

Oxidative stress related markers in the “VITA” and the centenarian projects

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Abstract

Oxidative stress seems to play an important role in the pathophysiology of Alzheimer's disease (AD). At present there are no easily accessible biochemical markers for AD. We performed activity assays for platelet MAO-B and erythrocyte Cu/Zn-SOD as well as Western blotting for these two proteins. Moreover, we assessed plasma lactoferrin and performed RFLP-analysis for the MAO-B-intron-13-polymorphism in patients from the Vienna-Transdanube Aging (VITA) and from the so called centenarian project. The first one, VITA, is a community-based cohort study of all 75 years old inhabitants of a geographical region of Vienna. The centenarian project investigates chronic care in-old patients suffering from AD. In both sexes platelet MAO-B activity increased significantly in the AD group, and Cu/Zn-SOD activity decreased, but the latter effect was significant only in females. No significant difference was found regarding plasma lactoferrin. No correlation was found between MAO-Bi13 and MAO-B platelet activity or allele MAO-Bi13 and disease frequency. These results point to the possibility that a combination of MAO-B and SOD activity levels might be useful tools for an early diagnosis of AD.

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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder accounting for about 60% of dementia cases in the elderly [14]. Aging is the most important risk factor for AD. While the prevalence among those 60–64-years-old is about 1% it increases up to 40% in those aged 85 and older [70]. The increasing life expectancy in our population makes AD a growing public health problem. Extracellular senile plaques consisting mainly of amyloid β (A β) and neurofibrillary tangles (NFT), intracellular bundles of hyper-

phosphorylated tau-protein and ubiquitin, are the hallmarks of the histopathological diagnosis of AD since the first case report by Alois Alzheimer in the year 1907 [4].

However, the mechanisms of cell death in AD are still largely unknown. Multiple etiologies including environmental exposure and genetic vulnerability (e.g. Apo E genotype [58]) are suggested to account for the age-related decline in neuronal viability and function. Reactive oxygen species (ROS) seem to play an important role in the pathophysiological cascade leading to AD [15]. Oxygen radicals are chemically unstable and highly reactive compounds, which are formed during normal cellular metabolism and within pathophysiological cascades. Due to their reactivity, they may be responsible for cellular and tissue damage at any time their generation exceeds the endogenous ability to destroy them—a condition also known as oxidative stress

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(OS) [15]. The probability of the involvement of OS in AD and other neurodegenerative diseases is supported by the fact that neurons are extremely sensitive to attacks by free radicals, as a consequence of: (a) their low glutathion content, an important natural antioxidant [16], (b) the high proportion of polyunsaturated fatty acids in their cell membranes [34], (c) the fact that brain metabolism requires substantial quantities of oxygen and (d) because neurons are post-mitotic.

Though there are characteristic signs for the clinical diagnosis of AD [45], initial changes are often subtle and the diagnosis may be missed by primary care physicians [49]. A “threshold model” suggests that the clinical disease is manifested when the degenerative changes result in neuronal dysfunction that exceeds the compensatory capacity of the brain [9]. In accordance, it has been suggested that the biological onset of the disease may occur 20–40 years before physical and clinical manifestations are detected [12]. At present there are no easily accessible diagnostic markers, such as blood or urine tests, that could help in early diagnosis, screening, and evaluation of drugs in clinical testing. Several criteria are important for such AD biomarkers. The marker should relate to key pathologic events in AD brain, have adequate sensitivity and specificity, possess the ability to distinguish AD from non-AD dementia, and finally should have low cost and ease of performance [1].

In this study we investigated the effect of AD on three proteins which are related to OS localized in blood cells or plasma: platelet monoamine-oxidase-B (MAO-B), erythrocyte Cu/Zn-superoxide-dismutase (Cu/Zn-SOD) and plasma lactoferrin (LTF). The main goal of our study was to elucidate whether one or more of these markers could be used in the future as a diagnostic tool for AD.

2. Methods

2.1. Patients recruitment and evaluation

Subjects of the present study originate from two different studies. The first study investigates a community-based cohort of volunteers recruited via the VITA project, which has been organized, planned and financed by the Ludwig-Boltzmann-Institute of Aging-Research. All the

volunteers are non-institutionalized and institutionalized residents of the Vienna-Transdanube, a geographically defined, urban working class area of Vienna, consisting of two districts (21st and 22nd districts) on the left side of the river Danube. All the subjects asked to volunteer are exactly 75 years of age. The up to now 463 volunteers who consented to participate and appeared for examination have gone through general physical health check, questionnaire for education, psychosocial activities, memory complaints and psychological tests (e.g. Mini-Mental State Examination (MMSE), Fluid Object Memory Test (FULD), Clinical Dementia Rating (CDR) and Hamilton score) and a cranial magnetic resonance imaging with reconstruction of the hippocampal formation (all test and recruitment strategies are described in [24]).

