

HO-1/BVR-A System Analysis in Plasma from Probable Alzheimer's Disease and Mild Cognitive Impairment Subjects: A Potential Biochemical Marker for the Prediction of the Disease

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Abstract. Several studies showed increased oxidative and nitrosative stress in plasma from patients with Alzheimer's disease (AD), however, little and controversial knowledge has emerged about the antioxidant functionality of the heme oxygenase-1/biliverdin reductase-A (HO-1/BVR-A) system in blood. The current study reports increased levels of both HO-1 and BVR-A in plasma from probable AD patients, as a result of the increased oxidative environment. However, the increase of oxidative stress in plasma result also in the increase of BVR-A 3-nitrotyrosine levels and the decrease of BVR-A phosphotyrosine levels and reductase activity, suggesting that nitrosative stress play the prominent oxidative role in plasma during AD. Our data on HO-1/BVR-A status in plasma closely correlate with recent reports in hippocampus of subjects with AD and arguably its early form, mild cognitive impairment. Moreover, we show that alterations on HO-1/BVR-A system are tightly connected with cognitive decline indexed by Mini-Mental Status Exam scores. We hypothesize that the HO-1/BVR-A system status in plasma might reflect the ongoing situation in the brain, offering an important biochemical tool for the potential prediction of AD at the earliest stages of the disease.

Keywords: Alzheimer disease's, bilirubin, biliverdin reductase-A, heme oxygenase-1, oxidative/nitrosative stress

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INTRODUCTION

Alzheimer's disease (AD) is one of the most disabling dementing disorders in the elderly affecting about 20 million people worldwide, a number that is expected to increase since the average human life span is expected to rise over the next few years [1]. Pathological brain changes may occur as early as 20–30 years prior the onset of the clinical symptoms, and the symptomatic phase of AD can last from about 5 up to 12 years [2–4]. AD is sometimes preceded by a condition of mild cognitive impairment (MCI) that can be considered the intermediate phase between normal aging and the early form of AD [5]. Subjects with amnesic MCI show objective memory problems without any functional impairment in daily living activities. Individuals with MCI have an increased risk of developing AD [6]. Diagnostic criteria currently used for MCI and AD are based on a battery of neuropsychological, cognitive, and functional tests supporting a probabilistic diagnosis; however, the diagnosis of AD can be confirmed to be definite only by neuropathological examination at either biopsy or autopsy. One of the main difficulties that do not allow an early diagnosis of MCI and AD is the lack of one or more reliable biomarkers of the disease.

An increase of oxidative and nitrosative stress occurs in AD and is considered to play a significant role in the pathogenesis of the disease [7–9]. In specific areas of AD brain such as hippocampus, neurodegenerative processes are associated with abnormal levels of oxidative/nitrosative stress markers probably due to an imbalance between stressful insults and cell stress response. Additionally, an increase in DNA, lipid, and protein oxidation products has been reported in plasma samples collected from AD patients, even at early stages of the disease [10–14].

The activation of heme oxygenase-1/biliverdin reductase-A (HO-1/BVR-A) system represents one of the earlier events in the adaptive response to stress. Heme oxygenase is the main enzyme involved in heme metabolism yielding free ferrous iron, carbon monoxide, and biliverdin-IX alpha (BV). BV is then converted to the potent antioxidant bilirubin-IX alpha (BR) by biliverdin reductase (BVR-A). Heme oxygenase exists in two main isoforms, the inducible (HO-1) and the constitutive (HO-2) forms. HO-1 is induced by various stimuli, including oxidative and nitrosative stress, while HO-2 is involved in the physiological turnover of heme and is responsive to developmental factors and adrenal glucocorticoids. Similarly, two isoforms of BVR-A were described and named BVR-A and

BVR-B. Only BVR-A reduces BV into BR, whereas BVR-A-B prefers the other BV isoforms, such as BV- β , BV- γ , and BV- δ [15–17]. BVR-A was known for a long time as an enzyme that only converted BV to BR; however, recent studies revealed new important features related to its serine/threonine/tyrosine kinase activity, which is involved in various cellular functions [15, 16]. Bilirubin, the final product of the HO/BVR-A system is a lipophilic linear tetrapyrrole, abundant in blood plasma, which occurs uniquely in mammals. Numerous evidence supports that BR possesses strong antioxidant potential against free radicals due to both the direct scavenging activity and the activation of intracellular pro-survival pathways [18, 19].

Several authors reported that the HO-1/BVR-A axis is induced in brain during AD-related increase of oxidative stress [20–22]. Recent studies performed in the Butterfield laboratory showed that, despite the increased levels of both HO-1 and BVR-A as adaptive response to stress, a decrease in the HO-1/BVR-A system activity occurs in AD hippocampus and its early phase, MCI, due to protein oxidative and nitrosative damage [23–26]. While the situation is being clarified in the brain, little is known about the HO-1/BVR-A system in blood. Several authors report changes in HO-1/BVR-A expression quantity without, however, drawing a clear picture of functionality alterations in plasma of AD subjects [20, 27, 28]. Recently Mueller et al. by using protein microarray assay, identified a battery of proteins whose expression was altered in plasma of mild AD subjects. Among these, both HO-1, BVR-A, and BVR-B were spotted, thus suggesting members of the heme-degradation pathway could be considered putative biomarkers for AD [29]. Blood-related proteins often have been considered a potential powerful diagnostic tool for their high accessibility and easy method of collection [30]. Using plasma in combination with other biomarkers of AD such as cerebrospinal fluid (CSF) analysis and neuroimaging potentially could allow an accurate prediction of the disease even at early stages of progression [31–33]. However, analysis of AD-related proteome alterations in blood is still challenging due to the large dynamic concentration ranges of proteins and the presence of high abundant proteins that can overcome the disease-specific biomarkers, which may account for only 1% of proteins in the whole blood proteome [34].

