QUANTIFICATION OF NEUROMOTOR FUNCTION FOR DETECTION OF THE EFFECTS OF MANGANESE.

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Abstract

The effect of low level exposure to manganese (Mn) was examined in 297 subjects from southwest Québec. Blood manganese (MnB) levels as well as other possibly relevant variables were obtained. We tested equipment and analysis procedures that we have developed to quantify aspects of motor function thought to be affected by exposure to toxins, in particular, rapid alternating movements, rapid and precise pointing movements, and tremor. (1) The eurythmokinesimeter measures timing and precision of contacts between a hand-held stylus and a pair of metal targets (proximal/distal. This roughly approximates the finger-to-nose test of the UPDRS. Characteristics quantifying speed, precision and regularity of the movements were calculated, as well as multiple contacts due to tremor and an index based on Fitts' Law eliminating the effect of the trade-off between speed and precision. (2) The diadochokinesimeter accurately measures rapid rotation of the forearms (pronation/supination). Characteristics quantifying the range, speed, period, shape and regularity of the oscillatory movements were calculated, as well as the smoothness of the movement on a fine scale and the coordination between the two hands. (3) Postural tremor of the arm and hand was measured using the accelerometry-based "TREMOR" system of Danish Product Development. We used the amplitude and frequency characteristics provided by the TREMOR system: intensity, center frequency, frequency dispersion and harmonic index. Previous studies have shown that these tests are sufficiently sensitive to detect small differences in performance of different groups of subjects, with indications that some characteristics are also specific to particular conditions. In this study, significant though small effects related to age and gender were found in many of the characteristics. When effects of other variables are removed, low-level exposure to Mn was found to be associated with a decrease in ability to perform regular, rapid and precise pointing movements, a decrease in ability to attain high maximum rotation speeds in rapid alternating movements, and an increase in regularity of tremor oscillations. Moreover, the effects are age-related for levels of MnB $\geq$7.5µg/L.

Running head: QUANTIFICATION OF NEUROMOTOR FUNCTION

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Introduction

Qualitative clinical assessment of subtle neuromotor dysfunction can be unreliable. Detection of the presence or absence of effects on neuromotor function of *low* levels of exposure to toxins such as manganese (Mn) depends on quantitative tests that are sufficiently sensitive, specific and reliable. Of particular relevance is quantification of neuromotor tests that simulate those used in clinical examinations, especially those that reveal neuromotor deficits in subjects with *high* levels of exposure to Mn, such as rapid pointing tasks, rapid alternating movements and tremor (Mergler and Baldwin, 1997, and other references cited in that paper).

Equipment for the precise measurement of rapid alternating movements (pronation/supination) and rapid pointing movements has been developed over the last few years at our laboratory at UQAM. The reliability, sensitivity and specificity of these systems has been investigated for populations exposed to methylmercury and Mn and for patients with Parkinson's disease (Beuter *et al*., 1994; Beuter *et al*., 1998a,b). A commercially available computer tremor analysis system has also been evaluated in our laboratory recently (Edwards and Beuter, 1997), particularly for patients with Parkinson's disease who do not necessarily have pronounced symptoms.

On the basis of promising results from this previous work these three tests of different aspects of neuromotor function were used in a large study of low-level environmental exposure to Mn. Multivariate analyses, carried out on the relation between blood manganese (MnB) and the different neuro-outcomes examined in this study, showed that these neuromotor tests were particularly sensitive (Mergler *et al.*, 1998, this issue). Here, a detailed description of the equipment and testing procedures is provided, and the quantitative characteristics derived from the recordings are examined. Multivariate analyses including the results of these and other tests are reported in Mergler *et al.* (1998, this issue), and since a significant relation to MnB is found there, we employ univariate methods to identify the components of movement that are affected.

Materials and Methods

Study population

A careful selection procedure was used to produce a stratified random sample of 297 French-speaking people who were residents of a region of southwest Québec near a closed ferroalloy plant. Details of this procedure are described by Mergler *et al*. (1998, this issue). Aside from MnB levels, many other potentially confounding or otherwise relevant variables were recorded for each subject, including age (20 to 69 years), residential history, education, dietary habits, alcohol consumption, smoking, medical history, history of previous exposure, gender, etc. The sample was reduced by removing persons with sequelae to neurological illness and persons with heavy alcohol consumption (≥ 420 g/week or with alcohol dependancy). The final group for the study as a whole included 273 persons. For this part of the study, however, data for three of these was lost due to a power failure, so the sample size for the results below is 270.