The second study group contains patients with advanced AD according to the NINCDS-ADRDA criteria [45] staged in accordance with the Clinical Dementia Rating (CDR) [8,35]. These patients were recruited from the Chronic Care Geriatric Hospital Lainz, now called Geriatric Center “Wienerwald” in Vienna and are described as the centenarian study group. For detailed information the number of subjects, MMSE, FULD score and CDR score as well as smoking habits for both sexes can be viewed in Table 1. As it can be seen from Table 1, the VITA group is still considered as controls for dementia while the centenarian study is considered as progressive dementia indicated by mean MMSE. In the VITA group there will be re-testing of the subjects after 30 months in order to investigate incidental dementia, the results of which will be followed in the next stage study. The VITA and the centenarian study was carried out with the permission of the Ethics Committee of the city of Vienna, Austria.

2.2. Preparation of plasma, erythrocytes and platelets

Blood samples (20 ml) were drawn into Vacutainer® (Becton Dickinson, Franklin Lakes, NJ, USA) tubes containing ethylenediaminetetraacetic acid (EDTA) at 08:00 a.m. after 12 h fasting. The blood was pipetted carefully onto 15 ml Ficoll in room-temperature and centrifuged at $400 \times g$ for 30 min at room-temperature. After centrifugation, the upper layer containing plasma and platelets was collected into a new tube, and was centrifuged at $1500 \times g$

Table 1
Study variables compared in both genders in VITA and centenarian group

	VITA-female	VITA-male	Centenarian-female	Centenarian-male
<i>n</i>	362	253	119	44
Mean age (years \pm S.D.)	75.0 \pm 0 ^a	75.0 \pm 0 ^a	79.6 \pm 8.6	79.8 \pm 7.8
MMSE (score \pm S.D.)	27.8 \pm 1.8 ^a	27.8 \pm 1.7 ^a	8.2 \pm 5.1	6.8 \pm 5.2
FULD (score \pm S.D.)	42.8 \pm 5.8	42.9 \pm 4.9	n.d.	n.d.
CDR (score \pm S.D.; <i>n</i>)	0.073 \pm 0.22; (361)	0.062 \pm 0.21; (252)	2.286 \pm 0.76; (7)	n.d.
CDR > 1 (<i>n</i> ; % of total)	42; 11.6%	26; 10.3%	7; 100%	n.d.

n.d.: not done. ANOVA and Fisher-PLSD.

^a VITA vs. centenarian study ($P < 0.0001$).

for 20 min. The supernatant containing the plasma was collected in cryo-tubes as 1 ml aliquots, and frozen at -70°C . The pellet containing platelets was resuspended in 1 ml PBS and centrifuged at $1500 \times g$ for 15 min. This washing procedure was repeated three times, followed by resuspension of platelets in 1 ml PBS and freezing at -70°C . The erythrocytes from the lower phase of the Ficoll gradient were aliquoted into cryo-tubes and frozen at -70°C till process.

2.3. Preparation of genomic DNA

DNA was prepared from 2 ml EDTA-blood by the standard procedure of proteinase K. 2 ml blood was mixed with 2 ml of lysis buffer (0.32 M sucrose 10 mM Tris-HCl, pH 7.5, 1% Triton x-100, 5 mM MgCl_2), vortexed and centrifuged at $13,000 \times g$ for 20 s. The pellet was then resuspended in 4 ml PBS and centrifuged at $13,000 \times g$ for another 20 s, followed by resuspension in 2 ml buffer with non-ionic detergents and proteinase K (50 mM KCl, 10 mM Tris-HCl pH 8.3, 0.1 mg/ml gelatine, 0.45% NP 40, 0.45% Tween 20, 0.3 $\mu\text{g/ml}$ proteinase K) and incubated at 60°C for 1 h. Consequently the mix was incubated at 95°C for 10 min to denature the proteinase K. Finally the DNA was aliquoted into cryo-Vials (NUNK, Germany) and frozen at -70°C till requirement.

2.4. Protein concentration determination

The amount of protein was assayed according to the method of Bradford [13]. For calibration curve BSA standard was used (BioRad, Munich, Germany). The concentration was determined by measurement of the absorbance at 595 nm.

2.5. MAO-Bi13 polymorphism identification

For genotyping genomic DNA extracted from patients' blood was used. The restriction analysis of the genotype was conducted with primers flanking the gene region containing a polymorphism (A-G replacement) 36 bp upstream of the start position of exon 14 (NCBI SNP cluster id: rs1799836) [39]. We used 5'-TGT-CAT-CCC-TAT-TTG-AGG-TGT-3' as forward primer and 5'-TGT-GGA-GAA-TCA-CCA-GGC-TA-3' as reverse primer. The amplification volume was 25 μl containing: $1 \times$ PCR buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.5% Triton x-100), 1.5 mM MgCl_2 , 200 μM dNTP, 0.8 pmol/ μl Primer mix, 0.5 μl *Taq* polymerase final concentration. The cyler conditions were 4 min denaturation at 95°C , followed by 34 cycles at 94°C , 59.3°C and 72°C for 45 s each and at last the elongation at 72°C for 5 min. The PCR product of 633 bp was digested for allele detection with the restriction enzyme Tsp45I (NmuCI, MBI Fermentas) for 12 h. Allele detection was conducted by electrophoresis on 2.5% agarose gel, which was stained with ethidium bromide and visualized under

UV-light. The resulting different genotypes: G/G (503 and 130 bp), G/A (503, 298, 205 and 103 bp) and AA (298, 205 and 130 bp) showed characteristic bands at the above listed molecular weights on the gel.

