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**Title**

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**ABSTRACT**

**Study objectives.** To measure the levels of five neurodegenerative biomarkers in the cerebrospinal fluid (CSF) of patients with narcolepsy type 1 (NT1) with variable disease duration.

**Methods.** Following a standardized protocol of CSF collection and storage, we measured CSF total-and phosphorylated-tau, amyloid-beta 1-40 and 1-42, and neurofilament light chain proteins in 30 non-neurological controls and 36 subjects with NT1, including 14 patients with recent disease onset (i.e. ≤ 12 months, short disease duration group).

**Results.** CSF levels of all biomarkers were similar in NT1 subjects and controls. The comparison between NT1 with short and long disease duration only revealed slightly higher levels of CSF amyloid-beta 40 in the former group (median 9549.5, IQR 7064.2-11525.0 vs. 6870.0, IQR 5133.7-9951.2, p=0.043). CSF storage time did not influence the levels of the tested biomarkers.

**Conclusions.** The measurement of CSF total-tau, phosphorylated-tau, amyloid-beta 40 and 42, and neurofilament light chain proteins is not informative in NT1.

**Keywords**: amyloid, cerebrospinal fluid, narcolepsy, hypersomnia, sleepiness, neurodegenerative, tau, neurofilament protein.

**Statement of Significance**

In contrast with previous studies, here we demonstrated, in a sizeable cohort of patients with variable disease duration, that in narcolepsy type 1 the levels of several cerebrospinal fluid (CSF) neurodegenerative biomarkers are unremarkable. The findings argue against the utility of the current most common neurodegenerative CSF biomarkers in the diagnostic or prognostic evaluation of patients with narcolepsy type 1.

## INTRODUCTION

Narcolepsy Type 1 (NT1) is a rare central sleep disorder clinically characterized by hypersomnolence and loss of boundaries between REM sleep and wake.[1] Several lines of evidence suggest that an autoimmune process mediated by T cells,[2,3] which leads to a deficiency of hypothalamic hypocretin-1 (hcrt-1) signaling pathway, drives the pathogenesis of NT1.[4,5] A reduction of hcrt-1 levels in cerebrospinal fluid (CSF) below the threshold of 110 pg/ml distinguishes NT1 from other hypersomnias of central origin with high specificity.[6] Accordingly, the last update of the International Classification of Sleep Disorders (ICSD-3) introduced the detection of CSF hcrt-1 ≤110 pg/ml by Radio Immune Assay a major diagnostic criterion for NT1.[7]

The availability of CSF prompted the search for biomarkers other than hcrt-1in NT1 cohorts to monitor disease pathogenesis and clinical outcome. However, initial studies, focusing on proteins involved in Alzheimer's disease and other neurodegenerative disorders, including tau and amyloid-beta (Aβ) peptides,[8-13] yielded inconclusive and often divergent results. The small sample size of the cohorts, their clinical heterogeneity, including age, time lapse between the disease onset and lumbar puncture (LP), presence/absence of concomitant pharmacological treatment, combination of narcolepsy type 1 and 2, and eventually analytical and pre-analytical variables, which notoriously may interfere with biomarker dosages,[14,15] likely contributed to these inconsistencies.

In the present study, we systematically compared the CSF levels of five neurodegeneration biomarkers, namely total-tau, phosphorylated-tau, Aβ 1-40 and 1-42, and neurofilament light chain (NfL) proteins, between non-neurological controls and a large cohort of patients with NT1, stratified according to the time lapse from disease onset to LP.

## METHODS

### Patients and clinical evaluation

We analyzed CSF samples from 30 non-neurological controls and 36 patients with NT1, diagnosed at the Narcolepsy Center of the University of Bologna / Institute of the Neurological Sciences of

Bologna (ISNB). Each patient underwent a standardized assessment that included 48-hours polysomnography, multiple sleep latency test (MSLT), *HLA-DQB1*06:02* typing, and determination of CSF hypocretin-1. At the time of evaluation, all patients were drug-naive. NT1 diagnosis was established by the current criteria, including pathological CSF hcrt-1 levels.[7] The control group included age- and sex-matched subjects with a subjective complaint of excessive daytime sleepiness not confirmed at sleep studies (i.e. mean sleep latency at MSLT 14.6±3.1 min, number of early onset REM sleep period ≤2 in each case) and lacking any neurophysiological or neuroradiological evidence of central nervous system diseases or respiratory/motor sleep disorders potentially responsible for the symptom(s). None had cataplectic episodes during a standardized video recording under emotional stimulation.[16] The Local Ethics Committee approved the study protocol (CE-BI 17009), and all patients gave their written informed consent to participate in the study.

