Online supplement

Smoking, telomere length and lung function decline: a longitudinal population-based study

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Conflicts of interest

All authors have indicated that they have no financial conflict of interest.

Authors contributions

P.A.: Study design, interpretation of data, and manuscript writing.

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D.C.: Study design, interpretation of data, statistical analysis and manuscript writing.

B.L.: Study design, interpretation of data, and manuscript writing and coordination of the ECRHS study and data collection in French centres.

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All authors participated in the interpretation of the findings, reviewed the manuscript critically, and approved the final version.

Methods – *Study population and measurements*

The European Community Respiratory Health Survey (ECRHS) assessed lung function and lung function decline in adults from the general population. In addition to the core protocol, DNA from participants in ECRHS II in the centres of Paris, Grenoble and Montpellier was assayed to assess relative telomere length. The analysis was based on the follow-up data from these 3 French centres. The methods for ECRHS-France and spirometry in ECRHS have been described elsewhere (1, 2).

Briefly, between 1991 and 1993, each participating centre randomly selected 1500 men and 1500 women, representative of the age group 20-44 years, to answer a short postal screening questionnaire (ECRHS I). A random sample of respondents was invited to visit a local testing centre, to answer a more detailed administered questionnaire, provide a blood sample and undergo lung function assessment. Participants who completed the extended questionnaire were eligible for the follow-up surveys in 2000-2002 (ECRHS-II) and 2011-2013 (ECRHS-III), during which they answered extended questionnaires, provided a blood sample for IgE measurement and DNA extraction and again performed lung function assessment. Forced expiratory volume in 1 s (FEV1), and forced vital capacity (FVC) were measured with a water-sealed bell spirometer (Biomedin srl, Padova, Italy) in ECRHS II and with the portable ultrasonic EasyOne spirometer (ndd Medizintechnik, Switzerland) in ECRHS III. The maximum FEV1 and FVC were calculated from up to five technically acceptable blows in accordance with the American Thoracic Society criteria for reproducibility. Post-bronchodilator (BD) spirometry 15 minutes after administration of 200µg Salbutamol was performed only at ECRHS III. Total serum IgE and IgE specific to house dust mite, timothy grass, cat, and Cladosporium were measured centrally at Kings College London using the Pharmacia CAP system (Uppsala, Sweden).

Relative telomere length in peripheral leukocytes in participants in ECRHS II was measured by quantitative PCR using QuantStudio^{TM} 6 Flex Real-Time PCR System (Applied Biosystems, Foster, CA), as described previously (3). Briefly, the telomere repeat copy number to single-gene copy number (T/S) ratio was determined using the comparative Ct method ($T/S = 2^{-\Delta\Delta Ct}$), using 36B4 gene for normalization (acidic ribosomal phosphoprotein PO, a single-copy gene). Genomic DNAs from the 3 study centres were extracted centrally in Bichat Medical School from leucocytes in peripheral venous blood samples using the QIAamp blood kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol, and quantified with a Nano Drop ND-1000 Spectrometer (Thermo Fischer Scientific, Wilmington, Delaware). Each sample was run in triplicate, using the SYBR green method (Invitrogen, Cergy-Pontoise, France). Triplicates merged into a final mean value that exhibited a standard deviation of the three Ct values smaller than 0.1.

The study was approved by the appropriate ethics committee, and informed written consent was obtained from every participant.

All individuals who had performed spirometry and with telomere length assessed in ECRHS II (referred to as 'baseline') were eligible for this analysis.

Methods – *Definition of outcomes and covariates and statistical analysis*

Individuals with specific IgE values ≥0.35 kIU/L were considered as atopic. Current asthma was defined as a positive answer to the questions "Have you had an attack of asthma in the past 12 months?" or "Are you currently taking any medicines, including inhalers, aerosols, or tablets for asthma?". Smokers were categorized as "ex-smoker" if they had already stopped smoking at the previous survey (sustained quitters), whereas more recent quitters and current smokers were gathered as "smokers" and dichotomized according the cumulative number of pack years they had smoked at the time of the survey (ie cumulative pack-years smoked at baseline for the cross-sectional analysis at baseline, and cumulative pack-years smoked at the end of follow-up for the analysis of lung function at follow-up and lung function decline)l . For the sensitivity analysis taking into the tobacco consumption over the follow-up rather than the cumulative tobacco history at the end of follow-up, only current smokers at the end of follow-up were considered as smokers, and subjects who quitted smoking between baseline and follow-up (N=45) were excluded from the analysis.

