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Research Article

Measurements of Sputum Desmosines Using Isotope Dilution Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

An LC-MS/MS method for the absolute quantitation of total desmosine (DES/IDS) in human sputum using synthesized in house internal standard (IS: D4-IDES) was developed. The method involves acid hydrolysis, solid phase extraction (SPE) and liquid chromatographic separation and tandem mass spectrometric analysis (LC-MS/MS). The synthesized IS was characterized to ensure its suitability for the assay. The IS was found to contain at least 4 forms of deuterated IDES. D4-IDES was the most abundant derivative and its structure confirmed by mass spectrometry. The calibration curve showed good precision and accuracy from 0.05 (LLQ) to 20.0ng/mL. The intra- and inter-assay precision (%CV) and accuracy (%Bias) of the assay were determined. The precision intraassay ranged from 4.76 to 23.47% and the interassay were from 1.05 to 20.15%. whereas per the accuracy intraassay ranged from 20.0 to -20.0% and the interassay were from -13.33 to -3.33%. The reference range of total DES was established in forty sputum samples and concentrations of all sputum samples fall within the reportable range of the assay. These results demonstrate that the developed method provided a sensitive, reproducible and accurate quantitation of total desmosine in human sputum samples that could be used as a biomarker for monitoring elastin degradation in respiratory diseases.

Abbreviations

DES: Desmosine
IDES: Isodesmosine
Total DES/IEDS: total amount of DES and IEDS in free form or peptide forms
LC: Liquid Chromatography
MS: mass spectrometry
COPD: CHRONIC OBSTRUCTIVE PULMONARY DISEASE
D4-IDES: Deuterated Isodesmosine, 4 Hydrogen atoms exchanged by 4 Deuterium atoms
SPE: Solid Phase Extraction
LC-MS/MS: liquid chromatography tandem mass spectrometry
LLQ: lower limit of quantitation
%CV: Precision, % coefficient of variance
%Bias: Accuracey, % of error
CF: Cystic Fibrosis
HPLC: High performance liquid chromatography
NMR: Nuclear magnetic resonance
D2O: Di-deuterium oxide
THF: Tetrahydrofuran
DBU: (1,8-Diazabicyclo[5.4.0]undec-7-ene)
DAD: Diode Array Detector
MRM: Multi reaction monitoring
FWMH Full width at half maximum
FA: Formic Acid
uDES: Urinary desmosine
bDES: blood desmosine
AAT: alpha anti-trypsin

Introduction

The World Health Organization has classified Chronic Obstructive Pulmonary Disease (COPD) as the most common chronic disease among children, expecting that one third of all deaths worldwide will be attributed to COPD by 2030 [1]. A common denominator was discovered among respiratory lung diseases, namely lung elastin degradation. Which in turn results in two major metabolites, two non-traditional amino acids known as Desmosine (DES) and Isodesmosine (IDES), in which both act as crosslinking networks of elastin [2,3]. The chemical structures of the two isomers shown in figure 1.

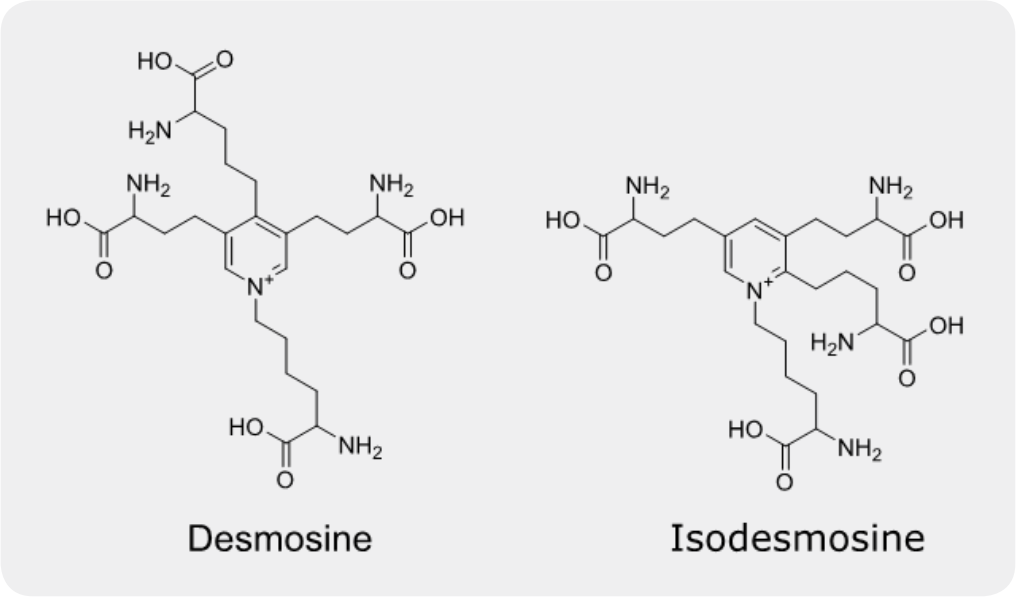

Figure 1: *The Chemical Structures of two major metaboloites of elastion degradation, Desmosine and Isodesmosine*

Elastin degradation gain potential as a target for pharmacological intervention in lung diseases [4-9]; elastin degradation has turned into a promising biomarker for both diseases progression as well as patient response to treatment. Therefore, a reliable and sensetive assays are needed to enhance certainty of elastin degradation clinical validity as a biomarker for COPD. over the past 30 years many analytical methods developed to quantify desmosine and isodesmosine as biomarkers of elastin degradation due to its uniqueness to human mature cross-linked elastin; simultaneously elastin degradation reflects/correlates to/with the respiratory disease status.