We hypothesize that, even if the brain proteome profile is relatively different from the blood protein profile, the HO-1/BVR-A system status in plasma could mime

the ongoing situation in the brain. A thorough analysis of the HO-1/BVR-A quantity and post-translational modifications (PTMs) in blood-related biofluids might offer an important biochemical tool for the prediction of AD, even in the early stages of the disease, and might represent a potential window of pharmacological intervention.

MATERIALS AND METHODS

Subjects

For this study, 36 subjects (12 with probable AD (pAD), 12 with MCI, and 12 cognitively normal persons) were enrolled. Complete clinical and neuropsychological evaluation was performed at the Department of Gerontology and Geriatrics, University of Perugia. Diagnosis of pAD was according to the National Institute of Neurological and Communicative Disease and Stroke and Alzheimer's disease (NINCDS-ADRDA) criteria [35]. MCI diagnosis was made following components of Petersen criteria [36]. Cognitively normal subjects (CTR) were enrolled among relatives of patients and subjects admitted to the Day Hospital of the same department for routine evaluation of the health status. All subjects underwent a thorough clinical, neurological, and neuropsychological evaluation; the neuropsychological assessment included the Mini Mental State Examination (MMSE), while the Clinical Dementia Rating scale measured the severity of dementia. The functional ability of subjects enrolled was evaluated on the basis of the activities of daily living and the instrumental activities of daily living (IADL). Subjects anxiety or depression (evaluated by the Geriatric Depression Scale), Hachinski ischemic score, cholesterol, erythrocytes sedimentation, and C-reactive protein was taken in consideration. The investigation conforms to the principles outlined in

the Declaration of Helsinki. All the subjects gave the written informed consent for blood donation. Blood was immediately centrifuged and plasma stored at -80°C until analyses. Subjects' demographic and clinical data are reported in Table 1 and Supplementary Table 1.

Samples preparation

Plasma (100 μl) samples from each subject were depleted of the major plasma proteins (albumin and Immunoglobulins G) using PROT-BA depletion kit according to manufacturer instructions (Sigma-Aldrich, St. Louis, MO, USA). Protein concentration after albumin and Immunoglobulins G depletion was determined by using the Comassie protein assay (Pierce, Rockford, IL, USA).

Western blot

HO-1, BVR-A, and their PTMs were evaluated by western blot analysis. In brief, an equal amount of proteins (40 μg) for each sample was separated by a 12.5% SDS-PAGE and electroblotted (1 h at 100 V) to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) using 25 mM tris, 192 mM glycine, and 20% (v/v) methanol. Equal protein loading was confirmed by 0.2% v/v Ponceau S in 7% acetic acid blot staining. Blotted membranes were blocked with 5% no-fat dried milk and challenged with appropriate primary antibodies, namely anti HO-1 mouse monoclonal antibody (1 : 500) (Stressgen, Ann Arbor, MI, USA), BVR-A mouse monoclonal (1 : 500) (Sigma-Aldrich, St. Louis, MO, USA), anti DNP (Millipore, Billerica, MA, USA), anti 4-hydroxynonenal (HNE) (Alpha Diagnostic International, San Antonio, TX, USA), anti 3-nitro-tyrosine (3NT), anti pTyr, and anti pSer/Thr (Millipore, Billerica, MA, USA), for 1 h at

Table 1
pAD, MCI and CTR subjects demographic and clinical data

	Controls (Avg)	MCI (Avg)	pAD (Avg)
Number of samples	12	12	12
Gender	6 F, 6 M	4 F, 8 M	9 F, 3 M
Age	79.8 \pm 7.4	79.2 \pm 5.4	82.2 \pm 4.0
Mini Mental Status Exam	28.2 \pm 2.0/30	24.3 \pm 2.9/30	18.2 \pm 5.6/30 (n = 11)
Activities of daily living	5.5 \pm 0.5/6	4.8 \pm 1.4/6	4.9 \pm 0.9/6 (n = 11)
Instrumental activities of daily living	6.5 \pm 1.5/8	4.7 \pm 2.3/8	2.9 \pm 1.9/8 (n = 11)
Geriatric Depression Scale	5.9 \pm 4.5/15	4.9 \pm 4.9/15	4.7 \pm 2.4/15 (n = 10)
Clinical Dementia Rating	0	0.5	1 (n = 11)
Hachinski Ischemic Score	1.6 \pm 0.7	1.9 \pm 1.2 (n = 11)	2.1 \pm 1.4 (n = 10)
Cholesterol levels	191.4 \pm 58.8 (n = 10)	194.3 \pm 53.4	218.5 \pm 37.9
Erythrocytes Sedimentation (0–30 normal values)	19.2 \pm 10.5 (n = 9)	23.3 \pm 9.7 (n = 9)	26.7 \pm 13.7 (n = 11)
C-Reactive Protein (0–0.5 normal values)	0.2 \pm 0.1 (n = 9)	0.8 \pm 1.0 (n = 9)	0.5 \pm 0.4 (n = 11)

Table 2
Results of HO-1/BVR-A expression levels and PTM levels alterations in hippocampus and plasma samples of MCI and pAD subjects compared to age-matched CTR

Groups of comparison	HO-1 quantity	HO-1 oxidation	HO-1 phosphorylation	BVR-A quantity	BVR-A oxidation	BVR-A phosphorylation	BVR-A activity
<i>Hippocampus</i>							
MCI versus CTR	≈ [25]	↑ HNE [26]	= [26]	↑ [24]	↓ PC [23] ↑ 3NT [23]	↓ pSer/Thr [24] ↓ pTyr [24]	↓ [24]
AD versus CTR	↑ [26]	↑ PC [26] ↑ HNE [26]	↑ pSer [26]	↑ [24]	↓ PC [23] ↑ 3NT [23]	↓ pSer/Thr [24] ↓ pTyr [24]	↓ [24]
<i>Plasma</i>							
MCI versus CTR	↑	=	=	≈	↑ 3NT	↓ pTyr	=
pAD versus CTR	↑	=	=	↑	↑ 3NT	↓ pTyr	↓

[] reference number; ≈ no significant changes; = no changes; ↑ increased quantity; ↓ decreased quantity.

room temperature. Unbound antibodies were removed by washing twice with tris-buffered saline containing 0.1% Tween 20, for 5 min. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody diluted 1 : 5000 (Sigma-Aldrich, St. Louis, MO, USA). Protein bands were visualized with ECL Plus™ (Amersham Biosciences, Pittsburgh, PA, USA) according to the manufacturer's protocol. Blots were scanned by GS880 densitometer (Bio-Rad, Hercules, CA, USA) and quantified by Quantity One image software (Bio-Rad, Hercules, CA, USA).

