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Discriminant analysis to study trace elements in biomonitoring: an application on neurodegenerative diseases

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Summary. - Quantification of 26 elements was performed in blood of patients affected by neurodegenerative pathologies, i.e., Alzheimer's disease (AD), Parkinson's disease (PD) and multiple sclerosis (MS), and of a control group to study the potential role of blood elements as markers for the different neurodegenerations. A multivariate discriminant analysis (stepwise method) was applied to determine the best set of variables to discriminate among subjects with different health status. Preliminary results show three classification functions of seven elements, namely Ca, Co, Cu, Fe, Ni, Pb and Zr.

Key words: neurodegenerative diseases, Alzheimer's disease, Parkinson's disease, multiple sclerosis, trace elements, discriminant analysis.

Riassunto *(L'analisi discriminante nello studio degli elementi in traccia nel biomonitoraggio: applicazione alle patologie neurodegenerative).* - La quantificazione di 26 elementi è stata eseguita nel sangue di pazienti affetti da patologie neurodegenerative, quali la malattia di Alzheimer, il morbo di Parkinson e la sclerosi multipla, e di un gruppo di controllo per studiare il potenziale ruolo degli elementi nel sangue come marcatori per le diverse patologie. Un'analisi discriminante multivariata (con il metodo *stepwise*) è stata applicata per determinare il miglior gruppo di variabili in grado di discriminare tra i differenti stati di salute dei soggetti. Sono state ottenute tre funzioni di classificazione costituite da sette elementi: Ca, Co, Cu, Fe, Ni, Pb e Zr.

Parole chiave: malattie neurodegenerative, malattia di Alzheimer, morbo di Parkinson, sclerosi multipla, elementi in traccia, analisi discriminante.

Introduction

Neurotoxic substances are thought to play a major role in several neurodegenerative diseases like Alzheimer's disease (AD), multiple sclerosis (MS), Parkinson's disease (PD). The association among certain neurodegenerative disorders and the chronic exposure to low doses of neurotoxins has been also demonstrated. Some reports have shown Al, Ca, Fe and Mg implicated in neuropathologies [1-3]. A clear increase of Al concentration in PD specific brain regions and, at the same time, a decrease in Mg concentration in the basal ganglia may be involved in the central nervous system degeneration in PD [4]. Aluminium toxicity has been recognized to reduce the enzyme activity and to increase the number of neurofibrillary tangles in AD [5]. Deposition of Fe observed in Lewy bodies may be related to an involvement of this metal in the ethiology of PD [3]. Abnormalities in the metabolism of the transition metals Cu and Fe have been demonstrated to play a crucial role in the pathogenesis of various neurodegenerative diseases; in particular, alterations in specific Cu- and Fe-containing metalloenzymes have been observed [6]. For Ca, one of the most important intracellular messengers in the brain, several studies have reported a destabilization in intracellular homeostasis associated with β-amyloid peptide neurotoxicity and with mitochondrial dysfunction in AD [7, 8]. Oxidative stress has also been established as a feature of these pathologies. It seems to provide a critical link of environmental factors and heavy metals with endogenous and genetic risk factors in the pathogenic mechanism of neurodegeneration [9].

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The application of multivariate analysis, and especially discriminant analysis, to the study of trace elements in food and environmental fields has been largely used [10-12]. In the clinical field, discriminant analysis has been tentatively used to improve the predictive value of tomography images in differential diagnosis between AD and frontotemporal dementia [13]. Similarly, the need for non-invasive, specific and sensitive test led to study whether levels of some proteins considered markers of neuronal degeneration were useful to discriminate between patients and control groups [14].

In this investigation a stepwise discriminant analysis was preliminarly applied to study elements able to discriminate among the three groups under study.

Materials and methods

Patients and controls

The population sample, selected according to definite criteria as previously described, included ca. 300 subjects [15-17]. In particular, the AD group consisted of 60 cases (40 females / 20 males), the PD group of 71 (18 females / 53 males), the MS group of 60 (38 females / 22 males) and control group comprised 109 individuals (37 females / 72 males).