Most of the previous studies focused on the measurement of desmosine in urine [10-26] due to the low urine matrix complexity and the high concentrations of desmosines predicted in urine compared to plasma or sputum. On the otherhand, urinary desmosine represents the whole body elastin turnover [4,5] which makes uDES (Urinary Demsosine) less directly associated to the pathological processes of lung disease.

Concentrations of desmosine are significantly high in patients with respiratory diseases such as asthma[17,27-30], (CF) cystic fibrosis [18,25,26,31-35], COPD [5,32,36-38], alpha-antitrypsin deficiency [5,32,39-47], and bronchiectasis [32,48-51] compared to healthy subjects unless they smoke; where, elevated levels of desmosine were observed in healthy smokers [21,32,38,52-57].

Different methodologies were used to measure desmosines, radioimmunoassay [58] immunoassay methods [37,58-60] capillary electrophoresis [32,34,61-63] electrokinetic chromatography [12,33,34,64], nuclear magnetic resonance (NMR) [65,66]. and high-performance liquid chromatography, HPLC [11,31,38,67], liquid chromatographymass spectrometry (LC-MS), and LC-tandem mass spectrometry (LC-MS/MS) [5,10,33,68-72] MALDI iontrap mass spectrometry [66,73], Isotope dilution tandem mass spectrometry provided the best specificity and sensitivity measuring desmosines.

LC-MS based desmosine methods lack to a prober internal standard until Thibault *et al*. 200916 developed a sensitive nanoLC-MS/MS method using heavy desmosine (D4-DES) as internal standard for absolute quantitation, compensating potential matrix effects. However, a derivativization step together with the use of nano-flow liquid chromatography made the use of this method in routine clinical studies difficult.later on, using D5-DES isotope dilution LC-MS/MS method with microflow suitable for high throughput clinical assays two methods for absolute quantitation of total urinary & plasma desmosines were published [10,72]. Using these validated methods, a combined study was subsequently published17 to verify the clinical application of uDES and bDES as phenotyping biomarkers for COPD. In addition, the correlation of uDES and bDES levels with the smoking status, disease frequency, and lung function was investigated.

Whereas for sputum desmosines, different methodologies were used. Ma *et al*. showed that total DES/IDS in sputum (both induced and spontaneous) were between 0.03-0.58ng/mL (5.3-207.69pg/mg protein) in patients with COPD (normal AAT levels) using a LC-MS/MS method 5. Boschetto *et al*. also showed that total DES/IDS in induced sputum of COPD patents with emphysema did not differ from the ones without emphysema (overall ranged between 5.8 to 13.1ng/mg protein) using capillary electrophoresis. In cystic fibrosis, Laguna *et al* reported recently that spontaneous sputum desmosine levels ranged between 5-128pmol/mL using RIA36.

Low concentrations in addition to technical difficulties hindered the accurate quantitation of sputum desmosines, despite its obvious association with lung pathologies. Therefore, a reliable and highly sensitive method quantifting desmosines- suitable for clinical use- is needed. In the present study; we renounce the ion-pairing reagents used as mobile phase modifiers in previous methods. The ion-pairing reagents resulted in significant ion-suppression effect on measured analytes intensities. Overcoming this effect has resulted in the improvement of sensitivity levels of the method allowing us to detect and accurately quantify sputum desmosine in healthy volunteers. As a result, an accurate and precise LC-MS/MS method for the quantitation of sputum desmosines was developed using in house synthesised internal standard (D4-IDES).

Experimental Details

Chemicals

Desmosine and Isodesmosine were purchased from Elastin Products Company Inc. (Owensville, MO). D4-IDES Internal Standard was synthesized in our lab, HPLC gradient grade acetonitrile (ACN), and Formic acid (FA) (analytical reagent grade, 98%) were from Fisher Scientific. Tetrahydrofuran (THF), butanol (Chromasolv grade), Heavy water (D2O) (part # 151882-10X1ML), DBU (1,8-Diazabicyclo[5.4.0]undec-7-ene) were all purchesed from Sigmal-Aldrich. Acetic acid, 37% HCl were from VWR. Finally, Milli-Q water, Millipore, USA)

Equipment and Materials

Agilent 1290 Infinity II UHPLC system from (Agilent Technologies, USA) equipped with a diode array detector (DAD). Agilent 6460 Triple quadrupole Mass Spectrometer with Jet Steam ESI source, Agilent Technologies, USA). High SpeedVac Model H/T12MM (CHINCAN, China). Agilent Polaris C18 HPLC

Column, 3µm, 50 × 1.0mm. and SPE columns, C18, 40-60µm, 120Å, 500mg/6mL (StarLab, China part # SLSPE5006C18), LCMS certified high recovery glass vials (Agilent part # 5183-2030).

Clinical Sample Collection

Human sputum samples were collected from King Fahd Hospital-Medina, KSA, 87 apparently healthy volunteers and Asthma/COPD patients only 40 samples were used in this pilot study. All samples were frozen at -80˚C until further analysis. All study participants gave their written informed consent and Taibah University Ethical approval board approved the original study.

