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Paraoxonase and Superoxide Dismutase Gene Polymorphisms and Noise-Induced Hearing Loss

Giuliana Fortunato,[1] Elio Marciano,[2] Federica Zarrilli,[3] Cristina Mazzaccara,[1] Mariano Intrieri,[1,3] Giuseppe Calcagno,[1] Dino F. Vitale,[4] Paolo La Manna,[5] Claudio Saulino,[2] Vincenzo Marcelli,[2] and Lucia Sacchetti[1*]

Background: **Noise-induced cochlear epithelium damage can cause hearing loss in industrial workers. In experimental systems, noise induces the release of free radicals and may damage the cochlear sensorial epithelium. Therefore, genes involved in regulating the reactive oxygen species manganese-superoxide dismutase (SOD2) and the antioxidant paraoxonase (PON) could influence cochlea vulnerability to noise. We evaluated whether susceptibility to noise-induced hearing loss (NIHL) is associated with *SOD2*, *PON1*, and *PON2* polymorphisms in workers exposed to prolonged loud noise.**

Methods: **We enrolled 94 male workers from an aircraft factory in the study. The *SOD2* gene was screened by denaturing reversed-phase HPLC, and the *PON1* (Q192R and M55L) and *PON2* (S311C) polymorphisms were analyzed by PCR amplification followed by digestion with restriction endonucleases.**

Results: **Three known (A16V, IVS3-23T/G, and IVS3-60T/G) and two new *SOD2* polymorphisms (IVS1+8A/G and IVS3+107T/A) were identified. Regression analysis showed that *PON2* (SC+CC) [odds ratio (OR) = 5.01; 95% confidence interval (CI), 1.11–22.54], *SOD2* IVS3-23T/G and IVS3-60T/G (OR = 5.09; 95% CI, 1.27–20.47), age (OR = 1.22; 95% CI, 1.09–1.36), and smoking (OR = 49.49; 95% CI, 5.09–480.66) were associated with NIHL. No association was detected for *PON1* (QQ+RR) and *PON1* (LL) genotypes.**

Conclusions: **Our data suggest that *SOD2* and *PON2* polymorphisms, by exerting variable local tissue antioxidant roles, could predispose to NIHL. However, caution should be exercised in interpreting these data given the small sample size and the difficulty in matching cases to controls regarding the overwhelming risk factor, i.e., smoking at least 10 cigarettes/day.**

Cochlear epithelium damage attributable to noise is a major cause of permanent hearing loss in industrial workers. It has been demonstrated in experimental systems that noise, by inducing the local release of free radicals, may damage the cochlear sensorial epithelium *(1)*. Consequently, genes involved in the regulation of reactive oxygen species, such as superoxide dismutase (*SOD*)[6] genes, may affect the vulnerability of the cochlea to noise-induced hearing loss (NIHL) *(2)*.

SOD enzymes catalyze the conversion of superoxide radicals to hydrogen peroxide: manganese SOD within the mitochondrion, copper-zinc SOD in the cytosol, and extracellular SOD in the extracellular compartment *(3)*. Manganese SOD is a homotetramer, and each of its subunits is encoded by the *SOD2* gene on chromosome 6q25. The gene spans five exons and produces a 222-amino acid protein whose first 24 amino acids represent the mitochondrial targeting sequence *(4, 5)*. A limited number of polymorphisms have been identified in the *SOD2* gene, C47T being the most widely studied. C47T is located at position 16 in the mitochondrial targeting

[1] Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II and CEINGE scarl, Napoli, Italy.
[2] Dipartimento di Neuroscienze e Scienze del Comportamento, Università di Napoli Federico II, Napoli, Italy.
[3] Dipartimento di Scienze e Tecnologie per l'Ambiente e il Territorio, Facoltà di Scienze MM FF NN, Università del Molise, Isernia, Italy.
[4] Fondazione Salvatore Maugeri, IRCCS Istituto di Campoli Telese, Benevento, Italy.
[5] Alenia Aereonautica, Stabilimento Pomigliano D'Arco, Napoli, Italy.
* Address correspondence to this author at: Dipartimento di Biochimica e Biotecnologie Mediche, Università di Napoli Federico II and CEINGE scarl, via S. Pansini 5, 80131 Napoli, Italy. Fax 39-081-7462404; e-mail sacchetti@dbbm.unina.it.

