COPD: recognizing the susceptible smoker

Hoonhorst, Susan

Chapter 5

Advanced glycation endproducts and their receptor in different body compartments in COPD

Susan Hoonhorst, Adèle Lo Tam Loi, Eef Telenga, Maarten van den Berge, Leo Koenderman, Jan-Willem Lammers, H. Marike Boezen, Antoon van Oosterhout, Monique Lodewijk, Wim Timens, Dirkje S. Postma, Nick H.T. ten Hacken

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ABSTRACT

Background: Inflammation and oxidative stress caused by cigarette smoking contribute to chronic obstructive pulmonary disease (COPD). Smoking and oxidative stress lead to accelerated formation and accumulation of advanced glycation end products (AGEs), causing local tissue damage either directly or by binding the receptor for AGEs (RAGE). This study assessed the association of AGEs or RAGE in plasma, sputum, bronchial biopsies and skin with COPD and lung function, and their variance between these body compartments.

Methods: Healthy smoking and never-smoking controls (n=191, age 18-40 years) and COPD patients (n=97, GOLD stage I-IV) were included. Autofluorescence (SAF) was measured in the skin, AGEs (pentosidine, CML and CEL) and sRAGE in blood and sputum by ELISA, and in bronchial biopsies by immunohistochemistry.

Results: Higher SAF and lower sRAGE levels associated with COPD and lower lung function ($p < 0.001$; adjusting for relevant covariates). Lower plasma sRAGE levels significantly and independently predicted higher SAF values ($p<0.001$).

Conclusion: In COPD, AGEs accumulate differentially in body compartments, i.e. they accumulate in the skin, but not in plasma, sputum and bronchial biopsies. The association between lower sRAGE and higher SAF levels supports the hypothesis that the protective mechanism of sRAGE as a decoy-receptor is impaired in COPD.

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by chronic airflow limitation, accompanied by persistent inflammation of the airways, mainly caused by cigarette smoking. Both smoking and inflammation are associated with oxidative stress leading to accelerated formation and accumulation of advanced glycation end products (AGEs) (1,2).

AGEs are a heterogeneous and complex group of compounds that are irreversibly formed by non-enzymatic glycation and oxidation of proteins and lipids (3). They accumulate in tissues with ageing, and under oxidative stress and inflammatory conditions their formation and accumulation increases. Therefore, accumulation of AGEs can be used as a read-out system for exposure to oxidative stress during life. This is particularly true in tissues with slow turnover, more than in tissues or products from tissues with rapid turnover. The best known AGEs are N^{ε}-(carboxymethyl)lysine (CML), N^{ε}-(carboxyethyl)lysine (CEL) and pentosidine. AGEs cause local tissue damage by affecting protein structure, by formation of crosslinks between molecules, or by binding the receptor for AGE (RAGE) (4,5). RAGE is a member of the immunoglobulin superfamily and is a pattern-recognition receptor on cell surfaces. Ligation of RAGE triggers inflammatory responses, induces oxidative stress, and in turn causes RAGE over-expression. This finally leads to increased tissue remodeling (5). Interestingly, expression of RAGE in the lung has shown to be relatively high when compared with other tissues (6).

A few studies have indicated that AGEs are involved in the pathology of COPD. One study showed increased accumulation of AGEs in lung parenchyma and small airways of COPD patients (7). We and others found increased AGEs accumulation in the skin of COPD patients compared to healthy smoking and never-smoking controls (8,9). Furthermore, plasma CML levels in COPD are elevated compared to non-COPD controls (9), suggesting a systemic component that may contribute to AGEs accumulation outside the lung, and to extra-pulmonary manifestations of COPD. Regarding RAGE, it has been shown that immunostaining of the receptor is increased in bronchial biopsies and lung parenchyma of COPD patients (7,10). Importantly, RAGE also exists as soluble form (sRAGE). It has been postulated that sRAGE can act as a decoy receptor by clearance of circulating AGEs, in this way preventing ligation of membrane bound RAGE. This possible 'protective' mechanism may be reduced in COPD, as levels of sRAGE have found to be lower in COPD patients than in non-COPD controls (11-15).

Taken together, studies so far suggested that the AGE-RAGE axis is involved in the pathology of COPD. In the current study we evaluated both AGEs and (s)RAGE levels in plasma, sputum, bronchial biopsies and the skin in the same study subjects. Young (18-40) and old (40-75) smokers and never-smokers, and mild-to-very severe COPD patients were included. We studied whether the expression of AGEs or RAGE in the different tissues was associated with COPD and lung function values, and whether the expression of AGEs and/or RAGE levels in different tissues were associated.

METHODS

Subjects

Data were collected from two studies performed in Groningen and Utrecht, the Netherlands

(Clinicaltrials.gov: NCT00807469 and NCT00848406 (A multi-center study (16)) and NCT00848406). All participating subjects gave peripheral blood and performed an AGE-reader measurement, while a subgroup of subjects underwent sputum induction and bronchoscopy with collection of bronchial biopsies. All measurements were obtained by using standardized protocols. The studies were approved by the medical ethics committees of University Medical Centers Groningen (UMCG) and Utrecht (UMCU), the Netherlands.

Mild to very severe COPD patients (40-75 years, >10 packyears), as classified by the Global Initiative for Chronic Obstructive Lung Disease (GOLD) (17) were recruited from outpatient clinics of UMCG and UMCU. Old (40-75 years) and young (18-40 years) healthy smokers and healthy never-smokers were recruited by advertisements. Old smokers had a smoking history >10 packyears and young smokers >0.5 packyears. All never-smoking subjects had smoked <0.5 packyears. Healthy participants had no history of pulmonary diseases and showed normal spirometry. Exclusion criteria for all groups were alpha-1 antitrypsin deficiency and a doctors' diagnosis of asthma.

Determination of AGEs and RAGE in peripheral blood samples and sputum

Blood was collected in tubes containing EDTA and was immediately placed on ice. After centrifugation (twice at 2000 rcf_{max}, 10 min, 4°C) samples were stored at -80°C until analysis. Sputum induction was performed according standard protocols (detailed methods in online supplement). Sputum samples were centrifuged (10 min, 450g, 4°C) and the supernatant was stored at -80°C until analysis.

In plasma and sputum samples, ELISA was performed to determine levels of total sRAGE (cleaved and secreted forms) (RAGE DuoSet; R&D Systems, Minneapolis, MN, USA), CEL (Cell Biolabs Inc. San Diego, CA, USA), CML (Cell Biolabs Inc. San Diego, CA, USA) and Pentosidine (Uscn Life Science Inc., Wuhan, China), all according to the manufacturer's instructions.