Deuterated Desmosine Synthesis and Purification

We followed previously described protocol [15] for the synthesis of desmosine dueterated derivatives . Briefly, 50µg of IDES reconsitituted in 0.1mL of heavy water (D2O), 5µL of DBU catalyzed the reaction while stirred at 70˚C for 36 h in a closed vessel. Thereafter, two clean up steps are needed to remove the BDU using 1mL of chloroform four times. Then unreacted heavy water was removed by 3 cycles of evaporation/ reconstitution in 1mL H2O. The purified D4-IDES dissolved in 1mL of 0.2% formic acid to inactivate the remaining traces of DBU and thus prevent the deuterium exchange, bubbled with nitrogen and stored. D4-IDES was subsequently analyzed by MALDI-TOF and with direct injection in LC-MS system to confirm its purity. See figure S1 in supplimental information figures.

Desmosines Extraction:

To each 250µl of sputum sample placed in 2mL eppendourf vial, 50µl of deuterated desmosine (Internal standared (final conc. of IS (0.5µg/ml)) was added. Then 250µL of concentrated HCl (12N) was added. Samples vials were secured with a lid lock clips and placed in a heating block for 18 hours at 110°C. Following up to digestion step to hydrolyze peptide bonds and release bonded desmosines. The mixture was allowed to cool-down for 1 hour, then filtered throght SPE columns to remove contaminants (potential matrix effect) and also concentrate analytes. C18 SPE columns were conditioned in two consecutive steps applying MilliQ water followed by the same volume of (butanol/acetic acid/water=4:1:1) mixture. Samples were loaded then eluted after the washing step, using MilliQ water; as described on the manifacturers manual. Eluted desmosines then dried and reconstituted in 40µL of 0.1% FA. The reconstituted mixture was vortexed for 10 seconds and sonicated for 5 minutes and then were spun at 14,000xg for 10 minutes prior to transferring the supernatant to LCMS high recovery Agilent vials.

LC-MS/MS Analysis

Mass spectrometric Analysis was carried out using (MRM) multi reaction monitoring procedure. LC-MS/MS parameters were optimized to achieve the maximum ion abundances, The mass spectrometer was set on positive ion mode, ion spray voltage at 4kV. The scan time was set at 500msec and mass resolution was set at 0.7Da FWMH), MS/MS spectra were acquired for the precursor ions (m/z= 526→481 for IDES and 530→485 for D4-IDES). The fragmentation pattern of D4-IDES was identical to that of IDES except the mass shift due to deuterium atoms. On the HPLC system, 10µL of each sample was injected onto

an Agilent Polaris C18 column (3µm, 50mm x 1.0mm). Samples were separated and eluted by gradiente mobile phases A (100 water/ 0.1% Formic acid), and B (98% acetonitrile/0.1% Formic acid) using a flow rate 250µl/min, employing a linear gradient from 5%B to 60%B in 4 minutes, then in 2 minutes to 90% B maintained for 2 minutes to clean up the column followed by 3 minutes back to 5%B to restore to initial conditions and recalibrate the column before the next injection.

Data Analysis

Agilent MassHunter Qualitative Analysis B.06.00 was used to measure chromatogram peak areas for IDES and D4-IDES. Three standard curves generated over three days and statistical values standard deviation (SD), precision %CV and accuracy (%Bias) were calculated. All data are shown in supplemental information tables S1-S3 and interassay table S4 in addition to calibration curve shown in Figure 2.

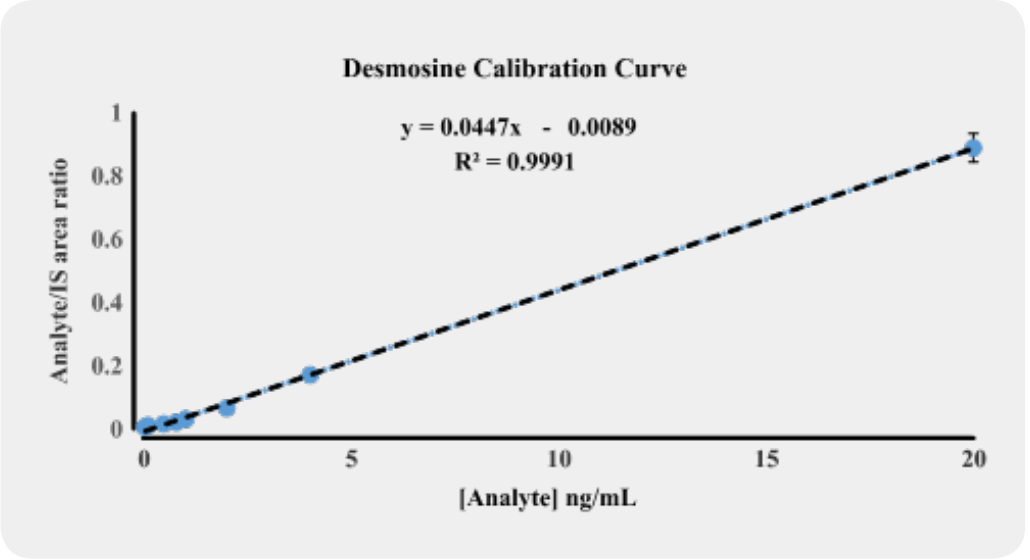

Figure 2: *A typical standard curve ranges from 0.05 to 20 ng/mL*

Results and Discussion

Improvement of Assay Sensitivity

In order to improve the assay sensitivity, we employed two approaches- (1) decrease LC-column diameter from 2.0 mm to 1.0mm and (2) change the mobile phase compositions, omitting the use of ion-pairing modifiers used previously [10,76]. A simplified workflow for sputum desmosine similar to the one used with urine and plasma methods was used. In brief, internal standard is spiked in from the first instance with sputum samples then acid-hydrolyzed at 108°C for 18 hrs. desmosines extracted using SPE columns and reconstituted in 0.1% FA prior to analysis. A steep gradient was used to ensure that desmosine and isodesmosine co-eluted see figure S3 in supplimental information. The MRM transitions monitored were 526/480 for DES/IDS and 530/485 for D4-IDS.

