Published in final edited form as:

Neurotoxicol Teratol. 2013 ; 38: 21–28. doi:10.1016/j.ntt.2013.04.003.

Lead Exposure and Fear-Potentiated Startle in the VA Normative Aging Study: A Pilot Study of a Novel Physiological Approach to Investigating Neurotoxicant Effects

Rachel Grashow^a, Mark W. Miller^{b,c}, Ann McKinney^b, Linda H. Nie^d, David Sparrow^{b,e}, Howard Hu^f, and Marc G. Weisskopf^a

^aDepartment of Environmental Health, Harvard School of Public Health, Boston, Massachusetts, USA

^bVeterans Affairs Boston Healthcare System, Boston, Massachusetts, USA

^cDepartment of Psychiatry, Boston University School of Medicine

^dSchool of Health Sciences, Purdue University, West Lafayette, Indiana, USA

^eDepartment of Medicine, Boston University School of Medicine, Boston, Massachusetts, USA

^fDalla Lana School of Public Health, University of Toronto, Toronto, Ontario, Canada

Abstract

Background—Physiologically-based indicators of neural plasticity in humans could provide mechanistic insights into toxicant actions on learning in the brain, and perhaps prove more objective and sensitive measures of such effects than other methods.

Objectives—We explored the association between lead exposure and classical conditioning of the acoustic startle reflex (ASR)—a simple form of associative learning in the brain—in a population of elderly men. Fifty-one men from the VA Normative Aging Study with cumulative bone lead exposure measurements made with K-X-Ray-Fluorescence participated in a fear-conditioning protocol.

Results—The mean age of the men was 75.5 years (standard deviation [sd]=5.9) and mean patella lead concentration was 22.7 μg/g bone (sd=15.9). Baseline ASR eyeblink response decreased with age, but was not associated with subsequent conditioning. Among 37 men with valid responses at the end of the protocol, higher patella lead was associated with decreased awareness of the conditioning contingency (declarative learning; adjusted odds ratio [OR] per 20 μg/g patella lead=0.91, 95% confidence interval [CI]: 0.84, 0.99, p=0.03). Eyeblink conditioning (non-declarative learning) was 0.44sd less (95% CI: −0.91, 0.02; p=0.06) per 20 μg/g patella lead after adjustment. Each result was stronger when correcting for the interval between lead measurement and startle testing (awareness: OR=0.88, 95% CI: 0.78, 0.99, p = 0.04; conditioning: −0.79sd less, 95% CI: −1.56, 0.03, p = 0.04).

Conclusions—This initial exploration suggests that lead exposure interferes with specific neural mechanisms of learning and offers the possibility that the ASR may provide a new approach to physiologically explore the effects of neurotoxicant exposures on neural mechanisms of learning in humans with a paradigm that is directly comparable to animal models.

Corresponding Author: Rachel Grashow, Department of Environmental Health, Harvard School of Public Health, Landmark Center, 401 Park Drive, 3rd Floor, Boston, MA 02215, USA. Phone: 617-384-8876. Fax: 617-384-8994. rgrashow@hsph.harvard.edu.

Conflict of Interest Statement:

The authors declare they have no competing financial interests.

Keywords

lead; aging; acoustic startle response; psychophysiology; behavioral toxicology

1. Introduction

Physiological measures of neural responses such as nerve conduction velocity or auditory brain stem responses have been used as a direct, objective assessment of nervous system function. While such measures reflect basic neural processes, they do not assess plasticity in the brain—the ability of the brain to adapt and change with experience. Such neural plasticity is widely believed to underlie behavioral learning and memory [35,49], functions which, at the behavioral level, have been found to be affected by neurotoxicant exposures [57,67,70]. The acoustic startle reflex is a basic brain reflex that can exhibit such plasticity under different conditions. Thus, modification of the acoustic startle reflex could provide researchers with a means to probe how the plasticity of the brain is affected by different exposures, including chemical toxicants.

The startle reflex refers to a set of physiological responses elicited by a sudden intense auditory stimulus, and can be measured using electromyography (EMG) of the eyeblink muscles. This response occurs via a simple neural circuit involving as few as three synapses, and is essentially the same in rodent models, non-human primates and humans [19,39]. The startle response is increased by fear [27], which is thought to reflect a heightened state of alertness in the face of an impending threat to the health and safety of the animal. Classical conditioning of the acoustic startle reflex takes advantage of this fear-enhanced startle and is used to probe basic learning mechanisms in the brain. In this paradigm the startle response is initially elicited in the presence of one of two innocuous stimuli, which have no effect on the amplitude of the startle response. Next, one of the two stimuli (the conditioned stimulus, or CS+) is paired with a mild electric shock (the unconditioned stimulus; US), which itself evokes a fearful state. After this pairing, when the shock (US) is no longer given, the magnitude of the startle response in the presence of the CS+ is increased, reflecting that the subject has learned that the CS+ predicts the US (the unpaired innocuous stimulus is referred to as the CS−). This paradigm can be used to probe two dissociable types of associative learning: declarative and non-declarative learning [6,42,64]. Declarative learning is reflected in the ability to verbally identify which CS was paired with the US. In contrast, non-declarative learning does not require conscious control and is manifested by the increased startle response to the CS+ following pairing with the US compared with the startle response to the CS−.

Fear-conditioning of the startle response is a paradigm that has been studied extensively in animal models to investigate basic aspects of learning in specific neural circuits [19,20,28,52]. It has also been used in humans to investigate deficits in different pathological conditions. For example, fear conditioned startle has been used to better understand the cognitive deficits and brain changes associated with posttraumatic stress disorder [48], schizophrenia [56], and Alzheimer's disease [32]. Thus far, this paradigm has not been used in humans in the context of environmental neurotoxicant exposure, even though many of the pathological conditions that show altered fear-potentiated startle responses share symptom manifestations with many neurotoxicant exposures. For example, anxiety, attention dysfunction, mood and memory have all been associated with lead, manganese and other environmental exposures [9,46,47,57].

