

Gene promoter methylation is associated with lung function in the elderly

The normative aging study

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Abbreviations: %5mC, percentage of 5-methylcytosine; CI, confidence interval; COPD, chronic obstructive pulmonary disease; CRAT, carnitine O-acetyltransferase; F3, coagulation factor-3, tissue factor; FEV₁, forced expiratory volume in one second; FVC, forced vital capacity; GCR, glucocorticoid receptor; CAM, intercellular adhesion molecule; IFN γ , interferongamma; IL6, interleukin-6; iNOS, inducible nitric oxide synthase; IQR, interquartile range; MMEF, maximum midexpiratory flow; mRNA, messenger RNA; NAS, Normative Aging Study; NF κ B, nuclear factor-kappa-light-chain-enhancer of activated B cells; NO, nitric oxide; OGG1, 8-oxoguanine DNA glycosylase 1; RNS, reactive nitrogen species; ROS, reactive oxygen species; SD, standard deviation; TFBS, transcription factor binding sites; TLR, toll-like receptor; UCSC, University of California Santa Cruz

Lung function is a strong predictor of mortality. While inflammatory markers have been associated with lung function decline, pathways are still poorly understood and epigenetic changes may participate in lung function decline mechanisms. We studied the cross-sectional association between DNA methylation in nine inflammatory genes and lung function in a cohort of 756 elderly men living in the metropolitan area of Boston. Participants donated a blood sample for DNA methylation analysis and underwent spirometry at each visit every 3 to 5 y from 1999–2006. We used separate multivariate mixed effects regression models to study the association between each lung function measurement and DNA methylation within each gene. Decreased CRAT, F3 and TLR2 methylation was significantly associated with lower lung function. One interquartile range (IQR) decrease in DNA methylation was associated with lower forced vital capacity (FVC) and forced expiratory volume in one second (FEV₁), respectively by 2.94% ($p < 10^{-4}$) and 2.47% ($p < 10^{-3}$) for F3 and by 2.10% ($p < 10^{-2}$) and 2.42% ($p < 10^{-3}$) for TLR2. Decreased IFN γ and IL6 methylation was significantly associated with better lung function. One IQR decrease in DNA methylation was associated with higher FEV₁ by 1.75% ($p = 0.02$) and 1.67% ($p = 0.05$) for IFN γ and IL6, respectively. These data demonstrate that DNA methylation may be part of the biological processes underlying the lung function decline and that IFN γ and IL6 may have ambivalent roles through activation of negative feedback.

Introduction

Epigenetics describes both modifiable and stable changes in gene expression control that do not depend on the underlying nuclear sequence.¹ The best understood of the epigenetic mechanisms is DNA methylation, which involves the addition of methyl groups to cytosine to form 5-methylcytosine. Low methylation in regulatory sequences, such as regions dense in CpG dinucleotides named CpG islands and in neighboring sequences named CpG island shores, has been shown to be associated with active genes or with genes that are poised to

be activated.²⁻⁴ Conversely, hypermethylation usually results in lower gene expression. DNA methylation is known to change through aging⁵ and has been associated with age-related diseases including cancer,^{6,7} atherosclerosis⁸ and cardiovascular diseases.^{9,10}

Lung function is tightly related to aging, and starts to decline in the third decade of life,¹¹ but with different rates of decline across individuals.¹² Lung function is one of the strongest predictors of cardiorespiratory and cardiovascular health¹³ and

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mortality,¹³⁻¹⁵ though the causal pathways are poorly understood.^{13,16} Faster lung function decline has been associated with increased risks of hospitalizations related to chronic obstructive pulmonary disease (COPD),¹⁷ which is a leading cause of mortality in all countries.¹⁸ By the year 2020, the prevalence of COPD is expected to be the third leading cause of worldwide mortality and the fifth leading cause of morbidity.¹⁹

Persons with impaired lung function have been found to have higher levels of inflammatory markers such as fibrinogen,²⁰ C-reactive protein²¹ and IL6,²² as well as oxidative stress markers.²³ Although inflammation is considered central to the pathogenesis of airways diseases, the mechanisms responsible for accelerated lung function decline remain unclear. DNA methylation can be meta-stable, and can be propagated through cell division. This can represent a form of cellular memory that determines the levels of inflammation and oxidative stress. However, whether changes in DNA methylation are associated with lower lung function or accelerated lung function decline has not been investigated.

We leveraged the prospective collection of data and biospecimens in the Normative Aging Study (NAS), in which participants' lung function was regularly monitored over a 7 year span, to examine the relationships between DNA methylation in nine genes related to inflammation and oxidative metabolism, and lung function. We hypothesized that decreased methylation of genes related to inflammation and oxidative stress would be associated with lower lung function. Given that age is a major risk-factor for decreased lung function²⁴ and for changes in DNA methylation,⁵ we further hypothesized that age could modify the relationship between DNA methylation and lung function.