2.6. Platelet MAO-B activity

MAO-B activity in platelets was analyzed according to a modification of the method described by Wurtman and Axelrod [72]. ^{14}C phenylethylamine (PerkinElmer NEN, Boston, MA, USA, final concentration: 10 μM , specific activity: 44.13 mCi/mmol) was incubated with 400 μg of platelet protein preparation in phosphate buffer (pH 7.4) in 250 μl total volume for 10 min at 37°C . The reaction was stopped by adding 200 μl of 1N HCl and the reaction products were extracted into 2 ml of ethylacetate. Blanks were prepared by adding 200 μl of 1N HCl before incubation. After centrifugation ($1000 \times g$ for 5 min) 1 ml of the organic phase was pipetted into a scintillation vial containing 4 ml scintillant. The amount of product formed was determined by liquid scintillation counting (Liquid Scintillation Counter, LS 1801, Beckmann Coulter, Unterschleissheim-Lohhof, Germany). Enzyme activity was expressed in nmols of product pro mg protein multiple minutes at 37°C . Platelet MAO activity was linear with respect to protein concentration and the time of incubation. For the preparation of standard curves and evaluation of the radiometric assay human MAO-B was used (Gentest, Woburn, MA, USA).

2.7. Erythrocyte SOD activity

For the measurement of erythrocyte SOD (Cu/Zn-SOD, SOD1) activity 250 μl red blood cells were hemolysed with 1.75 ml cold water (4°C). Extracts were prepared by adding 2 ml ethanol and 1.2 ml chloroform to the hemolysate, shaking for 1 min and centrifuging for 10 min at $1800 \times g$. The top clear layer was collected and diluted 20 times with phosphate buffer for SOD activity measurement. SOD activity was determined using Dojindo SOD Assay Kit (Dojindo Laboratories, Kumamoto, Japan) [56]. In this assay water soluble tetrazolium (WST), the sodium salt of 4-[3-(4iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, was used as a detector of superoxide radicals generated by xanthine oxidase and hypoxanthine, in the presence of a range of concentrations of an SOD standard (Sigma co., Saint Louis, Missouri, USA) and the patient erythrocyte SOD preparations. SOD activity was assessed by the ability to inhibit the reaction of superoxides with WST to form a yellow (440 nm) formazan dye. After 20 min incubation the reaction was stopped and the absorbance was read in a spectrophotometer plate reader (Dynatech MR4000) and the SOD activity calculated by relating to inhibition by the SOD standard measured at the same time.

2.8. Western blot for platelet MAO-B and erythrocyte SOD

For Western blot analysis proteins in tissue homogenates were separated by SDS-PAGE and transferred to nitrocellulose. Blots were incubated in blocking solution (low salt washing buffer with 5% milk and 0.5% Tween), followed by a 1:1000 dilution of anti-MAO-antibody (biomol, Hamburg, Germany) or anti-SOD-antibody (The Binding Site, Heidelberg, Germany), respectively. Blots were washed and incubated in a 1:2000 dilution of HRP-conjugated anti-chicken or anti-sheep antibody, respectively. The immunoreactive bands were visualized with ECL chemiluminescence kit (Amersham, Freiburg, Germany). Bands were visualized with ChemiDoc camera and analyzed via its software provided by the manufacturer (BioRad, Hercules, CA, USA).

2.9. LTF plasma levels

LTF levels in plasma were measurement by an enzyme-linked immunoassay of the sandwich type. We used the BIOXYTECH^R Lactof EIATM kit (OxisReasecrhTM, Portland, OR, USA) according to the company protocol. As this ELISA is very sensitive we used 100 μ l diluted plasma (1:15 dilution with sample diluting buffer).

2.10. Statistic analysis

The analysis of variance (ANOVA) was applied to the data, using the StatView computer program (Stat View 5.0. software, SAS Institute Inc., Cary, NC, USA) on a PC computer. For correlation studies the Bartlett-test was used with the above program. For non-parametric tests, Mann–Whitney *U*-test was used. For allele frequency study the σ^2 test and Fisher's exact tests were used. Statistical significance was set at $P < 0.05$ for all tests.

3. Results

3.1. Platelet MAO-B activity

Platelet MAO-B activity is a stable factor in the individual without great diurnal variation. Vitamin B12 deficiency (pernicious anaemia) increases the activity therefore

plasma levels of Vitamin B12 have been measured (data not shown). In several studies MAO-B activity in brain tissue as well as platelet were tested for its correlation with age with no clear results but rather clear elevation in AD and PD [25,47,51,66]. In accordance to previous studies, we found a 15–18% higher platelet MAO-B activity in women and a significant lower activity in current smokers (data not shown) [50]. We were also able to show a highly significant increase of platelet MAO-B activity in the centenarian group comparing to the VITA with MMSE higher than 24 (Fig. 1A). Comparison between subjects of the VITA group with a MMSE lower than 24 (defined as demented) and the centenarian group (demented) still gave a significant ($P = 0,0004$) difference in platelet MAO-B activity (Fig. 1B). In agreement, comparing the 2 subjects of the VITA group with a MMSE lower than 21 showed similar elevated platelet MAO-B activity to the centenarian group (data not shown). Interestingly, the platelet MAO-B activity within the two groups (VITA and Centenarian) correlated to the MMSE (Scatter plot, Fig. 1 gradients a and b), as well when only subjects with CDR = 0.5, 1 and 2 (considered as mild demented) were considered (gradient d). When analyzing the correlation between MMSE and MAO-B activity only in the VITA group the MAO-B activity was not changed with MMSE (gradient c). In addition, when comparing the so-called non-demented (CDR = 0) and mild demented (CDR = 0.5, 1 and 2) subjects of the VITA project we saw no difference in MAO-B activity between the groups (Fig. 1D).