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[6] Nonstandard abbreviations: SOD, superoxide dismutase; NIHL, noise-induced hearing loss; DHPLC, denaturing reversed-phase HPLC; PON, paraoxonase; TRAP, total radical-trapping antioxidant plasma; dB, decibels; OR, odds ratio; and CI, confidence interval.

sequence and causes the replacement of an alanine with a valine (A16V) *(3)*. It has been studied in association with various diseases, with discordant results *(6–9)*. To date, three intronic, non-disease-related *SOD2* polymorphisms have been detected *(10)*.

To screen the PCR products of the *SOD2* gene, we developed a denaturing reversed-phase HPLC (DHPLC) procedure that is highly sensitive (96%) and less expensive than the reference method of direct sequencing *(11)*. An additional advantage of the DHPLC procedure is that the post-PCR analysis can be automated, thereby saving time.

Paraoxonases (PONs) exert antioxidant activity and may protect against diseases such as atherosclerosis, diabetes, Alzheimer dementia, and Parkinson disease *(12–16)*. The *PON* gene family consists of *PON1*, *PON2*, and *PON3* on chromosome 7q21-q22 *(17)*. *PON1* and *PON3* are closely associated with apolipoprotein A-1 in HDL and may enhance its antiatherosclerotic properties *(18)*. *PON2* is ubiquitously expressed in tissues throughout the body *(19)* and may exert its antioxidant effect at a cellular level. Polymorphisms have been detected at codons Q192R and M55L in the *PON1* gene and at codon S311C in the *PON2* gene *(20, 21)*. Rare *PON3* point mutations (<1%) have been detected in apparently healthy heterozygotes *(18)*. *PON1* (55) L, *PON1* (192) R, and more recently *PON2* (311) C variants have been implicated in the oxidative damage associated with the pathogenesis of neurodegenerative diseases such as Alzheimer disease and Parkinson disease *(22, 23)*.

The aim of this study was to determine whether susceptibility to NIHL is related to *SOD2*, *PON1*, and *PON2* polymorphisms in workers employed at the Alenia Aereonautica aircraft factory. We also investigated routine biochemical indices and total radical-trapping antioxidant plasma (TRAP) activity to test their association with hearing loss evaluated through audiometric tests.

Materials and Methods

PARTICIPANTS

The overall cohort consisted of 252 men from the Campania Region working at Alenia Aereonautica (Pomigliano D'Arco, Naples, Italy); the age range was 29–58 years. All men were exposed to sound pollution ranging from 61.2 decibels [dB (A)] to 98.0 dB (A). The following information was collected for all participants: medical histories, presence of metabolic diseases, and lifestyle and smoking habits [smokers (>10 cigarettes/day) and combined nonsmokers/light smokers (≤10 cigarettes/day) because the medical records did not distinguish light smokers from nonsmokers]. All participants underwent an audiometric examination. Exclusion criteria were presence of cardiovascular events, diabetes, hyperlipidemia, and unmeasurable audiometric data because of poor collaboration by the participant. The inclusion criterion was exposure to a mean (SD) noise level equivalent to 92.4 (4.1) dB (A) for 20 years and use of the same noise-protection equipment. Ninety-four workers of 252, selected on the basis of the above stringent criteria, were enrolled in the study and underwent the genetic, biochemical, and audiometric analysis. The study was approved by the Ethics Committee of our Medical School, and informed consent was obtained from each individual.

AUDIOMETRIC EXAMINATION

Participants underwent otoscopy and tonal audiometric examination in a sound isolation cabinet. They were exposed to pure tones at 125, 250, 500, 1000, 2000, 3000, 4000, 6000, and 8000 Hz via earphones (air conduction) and pure tones at 250, 500, 1000, 2000, 3000, and 4000 Hz via a vibrator pressed against the mastoid portion of the temporal bone (bone conduction). The faintest pure tone that an individual could hear at each frequency was plotted on a graph (audiogram), and the hearing level was established. Normal hearing was diagnosed as follows: hearing any tone ≤25 dB, according to the Occupational Safety and Health Administration [46 FR 4078 (1981a) and January 1, 2003 (66 FR 52031–52034)]. According to audiometric results, 63 individuals had NIHL and 31 had normal hearing. Fig. 1 shows the mean audiograms of both groups.

