

Original Article

Total-tau and Phospho-tau_(181Thr) in Cerebrospinal Fluid of Neurologically Intact Population Increase with Age

(tau protein / phospho-tau protein)

J. JAWORSKI¹, M. PSUJEK², H. BARTOSIK-PSUJEK¹

¹Chair and Department of Neurology, ²2nd Department of Anaesthesiology and Intensive Care, Medical University of Lublin, Poland

Abstract. Tau protein is a microtubule-associated molecule playing a crucial role in maintenance of neuronal integrity and in many neurodegenerative processes; its pathology has become a hallmark feature at the tissue level. The aim of the study was to estimate total tau and phospho-tau (Thr181) concentrations in cerebrospinal fluid of healthy population. Cerebrospinal fluid samples were taken from 129 subjects (age 18–77 years) without known neurologic or psychiatric condition. Both total-tau and phospho-tau levels showed significant correlation with age, which was more pronounced in older population.

Introduction

Since original description of tau proteins as factors facilitating microtubule assembly *in vitro* (Weingarten et al., 1975), the protein became one of the most interesting topics in the field of neurodegenerative disorders. Its importance was underlined by the fact of finding pathological aggregates of hyperphosphorylated tau in brains of patients with Alzheimer's disease (AD) (Braak and Braak, 1991) and other forms of dementias: e.g. corticobasal dementia (Paulus and Selim, 1990) and progressive supranuclear palsy (Hof et al., 1992). Secondly, tau proteins found in cerebrospinal fluid gave hope for the effective early biomarker to be used in different pathological conditions. So far, many reports have been published on the tau levels in cerebrospinal fluid (CSF) of patients with AD (Schönknecht et al., 2003), mild

cognitive impairment (Riemenschneider et al., 2002), fronto-temporal dementia (Shoji et al., 2002), Lewy body dementia (Vanderstichele et al., 2006), vascular dementia (Skoog et al., 1995), Creutzfeldt-Jakob disease (CJD) (Skinningsrud et al., 2008), multiple sclerosis (Brettschneider et al., 2005), amyotrophic lateral sclerosis (Paladino et al., 2009) and stroke (Hesse et al., 2001).

Most of the works focused on AD and mild cognitive impairment pathology, and in this field the results were most consistent, reporting increased concentrations of total-tau (t-tau) as well as phospho-tau (p-tau) that correlated with disease severity (Ibach et al., 2006). Generally it is postulated that t-tau concentrations in CSF reflect axonal loss, most evident in rapidly progressive disease, e.g. CJD and stroke (Hesse et al., 2001). In contrast, in chronically progressive disorders, tau pathology involves aberrant phosphorylation and aggregation. These processes are reflected by increased concentrations of t-tau and p-tau in CSF.

On the clinical ground, CSF analysis of t-tau and p-tau was adopted in differential diagnosis of AD and depression (elevated t-tau in AD), AD and CJD (much higher values of t-tau in CJD reaching several thousand pg/ml, lower values of p-tau in CJD) (Riemenschneider et al., 2003; Skiningsrud et al., 2008), AD and vascular dementia (elevated t-tau and p-tau in AD) (Schönknecht et al., 2003).

Tau proteins are encoded by a single gene located on the long arm of chromosome 17 at band 17q21, but alternative splicing of pre-mRNA yields six tau isoforms, differing in the number of translated exons. Tau protein belongs to the family of microtubulin-associated proteins (MAP) promoting tubulin polymerization and stabilization of microtubules. It is mainly, but not exclusively, localized in neuronal axons; therefore, after disruption of neuronal integrity tau is released to extracellular matrix and consequently its concentration in CSF increases (Avila et al., 2004). Tau protein undergoes many posttranslational modifications, the most important of which is phosphorylation. However, abnormally increased tau phosphorylation affects microtubule binding and promotes its aberrant aggregation (Buée et

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Corresponding author: Jacek Jaworski, Chair and Department of Neurology, Medical University of Lublin, ul. Jaczewskiego 8, Lublin, Poland. e-mail: jjawor@onet.eu

Abbreviations: AD – Alzheimer disease, CJD – Creutzfeldt-Jakob disease, CSF – cerebrospinal fluid, MAP – microtubulin-associated proteins, MMSE – Mini Mental Score Examination, SD – standard deviation, t-tau – total tau, p-tau – phospho-tau.

al., 2000). Both these effects lead to neuronal death, due to the loss of function as well as gain of toxic functions of tau aggregates. There are data suggesting that different pathological conditions relate to the variable tau phosphorylation pattern and intensity (Johnson and Stoothoff, 2004).

