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Master's Thesis in Medicine

Alzheimer's disease: Identification of oxidative stress biomarkers in red blood cells

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I. Abstract

Background: Oxidative stress plays a significant role in the physiological aging process and in some pathological conditions, such as Alzheimer's disease (AD). Studies suggested that biomarkers of oxidative and nitrosative stress are elevated in brain of AD patients and that oxidative damage is one of the earliest events in AD. In this experimental study, we focused on protein carbonylation and S-nitrosylation, which are irreversible and reversible post-translational modifications, respectively. The aim of this project was to identify protein markers of oxidative stress as an early event in AD pathogenesis. We hypothesized first that levels of carbonylated and nitrosylated membrane proteins of red blood cell (RBC) – including spectrin –, are increased in patients with cognitive impairment (CI) compared to controls, as based on measurements made in human autopsy brain tissue. The CI group includes patients with mild cognitive impairment (MCI) and Alzheimer's disease (AD). Secondly we split the CI group in MCI and AD groups, and hypothesized that AD contains less oxidized RBC proteins than MCI, supporting the idea that oxidative stress decreases with the disease progression.

Method: We analysed blood samples from a cohort of one hundred and twenty subjects aged 49 to 85 years, including 69 cognitive impairment (CI) subjects and 51 age-matched controls. The CI group was composed of MCI and AD patients. RBC membrane proteins were extracted and separated by one-dimensional electrophoresis. Fluorescent detection was then performed by using anti-dinitrophenyl (DNP) and anti-S-nitrocysteine antibodies to detect carbonylated and S-nitrosylated proteins, respectively. Then we quantified total RBC-membrane protein content and RBC-spectrin proteins, as well as their oxidation levels. Finally, we compared the levels of oxidation in CI and control groups. Further comparisons were performed in groups with/without established molecular biomarkers of AD risk, i.e. increased *p*-tau/A$\beta_{1\text{-}42}$ ratio in cerebrospinal fluid (CSF) and APOEε4 allele carriers. Two-dimensional gel-electrophoresis of selected RBC-membrane extracts was performed to test for differences in protein content and oxidation.

Results: The data revealed that protein carbonylation and S-nitrosylation were significantly decreased in cases of CI, as determined by a clinical dementia rate (CDR) score compared to controls. Oxidized and nitrosylated proteins also tended to decrease significantly in the group with pathological *p*-tau/A$\beta_{1\text{-}42}$ ratio. Finally, S-nitrosylated β spectrin was found decreased in APOEε4 carriers when compared to APOEε4 non-carriers. Two-dimensional gel-electrophoresis of selected RBC-membrane extracts revealed the presence of heatshock protein 27 (hsp27) and an elongation factor 2 (EF2) in CI samples.

Conclusion: The results showed that the blood of CI patients contained less oxidized proteins than controls. This discovery might suggest that oxidative stress has a role in the beginning or before the dementia development, and suggests an activation of stress response in the blood, possibly by activation of heatshock proteins. Oxidative damages in blood are not accumulated as it was found in autopsy brain tissue, but may be eliminated due to the RBC turnover and microvesiculation process, thus decreasing the levels of marker proteins. The relation between oxidative stress in blood and AD should be more studied by measuring the capacity of antioxidant mechanisms and following the progression of the oxidative protein pattern in the same patients. Such an understanding may be necessary in order to develop the diagnostic tools to evaluate the role of redox mechanisms in peripheral tissues as well as for targeting and designing antioxidant therapies.

Key Words: Alzheimer's disease, oxidation, blood, spectrin, marker protein

II. Introduction

a. Alzheimer's disease

Epidemiology and clinic

According to the World Health Organization (WHO) Alzheimer's disease (AD) is the most common form of dementia [2, 19], counting about 2/3 of dementia cases [22, 50]. The other cases include fronto-temporal dementia, vascular dementia, dementia with Lewy bodies, Parkinson's disease, Huntington's disease, etc. Dementia diagnostic is based on CIM-10 classification [22, 50]. Its prevalence worldwide is as high as 24 million and it's predicted to rise due to the population ageing [2, 19]. It affects about 0.5% of population aged 65 years and 8 % of population aged 85 years. [22, 50]. The incidence rate increases exponentially with age and the peak incidence is between 70 and 80 years old. [22] Therefore dementia will become a growing public-health problem. In brief, this neurodegenerative disease related to age consists in a deterioration of memory, other cognitive domains and behaviour [2, 22] with a life span between three and nine years after diagnosis [2].

AD is divided into two subsets according to the age of onset. First an early onset form (< 65 y/o), which has a hereditary background and is generally associated with a more rapid disease decline and a Mendelian pattern of inheritance. Secondly, a late onset form (≥ 65 y/o), which is more frequent and is the sporadic form [15, 50].

The first symptoms of AD are usually a deficit of short-term memory and mild confusion, followed by a progressive decline of the memory and the other cognitive functions, including temporo-spatial disorientation, aphasia, apraxia and agnosia. Moreover, the patient can suffer from executive function disorders and neuropsychiatric symptoms [3, 34, 50]. These functions are evaluated with medical history, clinical examination and neuropsychological tests, such as the mini mental state examination (MMSE) [22].

Diagnostic criteria

The NINCDS-ADRDA Alzheimer's Criteria were proposed in 1984 by the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA). They assign the diagnosis of possible, probable and definite Alzheimer's disease [10]. These diagnostic criteria are based on clinical examination and neuropathological features. Not only specific criteria have to be present, but also diseases than can cause similar cognitive impairment have to be excluded. Therefore we have to exclude neurological, neuropsychological and systemic diseases, as well as delirium and major depressive disorder [50]. We must be aware that the definite diagnosis is obtained only by a post-mortem histological examination [10].

We have to mention a state between normal cognition and dementia, which has been defined as mild cognitive impairment (MCI). This group of people doesn't suffer from dementia, but is at risk of developing AD [22]. For more details, conversion from MCI to AD category is based on clinical criteria only, and not on valid biomarkers. Recent findings have shown that conversion from MCI to AD dementia can be predicted by cued measures, such as the Clinical Dementia Rating scale (CDR) score [53] and the CDR scale sum of boxes (CDRSOB) [39]. Furthermore, CDR score and CDRSOB demonstrated good reliability to stage dementia severity [39]. The proposed guidelines for CDRSOB use suggest that cognitive impairment begins with CDRSOB = 0.5, mild AD with 4.5 ≤ CDR score ≤ 9.0, mild AD with 9.0 ≤ CDR score ≤ 15.5, and severe AD with 15.5 ≤ CDR score ≤ 18.0 [42].

Biomarkers and neuroimaging

Biomarkers of Alzheimer's disease are useful to determine disease risk and to increase the specificity of diagnosis. They include measurements from cerebrospinal fluid (CSF) and blood. The accurate biomarkers in the cerebrospinal fluid for predicting incipient mild AD are the elevated levels of *p*-tau^{181} and total tau protein, as well as the diminution of the level of Aβ_{42} [2, 60]. Therefore increased *p*-tau^{181}/Aβ_{42} ratio is considered pathologic and specific for AD diagnosis [59, 60]. Furthermore it is recommended in differential diagnosis of AD. Concerning APOE genotyping, it is not recommended as a routine screening [43].

Besides these CSF biomarkers, we have other useful tools as predictor of AD occurrence. These tools include magnetic resonance imaging (MRI) as structural imaging and positron emission tomography (PET) scan or single-photon emission computerized tomography (SPECT) scan as functional imaging. Structural and functional neuroimaging studies showed a cortical volume loss predominantly in the temporo-parietal cortex and limbic regions – the hippocampus and the amygdala [15, 49, 50]. While structural MRI can show brain atrophy in typical regions (**Figure 1**), functional MRI can show a decrease in blood flow. A decreased metabolism and protein aggregates can be observed in PET/SPECT scan before visible brain injuries in structural imaging (**Figure 2**) [22].

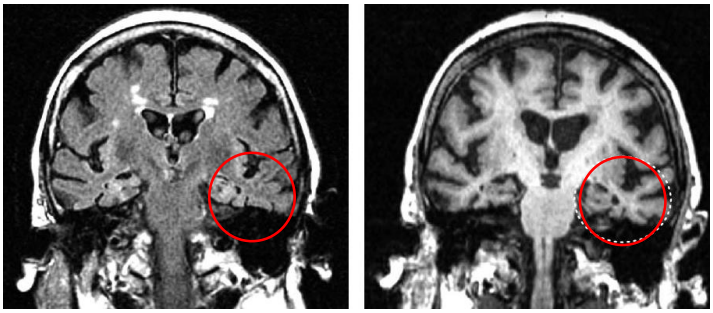

Figure 1. Brain structural MRI in Alzheimer's patient. The MRI in 2001 (right) shows a progressive cortical atrophy in the left temporal region, compared to the MRI in 1998 (left) in the same patient with AD [61]. *http://www.jle.com*

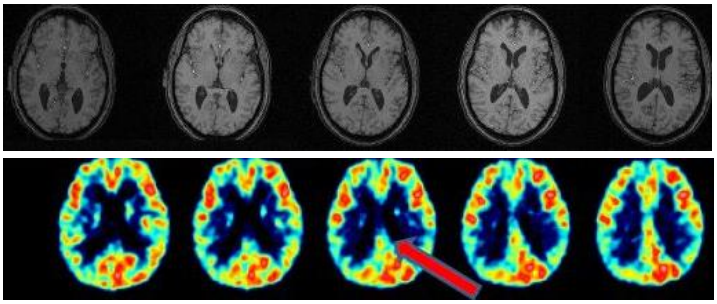

Figure 2. Brain ^{18}F-FDG PET of a patient with Alzheimer's disease (at the bottom) compared to structural MRI (on the top). ^{18}F-FDG is a marker for the uptake of glucose by a tissue and reflects tissue metabolism. This PET shows a typical reduced metabolism of the brain compatible with mild AD, while the MRI doesn't show yet any cortical atrophy.

Physiopathology

Concerning molecular lesions in the aging brain, it has been discovered an accumulation of misfolded proteins which results in inflammatory and oxidative damages. It has been established that an imbalance between production and clearance leads to proteins aggregation, which can be deleterious for neuronal cells [2]. Histopathological hallmarks of AD are characterized by extracellular β-amyloid (Aβ) deposition, intracellular neurofibrillary tangles (NFT) and neuronal loss [3, 50]. The neuronal loss affects particularly the limbic system and the associative neocortex [50]. The β-amyloid accumulation forms amyloid senile plaques extracellularly. The principal isoform in brain is β-amyloid 42 (Aβ_{42}). This supports the amyloid hypothesis, which suggests that β-amyloid is toxic to cells and may be one of the initiating factors in AD. The other pathological accumulation – the NFT – is formed by an insoluble hyper-phosphorylated tau protein (*p*-tau), which is a microtubule-associated protein, particularly the isoform 181 (phosphorylated at threonine 181). These inclusions are found in

pyramidal neurons, contrary to the extracellular deposits of β-amyloid [2]. Other pathological conditions where protein tau is hyper-phosphorylated include tauopathies, such as Huntington's disease, frontotemporal dementia, etc. [50]. Braak et al. [48] have established in 1991 a neuropathological staging of AD according to the distribution pattern of neurofibrillary tangles (See the resume in **Figure *3***). Then increase in *p*-tau/Aβ_{42} ratio has been established as a signature to AD and is assumed to happen before cognitive impairment. The decreases in β-amyloid is due to abnormal processing of the peptide, leading to amyloid aggregation in brain and thereby neuronal loss. On the other hand, the elevation in tau protein reflects also neuronal loss and brain injury [42].

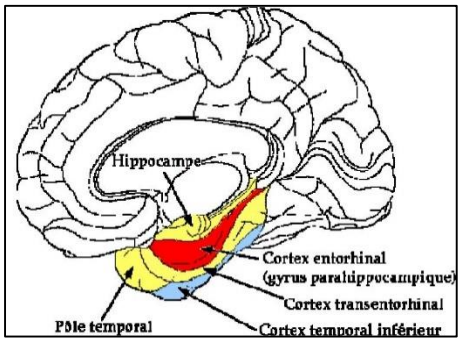

Figure 3. Distribution of neurofibrillary tangles. The accumulation of protein tau in AD begins in the temporal region with molecular lesions in the entorhinal and transentorhinal cortex (stages I-II). Then the limbic system is affected, including the hippocampus and the amygdala (stages III-IV). In advanced stage of disease, the neocortex is involved (stages V-VI). http://www.unil.ch *Course of Prof. J. F. Démonet, department of Neurology, Lausanne – CHUV.*

Risk Factors of Alzheimer's disease

There are genetic and non-genetic risk factors. Epidemiological studies indicate that less than 10% of AD patients have autosomal dominant inheritance, which is related to the early onset of AD [3]. Concerning this inheritance, three genes are known to be involved, including amyloid precursor protein (*APP*) and presenilin 1 and 2 (*PSEN 1 and 2*) genes. These two latter are involved in the breakdown of APP generating excess of Aβ_{42}, and leading to amyloid aggregation and plaques formation [19, 41]. In contrary, sporadic Alzheimer's disease – the late onset form – is related to environmental factors and variations in multiple genes. Researchers have established that the APOE gene is susceptible of increasing the risk of sporadic AD. This gene leads to the production of apolipoprotein E, which has a role in the transport of lipids in the CSF and to the neurons. Moreover there are three major variants, namely APOEε2, APOEε3 and APOEε4 alleles. It has been revealed that the APOEε4 allele is the major risk factor for AD and that homozygotes have greater risk than heterozygotes. Finally people with this APOE polymorphism inherit the risk of developing Alzheimer's disease and not the disease itself.

Apart from age as the main risk of cognitive decline [2], a lot of non-genetic risk factors have been identified. These factors involve cardiovascular disease, cerebrovascular disease, traumatic brain injury, smoking, pollutants, dietary protein, type II diabetes, elevated blood pressure, low and high body weight, etc. They can induce cerebrovascular lesions by oxidative stress and Aβ deposition, increasing the risk of dementia and AD [15, 22, 63].

Treatment and protective factors

Nowadays there is no curative treatment available [3], but there are two classes of pharmacological treatments that are indicated in AD to slow down memory impairment. The first class are the cholinesterase inhibitors – donepezil, rivastigmine and galantamine – which increase the

acetylcholine neurotransmitter and are given to mild AD patients (MMSE 10-26). The second class of drugs are the NMDA receptor antagonists – the memantine – which target the glutamate neurotransmitter and are indicated for severe AD patients (MMSE 3-19) [3, 4, 50].

