IL18 and *IL18R1* polymorphisms, lung HRCT and fibrosis: a longitudinal study in coal miners

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ABSTRACT

Interleukin (IL)-18 has been suggested to play a role in the development of inflammatory and fibrosing lung diseases.

Associations of polymorphisms in *IL18* (G-656T, C-607A, G-137C, T113G, C127T) and its receptor *IL18R1* (C-69T) with Coal Workers' Pneumoconiosis (CWP) were studied in 200 miners who were examined in 1990, 1994 and 1999. Coal dust exposure was assessed by job history and ambient measures. The main health outcome was lung computed tomography (CT) score in 1990. Internal coherence was assessed by studying CT score in 1994, 4-year change in CT score and CWP incidence and prevalence.

CT score in 1990 was a good predictor of *x* ray grade in 1999, and therefore an appropriate subclinical quantitative trait. The *IL18* –137 C allele was associated with lower CT score in 1990 and 1994 (1.24 *vs.* 1.69, p=0.2; 1.57 *vs.* 2.46, p=0.02), slower progression of CT score between 1990 and 1994 and lower pneumoconiosis prevalence in 1999 relative to the G allele (0.33 *vs*. 0.77 p = 0.03 and 8.2 *vs*. 19.6% p=0.02). Smoking or dust-adjustment, stratification on *IL18R1* genotype and adjustment for haplotype effects did not change the conclusions.

Results suggest a role for *IL18* in reducing the development of this fibrosing lung disease.

Keywords: *IL18* – *IL18R1* — epidemiology – computed tomography — genetics.

Introduction

Interleukin-18 (IL-18) is a recently described lymphokine involved in neutrophil activation, reactive oxygen species (ROS) synthesis [1], proinflammatory cytokine production, NF-kB activation, and degranulation [2]. A role for IL-18 in pulmonary inflammation has been suggested by studies in rodent models, but its importance is not clearly understood. In epidemiological genetic studies, the *IL18* C-607A polymorphism (SNP) was significantly associated with higher prevalence of sarcoidosis in Japanese subjects [3] but not in Dutch subjects [4]. The *IL18* A105C SNP was significantly associated with asthma [5], the G-allele of the *IL18* promotor variant (-137G/C) was associated with an increased risk for atopic asthma in the SAPALDIA cohort study [6], and the *IL18* G–656A, G-137C, T-133G, T113G and C127T SNPs were significantly associated with high IgE levels, specific sensitization to common allergens, and seasonal allergic rhinitis in 105 Caucasian families [7]. However, these findings have not been replicated [8].

Davis *et al.* [9] proposed that IL-18 plays an early role in the reiterative process of macrophage-lymphocyte interaction following silica exposure in mice, leading to chronic inflammation, tissue injury, and collagen production. Kitasato *et al.* [10] reported markedly elevated levels of IL-18 in the serum and bronchoalveolar lavage of patients with idiopathic pulmonary fibrosis compared to controls. Coal workers' pneumoconiosis (CWP) is another inflammatory and fibrosing lung disease caused by chronic inhalation of particles. The overall hypothesis of the present study was that *IL18* and *IL18R1* polymorphisms contribute to the pathogenesis of CWP. To test this hypothesis, we investigated the associations of *IL18* and *IL18R1* SNPs with CT score, a quantitative subclinical phenotype predicting the occurrence and the evolution to the disease [11,12,13], and with disease prevalence. The primary health outcome for the study was the computed tomography (CT) score at the first survey, when nearly all miners were active. The internal coherence of the results was tested by studying

associations with CT score at the second survey and with CWP prevalence at the end of the follow-up. Looking at CT score change between 1990 and 1994 was considered largely as a measure of the activity of the disease between both surveys. We also tested the interactions of polymorphisms in *IL18* and its receptor with coal dust exposure (i.e., gene X environment interaction), and between *IL18* and *IL18R1* polymorphisms (i.e., gene X gene interaction) on lung CT score and disease prevalence.

