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Research article

Interplay between oxidative stress, neuroinflammatory cytokines and melatonin in Alzheimer's disease: Insights from cerebrospinal fluid analysis

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1. ABSTRACT

Background: Among neurodegenerative disorders Alzheimer's disease (AD) displays the highest prevalence and the projected increase in its incidence will require new advances in early diagnosis and treatment, particularly for distinguishing AD from other dementias. While beta-amyloid (Aβ) and tau biomarkers are currently used to discriminate AD from other tauopathies and dementias, additional indicators could enhance patient stratification for specific dementia types. The present study was designed to find potential associations among the classic neurologic markers, Aβ, total and phospho-tau (T-tau and P-tau), with other biomarkers including melatonin and its oxidative-derived metabolite, Formyl-N-acetyl-5-methoxykynurenamine (AFMK) levels, assayed in patients' cerebrospinal fluid (CSF) taken previously for diagnostic purposes. Other factors previously associated with the aetiology of AD, including redox indicators or proinflammatory biomarkers, were also included.

Methods: The cross-sectional study included a cohort of 148 patients showing signs of dementia. A group of age-matched patients without neurological disorders were used as controls. CSF levels of Aβ, T-tau and P-tau were assayed, and patients were further classified according to threshold CSF levels of the three markers protein following the criteria of NIA-AA.

Results: Correlational and group analysis showed a positive association between oxidative stress and neuronal damage. TNF-α negatively correlated with CSF Aβ levels (amyloid plaques) while only RANTES/CCL5 correlated positively with T-tau and P-tau. Qualitative analysis of the proinflammatory cytokines assayed showed a higher detection level in Aβ-positive patients. Regarding melatonin in the CSF, indolamine levels did not correlate with its major oxidative-derived metabolite, i.e., AFMK. However, melatonin CSF levels were significantly reduced in AD patients but not in OT. On the contrary, AFMK showed the opposite pattern, with higher levels in samples from patients displaying high T-tau and P-tau levels. Neuroinflammation was associated with Aβ deposits (low concentration in CSF), while oxidative stress significantly correlated with high T-tau and P-tau levels. Finally, among all the parameters assayed in CSF samples from the cohort studied, P-tau, in combination with antioxidant capacity, offered the best ROC curve for the diagnostic capacity to discriminate between AD and OT, showing an 85 % specificity.

Conclusion: While oxidative stress is instead associated with high T- and P-tau levels, higher neuroinflammatory cytokines correlate with low CSF Aβ levels. An intriguing lack of correlation between neuroinflammation and melatonin found in this study could be as a result of sample size and requires further studies with a larger sample size. Even though indolamine levels in CSF drop significantly in AD, they do not correlate with AFMK, suggesting a different kynurenine synthesis source. None of them appear to discriminate between AD and OT. Finally, among all the parameters

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assayed in this study, P-tau in combination with antioxidant capacity, offered the best ROC curve for the diagnostic ability capacity to discriminate between AD and OT, showing an 85 % specificity. This study holds the potential to significantly improve patient stratification and contribute to the early diagnosis and treatment of Alzheimer's disease.

1. Introduction

Worldwide, roughly 25 million people are affected by dementia, 70 % of which are possibly due to Alzheimer's disease (AD), therefore creating a profound impact on families and healthcare systems. According to Livingston and co-workers [1] the number of people living with dementia in 2019 is over 57 million. Due to a rising life expectancy in developed countries [2,3], estimations project an increase in incidence up to 153 million by 2050, thus requiring an adjustment for new advances in both early diagnosis and more effective treatments [4,5]. Among the different neurodegenerative processes associated to aging, in addition to AD, the most common pathologies include frontotemporal dementia, Parkinson's disease, amyotrophic Lateral Sclerosis, Huntington's disease, vascular dementia, or dementia with Lewy bodies [6].

Preclinical stages of AD starts long before symptoms appear, affecting brain regions such as hippocampus and entorhinal cortex. A constellation of biological factors is involved in the aetiology of AD, but the accumulation of both beta-amyloid(Aβ) and hyperphosphorylated tau (P-tau) versus total tau (T-tau), are considered as the first steps in the molecular events triggering this pathology [7]. In addition to being used as biomarkers, Aβ proteins have been considered driving forces of Alzheimer's disease, thus configuring the so-called 'Amyloid hypothesis' [8]. This hypothesis states a cascade of molecular events triggered by the accumulation of the $A\beta_{42}$ fragment. This would result in abnormal changes of Aβ in CSF, which precedes downstream markers of neuronal damage. However, the failure of several clinical trials based exclusively on reducing the accumulation of these specific proteins have exhibited deficiencies in these theories, therefore showing a higher level of aetiological complexity, with the presence of other biological variables, including oxidative stress and neuroinflammation as the primary mediators of neuronal damage [9–15].

These pathophysiological processes could be of paramount importance, particularly in AD, which lacks adequate markers for either live (not postmortem) diagnosis, staging, or for differentiating AD from other dementias. Therefore, oxidative stress and neuroinflammation offer mechanistic importance and an opportunity for finding new co-biomarkers as diagnostic tools for early detection in either plasma or cerebrospinal fluid (CSF). To this aim, biomarkers in CSF have been proposed for discrimination and clinical diagnosis, selecting cut-off points for Aβ, T-tau, and P-tau, indicators of the pathological or non-pathological presence of neurofibrillary tangles and senile plaques [16].

Throughout aging, as well as along the stages of different neurodegenerative diseases, a rise in reactive oxygen and nitrogen species in the CNS occurs due to various causes, including a high metabolic rate, an unusually elevated concentration of transition metals, and a high content in polyunsaturated fatty acids [17], prone to be oxidized and in turn, generate lipoperoxidation [18]. In the specific case of AD, numerous studies have reported high levels of these reactive species in the brains of patients [19]. On the other hand, neurodegenerative diseases present a critical inflammatory component characterized by the activation of microglia, the production of proinflammatory cytokines, and the generation of reactive species, phenomena that unbalanced stimulate neuronal death [20].

Finally, the neuroindolamine melatonin has also been reported as an essential neuroprotector against neurodegenerative disorders and pathological aging [20]. Melatonin, mainly -but not exclusively-produced by the pineal gland, has numerous functions both at the central nervous system level and at the organism level, synchronizing the endogenous rhythms of body temperature, glucose homeostasis, immune response, and antioxidant defence to the circadian light cycle -darkness [21]. In numerous preclinical studies, melatonin has been shown to reduce cognitive damage in neurodegenerative diseases such as Parkinson's or AD [22,23]. In most of these studies focused on neuroprotection, the antioxidant function of melatonin has been emphasized since the indolamine is capable of directly scavenging free radicals and generating an indirect increase in the antioxidant response [24,25]. Interestingly, melatonin oxidative derivatives, including N-Acetyl-N-formyl-5-methoxykynurenamine (AFMK), also show an essential antioxidant activity [26], thus demonstrating a sort of antioxidant chain reaction [27]. Furthermore, in addition to the antioxidant capacity, it is well-known that melatonin and its oxidative derivatives also display anti-inflammatory actions through multiple pathways [28–30], thus enhancing the protective actions of the indolamine [31]. Collecting evidence of the antioxidant and anti-inflammatory actions of melatonin has led to propose melatonin not only as a crucial neuroprotective agent with therapeutic possibilities [32–34] but also to trigger the launch of several clinical trials, including some phase II and III [35]. Even more, the indication that melatonin serum and CSF levels are decreased in AD patients [36], which might reveal early pathophysiological changes [37,38], points out the interest in considering melatonin and its oxidation derivatives in CSF as potential biomarkers.