Exposure to lead is one of the most studied environmental toxicants with respect to effects on cognitive function. This has been most extensively explored in occupationally exposed

adults [3,57] and children [7,12,44]. Also among non-occupationally exposed adults, cumulative exposure to lead has been previously found to be associated with worse cognitive performance [4,57,66]. Lead could affect cognitive function via a number of putative mechanisms. On the cellular level, lead ions cross the blood brain barrier and have several effects, many of which relate to the ability of lead to substitute for calcium ions [55,67]. Thus, lead interferes with processes such as calcium dependent second messenger signaling and synaptic transmission [10,55]. Lead may specifically inhibit the N-methyl-D-aspartate (NMDA) receptors that are implicated in the cellular process of long term increases in synaptic efficacy (potentiation), learning in the hippocampus [50,67] and the acoustic startle response itself [45,63]. Lead may also interfere with voltage gated calcium channels, which could disrupt long term potentiation in the amygdala [58,65]—the presumed locus of cellular learning underlying fear conditioning [33,39,43,53]. In addition to reducing synaptic plasticity, lead may also inhibit neurogenesis [26,59], a process involved in learning, injury recovery and mood disorders. Taken together, lead could impact cognitive function via multiple pathways and mechanisms to affect learning.

A few rodent studies have explored the effects of lead exposures on the ASR. One study found that chronic lead exposure reduced sensorimotor gating or the extent to which sounds played prior to the startle stimulus reduce the ASR, called pre-pulse inhibition [15]. Another study showed no effect of lead exposure on the basic ASR, without measuring any associative conditioning or startle modification protocols [22]. Another study in rats examined the effect of lead exposure on cued (a tone) and contextual (chamber) fear-conditioning [54]. Lead exposure did not affect initial behavioral reactivity to the chamber or tone-shock pairing, but did reduce extinction of the learned fear-response to both the cued and contextual conditioned stimuli. To explore the use of the fear-potentiated startle paradigm as a physiological indicator of neurotoxicant effects on learning in humans, we conducted a study to determine whether cumulative environmental lead exposure was associated with impaired learning in the fear-conditioned startle paradigm among elderly men in the Department of Veterans Affairs (VA) Normative Aging Study (NAS), a cohort of elderly men from the greater Boston area with lead exposures similar to the general population. Given lead's effects on cognitive function, we hypothesized that higher long term cumulative lead exposure would reduce both declarative and non-declarative learning as measured using classical conditioning of the fear-potentiated startle response.

2. Materials and Methods

2.1 Study population

The study sample was a subgroup of participants in the NAS, an ongoing longitudinal study established in 1963. The NAS recruited community-dwelling healthy men from the greater Boston, MA area between the ages of 21 and 80 years of age. These men complete medical examinations and questionnaires every three to five years with a low attrition rate (<1% annually). Further detail on this study can be found elsewhere [36,66]. Starting in 1991 a subgroup of this population (876 members or 68% of all active study participants) agreed to have their bone lead concentration measured using K-shell x-ray fluorescence (KXRF).

Recruitment for this acoustic startle study was by mailed letters and direct invitation at regularly-scheduled NAS visits from April 2005- May 2007. Mailed letters were targeted to NAS participants whose most recent patella lead measurements were in the highest and lowest quintiles, thus the sample was slightly enriched for patella lead level extremes but also included individuals with intermediate lead concentrations. A total of 56 elderly men agreed to participate (overall participation: 52%). The first 35 men recruited were administered a hearing screen and 11 were excluded because of some hearing loss in the high frequency range. However, because i) the US was not auditory, ii) the acoustic startle

probe was broad-band noise, and iii) we could assess baseline responsiveness to the startle probe, we stopped excluding participants based on this screen in order to improve recruitment. Two subjects were excluded due to equipment malfunction, and two others were excluded because they fell asleep during the first protocol block. Finally, one participant without a lead measurement was inadvertently recruited and so could not be included in analyses. Thus, the final study sample was 51 men.

2.2 Bone lead KXRF Measurements

Each subject's bone lead concentration was measured at two sites: the midtibial shaft and the patella using an ABIOMED KXRF instrument (ABIOMED, Danvers, MA). Both sites were cleaned with a 50% solution of isopropyl alcohol, after which a 30-minute measurement was recorded. The tibial midshaft was determined to be the midpoint between the medial malleolus and the tibial plateau. The KXRF beam collimator was positioned 30° in the lateral direction of the patella, and perpendicular to the flat bony surface of the tibia. Tibia and patella lead measurements reflect cumulative lead exposure with slightly different time frames: patella lead has a half-life of approximately eight years, while tibia lead half-life is on the order of decades [69]. An average of 7.4 ± 2.7 years elapsed between the XRF measurements and the fear-conditioned startle experiment.

2.3 Fear conditioning

2.3.1 Testing procedure—Subjects participated in a classical conditioning procedure that consisted of five blocks: pre-acquisition, acquisition, and three post-acquisition blocks. Subjects were informed that electric stimuli and loud sounds would be delivered, but not informed of the CS-US relationship. Each block consisted of four presentations each of the CS+ and the CS− (totaling 8 CS presentations), shown as a row of either “O”s or asterisks (*) on the computer screen in 72 pt font, presented for eight seconds. Each presentation of the CS+ or CS− was separated by 50 second inter-trial intervals (ITI). Subjects were instructed to view each CS for the entire presentation duration. In each of the five blocks, nine startle probes (105 dB broadband noise for 50 ms with immediate rise time) were presented: three during each of the CS stimuli and three during ITIs. Startle probes were presented 2, 4, or 6 seconds after CS onset or 20, 25, or 30 seconds into the ITI. The order of the presentations was arranged by blocks into two pseudorandom orders and each participant was randomly assigned to one. The two protocols were counterbalanced to control for serial position effects of startle probe, US and CS type position.

Following the pre-acquisition block, shock electrodes were attached to the 2nd and 4th fingers of the non-dominant hand during a five-minute rest period. The remaining blocks followed in sequence with no breaks in between. In the acquisition block, either the “O”s or asterisks (*) were randomly assigned to be paired with a mild electric shock (the US; 5.0 mA at 10 Hz for 500 ms). During the acquisition block, the US was administered during each of the four presentations of the CS (CS+) assigned to be paired with the US, occurring at 1, 3, 5 or 7 s after CS+ onset. The US was not delivered during presentations of the other CS (CS−). Following the acquisition block, three post-acquisition blocks were administered during which no US were delivered.

Display and timing of the visual stimuli (CS), startle probes, and US were controlled using Superlab software (Cedrus Corporation, San Pedro, CA). Acoustic startle probes were generated by a Coulbourn Instruments white noise generator (Coulbourn Instruments, Allentown, PA) and amplified by an audio mixer-amplifier. Startle probes were played binaurally through a set of headphones.