Carbonylation

Samples were post-derivatized with dinitrophenylhydrazine (DNPH) on the membrane and probed with anti-DNP antibody to identify the carbonylated proteins. The nitrocellulose membranes were equilibrated in solution A (20% (v/v) methanol and 80% (v/v) wash blot buffer (phosphate buffered saline solution containing 0.04% (v/v) tween 20 and 0.10 M NaCl) for 5 min, followed by incubation of membranes in 2N HCl for 5 min. The proteins on blots were then derivatized in solution B (0.5 mM DNPH in 2N HCl) for 10 min. The membranes were successively washed 5 min per time in 2N HCl for three times, wash blot buffer/methanol (50/50) for five times, and finally wash blot buffer for two times. The DNP adducts were detected immunochemically as described above.

Immunoprecipitation

The immunoprecipitation was performed as described previously [23]. The antibodies were added directly to cell lysates, previously depleted, with IP Buffer (NaCl 0.15 M, NP-40 0.5%, tris-HCl 50 mM

pH 7.2, protease inhibitors), and the mixture was incubated on a rotary mixer overnight at 4°C. The antigen/antibody complexes were precipitated by adding protein-A-conjugated agarose beads, mixed on a rotary mixer for 1 h at room temperature. Beads were then centrifuged and washed with the washing buffer (pH 8, 50 mM tris HCl, 150 mM NaCl, 0.1% tween 20) three times. Proteins were solubilized in sample buffer for post-western blot analysis.

BVR-A activity assay

To test BVR-A activity in plasma samples from AD and MCI patients, the biliverdin reductase assay kit (Sigma-Aldrich, St. Louis, MO, USA) was used, following the standard protocol with minor changes. Briefly, 150 µg of previously depleted samples were prepared for the assay and loaded in the 96 well plate. Furthermore, BVR-A positive control solution (2.5, 5, 10, 15, 20 µl) was included in the assay for generation of a standard curve. Then, 50 µl of assay buffer and 150 µl of working solution (containing NADPH, substrate solution, and assay buffer) were added to standard and sample on the plate. This latter was placed on the UV-VIS plate reader Multiskan EX (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C and read every minute for 10 min. The reading after 5 min that presented a linear reaction rate was chosen for BVR-A activity calculation.

HPLC

For bilirubin extraction from plasma samples, 600 µl of chloroform and 200 µl of ethanol were added to 200 µl of plasma. The mixes were vortexed for 1 min and centrifuged at 3500 g for 10 min. The lower phase containing bilirubin was collected and dried with

nitrogen. The precipitate was re-suspended in 100 μ l of DMSO used for the investigation. A Waters 600 and a Waters 996 Photodiode (Waters Corporation, Milford, MA, USA) were used for HPLC analysis. The column used was a Hyper BDS column C18, 250 mm lengths and 4.6 mm diameter 5 μ m. Solution A was prepared with acetonitrile/water 80/20, while solution B was prepared with ammonium acetate 0.1 mM/acetonitrile 80/20. The column was equilibrated with A/B 95/5 for 5 min, followed by linear gradient of B increase from 5 to 80 in 20 min, and a final equilibration step with A/B 95/5. The flow rate was 1 ml/min. Bilirubin detection was conducted at 450 nm. A calibration curve, using bilirubin IX alpha standard, was built before the plasma analysis.

Statistical analysis

Data are expressed as mean \pm SD of n independent samples. All statistical analyses were performed using a non-parametric one-way ANOVA with post hoc t -test. $p < 0.05$ was considered significantly different from control. Pearson correlations were calculated to test the linear association between cognitive test scores and markers of oxidative damage.

RESULTS

HO-1 protein quantitation and PTMs in plasma from pAD and MCI patients

The first parameter taken in consideration in the analysis of HO-1/BVR-A system in plasma of MCI and

pAD patients was the measurement of HO-1 protein quantity. Previous studies from our laboratory showed an increase of HO-1 in hippocampus of MCI and pAD subjects in response to environmental increased oxidative/nitrosative stress [23–25]. Although several authors described increased oxidative stress at the systemic level (both in plasma/serum and CSF) in AD subjects [37–41], data about the systemic HO-1 expression are still controversial [20, 27–29].

In the current study, plasma samples from three groups of patients, namely pAD, MCI, and age-matched CTR (12 samples each) were assessed for the analysis of HO-1/BVR-A quantity. Each sample was previously depleted for the most abundant proteins, IgG and albumin. As shown in Fig. 1A, HO-1 quantity significantly increased in both MCI and pAD compared to CTR samples. Interestingly, the trend of increase paralleled the severity of the pathology with MCI having 70% and AD 130% increase compared to CTR (Fig. 1A). After the expression evaluation, each sample was tested for protein specific PTMs. Previous studies from the Butterfield laboratory revealed that increased quantity of HO-1-bound HNE and HO-1 protein carbonyls was present in hippocampi from AD and MCI subjects, respectively [26]. Furthermore, in the same samples, increased quantity of pSer/Thr-HO-1 was detected in AD, suggesting impaired functionality of the protein in pathological conditions. In the current study we found no significant alterations in either HO-1 oxidation (Supplementary Figure 1) or HO-1 Ser/Thr phosphorylation in pAD/MCI plasma. Our results indicated that, in contrast with the hippocampus, plasma HO-1 might preserve its activity under severe pathologic conditions.