Determination of AGEs and RAGE in bronchial biopsies

Bronchial biopsies were taken from subsegmental carinae of the right lower lobe. Biopsies were fixed in 4% neutral buffered formalin, processed and embedded in paraffin and cut in 3 µm sections. After antigen retrieval, sections were incubated with the primary monoclonal antibody against AGEs (anti-AGEs (clone 6D12), 1:750, Cosmo Bio Co, Ltd, Tokyo, Japan) or RAGE (anti-RAGE (ab7764), 1:1500, Abcam, Cambridge, UK). Immunohistochemical stainings were performed using the DAKO autostainer (DAKO, Glostrup, Denmark). Quantification of both stainings was performed by calculating the percentage positive and strong positive pixels of the total amount of pixels in whole biopsies, using ImageScope (Aperio Technologies, version 11.2.0.780). Detailed immunohistochemistry and quantification procedures are presented in the online supplement.

Measurement of AGEs using Skin autofluorescence in the skin

Skin autofluorescence (SAF) was assessed non-invasively by the AGE-Reader™ (DiagnOptics B.V., Groningen, The Netherlands) (18). Technical details of this device have been extensively

described elsewhere and briefly in the online supplement. (19). In short, the volar surface of subject's forearm was positioned on top of the device and three consecutive measurements were performed for each subject. In all analyses, SAF was expressed as the mean of these three measurements in arbitrary units (AU).

Statistical analysis

Differences in expression of AGEs and RAGE between groups were analyzed by Kruskal-Wallis test, followed by Mann-Whitney U tests if significant. Associations with COPD were examined by multiple regression analyses with AGEs or RAGE expression as dependent variables, and COPD or lung function values as predictor variables. Associations of AGEs and RAGE between different compartments were additionally analyzed by multiple regression models. All models were adjusted for co-variates that associate with AGEs formation, including age, gender, packyears, BMI, LDL cholesterol, and triglycerides. Benjamini Hochberg corrections were applied to correct for multiple testing (20). Regression models were considered valid if the residuals were normally distributed. Statistical analyses were performed using the statistical program IBM SPSS Statistics version 20.

RESULTS

Subject characteristics

In total, 108 young controls (including 36 never-smokers and 72 smokers), 83 old controls (including 28 never-smokers and 55 smokers) and 97 COPD patients (32 GOLD I, 25 GOLD II, 24 GOLD III, 16 GOLD IV) were included. Group characteristics are presented in Table 1.

Table 1. Group characteristics

	Young healthy	Old healthy	COPD
	n=108	n=83	n=97
Age, years	25 (66)	54 (8.9)	62 (7.6)
Males, n (%)	54 (50)	60 (72)	68 (70)
Current smokers, n (%)	72 (67)	54 (65)	51 (53)
Cigarettes per day	9 (6.8)	16 (7.1)	11 (7.9)
Packyears	3.1 (4.8)	20 (18.0)	38 (16.8)
BMI, kg/m^2	23.0 (2.8)	25.3 (3.6)	25.5 (4.7)
FEV_1,%pred	108 (9.5)	110 (13.5)	62 (27)
FEV_1/FVC (%)	85 (5.7)	79 (5.0)	48 (14)
RV/TLC (%)	23.3 (5.1)	31.1 (4.4)	45.8 (11.3)
FEF_{25-75}, %pred	101 (18.7)	100 (30.4)	24 (17)
TLCOc/VA, %pred	97 (13.3)	98 (12.6)	67 (24)
LDL cholesterol, mmol/L	2.6 (0.8)	3.6 (1.0	3.4 (1.0)
Triglycerides, mmol/L	1.0 (0.8)	1.4 (1.0)	1.2 (0.7)
Fasting glucose, mmol/L	5.2 (1.3)	5.6 (0.7)	5.8 (0.7)
Creatinine, µmol/L	73.7 (10.9)	80.2 (14.5)	82.7 (14.8)

Data are presented as mean (sd) unless otherwise stated. n=number, BMI=body mass index, FEV_1=forced expiratory volume in one second, FVC=forced expiratory volume, RV=residual volume, TLC=total lung capacity, FEF=forced expiratory flow, $TLCO_c$/VA=transfer coefficient for carbon monoxide, LDL=low density lipoprotein.

AGEs

Expression of AGEs in plasma, sputum, bronchial biopsies and the skin is presented in Figure 1 and Table 2.

In plasma (Figure 1A), CEL levels were significantly higher in young healthy subjects than in old healthy subjects and COPD patients. Furthermore, plasma CML levels were significantly higher in COPD patients than young and old subjects, and higher in the young group than in the old healthy group. Plasma pentosidine levels did not differ between groups. In sputum (Figure 1B), CEL and CML levels did not differ between groups, whereas pentosidine levels were too low to be detected; only 11 sputum supernatant samples of the total 182 samples were above the detection limit of 1.45 ng/ml. AGEs immunopositivity In whole bronchial biopsies, was not differently expressed between groups, (Figure 1C), neither were quantitative analyses in the intact and basal epithelium, smooth muscle and connective tissue (Figure 1, online supplement). However, accumulation of AGEs in the skin was significantly different between all groups, with highest SAF values in COPD patients and lowest values in the young group (Figure 1D)

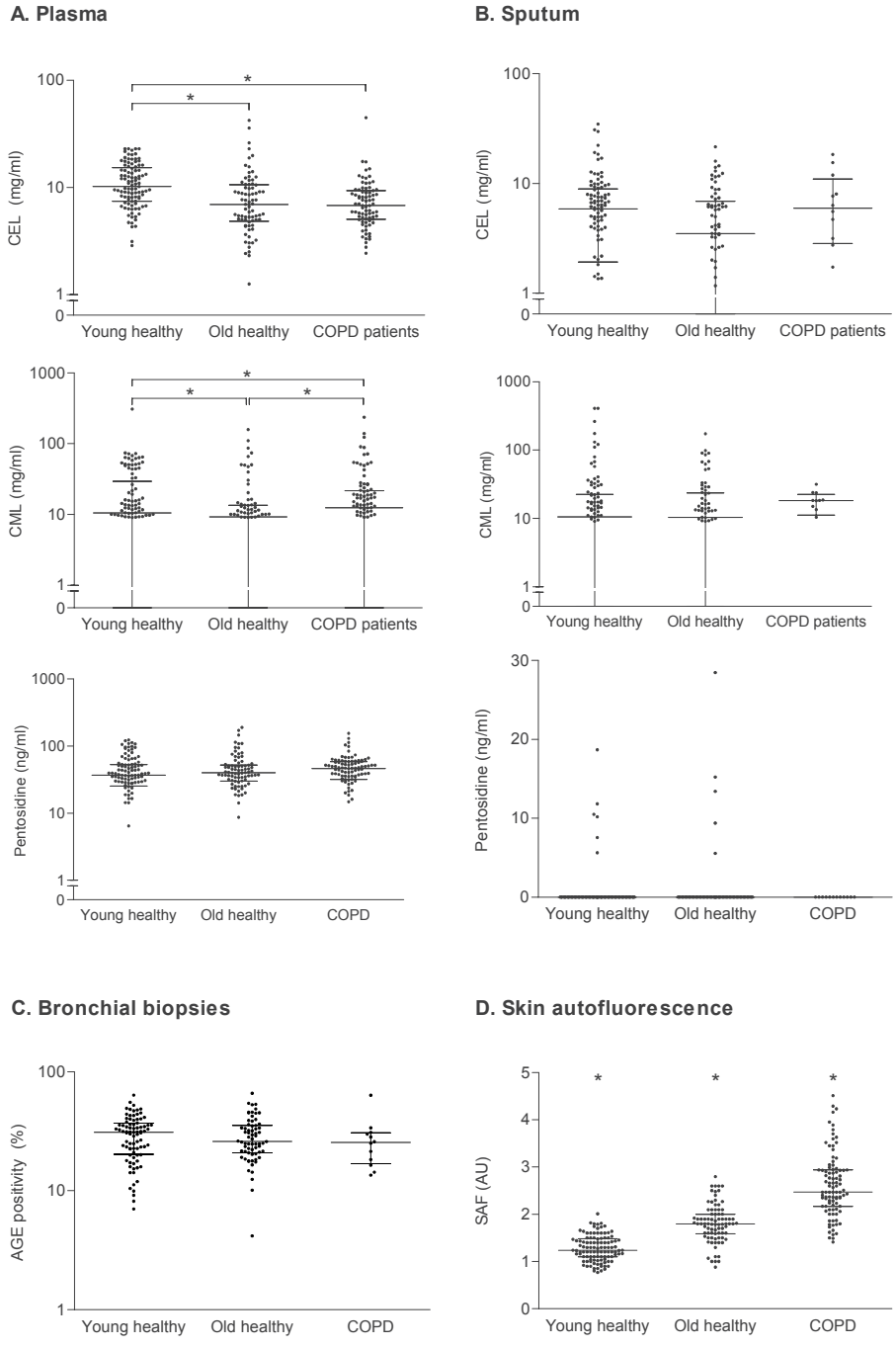
In all measurements, levels of AGEs did not differ between the COPD severities (GOLD I-IV, Table 1, online supplement). Additionally, AGEs levels are presented separately for healthy never-smokers and smokers (Table 1, online supplement).