Methods

Study sample

The study design has been described elsewhere [14]. Briefly, unrelated coal miners (aged 34-50 years) were recruited in 1990 to be contrasted by exposure and chest *x* ray including: 80 subjects heavily exposed to underground coal dusts (≥10 years at the coal face) with chest *x* ray classified 0/1 or 1/0 and without health alteration from any other diseases; 80 healthy subjects exposed to underground coal dusts with normal chest *x* ray classified 0/0; and 80 healthy subjects slightly exposed with normal chest *x* ray. The three groups were matched for age and smoking habits [12]. Miners were re-examined in 1994 and in 1999. The study sample includes 200 coal miners for whom genetic, environmental, and health data were available from 1990 to 1999; no differences regarding genotype, exposure, and health data were found with those not included in the analyses (n=40). In 1990, 96% of the miners were active; the proportion of retired miners rose from 24 to 88% between 1994 and 1999. The appropriate ethical committee approved the study and written consent was obtained from all subjects.

Environment

Besides smoking, detailed information on high or low current exposure based on job description, and cumulative exposure were recorded [15]. High current exposure refers to miners working at the coal face, mining, stope or drift advance; and low exposure refers to those working at ventilation maintenance, pumping, haulage, shaft, stock equipment, or safety. Cumulative personal exposure to dust was estimated from each person's job history and from dust measurements at various sites of the coal mine. The estimates were summations of each dust measurement (mg/m^3) for the respective time spent in each job. Estimated cumulative exposures to dust were calculated until 1999 and expressed as mg/m^3 x year.

Radiographic examination

CT scans were performed in 1990 and 1994 for all subjects as already described [12]. Briefly, the lungs were divided into six areas: the upper zones above the carina, the middle zones between the level of the carina and the lower pulmonary veins, and the lower zones below the level of the lower pulmonary veins. Micronodules were defined as "opacities" less than 7 mm in diameter, and nodules were defined as opacities from 7 to 20 mm in diameter. Micronodules, nodules and other abnormalities, such as "emphysema" and profusion of shadows were recorded according to criteria of the consensus meetings for CT scans established by the Society for Thorax Imagery and the Thorax Club in September 1989 and June 1990, and according to the pathology standards for Coal Workers' Pneumoconiosis defined by the College of American Pathologists. Profusion of abnormalities was graded from 0 to 3 (absent, rare, intermediate, high profusion), and was estimated for the six lung zones (upper, middle, lower parts of both lungs), giving a profusion score of 0 to 18. Analyses were based on the mean profusion score obtained and dividing this score by 6. The readings of the scans for the first and the second examinations (1990 and 1994) were done at the same time by two experienced radiologists blinded to individual exposure and *x* ray findings. If there was a discrepancy, a consensus was reached.

At each survey (1990, 1994 and 1999), chest *x* rays taken in the yearly medical examination were interpreted by two independent and experienced physicians according to the International Labour Office (ILO) standardised classification of radiographs of pneumoconiosis [16]. The films were presented in a random order, without any information about the occupational and medical history of the subjects. For statistical analyses, the 12 point ILO profusion grades were collapsed to three points: 0/- and 0/0, 0/1 and 1/0, and ≥ 1/1. Subjects with a profusion grade of <1/1 were considered as not having pneumoconiosis, those with a profusion grade of 0/- or 0/0 were considered as having normal chest *x* ray, and those

with a profusion grade of 0/1 or 1/0 were suspected to be in the process of evolution to pneumoconiosis. Pneumoconiosis was defined by a grade ≥ 1/1.

Genotyping

IL18 -137, -607, +113, and +127 SNPs were genotyped in 2004 as previously described [17]. *IL18* -656 and *IL18R1* –69 SNPs were genotyped by allelic discrimination using TaqMan probes. Primers and TaqMan probes were designed using Primer Expression v2.0 (Applied Biosystems). The forward and reverse primers for *IL18R1* are 5'-ttttttaaaaatctgtgtgccagaa – 3' and 5'- cagccaaagctttcaaacaaaa – 3', respectively and TaqMan probes are 5'- ttatgaaAgtttaaaaatc-Fam-3' and 5'-ttatgaaGgtttaaaaat-Vic-3'. The forward and reverse primers for *IL18* -656 are 5'-taggtcagtctttgctatcattcca-3' and 5'-acactttctgcaacagaaagtaagct -3', respectively and TaqMan probes are 5'- aattttggtaTccctctc-Fam-3' and 5'- aattttggtaGccctct-Vic-3'. For both assays, primers, probes, and TaqMan Universal MasterMix with no AmpErase UNG (Applied Biosystems) were utilized according to manufacturer's standard protocol in a Prism 7000 Sequence Detection System (Applied Biosystems). Genotypes were determined by manual clustering using Prism 7000 sequence detection software version 1 (Applied Biosystems).