DNA ANALYSIS

Genomic DNA was extracted from peripheral blood samples by standard procedures *(24)*. The *SOD2* gene exons, including intron/exon junctions, of each DNA sample were amplified with PCR using five primer pairs designed based on the human *SOD2* sequence (EMBL accession no. S77127). The PCR products were screened with a DHPLC procedure (Wave System 3500; Transgenomic) devised in our laboratory. The primer sequences, PCR product sizes, PCR annealing temperatures, and DHPLC conditions are listed in Table 1. The primers for exon 2 are described elsewhere *(8)*. In each run, six control samples (one wild-type and five bearing polymorphisms) were tested together with the DNA of the study participants. The control DNAs had been typed previously by sequence analysis with fluorescent dye-terminator cycle sequencing on an automated sequencer (ABI 373A; Applied Division, Perkin-Elmer).

Here we use the den Dunnen and Antonarakis nomenclature *(25)* for *SOD* polymorphisms. The *PON1* polymorphisms Q192R and M55L and the *PON2* polymorphism S311C were determined by PCR amplification followed by digestion with restriction enzymes *Alw*I, *Nla*III, and *Dde*I, respectively *(20, 21)*. The PCR products were resolved on a 4% metaphor gel and visualized by staining with ethidium bromide. *PON* genotypes were assessed independently by two observers. In each *PON* polymorphism, three control DNA samples (preliminarily verified by sequence analysis) were tested at the same time as the DNA samples from study participants.

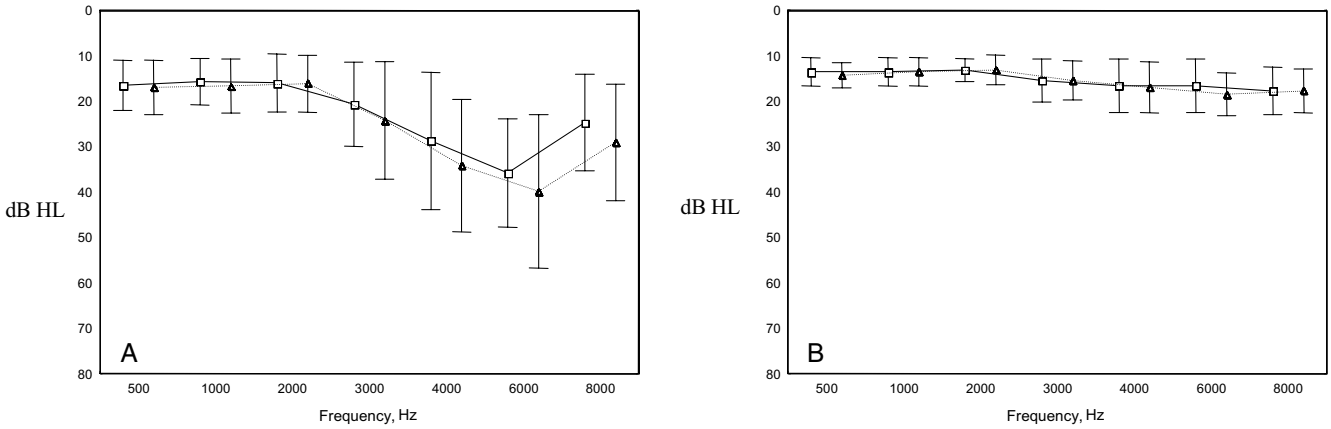

Fig. 1. Mean hearing threshold (*dB HL*) and SD (*error bars*) in individuals with hearing loss (*A*) and in individuals with normal hearing (*B*), evaluated by audiometric examination in 94 workers exposed to similar levels of sound pollution [mean (SD) = 92.4 (4.1) dB (A)] for 20 years. Normal hearing was diagnosed at this cut-point: hearing any tone ≤25 dB. □, right ear; △, left ear.

samples and biochemical measurements

Venous blood was sampled from all participants after an overnight fast. Serum total cholesterol, triglycerides, and glucose were measured enzymatically with a standard technique on an automated analyzer (Hitachi 747; Boehringer Mannheim). The HDL-cholesterol concentration was determined enzymatically by measuring cholesterol in the supernatant after precipitation with phosphotungstate. LDL-cholesterol was calculated according to the Friedewald formula *(26)*.