Table 2. AGE and RAGE expression in young and old subjects, and COPD patients

	Young healthy	Old healthy	COPD GOLD I-IV	Kruskal-Wallis
	<40 years	*>40 years*	*>40 years*	*p-value*
Plasma	*n=105*	*n=82*	*n=95*	
CEL, µg/ml	10.2 (7.4-15.4)	7.0 (4.8-10.6) §	6.8 (5.1-9.4) §	**0.000***
CML, µg/ml	10.5 (0.0-29.5)	9.2 (0.0-13.5) §	12.5 (0.0-21.6) §‡	**0.042***
Pentosidine, ng/ml	36.8 (25.3-53.1)	39.9 (30.1-52.0)	46.1 (31.8-58.5)	0.181
sRAGE, pg/ml	795 (614-1089)	805 (617-1032)	414 (292-592) §‡	**0.000***
Induced sputum	*n=97*	*n=73*	*n=12*	
CEL, µg/ml	5.9 (1.9-8.9)	3.50 (0.0-6.9)	6.0 (2.9-11.0)	0.064
CML, µg/ml	10.6 (0.0-22.7)	10.4 (0.0-23.7)	18.3 (11.2-22.5)	0.409
Pentosidine, ng/ml	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.650
sRAGE, pg/ml	78.1 (0.0-160.2)	114.0 (0.0-240.5)	110.9 (49.7-161.8)	0.117
Bronchial Biopsies	*n=85*	*n=68*	*n=12*	
AGEs, positivity (%)	31.1 (20.3-36.9)	25.9 (20.9-35.4)	25.5 (16.9-30.7)	0.418
RAGE, positivity (%)	9.8 (6.5-15.1)	8.2 (5.7-11.8)	8.0 (4.2-11.4)	0.204
Skin	*n=107*	*n=83*	*n=96*	
AGE-reader, SAF	1.2 (1.1-1.5) *	1.8 (1.6-2.0) *	2.5 (2.2-2.9) *	**0.000***

Data are expressed as medians (IQR). * $p<0.05$ between all groups, § $p<0.05$ compared with the young healthy group, ‡ compared with the old healthy group. CEL= N^{ε}-(carboxyethyl)lysine, CML= N^{ε}-(carboxymethyl)lysine, RAGE= receptor for advanced glycation endproducts, AGEs= advanced glycation endproducts, SAF= skin autofluorescence.

Figure 1. AGEs expression in plasma, sputum, bronchial biopsies and the skin



AGEs levels in A) plasma, B) sputum, C) bronchial biopsies and D) skin (SAF). Horizontal lines represent median values with interquartile ranges, * $p<0.05$ between groups.

Table 3. Associations of AGEs and RAGEs with COPD and lung function in the total population

A. Associations of AGEs with COPD and lung function

		Predictor variables									
		COPD, n/y		FEV1, % predicted		FEV1/FVC (%)		RV/TLC (%)		FEF25-75, %predicted	
Dependent variables		β	p-value	β	p-value	β	p-value	β	p-value	β	p-value
Plasma	CEL	0.009	0.924	-0.465	0.642	0.581	0.562	0.117	0.204	0.0.41	0.635
	CML	0.093	0.327	-0.081	0.283	-0.088	0.337	0.052	0.572	-0.027	0.761
	Pentosidine	-0.035	0.711	-0.020	0.788	0.069	0.451	0.093	0.314	-0.011	0.901
Sputum	CEL	0.093	0.311	-0.139	0.101	-0.044	0.691	0.11	0.353	-0.059	0.518
	CML	0.029	0.757	-0.030	0.728	0.043	0.706	0.021	0.862	0.000	0.998
	Pentosidine	-0.025	0.792	-0.124	0.152	0.022	0.844	-0.024	0.841	-0.053	0.568
Bronchial biopsies	AGE positivity	-0.065	0.550	0.139	0.155	0.075	0.551	-0.126	0.420	0.094	0.379
Skin	SAF	**0.426**	**<0.001**	**-0.302**	**<0.001**	**-0.357**	**<0.001**	**0.265**	**<0.001**	**-0.347**	**<0.001**

B. Associations of RAGE with COPD and lung function

		Predictor variables									
		COPD, n/y		FEV1, % predicted		FEV1/FVC (%)		RV/TLC (%)		FEF25-75, %predicted	
Dependent variables		β	p-value	β	p-value	β	p-value	β	p-value	β	p-value
Plasma	sRAGE	**-0.422**	**<0.001**	**0.310**	**<0.001**	**0.405**	**<0.001**	**-0.236**	**0.002**	**0.297**	**<0.001**
Sputum	sRAGE	-0.110	0.194	0.084	0.289	0.012	0.908	-0.015	0.895	-0.041	0.622
Bronchial biopsies	RAGE positivity	-0.083	0.365	-0.053	0.529	0.04	0.715	0.241	0.060	-0.038	0.678

Values in bold represent significant associations. β= standardized regression coefficient for predictor variables. Models in Table A were adjusted for age, gender, packyears, BMI, LDL cholesterol and triglycerides; models in Table B were adjusted for age, gender and packyears. FEV_1=forced expiratory volume in one second, FVC=forced expiratory volume, RV=residual volume, TLC=total lung capacity, FEF=forced expiratory flow, CEL=N^{ε}-(carboxyethyl)lysine, CML=N^{ε}-(carboxymethyl)lysine, AGEs=advanced glycation endproducts, SAF=skin autofluorescence, RAGE=receptor for advanced glycation endproducts.

