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**Infrared spectroscopy : a reagent-free method to distinguish Alzheimer's disease patients from normal ageing subjects**

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## STATEMENT

**Background** : The advent of new therapies in the treatment and prevention of Alzheimer's disease (AD) requires early and differential diagnosis of AD. Fourier transform-infrared (FT-IR) spectroscopy is a rapid method for investigation of chemical changes. Our aim was to detect AD-related chemical modifications in plasma by FT- IR spectroscopy.

**Translational significance** : AD patients displayed distinct hierarchical classification in the whole mid-infrared region when compared to cognitive normal controls.

## ABSTRACT

Physiopathogenesis of Alzheimer's disease (AD) is related to various biochemical mechanisms that may be reflected by changes in plasma components. In the present study, Fourier transform-infrared (FT-IR) spectroscopy was used to identify these biochemical variations by monitoring spectral differences in the plasma of 40 AD patients compared to those of 112 control subjects. A hierarchical classification in the whole mid-infrared region allowed a clear separation between AD and controls (C) that was optimized by using a restricted spectral range (1480-1428  $\text{cm}^{-1}$ ). Spectral changes confirmed vibration differences between AD and C mostly related to modified lipid, and nucleic acid structures involved in oxidative stress-dependant processes of AD. Moreover, the analysis of samples in the 1480-910  $\text{cm}^{-1}$  region allowed the distinction between C and AD with an accuracy of 98.4% and showed two sub-groups  $C_1$  and  $C_2$  within the C group. Interestingly, the  $C_1$  sub-group was located closer to the AD group than the  $C_2$  sub-group, suggesting biochemical differences within the non-demented subjects. Biochemical studies revealed a significant increase in a specific marker of oxidative stress, F8-isoprostanes (8-epi-  $\text{PGF}2\alpha$ ) levels in the plasma of AD patients as compared to total controls and sub-group  $C_2$  but not sub-group  $C_1$ . Thus, these results suggest that use of FT-IR spectroscopy could be valuable to distinguish AD patients from normal ageing subjects.

**Running head :** FT-IR spectroscopy and Alzheimer's disease

**Abbreviations** FT-IR = Fourier-transform infrared; AD = Alzheimer's disease; OHdG = 8-hydroxydeoxyguanosine; 8-epi-PGF<sub>2</sub>α = 8-epi-prostaglandin F<sub>2</sub>α; CSF = cerebrospinal fluid; MMSE = Mini-Mental State Examination; MDA = malondialdehyde.

## **INTRODUCTION**

Alzheimer's disease (AD) is the major cause of cognitive impairment in the elderly and accounts for most of cases of dementia [1]. As life expectancy is regularly increasing, AD is becoming a greater health problem. The pathological hallmarks of AD are intracellular protein deposits, neurofibrillary tangles in the cortical regions of the brain, and abnormal extracellular neuritic plaque deposits of amyloid β-peptide (Aβ) leading to severe cortical atrophy [2].

Various mechanisms have been proposed to explain the pathogenesis of AD including amyloid metabolism, oxidative stress, inflammation, and lipid dysregulation [3]. Oxidative stress and free radical damage have been recognized as important factors in the biology of ageing and of many age-associated degenerative diseases such as Parkinson disease and AD [4]. Brain tissue is highly susceptible to free radical damage because of its high oxygen consumption, the high level of polyunsaturated fatty acids in neuronal membranes, and the low level of endogenous antioxidants [5]. Moreover, amyloid β-protein is known to play an important role in the oxygen radical formation [6]. Amyloid can directly induce free radical formation and a strong correlation between the intensity of free radical produced by amyloid and neurotoxicity has been found [7]. Inflammatory mechanisms have also been linked to age-associated cognitive decline, including both AD and vascular dementia [8]. Inflammatory proteins are thought to exacerbate the pathogenic process by

stimulating the production of A $\beta$ , thus facilitating its aggregation into plaques and increasing its neuronal cytotoxicity [9].