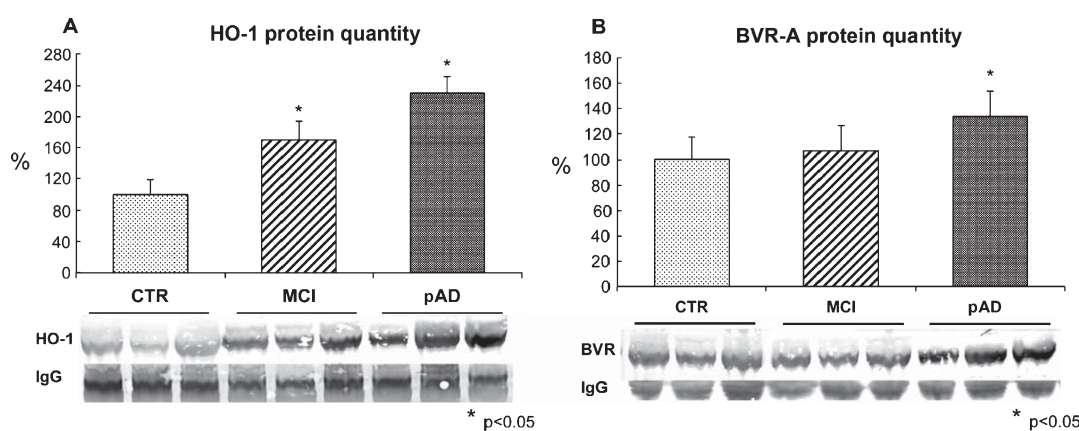


Fig. 1. HO-1 (A) and BVR-A (B) quantity in CTR, MCI, and pAD plasma samples. Densitometric values shown in the histograms are given as percentage of CTR, set as 100%. Data are expressed as mean \pm SD of 12 individual samples per group. * $p < 0.05$.

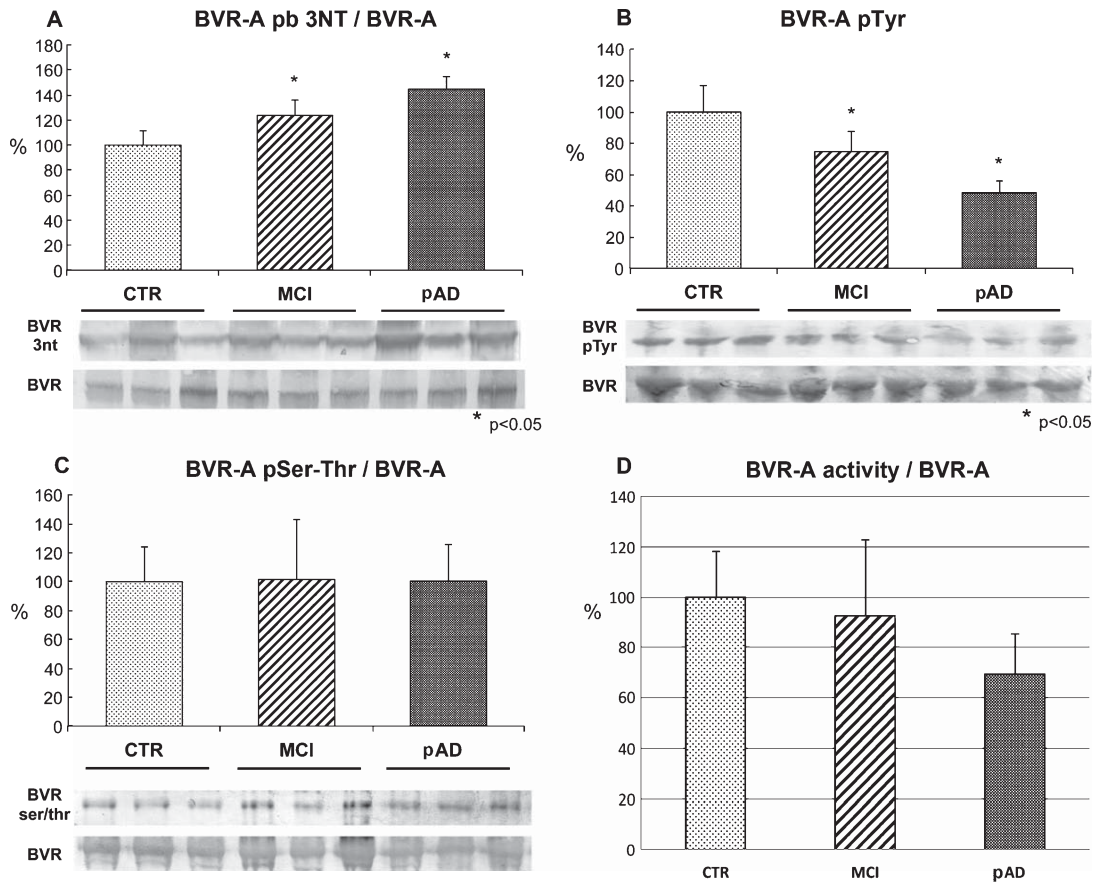


Fig. 2. BVR-A protein bound-3NT (A), phosphotyrosine (B), phosphoserine/threonine (C), and reductase activity (D) quantity in CTR, MCI, and pAD plasma samples. All the data from PTMs quantitation are normalized for BVR-A protein quantity. Densitometric values presented in the histograms are given as percentage of Control, set as 100%. Data are expressed as mean \pm SD of 12 individual samples per group. * $p < 0.05$.

