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Antioxidant Polymorphisms and Susceptibility to Solvent- Induced Hearing Loss in Factory Workers

Robert Udell Glazier
Yale University

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Robert Udell Glazier, Jr.

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ABSTRACT

ANTIOXIDANT GENE POLYMORPHISMS AND SUSCEPTIBILITY TO SOLVENT-INDUCED HEARING LOSS IN FACTORY WORKERS.

Robert U. Glazier, Peter M. Rabinowitz, and Martin D. Slade. Department of Occupational and Environmental Health, Yale University, School of Medicine, New Haven, CT.

Occupational exposure-related hearing loss is a significant health concern for affected workers. Organic solvent exposure has emerged as an important contributor to hearing loss. It is thought that hearing loss related to solvent and noise exposure is mediated by reactive oxygen species (ROS). The glutathione associated enzymes and the manganese superoxide dismutase enzymes (SOD2) are important components of the cochlear hair cell's defense against oxidative stress. This study is aimed to determine whether polymorphisms within the glutathione S-transferases (GST) P1 and GSTM1, glutathione peroxidase 1 (GPX1), and SOD2 are associated with hearing status in solvent exposed factory workers. Genotypes for the GSTM1 + vs. null, GSTP1 Ile105Val, GPX1 Pro198Leu, SOD2 Val16Ala polymorphisms along with hearing status were determined in factory workers exposed to organic solvents. Hearing tests consisted of pure tone audiometric (PTA) thresholds from 3-6 kHz and distortion product otoacoustic emissions (DPOAEs) for 3-6 kHz. Bivariate and multivariate regression analysis was undertaken to assess for association between polymorphisms and hearing outcomes. The GSTP1 Val/Ile genotype at position 105 was associated with higher PTA thresholds (β=12.41, P value= 0.01) from 3-6 kHz in workers below age 22-43. The analysis showed a protective association of the SOD2 Ala/Val genotype (β= -26.42, P value= 0.025) and The GPX1

Leu/Leu genotypes (β=47.81, P value= 0.034) with audiometric thresholds from 3-6 kHz in individuals above age 43. This small cross-sectional study suggests that polymorphisms within the antioxidant system may alter susceptibility to hearing loss in workers exposed to organic solvents. These results also suggest the mechanisms by which this affect are mediated are complex and should be further investigated.

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INTRODUCTION

Sensorineural Hearing Loss as Public Health Issue

It is reported that 28 million Americans suffer from sensorineural hearing loss[1]. Similarly, worldwide hearing impairment is reported to affect more than 10 percent of the adult population of industrialized nations[2]. Beyond the difficulty of day-to-day living for sufferers, hearing impairment carries with it significant socioeconomic implications for the afflicted and significant costs to national economies. Studies show that the hearing-impaired are much less likely to be employed and earn less than their normal-hearing counterparts[3]. The economic impact of hearing impairment represents a significant portion of the 156 to 187 billion dollars spent in the United States annually on communication disorders[3].

Mechanisms of Sensorineural Hearing Loss

It is also reported that at least 10 million of those suffering hearing deficits may be affected due to elevated noise exposure of an occupational or recreational origin[1]. There is a growing body of evidence that suggests that multiple environmental and genetic factors may play a role in the development of sensorineural hearing loss. Along with noise exposure within the occupational setting, chemical exposure to organic solvents has emerged as an important suspected cause of clinically significant hearing loss[4].

Both solvents and noise are thought to mediate hearing loss by the generation of reactive oxygen species (ROS)[5, 6]. In the process of sound transduction, the stria vascularis must continually maintain an electrochemical gradient between the endolymph and the outer hair cells via ion pumps; maintenance of this gradient and the tuning of the basilar

membrane by the outer hair cells are energy demanding processes[7]. Normal aerobic metabolism results in the generation of the highly reactive superoxide anion radical and subsequent derived reactive oxygen species. The ROS generated under levels sub-pathologic sound stimulation are normally adequately handled by the cells' natural antioxidant system[8, 9] (see **Brief Review of Radical Generation and Handling** below). However, when noise intensity reaches pathologic levels, acoustic overstimulation leads to an imbalance between ROS generation and handling resulting in a disruption of cochlear homeostasis. As described by Kopke et al., initial increases in sound are met with an increase in uptake of glucose and in cochlear tissues and a 20% increase in perilymphatic oxygenation. Further increase in sound leads to an unexpected decrease in endolymph oxygenation, cochlear blood flow, and glucose uptake[6]. This described radical generation and decrease in circulation was observed by Yamane et al. in 1999 when guinea pigs were exposed to 3 hours of "rock-n'-roll" music at 120-125 dB sound pressure level (SPL); elevated superoxide levels in the marginal cells, and empty capillaries within the stria vascularis were observed at 5 minutes post-exposure[10].

Direct cellular damage and ROS generation in noise exposed outer hair cells were first observed and reported in 1999[11]. Nicotera et al. utilized dichlorofluorescein (which stains for reactive oxygen species and/or the peroxynitrite radical (ONOO-)) and found evidence of elevated ROS in the nuclear region of the outer hair of chinchillas exposed to impulse and continuous noise[11]. Furthermore, staining of these noise exposed chinchilla cochleae with propidium iodine and acridine orange revealed hair cell death by apoptosis and necrosis[11]. Evidence for the observed ROS as a cause, rather than the result, of

cellular damage emerged in 1996[12] and 2005[13]. When superoxide generating compounds were infused into the perilymph it led to a decrease in the compound action potential sensitivity (a measure of physiologic response to sound)[12] , permanent threshold shifts (PTS) [13], and outer hair cell death[13].

Organic Solvents and Hearing Loss

Within the realm of hearing conservation programs in the industrial setting, noise has been the primary exposure of concern. Evidence in recent decades has led to the identification of neurotoxic agents commonly encountered in many workplaces that may be contributing to occupational hearing loss[14]. Organic solvents are but one class of these agents common in many workplaces that have proved ototoxic in animal and human studies and have been the subject of focused investigation[15-23].