The diagnosis of AD is drawn on the basis of specific pathological features found either on brain biopsy or after post-mortem examination of the brain. In other cases, the diagnosis is based on clinical history and on physical and neurological examination according to the NINCDS-ADRDA criteria [10]. The diagnosis is then considered as probable or possible. Therefore, the emergence of new therapies in the treatment and prevention of AD disease requires early and differential diagnosis of AD. To date, efficient diagnostic techniques for detecting early biochemical changes in tissues or cells of patients susceptible to develop AD are scarce. Among the available AD biomarkers, the cerebrospinal fluid (CSF) protein biomarkers are recognized as the most accurate owing to the direct contact between CSF and the central nervous system [11]. However, these tests require CSF which cannot be routinely collected in the evaluation of AD. Several serum biochemical markers for AD have been proposed but the current available tests lack sensitivity and specificity for AD diagnosis [12]. Given the multiplicity of pathophysiological processes implicated in AD, the diagnostic accuracy of biomarkers may be improved by combining several serum markers [13]. Recently, Ray et al [14] reported a blood molecular test including 18 signaling proteins that could separate AD patients from controls with close to 90% accuracy and that also could identify patients with mild cognitive impairment. Although, this biomarker profile is of great interest for the future, routine use of this test is questionable because it requires an expensive arrayed sandwich Elisa.

Fourier-transform infrared (FT-IR) spectroscopy is a physical technique that may potentially play a critical role in this area of diagnosis. The spectroscopic approach is very convenient since no reagent and only small amounts of samples are required [15]. This method measures the chemical variations of the structure and composition of biological materials at the molecular level [16]. Indeed, this technique is based on the specific absorption

spectra of all organic molecules in the mid-infrared region (4000 to 500  $\text{cm}^{-1}$ ). Therefore, FT-IR plasma spectra should contain the whole sample metabolic information. As FT-IR spectroscopy is extremely accurate and highly reproducible for biological sample analysis, it has been used for the identification and quantification of several metabolites [17,18]. Recently, the detection of bovine spongiform encephalopathy-related signatures in blood was revealed using this technique [19]. However, this method is limited to screen plasma sample contents for discriminatory classifications. In fact, the use of mathematical algorithms such as Ward's method is needed to segregate the spectra within that dataset according to similarities and differences in the spectra [20]. Then several classes may arise from the spectral data and the distance between the classes corresponds to the heterogeneity. Heterogeneity between classes is proportional to their absorption differences. Thus, heterogeneity has been used to efficiently differentiate patients with a defined metabolic trouble from normal subjects [21]. To our knowledge FT-IR has never been used to discriminate populations with restricted knowledge of the subjects' metabolic situation.

In the present study, we utilized this method to compare and sort plasma contents on the basis of complete (4000 to 500  $\text{cm}^{-1}$ ) or defined regions of plasma spectra of AD patients and control subjects. We asked whether the heterogeneous pathophysiology of AD might be reflected in plasma and whether patients with AD could be distinguished from a healthy population with normal cognitive functions. In the same way, we performed analysis of biochemical parameters that could be modified by AD.

## **METHODS**

### **Patients**

The study was approved by the ethics committee of the university hospital of Bordeaux. AD patients were recruited in the memory center of the walking clinic. They were diagnosed with probable (or possible for one of them) dementia of Alzheimer's type according to the NINCDS-ADRDA criteria. Diagnosis were established by a trained

neurologist after a medical examination, a comprehensive psychological assessment with a mean duration of one hour and a half, cerebral imaging and biological assessment. All of these tests were performed within one day, together with diet questioning. The MMSE score has been used to determine the cognitive level assessment [22]. Patients with signs of fronto-temporal dementia or Lewy bodies were not included in order to restrict the study only on those with AD. Inclusion of the patients was made on the basis of an MMSE score  $> 18$  so that the informed consent to participate in the study could be obtained. Forty AD patients were included in this group. Nineteen AD patients were on cholinergic therapy. All AD patients were living in the community.