As protective factors we know that physical activity can promote brain vascularization and is suggested to protect against the onset and the progression of age-associated dementia [3, 10, 15]. Furthermore, some rodent studies have indicated that physical activity decreased the rate of amyloid plaque formation in brain and had anti-oxidant effects by increasing superoxide-dismutase (SOD) and heat shock protein 70 (Hsp70) [6, 22]. We can also mention the Mediterranean diet characterized by nutrients with anti-inflammatory and antioxidant effects, including plant foods, fish, olive oil and low intake of red meat (saturated fats) [9, 15, 22]. Finally randomized controlled trials suggested that cognitive activities, even in late life, have a role in decreasing the risk to develop dementia [11, 22].

Concerning the dietary therapies, prospective assessments found that intake of fruit and vegetable in healthy elderly was associated with an improved antioxidant status, lower level of OS biomarkers and less cognitive decline in comparison to subjects with poor fruit and vegetable consumption [5, 7, 8]. Research projects suggested that moderate intake of unsaturated fats (omega-3 fatty acids), β-carotene, vitamin E, C, B12 and B9 may lower the risk of AD and improve cognitive performance by influencing plasticity synaptic [3, 4, 9, 50]. More specifically, the effect of high-dose vit E has been studied in a randomized double-blind placebo-controlled study and has shown significant delay on loss of ability to perform basic activities and severe dementia [18]. In vitro studies suggest that vit E reduces lipid peroxidation and apoptosis linked with Aβ deposition [22]. After all, the use of more than a single molecule could be better to reach a therapeutic effect. For instance, it is known that vit C is necessary to reduce the vit E radical (oxidized) to the antioxidant form [18, 23]. However we have to keep in mind that several research projects didn't find any association between nutrition and cognitive change [9]. In future, the relation between anti-oxidative nutrition and AD might be confirmed by long-term placebo-controlled studies to find effective dietary therapies.

b. Oxidative stress hypothesis

Free radical reactions are ubiquitous and are supposed to be implicated in the aging process. The free radical theory of aging was proposed in 1954 by Harman [23] and postulates, among others, that the life expectancy can be extended by decreasing the rate of free radicals [18, 23].

Metabolic processes that produce reactive particles are oxidation, S-nitrosylation, s-glutathionylation and lipid peroxidation [15, 23, 24]. These particles, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), are formed during normal metabolic reactions and have a physiological role is the regulation of intracellular signalling [15, 23, 24]. However these instable by-products are highly reactive and can damage different molecules and may lead to cell dysfunction or cell death [15, 19]. To protect from oxidative damages, the body developed defense mechanisms. These antioxidant mechanisms decreased with aging, which results in a loss of balance between the antioxidant and the reactive species levels. Therefore ROS are produced in excess, which induces cellular and mitochondrial damages [15, 18, 23]. The antioxidant system includes the following entities: an endogenous antioxidant, namely the glutathione (GSH); enzymes such as glutathione reductase and peroxidase, superoxide dismutase (SOD), catalase, thioredoxin peroxidase, peroxiredoxins, etc.; antioxidants from diet including tocopherols (vitamin E), the antioxidant β-carotene, ascorbic acid (vitamin C) and retinol (vitamin A); metal ions and cofactors. For example $Mg^{2+,}$ Zn^{2+}, NADH, uric acid, etc.; metal-binding proteins such as ferritin, transferrin and ceruloplasmin [15, 17]. In addition, chaperons, ubiquitin and proteasome normally prevent misfolded

and damaged proteins from aggregating, avoiding cellular impaired function [26]. We can suppose that the tendency for proteins to become misfolded and damaged with age is linked to an increased oxidative stress and a decreased function of defense systems.

In medicine, oxidation plays an important role in some human diseases such as Alzheimer's disease, Parkinson's disease, diabetes, cancer, inflammatory bowel disease, etc. Although the relationship between oxidative stress and these diseases remains uncertain, high levels of oxidised proteins have been observed in these pathological conditions [17, 35, 37]. The oxidative stress hypothesis implicated in the pathogenesis of AD is based on the damages caused by the by-products of oxidative and nitrosative metabolic reactions [18, 23, 24]. Several post-mortem human studies have identified an increased level of biomarkers of oxidative stress in AD brains compared to controls. More precisely, the by-products are elevated in the fronto-temporal cortex and hippocampus, which are typical regions with AD molecular lesions [18]. These different biomarkers include lipid peroxidation, DNA/RNA oxidation and protein oxidation (*i.e.* carbonylation and nitrosylation) [18, 23, 34, 35, 56].

There is scientific controversy whether oxidative stress in the central nervous system occurs at an early stage of Alzheimer's disease, contributing to its development [1, 2, 26], or whether it results from histopathological changes described previously [18]. However, experimental models have shown that markers of oxidative/nitrosative stress precede the onset of histopathological changes [1, 51] and it has been found in human brain that an increase in β-amyloid deposition during the progression of dementia is associated with decreased oxidative damage [1]. Apparently oxidative damage to protein is an early event in AD pathogenesis, which means it is more important at the beginning of disease and decreases with the progression of the neuronal lesions [2, 26]. Therefore, on the basis of previous studies on experimental models, we supposed that increased OS might have a role in accelerating the accumulation of amyloid and tau proteins and it decreases with time.

Protein modifications

ROS and by-products of OS can induce reversible and irreversible protein modifications. Protein reversible alterations, such as S-nitrosylation, may have a harmless role of modulation of protein function, contrary to irreversible protein modifications, such as carbonylation, which can lead to protein dysfunction, inactivation and accumulation. Some protein aggregates in brain tissue are associated with neurodegenerative diseases, such as Parkinson's disease and Alzheimer's diseases [26]. A lot of oxidized proteins in AD brain have been found, including cytoskeletal proteins, proteins belonging to the ubiquitin-proteasome system (UPS) and other types [13, 16, 19]. The cytoskeletal proteins identified in carbonylated and nitrosylated states were spectrins, actins, tubulins, glial fibrillary proteins and other neurofilament proteins [16, 17, 35, 36, 37, 38, 57].

We mention first the cysteine modifications because of its role in the misfolding and function of proteins. There are a subset of protein thiols (R-Cysteine-SH), which may exist as reactive thiolate anions (R-Cys-S^-), that are more readily oxidised by ROS and RNS [17]. This reaction between reactive cysteine thiols and nitrogen species is named S-nitrosylation (**Figure 4**). Furthermore it is a reversible post-translational protein modification, leading to a covalent bond, and it regulates the activity of many targets. S-nitrosylation plays a dynamic role in neuronal signal transduction and its dysregulation has been associated with a variety of pathological conditions, including our disease of interest, Alzheimer's disease [20, 25].

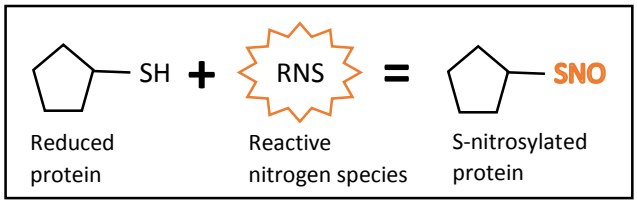

Figure 4. Cysteine thiol nitrosylation by reactive nitrogen species (RNS). The S-nitrosylated protein is referred to as a SNO-protein.

On the other side we focused our project on carbonylation of proteins, which is the most widely used biomarker for oxidative damage. Unlike S-nitrosylation, carbonylation of proteins is a non-enzymatic and irreversible modification. The introduction of carbonyl groups (ketones and aldehydes) into proteins can be triggered directly by ROS or by-products of oxidative stress, just as S-nitrosylation. This irreversible reaction alter the protein conformation and leads to a protein dysfunction or inactivation. If the cell is unable to repair or degrade theses protein carbonyls, it can be deleterious. It has been suggested that carbonylation targets the modified proteins in order to be eliminated by the proteasome system. However, excessive oxidation leads to heavily carbonylated proteins, which are resistant to proteolytic degradation by the proteasome. These aggregates in human tissues become toxic species and are associated with some neurodegenerative diseases (*e.g.*, amyloid precursor protein in AD) [26, 35]. What is more, elevated levels of protein carbonyls have already been observed in the brain of persons with mild cognitive impairment and AD patients [37, 58].

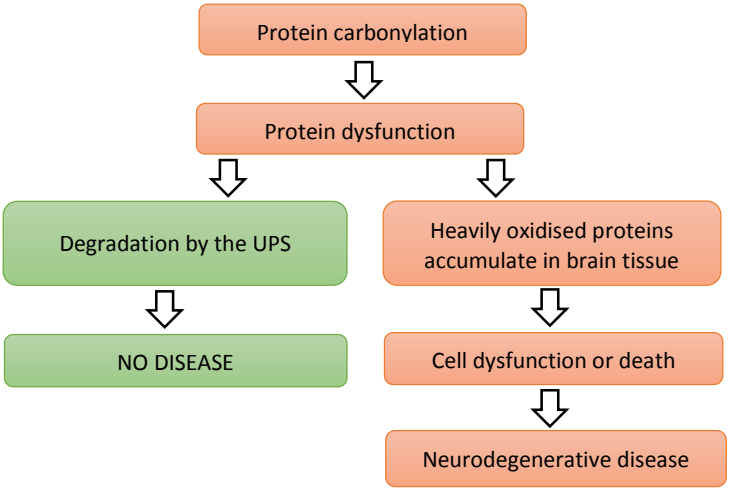

Figure 5. Pathogenic cascade of carbonylation. Irreversible protein carbonylation leading to protein aggregates and neurodegenerative disease, in case where the chaperones and the ubiquitin-proteasome system (UPS) didn't eliminate the carbonylated proteins.

c. Role of the erythrocyte spectrin

Several studies showed a role of brain spectrin in Alzheimer's disease. In our lab, Zeinab El Hajj, PhD student, has shown that brain spectrin was more carbonylated and nitrosylated in AD and MCI subjects in comparison to controls. In addition, it has been reported that the β spectrin is an integral component of β-amyloid plaques in some sporadic AD cases, supporting the hypothesis that an abnormality of spectrin may play a role in the pathogenesis of AD [38]. The spectrin in the RBC membrane cytoskeleton has the same role of maintenance of membrane integrity and the same structure as the spectrin in neuronal membrane cytoskeleton [38]. For these reasons, we are interested in red blood cell spectrin as a biomarker of AD.

The composition of the RBC-membrane includes a lipid bilayer, transmembrane proteins, and a filamentous meshwork forming the membrane cytoskeleton [27, 28, 29, 30, 31] as shown in **Figure 6**. The erythrocyte membrane proteins are divided into two parts as following: firstly, the transmembrane proteins, which penetrate the hydrophobic lipid bilayer. The main protein is band 3 (the anion exchanger) which serves as anchoring site for the membrane skeleton. Glycophorin C also serves as a binding site; secondly, the peripheral membrane proteins, which interact with other molecules at the membrane surface. These proteins are mainly skeletal proteins such as spectrin,

actin and proteins 4.1. These latter are responsible for maintaining the cellular shape and structural integrity of the membrane. Concerning our protein of interest – the spectrin –, it is a tetramer of two α– and two β–subunits. It has an important role in the stability of surface membrane proteins and in RBC deformability [31].

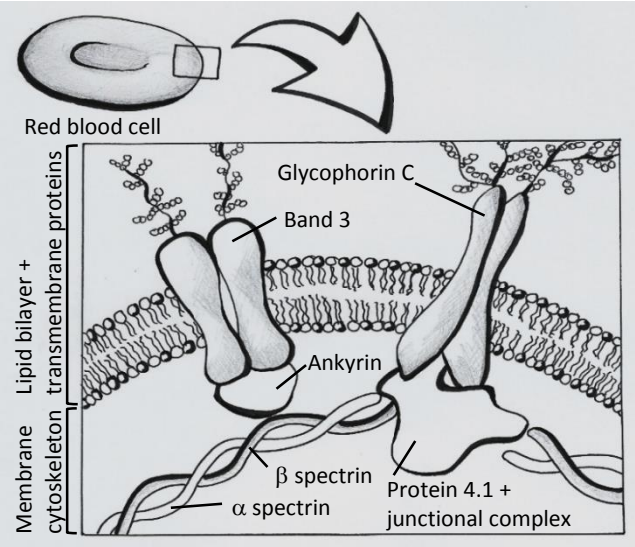

Figure 6. Simplified schema of RBC-membrane structure. The membrane skeleton is attached to the transmembrane proteins at two sites: 1) by ankyrin, which links the β spectrin to band 3; 2) by protein 4.1, which binds spectrin, a junctional complex (F-actin, dematin, tropomodulin, tropomyosin and adducin) and glycophorin C.

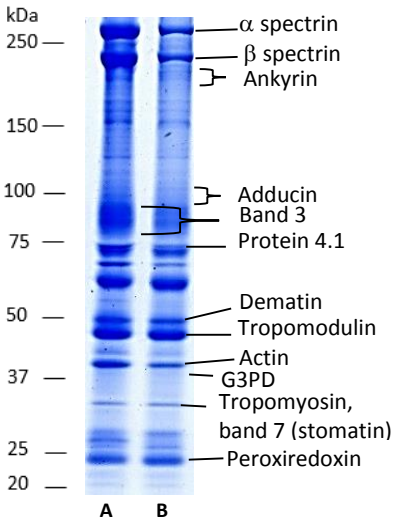

Figure 7. Coomassie blue stained-gel after SDS-PAGE of RBC-membrane extracts. A: membrane proteins extract. B: cytoskeletal proteins extract. Indicated proteins are placed according to RBC analyses of Dr. Julien Delobel, PhD.

RBC-membrane proteins have been studied for years by polyacrylamide gel electrophoresis [27, 31]. In this project, we identified our protein bands in 1-DE gels with the help of the PhD thesis of Dr. Julien Delobel (laboratory of the Regional Blood Transfusion Service in Epalinges, Vaud), who analysed the RBC-membrane proteins by LC-MS/MS. Some of the validated proteins are indicated on the Coomassie blue stained-gel in **Figure 7**.