Startle recording and scoring parameters were based on previously published guidelines [8]. The startle eyeblink responses were recorded using Beckman Ag/AgCl miniature electrodes. Electrodes were positioned below the left eye over the orbicularis oculi muscle [25]. The raw electromyography (EMG) signal was amplified using a Coulbourn bioamplifier with low- and high frequency cutoff values of 9 and 1000 Hz, respectively. The signal was rectified and integrated using a contour-following integrator with a time constant setting of 20 ms. Digital sampling at 1000 Hz started 50 ms before startle probe onset and ended 250 ms after the probe offset. Sampling, digitization and storage of the physiological data were executed on a computer using LabTech Notebook Pro software (LabTech Corporation, Tampa, FL). The initiation and termination of the physiological data collection was controlled by a transistor-transistor logic (TTL) signal from the computer running the Superlab stimulus presentation program. Startle response data were reduced offline using a program to score the magnitude of eyeblinks in arbitrary analog-digital (A–D) units [17].

Startle scoring was done using the WINSTAR startle scoring program [17], and SPSS (SPSS, Chicago, IL). Baseline muscle activity for each startle was calculated by averaging the 50ms of EMG signal recorded before the onset of the startle stimulus. If baseline showed more than a $\pm 10 \mu\text{V}$ amplitude change or if the mean amplitude during the baseline interval was unstable (i.e. showed more than a $5 \mu\text{V}$ change), the trial was rejected. Peak response was considered to be the highest magnitude EMG recording within 200 ms after probe presentation. If onset of response occurred after 150 ms or there was no discernible peak during the 200 ms after the probe, the trial was scored as a non-response. We calculated averaged responses excluding non-response and rejected trials. This is referred to as startle amplitude (as opposed to magnitude, which includes non-responses as zeros) [8].

Following the testing procedure declarative awareness about the US-CS relationship was assessed via interview. Participants were considered aware if they were able to correctly report which of the conditioned stimuli had been paired with the shock.

2.3.2 Cortisol level measurements—Endogenous cortisol levels have been shown to be associated with fear-potentiated startle responses in animals and humans [11,30]. Baseline cortisol level in saliva was measured after EMG electrodes were attached before the pre-acquisition block and then after the third post-acquisition block. Saliva was collected using Salivette cotton swabs (Sarstedt, Germany). Subjects were instructed to chew a dental-cotton roll for 30–45 seconds, which was then placed into a polystyrene tube stored at -20°C and assayed at Technical University of Dresden (Dresden, Germany). After thawing, and centrifuging, salivary cortisol concentrations in supernatant were measured using chemiluminescence-immunoassay with high sensitivity (IBL International, Hamburg, Germany). The intra and interassay coefficients for cortisol were below 7%.

2.3.3 Statistical analysis—Statistical analysis was performed using SAS 9.2 (SAS Institute Inc., Cary, NC). Individual startle responses were normalized within each block by creating a z-score using the mean and standard deviations of all startle responses (CS+, CS–, ITI) within the block. Differential startle amplitude (DSA; average CS+ z-score minus average CS– z-score within a given block) was calculated as an index of non-declarative learning. Ordinary least squares regression was used to assess associations with the DSA. Logistic regression was used to calculate odds ratios (OR) and 95% confidence intervals (CI) for awareness of the proper CS+–US contingency, the index of declarative learning. We show effect estimates per 20 $\mu\text{g/g}$ patella lead and 15 $\mu\text{g/g}$ tibia lead, which are the interquartile ranges (IQR) in the entire NAS cohort [66].

Covariates considered were age at startle test, education (12 or fewer years, 13–15 years, and 16 years or more), smoking (ever/never), financial stability and use of hearing aids.

Financial stability was a multiple choice survey question in which subjects were asked to choose the statement that best described their present situation: “I really can’t make ends meet with the income I now have”, “I just about manage to get by with the income I now have”, “I have enough to get along, and even a little extra”, and “I can pretty much buy anything I want with the income I now have”. Due to small numbers in the lowest stability category, “can’t make ends meet” was combined with “just manage to get by”. Two subjects missing financial stability data were included in the most common response category, “I have enough to get along”.

We additionally considered use of anti-hypertensive medications (use of α -blockers, β -agonists, diuretics, calcium channel blockers, or angiotensin-converting enzyme [ACE] inhibitors) and both baseline cortisol and change over the protocol in cortisol levels, as these can affect ASR [21,41]. However, these were not included in the main analyses since lead exposure has been found to be associated with hypertension [14,36] as well as HPA axis dysfunction and cortisol level production [37,62], and so these variables may reflect mediators of the association between lead and both startle conditioning and contingency awareness. In models with baseline cortisol the time of day (morning or afternoon) was also included as a term in the model as cortisol levels vary substantially diurnally.

To mitigate against undue influence of the highest patella and tibia bone lead level, we ran additional analyses after reassigning the highest concentration to the next highest level (Winsorization; [68]). Because the interval between bone lead measurement and the startle protocol was on the order of the half-life of lead in patella bone, we also ran additional analyses using an exponential decay model to estimate patella lead concentration at the startle testing date using an assumed half-life for lead in bone of 8 years [38]. We ran additional models to determine whether missing startle data was associated with age, lead and learning covariates. A p -value < 0.05 was considered statistically significant.

3. Results

3.1 Descriptive Statistics

The mean (sd) age of the participants at the time of startle testing was 75.5 (5.9), and the mean (sd) years of education was 14.8 (2.7). Among the 51 participants the mean (sd) patella and tibia lead concentrations were 22.7 (15.9) and 17.7 (11.8) $\mu\text{g/g}$ bone, respectively. Table 1 shows the bone lead concentrations by demographic characteristics of the participants. The mean (sd) latency of startle responses was 29.8 ms (13.6). Mean startle magnitude in μV by block across all conditions is shown in Figure 1. Habituation over the blocks can be seen.

3.2 Regression Analysis

We first explored baseline responsiveness to the startle probe across all conditions (CS+, CS−, ITI) in the pre-acquisition block. Neither tibia nor patella lead were associated with differences in baseline startle amplitude in unadjusted models (per IQR patella: $\beta = -92.8$; 95% CI: $-247.2, 61.6$; $p = 0.24$; per IQR tibia: $\beta = -50.9$; 95% CI: $-211.5, 109.8$; $p = 0.53$). In contrast, age showed a significant association with baseline startle. Startle magnitude decreased 26.6 A–D units (95% CI: $-46.54, -6.61$; $p = 0.01$) per year of age. In models of baseline startle amplitude that included lead and age terms together, tibia and patella lead were still not associated with baseline startle amplitude, while the parameter estimates for age and baseline amplitude remained essentially unchanged. Startle response amplitudes increased during the acquisition block, but this increase did not differ by lead level for any of the conditions (CS+, CS−, ITI; data not shown). Use of hearing aids, financial stability, smoking and years of education were not associated with baseline responsiveness or response amplitude during the acquisition block.

