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Peripheral markers in neurodegenerative patients and their first-degree relatives

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ABSTRACT

We have determined various biomarkers in the peripheral blood of Alzheimer, Parkinson and vascular dementia patients by comparing the samples with those of first-degree relatives and control subjects. Our results, together with correlation studies using data from the Mini-Mental State Examination (MMSE), suggest that the clinical evaluation of the nitrite (NOx) concentration in Alzheimer patients should be complemented by assays of protein carbonyls (PCs) levels, the ratio of reduced to oxidized glutathione (GSH/GSSG) in plasma, PCs in erythrocytes and PCs and calcium content in leukocytes. For Parkinson patients it would be useful to determine NOx, thiobarbituric-acid reactive substances (TBARS) and PCs in erythrocytes, and NOx and TBARS en leukocytes. For vascular-demented (VD) patients, determination of NOx, Cu, and GSH/GSSG in plasma and TBARS, and PCs in erythrocytes together with PCs in leukocytes should be assayed. Relatives of Alzheimer patients showed alterations in plasma Se and Zn concentrations, catalase (CAT) activity in erythrocytes and calcium content in leukocytes as possible predictive markers of the disease. Relatives of Parkinson patients showed elevated levels of NOx in leukocytes. In the case of vascular-demented patients we suggest NOx, GSH/GSSG and α-tocopherol in plasma, the CAT/superoxide dismutase ratio in erythrocytes and TBARS, GSSG and glutathione reductase in leukocytes as predictive markers. Large-scale longitudinal population-based studies using these suggested biomarkers are necessary in order to assess their level of reliability and specificity in clinical practice.

1. Introduction

The assay of oxidative stress parameters has provided preliminary insight into the pathogenesis of many human diseases by demonstrating the involvement of free radical-dependent mechanism(s) of action, the precise details of which remain to be elucidated. In this connection oxidative stress biomarkers (OSBs) have been studied in blood and other biological samples of neurological patients with a view to using them as indicators of diagnosis and/or disease progression. There is a large body of evidence demonstrating an increase in reactive oxygen species (ROS) and nitrogen reactive species (RNS) in the central nervous system of patients suffering from a number of illnesses including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis, Huntington's disease and vascular dementia (VD) among others [1,2]. Nevertheless, to date it has been extremely difficult to establish the direct involvement of free radicals in the ethiology and/or the pathogenesis of these disorders owing to the short lifetimes of ROS and RNS and the lack of sufficiently sensitive technology to detect them in situ in biological systems [3].

Independently of the exact role of ROS and RNS as causal/etiologic factors or as consequences of primary disease, the search in recent decades for reliable indicators of the characteristics of neurological disorders and for an index of the progression of disease has given rise to the determination of several OSBs [1,4–6]. The main approach has been aimed at determining the clinical utility of the decreased antioxidant levels and/or the alterations observed in the activities of the antioxidant enzymes. One of the most promising findings so far in relation to OSBs and neurological disorders is the increase in ROS generation consistently associated with familiar amyotrophic lateral sclerosis [7]. Lustig et al. [8,9] were the first to demonstrate elevated levels of Cu,Zn-superoxide dismutase (SOD) in erythrocytes of patients with AD or VD. This finding was confirmed and extended to the patients' first-degree relatives by Serra et al. [10]. Previously it was demonstrated that fibroblasts from AD patients also exhibited increased SOD activity [11]. Subsequent investigations by Famulari et al. [12] proved that SOD was a leader marker that apparently depends on the clinical stage in patients with AD or VD. SOD measurements together with the determination of catalase (CAT) activity, glutathione content and antioxidant capacity were also used to distinguish between AD dementia and cognitive impairment of vascular origin [12] or the diagnosis of dementia within a population of diabetic subjects [13,14]. These findings, however, were based on a

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relatively small number of cases and were not independently confirmed. Other research has been carried out on individual OSBs with promising results. A complete overview of the main findings in the field of ROS/RNS in AD and other cognitive disorders has recently been published by Mangialasche et al. [1], though other authors have not been able to reproduce the results and others have come to opposite conclusions [1,2,5,15–17].

As mentioned before, the precise nature of the mechanism(s) responsible for selective dysfunction and neuronal death in neurological diseases remains unclear [1,5]. Aging is the major risk factor and even in otherwise healthy persons determines a shift from redox balance to oxidative and nitrosative damage [1,2,5,16]. The majority of research groups concur that ROS and RNS play a crucial role in neurodegeneration, likely as causative factors rather than consequent alterations of the disease [1,2,5,16,18–21]. Although ROS/RNS overproduction (or accumulation) may not constitute the primary initiating event in neuronal death, it is generally agreed that oxidative stress is an early marker of neurological disease and that early detection of oxidative/nitrosative stress therefore has great preventive value. Determination of peripheral OSBs opens the possibility of anticipating neurological disorders in susceptible populations, an important step in view of the fact that the preclinical stage commences many years before the clinical diagnosis when future sufferers appear cognitively normal. A number of research groups consider that more reliable and non-invasive OSBs will be crucial in the anticipated diagnosis of increased predisposition to neurological pathologies [2,5,16].

In view of the above we aimed (i) to determine a set of non-invasive OSBs that reflect the redox status in peripheral blood of patients suffering from three of the most frequently observed neurological disorders (AD, PD and VD) sub-classified according to their clinical stage; and (ii) to compare these values with those measured in a population of first-degree relatives in order to investigate the putative clinical utility for neurological risk assessment within this population.

2. Experimental procedure

2.1. Chemicals

All the chemicals used were of analytical grade and obtained from Sigma Chem. Co. (Buenos Aires, Argentina), Merck (Darmstadt, Germany) or Carlo Erba (Milan, Italy).

2.2. Study population and ethical considerations

A total of 619 subjects (control, patients, and relatives) were recruited within the same geographical area (La Plata city and its surroundings). The collection of samples was performed during the years 2008 to 2010 with the assistance of three public (National University) hospitals and four private health institutions. The control group was composed of volunteers recruited in the same geographical area, most of whom undergo mandatory workplace health checks for insurance purposes. First-degree relatives were sisters, brothers, daughters and sons of the patients. They were defined as R-AD, R-PD and R-VD, respectively. All of these subgroups (control and relatives) were disease-free as determined through clinical and routine laboratory tests, and had no neurological disorder or any other evident dysfunction that could interfere with the intake, absorption or metabolism of dietary antioxidants. Subjects with a non-standard (non-balanced) diet – evaluated by means of a food sub-questionnaire – were excluded from the study. The control group was divided into two sub-populations of different mean ages designated as younger control group (YCG) and elderly control group (ECG). Healthy elderly volunteers were recruited by advertising among the general population and were subjected to examination to determine their clinical condition. Only those volunteers with no clinical evidence of metabolic, neurological, or psychiatric disorders were included in the study.

