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Research Article

Ceruloplasmin/Transferrin Ratio Changes in Alzheimer's Disease

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The link between iron and Alzheimer's disease (AD) has been mainly investigated with a focus on the local accumulation of this metal in specific areas of the brain that are critical for AD. In the present study, we have instead looked at systemic variations of markers of iron metabolism. We measured serum levels of iron, ceruloplasmin, and transferrin and calculated the transferrin saturation and the ceruloplasmin to transferrin ratio (Cp/Tf). Cp/Tf and transferrin saturation increased in AD patients. Cp/Tf ratios also correlated positively with peroxide levels and negatively with serum iron concentrations. Elevated values of ceruloplasmin, peroxides, and Cp/Tf inversely correlated with MMSE scores. Isolated medial temporal lobe atrophy positively correlated with Cp/Tf and negatively with serum iron. All these findings indicate that the local iron accumulation found in brain areas critical for AD should be viewed in the frame of iron systemic alterations.

1. Introduction

Alzheimer's disease (AD) is a heterogeneous and progressive neurodegenerative disorder representing the most common cause of dementia in the elderly. The disease results from a complex interaction between predisposing genes, biochemical variables, and environmental factors [1]. The variant E4 of the APOE gene (APOE4), for example, is widely recognized to contribute to the risk of developing the more common late-onset AD [2], while mutations in the genes that encode presenilin 1 and 2 and the amyloid precursor protein (APP) are known to be causative factors of a small percentage of familial and early-onset AD cases.

There is also total agreement on the existence of a link between AD and oxidative stress phenomena triggered by transition metals. However, these phenomena have been generally viewed as originating locally within specific brain areas, and autonomously, that is, independently of systemic influences. In this frame, authors have reported enhanced iron concentrations in AD brains, both in autopsy brain tissues and in cerebrospinal fluid (CSF) [3, 4], especially in the basal ganglia (in vivo: [5, 6] autopsy: [7]), in the hippocampus (in vivo: [6, 8]; autopsy: [9, 10]), neocortex (autopsy: [7, 9, 11]), and around the senile plaques of amyloid beta (Aβ) or the neurofibrillary tangles commonly found in AD brains [12–15]. Authors have also reported altered local concentrations of specific proteins regulating iron levels, such as ceruloplasmin [16–18], transferrin [7, 19], and ferritin [19].

Recently, a wider and somewhat complementary view has emerged suggesting a relationship of AD with systemic, rather than autonomous, changes of metal metabolisms. Some pilot studies based on this view have focused primarily on the genes of hemochromatosis (HFE) and of transferrin, since they play a key role in iron homeostasis, but investigations on circulating markers of iron metabolism are still relatively scanty.

In the present study, we focused on this aspect and studied systemic variations of markers of iron metabolism, such as serum iron, ceruloplasmin, transferrin, transferrin saturation, and ratio of ceruloplasmin to transferrin (Cp/Tf), in a sample of AD patients and in one of healthy elderly subjects.

2. Subjects and Methods

Forty-nine AD patients (mean age 75.6) and 46 cognitively normal individuals (mean age 71.2) were included in the study (see Table 1). The two samples were slightly different in sex and age; consequently, when appropriate, all statistical analyses were adjusted for these two confounders. The AD patient sample consisted of individuals with a clinical diagnosis of probable AD based on NINCDS-ADRDA criteria [20] and with a Mini-Mental State Examination (MMSE) [21] score of 25 or less. All AD patients underwent neurologic, neuroimaging (magnetic resonance imaging: MRI or computed tomography: CT), and neuropsychological evaluation, as well as routine laboratory tests. Average disease duration (from symptom onset) was 27 (range 6–96) months.

The control sample consisted of elderly volunteers with no clinical evidence of neurologic or psychiatric disease.

Twelve AD patients had either poor quality MRI or reasons not to take it (e.g., metal prostheses). In these cases, a CT scan was taken to rule out other causes of dementia. Six AD patients did not complete the entire battery of neuropsychological tests. The same AD cohort had been investigated in a previous study [22].

All controls underwent a neurologic examination and MMSE, but only 12 consented to MRI.

Criteria for exclusion of both patients and controls were conditions known to affect metal metabolism and biological variables of oxidative stress, for example, diabetes mellitus, inflammatory diseases, Hodgkin's disease, recent history of heart or respiratory failure, chronic liver or renal failure, malignant tumors, and alcohol abuse, assessed by tests including complete blood count, erythrocyte sedimentation rate (ESR), cholesterol, triglycerides, ferritin and fibrinogen levels, serum protein electrophoresis, renal function (creatinine, blood urea nitrogen), fasting glucose, electrolytes, vitamin B12, and folic acid and uric acid levels; thyroid function tests (thyroxin, triiodothyronine, and thyrotropin levels); liver enzymes (transaminases), cardiac enzymes (creatine phosphokinase, lactate dehydrogenase), bilirubin levels; serology for syphilis, and urine analysis.

