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Analysis of the Relationship Between Pineal Hormone Melatonin Level and Occupational Mercury Exposure in Ex-miners with Machine Learning Methods

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Abstract

The aim of this study was an analysis of the relationship between pineal hormone melatonin level and long-term occupational exposure to elemental mercury vapour (Hg°) of ex-mercury miners. Melatonin (MEL) is a hormonal product of the pineal gland. Hg accumulation in the pineal gland in ex-miners could modify the synthesis of melatonin and there are no data available in the scientific literature on the possible effects of Hg on melatonin excretion.

Regression/model trees were used to perform the analyses and investigate the interactions between melatonin secretion and excretion on one hand, and various indicators of occupational Hg exposure and some other medical variables on the other. The results point us towards some previously unknown factors that influence the excretion of melatonin and support some assumptions that melatonin also has antioxidative effect. In the process of antioxidation activity melatonin decomposes and we assume this is the reason for decreased excretion of melatonin in mercury miners.

1 Introduction

The toxic effects of mercury are well known and in the case of occupational exposure to Hg° the most frequent symptoms and signs include erythrism, increased irritability, depression, insomnia, psychotic disturbances, tremor and renal impairment (WHO, 1976, 1991). High Hg and Se retention and co-accumulation (near 1:1 molar ratio) have been found in the brain, endocrine glands and also pineal gland in ex-miners from the Idrija

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Mercury Mine even several years after exposure (Kosta et al., 1975; Falnoga et al., 2000). In our recent study (Kobal et al., 2004) elevated melatonin (MEL) level in blood of ex-mercury miners was found. Hg accumulation in the pineal gland in retired miners could modify the synthesis of melatonin and there are no data available in the scientific literature on the possible effects of Hg on melatonin excretion. Melatonin, N-acetyl-5-methoxytryptamine, is a hormonal product of the pineal gland. Its synthesis is higher at night than during the day. Once melatonin is produced in the pineal gland it is quickly released into the vascular system. The interactions of melatonin membrane-bound receptors are believed to mediate the endocrine and circadian rhythm effects of melatonin. Recently, the pineal hormone melatonin was found to have a potent-free-radical scavenging activity and the protective effect of melatonin towards peroxidative damage was found in some in vivo and in vitro studies (Reiter et al., 1995). Hypothetically, this melatonin activity could play a key role in interaction with known Hg free radical generation (Jansson and Harms-Ringdahl, 1993; Lund et al., 1993).

In studies (Kobal et al., 2004; Kobal-Grum et al., 2004), no association between melatonin levels in blood and urine, and observed external and biological indices of past occupational exposure were found. The aim of the present study was to investigate the possible associations between melatonin levels in blood and urine, and indicators of miners past occupational Hg° exposure in combination with some additional variables.

The rest of the paper is organized as follows. The next section describes the study population, the data collected, and the data analysis methodology. Section 3 gives the results of this study, while in the last section discussion and conclusions are presented.

2 Material and methods

2.1 Study population

Initially, 120 males were examined in the study. After the selection procedure, the study population comprised 53 mercury miners and 53 workers from the control group. The mean age of miners was 45 (range 29–62), of controls 43 (range 30–62) years. The study group of miners comprised 33 active miners not exposed to Hg° in the previous 8 to 60 months, and 20 retired miners who prior to the present observation were not exposed to Hg° from 32 to 336 months. In their work miners had been intermittently exposed to Hg°. The Hg° concentrations in the air varied, depending on specific workplaces, from 0.05 to over 1.00 mg/m^3. During exposure to Hg°, all miners used personal protective equipment, i.e., half masks or Racal helmets with Hg° absorbing filters. No individuals in the study were occupationally exposed to cadmium or lead.

2.2 Medical examination

The medical examination included a medical history, some behavioral and biological risk factors for cardiovascular disease (CVD) (Leparski and Nüssel, 1987) and an evaluation of clinical neurological status. A dental amalgam score was calculated with the methodology proposed by (Aposhian et al., 1992). The examination also included blood and urine sampling for determination of: 1) total blood mercury (B-THg) and urine mercury (U-

Hg), 2) pineal hormone melatonin in blood (B-MEL) and urine (U-MEL), 3) Se levels in blood, plasma and urine, 4) CuZn-SOD, CAT, GSH-Px activity in erythrocytes as indices of free radical activity, 5) malondialdehyde (MDA), a lipid peroxidation product, and 6) lipid profile and other basic laboratory analyses including cadmium (Cd) and lead (Pb) in blood. Psychological examination was also carried out (Kobal-Grum et al., 2004).

