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The "History" of Desmosines: Forty Years of Debate on the Hypothesis That These Two Unnatural Amino Acids May Be Potential Biomarkers of Chronic Obstructive Pulmonary Disease

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Abstract

Desmosine and isodesmosine (collectively known as desmosines), two unnatural amino acids unique to mature elastin in humans, have been widely discussed as being potential biomarkers of disorders, which involve connective tissue and whose clinical manifestations result in elastin degradation. In particular, experimental data accumulated over the last 40 years have demonstrated that patients with chronic obstructive pulmonary disease (COPD) excrete higher amounts of urinary desmosines than healthy controls. Based on this evidence, it has been speculated by several authors that these cross-links may be potential biomarkers of COPD with clinical significance. Nevertheless, a strict correlation between the amount of these amino acids and the severity of the disease still has to be demonstrated. For this reason, the debate on the opportunity to consider desmosines as biomarkers of COPD is still open, and the development of sophisticated methods aimed at obtaining very precise measurement of their concentration is still considered technically challenging. The aim of this chapter is to trace the history of this debate through the presentation and discussion of a large number of articles dealing with the detection and quantification of desmosines in different biological fluids, from early years until the present.

Keywords: unnatural amino acids, desmosines, COPD, biological fluids, biomarkers

1. Introduction

Elastin is a highly elastic protein in connective tissue that allows many tissues in the body to resume their shape after stretching or contracting. Being the main component of the elastic fiber, it provides resiliency to numerous pulmonary structures including the alveolar wall, thus influencing the characteristic shape of alveoli. The strong hydrophobicity of elastin, together with a good number of intermolecular cross-links, produces a highly insoluble network of polypeptide chains working as a perfect elastomer in an aqueous environment [1, 2].

Elastin is synthesized and secreted from fibroblasts and vascular smooth cells as a soluble 75-kDa precursor called tropoelastin, containing 12–13 repeats of alternating hydrophobic and cross-linking domains. While glycine, alanine, proline and valine are the predominant residues of the hydrophobic domains in a random-coil organization, polyalanine tracts containing lysine residues embedded in a rigid α-helical structure are characteristic of the cross-linking domains.

The formation of cross-links occurs extracellularly generating two amino acid isoforms, known as desmosine and isodesmosine (collectively, desmosines or DESs), whose content may be used as a quantitative measurement of insoluble elastin formation [1–4].

Given their uniqueness to mature elastin in mammals, DESs have been discussed as potential biomarkers of disorders, which involve connective tissue and whose clinical manifestations result in elastin degradation. In fact, being the desmosine-containing peptides derived from the destruction of the elastic fibers excreted in the urine, their determination in the body fluids may represent an indirect measurement of extracellular matrix degradation or of elastase activity [5, 6].

Connective tissue destruction is a major problem in chronic obstructive pulmonary disease (COPD), a class of disorders characterized by massive destruction of the elastic fibers of the alveoli with disabling airflow limitation, productive cough and dyspnea [7, 8]. According to the protease/antiprotease hypothesis, elastolytic proteases, in particular human neutrophil elastase (HNE), are responsible for the digestion of alveolar elastin. If, for any reason, the level of this proteolytic enzyme overcomes that of α1-antitrypsin (AAT), the most important defense barrier in the lower respiratory tract, elastin present in the alveolar walls will be degraded with consequent loss of lung functions [9].

One of the current emerging challenges of COPD is the research of parameters that may elucidate the events associated with a given disease condition. The detection of reliable biomarkers that can be correlated with the clinical outcome of this disorder obviously plays a special role in this setting. In spite of the increasing number of biomarkers proposed, currently a useful biomarker for COPD is still lacking. However, being this disorder characterized by the uncontrolled degradation of the extracellular matrix with abnormal excretion of elastin-derived fragments containing DESs, substantial interest has been placed in the development of reliable assays to measure their concentration in body fluids. The fact that these peptides are quantitatively excreted in urine provides the rationale for understanding why DESs may represent an indirect measurement of extracellular matrix degradation.

The question of whether these amino acids may actually be considered surrogate markers of elastin degradation has been extensively debated [4, 6, 10–12]. Experimental evidences accumulated over the last 40 years have demonstrated that smokers as well as patients with COPD or other destructive lung diseases excrete in their urine amounts of DESs higher than non-smokers and healthy controls [13–17]. On the basis of these data, it has been speculated by several authors that these cross-links may be considered clinically significant biomarkers of COPD [11, 12]. As a consequence, efforts have been devoted by different research groups in the world to the determination of DESs excreted in a variety of biological fluids which include urine, plasma, and induced sputum. Despite a large number of articles describing the application of different techniques for the screening and quantification of DESs has been published so far, only in few cases a strict correlation between the amount of these amino acids and the severity of the disease has been demonstrated [13–17]. In particular, what has emerged in the course of the last years is that only very accurate desmosine determinations can help researchers in understanding which is the degree of elastin degradation in COPD at different stages of severity and may allow these amino acids to become a reliable tool either in the differential diagnosis or in the clinical management of the disease [10, 12, 18].