Patients and controls with abnormal values of thyroid, liver, kidney, and cardiac functions were also excluded from the study.

The study was approved by the local IRB, and all participants or legal guardians gave written, informed consent.

2.1. Biochemical and Molecular Investigations. Sera from fasting blood samples were collected in the morning and rapidly stored at –80°C. Methods for measuring biological variables of metals and oxidative stress are described in detail elsewhere [23]. Briefly, hydroperoxide content was assessed by d-ROMs test (Diacron, Italy) and expressed in arbitrary units (U.CARR), 1 U.CARR corresponding to 0.08 mg/100 mL of hydrogen peroxide. Normal range was between 230 and 310 U.CARR [23]. Transferrin and ceruloplasmin were measured by immunoturbidimetric assays (Roche, Diagnostic, Germany) utilizing a goat antihuman transferrin antibody in TRIS/HCl buffer and a rabbit antihuman ceruloplasmin antibody in phosphate buffer and iron using Ferene [20, 21].

All biochemical measures were automated on a Hitachi 912 analyser (Roche Diagnostics) and performed in duplicate. APOE genotyping was performed according to established methods [24].

Table 1: Characteristics of the study groups.

	AD patients	Controls	Overall significance
Number of subjects	49	46	
Sex M/F	11/38	25/21	$\chi^2 = 10.15$; df = 1 $P = .001$
Age (years) Mean (SD)	75.6 (7.7)	71.2 (10.8)	t test; $P = .023$
ApoE $\varepsilon 4$ allele frequency (%)	19.6	4	Pearson's = 6.4 $P = .012$
MMSE Mean (SD)	19 (3.9)	28.2 (1.2)	t test, $P < .001$
Disease duration (months) Median (min-max)	27 (6–96)		

2.2. MRI Evaluation. Brain MRI was performed using a 1.5 Tesla superconductor magnet. The imaging protocol consisted of axial T2 W double Spin Echo (SE) sequences and T1 W SE images in axial, coronal, and sagittal planes, with 5 mm slice thickness and intersection gap = 0.5 mm. MR images were evaluated by two experienced neuroradiologists, blind to the patients' diagnoses and laboratory results, with an agreement of about 95%. Atrophy and white matter lesions were graded following standardized visual rating scales on plain MRI [25–27]. The degree of medial temporal lobe atrophy (MTA) was evaluated with a ranking procedure and validated by linear measurements of the medial temporal lobe including the hippocampal formation and surrounding spaces occupied by CSF, following standardized criteria (five-point rating scale of MTA) [26]. Generalized brain atrophy (ventricular and sulcal atrophy) was rated as present (=1) or absent (=0; global atrophy). The visual rating scale of white matter changes included the anatomical distribution as well as the severity of the lesions. Based on anatomical distribution, a distinction was made between areas of periventricular hyperintensities (PVH) (caps and rims) and deeper hyperintensities (including frontal, parieto-occipital, and temporal white matter hyperintensities: DWMH, basal

ganglia hyperintensities: BGH, and infratentorial hyperintensities: ITH). Large vessel cortical infarcts in the anterior, posterior, and medial cerebral artery were also evaluated. Presence of mass lesions and lobar hemorrhages was an exclusion criteria from the study [27, 28].

2.3. Ultrasonographic Examination of the Cerebral Vessels. The carotid and vertebral arteries were studied by means of color-coded duplex ultrasonography (7.5 MHz probe; Acuson, Aspen, USA) according to standardized criteria [29]. Detailed description of the sonographic procedures has been previously reported [30]. Particular attention was paid to detecting both presence and degree of stenosis of atherosclerotic plaques in the carotid and vertebral arteries. The intima and media thickness (IMT) from the distal portion of the CCA was investigated in detail in each patient according to previous studies [31]. Intracranial vessels were also examined by transcranial Doppler (TCD Multidop T TCD-DWL, Germany), according to previously described methods [32].

2.4. Statistical Analyses. AD and controls were described in terms of main demographic, genetic, and cognitive characteristics and statistically compared with either T- or χ^2-square tests. Both age and sex effects were considered in the statistical analyses. Correlation analyses between biological variables, MMSE scores, MRI atrophy, and vascular measurements (Spearman's rho) were performed. A P value less than .05 was considered significant in all statistical analyses. The t test was used when the homogeneity of variances could be assumed. When the variances were statistically heterogeneous, the Games-Howell procedure was applied.

We used the SPSS 11.5 for Windows statistical software package (SPSS Inc., Chicago).