The aim of the study was to investigate t-tau and p-tau (181Thr) concentrations in CSF of neurologically and psychiatrically intact individuals in order to establish reference values for the ongoing research and for clinical practice. Reference values of t-tau protein in large cohorts have been reported (Sjögren et al., 2001; Shoji et al., 2002; Burkhard et al., 2004); however, to our best knowledge there are no published data based on a large sample regarding reference values for tau phosphorylated on threonine 181 in CSF of neurologically and psychiatrically healthy population.

Material and Methods

Our study was approved by the local Ethics Committee of the Medical University of Lublin. All subjects were informed on the nature of the study and gave their written consent.

The study group consisted of 129 healthy individuals, 57 males and 72 females. Mean age of the whole population was 44.0 ± 12.8 years (range 18–77). Mean age of male subjects was 42.7 ± 13.1 years, mean age of female individuals was 45 ± 12.6 years. Mean age in both gender groups did not differ significantly (unpaired *t*-test, $P = 0.3$).

To avoid diurnal variation, samples of CSF were collected in the morning, during spinal anaesthesia prior to surgical procedures due to lower limb varices or urinary conditions. No medication was injected to intrathecal space before CSF was drawn. Spinal catheter was introduced through L3/L4 or L4/L5 interspace. Three ml of clear CSF were collected and stored in polypropylene tubes. For the future assays CSF was centrifuged at 3000 *g* for 10 min and frozen at the temperature of -80 °C. Samples were thawed just prior to the ELISA procedure. For each patient t-tau and p-tau concentrations were assayed simultaneously from the same CSF sample by an independent technician.

On the basis of medical history neurological as well as psychiatric conditions were excluded. None of the patients reported memory impairment. All subjects were tested with Mini Mental Score Examination (MMSE) query and scored 28 points or higher. Medical history and MMSE score were obtained the day before spinal tap during routine anaesthetic examination.

Protein concentrations were calculated at room temperatures and kits were brought to the rooms just before assays were performed. Total-tau concentrations were assayed in duplicate samples with INNOTEST® hTAU Ag kit from Innogenetics using the sandwich ELISA method. Detection threshold was 60.0 pg/ml. The assay was designed to detect both phosphorylated and unphosphorylated tau residues.

Phospho-tau concentrations were assayed in duplicates with INNOTEST® PHOSPHO-TAU_(181P) (Innogenetics NV, Gent, Belgium) using the ELISA sandwich method. Detection threshold was 15.6 pg/ml. The assay was designed to detect only phosphorylated on threonine₁₈₁ forms of tau protein. For both assays the absorbance was measured with a spectrophotometer at 450 nm. Standard blank was a sample diluent alone. Intra-assay variability was $< 10\%$.

Among all measurements of t-tau protein seven samples showed results below the detection threshold of 60 pg/ml and thus were rejected. Two measurements were then discarded because they were identified as outliers, beyond 3SD in probability density function for t-tau protein distribution. Finally, 120 measurements of t-tau protein were accepted for further statistical analysis.

Among all measurements of p-tau protein 19 samples showed results below the detection threshold of 15.6 pg/ml, and thus were rejected. Two more measurements had to be excluded from further analysis as outliers, beyond 3SD in probability density function for p-tau distribution. Finally, 108 measurements were accepted for statistical analysis. Calculations of p-tau/t-tau ratio demanded both measurements to be acceptable. In the study population both results could be accepted in 103 patients.

Probability density functions for both t-tau and p-tau proteins showed significant skewness and kurtosis, which was the cause not to assume Gaussian distribution in normality tests. Skewness, with regards to both proteins, was forced by the fact of detection threshold in the study (t-tau skewness 1.247; p-tau skewness 0.88). The left tail of probability density function has been cut because values expressed on the X axis could not reach the level below the remainder of detection threshold (60 pg/ml for t-tau; 15.6 pg/ml for p-tau) and mean value. Additionally, protein distribution showed that normality was significantly influenced by kurtosis (t-tau 1.22; p-tau -0.1).

Statistical analysis was performed using GraphPad Prism® 5.01. *P* value 0.05 was considered significant. As both tau and phospho-tau concentrations did not pass normality tests, non-parametric tests (Mann-Whitney) were adopted and values were presented as medians, interquartile range, minimum, maximum, 25th (Q1) and 75th (Q3) quartiles. Normality was tested with D'Agostino-Pearson test.