Predicted FEV1 and FVC values were calculated using the Global Lungs Initiative (GLI) reference equations (4). Airflow obstruction was defined either according the Global Initiative for Chronic Obstructive Lung Disease (GOLD) fixed cut-off (FEV1/FVC<0.70) or as FEV1/FVC<lower limit of normal (LLN) according to the GLI.

Absolute change in FEV1 (FEV1 decline) was expressed per year of follow-up, with ECRHS II considered as "baseline" (pre-bronchodilator ECRHS III value minus ECRHS II value - ie, a negative value represents decline). To account for possible bias related to change in spirometer between the surveys, the association between telomere length and lung function decline was assessed with (i) uncorrected FEV1 at follow-up, and in sensitivity analyses (ii) with FEV1 at follow-up corrected assuming a fixed additive correction term (obtained from Gerbase et al. in a quasi-experimental comparison of spirometers), and (iii) with FEV1 corrected using a correction term obtained from internally-derived spirometer-specific reference equations, integrating sex, age, height and spirometer type, as proposed by Bridevaux et al. (5). In another sensitivity analysis, change in FEV1 was expressed as change in FEV1%predicted, reconstructed for a period of 10 years: (FEV1%predicted (from Global Lungs Initiative (GLI) reference equations at ECRHS III minus FEV1%predicted at ECRHS II) divided by length of follow-up * 10 years) (4).

The $\chi 2$ test for qualitative variables or analysis of variance for continuous variables were used to analyse differences between groups. In addition, multiple linear regression analyses or logistic regressions were used, with lung function or airway obstruction as outcome and smoking group,

centre, gender, age, body mass index (BMI) and physical activity as covariates. We tested for possible interactions between smoking and age/gender/BMI/physical activity, but there was no suggestion for any modifying effect of these variables on the association between smoking and lung function decline. Tertile of relative telomere length at baseline (ECRHS II) (with the first tertile consisting of individuals with the shortest telomere lengths) was considered either as a covariate or to define strata for the stratified analyses. All analyses were performed on SAS 9.3 release (SAS Institute Inc.).

Results - ***Descriptive data of the study population***

Of the 584 participants with complete data for lung function and satisfying reproducibility criterion, relative telomere length at baseline, 448 (76.7%) participated in ECRHS-III and had lung function measured. The mean follow-up time was 11.1 years (SD 0.7; range 9.2-13.1). A comparison of baseline characteristics between participants and non-participants of the follow-up no significantly difference for smoking, age, sex ratio, body-mass index, physical activity, FEV1%predicted and telomere length. The baseline characteristics of the participants with follow-up data are shown in Table E1. At baseline, the participants (48.4% male) were aged 30 to 57 years (mean 45.7, standard deviation (SD) 6.8), 37.0% were current smokers, 7.6% had asthma (Table E1); 4.0% had airflow obstruction (pre-BD FEV1/FVC< LLN), and the mean FEV1%predicted was 99.1%% (SD 13.4) (Table E2).

The mean annual decline in FEV1 was 43.6 ml/year (SD27.6) in the total sample. It was 38.5 ml/year (SD27.5) after applying the fixed correction derived from the study by Gerbase et al. to take into account the change in spirometer between baseline and follow-up, and 30.4 ml/year (SD27.4) after applying the spirometer-specific individualized correction as proposed by Bridevaux et al. (5, 6).

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Online Table E1. Characteristics of the study population, by tertiles of telomere length