In addition to conventional clinical exams, all the subjects underwent examination of vessels (endocraneal, carotid, and vertebral arteries) by color-coded duplex ultrasonography, MRI, and TAC. They were also laboratory-tested for hepatic and renal functions, lipid profile in peripheral blood, and hematological condition. In addition, all the experimental subjects underwent a neurological examination together with an extensive neuropsychological test battery including the Mini-Mental State Examination (MMSE) or Folstein's test [22], Clinical Dementia Rating, Hachinski score (HS) [23], visuo-spatial, phonologic and verbal fluency tests, clock test, and Addenbrook test [22,24,25]. Patients were classified according to the Global Deterioration Scale [26,27]. In classifying AD and PD patients, special attention was paid to fully complying with the criteria established by the National Institute of Neurological and Communicative Disorders and Stroke, the Alzheimer's Disease Association and Related Disorders [27], and the American Psychiatric Association [28]. Parkinson patients were also scored according to Hoehn and Yahr [29]. In view of their well-known difficulties in performing tests to evaluate visual and spatial perceptions (as well as having impaired attention and writing abilities and even failures in memory, praxis, gnosis, motor coordination processes and reasoning, especially at advanced stages of their illness [22,24,25,29]), patients in the PD group were assessed for cognitive performance. On the basis of the results of the neurological exams, the recruited patients within each pathology type were sub-classified into those suffering mild (M), intermediate (I) or severe (S) conditions.

The study was approved by the local Institutional Review Board and Bioethical Committee (Medical School of La Plata University; COBIMED; certificated protocol # 0800-002982/09-000) and the Ethical Committee of the San Roque Hospital (La Plata, Argentina). All procedures were in accordance with the guidelines regarding ethical issues of "Non-invasive Studies of Human Brain Function" and the "Code of Ethics of the World Medical Association" (Declaration of Helsinki) [30]. Studies were undertaken with the understanding and written consent of each subject (patients and controls) and of each patient's first-degree relatives.

2.3. Samples

Blood was collected by venipuncture after 12 h fasting into heparinized (10 mUI/mL) sterile polystyrene test tubes and centrifuged at 2 °C, 1000×*g* for 15 min. Determinations were performed on intact blood, plasmas, leukocyte homogenates or purified erythrocyte membranes (ghosts). Plasmas were carefully aspirated and stored at −80 °C under nitrogen atmosphere until the analyses were performed. Aliquots for glutathione analyses were immediately treated with equal volumes of $HClO_4$ (12% W/V) and N-ethylmaleimide (40 mM final concentration) [31] in order to preserve the ratio of oxidized to reduced glutathione. Appropriate aliquots of blood samples were also subjected to leukocyte isolation [32]. Pelleted white cells were washed in cold PBS (2 °C) and sonicated (three 30-s bursts at 50% output in a Heat Systems Ultrasonic sonicator model W-220 F from Plainview, NY) to obtain cellular homogenates. PBS-washed erythrocytes were prepared and treated to obtain hemolyzates [12] and erythrocyte ghosts [33].

2.4. Non-enzymatic biomarkers

The sum of nitrates and nitrites (NOx) was measured as the main metabolic end-products of nitric oxide (NO) and peroxinitrite anion ($ONOO^-$) [34]. Protein carbonyls (PCs) were determined as a biomarker of oxidative damage to proteins [35]. The FRAP (Ferric Reducing Ability of Plasma) assay [36] was expressed as μM of equivalent TROLOX® or α-tocopheryldiacetate. The samples were previously treated with uricase (Sigma Chem. Co. Buenos Aires, Argentina) in a ratio of 1 U/μL plasma dissolved in phosphate buffer 50 mM/EDTA 1 mM/glicerol 25 mM, pH 8.50. Lipid peroxidation was assayed as tiobarbituric acid-reactive substances (TBARS) with tetraethoxypropane as standard by a fluorometric method [37]. Vitamin E (α-tocopherol) was measured after

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extraction [38] using an HPLC technique [39] able to detect and quantify both α- and γ-tocopherols (results expressed in μM concentration). Total glutathione (the sum of reduced -GSH- and oxidized -GSSG- forms) was measured by using an enzymatic recycling method [40] and the results were expressed as mM concentration. To calculate the GSH/GSSG ratio, samples were re-analyzed after derivatization with divinyl-pyridine (3 mM final concentration). To determine the concentration of Cu, Zn and Se the plasma samples (150 μL) were treated (3 mL) with a mixture of H_2SO_4:HNO_3 (1:2) in graphitized boron-silicate test tubes overnight at 100 °C. After cooling, the samples were diluted to 15 mL with pure water (Carlo Erba), filtered through 0.45 μ Sartorius membranes (CA, USA) and analyzed in a Spectra AA 300/400 (Mulgrave, Victoria, Australia) [41]. Calibration curves were constructed using the standard solutions provided by Sigma Chem. Co. (USA) or Merck (Darmstadt, Germany). Representative intra- and inter-assay coefficients of variations for these measurements were 7.7, 6.1 and 8.2, respectively. Calcium content was determined after sample mineralization by atomic absorption in a Shimatzu Atomic Absorption Spectrophotometer AA-630-12 (Shimatzu Corp., Kyoto, Japan) [42]. The peroxidation induction test (PIT) was performed on pure erythrocyte membranes (ghosts) by incubating 2 mg of membrane proteins with 3 mL of TRIS/ClH buffer 10 mM (pH 7.4) containing ACCN (Alldrich Chem. Co, Darmstadt, Germany) 50 mM for 60 min at 37 °C under vigorous shaking. The reaction was stopped by the addition of 1 mL of fosfotungstic acid 10% W/V in distilled water (Biopur, Rosario, Argentina) and centrifugation at 4000 g for 10 min. Supernatants were tested fluorimetrically to determine the TBARS concentration [37] and the final results were subtracted from those of the control vessels without the addition of ACCN in order to measure only induced peroxidation.

2.5. Enzymatic biomarkers

The activities of various enzymes of the antioxidant defence system were determined in erythrocyte hemolyzates and peripheral leukocyte homogenates. Catalase (CAT) activity was measured using an UV kinetic method [43]. The results were expressed as Spa (*k* min-1/mg of protein). Superoxide dismutase activity (Cu,Zn-SOD and Mn-SOD) was determined by the ability of the enzyme to inhibit the auto-oxidation of pirogallol [44]. Results were expressed as U/min.mg of protein. Glutathione peroxidase (GPx), glutathione-S-transferase (GST), and glutathione reductase (GR) activities were determined by the methods described by Flohe and Gunzler [45], Habig et al. [41], and Carlberg and Mannervik [46], respectively.

2.6. Other determinations

Total protein content was determined by the method of Bradford [47]. Other biochemical components (cholesterol, total lipids, phospholipids, triacylglycerides, hemoglobin) were analyzed using commercial enzymatic kits from Wienner Lab. (Rosario, Argentina).