Statistical analysis

The statistical calculations were carried out on concentration values obtained as reported in Bocca *et al.* [18]. Outliers, values above and below 3 times the standard deviation, were excluded from the analysis. The missing data were substituted by the respective means. In order to investigate a possible age effect on elements concentration, the control group was split in subjects over and under 45 yrs old and the mean levels were compared. The statistical analysis applied was a multivariate discriminant analysis (MDA) carried out using the statistical package SPSS - 12.0.1 version for Windows (SPSS Inc., Chicago, IL, USA).

Discriminant analysis is a well-known multivariate statistical classification technique used to determine which variables discriminate between two or more groups, given several quantitative (independent) variables and a categorical (dependent) variable. The method extracts n-1 discriminant functions, n being the number of groups to discriminate among, which are linear combinations of the original quantitative variables selected. These functions may be used to calculate a set of discriminant scores that are employed to predict the status of a new observation. In this study a forward stepwise procedure, guided by the respective "F to enter", has been applied to reduce the original number of variables. The F value indicates the statistical significance of a variable in the discrimination among the four groups [19]. The model parameters are Wilks' *Lambda*, an index of the discriminating power ranging between 0 and 1 (the lower the value the higher its discriminating power); *eigenvalues*, a measure of the variance in the dependent variable for each function; canonical correlations, a measure of the association between the groups formed by the dependent and the given discriminant function (the larger this value, the higher is the correlation between the discriminant functions and the groups). The first discriminant function (DF1) maximizes the differences between the values of the dependent variable. The second function (DF2), orthogonal to the first, maximizes the residual differences between values of this variable. And so on. The DF1 will be the most powerful differentiating dimension, but later functions may also represent additional significant dimensions of differentiation. Because of the different size of the groups under study, the predictions were accordingly adjusted using *a priori* probabilities classification. The predictive validity of the model has been assessed by a leave-one-out cross validation method.

Results and discussion

A MDA was applied, as indicated in the previous section, and four levels in the categorical variable, namely, AD, PD, SM and control groups, as well as 26 elements concentrations as independent variables were used. To eliminate the variables that provided superfluous information at a 99% level, a F to enter = 8 in the forward stepwise procedure with tolerance of 0.01 was applied. Because of the four levels of the categorical variable, three significant discriminant functions of classification were obtained. Moreover, seven elements, i.e., Ca, Co, Cu Fe, Ni, Pb and Zr were selected. Model parameters, in terms of percentage of explained variance, *eigenvalues*, canonical correlation values and Wilks' *Lambda* were reported in Table 1. According to these results only the first and the second functions were the most discriminating ones. A total Wilks' *Lambda* value of 0.186 ($p < 0.001$) showed a good discriminant power of the model. The DF1 explained 65.9% of the total variance with a good correlation value (0.793), therefore it was the best discriminating between control, AD and SM groups, and PD patients (see Fig. 1).

In Table 1, the matrix structure coefficients, showing the correlations of each variable in the model with each discriminant function were also reported. The structure coefficients are global (not partial) coefficients, similar to correlation coefficients, and

reflect the uncontrolled association of the discriminating variables with the categorical variable. Coefficients less than 0.30 were omitted. The DF1 was mainly correlated to high concentrations of Ni (0.680) and low concentrations of Ca (-0.544) and Fe (-0.425) (negative correlation). This means that cases with a positive score on DF1 tended to have higher concentrations of the former and lower concentrations of the later elements, as showed in Fig. 1. In Table 2, the standardized discriminant coefficients used to compare the relative importance of the independent variables, i.e., the element blood levels in predicting the dependent variable, were listed. The higher their absolute value, the greater is their unique contribution to the discriminant power. It was possible to assess that Ni (0.578), Co (0.425) and Ca (-0.368) were the most important discriminating elements in the DF1.

The DF2 explained 27.8% of the total variance (DF1 + DF2 = 93.7%) with a canonical correlation value equal to 0.646, and resulted to give a useful contribute in the discrimination. The DF2 appeared mainly associated with high concentrations of Cu (0.713) and Zr (0.346). Therefore, cases with a positive score on DF2 tended to have higher concentrations of these two elements. Although the third function showed only a slight increase in total variance (6.2%) and a low correlation value (0.372), it was considered in the classification procedure, but it was not graphically represented.