For this reason, the debate on the opportunity to consider DESs as surrogate markers of COPD is still open, and the development of increasingly sophisticated techniques aimed at obtaining very precise measurement of their concentration is considered technically challenging.

Indeed, the significant step forward of both sensitivity/specificity and degree of reproducibility of results provided by these technological advancements allowed researchers to fully utilize the power of such data sets thus improving the understanding of mechanisms involved in elastin degradation. Among the variety of research groups in the world that have focused their attention on DESs determination, a great contribution was provided by our own team. Novel strategies have been developed for the accurate determination of these amino acids in urine, plasma and sputum of a large number of smokers and patients affected by pulmonary diseases [3, 4, 6, 11, 13, 14, 16–19].

Aim of this chapter is to trace the history of this debate through the presentation and discussion of a large number of articles dealing with the detection and quantification of DESs in different biological matrices from early years until the present.

2. Biosynthetic pathway of desmosines

The biosynthesis of these cross-links involves the oxidative deamination of the ε-amino group of four lysine residues, by means of lysyl oxidase, a copper-dependent enzyme. The aldehydic residues which yield from this oxidation can then participate in Schiff-base reaction with the ε-amino group of other lysine residues or in aldol condensations with other similar aldehydic residues. These reactions result in the cyclization of the side-chain groups of the four lysines with the formation of DESs, two isomeric pyridinium cross-links which are characteristic of insoluble elastin [20]. The schematic view of these reactions, together with the final structure of these two amino acids, is shown in **Figure 1**.
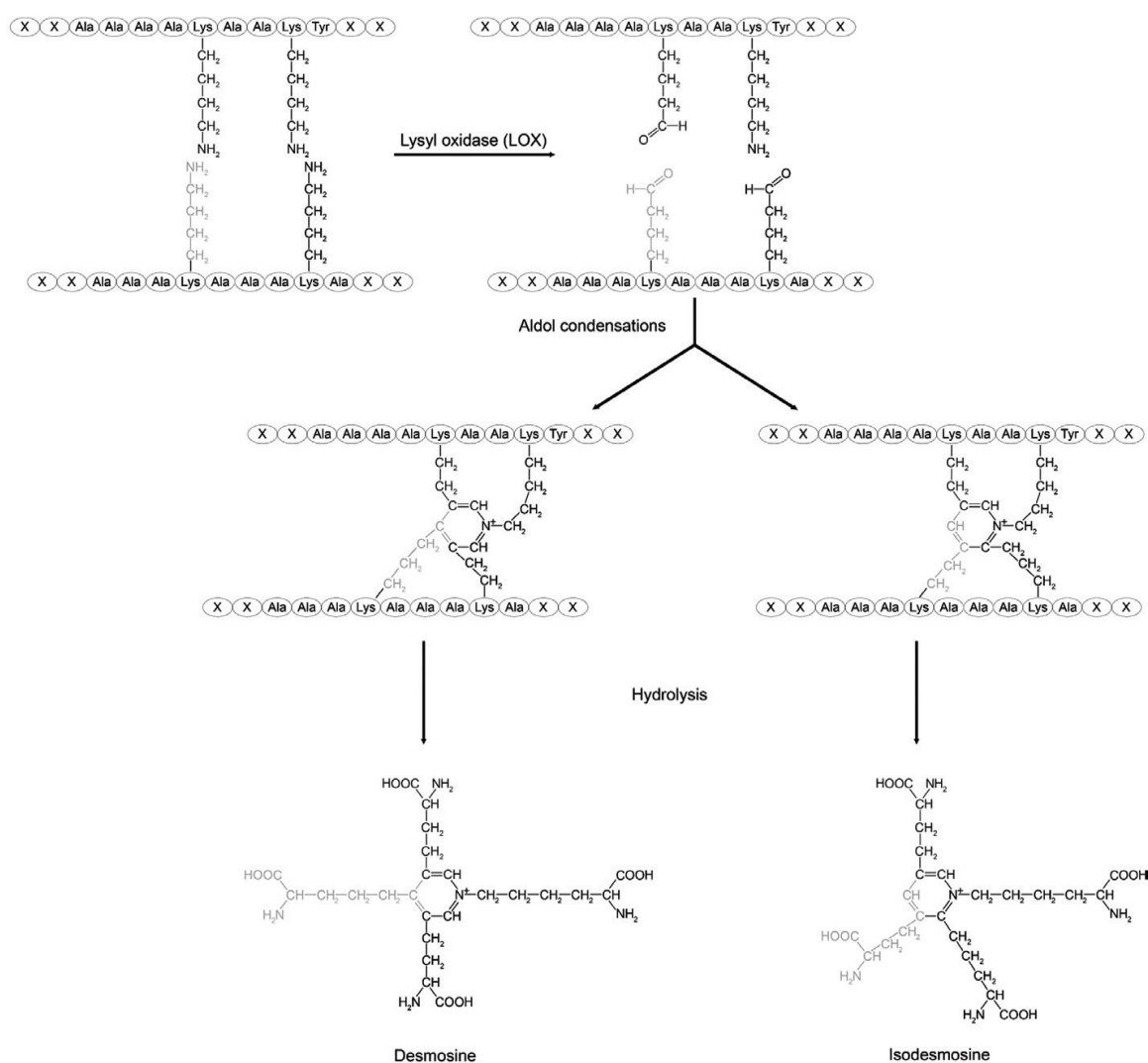
Figure 1. A schematic view of biosynthetic reaction of DESs and IDES.