3.2. MAO-Bi13 polymorphism

No significant difference was observed in the allele frequencies for MAO-Bi13 polymorphism between VITA and centenarian-group (Table 2). In addition, comparison of the frequency of the MAO-Bi13 mutation, in the VITA project only, between the non-demented (CDR = 0) and the mild demented (CDR = 0.5, 1 and 2) subjects resulted in no significant difference. However, the frequency of the A allele seemed to be slightly higher in the demented group (Table 2). In contrast to previous studies in which MAO-B activity in the brain was correlated to MAO-Bi13 polymorphism [6], we could not find any correlation between MAO-Bi13 polymorphism and platelet MAO-B activity in both genders (Fig. 2).

Table 2

The allele frequency distribution of the MAO-B intron 13 polymorphism in the VITA-group and the centenarian group

	VITA-all	VITA-group		Centenarian-study
		CDR = 0	CDR = 0.5, 1 and 2	
A/A genotype, <i>n</i> (%)	42 (32)	31 (29)	11 (45.8)	26 (36.6)
G/A genotype, <i>n</i> (%)	45 (34.4)	38 (35.5)	7 (29.2)	29 (40.8)
G/G genotype, <i>n</i> (%)	44 (33.6)	38 (35.5)	6 (25)	16 (22.6)
Allele frequency (A)	0.492 ^a	0.467 ^b	0.604	0.570

^a $\sigma^2=2.248$, *: $P = 0.1338$, Fisher's exact $P = 0.145$ VITA vs. Centenarian.

^b $\sigma^2=0.139$, *: $P = 0.7097$, Fisher's exact $P = 0.728$ VITA-CDR0 vs. VITA-CDR0,1,2.