The relation between oxidative stress and red blood cell in neurodegenerative disease has already been studied. Scientists compared erythrocytes from AD subjects with erythrocytes from healthy donors, and concluded that erythrocyte aging process, characterized by the appearance of IgG, is disturbed in Alzheimer's patients [32]. In other research projects, RBC morphology in AD subjects was altered as compared to their age-matched controls. Changes in the level of membrane and cytoskeletal proteins were linked to the alteration of RBC morphology in AD subjects [33]. Interestingly, S-nitrosylation of RBC-cytoskeleton proteins, especially α and β spectrins, has been linked to the improvement in RBC deformability, which is linked to the structural and functional integrity of the RBC cytoskeleton [25]. These discoveries encourage us the development of diagnostic tools in blood, especially changes in RBC-membrane proteins.

d. Aims of the project

The finding of oxidative damage in neurodegenerative diseases [17, 35, 37] raises the therapeutic possibilities that antioxidants might be useful in slowing the progression of these illnesses [37]. For this reason among others, we decided to study the relationship between the levels of oxidative stress and mild cognitive impairment or Alzheimer's disease (the cognitive impairment group). By-products of oxidative processes can damage different molecules, such as lipids, DNA, RNA and proteins [15, 23, 24]. In this project, our analyses are focused on protein modifications, that yield nitrated and carbonyl derivatives. Some discoveries support the choice of our target, showing many of oxidized proteins in AD brain, including cytoskeletal proteins, proteins belonging to the UPS and other types [13, 16, 19]. Furthermore, studies interestingly demonstrated that cytoskeletal proteins were in carbonylated and nitrosylated states; spectrins, actins, tubulins, glial fibrillary proteins and other neurofilament proteins were identified [16, 17, 26, 35, 36, 37, 38, 57]. We choose to analyse the spectrin in the RBC-membrane cytoskeleton because it has almost the same role and structure as the spectrin in neuronal membrane cytoskeleton [29]. We compared the state of carbonylation and nitrosylation of total red blood cell membrane proteins – as well as α and β spectrin– in controls, MCI and AD (CI group). We also compared the state of oxidation with other variables of AD diagnostic, such as *p*-tau181/Aβ_{42} ratio and APOEε4 genotyping.

In practice, we extracted membrane and cytoskeletal proteins from whole blood of 120 subjects. After the extraction we analysed the RBC-membrane and cytoskeleton by one- and two-dimensional electrophoresis, followed by either a Coomassie blue and silver nitrate staining or immunoblot. For immunoblotting technique, we used primary antibodies against carbonylation and S-nitrosylation modification. The selected antibodies were anti-DNP and anti-S-nitrocysteine, which have been used a lot of times in Riederer's laboratory [12, 16]. Finally an infrared detection was performed to identify protein modifications.

On the basis of several studies cited before [1, 2, 26, 51], we supposed firstly that the CI group might have a more important oxidative level compared to controls. Secondly we hypothesized that increased oxidative stress could be an early event in cognitive impairment, supposed to be related to the accelerated aging process in AD and the accumulation of β-amyloid and *p*-tau, and finally decreasing with the progression of the disease.

In conclusion, for most neurodegenerative disorders, diagnostic tools and biomarkers to reliably identify the first signs of disease are still missing [3]. With this project, the OS as an early event in AD pathogenesis is not clearly observed, since MCI and AD are firstly considered as one same group. Nevertheless we would like to contribute to the development of a reliable protocol to identify biomarkers of oxidative stress in Alzheimer's disease, and later, develop diagnostic tools in peripheral tissues. In future, the oxidative stress hypothesis could help to develop optimal nutrition or other antioxidant therapies to increase the defense mechanisms against protein aggregate-related diseases.

III. Materials and methods

a. Subjects

The cohort of subjects is currently analysed in a separate study on inflammatory markers conducted by the Neuropsychiatry and Biomarkers research group of University Service of Advanced Age Psychiatry (SUPAA). This study was approved by the Cantonal Human Research Ethics Commission of Vaud (CER-VD). All participants gave written informed consent for the blood and cerebrospinal fluid analysis. The cohort contained one hundred and twenty patients aged 49 to 85 years, including 69 subjects with various levels of cognitive impairment (CI) and 51 healthy age-matched controls.

The inclusion and exclusion criteria were based on the neuropsychological tests, the Lawton Instrumental Activities of Daily living (IADL) [47] and the Clinical Dementia Rating Scale (CDR) [53] score. Concerning the neuropsychological examination, the major cognitive domains were evaluated, such as memory, language, attention and executive functioning. This latter assessment consisted of the Mini Mental State Examination (MMSE) [52], the Buschke Double Memory Test [54], the digit span task [45], the Stroop Test [Stroop J.R., 1935], the letter fluency task [46] and the Trail Making Tests A and B [55].

First of all, the participants in the CI group have been recruited among outpatients with cognitive impairment referred to the Memory Centre, University Hospital of Lausanne (CHUV). For their inclusion into the study, subjects underwent a collection of medical history as well as neuropsychological and clinical examination. The patients scoring ≤ 1.5 SD below gender-, age- and education-adjusted norms in the verbal memory tasks, together with a CDR score = 0.5 or = 1, were considered to have memory impairment; AD patients have not been differentiated from MCI patients. For the control group, healthy community-dwellers have been recruited by advertisement or among spouses of memory clinic patients. These participants had no evidence of cognitive impairment and had a CDR score = 0. The exclusion criteria were any kind of neurological or psychiatric diseases, as well as severe or unstable medical illness.

Biomarkers have been measured in cerebrospinal fluid, such as total tau, *p*-tau^{181} and amyloid $\beta_{1\text{-}42}$. For these measurements, CSF samples obtained from the same patients were analyzed with assays using ELISA technology (Innogenetics/Fujirebio Europe N.V., Belgium). Furthermore people carrying the established genetic risk factor for sporadic AD – the APOEε4 allele – were identified by APOE genotyping on blood with the LightCycler (Roche Diagnostics, Basel, Switzerland). Finally these parameters have been used as variables for our statistical analysis.

b. Reagents

The following reagents have been used: nanopurified water (Barnstead, USA), deionised water, Na_2HPO_4 (Merck, Germany), protease inhibitor cocktail (PIC) (Sigma, USA), n-Dodecyl β-D-maltoside (DDM) (AppliChem GmbH, Germany), Tris-HCl (Sigma, USA), aminocaproic acid (Sigma, USA), EDTA (Pharmacia Biotech AB, Sweden), deoxycholate (DC) (Fluka, Switzerland), NaCl (Merck, Denmark), bovin serum albumin (BSA) (Sigma, USA), 2-Mercaptoethanol (Sigma, Germany), sodium dodecyl sulfate (SDS) (Sigma, Germany), glycérol (Merck, Germany), bromphenol blue (Fluka, Switzerland), acrylamide (Merck, Germany), N,N'-Methylenebisacrylamide (Merck, Germany), TEMED (National Diagnostics, USA), ammonium persulfate (Sigma, Germany), glycine (Biosolve Chimie, France), DeStreak rehydratation olution (GE Healthcare, Sweden), IPG Buffer (GE Healthcare, Sweden), urea

(Applichem, Germany), glycerol (Merck, Germany), DTT (Sigma, Germany), Iodoacetamide (Fluka, Switzerland), colloidal coomassie blue G-250 stain (National Diagnostics, USA), silver nitrate (Fluka, Switzerland), acetic acid (Brenntag, Germany), sodium thiosulfate (Acros, UK), sodium carbonate (Acros, UK), formaldehyde (Acros, UK), Tween 20 (Fluka, UK), Triton X-100 (Chemica, Switzerland), non-fat dry milk, methanol (Merck, Germany), 2,4-dinitrophenylhydrazine (DNPH) (Cell Biolabs Inc, STA-308, USA) and HCl (Merck, Germany).

c. Preparation of blood samples

All participants underwent venous punctures, during which 30 ml of whole blood was collected in EDTA tubes (Sarstedt, Germany) and stored frozen at -80°C. These punctures were performed precisely between 8:30 and 9:30 a.m. after overnight fasting. Blood samples have been thawed once for previous analysis, and again for our oxidative stress analysis, which has been carried out in a blind fashion.

First of all, we proceeded to the extraction of erythrocyte membrane proteins. Our protocol was established on the basis of the procedures of the Research and Development team at the Regional Blood Transfusion Service in Epalinges (SRTS VD). Whole blood samples were thawed and 1.5 ml was placed in three volumes of a hypotonic solution containing 10% phosphate-buffered saline (PBS) (5mM Na_2HPO_4, 155 mM NaCl, nanopure water, pH 7.4) and 5 µl of protease inhibitor cocktail (PIC) during 1 hour at 4°C under agitation. After the hypotonic lysis, the solution was spun down in a rotor 50.1 at 27'000 rpm (75'000 g) for 30 min at 4°C in order to separate the membrane and cytoplasmic contents. The membrane pellet was transferred to 1.5 ml conical tubes (Eppendorf) and centrifuged at 13'200 rpm (16'100 g) for 30 min at 4°C. Afterwards, the red blood cell membranes were washed in 0.1 x PBS several times to remove soluble haemoglobin from ghost cells.

The latter part of the membrane extraction consisted in incubating the ghosts with 5 volumes of a non-ionic detergent-containing buffer (7.8 mM n-Dodecyl β-D-maltoside DDM, 50 mM Tris-HCl, 750 mM aminocaproic acid, 0.5 mM EDTA, pH 7.0) at 13'200 rpm for 30 min at 4°C. The soluble content, which contained the integral membrane proteins, was removed in 1.5 ml tubes. Finally the cytoskeleton was extracted by incubation of the remaining pellet with 5 volumes of an ionic detergent-containing buffer (1% deoxycholate DC, 50mM Tris-HCl, 150mM NaCl, pH8.1) at 13'200 rpm for 30 min at 4°C.

After extraction the protein concentration was determined using the Bradford Protein assay (Bio-Rad, Germany) with a 0 to 20 µg Bovin Serum Albumin (BSA) calibration curve. Then the samples were diluted to a concentration of 2mg/ml with 5x SDS mix (15% Tris, 15% 2-Mercaptoethanol, 10% Sodium Dodecyl Sulfate SDS, 10% Glycerol, 5% Bromphenol Blue, pH 6.8). After all, proteins were denatured at 95°C for 5 minutes and stored at -20°C after adding 0.1 µl of PIC.

d. One and two-dimensional electrophoresis

Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) consists in separating charged molecules in an electric field. Here we have separated the proteins by one- (1-DE) and two-dimensional (2-DE) gel electrophoresis. The Sodium Dodecyl Sulfate (SDS) is an anionic detergent used to denature proteins and cover them with negative charges, allowing not only the protein migration toward the anode, but also the separation by molecular mass.

Concerning the 1-DE, the polypeptides were separated according to their molecular mass through acrylamide gradient gels of 3.6% - 15%. These latter were made of acrylamide solution (39% acrylamide, 1% N,N'-Methylenebisacrylamide), separating buffer (9.1% Tris-HCl, 0.2% SDS, pH 8.8), stacking buffer (3% Tris-HCl, 0.2% SDS, pH 6.8), TEMED, ammonium persulfate and nanopure water. Once the gels have been made, 20 μg of membrane and cytoskeleton extracts, defined as DDM and DC extracts respectively, were loaded into each well. Then the electrophoresis was run at 140 V for approximately 2 hours and 30 minutes in a running buffer (0.3% Tris-HCl, 1.44% Glycine, 0.1% SDS, deionised water).

The 2-DE procedure in our lab has been previously described [14, 16]. In short, the separation of the membrane proteins was made by isoelectric focusing (IEF) pH 3-10 in the first dimension and by molecular mass in the second dimension. First, Immobiline™ Dry-Strips (pH 3-10 NL 7 cm length, GE Healthcare, Sweden) were rehydrated with 150 μg of desalted blood samples in 125 μl DeStreak solution and 0.5 % IPG Buffer pH 3-11 NL for 16 hours. The first dimension was performed on Ettan™ IPGPhor™ 3 system (GE Healthcare, Sweden) with the following running conditions: 300 V at 200 Volt-hours (Vh), from 300 V to 1000 V at 300 Vh, from 1000 V to 5000 V at 4000 Vh and 5000 V at 1250 Vh. The next step was performed by equilibrating the Dry-Strips in an equilibration buffer (6 M Urea, 2 % SDS, 0.375 M Tris-HCl, 20 % Glycerol) with 130 mM DTT for 15 minutes, and then in a solution with equilibration buffer and 135 mM iodoacetamide for 15 minutes. For the second dimension, mini-gels were prepared (13% acrylamide separating gel, 4 % stacking gel) and placed in a running buffer (0.3 % Tris-HCl, 1.44 % Glycine, 0.1% SDS, deionised water). Finally the electrophoresis was run for 1 hour 30 minutes at 140 V.

After electrophoretic separation, gels were either stained – with Coomassie blue or silver nitrate –, or the proteins were transferred from the gel to a nitrocellulose membrane (Bio-Rad, Germany) for Western blotting.

e. Coomassie blue staining

To visualize the general composition of membrane proteins, the gels were incubated in the Coomassie blue overnight. The next morning gel was washed with nanopure water until getting visible bands. Finally they were scanned with the Magic Scan program.

For the establishment of our protocol, this step has initially been made in order to adapt the protein loading in wells. The loading has to be adequate and equal for all the wells, so we can compare the antibody detection after the Western Blot.

f. Silver nitrate staining

Silver staining was used for the 2D polyacrylamide gel electrophoresis. This staining increases the sensitivity for the detection of the protein bands about 50 times compared to Coomassie blue.

First, gels were washed for 30 minutes in a solution containing 50% methanol, 10% acetic acid, nanopurified water. Secondly they were washed for 15 minutes in 5% methanol. After washing the gels 3 x 5 min with deionised water, they were incubated for 2 min with 0.02% sodium thiosulfate. After 3 washes of 30 seconds, gels were incubated for 30 min in a solution of 0.2% silver nitrate. Then 3 rinses of 1 minute were performed and gels were incubated in a development solution (3% sodium carbonate, 50 μl formaldehyde, 2 ml of solution C) for 5-10 min. Finally, staining reaction was stopped in a 1.4 % EDTA solution for 10 min and a last rinse of 10 min was performed.

g. Western Blot and Infrared detection

Western blot is a technique used to detect proteins of interest, which have underwent an electrophoresis, an electrotransfer and finally an incubation with specific antibodies and labeled secondary antibodies. The electrotransfer consists in a transfer of the proteins from the polyacrylamide gel to a 0.45 µm nitrocellulose membrane (Bio-Rad, Germany) for 1 hour and 40 minutes at 90 V. More precisely, the nitrocellulose membrane is placed on the gel, which are both put between two filter papers (Dustcher SA, France), and then placed in a transfer buffer (24.9 mM Tris-HCl, 191.8 mM Glycine, 20% methanol, 0.01 % SDS, deionised water). Considering the electric current for the protein migration, the membrane was oriented towards the anode.

Riederer et al. demonstrated satisfying results with their Infrared labelling protocol [12, 13, 14]. We used this technique to identify carbonylated and nitrosylated proteins, as biomarkers of oxidative and nitrosative stress respectively. A direct detection (**Figure *8***) was used with two secondary antibodies labeled with infrared fluorescent dyes, namely IRDye® 800 CW and IRDye® 700 DX (Rockland, Gilbertville PA, USA), followed by a measure of the signal by digital imaging.