3. Methodological aspects in the determination of desmosines

The first reports dealing with the determination of DESs in elastin appeared in the 1960s when such amino acids were isolated from purified elastin of bovine *Ligamentum nuchae* [21]. Given their peculiarity in humans, the question of whether these amino acids could be used as clinically significant biomarkers of extracellular matrix disorders was of primary importance. In light of this, it became immediately evident that the only way to answer this question would have been the availability of strategies aimed at calculating the concentration of DESs in different matrices. The research of such strategies resulted in the development of a wide variety of techniques and, as a consequence, in the publication of a large number of articles describing their application to different tissues and fluids. This paragraph is planned to show that while the low technological content of methods available in the 1960s could provide poor results in terms of DESs' clinical significance, the impressive advances achieved with the progression of technology have completely changed the scenario. It will appear that amino acid analysis and

the immunochemical and early chromatographic methods, with their intrinsic limitations in terms of specificity and/or sensitivity, have been mainly used to the analysis of tissue hydrolyzates and/or urine. By contrast, thanks to the tremendous increase in sensitivity/specificity of modern methodologies, free and total DESs can currently be detected in all available tissues/fluids. This overall improvement has strengthened the firm belief that DESs might indeed be possible biomarkers of elastin-degrading disorders, in particular lung disorders.

3.1. Amino acid analysis

From a chronological point of view, amino acid analysis was the first method developed for desmosine detection [22–24]. The rationale was that, being amino acids, DESs necessarily were ninhydrin-positive. In brief, desmosine-containing elastin fragments were submitted to a conventional acid hydrolysis and the amino acids separated by ion exchange chromatography prior to be detected through the colorimetric reaction with ninhydrin. Despite appearing as two well-resolved peaks that were integrated with precision, DESs in real samples could be separated only by applying elution conditions that were not coincident with those used for standard compounds. In addition, the quantification of DESs required a chromatographic platform that was not identical to that applied for conventional amino acid analysis. This changeover of platforms was obviously quite laborious and time-consuming for those laboratories in which analysis of elastin hydrolyzates occurred infrequently. To overcome this drawback, an improved procedure was developed which provided excellent resolution and quantification of DESs without the necessity of systems changeover [25]. Despite this improvement, the methodological approach based on amino acid analysis was characterized by a number of limitations, the most important being the poor sensitivity and the need to perform on samples a series of preliminary steps which made the procedure very complex. Nevertheless, amino acid analysis was successfully used for a variety of applications including: (i) determination of DESs concentration in elastin isolated from uterus and skin of young animals and humans [26]; (ii) measurement of elastin turnover in hamsters [27]; (iii) determination of the primary sequence around elastin cross-linking sites and correlation of this information to possible structural "signals" which might modulate or otherwise direct cross-link formation [28]. Changes in elution buffers [29, 30] or in elution mode [31, 32] further improved resolution and sensitivity in the analysis of tissue hydrolyzates.