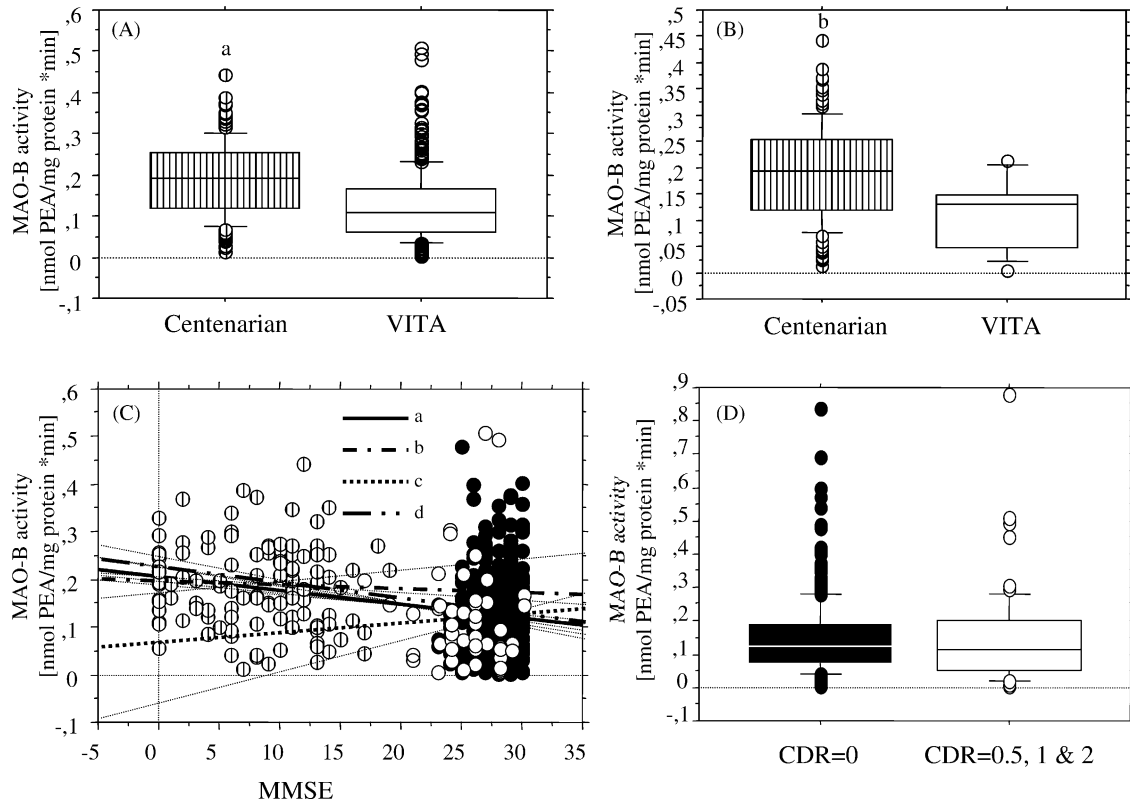


Fig. 1. Platelet MAO-B activity level in the VITA and centenarian group. (A) Comparison between the VITA ($n = 382$) with MMSE > 24 and the centenarian ($n = 135$) group with MMSE < 24. (B) Comparison between the VITA ($n = 22$) with MMSE < 24 and the centenarian ($n = 135$) group with MMSE < 24. (C) Correlation between platelet MAO-B activity and MMSE in VITA with CDR = 0.5, 1 and 2 (white), VITA with CDR = 0 (black) and centenarian group (stripped): (a) $R^2 = 0.085$ Bartlett-test $P < 0.0001$ (VITA vs. centenarian); (b) $R^2 = 0.002$ Bartlett-test $P = 0.867$ (centenarian); (c) $R^2 = 0.002$ Bartlett-test $P = 0.681$ (VITA); (d) $R^2 = 0.088$ Bartlett-test $P = 0.0004$ (CDR = 0.5, 1 and 2). (D) Comparison between MAO-B activity and CDR score (CDR = 0, $n = 480$; CDR = 0.5, 1 and 2, $n = 58$) in the VITA group (Mann–Whitney U test, $P = 0.1542$). In (A)–(C) the points above and under the graphs represents outliers subjects which does not appear to fit to the average value. ANOVA and Fisher-PLSD: (a) $P < 0.0001$; (b) $P < 0.0005$ VITA vs. centenarian group.

3.3. MAO-B Western blot

Western blot analysis was performed for platelet homogenates with a very high and a very low platelet MAO-B activity. No correlation ($P = 0.2149$) could be found be-

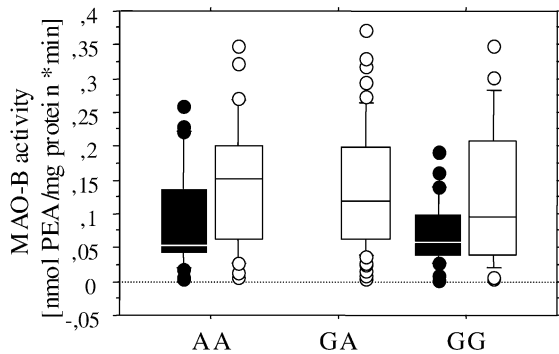


Fig. 2. MAO-B intron 13 polymorphism influence on MAO-B activity in males (black) and females (white). Males: AA, $n = 29$; GG, $n = 26$. Females: AA, $n = 37$; GA, $n = 72$; GG, $n = 33$. The points above and under the graphs represents outlier’s subjects, which does not appear to fit to the average value.

tween platelet MAO-B activity and MAO-B protein levels in platelet (Fig. 3).

3.4. Erythrocyte SOD activity

Erythrocyte Cu/Zn-SOD activity in females with AD (centenarian group) were significantly decreased compared to the VITA subjects. In males lower activity was apparent but did not reach statistical significance (Fig. 4A). It is noteworthy, that comparison of VITA subjects with MMSE lower than 24 (defined as dementia) showed similar values to those of the demented group ($P = 0.5665$) (Fig. 4B). A significant negative correlation appeared between Cu/Zn-SOD activity and MMSE in both groups (Fig. 4C). In addition, no significant differences were observed when we compared Cu/Zn-SOD activity in the VITA project only, between the non-demented (CDR = 0) and the mild demented (CDR = 0.5, 1 and 2) (Fig. 4D).

3.5. Erythrocyte SOD Western blot

Analysis of Cu/Zn-SOD protein levels in erythrocytes was performed for samples with a very high or very low

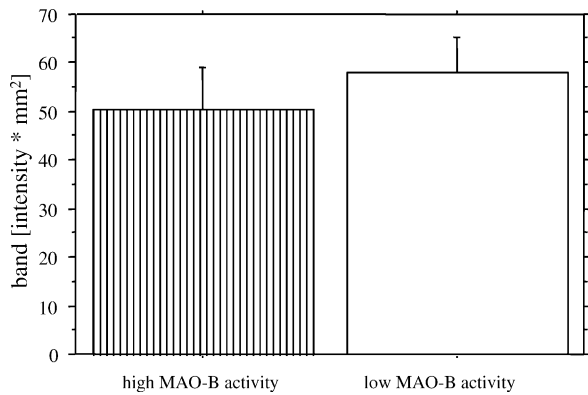


Fig. 3. Western blot for platelet MAO-B. Densitometry analysis of bands corresponding to high platelet MAO-B activity ($n = 8$) and low platelet MAO-B activity ($n = 8$). ANOVA and Fisher-PLSD: $P = 0.2149$.

Cu/Zn-SOD activity. The high activity group showed also a significantly ($P = 0.0246$) higher amount of Cu/Zn-SOD protein (Fig. 5).

3.6. Plasma LTF

Another biochemical marker in blood tested was the iron chelator, LTF. We saw only a slight lower level of plasma LTF in the centenarian group compared to the VITA group, but this result was not significant (Fig. 6A). Studying the effect of mild dementia (CDR = 0.5, 1 and 2) compared to non demented (CDR = 0) on the LTF plasma level in the VITA group showed no difference (Fig. 6B). Other parameters tested (e.g. MMSE, UPDRS test, etc.) for correlation with LTF plasma levels did not show any significance (data not shown).