One hundred and twelve age and sex-matched control subjects over 65 years old and living at home in Bordeaux were included. They were visited at home by a psychologist trained for home interviews. They were selected if they were free from any memory complaint, central neurological disease or symptom. Cognitive function was evaluated by the MMSE. All controls randomly selected had an MMSE score above 24. AD patients and controls were included after giving their written informed consent. During the visit, both patients and controls underwent blood sampling, and an interview was conducted to collect sociodemographic data, living conditions and nutritional habits. A comprehensive functional assessment including personal medical history, current symptoms and diseases, and neurosensory impairments was performed. All blood samples were collected from the AD and control subjects between January 2004 and November 2005.

From the interview data, subjects with signs of Parkinson's disease, diabetes mellitus, infections or inflammatory disease during the previous two months were excluded. Moreover, none of the patients or controls was taking any other drugs such as statins that could affect the oxidant/antioxidant and lipidic status and subsequently the absorption of chemical groups related with oxidative processes [23].

After overnight fasting, blood samples were drawn in heparinized vacutainers. After centrifugation at 1000 g for 10 min, plasma was immediately stored at  $-20^{\circ}\text{C}$  until analysis.

Biochemical markers and spectra of each AD and corresponding controls were performed the same day.

### **Biochemical analyses**

Glucose and cholesterol parameters that could be modified by ageing and/or AD were determined in an autoanalyzer (LX20, Beckman-Coulter, Villepinte, France).

Plasma glucose was assayed by an automatic enzymatic method using hexokinase, ATP, glucose-6-phosphate dehydrogenase and NADP<sup>+</sup>. Plasma cholesterol was performed by using a reaction type Trinder with cholesterol esterase, cholesterol oxidase, peroxydase and 4-aminoantipyrine with phenol for revelation that is measured at 520 nm.

Oxidant and antioxidant status were evaluated by determining malondialdehyde (MDA), 8-epi-PGF<sub>2</sub>α, OHDG levels that are catabolites of fatty acid or nuclei acid oxidation, and vitamins A and E that are the main intracellular antioxidants.

- MDA levels were determined by a modified method of Carbonneau et al [24]. Briefly, basic hydrolysis released MDA bound to amino groups. The protein-free extract obtained after acid treatment was reacted with thiobarbituric acid 42 mmol/l (TBA), and 10 μl of the MDA/TBA adduct was separated from interfering chromogens by HPLC (Waters, Ontario, Canada) using a C18 μBondapak column with a 60/40 (vol/vol) mixture of 25 mmol/l phosphate buffer, pH 6.5 and methanol as a mobile phase. The flow rate of the solvent was 1.5 ml/min. The fluorimetric detector was set at 515 nm for excitation and 553 for emission. The standard used was 1,1,3,3-tetraethoxypropane (Aldrich-Chemic, Steiheim, Germany).

- Plasma 8-epi-prostaglandin F<sub>2</sub>α (8-epi-PGF<sub>2</sub>α) levels were measured in plasma samples by using a commercially available ELISA kit (OxisResearch, Tebu, France). Briefly, after hydrolysis of phospholipids, 8-epi-PGF<sub>2</sub>α in the samples or standards binds to the specific anti-8-epi-PGF<sub>2</sub>α antibody coated on the plate. Binding is revealed with the binding of the 8-epi conjugated to horseradish peroxidase (HRP) on the free antibody sites. The



peroxidase activity results in color development when the substrate was added. The intensity of the color is proportional to the amount of 8-epi-HRP bound and inversely proportional to the amount of 8-epi in the samples or standard.

- Plasma 8-hydroxydeoxyguanosine (8-OHdG) levels were measured using a competitive enzyme-linked immunosorbent assay ELISA (Bioxytech, OXIS, Tebu, France).

- Vitamin A and E analysis was performed using an HPLC technique as previously described [25].

### **FT-IR measurements.**

For FT-IR spectra acquisitions, 30 microliters of plasma were diluted with 120  $\mu$ l of water (1/5; v/v), and homogenized with an agitator for 10 sec. Then, 35  $\mu$ l of each suspension were loaded within the cell limits of a ZnSe (zinc selenide) wheel (optical plate) suitable for absorbance FT-IR measurements of up to 15 samples (Bruker). The wheel was placed in a drying vacuum to evaporate unbound water. Then the wheel was introduced in the Bruker IFS 28/B FT-IR spectrometer (Bruker, Karlsruhe, Germany) equipped with a DTGS (deuterated triglycine sulfates) detector.