TRAP activity was measured with a spectrophotometric end-point method on a Cobas centrifugal analyzer (Hoffmann-La Roche) *(27)*. The synthetic water-soluble tocopherol analog Trolox™ (Hoffmann-La Roche) was used for calibration. The intra- and interassay imprecision coefficients (CVs), evaluated on a plasma pool, were 1.7% and 3.2%, respectively. TRAP activity is indicative of the antioxidant defense of plasma against free radicals and is based particularly on albumin, urate, ascorbate, bilirubin, α-tocopherol, and β-carotene.

statistical analysis

Allele frequencies were calculated by allele counting, and the departure from Hardy–Weinberg equilibrium was evaluated by χ^2 analysis. Linkage disequilibrium between the different polymorphisms in each *PON1* and *SOD2* gene was evaluated, and its significance was tested by the Fisher test. Associations of the *PON1*, *PON2*, and *SOD2* gene polymorphisms with continuous variables were tested by one-way ANOVA, and with categorical variables by the χ^2 test. Bonferroni correction for multiple comparisons was performed when required *(28)*. To check for risk genotypes, we combined *PON1* (55) (M/M+M/L) vs L/L, *PON1* (192) (Q/Q) vs (Q/R+R/R), and *PON2* (311) (S/S) vs (S/C+C/C) to obtain groups of similar size. We assessed heterozygosity vs homozygosity for each *SOD2* intronic polymorphism. For the A16V *SOD2* polymorphism, we combined the V/V vs (AA+AV) risk genotypes.

We used logistic regression analysis to compare the NIHL group vs the control group for biochemical indices, age, smoking, and genotypes and calculated the odds ratios (ORs) and the 95% confidence intervals (95% CIs). To test whether the 0% non-/light smokers in the control group affected the data, we removed smokers from the NIHL group and compared the genotypes in the two groups (controls and NIHL, both non-/light smokers) by logistic regression analysis. We then forced the statistics by including a fictitious smoker among the controls and again compared the genotypes and other variables in the

Table 1. PCR and DHPLC conditions for *SOD2* gene screening.

	PCR conditions				
	Primers, 5′-3′				
Exon	Forward	Reverse	Annealing temperature, °C	Product size, bp	DHPLC temperatures, °C
1	5′-TTCGGCAGCGGCTTCAG-3′	5′-TGCGGCCACTGTGGCCATTG-3′	62	167	65, 66, and 67
2	5′-CAGCCCAGCCTGCGTAGA-3′[a]	5′-TATGGGGCCTGGCTCCCT-3′	65	335	64 and 65
3	5′-CTGGTCCCATTATCTAATAGCTTAC-3′	5′-ATCACTTGAACCCAGGAAGC-3′	53	418	57 and 58
4	5′-TGTTGTCTAATTTCTTGGGCC-3′	5′-AAGACTCTGGGTGTTATCTGTTAAG-3′	52	434	56 and 57
5	5′-AAAGTTGAAATTGAGAAGATGC-3′	5′-GCTTAACATACTCAGCATAACG-3′	50	250	56, 57, and 58

[a] As described elsewhere *(8)*.

two groups (controls including a smoker and NIHL including both smokers and non-/light smokers) by logistic regression analysis. Statistical analyses were performed with the SPSS for Windows software (Ver. 11.0).

Results

There were no statistically significant differences between NIHL and control individuals regarding biochemical findings (Table 2), although total cholesterol, HDL, and LDL were slightly higher in NIHL individuals than in controls. However, the NIHL group was older ($P < 0.001$) and included a significantly higher percentage of smokers ($P < 0.001$).

The genotype distributions of the *PON1* M55L and Q192R and *PON2* S311C polymorphisms in the whole cohort and in the NIHL and control individuals are shown in Table 3. The relative frequencies of the *PON1* Q192R polymorphism did not differ significantly between NIHL individuals and controls, whereas the *PON1* (55) L allele was more frequent in NIHL individuals than in controls ($P = 0.005$), and *PON2* (311) (C/C) was present only in NIHL individuals.

