

**Monoamine oxidase activity in placenta in relation to manganese, cadmium, lead,  
and mercury at delivery.**

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## Abstract

**Background:** Environmental prenatal exposure to potentially neurotoxic metals poses a particular challenge in regard to study of early toxic effects. Monoamine oxidase activity, shown to be influenced by metals in experimental studies, could be useful biomarker in humans.

**Objectives:** To examine the relationship between blood metals concentrations at delivery and placenta MAO activity.

**Methods:** The study was performed in 163 pregnancies. Maternal and cord blood samples were obtained at delivery for manganese (Mn), lead (Pb), and cadmium (Cd) determination. Mercury (Hg) was also analysed in maternal hair. Placental samples were stored immediately after expulsion and total MAO activity was measured.

**Results:** MAO activity was significantly positively correlated with maternal and cord blood Mn concentrations in subjects with high MAO activity. In subjects with low MAO activity, maternal hair Hg was negatively correlated with MAO.

**Conclusion:** Our results suggest the use of placental MAO as potential surrogate marker of Mn toxicity in newborn and its correlation with psychomotor development should be further investigated.

**Keywords:** manganese, lead, cadmium, mercury, monoamine oxidase, pregnancy

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## 1. Introduction

The environmental exposure to manganese (Mn) poses a particular challenge: it is both an essential element and a potent cumulative neurotoxin. Considering the higher demand and increasing blood Mn levels during pregnancy, as well as its active transport across the placenta [1], there is a need for hazard assessment in regard to fetal exposure. In animal, Mn has been reported to disturb the dopamine metabolism *via* direct oxidation of dopamine, inhibition of its synthesis, and inhibition of monoamine oxidase activity in brain mitochondria [2, 3]. Platelet MAO activity was reported to be related to psychomotor skills and personality/behaviour in humans [4]. Although MAO activity is genetically determined, some environmental factors such as smoking [4], cadmium (Cd) [5], lead (Pb) [6] and mercury (Hg) [7] were correlated with MAO activity in humans and experimental animals. Environmental exposures interfering with neurotransmitters metabolism in foetus can influence further brain development [8].

Mn accumulates specifically within mitochondrial matrix where it can interfere with some enzymes including the monoamine oxydase (MAO) [3]. MAO is a flavo-enzyme involved in the oxidative deamination of amine neurotransmitters, including serotonin, dopamine and noradrenaline and exists as two isoenzymes, MAO-A and MAO-B [9]. These enzymes differ in substrate specificity [10, 11], and tissue expression. MAO A is the predominant isoenzyme in the placenta [12], although MAO B is also present at low levels and related to platelet and lymphocytes' MAO B expression in the placenta [13].

We hypothesized that increased metals levels in pregnant women and in newborn can interfere with MAO activity, which can be used as surrogate peripheral biomarker of neurotoxic effects of environmental exposures in human population. Placental tissue was used for MAO activity analyses for reason of high MAO content, its accessibility and easy feasibility for sampling at epidemiological scale. Thus, our objective was to examine the relationship between Mn and also Hg, Cd and Pb at delivery and placental MAO activity.

## **2. Methods**

### **Subject recruitment**

This study was realized among 163 from the 2002 pregnant women who participated in prospective mother-child cohort EDEN (Etude des Déterminants pré- et postnataux du développement et de la santé de l'Enfant) conducted in Centre Hospitalier Universitaire (CHU) of Nancy and Poitiers (France). All French speaking women with pregnancy before 24 weeks of amenorrhea were enrolled. Multiple pregnancies, diabetics prior to pregnancy and women planning to move out the region were excluded. The 163 women included in this study were the first recruited in EDEN study.

The study protocol was approved by the Ethic Committee of the Bicêtre Hospital. Written informed consents were obtained from the mother for herself at inclusion and for her newborn child after delivery.

### **Biological sampling**

One whole blood sample was obtained from pregnant women between 24 and 28 weeks of pregnancy and one cord blood sample was obtained at delivery for analysis of metals (lead, manganese, cadmium,). At delivery, placental samples (one central cotyledon on the maternal side) were collected immediately after expulsion, placed in a polystyrene box, and stored at  $-80^{\circ}\text{C}$ . In the days following birth, maternal hair samples were collected from the occipital region of the head close to the scalp. The lock of hair was then stapled at the base and stored in identified ziploc bags until Hg analysis. All data about pregnancy complications were obtained by research assistant from medical records.