To achieve a satisfactory signal-to-noise ratio for each spectrum, 64 interferograms were co-added, averaged, apodized with the Blackman-Harris 3-Term function and then Fourier-transformed. Spectra showing high absorption of water vapor and/or peak absorbance intensities outside the chosen limits (i.e. absorbance between 0.9 and 1.1) were excluded from the data set according to the manufacturer's protocol. The spectra were acquired at a resolution of 2  $\text{cm}^{-1}$ . Neither baseline correction nor normalization of spectra was done in our experiments.

Experiment was performed in triplicate for 101 plasma (25 AD and 76 controls). As we have withdrawn 11 measures on the 303 obtained, 101 spectra, each corresponding to the average of at most three samples, were included in the analysis of spectra.

## **IR data evaluation.**

The first and second derivative spectra were calculated. A multivariate statistical method based on first derivative spectra made it possible to separate spectra into different classes by hierarchical grouping or cluster formation [20, 26, 27]. This "best" union procedure can be based on some measure of similarity or distance. The most straightforward way of computing distance between objects in a multi-dimensional space is to compute Euclidean distances (geometric distances). For evaluating how two clusters are sufficiently similar to be linked together, Ward's algorithm was used in the Statistical 6.0 software [20]. This method uses an approach of analysis of variance (heterogeneity) to evaluate the distances between clusters. In fact, Ward's algorithm fuses those two clusters that yield the least increase in variance within the new clusters and therefore will construct the most homogeneous groups. The heterogeneity was calculated as the sum of the squared distances from the center of the cluster to each member of the cluster [28]. Therefore, the less similar any two spectra are, the greater is their heterogeneity. The method for spectra acquisition being a quantitative method, heterogeneity values between groups of spectra are proportional to their absorption differences. To be significant, heterogeneity value between two clusters must be higher than the sum of the cluster's heterogeneity [29]. Heterogeneity values between clusters were automatically determined by the software Statistica 5.0 (StarSoft) and Pearson's correlation was applied to determine the significance of the heterogeneity values. The results are presented as dendrograms that graphically represent the cluster analysis groups (the horizontal axes show the fusion levels).

Spectra classifications were performed on restrictive regions  $1480\text{-}910\text{ cm}^{-1}$  according to the results of the hierarchical classifications. Moreover, to enhance the resolution of superimposed bands (e.g. overlapping components) and subsequently to gain more insight into detailed structures, the second derivatives of the original spectra were calculated using the Savitsky-Golay algorithm combined with 9 smoothing points [30]. We also used all combinations of at least two spectra regions to determine the most effective classification.

Recording of spectra, data storage and all other manipulations, such as integration of peak areas were performed using the Opus 4.0 software (Bruker).

Validity of the hierarchical classification was assessed by including nine other plasma spectra (3 AD and 6 controls) in the 101 plasma spectra database.

Accuracy of the hierarchical classification was determined by analyzing an additional 12 samples of AD patients (true positive) and an additional 30 samples of normal cognitive patients (true negative).

### **Statistical analysis.**

Statistical analysis of data was conducted with a statistical software package (Stat-View, SAS Institute, Cary, USA). Results are expressed as mean  $\pm$  SD. The values obtained after integration of raw spectral band area were compared using Student's unpaired *t*-test. A *p* value less than 0.05 was considered significant. Statistical comparisons between clinical characteristics and biochemical markers in AD patients and controls were performed with the Mann-Whitney U test. To get better statistical significance in these comparisons, we applied the Bonferroni correction and considered a *p* value = 0.003 as significant.

## **RESULTS**

### **Clinical and biological data.**

General characteristics of patients and controls are presented in Table I. The mean age and sex ratio were similar in both AD and total controls. The mean body mass index of AD patients was significantly lower than control people ( $p < 0.0002$ ), but it was in the normal weight range. As expected, the MMSE score was significantly lower in AD patients than in controls ( $p < 0.0001$ ).