The discriminant functions appeared to have a good classification with about 70.3% of original cases correctly classified and 69.3% of cases using the cross-validation procedure. Classification data for each group were reported in Table 3. When looking at the cross-validated data AD (group no. 2) was the worst classified group (38.3%), with 21/60 cases misclassified into the control group and 15/60 into the MS group. MS patients (group no. 4) were also misclassified in 22/60 cases (46.7%) into the control group. These classifications are represented in the scatter plot of Fig. 1 for the two most important discriminant functions.

The DF1 mostly discriminated control, AD and MS groups (showing negative score values) from PD group (high positive score values). In fact this last group, although in a scattered way, was well separated from the other pathologies, with high classification percentage (74.6%). Thus, the PD patients were characterized by higher blood levels of Ni and Co, and lower concentrations of Ca and Fe. The high Ni and Co levels associated with PD could be also connected with environmental and/or lifestyle factors, like smoking [20]. Cobalt, in particular, could be involved in the aggregation of α-synuclein to form fibrils, the major constituents of Lewy bodies. [21]. Furthermore, both these elements are known to interfere with the ionic channels involved in the transfer of Ca, a key element in changes in the brain [22, 23]. As regards to Ca, its negative correlation with DF1 was a signal of lower Ca levels in the blood of PD patients. This could apparently disagree with the increased Ca influx into nerve cells, causing cell death and neuronal dysfunction as observed in some neurological diseases

Table 1. - Matrix structure coefficients, percentage of variance, *eigenvalues*, canonical correlations and Wilks' *Lambda* of the final model for whole blood

Element	Function		
	1	2	3
Ni	0.680*(*)*	-	-
Ca	- 0.544*(*)*	-	-
Fe	- 0.425*(*)*	-	-
Cu	-	0.713*(*)*	-
Zr	-	0.346*(*)*	-
Pb	-	-	0.787*(*)*
Co	0.435	-	- 0.519*(*)*
% of variance	65.9	27.8	6.2
Eigenvalues	1.694	0.715	0.160
Canonical correlation	0.793	0.646	0.372
Wilks' *Lambda*	0.186		

(*) : largest absolute correlation between each variable and any discriminant function; - : coefficients < 0.30.

Table 2. - Standardized discriminant coefficients for whole blood

Element	Function		
	1	2	3
Ca	- 0.368	- 0.119	- 0.065
Co	0.425	0.160	- 0.525
Cu	- 0.017	0.793	0.420
Fe	- 0.316	- 0.417	- 0.137
Ni	0.578	- 0.172	- 0.089
Pb	0.298	- 0.225	0.749
Zr	- 0.008	0.560	0.022

Table 3. - Classification matrix for blood

Type of classification	Group	Predicted group membership								Total	
		1		2		3		4			
		(no.)	(%)	(no.)	(%)	(no.)	(%)	(no.)	(%)	(no.)	(%)
Original	1	105	96.3	1	0.9	0	0.0	3	2.8	109	100
	2	21	35.0	25	41.7	1	1.7	13	21.7	60	100
	3	11	15.5	4	5.6	53	74.6	3	4.2	71	100
	4	22	36.7	10	16.7	0	0.0	28	46.7	60	100
Cross-validated	1	104	95.4	1	0.9	0	0.0	4	3.7	109	100
	2	21	35.0	23	38.3	1	1.7	15	25.0	60	100
	3	11	15.5	4	5.6	53	74.6	3	4.2	71	100
	4	22	36.7	10	16.7	0	0.0	28	46.7	60	100

Groups: 1 controls; 2 Alzheimer; 3 Parkinson; 4 multiple sclerosis.