TABLE 4. Associations between AGEs and RAGE expression in different tissues

		Plasma								Sputum								Bronchial biopsies				Skin	
		CEL		CML		Pentosidine		sRAGE		CEL		CML		Pentosidine		sRAGE		AGE positivity		RAGE positivity		SAF	
Predictor variables		*B*	*p*	*B*	*p*	*B*	*p*	*B*	*p*	*B*	*p*	*B*	*p*	*B*	*p*	*B*	*p*	*B*	*p*	*B*	*p*	*B*	*p*
Plasma	CEL	---	---	0.42	0.34	-0.39	0.27	3.21	0.47	0.07	0.46	0.99	0.20	0.02	0.59	-3.70	0.21	0.43	0.08	0.28	0.04	0.00	0.91
	CML	0.01	0.34	---	---	0.11	0.07	-1.01	0.13	0.02	0.22	-0.13	0.34	0.00	0.77	0.51	0.28	0.07	0.03	0.00	0.78	0.00	0.72
	Pentosidine	-0.02	0.27	0.13	0.07	---	---	-1.84	0.01	0.04	0.01	-0.13	0.38	0.01	0.44	0.46	0.37	0.04	0.20	-0.01	0.51	0.00	0.81
	sRAGE	0.00	0.47	-0.01	0.13	-0.01	0.01	---	---	0.00	0.21	-0.01	0.57	0.00	0.46	0.04	0.30	-0.01	0.09	0.00	0.64	**0.00**	**0.00***
Sputum	CEL	0.06	0.46	0.62	0.22	1.14	0.01	-6.92	0.21	---	---	**2.93**	**0.00***	**0.17**	**0.00***	-3.30	0.23	0.51	0.01	-0.07	0.53	0.00	0.91
	CML	0.01	0.20	-0.05	0.34	-0.04	0.38	-0.33	0.57	**0.03**	**0.00***	---	---	0.00	0.50	-0.63	0.03	0.03	0.07	0.00	0.72	0.00	0.51
	Pentosidine	0.10	0.59	-0.25	0.77	0.58	0.44	6.77	0.46	**0.47**	**0.00***	0.92	0.50	---	---	-5.94	0.21	0.12	0.70	-0.19	0.27	-0.01	0.50
	sRAGE	0.00	0.21	0.02	0.28	0.01	0.37	0.18	0.30	0.00	0.23	-0.06	0.03	0.00	0.21	---	---	0.00	0.96	0.00	0.95	0.00	0.63
Bronchial biopsies	AGE positivity	0.06	0.08	0.59	0.03	0.33	0.20	-4.99	0.09	0.14	0.01	0.92	0.07	0.01	0.70	0.09	0.96	---	---	0.10	0.07	0.00	0.39
	RAGE positivity	0.13	0.04	-0.13	0.78	-0.29	0.51	-2.52	0.64	-0.06	0.53	0.32	0.72	-0.06	0.27	-0.19	0.95	0.30	0.07	---	---	0.00	0.85
Skin	SAF	-0.10	0.91	-1.50	0.72	0.91	0.81	**-154.97**	**0.00***	0.18	0.91	9.76	0.51	-0.62	0.50	23.76	0.63	3.15	0.39	0.37	0.85	---	---

B=regression coefficient for predictor variables. All models are adjusted for age, gender, packyears, BMI, LDL cholesterol, and triglycerides. * significant p-value after Benjamini Hochberg correction for multiple testing. CEL=N^{ε}-(carboxyethyl)lysine, CML=N^{ε}-(carboxymethyl)lysine, sRAGE=soluble receptor for advanced glycation endproducts, AGE=advanced glycation end products, RAGE=receptor for advanced glycation endproducts, SAF= skin autofluorescence.

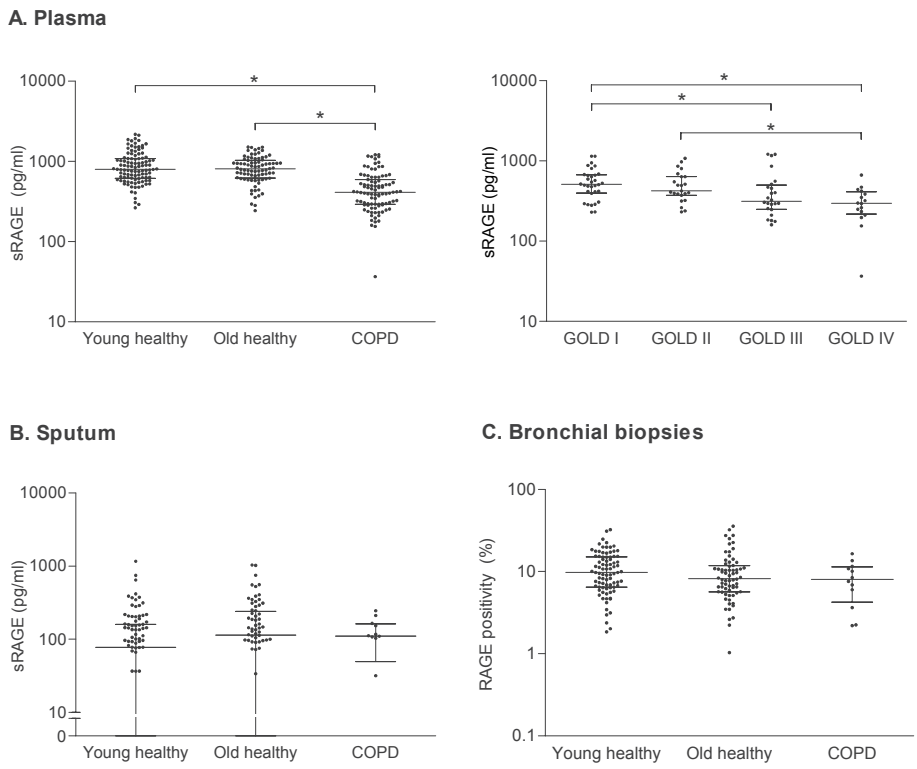
RAGE

Levels of RAGE in plasma, sputum and bronchial biopsies are presented in Figure 2 and Table 2.

In plasma, sRAGE levels were significantly lower in COPD patients than in young and old healthy subjects (Figure 2A). In addition, COPD GOLD stage III patients had lower sRAGE levels than GOLD stage I patients, and GOLD stage IV patients had lower sRAGE levels than GOLD stage I and II (Figure 2A and Table 1 in online supplement). No differences were found between young and old healthy subjects. RAGE levels in sputum and RAGE immunopositivity in whole sections from bronchial biopsies did not differ between groups (Figure 2B and 2C). When studying different parts of the bronchial biopsies (intact and basal epithelium, smooth muscle, connective tissue) no group differences were found (Figure 1, online supplement).