Biological analyses in Table II showed that AD glucose and cholesterol levels were within the normal physiological range. By contrast, oxidative stress markers such as 8-iso-PG $\alpha$  were

significantly higher in AD as compared to total controls and to the sub-group C<sub>2</sub> ( $p < 0.001$ ). 8-OHdG and MDA levels tended to increase in AD compared to total controls and compared to the sub-group C<sub>2</sub>. Conversely, vitamin E levels tended to decrease in AD compared to the sub-group C<sub>2</sub>.

### **Hierarchical classification**

In a first set of experiments, a pool of 76 normal ageing patients and a group of 25 patients with AD were analyzed. Hierarchical classification of the 101 sample spectra in the whole mid-infrared region (4000-500  $\text{cm}^{-1}$ ) revealed two main clusters different by a heterogeneity value of 450.5 (Fig 1A). The first one (AD) corresponded to the AD patients with an internal heterogeneity of 18.3 whereas the other corresponded to the total control group (C) with an internal heterogeneity of 115.7. Thus, the 4000-500  $\text{cm}^{-1}$  region enabled to separate AD population from controls. However, because the spectral window leading to a clear distinction between AD and controls was too large, we studied other smaller spectral windows that could be as equally effective. Among all the investigations, the 1480-1428  $\text{cm}^{-1}$  range (Fig. 1B) allowed the most accurate separation (heterogeneity of 1129.1) between the AD group (internal heterogeneity of 36.0) and the control group (internal heterogeneity of 117.6).

Moreover, the value of the internal heterogeneity of 115.7 of the control group suggested that this group was not homogeneous and could be further fragmented into sub-groups. The 1480-910  $\text{cm}^{-1}$  range provided heterogeneity within the pool of total controls (Fig. 2A). On this spectral window, two clusters appeared with an interclass heterogeneity value of 571.9. The first one with an internal heterogeneity of 449.4 corresponded to the 25 samples of the AD group (internal heterogeneity of 67.2) and to 32 samples of the control group (internal heterogeneity of 56.9) representing the sub-group C<sub>1</sub>. The second cluster with an internal heterogeneity of 141.7 included 44 samples of the control group representing the sub-group C<sub>2</sub>. Although, the interclass heterogeneity value was lower than the sum of the internal

cluster's heterogeneity values of these clusters and was not statistically significant, the analysis of other spectral windows yielded no such pairs of clusters. Moreover, the differentiation of the control group in 2 sub-groups observed with the FT-IR data was not correlated with clinical data since no difference was found in these subgroups for age ( $74.94 \pm 4.98$  for  $C_1$  vs  $75.41 \pm 4.65$  for  $C_2$ ), sex (51 for  $C_1$  vs 52 for  $C_2$ ), BMI ( $27.38 \pm 3.51$  for  $C_1$  vs  $27.11 \pm 3.77$  for  $C_2$ ) and MMSE ( $27.00 \pm 2.52$  for  $C_1$  vs  $27.02 \pm 3.77$  for  $C_2$ ). To confirm that the subdivision of the control group was related to the spectral differences within the 1480-910  $\text{cm}^{-1}$  region, we performed a validation set with new added subjects.

### **Validation test**

We further introduced in the previous database nine plasma spectra corresponding to three AD patients and six controls. First the new spectra were added separately. The three spectra from AD patients landed in the AD cluster without modifying the positions of the other clusters. Similarly, the same result was obtained when the six plasma spectra of controls were added. Then, when all of these spectra were introduced as a single set, the three clusters AD,  $C_1$  and  $C_2$  remained homogenous. Heterogeneity values obtained with the added samples were similar to those obtained without the added samples but the heterogeneity value of cluster AD/ $C_1$ - $C_2$  (584.6) was above the sum of the internal heterogeneity values of the two clusters (437.3 and 129.5, respectively) indicating a statistical significance [29] ( Figure 2 B).

### **Accuracy**

After validation of the data set, we calculated the accuracy of the method by using the number of true positive, true negative, false positive and false negative over a total of 42 additional samples (12 AD and 30 C).