2.3 Assessment of occupational Hg° exposure

Environmental and biological data on the group of miners studied were collected from 1959 to 2000 from workload records, daily reports on Hg° measurements in the workplace and personal medical records and biological monitoring data. On the basis of collected data, the external exposure indices and biological exposure indices (internal dose) were evaluated. The following environmental indices of Hg° were calculated for each miner: 1) years of work in the mercury mine (years of exposure), 2) cycles of exposure (intervals of work exposed to Hg°), 3) average time-weighted (ATW) air Hg° concentration in mgHg°/m^3 air, and 4) integrated exposure intensity (IEI) of the whole exposure period expressed in mgHg°/m^3 hours. Since 1959, the miners were biologically monitored by means of urine mercury (U-Hg) analyses. Biological exposure indices were evaluated on the basis of 5452 U-Hg measurements performed during the cycles of miners' exposure to Hg°. Based on these data, the following biological indices of occupational exposure were calculated: 1) geometric mean of cycles U-Hg level, calculated from all urine samples determined during the cycles of exposure expressed in μgHg/L, and 2) geometric mean of cycles peak U-Hg level, calculated from all cycles peak U-Hg levels determined during the cycles of exposure expressed in μgHg/L. The actual background exposure to inorganic and methyl mercury was evaluated by determination of B-THg and U-Hg. Background exposure to Cd and Pb was also evaluated.

2.4 Blood and urine sampling

Venous peripheral blood samples were collected between 6:30 and 7:30 a.m. in 7 mL sterile Vacutainer tubes containing lithium heparin as an anticoagulant. An aliquot for melatonin determination was taken and stored at –40°C until the analysis. The rest of the blood was centrifuged at 1000g for 15 minutes at 4°C and plasma was separated. Erythrocytes were washed four times with isotonic saline. Centrifugation for each wash was carried out at 1000g and 4°C for 10 minutes. Washed erythrocytes were stored at –40°C. 8 hour urine (night-morning urine) was collected in metal-free polypropylene tubes during the night (22 p.m. – 6 a.m.). Blood and urine samples were brought to the laboratory within 1 hour after collection at 4°C.

2.5 Biological analyses

Blood and urine MEL was determined by the ELISA (Enzyme-Linked Immunosorbent Assay) method (IBL-Hamburg). The 6-sulphatoxymelatonine ELISA kit provides material for quantitative measurement of Me-SO_4 in urine.

Total mercury in whole blood (B-THg) was determined by cold vapor atomic absorption spectrometry (CV AAS). Plasma and urine selenium (P-Se and U-Se), Cd and Pb in

blood were determined by Zeeman graphite furnace atomic absorption spectrometry (GF AAS) using a palladium chemical modifier. Analyses of other biological variables are presented in previous publications (Kobal et al., 2004; Kobal-Grum et al., 2004).

2.6 Data analysis methodology

2.6.1 Statistical analyses

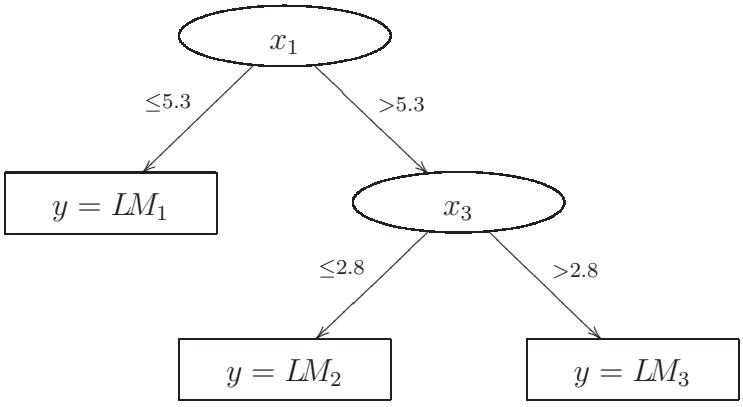
The group differences in all observed parameters were evaluated by applying an analysis of variance using one-way ANOVA software. The relationship between exposure and other variables was evaluated by means of Pearson's correlation coefficient, which reflects the degree of linear relation between two sets of data.

