070	11.44	103	23	0.1299
	Non-smoker	9	0.06945	0.02440–0.16730	7.56	68		

Reversed-phase concentration is proposed to obtain the protein profile present in EBC samples by MALDI-MS analysis. Dermcidin and S100A9 seem to be highly expressed in EBC samples from lung cancer patients thus pointing to their possible role in the disease development and their possible use as early markers for lung cancer screening procedures.

According to our results, neither lyophilization nor ultracentrifugation is appropriate and no protein could be identified. Reversed-phase chromatography was effective for preprocessing EBC and obtaining a reduced protein profile of the proteome. It is expected that those proteins with a crucial role in the disease development are the most expressed in the EBC, so they should be easily detected even in small volume samples.

Storage of the samples showed to be an important parameter to be considered. The duration of the frozen storage affects protein stability and accordingly the number of proteins identified by MALDI-MS. Protein identification and ELISA detection decreased with time thus highlighting the need of the early analysis of the samples. EBC samples should be immediately frozen after collection and stored at − 70 °C until biomarker determination is performed, but even after taking these precautionary measures, some mediators are not stable after long-term storage [19]. Similar situation was described when analysing the proteome of serum samples after 1 year of cryostorage. The most abundant proteins were identified by MALDI-MS even after 1 year, with no detectable peptide loss, but it is suggested that the detection of low abundant proteins could not be optimal after very long periods of time [22].

We suggest some tips for processing EBC samples and MALDI-MS detection: (1) two periods of 10 min can

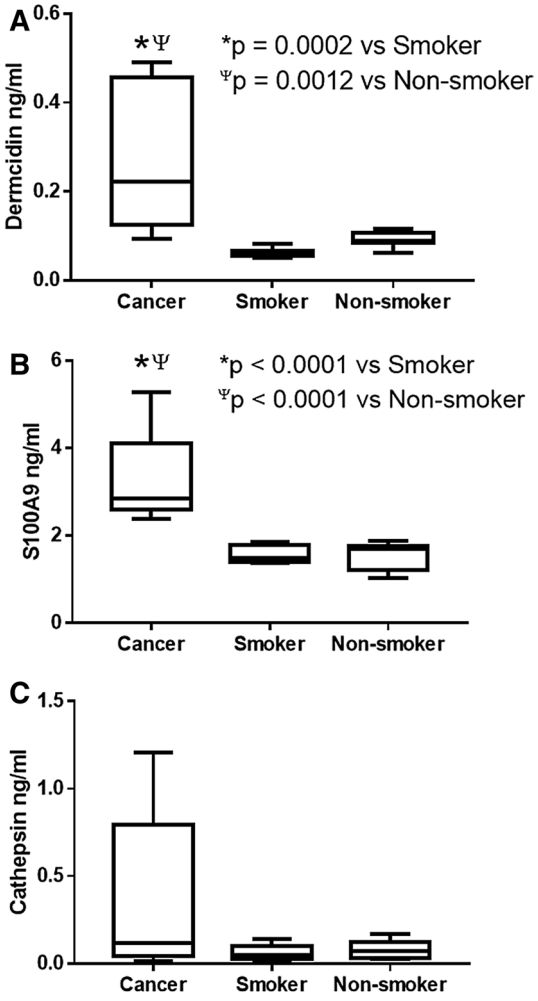

Fig. 3 Biomarker quantification using ELISA. **a** Dermcidin (ng/ml) expression in the three studied groups: cancer, smoker and non-smoker. **b** S100A (ng/ml) expression in the three studied groups: cancer, smoker and non-smoker. **c** Cathepsin G (ng/ml) expression in the three studied groups: cancer, smoker and non-smoker

increase the EBC volume recovery and protein obtained, (2) storage period should not surpass 1 year and (3) reversed-phase chromatography protocol is effective to identify proteins profiles in EBC samples.

Keratins have been described as the major components in EBC samples [23] and were discarded for analysis as considered contaminations [20]. They may derive from ambient air or the upper airway [13]. Proteins suspected to derive from salivary origin were also discarded [20]. According to the literature, even after following a proper sample collection and handling, small volumes of saliva may still be present in EBC thus triggering the detection of saliva related proteins [24].