Results

Participants were 73.3 ± 6.7 y-old on average. From the 756 participants, 368 (49%) had one visit, 290 (38%) had two visits, 97 (13%) had three visits and 1 had four visits (0.13%). Most of the participants were former smokers (67.2%) or never smokers (28.6%). Mean levels \pm SD of forced vital capacity (FVC), forced expiratory volume in one second (FEV_1), FEV_1/FVC and maximum mid-expiratory flow (MMEF) are shown in **Table 1**. From the 388 participants with more than one visit, the average time between the first and last visit was 4 y and 7 mo and the average change in lung function during this time was 0.007 L for FEV_1 , 0.08 L for FVC, -1.5 for FEV_1/FVC and -33.1 L/min for MMEF. The distribution of blood DNA methylation for each of the nine genes (Carnitine O-acetyltransferase (CRAT), coagulation factor-3 (F3), glucocorticoid receptor (GCR), intercellular adhesion molecule (ICAM), interferon-gamma ($IFN\gamma$), interleukin-6 (IL6), inducible nitric oxide synthase (iNOS), 8-oxoguanine DNA glycosylase 1 (OGG1) and toll-like receptor-2 (TLR2)), expressed as the percentage of 5-methylcytosine (%5mC), is described in **Table 2** and in **Table S1** in the online **Supplement**. In general, the correlations between the individual positions within each gene were low (<0.46), except between position 1 and 2 within CRAT, ICAM, $IFN\gamma$ and IL6 where the correlation was higher than 0.7 (**Table S2**).

Association between white blood cells type and DNA methylation. DNA methylation may vary by white cell type, and hence variations from sample to sample in e.g., the fraction of cells that are lymphocytes may create differences in methylation levels (measured in mixed cells) that do not reflect differences in underlying levels of methylation in the participants. Upon examination, all genes except CRAT and ICAM exhibited associations with the proportion of one or more cell types in the blood count (**Table 3**). GCR, IL6 and TLR2 were negatively associated with percent neutrophils and positively associated with percent lymphocytes. Conversely, $IFN\gamma$ and iNOS methylation were positively associated with percent neutrophils and monocytes (only iNOS), and negatively associated with percent lymphocytes. F3 methylation was positively associated with percent lymphocytes and negatively with percent monocytes. OGG1 methylation was positively associated with percent basophils. Results for the individual positions within each gene were similar (**Table S3**). However, further associations with individual positions were observed, including negative association of F3 and ICAM methylation with percent eosinophils, and of $IFN\gamma$ and OGG1 with percent monocytes. Hence, for analyses with lung function, methylation levels were standardized for cell type.

Cross-sectional association between DNA methylation and lung function. Because lung function decrease with age was very slow and most participants had only 1 or 2 measurements in the study period, we were only able to examine the cross-sectional association of methylation with lung function. Because persons with a second visit may have represented a healthier subset of the overall population, we used inverse probability weighting to adjust for the potential of survival bias. These analyses excluded persons with chronic lung disease. Decreased DNA methylation in the mean of all positions for CRAT, F3, iNOS, OGG1 and TLR2 was associated with lower lung function (**Table 4**). An IQR %5mC decrease in the mean of all positions tested for F3 and TLR2 was associated with 2.94% lower FVC ($p < 10^{-4}$) and 2.47% lower FEV_1 ($p < 10^{-3}$), and with 2.10% lower FVC ($p < 10^{-2}$) and 2.42% lower FEV_1 ($p < 10^{-3}$), respectively. Decreased DNA methylation of TLR2 was also associated with 2.86% lower MMEF ($p = 0.02$). Similarly for CRAT and OGG1, decreased DNA methylation was significantly associated with lower FEV_1 and FEV_1/FVC and with lower FVC, respectively. Associations between iNOS and FVC and FEV_1 were borderline significant ($p = 0.07$). For ICAM, $IFN\gamma$ and IL6, associations with lung function were in the opposite direction: decreased DNA methylation was associated with better lung function. Especially, an IQR %5mC decrease in the mean of all positions tested for $IFN\gamma$ and IL6 was associated with 1.75% and 1.67% higher FEV_1 ($p < 0.05$), respectively. Decreased DNA methylation of $IFN\gamma$ was also associated with 3.72% higher MMEF ($p < 10^{-2}$). Associations between ICAM and FEV_1/FVC and MMEF were borderline significant ($p = 0.07$). We did not observe any significant association between DNA methylation of GCR and lung function.