2.6.2 Machine learning methods

Machine learning methods were used in order to find possible explanations of associations between the target variables (concentrations of melatonin in urine and blood) and biological indicators of occupational Hg$^\circ$ exposure in combination with other variables. More specifically, we used model trees (Quinlan, 1992), which are a generalization of regression trees (Breiman et al., 1984). Regression trees are a formalism for representation of piece-wise constant functions, while model trees are more general and are a formalism for representation of piece-wise linear functions. In the following, we only discuss model trees since regression trees are just a special case. Like classical regression equations, model trees predict the value of a dependent variable (called a class) from the values of a set of independent variables (called attributes). Data represented in the form of a table can be used for learning or construction of a model tree. In the table, each row (example or subject) has the form (x_1, x_2, ..., x_N, y), where x_i are values of N attributes (e.g., subjects' age, daily consumption of alcohol, etc.) and y is the value of the class (e.g., the melatonin concentration in blood). Unlike classical regression approaches, which find a single equation for a given set of data, model trees partition the space of examples into axis-parallel rectangles and fit a model to each of these partitions. A model tree has a test in each inner node which tests the value of a certain attribute, and in each leaf a model for predicting the class: the model can be a linear equation or merely a constant. An example of a model tree can be seen in Figure 1. Given a new example (subject) for which the value of the class (y) should be predicted, the tree is interpreted from the root (x_1). In each inner node, the prescribed test is performed and according to the result, the corresponding left or right sub-tree is selected. When the selected node is a leaf, the value of the class for the new example is predicted according to the model in the leaf (LM_1, LM_2, or LM_3).

Model tree construction

The first step in the construction of a model tree is the construction of the initial regression tree. Regression tree construction proceeds recursively, starting with the entire set of training examples in one node. At each step, the most discriminating attribute is selected as the root of the (sub)tree and the current training set is split into two subsets according to the values of the selected attribute. Selection of the most discriminating attribute and its



$LM_1 = 0.24x_2 + 2.54x_3 + 2.54$
$LM_2 = 4.04x_3 - 0.24$
$LM_3 = 18.5$

Figure 1: An example of a model tree with three leaves (LM_1 to LM_3).

split value is based on treating the standard deviation of class values of training examples at the current node as a measure of error at this node and maximizing the expected error reduction if that node is split in two nodes. Tree construction stops when the variance of class values of all examples in a node is small enough, or if only a small number of examples remain in the node. Such nodes are called leaves and are labeled with a constant value (mean class value of examples in a leaf) for predicting the class value.

The second step in model tree construction is calculation of linear models for each interior node (all nodes except leaves). Linear models are calculated using standard regression techniques, but only attributes tested in the subtree below the current node are used. The resulting linear model is simplified by dropping attributes one by one (greedy search) for as long as the error estimate decreases.

The next step is pruning of the tree, which reduces its size and improves accuracy. The tree is pruned back from the leaves. If the estimated error of a subtree is bigger than the estimated error of the linear model in the current node, the subtree is discarded and the previously internal node becomes a leaf with a linear model attached.

The last step in model tree construction is smoothing of the tree. Smoothing compensates for sharp discontinuities that occur between adjacent models at the leaves of the pruned tree. The idea is that the class value predicted by a leaf model is combined with the values predicted by models along the path to the root of the tree where the final prediction is computed. However, the same effect can be obtained by appropriately modifying linear models at the leaves. Additional details of model tree construction are concerned with dealing with non-numerical attributes and missing values; these can be found in (Wang et al., 1997) or (Witten and Frank, 1999).

2.6.3 Machine learning experimental setup

There are a number of systems for inducing regression and model trees, such as RETIS (Karalič, 1992) and M5 (Quinlan, 1992). The latter is one of the most well-known systems

for regression and model tree induction. We used the M5' system (Wang and Witten, 1997), a re-implementation of M5 within the WEKA software package (Witten and Frank, 1999). The parameters of M5' were set to their default values.