### **Analysis of blood manganese, lead, cadmium, and hair mercury**

Maternal and cord blood were sampled between 24-28 weeks of amenorrhea and at delivery respectively and kept at  $-80^{\circ}\text{C}$  until analysis. Determination of Mn, Cd, and Pb was performed in maternal and cord blood by AAS using a Zeeman effect corrector (4100 ZL Perkin Elmer) as was previously described [14, 15]. Standard Cd containing blood solution Seronorm STE level 1 (Fumouze diagnostics) was used to evaluate the accuracy. Solution Palladium (Merk) and magnesium sulphate were used as modifier. Total hair Hg (HHg) concentration was determined in maternal hair by cold vapor AAS (Perkin Elmer AA 600). Prior to analyses, hair samples were digested with nitric acid for 2 hours at  $70^{\circ}\text{C}$ , and then oxidized with potassium permanganate at  $90^{\circ}\text{C}$  for 15 minutes. HHg concentrations are expressed in  $\mu\text{g/g}$ . All blood metals concentrations are expressed in  $\mu\text{g/L}$ .

### **MAO activity assay**

During delivery placenta was sampled and stored at minus  $80^{\circ}\text{C}$ . MAO activity was measured, in placental microsomes, using fluorometric method as described by Krajl [16] and Chakrabarti et al [17]. Before MAO analysis, placenta sample was vigorously rinsed with distilled water to remove all the blood. Briefly, around 1 g of the placenta was homogenized in phosphate buffer (50mM  $\text{NaH}_2\text{PO}_4$ , 120mM  $\text{NaCl}$ , pH 7.8) at proportion of 10 v/w. Triton X-100 (50 $\mu\text{L}$  per mL of sample) was then added and placental microsomes were obtained in the supernatant after ultracentrifugation 30 min at

27000 g.. Supernatant was added (1:100) to phosphate buffer (pH 7.8) and to saline solution (0.9%). The mitochondrial MAO reaction was initiated by the addition of the MAO substrate, kynuramine dihydrobromide (2.2 mM) (Sigma-Aldrich, St-Louis, MO), followed by a 30 minute incubation at 37°C. The reaction was stopped by adding perchloric acid (5M). After centrifugation 2000 g for 10 minutes, the supernatant was diluted with NaOH (1M) and the fluorescence was read at 318nm excitation and 380nm emission on a spectrofluorometer (RF-551 Shimadzu, Japan). The concentration of the reaction was determined from a standard curve (0 – 30 nM) of 4-hydroxyquinoline (4-HOQ; Sigma-Aldrich, St-Louis, MO). The activity was reported to total proteins determined by Bradford method [18] and expressed in nmol of 4-HOQ /g of proteins. To determine what isoform contributes to the total MAO activity in our study, before to start our analyses we realised an inhibition essay with clorgyline and deprenyl (data not shown) showing that MAO-A activity predominates.

All samples were analysed in duplicate and blank was added to each 10 samples.

### **Quantification of MAO-A mRNA expression in placenta**

In order to explore the relationship between metals and MAO-A mRNA expression, we randomly selected 20 placental tissues. All real-time PCR reactions were performed in our laboratory. Total RNA was isolated from placenta tissues using a phenol and guanidine isothiocyanate RNA extraction method (Trizol; Invitrogen, Carlsbad USA) following the instructions of the supplier. To avoid any contamination of the RNA by genomic DNA, DNAase treatment was performed using RNase-Free DNase 1 (Qiagen). Total RNA (1 µg) was reverse transcribed using Oligo(dT)15 Primer (Promega, Madison

USA) in presence of Omniscript Reverse Transcriptase according Qiagen protocol (Omniscript RT kit; Qiagen). Quantative real-time PCR was carried out using the Eppendorf RealPlex System according to the shuttle protocol for TAKARA SYBR Green kit. Relative gene expression was analysed according to  $2^{-\Delta\Delta Ct}$  Method. MAO-A primers were designed by Beacon Design 4.0. The forward sequence for MAO-A was GCCAGGAACGGAAGTTTGTAGG and reverse sequence was AATGTTCATGGTTCAGCGTCTC (150 bp). Human glyceraldehyde-3-phosphate dehydrogenase gene (hGAPDH) was used as housekeeping gene. All primers were purchased from Integrated DNA Technologies, Inc (Coralville, USA).