Similar associations were found in the secondary analyses looking at individual positions within each gene (**Table S4**), but only some of the positions showed significant associations:

Table 1. Lung function and characteristics of the 756 men participating to the Normative Aging Study, 1999–2006, Boston

	All visits (n = 1243)	1st visit (n = 756)	2nd visit (n = 388)	3rd visit (n = 98)
Outcomes				
FVC ± SD, liters	3.3 ± 0.7	3.3 ± 0.7	3.4 ± 0.7	3.5 ± 0.7
FEV ₁ ± SD, liters 1 st sec	2.5 ± 0.6	2.5 ± 0.6	2.5 ± 0.6	2.6 ± 0.6
FEV ₁ /FVC ± SD	74.9 ± 8.0	75.4 ± 8.0	74.0 ± 8.3	75.1 ± 6.6
MMEF ± SD, liters min	239.7 ± 107.6	246.9 ± 112.2	228.7 ± 101.7	228.6 ± 88.1
Adjustment factors				
Age ± SD (p25, p75), yr	73.3 ± 6.7 (68, 78)	72.2 ± 6.8	74.8 ± 6.4	75.9 ± 5.4
Race, n (%)				
Black	23 (1.9)	14 (1.8)	6 (1.6)	3 (3.1)
White	1207 (97.1)	734 (97.1)	378 (97.4)	94 (95.9)
missing	13 (1.0)	8 (1.1)	4 (1.0)	1 (1.0)
BMI ± SD, kg/m ²	28.0 ± 4.1	28.2 ± 4.1	27.7 ± 4.1	27.4 ± 3.7
Height ± SD, cm	173.3 ± 7.2	173.5 ± 6.9	173.3 ± 7.5	172.4 ± 7.9
Weight ± SD, kg	84.3 ± 14.2	85.1 ± 14.2	83.5 ± 14.3	81.7 ± 19.7
Education, n (%), y				
<12	46 (3.7)	30 (4.0)	13 (3.4)	3 (3.1)
12	304 (24.5)	183 (24.2)	96 (24.7)	25 (25.5)
13–15	361 (29.0)	214 (28.3)	112 (28.9)	34 (34.7)
>15	525 (42.2)	322 (42.6)	167 (43.0)	36 (36.7)
missing	7 (0.6)	7 (0.9)	0 (0.0)	0 (0.0)
Smoking status, n (%)				
Never	366 (29.4)	216 (28.6)	115 (29.6)	35 (35.7)
Current	50 (4.0)	32 (4.2)	16 (4.1)	2 (2.0)
Former	827 (66.5)	508 (67.2)	257 (66.2)	61 (62.2)
Packs years* ± SD	20.5 ± 25.7	21.7 ± 26.9	19.8 ± 24.6	14.4 ± 19.7
Season, n (%)				
Spring (March–May)	290 (23.3)	181 (23.9)	95 (24.5)	14 (14.3)
Summer (June–Aug)	351 (28.2)	213 (28.2)	105 (27.1)	32 (32.7)
Fall (Sept–Nov)	407 (32.7)	234 (30.9)	134 (34.5)	39 (39.8)
Winter (Dec–Feb)	195 (15.7)	128 (16.9)	54 (13.9)	13 (13.2)
Day of the week, n (%)				
Tuesday	71 (5.7)	71 (9.4)	0 (0.0)	0 (0.0)
Wednesday	333 (26.8)	167 (22.1)	120 (30.9)	0 (0.0)
Thursday	617 (49.6)	329 (43.5)	235 (60.6)	45 (45.9)
Friday	222 (17.9)	189 (25.0)	33 (8.5)	53 (54.1)
Blood count				
Neutrophils ± SD, %	62.2 ± 8.6	62.0 ± 8.6	62.6 ± 8.6	61.6 ± 8.0
Lymphocytes ± SD, %	25.5 ± 7.9	25.6 ± 7.8	25.0 ± 7.9	26.4 ± 7.7
Asthma, n (%)	83 (6.7)	45 (6.0)	31 (8.0)	7 (7.1)
Chronic bronchitis, n (%)	81 (6.5)	52 (6.9)	24 (6.2)	5 (5.1)
Emphysema, n (%)	42 (3.4)	29 (3.8)	11 (2.8)	2 (2.0)
Positive methacholine challenge test, n (%)	115 (10.9)	73 (11.5)	36 (10.9)	6 (6.7)
Missing	184 (14.8)	119 (15.7)	57 (14.7)	8 (8.2)
Corticosteroids, n (%)	90 (7.2)	50 (6.6)	33 (8.5)	7 (7.1)
Sympathomimetic α and β, n (%)	95 (7.6)	54 (7.1)	37 (9.5)	4 (4.1)
Anticholinergic, n (%)	30 (2.4)	14 (1.9)	14 (3.6)	2 (2.0)

*Among current or former smokers.

