ERJ Express. Published on October 17, 2011 as doi: 10.1183/09031936.00064611

Total desmosines in plasma and urine correlate with lung function

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Word count: 2893/3000

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Abstract

Objectives: To evaluate the relationship between the matrix degradation biomarkers, desmosine and isodesmosine (desmosines), and lung function.

Methods: Plasma and creatinine-corrected urinary total desmosines (P- and U-desmosines), lung function and carbon monoxide diffusion capacity (D_LCO) were measured in a cohort of subjects from the Swedish Twin Registry.

Results: Concentrations of U- and P-desmosines were measured in 349 and 318 subjects, respectively; approximately one-third of subjects had chronic obstructive pulmonary disease (COPD). Age, female gender, body mass index (BMI) and smoking were significantly associated with U-desmosines in a multiple linear regression analysis. In the overall population, after adjustments for age, gender, height, BMI and smoking, concentrations of U-desmosines were significantly correlated with all lung function measures, and P-desmosines with forced expiratory volume in 1 s and D_LCO ($P < 0.05$). With the exception of residual volume versus P-desmosines, relationships between concentrations of desmosines and lung function measures were markedly stronger in subjects with COPD compared with those without COPD.

Conclusions: These cross-sectional data showing associations between desmosines and several lung function variables suggest that desmosines, particularly U-desmosines, could be a useful biomarker of COPD status.

Abstract word count: 184/200

Keywords: Biomarkers, Chronic obstructive pulmonary disease, Cigarette smoking, Elastin, Emphysema, Lung function tests

Introduction

Chronic obstructive pulmonary disease (COPD) affects the fundamentals of daily living [1] and exacerbations in particular reduce patients' quality of life [2, 3]. The disease is characterised by progressive airflow limitation [4] and clinical symptoms of dyspnoea, cough and sputum production [4, 5]. Classification of COPD is usually based on the severity of airflow obstruction, as assessed using the forced expiratory volume in 1 s (FEV_1) [5]. However, there is a lack of biomarkers to measure disease activity and disease progression in COPD [6]. Matrix degradation is a key feature of COPD, leading to lung destruction, emphysema and impaired pulmonary function. The elasticity and resilience of the lungs are mainly provided by elastin, which is synthesised as a soluble precursor, tropoelastin. The elastin fibres are formed by crosslinking through post-translational modification of lysine residues by lysyl oxidase, to form inter- and intramolecular covalent crosslinks [7]. The crosslinking structures, desmosine and isodesmosine (collectively referred to as desmosines), are unique to mature elastin. They are released, either in free form or conjugated to peptides, as a result of elastin degradation, and can be found in sputum, blood and urine [8–10]. Desmosine and isodesmosine have therefore been proposed as biomarkers of lung matrix degradation in COPD (COPD Foundation, Biomarker Qualification Workshop, Bethesda, MD, January 27-28, 2011; see also Stone et al 1995 [11]).

Most published studies have reported urinary desmosines (U-desmosines) rather than plasma desmosines (P-desmosines), probably because of the higher concentrations found in the urine. However, recent publications show that P-desmosines might be the preferred analyte, in so far as P-desmosines have demonstrated a clearer distinction between COPD patients and healthy subjects [9, 10].

There is accumulated evidence that the urinary excretion of desmosines is significantly higher in smokers than in non-smokers and also higher in COPD patients than in subjects with normal lung function (see review by Luisetti et al. [12] and references cited therein). However, different studies have shown slightly different results; the lack of consistency may be due to the small size of the studies, the use of carefully selected subjects or the use of many different analytical methods. The hypothesis tested in the present study was that there would be correlations between desmosines in urine and plasma, and lung function. Desmosines were measured using a highly specific and sensitive analytical method based on liquid chromatography combined with tandem mass spectrometry (LC-MS-MS).

Materials and methods

Study population

The current study was approved by the Ethics Committee at Karolinska Institute, No. 03-461. All twins signed informed consent. The study was based on the Swedish Twin Registry (STR), which contains information on more than 80,000 twin pairs (n = 160,000) born from 1886 to 2000. Between 1998 and 2002, all living twins in the STR born in 1958 or earlier were contacted using a computer-assisted telephone interview developed for the SALT (Screening Across the Lifespan Twin) study [13]. The interview included a checklist of common diseases and respiratory symptoms, as well as smoking habits. From a population of 26,516 twin pairs (n = 53,032), where both twins participated in the telephone interview, a subgroup was selected (515 pairs, n = 1030). These twins were invited to participate in more in-depth measures of lung function. The selection of subjects was initially conducted for a heritability study as described previously, in a manner that disease concordant and discordant twins were prioritised over symptom-free twin pairs [14]. The selection was also based on

feasibility aspects such as geography, budget and personnel. In total, 392 twins (38%) of 1030 twins accepted the invitation to participate.