Results and Discussion

Median, minimum, maximum and quartile values for t-tau, p-tau concentration as well as p-tau/t-tau ratio in the study group are depicted in Table 1. Our whole study population was divided into two age categories: 18–44 years of age and 45–77 years of age (age limits were chosen arbitrary to achieve a comparable number of measurements in both groups). Median concentration of t-tau and p-tau in both groups differed significantly

Table 1. Concentrations of t-tau and p-tau in CSF in the whole healthy population and in separate age categories

Age categories (years)	t-tau			p-tau			p-tau/t-tau ratio		
	t-tau	p-tau	p-tau/t-tau	t-tau	p-tau	p-tau/t-tau ratio	t-tau	p-tau	
Number of values	120	108	103	63	57	51	57	48	55
Minimum	61.80	15.63	0.06387	61.80	71.84	15.63	17.63	0.06387	0.1474
25% Percentile	107.0	24.81	0.1997	95.61	138.2	19.66	35.84	0.1856	0.2147
Median	146.3	38.86	0.2353	118.3	172.7	29.58	46.66	0.2180	0.2424
75% Percentile	202.8	61.11	0.2966	161.9	225.3	45.67	66.26	0.2696	0.3004
Maximum	404.0	109.8	0.7709	404.0	376.1	109.8	105.4	0.7709	0.7145

Values are depicted as median and percentiles due to the lack of normal distribution. (t-tau in pg/ml, p-tau in pg/ml, p-tau/t-tau ratio without units)

(Mann-Whitney test, $P = 0.0001$) with lower concentrations in younger population (see Table 1).

In the whole study population, a moderate positive correlation of both proteins with age was detected. Spearman coefficient for t-tau/age correlation was $r = 0.44$ (95% CI 0.27–0.58, $P < 0.0001$). Spearman coefficient for p-tau/age correlation was $r = 0.36$ (95% CI 0.19–0.52, $P < 0.0001$). The non-linear regression curve for t-tau/age correlation is depicted in Fig. 1 ($r^2 = 0.17$).

Linear regression for p-tau/age correlation is shown in Fig. 2 ($r^2 = 0.08$).

Considering both age categories separately, the positive correlation of t-tau and p-tau with age was preserved only in the older age group (age 45–77), Spearman correlation (t-tau $r = 0.33$, $P = 0.01$; p-tau $r = 0.39$, $P = 0.003$). In younger population t-tau and p-tau concentrations were independent of age.

A weak correlation was found between the p-tau/t-tau ratio and age (Spearman test $r = 0.20$; $p = 0.04$). In any case, regression analysis showed that the slope of the regression line was not significantly different from zero, assuming constant value irrespective of age. Accordingly, there was no difference in the phosphorylation ratio in both age categories in non-parametric *t*-test ($P = 0.07$) (Fig. 3).

A significant correlation was found between t-tau and p-tau (Spearman $r = 0.73$, $P < 0.0001$) in the whole study population. The regression line is shown in Fig. 4 ($r^2 = 0.24$).

Analysis performed separately in both gender groups did not reveal significant differences in t-tau, p-tau levels nor p-tau/t-tau ratios. Accordingly, similar correlation coefficients were observed in both gender groups (data not shown).

Similar reference values in healthy subjects for t-tau protein were reported before (Burkhard et al., 2004), but to our best knowledge there was no report on p-tau (181 Thr) reference values neither on p-tau/age relation in large healthy population. The role of tau phosphorylation is particularly important in chronic neurodegenerative diseases as analysis of p-tau concentration in CSF can support clinicians with a useful marker of pending progressive disability and help with treatment decisions (Martínez-Yélamos et al., 2004). Concentration of p-tau in CSF probably reflects the degree of tau phosphorylation, not only axonal damage, especially as a significant correlation was found between t-tau and p-tau. In the studies concerning stroke (Hesse et al., 2001) and CJD (rapidly progressive encephalopathy) (Riemenschneider

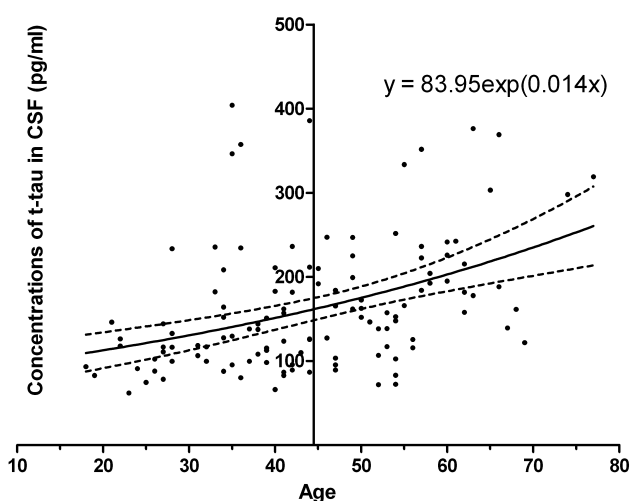


Fig 1. Age/total-tau correlation and regression line with 95% CI. Vertical axis is located between respective age categories. In upper right corner formula that best describes shape of regression line.