The application of amino acid analysis to the detection of DESs in human urine was a step forward in the generation of results that could be considered relevant from a diagnostic point of view. For example, the analysis of urine from patients affected by Marfan syndrome during the early development of the disorder revealed that these subjects excreted a consistently lower amount of DESs than that of controls [33]. This was the first evidence of altered elastin cross-linking in a heritable connective-tissue disease. These data were correlated with a low lysyl oxidase activity and/or with an attenuation of the conversion of precursor aldehydes and lysyl cross-links into desmosines. Another study carried out on urines of cystic fibrosis (CF) patients chronically infected with *Pseudomonas aeruginosa* aimed at understanding whether destruction of the lung elastic fibers was an ongoing process in this disorder and whether proteolysis could contribute to the pathological changes in both airways and pulmonary parenchyma [34]. The amino acid analysis showed that the urinary content of DESs was

significantly higher in patients than in the age-matched control group and that DESs excretion was significantly correlated with the disease severity. These observations, together with the evidence that fibers were fragmented and distorted, were the proof that destruction and re-synthesis of elastic fibers are a chronic process in patients with CF.

3.2. Immunochemical methods

The application of immunochemical methods for detection of DESs originally started around the 1980s as soon as polyclonal antibodies against these cross-links became available. Although at the beginning RIA and ELISA assays suffered for poor specificity, they have been widely applied for at least 20 years. Their success was attributable to the remarkable increase in sensitivity that made these approaches, at least for a while, a valid alternative to chromatographic ones.

3.2.1. RIA

Initially developed to study the mechanisms of elastogenesis in cell cultures, DESs determination *via* RIA assay was also helpful for a better understanding of in vivo elastogenesis [35]. In this context, the levels of urinary DESs have been used as a measure of elastin catabolism in vivo to study the progression over months of experimental emphysema induced in hamsters by a single intratracheal injection of elastase [36]. DESs excretion was found to increase progressively during the first 24 h after injection, and to normalize after 6 days. This behavior seemed to suggest that the late progression of elastase-induced emphysema was not accompanied by increased elastolysis.

A remarkable improvement in the original RIA assay was the binding of antibodies to magnetic particles. Applied to urine of CF patients, this modified procedure allowed to show that urinary DESs in CF were higher than in controls [37]. Although a few interfering compounds present in the samples competed with DESs for the antibody, thus impoverishing method precision, this was a clear message that DESs measurement could be used to discriminate the two cohorts.

Although with conflicting results, other numerous lung disorders have been investigated by monitoring with RIA the levels of urinary DESs. These assays have been used: (i) to demonstrate that the amount of urinary DESs excreted by healthy non-smokers over 24 h was around 10-fold lower than that of smokers with evidence of COPD [38, 39]; (ii) to compare urinary DESs excretion in homozygous AAT-deficient patients with emphysema; patients with interstitial lung diseases and healthy subjects [40]; (iii) to detect DESs levels in patients with adult respiratory distress syndrome [41] and in patients with acute lung injury [42] and (iv) to assess elastin maturation during the development of human lungs [43].

3.2.2. ELISA

The sake of rapid, specific, safe and sensitive immunochemical assays resulted in the development of ELISA methods. Although the early approaches suffered of poor specificity due

to the cross-reactivity toward pyridoline of antibodies against desmosines [44, 45], optimization in the production of antibodies allowed to overcome this problem. The design of an anti-desmosine antiserum characterized by high specificity and sensitivity had a positive impact on the precision of DESs detection in tissue hydrolyzates [46, 47]. A very specific indirect competitive ELISA test was also used to compare the urinary content of DESs in COPD patients with that of healthy controls. The finding that the amount of DESs excreted by the former cohort was significantly higher than that of controls, while not being a novelty, was a confirmation that these cross-links could be potential indicators of lung status [48]. Of great attractiveness was the observation that DESs concentration was higher in patients which showed no evidence of emphysema (or with only mild emphysema) than in those with moderate-to-severe emphysema. This allowed to reason that urinary DESs could be a remarkable tool from a clinical point of view, being potential markers for the identification of subjects at risk of developing emphysema and for assessing the efficacy of therapeutic interventions.