Pryor et al. and Rebert et al. first reported of an association between solvent exposure and hearing loss in animals in 1983[15, 23]. A review of previous case reports and a case series of Scandinavian workers who were exposed to noise and solvents with hearing thresholds much worse than would have been predicted by noise exposure alone was published by Barregard and Axelsson in 1984[24]. In 1986, a 20-year longitudinal study of 319 workers in a Swedish timber processing factory was published by Bergstrom and Nystrom[25]. When the workers in the chemical department of the company who were exposed to lower levels of noise (80-90dBA) were compared to workers outside the chemical department where noise exposure was higher (95-100dBA), it was discovered that 23% of chemical workers demonstrated pronounced hearing loss while 5-13% of workers from areas outside the chemical area of the plant exhibited hearing loss.

Subsequently, the direct ototoxic effects of solvents in animals and humans have been well confirmed[14, 16-20, 22, 26-30]. Most recently, Fuente et al. reported a negative effect of solvent exposure on measured pure tone audiometric thresholds in factory workers[31].

In addition to these studies that have shown organic solvents experimentally to be ototoxic alone, there is some evidence solvents may synergistically interact with noise to potentiate sensorineural hearing loss[32-41]; this relationship is significant as many workplaces in which there are noise levels warranting a hearing conservation program also expose workers to potentially ototoxic levels of solvents. It has also been suggested that solvent exposure and concomitant noise exposure may be an unrecognized risk, even when exposure levels are below standards set by the Occupational Safety and Health Administration (OSHA) and American Conference of Governmental Industrial Hygienists (ACGIH)[42].

Organic Solvents and Reactive Oxygen Species

The full extent of the mechanisms by which solvents induced cochlear pathology is an area of active investigation. Importantly, there is evidence that some organic solvents can contribute to oxidative stress and may potentiate the effects of noise on hearing in this way[43-47]. The organic solvents to which the workers in our study population are exposed in this study are: methyl ethyl ketone (MEK), toluene, and xylene. McDermott et al. demonstrated that cells in culture exposed to MEK, toluene, and xylene have increased levels of membrane permeability, decreased reduced glutathione levels, and increased levels of cytosolic Ca^{++}[43]; these changes likely represent increased ROS burden. In vitro experiments of hair cells show a similar increase in intracellular free

Ca^{2+} when the cultures were exposed to acoustic trauma and mechanical disruption designed to mimic noise[48, 49]. There are several examples of Ca^{2+} mediated downstream effects of oxidative stress[50]. ROS have been shown to increase the levels of intracellular Ca^{2+} by interfering with the proteins responsible for Ca^{2+} transport out of the cell[50]. Alternatively, increased free cytosolic Ca^{2+} has been shown to increase ROS levels in cells expressing xanthine reductase[50]. Rising Ca^{2+} activates calpain enzymes that convert xanthine reductase into xanthine oxidase, which in turn generates O_2^- radicals[50].

Animal model studies have yielded similar increases in intracellular free Ca^{2+} concentration in rat cochlea hair cells when exposed to levels of toluene similar to those expected in workers exposed at or below recommended occupational limits[51, 52]. In rat studies, Mattia et al. reported an elevation of ROS in the livers and central nervous system[46, 47] in animals exposed to toluene; xylene exposure led to reduced levels of hepatic reduced GSH[53].

Evidence of solvent-induced oxidative stress in humans is reported by Halifeoglu et al.; workers exposed to paint thinner (containing toluene) showed increased plasma malondialdehyde (a marker of lipid peroxidation) and elevated GPX and SOD enzyme levels compared to contols[44]. The suggested implication of the effect of ROS generated by these solvents is bolstered by the fact that toluene and xylene have exhibited direct ototoxicity in several animal studies[16, 17, 26, 30, 54, 55] and that ROS in cochlear tissues have been repeatedly been shown to affect hearing[12, 56].

While the delivery route of these organic solvents has not been definitively proven, Campo et al. showed that rats treated with toluene via inhalation had toluene

contaminated organs of Corti, suggesting direct tissue toxicity[19]. Taken with the fact that the outer hair cells are the most susceptible to solvent-induced injury, these results point to a hematogenous route of intoxication from the stria vascularis via the outer sulcus and lipid rich membranes composed of Hensen's cells and Dieters cells, onto the outer hair cells[19].

Brief Review of Radical Formation and Handling

The cellular antioxidant system is composed of numerous enzymes and substrates which act to catalyze the detoxification and conjugation of ROS. Within the cochlear tissues, animal studies have identified the presence of two major pathways composed of three classes of antioxidant system enzymes: The superoxide dismutase (SOD) and catalase enzymes[6] and the glutathione (GSH) related enzymes[57, 58].

Superoxide (O_2^-) is generated by the reduction of oxygen in aerobic respiration and other normal cellular reactions (e.g. xanthine oxidase action). The superoxide dismutase (SOD) family of antioxidant enzymes convert the generated superoxide anion into H_2O_2 and O_2 ($2O_2^- + 2\ H \rightarrow H_2O_2 + O_2$)[50]. Site specific haplotypes of SOD reside and function within the mitochondria, cytoplasm, and extracellular space that serve this function only within their respective compartments: manganese SOD (MnSOD or SOD2) in the mitochondria, copper/zinc SOD (CuZnSOD or SOD1) in the cytoplasm, and extracellular SOD (ECSOD or SOD3)[59]. The catalase (CAT) enzymes present in cellular peroxisomes act to convert the H_2O_2 generated by SOD into O_2 and H_2O ($2H_2O_2 \rightarrow O_2 + H_2O$)[50].

Reactions of unreduced generated superoxide and intermediate ROS such as H_2O_2 with susceptible cellular molecules also occur that initiate self perpetuating free radical damage to cellular structures such as peroxidation of membrane lipids. This oxidative stress has detrimental effects on energy production and membrane permeability as well as downstream signaling events that can initiate programmed cell death.

