

## Plasma malondialdehyde as biomarker for oxidative stress: reference interval and effects of life-style factors

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Malondialdehyde (MDA) is one of the most frequently used indicators of lipid peroxidation. To generate reliable reference intervals for plasma malondialdehyde (P-MDA), a reference sample group was established in Funen, Denmark. The group consisted of 213 individuals (107 men, 106 women), ages 20–79 years. P-MDA was measured in EDTA-treated plasma after derivatization by thiobarbituric acid (TBA) and separation on HPLC. UV detection was performed at 532 nm. A reference interval was calculated as recommended by IFCC with REFVAL 3.42. The estimated reference limits (0.025 and 0.975 fractals) for the group were 0.36 and 1.24  $\mu\text{mol/L}$ . The data were analyzed for gender- and age-related differences. Analysis of variance showed no interaction between gender and age, but separate analyses showed an independent effect of gender ( $P = 0.03$ ), but not of age ( $P = 0.11$ ). Daily smokers had a slightly higher average concentration of P-MDA than nonsmokers ( $P = 0.05$ ), and P-MDA correlated with daily exposure to cigarette smoke ( $r = 0.162$ ;  $P = 0.03$ ). A positive correlation was also demonstrated between P-MDA and weekly alcohol consumption ( $r = 0.153$ ;  $P = 0.03$ ). Within-subject and day-to-day variations of P-MDA indicated that the potential of P-MDA as a biomarker for individuals is questionable. However, on a group basis, the present data support that P-MDA may be a potential biomarker for oxidative stress.

INDEXING TERMS: lipid peroxidation • reference intervals • HPLC

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Clinical research in the area of lipid peroxidation has been hampered by the lack of a valid biomarker. One of the most frequently used biomarkers providing an indication of the overall lipid peroxidation level is the plasma concentration of malondialdehyde (P-MDA), one of several byproducts of lipid peroxidation processes.<sup>1</sup> Proper utilization requires reliable reference intervals from large unselected human populations and thorough evaluation of the influence of age and gender, as well as other variations within the population.

One of the prominent risk factors for increased lipid peroxidation is smoking. Because of the presence of free radicals in cigarette smoke [1, 2], increases in P-MDA may occur [3–5]. Nevertheless, others failed to identify any correlation between smoking status and P-MDA [6–8]. In interpreting this evidence as well as other published P-MDA results, the size of the studies, the selection of subjects, and the quality of the P-MDA assay must be taken into account.

We have quantified P-MDA by the thiobarbituric acid (TBA) test. TBA-reactive substances (TBARS) formed in plasma, urine, or tissue samples after a calibrated sample pretreatment procedure primarily consist of MDA, which forms a red adduct with two molecules of TBA (MDA-TBA<sub>2</sub>) [9]. The adducts are separated by an HPLC method originally described by Wong et al. [10] and Carbonneau et al. [11], but modified for improvement of the selectivity from nonidentified substances in plasma. Reference values for P-MDA for several individuals, with a selection between different age groups as well as gender, have been reported only in a selected group of blood donors [12]. The aim of this study was to establish and validate an HPLC-based method for analysis of P-MDA, to determine

<sup>1</sup> Nonstandard abbreviations: P-MDA, plasma malondialdehyde; TBA, thiobarbituric acid; and TEP, 1,1,3,3-tetraethoxypropane.

a reference interval in a population-based sample group, and to assess the possible influence of smoking on P-MDA.

### Materials and Methods

#### ESTABLISHMENT OF REFERENCE SAMPLE GROUP

The reference sample group was established on the basis of a randomly selected group of 480 persons on Funen, Denmark. The selection was performed by the Danish Central Personal Registry (CPR) from criteria of equal number of men and women, ages 20–79 years, and an equal participation from individuals living in urban (Odense; 94% urbanization) and rural (Otterup and Sønderlø; 46% urbanization) municipalities. Two-hundred forty of the randomly selected persons received a standard questionnaire and were invited to participate in the investigation. The questionnaire contained detailed questions regarding former and present work, life-style factors, exposure to toxic chemicals, exposure to air pollution, use of prescribed drugs, and dietary and cooking habits. Persons who did not respond to the first invitation after 3 weeks received a written reminder. If the second letter remained unanswered within another 2 weeks or the person did not want to participate, a new person from the same subgroup, gender, and age decade was given the invitation to participate. No exclusion criteria were used. At the request of the Regional Ethical Review Committee, no unsolicited telephone contact was made. The overall rate of participation in the groups from 20–79 years was ~40%.