3.3. High-performance liquid chromatography

With the development of high-performance liquid chromatography (HPLC), a marked improvement in terms of resolution and robustness was contributed to the chromatographic platform. This big difference allowed HPLC methods to be used, for the first time, for simultaneous detection of DESs and other cross-links. These methods have been mostly dedicated to the analysis of DESs in tissues, being tailored for specific applications which range from the determination of DESs concentration on hamsters aorta [49] to their detection in hamsters lungs [50] or to the estimate of the amount of tissue elastin in human and dog aorta [30]. A great deal of research was also focused on the age-related changes in the content of elastin and collagen cross-links. To this aim, human aorta [51], human yellow ligament [52] and bovine *Ligamentum nuchae* or rat aorta have been often used as sources of DESs: desmopyridine and isodesmopyridine. These studies were designed to understand the correlation of the elastic properties of this tissue with age [53] and to explore possible defects in elastin metabolism [54–56]. To find a biochemical explanation for the dilatation of vein wall, DESs and 4-L-Hydroxyproline have been quantified by HPLC in specimens of normal and varicose, dilated and non-dilated veins [57]. These determinations showed that the levels of cross-links were reduced in dilated vein, thus proving that dilatation may be related to elastin metabolism. To investigate the biochemical basis of alterations present in upper esophagical sphincter of patients with Zenker's diverticulum, the same cross-links were detected in samples of cricopharyngeal muscle [58].

Despite their above-mentioned features, when HPLC procedures were applied to urine, it appeared immediately evident that the interfering substances present in this matrix spuriously increased DESs levels, thus being a strong limit for their quantification. After exploring a number of routes to implement resolution, RP-HPLC with isotope dilution was suggested as an affordable procedure to overcome this problem. This approach, successfully applied to investigate a variety of disorders, led to the achievement of significant biochemical insights, in particular from detection of DESs in urine of smokers with/without COPD or with/without rapid decline of lung function and of patients with CF [59, 60].

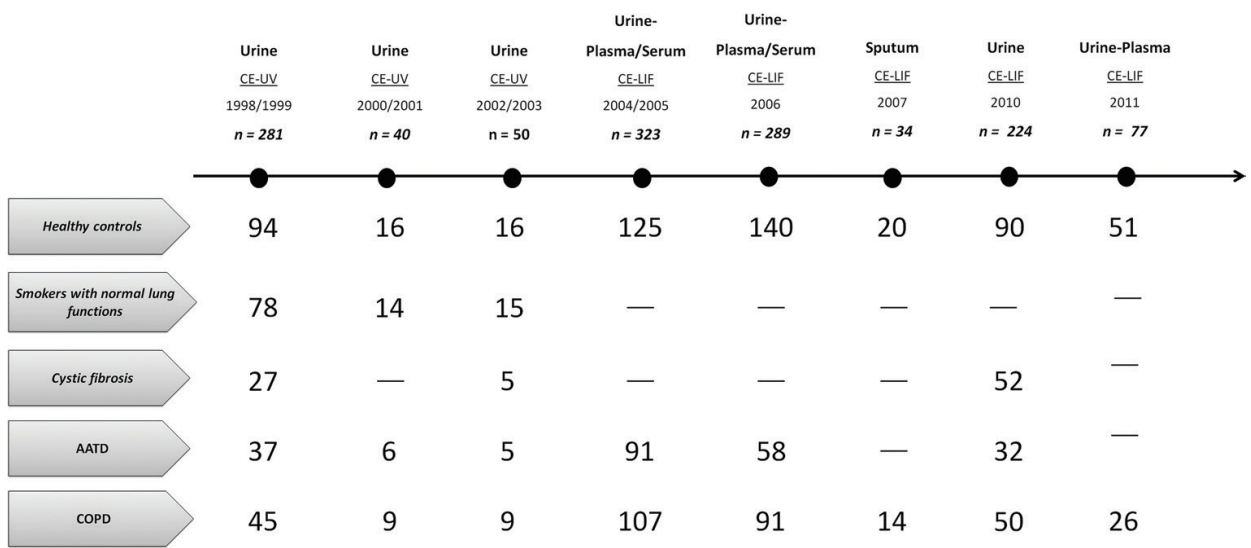### 3.4. Electrophoresis and capillary electrophoresis

Pioneering experiments carried out in the 1980s demonstrated that electrophoretic procedures could be helpful for detecting DESs in biological samples [61]. While 1-DE allowed a rapid detection of small amounts of these cross-links in hydrolyzates of elastin from *Lingamentum nuchae*, the complete separation of desmosine, isodesmosine and merodesmosine was achieved by applying a thin-layer methodology. The same procedure was used to measure cross-linked elastin synthesis in hamsters with pulmonary fibrosis induced by bleomycin [62]. The synthesis of cross-linked elastin was found to be significantly elevated in animals at 1–3 weeks after exposure to bleomycin. The message associated with this increase was that this tissue component was, most likely, an important part of the fibrotic response of the pulmonary parenchyma. The 2-D fingerprint (first dimension ascending chromatography, second one electrophoresis) of peptides produced by elastase digestion of elastin was another useful approach for DESs detection.