Table 2. Descriptive statistics of DNA methylation (percentage of 5-methylcytosine) by visit for 756 men participating to the Normative Aging Study, Boston, 1999–2006

Gene	All visits (n = 1243)			1st visit (n = 756)	2nd visit (n = 388)	3rd visit (n = 98)
	n	mean ± SD (p25–p75)	IQR	mean ± SD	mean ± SD	mean ± SD
CRAT	1150	3.2 ± 1.1 (2.4–3.9)	1.5	3.1 ± 1.0	3.3 ± 1.2	3.8 ± 0.9
F3	1093	2.4 ± 1.2 (1.6–3.0)	1.4	2.2 ± 1.2	2.6 ± 1.2	3.2 ± 1.0
GCR	1034	47.2 ± 5.8 (44.0–50.2)	6.2	47.0 ± 5.7	47.4 ± 6.4	48.1 ± 3.2
ICAM	926	4.3 ± 1.9 (3.1–5.2)	2.1	4.4 ± 1.9	4.3 ± 2.0	4.2 ± 1.0
IFN γ	1196	84.7 ± 5.3 (82.0–88.2)	6.2	84.4 ± 5.5	85.1 ± 5.0	85.2 ± 4.2
IL6	1202	43.4 ± 10.4 (37.1–49.3)	12.2	43.7 ± 10.7	42.7 ± 10.1	43.1 ± 9.4
iNOS	792	69.7 ± 6.4 (65.9–74.1)	8.2	70.1 ± 6.6	68.6 ± 6.1	71.4 ± 5.3
OGG1	745	2.3 ± 1.2 (1.4–2.9)	1.5	2.1 ± 1.1	2.3 ± 1.3	3.4 ± 1.2
TLR2	996	3.1 ± 1.3 (2.1–3.8)	1.7	3.1 ± 1.2	3.0 ± 1.4	3.1 ± 1.4

Table 3. Unadjusted association between white blood cells counts (%) and DNA methylation (percentage of 5-methylcytosine) in 1243 visits undertaken by 756 men participating to the Normative Aging Study, Boston, 1999–2006

Gene	White blood cell (%)									
	Neutrophils		Lymphocytes		Basophils		Monocytes		Eosinophils	
	β	p value	β	p value	β	p value	β	p value	β	p value
CRAT	-0.03	0.43	0.06	0.14	-0.75	0.23	-0.08	0.63	-0.25	0.11
F3	-0.06	0.20	0.11	0.03	0.32	0.66	-0.57	<10⁻²	-0.11	0.53
GCR	-0.60	<10⁻²	0.98	<10⁻⁴	-0.31	0.93	-1.48	0.10	-1.63	0.07
ICAM	0.06	0.40	-0.004	0.96	0.91	0.43	-0.28	0.36	-0.53	0.07
IFN γ	2.64	<10⁻⁴	-3.10	<10⁻⁴	-0.63	0.82	-1.28	0.10	0.54	0.48
IL6	-0.75	0.03	1.12	<10⁻²	-1.59	0.73	0.003	1.00	-0.83	0.54
iNOS	0.68	0.02	-1.02	<10⁻³	-2.06	0.62	2.54	0.02	0.61	0.57
OGG1	-0.02	0.68	0.03	0.62	2.16	0.02	-0.25	0.26	0.22	0.30
TLR2	-0.16	<10⁻²	0.18	<10⁻³	0.46	0.58	0.18	0.38	-0.06	0.78

β is expressed for a 10% increase in percent of white blood cells type.

position 2 in CRAT, positions 3–5 in F3, position 1 in IL6 and iNOS, positions 1, 2 and 4 in OGG1, position 1 and 3–5 in TLR2. The only case where a significant association was seen with an individual position that was not apparent using the mean of all positions was for ICAM, where decreased methylation at positions 1 and 3 was significantly associated with higher FEV₁, FEV₁/FVC and MMEF.

Modification by age of the association between DNA methylation and lung function. Of the genes with significant main effects the following associations were modified by age ($p \leq 0.05$): mean TLR2 and FVC, position 5 in TLR2 and FVC and FEV₁, position 4 in OGG1 and FEV₁, mean IFN γ and FEV₁, position 4 in F3 and FEV₁/FVC (Table S5). For these genes and positions, the association was stronger in older people. Hence decreased methylation in TLR2 and OGG1 was associated with a greater decrement in lung function in older people than in younger ones, while decreased methylation in IFN γ and F3 was associated with a greater increment in lung function in older people than in younger ones. There were some genes/positions for which there was no main effect, but where a significant effect modification was seen with age. However, in these cases the effect of age was not consistent either across

genes or across positions within the genes, such that the association between DNA methylation and lung function was sometimes more deleterious for younger and sometimes more deleterious for older participants.