2.7. Statistical analysis

All values represent the mean of the individual determinations indicated as *n* in Table 1 (each assayed in duplicate) ± standard deviation (SD). We corroborated that log-transformed data were almost normally distributed. Data were analyzed by ANOVA plus Tukey test. In addition, the differences in the mean values of experimental parameters were also tested by the Student's *t*-test after ascertaining the homogeneity of variances (Kolmogórov-Smirnov). To check the influence of smoking habits we used -as a first approach- the adjustment {[ESM − (CSM)² / CNSM]. 100}/ ESM; where E is the patient or first-degree relative; C the corresponding control group (YCG or ECG); SM, smoker participants (less than 10 cigarettes a day) and NSM, non-smokers. The smoking habit in this study accounts for approximately ten percent of the differences observed within (or inter-) subgroups, thus having no significant impact on the results compared with those obtained when the smoking habit was ignored. For simplicity, mean values were used for all data within a group. However, in some cases the experimental parameter under comparison was significantly different between women and men of the same sub-group. The influence of confounding factors such as sex or smoking habit was taken into consideration in the multiregression statistical analyses. Data plotting, including linear and non-linear fitting regressions, were performed with the aid of Systat (version 15.0 for Windows) from SPSS Science (Chicago, IL), or Sigma Scientific Graphing Software (version 11.0) from Sigma Chem. Co. (St. Louis, MO). Correlation studies were performed using the method of Pearson [48] and equivalent conclusions were obtained with the non-parametric Spearman's (r_s) correlation rank test. Comparisons between the experimental parameters and the illness stage take account of the fact that the clinical stage is a discontinuous/categorical variable assigned three grades (M, I or S), for which reason we use the expression *parallel* (rather than *correlate*) with the clinical condition. A level of significance of $p<0.05$ is indicated by superscript letters (values that do not share the same superscript differ significantly from one another).

3. Results

3.1. Main characteristics of the group studied

Table 1 shows the composition of the group studied. The number of sex- and age-discriminate volunteers within each category was not statistically different. Due to possible differences in some biochemical parameters we included two control groups, one for young (YCG) and one for elderly (ECG) people. These control sub-groups were created in order to facilitate the statistical comparison between first-degree relatives and patients. As mentioned previously, we chose the MMSE as a representative index of cognitive performance. Early evidence clearly indicated that the MMSE correlates almost linearly with the results obtained via other commonly used neurological tests [49,50], a conclusion recently corroborated by other authors [25] and also by our group (data not shown); we have consequently incorporated MMSE scores into the current study for statistical purposes. As expected, in all groups the progression of the disease from the mild to the advanced clinical condition was progressively longer. Table 1 shows that performance in the MMSE clearly deteriorates from the mild to the severe condition. YCG and ECG showed significant differences in their mean ages (pairwise comparison, Tukey test, $p<0.01$, Table 1); however, they exhibited almost the same MMSE scores. Other demographical, nutritional and socio-cultural conditions (including years of education, not shown in Table 1) did not differ among groups. The proportion of smokers was higher in ECG and YCG than in other groups; however, taking this factor into account in the statistical analysis did not significantly modify the final conclusions.

3.2. Biomarkers in patients with Alzheimer's disease

Table 2 shows various OSBs determined in plasma, erythrocytes and leukocytes obtained from the three experimental groups of patients and the corresponding control group (ECG); some of these markers were published in a previous paper from our laboratory [51]. Statistical significances ($p<0.05$ or less) are denoted with superscript letters.

The production of nitrates plus nitrites (NOx) was considered to be the result of iNOS activation since they are the metabolic end-products spontaneously produced by NO dismutation. The plasma and erythrocyte samples obtained from AD patients showed higher levels of NOx, particularly in the intermediate and severe stages, with plasma increasing in parallel with the progression of the illness. TBARS are indicators of lipid peroxidation and were also higher at all clinical stages though only in erythrocytes was the accumulation of TBARS clearly associated with the progression of the illness. The findings were similar for protein carbonyls (PCs), with an even more marked increase in production in

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Table 1
Main characteristics of the groups examined.

Experimental groups	MMSE	Disease duration	Σ *n* (F+M)	*n* F	*n* M	Mean age (Σ years ± SD)		
	(Σ ± SD)	(Σ years ± SD)				Σ F	Σ M	∑ (F+M)
Alzheimer disease (AD)								
Mild (M-AD)	22.2 ± 3.1	3.1 ± 1.1	39	22	17	69.6 ± 5.4	70.3 ± 6.3	70.0 ± 5.6
Intermediate (I-AD)	15.9 ± 1.5	5.1 ± 2.4	36	21	15	75.2 ± 6.0	77.5 ± 4.8	76.4 ± 5.4
Severe (S-AD)	11.6 ± 2.2	7.8 ± 3.5	35	21	14	76.8 ± 4.4	78.4 ± 5.0	77.6 ± 4.6
Total AD			110	64	46	74.4 ± 5.1	76.1 ± 5.2	74.7 ± 4.1
Fist-degree-relatives AD (R-AD)			76	41	35	54.0 ± 6.1	51.2 ± 5.3	53.1 ± 5.7
Parkinson disease (PD)								
Mild (M-PD)	24.6 ± 2.9	4.5 ± 3.3	33	19	14	66.1 ± 3.8	68.5 ± 5.4	66.9 ± 4.4
Intermediate (I-PD)	22.1 ± 1.5	6.5 ± 2.7	29	16	13	69.6 ± 6.1	68.0 ± 4.7	69.1 ± 5.6
Severe (SPD)	17.4 ± 3.3	9.0 ± 2.7	25	15	10	75.2 ± 4.7	73.6 ± 3.3	74.5 ± 4.1
Total PD			87	50	37	70.1 ± 4.8	70.2 ± 4.5	70.0 ± 4.6
Fist-degree relatives PD (R-PD)			46	29	17	45.8 ± 4.4	41.9 ± 5.0	43.9 ± 4.6
Vascular dementia (VD)								
Mild (M-VD)	22.2 ± 3.4	5.2 ± 3.0	48	25	23	74.3 ± 6.1	72.8 ± 7.0	73.7 ± 6.4
Intermediate (I-VD)	17.4 ± 2.5	7.1 ± 2.9	32	20	12	77.5 ± 4.3	75.4 ± 5.1	76.6 ± 4.6
Severe (S-VD)	9.9 ± 1.8	8.7 ± 2.1	30	18	12	79.8 ± 3.2	80.1 ± 2.5	80.0 ± 2.8
Total VD			110	42	32	77.4 ± 4.4	76.4 ± 4.7	77.0 ± 4.5
First-degree relatives VD (R-VD)			56	29	27	38.5 ± 6.6	41.3 ± 5.0	39.9 ± 5.8
Control groups (CG)								
Younger control (YCG)	29.5 ± 0.8		55	29	26	42.2 ± 4.3	41.6 ± 3.9	42.0 ± 4.1
Elderly control (ECG)	27.8 ± 1.1		79	38	41	78.5 ± 3.0	77.1 ± 4.4	77.8 ± 3.7
Total controls			134	67	67			
Total first-degree relatives			178	99	79			
Total patients			307	177	130			
Total subjects			619	343	276			

"Σ" Indicates the sum of the variables involved. MMSE: Mini-Mental State Examination test. F; female. M; male. None of the patients was institutionalized. Disease duration was computed from the day of diagnosis. Scholar level and alcohol consumption were not statistically different among groups. Smokers (<10 cigarettes/day) proportion was higher in both control groups respect to the other groups.