Urine samples were obtained from 349 subjects for measurement of desmosines, and data on spirometry, body plethysmography and diffusion capacity for carbon monoxide (D_LCO) were also collected in these subjects. In addition, blood samples for P-desmosines determination were obtained from 318 of these subjects.

Pack years were quantified from information in the SALT telephone interview and subsequently confirmed using a questionnaire at the clinic; smoking status was defined as current smoker, ex-smoker, never-smoker or occasional (social) smoker. The question was *'Are you smoking or have you previously been smoking?'* Respondents chose from four alternative responses: 1) No, 2) Current smoker, 3) Have previously been smoking, 4) Only smoking at parties/have only been smoking at parties. Subjects giving the last response were defined as occasional smokers. The distribution, based on smoking habits and disease status, of subjects with urine samples available for determination of desmosines is presented in Table 1.

Determination of urine and plasma desmosines

The concentration of the sum of total (free plus peptide-bound) desmosine and isodesmosine in urine (U-desmosines) and plasma (P-desmosines) was measured by LC-MS-MS (further details are available in the online supplementary information).

Creatinine concentration in urine was measured by liquid chromatography with ultraviolet detection (LC-UV) [15]. Urinary levels of desmosines were normalised by creatinine concentration and are reported as nmol desmosines per mmol creatinine.

Statistical methods

The median and range for levels of U- and P-desmosines were calculated and presented for descriptive purposes. The correlation between the two was assessed using the Spearman rank correlation.

The relationship between U- and P-desmosines and patients' baseline characteristics (gender, age, smoking status, body mass index [BMI], and Global Initiative for Chronic Obstructive Lung Disease [GOLD] disease severity) were also assessed, using the Spearman rank correlation for continuous variables and the Mann-Whitney U-test or Kruskal-Wallis test for categorical variables.

A general linear model was used to compare log U-desmosines and log P-desmosines in GOLD stage categories, with adjustments for age and gender. Subjects without COPD were the reference category. The anti-log values (i.e. geometric means) were calculated to facilitate the interpretation of the log transformed measures.

Multiple linear regression analysis was used to explore whether the following factors were associated with desmosine concentrations: age, gender, height, smoking status and BMI. Gender and smoking (current and occasional smokers vs. never smokers and former smokers) were modelled as dichotomous variables, age, height and BMI were modelled as continuous variables, and levels of U- and P-desmosines were included as dependent variables.

Multiple linear regression was also used to assess the relationship between U- and P-desmosines and lung function measures (forced expiratory volume in 1 second [FEV_1], percentage predicted FEV_1 [FEV_1%pred], forced vital capacity [FVC], percentage predicted FVC [FVC%pred], residual volume [RV], RV/total lung capacity [RV/TLC] and D_LCO), with lung function measures as the dependent variables. Predicted values for FEV_1

and FVC were calculated using published reference equations [16]. A separate analysis on the relationship between desmosines and lung function was conducted to compare those with and without COPD (defined as $FEV_1/FVC<0.70$ according to GOLD [17]). In both analyses, the data were adjusted for confounding factors, i.e. factors found to be independently associated with desmosines in the first part of the analysis. In the first step, age, gender and height were assessed, and in the second step, smoking and BMI were added.

Due to positively skewed distributions, log-transformed (natural logarithm) U- and P-desmosines values were used in all multivariate analyses. The relationships between lung function measures and desmosines are presented as standardised beta-coefficients, in order to increase the comparability between the lung function measures.

The relationship between concentrations of desmosines and lung function was also studied within twin pairs of the same gender. The desmosines and lung function values of the first twin were subtracted from the corresponding values in the second twin. Relationships between the differences were assessed using Spearman rank correlations.

The PASW statistics software (Version 18) was used for all calculations (www.spss.com).

Results

Factors associated with concentrations of desmosines

U-desmosines were measured in 349 subjects, while P-desmosines were measured in 318 of these subjects. The Spearman correlation between U- and P-desmosines was r=0.70 ($P < 0.001$). The median levels of U- and P-desmosines according to patients' baseline characteristics are presented in Table 2. Age was significantly correlated with both U-desmosines (Spearman's r=0.53; $P < 0.001$) and with P-desmosines (r=0.55; $P < 0.001$).

The correlations with age were observed in both men and women, in smokers and non-smokers and in subjects with and without COPD ($P < 0.005$ for all groups).