With the detection of 12 over 12 AD positive samples and 29 over 30 C samples, a sensitivity of 100% with a 73.5-100 as 95% confidence interval and a specificity of 96.7% with a 82.8-

100 as 95% confidence interval were achieved. The weighted arithmetic mean of these two values, frequently called accuracy, reached 98.4%.

### **IF spectral analysis.**

To investigate the differences related to metabolic changes, we calculated and compared the mean spectra obtained from the control group C<sub>2</sub> (spectra 1), the control group C<sub>1</sub> (spectra 2) and the AD group (spectra 3). The variation in intensity or/and the lack of some bands in the spectra reflect chemical differences between the groups.

We analyzed the mid-infrared region 1480-910 cm<sup>-1</sup> where several peaks are related to the vibrational modes of sugar and phosphate skeletal motions of nucleic acids [31]. Since it is difficult to separate some peaks with the first derivate, we used a second derivative procedure to separate the superimposed bands and to visualize shoulders imperceptible on the original spectra. Figure 3 reveals three regions which appeared of great interest. First, the 1480-1140 cm<sup>-1</sup> region presented six peaks centered respectively at 1455, 1402, 1343, 1315, 1242 and 1155 cm<sup>-1</sup> that tended to be lower in the C<sub>1</sub> and AD groups as compared to C<sub>2</sub> controls. The first band centered at 1455 cm<sup>-1</sup> corresponds to the CH<sub>2</sub> bending vibrations of mainly fatty acids [32]. The second band at 1402 cm<sup>-1</sup> is due to COO<sup>-</sup> symmetric vibrations of amino-acid side chains and fatty acids [33]. The relatively strong bands at 1343 and 1315 cm<sup>-1</sup> are mainly related to the CH<sub>2</sub> side chain vibrations in lipids and proteins [33]. The last bands at 1242cm<sup>-1</sup> and 1155 cm<sup>-1</sup> respectively correspond to the asymmetric phosphate stretching vibrations [33] and to the stretching vibration of the CO group of the nucleic acid ribose [32].

Beyond 1140 cm<sup>-1</sup>, the spectral profiles between 1140 and 1095 cm<sup>-1</sup> range displayed three peaks centered at 1128, 1119 and 1105 cm<sup>-1</sup> in spectra 1, 2 and 3. There were slight differences in intensity in these peaks in spectra 1 and 2 whereas a strong change in the shape of the peak centered at 1128 that becomes broader with a shoulder at 1119 cm<sup>-1</sup> was observed in spectra 3. These peaks correspond to the phosphate stretching vibrations of the

phosphodiester backbone and the stretching vibrations associated with the CO group of deoxyribose [34].

Similarly, between 1063 and 1037  $\text{cm}^{-1}$  the three spectra presented a peak at 1053  $\text{cm}^{-1}$  which changes in shape only on spectrum 3. The change was accompanied by the emergence of two shoulders at 1045 and 1041  $\text{cm}^{-1}$ . This band is mainly assigned to the CO stretching vibrations in deoxyribose /ribose of nucleic acids [35].

In the last region, between 1020 and 930  $\text{cm}^{-1}$ , the three spectra exhibited different patterns. The five peaks at 1012, 994, 987, 970 and 936  $\text{cm}^{-1}$  respectively, tended to decrease in intensity in spectra 2 and 3. This region is generally assigned to the CO stretching vibrations in osidic and protein structures and to the symmetric stretching mode of dianionic phosphate monoester of nucleic acids especially for DNA [36].

All these data were confirmed by quantitative analysis of absorbance band areas of the raw spectra (Table III).

Taken together, these findings indicate that the best differences between the three groups can be obtained on a dendrogram using two spectral ranges, i.e. 1140-1037  $\text{cm}^{-1}$  and 1480-1428  $\text{cm}^{-1}$  in a proportion 1:2. Figure 4 shows that sub-groups  $C_1$  (heterogeneity of 81.7) and  $C_2$  (heterogeneity of 171.7) were well separated by a linkage distance of 326.9, whereas the linkage distance between the total control group and AD (heterogeneity of 109.4) was 3199.