Figure 2 shows the DSA by test block among participants who had non-zero responses in each of the five startle blocks. As expected, DSA was essentially 0 in the pre-acquisition block before any CS-US pairing and increased (indicating a larger response to the CS+ than the CS-) after the acquisition block. DSA was significantly different from 0 in the first post-acquisition block (DSA: = 0.78, 95% CI: 0.61, 0.99; $p=0.043$) and third post-acquisition block (DSA: = 0.90; 95% CI: 0.71, 1.14, $p=0.005$). The results were virtually the same when considering all participants ($n=51$) regardless of whether they were missing responses for some blocks. Baseline startle was not associated with DSA in any of the post-acquisition blocks, nor was pseudorandom order assignment for CS+ and CS- startle presentation.

In unadjusted models, associations between patella lead and DSA were apparent only in the third and final post-acquisition block (Table 2); higher patella lead was associated with smaller DSA. Adjustment for age, smoking status, education, financial stability, and hearing aid use resulted in only a slightly reduced effect estimate (Table 2). The p -value was slightly larger ($p=0.06$), which is in part related to the slightly diminished statistical power as a result of having additional variables in the model. None of these other variables (including age, $p=0.71$), however, showed an association with DSA in this model (all $p>0.15$). In analyses that used patella lead concentrations corrected for the interval between lead measurement and the startle protocol, the association between patella lead and DSA (per IQR = -0.79 , 95% CI: -1.56 , -0.03 , $p = 0.04$) was stronger (Table 2). When additional adjustment for baseline startle amplitude was made, results were essentially unchanged (data not shown). We did not find an association with tibia lead.

At the end of the testing session, 39 out of 51 participants (76.5%) correctly identified which of the conditioned stimuli had been paired with the shock. In unadjusted models patella lead was associated with this contingency awareness (Table 3). Results of fully-adjusted models of awareness with patella lead were similar (Table 3), and none of the other variables, including age ($p=0.92$), were associated with awareness (all $p<0.45$). In analyses that used patella lead concentrations corrected for the interval between lead measurement and the startle protocol, the association between patella lead and awareness were similar (Table 3). The same pattern held in models restricted to participants who had startle responses in all three of the post-acquisition blocks (data not shown). Baseline startle amplitude was not related to awareness, nor did adjusting for baseline meaningfully affect the results. Awareness was not associated with tibia lead.

Twenty-eight out of 51 subjects in this study were taking anti-hypertensive medications. In a fully-adjusted model that included use of these medications, the medication use term was not significantly associated with DSA and the estimated patella lead term in the 3rd post-acquisition block was not changed (per IQR = -0.79 ; 95% CI: -0.155 , -0.3 , $p = 0.04$). Tibia lead was unrelated to DSA in models that included hypertension medication use. Beta-blockers and alpha-agonists have received particular research attention in the context of fear conditioning [18,21,29], therefore, we also considered a term just for use of either of these medications ($n=18$). As expected, the use of one of these medications was significantly associated with less DSA (= -0.65 , 95% CI: -1.18 , -0.12 , $p = 0.02$). Furthermore, the addition of this term to the model strengthened the association with patella lead and improved the p -value (per IQR = -1.03 , 95% CI: -1.77 , -0.30 , $p = 0.01$). Fully adjusted models of lead and awareness of reinforcement contingency were not substantively affected by inclusion of either the broad or limited antihypertensive medication use variables.

When baseline cortisol was included in the fully adjusted model of DSA (including a term for use of beta blockers or alpha agonists), the patella lead term was slightly reduced, but remained significant (per IQR = -0.88 , 95% CI: -1.65 , -0.11 , $p = 0.03$). Baseline cortisol was not associated with DSA (per nmol/L = -0.02 ; 95% CI: -0.07 , 0.03 , $p = 0.33$).

Essentially the same was seen when change in cortisol was included in the model (per IQR patella lead = -0.84 , 95% CI: $-1.59, -0.06$, $p = 0.03$), although the change in cortisol term approached significance (per nmol/L = 0.07 , 95% CI: $-0.01, 0.14$, $p = 0.07$). The lead terms in fully adjusted models of awareness that included either baseline cortisol or change in cortisol were not significantly different from models without cortisol terms.

In sensitivity analyses, results were similar after Winsorization [68] of the highest tibia and patella lead concentration, and in analyses that excluded participants who used hearing aids.

4. Discussion and conclusions

The results of this study suggest that cumulative non-occupational exposure to lead is associated with deficits in two dissociable types of neural mechanisms of learning: declarative or semantic learning, and non-declarative learning. Intriguingly, these associations appeared independent of age and, in fact, lead exposure appeared to be a more important risk factor than age in this study sample. That the findings were specific for patella rather than tibia bone lead may suggest that these forms of learning are sensitive to more recent lead exposure. Patella bone lead has a half-life of approximately eight to ten years in contrast to the half-life of decades for lead in tibia. Our results are qualitatively similar to those found using the fear-conditioned startle reflex in elderly subjects with Alzheimer's disease. Both Hoefer et al. [34] and Hamann et al., [32] found that elderly subjects with probable Alzheimer's disease showed a failure to acquire conditioned fear responses.

That these findings were specific to neural learning mechanisms and not simply the result of reduced functionality of the ASR circuit is argued for by the fact that lead was not related to baseline startle levels, nor were baseline startle levels related to learning. A similar result was seen in Ferguson et al. [22] where lead exposure did not alter simple acoustic startle responses in rodents and in Salinas et al.[54], where lead exposed rats and controls showed similar responsivity to initial stimulus presentations but later differences in conditioning extinction. It is also unlikely that the results presented here are solely due to sensory deficits affecting the perception of the US. Startle amplitudes recorded in response to trials during the acquisition block went up, as expected given that the US was introduced during this block. However the level of this increase was not associated with lead exposure.