Limits of Quantification

Nine calibrators used to construct the calibration curve for desmosines with concentrations ranged between 0.05 to 20.0ng/mL. Based on three analytical runs all statistical calculations were elaborated. The intra-assay precision (%CV) and accuracy (%Bias) for each of the eleven calibrators (0.05 - 20ng/mL) (n=3) are shown in supplimental information tables (Table S1, S2, and S3) with %CV ranging from 4.76 to 23.47% and %Bias from -20.0 to 20.0%. The inter-assay precision (%CV) and accuracy (%Bias) results are shown in (Table S4). The %CV ranged from 1.05 to 20.15% and %Bias from -13.33 to 3.33%.

Sputum Total Desmosines Concentrations in Two Clinical Cohorts

This method utility was assessed measuring total sputum DES/IDS in healthy volunteers and patients with COPD. The first cohort consists of a total of 20 sputum samples from healthy volunteers, and, the second cohort consists of 20 sputum samples from COPD patients. Detailed results are shown in supplimental information tables (Table S5). All concentrations of sputum total DES/IDS measured fall above the LLOQ, ranging from 0.047 to 0.380 ng/mL. Results are summarised in Box and Whisker plot figure 3.

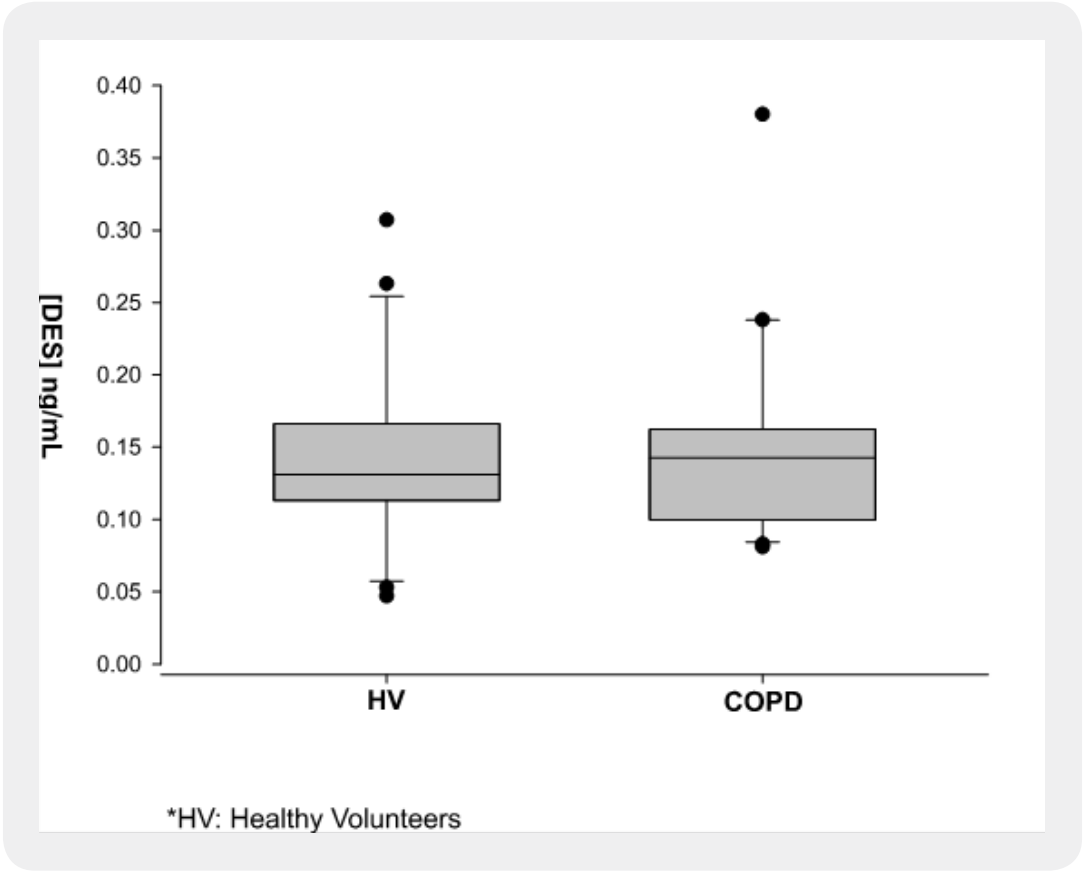

Figure 3: *Box and whisker plot showing Sputum total DES/IDS levels in healthy individuals and COPD patients*

Conclusion

In summary, an isotope dilution liquid chromatography mass spectrometric method with improved sensitivity, reproducibility and accurate quantification for the measurement of total sputum DES/IDS which, could be used as a biomarker for monitoring elastin degradation in diseases such as Asthma and COPD was developed.