**CSF biochemical analysis**

CSF was taken at the L3-L4 or L4-L5 lumbar space during the diagnostic spinal tap for CSF hcrt-1 assay, following a standard procedure, centrifuged at 1000xg for 10 minutes, divided into aliquots, and stored in polypropylene tubes at –80 °C until analysis. In each case, CSF was collected between 8:00 a.m. and 10:00 a.m.

The same senior technician handled and analyzed the samples at the Neuropathology Laboratory of the ISNB. CSF total-tau (t-tau), phosphorylated-tau (p-tau), Aβ1-42 and Aβ1-40 (hence abbreviated as Aβ42 and Aβ40, respectively) levels were analyzed using commercially available ELISA kits (INNOTEST htau-Ag, INNOTEST phosphorylated-Tau181, INNOTEST Aβ1–42 and INNOTEST Aβ1–40; Innogenetics/Fujirebio) according to the manufacturer's instructions. NfL protein levels were analyzed using a commercially available ELISA kit (IBL, Hamburg, Germany), as previously described.[17] The ratio of Aβ42 to Aβ40 was calculated according to the formula [(Aβ42)/(Aβ40)x10].

The mean intra-assay coefficients of variation (CVs) of the assays were 4.7% for t-tau, 1.1% for p-tau, 2.9% for Aβ42, 4.8% for Aβ40 and 4.4% for NfL, whereas the mean inter-assay CVs were 5.0% for t-tau, 2.9% for p-tau, 7.2% for Aβ42, 5.8% for Aβ40 and 6.5% for NfL.

Concerning pre-analytical variables, we collected for each case the CSF storage time (i.e., the interval between LP and each biomarker analysis) and the time interval between clinical onset and LP. We defined NT1 of "recent onset" when the elapsed time from the disease onset to LP was within a window of 12 months.[18] CSF hcrt-1 levels were measured by radioimmune assay ($I^{125}$ RIA kits from Phoenix Pharmaceuticals; Belmont, CA) according to the manufacturer's instructions.

**Statistical analysis**

Statistical analysis was performed using the SigmaPlot 12.5 (Systat Software Inc) software. Based on the presence or not of a normal distribution of the values, data were expressed as mean ± standard deviation (SD) or median and interquartile range (IQR), respectively. Depending on the data distribution the Mann-Whitney U test or the two-tailed t-test were used, as appropriate, to test differences between two groups of continuous variables, while the one-way ANOVA (followed by Tukey's or Bonferroni's post hoc test) or the Kruskal-Wallis test (followed by Dunn's posthoc test) were applied for multiple group comparisons. The chi-square test was adopted for categorical variables. Spearman's correlation was used to determine the relationship between two continuous or ordinal variables. We tested the effect of storage time on CSF biomarker results using univariate linear regression models. A $p$ value of $<0.05$ was considered statistically significant.

**RESULTS**

The study cohort included middle-aged participants (mean age 40.7±7.3 years) with similar gender distribution (male: female ratio 1.3). Fourteen of the 36 NT1 patients (38.9%) had a recent disease onset, while the interval between clinical onset and LP in the whole group was 9.7±10.2 years (mean±SD). All NT1 patients had CSF hcrt-1 levels below the 110 pg/mL threshold, and all but one

carried the *HLA-DQB1*06:02* allele (Table 1). Sleep polysomnographic measures revealed more numerous awakenings during sleep, an increased percentage of N1 sleep stage and, conversely, a reduction of N3 sleep stage in NT1 patients with a recent onset than in controls and NT1 patients with a prolonged clinical course (Table 2).

All tested CSF biomarkers as well as the Aβ42/Aβ40, t-tau/Aβ42 and p-tau/Aβ42 ratios showed similar values in the NT1 and control groups (Table 3). Moreover, there was no correlation between CSF biomarkers levels and polysomnographic sleep parameters. Notably, CSF biomarker levels in both groups were within the range of our "in house" reference values, that are based on the analysis of 50 subjects who underwent LP due to tension-type headache, non-inflammatory polyneuropathies, or subjective complaints (IQR t-tau 152-256 pg/ml, IQR p-tau 32-45 pg/ml, IQR Aβ42 698-1175 pg/ml, IQR Aβ40 5831-10550 pg/ml, and IQR NfL 881-1680 pg/ml).

The comparison between NT1 patients with recent onset and those with a longer course revealed no significant differences in any biomarker results, except for higher median levels of Aβ40 in the former group (9549.5 pg/ml, IQR 5133.7-9951.2 vs. 6870.0 pg/ml, IQR 5133.7-9951.2, p=0.043) (Figure 1). Moreover, the Aβ42/Aβ40 ratio, which allows the normalization of the Aβ peptide levels, did not significantly differ between the two groups.

Regarding pre-analytical variables, the overall median CSF storage time was slightly shorter in controls than in patients with NT1 (15.5 vs 23.0 months, p=0.009). However, on univariate linear regression models, including the storage time as the independent variable and the concentrations of each biomarker as the dependent variable, the latter were not influenced by the time lapse between LP and the biomarker analysis.