Overall, data from numerous studies support the use of these parameters as co-biomarkers which, in addition to the widely used Aβ and T-tau and P-tau, could help in a more efficient subgrouping of AD patients [32,34,37–39]. Accordingly, the main goal of the present study was to investigate the association among melatonin, AFMK, neuroinflammation indicators and CSF biomarker levels (i.e. Aβ, T-tau and P-tau) and test the diagnostic value of these biomarkers as tools to discriminate the dementia subtypes, as well as to provide some insights into the interrelationship between the abovementioned variables throughout the neurodegeneration process in AD patients.

2. Material and methods

2.1. Patients and age-matched controls

The assays were conducted with a total of 148 CSF samples, from advanced aged patients with a possible Alzheimer's-type dementia. Patients with neurodegenerative symptoms were derived from the Neurology Service for subsequent biochemical analysis of the biomarkers Aβ, T-tau and P-tau to consolidate a possible diagnose of Alzheimer's type dementia. All the studies conducted were previously approved by the Ethical Committee Board (Internal Reference #2023.578 of the Ethical Committee Board at the 'Hospital Universitario Central de Asturias', HUCA), following the ethical principles for Medical Research involving human subjects adopted by the 'World Medical Association Declaration of Helsinki". The Board approved the waiver of informed consent, since only a small fraction of samples for diagnostic assessment were used. Samples were extracted by lumbar puncture at early morning 9 a.m. and processed at the Central University Hospital of Asturias (HUCA). Patients with cancerous diseases were not further considered. For the present study, once the necessary volume for diagnosis was employed, aliquots of each sample were stored at −80 °C for further analysis. The Aβ values, as well as T-tau and P-tau values, were measured as markers for the possible diagnosis of AD, using the Luminex 200 flow cytometer and the respective commercial kits INNOTEST® β-AMYLOID (1–42), INNOTEST® hTAU and INNOTEST® PHOSPHO-TAU (181P). Reference values (pg/ml) used as positive 'cut-offs' for the disease were: Aβ < 500, t-tau >500 and p-tau >75, based on clinical [40,41] and meta-analysis studies [42]. Patients were accordingly grouped into the following cohorts analysed in a cross-sectional study: (i) controls (NDC) were those with CSF biomarkers levels of Aβ > 500, T-tau <500 and P-tau <75, ii) Alzheimer's disease patients (AD) were those with CSF biomarkers levels of Aβ < 500, T-tau >500 and P-tau >75, who showed low values of CSF Aβ and high values of CSF for both T-tau and P-tau, which confirms the deposit of senile plaques and neurofibrillary tangles, both pathological features of AD; and iii) other tauopathies (OT) were those with CSF biomarkers levels of Aβ > 500, T-tau >500 and P-tau >75 which discard the AD disease but confirms a tauopathy process. When it was found appropriate, analysis was performed using grouping according exclusively to Aβ or T-tau and P-tau cut-off points to correlate both biomarkers with oxidative stress and immunological parameters (neuronal damage group T-tau >500, P-tau >75 and Aβ positive group Aβ < 500). For correlational studies, when trying to find whether there is any interaction between the variables analysed, all the samples -including those control subjects out of the 'cut-off' points-were considered.

2.2. Antioxidant capacity discoloration test of radical 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) free cation

The determination of the antioxidant capacity was carried out by the cation discoloration test ($ABTS^{+}$) described by Refs. [43,44] with some modifications. An ABTS 7 mM and sodium persulphate ($Na_2S_2O_8$) 2.45 mM solutions were mixed in equal volumes, and the resulting mixture was incubated in dark overnight (18–20 h). Before carrying out the test, the $ABTS^{\bullet+}$solution was diluted (25 times) with PBS, to obtain an absorbance of 0.7 (± 0.1) at 734 nm in the Cary-50 spectrophotometer, (Agilent Technologies, California, USA). Ascorbic acid was used as external standard to estimate the antioxidant capacity of the samples. Finally, the values are shown as percentage of inhibition per 10 μL of sample. Samples were run in duplicate. Number of patients used were: NDC, n = 45, AD, n = 27 and OT, n = 27. For the correlational analysis, all the samples -including those out of the 'cut-off' points-were considered.

2.3. Lipoperoxidation. Determination of hydroperoxides using the diphenyl-1- pyrenylphosphine fluorescence probe (DPPP)

Lipid peroxidation was assayed using the DPPP probe, following the protocol previously described [45], with slight modifications. Fluorescence was measured with a Sinergy H4 hybrid reader (Bio-Tek, Vermont, USA), using excitation and emission wavelengths of 351 and 380 nm respectively and with a 65 % gain. Hydrogen peroxide was used as external calibration standard. Number of patients used were: NDC n = 39, AD n = 22 and OT n = 23. For the correlational analysis, all the samples -including those out of the 'cut-off' points-were considered.

2.4. Cytokines levels in CSF

Luminex 200™ flow cytometer was used to determinate immunological markers. The ProcartaPlex™ system from Thermo Fisher Scientific was designed for detecting TNF-α, RANTES/CCL5, MIF, IL-6 and IL-1β quantification. In the case of TGF-β quantification, it was carried out using a specific kit (TFG Beta-1 human ProcartaPlex™ Simple Kit) to acidify the samples prior to the analysis. A total of 88 CSF samples were analysed, divided in NDC n = 25, AD n = 23 and OT n = 13. Patients' samples which did not pass the cut off points were used for correlational studies. Among the cytokines assayed, only MIF showed fully detectable values in most of them (n = 80). Thus, to standardize all the samples including those showing values under LOD/LOQ, and to correlate neuroinflammatory cytokines in Alzheimer, patients were further grouped only considering as positive or negative for Aβ. Then, it was determined whether any of the cytokines with detectable levels were preferentially correlated to a specific group of patients. For the correlational analysis, all the patients' samples were used.

2.5. Melatonin and metabolites

Sample extraction was performed following the protocol described by Hevia et al. [46]. Melatonin and AFMK levels were determined by LC/MS-MS (Agilent 1290 Infinity II 2D-HPLC system coupled to a triple quadrupole mass spectrometer Agilent 6460) and

following the protocol described by Ref. [47]. External calibration was made to calculate the melatonin and AFMK concentration. For Melatonin assays, sample size used were: NDC n = 27, AD n = 15 and OT n = 16. For AFMK analysis, sample size used were: NDC n = 21, AD n = 14 and OT n = 13. For the correlational analysis, all the samples -including those out of the 'cut-off' points-were considered.

2.6. Statistical analysis

Normality data was tested by Shapiro Wilk, D'Agostino and Kolmogorov Smirnov test. Data comparison between groups was performed by ANOVA test followed by a Newman-Keuls post hoc analysis. For data that did not follow a normal distribution, the Kruskal Wallis test was carried out for the comparison between groups. Unpaired T student test, for normally distributed data or Mann-Whitney test (non-normal distributed data) were carried out for comparisons between two groups.