Table 2
Biomarkers in plasma, erytrhocyte and leukocyte lysates from neurological patients classified according to their clinical stage.

Samples and biomarkers	Control ECG	Patient sub-groups and clinical stages								
		Alzheimer (AD)			Parkinson (PD)			Vascular dementia (VD)		
		Mild	Intermediate	Severe	Mild	Intermediate	Severe	Mild	Intermediate	Severe
Plasma										
NOx (μM)	26.7 ± 2.9^{a}	27.5 ± 2.2^{a}	29.8 ± 2.2^{b}	33.3 ± 2.0^{c}	25.8 ± 2.5^{a}	27.4 ± 2.0^{a}	28.1 ± 1.9^{a}	32.1 ± 1.9^{b}	35.4 ± 2.0^{c}	38.6 ± 1.9^{d}
TBARS (μM)	2.2 ± 0.1^{a}	2.5 ± 0.2^{a}	2.7 ± 0.2^{a}	3.0 ± 0.1^{b}	2.8 ± 0.2^{a}	2.7 ± 0.2^{a}	3.1 ± 0.1^{b}	3.0 ± 0.2^{b}	3.3 ± 0.1^{b}	3.5 ± 0.2^{b}
PCs (nmoles /mg prot.)	0.9 ± 0.1^{a}	1.5 ± 0.1^{b}	2.6 ± 0.2^{c}	3.5 ± 0.2^{d}	1.0 ± 0.1^{a}	1.8 ± 0.2^{b}	2.3 ± 0.1^{b}	2.5 ± 0.1^{b}	2.4 ± 0.2^{b}	2.8 ± 0.1^{b}
α-Tocopherol (μM)	24.5 ± 2.9^{a}	20.2 ± 2.0^{a}	18.5 ± 1.8^{b}	15.3 ± 1.5^{c}	23.5 ± 1.8^{a}	25.0 ± 2.2^{a}	22.8 ± 1.7^{a}	19.6 ± 1.4^{b}	18.0 ± 0.9^{b}	16.5 ± 1.1^{b}
FRAP (μM)	989 ± 74	702 ± 56^{b}	655 ± 68^{b}	660 ± 71^{b}	977 ± 101^{a}	842 ± 53^{b}	823 ± 49^{b}	725 ± 43^{b}	712 ± 67^{b}	640 ± 39^{c}
Cu (μM)	15.6 ± 1.5^{a}	16.0 ± 1.1^{a}	17.2 ± 1.3^{a}	20.2 ± 1.1^{b}	15.9 ± 0.8^{a}	18.6 ± 0.9^{b}	19.5 ± 1.0^{b}	17.7 ± 0.8^{b}	23.6 ± 1.1^{c}	26.4 ± 0.8^{d}
Se (μM)	1.3 ± 0.03^{a}	0.8 ± 0.01^{b}	0.7 ± 0.02^{b}	0.7 ± 0.04^{b}	1.4 ± 0.1^{a}	1.3 ± 0.05^{a}	1.2 ± 0.1^{a}	1.2 ± 0.05^{a}	1.1 ± 0.1^{a}	1.4 ± 0.04^{a}
Zn (nM)	16.9 ± 0.8^{a}	14.1 ± 0.7^{b}	13.0 ± 0.5^{c}	12.5 ± 1.0^{c}	15.3 ± 1.1^{a}	16.2 ± 0.8^{a}	15.8 ± 1.1^{a}	14.5 ± 0.9^{a}	16.0 ± 1.3^{a}	15.1 ± 1.5^{a}
Erythrocytes										
NOx (μmoles /g Hb)	0.9 ± 0.04^{a}	1.1 ± 0.1^{a}	1.6 ± 0.04^{b}	1.8 ± 0.1^{b}	1.3 ± 0.1^{b}	1.9 ± 0.1^{c}	3.0 ± 0.2^{d}	1.5 ± 0.02^{b}	1.5 ± 0.1^{b}	1.8 ± 0.1^{b}
TBARS (nmoles /g Hb)	115 ± 10^{a}	124 ± 8^{a}	139 ± 12^{b}	161 ± 9^{c}	119 ± 14^{a}	137 ± 15^{b}	158 ± 10^{c}	137 ± 12^{b}	142 ± 10^{b}	157 ± 7^{c}
PIT (pmoles MDA/ min.mg prot.)	1.9 ± 0.04^{a}	2.5 ± 0.03^{b}	3.6 ± 0.1^{c}	4.0 ± 0.1^{c}	2.0 ± 0.2^{a}	2.4 ± 0.1^{b}	2.7 ± 0.1^{b}	3.0 ± 0.2^{b}	3.8 ± 0.1^{b}	4.9 ± 0.2^{b}
PCs (nmoles /mg prot.)	2.1 ± 0.1^{a}	2.8 ± 0.03^{b}	3.3 ± 0.1^{c}	4.2 ± 0.1^{d}	2.2 ± 0.1^{a}	2.8 ± 0.1^{b}	3.0 ± 0.04^{b}	2.0 ± 0.1^{a}	2.7 ± 0.1^{b}	3.4 ± 0.1^{c}
CAT (*k*/g Hb)	136 ± 12^{a}	159 ± 8^{b}	174 ± 10^{c}	197 ± 7^{d}	141 ± 17^{a}	166 ± 8^{b}	173 ± 11^{b}	135 ± 14^{a}	141 ± 12^{a}	150 ± 9^{a}
SOD (U/g Hb)	1404 ± 98^{a}	1513 ± 72^{a}	1688 ± 65^{b}	1911 ± 73^{c}	1471 ± 63^{a}	1572 ± 55^{b}	1541 ± 64^{b}	1533 ± 71^{b}	1614 ± 64^{c}	1742 ± 59^{d}
CAT/SOD (*k*/U).10^{3}	97 ± 5^{a}	105 ± 5^{a}	103 ± 4^{a}	103 ± 6^{a}	95 ± 7^{a}	106 ± 4^{a}	112 ± 5^{b}	88 ± 4^{b}	87 ± 5^{b}	86 ± 4^{b}
CAT/GPx (k/U).	9.1 ± 0.1^{a}	9.6 ± 0.1^{a}	9.5 ± 0.2^{a}	8.9 ± 0.2^{a}	9.2 ± 0.2^{a}	9.7 ± 0.1^{a}	10.3 ± 0.1^{b}	7.1 ± 0.1^{b}	5.9 ± 0.1^{c}	5.7 ± 0.2^{c}
Leukocytes										
NOx (pmoles /mg prot.)	10.7 ± 0.4^{a}	9.8 ± 0.2^{a}	10.3 ± 0.1^{a}	11.2 ± 0.2^{a}	14.3 ± 0.2^{b}	18.1 ± 0.3^{c}	23.0 ± 0.3^{d}	9.8 ± 0.4^{a}	10.2 ± 0.3^{a}	10.5 ± 0.5^{a}
TBARS (pmoles /mg prot.)	68 ± 4^{a}	72 ± 5^{a}	76 ± 6^{a}	89 ± 4^{b}	75 ± 5^{a}	87 ± 4^{b}	101 ± 6^{d}	81 ± 6^{b}	121 ± 7^{c}	142 ± 9^{d}
PCs (nmoles /mg prot.)	1.5 ± 0.03^{a}	2.5 ± 0.1^{b}	3.2 ± 0.2^{c}	3.9 ± 0.1^{d}	2.8 ± 0.1^{b}	3.0 ± 0.1^{b}	2.9 ± 0.05^{b}	3.1 ± 0.1^{b}	3.9 ± 0.05^{c}	4.5 ± 0.1^{d}
Ca (μmoles/mg prot.)	108 ± 7^{a}	137 ± 8^{b}	156 ± 9^{c}	174 ± 9^{d}	105 ± 11^{a}	117 ± 8^{a}	109 ± 6^{a}	112 ± 5^{a}	118 ± 10^{a}	101 ± 14^{a}
CAT (*k*/mg prot.)	485 ± 23^{a}	588 ± 25^{b}	723 ± 31^{c}	818 ± 44^{d}	472 ± 31^{a}	494 ± 22^{b}	527 ± 18^{d}	496 ± 34^{a}	480 ± 25^{a}	501 ± 30^{a}
SOD (U/mg Prot.)	1370 ± 84^{a}	1781 ± 103^{b}	1811 ± 76^{b}	1575 ± 92^{c}	1544 ± 79^{b}	1531 ± 85^{b}	1610 ± 102^{b}	1647 ± 102^{b}	1811 ± 94^{c}	1998 ± 101^{d}
CAT/SOD (*k*/U).10^{3}	354 ± 14^{a}	330 ± 16^{b}	399 ± 12^{c}	519 ± 23^{d}	305 ± 27^{b}	322 ± 18^{b}	327 ± 16^{b}	301 ± 15^{a}	265 ± 10^{c}	251 ± 12^{d}