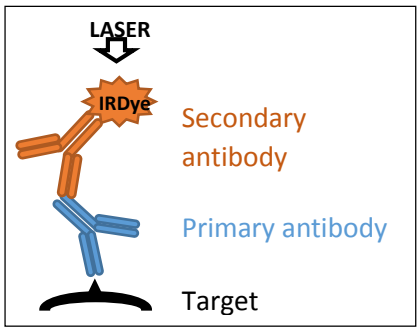

Figure 8. Fluorescent detection method for Western blot. Here we have used polyclonal and monoclonal primary antibodies which bind carbonylated and S-nitrosylated proteins, as well as β-actin. Then we have added labeled secondary antibodies directed against the primary antibodies. Finally digital imaging revealed directly fluorescent signal.

Detection of S-nitrosylated proteins

All steps have been made under agitation and at room temperature. The first step was to block non-specific binding sites of the antibodies to the nitrocellulose membrane. This latter was blocked with 5% non-fat dry milk diluted in 1 x PBS – 0.015% Tween-20 (PBST) for at least 30 minutes. Then the membrane was washed with PBS and incubated with primary antibodies in 2.5% milk diluted in PBST for 1 hour. The following primary antibodies have been used: rabbit polyclonal anti-S-nitrocysteine antibody (Abcam n° 50185, Cambridge, UK) with 1:2000 dilution and mouse monoclonal anti-β-actin antibody (Abcam n° 6276, Cambridge, UK) with 1:10 000 dilution.

After further rinses, secondary antibodies in 2.5% milk diluted in PBST + 0.1% Triton X-100 (to minimize background) were added and incubated for 1 hour. As secondary antibodies, we have used IRDye 800 CW Goat Polyclonal anti-rabbit antibody (Rockland, Gilbertville PA, USA n° 611-632-122) with 1:10 000 dilution and IRDye 700 DX Goat Monoclonal anti-mouse antibody (Rockland, Gilbertville PA, USA, n° 611-130-122) with 1:10 000 dilution.

The last step is the washing of the membrane with PBS. After all, labeled antibodies were detected by scanning the membrane with LI-COR Odyssey® CLx Infrared Imaging System (LI-COR Biosciences GmbH, Germany).

Detection of carbonylated proteins

To detect protein carbonyls, we have used OxySelect™ Potein Carbonyl Immunoblot Kit (Cell Biolabs Inc, STA-308, USA) with the rabbit polyclonal anti-Dinitrophenol (DNP) antibody, 1:1000 dilution (Cell Biolabs Inc, n° STA-308, USA). The OxiBlot method allows a detection of oxidized proteins, which

need a conjugation with 2,4-dinitrophenylhydrazine (DNPH) and the use of the anti-DNP antibody as seen in **Figure *9***. This method is widely used and has been largely described [17, 26].

Before the immunoblotting, the derivatization of oxidized proteins was performed. Firstly, the nitrocellulose membrane was equilibrated in 20% methanol diluted in Tris-buffered saline (TBS) (20 mM Tris-HCl, 0.15 M NaCl, pH 7.4) for 5 minutes. Secondly, the membrane was washed in 2N HCl for 5 minutes and was incubated with 1 x DNPH solution for exactly 5 minutes. Finally we have proceeded to three rinses with 2N HCl and five last rinses with 50 % methanol diluted in TBS.

Concerning the immunoblotting, the protocol was the same as for the nitrosylated protein detection, apart from the following changes in reagents and antibodies: the PBS and PBST was substituted by TBS + 0.05% Tween 20 (TBST) and anti-DNP antibody was used instead of the anti-S-nitrocysteine antibody.

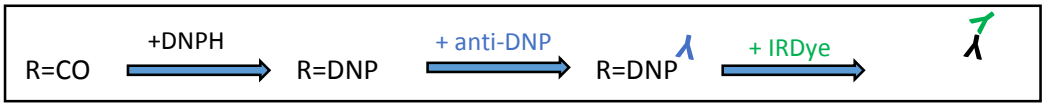

Figure 9. Schema of derivatization and Western blot of carbonylated proteins. The derivatization consists in replacing the carbonyl groups with the DNPH. This step is followed by the immunoblotting using an anti-DNP antibody and a secondary antibody labeled with infrared dye.

h. Protein quantification and statistical analysis

ImageJ program was used to assess the intensity of immunostaining and therefore to quantify the proteins on SDS-PAGE gel bands. The quantification was assessed on whole migration lanes, and then on α and β spectrin separately. The values were normalized with a housekeeping protein – the β-actin – and entered in an Excel database.

Statistical analysis were performed on IBM SPSS Statistics Version 20.0 with a significance level set at p-value < 0.05 with the selected 95% confidence intervals. Firstly we used the Student's t-test with Levene's test for equality of variances to compare binary variables. Three different variables have been used, including the global CDR score, p-tau$_{181}$/A$\beta_{1\text{-}42}$ ratio and APOE polymorphism. The first variable of CDR score permitted to compare control subjects with CDR score = 0 and patients with cognitive impairment (CI) with a CDR score ≥ 0.5. Note that the cognitive impairment group included both MCI and AD patients. The second variable included a normal p-tau$_{181}$/A$\beta_{1\text{-}42}$ ratio < 0.07794, which has been defined according to previous determined biomarker cut-offs at the Leenards center, and an increased p-tau$_{181}$/A$\beta_{1\text{-}42}$ ratio > 0.07794, which has been considered specifically pathologic for AD diagnosis [59, 60, 66]. Finally we compared APOEε4 carriers to non-carriers.

In a second part, our data have been analysed using the one-way analysis of variance (ANOVA) followed by the Tukey post-hoc tests for multiple comparisons. Thus we have compared controls, mild cognitive impairment and Alzheimer's disease, using the Clinical Dementia Rating Scale Sum of Boxes Score (CDRSOB) to stage dementia severity [39]. Indeed, the proposed guidelines suggest that the controls have a score = 0, MCI is associated with a score between 0.5 and 4.5, and mild AD begins with a CDRSOB score = 4.5. Please note that we consider MCI and AD as independent groups.

In a third part, we excluded the control subgroup which had a CDR score equal to zero. Then we determined the MCI and AD groups according to the molecular biomarker, p-tau^{181}/Aβ_{42} ratio, and not according to the CDR score as previously. MCI had a p-tau^{181}/Aβ_{42} ratio below 0.07794 and AD greater than 0.07794, which is known to be associated with Alzheimer's disease [60].

IV. Results

a. 1-DE gels: Coomassie blue stain and Western blots

One hundred and twenty blood samples were prepared to obtain two different extracts of proteins: an integral membrane extract (DDM extract) and a cytoskeletal extract (DC extract). Then SDS-PAGE was run and gels were stained with Coomassie blue or immunostained with anti-DNP and anti-S-nitrosylation antibodies (**Figure *10***).

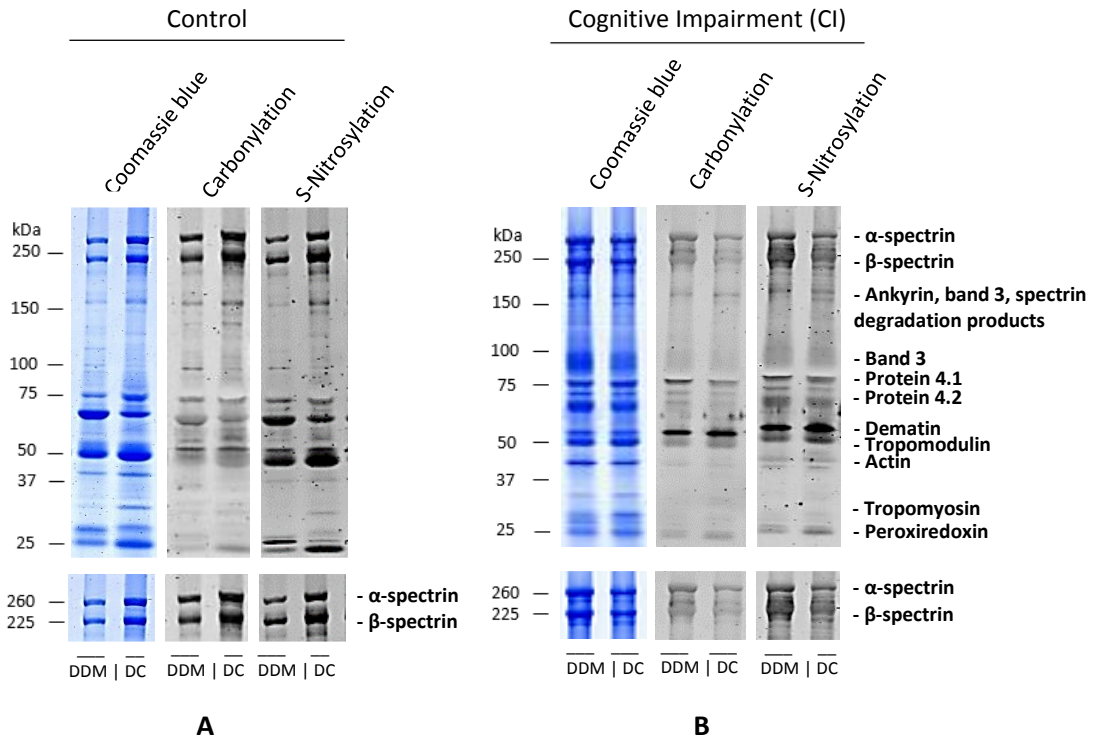

Figure 10. Red blood cell membrane proteins separated by SDS-PAGE. Panel A includes membrane extracts from a representative control subject and panel B includes membrane extracts from a selected CI patient. The control subjects have been selected by their CDR score = 0 and the absence of cognitive impairment. The CI patients have a CDR score ≥ 0.5, and include both MCI and AD patients. Samples were first separated by 1-DE 3.6 % - 15 % gradient gels with 20 μg of proteins per well. A Coomassie blue staining was performed for each sample. Then Western blot was made to detect carbonylated and nitrosylated proteins with the following primary antibodies: anti-DNP antibody and anti-S-nitrosylation antibody. A normalisation was performed with a housekeeping protein – the β-actin. Molecular mass is indicated on the left in kDa. Proteins of interest are indicated on the right. DDM: integral membrane fraction. DC: cytoskeletal fraction. Note that the peroxiredoxin is lesser carbonylated and nitrosylated in the CI patient, suggesting that peroxiredoxins are more active in the CI blood.

By comparing the Western blots, differences in the composition of RBC-membrane proteins were observed between controls and CI subjects. Interestingly, α and β spectrin were lesser carbonylated and S-nitrosylated in the CI group compared to the control group. In addition, some immunostained proteins detected on the whole migration lanes are quantitatively lesser immunostained in CI group (**Figure *10***). On the contrary, a band of about 50 kDa was more intensely stained in CI patients (**Figure *10***). Although, these proteins have not been identified by mass spectrometry (MS) we can make an educated guess, (**Table *1***) by comparing the protein bands with those reported in the literature (PhD thesis of Julien Delobel and references [28, 30, 31]). Please observe the difference in carbonylation and S-nitrosylation between control and CI samples of a protein of 25kDa (possibly peroxiredoxin), suggesting lesser oxidation.

Molecular weight of the unknown bands (kDa)	Suspected membrane proteins
About 150 kDa	Ankyrin, band 3, syndeins and spectrin degradation products
Between 50-75 kDa	Protein 4.1 a and b and 4.2
About 50 kDa	Dematin, tropomodulin and actin
Between 25-37 kDa	G3PD, band 7 (stomatin), tropomyosin and glycophorins A, B, C
About 25 kDa	Peroxiredoxin

Table 1. List of suspected proteins on the different bands of RBC-membrane SDS-PAGE. Bands on SDS-PAGE of RBC-membrane proteins from CI patients are different from controls. These visible bands have not been analysed by MS, but suppositions were made about the corresponding proteins.

b. 1-DE: Protein quantification and statistics

The detection levels of protein carbonylation and nitrosylation were illustrated in the following graphs (**Figure *11 - 14***). In addition, total carbonylation and S-nitrosylation were divided by the total proteins detected on Coomassie blue stained gels. These four measures were made on the total red blood cell membrane extracts (DDM extract), the cytoskeleton extracts (DC extract), and both α spectrin and β spectrin subunits.

In the first part, Student's test with Levene's test for equality of variances were used. Two groups were compared to each other according to three different variables. The 1st variable was defined by *p*-tau^{181}/A$\beta_{1\text{-}42}$ ratio as a negative measure (if < 0.07794) and a positive measure (if > 0.07794) for the specific diagnostic of AD. The 2nd variable contained APOEε4 carriers, including homozygotes and heterozygotes, and non-carriers. The 3rd variable was determined by the global CDR score, which defines a CI group – including MCI and AD patients – and a control group. In the second part, One-Way ANOVA with Post Hoc tests were used to compare three groups, namely Alzheimer's disease (AD) group, mild cognitive impairment (MCI) group and a control group. These variables were defined according to the CDRSOB1 scale described before [39]. More precisely, these guidelines suggest that controls have a CDRSOB score = 0, MCI has a score between 0.5 and 4.5, and mild AD begins with a score = 4.5. In the third part, we excluded the control subgroup (CDR=0) and determined the MCI and AD groups according to the molecular biomarker as described previously. First we compared MCI and AD by using the Student's test, and then we compared the three groups – controls, MCI and AD subjects – by using one-way ANOVA. In the last part with the help of the statistician, we assessed the categorical outcomes with logistic regressions analyses in order to consolidate our results and analyse the independence between the groups which have been analysed.

Part I. Comparison of controls and pathological groups defined by biomarkers, genetic and clinical dementia score

CSF Biomarkers: *p*-tau^{181}/Aβ_{42} ratio analysed with Student's test

The quantification of carbonylated proteins divided by total protein (carbonyl/total) in the integral membrane (DDM) and cytoskeletal (DC) extracts was revealed significantly decreased (*p*-value = 0.014 and 0.012 respectively) according to *p*-tau^{181}/Aβ_{42} ratio. The levels of nitrosylated and carbonylated proteins also decreased significantly in the cytoskeletal extract (DC) in the pathological *p*-tau^{181}/Aβ_{42} ratio (*p* = 0.04 and 0.021, respectively) (**Figure *11 A and B***).