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n/a

Conflicts of Interests

The article is free from any such conflicts between authors or with others in any aspect.

Bibliography

1. W. H. O. (WHO) Chronic obstructive pulmonary disease (COPD).

2. Foster, J. A., Rubin, L., Kagan, H. M., Franzblau, C., Bruenger, E. & Sandberg, L. B. (1974). Isolation and Characterization of Cross- Linked Peptides from Elastin. *J. Biol. Chem., 249*(19), 6191-6196.

3. Brown-Augsburger, P., Tisdale, C., Broekelmann, T., Sloan, C. & Mecham, R. P. (1995). Identification of an Elastin Cross-linking Domain That Joins Three Peptide Chains Possible Role in Nucleated Assembly. *The Journal of Biological Chemistry, 270,* 17778-17783.

4. Ma, S., Lieberman, S., Turino, G. M. & Lin, Y. Y. (2003). The detection and quantitation of free desmosine and isodesmosine in human urine and their peptide-bound forms in sputum. *Proc Natl Acad Sci U S A., 100*(22), 12941-12943.

5. Ma, S., Lin, Y. Y. & Turino, G. M. (2007). Measurements of Desmosine and Isodesmosine by Mass Spectrometry in COPD. *Chest, 131*(5), 1363-1371.

6. Luisetti, M., Ma, S., Iadarola, P., Stone, P. J., Viglio, S., Casado, B., Lin, Y. Y., Snider, G. L. & Turino, G. M. (2008). Desmosine as a biomarker of elastin degradation in COPD: current status and future directions. *Eur Respir J., 32,* 1146-1157.

7. Rennard, S., Turino, G. M., Lin, Y. Y., He, J., Cantor, J. O. & Ma, S. (2012). Elastin Degradation: An Effective Biomarker in COPD. *Copd., 9,* 1-4.

8. Turino, G. M., Lin, Y. Y., He, J., Cantor, J. O. & Ma, S. (2012). Total synthesis of COPD biomarker desmosine that crosslinks elastin. *Copd., 9,* 435-438.

9. Hu, J., Van den Steen, P. E., Sang, Q. X. A. & Opdenakker, G. (2007). Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases. *Nature Reviews Drug Discovery, 6,* 480-498.

10. Albarbarawi, O., Barton, A., Lin, Z., Takahashi, E., Buddharaju, A., Brady, J., Miller, D., Palmer, C. N. & Huang, J. T. (2010). Measurement of Urinary Total Desmosine and Isodesmosine Using Isotope-Dilution Liquid Chromatography-Tandem Mass Spectrometry. *Analytical Chemistry, 82*(9), 3745-3750.

11. Stone, P. J., Bryanrhadfi, J., Lucey, E. C., Ciccolella, D. E., Crombie, G., Faris, B., Snider, G. L. & Franzblau, C. (1991). Measurement of Urinary Desmosine by Isotope Dilution and High Performance Liquid Chromatography: Correlation between Elastase-induced Air-Space Enlargement in the Hamster and Elevation of Urinary Desmosine. *Am. Rev. Respir. Dis., 144*(2), 284-290.

12. Viglio, S., Zanaboni, G., Luisetti, M., Trisolini, R., Grimm, R., Cetta, G. & Iadarola, P. (1998). Micellar electrokinetic chromatography for the determination of urinary desmosine and isodesmosine in patients affected by chronic obstructive pulmonary disease. *Journal of Chromatography B: Biomedical Applications, 714,* 87-98.

13. Fill, J. A., Brandt, J. T., Wiedemann, H. P., Rinehart, B. L., Lindemann, C. F., Komara, J. J., Bowsher, R. R., Spence, M. C. & Zeiher, B. G. (2006). Urinary desmosine as a biomarker in acute lung injury. *Biomarkers: Biochemical Indicators of Exposure, Response, and Susceptibility to Chemicals, 11*(1), 85-96.

14. Sato, T., Kajikuri, T., Saito, Y., Chikuma, M. & Nagai, S. (2008). Determination of desmosine in bronchoalveolar lavage fluids by time-resolved fluoroimmunoassay. *Clin Chim Acta., 387*(1-2), 113-119.

15. Boutin, M., Ahmad, I., Jauhiainen, M., Lachapelle, N., Rondeau, C., Roy, J. & Thibault, P. (2009). NanoLC-MS/MS Analyses of Urinary Desmosine, Hydroxylysylpyridinoline and Lysylpyridinoline as Biomarkers for Chronic Graft-versus-Host Disease. *Analytical Chemistry, 81*(22), 9454-9461.

16. Boutin, M., Berthelette, C., Gervais, F. G., Scholand, M. B., Hoidal, J., Leppert, M. F., Bateman, K. P. & Thibault, P. (2009). High-Sensitivity NanoLC-MS/MS Analysis of Urinary Desmosine and Isodesmosine. *Analytical Chemistry, 81,* 1881-1887.

17. Huang, J. T., Chaudhuri, R., Albarbarawi, O., Barton, A., Grierson, C., Rauchhaus, P., *et al.* (2012). Clinical validity of plasma and urinary desmosine as biomarkers for chronic obstructive pulmonary disease. *Thorax, 67,* 502-508.