Sensitivity analyses. The sensitivity analyses including participants with chronic lung diseases showed similar associations between lung function and DNA methylation as the main analyses, with only slight variations in significance (Table S5). The association with lung function was no longer significant for the mean of OGG1 and decreased DNA methylation in the mean of ICAM became significantly associated with better lung function. For the individual positions, the associations were consistent with those observed in the main analysis (Table S6).

When adjusting for the proportions of different types of white blood cells that were associated with gene's methylation (Table 3), the associations between the mean of ICAM methylation and lung function were no longer borderline significant ($p = 0.15$ with FEV₁/FVC and $p = 0.12$ with MMEF). Furthermore, the individual positions of ICAM that had been significantly associated with lung function in the main analysis appear to be borderline significant in the sensitivity analysis ($p = 0.07$ for pos 1 and FEV₁/FVC, $p = 0.05$ for pos 3 and FEV₁ and MMEF).

Table 4. Percent change in lung function associated* with an interquartile range decrease in gene-specific DNA methylation (percentage of 5-methylcytosine) in 510 men without any chronic respiratory condition† participating to the Normative Aging Study, Boston, 1999–2006

Gene	n observations/n subjects	FVC		FEV ₁		FEV ₁ /FVC [‡]		MMEF	
		%	p value	%	p value	change	p value	%	p value
CRAT	755/479	-0.99	0.21	-1.68	0.04	-0.51	0.04	-1.51	0.33
F3	716/466	-2.94	<10⁻⁴	-2.47	<10⁻³	0.34	0.13	1.11	0.41
GCR	689/441	-0.78	0.22	-1.22	0.07	-0.31	0.14	-1.01	0.42
ICAM	621/413	0.31	0.68	0.88	0.29	0.45	0.07	2.94	0.07
IFN γ	784/489	1.32	0.07	1.75	0.02	0.36	0.12	3.72	<10⁻²
IL6	793/486	1.09	0.17	1.67	0.05	0.38	0.15	2.90	0.07
iNOS	517/363	-1.83	0.07	-1.93	0.07	-0.03	0.93	-0.31	0.87
OGG1	513/367	-1.37	0.05	-1.36	0.08	0.02	0.92	0.14	0.92
TLR2	662/431	-2.10	<10⁻²	-2.42	<10⁻³	-0.24	0.23	-2.86	0.02

*Results were adjusted for age (continuous), race (white/black), log(height), standardized weight (linear and quadratic term), % neutrophils, % leucocytes, education level (<12, 12, 13–15 and >15 y), smoking status (former/current/never), cumulative smoking (continuous), season of the medical exam (indicator variable), day of the week, corticosteroids (Y/N), sympathomimetic α and β (Y/N), anticholinergics (Y/N). †Subjects with asthma, chronic bronchitis, emphysema or positive methacholine test were excluded. ‡Absolute change in FEV₁/FVC.

Also, the mean of OGG1 was no longer associated with FVC ($p = 0.08$).

When further adjusting for cardiovascular diseases, diabetes and hypertension, the mean of OGG1 was no longer associated with FVC ($p = 0.08$) and the mean of IL6 was no longer associated with FEV₁ ($p = 0.07$). The associations between the mean of iNOS and FVC and FEV₁ were a bit stronger ($p = 0.04$ and $p = 0.05$ respectively). Results for the individual positions were consistent with the main analysis.

Discussion

In the present cohort of elderly men, we found that decreased DNA methylation in the mean of all positions tested for CRAT, F3 and TLR2 was associated with lower lung function metrics, especially FVC and FEV₁, which are related to large airways. We also observed borderline-significant associations in the same direction for mean iNOS and OGG1 methylation. In contrast, decreased DNA methylation in the mean of all positions tested for IFN γ and IL6 was associated with better lung function in both large and small airway markers (MMEF). We also observed borderline-significant associations in the same direction for mean ICAM.

We found that decreased F3 methylation was associated with lower lung function. The F3 gene encodes coagulation factor III, a cell surface glycoprotein with major roles in initiating the blood coagulation cascade, chemokine production,²⁵ pro-inflammatory effects and innate immunity.^{25,26} There is no experimental data to support the assumption that methylation in F3 promoter regulates the gene. However, the sequence we analyzed was previously suggested to have that role based on sequence characteristics.²⁷ F3 is expressed in monocytes and neutrophils, which are both involved in adhesion, migration and spreading and tend to accumulate at inflammatory sites.^{28,29} Consistent with our results, higher F3 levels in whole blood have been shown in patients with COPD compared with healthy subjects.²⁶ Moreover, inflammation also promotes coagulation which accentuates the

coagulation-inflammation cycle;³⁰ hypomethylation of F3 therefore constitutes a prothrombic and proinflammatory state that may also increase the risk for cardiovascular events, which are a major cause of mortality in COPD.