Samples of plasma, erythrocyte or leukocyte lysates from Alzheimer (AD), Parkinson (PD) or vascular demented (VD) patients were analyzed as described in the experimental part. Values with different superscript letters within a group and compared to EGC were statistically significant ($p<0.05$ or less).

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parallel with the duration/severity of the illness for the three biological materials analyzed (Table 2). In plasma, the FRAP assay clearly showed a lower ratio of reducing to pro-oxidant agents, though this was unrelated to the clinical stage of the disease. This decrease in reducing agents was due at least in part to a reduction in α-tocopherol, the main lipophilic antioxidant. Susceptibility to induced peroxidation (explored by the peroxidation-induced test or PIT) was clearly higher in erythrocyte ghosts isolated from AD patients. Copper content was higher only at severe stages of AD whereas the level of Se and Zn was lower from the very beginning of the illness, with no evident association with clinical stage or illness duration. Enzymes of the antioxidant defense system were also altered. Catalase (CAT) and superoxide dismutase (SOD) activities were higher in both erythrocyte and leukocyte lysates; however, the increment in CAT in leukocytes was proportionally higher as indicated by the progressive increase of the CAT/SOD ratio associated with the progression of the illness (Table 2). The CAT/G-Px ratio, on the other hand, did not change significantly. A noteworthy finding was that the calcium content in leukocyte preparations from AD patients increased in parallel with the progression of the illness.

3.3. Biomarkers in patients with Parkinson's disease

The pattern of changes observed with Parkinson's disease patients was clearly different from that of the AD group.

The concentration of NOx did not change in plasma, whereas there were significant increments in erythrocytes and more particularly in leukocytes (37, 69 and 114% over control data for mild, intermediate, and severe stages, respectively). An increase in TBARS was also observed in blood cells along the progression of the illness. The increment in protein carbonyls (PCs), however, was not associated with the clinical stage of the patients. Plasma values measured by the FRAP assay were not parallel to the plasma α-tocopherol concentrations and the changes in the two parameters appear to indicate that they are poor biomarkers of the progress of the disease. No marked changes in PIT values in erythrocytes ghosts were found along the evolution of the clinical condition of PD patients. Plasma oligoelement concentrations behaved differently: whereas Cu increased, no significant modifications occurred in Se and Zn. The activities of CAT and SOD in erythrocytes and the CAT/SOD and CAT/G-Px ratios increased slightly with no evident association with the clinical stage.

3.4. Biomarkers in patients with vascular dementia

The increases observed in plasma NOx were particularly significant in this group of patients (20, 33 and 45% over control data for mild, intermediate, and severe stages, respectively). Changes were in the same direction in both blood cell types although of less intensity than those observed in plasma. Conversely, TBARS increments in erythrocytes and leukocytes were greater than changes in plasma. FRAP and α-tocopherol levels in plasma were both lower and the peroxidability of the erythrocyte ghosts (PIT) increased significantly from the mild to the severe condition as reflected in the higher TBARS levels in all samples, particularly red blood cells. PCs formation was higher in all samples studied; however, accumulation of carbonylated proteins was more evident in leukocytes, such change evidently being associated with the progression of the illness (from 106 to 200% over control data). The increase in the plasma Cu concentration was the highest of all experimental groups (103% over control values in severe VD patients). On the contrary, Se and Zn concentrations were not significantly modified. CAT activity increased in erythrocyte but not in leukocyte preparations; however, SOD increased in both type of blood cells. The CAT/SOD ratio remains consistently elevated in erythrocytes whereas it decreased with illness progression in leukocytes.

3.5. Biomarkers associated with glutathione metabolism in AD, PD and VD patients

In view of the great importance of glutathione as an antioxidant within the hydrophilic cellular compartment, the total concentration and the ratio between reduced (GSH) and oxidized (GSSG) forms were determined (Table 3). In AD and VD patients the content of total glutathione measured in all type of samples decreased at the severe stage of the illnesses. Oxidized glutathione content also increased progressively along clinical evolution in these patients, with a concomitant decrease in the GSH/GSSG ratio in blood, plasma and erythrocytes. With the exception of glutathione transferase, the enzyme activities involved in glutathione metabolism were also altered. GR and GPx were both higher in AD, PD and VD patients. Except in the PD group, the activities of these enzymes increased in parallel to the illness progression (Table 3).

Table 3
Biomarkers involved in glutathion metabolism determined in samples from neurological patients classified according to their clinical stage.