### **Statistical analysis**

All statistical analyses were performed using SAS/STAT version 9.1. Firstly, we explored all potential factors influencing metals concentrations and MAO activity such as gestational age, newborn gender, maternal age, smoking and alcohol consumption as well as pregnancy outcomes (gestational age at delivery, birth weight, pregnancy induced hypertension, gestational diabetes). Correlation analysis was used for continuous variables and ANOVA for categorical variables. None of these variables were significantly related to MAO activity. Only smoking during third trimester shown a trend for negative association with MAO activity (Student  $p=0.10$ ), but not with Mn concentrations. Cord blood Mn levels were significantly higher in boys (mean 33.9 versus 29.7  $\mu\text{g/L}$ ,  $p=0.05$ ). Finally, linear regression models were used to test the relationship between MAO activity (dependent variable) and Mn concentrations (independent variables) with adjustment for smoking status and Pb and Cd concentrations

in blood. The same models were tested in boys and girls to explore gender differences. The normality of residuals was estimated post-hoc to assure the adequate modelling. Non-linear models testing quadratic and threshold [19] relationships were also explored. Blood Pb data, MAO activity, and Cd concentrations in cord blood were log-transformed to achieve the normal distribution. Spearman correlation analysis was used to explore the relationship between Mn and MAO-A mRNA expression. Taking into account the bimodality of distribution of MAO activity (Figure 1), due probably to genetic polymorphism, we performed the same analyses in two subgroups separated by the least frequent value (7.5 nmol/g protein) between two modes. All subjects with MAO activity below 7.5 nmol/g of protein were considered as subjects with “low MAO activity”, and those with MAO higher than 7.5 nmol/g of protein were considered as subjects with “high MAO activity”

### **3. Results**

#### **3.1 Population characteristics**

The age of pregnant women at admission for delivery ranged from 19 to 39 years with a mean of 29 years (Table1). Sixteen percent of women smoked during the third trimester. Mean body mass index in the population was  $23.2 \pm 4.6 \text{ kg/m}^2$ . Eleven deliveries (4.6%) were preterm (between 28 and 38 weeks). Birth weight ranged from 1170 g to 4960 g with a mean of 3382 g. Forty nine percent of newborns were boys. One pregnancy was terminated by foetal death intrapartum.

#### **3.2 Biomarkers measurements**

Table 2 shows the descriptive statistic of metals concentrations and total MAO activity in placental tissue. HHg measurements were available for 145 pregnant women. The maternal HHg was ranged from 0.13 to 64.1  $\mu\text{g/g}$  with average of 1.1  $\mu\text{g/g}$ . The relative expression of MAO-A RNA done in 20 placental samples ranged from 0.75 up to 144.0  $2^{-\Delta\Delta\text{Ct}}$ . Correlations between metals in maternal and cord blood are shown in Table 5. Maternal and cord blood Mn shown high linear correlation, without threshold determined by non linear modelling. The quadratic model was also tested to explain Mn in cord blood by maternal Mn, but the significance of this model was much less ( $p=0.03$ ) than that of the linear model ( $p<0.0001$ ).

#### **3.3 Relationship between MAO activity and metals concentrations**

Simple correlation analysis showed that placental MAO activity was positively correlated with Mn concentration in both, mother and cord blood (Tables 3 and 4). This correlation was more pronounced in males ( $r=0.26$ ,  $p=0.02$  and  $r=0.21$ ,  $p=0.05$ , respectively for

maternal and cord MnB, but the same not significant trend was observed in females ( $r=0.16$  and  $r=0.11$ , respectively). The gender\*Mn interaction was not significant.

HHg showed low correlation with MAO activity ( $r=-0.02$ ,  $n=145$ ).

MAO activity was negatively correlated with Pb and Cd in cord blood, but not in maternal blood (Table 3).