RAGE levels in healthy never-smokers and smokers are presented in Table 1 in the online supplement.

Figure 2. RAGE expression in plasma, sputum and bronchial biopsies



RAGE levels in A) plasma, B) sputum, and C) bronchial biopsies. Horizontal lines represent median values with interquartile ranges, * $p<0.05$ between groups.

Associations between COPD, lung function and AGEs and RAGE

Table 3(A-B) shows the results of multiple regression analyses with COPD or lung function values as predictors of AGEs and RAGE expression in the different compartments.

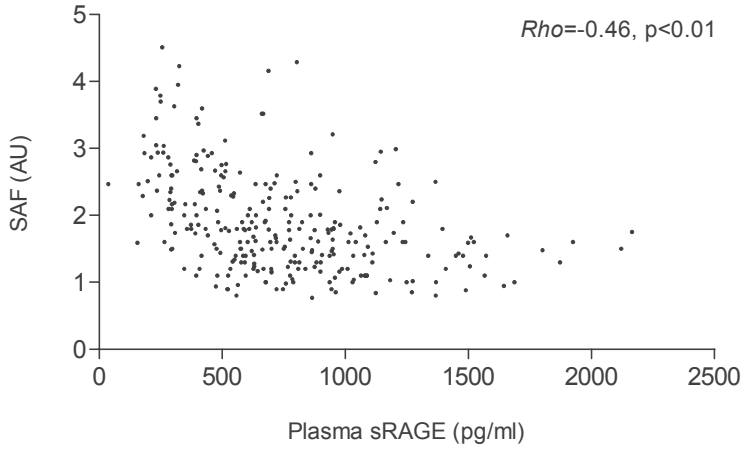
In established COPD, a lower FEV_1 %predicted, FEV_1/FVC, FEF22-75 % predicted, as well as a higher RV/TLC were associated with a higher SAF, independently of age, gender, number of packyears, BMI, LDL cholesterol and triglycerides (Table 3A). No associations were observed between COPD or lung function values on one hand and AGEs in plasma, sputum and bronchial biopsies on the other hand.

Regarding RAGE, both established COPD and impaired lung function values were associated with higher levels of soluble RAGE in plasma (Table 3B). No associations were found of COPD or lung function values with RAGE levels in sputum and bronchial biopsies.

Associations of AGEs and RAGE expression between different compartments

Results of multiple regression analyses are presented in Table 4, reflecting associations between AGEs and RAGE expression in the different tissues after adjustment for age, gender, packyears, BMI, LDL cholesterol and triglycerides. Lower plasma sRAGE levels were significantly associated with higher SAF values. Furthermore no significant associations were found.

Figure 3. Associations between sRAGE and SAF



Rho=correlation coefficient, SAF=skin autofluorescence, sRAGE is soluble receptor for advanced glycation endproducts. Association after adjustment for age, gender, packyears, BMI, LDL cholesterol and triglycerides was in B=0.00, p=<0.01.

DISCUSSION

In this study we investigated a large COPD and a non-COPD control population with respect to the accumulation of AGEs and expression of its receptor RAGE in different body compartments including plasma, induced sputum, bronchial biopsies and the skin. We performed this study in COPD, a chronic disease which for a long time has been associated with chronic oxidative stress, the most important accelerator of AGES formation. Our study shows that SAF values in the skin were higher in COPD than in young and old non-COPD controls, whereas the expression of AGEs in bronchial biopsies was not different between the groups. In addition, sRAGE levels in plasma were lower in COPD patients. Of interest, lower sRAGE associated with higher SAF, fitting the hypothesis of a 'protective' function of sRAGE by acting as a decoy-receptor preventing accumulation in the skin.

In COPD, oxidative stress is thought to be continuously increased as a consequence of ongoing inflammation (endogeneous component) and chronic smoking (exogeneous component). This continuous exposure to oxidative stress, both locally in lung tissue as well as systemically in peripheral blood, might lead to increased accumulation of AGEs inside and outside the lung. In the current study we demonstrated that AGEs accumulation was elevated in the skin of COPD patients, a finding that we and others have observed before (8,9). Interestingly, SAF values were comparable between the different severity stages of COPD. This suggests that AGEs formation is not increased during disease progression, but may be accelerated in the induction phase of COPD. Our data may suggest the following processes: AGEs accumulate due to oxidative stress responses to some extent during aging in healthy smokers, whereas this is accelerated with chronic smoking in healthy smokers. The highest AGEs would be expected in 'susceptible' smokers, i.e. subjects who develop COPD. Here, AGEs accumulate due to a combination of ageing and disease-related exaggerated response to smoking and associated local and systemic oxidative stress.

In contrast with our findings in the skin, AGEs expression in bronchial biopsies was not different between COPD patients and non-COPD controls and did not associate with lung function values in the total population. This contradicts a previous study showing higher AGEs expression in the lung parenchyma and small airways of COPD patients as compared to non-COPD controls (7). In an effort to replicate these findings we analyzed immunopositivity of our bronchial biopsies in numerous ways, e.g. by quantifying AGEs in different parts of the bronchial biopsies and by using different antibodies (against total amount of AGEs, CML and pentosidine). However, no differential expressions in COPD patients were observed. There are several explanations for our negative finding in bronchial biopsies. First, we collected biopsies from the central airways whereas oxidative stress might predominantly exist in the peripheral airways, and thereby also AGEs formation. Unfortunately, studies comparing oxidative stress in central and peripheral airways are scarce. One study showed that isoprostane levels in ELF from the peripheral airways were higher than from the central airways, both in smokers with and without airway obstruction (21). We also checked expression of AGEs in peripheral lung tissue sections of smokers and non-smokers with and without COPD but did not find differences between these (small) groups. Secondly, accumulation of AGEs in the lung might be limited because of the relatively high turn-over rate of cells and extracellular matrix

(22). For example, the turn-over rate of epithelium from the tracheo-bronchial wall in adult rodents is estimated to be more than 100 days (23), in contrast to about 20 years of the dermis and an infinite turnover time of the ocular lens, both organs in which AGES are stored (24). Finally, a quantification problem may contribute to a lower expression of AGEs in the lungs of COPD patients, as extracellular matrix proteins are reduced in the central airways of COPD patients (25). Obviously, more research at AGEs accumulation is needed in both the central and peripheral airways before definitive conclusions can be drawn regarding our conflicting results.

In plasma, we demonstrated that CML, CEL and pentosidine levels were comparable between COPD patients and non-COPD controls, after correction for confounding factors. Our results are in line with three previous studies in COPD investigating plasma CML levels and showing no differences between COPD and non-COPD controls (12,14,26). In contrast, one study showed comparable pentosidine levels as well, but lower CML levels and higher CEL levels in COPD patients (9). The latter is surprising since CML and CEL both are formed by the same pathway, namely via reactive carbonyl compounds. One explanation might be that another technique was used, namely mass spectrometry. One also have to realize that AGEs are very volatile, hence measure a 'snap shot' in time only and results can be affected by food intake and smoking as well (1,27).