Samples and bimarkers	Control ECG	Patient sub-groups and clinical stage								
		Alzheimer (AD)			Parkinson (PD)			Vascular dementia (VD)		
		Mild	Intermediate	Severe	Mild	Intermediate	Severe	Mild	Intermediate	Severe
Blood										
Total glutathion (μM)	929 ± 130^{a}	899 ± 97^{a}	902 ± 87^{a}	776 ± 96^{b}	915 ± 76^{a}	891 ± 114^{a}	873 ± 122^{a}	808 ± 74^{a}	753 ± 81^{b}	744 ± 62^{b}
GSSG (μM)	144 ± 11^{a}	172 ± 9^{b}	195 ± 6^{c}	222 ± 8^{d}	138 ± 12^{a}	129 ± 15^{a}	141 ± 11^{a}	163 ± 8^{b}	184 ± 10^{c}	202 ± 16^{d}
GSH/GSSG	5.5 ± 0.4^{a}	4.2 ± 0.2^{b}	3.6 ± 0.1^{c}	2.5 ± 0.1^{d}	5.6 ± 0.2^{a}	5.9 ± 0.3^{a}	5.2 ± 0.3^{a}	3.9 ± 0.1^{b}	3.0 ± 0.1^{c}	2.6 ± 0.1^{d}
Plasma										
Total glutathion (μM)	662 ± 23^{a}	651 ± 45^{a}	648 ± 33^{a}	504 ± 26^{b}	674 ± 41^{a}	645 ± 33^{a}	637 ± 52^{a}	519 ± 24^{b}	498 ± 19^{b}	456 ± 22^{c}
Total glutathion (nmoles/mg.prot.)*	9.3 ± 0.8^{a}	9.5 ± 0.5^{a}	9.3 ± 0.6^{a}	7.1 ± 0.4^{b}	9.4 ± 0.6^{a}	9.1 ± 0.7^{a}	9.0 ± 0.8^{a}	7.3 ± 0.5^{b}	7.0 ± 0.5^{b}	6.4 ± 0.7^{b}
GSSG (μM)	85 ± 7^{a}	96 ± 9^{a}	131 ± 12^{b}	149 ± 8^{c}	80 ± 5^{a}	91 ± 8^{a}	106 ± 9^{b}	129 ± 11^{b}	148 ± 7^{c}	164 ± 12^{d}
GSH/GSSG	6.8 ± 0.5^{a}	5.7 ± 0.4^{a}	3.9 ± 0.1^{b}	2.3 ± 0.1^{c}	7.4 ± 0.4^{a}	6.0 ± 0.3^{a}	5.0 ± 0.2^{b}	3.0 ± 0.3^{b}	2.3 ± 0.3^{b}	1.8 ± 0.1^{c}
Erythrocytes										
Total glutathione (μmoles/g Hb)	2.9 ± 0.2^{a}	2.0 ± 0.1^{b}	1.8 ± 0.1^{b}	1.6 ± 0.1^{c}	2.6 ± 0.2^{a}	2.5 ± 0.3^{a}	2.3 ± 0.3^{a}	2.0 ± 0.1^{b}	1.8 ± 0.1^{b}	1.8 ± 0.2^{b}
GSSG (μmoles/g Hb)	0.3 ± 0.04^{a}	0.5 ± 0.02^{b}	0.7 ± 0.01^{c}	0.8 ± 0.02^{c}	0.4 ± 0.02^{a}	0.4 ± 0.03^{a}	0.5 ± 0.04^{a}	0.7 ± 0.1^{b}	0.9 ± 0.04^{c}	1.3 ± 0.1^{d}
GSH/GSSG	8.7 ± 0.3^{a}	3.0 ± 0.1^{b}	1.6 ± 0.1^{c}	1.0 ± 0.1^{d}	5.5 ± 0.2^{b}	5.3 ± 0.2^{b}	3.6 ± 0.1^{c}	1.8 ± 0.1^{b}	1.0 ± 0.04^{c}	0.4 ± 0.02^{d}
GR (U/g Hb)	15.7 ± 2.4^{a}	18.8 ± 1.6^{b}	19.1 ± 2.2^{b}	24.3 ± 1.5^{c}	16.6 ± 2.2^{a}	15.1 ± 1.9^{a}	15.5 ± 2.3^{a}	18.5 ± 1.4^{b}	22.2 ± 2.0^{c}	25.8 ± 1.9^{c}
GST (U/g Hb)	34.4 ± 2.9^{a}	33.2 ± 2.3^{a}	35.4 ± 3.0^{a}	34.1 ± 2.7^{a}	33.6 ± 2.7^{a}	31.5 ± 2.3^{a}	34.1 ± 2.2^{a}	34.0 ± 1.8^{a}	32.8 ± 2.5^{a}	35.7 ± 2.0^{a}
GPx (U/g Hb)	14.9 ± 2.6^{a}	16.5 ± 1.1^{b}	18.3 ± 0.9^{c}	22.2 ± 0.7^{d}	16.5 ± 1.1^{a}	17.2 ± 1.0^{a}	16.8 ± 0.9^{a}	19.0 ± 0.8^{b}	23.7 ± 1.1^{c}	26.2 ± 0.8^{d}

Samples of blood, plasma, or erythrocyte lysates from Alzheimer (AD), Parkinson (PD) or vascular demented (VD) patients were analyzed as described in the experimental part. * Plasma protein contents were significantly lower in S sub-group of AD and PD patients compared to ECG. Values with different superscript letters within the same sub-group and compared to ECG were statistically significant ($p<0.05$ or less).

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3.6. Correlations between biomarkers and cognitive performance

Various non-enzymatic biomarkers (mainly determined in plasma) were analyzed to test their possible correlation with the values obtained using MMSE, one of the most frequently used indices associated with the clinical condition of patients with neurodegenerative disorders (Table 4). The results of the analysis are consistent with our earlier comments and indicate that there are several markers that could be associated with only one pathological condition. Plasma PCs and calcium content in leukocytes correlate negatively with MMSE scores, whereas the plasma concentration of α-tocopherol correlates positively. Within the PD group, the NOx concentration in plasma and NOx and TBARS in leukocytes correlate negatively with MMSE scores. The copper level in plasma was a non-shared marker negatively associated with MMSE scores for the VD patients.

3.7. Predictive values of biomarkers within the first-degree relatives of neurological patients

Tables 5 and 6 show the data obtained for the oxidative stress biomarkers measured in the groups of first-degree relatives of AD, PD and VD patients. R-AD exhibited lower values for the plasma FRAP assay, Se and Zn plasma concentrations and the CAT/SOD ratio in leukocytes, whereas the plasma PCs content, CAT activity in erythrocytes and CAT and SOD activities in leukocytes were all higher. The concentration of calcium in leukocytes was significantly higher (25% over control data). R-PD was the group with the lowest number of changes compared to the respective control group. The NOx concentration was higher in erythrocytes and leukocytes and the CAT/SOD ratio lower in leukocytes. The main changes registered in first-degree relatives were observed for the R-VD group: plasma NOx, PCs and Cu concentrations were all higher whereas the FRAP assay and α-tocopherol concentrations were lower. In addition, the levels of NOx and TBARS, and SOD activity in leukocytes were higher, and the CAT/SOD ratios lower than those of the corresponding control group.