On one hand analysis were conducted between controls, AD patients and OT to compare antioxidant capacity, hydroperoxides concentration, melatonin and AFMK concentration. On the other hand, analysis between positive Aβ group (low CSF Aβ) and neuronal damage group (high CSF T-tau and P-tau) were conducted to compare oxidative stress (antioxidant capacity, hydroperoxides, melatonin and AFMK) and immunological parameters.

Correlations were analysed using Spearman coefficient for those data that did not follow a normal distribution, non z transformed data was employed in the correlational studies. The following correlations were tested: correlation between hydroperoxides, T-tau and P-tau, correlation between Aβ, T-tau and P-tau, correlation between Aβ and cytokines and correlations between oxidative stress, immunological parameters, melatonin and AFMK.

For qualitative analysis, Fisher's exact test was performed. The diagnostic evaluation of the tests was carried out by means of the elaboration of the ROC curves and the calculation of the area under the curve (AUC). P Values under <0.05 were considered statistically significant. Statistical analyses were performed by using GraphPad Prism 8 statistical package.

2.7. Data availability statement

All raw data, correlation data, and plots have been made openly accessible in the repository of our university at https://hdl.handle.net/10651/76068 and also at the Mendeley dataset https://data.mendeley.com/drafts/2t8wjy8v4g/1

3. Results

3.1. Characteristics of the study participants

The cohort of patients included in this study showed a similar percentage ratio of men/women (NDC 53/47 %, AD 44/56 % and OT 43/57 %, respectively) as well as a virtually the same mean age (roughly 70 years old). Sex, age, neurological and biochemical features are shown in Table 1. Cut-off points for Aβ, T-tau and P-tau are displayed in Table 2. Patients were grouped according to the criteria specified above in M&M section.

3.2. Comparison of oxidative stress parameters in AD and other related tauopathies

Fig. 1 shows the antioxidant capacity in the CSF from a cohort of patients previously grouped following the canonical threshold levels (see Table 2) in Aβ and T- and P-tau (Fig. 1A and B), and the antioxidant capacity in patients grouped according to the specific levels of Aβ (Fig. 1C) or tau (Fig. 1D). Surprisingly, antioxidant capacity is increased in patients with other tauopathies (Aβ >500, T-tau >500) but a reduction in AD group was found when it was specifically compared to NDC patients. Furthermore, this decrease observed is more pronounced grouping the patients according to high or low CSF Aβ levels, based exclusively on the clinical Aβ cut-off point (Fig. 1C).

Regarding the levels of hydroperoxides, no statistically significant differences were observed in the comparison between the three abovementioned groups of patients, despite the trend of an increase observed in AD/OT groups compared to NDC group (Fig. 2A). However, when clustering samples considering exclusively neuronal damage (i.e. high for both T and P-tau) rather than AD/OT patients subgrouping, a significant increase between controls and T- and P-tau high was observed (Fig. 2B), probably due to an increase in sample size. This finding led us to analyse the potential correlation between hydroperoxides and both, total and phosphorylated tau (T

Table 1
Summary of sex ratio, age mean and neurological parameters from patients' cohort included in the present study.

	Non-demented controls (NDC, n=47)	Alzheimer's disease (AD, n = 27)	Other tauopathies (OT, n=28)
Gender (M/F, n)	25/22	12/15	12/16
Age (Mean ± SD)[a]	67.94 ± 8.33	68.69 ± 8.05	69.96 ± 6.03
MMSE[b]	26.29 ± 5.70	18.57 ± 60	24.81 ± 3.50
CSF T-tau	226.6 ± 84.37	859.2 ± 275.2	772.9 ± 193.90
CSF P-tau	36.92 ± 10.86	138.7 ± 38.35	112.9 ± 23.41
CSF Aβ (1–42)	1056 ± 175.80	315.3 ± 68.58	679.6 ± 160.80

[a] All data are expressed as mean ± SD.
[b] Mini-mental state examination.

Table 2
Standard 'cut-off' biochemical parameters of beta-amyloid (Aβ), total tau (T-tau) and phosphorylated tau (P-tau) used for patients' stratification.

	Aβ (pg/ml)	T-tau (pg/ml)	P-tau (pg/ml)
AD (n=27)	<500	>500	>75
	>500	<500	<75
NDC (n=47)			
	>500	>500	>75
OT (n=28)			