A separate model tree for concentration of melatonin in blood (B-MEL) and in urine (U-MEL) was induced on the following features:

- group (active but not exposed to Hg$^\circ$ ex-mercury miners – underground work, retired ex-mercury miners – underground work, and controls – work in the open),
- age,
- number of cigarettes per day,
- number of years smoking,
- number of years drinking alcohol,
- alcohol consumption in ml/day,
- total working time,
- body mass index (BMI),
- systolic and diastolic blood pressure,
- tremor frequency and character,
- albumin in urine g/mol creatinine (a potential marker of the effect of Hg exposure),
- $\alpha_1\mu$ globulin in urine g/mol creatinine,
- total Hg in plasma,
- Se-Hg ratio in plasma,
- Se in plasma (P-Se)in μg/L and in urine (U-Se) in μg/g creatinine,
- corrected Hg in urine to 1L,
- cadmium and lead in blood (B-Cd and B-Pb),
- SOD, catalase, and glutation peroxidase in erythrocytes,
- LDL and LP-a lipids in serum,
- LOOH, U-8OHdG, and MDA markers,
- integrated exposure intensity (IEI) score,
- number of work cycles of Hg exposure,
- average time-weighted (ATW) air Hg$^\circ$ concentration,
- geometric mean of cycles U-Hg level in μg/L,
- the geometric mean of peak cycles U-Hg level in μg/L,
- cumulative U-Hg and cumulative peak U-Hg levels in μg/L,
- time since last exposure in days (exposure-free interval), and
- presence of prenatal exposure to Hg.

Table 1: Indices of past occupational Hg° exposure in miners.

	Mean	SD	Range
Years of exposure	14.9	5.5	7 – 31
Cycles of exposure	41	21	13 – 119
ATWE (mg Hg°/m^3)	0.29	0.08	0.13 – 0.47
IEI (score)	1428	2108	105 – 10907
Cycles U-Hg level (μg/L)	53.1	20.5	20 – 120
Cycles peak U-Hg level (μg/L)	77.2	23.0	40 – 134
Cumulative U-Hg level (μg/L)	6584	4444	1286 – 21390
Cumulative peak U-Hg level (μg/L)	3900	2196	794 – 11365

Table 2: Characteristics of the observed groups.

	Miners		Controls		
	Mean	SD	Mean	SD	p-value
Dental amalgam score	12.8	12.4	12.5	10.9	NS
Fish meals per week	0.52	0.96	0.59	0.88	NS
Cigarettes per day[a]	21.6	7.3	20.5	9.5	NS
Alcohol (ml/day)[b]	35.2	40.2	22.4	18.6	NS
Body mass index (kg/m^2)	27.8	4.1	27.4	4.1	NS
Blood pressure[c]					
Systolic (mmHg)	134.4	13.3	125.9	13.8	$<$0.01
Diastolic (mmHg)	87.9	8.8	81.2	11.1	$<$0.01
U-albumin (g/mol creat.)	1.36	1.78	0.85	0.44	$<$0.05
U-$\alpha_1\mu$ globulin (b/mol creat.)	1.39	2.67	0.61	0.35	$<$0.05

[a] % of smokers: miners 59 , controls 41

[b] % of alcohol consumers over 20 ml/day: miners 28 , controls 19, Pearson's χ^2 NS

[c] hypertension over 140/90 mmHg: miners 22 , controls 12, Pearson's χ^2 NS

Table 3: Mercury, selenium, melatonin, and peroxidative products levels in blood and urine.

	Miners		Controls		
	Mean	SD	Mean	SD	p-value
B-THg (μg/L)	2.5	1.5	2.5	1.2	NS
U-Hg (μg/L)	2.1	1.4	1.4	1.1	$<$0.01
P-Se (μg/L)[a]	71.4	12.4	77.3	13.2	$<$0.05
U-Se (μg/g creat.)	16.5	6.6	14.0	6.9	$<$0.05
B-MEL (ng/L)	44.3	38.7	14.9	9.2	$<$0.01
U-MEL sulphate (μg/L)	31.8	40.7	46.9	43.2	$<$0.01
U-MDA (μmol/mmol creat.)	0.32	0.31	0.19	0.30	$<$0.01

For abbrevations see materials and methods section.

[a] Negative correlation with biological past Hg° exposure indices: $r = -0.38, p < 0.01$

Table 4: Linear regression model (model tree with a single leaf) constructed by M5', describing the concentration of melatonin in blood (B-MEL); correlation coefficient = 0.57. The numbers in brackets (if present) correspond to the minimal, maximal, average, median values, and the relative importance factor of each numerical attribute. Relative importance factor is the product of an average value and a corresponding coefficient in the model. For nominal attributes, only number of subjects for which the condition is fulfilled and the number of all subjects are given.