Test procedures

1. **Eurythmokinesimetry (EKM)**

The eurythmokinesimeter (Beuter *et al*., 1998a) quantifies eye-hand coordination in a pointing task. It is composed of two rigidly mounted metallic squares (side: 10 cm) each containing 3 electrically isolated concentric circular areas (radii: 1.4, 4.4, and 11.5mm). The system is placed in front of the subject at a distance such that when touching the distal target, the subject's arm is almost completely extended. Inclination of the target plane is kept constant at 30 degrees from the horizontal. Subjects are asked to hold the stylus like a pen and touch alternately the center of each target as quickly and precisely as possible. The ability of subjects to see the targets properly is verified beforehand by asking them to read typewritten letters on a sheet of paper placed temporarily over the targets. Subjects are given sufficient rest time between trials to avoid fatigue. Information on location and timing of contacts between stylus and target is recorded for 30 seconds per trial. This raw data is downloaded to a personal computer and further analysis is done using the Splus software (MathSoft, Seattle, Wa).

In this study, performance was characterized by the three characteristics that follow. Eight values for each characteristic (2 trials, 2 hands, 2 targets) were obtained for each subject, but for analysis were combined into two, one average measure for each target.

EKM Characteristics

¬ **Multiple contacts:** [number of contacts] - [number of target areas contacted], averaged over events, i.e., the frequency of rebound contacts. Larger values indicate more tremor.

¬ **Irregularity:** standard deviation of intervals between events. Larger values indicate less regular timing of movement.

¬ **Fitts' Law constant:** a measure of ability independent of the compromise between speed and accuracy (see below). Larger values indicate more difficulty (Beuter *et al*., 1998a).

Fitts' Law asserts that the time required to make a rapid movement (t) is proportional to an "index of difficulty" involving the precision of the movement (Fitts, 1954). The "constant" of proportionality in Fitts' Law, which may be different for each subject, can be calculated from the EKM data as the average over events of

$$\mathbf{k = t / \log(2A/W)},$$

where **t** = transit time, **A** = distance between target centers = 25cm and **W** = approximate distance between the location of contact(s) and the target center. **W** is calculated as the mean of the maximum and minimum distances possible in one target area (central circle or annular regions around it) in case of contact on a single target area, and as the distance of the boundary between areas in case of contacts on two areas in the same event.

This should measure inherent ability independent of the subject's choice in the speed/accuracy trade-off (the lower **k** is, the better the performance). In principle, the value of **k** should not vary for a given subject, but it should reflect differences in ability between subjects, in the sense of different accuracy for the same speed or different speed for the same accuracy. In practice, the values do vary across events in a trial for a given subject, but the within-subject variability is reduced by taking the average over the many events of the trial. For this particular task, the range of the "index of difficulty" (which measures accuracy) was found to be rather narrow (see Fig. 1). Thus, the value of **k** is more sensitive to differences in duration of movement than to differences in accuracy based on target areas contacted. It can be interpreted as a measurement of speed with a small adjustment to account for accuracy.

2. **Diadochokinesimetry (DIADO)**

The diadochokinesimeter (Beuter *et al.*, 1994; Beuter *et al.*, 1998b) quantifies diadochokinesia, the ability to perform rapid alternating movements (RAMs). Subjects are asked to keep their elbows by the trunk and flexed at about 90 degrees while rotating 2 hand-held foam spheres connected via flexible rods to optical encoders inside the unit. Angular displacement is recorded at a rate of 200 samples per sec for 5 sec with a resolution of 0.36 degree. The multidirectional joints connecting the rods to the unit, an improvement over previous similar systems, allow even subjects with severe movement disorders to complete the test.

Two trials were performed under each of four conditions (see Fig. 2):

- normal cadence, with both hands moving simultaneously (NC2),
- fast cadence, with only the right hand moving, the left held in position (FC1),
- fast cadence, with only the left hand moving, the right held in position (FC1),
- fast cadence, with both hands moving simultaneously (FC2).