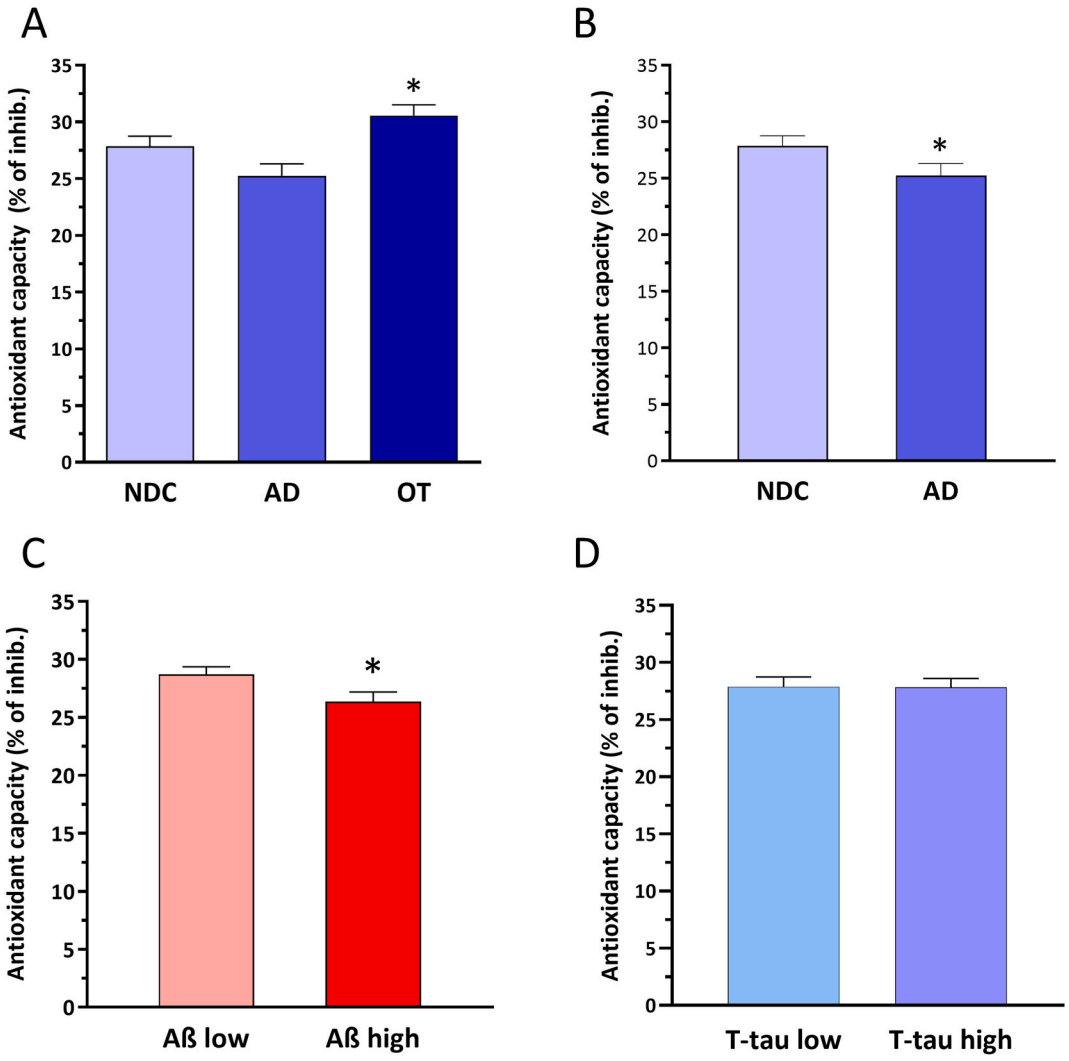

Fig. 1. The association between oxidative stress parameters and neuronal damage in AD and other related tauopathies (two tailed *t*-test and ANOVA were used). Antioxidant capacity (as % of inhibition) assayed in samples from non-demented controls (NDC), Alzheimer's disease (AD) or other tauopathies patients (OT) (A).Antioxidant capacity in NDC and in patients with low CSF Aβ levels (AD) (B). Antioxidant capacity in patients grouped according to Aβ levels (C) or to T-tau (D). Aβ low, β-amyloid >500 pg/ml; Aβ high (β-amyloid <500); T-tau low (T-tau<500 pg/ml); T-tau high (T-tau>500 pg/ml). Values are represented as Mean ± SEM. NDC, n = 45; AD, n = 27; OT, n = 27; Negative amyloid, n = 72; Positive amyloid, n = 57. Two-tailed *t*-test and ANOVA were used. T-test P values: A, p = 0.0127; B, p = 0,0341; C, p = 0,0291; D, p = 0,9618.

and P-p-tau). A low but statistically significant correlation was found between T-tau or P-tau with hydroperoxides (Fig. 2C and D, respectively), but no correlation was found with antioxidant capacity (Supplementary Fig. 1 and Supplementary Tables 1 and 2).

As shown in Supplementary Fig. 1, the three core CSF biomarkers correlated well each other. However, no correlation was observed between Aβ and hydroperoxides nor with antioxidant capacity.

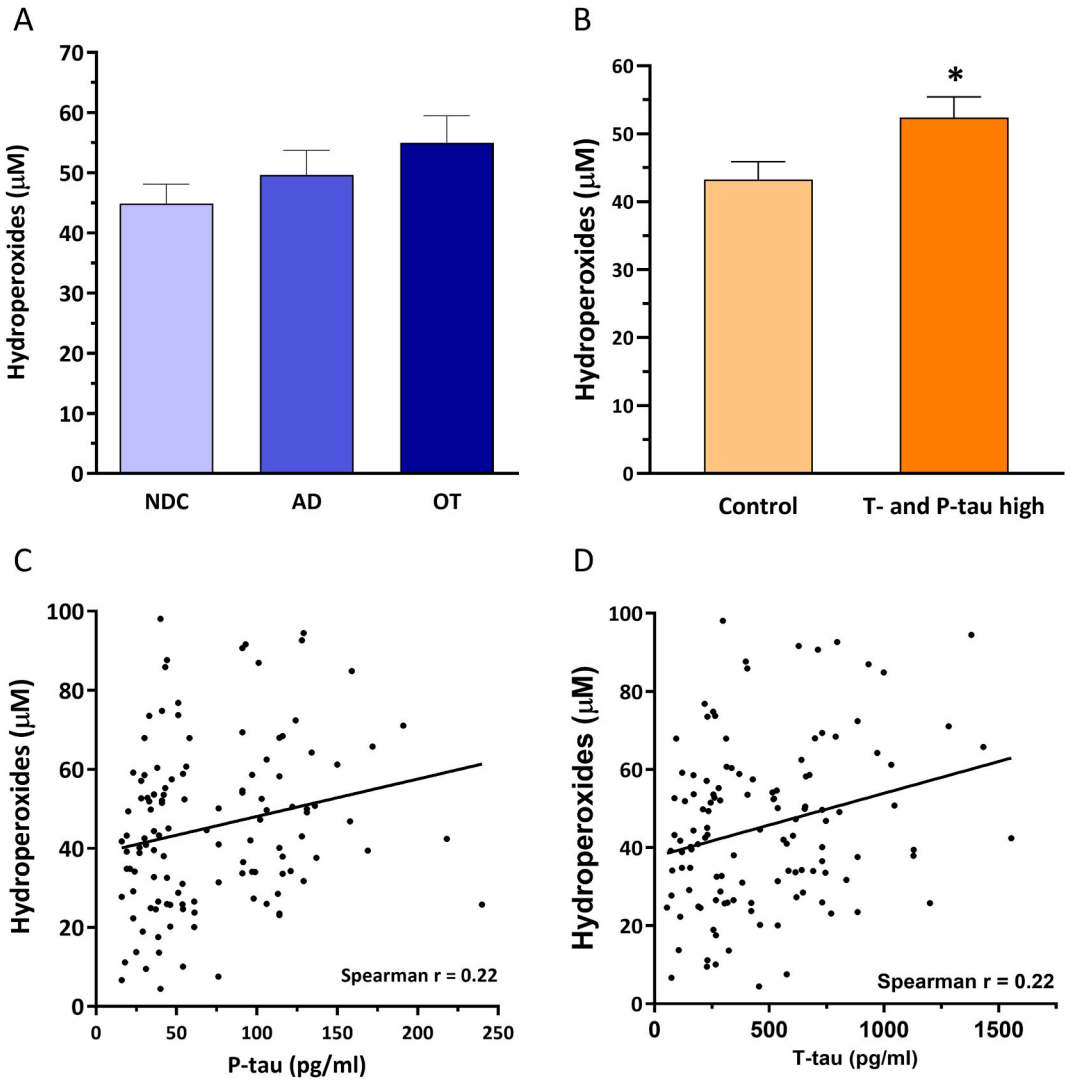

Fig. 2. Hydroperoxides levels assayed in samples from non-demented controls (NDC), Alzheimer's disease (AD) and other tauopathies (OT) in CSF patient's samples. Hydroperoxides concentration mean representation in NDC, AD and OT (A) and hydroperoxides levels from control samples (T-tau <500 pg/ml and P-tau <75 pg/ml) or from patients' samples displaying T- and P-tau high (T- and P-high) (neuronal damage, T-tau >500 and P-tau >75) (B). Correlational studies between hydroperoxides and T-tau (C) or P-tau (D). Values are represented as mean ± SEM and correlation is represented by spearman correlation. Control, n = 39; Alzheimer's disease, n = 22; Other tauopathies, n = 23. T-test P values: A, p = 0.1; B, p = 0.0259. Non-parametric Spearman 'r' correlations p values: C, p = 0.0159; D, p = 0.0214.

3.3. *Neuroinflammatory cytokines levels in CSF*

Among the neuroinflammatory cytokines included in the assay, only undetected levels of TNF and IL-6 were more significantly associated to patients positive for Aβ plaques (Aβ< 500 pg/ml in CSF). Results are shown in Fig. 3. Contrary to what occurred with hydroperoxides, no differences were found when patients were grouped according to T and P-tau levels (data not shown).