The overall non-declarative learning (DSA) exhibited by study subjects varied over the post-acquisition blocks and was largest in the third and final block. This is not atypical of conditioning paradigms as responses to the CS– in initial post-acquisition trials are often also increased but then drop off revealing a more robust DSA [1,13,51]. The significant associations between lead and non-declarative learning that were only apparent in the third post-acquisition block may be related to the more robust expression of conditioning in that block. Alternatively, lead may not interfere with the immediate formation of an association, but rather its consolidation thus enhancing loss of the memory as can be seen when the extinction phase closely follows the acquisition phase as in our protocol [5]. We also cannot rule out that the significant finding in the final block is the result of chance.

Lead has been found to be associated with hypertension [14,36]. Such an association could account in part for how lead could affect conditioning since some anti-hypertensive medications can blunt autonomic responding [21,41]. However, use of these medications did not reduce the association between lead and conditioning suggesting that this is not the case. The association with lead was stronger when use of alpha-agonists or beta-blockers was included in the model, which may indicate that inclusion of this variable accounted for an additional portion of the variance seen in conditioning responses. Adding cortisol level variables to the model did reduce the association between lead and conditioning slightly, but

the association was still significant. Previous studies have found that lead can result in dysregulation of the HPA axis and cortisol production [16,24,31], thus part of the effect of lead on fear conditioning may be mediated by its effects on cortisol.

To date, we are aware of only a few studies—all in rodent models—that have used the acoustic startle reflex to explore the effects of environmental toxicants, specifically manganese, mercury and lead [15,22,54,60,61]. However, to our knowledge, this is the first study to use classical conditioning of fear-potentiated startle—or any other modification of the startle reflex—to explore neurotoxicity of environmental toxicants in humans.

There are limitations of this study that should be considered. First, although few conditioning studies have been done among elderly subjects, these have found that the elderly are less responsive to startle probes [23,34,40] than younger populations. Moreover, in one study on conditioning of a skin conductance response, healthy elderly adults showed a reduced potentiation of the startle response, and a reduced awareness of the reinforcement contingency when compared to younger subjects [42]. Reduced responding to startle stimuli among our participants likely would not have affected our results greatly since we did not find an association between baseline startle response and learning. However, to the extent that reduced responding led to non-responses, our ability to detect differences by lead exposure levels would have been reduced. Reduced learning among the elderly could have limited our ability to detect associations with lead exposure. Indeed, we found significant associations between lead and DSA in the post-acquisition block that showed the greatest degree of overall DSA (Figure 2).

In order to boost recruitment numbers, we did not uniformly exclude participants based on formal hearing tests. However, hearing loss tends to occur at high frequencies [2] while our startle probes were broadband noise. In addition, any hearing loss would not affect responsiveness to the shock because it was not auditory. Most importantly, even if some hearing loss reduced responsiveness to the startle probes, we did not find that this baseline responsiveness was related to learning, nor were the use of hearing aids significant in adjusted models.

Another limitation of this study is that in some cases several years passed between the XRF bone lead measurements and the startle testing. It is possible that lead exposures could have occurred after the bone lead measurement, but before the startle protocol, and these could have influenced fear conditioning. However, not only would such exposures be expected to be a very small fraction of a subject's measured bone lead given the downward trends in environmental lead levels over time in the US, but also as additional exposure not captured by our bone lead measurement, this would be expected to bias our estimates toward the null. This could contribute to why we don't see any associations with tibia lead, but is unlikely to account for the association we see with patella lead. The differing intervals between bone lead measurement and startle protocol, however, does introduce some misclassification of how the bone lead measurement reflects actual bone lead at the time of the startle protocol. To address this, we used a model of exponential decay of patella lead to estimate patella lead on the startle testing date and in fact found slightly stronger results as would be expected if we were removing some measurement misclassification.

Our findings suggest that lead impairs neural mechanisms responsible for learning that occurs during classical conditioning of the fear-potentiated startle protocol. Although this is the first exploration of the use of classical conditioning of the fear-potentiated startle reflex—or any other modification of the startle reflex—to explore actions of environmental neurotoxicants in humans, our findings suggest that physiological tests such as these may provide a useful way to investigate neurotoxicity in environmental epidemiology studies.

They offer an objective assessment of critical brain functions, and can aid in understanding effects of neurotoxicant exposures at the level of specific neuronal circuits. The highly conserved neural basis of these reflexes across species can facilitate translational work in animal models, thus increasing the opportunity to uncover the mechanisms of toxicity and locate site-specific areas affected by different exposures.

Acknowledgments

This study was supported by funds from the US National Institute of Environmental Health Sciences (NIEHS; grants K01 ES012653, T32 ES007069, P30 ES017885, R01 ES005257, R01 ES007821, P01-ES000002), the US National Institute of Mental Health (grant MH-66324), and a VA Research Career Scientist award to David Sparrow. The Normative Aging Study is supported by the Cooperative Studies Program/Epidemiology Research and Information Center of the US Department of Veterans Affairs and is a component of the Massachusetts Veterans Epidemiology Research and Information Center (MAVERIC), Boston, MA. The KXRF was developed by ABIOMED, Inc. (Danvers, MA) with US National Institutes of Health grant support (ES03918-02). All participants provided written informed consent, and study protocols were approved by human research committees at the Harvard School of Public Health and the VA Boston Healthcare System.

Units and Abbreviations

ASR	Acoustic startle reflex
CI	Confidence interval
CS	Conditioned stimulus
DSA	Differential startle amplitude
EMG	Electromyography
IQR	Interquartile range
KXRF	K-X-Ray-Fluorescence
NAS	Normative Aging Study
NMDA	N-methyl-D-aspartate
OR	Odds ratio
US	Unconditioned stimulus

References

1. Alvarez RP, Johnson L, Grillon C. Contextual-specificity of short-delay extinction in humans: renewal of fear-potentiated startle in a virtual environment. *Learn Mem.* 2007; 14:247–53. [PubMed: 17412963]
2. Aydelott J, Leech R, Crinion J. Normal adult aging and the contextual influences affecting speech and meaningful sound perception. *Trends Amplif.* 2010; 14:218–32. [PubMed: 21307006]
3. Balbus-Kornfeld JM, Stewart W, Bolla KI, Schwartz BS. Cumulative exposure to inorganic lead and neurobehavioural test performance in adults: an epidemiological review. *Occup Environ Med.* 1995; 52:2–12. [PubMed: 7697135]
4. Bandeen-Roche K, Glass TA, Bolla KI, Todd AC, Schwartz BS. Cumulative lead dose and cognitive function in older adults. *Epidemiology.* 2009; 20:831–9. [PubMed: 19752734]
5. Barad M. Is extinction of fear erasure or inhibition? Why both, of course. *Learn Mem.* 2006; 13:108–9. [PubMed: 16585787]
6. Bechara A, Tranel D, Damasio H, Adolphs R, Rockland C, Damasio AR. Double dissociation of conditioning and declarative knowledge relative to the amygdala and hippocampus in humans. *Science.* 1995; 269:1115–8. [PubMed: 7652558]
7. Bellinger D. Very low lead exposures and children's neurodevelopment. *Curr Opin Pediatr.* 2008; 20:172–7. [PubMed: 18332714]