TLR2 and iNOS, which are also related to inflammation, were associated with lower FVC and FEV₁ in the present study. The TLR2 gene encodes a membrane protein that regulates the activation of innate immunity. The highest constitutive expression of TLR2 is observed in peripheral blood leukocytes,³¹ but TLR2 is also strongly expressed in the lungs. Consistent with our results, other studies have reported higher expression of TLR2 in monocytes³² and neutrophils³³ in COPD patients compared with healthy patients, and a positive association between TLR2 expression and inflammatory lung diseases has been demonstrated in mice.³⁴ Decreased lung function is characterized by airflow obstruction and inflammatory responses involving neutrophils production through the activation of TLR2, TLR4 and TLR9. TLRs detect pathogen- or damage-associated molecular patterns and trigger the production of NF κ B cells which then stimulate the production of inflammatory chemokines and cytokines.³³ These events also stimulate the production of iNOS, the inducible form of nitric oxide (NO) synthase, which releases NO. iNOS methylation has been associated with gene silencing.³⁵ Increased iNOS production is seen in inflammation as well as in the bronchi³⁶ and in induced sputum³⁷ of COPD patients compared with healthy subjects, consistent with our finding of lower iNOS methylation in subjects with lower lung function. Brindicci et al. showed increased iNOS protein expression at all stages of COPD, whereas iNOS mRNA was increased at earlier stages of COPD, but decreased in patients with severe disease. NO is a free radical that can interact with Reactive Oxygen Species (ROS) to form Reactive Nitrogen Species (RNS), which may both have deleterious effects for cell components and enhance inflammation, and lead to lung disorders such as COPD.^{38,39}

We also found that lower CRAT and OGG1 (mean and positions 2 and 4) methylation was associated with lower lung function. Oxidative stress has been identified as one of the mechanisms

responsible for decreased lung function, including in persons free of COPD. Previous work in this cohort has shown that statin use, which in addition to lowering cholesterol has antioxidant activity, was associated with higher than predicted lung function, independent of chronic respiratory disease.⁴⁰ OGG1 encodes a DNA glycosylase enzyme expressed in the lungs and involved in excision of 8-oxoguanine resulting from ROS exposure. Free radicals resulting from lung inflammatory responses can contribute to lung damage and decreased lung, while also resulting in oxidative stress that may activate OGG1 as suggested by our results. In contrast, Liu et al. found no association between OGG1 polymorphisms and COPD susceptibility.⁴¹ CRAT is an enzyme playing an important role in metabolic processes. Carnitine decrease has been associated with insulin resistant states and aging.⁴²

We found that decreased methylation of ICAM, IFN γ and IL6 was associated with a better lung function. As these three genes are related to pro-inflammatory processes, we would have expected lower DNA methylation to be associated with lower lung function. ICAM encodes a cell surface glycoprotein that is more expressed during inflammatory responses.⁴³ IFN γ is a cytokine, whose hypomethylation has been shown to suppress gene expression.⁴⁴ IFN γ is a mediator in innate and adaptive immunity that regulates a variety of pro-inflammatory parameters. However, it also has anti-inflammatory properties, which confer an ambivalent role,⁴⁵ and reduced IFN γ production has been shown in asthmatic patients.⁴⁶ In the same cohort we investigated here, serum levels of IFN γ and IL6 were not associated with pulmonary function of healthy subjects⁴⁷ although another study found serum level of IL6 associated with impaired FEV₁.⁴⁸ IL6 encodes a protein that acts as both a pro-inflammatory and anti-inflammatory cytokine.⁴⁹ The consistent results between IFN γ and IL6 might reflect their concomitant variation in pro- and anti-inflammatory processes. Inflammatory processes involve the production of a myriad of signaling cells, which makes difficult disentangling the specific role of each gene.