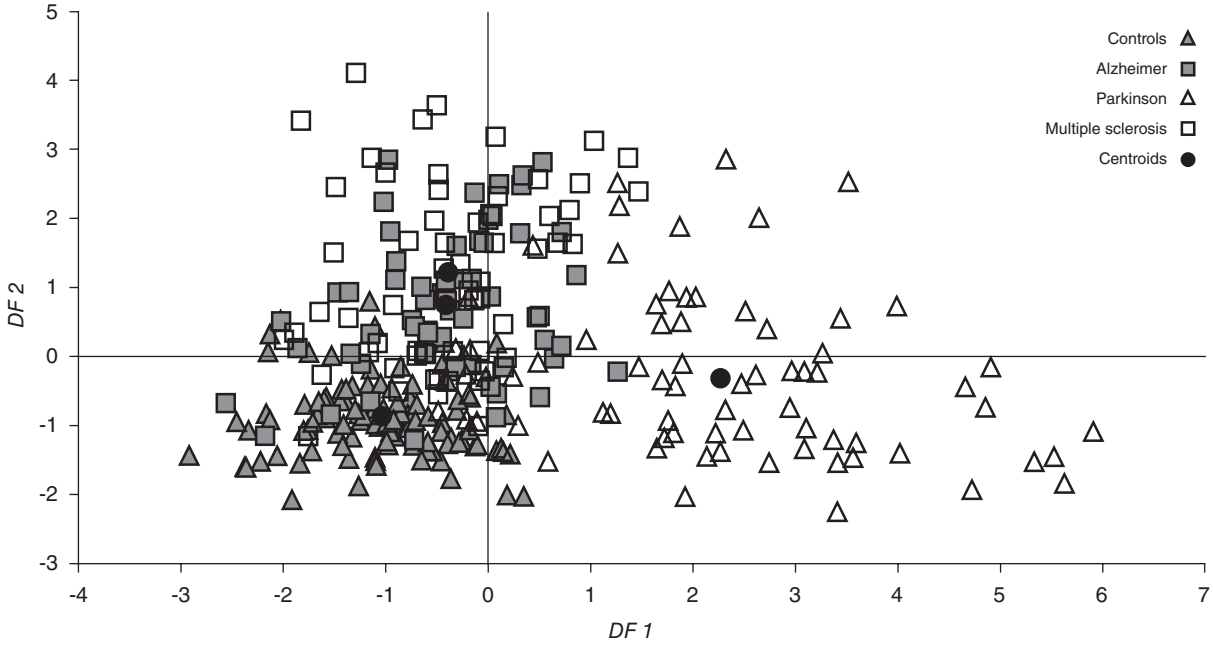

Fig. 1. - Plot of DF1 *vs* DF2 for whole blood. DF: discriminant function.

[24, 25]. Similarly, the Fe negative correlation with DF1, i.e, the low blood Fe content in PD patients, seems difficult to explain considering the high concentration of this element found in patients brain affected by several neurodegenerative disorders [26]. All these considerations could justify lower presence of Ca and Fe relieved in peripheral body fluids.

As concern DF2, a weak separation of the control group (negative values) from AD and SM groups (positive values) could be observed. The high discriminant weights observed for Cu (DF2) could confirm the role played by this metal in AD as responsible of the abnormal aggregation of amyloid-β (A-β) protein [27, 28]. However, Cu is a cofactor of

Cu/Zn-superoxide-dismutases which play a key role in the cellular response to oxidative stress by scavenging reactive oxygen species and it also is a component of cytochrome c oxidase, which catalyzes the reduction of oxygen to water, essential step in cellular respiration. [29, 30]. The possible involvement observed for Pb in DF3 (standardised coefficient, 0.749) could be related with environmental exposures identified as an additional risk for the onset of AD. Moreover, it is well known that exposures to Pb at levels lower than those associated with evident toxicity can cause mild intellectual impairment in childhood [31].

Conclusions

These preliminary results give evidence of a possible application of MDA to individuate biomarkers characterizing the Alzheimer's, Parkinson's and multiple sclerosis's patients as proved by the data on Ca, Co, Cu, Fe, Ni, Pb and Zr in blood.

The statistical analysis presents some limits (as the classification results showed) mainly related to the small sizes of the groups under study, although *i)* the population samples are homogeneous in terms of recruitment criteria, and *ii)* the elements quantifications were carried out in the same laboratory with standardized and reliable analytical procedures.

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