18. Laguna, T. A., Wagner, B. D., Starcher, B., Luckey Tarro, H. K., Mann, S. A., Sagel, S. D. & Accurso, F. J. (2012). Urinary desmosine: A biomarker of structural lung injury during CF pulmonary exacerbation. *Pediatric Pulmonology, 47,* 856-863.

19. Ongay, S., Sikma, M., Horvatovich, P., Hermans, J., Miller, B. E., Ten Hacken, N. H. T. & Bischoff, R. (2016). *Chronic Obstr Pulm Dis., 3*(2), 560-569.

20. Kim, C., Ko, Y., Kim, S. H., Yoo, H. J., Lee, J. S., Rhee, C. K., *et al.* (2018). Urinary desmosine is associated with emphysema severity and frequent exacerbation in patients with COPD. *Park, Respirology, 23*(2), 176-181.

21. Davies, S. F., Offord, K. P., Brown, M. G., Campe, H. & Niewoehner, D. (1983). Urine Desmosine is Unrelated to Cigarette Smoking or to Spirometric Function. *The American Review of Respiratory Disease, 128*(3), 473-475.

22. Janoff, A. (1984). Urine desmosine excretion. *The American Review of Respiratory Disease, 129*(3), 511-512.

23. Pelham, F., Wewers, M., Crystal, R., Buist, A. S. & Janoff, A. (1985). Urinary Excretion of Desmosine (Elastin Cross-Links) in Subjects with PiZZ Alpha-1-Antitrypsin Deficiency, a Phenotype Associated with Hereditary Predisposition to Pulmonary Emphysema. *The American Review of Respiratory Disease, 132*(4), 821-823.

24. Tenholder, M. F., Rajagopal, K. R., Phillips, Y. Y., Dillard, T. A., Bennett, L. L., Mundie, T. G. & Tellis, C. J. (1991). Urinary Desmosine Excretion as a Marker of Lung Injury in the Adult Respiratory Distress Syndrome. *Chest, 100,* 1385-1390.

25. Wagner, B. D., Accurso, F. J. & Laguna, T. A. (2010). The applicability of urinary creatinine as a method of specimen normalization in the cystic fibrosis population. *Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society, 9*(3), 212-216.

26. Laguna, T. A., Williams, C. B., Nunez, M. G., Welchlin-Bradford, C., Moen, C. E., Reilly, C. S. & Wendt, C. H. (2018). Biomarkers of inflammation in infants with cystic fibrosis. *Respiratory Research, 19,* 6.

27. Akers, S., Kucich, U., Swartz, M., Rosen, G., Glass, M., Rosenbloom, J., Kimbel, P. & Weinbaum, G. (1992). Specificity and Sensitivity of the Assay for Elastin-derived Peptides in Chronic Obstructive Pulmonary Disease. *Am. Rev. Respir. Dis., 145,* 1077-1081.

28. Cataldo, D., Munaut, C., Noel, A., Frankenne, F., Bartsch, P., Foidart, J. M. & Louis, R. (2000). MMP-2- and MMP-9-Linked Gelatinolytic Activity in the Sputum from Patients with Asthma and Chronic Obstructive Pulmonary Disease. *Int Arch Allergy Immunol., 123,* 259-267.

29. Coultas, D. B. (1998). Passive smoking and risk of adult asthma and COPD: an update. *Thorax, 53,* 381-387.

30. Demedts, I. K., Brusselle, G. G., Bracke, K. R., Vermaelen, K. Y. & Pauwels, R. A. (2005). Matrix metalloproteinases in asthma and COPD. *Curr Opin Pharmacol., 5,* 257-263.

31. Stone, P. J., Konstan, M. W., Berger, M., Dorkin, H. L., Franzblau, C. & Snider, G. L. (1995). Elastin and collagen degradation products in urine of patients with cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine, 152,* 157-162.

32. Viglio, S., Iadarola, P., Lupi, A., Trisolini, R., Tinelli, C., Balbi, B., Grassi, V., *et al.* (2000). MEKC of desmosine and isodesmosine in urine of chronic destructive lung disease patients. *Luisetti, Eur Respir J., 15,* 1039-1045.

33. Ferrari, F., Fumagalli, M., Piccinini, P., Stolk, J., Luisetti, M., Viglio, S., Tinelli, C. & Iadarola, P. (2012). Micellar Electrokinetic Chromatography with Laser Induced Detection and liquid chromatography tandem mass-spectrometry-based desmosine assays in urine of patients with Chronic Obstructive Pulmonary Disease: A comparative analysis. *J Chromatogr A., 1266,* 103-109.

34. Viglio, S., Stolk, J., Luisetti, M., Ferrari, F., Piccinini, P. & Iadarola, P. (2014). From micellar electrokinetic chromatography to liquid chromatography-mass spectrometry: Revisiting the way of analyzing human fluids for the search of desmosines, putative biomarkers of chronic obstructive pulmonary disease. *Electrophoresis, 35,* 109-118.

35. Laguna, T. A., Wagner, B. D., Luckey, H. K., Mann, S. A., Sagel, S. D., Regelmann, W. & Accurso, F. J. (2009). Sputum Desmosine During Hospital Admission for Pulmonary Exacerbation in Cystic Fibrosis. *Chest, 136*(6), 1561-1568.

36. Boschetto, P., Quintavalle, S., Zeni, E., Leprotti, S., Potena, A., Ballerin, L., Papi, A., *et al.* (2006). *Thorax, 61,* 1037-1042.