## **DISCUSSION**

The present study shows that FT-IR spectroscopy is a powerful technique for determining spectral content changes in plasma of AD patients as compared to normal cognitive controls with an accuracy above 95%. Particularly, the whole mid region displayed an interesting pattern in which spectral differences could be associated with the mechanisms of AD pathophysiology.

To date, several independent hypotheses have been proposed to link the pathological lesions and the neuronal cytopathology such as apoE genotype, hyperphosphorylation of cytoskeletal proteins oxidative stress, abnormal cell cycle re-entry, inflammation and amyloid  $\beta$  metabolism [37]. However, none of these theories taken alone is sufficient to explain the diversity of biochemical and pathological abnormalities. Actually, oxidative stress which is clearly associated with ageing, is considered as a major mechanism responsible for the onset and progression of AD [38]. Free radical oxidative damage is a non specific process and can affect simultaneously multiple targets including lipids, proteins and nucleic acids [39]. Our results of hierarchical classification showed that the restricted range 1480-1428  $\text{cm}^{-1}$  allowed the best separation between AD patients and total controls. In this region, we observed in the AD average FT-IR spectra a decrease in intensity of the peak centered at 1455 $\text{cm}^{-1}$  resulting from the deformation and stretching vibrations of  $\text{CH}_2$  groups particularly in fatty acids. The brain is known to have a high content of easily peroxidizable unsaturated fatty acids (especially arachidonic and docosahexaenoic acids) and a high content of iron that are key factors for neuronal membrane lipoperoxidation [40]. Determination of plasma markers of lipoperoxidation confirmed our FT-IR results. The significant increase in F8 isoprostanes levels found in plasma AD patients are in accordance with other studies [41,42]. Surprisingly, MDA levels had only a tendency to increase in AD patients: statistical significance could not be reached. This discrepancy in these lipid oxidative markers could be explained by the specificity of the two markers. MDA is the end product of unsaturated fatty acid oxidation and is not a specific breakdown product [43]. Furthermore it can be generated by thromboxane synthase [44]. Isoprostanes are specifically generated from arachidonic acid, which is the major unsaturated fatty acid in the neuronal membranes [45]. A recent study, using a transgenic mouse model of AD demonstrated that brain trauma not only increased the amount of amyloid beta deposition but showed enhanced formation of isoprostanes long before the plaques were deposited [46]. Others have shown a direct correlation between



plasma and cerebrospinal fluid isoprostane levels, suggesting that plasma isoprostane levels may reflect brain oxidative stress [47]. Subsequently, isoprostanes were considered as stable markers of oxidative lipid damage in AD patients.

Importantly, we noticed that in the hierarchical classification between 1480 and 910  $\text{cm}^{-1}$ , in addition of the two distinct clusters corresponding to AD and controls, two sub-groups  $C_1$  and  $C_2$  could be evidenced within the control group. The peaks present in this region are mainly the signature of the deoxyribose-phosphate backbone of DNA and of alterations in the osidic structures of nucleic acids [48]. DNA represents a major target for free radicals when the capacity of intracellular free radical scavengers and antioxidants is overcome [49]. Some studies *in vitro* have shown that the hydroxyl radicals attack the deoxyribose moiety, giving rise to a variety of products resulting from hydrogen abstractions of the pentose ring and resulting in the loss of phosphoric acid and strand breaks [50]. These modifications are revealed on the spectra of AD patients by the shifts of the absorption maxima at 1128, 1119 and 1105  $\text{cm}^{-1}$  assigned to PO symmetric variations in nucleic acids and by the decrease in 1053 and 936  $\text{cm}^{-1}$  peaks, corresponding to the C-C/C-O stretching vibrations of the deoxyribose moiety of the DNA backbone [51]. Although many repair enzymes systems have evolved to remove and replace the damaged nucleosides and repair the broken strands, there is a high rate of damage of DNA as shown by the high levels of 8-OHdG that we found in AD plasma samples as compared to total controls and sub-group  $C_2$ . Numerous studies have shown that AD brain has higher levels of DNA breaks [52,53] confirming that oxidative DNA is indeed a feature of AD brain tissues or peripheral fluids. The cumulative damage to DNA is suspected to contribute to progressive neuronal cell loss since non repaired DNA damage can trigger programmed cell death. Moreover, Migliore et al showed significant levels of 8-OHdG in patients with mild cognitive impairment [54]. This suggests that abnormalities in oxidative processes occur at the early stages of AD and that ROS-mediated damage is not just secondary to the neurodegenerative process. Thus, oxidative stress is present before the diagnosis of AD can be established. In our study, the F8-