Results of the multiple linear regression analyses to explore factors independently associated with concentrations of desmosines are shown in Table 3. Log U-desmosines were associated with age, gender, height (women only), smoking (men only) and BMI (women only). Log P-desmosines were associated with age, gender and BMI. The relationship between log P-desmosines and smoking was not significant in the multivariate analysis ($P = 0.08$).

Desmosines and lung function

Mean (±SD) FEV_1%pred was 93.4 ± 17.3% in men and 99.8 ± 6.5% in women. The proportion of subjects with COPD was 36% in men and 31% in women. The median values of U-desmosines and P-desmosines were higher in subjects with GOLD stage I-III disease, compared with those without COPD (Table 2). After adjustments for age and gender in a general linear model, the log U-desmosines values were significantly higher in GOLD stage II patients, compared with those without COPD ($P = 0.003$). There was also a significant difference in log P-desmosines values between GOLD stage III patients and those without COPD, after adjustments for age and gender ($P = 0.032$). In terms of geometric mean values, the age- and gender-adjusted U-desmosines values were 2.63, 2.49, 2.94 and 2.73 nmol/mmol creatinine, respectively, for no COPD, GOLD I, GOLD II and GOLD III. The corresponding values of P-desmosines were 0.48, 0.48, 0.52 and 0.61 nmol/L.

The relationships between FEV_1%pred, RV/TLC and D_LCO and log U-desmosines are depicted in Fig. 1. After adjustments for age, gender, height, BMI and smoking, log U-desmosines were significantly and inversely associated with FEV_1, FVC and D_LCO, and positively associated with RV and RV/TLC (Table 4). The relationships between

U-desmosines and lung function variables were generally stronger for men than for women. Log P-desmosines were significantly associated with FEV_1 and D_LCO.

Results of the analysis to compare subjects with and without COPD are shown in Table 5. With the exception of RV and P-desmosines, relationships were markedly stronger in subjects with COPD after adjustment for age, gender, height, BMI and smoking. All measures of lung function were significantly associated with U-desmosines in this group. When the analysis was restricted to women with COPD, all lung function measures, except for RV, were significantly associated with U-desmosines, after adjustment for age, height, BMI and smoking (data not shown). Except for a significant inverse relationship between D_LCO and P-desmosines, there were no significant relationships in subjects without COPD.

Comparisons within twin pairs of the same gender are provided in the supplementary materials.

Discussion

In this study we report the association between U- and P-desmosines and different lung function measures (FEV_1, FVC, RV, RV/TLC and D_LCO). The correlations with lung function parameters were much more pronounced in COPD subjects compared with those without COPD.

Previous studies on the correlation between desmosines and lung function have not demonstrated consistent results. For example, Gottlieb et al. [18] studied apparently healthy, smoking, adult men and found significantly higher U-desmosine excretion in rapid FEV_1 decliners than in slow decliners, and a significant correlation between desmosine excretion and the rate of FEV_1 decline over 6.3 years. In contrast, Boutin et al. [19] found significantly lower levels of U-desmosine in COPD patients with rapid FEV_1 decline compared with slow

decliners over 15 years. Another study by Viglio et al. [20] showed a correlation between U-desmosine and FEV_1% predicted in a group of patients with destructive lung disease (cystic fibrosis, bronchiectasis, COPD, α_1-antitrypsin deficiency), but Ma et al. [9] could not demonstrate any correlation between desmosines and various lung function parameters. One explanation for the different results may be the different methods used, the small sample sizes (usually <20 subjects) and varying correction for other, potentially confounding, factors. Another explanation for inconsistent results between studies could be that the relationship between desmosines and lung function may be different in different populations depending on disease status and disease severity. This is supported by our finding that the correlation with FEV_1 was only seen in the subjects fulfilling the COPD criteria.

Adjusted for age and gender, 1 standard deviation of U-desmosine (0.86 nmol/mmol creatinine) corresponded to approximately 3% units lower FEV_1%pred and about 1 ml/min/mmHg lower D_LCO; the relationships were even stronger among men. There was a significant association between U-desmosines and several lung function parameters (FEV_1, FVC, D_LCO, RV and RV/TLC) after adjustment for age, gender, height, BMI and smoking status, while P-desmosines were significantly associated only with FEV_1 and D_LCO. The finding that the correlations with lung function are stronger for U-desmosines compared with P-desmosines are not completely in line with the data from Ma et al. [9] which indicate that P-desmosine gives a better separation between COPD patients and healthy subjects. In the current study, the relationships with lung function were mainly seen in subjects with COPD. In this context, it is interesting to note that the pathophysiology of COPD involves neutrophil-driven inflammation and lung matrix degradation [21]. Desmosines in healthy subjects may be more strongly determined by the normal, age-related elastin degradation from all elastin-containing organs. Elastin is a major component not only of the lungs but also serves an important function in arteries, as a medium for pressure wave propagation to