The glutathione peroxidases (GPX) are a class of selenium dependant enzymes which catalyze the breakdown of H_2O_2 by reduction and the formation of conjugated glutathione (GSSG) from reduced glutathione (GSH) ($H_20_2 + 2GSH \rightarrow GSSG + 2H_2O$)[60]. The ubiquitously expressed glutathione peroxidase 1 (GPX1) has a single selenocysteine residue on each of its four identical subunits and is one of the most important antioxidant enzymes in humans.

Glutathione-S-transferase (GST) enzymes catalyze the conjugation of electrophilic radicals to the sulfhydryl residues of reduced glutathione (GSH + Xradical → GSradical +HX) [61] enzymes, thus neutralizing free radical species and halting free radical chain reaction damage. These radicals include endogenous species as well as xenobiotic molecules.[62] Epoxides, activated alkenals, and organic hydroperoxides that result from oxidative metabolism are regarded as the major "natural" substrates of the GST.[62]

Solvents, Reactive Oxygen Species, and Genetic Polymorphisms

With the available evidence that noise-induced and the likelihood that solvent-induced hearing loss are both mediated by ROS, it would follow that the cellular antioxidant molecules would play an important role in modifying the effects of noise and solvents in

cochlear pathology. The antioxidant enzymes GST, GPX, and SOD all play a role in the detoxification of ROS. Indeed animal studies have shown that knockout mice expressing GPX1-/-[63] and SOD1-/-[64] were more susceptible to noise-induced hearing loss (NIHL) than wild type controls. In the studies of toluene cited above there was also an observed decrease of GSSH in tissues showing increased ROS[46]. A similar decrease in GSSH/GSSG was observed in in vitro assays of cells treated with MEK, toluene, and xylene[43]. Depending on the mechanisms of solvent detoxification, activation, and conjugation, a gain or loss of function in enzymes catalyzing these steps could lead to elevated or diminished toxicity. Studies have shown that genetic polymorphisms in genes responsible for solvent metabolism, ROS generation, and antioxidant function can modify risk for developing chronic solvent encephalopathy (CSE)[65]. Genetic polymorphisms for CYP2E1, EPHX1, and GSTP1 modified risk of developing CSE in organic solvent-exposed workers when compared to non-exposed controls[65]. A similar study showed that GSTM1 null individuals that smoked tobacco were at an elevated risk for CSE; this elevated risk was not seen among non-smoking GTSM1 null individuals[66].

SOD2 and Sensorineural Hearing Loss

Much of the previous investigation into superoxide handling and hearing status has centered on the cytosolic SOD1. SOD1 has been shown in several animal studies to be associated with noise- and age-related hearing loss[64, 67-69]. Studies in which targeted deletions of SOD1 have been performed in mice have shown an increased susceptibility to presbycusis[68, 69] and noise-induced hearing loss[64] in SOD1 knockouts. While deficiency of SOD1 predisposes to hearing loss, studies have shown that over-expression

has little or no protective effect against age- and noise-induced hearing loss[69, 70]. Interestingly, one study in which transgenic C57BL/6-TgN_SOD1_3Cje mice over-expressing SOD1 were compared to non transgenic C57BL/6-TgN_SOD1_3Cje littermates showed no difference in auditory brainstem responses (ABR) after noise exposure. Cohorts of the same SOD1 normal and SOD1 over-expressing mice did not show any difference in ABR when aged 2-7months. However, the transgenic mice did show an increase in mitochondrial DNA-deletion levels (a marker for age-related damage) in acoustic nerve tissue; this led Coling et al. to suggest the role of SOD1 in ROS homeostasis may be more complex than previously appreciated or presumed.[70]

The SOD2 Val16Ala Single Nucleotide Polymorphism

As noted previously, SOD2 catalyzes the reduction of superoxide within the mitochondrion. The SOD2 enzyme is a homotetramer of identical subunits, each of which are encoded on chromosome 6q25. The gene encodes a 222 amino acid polypeptide of which the first 24 amino acids encode a mitochondrial targeting sequence[71, 72]. There are relatively few polymorphisms in the SOD2 gene, this is likely due to SOD2 occupying a critical role in the prevention of cellular oxidative damage[73]. One notable, and the most studied, SOD2 single nucleotide polymorphism (SNP) is the Val16Ala variant that occurs when T transitions to a C in base 47 within the mitochondrial signaling domain[74]. Compared to the Val genotype, more active SOD2 is translated from the Ala expressing alleles and these variants have been shown to be more efficiently transported into the mitochondrial matrix which may lead to higher levels of functional SOD2 in the mitochondria[75-77]. Interestingly, studies of both variant alleles

have suggested individual association with differing pathologies. The homozygous Val variant has been shown to be associated with non-familial idiopathic cardiomyopathy[75] and non-small cell lung carcinoma[78]. The Ala variant has been associated with motor neuron disease[79], Parkinson's disease[74, 80], increased shrinkage of grey matter in alcoholics[81], urolithiasis[82] breast cancer[83, 84], longevity[85], and prostate cancer [86].

GSTM1 and GSTP1 GPX1 Polymorphisms

Glutathione S-transferase P1 and M1 (GSTP1 and GSTPM1) have higher measured activities in the cochlea than in other sensorineural tissue[57]. It is possible that this high activity is an indication of the importance of their role in controlling oxidative damage in the cochlear tissues. It is reported that between 40-60% of the population lacks a functional copy of the GSTM1 gene[87-89]. This GSTM1 null genotype has been associated with elevated risk of lung cancer[90, 91], breast cancer (given alcohol consumption)[92], and colorectal cancer[93]. The GSTP1 Ile105Val polymorphism has been reported to affect the enzyme's substrate affinity and heat stability[94]. This variant also demonstrates increased catalytic efficiency with aromatic epoxides[95] and is reported to elevate risk for testicular and bladder cancer[96].