Statistical methods

Standard statistical tests (χ^2 or Fisher exact test when appropriate, and logistic regression for qualitative variables; analysis of variance and multiple regression analysis for quantitative variables) were performed with the SAS statistical software (2001). Significance was assessed at the 5% two sided level.

All analyses were first conducted considering each *IL18* SNP separately and the main outcome CT score in 1990, where 96% of the miners were active. Associations of each SNP with CT score in 1994, change in CT score between 1990 and 1994, and 1999 CWP incidence

and prevalence were also investigated to test the coherence of the results, and the activity of the disease (change in CT score between 1990 and 1994). No a priori adjustment was done. CT score was not normally distributed; but non-parametric Kruskal-Wallis and standard parametric tests (analysis of variance and Student's t-Test) gave similar p values. Analyses were conducted considering subjects heterozygous and subjects homozygous for the variant allele, and variant allele carriers as other authors [3-7]. *IL18* haplotype analysis was then performed using a maximum likelihood method for haplotype-phenotype association as implemented in the THESIAS program (http://genecanvas.ecgene.net/) [18]. The most frequent haplotype (*IL18* –607C/*IL18* –137G (CG)) was used as the reference.

Interaction between genetic polymorphisms and exposure to coal mine dusts, or between both genetic polymorphisms (*IL18* and its receptor *IL18R1*) on health outcomes (CT score and pneumoconiosis prevalence) were statistically tested using multivariate linear or logistic regression models.

Results

The characteristics of the 200 miners included in the analyses are summarized in Table 1. The mean age of miners in 1990 was 43 years. More than half of the miners were current smokers, and 48.5% were highly exposed to coal mine dusts in 1990. Sixty-eight percent were born in France, and only 1.5% had their geographical origin from non-European countries. Among all coal miners, the CT score increased by ~40% between 1990 and 1994, and the prevalence of pneumoconiosis rose from 3.5% to 14% between 1994 and 1999.

CT score and *x* ray grade were highly associated in 1990 and in 1994. Mean CT score values (SD) in 1990 were 0.71 (1.19), 2.98 (2.20) and 3.09 (2.61) in miners with *x* ray grade of 0/0, 0/1 and 1/0 respectively (trend test, $p<0.0001$). In 1994, mean values (SD) were 1.07 (1.38), 3.87 (2.95) and 4.39 (3.60), with the 7 pneumoconiotic miners (1/1 or more) having a mean value (SD) of 7.43 (4.20) (trend test, $p<0.0001$). CT score and cumulative coal dust exposure (mg/m^3 x year) were highly correlated in 1990 and in 1994: $r=0.35$, $p<0.0001$ and $r=0.29$, $p<0.0001$ respectively.

In 1999, 26 (92.9%) of the 28 pneumoconiotic miners were those heavily exposed to underground coal dusts with chest *x* ray classified 0/1 or 1/0 in 1990, 2 (7.1%) were those exposed to underground coal dusts with normal chest *x* ray classified 0/0 in 1990. No miner slightly exposed with normal chest *x* ray in 1990 had pneumoconiosis in 1999. Figure 1 shows *x* ray grade in 1999 acccording to CT score in 1990. At the end of the 10-year follow-up, 46 subjects had worsened x ray findings and 28 of them were pneumoconiotic. CT score in 1990 was significantly higher in miners with worsened x ray findings in 1999 as compared with those who did not (3.67 ± 2.39 (n=46) *vs.* 0.81 ± 1.22 (n=154), $p < 0.0001$), and in miners who developed pneumoconiosis as compared with others (4.18 ± 2.68 (n=28) *vs.* 1.03 ± 1.41 (n=172), $p < 0.0001$).