The screening of the *SOD2* gene by DHPLC revealed three known polymorphisms (A16V, IVS3-23T/G, and IVS3-60T/G) and two novel polymorphisms (IVS1+8A/G and IVS3+107T/A). Their relative frequencies did not differ between NIHL and control individuals (Table 4). The genotype distributions of the *PON1*, *PON2*, and *SOD2* polymorphisms evaluated in the study cohort (n = 94) and in individuals with normal hearing (n = 31) were in Hardy–Weinberg equilibrium. Despite a significant linkage disequilibrium ($P = 0.003$), which favored the simultaneous presence of the R and L alleles, there was no statistically significant association between the different *PON1* 55/192 haplotypes and NIHL, except for the MMQQ haplotype, which was not found in NIHL individuals ($P = 0.001$). Concerning *SOD2*, linkage disequilibrium was found between *SOD2* IVS1+8A/G and *SOD2* A16V ($P = 0.003$); *SOD2* A16V and *SOD2* IVS3+107T/A ($P < 0.001$); *SOD2* IVS3+107T/A and *SOD2* IVS3-23T/G, IVS3-60T/G ($P < 0.001$); and *SOD2* A16V and *SOD2* IVS3-23T/G, IVS3-60T/G ($P < 0.001$), which favored the simultaneous presence of A alleles of both *SOD2* IVS1+8/*SOD2* 16 and *SOD2* 16/*SOD2* IVS3+107, and the A allele of *SOD2* IVS3+107 with the G allele of *SOD2* IVS3-23/*SOD2* IVS3-60. However, there were no significant associations between the different *SOD2* haplotypes and NIHL.

ANOVA did not reveal any significant associations between biochemical markers and *PON1*, *PON2*, or *SOD2* polymorphisms in either NIHL individuals or controls (data not shown). To eliminate the effects of age and

Table 3. Genotype distributions of *PON1* (55), *PON1* (192), and *PON2* (311) polymorphisms in the enrolled cohort.

	Total cohort	Individuals with normal hearing	NIHL individuals
PON1 (55)			
n	91	30	61
Genotype, n (%)			
L/L	42 (46)	12 (40)	30 (49)
M/L	44 (48)	13 (43)	31 (51)
M/M	5 (6)	5 (17)	
P		0.005	
PON1 (192)			
n	91	30	61
Genotype, n (%)			
Q/Q	40 (44)	14 (47)	26 (43)
Q/R	34 (37)	13 (43)	21 (34)
R/R	17 (19)	3 (10)	14 (23)
P		0.315	
PON2 (311)			
n	89	28	61
Genotype, n (%)			
S/S	60 (67)	21 (75)	39 (64)
C/S	25 (28)	7 (25)	18 (29)
C/C	4 (5)		4 (7)
P		0.313	

Table 2. Physical and biochemical characteristics of the enrolled cohort.

Characteristics	Total cohort[a] (n = 94)	Individuals with normal hearing (n = 31)	NIHL individuals (n = 63)
Mean (SD) age, years	43 (7)	39 (5)	46 (7)[b]
Mean (SD) glucose, mmol/L	5.04 (1.00)	4.90 (1.07)	5.11 (0.96)
Mean (SD) urea, mmol/L	6.35 (1.30)	6.42 (1.42)	6.32 (1.25)
Mean (SD) creatinine, mmol/L	76.98 (8.15)	77.48 (7.65)	76.73 (8.43)
Mean (SD) total cholesterol, mmol/L	5.08 (1.00)	4.82 (0.99)	5.21 (0.99)
Mean (SD) HDL-cholesterol, mmol/L	1.02 (0.22)	0.98 (0.20)	1.04 (0.22)
Mean (SD) LDL-cholesterol, mmol/L	3.25 (0.87)	3.06 (0.78)	3.34 (0.90)
Mean (SD) triglycerides, mmol/L	1.78 (0.89)	1.71 (1.03)	1.81 (0.82)
Mean (SD) TRAP, mmol/L	1.29 (0.05)	1.29 (0.05)	1.29 (0.05)
Smokers (>10 cigarettes/day), %	34	0	51[c]
Non-/light smokers (≤10 cigarettes/day), %	66	100	49

[a] All participants were exposed to a mean (SD) noise level of ~92.4 (4.1) dB (A) for 20 years.
[b] $P < 0.001$ vs controls by *t*-test.
[c] $P < 0.001$ vs controls by χ^2 test.