Glutathione peroxidase 1 (GPX1) is ubiquitously expressed in human cells and is elevated in metabolically active tissues. The Pro198Leu variant of GPX1 is not reported to have any diminished enzymatic activity[97] but has been identified with risk for lung[98] and breast[99] cancer as well as selenium deficiency, Keshan disease[100], thoracic aortic aneurism[101], prostate cancer (decreased risk in heterozygotes)[102], metabolic syndrome in Japanese men[103], hypertension[104], bladder cancer recurrance[105], and longevity[85].

Statement of Purpose of Specific Hypothesis and Specific Aims of the Thesis

The evidence for the role of ROS in the development of solvent-induced hearing loss (SIHL) begs the question whether such a relationship could be modified by genetic antioxidant factors as it has been demonstrated in NIHL. Identification of defined antioxidant genetic polymorphisms as risk factors for the development of SIHL would serve to support the reasoning that SIHL is mediated through ROS. Evidence of this type could also serve as an important tool in tailoring prevention strategies against SIHL in susceptible individuals.

To our knowledge, no study has been published to assess the effect that antioxidant gene polymorphisms may have in the development of sensorineural hearing loss in workers exposed to solvents. It is our hypothesis that polymorphisms of GSTM1, GPX1, GSTP1, and SOD2 in which functional studies have shown a change in activity or expression will be associated with hearing status in factory workers exposed to ototoxic solvents. We aim to detect any association by statistical analysis controlling for known hearing loss associated variables. We also aim to investigate the relationship between antioxidant gene polymorphisms and solvent-induced hearing loss to test if the ROS mediated mechanism of SIHL is supported by our analysis.

METHODS

This study was designed as a cross-sectional pilot study. A total of 93 (11 female and 82 male) industrial workers were recruited for the purposes of the study. All of the participants were employed full time at a factory that produced large rolls of

polyurethane coated fabric. The polyurethane is applied in a liquid form onto wide rolls of synthetic fiber fabrics. Workers from every work area were invited to participate as subjects in the study. Though workers from all areas in the factory participated, the majority of the workers at the plant that enrolled were production laborers.

Information detailing the purpose of the study as well as the nature of the study protocols was given to the participants in both written and oral form. Informed consent was obtained from all participants in keeping with the guidelines of the study protocol approved by the Yale Human Investigation committee. All subjects were assigned an anonymous numerical identifier and the coded key with subject's names was kept in a password-protected file on a network isolated computer in a locked room.

Genetic analysis was performed by Dr. Adam Wisnewski and members of his laboratory. Samples of whole blood were collected in yellow top acid-citrate-dextrose tubes. DNA was extracted and purified from 200 µl of whole blood using a commercial kit from Qiagen®. PCR was used to amplify all desired gene segments and products electrophoresed against a base pair standard in 3% agarose gels with ethidium bromide.

GTSM1 Analysis

A direct PCR was used to determine the presence (or absence) of the GSTM1 gene. The following 3 primers were used to simultaneously amplify GSTM1 and GSTM4 (as an internal positive control). GSTM1 5'-CGC CAT CTT GTG CTA CAT TGC CCG-3' 5'-ATC TTC TCC TCT TCT GTC TC-3' 5'- TTC TGG ATT GTA GCA GAT CA -3'. Amplification results in the following bands: 158bp band for GSTM4, and a 231bp band

for GSTM1, if present. PCR mixture consisted of: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.0 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 pM of each primer, 200-400 ng gDNA, 1.25 U Taq. Polymerase. PCR cycling conditions were 94°C 2min, (94°C 1min + 72°C 1min) × 30cycles, 72°C 7min.

GSTP1 Analysis

Identification of SNP at amino acid 105 which results in the replacement of the isoleucine with a valine was accomplished as described by Lin et al [106]. The primers 5'- GTA GTT TGC CCA AGG TCA AG -3' 5'- AGC CAC CTG AGG GGT AAG -3' and the same PCR mixture composition were used to amplify a 433bp band that was digested with 5U Alw 26I and run on a 3% agarose gel. The "wild type" allele (GSTP1 +/+) has one restriction cut site and yields a 329bp band and a 104bp band. On digestion, the polymorphic (null) allele (GSTP1 -/-) yields bands of 222bp, 107bp, and 104bp in length. Heterozygotes (GSTM +/-) showed all 4 bands: 329bp, 222bp, 107bp, and 104bp. PCR reagent composition were the same as for GSTM1. PCR cycling conditions: 94°C 2min, (94°C 1min + 72°C 1min) × 30cycles, 72°C 7min.

GPX-1 Analysis

The GPX SNP at codon 198 which changes a proline to leucine was detected using methods described by Hu et al[99]. The PCR primers 5'- TGT GCC CCT ACG CAG GTA CA -3' 5'- CCA AAT GAC AAT GAC ACA GG -3' flank the polymorphic region and yield a product of 337bp. The polymorphic leucine at codon 198 creates a site cleavable by Apa 1 restriction enzyme. Upon Digestion of DNA by Apa 1, homozygous wild type (GPX1 +/+) yield the single 337bp band. Homozygous polymorphic alleles (GPX -/-)

yield two bands at 79bp and 258bp. Heterozygous samples (GPX +/-) show all 3 bands: 79bp 258bp and 337bp. PCR reagent mixture was identical to that used in GSTM1 and GSTP1. PCR cycling conditions: 94°C 3min, (94°C 0.5min + +58°C 1min + 72°C 1.5min) × 35cycles, 72°C 10min.