Next, a nonparametric, Spearmen correlational studies between detectable and quantifiable cytokines, Aβ and T and P-tau were calculated (See Supplementary Tables 1 and 2). As it is shown, partial but significant correlations between TNF-α, RANTES/CCL5 and IL-6 with Aβ were found, Spearman correlation coefficient −0.49, −0.45 and −0.59 respectively (Fig. 4A–C, respectively). Other pro-inflammatory cytokines such as TGF-β or MIF did not correlate with neither Aβ nor T and P-tau.

It is noteworthy to mention that when patients were stratified according to Aβ (<500 or >500 pg/ml) or T- and P-tau (T-tau <500 or >500, P-tau <75 or P-tau >75), most of the cytokines showed a medium-high correlation among them (Supplementary Fig. 2), but only in the subgroup of patients displaying low Aβ (Supplementary Fig. 2A) or high T-tau (Supplementary Fig. 2D). No significant correlations were observed among the cytokines and the redox parameters, except for RANTES/CCL5 in patients with normal levels of either Aβ and T- and P-tau.

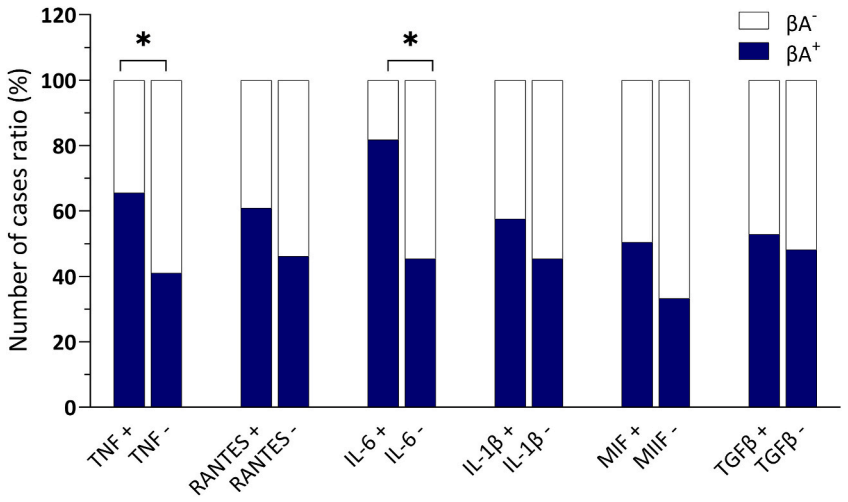

Fig. 3. Distribution of cases (ratio %) according to the qualitative detection of proinflammatory cytokines in patients' CSF samples, previously grouped depending on the Aβ levels. Additionally, in the case of cytokines, '+' indicates that LOD/LOQ were found over the limit of detection of the assay, while '-' '-' denotes that levels were below LOD. P values for Fisher's exact tests: p = 0.0453 for TNF-α; p = 0.332 for RANTES; p = 0.0492 for IL-6; p = 0.3786 for IL1B; p = 1 for MIF; p = 0.8269 for TGFB.

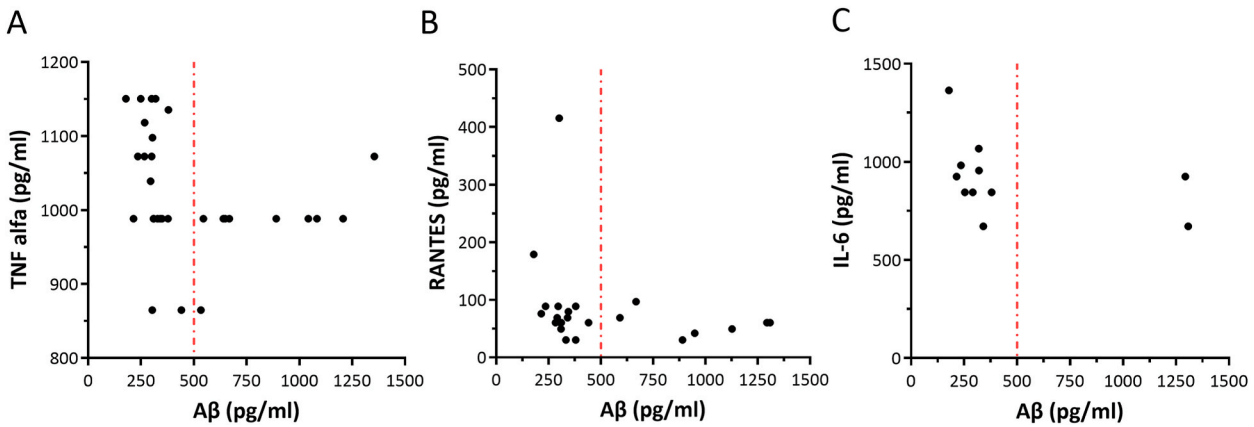

Fig. 4. Correlations between detectable and quantifiable pro-inflammatory cytokines and Aβ values. As a reference line, cut-off point for Aβ (500 pg/ml) used for patient grouping is shown in each plot (–□ –). Aβ correlations with TNF-α (A), RANTES (B) or IL-6 (C). P values for nonparametric Spearman correlations: p = 0.005 for TNF-α (n = 32); p = 0.03 for RANTES (n = 23); p = 0.06 for IL6 (n = 11).

3.4. Correlation of CSF levels of melatonin and AFMK with oxidative stress parameters in AD and other tauopathies

Considering the classic neuroprotective effect exerted by melatonin against neurodegeneration, we then assayed melatonin and its oxidative metabolite, AFMK. Other oxidative metabolite derived from melatonin, namely N1-acetyl-5-methoxykynuramine (AMK), which is also produced after AFMK reaction with free radicals [48,49], was not detected in CSF samples from patients included in this study. Delving into the relation between melatonin and neuronal damage, the indolamine levels showed a significant decrease in positive AD patients, both quantitative and qualitative (Fig. 5A and B, respectively). AFMK levels didn't show a drop in AD patients, as it could be expected by observing melatonin levels. Quite the contrary, a slight but significant increase was observed in the group of other tauopathies (OT, Fig. 5C), while in AD patients the increase was not statistically significant, likely due to a larger dispersion of data. As it occurs with oxidative stress parameters, when samples were grouped according exclusively to neuronal damage, an increase in AFMK in the neuronal damage group was observed (Fig. 5D). Fig. 6 shows the violin plots with the levels of both, melatonin (Fig. 6A and B) and AFMK (Fig. 6C and D) in patients grouped according exclusively to Aβ or T-tau and P-tau.