Subjects are given sufficient rest time between trials to avoid fatigue. For each active hand and each trial, 7 characteristics were calculated. The first 6 of these are a subset of the 10 characteristics suggested by Okada & Okada (1983), to which we have added coherence as a measure of coordination between the hands (Beuter *et al.* 1998b). The remaining characteristics suggested in these two papers were excluded from consideration due to high correlations with other characteristics, lack of reliability across trials or both (Beuter, *et al*. (1998b). Means over the two trials and two hands were used for analysis.

DIADO Characteristics

- **Duration:** Mean duration of a pronation/supination cycle (in units of 100 msec, after

Okada and Okada, 1983). Larger values mean slower movement.

¬ **Range:** Mean of total angular displacement (pronation and supination) per cycle (in degrees). Larger values indicate greater range.

¬ **Maximum slope:** Maximum slope (velocity) per movement, averaged over each ascending and descending part of all the pronation and supination movements of a trial (in degrees per sampling point; multiply by 200 to obtain degrees per second). Larger values indicate faster movement.

¬ **Similarity in shape:** It is the weighted mean of "distances" between all pairs of cycles in which nearby cycles are weighted more heavily. "Distances" are based on seven characteristics characterizing the shape of each cycle. The larger the number the more irregular the performance. (The logarithm is used for analysis, since its distribution is approximately log-normal).

¬ **Smoothness:** Fine scale irregularity of movement, calculated from oscillations in the second differences of the signal. The larger the number the more irregular the performance. (The logarithm is used for analysis, since its distribution is approximately log-normal).

¬ **Sharpness:** Mean absolute value of velocity relative to peak velocity for ascending and descending segments and averaged across all cycles. If the peak velocity is high and sustained for a very short time with most of the rest of the time spent in relatively slow movement or none, then the value will be large. Conversely, if much of the time is spent ramping up to maximum velocity and ramping down again, the value will be small. Thus, this measure is sensitive to the ability to rapidly stop and start movements. The larger the number the less difficulty there is at the turn.

¬ **Coherence:** The height of the highest peak in spectral coherence between zero and six Hz (i.e., up to the fastest cadence observed for pronation-supination movements). This reflects the coordination of the oscillations in the two hands. A high coherence value means that the two hands are synchronized (though not necessarily in phase). In the conditions in which one hand is immobile, a value different from zero indicates the presence of synkinesia, so for this condition, larger values are considered worse. For the 2-hand conditions, we used log(1-coherence) for analysis, since the bulk of the distribution is squeezed up against the maximum value of 1, and this transformation again makes larger values worse.

3. **Tremor**

We used the TREMOR system developed by Danish Product Development, Ltd. (DPD) to measure acceleration of postural tremor (DPD, 1994; Edwards and Beuter, 1997). Subjects look at a light stylus held in one hand horizontally 10 cm in front of the navel. Two axis micro-accelerometers embedded in the tip of the stylus measure accelerations in orthogonal directions for 24.6 seconds. Tremor is recorded in the two hands successively. Fourier transforms are calculated from the two time series and are combined to give a single power spectrum, which is displayed graphically by the system (Fig. 3).

Four characteristics are calculated and reported by the system for each hand and these are then combined into a single "tremor index" for each hand. We omitted the tremor index as it is a composite of the other four. For subsequent analysis, we used the mean over the two hands of each of the four retained characteristics.

TREMOR Characteristics

¬ **Tremor intensity (amplitude):** the root mean square of acceleration recorded in the 0.9 Hz to 15 Hz band (m/s^2). (The logarithm was used for analysis as its distribution is approximately log-normal). Larger values indicate more tremor.

¬ **Center frequency:** the median frequency (Hz) of the acceleration in the 0.9 Hz to 15 Hz band: 50% of the area under the spectrum is at frequencies above the center frequency and 50% is below. Normal physiological tremor tends to fall near 7 Hz in this test.

¬ **Dispersion about center frequency:** the distance (Hz) to either side of the center frequency that contains 68% of the spectral power. Pathological tremors tend to be regular and thus have spectral power in a narrow frequency band.

¬ **Harmonic index:** a measure of how similar the tremor oscillations are to a sinusoid. Normal physiological tremor is irregular and has a low harmonic index. For analysis, we use -log(1-HI) (see Edwards and Beuter, 1997,1998). Larger values are more regular.

Statistical Analyses

Simple and multiple regression analyses were performed in order to examine the relation between each of the characteristics and the variables: gender, age, educational level, smoking status, alcohol consumption, blood lead, serum iron and MnB. Because of their distribution (Baldwin et al. 1998, same issue), log values were used for the bioindicators. If one of the values was missing, the individual was excluded.