SOD2 Analysis

Identification of the Val-16-Ala SNP (a T to C transition in the mitochondrial signaling domain) was effected by the methods previously described by Lin et al[106]. The primers used were: 5'- GCA CCA GCA GGC AGC TGG CGC CGG-3' and 5'- TGC GCG TTG ATG TGA GGT TCC AG -3'. A single base pair mismatch was introduced into the forward primer to create a Nae 1 site in the Alanine coding allele. The PCR products were purified through use of a PCR clean-up column to remove primer dimmers, and then digested with 5U Nae 1. The homozygous Val allele (SOD2 -/-) shows a band at 112bp. The heterzygotes (SOD2 +/-) show 3 bands at 112bp, 90bp, and 22bp. Homozygous Ala individuals (SOD2 +/+) only show 2 bands at 90bp and 22bp.

Hearing Tests

Data was collected in the form of a hearing test battery designed to measure several parts of the auditory system. All audiometric tests were performed by certified audiologists Frank Nusdeu and Adrian Fuente. An otoscopic exam and tympanometry test was performed to ensure the absence of external ear obstruction such as cerumen impaction and/or other middle or external ear pathology. Tympanometry was measured by a GSI 37 auto-tympanometry middle ear analyzer. Subjects were required to have type A results bilaterally[107]. Subjects with abnormal otoscopy, or tympanometric results falling

outside said parameters, were eliminated from the study. The rest of the test battery consisted of conventional pure tone audiometry (PTA) (from 500-8000Hz), very high frequency PTA (12 and 16 kHz), dichotic digits test, and distortion product otoacoustic emissions (DPOAEs)(from 2000-8000 Hz). The DPOAE, and PTA tests were conducted in an Acoustic Systems® double walled sound booth to effectively eliminate ambient noise. Air conduction PTA thresholds from 500 Hz to 8000 Hz, and high frequency PTA thresholds, at 12 and 16 kHz, were measured with an Interacoustics® AC40 clinical audiometer. Telephonics® TDH-39P headphones were used for conventional PTA thresholds (500-8000 Hz) and Koss® R/80 headphones for high-frequency PTA thresholds. DPOAEs were measured utilizing Madsen Capella equipment connected to a Dell notebook computer. DPOAEs were tested as follows: two continuous tones were presented monaurally to each ear at differing frequencies designated f1 and f2, the ratio of frequencies was maintained at f2/f1=1.22. The tone at f2 was 10 dB lower than the tone at f1. The primary tone f2 was presented at 5 frequencies (2-8 kHz). The 2f1-f2 DPOAEs levels and corresponding measured noise floor were registered as a product of f2. DPOAE amplitudes measuring 3 dB or more above the noise floor were considered a response. All tests were performed by a certified audiologist at the facilities of Occupational and Environmental Medicine Program, Yale University.

Exposure Assessment

Written bilingual (English and Spanish) questionnaires were given to the participants prior to audiometric testing to assess self-reported noise and solvent exposures. Preparation and administration of these questionnaires was performed by the Department

of Occupational and Environmental Medicine at Yale University School of Medicine. Exposure data was collected for current and prior employment as well as relevant recreational and military activities. Questions relating to pertinent medical and social history, along with other known risk factors for hearing loss were also included. Measurements of previous environmental solvents (toluene) were obtained from the factory records. Air samples for solvents have been taken by using charcoal tube and personal sampling pumps. Collection was undertaken around areas occupied by workers, including the quality control laboratory, the machines processing polyurethane coated fabrics, and the mixing shed. Conventional air sampling techniques were used to collect samples (NIOSH Sampling and Analytical Methods). Air sample analysis was accomplished by gas chromatography-flame ionization detection. The recorded concentrations ranged between 0.1 ppm and 26 ppm (average concentration for the nine workstations sampled was 4.71 ppm).

This factory was chosen because of known solvent use among the production laborers. Noise exposures at this site had not been measured at levels that would require a hearing conservation program (85 >dBA, time weighted average for 8 hrs)[108]. To best control for any effect of noise on hearing status, noise exposure indices were calculated based on a workers self reported level of ear protection use during noise exposure from previous and current employment as well as recreational noise exposure.

Solvent exposure was assessed by the workers' self-report of contact with solvents along with the self-report of episodes of symptomatic acute solvent intoxication. Additionally, an experienced industrial hygienist familiar with the plant assigned a solvent exposure

number to each participant based on job title and work area. Workers that had worked in multiple areas of different solvent levels were assigned a group using a time weighted average of exposure duration. The numbers ranged from 1-3 with 1 being the least exposed. This number was multiplied by the number of years the subject had been working at the factory to create an exposure index product for the exposure limited to the factory site.

A total of 94 subjects were recruited for the study. One individual had abnormal tympanometric test results and could not be included; 93 were used for the analysis.

Statistical Analysis

Genetic test results were linked by the author with questionnaire and hearing test data from medical charts into a computer database by anonymous numerical identifiers. Statistical analysis was performed using SAS 9.1 statistical software package (SAS Institute, Cary, NC, USA). Binaural averages were calculated for DPOAEs and PTA thresholds (high frequency and very high frequency) and dichotic digits. Combined binaural DPOAEs averages for frequencies 3000, 4000, and 6000 Hz were calculated for each subject. A binaural average of PTA thresholds at 3000, 4000, 6000 Hz was also calculated for each subject. Linear regression was performed to determine the association between hearing status and the predictors.

Initially, bivariate analyses were conducted separately for each of the above listed hearing outcomes with the following variables: age, race, eye color, solvent group, solvent group index, recreational noise exposure, years at factory, experienced acute

solvent intoxication, cigarette use (pack-years), alcohol use (average drinks/week multiplied by years), outside of work solvent exposure index, and genotypes for GSTM1, GSTP1, GPX1, and SOD2.

Table 1. Variables used in model construction.