help blood flow, and is particularly abundant in large elastic blood vessels such as the aorta. Elastin is also important in other tissues such as elastic ligaments, the skin and the bladder. Therefore, it is possible that degradation from tissues other than the lung may have contributed to the associations seen in this study. However, since a number of different tissues are affected in COPD, this may also be relevant to the pathophysiology of the disease. It is difficult to assess how much lung elastin constitutes the total amount in the body; estimates vary from 1.3% to 24% [22–24]. It has also been estimated that the normal rate of lung elastin turnover accounts for only about 19% of the desmosines excreted in urine [11].

The present study also demonstrated a strong correlation between age and either U- or P-desmosines. This is consistent with the finding that increased elastolysis and degradation of elastin are features of normal ageing [25] and the concept of 'senile emphysema', normal physiological ageing of the lung associated with dilatation of alveoli, enlargement of airspaces and loss of supporting tissue for peripheral airways [26]. It should be noted, however, that previous studies have only shown a correlation between age and urinary desmosine in the presence of another risk factor. Stone and coworkers [11] reported a positive correlation between age and urinary desmosine excretion (creatinine normalised) in a small group of current smokers ($n = 13$) but they found no significant correlation in a group of never-smokers ($n = 22$). A strong correlation was also found between U-desmosines and age in patients with pseudoxanthoma elasticum but not in healthy subjects [27].

Smoking was significantly associated with levels of U-desmosines ($P < 0.001$) and non-significantly ($P = 0.08$) with P-desmosines, after adjustments for age, gender and BMI. Interestingly, no significant association with smoking was observed in the unadjusted analysis; however, smokers were somewhat younger than non-smokers and age was a very strong determinant of desmosine levels. The results illustrate the importance of adequately

correcting for other risk factors, especially age, in studies of desmosines. U- and P-desmosines were also significantly associated with gender, with higher concentrations in women than in men. Furthermore, women showed weaker correlations between desmosines and lung function than men. However, it should be noted that FEV_1 was lower in men than in women (FEV_1%pred: 93.4 ± 17.3 vs. 99.8 ± 6.5 %), and many women had relatively high FEV_1. As the relationship between desmosines and lung function was observed mainly in subjects with COPD, the stronger correlation in men seems to be related to degree of lung function impairment rather than gender differences. BMI also showed association with desmosines in this study. Potential explanations for this association may be either increased mass of elastic tissues such as skin and blood vessels, or increased systemic inflammation in subjects with high BMI.

One of the limitations of this study was the generally mild level of COPD in study subjects (according to the current GOLD classification) [17]. Relationships between lung function and desmosines may be stronger in a more severe cohort. The use of twins for the study may also have influenced the outcome as the selection procedure may have resulted in an over-representation of certain groups, or because genetic factors may influence the relationships studied. The results therefore need to be confirmed in additional COPD cohorts with varying degrees of disease severity. Furthermore, longitudinal studies are needed to address whether an increase in desmosine is associated with a decrease in lung function and other measures of disease progression, as has been suggested [12]. In addition, although one of the most accurate analytical techniques available, tandem mass spectrometry requires relatively expensive instrumentation and skilled personnel, which may limit a wider distribution of the method.

In summary, this study, which to our knowledge is the largest study on desmosines and lung function, demonstrates that both U- and P-desmosines are correlated with a number of lung function measurements in subjects with COPD. The finding that desmosines can be independently influenced by a number of factors other than lung function (age, gender, height, smoking and BMI) emphasises the need to correct for these factors to avoid confounding results.

Acknowledgements

This study was supported by AstraZeneca and grants from the Swedish Research Council and the Karolinska Institute. The data collection in SALT was supported by grants from the Swedish Research Council and NIH grant AG 08724. We thank Henrik Lindberg and Anders Blomgren, AstraZeneca R&D Lund, for technical assistance with the measurement of desmosines in urine and plasma. Editorial support was provided by Dr Nicky French from Complete Medical Communications, funded by AstraZeneca.