For the Fitts' constant, the 90th percentile was used as a cut-off point and a comparison of the prevalence of persons with results $\geq$ the 90th percentile was examined with respect to age category (<50years, $\geq$50years) and MnB category (<7.5µg/L, $\geq$7.5µg/L), using a contingency table with the Fisher Exact Test. The relative risk was calculated.

Results

Fig. 4 shows a scatter plot of Fitts' constant from the EKM versus age, illustrating the overall variability amongst subjects and the variation with age.

Table 1 shows the results of multiple regression analyses for the EKM characteristics (multiple contacts, irregularity and Fitts' constant) with respect to gender, age, educational level, smoking status, alcohol consumption, blood lead, serum iron and MnB. For all of the characteristics, performance decreases with age, while women display more multiple contacts and more irregularity. There may be some effect related to education for multiple contacts.

Irregularity and Fitts' constant increase significantly with MnB. Since a manganese-age interaction has been shown with these EKM results (Mergler *et al.*, 1998, this issue), this relation was further examined. Figure 5 shows the results for these characteristics, adjusted for gender, educational level, smoking status, alcohol consumption, blood lead and serum iron, with respect to age decade, for those with lower MnB (<7.5µg/L) and higher MnB (≥7.5µg/L). An increase in irregularity with age is observed for those in the lower MnB category (n =136; r_2 = 0.06; F= 8.28; p<0.01), while for those in the higher MnB category, this relation is much stronger (n =124; r^2 = 0.12; F= 16.2; p<0.0001). For the Fitts' constant, no relation is observed with age in the lower MnB category (n = 139; r^2 < 0.01; F= 0.90; p = 0.34), while a very significant relation is present for the higher MnB category (n =124; r^2 = 0.09; F= 12.5; p<0.001).

The combined MnB-age effect was further examined using the 90th percentile score from the Fitts' constant; for those in the lower MnB category, the prevalence of persons under 50 years of age, with scores equal to or above the 90th percentile is 7.2% (n = 6), while for those 50 years or above, it is 8.5 % (n = 5); for those in the upper MnB category, the prevalence for those under 50 years of age is 6.8% (n = 6) and for those 50 years or older, it is 26.2% (n = 11). The differences are significant (Fisher Exact test: p<0.01); the Relative Risk is 2.04 [95% confidence interval: 1.06 - 3.93].

Table 2 also shows the results of the multiple regression analyses for the DIADO. Gender differences are present for almost all characteristics, with women performing more slowly than men but with sharper turns, greater range and less irregular movement. There is an increase in cycle duration with age, and a decrease in sharpness, but range and coherence increase (FC2) and the movements are smoother (FC1 and FC2). The maximum slope is unaffected by age, but is significantly associated with log MnB for maximum cadence with each hand separately, and a tendency is present (p = 0.07) for maximum cadence with both hands together. MnB did not influence the age relations for the DIADO parameters. There may be some effect related to smoking for sharpness and coherence.

Multiple regression analyses for the TREMOR system characteristics (Table 3) show that tremor intensity and center frequency were significantly higher among men, while the center frequency of tremor decreased significantly with age and the tremor oscillations became more regular (decreased frequency dispersion and increased harmonic index). Center frequency was lower among smokers, and tremor oscillations became more regular with increasing log MnB (frequency dispersion decreased and harmonic index increased). The age relations for frequency dispersion and harmonic index were influenced by MnB (Figure 6). For frequency dispersion, no relation with age was observed for those in the lower MnB category ($n = 140$; $r^2 = 0.01$; $F= 1.30$; $p = 0.26$), while a significant relation was observed for those in the higher MnB category ($n = 124$; $r^2 = 0.4$; $F= 5.38$; $p=0.02$). Similarly, harmonic index showed no relation with age for those in the lower MnB category ($n = 140$; $r^2 = 0.01$; $F= 1.74$; $p = 0.19$) but a very significant relation for those in the higher MnB category ($n = 124$; $r^2 = 0.14$; $F= 19.7$; $p<0.001$).

Discussion

These results indicate that the instruments, procedures and analyses used here are able to detect subtle differences in neuromotor abilities. There are small, though highly significant differences in average performance on many aspects of movement between men and women. Why gender related differences exist is not clear; it will be interesting to examine these in detail in future studies.