8. Blumenthal TD, Cuthbert BN, Filion DL, Hackley S, Lipp OV, van Boxtel A. Committee report: Guidelines for human startle eyeblink electromyographic studies. *Psychophysiology*. 2005; 42:1–15. [PubMed: 15720576]
9. Bouchard D, Mergler M, Baldwin M, Panisset H, Roels. Neuropsychiatric symptoms and past manganese exposure in a ferro-alloy plant. *Neurotoxicology*. 2007; 28:290–7. [PubMed: 16962176]
10. Braga MF, Pereira EF, Albuquerque EX. Nanomolar concentrations of lead inhibit glutamatergic and GABAergic transmission in hippocampal neurons. *Brain Res*. 1999; 826:22–34. [PubMed: 10216193]
11. Campeau S, Falls WA, Cullinan WE, Helmreich DL, Davis M, Watson SJ. Elicitation and reduction of fear: behavioural and neuroendocrine indices and brain induction of the immediate-early gene c-fos. *Neuroscience*. 1997; 78:1087–104. [PubMed: 9174076]
12. Canfield R, Henderson CJ, Cory-Slechta D, Cox C, Jusko T, Lanphear B. Intellectual impairment in children with blood lead concentrations below 10 microg per deciliter. *N Engl J Med*. 2003; 348:1517–26. [PubMed: 12700371]
13. Carter RM, Hofstotter C, Tsuchiya N, Koch C. Working memory and fear conditioning. *Proc Natl Acad Sci U S A*. 2003; 100:1399–404. [PubMed: 12552137]
14. Cheng Y, Schwartz J, Sparrow D, Aro A, Weiss ST, Hu H. Bone lead and blood lead levels in relation to baseline blood pressure and the prospective development of hypertension: the Normative Aging Study. *Am J Epidemiol*. 2001; 153:164–71. [PubMed: 11159162]
15. Commissaris RL, Tavakoli-Nezhad M, Barron AJ, Pitts DK. Effects of chronic low-level oral lead exposure on prepulse inhibition of acoustic startle in the rat. *Neurotoxicol Teratol*. 2000; 22:55–60. [PubMed: 10642114]
16. Cory-Slechta DA, Virgolini MB, Thiruchelvam M, Weston DD, Bauter MR. Maternal stress modulates the effects of developmental lead exposure. *Environ Health Perspect*. 2004; 112:717–30. [PubMed: 15121516]
17. Curtin, JJ. Unpublished computer program. Florida State University; Tallahassee, FL: 1996. Winstar: a windows-based program for scoring peak analog startle responses.
18. Davies MF, Tsui J, Flannery JA, Li X, DeLorey TM, Hoffman BB. Activation of alpha2 adrenergic receptors suppresses fear conditioning: expression of c-Fos and phosphorylated CREB in mouse amygdala. *Neuropsychopharmacology*. 2004; 29:229–39. [PubMed: 14583739]
19. Davis M, Antoniadis E, Amaral D, Winslow J. Acoustic startle reflex in rhesus monkeys: a review. *Rev Neurosci*. 2008; 19:171–85. [PubMed: 18751523]
20. Davis M, Gendelman D, Tischler M, Gendelman P. A primary acoustic startle circuit: lesion and stimulation studies. *J Neurosci*. 1982; 2:791–805. [PubMed: 7086484]
21. Davis M, Redmond DE Jr, Baraban JM. Noradrenergic agonists and antagonists: effects on conditioned fear as measured by the potentiated startle paradigm. *Psychopharmacology (Berl)*. 1979; 65:111–8. [PubMed: 117478]
22. Ferguson SA, Holson RR, Gazzara RA, Siitonen PH. Minimal behavioral effects from moderate postnatal lead treatment in rats. *Neurotoxicol Teratol*. 1998; 20:637–43. [PubMed: 9831125]
23. Ford JM, Roth WT, Isaacks BG, White PM, Hood SH, Pfefferbaum A. Elderly men and women are less responsive to startling noises: N1, P3 and blink evidence. *Biol Psychol*. 1995; 39:57–80. [PubMed: 7734630]
24. Fortin MC, Cory-Slechta DA, Ohman-Strickland P, Nwankwo C, Yanger TS, Todd AC, Moynihan J, Walton J, Brooks A, Fiedler N. Increased lead biomarker levels are associated with changes in hormonal response to stress in occupationally exposed male participants. *Environ Health Perspect*. 2012; 120:278–83. [PubMed: 22112310]
25. Fridlund AJ, Cacioppo JT. Guidelines for human electromyographic research. *Psychophysiology*. 1986; 23:567–89. [PubMed: 3809364]
26. Gilbert ME, Kelly ME, Samsam TE, Goodman JH. Chronic developmental lead exposure reduces neurogenesis in adult rat hippocampus but does not impair spatial learning. *Toxicol Sci*. 2005; 86:365–74. [PubMed: 15788721]
27. Grillon C, Ameli R, Woods SW, Merikangas K, Davis M. Fear-potentiated startle in humans: effects of anticipatory anxiety on the acoustic blink reflex. *Psychophysiology*. 1991; 28:588–95. [PubMed: 1758934]