Results for iNOS, OGG1, IL6 and ICAM sometimes differed among the individual positions. We searched for Transcription Factor Binding Sites (TFBS) in these genes using the University of California Santa Cruz (UCSC) genome browser (genome.ucsc.edu). There was no TFBS and no SNP near our target iNOS sequence. For OGG1, the CpG positions analyzed were in proximity of a variety of TFBS including: HA-E2F1, NRSF, Pol2, Pol2-48H, AP-2Alpha, AP-2Gamma, TAF7, TAF1, TBP, P300, CTCF, HMGN3, NFkB, SMC3, CCNT2 (positions 2–4), Sin3Ak-20 (positions 2–4) and HEY1 (position 4). For IL6, TFBS (BAF155, Inil, c-Myc, BAF170, Max, NRSF and Nrf1) were present for position 1 and one SNP was observed (C/T), whereas position 2 was free of TFBS and SNP, which may explain the different results we observed for these two positions. For ICAM, all positions were SNPs free and TFBS located near positions 1 and 2 were similar (NFkB, Pol2-4H8, PAX5-C20, EBF, Pol2, IRF4, BCL11A, PAX5-N19, TCF12, E2F6, ELF1, MEF2a, p300, EBF1 (position 2 only)). However, TFBS Pol2-4H8, PAX5-C20, EBF, Pol2, PAX5-N19, E2F6, ELF1, MEF2a and EBF1 were not observed near position 3 and different TFBS were observed (POU2F2, BCL3, STAT1, PU.1,

SP1, Inil, EBF1, HEY1, BATF, BAF155, TAF1 and STS2). This might help to explain the differences we saw between position 3 and positions 1–2 (Table S4).

We found that age significantly modified the associations between lung function and some of the genes. The associations between DNA methylation and lung function were somewhat stronger for older people for genes with main effects. For genes without main effects, the patterns were not consistent either across genes or across positions within genes (Table S5). This suggests the association of methylation of some genes with lung function may get stronger with age. Few studies have described intra-individual changes over time in DNA methylation.^{50,51} Interestingly, Madrigano et al. found that longitudinal change in age was associated with decreased methylation in GCR and INOS and with increased methylation in CRAT, F3, IFN γ and OGG1 in the same cohort as we investigated here.⁵⁰ They also showed that the cross-sectional effect of age (between individuals) was associated with increased TLR2 methylation, whereas the longitudinal effect of age (within individuals) was associated with decreased TLR2 methylation, which means that the effect of age on DNA methylation might be difficult to disentangle. The changes they observed in DNA methylation were quite small, and given this and the small changes in lung function between visits, we did not think it feasible to examine the association of changes in methylation and changes in lung function.

While our study cannot draw conclusions on how gene expression relates to lung function, our results in an aging healthy population confirm that inflammation and oxidative stress are key features that drive lung function decline and that methylation of inflammatory genes is part of these processes, which involve a complex cascade of interactions between signaling cells. However, the present study has a number of limitations. While our results demonstrate that methylation patterns were associated with lung function characteristics, whether methylation patterns is a cause or a consequence of the changes in lung function cannot be determined in our study design. We currently do not have enough years of follow-up to examine methylation and change in lung function. DNA methylation may be a biomarker of lung function or may mediate the effects of environmental factors such as smoking or air pollution that are associated with lung function decline. How methylation patterns of these genes relate to changes in lung function throughout the life course and how the DNA methylation relates to expression for these genes requires further investigation.

In this study, DNA methylation was measured in white blood cells, and while these cells infiltrate the lungs, it is not clear to what extent the observed changes in white blood cells DNA methylation reflect similar changes in lung tissues and airways. Several studies have shown elevated levels of inflammatory cytokines in circulating blood associated with COPD, suggesting an overspill of inflammatory mediators from peripheral lung tissues,⁵² but this might also be due to other inflammatory diseases commonly seen with aging and in subjects with impaired lung function. However, a recent study found elevated circulating levels of surfactant protein D, which is specific from lung tissues and therefore provides more evidence for the overspill hypothesis.^{53,54}

Blood is an easily accessible biological sample and because the neutrophilic inflammation is an early component of lung function decline and because the genes under study are known to be expressed in neutrophils, measuring DNA methylation in white blood cells may be a relevant marker of inflammatory processes in the lungs.

Since we performed multiple tests with 4 related outcomes and 34 DNA methylation exposures (9 related genes with several positions for each), we would expect 7 false positives, which means that among the 48 significant associations we observed, 7 of them might be wrong. Moreover, since our cohort consists of mainly white elderly men, our findings may not be generalizable to other populations. How DNA methylation would be associated with lung function in younger population, women or in other ethnicities remain to be determined.

Epigenetic mechanisms such as DNA methylation are increasingly recognized to play a role in chronic diseases. For the first time, methylation differences in CRAT, F3, IFN γ , IL6, INOS and TLR2 genes have been associated with lung function characteristics, particularly with large airways flow, in a cohort of elderly men. These associations were somewhat modified by age. Our results provide new insights regarding the biological processes related to changes in lung function which strongly determine mortality by complex mechanisms that are not fully understood.