		Tertiles of telomere length at baseline				
		All	**Tertile1 (shortest)**	**Tertile2**	**Tertile3 (longest)**	
		n=448	**n=148**	**n=147**	**n=153**	
Characteristics at baseline*	n	% or mean±sd	% or mean±sd	% or mean±sd	% or mean±sd	p for trend†
Centre						0.22
Grenoble	213	47.5	44.6	53.1	45.1	
Montpellier	66	14.7	16.9	15.6	11.8	
Paris	169	37.7	38.5	31.3	43.1	
Sex, male	217	48.4	48.7	47.6	49.0	0.95
Age, years	448	45.7±6.8	47.7±6.1	45.3±7.1	44.0±6.7	<0.0001
Smoking status						0.04
Never smokers	169	38.6	33.8	45.1	37.1	
Ex-smokers	107	24.4	31.7	16.9	24.5	
Smokers ≤ 15 Pack-yrs	81	18.5	14.5	17.6	23.2	
Smokers > 15 Pack-yrs	81	18.5	20.0	20.4	15.2	
Cumulative Pack-Years						
In total sample, Pack-yrs	438	10.5±15.	11.2±14.6	9.9±13.9	10.3±16.7	p=0.63
In Ex+Smokers, Pack-yrs	269	17.1±16.	16.9±15.0	18.1±14.3	16.4±18.5	p=0.82
In smokers> 15 PY, Pack-yrs	81	30.0±15. 0	30.0±11.9	31.0±12.9	28.7±20.5	p=0.77
BMI, kg/m2	448	23.9±3.5	23.6±3.4	24.4±3.6	23.8±3.6	0.64
<25 kg/m^2	306	68.3	75.0	61.2	68.6	0.09
[25-30[kg/m^2	116	25.9	22.3	30.6	24.8	
≥30 kg/m^2	26	5.8	2.7	8.2	6.5	
Physical activity						
None	154	34.5	27.7	42.5	33.5	0.29
1/2 hour per week	61	13.7	15.5	11.6	13.8	
1 hour per week	86	19.3	21.6	17.1	19.1	
>1 hour per week	145	32.5	35.1	28.8	33.6	
Respiratory symptoms in last 12						
Wheeze	83	18.5	17.6	14.3	23.5	0.18
Night chest tightness	79	17.6	19.6	17.6	15.7	0.37
Attack of shortness of breath	32	7.2	6.8	6.1	8.5	0.54
Woken by shortness of breath	30	6.7	10.1	2.7	7.2	0.32
Chronic cough or phlegm	30	6.7	5.4	7.5	7.2	0.54
Asthma ever in life	70	15.6	18.9	12.2	15.7	0.45
Current Asthma	34	7.6	9.5	5.4	8.0	0.63
Self-reported comorbidities						
No comorbidity	250	56.2	54.4	53.7	60.3	0.31
Depression	25	5.6	8.9	2.7	5.3	0.18
Diabetes	6	1.4	1.4	1.4	1.3	0.98
Migraine/recurrent headaches	83	18.8	21.1	18.1	17.3	0.41
Cancer	4	0.9	1.4	0	1.3	0.99
Cardiovascular diseases	46	10.4	12.9	11.0	7.4	0.12
Atopy	132	30.1	27.6	33.6	29.3	0.75

Table E1 (continued)

Characteristics at follow-up*	n	% or mean±sd	% or mean±sd	% or mean±sd	% or mean±sd	p for trend†
Mean Age, years	448	56.6±6.8	58.6±6.0	56.3±7.1	55.0±6.7	<0.0001
Change in pack-years between baseline and follow-up (ECRHS III)	439	1.6±3.8	1.5±3.6	1.6±3.6	1.5±4.2	0.96
Cumulative total pack-years at						
- **In all participants at ECRHS III**	439	12.3±17.	13.2±16.8	11.4±16.1	12.2±19.1	0.63
- **In Ex+Smokers at ECRHS III**	275	19.6±18.	19.3±17.2	20.2±16.8	19.4±21.0	0.97
- **In Smokers>15PY at ECRHS III**	76	36.1±19.	36.9±16.0	35.8±15.6	36.0±26.4	0.87
Change in BMI between baseline and follow-up (ECRHS III)	447	1.7±2.2	1.8±2.0	1.8±2.4	1.5±2.1	0.25

***** Characteristics of the sample of participants with telomere length assessed at baseline and lung function data available at baseline and follow-up. Smoking status at baseline defined according to current status and cumulative pack years of smoking at ECRHS II. Smoking status at follow-up defined according to current status and cumulative pack years of smoking at ECRHS III. Predicted value for FEV1 and FVC and lower limit of normal (LLN) obtained from GLI reference equations. † p values are for test of trend across the tertiles, apart for centre, smoking, BMI, and physical activity, for which p values are for chi-square test.

Online Table E2. Lung function and airflow obstruction at baseline and follow-up, and lung function decline by tertiles of telomere length at baseline*