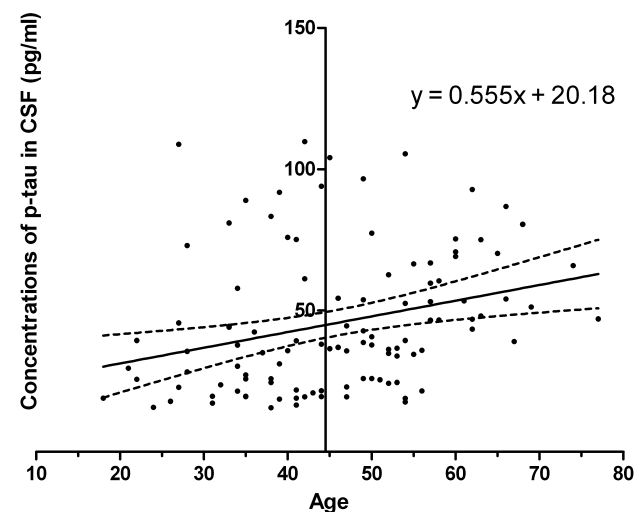


Fig 2. Age/phospho-tau correlation and regression line with 95% CI. Vertical axis is located between respective age categories. In upper right corner formula that best describes shape of regression line.

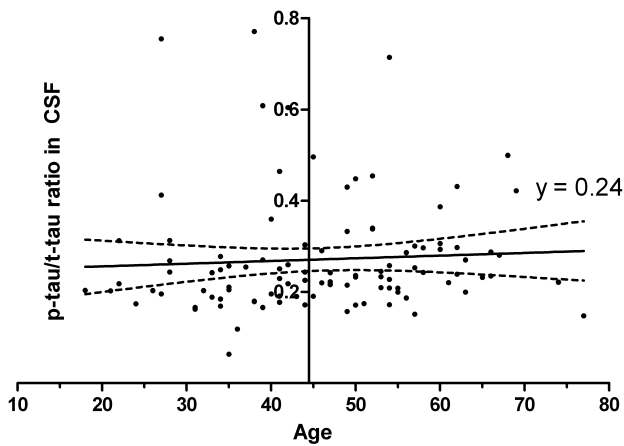


Fig 3. Correlation between age and phospho-tau/total-tau ratio assumes constant value. Regression line with 95% CI. Vertical axis is located between respective age categories.

et al., 2003; Skinningsrud et al., 2008), increased t-tau levels in CSF were not paralleled with p-tau increase. In these processes a predominant feature is axonal damage, not neurodegeneration. In contrast, patients with AD, progressive mild cognitive impairment and probably multiple sclerosis exhibit increased levels of p-tau and increased p-tau/t-tau ratios, which is regarded as a marker of higher degree of tau phosphorylation (Riemenschneider et al., 2003). There are studies suggesting different phosphorylation intensity in different pathological conditions (Vanmechelen et al., 2000).

Tau protein undergoes many posttranslational modifications including phosphorylation, glycosylation, ubiquitinylation, deamination, oxidation, nitration, cross-linking and glycation (Avila et al., 2004). Glycosylation of tau may be important in nuclear localization of the protein. In hyperphosphorylated tau N-glycosylation was reported, whereas in unmodified protein there was mainly O-glycosylation. There is evidence that N-glycosylation inhibits translocation of tau into the nucleus (Lefebvre et al., 2003). Ubiquitinylation is a process that precedes ATP-dependent degradation of various proteins. Ubiquitinated tau was mainly found in inclusion bodies in Pick's and Parkinson's disease and in paired helical filaments in AD. Glycation is a process that involves non-enzymatic condensation of aldehyde or ketone groups with NH_2 groups of lysine. This modification may cause cross-linking of proteins. Tau from paired helical filaments is glycated and this may promote formation of more complex aggregates – neurofibrillary tangles. Other posttranslational modifications of tau include truncation and deamination – both could facilitate pathological aggregation. The presence of cysteine residues may enable formation of S-S bonds through oxidation. Depending on the number of cysteine (one in isoforms lacking or two residues in isoforms containing exon 10) S-S bonds can cross-link different molecules or appear as intramolecular linkage. Nitration of tyrosine can also facilitate dityrosine cross-linking (Avila et al., 2004).