Variable	Categorical	Continuous Range
GSTM1	Present (+) Null (-)	
GSTP1	Ile/Ile (+/+) Ile/Val (+/-) Val/Val (-/-)	
SOD2	Ala/Ala (+/+) Ala/Val (+/-) Val/Val (-/-)	
GPX1	Pro/Pro (+/+) Pro/Leu (+/-) Leu/Leu (-/-)	
Eye Color	Brown Blue Green Other	
Hair Color	Brown Black Blond Auburn	
Race	Black White Pacific Islander Asian Native American	
Ethnicity	Hispanic Non-Hispanic	
Age (years)		22-67
Solvent Group	1 Least Exposed 2 Medium Exposed 3 Most Exposed	
Years at Factory		0-31
Years × Solvent Group Index		0-90
Cigarettes Use Years		0-49
Cigarette Pack × Year		0-49
Alcoholic Drinks per Week × Years		0-500
Recreational Noise Exposure Index		0-450
Noise Exposure Index		0-175
Acute Solvent Intoxication	Yes No	

Next, a set of four multivariate models were analyzed for each of the outcomes. To better control for age and to explore the effect of ethnicity additional models were constructed. In total the logistic models for each outcome were composed of: entire data set, age stratified data set (age: 22-43yrs, 44-67yrs), Hispanics only and age stratified Hispanics (age: 22-43yrs, 44-67yrs) for each of the hearing outcomes. Initial model speculation included variables that have been reported or are suspected to have an impact on hearing status. These variables are summarized in table 1. A backward elimination strategy was employed using a significance level-to-stay of $p = 0.05$.

RESULTS

Pure Tone Audiometry Thresholds

Table 2 summarizes the outcomes for the included variables for both bivariate and age stratified multivariate analysis. In the multivariate model for subjects 43 years and younger, a significant association between SOD2 genotype and high frequency (3,4,6 kHz) audiometric threshold value is observed. The homozygous valine at position 16 genotype is associated with lower thresholds measured with high frequency pure tone audiometry with a β of -20.11 and p-value of 0.023. In the same model, a significant protective association of the heterozygous SOD2 genotype (Val/Ala) with high frequency (3,4,6 kHz) pure tone audiometric thresholds was also observed with β value of -16.51 and p-value of 0.009.

Table 2. Cohort variables correlation with pure tone audiometric thresholds binaural average at 3,4,6 kHz

	Bivariate Analysis		Multivariate Models					
Variable	All Ages		All Ages		Age ≤ 43		43 < Age	
	β	P value	β	P value	β	P value	β	P value
GSTM1 (null) (reference)	1.1	0.80						
GSTM1 (present)								
GSTP1 -/- (Val/Val)	-7.36	0.25			0.62	0.91		
GSTP1 +/- (Val/Ile)	-4.2	0.37			12.41	0.01		
GSTP1 +/+ (Ile/Ile) (reference)								
GPX1 -/- (Leu/Leu)	-16.19	0.02					-47.81	0.034
GPX1 +/- (Pro/Leu)	-10.0	0.01					-8.22	0.24
GPX1 +/+ (Pro/Pro) (reference)								
SOD2 -/- (Val/Val)	1.9	0.7			-20.11	0.023	-18.95	0.15
SOD2 +/- (Ala/Val)	-0.94	0.85			-16.51	0.009	-26.42	0.025
SOD2 +/+ (Ala/Ala) (reference)								
Age	1.06	<.0001	1.02	>0.0001	0.91	0.012		
Hispanic	6.6	.12						
Non-Hispanic								
White (reference)								
Black	-9.6	0.15			-37.33	0.0037	23.64	0.31
Asian	-17.7	0.09			-3.15	0.74	59.62	0.026
Native American	-14.1	0.43						
Years at Factory	0.81	0.006			-2.72	0.0055	8.52	0.005
Alcoholic Drinks per Week×Yrs.	0.04	0.02			-0.09	0.032	0.098	0.002
Cigarette Pack×Years	0.04	0.84						
Noise Exposure Index	0.10	0.07			0.31	0.018		
Recreational noise protection	0.02	0.51						
Solvent Exposure Group	4.86	0.046	5.03	0.012			47.13	0.003
Years × Solvent Exposure Group	0.30	0.004					-2.61	0.010
Acute Solvent Intoxication	-2.49	0.71						
Hair Color Black (reference)								
Hair Color Blond	-1.4	0.94						
Hair Color Auburn	-3.7	0.62						
Hair Color Brown	3.1	0.66						
Eye Color Brown (reference)								
Eye Color Blue	-4.0	0.5					72.0	0.006
Eye Color Green	7.11	0.42					23.5	0.041
Eye Color Other	-1.4	0.86					-3.42	0.78

Table 3. Cohort variables correlation with DPOAEs, Binaural Average at 3,4,6 kHz

	Bivariate Analysis		Multivariate Models					
Variable	All Ages		All Ages		Age ≤ 43		43 < Age	
	β	P value	β	P value	β	P value	β	P value
GSTM1 (null)	-0.2	0.87						
GSTM1 (present)								
GSTP1 -/- (Val/Val)	-0.73	0.71						
GSTP1 +/- (Val/Ile)	0.61	0.66						
GSTP1 +/+ (Ile/Ile) (reference)								
GPX1 -/- (leu/Leu)	3.0	0.17						
GPX1 +/- (Pro/Leu)	1.3	0.31						
GPX1 +/+ (Pro/Pro) (reference)								
SOD2 -/- (Val/Val)	0.33	0.86						
SOD2 +/- (Ala/Val)	-1.3	0.44						
SOD2 +/+ (Ala/Ala) (reference)								
Age	-0.27	<.0001	-0.25	<0.0001	-0.48	0.0009		
Non-Hispanic (reference)								
Hispanic	1.15	0.4						
White (reference)								
Black	0.9	0.67						
Asian	7.68	0.02						
Native American	1.8	0.74						
Years at Factory	-0.2	0.03			0.70	0.0074		
Alcoholic Drinks per Week×Yrs.	-0.006	0.31						
Cigarette Pack×Years	-0.08	0.19						
Noise Exposure Index	-0.02	0.32						
Recreational noise protection	-0.01	0.17						
Solvent Exposure Group	-0.9	0.24						
Years × Solvent Exposure Group	-0.06	0.05						
Acute Solvent Intoxication	-1.82	0.41						
Hair Color Black (reference)								
Hair Color Blond	-1.9	-0.33						
Hair Color Auburn	2.3	0.26						
Hair Color Brown	1.1	0.57						
Eye Color Brown (reference)								
Eye Color Blue	-4.3	0.04						
Eye Color Green	-1.3	0.60						
Eye Color Other	1.39	0.55						

This protective association between the SOD2 genotypes with at least one valine allele was also seen in the model composed of subjects older than 43 years (β = -18.95 and β = -26.42 for SOD2 Val/Val and Val/Ala respectively), although only the heterozygous genotype association demonstrated significance (p-value .026).