Myeloperoxidase is known to be part of the host defence system of polymorphonuclear leucocytes and was proposed as marker of oxidative stress of airway inflammation [25]. It was found increased in sputum supernatant of smokers compared with non-smokers, while no differences were observed between smokers and COPD stage 0 [26]. Lactotransferrin has been described as a glycoprotein with antibacterial, antioxidant and anti-carcinogenic effects, expressed not only in the respiratory tract but also in the salivary gland [20]. No differences in expression between lung cancer and control patients were found in an analysis of tissue specimens looking for novel biomarkers for diagnosis [27]. Azurocidin is a monocyte chemotactic protein with antibacterial activity. It is an important multifunctional inflammatory mediator found to be differentially expressed in lung cancer tissues [27] and bronchoalveolar fluid [28], but not previously detected in EBC samples. Lysozyme C was detected in EBC from asthmatic patients [11] and healthy controls [20]. It has primarily a bacteriolytic function, and is described as expressed in the respiratory tract but also may come from the salivary gland [20]. Transthyretin is a normal serum protein synthesized primarily in the liver, the choroid plexus and the retina. Immunostaining of lung cancer and normal lung tissues specimens showed no statistically significant relationship between transthyretin expression and lung cancer stages [29], so the role of transthyretin in carcinogenesis could not be elucidated.

Among the proteins identified in our work, three biomarkers were selected based on their possible role in lung cancer development [20, 21, 30]. Cathepsin G activity was suggested to be related to the grade of tumour differentiation and particular clinical stages of disease [31]. Cathepsin G binds to the cancer cell surface and enters tumour endosomes competing for Neutrophil Elastase binding sites. Consequently, Cathepsin G acts by inhibiting the tumour promoting effects of Neutrophil Elastase [32]. According to our results, Cathepsin G was hardly detected by ELISA, with very low levels of expression and no significant differences between cancer and control groups, showing not to be an appropriate marker for lung cancer prognosis. Dermcidin has antimicrobial activity and have been reported in EBC of healthy volunteers [12], patients with asthma [11] and lung cancer [21, 30] thus supporting the findings of our study. Dermcidin in lung cancer patients was higher in those individuals with weight loss, and negatively related to survival in medium-to-advanced stages of disease (II–IV) [33]. A glycosylated form of the N-terminal peptide may be associated with cachexia (muscle wasting), which is characteristic of cancer patients [34]. S100A9 is increased in patients with non-small cell lung cancer (NSCLC) [35] and correlates with tumour differentiation degree [36]. mRNA expression of S100A9 was found to be a negative prognostic marker in NSCLC patients [37]. Recent studies have reported that S100 protein may be associated with tumour metastasis [38] and suggested that S100A9 may serve as useful clinical target for anti-metastasis therapy by suppressing cancer cells

invasive and migratory capabilities [39]. Dermcidin and S100A9 may be potential markers for poor prognosis of the disease considering (1) that both markers can be detected in EBC, (2) that their expressions are elevated in samples from lung cancer patients, and (3) the importance of their levels in lung cancer development.

This study has some limitations related to the small sample size that could be biasing the results. New patient recruitment would be necessary to analyse Dermcidin and S100A9 detection and confirm if the observed results can be replicated. On the contrary, due to the high percentage of stage IV patients, it is not currently possible to discern if the suggested biomarkers are able to detect early stages of disease. Consequently, higher numbers of patients in each cancer stage would help to determine if those markers are detectable in each stage or if their expression level changes with disease progression.

Conclusion

Exhaled breath condensate from lung cancer patients contained statistically higher concentrations of Dermcidin and S100A9, pointing to their potential use as biomarkers for lung cancer. Screening and prospective studies enrolling asymptomatic patients should be performed in the future to evaluate if Dermcidin and S100A9 markers could identify a preclinical lung disease condition.

In conclusion, EBC analysis combined with MS technique may become a valuable method for lung cancer screening, and Dermcidin and S100A9 may serve as biomarkers for lung cancer diagnosis or prognosis.

Acknowledgements We thank M. Blanco-Calvo for critical reading of the manuscript and also Jesús Mateos-Martín for helping with MALDI-MS processing.

Authors' Contribution LN-N, LM-P and CM-M designed the study and protocol. LN-N and LM-P drafted the present manuscript and performed the statistical analyses. LN-N performed EBC collection and processing. All authors contributed to the data acquisition, interpretation, and drafting of the analyses, critical review, and final approval of the manuscript.

Funding This research was funded by Xunta de Galicia, INCITE 09 916 360 PR

Compliance with Ethical Standards

Conflict of interest All authors confirm that they do not have any conflicts of interest associated with this manuscript.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent Informed consent was obtained from all individual participants included in this study.