BVR-A protein quantitation, PTMs, and activity in plasma from pAD and MCI patients

Similarly to HO-1, BVR-A protein quantity increased in both pAD and MCI samples, but the induction of expression was slightly lower. Indeed, we observed an increase of 10% in MCI (not significant) and of 36% in pAD compared to CTR (Fig. 1B). Previous studies from the Butterfield laboratory showed increased BVR-A levels in the hippocampus of AD and MCI subjects, however, it was also shown that BVR-A underwent nitrosative stress modifications during AD and MCI exhibiting altered quantity of protein carbonyls and protein nitration [24]. As a consequence of the increased oxidative and nitrosative environment in AD that resulted in increased BVR-A oxidation and nitration, were also found a decrease in BVR-A phosphorylation on both Tyr and Ser/Thr residues [24]. Current data in plasma samples are consistent with these findings, since we observed

increased amounts of protein bound 3-NT, of 24% and 44% respectively, in BVR-A in plasma from both MCI and pAD patients compared to CTR (Fig. 2A), and lower levels of pTyr-BVR-A in MCI and pAD plasma (78% and 43%, respectively) compared to CTR (Fig. 2B). No alteration was found for pSer/Thr (Fig. 2C). BVR-A reductase activity is a direct consequence of protein PTMs (e.g., oxidation and phosphorylation). Previous studies confirmed that increased oxidation and decreased phosphorylation of BVR-A resulted in decreased activity in both MCI and AD compared to CTR hippocampus [24]. As shown in Fig. 2D, a significant reduction in BVR-A plasma activity, normalized on protein quantity, was only found in pAD subjects compared to CTR (69%), whereas in MCI the BVR-A activity (92%) is similar to CTR. These data suggest that only in pAD plasma does the amount of BVR-A altered oxidation/phosphorylation affect protein enzymatic activity.

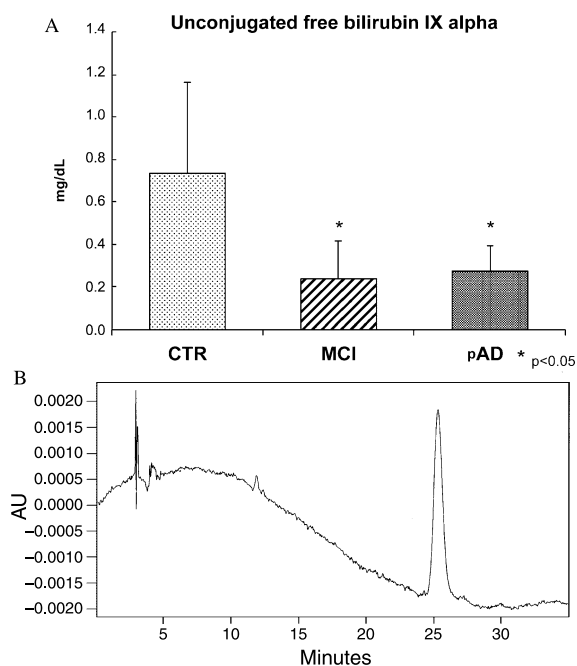


Fig. 3. A) Unconjugated bilirubin IX alpha levels in CTR, MCI, and pAD plasma samples. Data are expressed as mean \pm SD of 12 individual samples per group. * $p < 0.05$. B) Representative image of HPLC time course with bilirubin absorbance peak.

Bilirubin levels in AD and MCI plasma

Plasma BR is an important player in response to oxidative stress. Bilirubin is the end product of HO-1/BVR-A pathway in several organs (such as brain or liver) and in peripheral cells. In this study, we report decreased amount of unconjugated bilirubin plasma from 0.73 mg/dL in CTR samples to 0.23 mg/dL in MCI and 0.27 mg/dL in pAD (Fig. 3A, B). These findings suggest that BR reduction is an early event in AD pathogenesis and might have a role in transition from MCI to AD.

Correlation data

In order to determine possible relationships among protein quantity, protein oxidation and protein phosphorylation of HO-1 and BVR-A in plasma samples of each group, we performed correlation analysis. A positive correlation ($p < 0.01$, $r = 0.53$) was shown for HO-1 and BVR-A protein quantity of pAD plasma compared to CTR plasma (Fig. 4A). HO-1, presumably due to the lack of PTM, did not show any kind of correlation. Conversely, we found positive correlation data in pAD compared to CTR for BVR-A protein quantity with BVR-A nitration ($p < 0.01$, $r = 0.63$; Fig. 4B),

and negative correlations for BVR-A protein quantity with BVR-A pTyr ($p < 0.01$, $r = -0.53$; Fig. 4C) and BVR-A nitration with BVR-A pTyr ($p < 0.01$, $r = -0.73$; Fig. 4D). Moreover we found a negative correlation ($p < 0.05$, $r = -0.48$) for BVR-A nitration with BVR-A pTyr in MCI compared to CTR plasma samples (Fig. 4E).

A second group of correlation analyses involved the alterations in expression and in PTM of HO-1/BVR-A and the parameters of cognitive decline in order to verify if our findings follow the progression of the disease. We show a negative correlation for MMSE with HO-1 quantity ($p < 0.001$, $r = -0.63$; Fig. 5A), BVR-A quantity ($p < 0.001$, $r = -0.54$; Fig. 4B), and BVR-A nitration ($p < 0.001$, $r = -0.64$; Fig. 5D), and a positive correlation for MMSE with BVR-A phosphorylation on tyrosine residue ($p < 0.001$, $r = 0.68$; Fig. 5C) in the comparison of CTR, MCI, and pAD. Similar results have been obtained for HO-1 protein quantity, BVR-A protein quantity, and PTM with IADL index (Supplementary Figure 2) always comparing CTR, MCI, and pAD data.

DISCUSSION

The biochemical changes that precede AD may be present up to 20 years before the clinical manifestation of the disease [2–4]. Consistent with this notion, brains from a number of cognitively intact individuals at autopsy reveal an extensive senile plaques and neurofibrillary tangle load, indicating that the pathologic alterations occur before the symptomatic state [42]. Any effort focused on detecting pathology of AD, particularly in individuals that might have a greater risk of developing AD over the course of their life, will allow clinicians and scientist to improve the understanding of the natural history of AD. In addition, new diagnostic strategies might have an important role in determining specific molecular targets for the early diagnosis of AD. The novelty of our study is to propose the HO-1/BVR-A system as a promising biomarker for the evaluation of the progression of AD. In the last few years, by using genomic, proteomic, and metabolomic approaches, several promising molecular biomarkers have been identified [30].