In the early 1990s, capillary electrophoresis (CE) was developed as a modern approach to obtain high efficiency, fast analysis times and excellent flexibility in changing the selectivity of the separation. As for other techniques described in previous paragraphs, capillary zone electrophoresis (CZE) was initially applied to detect DESs in elastin hydrolyzates but, although analysis was fast, the peaks of the two amino acids could not be completely separated under the conditions used [63]. Although several attempts to improve resolution have been made, it became soon clear that CZE could not become the "gold standard" for the detection of DESs in human matrices for at least two reasons. First, separating the two analytes, whose peaks were largely overlapping, was not possible due to the strict similarity of DESs structures, and second, the sensitivity of the method, although promising for the analysis of hydrolyzates and urine, was not suitable to allow the investigation of other important matrices (plasma, sputum). Nevertheless, the application of this method to study elastin content of abdominal aorta and aortic function in rats exposed to Vitamin D during gestation and in postnatal period led to the finding that the mean content of DESs was higher in control rats than in those treated with high/low Vitamin D doses [64]. By addition of ionic and/or nonionic detergents to the BGE, the separation mode was switched to micellar electrokinetic chromatography (MEKC) in an effort to verify whether the formation of micelles was a suitable tool to obtain the differential migration of DES and IDES. The efficacy of MEKC was explored on urine of healthy controls and COPD patients. The finding that DESs levels were higher in patients than in controls, while not being surprising, was a sort of "proof of the pudding" that the path taken was the right one. Further confirmation of the robustness of this approach came from the analysis of a large number of urine samples from patients with a variety of pulmonary diseases ranging from stable COPD [14] to subjects with acute exacerbation of COPD [13, 14], with AAT deficiency [14]; bronchiectasis [14] and cystic fibrosis [14, 65]. Taken together, the results of these analyses generated a clear picture of DESs excretion in all subjects characterized. They allowed to speculate that the level of these cross-links, being able to report the airway inflammation or to evaluate the efficacy of replacement therapy, could indeed reflect the lung conditions. Unfortunately, to meet the requirements of sensitivity needed for the use of this approach, a preliminary concentration step of urines, with related drawbacks, was

mandatory. In an effort to improve sensitivity, a new procedure was developed in which urinary DESs were labeled with a fluorescent probe to be visualized with a LIF detection system [66]. Due to heavy similarity of their final structure, the two labeled desmosines could not be resolved at all by the electrophoretic system. Despite this potential limitation, the method showed to be reliable and allowed DESs to be quantified with precision as the sum of the two isomers. Since its development and for at least a decade, the high sensitivity, the good accuracy and robustness of MECK-LIF have encouraged its utilization for the determination of DESs in a wide variety of human fluids. As a matter of fact, to the best of our knowledge, this is the protocol applied for screening the largest number of real samples ever investigated [4]. The scheme of **Figure 2** indicates the number of samples analyzed per year and all different matrices in which DESs have been detected by MEKC and CE-LIF.

3.5. LC-MS

Despite being very sensitive, MEKC-LIF suffered for a limit in the quantitation of DESs. The procedure, based on the integration of peak corresponding to the two amino acids, was not free from errors which derived from the possible presence, under this peak, of small amounts of interfering substances. The advent of mass spectrometry-based approaches completely circumvented these technical limitation. In fact, the possibility of monitoring only selected ions and their fragments may lead to a higher chance for better analyses in terms of specificity and sensitivity. These technological advances have strongly increased the popularity of this approach, allowing liquid chromatography-mass spectrometry (LC-MS) to merge as the most popular protocol to date applied in the DESs field. In addition, only the extreme sensitivity and specificity of these techniques allowed, for the first time, very minute amounts of free DESs to be observed in urine after a simple clean up of the specimens. An example is the accurate measurement of DESs in urine and sputum of healthy volunteers and patients with

	Urine	Urine	Urine	Urine-Plasma/Serum	Urine-Plasma/Serum	Sputum	Urine	Urine-Plasma
	CE-UV	CE-UV	CE-UV	CE-LIF	CE-LIF	CE-LIF	CE-LIF	CE-LIF
	1998/1999	2000/2001	2002/2003	2004/2005	2006	2007	2010	2011
	n = 281	n = 40	n = 50	n = 323	n = 289	n = 34	n = 224	n = 77
Healthy controls	94	16	16	125	140	20	90	51
Smokers with normal lung functions	78	14	15	—	—	—	—	—
Cystic fibrosis	27	—	5	—	—	—	52	—
AATD	37	6	5	91	58	—	32	—
COPD	45	9	9	107	91	14	50	26