Nonparametric Sperman correlations between melatonin, antioxidant capacity or hydroperoxides were calculated for samples obtained from patients showing neuronal damage (i.e., positive for Alzheimer's or for other tauopathies, Fig. 7). As it might be expected, melatonin displayed a positive correlation with total antioxidant capacity (Fig. 7A). However, pineal indolamine also showed a positive correlation with hydroperoxide content (Fig. 7B). Finally, a positive relation between antioxidant capacity and hydroperoxide levels was found in the analysed samples (Fig. 7C). When all the samples (including NDC) were analysed, no correlation was found

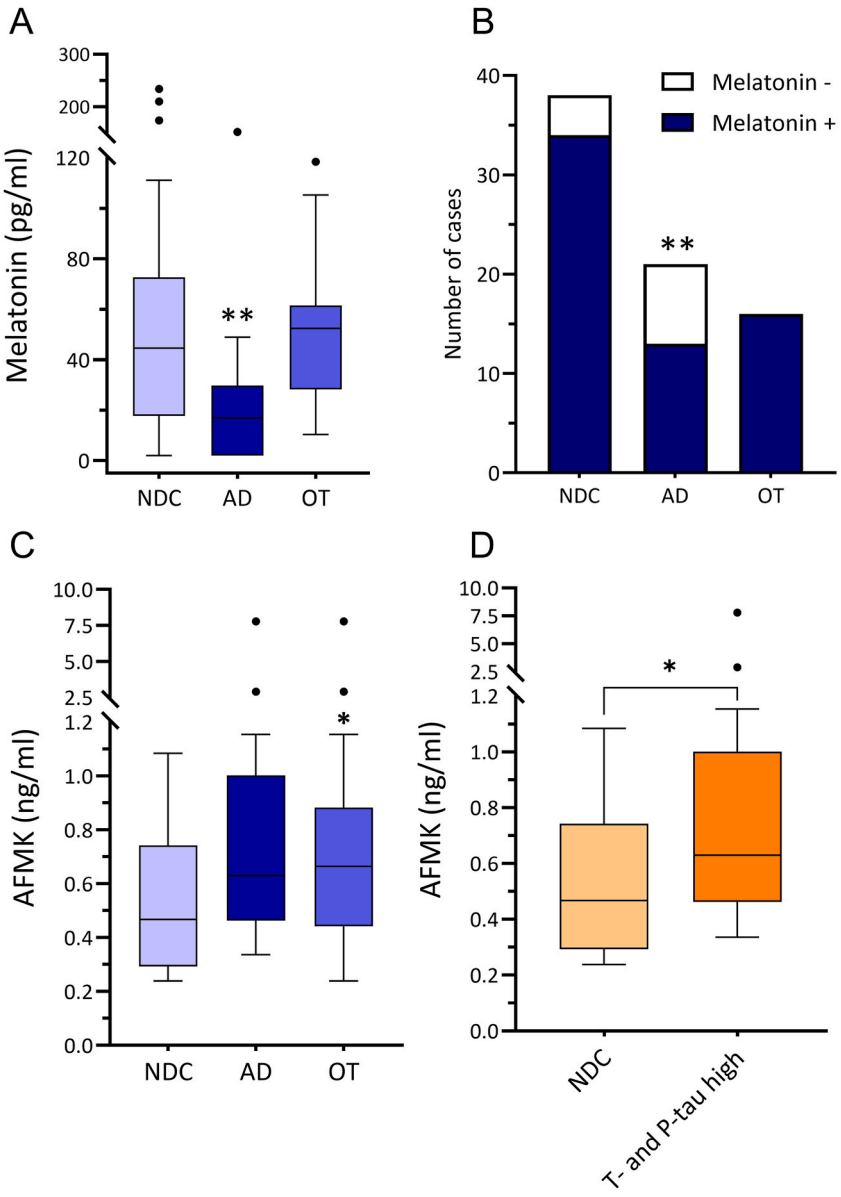

Fig. 5. Melatonin and AFMK levels in CSF samples from non-demented controls (NDC), Alzheimer's disease (AD) and other tauopathies (OT). Melatonin concentration median representation (A). Melatonin qualitative analysis, representing ratios of detectable vs not-detectable samples (B). AFMK concentration median representation (C). AFMK qualitative analysis, representing the ratio of detectable *vs* not-detectable samples (D). For melatonin assay, NDC, n = 27, AD = 15, OT = 16. For AFMK assay, NDC, n = 21; AD, n = 14; OT, n = 13. Control, n = 21; T-tau and P-tau (T- and P-high, neuronal damage), n = 26. Median instead of mean was used in A and C since data did not follow a normal distribution. P values for Mann-Whitney, Krustal-Wallis, and Fisher's exact test: p = 0.01 (A), p = 0.0031 (B), p = 0.0188 (C) and p = 0.0402 (D).

among these parameters (Supplementary Fig. 1). AFMK did not follow any significant trend with melatonin or with redox parameters in any group.

In patients with either, low Aβ or high T-tau, melatonin levels showed different correlations with the neuroinflammatory cytokines assayed, depending on the group of patients (Supplementary Fig. 2). Thus, in patients displaying low Aβ CSF levels, both melatonin and AFMK followed inversed patterns with IL-1B and RANTES, but they differed in the correlation with MIF and TGF-β (Supplementary Fig. 2A). In NDC patients -high CSF levels of Aβ− (Supplementary Fig. 2B), only AFMK showed a mild correlation with TGF-β and RANTES, while melatonin did not follow any significant trend with any neuroinflammatory cytokine in these patients. On the contrary, in patients' samples with normal levels of T and P-tau, melatonin and AFMK positively correlated with TGF-β (Supplementary Fig. 2C). Patients' samples with signs of neuronal damage (high content of T-tau) showed again a negative association between melatonin and IL-1B or MIF and less clear with TNF-α, while in this group of patients, AFMK negatively correlated with IL-1B (Supplementary

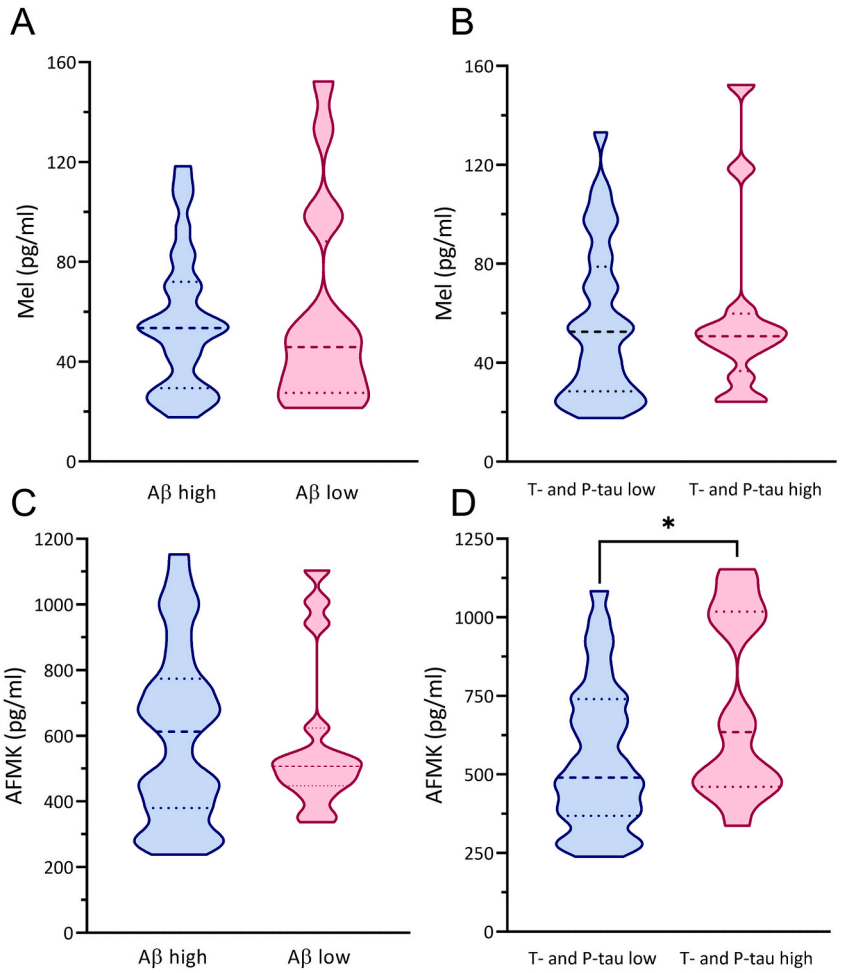

Fig. 6. CSF levels of melatonin and AFMK in patients grouped according to the levels of Aβ, T and P-tau. Levels of melatonin (pg/ml) in CSF samples from patients showing high (>500) or low Aβ (<500) (A) or high T and P-tau (>500 T-tau and >75 P-tau) (B). Levels of AFMK (pg/ml) in CSF samples from patients showing high (>500) or low Aβ (<500) (C) or high T and P-tau (>500 T-tau and >75 P-tau) (D). *, $p < 0.05$ (non-parametric median comparation). Violin plots show median (——), Q1 and Q3 (….). Statistically detected outliers (ROUT method; n = 2 for melatonin; n = 2 for AFMK)) were eliminated.