Besides their harmful local effects in tissue, AGEs can interact with RAGE thereby triggering intracellular signaling in pro-inflammatory pathways. Two previous studies showed that immunostaining of RAGE was increased in bronchial biopsies and in lung parenchyma of COPD patients (7,10), but we observed no differences with non-COPD controls in the current study. RAGE also exists as a soluble form, generated as a splice variant of the advanced glycosylation end product-specific receptor (AGER) gene or by proteolysis of the receptor from the cell surface. In line with previous studies (11-15), we demonstrated lower levels of sRAGE in plasma of COPD patients and these reduced levels were associated with lower lung function values in the total population. Of interest, we demonstrated for the first time that lower sRAGE levels were associated with increased SAF values. This finding supports the idea that sRAGE acts as a decoy receptor. AGEs binding to sRAGE may lead to clearance of AGEs preventing to accumulate in body tissues, a protective mechanism that apparently is impaired in COPD patients. There are indications that lower sRAGE levels are genetically determined, as a single nucleotide polymorphism (SNP) in the AGER gene associates with lower sRAGE levels (11). In this perspective, impaired sRAGE levels might contribute to higher levels of AGEs in tissues specifically in COPD.

Finally, we assessed levels of AGEs and RAGE in sputum supernatant, which has not been studied in COPD before. We hypothesized that AGEs and RAGE levels in sputum might reflect expression in the lung. No differential levels of both AGEs and RAGE in induced sputum from COPD patients and non-COPD controls were found, nor associations with expression in bronchial biopsies. This observation fits with the comparable expression of AGEs and RAGE between COPD and healthy individuals as observed in our bronchial biopsies. An explanation may be that AGEs are released in more peripheral airways and not captured in sputum, as one study in COPD demonstrated that CML was elevated in the epithelial lining fluid (ELF) collected

in the peripheral airways, but not in the central airways (28). Since this is the first study of AGEs and RAGE in sputum, further research is needed.

This study is unique because of its large population of healthy smokers and never-smokers and a large group of COPD patients of all severities, as well as the availability of different tissues from each participant. Unfortunately, only a subgroup of COPD patients performed sputum induction and a bronchoscopy which may have affected the statistical power of this study. Another limitation is that our study had a cross-sectional design. Longitudinal studies are needed to investigate changes in AGEs and RAGE levels in the different tissues over time and to further assess the potential contributing role of AGEs and RAGE in the development of COPD.

To summarize, there is growing evidence in the literature for an AGEs - RAGE interaction in the pathology of COPD. Our study contributes to this insight since we show an increased AGEs accumulation in the skin of COPD patients compared to non-COPD smokers and never-smokers. Moreover, we did not observe differences between COPD and non-COPD controls in central bronchial biopsies, indicating that accumulation of AGEs is not similar in different body compartments. No further associations were found between AGEs and RAGE in the different compartments that were investigated. Interestingly, we demonstrated that lower sRAGE levels associate with higher AGE accumulation in the skin. This fits the hypothesis of a 'protective' function of sRAGE by acting as a decoy-receptor preventing accumulation in the skin.

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Chapter 5

Supplement

METHODS

Sputum induction

Sputum induction was performed according to the method described elsewhere with some modifications (1). In short, 4,5% hypertonic saline was nebulized with an ultrasonic nebulizer (Ultraneb, DeVillbiss, Somerset, PA, USA). Patients inhaled for three periods of five minutes and were encouraged to cough and expectorate sputum after each period. The volume of the whole sputum sample was determined and an equal volume of 0.1% dithiothreitol (Sputolysin; Calbiochem, La Jolla, CA, USA) was added. The samples were agitated during 15 minutes in a shaking water bath for 15 minutes at 37°C to complete homogenization and then filtered through a 48 µm nylon gauze. The filtered sample was centrifuged (10 min, 450g, 4°C) and the supernatant was stored at -80°C until analysis.

Bronchial biopsies

After administering of local anaesthesia (lidocain 2-4%), a flexible bronchoscope was introduced and bronchial biopsies were taken from subsegmental carinae of the right lower lobe. Biopsies were fixed in 4% neutral buffered formalin, processed and embedded in paraffin and cut in 3 µm sections. Quality of biopsies was verified by hematoxylin and eosin (HE) staining. Sections were deparaffinized in xylene (2x10 min), rehydrated in alcohol dilations (2x100%, 2x96%, and 1x70%), and rinsed in demi-water. For AGEs staining, antigens were retrieved by incubating the slides in 0,1M Tris-HCl pH 9.0 buffer at 80°C overnight. For RAGE staining, Citrate 10mM pH 6.0 buffer was preheated, slides were placed in a plastic container and were heated in microwave for 15 min at 400W. After antigen retrieval the slides were cooled down at room temperature (RT) and were washed with PBS. All sections were incubated with 0.3% hydrogen peroxide H_2O_2 (Merck, Germany) in PBS (500µl H_2O_2 30% in 50ml PBS) for 30 min at RT to block endogenous peroxidase activity. After three washes with PBS, sections were incubated with the primary monoclonal antibody against AGEs (anti-AGEs (clone 6D12), 1:750, Cosmo Bio Co, Ltd, Tokyo, Japan) or RAGE (anti-RAGE (ab7764), 1:1500, Abcam, Cambridge, UK) diluted in PBS/1%BSA for 1 hour at RT. For AGEs staining, sections were washed in PBS for three times and incubated with the secondary antibody (Envision™ Detection Systems Peroxidase (DAKO)) for 30 min at RT. For RAGE staining, sections were washed in PBS for 3 times and incubated with the secondary peroxidase labeled rabbit anti-goat antibody (DAKO, 1:100 diluted in PBS/1% BSA + 1%AB serum) for 30 min at RT. After washing with PBS 3x, sections were incubated with the tertiary peroxidase labeled goat anti-rabbit antibody (DAKO, 1:100 diluted in PBS/1% BSA + 1%AB serum) for 30 min at RT. After washing the sections for three times in PBS for three times, peroxidase activity was visualised by incubating the slides in DAB (3-3'DiaminoBenzidine) together with 50 µl of hydrogen peroxide for 10 min at RT. Sections were rinsed in demi water. Finally, the sections were counterstained with haematoxilin for approximately 2 min, rinsed in tap water, dehydrated in alcohol (70%, 96% and 100%), dried, and mounted with mounting medium and covered with a coverslip. Both immunohistochemical stainings were performed using the DAKO autostainer (DAKO, Glostrup, Denmark). Quantification of both stainings was performed by calculating the percentage positive and strong positive pixels of the total amount

of pixels in whole biopsies, using ImageScope (Aperio Technologies, version 11.2.0.780).