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Table 1. Smoking status and lung function variables according to GOLD stage

	Lung function			
	Normal (*n* = 234)	**GOLD I (*n* = 67)**	**GOLD II (*n* = 43)**	**GOLD III (*n* = 5)**
Never smoker, n (%)	96 (41)	12 (18)	6 (14)	0
Ex-smoker, n (%)	95 (41)	29 (43)	19 (44)	3 (60)
Current smoker, n (%)	32 (14)	18 (27)	17 (40)	2 (40)
Occasional smoker, n (%)	11 (5)	8 (12)	1 (2.3)	0
FEV_1 (L)	2.9 ± 0.68	2.6 ± 0.60	2.0 ± 0.46	1.3 ± 0.24
FEV_1%pred	103 ± 12.7	98 ± 11.3	70 ± 6.5	43 ± 5.2
FVC (L)	3.8 ± 0.94	4.0 ± 0.93	3.3 ± 0.75	3.2 ± 1.16
FVC%pred	113 ± 14.6	121 ± 14.4	98 ± 13.9	87 ± 22.0
RV (L)	2.1 ± 0.52	2.4 ± 0.51	2.8 ± 0.75	3.5 ± 1.08
RV/TLC	0.36 ± 0.06	0.38 ± 0.06	0.46 ± 0.07	0.52 ± 0.04
D_LCO (ml/min/mmHg)	24 ± 6.6	22 ± 5.8	19 ± 6.7	18 ± 3.4

Values are mean ± standard deviation or *n* (%)

GOLD (Global initiative for chronic Obstructive Lung Disease): Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease (updated 2010, available at www.goldcopd.org) uses the following spirometry criteria for grading of COPD severity: GOLD stage I (mild) FEV_1%pred ≥ 80; stage II (moderate) FEV_1%pred <80 and ≥ 50; stage III (severe) FEV_1%pred <50 and ≥30; stage IV (very severe) FEV_1%pred <30.

No subjects fulfilled criteria for GOLD IV.

FEV_1, forced expiratory volume in 1 second; FEV_1%pred, percentage predicted FEV_1; FVC, forced vital capacity; FVC_1%pred, percentage predicted FVC_1; RV, residual volume; RV/TLC, RV/total lung capacity; D_LCO, carbon monoxide diffusion capacity.

Table 2. Concentrations of desmosines in relation to baseline characteristics of the study cohort

		U-desmosines (nmol/mmol creatinine)		P-desmosines (nmol/L)	
		n	Median (range)	*n*	Median (range)
Gender	Men	128	2.2 (1.3-6.0)	122	0.47 (0.16-1.3)
	Women	221	2.8 (1.5-5.7)	196	0.48 (0.28-1.4)
Age (years)	46-55	104	2.2 (1.4-4.1)	98	0.41 (0.16-0.76)
	55-59	83	2.4 (1.3-4.2)	75	0.43 (0.28-0.87)
	60-64	77	2.8 (1.8-5.2)	72	0.51 (0.32-1.3)
	65-69	41	3.0 (1.7-6.0)	34	0.58 (0.37-1.1)
	70-87	44	3.7 (2.3-5.7)	39	0.71 (0.41-1.4)
Smoking habits	Current smokers	69	2.7 (1.5-6.0)	61	0.51 (0.16-1.3)
	Occasional smokers	20	2.5 (1.5-4.0)	20	0.47 (0.31-0.68)
	Ex-smokers	146	2.6 (1.4-5.2)	138	0.48 (0.28-1.2)
	Never smokers	114	2.5 (1.3-5.7)	99	0.46 (0.28-1.4)
BMI (kg/m^2)	< 25	187	2.5 (1.4-6.0)	170	0.46 (0.16-1.3)
	25-30	131	2.7 (1.3-5.7)	120	0.48 (0.28-1.4)
	≥ 30	31	2.9 (1.9-5.0)	28	0.53 (0.37-1.3)
Disease severity (GOLD)	No disease	234	2.5 (1.3-5.7)	212	0.46 (0.16-1.4)
	GOLD I	67	2.6 (1.5-5.0)	61	0.49 (0.30-1.3)
	GOLD II	43	2.9 (1.7-6.0)	40	0.55 (0.33-1.2)
	GOLD III	5	2.8 (2.0-4.1)	5	0.64 (0.47-1.1)

GOLD: Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease (updated 2010); available at www.goldcopd.org.

No subjects fulfilled criteria for GOLD IV.

BMI, body mass index.