References

1. Sidorenko GI, Zborovskii EI, Levina DI (1980) Surface-active properties of the exhaled air condensate (a new method of studying lung function). Ter Arkh 52:65–68
2. Shibata A, Katsunuma T, Tomikawa M et al (2006) Increased leukotriene E4 in the exhaled breath condensate of children with mild asthma. Chest 130:1718–1722. https://doi.org/10.1378/chest.130.6.1718
3. Vaughan J, Ngamtrakulpanit L, Pajewski TN et al (2003) Exhaled breath condensate pH is a robust and reproducible assay of airway acidity. Eur Respir J 22:889–894
4. Montuschi P, Corradi M, Ciabattoni G et al (1999) Increased 8-isoprostane, a marker of oxidative stress, in exhaled condensate of asthma patients. Am J Respir Crit Care Med 160:216–220
5. Robroeks CM, Rijkers GT, Jobsis Q et al (2010) Increased cytokines, chemokines and soluble adhesion molecules in exhaled breath condensate of asthmatic children. Clin Exp Allergy 40:77–84. https://doi.org/10.1111/j.1365-2222.2009.03397.x
6. Nunez-Naveira L, Marinas-Pardo LA, Amor-Carro O, Montero-Martinez C (2012) Determination of ELISA reproducibility to detect protein markers in exhaled breath condensate. J Breath Res. https://doi.org/10.1088/1752-7155/6/4/046003
7. Conrad DH, Goyette J, Thomas PS (2008) Proteomics as a method for early detection of cancer: a review of proteomics, exhaled breath condensate, and lung cancer screening. J Gen Intern Med 23(Suppl 1):78–84. https://doi.org/10.1007/s11606-007-0411-1
8. Torre LA, Bray F, Siegel RL et al (2015) Global cancer statistics, 2012. CA Cancer J Clin 65:87–108. https://doi.org/10.3322/caac.21262
9. Pallis AG, Fennell DA, Szutowicz E et al (2011) Biomarkers of clinical benefit from anti-epidermal growth factor receptor agents in patients with non-small-cell lung cancer. Br J Cancer 105:1–8. https://doi.org/10.1038/bjc.2011.207
10. Dalaveris E, Kerenidi T, Katsabeki-Katsafli A et al (2009) VEGF, TNF-alpha and 8-isoprostane levels in exhaled breath condensate and serum of patients with lung cancer. Lung Cancer 64:219–225. https://doi.org/10.1016/j.lungcan.2008.08.015
11. Bloemen K, Van Den Heuvel R, Govarts E et al (2011) A new approach to study exhaled proteins as potential biomarkers for asthma. Clin Exp Allergy 41:346–356. https://doi.org/10.1111/j.1365-2222.2010.03638.x
12. Kurova VS, Anaev EC, Kononikhin AS et al (2009) Proteomics of exhaled breath: methodological nuances and pitfalls. Clin Chem Lab Med 47:706–712. https://doi.org/10.1515/CCLM.2009.166
13. Hoffmann HJ, Tabaksblat LM, Enghild JJ, Dahl R (2008) Human skin keratins are the major proteins in exhaled breath condensate. Eur Respir J 31:380–384. https://doi.org/10.1183/09031936.00059707
14. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
15. Rabilloud T, Vuillard L, Gilly C, Lawrence JJ (1994) Silver-staining of proteins in polyacrylamide gels: a general overview. Cell Mol Biol 40:57–75
16. Dwyer TM (2004) Sampling airway surface liquid: non-volatiles in the exhaled breath condensate. Lung 182:241–250
17. Banks R, Selby P (2003) Clinical proteomics—insights into pathologies and benefits for patients. Lancet 362:415–416. https://doi.org/10.1016/s0140-6736(03)14096-2