Genotype and allele frequencies

Minor allele frequencies were 0.42 for *IL18* G–656A and C–607A, 0.285 for *IL18* G–137C, T+113G, and C+127T, and 0.347 for *IL18R1* C-69T*;* all fit predictions for Hardy-Weinberg equilibrium (all $p > 0.6$). Complete linkage disequilibrium was observed between *IL18* –656 and –607 genotypes, and between *IL18* –137, +113 and +127 genotypes; three haplotypes were found: *IL18* –607C/*IL18* –137G (CG), 58.0%; AC, 28.5%; and AG, 13.5%. All miners homozygous for the *IL18* –137 C allele were homozygous for the *IL18* –607 A allele, and 63.7% of miners homozygous for the *IL18* –137 G allele were homozygous for the *IL18* –607 C allele ($p<0.0001$ for association). No differences in genotype or allele distributions were observed according to the geographical origin of the miners (data not shown).

Association of IL18 –607, IL18 -137 and IL18R1 SNPs with stage of pneumoconiosis

No significant association was found between *IL18* or *IL18R1* SNPs with CT score in 1990 (Table 2). Further, no significant associations were found between *IL18* C–607A or *IL18R1* C-69T genotype with CT score in 1994, change in CT score between 1990 and 1994, and pneumoconiosis incidence or prevalence.

Lower CT score in 1990, significantly lower CT score in 1994 and slower progression of CT score were found in *IL18* –137 C carriers (i.e. miners homozygous or heterozygous for -137C). No *IL18* –137 C carrier had pneumoconiosis in 1994, and the *IL18* –137 C allele was significantly associated with lower disease prevalence in 1999.

No interaction was observed with coal dust exposure on CT score in 1990 and in 1994, change in CT score, and on disease prevalence in 1999 (data not shown). Analyzing smoking or dust-adjusted CT score and disease prevalence, or stratifying on *IL18R1* C-69T genotype did not change the conclusions.

Haplotype analyses

No association of *IL18* -607/-137 haplotype AC with CT score in 1990 was found (Table 3). *IL18* -607/-137 haplotype AC was associated at borderline significance with CT score in 1994, was significantly associated with a slower progression of CT score between 1990 and 1994 and with a lower prevalence of disease in 1999. No significant association was found with haplotype AG.

Discussion

We tested the hypothesis that polymorphisms in *IL18* and its receptor contribute to the pathogenesis of CWP, an inflammatory and fibrosing lung disease. Results show significant associations of *IL18* –137 C allele with CT score in 1994, slower progression of CT score between 1990 and 1994, and lower pneumoconiosis prevalence in 1999 relative to the G allele. Adjusting for haplotype effects confirmed the results. Further, analyzing smoking or dust-adjusted CT score or disease prevalence, or stratifiyng on *IL18R1* genotype did not change the conclusions.

Computed tomography is not used as a standard method to assess pneumoconosis, although it is a sensitive tool to evaluate lung parenchyma [11]. The determination of genetic factors is greatly enhanced by considering subclinical quantitative phenotypes [19]. CT has been proposed as screeening method to distinguish normal from early pneumoconiosis [13]. In the present study, the predictive value of CT score, as an appropriate subclinical quantitative phenotype was confirmed. Results with a 10-year follow-up confirm and extend those reported only on a 4-year period [12]. As mines have been totally closed in France, it was not possible to build a replication sample. Furthermore, the use of CT in coal mining is still limited at an international level. Coal worker's pneumoconiosis however remains a major occupational disease in terms of public health burden worldwide and it is important to better understand the genetic modifyers of this environmental disease.

The present study has some limitations. We did not consider all of the *IL18* and *ILR1* SNPs, nor other genes involved in the IL-18 pathway which might contribute, alone or in combination, to IL-18 variability and in turn affect coal workers' pneumoconiosis susceptibility. However, the SNPs that were analyzed are those with functional significance that have been previously published. Further, the sample size of the population studied was small. A precise assessment of the power could not be performed prior to the study; variations

of CT score across the general population are not known, as it is not possible to perform CT scan on a large scale for ethical and technical reasons. However, it was anticipated that the contrasted disease status chosen by design, and the availability of such a sensitive score would increase the power to detect differences compared with classical designs based on random samples with only chest *x* ray measures.