Analysis of the questionnaires indicated that the participants did not deviate in any significant way from the general Danish population. Approximately 60% of our reference population was employed (the remaining participants were attending school or university, retired, or unemployed). Forty-three percent were smokers, 23% had stopped smoking, and 32% had never smoked.

#### STATISTICS

All statistical analyses of relations between P-MDA and age, gender, and life-style factors were performed by the statistical package SPSS for Windows, Version 6.1.3 (SPSS, Chicago, IL). Analysis was performed on  $\log_e$ -normalized data. Correlations were calculated as Pearson's correlation coefficients. For comparison of means we used an independent *t*-test. Reference intervals were calculated as recommended by IFCC [13], with REFVAL 3.43 [14]. The data were standardized to zero mean and unit variance before exponential and modulus transformation to adjust for skewness and kurtosis. The final distribution was not significantly different from gaussian (Anderson–Darling's  $A^2 = 0.255$ ,  $P \approx 1.0$ ).

#### PREANALYTICAL FACTORS

Each participant was invited to come to either the Department of Environmental Medicine at Odense University, or the local health center in Sønderlø. A home visit was also

offered as an alternative. We reviewed the completed questionnaire. All sampling of blood was performed between 1400 and 1800 h during the months of September and October, 1994. All blood samples were obtained by the same phlebotomist. The participants were placed in a supine posture and blood was drawn from a cubital vein into 10-mL Venoject tubes with 0.1 mL of 0.47 mol/L EDTA as anticoagulant (Terumo Europe, Leuven, Belgium). Blood samples were kept at 5 °C until centrifugation at 1000g (3 h at maximum). Plasma was distributed into sterile Cryo Vials (Greiner laborotechnik, Frickenhausen, Germany) in volumes of 500  $\mu$ L and was immediately frozen to –80 °C until analysis.

#### CHEMICALS AND REAGENTS FOR ANALYTICAL HPLC

2-TBA, potassium dihydrogen phosphate, potassium hydroxide, sodium hydroxide, and orthophosphoric acid 85% were of analytical grade and purchased from Merck (Darmstadt, Germany). 1,1,3,3-Tetraethoxypropane (TEP) was purchased from Sigma (St. Louis, MO). Methanol was of HiPerSolv grade and obtained by BDH Laboratory Supplies (Poole, UK). All water used was demineralized twice and filtered through a Milli-RO 10 Plus and a Milli-Q Plus plant (final pore size 0.2  $\mu$ m; Millipore, Bedford, MA). A 10 mmol/L potassium dihydrogen phosphate solution was prepared and adjusted to pH 6.8 with 2.0 mol/L potassium hydroxide. The solution was filtered by vacuum through a 0.45- $\mu$ m nylon 66 membrane (Supelco 5–8060; Bellefonte, PA). A 42 mmol/L 2-TBA solution was prepared by dissolving 0.6 g of 2-TBA in 80 mL of water, and then stirring and heating to 35–40 °C. The solution was cooled to room temperature and filled with water to 100 mL. The solution was stable for 2 days at 5 °C. The mobile phase consisted of 60:40 (by vol) 10 mmol/L potassium dihydrogen phosphate, pH 6.8:methanol. The mobile phase was degassed by vacuum and sonification before use.

#### HPLC INSTRUMENTATION AND CONDITIONS

A Kontron HPLC system (Kontron Instruments, Zürich, Switzerland) consisting of a Kontron HPLC pump 420, a Kontron HPLC 360 autosampler with a 50- $\mu$ L injection loop, and a Kontron UV detector 430 equipped with a 3- $\mu$ L flow cell was used. The system was controlled through a Kontron Multiport Module and a personal computer (Victor 433D). The column was a LiChroCART® 250–4 packed with 5- $\mu$ m LiChrospher® 100 RP-18 (Merck). The column was equipped with a guard column: LiChroCART 4–4 packed with 5- $\mu$ m LiChrospher 100 RP-18. The elution was carried out at a flow rate of 0.5 mL/min. The column effluent was quantified at a wavelength of 532 nm.