The relation between oxidised α spectrin and the CSF biomarkers showed significant decreased levels of global nitrosylation (*p*-value = 0.012) and nitrosyl/total (*p*-value = 0.047) in the pathological *p*-tau^{181}/Aβ$_{42}$ ratio group (**Figure *11 C***). The same trend was observed between the β spectrin and the pathological ratio (*p* = 0.025 for the S-nitrosylation and *p* = 0.064 for S-nitrosyl/total) (**Figure *11 D***). It seemed noteworthy that cytoskeleton extracts revealed a more pronounced staining compared to the membrane fractions.

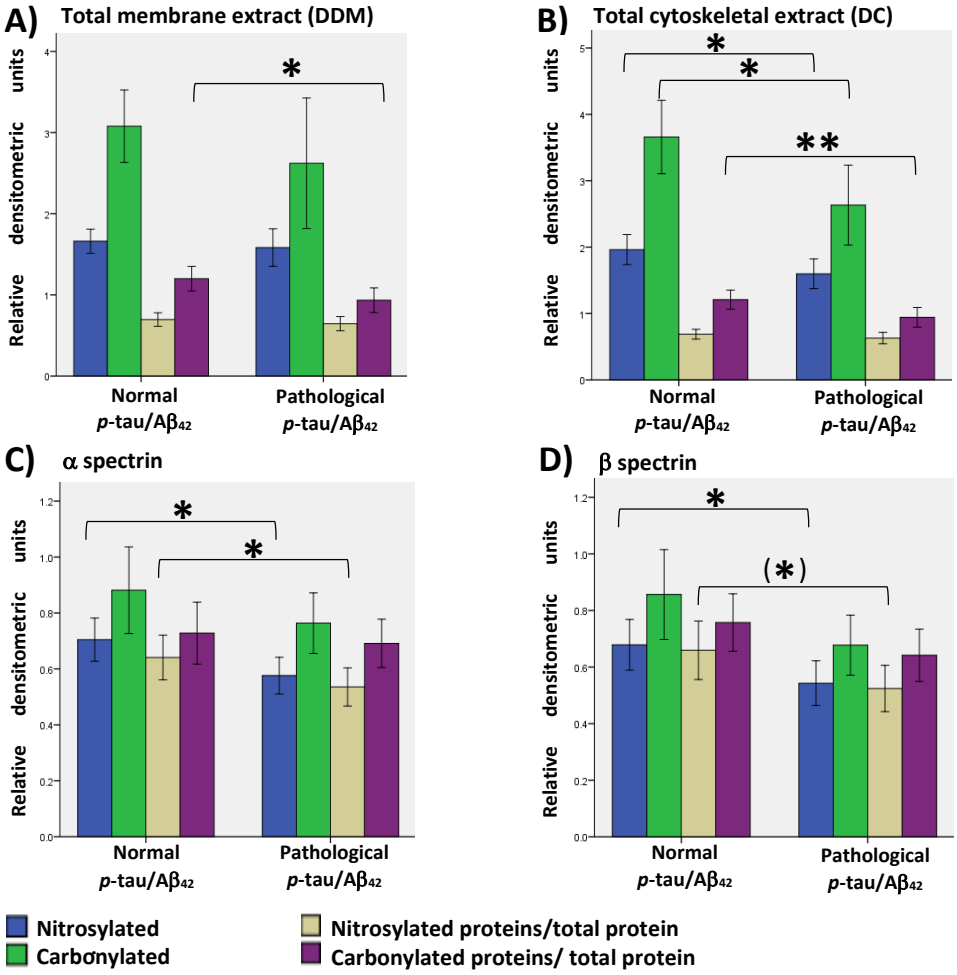

Figure 11. Quantification by densitometry and statistical analysis by Student's test of RBC membrane proteins according to the p-tau/Aβ$_{42}$ ratio. Normal *p*-tau^{181}/Aβ$_{1\text{-}42}$ ratio has been defined with a value < 0.07794, and pathological *p*-tau^{181}/Aβ$_{1\text{-}42}$ ratio with a value > 0.07794, according to previous studies on this cohort at the Leenards Center. Statistical significance (p < 0.05) is identified by an asterisk * on the histograms, high significance (p < 0.01) by two asterisks ** and decreasing trend (0.05 < p-value < 0.1) by an asterisk between parentheses (*).

Genetic risk factor: APOEε4 analysed with Student's test

No significant differences in the total proteins of RBC (DDM and DC extracts), neither in the α spectrin were observed between the APOEε4 carriers and non-carriers (*not shown*). In contrast, β spectrin was significantly less nitrosylated in the group of APOEε4 carriers with a *p*-value = 0.001 (**Figure *12***).

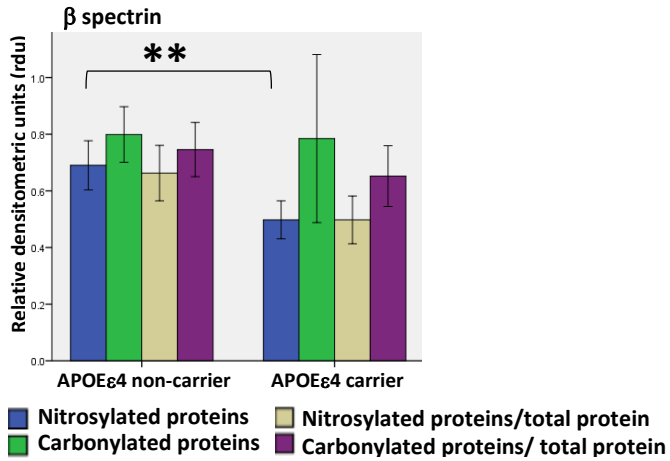

Figure 12. Quantification by densitometry and statistical analysis by Student's test of RBC membrane proteins according to APOE genotype. High statistical significance ($p < 0.01$) is reported with two asterisks **.

Clinical dementia rate score analysed with Student's test

Significant differences were also observed between the controls and the CI determined by the CDR score. First of all, high significant decreased in carbonylated proteins/total in the membrane (DDM) and cytoskeletal (DC) extracts were found in the CI group compared to controls, with respectively p = 0.007 and p =0.009. The carbonylated proteins of RBC were also significantly reduced in the DDM and DC extracts (p = 0.024 and p = 0.035).

Interestingly different protein modifications were found in the two spectrins, which are significantly less nitrosylated in the CI group. We have observed a significant decrease in the nitrosylated α and β spectrins (p = 0.031 and p = 0.005) and a decreasing trend in the nitrosylated spectrins/total (p = 0.061 and p = 0.071).

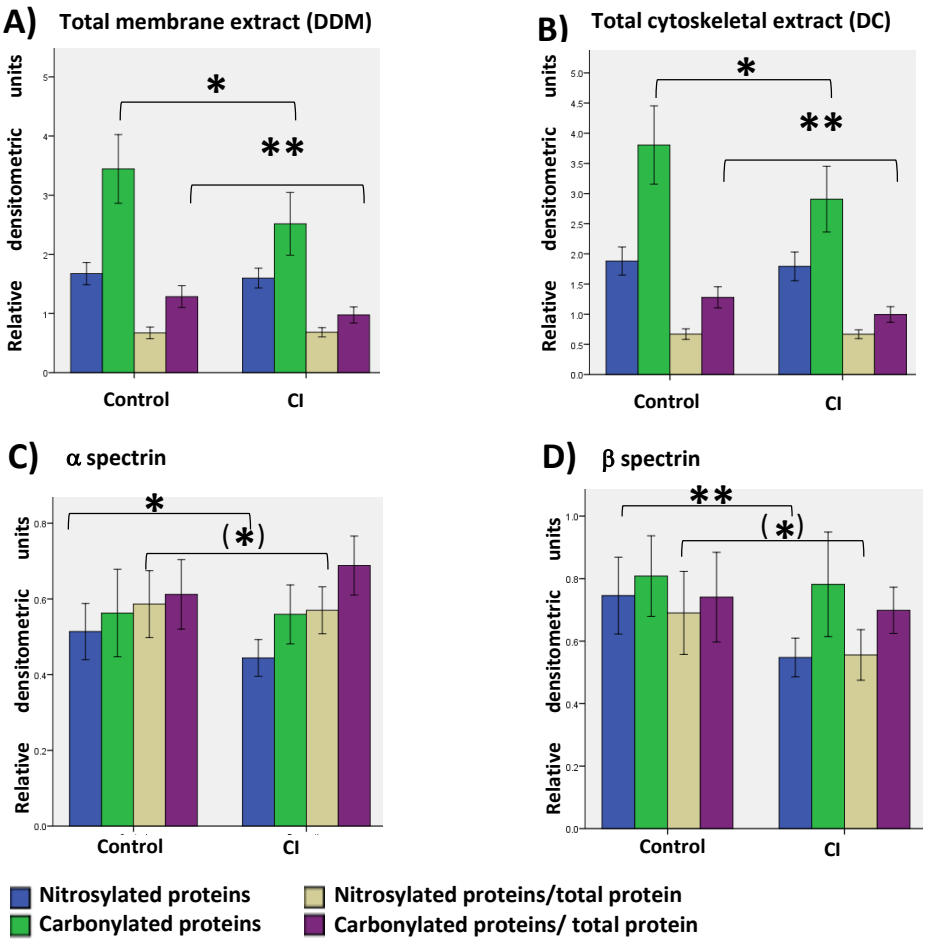

Figure 13. Quantification by densitometry and statistical analysis by Student's test of RBC membrane proteins according to the clinical dementia rate score. The control subjects have been selected by their CDR score = 0 and the absence of cognitive impairment. The CI patients have a CDR score ≥ 0.5, and included both MCI and AD patients. Statistical significance ($p < 0.05$) is reported with an asterisk *, high statistical significance ($p < 0.01$) with two asterisks ** and decreasing trend ($0.05 < p < 0.1$) by an asterisk between parentheses (*).

Part II. Comparison of MCI and AD with controls

Clinical dementia rate sum of boxes score analysed with One-Way ANOVA

In this second analytical part, we compared control, MCI and AD subjects according to the CDRSOB score. The significance level of these analyses was less reached compared to the first analytical part with the Student's t-test due to the lower statistical power of ANOVA test and the new smaller groups. First we discovered a significant decrease in carbonyl/total of DDM extract between controls and MCI ($p = 0.042$). A decreasing trend in carbonyl/total and total carbonylation was also observed between controls and AD ($p = 0.057$ and 0.095 respectively). Then the carbonyl/total of the cytoskeleton fraction (DC) tended towards a diminution in MCI and AD compared to controls ($p = 0.059$ and 0.054, respectively). Furthermore, while total S-nitrosylated α spectrin had a decreasing trend in MCI in comparison to controls ($p = 0.054$), total S-nitrosylated β spectrin was significantly decreased ($p = 0.008$).

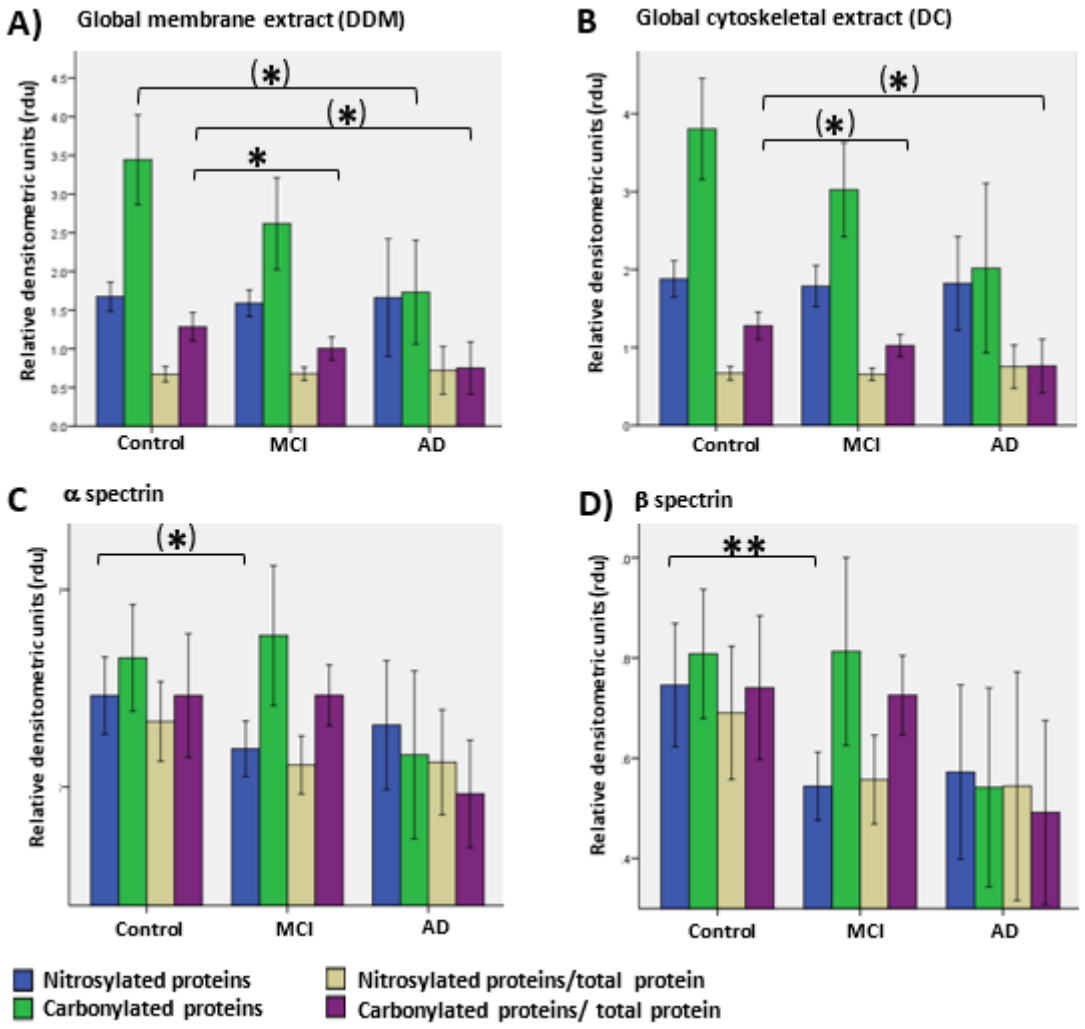

Figure 14. Quantification by densitometry and statistical analysis by One-Way ANOVA of RBC membrane proteins according to the CDRSOB score. Controls have been selected with a CDRSOB score = 0, MCI had a score between 0.5 and 4.5, and AD had a score between 4.5 and 18. Statistical significance ($p < 0.05$) is identified by an asterisk * on the histograms, high significance ($p < 0.01$) by two asterisks ** and decreasing trend ($0.05 < p < 0.1$) by an asterisk between parentheses (*).