Few epidemiological genetic studies have examined the associations of *IL18* A–607C and G–137C SNPs with chronic inflammatory and fibrosing lung diseases, and none have simultaneously investigated the role of environmental factors and polymorphism in *IL18R1*. Furthermore, none has considered CT score. Kruse *et al.* [7] reported a significant association of *IL18* 137C allele with high serum IgE levels, specific sensitization to common allergens and seasonal allergic rhinitis in 105 German families; the *IL18* A–607C SNP was unrelated to these phenotypes. In populations recruited in the same areas, the *IL18* A–607C and G-137C SNPs were unrelated to bronchial asthma in 230 children compared to 270 controls [8]. Recently, a significant association of *IL18* –137G allele with increased risk for atopic asthma in the SAPALDIA cohort study was found [6]. Takada *et al.* [3] reported significant higher frequency of the *IL18* –607C allele in 119 Japanese sarcoidosis patients as compared to 130 controls, and no association was found for the *IL18* G-137C SNP. The significant association was not replicated in a population of Dutch Caucasians, where 133 sarcoidosis patients were compared to 103 controls [4]. The inconsistency between studies may be due to differences in *IL18* -607A and –137C allelic frequencies between populations, or in onset or biological pathways during disease progression. Further, the pleiotropic role of IL-18 with varying effects according to the cytokine milieu (i.e. Th2 cytokines when considering atopic phenotypes, or Th2 and Th1 cytokines when considering asthma [20]) could also partly explain these differences.

In our study, only the *IL18* G–137C SNP was significantly associated with pneumoconiosis phenotype and prevalence. Taking into account coal dust exposure, which is the first cause of the disease, and stratifying on *IL18R1* genotype did not change the conclusions. Genotype and allelic frequencies were very close to those reported in previous studies in Caucasians [4, 6-8]. Haplotypes found in our study were identical to those reported by Giedraitis *et al.* [21] in a Swedish population. They found that the haplotype –656T/-607A/-137C/+113G/+127T (-607/-137 AC in our study) was clearly associated with lower promoter activity and lower *IL18* gene expression than haplotypes CG and AG. Further, in C carriers at position –137, no correlation was found between IL-18 and interferon-gamma (IFN-γ) mRNA levels whereas a strong correlation was found in those homozygous wildtype with GG at -137.

Our results were also consistent with the study of Wei *et al.* [22] in which markedly reduced incidence and severity of collagen-induced arthritis was found in IL-18$^{-/-}$ mice compared to wild-type mice. This was accompanied *in vitro* by significantly reduced proinflammatory cytokine production including IFN-γ. Further, significantly reduced lung collagen was observed in IFN-γ deficient mice exposed to silica as compared to wild-type mice [9]. Pneumoconiosis is another collagen-related disease, including the activation of alveolar macrophages, the ROS synthesis and the proinflammatory cytokine production such as TNF and NF-kB; and IL-18 is involved in all of these steps [1-2].

In summary, we found that the *IL18* G–137C SNP was associated with lower CT score, slower progression of CT score and lower pneumoconiosis prevalence. Further, smoking or dust-adjustment, stratification on *IL18R1* genotype and adjustment for haplotype effects did not change the conclusions. The results are consistent, support the biological and functional significance of *IL18* and suggest its potential role in reducing the development of this inflammatory and fibrosing lung disease. However, it is premature to consider any

clinical application of the findings and replication of our findings in additional populations is warranted.

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Table 1.- Characteristics of coal miners in 1990*

	Value
Age (years, mean (SD))	42.6 (3.5)
Smoking habits, n (%)	
Non-smokers	49 (24.5)
Ex-smokers	42 (21.0)
Current smokers	109 (54.5)
Pack-years (mean (SD))	12.7 (11.1)
Current coal dust exposure (based on job description), n (%)	
No exposure (retirement)	8 (4.0)
Low exposure	95 (47.5)
High exposure	97 (48.5)
Cumulative coal dust exposure (mg/m^3 x year, mean (SD))	52.5 (39.2)
Geographical origin, n (%)	
France	135 (67.5)
Other European countries	62 (31.0)
North Africa	3 (1.5)
Computed Tomography (CT) scan micronodule score (mean (SD))	1.47 (1.97)
Chest *x* ray grade, n (%)	
0/0	134 (67.0)
0/1	45 (22.5)
1/0	21 (10.5)
CT scan micronodule score in 1994 (mean (SD))	2.02 (2.65)
Chest *x* ray grade in 1994, n (%)	
0/0	144 (72.0)
0/1	31 (15.5)
1/0	18 (9.0)
1/1 or more (pneumoconiotic)	7 (3.5)
Chest *x* ray grade in 1999, n (%)	
0/0	134 (67.0)
0/1	17 (8.5)
1/0	21 (10.5)
1/1 or more	28 (14.0)

*Unless otherwise stated.