With respect to glutathione-related biomarkers (Table 6), only the R-VD group exhibited lower values of GSH/GSSG in blood and erythrocytes, with higher GSSG content only in erythrocytes. Glutathione reductase activity was higher in red blood cells. These changes were all specific to the R-VD group whereas the other biomarkers tested (Table 5) were mostly shared by more than one group. It is important to note that determination of one biomarker also depends on the type of sample analyzed. For example the GSH/GSSG ratio was lower in the blood of the R-VD group but not in plasma.

Table 4
Correlation coefficients between MMSE and various non-enzymatic biomarkers for the three experimental groups of patients.

Parameters	MMSE		
	Alzheimer	Parkinson	Vascular dementia
Years[a]	−0.77*	−0.45*	−0.49*
Plasma			
NOx	−0.53*	−0.09	−0.61*
TBARS plasma	−0.15	−0.13	−0.12
PCs plasma	−0.39*	−0.17	0.06
Cu	−0.18	0.08	−0.74*
Se	0.15	0.11	0.05
Zn	0.21	0.06	0.14
GSH/GSSG	0.62*	0.12	0.39*
Erythrocytes			
NOx	−0.17	−0.48*	−0.11
TBARS	−0.18	−0.37*	−0.31*
PCs	−0.46*	−0.30*	−0.52*
Leukocytes			
NOx	0.06	−0.57*	0.08
TBARS	−0.05	−0.51*	−0.14
PCs	−0.55*	−0.19	−0.66*
Ca	−0.79*	−0.07	0.11

Correlation coefficients were obtained as described in the Material and method section using the individual values of each parameter test within a determined experimental group of patients. Significance was denoted by * ($p<0.05$ or less). [a], Years of evolution from the date of diagnosis.

Table 5
Biomarkers in plasma, erythrocyte and leukocyte lysates determined in samples from first-degree relatives (R) of neurological patients.

Samples and biomarkers	Control YCG	First-degree relatives		
		Alzheimer	Parkinson	Vascular dementia
		R-AD	R-PD	R-VD
Plasma				
NOx (μM)	22.2 ± 1.7^a	23.6 ± 1.2^a	21.7 ± 0.9^a	27.4 ± 1.0^b
TBARS (μM)	1.9 ± 0.1	1.7 ± 0.2	1.7 ± 0.1	2.0 ± 0.2
PCs (nmoles /mg prot.)	0.7 ± 0.1^a	1.2 ± 0.03^b	0.9 ± 0.1^a	1.4 ± 0.1^b
FRAP (μM)	1016 ± 61^a	901 ± 77^b	1105 ± 86^a	812 ± 47^c
Cu (μM)	14.8 ± 0.5^a	14.9 ± 0.4^a	15.1 ± 0.6^a	16.3 ± 0.5^b
Se (μM)	1.2 ± 0.02^a	0.9 ± 0.03^b	1.2 ± 0.1^a	1.1 ± 0.04^a
Zn (nM)	17.1 ± 0.5^a	15.2 ± 0.3^b	17.2 ± 0.3^a	17.0 ± 0.4^a
α-Tocopherol (μM)	26.4 ± 2.0^a	27.1 ± 1.5^a	25.8 ± 2.3^a	22.2 ± 1.7^b
Erythrocytes				
NOx (μmoles /g Hb)	0.7 ± 0.02^a	0.6 ± 0.1^a	1.0 ± 0.04^b	1.1 ± 0.1^b
TBARS (nmoles /g Hb)	96 ± 11	101 ± 14	89 ± 12	125 ± 16
PCs (nmoles /mg prot.)	1.5 ± 0.2	1.9 ± 0.2	1.8 ± 0.3	1.6 ± 0.1
CAT (*k*/g Hb)	124 ± 10^a	139 ± 8^b	118 ± 11^a	127 ± 14^a
SOD (U/g Hb)	1311 ± 33^a	1299 ± 41^a	1327 ± 50^a	1424 ± 38^b
CAT/SOD (*k*/U).10^3	94 ± 3	107 ± 5	89 ± 7	89 ± 5
CAT/GPx (k/U).	9.2 ± 0.4	9.9 ± 0.4	8.6 ± 0.3	8.9 ± 0.5
PIT (pmoles MDA/min. mg prot.)	1.3 ± 0.1^a	1.4 ± 0.2^a	1.3 ± 0.2^a	1.8 ± 0.1^b
Leukocytes				
NOx (pmoles /mg prot.)	9.1 ± 0.2^a	9.5 ± 0.4^a	12.2 ± 0.2^b	9.6 ± 0.3^a
TBARS (pmoles /mg prot.)	66 ± 5^a	71 ± 4^a	69 ± 6^a	75 ± 4^b
PCs (nmoles /mg prot.)	1.1 ± 0.1	0.9 ± 0.1	1.0 ± 0.04	1.3 ± 0.2
Ca (μmoles/mg prot.)	99 ± 7^a	124 ± 6^b	97 ± 9^a	96 ± 5^a
CAT (*k*/mg prot.)	477 ± 18^a	485 ± 12^b	465 ± 17^a	470 ± 11^a
SOD (U/mg Prot.)	1204 ± 62^a	1319 ± 41^b	1246 ± 33^a	1355 ± 36^b
CAT/SOD (*k*/U).10^3	396 ± 17^a	367 ± 11^b	373 ± 10^b	347 ± 9^b

Samples of blood, plasma, or erythrocyte lysates from first-degree relatives of Alzheimer (R-AD), Parkinson (R-PD) or vascular demented (R-VD) patients were analyzed as described in the experimental part. Values with different superscript letters were statistically significant ($p<0.05$ or less). Superscripts were omitted when there was no significant difference among data.

4. Discussion

Chronic neurodegenerative illnesses have a profound impact on human society. Early diagnosis in combination with new therapeutic strategies may delay or even prevent the irreversible consequences of these disorders. The issue of early diagnosis is particularly critical for patients from lower socioeconomic backgrounds who may lack access to current advanced diagnostic procedures. Available data indicate that two-thirds of neurodegenerative illnesses go undiagnosed [52]. The main challenge to early detection is the fact that the diagnosis of most neurodegenerative disorders is based mainly on clinical symptoms. Research into possible new biomarkers for disease prevention or evolution is therefore of major importance. According to the National Institutes of Health (NIH), a biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention." Furthermore it should ideally be both non-invasive and cost-affordable. Current diagnostic biomarkers such as those emerging from the molecular biology domain include determination of Aβ1-42 and phosphorylated Tau proteins in cerebrospinal fluid [53], positron emission tomography using the Pittsburg compound B [54], or cDNA microarray and

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Table 6
Biomarkers involved in glutathione metabolism determined in samples from first-degree relatives (R) of neurological patients.