Part III. Comparison of MCI and AD

In the third part, we excluded the controls (CDR=0) and discriminated MCI and AD subjects according to the molecular biomarker (*p*-tau^{181}/Aβ_{42} ratio < 0.07794 and > 0.07794, respectively). We tried to be more specific in our analyses with the restriction, but since the cohort decreased by 120 to 69 subjects, the significance also decreased and no significant results were obtained between MCI and AD subjects (the CI group) (*not shown*).

Then we analysed the controls, the MCI group and AD patients with ANOVA and Tukey tests for the multiple comparisons. Interestingly, we found globally significant differences between the controls and Alzheimer's disease subjects. In short, global RBC-integral membrane (DDM extract) and cytoskeletal (DC extract) proteins in the AD group were significantly less carbonylated than in the controls (*not shown*). In addition, α and β spectrins were found less nitrosylated in the AD group compared to the controls (*not shown*).

Part IV. Logistic regression analysis

In the last part, we assessed the categorical outcomes with logistic regressions analyses in order to consolidate our results. The generalized linear models have been used with additional variables including age, gender and APOEε4. Given that no significance has been found, more statistical analyses should be performed. More specifically, hierarchical analysis – which has more statistical power – will be preferred to the generalized additive model which has been used.

c. 1-DE: Summary of results

Our study's aim was to establish a protocol to measure biomarkers of oxidative stress in relation with neurodegenerative disease. The biomarkers that we chose were the protein modifications, more precisely red blood cell proteins in nitrosylated and carbonylated states. Then we compared the levels of oxidative stress biomarkers in blood from controls, MCI subjects and AD patients. The samples were analysed first by one-dimensional electrophoresis followed by western blot with infrared detection.

Part I. Oxidative levels of pathological groups compared to controls					
		Carbo	**Carbo/total**	**Nitro**	**Nitro/total**
Pathological *p*-tau^{181}/Aβ$_{42}$ ratio	DDM extract		↓		
	DC extract	↓	↓↓	↓	
	α spectrin			↓	↓
	β spectrin			↓	(↓)
Pathological APOEε4 carrier	DDM extract				
	DC extract				
	α spectrin				
	β spectrin			↓↓	
Pathological CDR score (CI group)	DDM extract	↓	↓↓		
	DC extract	↓	↓↓		
	α spectrin			↓	(↓)
	β spectrin			↓↓	(↓)
Part II. Oxidative levels of MCI and AD compared to controls					
		Carbo	**Carbo/total**	**Nitro**	**Nitro/total**
Mild Cognitive Impairment	DDM extract		↓		
	DC extract		(↓)		
	α spectrin			(↓)	
	β spectrin			↓↓	
Alzheimer's disease	DDM extract	(↓)	(↓)		
	DC extract		(↓)		
	α spectrin				
	β spectrin				
Part. III. Comparison of MCI and AD defined by *p*-tau^{181}/Aβ$_{42}$ ratio					
No significant results were obtained comparing MCI and AD, but AD subjects showed a significant decrease of carbonylated proteins in DDM and DC extracts, compared to the controls (CDR = 0). Furthermore, AD showed a decrease of nitrosylated α and β spectrins in comparison to the controls.					
Part IV. Logistic regression analysis					
No significant results were found with the generalized linear model including age, gender and APOEε4.					

Table 2. Summary table of results. The levels of oxidation in pathological groups were compared to controls and resumed in this table. Significant decrease ($p < 0.05$) in the pathological groups compared to controls is reported with an arrow ↓, high significant decrease ($p < 0.01$) with two arrows ↓↓ and a decreasing trend ($0.05 < p < 0.1$) with an arrow between

parentheses (↓). Empty cell translate no significance difference between the pathological group and controls. The level of carbonylated RBC proteins was abbreviated as "carbo" and divided by the total proteins, reported as "carbo/total". The level of nitrosylated RBC proteins was abbreviated as "nitro" and also divided by the total proteins, reported as "nitro/total". DDM: integral membrane extract. DC: cytoskeletal extract. Note the trend of spectrins to be less nitrosylated in pathological groups compared to controls, while DDM and DC extracts have a tendency to be less carbonylated.

Our first analyses with Student's test showed mainly significant decrease of carbonylation in the group with pathological *p*-tau^{181}/Aβ_{42} ratio – which is related to Alzheimer's disease diagnosis – and in the CI group determined by the CDR score. Concerning α and β spectrins in brief, they were particularly less nitrosylated in the same groups. In the second part, the results obtained by the One-Way ANOVA analysis were less significant, but also showed a global decreasing trend of carbonylated red blood cell proteins in the MCI group, and even more in the AD group, in comparison to the controls. Spectrins were less nitrosylated in the MCI subjects compared to controls subjects.

In conclusion our results suggested that levels of oxidative and nitrosative stress in blood decrease with the progression of the molecular lesions in brain and the decline of cognitive functions.

d. 2-DE gels: Silver nitrate and Western blot

Further results were obtained by Irene M. Riederer, who analysed the same RBC-membrane proteins by two-dimensional electrophoresis. 2D gels were stained with silver nitrate to see the global composition of proteins and were also prepared for Western blot in order to detect carbonylated and nitrosylated proteins (**Figure *15***). On the 2D silver-stained gels, some spots of proteins appeared differently in CI subjects and controls; proteins that were present on CI sample were absent in control sample, and vice versa. Afterward these proteins were not visible in carbonylated or nitrosylated state in Western blot. Four spots were analysed by MALDI-TOF MS in order to identify the differences in the composition of proteins.

We identified degradation products of band 3 anion transport protein on the four chosen spots (**Figure *15 A and D***). Interestingly, we found a heat shock protein of 71 kDa and an elongation factor 2 of 95 kDa on the spot number 4 in the CI patient (**Figure *15 D***). We can suppose that it is related to the heat shock response and the induction of the repair machinery to decrease the oxidative damages. One should do more immunoblotting to identify repetitive and significant differences between the two groups.

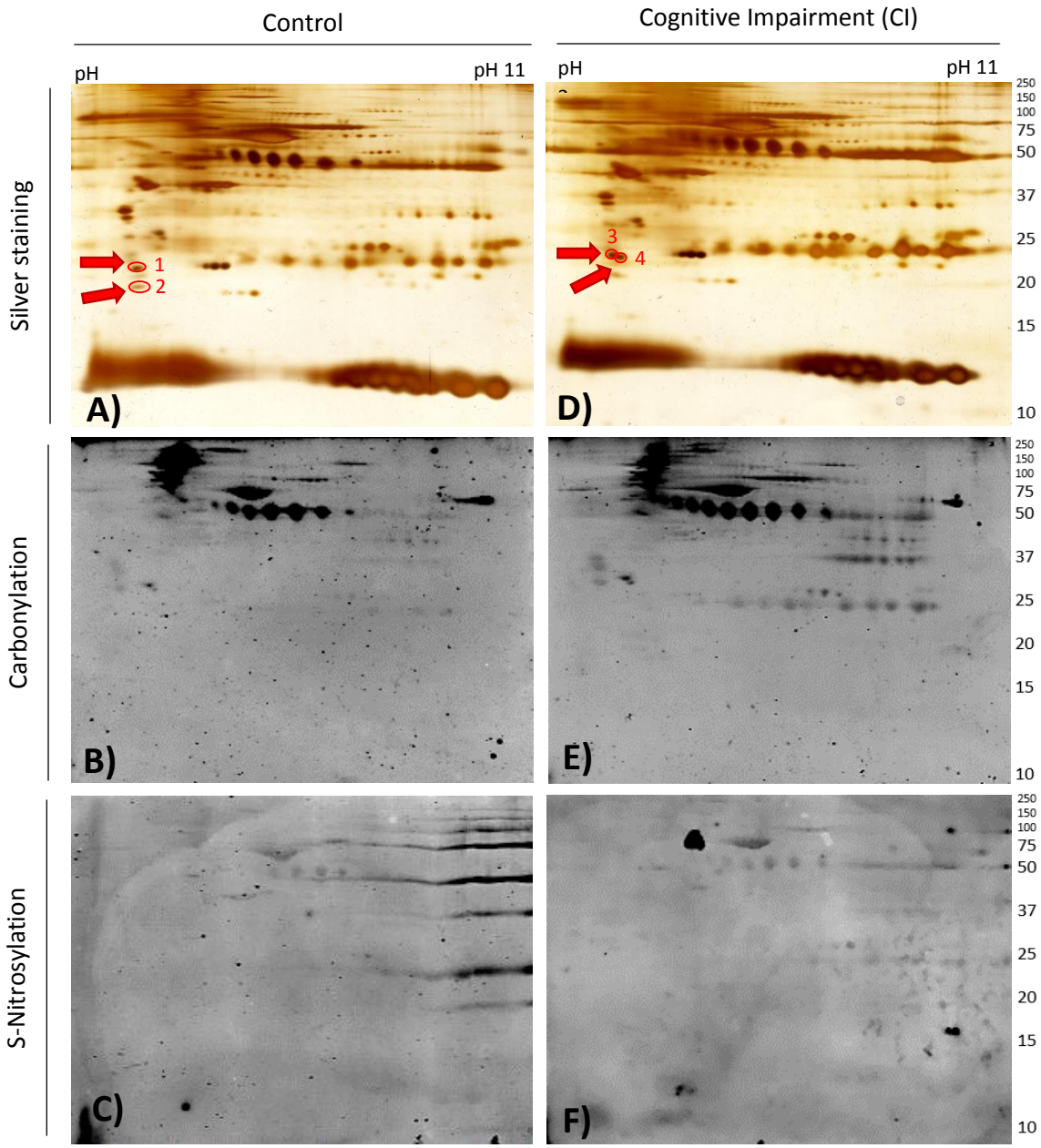

Figure 15. SDS-PAGE of RBC membrane extracts. Proteins were separated by 2-DE 4 % - 13 % gradient gels. Gels were stained with silver nitrate (A and D) or immunostained with anti-DNP (B and E) or anti-S-nitrocysteine antibody (C and F). The control subjects have been selected by their CDR score = 0 and the absence of cognitive impairment. The CI patients had a CDR score ≥ 0.5, and included both MCI and AD patients. One should note that panel A and B show different spots of proteins in CI patient and control subject (red circles and arrows). Four spots (1, 2, 3 and 4) have been selected for the analysis by MALDI-TOF MS. Degradation products of band 3 were identified on the four spots in control and CI subjects. In addition, a heat shock protein (hsp27) and an elongation factor 2 (EF2) were found on the spot n°4 that was lacking in the control sample.

V. Discussion

The first aim of our project was to establish a protocol in order to measure oxidation of red blood cell proteins. Then our main hypothesis stated that oxidative stress occurs at an early stage of Alzheimer's disease, contributing to AD pathogenesis. This hypothesis was already tested in some research projects [1, 2, 26] and we tested it in our project by analysing the oxidative stress in blood from controls, MCI and AD patients. Carbonylation and S-nitrosylation of integral membrane and cytoskeletal proteins of RBC were selected as biomarkers of OS. They were assessed by 1-DE and 2-DE, followed by fluorescent immunedetection as described in previous papers [12, 13, 14, 16].

We first analysed *p*-tau^{181}/Aβ_{42} ratio, the CSF biomarker which reinforces AD diagnosis when it is elevated [43, 59]. An increased level of *p*-tau^{181} and a decreased level of Aβ_{42} reflect respectively the levels of neurofibrillary tangles and β-amyloid deposition in brain tissue [2]. The analyses demonstrated that oxidative and nitrosative stress decreased in the group with positive CSF biomarker for Alzheimer's disease (**Figure *11***). Our results suggest that levels of oxidative stress in the erythrocytes decrease with the progression of the molecular lesions in brain. Therefore it suggests that oxidative/nitrosative stress occurs early in AD pathogenesis seen as an oxidative stress response in peripheral tissue. Experimental models on transgenic mice have already shown that markers of oxidative/nitrosative stress precede the onset of histopathological changes [51]. Moreover our results are in accordance with the study in human brain by Akihiko Nunomura et al., who found that an increase in Aβ plaques during the progression of AD was associated with a decreased level of oxidised nucleoside derived from RNA – another oxidative damage biomarker [1]. In conclusion our results concerning the histopathological hallmarks and oxidative damages are in accordance with previous papers supporting that oxidative stress levels decrease with progression of the neuronal lesions [2, 26]. Secondly we discovered that our results suggest also that levels of oxidative stress in blood decrease with the decline of cognitive functions according to the clinical features of the CDR and CDRSOB score. For example, β spectrin was found significantly more nitrosylated in controls compared to CI patients (both MCI and AD patients) determined by CDR and CDRSOB score (**Figure *13*** and **Figure *14*** respectively). Thus this trend might support our hypothesis suggesting oxidative stress is an early event in AD pathogenesis and decrease with the disease progression. However, more precisely, the OS seems to have a role before the establishment of cognitive impairment. Thirdly APOEε4 allele carriers seemed to have a β spectrin less oxidized compared to the non-carriers in our results (**Figure *12***). This APOE polymorphism is known to be a genetic risk factor for Alzheimer's disease, and several studies demonstrated an association between APOEε4 and diseases modulated by oxidative stress as related by Laia Jofre-Monseny et al. [67]. They provide that APOEε4 is associated with an increase in oxidative stress in post-mortem brains of AD subjects, which was not directly demonstrated by our discoveries in blood, given that nitrosylated β spectrin decreased in the presence of APOEε4 allele.

Comparing our results in blood and other recent projects in brain we noticed that the levels of oxidative stress biomarkers follow a different pattern. For example, proteins carbonyls have been observed at high levels in the brain of MCI and AD patients compared to controls independently of APOE genotype [37, 58]. Interestingly, Zeinab El Hajj, PhD student of Riederer's laboratory has shown that brain spectrin was more carbonylated and nitrosylated in frontal cortex of AD and MCI subjects in comparison to controls, supporting findings about protein carbonyls in AD brain [18, 37, 38, 58]. These findings suggest that brain tissue behaves differently from blood against oxidative damages, and we will try to explain these different mechanisms.