Skin autofluorescence

SAF was assessed non-invasively by the AGE-Reader™ (DiagnOptics B.V., Groningen, The Netherlands) (2). Technical details of this device have been extensively described elsewhere (3). In short, the AGE reader illuminates approximately 1 cm^2 of the skin, guarded against surrounding light, with an excitation light source between 300 and 420 nm (peak excitation flow ~350 nm). Only light from the skin is measured between 300 and 600 nm with a spectrometer using a 200-µm glass fiber. SAF was calculated by dividing the average light intensity emitted per nm over the 420- to 600-µm range by the average light intensity emitted per nm over the 300- to 420-µm range, using the AGE Reader software version 2.2. The volar surface of subject's forearm was positioned on top of the device, taking care to perform the measurement at normal skin site, i.e. without visible vessels, scars, or other skin abnormalities. SAF was averaged from three consecutive measurements for each subject, measured within a time period of approximately 2 minutes. In all analyses, SAF is expressed in arbitrary units (AU).

Table 1. AGE and RAGE expression in young and old never-smokers and smokers, and COPD GOLD stages

A. Young healthy never-smokers and smokers

	Young healthy never-smokers	Young healthy smokers
Plasma	*n=36*	*n=69*
CEL	9.0 (6.7-15.0)	11.4 (8.2-16.2)
CML	10.9 (0.0-25.3)	10.5 (0.0-38.1)
Pentosidine	48.7 (32.6-70.9)	35.3 (18.7-47.8)*
RAGE	878.8 (573.3-1113.5)	777.0 (628.8-1077.1)
Sputum	*n=34*	*n=63*
CEL	4.0 (0.0-9.5)	6.4 (3.8-8.2)
CML	14.8 (0.0-44.8)	0.0 (0.0-17.6)*
Pentosidine	0.0 (0.0-0.0)	0.0 (0.0-0.0)
RAGE	0.0 (0.0-97.1)	101.3 (0.0-195.1)*
Bronchial biopsies	*n=32*	*n=53*
AGEs, positivity (%)	31.3 (18.5-37.4)	30.6 (20.7-36.9)
RAGE, positivity (%)	8.8 (6.1-14.8)	10.8 (6.8-15.2)
Skin	*n=36*	*n=71*
AGE-reader	1.20 (1.04-1.40)	1.3 (1.1-1.5)

CEL= N^{ε}-(carboxyethyl)lysine, CML= N^{ε}-(carboxymethyl)lysine, RAGE= receptor for advanced glycation endproducts, AGEs= advanced glycation endproducts, SAF= skin autofluorescence. Values are expressed as median (IQR).

B. Old healthy never-smokers and smokers

	Old healthy never-smokers	Old healthy smokers
Plasma	*n=28*	*n=54*
CEL	5.37 (3.29-8.55)	8.56 (5.06-11.26)*
CML	10.97 (0.00-13.64)	0.00 (0.00-12.00)
Pentosidine	39.53 (28.29-45.51)	41.1 (30.12-66.48)
RAGE	810.8 (672.9-1118.5)	795.6 (609.3-953.3)
Sputum	*n=23*	*n=50*
CEL	0.00 (0.00-3.23)	6.11 (2.47-8.97)*
CML	10.40 (0.00-23.49)	11.46 (0.00-24.41)
Pentosidine	0.00 (0.00-0.00)	0.00 (0.00-0.00)
RAGE	115.2 (0.0-206.9)	114.0 (0.0-262.2)
Bronchial biopsies	*n=26*	*n=42*
AGEs, positivity (%)	28.2 (19.1-37.4)	25.4 (21.3-33.7)
RAGE, positivity (%)	6.9 (5.0-10.6)	9.5 (6.3-12.5)
Skin	*n=28*	*n=55*
AGE-reader	1.774 (1.43-2.00)	1.80 (1.60-2.10)

CEL= N^{ε}-(carboxyethyl)lysine, CML= N^{ε}-(carboxymethyl)lysine, RAGE= receptor for advanced glycation endproducts, AGEs= advanced glycation endproducts, SAF= skin autofluorescence. Values are expressed as median (IQR).

Table 1. AGE and RAGE expression in young and old never-smokers and smokers, and COPD GOLD stages

C. COPD severities

	COPD GOLD I	COPD GOLD II	COPD GOLD III	COPD GOLD IV	Kruskall-Wallis p-value
Plasma	*n=32*	*n=23*	*n=24*	*n=16*	
CEL	6.61 (4.92-9.05)	5.37 (3.67-7.48)	8.23 (5.92-9.58)	7.34 (5.94-13.39)	0.084
CML	13.76 (0.00-23.31)	9.25 (0.00-18.70)	13.06 (0.00-45.76)	14.47 (0.00-21.66)	0.508
Pentosidine	45.61 (30.29-59.85)	49.17 (30.28-58.88)	39.63 (30.50-49.28)	53.67 (39.81-66.58)	0.111
RAGE	510.02 (397.75-672.20)	423.9 (372.3-635.8)	314.40 (249.45-500.79)[§]	295.8 (216.5-412.2)[§‡]	**0.001***
Sputum	*n=0*	*n=10*	*n=2*	*n=0*	
CEL	-	13.75 (nvt)	13.75 (nvt)	-	0.086
CML	-	18.61 (nvt)	18.6 (nvt)	-	0.83
Pentosidine	-	0.00 (0.00-0.00)	0.00 (0.00-0.00)	-	1,000
RAGE	-	110.9 (77.4-176.3)	74.3 (nvt)	-	0.667
Bronchial biopsies	*n=0*	*n=10*	*n=2*	*n=0*	
AGEs, positivity (%)	-	23.6 (15.9-31.7)	26.7 (25.1-26.7)	-	-
RAGE, positivity (%)	-	7.3 (3.3-11.0)	12.4 (5.7-12.4)	-	-
Skin	*n=32*	*n=25*	*n=24*	*n=15*	
AGE-reader	2.42 (2.20-2.86)	2.47 (2.03-2.95)	2.71 (2.20-3.14)	2.51 (2.17-2.89)	0.612