MnB level was associated with a decrease in ability to perform regular, rapid and precise pointing movements, a decrease in ability to attain high maximum rotation speeds in rapid alternating movements and an increase in regularity of tremor oscillations. Although many separate statistical tests were performed here, multivariate analysis performed on all of the tests from the study which involved coordinated upper limb movements showed an effect of MnB (Mergler *et al.*, 1998, this issue). The observed changes are small in comparison to the overall variability, but the neuromotor tests used here were capable of measuring them. Moreover, the profile of changes is consistent with the literature on Mn exposure and with the action of Mn on the central nervous system (see Mergler and Baldwin, 1997, for review)

Overall, performance on most of the characteristics varied with age. However, MnB appears to affect the age relation for certain neuromotor characteristics. For Fitts' constant and irregularity from the EKM and frequency dispersion and harmonic index from the TREMOR system, at lower MnB levels (<7.5µg/L), the relation with age is no longer present or less pronounced as compared to the very significant age relation for those in the higher MnB category (≥7.5µg/L). These findings suggest that MnB modifies the relation between age and these neuromotor functions. On the basis of results from animal studies, accelerated ageing associated with Mn has been hypothesized (Donaldson, 1987; Stokes *et al.*, 1988; Weiss, 1996). The MnB-age interaction is particularly evident in the Fitts' constant, which reflects ability to perform rapid and precise pointing movements. Among persons 50 years of age and over, the prevalence of those in the upper 90th percentile is significantly higher for those in the higher MnB category, with a relative risk of about 2. The characteristics from these neuromotor tests are little affected by other parameters, such as education, smoking, alcohol intake, blood lead and serum iron.

In addition to the sensitivity of these tests, they are valuable for the development of profiles of neuromotor function. Our past experience in this field has taught us that three components were necessary to detect differences between populations. We needed: (1) highly reliable and precise quantitative instruments; (2) procedures that could reveal or unmask the presence of motor deficits even in their early and subtle form; and (3) sophisticated mathematical tools to analyze the results. These techniques will now provide information addressing a fourth need, to define "normal", "questionable" or "abnormal" motor profiles, such as the profile of motor changes related to exposure to Mn. We recommend that basic areas of motor function such as those reported here should be quantified in toxicity studies and included in these motor profiles.

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Table 1: Multiple regression analyses on each characteristic for the EKM test. Each row gives F-statistics for each factor as well as for the model as a whole. (PbB = blood lead; FeS = serum iron; MnB = blood manganese).

measure	n	gender	age	education	smoking	alcohol	log PbB	log FeS
EKM								
multiple contacts	263	-1.96*	3.30**	-2.26*	-1.18	-1.56	1.12	0.43
Fitts' Law constant	263	0.16	4.47***	0.85	1.09	1.35	-0.62	1.68+

+ p<0.10, * p < 0.05, ** p<0.01, *** p<0.001

Table 2: Multiple regression analyses on each characteristic for the DIADO test. Each row gives F-statistics for each factor as well as for the model as a whole. (PbB = blood lead; FeS = serum iron; MnB = blood manganese; other abbreviations: see text).

measure	n	gender	age	education	smoking	alcohol	log PbB	log FeS
DIADO								
duration								
NC2	263	-2.47*	2.62**	-0.13	1.63	0.42	-1.62	0.66
FC1	263	-5.35***	5.89***	-0.76	1.77	-0.36	-0.59	1.68+
FC2	264	-5.43***	5.89***	-0.71	1.76+	-0.37	-0.52	1.64
maximum slope								
NC2	263	2.52*	0.44	1.71	-0.55	0.11	1.73	-1.28
FC1	263	4.04***	-1.21	1.04	0.14	-0.50	0.54	-1.01
FC2	263	4.16***	-1.31	0.56	0.51	-0.40	-0.24	-0.78
FC1	262	-1.19	-5.42***	0.84	-2.15*	0.13	1.62	-1.09
FC2	262	-1.71+	-4.50***	-0.09	-1.22	-0.62	1.44	-0.15
range								
NC2	263	-2.67**	1.36	1.67	1.73	0.16	0.58	-0.69
FC1	264	-3.42***	1.70	0.98	0.77	-1.00	1.36	0.19
FC2	264	-2.53*	2.19*	0.31	1.47	-1.12	0.58	1.16
shapesim								
NC2	262	-0.46	-1.17	0.67	0.35	-0.73	0.04	-0.58
FC1	263	4.01***	0.20	-.20	0.83	-1.39	-0.79	-0.65
FC2	264	3.87***	-1.61	0.09	0.22	-0.11	- <0.01	0.28
smoothness								
NC2	263	2.82*	0.33	-1.80+	0.57	0.44	-1.86+	-0.68
FC1	264	5.00***	-2.54*	0.39	-0.42	-0.69	-0.62	-0.95
FC2	264	4.26***	-3.08**	0.67	-1.56	0.04	0.49	-1.33
coherence								
NC2	263	-1.16	0.70	0.33	-2.30*	-0.45	1.14	0.68
FC1	264	2.16*	-0.77	-0.75	-1.14	-0.31	1.85	0.28
FC2	264	3.15**	-2.64**	-0.42	0.55	0.20	0.93	-0.34