In a nutshell, studies on brain showed an increasing trend of oxidized proteins, while studies on blood demonstrated a decreasing level of oxidative biomarkers in neurodegenerative diseases. To explain this different trend of our blood biomarkers compared to the studies in the brain, we can suppose that oxidative damages of brain can't be well repaired or eliminated contrary to RBC damages. First of all, the average life span of erythrocytes is 120 days [62], which means that damaged membrane proteins are removed within 120 days too. Then, with the decrease in oxidative stress levels, the new erythrocytes produced should not contain oxidised proteins any more. Red blood cells have the capacity of regeneration, in contrary to the brain, where damages are permanent due to the limited capacity of neuronal cells regeneration. But this explanation is not sufficient to decrease significantly the oxidative state in the peripheral tissue compared to the central nervous system (CNS). First of all, the CNS is very sensitive to the oxidants because of its high lipid content, high consumption of oxygen and many oxidative reactions [21, 76]. The brain has his defense mechanisms against excessive oxidative stress, such as the blood, but its antioxidant system is clearly not sufficient. The antioxidant enzymes in the CNS include the SOD, glutathione peroxidase and catalase, and the non-enzymatic system includes vitamins, as well as proteins and amino acids. However these endogenous defenses are not sufficient and unfortunately antioxidant enzymes from blood do not cross the blood brain barrier (BBB). The BBB, consisting in endothelial cells connected by tight junctions, separates the blood from the brain and selects the molecules to pass in order to protect the CNS. Water, gazes and some other molecules can pass, but antioxidant enzymes can't pass across the BBB. Thus, the enzymes circulating in blood may not counteract increasing oxidation lesions in neurons [75, 76].

Then we refer to the hypothesis stipulating that the antioxidant defense – enzymatic and non-enzymatic – increases in response to an increased oxidative/nitrosative stress [21, 23, 70]. Enzymatic antioxidant defenses of the human body, such as the glutathione, the superoxide dismutase, the catalase and the peroxiredoxin, help to decrease the oxidative damages in blood [15, 17, 69, 70]. The peroxiredoxins are antioxidant enzymes which reduce hydrogen peroxide. But when the levels of peroxide increase, the peroxiredoxins become overoxidized and their enzymatic activity are reduced, generating an elevation in ROS for redox signalling. This inhibition is due to the formation of a sulfinic acid (SO_2H) by the overoxidation of the peroxidatic cysteine of the peroxiredoxin [69]. As observed in **Figure *10***, peroxiredoxin was less oxidized in the CI patient blood than in controls, suggesting that there was more enzymatic activity in the pathological situation, in order to reduce oxidized proteins. In the control situation, peroxiredoxins were stronger oxidized, meaning that oxidation was reducing enzymatic activity, allowing an oxidative burst. Finally we conclude to an elevation of the antioxidant capacity in the CI patient in response to the oxidative stress. It could be interesting to measure the levels of the glutathione and peroxiredoxins in the patients' blood in future, to assess the progression of its level with the progression of the disease. Thus we could see if OS is compensated by the antioxidant capacity, translating a balanced redox system.

Besides these antioxidant enzymes, our results of 2-DE gels showed an increase in heat shock protein (Hsp) 27 in CI patient's blood compared to the control (***Figure 15***). Thus we raised the possibility of the heat shock protein increase in pathological conditions, translating an elevation of OS response protein in CI patients (including MCI and AD). This response to ROS/RNS has already been observed [33]. Its role is to prevent cell dysfunction partly due to misfolded and aggregated proteins. Chaperones can induce pathways that lead to stress responses, which help to protect from a stress damage. They assist in refolding or degrading the misfolded proteins, in order to stabilize them and avoid aggregation [26]. More precisely, the activation of the proteasome is partly due to the Hsp27, and permits the degradation of the misfolded and oxidized proteins [71]. When these latter cannot be refolded by the chaperones, they are ubiquitinated for degradation by the proteasome.

Unfortunately, heavily oxidized proteins inhibit the UPS and cannot be degraded, leading to an aggregation and cell injury. Proteasome and chaperones dysfunction occurs in neurodegenerative disorders, probably due to the inhibition by oxidative stress [26, 71]. Furthermore heat shock proteins have an anti-apoptotic role, by a direct inhibition or by preventing the activation of caspase-3 [71]. Concerning Hsp27, it is constitutively expressed in the cortex of the brain and it is also inducible in glial and neuronal cells in response to stressors, including ischemia, hyperthermia and psychological stressors. Interestingly, some studies demonstrated that high serum levels of Hsp27 was a marker of myocardial ischemia and incipient chronic obstructive pulmonary disease. In conclusion, Hsp27 might modulate ROS and have neuroprotective effects. The relation between this chaperone protein and Alzheimer's disease is not well established, but phosphorylated Hsp27 was observed before in neurodegenerative diseases and is supposed to counteract the effects of oxidative damages in the CNS [71]. This hypothesis is compatible with our results obtained in the 2-DE gels.

Furthermore, we identified an additional spot in CI patient which was absent in control subject (**Figure *15***). The identification by MALDI-TOF MS showed an elongation factor 2 (EF2), which is a protein substrate of a Ca^{2+}/calmodulin-dependent enzyme, named EF2 kinase. The EF2 is involved in protein synthesis and its activity is inhibited when it is phosphorylated by the EF2 kinase. Philippe Marin et al. reported that glutamate in neurons – the major excitatory neurotransmitter – enhances Ca^{2+} influx, EF2 kinase activation and EF2 phosphorylation, leading to EF2 inhibition and therefore to protein synthesis inhibition. Researchers showed that EF2 kinase was activated in anoxic conditions and with overstimulation by glutamate. They discovered that the protein synthesis inhibition protected from excitotoxicity and delayed neuronal death [72, 73]. We can suppose then that EF2 plays a neuroprotection role in Alzheimer's disease.

Apart the differences between the brain and the blood, it is important to identify and understand the significant differences between normal aging and neurodegenerative diseases. Metabolic processes participating in physiological redox signalling produce reactive oxygen/nitrogen species, which are known to cause damages when the redox balance is lost [23, 69]. In fact, these damages increase with aging and even more in some diseases, while anti-oxidants systems decrease with aging [24]. Researchers are still studying the specific and reliable markers in Alzheimer's disease, comparing factors that prime in MCI and AD subjects compared to age-matched controls. Interestingly, although carbonylated proteins increase both during normal aging and neurodegenerative diseases, some proteins are selectively more oxidized in some diseases. For example, human brain copper-zinc SOD has an isoform which is heavily carbonylated in AD subjects compared to a normal aging brain in controls [26]. Moreover, some blood and brain biomarkers prime in MCI and AD subjects compared to normal aging controls (*i.e.* not accelerated aging). These studied biomarkers include several oxidized proteins, lipid peroxidation and nucleic acids oxidation [17, 18, 21, 23, 34, 35, 37, 56]. In this project we choose to study carbonylated and S-nitrosylated red blood cell membrane proteins, which have been associated with AD [20, 25, 37, 42]. Interestingly S-nitrosylation of RBC-cytoskeleton has been associated with the improvement in RBC deformability [25], which has been reported to be significantly decreased in AD subjects by Joy G Mohanty et al., causing some neurodegenerative lesions [33]. These discoveries lead us to suppose that nitrosylated proteins of red blood cell cytoskeleton decreases with the progression of AD, which is compatible with our results showing a decrease in nitrosylated α and β spectrins. Concerning the reason of the decrease in carbonylation, Julien Delobel, PhD of "Ma Vie Ton Sang laboratory", hypothesized that oxidative alteration can cause a cytoskeleton and membrane destabilization during blood storage. He also found that the state of carbonylation decreased in the DDM membrane extract after four weeks of storage. Julien Delobel supposed the elimination of the carbonylated proteins by the microvesiculation process,

which might be triggered by oxidative damages of RBC-cytoskeleton proteins [68]. This process consists in a membrane vesiculation, which releases microparticles, allowing the elimination of oxidized membrane and cytoskeletal proteins. This release of vesicles might have a protective role. Indeed, studies showed that RBC-membrane vesiculation correlates with spectrin oxidation in stored RBCs [70, 74]. This microparticulation is then supposed to be a defense mechanism of red blood cells against oxidative damages and could explain our results. In conclusion, nitrosylation and carbonylation can dysregulate RBC deformability and function, probably contributing to neuronal lesions in neurodegenerative diseases related to oxidative damages.

VI. Conclusion and outlook

Alzheimer's disease is one of the world's biggest problem due to the important number of people affected according to the World Health Organization (WHO). Although the cause is not clearly understood, some hypotheses are well established, such as the β-amyloid cascade hypothesis [2]. Moreover, much data points to a significant role of oxidative stress and inflammation as early events in the disease or appearing before cognitive disorder [23, 64]. In order to better understand the relation between the levels of oxidative stress and the cognitive impairment in Alzheimer's disease we processed to analyse the red blood cell protein modifications. This method, which gave significant results, points to new reliable biomarkers that can be used to set up diagnostic tools to measure changes in peripheral tissue in AD or other neurodegenerative diseases.

The results showed that the blood of MCI and AD patients (CI group) contained lesser oxidized RBC-proteins than controls. This discovery might suggest that oxidative stress is an early event in dementia [26]. In addition, we found that RBC-spectrin is affected by a decrease in S-nitrosylation, while the total RBC-membrane proteins are affected by a decrease in their carbonylated state. The lesser nitrosylation of spectrins may be correlated with the diminution of RBC deformability, oxygen exchange and consequently leading to brain damages [25, 33].

The next step will be to apply our protocol in the CSF to assess the trend of the oxidative stress in comparison to peripheral blood. Some studies in Julius Popp's laboratory analysed the inflammation biomarkers in CSF and found some significant differences between AD subjects and controls. As we know that inflammation and oxidative stress coexist in AD brain [64], we would like to see if OS also shows significant differences in oxidation levels in CSF. As concomitant analyses we can keep on with the 2-DE gels in order to detect a variance in the total, the carbonylated and the nitrosylated proteins in blood between controls, MCI, and AD. Then it would be interesting to perform a longitudinal study following the levels of oxidised proteins in relation with the AD progression in order to observe if the OS keeps on decreasing, supporting the hypothesis that OS decreases with the cognitive decline. Furthermore, the relation between OS in blood and AD should be more studied by measuring the capacity of antioxidant mechanisms, although a similar project has already been carried out and didn't find any significant results [65]. To estimate the antioxidant status in the subjects, we can use an antioxidant assay kit (Chemie Brunschwig AG, Basel), which measures the total antioxidant capacity of plasma and serum. We can also precisely measure the peroxiredoxin level, which appeared to be interesting in our results, or even the gene expression of the heat shock proteins. Finally the final confirmation would be to have the definite diagnosis of AD and measure oxidative stress levels in post-mortem brain tissue.

This project may be useful to develop diagnostic tools to measure oxidative stress in peripheral tissues and lead to a screening test in the future. Then we will probably need to understand better how antioxidants can prevent oxidative stress in neurodegenerative diseases in order to find optimal

nutrition or antioxidant therapies for this public-health concern. In conclusion our results allowed us to better understand the AD pathogenesis in relation with oxidative stress.

Study limitations

We obtained interesting and significant results in our study, however we have to mention some limitations of this project. First of all, there is the selection bias of the subjects, because the two main groups, namely the CI and control groups, have not been selected in the same way, although they were considered independent. As described before, the first group included patients who suffer from cognitive complaints and the second group included in part their spouses. Thereby our oxidative measures may not be representative of the dwellers-community.

Moreover, we have to keep in mind that the levels of oxidized proteins in blood are modulated by nutrition, exercise training, cognitive activities and diseases [5, 6, 11], thus, may lead to a variability of oxidative stress between subjects. Not only this information was not available for a more detailed analysis, but also the comorbidities were not controlled and considered having an impact in the oxidative parameters. In addition, APOEε4 groups have not been separated in heterozygotes and homozygotes. Concerning the statistical analysis, we observed a heterogeneity in the analysed groups. We considered that the groups were independent, but there are many variables that overlap, which lead to a difficult interpretation of our results. Further, we should have a larger cohort in order to detect minimal significant differences.

Finally we developed a new method to measure the levels of oxidative and nitrosative stress in blood, but some parameters have to be improved to be sure that our protocol reflects the real oxidative status in blood contributing to Alzheimer's disease. Nevertheless the oxidative stress is not AD specific and has been observed in a large panel of diseases, decreasing the chance to develop a specific screening test for AD. It is difficult to conclude a real correlation between OS and AD, as well it is difficult to understand the relation between clinical aging and molecular lesions. Given that the causality is not evident, we can just suppose that OS is related to the accelerated aging in Alzheimer's disease. Even with these limitations, our results followed a significant trend and provide us some possibilities for future prevention strategies.

VII. Abbreviations

1- and 2-DE = one- and two-dimensional electrophoresis
$A\beta_{42}$ = isoform 42 of β-amyloid
AD = Alzheimer's disease
APOEε4 = allele ε4 of apolipoprotein E
BBB = brain blood barrier
CSF = cerebrospinal fluid
DC = cytoskeletal membrane extract
DDM = integral membrane extract
EF = elongation factor
Hsp = heat shock protein
MCI = mild cognitive impairment
MMSE = mini mental state examination
MS = mass spectometry
NFT = neurofibrillary tangles
OS = oxidative stress
p-tau = phosphorylated tau protein
RBC = red blood cell
SDS-PAGE = Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis
SOD = superoxide dismutase
UPS = ubiquitin-proteasome system

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IX. References

1. Nunomura A, Perry G, Aliev G, Hirai K, Takeda A, Balraj EK, et al. Oxidative damage is the earliest event in Alzheimer disease. Journal of neuropathology and experimental neurology. 2001 Aug 6;60(8).
2. Querfurth HW, LaFerla FM. Alzheimer's Disease. New England Journal of Medicine. 2010 Jan 28;362(4):329–44.
3. Mohajeri MH, Leuba G. Prevention of age-associated dementia. Brain research bulletin. 2009;80(4-5):315–25.
4. Jorissen BL, Riedel WJ. Nutrients, age and cognition. Clinical Nutrition. 2002 Feb;21(1):89–95.
5. Polidori MC, Praticó D. High fruit intake is positively correlated with antioxidant status and cognitive performance in healthy subjects. Journal of Alzheimer's disease : JAD. 2009;17(4):921–7.
6. Rinaldi B, Corbi G, Boccuti S, Filippelli W, Rengo G, Leosco D, et al. Exercise training affects age-induced changes in SOD and heat shock protein expression in rat heart. Exp Gerontol. 2006 Aug;41(8):764–70.
7. Kang JH, Ascherio A, Grodstein F. Fruit and vegetable consumption and cognitive decline in aging women. Annals of Neurology. 2005 May 1;57(5):713–20.
8. Morris MC, Evans DA, Tangney CC, Bienias JL, Wilson RS. Associations of vegetable and fruit consumption with age-related cognitive change. Neurology. 2006 Oct 24;67(8):1370–6.
9. Gómez-Pinilla F. Brain foods: the effects of nutrients on brain function. Nature Reviews Neuroscience. 2008 Jul 1;9(7):568.
10. Colcombe S, Kramer AF. Fitness Effects on the Cognitive Function of Older Adults A Meta-Analytic Study. Psychological Science. 2003 Mar 1;14(2):125–30.
11. Hall CB, Lipton RB, Sliwinski M, Katz MJ, Derby CA, Verghese J. Cognitive activities delay onset of memory decline in persons who develop dementia. Neurology. 2009 Apr 8;73(5):356–61.
12. Riederer BM. Non-covalent and covalent protein labeling in two-dimensional gel electrophoresis. Journal of Proteomics. 2008 Jul 21;71(2):231–44.
13. Riederer IM, Herrero RM, Leuba G, Riederer BM. Serial protein labeling with infrared maleimide dyes to identify cysteine modifications. J Proteomics. 2008 Jul 21;71(2):222–30.
14. Riederer IM, Riederer BM. Differential protein labeling with thiol-reactive infrared DY-680 and DY-780 maleimides and analysis by two-dimensional gel electrophoresis. Proteomics. 2007 Jun;7(11):1753–6.
15. Riederer BM, Leuba G, ElHajj Z, Gerster F, Kraftsik R, Riederer IM. "Oxidation and proteasome dysfunction in aging and neurodegeneration", Acta Neuropathologica. 2013
16. Riederer IM, Schiffrin M, Kövari E, Bouras C, Riederer BM. Ubiquitination and cysteine nitrosylation during aging and Alzheimer's disease. Brain Research Bulletin. 2009 Oct 28;80(4–5):233–41.
17. Riederer BM. Oxidation proteomics: The role of thiol modifications. Current Proteomics. 2009;6:51–62.
18. Praticò D. Oxidative stress hypothesis in Alzheimer's disease: a reappraisal. Trends in Pharmacological Sciences. 2008 Dec;29(12):609–15.
19. Riederer BM, Leuba G, Vernay A, Riederer IM. The role of the ubiquitin proteasome system in Alzheimer's disease. Exp Biol Med (Maywood). 2011 Jan 3;236(3):268–76.