Here, we provided new lines of evidence in support of the HO-1/BVR-A system as a potential peripheral biomarker for MCI and AD. In addition, the pathogenic role of altered protein oxidation/nitration/phosphorylation on both HO-1 and BVR-A were also highlighted. The important role of plasma HO-1 and BVR-A as predictors of MCI and AD

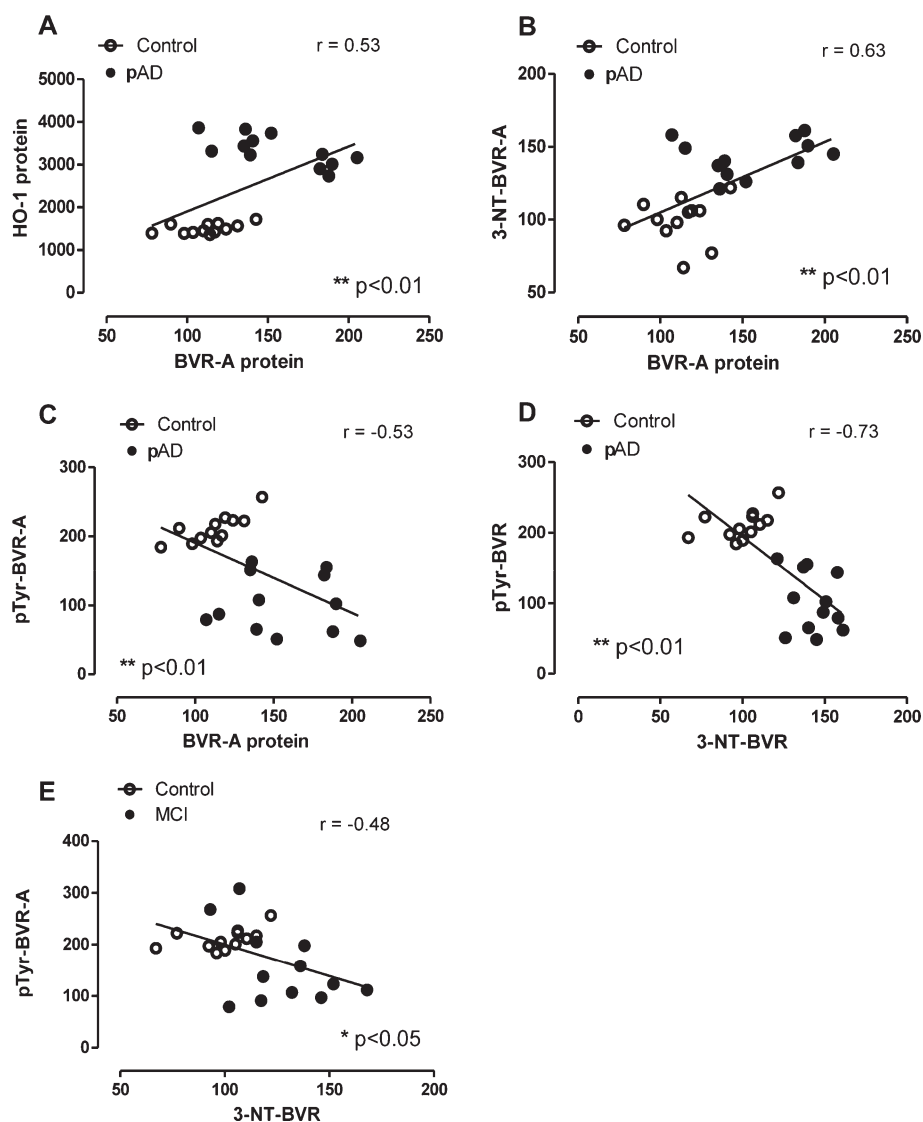


Fig. 4. Correlation graphs I. A positive correlation ($p < 0.01$, $r = 0.53$) is shown for HO-1 and BVR-A protein quantity of pAD patients compared to CTR plasma (A) and for BVR-A protein quantity with BVR-A nitration in pAD compared to CTR ($p < 0.01$, $r = 0.63$) (B) Negative correlations were found for BVR-A protein quantity with BVR-A pTyr ($p < 0.01$, $r = -0.53$) (C) and BVR-A nitration with BVR-A pTyr ($p < 0.01$, $r = -0.73$) (D) in pAD versus CTR samples. A negative correlation ($p < 0.05$, $r = -0.48$) was found for BVR-A nitration with BVR-A pTyr in MCI compared to CTR plasma samples (E).

is corroborated by the parallelism between the changes occurring in plasma and those in the brain [23, 24, 26] and the correlation between plasma alteration and cognitive decline.

The main role of oxidative and nitrosative stress in the pathogenesis of AD has been largely investigated in the brain of subjects affected by the disease during their lives [43]. However, brain studies provide only a final static observation of the mechanism impaired by increased pro-oxidant conditions, hence brain biopsies are useless to obtain the starting point

or the rate of their involvement. Over the last decade, a great interest is growing on biological fluids analysis, like CSF, plasma, and serum, in order to find a reliable biomarker to monitor the ongoing pathologic progression. Several authors have reported on plasma levels of protein oxidation and lipid peroxidation markers in AD patients compared to controls. Lipid peroxidation levels, indexed as increased levels of isoprostanes, in AD plasma samples have been the topic of intense discussion in the last decade since several groups showed no differences during AD