4. Discussion

Great deal of evidence is leading to the assumption that OS mechanisms are involved in the pathogenesis of AD [32,68]. Markers of oxidative damage have been found on proteins, DNA and lipids in brains of AD patients and in vitro models of $\text{A}\beta$ -toxicity and are associated with increased production of ROS [44]. The fact that age is a key risk factor in AD provides considerable support for the free radical hypothesis as the accumulating effects of the attacks by free radicals can be seen over the years [7]. The pathophysiological events leading to AD are mainly focused on the brain, however it is hypothesised that AD might be a systemic disease that affects several tissues in the body [28]. One of the hypothesis to the cascades of events in AD, is the early changes of reduced brain metabolism in AD which may then lead to the oxidative damage observed in aging [55]. Additionally, a specific oxidative change of reactive carbonyls was shown to be increased in AD patients [61]. A major problem of measuring direct OS markers is that they are very unstable, because they easily autooxidise. Therefore, we investigated

the possible involvement of enzymes and proteins related to OS, in an indirect way of producing or preventing ROS, such as platelet MAO-B activity, erythrocyte Cu/Zn-SOD activity and plasma LTF levels.

The first biochemical marker in blood tested in our study was the amine-metabolizing enzyme, MAO-B (MAO, E.C.: 1.4.3.4), a flavin containing enzyme localized in the outer mitochondrial membrane [31] and responsible for the oxidative deamination of neurotransmitters (noradrenaline, dopamine and serotonin) and exogenous amines (e.g. tyramine) [71]. During its catalytic activity it produces hydrogen peroxide (H_2O_2) according to the following equation: $\text{RCH}_2\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{RCHOH} + \text{NH}_3 + \text{H}_2\text{O}_2$. Hydrogen peroxide formed in the reaction is a possible source for OS [57]. Several previous studies reported increased activities of MAO-B in the brain and blood platelets of patients suffering from neurodegenerative diseases such as AD and Parkinson disease [2,3,11,19,23,29,51,62,73]. The increase in brain MAO-B might be due to transcriptional elevation of MAO-B protein [48] and predominant in plaque-associated astrocytes in neuropathologically verified AD brains with astrogliosis, respectively [37,59]. However, the reason leading to an increase in platelet MAO-B activity is still unknown, but may be related to transcriptional elevation of MAO-B protein and/or to changes in the cell milieu causing manipulation of the enzyme activity [6,18,65]. In accordance to previous studies [30,51,53] we could show a highly significant increase in platelet MAO-B activity in the centenarian group, which are all demented. Within the demented group this increase was correlated with the mental status test as the MMSE (Fig. 1). This indicates that the observed increase of platelet MAO-B activity takes place at a time point when, for the first time, clear clinical symptoms of dementia appear (e.g. MMSE between 23 and 16) and not in early development of dementia. A change in the ratio of the number of neurons to the number of supportive tissue in the degenerating brain areas could explain the increased activity of MAO-B in AD brains. However, the augmentation of MAO-B activity in platelets, which only express the B form of the enzyme, points to an additional, yet unknown factor which may act on transcriptional regulation [22] of the amount of enzyme and/or in the kinetic regulation of the molecular activity of MAO-B in platelets [10,65]. Our finding in which the activity of platelet MAO-B does not correlate with platelet MAO-B protein content as measured via Western blot analysis (Fig. 3) supports the latter mode of regulation possibly by altering the kinetics of the enzyme via post-translational mechanisms, which was not tested in this study.

Contradictory results have been published concerning the MAO-Bi13 polymorphism with regard to its association with Parkinson's disease [17,39,46] or its effect on MAO-B activity [6,27]. For the first time we investigated this polymorphism in AD, however, our results did not show any significant association with AD or any effect on platelet MAO-B activity (Fig. 2).