20. Nakamura T, Tu S, Akhtar MW, Sunico CR, Okamoto S-I, Lipton SA. Aberrant protein s-nitrosylation in neurodegenerative diseases. Neuron. 2013 May 22;78(4):596–614.
21. Rivas-Arancibia S, Gallegos-Ríos C, Gomez-Crisostomo N, Ferreira-Garcidueñas E, Briseño DF, Rodríguez-Martínez LN and E. Oxidative Stress and Neurodegenerative Disease. 2011 Dec 9 [cited 2015 Nov 9]; Available from: http://www.intechopen.com/books/neurodegenerative-diseases-processes-prevention-protection-and-monitoring/oxidative-stress-and-neurodegenerative-disease
22. Reitz C, Mayeux R. Alzheimer disease: epidemiology, diagnostic criteria, risk factors and biomarkers. Biochem Pharmacol. 2014 Apr 15;88(4):640–51.
23. Harman D. Free radical theory of aging. Mutation Research/DNAging. 1992 Sep 1;275(3):257–66.
24. Harman D. Free Radical Theory of Aging: An Update. Annals of the New York Academy of Sciences. 2006 May 1;1067(1):10–21.
25. Grau M, Pauly S, Ali J, Walpurgis K, Thevis M, Bloch W, et al. RBC-NOS-Dependent S-Nitrosylation of Cytoskeletal Proteins Improves RBC Deformability. PLoS ONE. 2013 Feb 12;8(2):e56759.
26. Dalle-Donne I, Aldini G, Carini M, Colombo R, Rossi R, Milzani A. Protein carbonylation, cellular dysfunction, and disease progression. Journal of Cellular and Molecular Medicine. 2006 Apr 1;10(2):389–406.
27. Molitoris BA, Dahl R, Hosford M. Cellular ATP depletion induces disruption of the spectrin cytoskeletal network. American Journal of Physiology - Renal Physiology. 1996 Oct 1;271(4):F790–8.
28. Luna EJ, Hitt AL. Cytoskeleton-plasma membrane interactions. Science. 1992 Jun 11;258(5084):955–64.
29. Winkelmann JC, Forget BG. Eythroid and nonerythroid spectrins. Blood. 1993;81(12):3173–85.
30. Bennett V. Spectrin-based membrane skeleton: a multipotential adaptor between plasma membrane and cytoplasm. Physiological Reviews. 1990 Oct 1;70(4):1029–65.
31. Goodman SR, Shiffer K. The spectrin membrane skeleton of normal and abnormal human erythrocytes: a review. American Journal of Physiology - Cell Physiology. 1983 Mar 1;244(3):C121–41.
32. Bosman GJ, Bartholomeus IG, de Man AJ, van Kalmthout PJ, de Grip WJ. Erythrocyte membrane characteristics indicate abnormal cellular aging in patients with Alzheimer's disease. Neurobiol Aging. 1991 Feb;12(1):13–8.
33. Mohanty JG, Shukla HD, Williamson JD, Launer LJ, Saxena S, Rifkind JM. Alterations in the red blood cell membrane proteome in alzheimer's subjects reflect disease-related changes and provide insight into altered cell morphology. Proteome Sci. 2010 Mar 3;8:11.
34. Butterfield DA, Hardas SS, Bader Lange ML. Oxidatively Modified Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and Alzheimer Disease: Many Pathways to Neurodegeneration. J Alzheimers Dis. 2010;20(2):369–93.
35. Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. Protein carbonyl groups as biomarkers of oxidative stress. Clinica Chimica Acta. 2003 Mar;329(1–2):23–38.
36. Dalle-Donne I, Rossi R, Giustarini D, Gagliano N, Lusini L, Milzani A, et al. Actin carbonylation: from a simple marker of protein oxidation to relevant signs of severe functional impairment. Free Radic Biol Med. 2001 Nov 1;31(9):1075–83.
37. Beal MF. Oxidatively modified proteins in aging and disease. Free Radical Biology and Medicine. 2002 May 1;32(9):797–803.
38. Sihag RK, Cataldo AM. Brain beta-spectrin is a component of senile plaques in Alzheimer's disease. Brain Res. 1996 Dec 16;743(1-2):249–57.
39. O'Bryant SE, Waring SC, Cullum CM, Hall J, Lacritz L, Massman PJ, et al. Staging Dementia Using Clinical Dementia Rating Scale Sum of Boxes Scores. Arch Neurol. 2008 Aug;65(8):1091–5.
40. McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM. Clinical diagnosis of Alzheimer's disease Report of the NINCDS-ADRDA Work Group* under the auspices of

Department of Health and Human Services Task Force on Alzheimer's disease. Neurology. 1984 Jan 7;34(7):939–939.
41. Lewczuk P, Kamrowski-Kruck H, Peters O, Heuser I, Jessen F, Popp J, et al. Soluble amyloid precursor proteins in the cerebrospinal fluid as novel potential biomarkers of Alzheimer's disease: a multicenter study. Mol Psychiatry. 2010 Feb;15(2):138–45.
42. Wagner M, Wolf S, Reischies FM, Daerr M, Wolfsgruber S, Jessen F, et al. Biomarker validation of a cued recall memory deficit in prodromal Alzheimer disease. Neurology. 2012 Jul 2;78(6):379–86
43. Hort J, O'Brien JT, Gainotti G, Pirttila T, Popescu BO, Rektorova I, et al. EFNS guidelines for the diagnosis and management of Alzheimer's disease. Eur J Neurol. 2010 Oct;17(10):1236–48.
44. Ning-Liu "How to Bolster Your Blot Confidence: New Approaches to Verification." Bioscience Technology. Accessed November 9, 2015. Available from : http://www.biosciencetechnology.com/articles/2013/10/how-bolster-your-blot-confidence-new-approaches-verification
45. Kaufman AS. Test Review: Wechsler, D. Manual for the Wechsler Adult Intelligence Scale, Revised. New York: Psychological Corporation, 1981. Journal of Psychoeducational Assessment. 1983 Jan 9;1(3):309–13.
46. D Cardebat BD. Formal and semantic lexical evocation in normal subjects. Performance and dynamics of production as a function of sex, age and educational level [in French]. Acta neurologica Belgica. 1990;90(4):207–17.
47. Lawton MP, Brody EM. Assessment of Older People: Self-Maintaining and Instrumental Activities of Daily Living. The Gerontologist. 1969 Sep 21;9(3 Part 1):179–86.
48. Braak H, Braak E. Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol. 1991;82(4):239–59.
49. Whitwell JL, Clifford RJ, Przybelski SA, Parisi JE, Senjem ML, Boeve BF, et al. Temporoparietal atrophy: a marker of AD pathology independent of clinical diagnosis. Neurobiol Aging. 2011 Sep;32(9):1531–41.
50. Furger P et al. Gériatrie – Psychiatrie. Surf Guidelines Médecine Interne Générale, 5th ed. Switzerland : D&F Sàrl/GmbH ; 2012. p. 1176-1186
51. Apelt J, Bigl M, Wunderlich P, Schliebs R. Aging-related increase in oxidative stress correlates with developmental pattern of beta-secretase activity and beta-amyloid plaque formation in transgenic Tg2576 mice with Alzheimer-like pathology. International Journal of Developmental Neuroscience. 2004 Nov;22(7):475–84.
52. Folstein MF, Folstein SE, McHugh PR. "Mini-mental state". A practical method for grading the cognitive state of patients for the clinician. J Psychiatr Res. 1975 Nov;12(3):189–98.
53. Morris JC. The Clinical Dementia Rating (CDR): Current version and scoring rules. Neurology. 1993 Nov; *43*(11), 2412-2414.
54. Buschke H, Sliwinski MJ, Kuslansky G, Lipton RB. Diagnosis of early dementia by the Double Memory Test Encoding specificity improves diagnostic sensitivity and specificity. Neurology. 1997 Jan 4;48(4):989–96.
55. Reitan RM. Validity of the trail making test as an indicator of organic brain damage. Perceptual and Motor Skills. 1958 Dec 1;8(3):271–6.
56. Subbarao KV, Richardson JS, Ang LC. Autopsy Samples of Alzheimer's Cortex Show Increased Peroxidation In Vitro. Journal of Neurochemistry. 1990 Jul 1;55(1):342–5.
57. Porchet R, Probst A, Bouras C, Dráberová E, Dráber P, Riederer BM. Analysis of glial acidic fibrillary protein in the human entorhinal cortex during aging and in Alzheimer's disease. Proteomics. 2003 Aug 1;3(8):1476–85.
58. Keller JN, Schmitt FA, Scheff SW, Ding Q, Chen Q, Butterfield DA, et al. Evidence of increased oxidative damage in subjects with mild cognitive impairment. Neurology. 2005 Apr 12;64(7):1152–6.
59. Blennow K, Hampel H, Weiner M, Zetterberg H. Cerebrospinal fluid and plasma biomarkers in

Alzheimer disease. Nature Reviews Neurology. 2010 Mar 1;6(3):131.
60. Hansson O, Zetterberg H, Buchhave P, Londos E, Blennow K, Minthon L. Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: a follow-up study. Lancet Neurol. 2006 Mar;5(3):228–34.
61. Philippon J. "Hydrocéphalie à Pression Normale". Psychologie & NeuroPsychiatrie Du Vieillissement 3, no. 1 (March 1, 2005): 53–61.
62. Franco RS. Measurement of Red Cell Lifespan and Aging. Transfus Med Hemother. 2012 Oct;39(5):302–7.
63. Kivipelto M, Solomon A. Alzheimer's disease — The ways of prevention. J Nutr Health Aging. 2008 Jan;12(1):S89–94.
64. Yao Y, Chinnici C, Tang H, Trojanowski JQ, Lee VM, Praticò D. Brain inflammation and oxidative stress in a transgenic mouse model of Alzheimer-like brain amyloidosis. Journal of Neuroinflammation. 2004 Oct 22;1(1):21.
65. Pulido R, Jiménez-Escrig A, Orensanz L, Saura-Calixto F, Jiménez-Escrig A. Study of plasma antioxidant status in Alzheimer's disease. Eur J Neurol. 2005 Jul;12(7):531–5.
66. Popp J, Lewczuk P, Frommann I, Kölsch H, Kornhuber J, Maier W, et al. Cerebrospinal fluid markers for Alzheimer's disease over the lifespan: effects of age and the APOEε4 genotype. J Alzheimers Dis. 2010;22(2):459–68.
67. Laia Jofre-Monseny A-MM. Impact of apoE genotype on oxidative stress, inflammation and disease risk. Mol Nutr Food Res 52:131-145 ([IF: 4.909]). Molecular nutrition & food research. 2008;52(1):131–45.
68. Delobel J, Prudent M, Rubin O, Crettaz D, Tissot J-D, Lion N. Subcellular fractionation of stored red blood cells reveals a compartment-based protein carbonylation evolution. Journal of Proteomics. 2012 Dec 5;76:181–93
69. Bak DW, Weerapana E. Cysteine-mediated redox signalling in the mitochondria. Mol Biosyst. 2015 Mar;11(3):678–97.
70. Çimen MYB. Free radical metabolism in human erythrocytes. Clinica Chimica Acta. 2008 Apr;390(1–2):1–11.
71. Franklin TB, Krueger-Naug AM, Clarke DB, Arrigo A-P, Currie RW. The role of heat shock proteins Hsp70 and Hsp27 in cellular protection of the central nervous system. Int J Hyperthermia. 2005 Aug;21(5):379–92.
72. Horman S, Browne GJ, Krause U, Patel JV, Vertommen D, Bertrand L, et al. Activation of AMP-Activated Protein Kinase Leads to the Phosphorylation of Elongation Factor 2 and an Inhibition of Protein Synthesis. Current Biology. 2002 Aug 20;12(16):1419–23.
73. Marin P, Nastiuk KL, Daniel N, Girault J-A, Czernik AJ, Glowinski J, et al. Glutamate-Dependent Phosphorylation of Elongation Factor-2 and Inhibition of Protein Synthesis in Neurons. J Neurosci. 1997 May 15;17(10):3445–54.
74. Tissot JD, Canellini G, Rubin O, Angelillo-Scherrer A, Delobel J, Prudent M, et al. Blood microvesicles: From proteomics to physiology. Advances in Integrative Medicine. 2013;1(1):38–52.
75. Gilgun-Sherki Y, Melamed E, Offen D. Oxidative stress induced-neurodegenerative diseases: the need for antioxidants that penetrate the blood brain barrier. Neuropharmacology. 2001 Jun;40(8):959–75.
76. Reiter RJ. Oxidative processes and antioxidative defense mechanisms in the aging brain. FASEB J. 1995 Apr;9(7):526–33.