28. Grillon C, Baas J. A review of the modulation of the startle reflex by affective states and its application in psychiatry. *Clin Neurophysiol.* 2003; 114:1557–79. [PubMed: 12948786]
29. Grillon C, Cordova J, Morgan CA, Charney DS, Davis M. Effects of the beta-blocker propranolol on cued and contextual fear conditioning in humans. *Psychopharmacology (Berl).* 2004; 175:342–52. [PubMed: 15007536]
30. Grillon C, Pine DS, Baas JM, Lawley M, Ellis V, Charney DS. Cortisol and DHEA-S are associated with startle potentiation during aversive conditioning in humans. *Psychopharmacology (Berl).* 2006; 186:434–41. [PubMed: 16052364]
31. Gump BB, Stewart P, Reihman J, Lonky E, Darvill T, Parsons PJ, Granger DA. Low-level prenatal and postnatal blood lead exposure and adrenocortical responses to acute stress in children. *Environ Health Perspect.* 2008; 116:249–55. [PubMed: 18288326]
32. Hamann S, Monarch ES, Goldstein FC. Impaired fear conditioning in Alzheimer's disease. *Neuropsychologia.* 2002; 40:1187–95. [PubMed: 11931922]
33. Hitchcock JM, Sananes CB, Davis M. Sensitization of the startle reflex by footshock: blockade by lesions of the central nucleus of the amygdala or its efferent pathway to the brainstem. *Behav Neurosci.* 1989; 103:509–18. [PubMed: 2544201]
34. Hoefler M, Allison SC, Schauer GF, Neuhaus JM, Hall J, Dang JN, Weiner MW, Miller BL, Rosen HJ. Fear conditioning in frontotemporal lobar degeneration and Alzheimer's disease. *Brain.* 2008; 131:1646–57. [PubMed: 18492729]
35. Howland JG, Wang YT. Synaptic plasticity in learning and memory: stress effects in the hippocampus. *Prog Brain Res.* 2008; 169:145–58. [PubMed: 18394472]
36. Hu H, Aro A, Payton M, Korrick S, Sparrow D, Weiss ST, Rotnitzky A. The relationship of bone and blood lead to hypertension. The Normative Aging Study. *JAMA.* 1996; 275:1171–6. [PubMed: 8609684]
37. Kim D, Lawrence DA. Immunotoxic effects of inorganic lead on host resistance of mice with different circling behavior preferences. *Brain Behav Immun.* 2000; 14:305–17. [PubMed: 11120598]
38. Kim R, Landrigan C, Mossman P, Sparrow D, Hu H. Age and secular trends in bone lead levels in middle-aged and elderly men: three-year longitudinal follow-up in the Normative Aging Study. *Am J Epidemiol.* 1997; 146:586–91. [PubMed: 9326437]
39. Koch M. The neurobiology of startle. *Prog Neurobiol.* 1999; 59:107–28. [PubMed: 10463792]
40. Kofler M, Muller J, Reggiani L, Valls-Sole J. Influence of age on auditory startle responses in humans. *Neuroscience Letters.* 2001; 307:65–68. [PubMed: 11427301]
41. Kumari V, Cotter P, Corr PJ, Gray JA, Checkley SA. Effect of clonidine on the human acoustic startle reflex. *Psychopharmacology (Berl).* 1996; 123:353–60. [PubMed: 8867875]
42. Labar KS, Cook CA, Torpey DC, Welsh-Bohmer KA. Impact of healthy aging on awareness and fear conditioning. *Behav Neurosci.* 2004; 118:905–15. [PubMed: 15506873]
43. Lang P, Davis M, Ohman A. Fear and anxiety: animal models and human cognitive psychophysiology. *J Affect Disord.* 2000; 61:137–59. [PubMed: 11163418]
44. Lanphear BP, Hornung R, Khoury J, Yolton K, Baghurst P, Bellinger DC, Canfield RL, Dietrich KN, Bornschein R, Greene T, et al. Low-level environmental lead exposure and children's intellectual function: an international pooled analysis. *Environ Health Perspect.* 2005; 113:894–9. [PubMed: 16002379]
45. Lee HJ, Choi JS, Brown TH, Kim JJ. Amygdalar nmda receptors are critical for the expression of multiple conditioned fear responses. *J Neurosci.* 2001; 21:4116–24. [PubMed: 11356900]
46. Lindgren KN, Masten VL, Ford DP, Bleecker ML. Relation of cumulative exposure to inorganic lead and neuropsychological test performance. *Occup Environ Med.* 1996; 53:472–7. [PubMed: 8704872]
47. Mergler D. Neurotoxic effects of low level exposure to manganese in human populations. *Environ Res.* 1999; 80:99–102. [PubMed: 10092399]
48. Morgan CA 3rd, Grillon C, Southwick SM, Davis M, Charney DS. Fear-potentiated startle in posttraumatic stress disorder. *Biol Psychiatry.* 1995; 38:378–85. [PubMed: 8547457]
49. Muller D, Nikonenko I, Jourdain P, Alberi S. LTP, memory and structural plasticity. *Curr Mol Med.* 2002; 2:605–11. [PubMed: 12420800]