Table 3. Multivariate analysis of factors associated with desmosines measures

	MEN				**WOMEN**				**ALL**			
Dependent	Ln U-desmosines (*n* = 128)		Ln P-desmosines (*n* = 122)		Ln U-desmosines (*n* = 221)		Ln P-desmosines (*n* = 196)		Ln U-desmosines (*n* = 349)		Ln P-desmosines (*n* = 318)	
	Beta	*P*	Beta	*P*	Beta	*P*	Beta	*P*	Beta	*P*	Beta	*P*
Age (per 1 year)	0.023	< 0.001	0.023	< 0.001	0.018	< 0.001	0.022	< 0.001	0.021	< 0.001	0.022	< 0.001
Gender (vs. men)	---	---	---	---	---	---	---	---	0.21	< 0.001	0.067	0.014
Height (per 1 cm)	-0.001	0.83	0.003	0.44	-0.006	0.02	0.00	0.92	---	---	---	---
Current smoking (vs. non-smokers)	0.210	< 0.001	0.089	0.09	0.002	0.94	0.017	0.64	0.092	< 0.001	0.054	0.08
BMI (per 1 kg/m^2)	-0.003	0.72	0.008	0.039	0.014	< 0.001	0.020	< 0.001	0.011	< 0.001	0.017	< 0.001

All independent variables were simultaneously entered into the multiple linear regression model. 349 individuals were available for the analysis of U-desmosines and 318 individuals for the analysis of P-desmosines.

BMI, body mass index; Ln, natural log.

Table 4. Standardised beta-coefficients for correlations between concentrations of desmosines and lung function measures

	MEN				**WOMEN**				**ALL**			
	Ln U-desmosines (*n* = 128)		**Ln P-desmosines (*n* = 122)**		**Ln U-desmosines (*n* = 221)**		**Ln P-desmosines (*n* = 196)**		**Ln U-desmosines (*n* = 349)**		**Ln P-desmosines (*n* = 318)**	
	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2	Model 1	Model 2
FEV_1	-0.29**	-0.20*	-0.17	-0.12	-0.12	-0.11	-0.11	-0.11	-0.17***	-0.14**	-0.11*	-0.10*
FEV_1%pred	-0.37***	-0.26*	-0.22*	-0.16	-0.13	-0.12	-0.10	-0.10	-0.23***	-0.18**	-0.14*	-0.13*
FVC	-0.27**	-0.22*	-0.06	-0.03	-0.19**	-0.14*	-0.20**	-0.16*	-0.17***	-0.15***	-0.09*	-0.07
FVC%pred	-0.34**	-0.28*	-0.08	-0.03	-0.24**	-0.18*	-0.24**	-0.19 $P = 0.052$	-0.27***	-0.23***	-0.16*	-0.12
RV	0.26*	0.16	0.06	0.03	0.03	0.08	-0.02	-0.002	0.14*	0.14*	0.02	0.03
RV/TLC	0.34***	0.24*	0.09	0.05	0.15*	0.15*	0.11	0.10	0.24***	0.22***	0.10	0.09
D_LCO	-0.33***	-0.14	-0.35***	-0.30***	-0.01	-0.06	-0.09	-0.17*	-0.14**	-0.13*	-0.18***	-0.21***

Multiple linear regression with the lung function variable as dependent variable.

Model 1: adjusted for age, gender (all subjects only) and height.

Model 2: + BMI, current smoking.

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

FEV_1, forced expiratory volume in 1 second; FEV_1%pred, percentage predicted FEV_1; FVC, forced vital capacity; RV, residual volume; RV/TLC, RV/total lung capacity; D_LCO, carbon monoxide diffusion capacity; BMI, body mass index.

Table 5. Standardised beta-coefficients for correlations between concentrations of desmosines and lung function measures in subjects with and without COPD

	No COPD ($FEV_1/FVC \geq 0.70$)		**COPD ($FEV_1/FVC < 0.70$)**	
	U-desmosines	P-desmosines	U-desmosines	P-desmosines
Men/women (n/n)	82/152	78/134	46/69	44/62
FEV_1	-0.02	0.02	-0.32^{***}	-0.22^{*}
FEV_1%pred	-0.03	0.06	-0.42^{***}	-0.29^{*}
FVC	-0.05	0.03	-0.29^{***}	-0.21^{*}
FVC%pred	-0.08	0.03	-0.41^{***}	-0.29^{*}
RV	0.07	0.01	0.23^{*}	0.01
RV/TLC	0.10	0.00	0.40^{***}	0.18
D_LCO	-0.02	-0.12*	-0.25^{**}	-0.29^{**}

Data adjusted for age, gender, height, BMI and current smoking. Multiple linear regression was used with the lung function variable as dependent variable.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

COPD, chronic obstructive pulmonary disease; FEV_1, forced expiratory volume in 1 second; FEV_1%pred, percentage predicted FEV_1; FVC, forced vital capacity; RV, residual volume; RV/TLC, RV/total lung capacity; D_LCO, carbon monoxide diffusion capacity; BMI, body mass index.