		Tertile of telomere length at baseline			
		All	**Tertile1 (shortest)**	**Tertile2**	**Tertile3 (longest)**
		n=448	n=148	n=147	n=153
		% or mean±sd	% or mean±sd	% or mean±sd	% or mean±sd	p for trend
Lung function at baseline (ECRHS II)						
FEV1%predicted	447	99.1±13.4	98.7±13.5	98.4±13.2	100.0±13.6	0.41
FVC%predicted	444	97.0±13.3	97.3±14.0	96.6±13.3	97.1±12.7	0.90
pre bronchodilator FEV_1/FVC	18	4.0	5.4	3.4	3.3	0.36
Lung function at follow-up (ECRHS III)						
FEV_1%predicted	448	94.0±14.9	93.4±15.3	93.8±15.2	94.8±14.2	0.42
FVC%predicted	426	97.6±13.7	97.4±13.5	97.4±14.8	97.9±12.9	0.75
pre bronchodilator FEV_1/ FVC	35	8.2	9.9	9.3	5.6	0.18
post bronchodilator FEV_1/FVC	17	4.2	5.2	3.8	3.7	0.55
FEV1 decline						
FEV1_FEV_1 decline, uncorrected,	448	-43.6±27.6	-44.3±26.4	-42.3±30.1	-44.1±25.5	0.94
FEV1 FEV_1 decline, Gerbase,	448	-38.5±27.5	-39.3±26.3	-37.3±30.8	-39.1±25.5	0.95
FEV1_FEV_1 decline, Bridevaux,	448	-30.4±27.4	-	-29.1±30.6	-31.0±25.4	0.97
FEV1% predicted decline, 10yrs	447	-4.63±8.1	-	-4.30±8.72	-4.70±7.29	0.83

* FEV1 decline was defined (i) using pre-BD FEV1 at follow-up uncorrected for change in spirometer between the surveys, (ii) or using FEV1 at follow-up corrected assuming a fixed additive correction term (1) (iii) or using a correction term obtained from internally-derived spirometer-specific reference equations (2) (iv) or expressed as change in FEV1%predicted, reconstructed for a period of 10 years. Predicted value for FEV1 and FVC and lower limit of normal (LLN) were obtained from GLI reference equations. See method details in the online supplement.

Online Table E3. Difference in annual FEV1 decline according to smoking and telomere length, obtained from the model including an interaction between smoking and telomere length.

	Tertile of telomere length at baseline		
	Tertile1 (shortest)	**Tertile2**	**Tertile3 (longest)**
Annual FEV_1 decline (ml/year)			
Estimate for the effect of telomere length in never smokers	4.9 (-5.0 to 14.9)	3.2 (-5.9 to 12.4)	(ref)
	Estimate for interaction showing the modified effect of smoking in subjects with short or medium telomere length*		Estimate for the effect of smoking in subjects with long telomeres
Ex-smokers	-2.5 (-16.1 to 11.2)	3.0 (-10.9 to 16.9)	-6.4 (-16.1 to 3.3)
Smokers ≤ 15 Pack-yrs	**-21.8 (-40.6 to -3.0)** †	-11.0 (-31.8 to 9.7)	5.9 (-7.0 to 18.8)
Smokers > 15 Pack-yrs	**-21.3 (-38.8 to -3.7)** †	-16.5 (-33.2 to 0.2) §	1.6 (-10.7 to 14.0)

Data are regression coefficients (95%CI). All models adjusted for age, gender, centre, BMI and physical activity. Smoking status is defined according to current status and cumulative pack-years smoked at the end of follow-up. *In this model, the difference in FEV1 decline is modelised as the sum of the estimate obtained for smoking, the estimate obtained for tertile of telomere length and the estimate of the interaction. For example, compared to non-smokers with long telomeres (reference), heavy smokers with short telomeres will have an additional loss of (1.6 +4.9 -21.3 =) -14.8 ml/yrs.
† $p<0.05$; § $p=0.06$;

Online Table E4. Difference in FEV1%predicted at follow-up in smokers as compared to never-smokers, stratified by tertiles of telomere length

		Tertile of telomere length at baseline		
		Tertile1 (shortest)	**Tertile2**	**Tertile3 (longest)**
FEV_1 %predicted at follow-up				
Never smokers	n=164	(ref)	(ref)	(ref)
Ex-smokers	n=151	-3.4 (-9.3 to 2.4)	-2.9 (-9.3 to 3.4)	1.3 (-4.2 to 6.8)
Smokers ≤ 15 Pack-yrs	n=49	-6.1 (-14.1 to 1.9)	-6.3 (-16.1 to 3.5)	1.8 (-5.4 to 9.0)
Smokers > 15 Pack-yrs	n=76	-14.9 (-22.3 to -7.4) ‡	-10.4 (-17.1 to -3.6) ‡	2.3 (-4.7 to 9.3)
p for trend		*0.0001*	*0.003*	*0.51*

Data are regression coefficient (95%CI). All models adjusted for age, gender, centre, BMI and physical activity. Smoking status is defined according to current status and cumulative pack-years smoked at the end of follow-up.
† $p<0.05$ ‡ $p<0.01$ § $p<0.10$, as compared to never –smokers.
Interaction Tertile telomere*smoking: p=0.07