## DISCUSSION

In this study, the assessment of a large set of neurodegenerative CSF biomarkers did not distinguish between patients with NT1 and non-neurological controls. Previous studies in this field reported discordant results. A single study showed increased levels of CSF Aβ42, t-tau and p-tau in patients

with NT1 and NT2 and a long disease duration.[9] To explain this unexpected finding some authors claimed a neurotoxicity from a chronic treatment with stimulants, i.e. amphetamines and methylphenidate.[19,20] Most frequently, however, studies found reduced CSF Aβ42 levels in patients NT1 in comparison to healthy subjects.[11-13] A marked reduction of CSF Aβ42 was also reported in a young patient who developed NT1 after H1N1 flu-vaccination.[8] However, the relatively young age of enrolled NT1 participants and the normal levels of CSF tau proteins (t-tau and p-tau)[11,13] make it unlikely that the low CSF Aβ42 reflects ongoing neurodegeneration in NT1. Given that reduced levels of CSF Aβ42 are also found in patients affected by CNS inflammatory disorders of various etiologies,[21-23,] some authors linked the finding to the inflammatory/autoimmune pathogenesis of NT1. Pursuing this hypothesis, Liguori and colleagues compared two subgroups of NT1 according to the duration of the disease and found a more pronounced CSF Aβ42 decrease in the group with the shorter clinical course (≤5 years).[12] In contrast, however, we found no difference in CSF Aβ42 levels between NT1 patients with recent onset and those with a longer disease course. Since the autoimmune pathogenic process in NT1 is thought to be more severe near clinical onset, our results strongly argue against the hypothesis of the reduction of CSF Aβ42 levels due to neuroinflammation.

As the only possible difference related to the clinical course, we found higher levels of CSF Aβ40 in NT1 group with recent disease onset. Recently, the disruption of nocturnal slow wave activity (SWA), which characterizes the sleep of NT1 patients,[24] has been correlated to increased levels of CSF Aβ40 in a dose-dependent manner.[25] In our study, however, we failed to demonstrate a statistically significant correlation between the CSF levels of Aβ40 and the polysomnographic sleep measures, including the absolute and percentage amount of N3 stage sleep. Nevertheless, the lower representation of N3 sleep suggests a more pronounced disruption of SWA in NT1 patients close to onset, a finding to be confirmed in larger studies.

It is well-established that a correct interpretation of the results of CSF Aβ42 assay relies on several factors. Firstly, among the commonly exploited neurodegenerative biomarkers, Aβ42 is the most susceptible to variation in the pre-analytical process.[15,26] To reduce this source of variability, we applied a standardized protocol of CSF collection and storage, had a single, experienced technician performing all the analyses, and excluded the possible influence of CSF storage time on biomarker results, using univariate linear regression analyses in both groups. Secondly, CSF levels of Aβ42 may also vary among individuals depending on the constitutional production of Aβ peptides.[27] For the correct interpretation of CSF Aβ42 results, increasing evidence recommends the evaluation of the CSF Aβ42/Aβ40 ratio,[15,28-30] which allows the normalization of CSF Aβ42 value concerning both the overall individual production of Aβ peptides and the confounding interference of preanalytical variables. In our NT1 cohort, the CSF Aβ42/Aβ40 ratio showed similar mean values in NT1 patients and controls. Finally, the concentration of Aβ shows physiological fluctuations over time as tested in healthy subjects.[31] In human APP transgenic mice, this variation has been related to diurnal fluctuations, given that the Aβ increases in the interstitial fluid during wakefulness and decreases during sleep.[32] In this study, we addressed the potentially confounding influence of the ultradian oscillation of CSF Aβ peptides by defining a standardized time window for CSF collection for both NT1 patients and controls.

The finding that NT1 patients have normal CSF levels of NfL, a biomarker found altered in a large variety of neurodegenerative disorders, is also consistent with the conclusion that the current neurodegenerative biomarkers lack sufficient sensitivity for the pathological process occurring in NT1. The latter result is also in line with that of the only previous study on this marker in NT1 patients.[13]

In conclusion, NT1 patients had normal CSF t-tau, p-tau, Aβ42, and NfL irrespective of disease duration. Similar results were obtained by evaluation of CSF Aβ42/Aβ40, t-tau/Aβ42, p-tau/Aβ42

ratios. Hence, our data do not support the use of these CSF biomarkers for the clinical assessment of NT1 patients.

## DISCLOSURE STATEMENT

**Financial disclosure**: none

**Non-financial disclosure**: G. Plazzi participates to advisory board of UCB Europe, Jazz pharmaceuticals, Bioprojet, IDORSIA. He is president of the Italian Sleep Medicine Association (AIMS) and vice president elected of the European Narcolepsy Network (EU-NN). Nothing to declare for the other co-authors.