#### SAMPLE PRETREATMENT

We added 100  $\mu$ L of plasma to a 10-mL Pyrex centrifugation tube containing 700  $\mu$ L of 1% orthophosphoric acid, and vortex-mixed for 10 s. We then added 200  $\mu$ L of 42

mmol/L 2-TBA solution, screwed the Teflon-lined cap on tightly, and vortex-mixed the sample for 10 s, and then heated it for 60 min in a water bath at 100 °C. Hereafter, the sample was kept on ice until 10 min before HPLC analysis. At that time the sample was vortex-mixed for 10 s, and 200  $\mu$ L was transferred into a 2.0-mL Microwtube (Simport Plastics, Québec, Canada) containing 200  $\mu$ L of 1:12 (by vol) 2 mol/L sodium hydroxide:methanol. The sample was vortex-mixed for 10 s and centrifuged for 3 min at 13 000g. We transferred 200  $\mu$ L of the supernatant to a 300- $\mu$ L glass vial, and injected a 50- $\mu$ L aliquot onto the column. A calibration solution was prepared from TEP solubilized in water. TEP undergoes hydrolysis to liberate stoichiometric amounts of MDA. Calibration curves from supplemented water samples were produced for each day of analysis. Calibration samples can be neutralized until 10 h before analysis. The 42 mmol/L 2-TBA was stable for 4 months, but was freshly prepared every second day. To avoid interfering peaks, after each use the tubes were immediately placed in a detergent solution and machine-washed and rinsed in Milli-Q-treated water. The tubes were then soaked in 1% HNO<sub>3</sub>, rinsed with Milli-Q water, flushed with 960 mL/L ethanol, then oven-dried. To maintain optimal separation performance and avoid buffer precipitation, the column was regenerated with 15 mL of Milli-Q water followed by 15 mL of methanol at a flow rate of 0.5 mL/min after each day of analysis. The guard column was replaced after ~300 injections.

## Results

### HPLC METHOD VALIDATION

Representative chromatograms for plasma from a healthy volunteer (A), a supplemented water-based calibrator (B), and a blank water sample (C) are shown in Fig. 1. Baseline separation of MDA-TBA<sub>2</sub> and two unidentified peaks (1 and 3) was achieved with the applied conditions (see Fig. 1A). About 15 min was required for each analysis. The retention times in minutes were 8.52 for peak 1, 9.67 for

the MDA-TBA<sub>2</sub> complex 2, and 10.77 for peak 3. No correlation was found between any of the unidentified peaks and the MDA-TBA<sub>2</sub> peak. The peak in the blank water sample was present in all blank samples, and the mean area of this peak was subtracted from the area of the plasma samples.

The linearity of the detector response to different concentrations of the compound was determined at plasma concentrations of 0.59, 1.09, 1.59, 2.09, and 2.59  $\mu$ mol/L for the MDA-TBA<sub>2</sub> complex. The calibration curve for the compound added to water and plasma was linear over the range investigated when peak areas were plotted against concentrations and applied to a least-squares regression equation (see Fig. 2). The regression coefficient *r* was 0.999 for the plasma samples and 0.998 for the water samples.

Before analysis of each series, calibration curves from supplemented water samples were prepared for three calibration concentrations covering the expected concentration range. The linear calibration curve was fitted through the data points by linear regression.

The within-day repeatability of the method was evaluated by repeated analysis (*n* = 10) of samples of supplemented plasma. Four concentrations of MDA were investigated: 0.5, 1.0, 1.5, and 2.0  $\mu$ mol/L. The mean background of MDA-TBA<sub>2</sub> response in blank samples was subtracted from all the supplemented samples. The average CVs were 1.6% (range 1.3–2.1%). The between-day reproducibility and accuracy of the method was assessed five consecutive days at three different concentrations in plasma (0.49, 0.75, and 1.32  $\mu$ mol/L). Accuracy is calculated as deviation in percent of the mean estimate from the 5 days of analysis with the supplemented value. The mean estimates, SDs, and CVs and deviations from supplemented values are given in Table 1.