Table 4. Genotype distributions of *SOD2* IVS1+8A/G, A16V, IVS3+107T/A, IVS3-23T/G, and IVS3-60T/G polymorphisms in the enrolled cohort.

	Total cohort	Individuals with normal hearing	NIHL individuals
SOD2 IVS1+8A/G			
n	93	30	63
Genotype, n (%)			
A/A	50 (54)	14 (47)	36 (57)
G/G	7 (7)	1 (3)	6 (10)
G/A	36 (39)	15 (50)	21 (33)
P		0.234	
SOD2 A16V			
n	90	29	61
Genotype, n (%)			
A/A	18 (20)	4 (14)	14 (23)
A/V	52 (58)	19 (65)	33 (54)
V/V	20 (22)	6 (21)	14 (23)
P		0.519	
SOD2 IVS3+107T/A			
n	93	31	62
Genotype, n (%)			
A/A	33 (35)	9 (30)	24 (38)
T/A	46 (50)	16 (53)	30 (48)
T/T	14 (15)	5 (17)	9 (14)
P		0.746	
SOD2 IVS3-23T/G; IVS3-60T/G			
n	93	30	63
$T^{-23}G^{-60}/T^{-23}G^{-60}$, n (%)	28 (30)	10 (32)	18 (29)
$T^{-23}G^{-60}/G^{-23}T^{-60}$, n (%)	47 (51)	11 (39)	36 (57)
$G^{-23}T^{-60}/G^{-23}T^{-60}$, n (%)	18 (19)	9 (29)	9 (14)
P		0.109	

smoking, we tested by logistic regression analysis, after adjustment for these confounding factors, the association between *PON1*, *PON2*, and *SOD2* genotypes, biochemical indices, and NIHL. As shown in Fig. 2, *PON2* (SC+CC) genotypes (OR = 5.01; 95% CI, 1.11–22.54), *SOD2* IVS3-23T/G, IVS3-60T/G (OR = 5.09; 95% CI, 1.27–20.47), age (OR = 1.22; 95% CI, 1.09–1.36), and smoking (after the inclusion of a fictitious smoker among the controls; OR = 49.49; 95% CI, 5.09–480.66) were associated with NIHL. After exclusion of smokers from the NIHL group, *PON2* and *SOD2* polymorphisms were still significantly associated with hearing loss, as evaluated by logistic regression analysis: *PON2* (SC+CC) genotype (OR = 5.00; 95% CI, 1.10–22.72) and *SOD2* IVS3-23T/G, IVS3-60T/G genotype (OR = 5.10; 95% CI, 1.20–20.40).

Discussion

Here we show that *PON2* (SC+CC) genotypes and the IVS3-23T/G, IVS3-60T/G *SOD2* polymorphisms are associated with NIHL irrespective of age and smoking habits. The ubiquitously produced PON2 acts as an antioxidant enzyme; thus, its overproduction is capable of lowering the oxidative state of cells induced by hydrogen peroxide *(19)*.

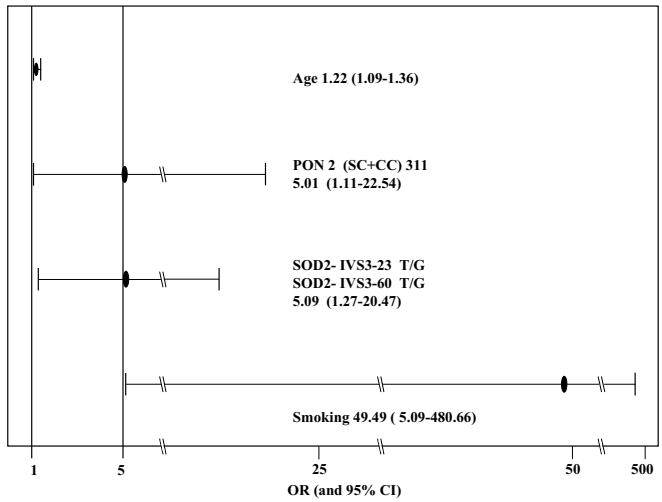

Fig. 2. ORs (*filled ovals*) and 95% CIs (*error bars*) obtained by logistic regression analysis of NIHL and *PON2* (S/C) and *SOD2* IVS3-23 T/G and IVS3-60 T/G polymorphisms, age, and smoking in the 63 NIHL individuals and in the 31 individuals with normal hearing after the inclusion of a fictitious smoker.