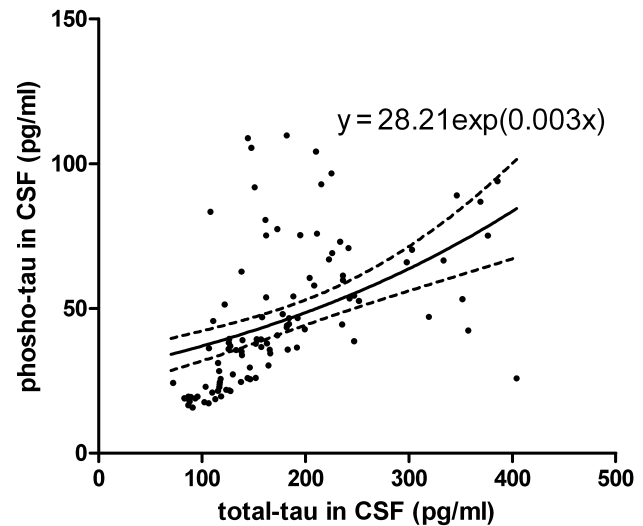


Fig 4. Correlation between total-tau and phospho-tau in the whole study population. Regression line with 95% CI. The formula that best describes shape of regression line is seen in upper right corner.

However, the predominant way of tau function regulation is phosphorylation. In adult brain tau isoforms include 80 Ser or Thr residues and five Tyr residues that can be phosphorylated – it consists of about 20 % of all residues (Johnson and Stoothoff, 2004). Tau may be phosphorylated by a number of kinases. The most important role in both physiological and pathological conditions is played by glycogen synthase kinase 3 β (GSK3 β). This kinase is present in high levels in brain and mainly localizes in neurons. Another important kinase is cyclin-dependent kinase 5 (Cdk5) activated by regulatory proteins p35 and p39, which are expressed almost only in postmitotic neurons (Buée et al., 2000). In physiological conditions phosphorylation plays an important role in regulating tau/microtubule interactions, thus influencing axonal transport. There is evidence that appropriate coordination of tau phosphorylation plays a crucial role in neuronal outgrowth and development (Johnson and Stoothoff, 2004). Pathological phosphorylation, on the other hand, leads to microtubule dysfunction, formation of aberrant tau filament structures and cell death. Given the considerable number of residues as potential phosphorylation sites, it is not justified to treat our results as an estimation of the degree of tau phosphorylation. Our study was designed to delineate reference values for clinical use of phospho-tau assay, as the assay we used in our study detected tau protein phosphorylated on Thr181.

Much higher t-tau and p-tau CSF concentrations in our older population point to normal aging as a factor of progressive neuronal loss, especially as positive tau/age correlation was sustained only in the older age category. There are many studies supporting neuronal loss and decline of brain volume during normal aging both at the tissue level (Jacobs et al., 1997; Šimić et al., 1997) and in neuroimaging studies (Good et al., 2001; Kruggel,

2006). Pathological studies also showed that t-tau levels in brain tissue were higher in younger population compared with the older one, suggesting that t-tau may be redistributed from brain tissue to CSF during normal aging (Mukaetova-Ladinska et al., 1996). On the other hand, higher p-tau concentrations in older population suggest that other pathological mechanisms are involved in normal aging, as apparently different processes are responsible for t-tau and p-tau increase in CSF. It seems that aberrant tau phosphorylation takes place not only in neurodegenerative disease processes, but also during normal aging. This conclusion should be considered in studies including older population (e.g. AD patients), who are generally above 50 years of age.

We found a positive correlation of both t-tau and p-tau with age, independently of gender, in the whole study group, which was previously described with respect to t-tau (Shoji et al., 2002). Interestingly enough, the regression line in the above-mentioned report was not linear, similarly to our results. In any case, after all subjects were split into two age categories, the positive correlation for both t-tau and p-tau with age remained significant only in the older population.

The results obtained for the p-tau/t-tau ratio with respect to age showed a constant value in the whole population. This factor could probably be more helpful as a marker of neuronal loss and disease activity in neurodegenerative diseases. The results suggest that unlike t-tau and p-tau concentrations, the p-tau/t-tau ratio is not influenced by normal aging or any physiological condition. On the other hand, different t-tau/p-tau correlation coefficients were described in various conditions, suggesting the degree of phosphorylation being a distinct feature of these processes (Vanmechelen et al., 2000).

To conclude, the established reference values will be adopted in ongoing studies. Future work would support the possible role of p-tau/t-tau ratio as a marker of neuronal loss and disease activity in different neurodegenerative processes. We hypothesize that the p-tau/t-tau ratio reflects pathological phosphorylation and could provide a measure to quantify this process in various neurological diseases.

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