The relative recovery for the MDA-TBA<sub>2</sub> complex was assessed (*n* = 10) at four concentrations: 0.5, 1.0, 1.5, and 2.0  $\mu$ mol/L. The peak area from a supplemented plasma sample was compared, after subtraction of the baseline

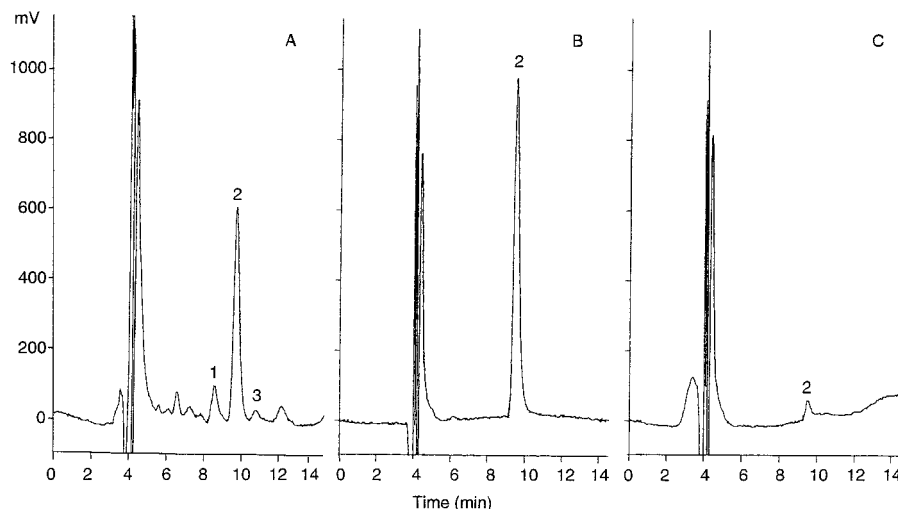


Fig. 1. Chromatograms of (A) plasma from a healthy volunteer containing 0.67  $\mu$ mol/L P-MDA (peak 2), (B) supplemented water sample containing 1  $\mu$ mol/L P-MDA, and (C) blank plasma.

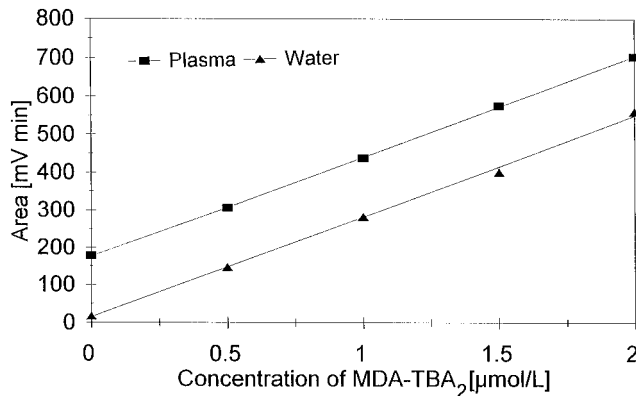


Fig. 2. Calibration curve of the MDA-TBA<sub>2</sub> complex, supplemented as TEP in water and plasma.

response, with the peak area from a supplemented water sample. The plasma sample was prepared with equal amounts of the TEP calibrator. The average recovery was 99% (range 96.7–103.4%). The limit of determination for P-MDA on the basis of a signal-to-noise ratio of 10:1 was 0.05 μmol/L. The limit of detection, from a signal-to-noise ratio of 3:1, was 0.02 μmol/L.

Stability of EDTA-treated plasma was investigated in a plasma pool used for control samples. The samples were stable at –80 °C for 6 months. We also compared the response from serum and from plasma collected in Venoject tubes with 0.13 mol/L sodium citrate, sodium heparin, and 0.47 mol/L tripotassium EDTA. The mean response of the MDA-TBA<sub>2</sub> complex from 13 fasting volunteers (7 men and 6 women, ages 20–33 years) was 1.16, 0.89, 1.27, and 0.26 μmol/L, respectively. Reproducibility calculated as CV was <10% only with EDTA as anticoagulant (data not shown).

Intraindividual variations were investigated in six healthy volunteers (3 women and 3 men, ages 21–53 years). Blood samples were obtained in EDTA-treated Venoject tubes from nonfasting individuals between 0800 and 0830 h for 6 consecutive days. To eliminate analytical variations, analysis was performed for all individuals in a single run. Within-subject variations are shown in Table 2 and the day-to-day variability for the group is shown in Table 3.