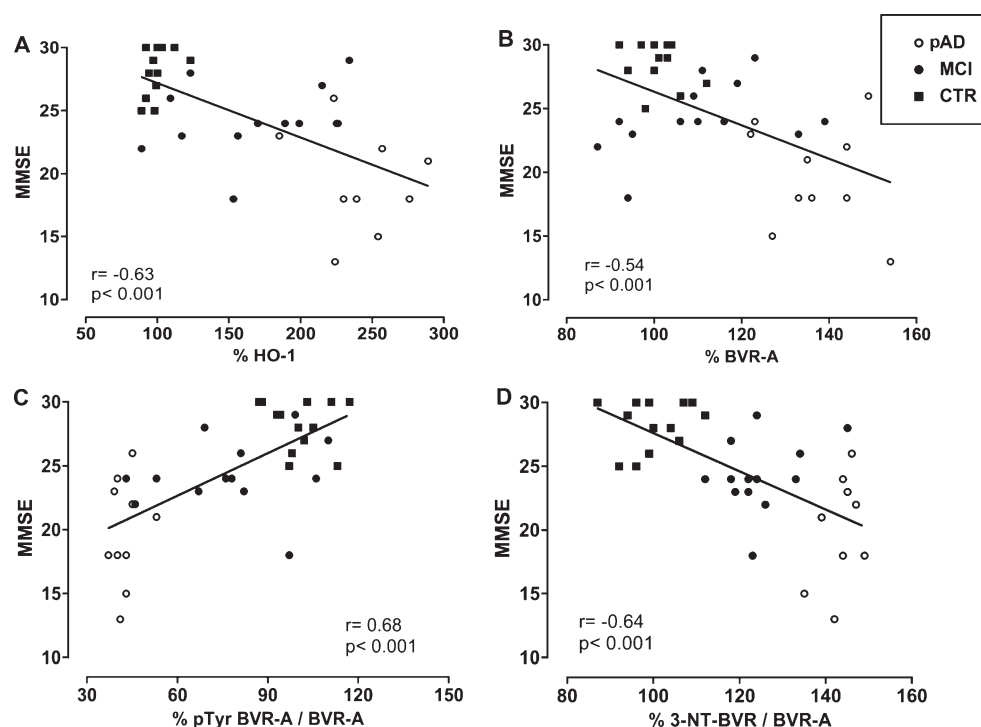


Fig. 5. Correlation graphs II. A negative correlation is shown for MMSE and HO-1 quantity ($p < 0.001$, $r = -0.63$) (A), BVR-A quantity ($p < 0.001$, $r = -0.54$) (B), and BVR-A nitration ($p < 0.001$, $r = -0.64$) (D), in the comparison of CTR, MCI, and pAD; a positive correlation is shown for MMSE with BVR-A phosphorylation on tyrosine residue ($p < 0.001$, $r = 0.68$) (C).

progression [41, 44–47], while, in contrast other studies showed increased F2-isoprostanes in plasma of AD and MCI [48, 49].

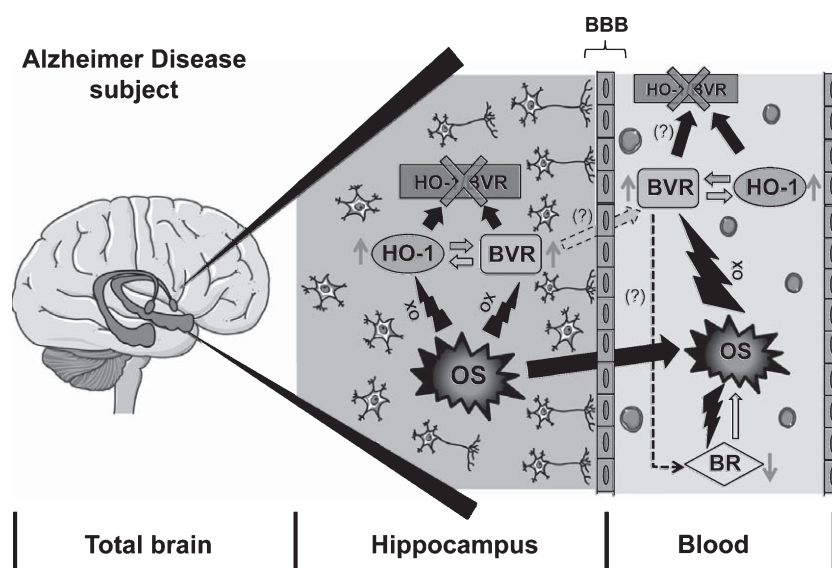
Conrad et al. and others showed increased total and protein specific carbonylation and increased total levels of protein bound 3-nitrotyrosine [50–52]. In addition, the Butterfield group reported on a significant decrease of plasma glutathione in AD patients compared with the control group, which was associated with a significant increase in oxidative stress markers (i.e., reduced glutathione, 4-hydroxy-2-nonenal, protein carbonyl content, and 3-nitrotyrosine) [20]. Recently Zaffrilla et al. and Korolainen et al. raised doubts about the increase of total carbonyls in plasma of AD patients showing no changes in their experimental setting [37, 53]. In support of the hypothesis that oxidative injuries may have an important role in the pathogenesis of AD, plasma antioxidant capacity is impaired in AD subjects as demonstrated by the significant depletion of some of the main players of the antioxidant response [20, 54]. In this study, we show a significant reduction of plasma unconjugated bilirubin in both pAD and MCI subjects compared to age-matched CTR. Unconjugated bilirubin is the main form

of the bile pigment endowed with strong antioxidant activity, and in the plasma it is complexed with saturating concentrations of human serum albumin (HSA), which serves as a carrier in the bloodstream. Both BR and BR-HSA were demonstrated to work as powerful scavenger against reactive oxidative and nitrogen species [55–59]. The frequently quoted reference interval of total plasma bilirubin concentrations in healthy adults is 0.342–1.710 $\mu\text{mol/dl}$, even if the BR levels are higher in men than in women and decreases with age [56, 57, 60–63]. Our data account for BR levels in the control subjects which are in the same order of magnitude as those of reference, whereas those in pAD and MCI are about 5 times lower than those observed in the matched controls, thus supporting the idea that the amount of BR is not enough to counteract the increased oxidative/nitrosative damage observed in the plasma of pAD and MCI subjects. Furthermore, our data are in agreement with the study of Kim et al. on AD subjects and provide a new level of comprehension suggesting that depletion of antioxidant capacity is an early event in the progression of AD being involved in the increased oxidative damage at peripheral and at brain level [54].