37. Cocci, F., Miniati, M., Monti, S., Cavarra, E., Gambelli, F., Battolla, L., Lucattelli, M. & Lungarella, G. (2002). *International Journal of Biochemistry and Cell Biology, 34,* 594-604

38. Stone, P. J., Gottlieb, D. J., O'Connor, G. T., Ciccolella, D. E., Breuer, R., Bryan- Rhadfi, J., *et al.* (1995). Elastin and Collagen Degradation Products in Urine of Smokers With and Without Chronic Obstructive Pulmonary Disease. *American Journal of Respiratory and Critical Care Medicine, 151*(4), 952-959.

39. Churg, A., Dai, J., Zay, K., Karsan, A., Hendricks, R., Yee, C., Martin, R., *et al.* (2001). Alpha-1-Antitrypsin and a Broad Spectrum Metalloprotease Inhibitor, RS113456, Have Similar Acute Anti-Inflammatory Effects. *Laboratory Investigation; a Journal of Technical Methods and Pathology, 81,* 1119-1131.

40. Churg, A., Wang, R. D., Xie, C. & Wright, J. L. (2003). α-1-Antitrypsin Ameliorates Cigarette Smoke-induced Emphysema in the Mouse. *American Journal of Respiratory and Critical Care Medicine, 168,* 199-207.

41. Kurucz, I., Nemeth, K., Meszaros, S., Torok, K., Nagy, Z., Zubovics, Z., Horvath, K. & Bodor, N. (2004). Anti-inflammatory effect and soft properties of etiprednol dicloacetate (BNP-166), a new, anti-asthmatic steroid. *Pharmazie, 59,* 412-416.

42. Stoller, J. K. & Aboussouan, L. S. (2005). α1-antitrypsin deficiency. *The Lancet, 365*(9478), 2225-2236.

43. Fregonese, L., Stolk, J., Frants, R. R. & Veldhuisen, B. (2008). Alpha-1 antitrypsin Null mutations and severity of emphysema. *Respiratory Medicine, 102*(6), 876-884.

44. Stockley, R. A. (2014). Alpha1-antitrypsin Review. *Clinics in Chest Medicine, 35*(1), 39-50.

45. Hatipoglu, U. & Stoller, J. K. (2016). α1-Antitrypsin Deficiency. *Clinics in Chest Medicine, 37,* 487-504.

46. Ma, S., Lin, Y. Y., Cantor, J. O., Chapman, K. R., Sandhaus, R. A. & Fries, M. (2016). Prevalence of Low Peak Inspiratory Flow Rate (PIFR) in Chronic Obstructive Pulmonary Disease (COPD) Patients at Discharge After an Exacerbation- Interim Findings from a Prospective Study. *Chronic Obstr Pulm Dis., 4.*

47. Hazari, Y. M., Bashir, A., Habib, M., Bashir, S., Habib, H., Qasim, M. A., Shah, N. N., Haq, E., Teckman, J. & Fazili, K. M. (2017). Alpha-1-antitrypsin deficiency: Genetic variations, clinical manifestations and therapeutic interventions. *Mutat Res., 773,* 14-25.

48. Stockley, R., De Soyza, A., Gunawardena, K., Perrett, J., Forsman-Semb, K., Entwistle, N. & Snell, N. (2013). Phase II study of a neutrophil elastase inhibitor (AZD9668) in patients with bronchiectasis. *Respiratory Medicine, 107*(4), 524-533.

49. Gray, R. D., MacGregor, G., Noble, D., Imrie, M., Dewar, M., Boyd, A. C., Innes, J. A., Porteous, D. J. & Greening, A. P. (2008). Early and Extended Early Bactericidal Activity of Linezolid in Pulmonary Tuberculosis. *American Journal of Respiratory and Critical Care Medicine, 178*(11), 444-452.

50. Gramegna, A., Amati, F., Terranova, L., Sotgiu, G., Tarsia, P., Miglietta, D., Calderazzo, M. A., Aliberti, S. & Blasi, F. (2017). Neutrophil elastase in bronchiectasis. *Respiratory Research, 18,* 211.

51. Chalmers, J. D., Moffitt, K. L., Suarez-Cuartin, G., Sibila, O., Finch, S., Furrie, E., Dicker, A., *et al.* (2017). Neutrophil Elastase Activity Is Associated with Exacerbations and Lung Function Decline in Bronchiectasis. *Fardon, American Journal of Respiratory and Critical Care Medicine, 195,* 1384-1393.

52. Janoff, A., Raju, L. & Dearing, R. (1983). Levels of Elastase Activity in Bronchoalveolar Lavage Fluids of Healthy Smokers and Nonsmokers. *The American Review of Respiratory Disease, 127,* 540-544.

53. Osman, M., Cantor, J. O., Roffman, S., Keller, S., Turino, G. M. & Mandl, I. (1985). Cigarette Smoke Impairs Elastin Resynthesis in Lungs of Hamsters with Elastase-induced Emphysema. *Am Rev Resp Dis., 132,* 640 - 643.

54. Churg, A., Zay, K., Shay, S., Xie, C., Shapiro, S. D., Hendricks, R. & Wright, J. L. (2002). Acute Cigarette Smoke–Induced Connective Tissue Breakdown Requires both Neutrophils and Macrophage Metalloelastase in Mice. *American Journal of Respiratory Cell and Molecular Biology, 27,* 368-374.