isoprostanes levels, representing one efficient marker of oxidative stress, were significantly higher in sub-group C<sub>1</sub> than in sub-group C<sub>2</sub>. Regarding the antioxidant levels of vitamin E in AD patients and in controls, we found that vitamin E levels tended to be lower in sub-group C<sub>1</sub> and in AD compared to the sub-group C<sub>2</sub>. This result could be of great interest since high levels of vitamin E have been found in plasma samples of mentally healthy centenarians [55], suggesting a protective role of these nutrients in the oldest-old. Consistently, we demonstrated in a previous study on the PAQUID (Personnes Agees Quid) cohort that the risk of AD was significantly increased in subjects having a low serum vitamin E [56]. In the chronic disease of AD, a latent stage has been described where some structural damages occur but no functional or behavioral changes can be noticed. This phase is followed by a prodromal stage called “mild cognitive impairment” during which structural damages continue to increase with mild functional or behavioral effects. Eventually, a clinical stage of dementia, characterized by substantial irreversible structural damages and extensive functional and behavioral deficits is set [57]. Other studies on the prodromal stage of AD showed that vitamin E levels were depleted to a similar extent as in AD [58]. As the pathologic processes of AD precede the clinically diagnosed dementia by as much as 2 or 3 decades, the sub-group C<sub>1</sub> could be considered as being at a higher risk of developing dementia, even though these patients had a normal MMSE. A follow-up of these patients will be necessary to determine whether they are prone to develop AD.

In conclusion, spectral analysis revealed biochemical modifications in AD plasma mainly related to oxidative stress, highlighting this important process in AD. Moreover, the hierarchical classification in clusters could be helpful to distinguish AD patient sera from non-demented ones.

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Figure 1. Classification scheme based on FT-IR spectra from 101 human plasma (confirmed demented patients: group AD and total controls: group C). Cluster analysis was performed using the first derivatives of the spectra in the 4000-500  $\text{cm}^{-1}$  infrared region (A) and in the 1480-1428  $\text{cm}^{-1}$  mid-infrared range (B). Ward's algorithm was applied.

Figure 2 - A. Classification scheme based on FT-IR spectra from 101 human plasma confirmed demented patients: group AD and sub-groups  $C_1$  and  $C_2$  of total controls. - B Classification scheme based on FT-IR spectra from 110 human plasma: 25 AD and 3 added ones, and 76 controls and 6 added ones. Cluster analysis was performed using the first derivatives of the spectra and considering the 1480-910  $\text{cm}^{-1}$  mid-infrared range. Ward's algorithm was applied.

Figure 3 - A. Mean FT-IR spectra of groups  $C_2$  (spectrum 1),  $C_1$  (spectrum 2) and AD (spectrum 3) studied in the whole mid-infrared region (4000-500  $\text{cm}^{-1}$ ). Spectra are shown offset for clarity. - B. Second derivative spectra of groups  $C_2$  (spectrum 1),  $C_1$  (spectrum 2) and AD (spectrum 3) studied in the 1480-910  $\text{cm}^{-1}$  mid-infrared range. Second derivative spectra are inverted and shown offset for clarity.

Figure 4. Classification scheme based on FT-IR spectra from 101 human plasma (confirmed demented patients: group AD and sub-groups  $C_1$  and  $C_2$  of total controls). Cluster analysis was performed using the first derivatives of the spectra and considering two unequally weighted spectral windows, i.e. 1140-1095 (weight 1) and 1480-1428 (weight 2)  $\text{cm}^{-1}$ . Ward's algorithm was applied.