3. Results

The two groups differed for MMSE scores ($P < .001$). No differences for fasting glucose, vitamin B12, folic acid and uric acid levels, ESR, bilirubin levels, cholesterol, triglycerides, albumin, complete blood count, and hypertension were detected between the two groups (data not shown). APOE $\varepsilon 4$ allele was more frequent in AD patients (Table 1).

None among the biological variables under study correlated with age, with the exception of ceruloplasmin ($r = 0.355$, $P = .01$) and peroxides ($r = 0.215$, $P = .039$), which both increased with age (these two variables were corrected for the age effect before entering the statistical analyses). No variations on sex effect were found in our data.

Table 2 reports the values of serum iron, ceruloplasmin, transferrin, transferrin saturation, and Cp/Tf in AD patients and controls. Transferrin saturation ($P = .033$) and Cp/Tf ($P < .001$) resulted increased in AD patients compared to controls (Table 2).

Cp/Tf correlated positively with peroxide levels ($r = 0.560$, $P < .001$) and negatively with serum iron concentrations ($r = -0.417$, $P < .001$). Elevated values of ceruloplasmin, peroxides, and Cp/Tf inversely correlated with MMSE scores (Table 3). Correlation analysis from the Doppler examination revealed that no AD patient had abnormal flow velocity in the cerebral arteries examined by Duplex sonography and TCD (results not shown). Moreover, none of the biochemical variables assayed correlated with ultrasound indicators of cerebrovascular burden, namely, presence of carotid plaque or stenosis, except for the IMT values that correlated with APOE $\varepsilon 4$ genotype (Spearman rho $= 0.36$; $P < .01$) confirming what was previously reported [20]. Analyses of MRI data showed that presence of supratentorial atrophy, (enlarged sulci, widened lateral, and third ventricles), and atrophy of the temporal lobes (reduction in hippocampal volume), was prominent in AD subjects, while control subjects' values were consistent with those found in normal aging. Among MRI measurements and serum oxidative-iron markers values, there was a negative correlation between isolated medial temporal atrophy (MTA) and serum iron and a positive one between Cp/Tf and MTA (Table 3). White matter changes and deep-seated ischemic changes did not correlate with any metal marker as well as peroxides.

Table 2: Serum levels of the investigated variables in AD patients and controls (means (SD)).

	AD patients ($n = 49$)	Controls ($n = 46$)	t test
Iron (ng/dL)	69.3 (27.79)	76.7 (23.6)	$P = .209$
Ceruloplasmin (mg/dL)	29.9 (5.8)	26.5 (4.6)	$P = .017$
Peroxides (U.CARR)	349 (63)	310 (56)	$P = .005$
Transferrin (g/L)	2.5 (0.52)	2.7 (0.35)	$P = .232$
Transferrin saturation (%)	33.9 (9.6)	30.5 (4.1)	$P = .03$
Cp/Tf	12.4 (3.1)	10 (2.1)	$P = .001$

After correction for age effect.

4. Discussion

The results of our comparison between AD patients and healthy controls show that major markers of circulating iron status, such as ceruloplasmin, Cp/Tf, transferrin saturation, and peroxides levels, are abnormal in AD patients. This finding demonstrates that systemic alterations of iron metabolism accompany the disease and indicate that the role attributed by existing literature to local iron accumulations in brain areas critical for AD should be rather viewed in the frame of a wider systemic alteration. In this scenario, antioxidant systems play of course a role. In the present AD study, as we did previously with stroke patients [33], we used the Cp/Tf ratio to represent the functionality of one of these systems, the Cp-Tf system [31], which is otherwise difficult to monitor biochemically [31].

Table 3: Correlations between outcomes of MMSE, MTA evaluation, and biological variables under study.

Spearman's rho correlations	MMSE	MTA
Iron	rho = 0.121 P = .364 n = 58	rho = −0.393 P = **.008** n = 45
Ceruloplasmin	rho = −0.265 P = **.040** n = 60	rho = 0.184 P = .225 n = 45
Peroxides	rho = −0.329 P = **.008** n = 64	rho = 0.184 P = .211 n = 48
Transferrin	rho = 0.113 P = .372 n = 65	rho = −0.269 P = .065 n = 48
Transferrin saturation	rho = −0.120 P = .336 n = 66	rho = 0.280 P = .051 n = 49
Ceruloplasmin/transferrin ratio	rho = −0.373 P = **.004** n = 59	rho = 0.300 P = **.048** n = 44

After correction for sex and age.