Figure legend

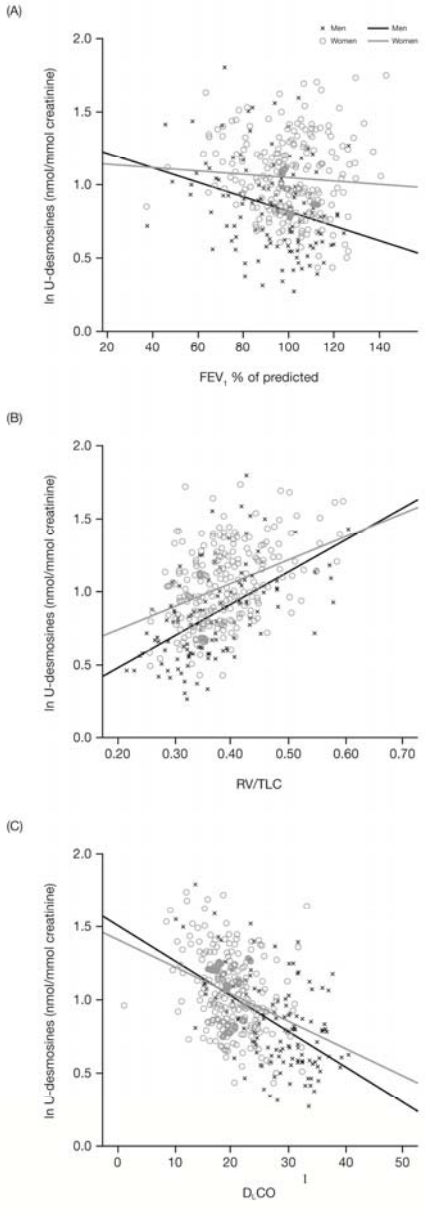
Fig. 1. Scatterplot with regression slopes of log U-desmosines in relation to:

(A) FEV_1%pred ($r^2 = 0.08$ for men and $r^2 = 0.005$ for women)

(B) RV/TLC ($r^2 = 0.29$ for men and $r^2 = 0.16$ for women)

(C) D_LCO ($r^2 = 0.26$ for men and $r^2 = 0.11$ for women).

FIGURE 1



FEV_1, forced expiratory volume in 1 second; RV/TLC, residual volume/total lung capacity; D_LCO, carbon monoxide diffusion capacity

Supplementary material

Supplementary methods

Determination of total U-desmosines

Urine was collected from 18:00 to 08:00 the following morning, with compulsory voids at these times. Samples were divided into 10- or 50-mL aliquots and stored frozen at -20ºC until analysed. A 100 µl sample of urine was mixed with 200 µL of the internal standard solution (D5-isodesmosine, 75 nmol/L) and 300 µL of concentrated hydrochloric acid (HCl). The tube was incubated at 100°C overnight. After cooling, the tube was centrifuged and the supernatant evaporated to dryness under vacuum in a Syncore Polyvap (Büchi Labortechnik AG, Switzerland). The residue was dissolved in 500 µL HCl (50 mmol/L) and applied to a column in an Oasis MCX µElution plate (Waters Corp., Milford, MA, USA) previously conditioned with 200 µL of methanol and 200 µL of HCl (50 mmol/L). After washing with 200 µL of methanol and 200 µL of HCl (50 mmol/L), the desmosines finally eluted with 200 µL HCl (2 mol/L) into a 96-well plate. The plate was placed in a SPD 2010 SpeedVac vacuum concentrator (Thermo Savant, Holbrook, NY, USA) and the solvent evaporated to dryness. Methylation of the carboxylic acid groups of desmosine was achieved by adding 200 µL HCl (1.5 mol/L) in methanol and leaving the sample at room temperature overnight. The HCl in methanol solution was prepared by drop-wise addition of acetyl chloride to methanol, with the methanol container immersed in ice and the solution stirred using a magnetic stirrer. Note: The reaction of acetyl chloride with methanol is exothermic and can be violent. After evaporation of the methanol to dryness, the sample was treated with 200 µL of a mixture of 5% acetic anhydride and 5% triethylamine in acetonitrile in order to acetylate the amino groups of the desmosines. The reaction mixture was allowed to stand at room temperature for 5 min and was then evaporated to dryness. The residue was dissolved in

200 µL acetonitrile (20%) and acetic acid (0.5%) in water and analysed by liquid chromatography combined with tandem mass spectrometry (LC-MS-MS).