An intriguing aspect of our previous research was the finding that the HO-1/BVR-A axis undergoes important PTMs in AD and MCI hippocampi [23–26]. This evidence obtained by studying an important brain area, such as the hippocampus, whose involvement in AD occurs at the earlier stages, prompted us to investigate whether or not the same PTMs were detected in both plasma HO-1 and BVR-A, and how these modification might alter proteins expression or activity. The results of this paper show that plasma levels of HO-1 increase with patient cognitive decline, in agreement with previous data reporting increased amount of HO-1 in AD lymphocytes compared with control [20]. However, the reports of HO-1 plasma levels in pAD subjects are controversial, since studies from Schipper et al. and Ishizuka et al. showed plasma HO-1 protein and mononuclear cell HO-1 mRNA levels significantly suppressed in AD subjects compared to CTR [27, 64]. In later studies, Schipper et al. proposed the existence of an HO-1 suppressor in AD plasma, alpha-1 anti-chymotrypsin (AAT), which inhibits HO-1 mRNA levels in the lymphocyte [65, 66]. The same inhibition does not occur in the central nervous system (CNS) of AD patients for the high AAT exposure to disease-related protein oxidation and nitration. Nonetheless, plasma, as well CNS, present increased amount of protein oxidation and nitration, and a previous study by Choi et al. reported that AAT and other inflammation proteins were increasingly oxidized in AD [50]. In addition, a recent research by Mateo et al. described

unaltered HO-1 serum quantity between CTR and AD subject [28]. A different situation from the brain was noticed in the analysis of plasma HO-1 PTMs. In fact, while we showed increased levels of protein bound-HNE in HO-1 from MCI and AD hippocampus and increased levels of protein carbonyls and pSer in AD hippocampus compared to CTR, none of these modifications were observed in plasma. We previously stated that HO-1 is less prone to nitration and pTyr alteration for the low number of tyrosine residue in its structure, elucidating the lack of increased 3NT and pTyr in AD and MCI plasma notwithstanding the NS stress [67].

A unique finding of this study was the similarity between BVR-A expression, PTMs, and activity in plasma samples and in brain of pAD/MCI subjects (Table 2). Indeed, BVR-A plasma data are closely related to BVR-A hippocampus data regarding increased protein quantity, increased protein nitration, decreased tyrosine phosphorylation, and decreased protein reductase activity. Previous studies by Maines et al. in 1999 reported the presence of BVR-A in infiltrating monocytes, macrophages, T cells, and neutrophils as well as in circulating lymphocytes, which might be the main source of plasma BVR-A [68]. However, another possible source of plasma BVR-A might be the CNS, where, for example, BVR-A is greatly induced by AD-related increase of oxidative stress. Within this frame, we hypothesize that during the progression from MCI to AD part of BVR-A is lost



Scheme 1. Putative scenario of the mechanisms leading to HO-1/BVR-A oxidative alterations in plasma of probable AD subjects. BBB, blood brain barrier; OS, oxidative stress.

from the brain, passing through the blood brain barrier and released into the peripheral system (Scheme 1). With regard to BVR-A protein quantity, we previously stated that the investigation of only BVR-A expression might be ambiguous with regards to BVR-A functionality. Indeed, the analysis of BVR-A PTMs and reductase activity provide deeper and more detailed information about its real status during AD pathology [23, 24]. Consistent with the hippocampus results, our current findings demonstrate increased amount of protein bound 3-NT in pAD and MCI plasma, while no alterations of protein carbonylation and protein bound-HNE were detected. These results confirm the prevalence of nitrosative stress-induced modifications than protein carbonylation or protein bound-HNE on BVR-A structure during AD, as already observed in hippocampus [23].

By the analysis of protein phosphorylation, we showed decreased tyrosine residue phosphorylation in MCI and pAD plasma but no alterations regarding serine/threonine residues. Phosphorylation of critical ser/thr/tyr residues plays a main role in the regulation of the protective and/or metabolic activities of BVR-A and, as previously seen in hippocampus [24], one of the outcome of BVR-A altered phosphorylation and nitrosative modifications is represented by its impaired reductase activity, which in this study has a 30% drop in pAD.

Interestingly, we show that in pAD plasma, where the pro-oxidant conditions are steadily higher than CTR, BVR-A quantity and activity follow the indirect relationship seen in hippocampus. Conversely, in MCI, the lack of significant increase of BVR-A levels is coupled with unchanged reductase activity levels. The differences between pAD and MCI might be related to the severity of the disease that results in different grades of protein induction, oxidative modification, phosphorylation, and finally protein activity. Correlation data among nitration, pTyr alterations, and BVR-A levels in pAD strengthen our conclusions. Moreover, correlations of HO-1 and BVR-A quantity and BVR-A PTM, with cognitive performance parameters such as MMSE and IADL in CTR, AD, and MCI confirm that the alteration of the HO-1/BVR-A system in plasma is dependent on disease stage, increasing with the severity of AD pathology.

BVR-A, being a ser/thr/tyr kinase involved in glucose metabolism through the phosphorylation of the insulin receptor substrate-1, could also contribute to the impaired glucose metabolism seen in AD-related disorders [15, 17]. Studies to demonstrate this notion are in progress.

In conclusion, in light of the results obtained in plasma and their tight correlation with hippocampus, we propose plasma BVR-A status, more than HO-1, as a reliable monitor of hippocampal BVR-A status and brain damage in pAD. Indeed, since plasma BVR-A alterations mostly copy the hippocampal ones, the analysis of plasma samples is supposedly a reasonable way to gain information on increased oxidative and nitrosative stress ongoing in the brain and conceivably predict AD onset and advancement. Plasma BVR-A status, as a potential biomarker, fulfills most of the criteria proposed for AD diagnosis [69]. In fact BVR-A seems to reflect fundamental aspects of CNS pathophysiology in AD as demonstrated by the correlation of plasma with hippocampus data. BVR-A changes are efficacious biomarker in early AD stages as proven by the occurrence of altered PTM in MCI. BVR-A monitors disease severity or rate of progression as exhibited by the different grade of alterations between MCI and pAD compared to CTR and the correlation of HO-1/BVR-A data with cognitive performance. Finally, the analysis of the HO-1/BVR-A system in plasma samples is noninvasive, relatively inexpensive, and readily available.

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