The GSTP1 Val/Ile at position 105 genotype demonstrated an adjusted regression coefficient suggesting an association as a risk factor for worse high-frequency PTA (β = 12.41 p-value 0.01. No model showed an association of GSTM status with PTA outcome.

Within the model of the older individuals, the GPX1 Leu/Leu at position 198 genotype demonstrated a powerful protective association for high frequency PTA with β = -47.81 (p-value 0.03). A less pronounced protective coefficient was observed with the GPX1 Pro/Leu genotype (β= -8.23), but the association did not demonstrate statistical significance with a p-value of 0.24. The same trend was observed in the bivariate analysis for GPX Pro105Leu allele with a β = -16.19 for Leu/Leu (P value = 0.02) and β = 10.0 for Pro/Leu (P value = 0.01).

Within the younger multivariate model, age, race, years at the factory, noise exposure index, and alcohol use also correlated with PTA thresholds in a statistically significant way. Within the older group model race, years at factory, solvent exposure group, years

× solvent exposure group index, and eye color also showed significant association with PTA outcome.

Distortion Products Otoacoustic Emissions

The models constructed with binaural averages of the distortion product otoacoustic emissions at 3, 4, and 6 kHz are summarized in table 3. Bivariate modeling showed DPOAEs were also significantly negatively associated with age (lower DPOAE values reflect worse hearing status) $\beta = -0.27$ (P value = <0.0001) along with years at the factory $\beta = -0.2$ (P value = 0.03), and the related solvent group time index $\beta=-0.06$ (P value = 0.05). A powerful protective effect of being of Asian race on DPOAEs was also observed with $\beta= 7.68$ (P value 0.02). Given the powerful negative effect of age observed in bivariate analysis in the tested outcomes, we stratified the study group into those at and above and below the median age of 44 for multivariate model analysis in hopes of detecting variables that might have a lesser but significant effect on the outcomes.

As a group, the multivariate model analysis showed a highly significant negative effect of age on the measured emissions in the entire study group with a β value = -0.25 and p-value = <0.0001 in the all ages multivariate model. The effect of age on the DPOAEs is more pronounced when modeled in the younger age group with β value = -0.48 and p-value = 0.0009. A similar effect of age was detected in the bivariate analysis. This model also showed an effect of years at the factory as having a positive effect on DPOAEs with a β value = 0.70 and p-value = 0.0074. No effect was observed in the model composed of the age > 43 group.

Very High Frequency PTA Thresholds and Dichotic Digits

Genetic markers did not demonstrate an effect on very high frequency hearing thresholds (12-26 kHz) in the study group with only the covariates age (β value = 1.81 and p-value = < 0.0001) and solvent group (β value = 8.80 and p-value = 0.047) being significantly associated with worse thresholds. The dichotic digits model only showed a (negative) effect of solvent group on test scores (β value = -1.10 and p-value = 0.0004).

DISCUSSION

The analysis revealed that several of the antioxidant gene polymorphisms were associated with significantly better or worse hearing outcomes. Interestingly, no single polymorphism showed a significant effect across all age groups and outcomes. However, significant associations were observed between improved PTA thresholds and the SOD2 Ala/Val genotype in both groups of the age stratified models. The SOD Val/Val genotype showed a similar association with the correlation reaching statistical significance in the younger age group and approaching significance in the older group. These results suggest that some antioxidant genotypes may modify susceptibility to sensorineural hearing loss.

This study is subject to limitations. Though that analysis yielded statistically significant associations of several exposure variables with hearing outcome, the sample size engenders statistical power probably most suitable for a pilot study. It is also worth noting that a relatively large number of models were constructed and tested. The design as a cross-sectional observational cohort only provides data at the time of collection with no measurement of workers hearing status over time with no collection of pre-employment hearing status. Noise, though not measured to such a level within the

factory that would require a hearing conservation program, could be heterogeneously distributed among working spaces in the factory and some workers could have been more exposed than others effecting hearing status in a subset of subjects. Also, efforts to characterize noise and ototoxic exposures that were unrelated to the occupational setting were dependent upon questionnaire answers; this presents a challenge in terms of quantifying exposure levels. Additionally, data collected by questionnaire relating to past exposures to noise, solvents, and other ototoxic agents is also subject to general recall bias. This study population was also particularly prone to confounding affects due to cigarette smoking and alcohol use.

The association of GSTP1 status and PTA outcomes showed an interesting protective effect of having the heterozygous Ile/Val genotype. The above-mentioned change in substrate specificity in the GSTP1 Ile105Val variant may in part explain the observed heterozygous disadvantage in regards to high frequency pure tone thresholds. It is possible that the change in the enzyme's heat stability and substrate preference significantly impacts the enzyme's ability to protect the cochlear tissues from solvent mediated damage when there is at least one copy of the Val expressing allele. The reasons for a lack of more dramatically elevated thresholds in the subjects expressing Val/Val are unclear. The lack of correlation of elevated threshold and Val/Val homozygotes could simply relate to the small number of individuals in the cohort that had a Val/Val genotype (12 (14%)); this observation could also be due to the more pronounced deficit stimulating a compensatory pathway.