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**Figure legend**

**Figure 1.** Comparison of CSF biomarker levels between NT1 patients with recent onset (green) and those with a longer course (orange). *p<0.05.

**Table 1.** Demographic features, results of HLA DQB1*06:02 typing and CSF hypocretin-1 values.

| | **NT1**<br><br>**n=36 [%]** | **Controls**<br><br>**n=30 [%]** | ***p* value** |
|---|---|---|---|
| **Mean age at LP – years ± SD** | 41.1±8.6 | 40.2±5.5 | 0.969 |
| **Female** | 16 [44.4] | 12 [40.0] | 0.909 |
| **HLA-DQB1*06:02** | 35 [97.2] | 3 [10.0] | <0.001 |
| **CSF hypocretin-1 pg/ml (IQR)** | 19.9 (0.7-47.9) | 358.5 (322.5-385.2) | <0.001 |
| **NT1 of recent onset (<12 months)** | 14 [38.9] | - | - |
| **Interval between clinical onset and LP – years ± SD** | 9.7±10.2 | - | - |

**Table 2.** Sleep polysomnographic results in NT1 with long duration and recent onset, and in controls.

| | Controls | NT1 | *p* value | NT1 with long duration | NT1 with recent onset | *p* value[¥] |
|---|---|---|---|---|---|---|
| **Total sleep time (min)** | 401.0 (325.0-431.6) | 369.5 (342.2-424.2) | 0.822 | 385.0 (349.7-430.5) | 350.2 (329.4-416.1) | 0.490 |
| **Sleep efficiency (%)** | 86.0 (77.0-90.4) | 83.4 (76.4-88.9) | 0.694 | 87.4 (75.9-90.8) | 80.0 (75.8-87.6) | 0.392 |
| **Awakenings (n)** | 27.0 (18.0-35.0) | 27.0 (24.0-44.0) | 0.147 | 26.0 (23.0-35.7) | 44.0 (25.0-54.5) | 0.101 |
| **N1 stage (%)** | 5.4 (3.7-6.9) | 10.1 (5.8-14.2) | <0.001 | 9.9 (5.6-11.9) | 13.0 (6.2-21.5) | <0.001[§] [#] |
| **N2 stage (%)** | 42.5 (34.9-50.3) | 41.0 (35.3-49.9) | 0.662 | 39.4 (35.1-50.3) | 41.1 (36.2-46.9) | 0.853 |
| **N3 stage (%)** | 29.9 (22.3-33.3) | 19.2 (13.9-30.9) | 0.004 | 24.7 (18.9-31.9) | 14.5 (12.2-20.5) | <0.001[§] * |
| **REM stage (%)** | 21.9 (17.4-25.9) | 24.9 (20.5-28.5) | 0.071 | 23.6 (19.9-26.4) | 27.5 (21.0-33.8) | 0.120 |

Data are expressed as median and interquartile range (IQR). [¥] ANOVA or the Kruskal-Wallis test compared controls vs NT1 with long duration vs NT1 with recent onset. [§]Controls vs NT recent onset *p* value <0.05, [#]Controls vs NT1 long duration *p* value <0.05, *NT1 long duration vs NT1 recent onset *p* value <0.05.

**Table 3.** CSF biomarker levels in the NT1 and control groups.

| | **NT1**<br><br>**n=36** | **Controls**<br><br>**n=30** | ***p* value** |
|---|---|---|---|
| **t-tau pg/ml** | 148.0 (117.2-235.7) | 164.5 (130.7-184.0) | 0.979 |
| **p-tau pg/ml** | 34.0 (26.0-48.2) | 35.5 (29.0-40.7) | 0.986 |
| **Aβ42 pg/ml** | 940.5 (776.0-1191.0) | 959.0 (835.5-1103.2) | 0.953 |
| **Aβ40 pg/ml** | 7421.0 (5820.2-10700.0) | 8359.0 (6699.7-10050.0) | 0.562 |
| **Nfl pg/ml** | 619.0 (478.7-826.2) | 658.5 (558.2-830.2) | 0.260 |
| **Aβ42/Aβ40** | 1.207 (1.097-1.360) | 1.140 (1.053-1.239) | 0.382 |
| **t-tau/Aβ42** | 0.160 (0.133-0.200) | 0.170 (0.142-0.189) | 0.718 |
| **p-tau/Aβ42** | 0.037 (0.031-0.042) | 0.036 (0.032-0.042) | 0.730 |
| **CSF storage time - months** | 23.0 (13.2-30.7) | 15.5 (9.5-22.2) | 0.009 |

Data are expressed as median and interquartile range (IQR)

Figure 1