55. Eisner, M. D. (2002). Environmental tobacco smoke and adult asthma. *Clin Chest Med., 23*(4), 749 - 761.

56. Jaakkola, M. S. & Jaakkola, J. J. (2002). Effects of environmental tobacco smoke on the respiratory health of adults. *Scand J Work Environ Health., 28*(Suppl 2), 52-70.

57. Thomson, N. C. (2017). 2017 ERS/ATS standards for single-breath carbon monoxide uptake in the lung. *The European Respiratory Journal, 49*.

58. Starcher, B., Green, M. & Scott, M. (1995). Measurement of Urinary Desmosine as an Indicator of Acute Pulmonary Disease. *Respiration, 62*(5), 252-257.

59. Luisetti, M., Sturani, C., Sella, D., Madonini, E., Galavotti, V., Bruno, G., Peona, V., *et al.* (1996). MR889, a neutrophil elastase inhibitor, in patients with chronic obstructive pulmonary disease: a double-blind, randomized, placebo-controlled clinical trial. *Eur Respir J., 9,* 1482-1486.

60. McClintock, D. E., Starcher, B., Eisner, M. D., Thompson, B. T., Hayden, D. L., Church, G. D., Matthay, M. A., Wiedemann, H. P., *et al.* (2006). *American Journal of Physiology - Lung Cellular and Molecular Physiology, 291,* L566-L571.

61. Iadarola, P., Fumagalli, M., Bardoni, A. M., Salvini, R. & Viglio, S. (2016). Recent applications of CE- and HPLC-MS in the analysis of human fluids. *Electrophoresis, 37*(1), 212-230.

62. Guzman, N. A., Blanc, T. & Phillips, T. M. (2008). Immunoaffinity capillary electrophoresisas a powerful strategy for the quantificationof low-abundance biomarkers, drugs, andmetabolites in biological matrices. *Electrophoresis, 29,* 3259-3278.

63. Choudhury, S. D., Allsop, T., Passman, A. & Norris, G. E. (2006). Use of a proteomics approach to identify favourable conditions for production of good quality lambskin leather. *Anal Bioanal Chem., 384,* 723-735.

64. Huang, J. & Kang, J. (2007). Separation and measurement of desmosine and isodesmosine in vascular tissue hydrolysates by micellar electrokinetic capillary chromatography with a mixed micelle system. *J Chromatogr A., 1175,* 294-296.

65. Dhital, B., Durlik, P., Rathod, P., Gul, E. N. F., Wang, Z., Sun, C., Chang, E. J., Itin, B. & S. Boutis, G. (2017). Ultraviolet radiation reduces desmosine cross-links in elastin. *Biochem Biophys Rep., 10,* 172-177.

66. Papaioannou, A., Louis, M., Dhital, B., Ho, H. P., Chang, E. J. & Boutis, G. S. (2015). Quantitative comparison of structure and dynamics of elastin following three isolation schemes by 13C solid state NMR and MALDI mass spectrometry. *Biochim Biophys Acta., 1854,* 391-401.

67. Stone, P. J., Lucey, E. C., Bryan-Rhadfi, J., Snider, G. L. & Franzblau, C. (1991). Isolation of Urinary Desmosine by HPLC, Amino Acid Analysis, and Quantification by Isotope Dilution. *Ann N Y Acad Sci., 624,* 355-357.

68. Kaga, N., Soma, S., Fujimura, T., Seyama, K., Fukuchi, Y. & Murayama, K. (2003). Quantification of elastin cross-linking amino acids, desmosine and isodesmosine, in hydrolysates of rat lung by ion-pair liquid chromatography-mass spectrometry. *Anal Biochem., 318,* 25-29.

69. Ma, S., Turino, G. M. & Lin, Y. Y. (2011). Quantitation of desmosine and isodesmosine in urine, plasma, and sputum by LC-MS/MS as biomarkers for elastin degradation. *J Chromatogr B Analyt Technol Biomed Life Sci., 879*(21), 1893-1898.

70. Lamerz, J., Friedlein, A., Soder, N., Cutler, P. & Dobeli, H. (2013). Determination of free desmosine in human plasma and itsapplication in two experimental medicine studies. *Anal Biochem., 436,* 127-136.

71. Ma, S., Turino, G. M., Hayashi, T., Yanuma, H., Usuki, T. & Lin, Y. Y. (2013). Stable deuterium internal standard for the isotope-dilution LC–MS/MS analysis of elastin degradation. *Anal Biochem., 440,* 158-165.

72. Albarbarawi, O., Barton, A., Miller, D., McSharry, C., Chaudhuri, R., Thomson, N. C., Palmer, C. N., Devereux, G. & Huang, J. T. (2013). Characterization and validation of an isotope-dilution LC-MS/MS method for quantification of total desmosine and isodesmosine in plasma and serum. *Bioanalysis, 5,* 1991-2001.

73. Rathod, P., Kaur, M., Ho, H. P., Louis, M. E., Dhital, B., Durlik, P., Boutis, G. S., Mark, K. J., Lee, J. I. & Chang, E. J. (2018). Quantitation of desmosine and isodesmosine in urine, plasma, and sputum by LC–MS/MS as biomarkers for elastin degradation. *Anal Bioanal Chem., 410,* 6881-6889.