Mn concentrations in maternal and cord blood were highly correlated with MAO-A mRNA relative expression in placental tissue (Tables 3 and 4).

Multiple regression analysis (Table 6) confirm the positive relationship between Mn concentrations in both mother's and cord blood and MAO activity in placenta after adjustment for Pb, Cd and smoking at third trimester. Cord blood Pb was also significantly associated with MAO activity, but negatively. When maternal HHg was introduced in these models, significance of previous models did not change.

We performed stepwise analysis to determine the best predictors of MAO activity in boys and girls including all available data for Mn, Pb, and Cd as well as smoking status at third trimester. This analysis showed that cord blood Mn and smoking were the best predictors in boys (model  $R^2=17\%$ , partial  $R^2=8\%$  for cord Mn) with positive association for Mn and negative for smoking. In girls, maternal blood Mn and cord blood Pb were the best predictors (model  $R^2=11\%$ , partial  $R^2=5\%$  for maternal Mn and partial  $R^2=6\%$  for cord blood Pb) with positive association for Mn and negative for Pb.

The test of non linear relationship between cord blood Mn, Pb, and MAO activity showed no significant threshold suggesting linear relationship between Pb and Mn, and MAO.

Correlation analysis (Table 7) between metals and MAO activity in subjects with high (>7.5 nmol/g protein) and low (<7.5 nmol/g protein) showed that Mn was highly correlated with MAO only in the group with high MAO activity. Inversely, in group with low MAO activity, only HHg was significantly and negatively correlated with MAO. The similar trend was observed for cord blood Cd. Separate Stepwise analysis in each group confirmed the high association of maternal Mn and MAO in high MAO group. In low MAO group, however, only cord blood Cd reached the significance level explaining 5% of the total variance while the effect of HHg became not significant.

## **Discussion**

This study is the first to correlate MAO activity in placenta with metals concentrations at delivery. Blood Pb levels and maternal hair Hg at delivery in our population were lower than in previously reported studies showing neurotoxic effects in children [20, 21]. For cord blood Cd, the levels were similar to a recent study reporting a significant association with children cognitive performance at 4 years [22]. Cord blood Mn concentrations in this study were similar to those correlated with psychomotor skills at 3 years [14].

The most striking finding is that Mn concentrations in both cord and maternal blood were positively correlated with MAO activity in placenta. Cord blood Pb was also inversely correlated with MAO activity in agreement with previous experimental reports [6]. It was shown previously that human placenta is characterised by high MAO expression and contains primarily MAO-A isoform [23], but the mRNA of MAO-B isoform is also expressed [24]. Thus, the observed correlation could be explained by the modification of MAO-A activity in relation to circulating levels of Mn. The explored correlation between Mn and MAO-A mRNA levels confirm this hypothesis and suggests the transcriptional modifications of this enzyme in relation to blood Mn. It should be noted that the promoter sequences of both, MAO-A and -B, genes share approximately 60 percent identity [25] that could explain the correlation between brain MAO-A and -B reported in human [26].

The correlation between Mn and MAO activity was more pronounced in male newborns. This could be explained by higher cord blood Mn in boys in our study in agreement with data from other newborn populations [14]. Interesting, the maternal smoking was inversely associated and predictive for placental MAO activity only in males according to

data on MAO-B from adults [7]. These observations suggest that gender related factors such as sexual hormones together with environmental factors may influence the monoamine metabolism in males since early life.

Since disruption of monoaminergic, especially dopaminergic, neurotransmission was hypothesised as major mechanism of toxicity of manganese at high dose [27], the use of biomarkers in relation to this mechanism in human populations became more frequent. Recent epidemiological studies using biomarkers such as prolactin in adults and newborns [28, 29], blood [14] and urine monoamines metabolites [30], and MAO-B and dopamine beta-hydroxylase in exposed adult subjects [31] were consistent with mechanistic hypotheses. However, few studies explored MAO as potential biomarker in general population. Stamler et al [7] reported recently a negative correlation between MAO-B and Hg in fish-eating adults, but blood Mn was not related to MAO-B activity in this population. This could be explained by highly efficient Mn excretion leading to low blood Mn levels in adults when compared to newborns. Knowing that Mn actively crosses over the placenta [1] and cord blood Mn concentrations are 3-fold higher than in adults, it is difficult to compare the findings reported in environmentally adults with these in newborns. Our results are in agreement with recently reported increase of serotonin metabolite in urine from Mn exposed workers [30].