B-MEL =		
23.72×	group=miner +	[21 / 106]
–0.52×	age +	[25 / 63 / 44.7 / 45 /–23.2]
9.04×	tremor character=2 +	[72 / 106]
–2.33×	$\alpha_1\mu$ globulin +	[0.14 / 15.6 / 0.89 /0.48 /–2.07]
0.64×	Se in plasma +	[44 / 106 / 73.7 / 73 / 47.1]
0.01×	cumul. peak U-Hg +	[0 /11365 /2021 / 794 / 9.7]
–12.04		

3 Results

3.1 Statistical analysis results

The indices of past occupational exposure to Hg° are presented in Table 1. The mercury miners were intermittently exposed to Hg° for periods of 7 to 31 years. The total number of exposure periods varied from 13 to 119. The cumulative U-Hg peak level varied from 794 to 11365 μg/L. The main characteristic of both observed groups are presented in Table 2. Miners showed some neurotoxic and nephrotoxic sequels of micromercurialism (Kobal et al., 2004; Kobal-Grum et al., 2004). A weak correlation ($r = 0.36, p < 0.01$) between systolic blood pressure and average past exposure U-Hg level was found.

The current blood and urine Hg concentrations were practically on the same level in miners and controls (Table 3). The mean concentration of B-MEL in miners (44.3 ng/L) was significantly higher ($p < 0.01$) than in the controls (14.9 ng/L). The mean value of U-MEL sulphate (31.8 μg/L) in miners was significantly lower ($p < 0.01$) than in the control group (46.9 μg/L). The mean P-Se in miners (71.4 μg/L) was significantly lower ($p < 0.05$) than in the controls (77.3 μg/L). Among antioxidative enzyme activities (data not presented), only catalase in erythrocytes was significantly higher ($p < 0.01$) in miners (3.14 MU/g Hb) than in the controls (2.65 MU/g Hb). Among the observed lipid peroxidative products, the mean concentration of U-MDA was statistically higher ($p < 0.01$) in miners (0.21 mmol/mmol creatinine) than in the controls (0.17 mmol/mmol creatinine).

3.2 Machine learning results

The model tree predicting the B-MEL level presented in Table 4 contains a single linear equation comprising group, age, tremor (evaluated with hand-writing test), $\alpha_1\mu$ globulin in urine, Se in plasma and cumulative peak U-Hg level. The model represents the miners group. Plasma Se level dominantly, while tremor and cumulative peak U-Hg level partially increased the melatonin concentration in blood. Age dominantly decreased the

Table 5: Linear regression model (model tree with a single leaf) constructed by M5', describing the concentration of melatonin sulphate in urine (U-MEL sulphate); correlation coefficient = 0.60. The numbers in brackets correspond to the minimal, maximal, average, median values, and the relative importance factor of each numerical attribute. Relative importance factor is the product of an average value and a corresponding coefficient in the model.

U-MEL sulphate =					
–1.12× age +	[	25 /	64 /	44.6 /	45 /–50.0]
–0.81× no of cigarettes per day +	[	0 /	40 /	9.4 /	0 /–7.66]
0.59× no of years smoking +	[	0 /	40 /	9.3 /	0 / 5.48]
–1.88× body mass index +	[	16.8 /	38.5 /	26.8 /	26.0 /–50.3]
0.51× systolic blood press. +	[	100 /	170 /	129.4 /	128 / 66.2]
1.34× Se in urine +	[	7.2 /	41.3 /	18.4 /	17.5 / 24.6]
8.89× Cd in blood +	[	0.23 /	9.51 /	1.58 /	11.1 / 14.0]
–0.69× Pb in total blood +	[	14.7 /	91.3 /	48.3 /	49.6 /–33.2]
–29.29× MDA +	[	0.001 /	1.26 /	0.19 /	0.081 /–5.57]
–0.01× cumul. peak U-Hg +	[	0 /	11365 /	1831 /	0 /–4.76]
79.64					

melatonin level in blood. Slight renal tubular disfunction (multicausal etiology) also tends to partially decrease the melatonin concentration in blood.