50. Neal AP, Guilarte TR. Molecular neurobiology of lead (Pb(2+)): effects on synaptic function. *Mol Neurobiol.* 2010; 42:151–60. [PubMed: 21042954]
51. Neumann DL, Waters AM. The use of an unpleasant sound as an unconditional stimulus in a human aversive Pavlovian conditioning procedure. *Biol Psychol.* 2006; 73:175–185. [PubMed: 16698165]
52. Pape HC, Stork O. Genes and mechanisms in the amygdala involved in the formation of fear memory. *Ann N Y Acad Sci.* 2003; 985:92–105. [PubMed: 12724151]
53. Sah P, Westbrook RF, Luthi A. Fear conditioning and long-term potentiation in the amygdala: what really is the connection? *Ann N Y Acad Sci.* 2008; 1129:88–95. [PubMed: 18591471]
54. Salinas JA, Huff NC. Lead and conditioned fear to contextual and discrete cues. *Neurotoxicol Teratol.* 2002; 24:541–50. [PubMed: 12127900]
55. Sanders T, Liu Y, Buchner V, Tchounwou PB. Neurotoxic effects and biomarkers of lead exposure: a review. *Rev Environ Health.* 2009; 24:15–45. [PubMed: 19476290]
56. Schaub C, Koch M. Blockade of NMDA receptors in the amygdala prevents latent inhibition of fear-conditioning. *Learn Mem.* 2000; 7:393–9. [PubMed: 11112798]
57. Shih RA, Hu H, Weisskopf MG, Schwartz BS. Cumulative lead dose and cognitive function in adults: a review of studies that measured both blood lead and bone lead. *Environ Health Perspect.* 2007; 115:483–92. [PubMed: 17431502]
58. Sigurdsson T, Doyere V, Cain CK, LeDoux JE. Long-term potentiation in the amygdala: a cellular mechanism of fear learning and memory. *Neuropharmacology.* 2007; 52:215–27. [PubMed: 16919687]
59. Verina T, Rohde CA, Guilarte TR. Environmental lead exposure during early life alters granule cell neurogenesis and morphology in the hippocampus of young adult rats. *Neuroscience.* 2007; 145:1037–47. [PubMed: 17276012]
60. Vezar T, Hoyk PAZ, Varga C, Naray M, Nagymajtényi L. Behavioral and neurotoxicological effects of subchronic manganese exposure in rats. *Environmental toxicology and pharmacology.* 2005; 19:797–810. [PubMed: 21783557]
61. Vezar T, Kurunczi A, Naray M, Papp A, Nagymajtényi L. Behavioral effects of subchronic inorganic manganese exposure in rats. *Am J Ind Med.* 2007; 50:841–52. [PubMed: 17918212]
62. Virgolini MB, Chen K, Weston DD, Bauter MR, Cory-Slechta DA. Interactions of chronic lead exposure and intermittent stress: consequences for brain catecholamine systems and associated behaviors and HPA axis function. *Toxicol Sci.* 2005; 87:469–82. [PubMed: 16049266]
63. Walker DL, Davis M. The role of amygdala glutamate receptors in fear learning, fear-potentiated startle, and extinction. *Pharmacol Biochem Behav.* 2002; 71:379–92. [PubMed: 11830172]
64. Weike AI, Schupp HT, Hamm AO. Fear acquisition requires awareness in trace but not delay conditioning. *Psychophysiology.* 2007; 44:170–80. [PubMed: 17241153]
65. Weisskopf MG, Bauer EP, LeDoux JE. L-type voltage-gated calcium channels mediate NMDA-independent associative long-term potentiation at thalamic input synapses to the amygdala. *J Neurosci.* 1999; 19:10512–9. [PubMed: 10575047]
66. Weisskopf MG, Proctor SP, Wright RO, Schwartz J, Spiro A 3rd, Sparrow D, Nie H, Hu H. Cumulative lead exposure and cognitive performance among elderly men. *Epidemiology.* 2007; 18:59–66. [PubMed: 17130688]
67. White LD, Cory-Slechta DA, Gilbert ME, Tiffany-Castiglioni E, Zawia NH, Virgolini M, Rossi-George A, Lasley SM, Qian YC, Basha MR. New and evolving concepts in the neurotoxicology of lead. *Toxicol Appl Pharmacol.* 2007; 225:1–27. [PubMed: 17904601]
68. Wilcox RR, Keselman HJ. Modern robust data analysis methods: measures of central tendency. *Psychol Methods.* 2003; 8:254–74. [PubMed: 14596490]
69. Wilker E, Korrick S, Nie LH, Sparrow D, Vokonas P, Coull B, Wright RO, Schwartz J, Hu H. Longitudinal changes in bone lead levels: the VA Normative Aging Study. *J Occup Environ Med.* 2011; 53:850–5. [PubMed: 21788910]
70. Wormley DD, Ramesh A, Hood DB. Environmental contaminant-mixture effects on CNS development, plasticity, and behavior. *Toxicol Appl Pharmacol.* 2004; 197:49–65. [PubMed: 15126074]

Figure 1.

Mean startle amplitude by block. Bars represent mean amplitude averaged across CS+, CS– and ITI conditions. Error bars indicate standard error.

Figure 2.

Differential startle amplitude (DSA) by protocol block for participants with qualifying startles for all post-acquisition blocks ($n = 33$). Error bars indicate the standard deviation. * indicates $p < 0.01$ and ** indicates $p < 0.001$ for differences from 0.

Table 1

Distribution of lead biomarker levels by participant characteristics

Characteristic	Patella lead (n = 51)	
	N (%)	Mean (µg/g)
Age (years)		
<70	11 (21.6)	15.7
70–74	12 (23.5)	17.1
75–79	15 (29.4)	20.8
80+	13 (25.5)	36.2
Education (years)		
<13	16 (31.4)	27.6
13–15	11 (21.6)	21.7
16+	24 (47.1)	20.0
Smoking status		
Never	13 (25.5)	19.1
Ever	38 (74.5)	24.0
Hearing Aids		
Yes	3 (5.9)	20.3
No	47 (92.2)	23.0
Missing	1 (1.9)	15
Financial stability		
“Can’t make ends meet with current income”	1 (1.9)	17.0
“Just about manage to get by”	8 (15.7)	18.8
“Have enough to get along, and even a little extra”	31 (60.8)	23.8
“Can buy anything I want”	8 (15.7)	22.8
Missing	3 (5.9)	24.0

Difference (95% confidence interval, CI) in conditioned learning (differential startle amplitude, DSA, in standard deviation units) per 20µg/g patella^a lead by post-acquisition block. Multivariate models are adjusted for age, smoking, education, hearing aid use, and financial stability.

Table 2

Protocol block	Unadjusted		Multivariate-adjusted		Interval-corrected ^b	
	Effect estimate (95% CI)	p-value	Effect estimate (95% CI)	p-value	Effect estimate (95% CI)	p-value
Post-acq. block 1 (n = 45)	-0.17 (-0.47, 0.14)	0.28	-0.01 (-0.34, 0.32)	0.90	0.06 (-0.46, 0.58)	0.82
Post-acq. block 2 (n= 40)	-0.02 (-0.54, 0.50)	0.94	-0.23 (-0.80, 0.34)	0.33	-0.24 (-1.16, 0.69)	0.62
Post-acq. block 3 (n= 37)	-0.56 (-0.98, -0.14)	0.01	-0.44 (-0.91, 0.02)	0.06	-0.79 (-1.56, -0.03)	0.04

^aThe interquartile range among the parent population [66]

^bMultivariate-adjusted model with interval correction done for patella lead with estimated half-life of 8 years.

Odds ratio (OR) and 95% confidence interval (CI) for contingency awareness per 20µg/g patella^a lead. Multivariate models are adjusted for age, smoking, education, hearing aid use, and financial stability (n=51).

Table 3

Patella	Unadjusted		Multivariate-adjusted		Interval-corrected ^b	
	Odds ratio (95% CI)	p-value	Odds ratio (95% CI)	p-value	Odds ratio (95% CI)	p-value
Per 20µg/g	0.93 (0.87, 0.99)	0.02	0.91 (0.84, 0.99)	0.03	0.88 (0.78, 0.99)	0.04

^aThe interquartile range among the parent population [66]

^bMultivariate-adjusted model with interval correction done for patella lead with estimated half-life of 8 years.