Figure 2. The scheme indicates the number of samples analyzed per year in all different matrices in which DESs have been detected by MEKC and CE-LIF.

previously diagnosed COPD [67]. A remarkable implementation of the analytical procedures, in terms of sensitivity and specificity, was indeed the coupling of ESI-MS to the HPLC system. This platform drove the researchers to look forward and explore its potential also for the analysis of matrices other than urine. Once applied to sputum and plasma of AATD- and non-AATD-related COPD patients, this approach resulted in the production of very precise data which demonstrated that the amount of DESs in these matrices was significantly different between the two cohorts [68]. In addition, the data allowed to hypothesize that it could be possible to differentiate patients with COPD of various phenotypes based on the levels of excreted DESs. A LC-MS/MS method with selected reaction monitoring (SRM) of transition ions was also standardized to obtain an accurate measurement, in all body fluids, of DESs as biomarkers for in vivo measurement of tissue elastin degradation in man and animals [69]. The data showed an increase of total DESs in sputum and plasma of COPD patients over normal controls along with an increase of free DESs in urine of these patients. The suggestion that the total/free DESs ratio could be a possible parameter useful for studying the course of COPD and the response to therapy was also an interesting speculation. The measurement of DESs in plasma, urine and sputum of COPD patients was also used as a useful tool to demonstrate therapeutic effects of different pharmacological interventions aimed at reducing elastin degradation in this disorder [70, 71]. The precision of LC-MS data seemed to indicate that DESs might indeed have a role as potential biomarkers for evaluating therapeutic effects of any treatment. The mentioned accuracy of the method was even increased by the use of deuterated DES (DES-d$_4$) as internal standard in the LC-MS/MS analysis [72] and with the advent of ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) with selected reaction monitoring [73]. This was a further methodological improvement that allowed to measure the level of DESs in small amounts of urine from patients with lymphangioleiomyomatosis, an elastin degrading disorder that, similarly to COPD, affects lung tissues. The LC-MS/MS analysis of DESs with the multiple reaction monitoring (MRM) acquisition modes for monitoring the transitions of interest was applied to urine of COPD patients, and to urine and blood of patients with COPD and asthma. This approach evidenced that while the elevation of urinary DESs levels was associated with the exacerbation status in COPD patients, blood DESs levels were strongly associated with age and were negatively correlated with lung diffusing capacity for carbon monoxide [12, 74, 75]. From among the recently introduced high-sensitive techniques, a prominent position is also held by nano-LC-MS/MS. It has been applied to detect DESs in urine of: (i) COPD rapid decliners; (ii) COPD slow decliners; (iii) healthy smokers and healthy nonsmokers and also to detect hydroxyl-lysyl-pyridinoline and lysyl-pyridinoline as biomarkers for Chronic Graft-versus-Host disease [76, 77]. These latter studies got the conclusion that the chemotherapy treatment had significant effect on the turnover of elastin and collagen.

Investigations performed to check the agreement between LC-MS and MEKC-LIF demonstrated the compatibility of the two methods although the latter showed the tendency to overestimate DESs levels (likely due to the presence of interferents co-eluting with DESs peak) compared to the former [78]. Based on these results, the conclusion was drawn that, despite the advent of very sophisticated LC-MS/MS techniques, MEKC-LIF may still be considered a valuable method for assessment of DESs concentration in clinical investigations.