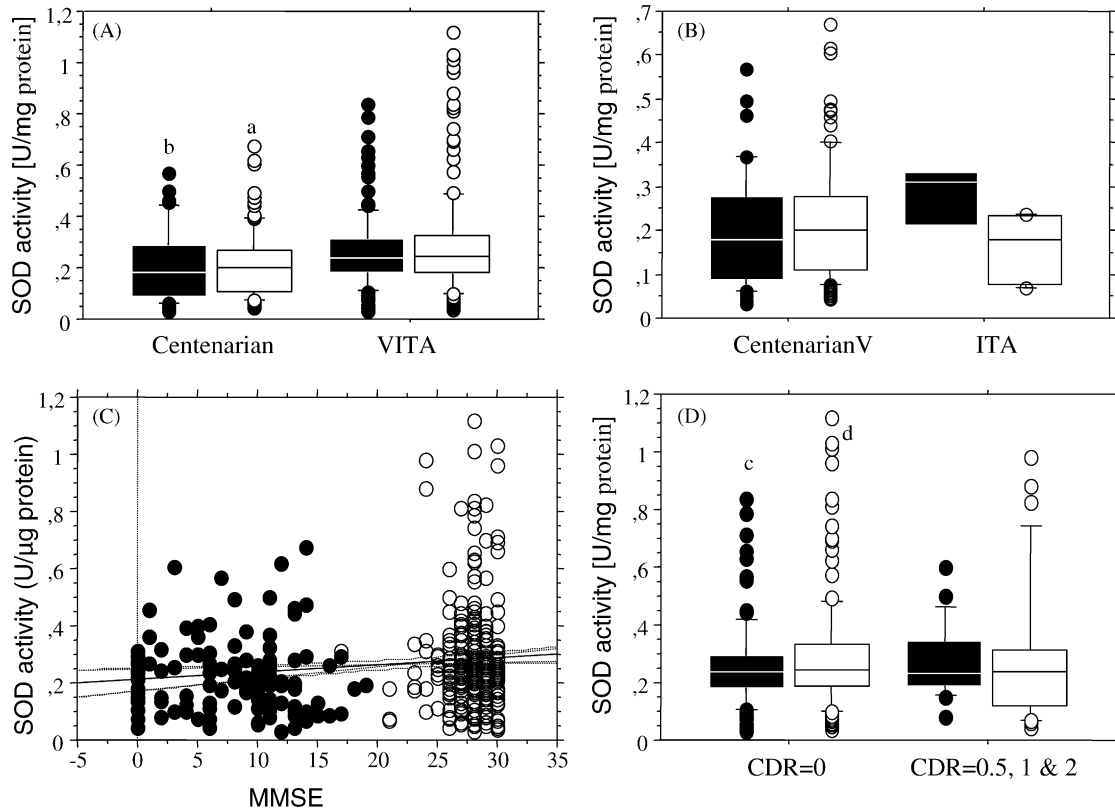


Fig. 4. Erythrocyte Cu/Zn-SOD activity in (A) males (black) and females (white) of the VITA and centenarian group (males: VITA $n = 120$, centenarian $n = 37$; females: VITA $n = 192$, centenarian $n = 107$). (B) Males and females of the VITA group with MMSE less than 24 (males, $n = 3$; females, $n = 6$) and the centenarian group. (C) Correlation between Cu/Zn-SOD activity and MMSE in VITA (white) and centenarian group (Black): $R^2 = 0.02$ Bartlett-test $P = 0.0124$ (VITA vs. centenarian). (D) CDR score in the VITA group (Mann–Whitney U Test, ^c $P = 0.534$, ^d $P = 0.456$). Males: CDR = 0; $n = 101$; CDR = 0.5, 1 and 2; $n = 19$. Females: CDR = 0; $n = 165$; CDR = 0.5, 1 and 2; $n = 27$. In (A)–(C) the points above and under the graphs represents outliers subjects which does not appear to fit to the average value. ANOVA and Fisher-PLSD: (a) $P < 0.005$; (b) $P = 0.064$ VITA vs. centenarian group.

The second marker tested was the superoxide metabolizing enzyme, Cu/Zn-SOD (SOD; E.C.: 1.15.1.1), which catalyses the breakdown of superoxide radicals to molecular oxygen and hydrogen peroxide [26]: $O_2^- + O_2^- + 2H \rightarrow$

$H_2O_2 + O_2$. This enzyme provides the first line of defence against oxygen toxicity. In humans three forms of SOD can be distinguished: cytosolic Cu/Zn-SOD (SOD1), mitochondrial Mn-SOD (SOD2) and extracellular SOD (SOD3) [33]. Human erythrocytes contain only the cytosolic Cu/Zn-SOD form. So far, there are contradicting results concerning the behaviour of Cu/Zn-SOD activity both in brain and erythrocytes in AD [20,36,42,43,52,60,63]. Due to the fact that the gene is located on chromosome 21, Cu/Zn-SOD is also a focus of research in down syndrome and specifically in dementia of down syndrome patients, which has close pathological hallmarks as AD [54]. In our study, we found a decrease in the Cu/Zn-SOD activity in AD patients, but this result was only statistically significant in women. Additionally, analysis of only the VITA results showed no significant difference. A slight decrease in female SOD activity was apparent in subjects with CDR = 0.5, 1 and 2 compared to the controls. This may be explained by gender differences. The decrease in SOD activity could be a result of several factors: (a) decrease in transcriptional process, such as shown in post-mortem CA1 hippocampal regions using in-situ hybridization [64], (b) increase in turn over of the enzyme or, (c) decrease in translational process since

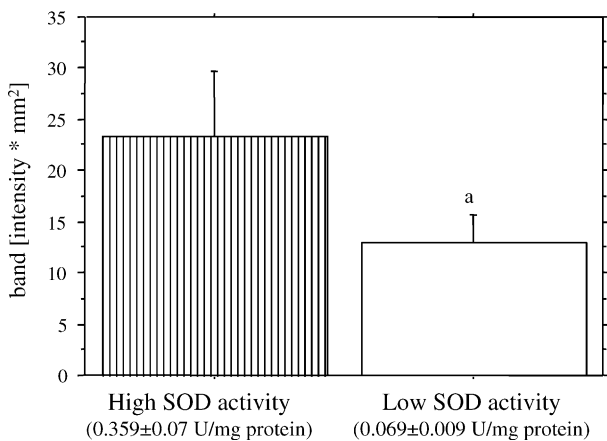


Fig. 5. Western blot for erythrocyte Cu/Zn-SOD. Densitometry analysis of bands corresponding to high erythrocyte Cu/Zn-SOD activity ($n = 8$) and low erythrocyte Cu/Zn-SOD activity ($n = 8$). ANOVA and Fisher-PLSD: $P = 0.0246$.