Fig. 2D).

3.5. Diagnostic capacity of the variables assayed

Among all the variables analysed in CSF of patients, total antioxidant capacity and P-tau (Fig. 8) presented the best diagnostic value to discriminate between positive AD and other tauopathies (Fig. 8A). Both parameters in conjunction manage to increase both the specificity and the sensitivity of diagnostic discrimination (Fig. 8). The rest of parameters included in this study (i.e. neuro-inflammatory cytokines or melatonin) were not able to discriminate both groups efficiently, according to their ROC curves (data not shown).

4. Discussion

The high prevalence of dementia, including AD, is challenging healthcare systems and has a substantial social impact. Accurate diagnostic currently requires a brain autopsy, highlighting the urgent need for improved tools to identify AD patients at early stages. It is well known that AD pathology begins 10–20 years before onset of clinical symptoms. To this aim, in the present study we evaluated the combination of redox parameters, pro-inflammatory cytokines and melatonin/AFMK levels as CSF biomarkers to complement conventional diagnostic methods to differentiate pre-mortem dementia patients. Here we report that while low CSF Aβ levels -denoting amyloid plaques-show a link with neuroinflammation, T- and P-tau are rather associated to oxidative stress. Even though, melatonin was significantly much lower in AD patients, but not in OT samples, neither melatonin nor AFMK could be used as diagnostic tools and

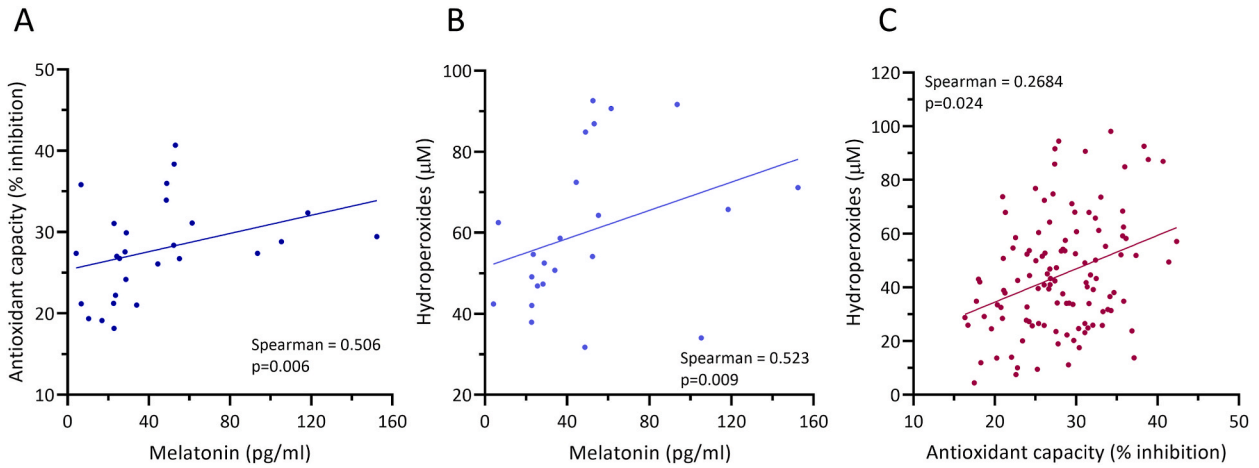

Fig. 7. Correlations between melatonin and oxidative stress parameters in samples from patients showing neuronal damage (T-tau>500, P-tau>75). Melatonin correlations with antioxidant capacity and hydroperoxides in neuronal damage group (positive Alzheimer and other tauopathies) (A, B, respectively). Antioxidant capacity correlation with hydroperoxides in all samples (C). Melatonin *vs* antioxidant capacity, n = 28; Melatonin vs hydroperoxides, n = 24; Antioxidant capacity vs hydroperoxides, n = 116. P values for nonparametric Spearman correlations: p = 0.006 (A), p = 0.009 (B); p = 0.024 (C).

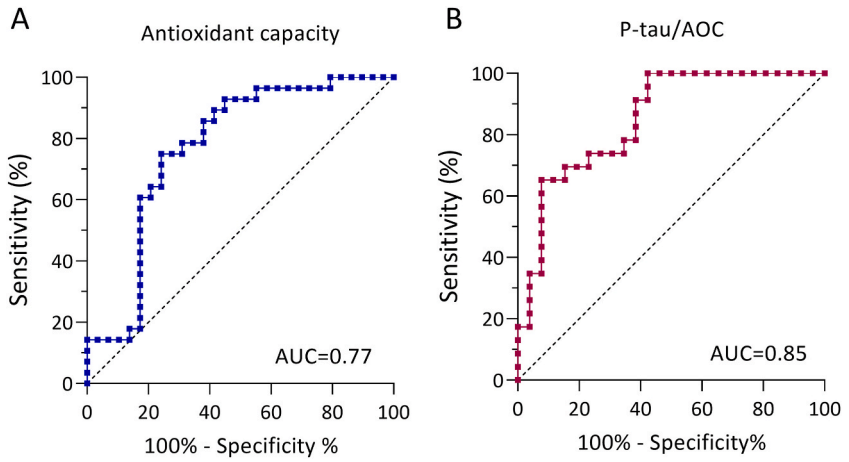

Fig. 8. Assessment of diagnostic capacity of variables aimed to discriminate between Alzheimer's disease and other tauopathies. ROC curve elaborated by using antioxidant capacity to discriminate between Alzheimer's disease (n = 26) and other tauopathies (n = 28) (A). ROC curve elaborated by ratio P-tau/AOC to discriminate between Alzheimer's disease (n = 26) and other tauopathies (n = 23) (B). Cut off −4295 with Specificity −77 % and Sensibility −74 %.

the best diagnostic assessment found, based on ROC curves, was the combination of P-tau and antioxidant capacity.