+ p<0.10, * p < 0.05, ** p<0.01, *** p<0.001

Table 3: Multiple regression analyses on each characteristic for the TREMOR test. Each row gives F-statistics for each factor as well as for the model as a whole. (PbB = blood lead; FeS = serum iron; MnB = blood manganese).

measure	n	gender	age	education	smoking	alcohol	log PbB	log FeS
TREMOR								
intensity	264	3.57***	-1.18	-0.69	-0.16	0.22	0.77	0.78
center frequency	264	2.62**	-3.58***	-0.42	-2.42*	1.17	0.88	0.79
frequency dispersion	264	0.83	-3.11***	-0.61	-1.58	-0.79	-0.94	-0.58
harmonic index	264	0.63	5.11***	0.22	0.83	-1.69+	-0.50	0.25

+ $p<0.10$, * $p < 0.05$, ** $p<0.01$, *** $p<0.001$

Figure Captions

Figure 1: Precision (index of difficulty) as a function of transit duration in the EKM test. Three subjects from another study with different values of Fitts' constant were selected for illustration. There are 8 measurements (2 trials, 2 hands, 2 targets) of precision and transit duration for each of the three subjects (represented by different symbols). The estimate of Fitts' constant for each subject is the average slope of lines from the origin through each point. Note that one subject (triangles) is less precise but that this subject's lower transit duration still gives a lower (better) Fitts' constant. The minimum and maximum precision values possible in this test are indicated by dotted lines.

Figure 2: Example of recordings from the DIADO for subject 1651. Angles are represented in degrees. Each of the four conditions lasted 5 sec and both hands are represented. Note that the movements become less regular in the condition with both hands at fast cadence.

Figure 3: Example of a tremor power spectrum from the TREMOR test for subject 1899. The center frequency (F50, solid vertical line) and the frequency dispersion (dotted lines) are marked on the spectrum. Amplitude (intensity) compared to the normal range is shown at right. The tremor intensity, center frequency, dispersion about center frequency and harmonic index are presented below. This subject has an unusually harmonic tremor, though not high in amplitude.

Figure 4: Scatter plot of Fitts' constant vs. age from the EKM (n=270). The regression line is also indicated. The p-value for a non-zero slope of the regression line is $<10^{-5}$. This illustrates the scale of the effect in relation to the overall variability among subjects.

Figure 5: Relation between EKM characteristics (adjusted for gender, educational level, smoking status, alcohol consumption, blood lead and serum iron) and age category by decade for the subjects with lower levels of MnB (<7.5 μg/L) and those with higher levels (≥7.5 μg/L). The bar

indicates one standard error from the mean. (a) irregularity vs. age category in the low MnB group; (b) irregularity vs. age category in the higher MnB group; (c) Fitts' constant vs. age category for the lower MnB group; (d) Fitts' constant vs. age category for the higher MnB group.

Figure 6: Relation between TREMOR characteristics (adjusted for gender, educational level, smoking status, alcohol consumption, blood lead and serum iron) and age category by decade for the subjects with lower levels of MnB (<7.5 µg/L) and those with higher levels (≥ 7.5 µg/L). The bar indicates one standard error from the mean. (a) frequency dispersion vs. age category in the low MnB group; (b) frequency dispersion vs. age category in the higher MnB group; (c) harmonic index vs. age category for the lower MnB group; (d) harmonic index vs. age category for the higher MnB group.