The reference intervals are defined by the 0.025 and 0.975 fractals, and the 0.90 confidence interval is calcu-

Conc., μmol/L	0.49	0.75	1.32
MDA-TBA <sub>2</sub>			
Mean	0.48	0.79	1.35
SD	0.02	0.05	0.08
CV, %	5.1	6.9	6.0
Deviation, %	2.0	5.3	2.3

Samples were from the same pool, analyzed once a day for 5 days. Deviation is from the supplemented value.

Table 2. Within-subject variation of P-MDA concentrations in six individuals from six consecutive days.

Individual	Mean, μmol/L	CV, %
1	0.39	23.6
2	0.59	5.9
3	0.43	15.3
4	0.31	30.0
5	0.44	20.8
6	0.58	14.0

lated for each of these fractals. Additional reference intervals were calculated for each age decade and for women and men separately (Table 4).

Analysis of variance was used to reveal relations between normalized P-MDA concentration in the three 20-year age groups and gender. The analysis revealed a significant effect from gender ( $P = 0.033$ ), but apparently not from age ( $P = 0.109$ ). No major interaction occurred between gender and age ( $P = 0.103$ ). Men had slightly but significantly higher P-MDA concentrations than women. In the reference sample group, 92 were smokers and 122 were nonsmokers. Smokers had a significantly higher

Table 3. P-MDA values and CV for a group of six individuals on six consecutive days.

Day	Mean, μmol/L
1	0.50
2	0.43
3	0.42
4	0.46
5	0.48
6	0.46
	<b>Group mean</b>
	0.46
	<b>Group CV, %</b>
	6.5

Table 4. Reference intervals for malondialdehyde (parametric estimate).

Age intervals	0.025 and 0.975 fractiles, 0.90 confidence intervals, μmol/L	
	0.025 μmol/L	0.975 μmol/L
20–39 years (n = 80)	0.33	1.15
40–59 years (n = 83)	0.39	1.18
69–79 years (n = 50)	0.39	1.40
20–79 years (n = 213)	0.36	1.24
Men (n = 107)	0.41	1.29
Women (n = 106)	0.33	1.22

P-MDA concentration (mean  $0.66 \mu\text{mol/L}$ ) than non-smokers (mean  $0.60 \mu\text{mol/L}$ ) ( $P = 0.05$ ). Correlation analysis revealed an association between P-MDA and the number of hours of daily exposure to cigarette smoke ( $r = 0.162$ ,  $P = 0.03$ ), but we found no clear correlation between P-MDA and the number of cigarettes smoked ( $r = -0.065$ ,  $P = 0.55$ ). P-MDA was significantly correlated with weekly alcohol consumption ( $r = 0.153$ ,  $P = 0.03$ ). Weekly alcohol consumption was defined as units consumed during 1 week before completing the questionnaire.

### Discussion

The formation of the MDA-TBA<sub>2</sub> adduct is probably initiated by a nucleophilic attack involving carbon-5 of TBA onto carbon-1 of MDA, followed by dehydration and a similar reaction of the intermediate MDA-TBA adduct with a second molecule of TBA [15]. Hydroperoxides as well as certain carbohydrates and amino acids could yield products that would also produce a genuine MDA-TBA<sub>2</sub> adduct upon heating with TBA [16], although the significance of these reactions is unclear. The anticoagulant used during blood sampling (see below), the type and strength of the acid used in the pretreatment procedure, and the duration of heating affect the amount of adduct produced. These facts, combined with differences in selectivity with the numerous analytical methods, makes the interpretation of previously reported P-MDA results difficult. Nonetheless, the TBA assay may offer an indication of the current status of fatty acid peroxide formation and decomposition, and it is still commonly used as a screening parameter. We therefore established a selective HPLC method that was optimized to produce acceptable levels of precision and accuracy, but not too arduous to use for population studies. We have modified the analytical methods originally described by Wong et al. [10] and Carbonneau et al. [11]. We found the best symmetrical peak shape and separation performance by using only 10 mmol/L potassium dihydrogen phosphate buffer (pH 6.8) and by reducing the flow rate to 0.5 mL/min. Although retention time was prolonged, the baseline separation from a nonidentified back peak was improved (peak 3, Fig. 1), eluting 1 min after the MDA-TBA<sub>2</sub> complex. No correlation was found between the areas of background peaks 1 and 3 and the area of the MDA-TBA<sub>2</sub> adduct. The sensitivity of the method is similar to the one reported by Young and Trimble and by Richard et al. [17, 18]. Further advantages of this method are that no extraction into organic solvents and tedious evaporation procedures are required, and the use of an isocratic eluant allows a higher number of samples to be analyzed per day.