Samples and biomarkers	Control	First-degree relatives (R)		
	YCG	Alzheimer	Parkinson	Vascular dementia
		R-AD	R-PD	R-VD
Blood				
Total glutathion (μM)	1012 ± 101	1157 ± 122	1080 ± 96	989 ± 123
GSSG (μM)	96 ± 18	105 ± 23	90 ± 23	111 ± 15
GSH/GSSG	9.5 ± 0.4[a]	10.0 ± 0.8[a]	11.0 ± 0.7[a]	7.9 ± 0.5[b]
Plasma				
Total glutathion (μM)	714 ± 33	725 ± 29	722 ± 31	718 ± 25
Total glutathion (nmoles/mg.prot.)	9.5 ± 1.0	9.3 ± 0.8	10.1 ± 1.2	10.0 ± 1.0
GSSG (μM)	72 ± 5	69 ± 7	70 ± 8	81 ± 7
GSH/GSSG	8.9 ± 0.5	9.5 ± 0.7	9.3 ± 0.6	7.8 ± 0.5
Erythrocytes				
Total glutathion (μmoles/g Hb)	3.1 ± 0.1	3.2 ± 0.2	3.0 ± 0.3	3.0 ± 0.2
GSSG (μmoles/g Hb)	0.3 ± 0.02[a]	0.3 ± 0.04[a]	0.3 ± 0.03[a]	0.6 ± 0.03[b]
GSH/GSSG	9.3 ± 0.4[a]	9.6 ± 0.5[a]	9.0 ± 0.6	4.0 ± 0.1[b]
GR (U/g Hb)	12.2 ± 0.8[a]	12.8 ± 0.9[a]	13.0 ± 0.5[a]	15.1 ± 0.7[b]
GST (U/g Hb)	36.5 ± 1.8	31.8 ± 2.2	33.3 ± 2.1	34.2 ± 1.9
GPx (U/g Hb)	13.4 ± 1.4	14.0 ± 1.1	13.8 ± 1.2	14.3 ± 1.5

Samples of blood, plasma, or erythrocyte lysates from first-degree relatives of Alzheimer (R-AD), Parkinson (R-PD) or vascular demented (R-VD) patients were analyzed as described in the experimental part. Values with different superscript letters compared to YCG were statistically significant ($p<0.05$ or less). Superscripts were omitted when there was no significant difference among data.

proteomics studies [55]. These procedures have been shown to correlate well with the clinical condition especially in the case of AD patients. Determination of the apoliprotein E ε4 allele is promising as a genetic risk factor in specific populations [56]. However, these and other biomarkers are usually prohibitively expensive for the majority of those affected, particularly in third-world countries, and/or are invasive [55]. More accessible biochemical markers such as the lipid profile over time could be determined for VD patients; and in general terms it is clear that more non-invasive, easily reproducible, reliable and low-cost biomarkers are required in order to be able to diagnose neurodegenerative disorders in their early stages. A correlation between the evolution of cognitive impairment and reliable biomarkers may also provide a partial solution to the problem posed by classical measurements in the behavioral domain, which are expensive, time-consuming and available only at specialized dementia clinics. A combination of biomarkers matching the characteristics defined by the NIH will serve to increase the sensitivity and specificity of diagnostic techniques [57].

In this paper we analyzed a battery of putative biomarkers using peripheral blood of patients with neurodegenerative disorders or their first-degree relatives. The involvement of oxidative stress was largely demonstrated for AD [2,6,20,34,58], PD [2,6,18,20,59,61,62] and VD [19,63–65]. Biomarkers assayed to characterize oxidative stress condition in animal models of neurodegenerative disorders demonstrate that a common mechanism may explain some of the complex underlying neurobiological processes in these three pathologies (AD, PD and VD) and other neurological conditions [1,5,66]. However, the adequacy of peripheral oxidative stress biomarkers in the early characterization of neurological disorders remains a matter of debate: some authors discourage their use whereas others consider them good tools for enhancing the accuracy of clinical evaluations in cases of cognitive decline and the prediction of clinical evolution at mild neurodegenerative stages [5,58,69].

A further problem associated with the use of biochemical markers in neurodegenerative patients relates to the type of biological sample analyzed. Blood is a very complex tissue that reflects physiological activity and pathology in body organs and systems, including the central nervous system [5,68]. In humans, about 500 mL of cerebrospinal fluid is daily absorbed into the blood [68], making it a suitable source of neurodegenerative disease biomarkers. Furthermore, the ease of venipuncture compared to lumbar puncture allows for repeatability, making it suitable for application in clinical trials to evaluate illness progression or the effect of treatment.

Oxidative stress biomarkers should be validated as reliable tools to implement in clinical practice alongside other blood tests already available to clinicians as risk factor measurements [5]. The key features of a biomarker should include its ability to detect the disease process during its early stages, ideally at the preclinical stage, and to show a variation or correlation with disease progression. Our findings that some markers are higher or lower in one or more of the illnesses studied are in agreement with data previously reported by other laboratories [1]. But more interesting is the finding of positive and negative correlations with disease progression and the fact that some biomarkers are significantly altered in first-degree relatives of the patients. This latter finding is of particular importance in view of the lack of methodologies for evaluating preclinical neurodegenerative conditions.

Despite the non-selective characteristics of the markers assayed, the easy and non-expensive, non-invasive determinations using peripheral blood samples largely justify their inclusion as promising laboratory tests, especially in populations with genetic predisposition to develop AD, PD, or VD. Other laboratories [1,4,5,47,59,60,62,65] and our group [69,70] report reliable biomarkers for evaluating neurodegenerative illness and distinguishing Alzheimer's disease from vascular dementia and other types of cognitive impairments. Furthermore, there is experimental evidence that oxidative damage is the earliest event in cognitive decline [67,70]. In addition, it is well known that cognitive scores, even within the normal range, predict death and institutionalization risk in the general population [71]. It is considered that a predictive battery of biomarkers such as those assayed in the present study would be a useful complement to evaluation by means of the MMSE.

In conclusion, we suggest assaying NOx, PCs and GSH/GSSG in plasma, PCs in erythrocytes and PCs and calcium content in leukocytes for AD patients; NOx, TBARS and PCs in erythrocytes, and NOx and TBARS in leukocytes for PD patients; and NOx, Cu, and GSH/GSSG in plasma, and TBARS and PCs in erythrocytes together with PCs in leukocytes for the group of VD patients. As predictive markers, the relatives of AD patients should be assayed for plasma Se and Zn concentration, CAT activity in erythrocytes, and calcium content in leukocytes. For R-PD we suggest NOx in leukocytes; and for relatives of VD patients the suggested assays are NOx, GSH/GSSG and α-tocopherol in plasma, CAT/SOD ratio in erythrocytes, and TBARS, GSSG and glutathione reductase in leukocytes.

Large-scale longitudinal population-based studies using these suggested biomarkers are necessary for an accurate assessment of their level of reliability and specificity in clinical practice.

Conflict of interest

The authors declare that there are no conlfict of interest.

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