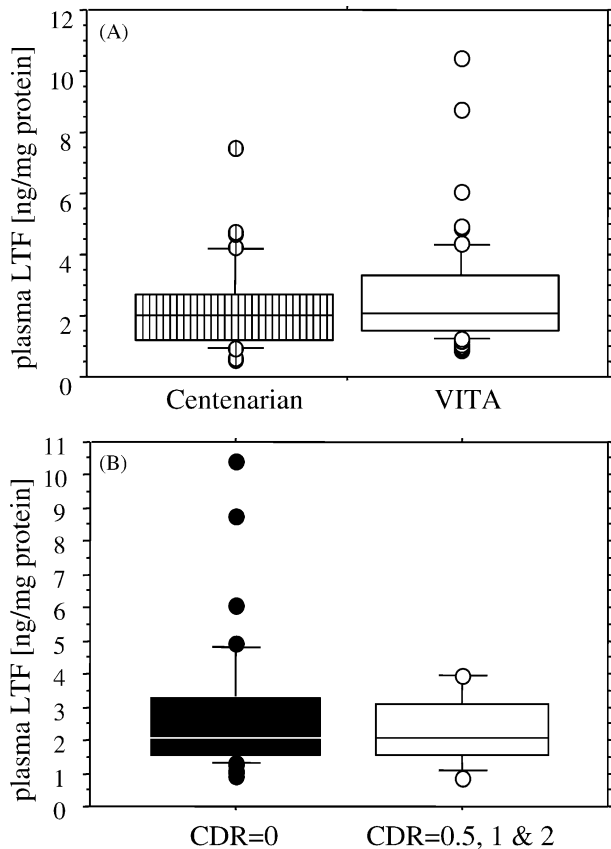


Fig. 6. Plasma LTF levels in (A) the VITA ($n = 60$) and centenarian ($n = 46$) group. ANOVA and Fisher-PLSD: $P = 0.1665$. (B) The VITA group with different CDR scores (CDR = 0, $n = 47$; CDR = 0.5, 1 and 2, $n = 13$). Mann–Whitney U test, $P = 0.774$. The points above and under the graphs represents outlier's subjects, which does not appear to fit to the average value.

we observed a significant reduction in SOD protein in the samples with low SOD activity (Fig. 5). A decreased activity of the “first line of defence” enzyme against the highly reactive superoxides suggests increased OS possibly leading to neurodegeneration. The inconsistencies in SOD activity between our result and other studies may be explained by the finding of Serra et al [60] in which SOD activity was found to be increased in subjects under the age of 70 and decreased in those above this age. However, this finding is in contrast to the ones by de Lustig et al. [20] who found the opposite. In addition in the above study no changes in SOD activity were observed in controls as consequence of age. Another reason to the contradictory results is the fact that each study was conducted with a different SOD activity assay where in some cases the colored substrate is sensitive to outside influence such as oxygen. We used a sensitive assay in which the substrate is stable to every external influence and it is better soluble than other substrates in aqueous medium [56,69]. The fact that in our sample the mean Cu/Zn-SOD activity of the VITA-group members with a MMSE lower than 24 tended to be similar to the demented

group suggests that the decrease in activity could be an early event in AD (Fig. 4B). Still, this last point is only an assumption, which will be tested in our second recruitment of the VITA subjects after 30 months from their first test.

The third marker tested in this study was the iron chelator, LTF. Lactotransferrin (LTF) is a glycoprotein belonging to the transferrin (TF) family. Both LTF and TF are composed of two lobes each possessing one iron-binding site with the capacity to bind reversibly one ferric ion, however LTF binds iron more tightly and acts as a powerful iron chelator with antioxidant properties [5]. LTF is practically absent from the normal human cerebral cortex though its distribution and concentration change during aging and in neurodegenerative disorders [38,40,41]. Plasma LTF, a strong natural iron-chelator, helps in averting OS, via inhibition of the Fenton reaction [21,67]. We found that plasma LTF was decreased in the centenarian group, but this result did not achieve statistical significance. Moreover, comparison of the LTF levels in the VITA group with different CDRs did not show any significant difference. This finding is in accordance with another study in which no statistical difference was found [68]. In contrast to our findings in blood, LTF expression was greatly up-regulated in both neurons and glia cells in affected AD tissue [38]. Still, this contradictory result may be as a consequence of the late stage of the disease where the resting neurons try to defend themselves from OS, while our results show still the early stages of the disease.

Taken together, MAO-B and SOD activities when combined may be used for early diagnosis for AD. In this study we can only present the first stage of the VITA study, which continues into its second stage of 30 months recalls, where, hopefully, early measurements will confirm themselves as early diagnostic tools for AD. Therefore, no clear-cut conclusion can be placed in this stage to whether these markers can differentiate between early-onset, late-onset and healthy subjects. However, the value of these markers should be further evaluated, particularly in different cohorts of patients in order to reproduce the validity of our data. Still these early results heave some light to a future where early diagnosis may be considered.

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