According to the Aβ hypothesis, the deposition of insoluble Aβ proteins and the formation of senile plaques lead to pathogenic processes in Alzheimer's disease, which in turn produce a decrease in the concentration of Aβ in CSF [50], reviewed by Ref. [51]). Progressive accumulation of Aβ promotes pro-inflammatory changes, switching from inactive to an activated microglial phenotype, characterized by the production of ROS and cytokines, thus stimulating the neuroinflammatory process [52]. Additionally, Aβ itself can generate free radicals and mitochondrial oxidative stress [53], which further causes amyloid aggregation and tau hyperphosphorylation [54]. This, in turn, leads to more oxidative stress, thus generating a vicious circle of Aβ aggregation-oxidative stress-neuroinflammation [55]. However, contrary to previous evidence, in the study presented here, Aβ did not show any significant trend with CSF redox parameters other than a higher antioxidant capacity observed in AD patients' samples. While it is accepted that oxidative stress and Aβ are interconnected, the role of tau in this relationship remains unclear. In this regard, the correlation observed between the CSF values of T- and P-tau and hydroperoxides supports the crucial role of the bidirectional vicious cycle in which oxidative stress promotes aggregation and hyperphosphorylation of tau and, on the other hand, p-tau itself is also able to stimulate ROS production, as it has been already demonstrated [56]. Further studies should determine whether redox parameters in CSF and serum are comparable since this is not necessarily the case for other neurodegenerative diseases [57].

The positive correlation between hydroperoxides and the antioxidant capacity points towards a cellular response triggered to

manage reactive species (mainly hydroperoxides). This correlation was observed in most of the samples with high concentrations of hydroperoxides in the three groups studied. The increase in the antioxidant response to reduce high oxidative levels has already been demonstrated in situations of physiological adaptation after intense physical exercise with the consequent rise in oxidative levels [51]. On the other hand, in studies focused on neurodegenerative diseases, a higher expression and translocation to the nucleus of the transcription factor Nrf2 has been reported, which in turn stimulates cellular antioxidant defences, by inducing the expression of several genes to respond against oxidative stress [58]. Despite the induction of an antioxidant response, the reactive species are not adequately purified, and there is a clear imbalance towards the formation of more radicals, being this relation not dependent on the type of disease, thus showing a transversal character.

A negative correlation between Aβ and neuroinflammatory cytokines was found, namely with TNF-α, IL-6, or RANTES, in agreement with other studies published. In the case of the cytoskeletal protein, only RANTES is positively associated. Signalling mediated by RANTES/CCL5 and its receptor CCR5 plays a crucial role in normal brain physiology and in pathologic conditions, specifically in AD. Our results show that RANTES/CCL5 stands out as one of the best neuroinflammatory biomarkers associated with dementia. Nevertheless, it should be noted that TGF-β was also associated with T and P-tau, but mainly in patients with low Aβ. Since TGF-β signalling has been associated with cellular senescence [59], to our knowledge, though melatonin has been reported in a few studies to be reduced in CSF of Alzheimer's patients, the present study is one of the first to make a different approach by correlating oxidative stress, neuroinflammatory cytokines, and AFMK with melatonin itself, aimed to introduce new parameters that would help to discriminate between patients with other dementias. The goal of the present study was to determine whether redox parameters and melatonin and its oxidative metabolite, AFMK, showed a significant variation in the CSF of patients displaying dementia and if this could be used as potential biomarkers to discriminate between AD patients and other tauopathies.

To our knowledge, this is one of a few studies focused on melatonin on CSF, which according to a recent report by Nous and co-workers, serum daytime melatonin reflects CSF levels. In most of these studies, melatonin was found reduced in AD samples. Here we made a different approach by them with correlating oxidative stress, neuroinflammatory cytokines, and AFMK. The decline in melatonin levels in CSF from positive AD patients indicates that once certain threshold levels of Aβ deposits are reached, antioxidant defence is compromised, independently of the Aβ levels assayed. Other studies have shown a correlation between decreased melatonin levels in AD patients and reduced antioxidant defence. According to classical studies, high local concentrations of hydrogen peroxide are the likely culprit [53]. Furthermore, the direct interaction of antioxidant systems with Aβ deposits and their aggregation state has also been reported [60], a phenomenon which does not occur in OT. Hence, no reduction of antioxidant capacity in CSF samples with high levels of T and P-tau was observed.

It would be expected that AFMK levels, as a major oxidative metabolite derived from melatonin reaction with free radicals [31], would show a linear regression with melatonin, but this was not the case in CSF, suggesting other sources for the kynurenine. In the OT group of patients, the rise of AFMK overlaps with the detection of significant melatonin levels, contrary to AD patients, in which one-third of them show undetectable values of the indolamine. How both melatonin and AFMK could be inversely related in dementia requires further studies as both could be considered potential co-biomarkers. This also points to AFMK as an indicator of oxidative damage rather than an antioxidant capacity marker. This kynurenine has recently been postulated as a potential marker in CSF using an untargeted metabolomic analysis [39].

Despite no apparent connection between redox and immunological parameters, the P-tau/AOC ratio variable and antioxidant capacity display good diagnostic power to discriminate between positive Alzheimer's disease and other tauopathies. The antioxidant capacity alone provided an acceptable discrimination with an AUC = 0.73, considerably increasing its sensitivity and specificity when combined with the P-tau variable. As described above, tau phosphorylation increases in Alzheimer's disease, mainly through amyloid action. At the same time, antioxidant capacity was observed to be higher in the group of other tauopathies rather than the positive Alzheimer's group due to the possible interaction of Aβ with hydrophilic antioxidant systems. In conclusion, the use of this biomarker described here could represent a substantial potential to be used in the differential diagnosis of Alzheimer's dementia vs another tauopathy. Contrary to the starting hypothesis, neither melatonin nor AFMK proved to be useful markers for discriminating between both groups of patients, though melatonin is much lower in AD and AFMK higher in OT. Further studies with a higher number of patients are required to confirm this apparent incongruency, probably due to a wide dispersion in melatonin CSF levels among aged patients.

5. Conclusions

In summary, this study supports evidence of a relation between neuronal damage and oxidative stress on one hand and between neuroinflammation and Aβ on the other. Furthermore, melatonin and AFMK are participating redox agents in the antioxidant response against oxidative damage, with the indolamine significantly low in AD patients and AFMK high in OT samples. This opposing behaviour provides a window opportunity for improving early diagnosis in AD vs other dementias and consequently should be explored. The absence of relationships between redox parameters and neuroinflammatory cytokines leads to the conclusion that, at least in patients with neurological symptomatology, immunological and oxidative markers do not help perform a differential diagnosis or follow-up specific neurodegenerative processes. This absence cannot be extrapolated to pre-symptomatic stages, in which a relation between the mentioned parameters may exist. The elevation of the antioxidant capacity in the OT group or the more significant phosphorylation rate of tau in the Alzheimer group postulates the P-tau/AOC ratio as a good marker for diagnosing Alzheimer's versus another type of tauopathies, potentially impacting clinical practice in the future.

CRediT authorship contribution statement

Francisco Artime-Naveda: Writing – original draft, Validation, Methodology, Formal analysis. **David Hevia:** Validation, Methodology, Formal analysis. **Rebeca Alonso-Arias:** Validation, Methodology, Formal analysis. **Carmen Martínez:** Resources, Data curation. **Isabel Quirós-González:** Methodology, Conceptualization. **Rafael Cernuda-Cernuda:** Methodology. **Alejando Alvarez-Artime:** Methodology, Formal analysis. **Iván Menéndez-Valle:** Resources, Data curation. **Rosa M. Sainz:** Writing – review & editing, Investigation, Funding acquisition, Conceptualization. **Juan C. Mayo:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Ethics declarations

The Ethical Board (HUCA) approved the waiver of informed consent, since only a small fraction of samples for diagnostic assessment were used in the present study.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2025.e41841.

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