The model tree predicting the U-MEL concentration is presented in Table 5. It also consists of a single linear equation comprising age, cigarettes consumption, BMI systolic blood pressure, U-Se, Cd and Pb in blood, U-MDA and cumulative peak U-Hg level. Age and BMI dominantly decreased melatonin sulphate excretion in urine. Pb level in blood, cigarette consumption per day, cumulative peak U-Hg level and U-MDA also decreased the excretion of melatonin sulphate in urine. Systolic blood pressure and Se concentration in urine were dominantly associated with melatonin sulphate excretion in urine.

4 Discussion and conclusion

Melatonin (N-acetyl-5-methoxytryptamine) is a hormone produced mainly in the pineal gland during the dark phase of the circadian cycle. Melatonin metabolises in the liver and is then excreted via urine as melatonin sulphate (U-MEL). The concentration of the metabolite in urine reflects the melatonin concentration in blood (B-MEL) (Reiter et al., 1995). The non-enzymatic reactions of melatonin with free radicals represent a new significant aspect of its biological role (Hardeland et al., 1993). B-MEL concentrations in the present study were significantly higher in miners ($p < 0.01$) in comparison to the controls. U-MEL sulphate tended to be lower in miners which could not be a consequence of lowered melatonin synthesis as B-MEL in miners was very high. To our knowledge such results indicating an increased B-MEL level in Hg° exposed miners have never been previously reported.

The increased level of U-MDA obtained in the present study could represent a moderate increase in lipid peroxidation which could be associated with inorganic Hg accumulation. Our results are in agreement with the results of some in vitro and in vivo studies

(Halliwel and Gutteridge, 1989; Lund et al., 1993; Benov et al., 1990) and partly also with the results of (Bulat et al., 1998) where in subjects under ongoing Hg° exposure increased levels of MDA in erythrocytes were found.

In the miners group, Se in plasma together with cumulative peak U-Hg are the most important variables which seem to stimulate the melatonin secretion in blood. It also seems that both variables reflect the higher co-accumulation and retention of Hg and Se (near equimolar ratio) in central nervous system and endocrine glands (Kosta et al., 1975; Falnoga et al., 2000) that occurred during the past occupational exposure. Age is the most important independent variable which decreases the melatonin synthesis and its concentration in the blood. This effect has also been observed in other studies (Iguchi et al. 1982; Sack et al., 1986). The drop in melatonin synthesis with age is also reflected in the decreased urinary excretion of the chief metabolite melatonin sulphate (Sack et al., 1986).

BMI represents a very important independent variable which reduces the urinary excretion of melatonin sulfate. The influence of BMI on urinary excretion of melatonin sulfate has not been previously elucidated. The increased blood pressure in miners could be hypothetically indirectly associated with higher melatonin sulphate excretion due to the higher melatonin level which acts as an inhibitor of nitric oxide synthase activity and thus on the nitric oxide, a endotetelial-derived relaxing factor, production (Pozo et al., 1994).

Factors that promote the formation of free radicals (Pb, Cd, Hg, and tobacco smoking) and affect peroxidative degradation of lipids and phospholipids of cellular membrane additionally decrease the concentration of melatonin in urine. In accordance with this is the negative influence of a lipid peroxidation product (MDA – malondialdehyde) on the melatonin concentration. This supports some assumptions that melatonin also has antioxidative effect. In the process of antioxidative activity melatonin decomposes and we assume this is the reason for decreased urinary excretion of melatonin sulphate in mercury miners in spite of higher melatonin level in blood.

It was surprising to find elevated melatonin synthesis in miners in the period after mercury exposure. Due to the long-term accumulation of mercury in the pineal gland, the opposite was expected. Sleep disturbances typically observed in miners during the state of increased mercury absorption or intoxication (Kobal, 1975a) could be the consequence of mercury interacting with melatonin synthesis in the pineal gland. For the time being, we have no plausible explanation for this highly interesting finding. Increased melatonin secretion could be an adaptive response to free radical production induced by accumulated Hg (Crawford and Davies, 1994). The mechanism of Hg interaction with the pineal gland is not known.

Machine learning methods enabled us to evaluate interactions between the secretion of melatonin into blood circulation and various indicators of occupational Hg exposure, some essential metals (Se) and non-occupational exposure to cadmium and lead, as well as some other independent biological variables (age, BMI, and systolic blood pressure). The results of this study are encouraging and point us towards further research of interactions of secretion and excretion of melatonin in subjects occupationally exposed to mercury.

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