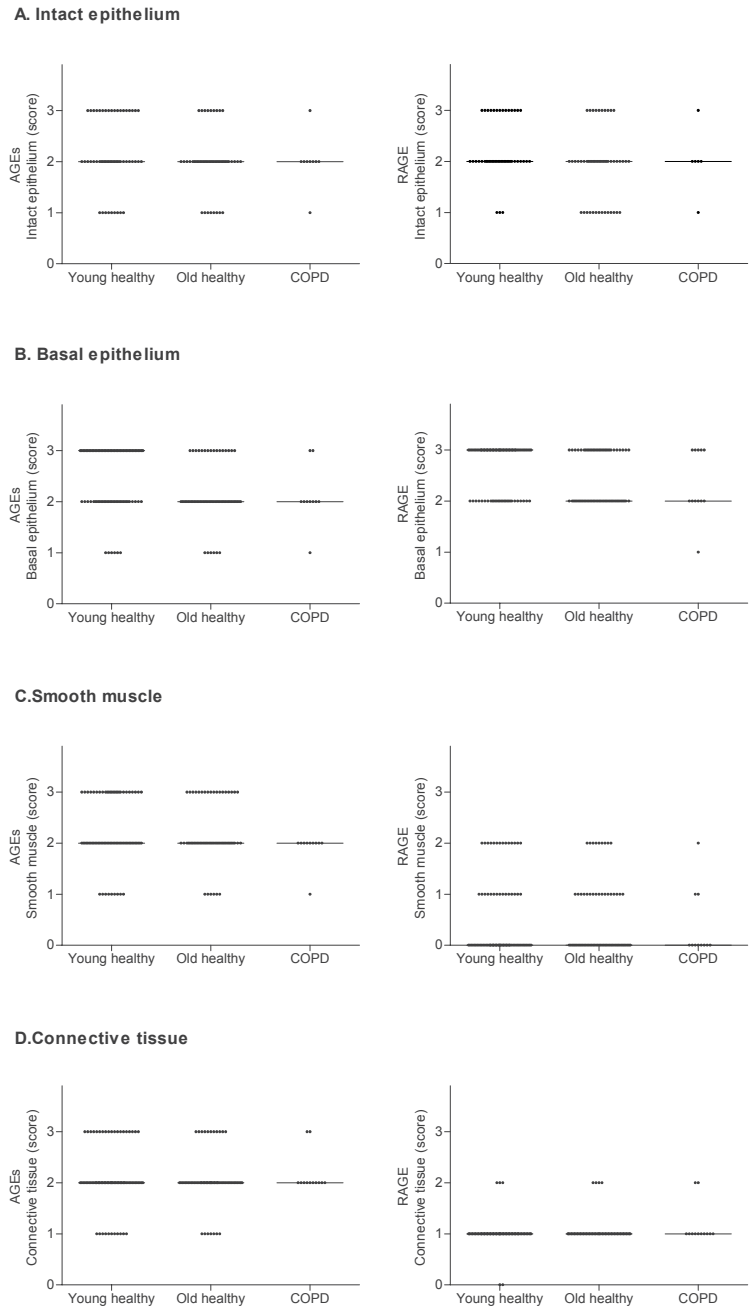
CEL= N^{ε}-(carboxyethyl)lysine, CML= N^{ε}-(carboxymethyl)lysine, RAGE= receptor for advanced glycation endproducts, AGEs= advanced glycation endproducts, SAF= skin autofluorescence. Values are expressed as median (IQR).

Table 2. Correlations of AGEs and RAGE between different compartments

		Plasma						Sputum								Bronchial biopsies						Skin	
		CML		Pentosidine		RAGE		CEL		CML		Pentosidine		RAGE		AGEs positivity		RAGE positivity		SAF	
		Rho	*p*	*Rho*	*p*	*Rho*	*p*	*Rho*	*p*	*Rho*	*p*	*Rho*	*p*	*Rho*	*p*	*Rho*	*p*	*Rho*	*p*	*Rho*	*p*
Plasma	CEL	**0.12**	**0.05**	**-0.12**	**0.06**	0.10	0.12	**0.28**	**<0.01**	0.07	0.38	0.09	0.25	-0.08	0.30	0.09	0.28	**0.16**	**0.05**	**-0.29**	**<0.01**
	CML	---	---	0.05	0.41	**-0.22**	**<0.01**	**0.16**	**0.03**	0.01	0.92	-0.01	0.90	-0.01	0.94	**0.21**	**0.01**	-0.00	0.99	0.04	0.55
	Pentosidine	---	---	---	---	**-0.16**	**0.01**	0.05	0.54	0.00	0.99	-0.03	0.67	-0.06	0.45	0.13	0.10	-0.05	0.52	**0.12**	**0.05**
	RAGE	---	---	---	---	---	---	**-0.15**	**0.06**	0.00	0.97	0.01	0.94	0.06	0.46	**-0.17**	**0.03**	-0.09	0.28	**-0.46**	**<0.01**
Sputum	CEL	---	---	---	---	---	---	---	---	**0.26**	**<0.01**	0.10	0.18	0.04	0.56	**0.20**	**0.02**	0.12	0.15	**-0.16**	**0.03**
	CML	---	---	---	---	---	---	---	---	---	---	0.02	0.83	**-0.29**	**<0.01**	-0.07	0.38	0.07	0.38	0.11	0.15
	Pentosidine	---	---	---	---	---	---	---	---	---	---	---	---	**-0.19**	**0.01**	-0.08	0.56	-0.10	0.26	-0.10	0.18
	RAGE	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-0.09	0.79	-0.03	0.72	0.10	0.18
Bronchial biopsies	AGEs positivity	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	**0.24**	**<0.01**	0.06	0.44
	RAGE positivity	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	---	-0.12	0.14

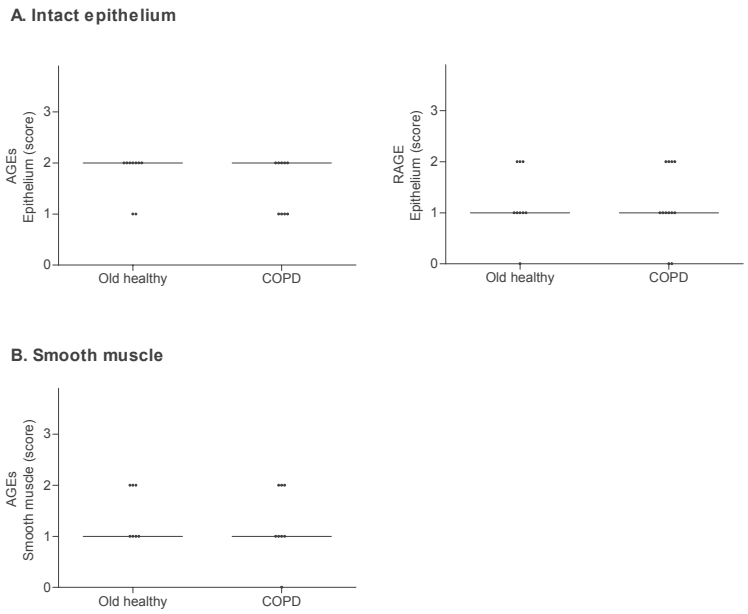
Spearman's rank correlations, *Rho*=correlation coefficient. CEL= N^{ε}-(carboxyethyl)lysine, CML= N^{ε}-(carboxymethyl)lysine, RAGE= receptor for advanced glycation endproducts, AGEs= advanced glycation endproducts, SAF= skin autofluorescence.

Figure 1. Quantitative analyses of AGEs and RAGE expression in bronchial biopsies



Quantitative analyses of AGEs (left panel) and RAGE (right panel) expression in A) intact epithelium, B) basal epithelium, C) smooth muscle, D) connective tissue of bronchial biopsies. Intensity of staining was scored by a 4-points scale: 0=negative staining, 1=weak positive, 2=positive, and 3=strong positive. Horizontal bars represent median values.

Figure 2. Quantitative analyses of AGEs and RAGE expression in peripheral airways



Quantitative analyses of AGEs (left panel) and RAGE (right panel) expression in A) epithelium and B) smooth muscle of the peripheral airways. Intensity of staining was scored by a 4-points scale: 0=negative staining, 1=weak positive, 2=positive, and 3=strong positive. Horizontal bars represent median values.

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