18. Mutlu GM, Garey KW, Robbins RA et al (2001) Collection and analysis of exhaled breath condensate in humans. Am J Respir Crit Care Med 164:731–737
19. Horvath I, Hunt J, Barnes PJ et al (2005) Exhaled breath condensate: methodological recommendations and unresolved questions. Eur Respir J 26:523–548. https://doi.org/10.1183/09031936.05.00029705
20. Lacombe M, Marie-Desvergne C, Combes F et al (2018) Proteomic characterization of human exhaled breath condensate. J Breath Res 12:021001. https://doi.org/10.1088/1752-7163/aa9e71
21. López-Sánchez LM, Jurado-Gámez B, Feu-Collado N et al (2017) Exhaled breath condensate biomarkers for the early diagnosis of lung cancer using proteomics. Am J Physiol Cell Mol Physiol 313:L664–L676. https://doi.org/10.1152/ajplung.00119.2017
22. Mateos J, Pintor-Iglesias A, Fernández-Puente P et al (2014) Cryoconservation of peptide extracts from trypsin digestion of proteins for proteomic analysis in a hospital biobank facility. J Proteome Res 13:1930–1937. https://doi.org/10.1021/pr401046u
23. Jackson AS, Sandrini A, Campbell C et al (2007) Comparison of biomarkers in exhaled breath condensate and bronchoalveolar lavage. Am J Respir Crit Care Med 175:222–227. https://doi.org/10.1164/rccm.200601-107OC
24. Cruickshank-Quinn C, Armstrong M, Powell R et al (2017) Determining the presence of asthma-related molecules and salivary contamination in exhaled breath condensate. Respir Res 18:57. https://doi.org/10.1186/s12931-017-0538-5
25. Romero PV, Rodríguez B, Martínez S et al (2006) Analysis of oxidative stress in exhaled breath condensate from patients with severe pulmonary infections. Arch Bronconeumol 42:113–119
26. Rytilä P, Rehn T, Ilumets H et al (2006) Increased oxidative stress in asymptomatic current chronic smokers and GOLD stage 0 COPD. Respir Res 7:69. https://doi.org/10.1186/1465-9921-7-69
27. Nigro E, Imperlini E, Scudiero O et al (2015) Differentially expressed and activated proteins associated with non small cell lung cancer tissues. Respir Res 16:74. https://doi.org/10.1186/s12931-015-0234-2
28. Hmmier A, O'Brien ME, Lynch V et al (2017) Proteomic analysis of bronchoalveolar lavage fluid (BALF) from lung cancer patients using label-free mass spectrometry. BBA Clin 7:97–104. https://doi.org/10.1016/j.bbacli.2017.03.001
29. Hao S, Sun S, Xiao X et al (2016) Selective expression of transthyretin in subtypes of lung cancer. J Mol Histol 47:239–247. https://doi.org/10.1007/s10735-016-9666-3
30. Chang WC, Huang MS, Yang CJ et al (2010) Dermcidin identification from exhaled air for lung cancer diagnosis. Eur Respir J 35:1182–1185. https://doi.org/10.1183/09031936.00169509
31. Maksimowicz T, Chyczewska E, Chyczewski L et al (1997) Activity and tissue localization of cathepsin G in non small cell lung cancer. Rocz Akad Med Bialymst 42(Suppl 1):199–216
32. Gregory AD, Hale P, Perlmutter DH, Houghton AM (2012) Clathrin pit-mediated endocytosis of neutrophil elastase and cathepsin G by cancer cells. J Biol Chem 287:35341–35350. https://doi.org/10.1074/jbc.M112.385617
33. Wang Q, Lu J-B, Wu B, Hao L-Y (2010) Expression and clinicopathologic significance of proteolysis-inducing factor in non-small-cell lung cancer: an immunohistochemical analysis. Clin Lung Cancer 11:346–351. https://doi.org/10.3816/CLC.2010.n.044
34. Mirza KA, Wyke SM, Tisdale MJ (2011) Attenuation of muscle atrophy by an N-terminal peptide of the receptor for proteolysis-inducing factor (PIF). Br J Cancer 105:83–88. https://doi.org/10.1038/bjc.2011.216
35. Feng P-H, Lee K-Y, Chang Y-L et al (2012) CD14 (+) S100A9 (+) monocytic myeloid-derived suppressor cells and their clinical relevance in non-small cell lung cancer. Am J Respir Crit Care Med 186:1025–1036. https://doi.org/10.1164/rccm.201204-0636OC
36. Huang H, Huang Q, Tang T et al (2018) Clinical significance of calcium-binding protein S100A8 and S100A9 expression in non-small cell lung cancer. Thorac Cancer 9:800–804. https://doi.org/10.1111/1759-7714.12649
37. Liu Y, Cui J, Tang Y-L et al (2018) Prognostic roles of mRNA expression of S100 in non-small-cell lung cancer. Biomed Res Int 2018:1–11. https://doi.org/10.1155/2018/9815806
38. Tian T, Li X, Hua Z et al (2017) S100A1 promotes cell proliferation and migration and is associated with lymph node metastasis in ovarian cancer. Discov Med 23:235–245
39. Lim SY, Yuzhalin AE, Gordon-Weeks AN, Muschel RJ (2016) Tumor-infiltrating monocytes/macrophages promote tumor invasion and migration by upregulating S100A8 and S100A9 expression in cancer cells. Oncogene 35:5735–5745. https://doi.org/10.1038/onc.2016.107