Materials and Methods

Study population. This study included 756 men examined between March 1999–December 2006, in the NAS, a longitudinal closed-cohort of aging established by the Veterans Administration in 1963.⁵⁵ Participants were free of known chronic medical conditions at enrolment, returned for examinations every 3–5 y, and were asked to give a DNA sample from 7-ml blood at each visit between 1999 and 2006. Height, weight and medication use were assessed and pulmonary disorders (asthma, chronic bronchitis, emphysema) and smoking history were collected through American Thoracic Society questionnaire.⁵⁶ Participants provided written informed consent and the study protocol was approved by the Institutional Review Boards of all participating institutions.

Lung function. Spirometric tests were performed as previously reported in reference 57, following a strict protocol in accordance with American Thoracic Society guidelines.

We used data from the most recent (1999–2000) methacholine challenge tests available for each subject at that visit.

DNA methylation analysis. Given the strong relation between aging and both DNA methylation and lung function and given the lack of studies reporting the links between DNA methylation and lung function, we leveraged an ongoing study on aging in which methylation was measured in nine genes: CRAT, F3, GCR, ICAM, IFN γ , IL6, iNOS, OGG1, TLR2. The aim of the cohort was to study respiratory and cardiovascular outcomes. In order to cover the broad range of these outcomes, we selected nine genes among those: (1) related to respiratory and cardiovascular outcomes; or related to inflammation and oxidative stress;

or known to be associated with aging and age-related diseases; and (2) expressed at variable degree in leukocytes,⁵⁸ the DNA source used in our study. The procedure for DNA methylation analysis is detailed in the supplementary material online. For each gene, we measured between 1–5 CpG sites (positions) and used the mean of DNA methylation at all positions for our baseline analysis. Individual positions were examined in a secondary analysis.

Statistical analysis. Lung function measurements FVC, FEV₁ and MMEF were log-transformed to increase normality and stabilize variance. A mixed linear model was used to account for the correlation among measurements within the same subject:

$$Y_{it} = \beta_0 + u_i + \beta_1 \text{DNA methylation}_{it} + \beta_2 X_{2it} + \dots + \beta_p X_{pit} + \varepsilon_{it} \quad (1)$$

where Y_{it} was the lung function measurement for subject i at visit t , β_0 was the overall intercept, u_i was the separate random intercept for subject i , X_{2it} – X_{pit} were the $p - 1$ covariates for subject i at visit t . Adjustment factors included in the model were age, height (log) and standardized weight (linear and quadratic term), race (white, black), education level (<12, 12, 13–15 and >15 y), cigarette smoking (current, former, never) and pack-years, chronic lung conditions (asthma, emphysema, chronic bronchitis), methacholine responsiveness, medication use in yes/no (corticosteroids, sympathomimetics α and β , anticholinergics), percent lymphocytes and neutrophils, season (indicator variables) and day of the week. A p value of < 0.05 was considered statistically significant. To exclude associations between DNA methylation and lung function merely due to differences in the proportions of white blood cell types, we adjusted all models below for percent neutrophils and lymphocytes. Separate models were fit for each combination of lung function and DNA methylation measures. An association between the dependent variable and a covariate was considered to be significant if the covariate had a p value < 0.05 in the model.

To determine if the association between DNA methylation and lung function was modified by age, models with an interaction term between DNA methylation and age, along with the main effects, were run. If there was a significant interaction ($p < 0.05$), effects estimates of methylation on lung function were then calculated as the effect of DNA methylation at the 25th and 75th percentile of the distribution of age. We present the estimated effect of methylation on lung function as the percent change in FVC, FEV₁ and MMEF and as the unit change in FEV₁/FVC for an interquartile range decrease in methylation.

We excluded from the main analysis participants with asthma, emphysema, chronic bronchitis or positive methacholine tests or with missing values for these chronic conditions. A sensitivity analysis further adjusted for these medical conditions was performed on the whole sample. In addition, we further adjusted for cardiovascular diseases, diabetes and hypertension. For each gene, we performed another sensitivity analysis adjusting the models for the cell type proportions of white blood cells that were associated with gene's methylation.

To adjust for the fact that healthier men are more likely to come back to subsequent visits, we used inverse probability weighting to correct for a potential survival bias.⁵⁹ We calculated

the probability of having a second visit or a third visit using logistic regressions given all relevant factors at the previous visit: age, education level, body mass index, smoking status and pack-years, hypertension, cholesterol, diabetes, FEV₁ (further adjusted for asthma, emphysema, chronic bronchitis and result of methacholine test for the sample including participants with these chronic conditions). The probability at first visit was 1. We then used the inverse of the predicted probabilities as the weights. We used SAS version 9.2 (SAS Institute, Cary, NC).

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Note

Supplemental material can be found at:
www.landesbioscience.com/journals/epigenetics/article/19216/

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