Within the older age group there was also a significant observed association of the GPX1 Leu/Leu genotype and favorable hearing thresholds. The level of the Leu expressing variant of this allele has been shown experimentally to be less inducible in response to selenium supplementation[99]. This variant has, however, not been shown to demonstrate an inherent decreased enzymatic activity. Further analysis is needed to mechanistically explain the observed association of this Leu allele with more favorable hearing thresholds.

The lack of association between any of the polymorphisms analyzed and the measured distortion product otoacoustic emissions is difficult to explain given the significant findings for the same SNPs associations with hearing thresholds. Otoacoustic emissions are generated by the outermost hair cells; it is possible that the pathway for the generation of the OAEs is differentially sensitive to solvents and/or oxidative stress compared to the tissue and pathway measured with hearing threshold values; indeed DPOAEs have been studied as a more sensitive indicator of hearing loss[109, 110]. It is true that a large proportion of the older workers in our set had undetectable DPOAEs (only 5 (12.5%) of subjects had detectible DPOAE's in the 44-67 age group), making detection of any effect less probable with the analysis. However, age overall did have its predicted effect on DPOAEs and PTA thresholds rendering the results of this analysis that were observed more plausible.

The lack of effect of any of the antioxidant SNPs on the dichotic digits test could be due to the nature of the test being designed to preferentially detect defects in central auditory processing rather than peripheral hearing loss. Also many of the study participants were

Spanish speaking and the test is administered using English spoken numbers and it is the suspicion of the author that spoken language confounded the results.

Perhaps the most striking finding in this study lies in the observed relationship of the SOD2 genotype with high frequency audiometric thresholds. In the age stratified multivariate model adjusting for the effects of age, race, noise and solvents in the cohort of workers, subjects that possessed a valine containing genotype at position 16 demonstrated superior high frequency pure tone audiometric thresholds compared to subjects expressing only alanine at the same position; this association was demonstrated in a statistically significant dose dependant fashion in the younger age group.

It is difficult to interpret epidemiological studies in light of results of laboratory investigation of the transport and functionality of the SOD2 Val-16 and Ala-16 allotypes here considered. One would expect the more functional and efficiently transported alanine variant of SOD2 to be associated with superior hearing status when compared to the less functional valine variant. It should be noted that the studies conducted by Sutton et al. into the transport and functionality of the SOD2 variants were conducted in rat and human liver cells and may not be entirely translatable to cochlear cells[77]. Indeed, discordant reports of disease associations have posed a challenge for proposing mechanisms for MnSOD modification of risk in disease[74, 75, 78-80, 82-84, 86]. It is important to recognize that antioxidant enzymes work in balanced concert to detoxify reactive oxygen species. MnSOD catalyzes the conversion of O_2^- into H_2O_2, which is itself another reactive species that requires conversion to inert species largely via the Catalase and GPX enzymes. It has been shown experimentally in vitro and in vivo that increasing

concentration of Cu/ZnSOD[111] and MnSOD[112] had a bell-shaped curve in relationship to exacerbation and protection of post-hypoxic tissue from reperfusion oxidative damage. Michiels et al. postulated that this observed effect may be due to imbalance between Catalase and SOD enzymes resulting in unfavorable concentrations of O_2^- and H_20_2 resulting in formation of highly reactive Hydroxyl radicals via the Haber-Weiss reaction[113]. McCord *et al*. explain the apparent hormetic nature of oxidative protection curves from increasing SOD activity by suggesting that lipid peroxidation resultant from oxidative damage is both initiated and terminated by O_2^{-}[114]; They conclude that the absolute reduction in the amount of O_2^- brought about by increased SOD would eliminate an important means of controlling the level of lipid peroxidation in cells under oxidative stress.[114] Additionally, it may be that in some tissues the level of H_2O_2 generated by the alanine variant is more damaging in the hearing tissues than the O_2^- it is produced from by the action of SOD2 within the mitochondria; this may be via the generation of hydroxyl radicals or by some other mechanism.

There are other means by which the Ala allele could possibly contribute to hearing loss; Associations of both alleles at this locus are appearing in the literature with regularity. It is very feasible that SOD2 is involved in a number of differing mechanisms leading to disease. For example, Kakko et al. demonstrated a small but significant association between the Ala allele and the degree of atherosclerosis in a Finnish cohort[115], given this report it is conceivable that this allele's effect on the cochlea could be of purely potentiating the probability of sustaining a vascular insult.

It is also possible, and even likely, that the genetic relationship of the SOD2 polymorphism Ala16Val is more complex than the results suggest, this is often true of single nucleotide polymorphisms[116, 117]. Linkage disequilibrium between the valine containing alleles and another variation that either increases risk of hearing loss with solvent, age, or noise exposure, or independently, cannot be ruled out as an explanation of this observed pattern.

The findings of this study provide additional support for the hypothesis that antioxidant genes play a role in the pathogenesis of hearing loss and provide the first evidence to suggest known functional single nucleotide polymorphisms may specifically modify risk in solvent-induced hearing loss. Our findings should encourage additional investigation into the relationship of antioxidant gene SNPs with hearing loss in solvent exposed workers.

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TABLE LEGEND

Table 1. Variables used in construction of the statistical regression models that were collected by participant questionnaire and genetic analysis of antioxidant gene polymorphisms.

Table 2. Statistically significant results from bivariate and multivariate analysis of variables with pure tone audiometry thresholds (average of both ears at 3, 4, 6 kHz) as the outcome. Higher Threshold values represent worse hearing status. The multivariate analyses include the entire data set and two models composed of age groups above and below (age $\leq$ 43 and 43<age) the mean age for the study group.

Table 3. Statistically significant results from bivariate and multivariate analysis of variables with distortion product otoacoustic emissions as outcome (average of both ears at 3, 4, 6 kHz). Higher DPOAE values represent better hearing status. The multivariate analyses include the entire data set and two models composed of age groups above and below (age $\leq$ 43 and 43<age) the mean age for the study group.