Our data showing that the *PON2* C allele is associated with NIHL (OR = 5.01; 95% CI, 1.11–22.54) suggest a genetic predisposition to this disorder. The pathogenesis of NIHL may involve the release of oxygen species consequent to chronic exposure to high sound levels that may damage Corti's organ. In fact, exposure to noise in animal models appears to increase the concentrations of superoxide radicals in the cochlear fluid as well as in the stria vascularis *(1, 29, 30)*. Thus, also in humans, Corti's ciliated cells might be damaged by local release of free radicals, which in turn may lead to a neurosensorial hearing loss, particularly in individuals bearing the *PON2* C allele. Our data are in agreement with the association between the *PON2* S311C polymorphism and Alzheimer dementia, a neurodegenerative disease in which oxidative stress may play an important role *(23)*. Concerning PON1, a HDL-associated enzyme produced mainly in the liver that plays a major role in such diseases as atherosclerosis *(12, 13)*, we did not detect any significant association between the *PON1* Q192R polymorphism and NIHL, and the significant increase of the *PON1* (55) (LL) genotype in NIHL ($P = 0.005$) disappeared after adjustment for age and smoking. These data, and the lack of differences in lipid indices and antioxidant status between NIHL and control individuals, indicate that atherosclerosis is not involved in the pathogenesis of NIHL.

In the rat cochlear labyrinth, the SOD2 enzyme protects against damage caused by free radicals *(31)*. Furthermore, SOD2-knockout mice have enhanced susceptibility to alterations caused by other mitochondrial enzymes and to diseases resulting from increased concentrations of mitochondrial reactive oxygen species *(32)*. We detected three known (A16V, IVS3-23T/G, and IVS3-60T/G) and two novel (IVS1+8A/G and IVS3+107T/A) polymorphisms

in the *SOD2* gene in our cohort. The frequencies of *SOD2* A16V genotypes matched those reported for other Caucasian populations *(6)* and did not differ between NIHL and control individuals. Similarly, this polymorphism is unrelated to degenerative diseases such as Parkinson disease *(9, 33)* and amyotrophic lateral sclerosis *(8)*. In contrast, the *SOD2* A16A genotype is associated with increased breast cancer risk *(6)*, a high degree of carotid atherosclerosis *(7)*, and to exudative age-related macular degeneration in Japanese individuals, in whom allele A occurs less frequently than in Caucasians *(34)*.

Finally, although it has been reported that A16A homozygotes may have higher SOD2 activity than V16V homozygotes *(35)*, in our cohort the number of individuals bearing the A allele did not differ between NIHL and control individuals. Thus, this polymorphism does not appear to influence susceptibility to noise-induced damage.

The heterozygous frequency of the two novel polymorphisms IVS1+8A/G and IVS3+107T/A did not differ between NIHL and control individuals; however, these polymorphisms could provide a tool for investigating other SOD2-related diseases and for linkage disequilibrium mapping. *SOD2* polymorphisms IVS3-23T/G and IVS3-60T/G showed a high heterozygosity, and their genotype frequencies were similar to those reported previously *(8)*. They were clearly associated with NIHL (OR = 5.09; 95% CI, 1.27–20.47); however, given their intron localization, it is unlikely that they are involved in the development of NIHL and they may function, instead, as markers that are in linkage disequilibrium with other polymorphisms.

Concerning the effect of smoking on NIHL, it has been reported that smokers have a greater risk of hearing loss than nonsmokers *(36, 37)*. Recently, Palmer et al. *(38)* reported an additive rather than a multiplicative interaction between smoking and noise exposure. They concluded that although workers exposed to long-term noise should be discouraged from smoking, the extra risk of smoking on hearing loss, in environments where noise levels are significant, is small relative to that of noise itself.

In conclusion, the association of *PON2* and *SOD2* polymorphisms with neurosensorial hearing could represent a marker of susceptibility to NIHL independent of the smoking effect, although the risk associated with smoking was surprisingly large in our study.

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