The derivatised sample extract was chromatographed on a coupled column system consisting of two Aquasil C18 columns (10 mm × 1 mm ID and 30 mm × 1 mm ID) (Thermo Scientific, Waltham, MA, USA). The LC equipment consisted of two Shimadzu LC-10ADvp pumps, an SCL-10Avp controller and a CTC PAL autoinjector (Shimadzu Corp., Kyoto, Japan). Mobile phase A was 0.5% acetic acid in water and mobile phase B was 0.5% acetic acid and 4.5% water in acetonitrile. The mobile phase flow rate was 250 µL/min and the injection volume was 20 µL. Gradient elution was performed, starting at 0% B, increasing to 40% B at 1.8 min, 100% B at 2.6 min and holding for 0.7 min. Derivatised desmosine and isodesmosine co-chromatographed under these conditions and eluted at a retention time of 2.3 min.

Desmosine and the internal standard were detected by multiple reaction monitoring (MRM) using a Sciex API 5000 triple quadrupole mass spectrometer (AB Sciex, Foster City, CA, USA). The instrument was operated in positive ion electrospray mode and MRM was performed by monitoring the precursor ion (M+) to product ion transition of m/z 750.4 to m/z 690.4 (desmosines) and m/z 755.4 to m/z 85.1 (internal standard). Quantification was performed by determining the peak area ratio of desmosines:internal standard. A standard curve was constructed from standard curve samples (5.0-100 nmol/L) and used to calculate the desmosine concentrations in the study samples. Standard curve samples were prepared by adding known amounts of desmosine to artificial urine (NaCl 100mmol/L, urea 150 mmol/L, creatinine 5 mmol/L and bovine serum albumin 300 mg/L in water; pH adjusted to 6 with NaH_2PO_4). The lower and upper limits of quantification were 5 and 100 nmol/L, respectively, and the coefficients of variation were 5-6% and 8-9%, respectively.

Determination of total P-desmosines

Blood was collected in 10-mL tubes (Venoject, Terumo Europe N.V. Leuven, Belgium) with 0.1 mL EDTA (K3) (0.47 mol/L, 21 W/V%) (Art nr: VT-100STK). Plasma was obtained by centrifugation at 500 g for 10 min at room temperature, then 200 µL was mixed with 40 µL of internal standard solution (D9-isodesmosine, 50 nmol/L) and 100 µL trichloroacetic acid (0.45 mol/L) was added to precipitate the plasma proteins. The sample was vortex mixed, centrifuged and the supernatant mixed with concentrated (37%) HCl (300 µL) and the sample incubated at 100°C overnight. Further sample preparation, including solid phase extraction and derivatisation, was identical to the procedure described for U-desmosines.

The LC-MS-MS method was also similar to that used for U-desmosines, although upgraded models of the equipment were used. Two Shimadzu LC-20AD XR pumps together with a Shimadzu CBM-20A controller and a Sciex triple quadrupole 5500 MS-MS instrument were used. The mobile phase flow rate was increased to 500 µL/min and under these conditions the derivatised desmosines eluted at a retention time of 0.5 min.

MRM was performed by monitoring the precursor ion (M+) to product ion transition of m/z 750.4 to m/z 690.4 (desmosines) and m/z 759.4 to m/z 699.4 (internal standard). For quantification, a standard curve in the range of 0.1-2.0 nmol/L was used. The lower and upper limits of quantification were 0.1 and 2.0 nmol/L, respectively, and the coefficients of variation were 5-6% and 8-9%, respectively.

Supplementary results and discussion

Differences within twin pairs

The relationships between desmosines and lung function were also explored by correlating the differences within twin pairs of same gender. This design gives a high degree of control for confounding factors, such as age, gender, shared genetic and environmental factors. The desmosines and lung function values of the first twin were subtracted from the corresponding values in the second twin. There were 122 same-gender twin pairs (38 male and 84 female pairs; 82 monozygotic, 37 dizygotic, 3 pairs with unknown zygocity). In 87 pairs, both twins were non-smokers, in 9 pairs, both were current smokers, and in 26 pairs, one individual was a smoker. There was no significant correlation between within-pair differences in U-desmosines and within-pair differences in forced expiratory volume in 1 second, forced vital capacity, residual volume/total lung capacity or carbon monoxide diffusion capacity. A total of 101 twin pairs of the same gender had information about P-desmosines. Similarly, there was no significant correlation between within-pair differences in P-desmosines and within-pair differences in lung function measures. The correlation between within-pair differences in U-desmosines and P-desmosines was r=0.42 ($P < 0.001$).

In contrast to the results for the entire cohort of twins, there was no evidence from the within-pair analysis for a correlation between desmosines and lung function. These correlations were only based on 122 observations (twin pairs of same gender), and the statistical power was therefore substantially lower. However, overmatching is a concern when comparing differences within twin pairs, especially for factors with a strong genetic component. Previous studies of twins suggest that pulmonary function is strongly influenced by genetic factors [14]. Whether desmosine concentrations are genetically determined is still unclear.