4. Clinical validity of desmosines as biomarkers of lung disorders

Although the recent technological advancements have made the measurement of DESs more common practice, the question of whether these cross-links are ready to be introduced into the biomarker "hall of fame" is still unanswered. In fact, it remains unclear whether these surrogate markers can predict effects or clinically relevant endpoints of pulmonary disorders. This is mainly due to a number of critical questions that, by making the clinical validity and utility of this assay controversial, need to be addressed. One of the major questions is the fact that elastic fibers, being present in many tissues including large elastic arteries and the dermis, are obviously not unique to lung interstitium. As a consequence, not necessarily an increase in elastin turnover could be primarily related to pulmonary diseases. Thus, the question arises whether increased DESs levels might be associated, for example, with accelerated elastin turn-over in the skin or major vessels. In this case, the use of DESs as markers of lung diseases will be inappropriate. However, the well-documented finding of DESs in sputum of individuals with pulmonary disorders and their good correlation with the lung conditions strongly supported the hypothesis that lung would be the major source of elastin cross-links in body fluids of these subjects. This being said, given that the analytical validation of a method is mandatory to guarantee the reliability of results, an equally important question is whether fully validated methods for DESs quantification are currently available. The rationale is that data of inadequate quality may lead to inaccurate patient monitoring and incorrect conclusions in clinical studies or over-/under-estimation of new drug effects. The immunochemical tools for testing DESs, while being able to differentiate patients with lung disorders from healthy controls, evidenced a number of drawbacks. First of all, the matrix analyzed for their determination was mostly urine, a fluid not devoid of potential limits. These included: (i) the use, in most cases, of single-point urine samples which are less representative than urine collected over 24 h; (ii) the variability over time of urinary excretion of DESs in individuals with pulmonary disorders and (iii) the effect (never taken into account) that decreased renal function might have on DESs excretion into urine. Moreover, based on the absolute values determined (which were much higher than those observed with amino acid analysis or HPLC), immunochemical methods appeared to overestimate DESs, most likely because of the presence in urine of cross-reacting substances. The obvious consequence of such spurious elevation was the masking of important differences between controls and patients. Last but not least, the low size of individuals analyzed for each set of experiments was another limitation to be reckoned with. Thus, in spite of the efforts made to refine the data, it appeared clear that these methods, while being a tool for the measurement of urinary DESs, were not sufficiently powerful for pointing to these cross-links as biomarkers helpful for clinical use. The horizon was widened by the methodological progresses achieved with the advent of HPLC and CE that allowed DESs to be detected in a larger variety of human fluids. In particular, a tremendous improvement in data accuracy and reliability came from the combination of direct DESs analysis by CE-LIF, with an increase in sample size. This method of analysis had apparently the potential to provide important information in the understanding of the pathogenesis of pulmonary disorders in which degradation of lung elastin is believed to be an ongoing part of the disease process. In addition, at least in part,

it fulfilled its goal. The improved consistency of results allowed incontrovertible proofs of DESs differences existing among different groups of subjects or different clinical phenotypes within the group of patients affected by the same disease to be evidenced. However, since the effect of confounding factors (including gender, age and body mass index) which influence urinary DESs excretion was not considered, the clinical utility and validity of urinary DESs measurements remained unproven.

The application of new experimental strategies promoted by the rapid state of flux of this field, while allowing to dig deeper into the catabolism of elastin through the quantification of more and more minute amounts of DESs, did not answer the question of whether these cross-links could be considered surrogate markers of pulmonary diseases, in particular of COPD. Moreover, the lack of consensus on what should be quantified (free or total DESs, or DES + IDES) and in which biological fluid (plasma, sputum and urine) might obscure the view of these cross-links as reliable biomarkers for COPD diagnosis or prognosis. Nevertheless, the standardized methodologies already developed, together with the implementation of sample size and the taking into account of possible confounding factors, seem to indicate that DESs are mature to be addressed as candidate for becoming in the near future the "gold standard" for the study of COPD. Another important aspect of DESs validation is the longitudinal behavior and the relationship with progression and severity of the disease. Large longitudinal studies are necessary to confirm their predictive power for patients' clinical outcome. Indeed, these studies would add to the understanding of whether, besides their association with COPD in cross-sectional studies, DESs could be related to FEV1 decline and to the worsening of diffusing capacity in longitudinal cases, and perhaps to changes in lung CT scan densitometry. This would certainly confirm their capacity for monitoring progression of disease severity and response in effective interventional trials. In this context, something has already been done. After adjustment for age, sex, height, body mass index, and smoking status convincing evidence has been gained that, while urinary DESs had a significant association with several lung function parameters (FEV1, FVC, RV, RV/TLC and DL, CO), plasma DESs correlated with FEV1, DL, and CO only. These correlations were much more pronounced in COPD subjects than in individuals without COPD. Of great interest was the finding that DESs can be independently influenced by a number of factors after adequately correcting for risk factors to avoid confounding results.

Thus, what has emerged from the scientific literature over the course of these years is that we are on the right path to utilizing these cross-links as valid tools or "biomarkers" in the differential diagnosis and clinical management of pulmonary diseases, COPD in particular. It remains to be seen whether DESs measurement could have an evidence-based role in stratifying patients for specific treatment or prognosis.

Appendices and nomenclatures

COPD	Chronic obstructive pulmonary disease
DESs	Desmosines
IDES	Isodesmosines
HNE	Elastase
AAT	α1-Antitrypsin
CF	Cystic fibrosis
HPLC	High-performance liquid chromatography
CE	Capillary electrophoresis
CZE	Capillary zone electrophoresis
MEKC	Micellar electrokinectic chromatography
LC-MS	Liquid chromatography-mass spectrometry
UPLC-MS/MS	Ultra-performance liquid chromatography coupled to tandem mass spectrometry

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