The previously reported findings of effects on P-MDA from use of anticoagulant by blood sampling has been somewhat contradictory. Richard et al. [18] found no differences in amount of MDA-TBA<sub>2</sub> adduct formed during the sample preparation by addition of EDTA as

antioxidant to blood samples collected with heparin as anticoagulant. Our findings of lowest P-MDA concentrations in EDTA compared with heparin-treated plasma are in agreement with the findings of Knight et al. [12]. Carbonneau et al. [11] found only minor differences in the concentrations of MDA in serum or plasma with EDTA/heparin as anticoagulants. The consistently lower MDA-TBA<sub>2</sub> concentrations observed in EDTA-treated plasma are probably related to EDTA chelation of iron in the TBA assay as well as its weak activity as an antioxidant [10]. The difference in findings of effects of EDTA could reflect the importance of having EDTA in the blood sampling tube to ensure immediate reduction in iron-initiated lipoperoxidation generated from the platelets. The higher content of MDA in serum vs plasma could be explained by lipoperoxides being formed during coagulation [10]. Thus, MDA measured in EDTA-treated plasma seems to generate the least interfered indication of the degree of lipid peroxidation at the time of blood sampling.

The within-subject variations (CV range 5.9–30%) revealed that P-MDA probably cannot be used as a biomarker or a diagnostic test on an individual basis. However, on a group basis, the small day-to-day variability seems more promising. We therefore suggest that P-MDA be used only as a biomarker for the degree of lipid peroxidation on a group basis.

The reference sample group in the study of Knight et al. [12] consists of blood donors with a carefully screened medical history. Our study deviates from this by its selection criteria, random selection from the general population, instead of healthy subjects generally used as controls in clinical studies. Other studies reporting total P-MDA collected in EDTA sample tubes and measured as a TBA adduct by HPLC do, however, report a similar group mean of  $0.6 \mu\text{mol/L}$  [10, 17]. Jiun and Hsien [19] reported a mean of  $0.9 \mu\text{mol/L}$  in a control group consisting of 26 men and 40 women, ages 19–69 years. Carbonneau et al. [11] reported a group mean for healthy controls of  $0.43 \mu\text{mol/L}$  in a group of 30 individuals, ages 23–70 years.

We found a significant correlation between P-MDA and the number of hours of exposure to cigarette smoke, but we found no correlation between P-MDA and the number of cigarettes smoked by the individual (inhalation was not taken into account). These findings, although supporting that P-MDA is a weak biomarker for individual exposure, may also indicate that the recorded number of cigarettes smoked by an individual may be a poor estimate for the actual exposure to the smoke toxins. Smokers are often exposed for longer periods to cigarette smoke from other smokers than are nonsmokers. Also, some smokers do not inhale the smoke from their own cigarettes. These factors may affect the relation between P-MDA and the exposure indicators. On a group basis, however, our findings of a significantly increased P-MDA in smokers is supported by the findings of Kalra et al. [4].

Cigarette smoke is known to increase production of

oxygen free radicals by polymorphonuclear leukocytes [20], and to decrease activities of some free radical scavengers [21, 22]. Alcohol's ability to induce lipid peroxidation has been related to hypotheses concerning damages caused directly or indirectly by ethanol or the major metabolite acetaldehyde. Current hypotheses include the direct impact of the free radicals derived by ethanol; ethanol's ability to generate formation of oxygen free radical species, which are able to start lipid peroxidation either directly or by exhausting antioxidative defense substances; and acetaldehyde's ability to stimulate lipid peroxidation either directly through free-radical formation or through depletion of the concentration of antioxidative substances [23]. The weak correlation seems in accordance with Vendemiale et al. [24]: increased plasma concentrations of glutathione and MDA after acute ethanol ingestion in humans (although our parameters are based on total consumption during 1 week before the blood sampling). Adjustment of the correlation analysis for smoking, by "number of daily smoked cigarettes," did not change the outcome.

In future studies, P-MDA may be used as a biomarker of oxidative stress in exposed groups, but smoking and alcohol consumption should be taken into account as possible potential confounders.

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