Table 2.- Association of *IL18* and *IL18R1* polymorphisms with stages of pneumoconiosis in coal miners

		CT score in 1990		CT score in 1994		1994-1990 CT score		CWP incidence, %				1999 CWP prevalence, %	
	n	mean ± SD	p-value*	mean ± SD	p-value*	mean ± SD	p-value*	1994-1990	p-value	1999-1994	p-value	%	p-value
IL18 C–607A													
CC	65	1.48 ± 2.26		2.31 ± 2.96		0.83 ± 1.58		4.6		14.5		18.5	
CA	102	1.56 ± 1.90		1.98 ± 2.47		0.42 ± 1.34		2.9		10.1		12.7	
AA	33	1.18 ± 1.57	0.6	1.61 ± 2.60	0.5	0.42 ± 1.41	0.2	3.0	0.9	6.3	0.4	9.1	0.4
CA or AA	135	1.47 ± 1.82	0.4	1.89 ± 2.49	0.4	0.42 ± 1.35	0.09	3.0	0.7	9.2	0.3	11.8	0.2
IL18 G–137C													
GG	102	1.69 ± 2.23		2.46 ± 2.95		0.77 ± 1.49		6.9		13.7		19.6	
GC	82	1.19 ± 1.57		1.46 ± 1.91		0.27 ± 1.22		0.0		7.3		7.3	
CC	16	1.50 ± 2.00	0.4	2.12 ± 3.46	0.05	0.62 ± 1.93	0.08	0.0	0.03	12.5	0.4	12.5	0.06
GC or CC	98	1.24 ± 1.64	0.2	1.57 ± 2.23	0.02	0.33 ± 1.35	0.03	0.0	0.01	8.2	0.2	8.2	0.02
IL18R1 C-69T													
CC	86	1.66 ± 2.17		2.29 ± 2.71		0.63 ± 1.32		2.3		14.3		16.3	
CT	89	1.40 ± 1.97		1.99 ± 2.83		0.58 ± 1.57		5.6		8.3		13.5	
TT	25	1.04 ± 0.98	0.7	1.24 ± 1.48	0.2	0.20 ± 1.32	0.5	0.0	0.5	8.0	0.4	8.0	0.6
CT or TT	114	1.32 ± 1.81	0.7	1.82 ± 2.60	0.2	0.50 ± 1.52	0.3	4.4	0.7	8.3	0.2	12.3	0.4

CWP: coal workers' pneumoconiosis (*x* ray grade ≥ 1/1). *: Kruskal-Wallis Test.

Table 3.- Association of *IL18* haplotypes with stages of pneumoconiosis in coal miners

		CT score in 1990		CT score in 1994		1994-1990 CT score		1999 CWP prevalence	
		d *	p-value	d	p-value	d	p-value	OR (95% CI)	p-value
Haplotypes									
-607/-137	CG	Reference		Reference		Reference		Reference	
	AC	-0.23 [-0.68 ; 0.22]	0.3	-0.52 [-1.06 ; 0.01]	0.06	-0.29 [-0.58 ; -0.001]	0.05	0.50 [0.25 ; 0.99]	< 0.05
	AG	0.18 [-0.45 ; 0.81]	0.6	0.06 [-0.79 ; 0.91]	0.9	-0.12 [-0.66 ; 0.43]	0.7	1.06 [0.46 ; 2.41]	0.9

CWP: coal workers' pneumoconiosis (*x* ray grade ≥ 1/1). OR = odds ratio (95% confidence interval).
*: difference compared to reference CG.

Figure legend

Figure 1.- Computed tomography score, a quantitative trait predicting the evolution to pneumoconiosis. Box plots of CT score in 1990 according to chest x ray grade in 1999. Box plots show the median (bar), the first and third quartile (box), the first and last decile (fences) and the minimum and maximum (stars) of CT score for each *x* ray grade category. Means of CT score are 0.72 ± 1.19, 1.35 ± 1.00, 2.76 ± 1.70, 3.58 ± 2.22 and 7.75 ± 2.63 in each category ($p < 0.0001$).