The biological significance of MAO-A activation in relation to blood Mn is not clear. However, two explicative hypotheses could be proposed. First hypothesis, the less probable, that the observed correlation reflects the start of “hormesis” curve, characteristic for essential elements with “∩-shape” dose response relationship, before plateau and can be followed by inhibition with increasing of dose. However, the

toxicokinetic of Mn during pregnancy including increase of Mn in maternal blood and active placental transport indicates that Mn deficit is unlikely in newborn.

The second explanation we propose is that MAO activation could reflect early toxic effect of Mn. Existing epidemiological findings could support this hypothesis: adverse effects on psychomotor scores [14] and prolactin increase [29] were reported at similar cord blood Mn levels in different populations. This observational study can not establish causality and support the direct involvement of Mn in MAO activity. However, our finding could indicate early changes either in monoamine levels (increase of substrates) or in mitochondrial function reflected by activation of MAO. Indeed, it was suggested recently that activation of MAO-A plays a key role in initiating of cell apoptosis [32, 33] through generation of reactive oxygen species. Also, it was reported that expression of MAO-A, but not MAO-B, is activated in response to inflammatory cytokines and by oxidative stress [34], which could be also induced by Mn [35]. In addition, the increase in MAO-A expression was correlated to neuropsychiatric signs in clinical and experimental portal-systemic encephalopathy, without any change in MAO-B, in parallel to the accumulation of Mn in brain [36].

In regards to cadmium, mercury, and lead effects on MAO, no epidemiological data are available in newborn. However, several experimental studies showed behavioural and neurotransmitter impairments associated to prenatal cadmium and lead [6, 37].

Monoamine oxidase activity was extensively studied in relation to behaviour and personal traits. Most available papers referred to the low platelet MAO activity as risk factor for asocial behaviour, alcoholism, and depression [38]. Stamler et al. [39] reported

recently the association between low platelet MAO-B activity and acquired color vision loss suggesting, together with their previous report [7], the use of MAO-B activity as peripheral biomarker of Hg toxicity in adults. In our study, the similar trend was observed only in subjects with relatively low MAO activity, but this association was relatively weak and not significant in the multivariate analysis. In regards to lead and cadmium, our findings suggest that low exposure to these metals could inhibit MAO in human newborns.

Genetic polymorphism of MAO-A promoter gene, closely related to MAO-A activity, has been reported to be associated with several behavioural disturbances in interaction with social risk factors [40]. Based on the hypothesis that MAO activity could reflect this polymorphism, we explored the association between metals and MAO activity in subjects with low and high activity. We observed a different association between manganese and MAO with significant correlation in high MAO group and no correlation in low MAO group. No evident association was observed for other metals. At date, no studies are available to provide any explanation for our observation in regards to interaction between manganese and MAO polymorphism.

In conclusion, MAO activity in placenta was positively correlated to manganese in maternal and cord blood at delivery. Other metals showed a modest association with MAO activity. Further investigations are needed to correlate this biomarker with psychomotor skills and behaviour with ultimate goal to establish safe Mn intake for growing foetus and child.

Table 1: Socio-demographic and pregnancy information of the pregnant women.

Table 2: Descriptive statistic of metal blood concentrations at delivery and MAO activity (nmol 4-HOQ/g of protein, log transformed) in placenta

Table 3: Correlation matrix for metals in maternal blood and MAO activity (nmol 4-HOQ/g of protein, log transformed) in placenta

Table 4: Correlation matrix for metals in cord blood and MAO activity (nmol 4-HOQ/g of protein, log transformed) in placenta

Table 5: Mother – cord correlations for metals (n=157)

Table 6: Monoamine oxidase activity (nmol 4-HOQ/g of protein, log transformed) in placenta in relation to maternal and cord metals concentrations (ppm) in all subjects

Table 7: Correlation matrix for metals at delivery and MAO activity (nmol 4-HOQ/g of protein, log transformed) in placenta in subjects with high and low MAO activity

Figure 1 Distribution of MAO activity.

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