In the Cp-Tf system, ceruloplasmin catalyzes the oxidation of ferrous iron (Fe^{2+}) into ferric iron (Fe^{3+}), by way of the following chemical reaction:

$$4\,Fe^{2+} + 4\,H^{+} + O_2 \longrightarrow 4\,Fe^{3+} + 2\,H_2O. \quad (1)$$

This is generally viewed as a "good" reaction both because its byproduct is innocuous water and because, once transformed into Fe^{3+}, iron binds to transferrin by which it is transported to cells. Ceruloplasmin-catalyzed iron oxidation and removal from circulation by transferrin is a very important process since it reduces the amount of Fe^{2+} available for participation in Fenton's reactions, whose byproducts are instead extremely vicious. In fact, ferrous iron can also convert hydrogen peroxide into the highly reactive radicals $OH^{\bullet}$ and $OOH^{\bullet}$:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \quad (2)$$

which, in turn, proceeds as follows:

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + OOH^{\bullet} + H^{+}. \quad (3)$$

In other words, if it were not oxidized and removed by the coordinated ceruloplasmin and transferrin actions, iron would cyclically produce highly reactive radicals, which the circulating blood would then distribute all over the organism. Nonetheless, it is important to keep in mind that if, on one hand, this mechanism avoids or reduces production of lethal oxidative species in general circulation and allows the vital transport of iron to cells, on the other hand, when iron moves into the cells, as it could be the case for neurons, it can still cause brain damage if a concomitant neuronal iron defective efflux does occur [34]. Evidence of a defective iron efflux from neurons, linked to APP, was reported in several studies on cell cultures and APP knockout mice (APP−/−), in which APP expression is experimentally suppressed [34, 35]. The ablation of APP, which is normally expressed both in the brain and in a limited number of non-neural tissues as platelets, liver, kidney, and heart [34, 35], produces metal disbalance in these organs and tissues. In particular, some authors [35] demonstrated an increase of copper concentrations in the liver (80%) and in the brain (40%) in APP−/− mice. Other authors [34] demonstrated that APP ablation causes an iron increase of 26% in the brain, 31% in the liver, and 15% in the kidney. They also showed that, like ceruloplasmin, APP catalytically oxidizes Fe^{2+} [34]. All together, these studies demonstrate that APP plays a role in copper and iron mobilization, indicating that its abnormalities in AD have either a systemic effect on those metals' homeostasis or a local effect on neuronal metal efflux. Our current results of increased brain atrophy (MTA scores) in AD patients are in support to this concept, as MTA scores associate to either decreasing levels of serum iron or increased values of Cp/Tf ratios. Both these results appear coherent with the picture of an abnormal activation of the Cp-Tf system in AD, the subtraction of iron from general circulation, and its probable internalization into neural cells. This eventually could cause neuronal death via generation of oxidative stress in Aβ transition metal(s) reactions [2].

Finally, in the same direction go also our results on MMSE scores, which worsen with increasing Cp/Tf ratios.

Ceruloplasmin increase and transferrin decrease concentrations were identified in diverse pathological conditions as an important defense mechanism reflecting the body's resistance to an oxidant insult [36]. This is the case, for example, in severe preeclampsia, where women have high levels of lipid peroxidation and serum ceruloplasmin and low levels of transferrin [37, 38], in acute stroke, where the Cp/Tf correlates with the severity of the clinical status [33], or in experimental hypercholesterolemia [36].

Ceruloplasmin increase and transferrin decrease concentrations are also reported as a sign of inflammation in a number of conditions spanning from cardiac to psychiatric. In fact, these proteins are acute phase reactants (positive and negative, resp.), produced by the liver together with several other proteins.

Another protein affecting iron homeostasis is HFE, a membrane protein which controls iron absorption by regulating the affinity of transferrin receptors on cell membrane [39]. Specific mutations of the HFE gene cause hemochromatosis, that is, an increased absorption of dietary iron and its consequent over deposition in tissues and organs [40]. Recently, a number of studies have shown various relationships between AD and mutations of the HFE, as well as the transferrin, genes but reported data are controversial and far from univocal interpretation [41–46]. In a very recent study of ours, we found that the synergy of altered markers of iron status (iron levels, transferrin, transferrin saturation, ferritin, and Cp/Tf ratio) and mutations of the HFE gene can increase the probability of developing AD (submitted).

Overall, Cp-Tf antioxidant system represents the serum capacity to sequester exchangeable metals (copper, iron) [36]

which make a labile metal pool prone to partake in reactions generating oxidative damage [47, 48]. Previous studies of altered levels of this labile metal pool in AD, especially referred to a serum increase of "free" copper, that is, the quantity of copper which is not bound to ceruloplasmin, sustain this concept [49, 50]. Support to this notion comes also from a study of our laboratory showing lower cortical responses in depressed patients who presented lower transferrin and higher free copper serum levels [51].

As a whole the data presented are in agreement with the past key works by both Loeffer's [16] and Castellani's [17] groups, who reported higher ceruloplasmin levels in brain areas critical for AD. However, the relationship between systemic processes and local effects in the brain is the main focus of our laboratory's work in progress.

Conflict of Interests

All authors have no conflict of interests.

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