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► **To cite this version:**

Josef Marksteiner, Christian Humpel. Glycogen-synthase kinase-3 $\beta$  is decreased in peripheral blood mononuclear cells of patients with mild cognitive impairment. *Experimental Gerontology*, 2009, 44 (6-7), pp.370. 10.1016/j.exger.2009.02.007 . hal-00493100

**HAL Id: hal-00493100**

**<https://hal.science/hal-00493100>**

Submitted on 18 Jun 2010

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## Accepted Manuscript

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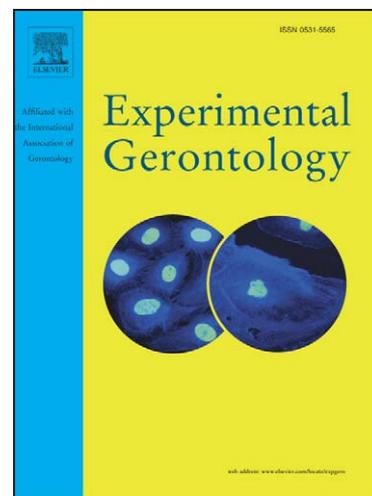
PII: S0531-5565(09)00031-X  
DOI: [10.1016/j.exger.2009.02.007](https://doi.org/10.1016/j.exger.2009.02.007)  
Reference: EXG 8586

To appear in: *Experimental Gerontology*

Received Date: 4 February 2009  
Accepted Date: 18 February 2009

Please cite this article as: Marksteiner, J., Humpel, C., Glycogen-synthase kinase-3 $\beta$  is decreased in peripheral blood mononuclear cells of patients with mild cognitive impairment, *Experimental Gerontology* (2009), doi: [10.1016/j.exger.2009.02.007](https://doi.org/10.1016/j.exger.2009.02.007)

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submitted to: Experimental Gerontology, Controversies, 020209

**Glycogen-synthase kinase-3 $\beta$  is decreased  
in peripheral blood mononuclear cells of patients  
with mild cognitive impairment**

Josef Marksteiner<sup>1</sup> and Christian Humpel

Department of General and Social Psychiatry, Innsbruck Medical University, Austria

<sup>1</sup> present address: Department of Psychiatry and Psychotherapy,  
Landeskrankenhaus Klagenfurt, Austria

Correspondence

Dr. Josef Marksteiner  
josef.marksteiner@kabeg.at

**Abstract**

Glycogen-synthase kinase-3 (GSK-3) plays a central role in Alzheimer's disease (AD). It is involved in the hyper-phosphorylation of Tau and the increased production of  $\beta$ -amyloid. Despite its eminent role, only one study has been published so far in AD blood samples, reporting an increase of GSK-3 $\alpha$  and -3 $\beta$  levels in white blood cells. In this study, we measured GSK-3 $\alpha$  and -3 $\beta$  by quantitative ELISA in peripheral blood mononuclear cells of patients with mild cognitive impairment (MCI), AD and depression in comparison to healthy subjects. In contrast to the previous study, we observed a significant reduction of GSK-3 $\beta$  levels in MCI patients and less pronounced in AD but not in depression. The data indicate that high GSK-3 brain activity is not reflected in peripheral blood mononuclear cells. Therefore, we conclude that more longitudinal studies have to be performed to clarify whether GSK-3 blood levels may qualify as disease specific biological markers.

The enzyme glycogen-synthase kinase-3 (GSK-3) plays an important role in Alzheimer's disease (AD) due to its role of hyper-phosphorylation of Tau (Hanger et al., 1992; Mandelkow et al., 1992). GSK-3 occurs in 2 forms, alpha and beta (Phiel et al., 2003; Bhat et al., 2002; Eldar-Finkelman, 2002) and both forms are widely distributed and found in circulating lymphocytes. GSK-3 is regulated by phosphorylation at serine 9 in GSK-3 $\beta$  and serine 21 in GSK-3 $\alpha$ . GSK-3 has been shown to co-localize with dystrophic neurites and neurofibrillary tangles (Yamaguchi et al., 1996; Hooper et al., 2007), and active GSK-3 is contained in neurons with pre-tangle changes (Pei et al., 1999). GSK-3 expression is up-regulated in circulating peripheral lymphocytes in both AD and MCI patients (Hye et al., 2005). The aim of the present study was to investigate GSK-3 $\beta$  by quantitative ELISA in our patient samples, in order to further examine whether GSK-3 $\beta$  could be established as a biological marker in peripheral blood samples.

Healthy subjects (n=12) and patients with AD (n=44), with MCI (n=57) and with depression (n=25) were assessed by the same diagnostic procedure. Psychiatrists clinically examined all subjects, performed a standardized neurological examination, reviewed medical records. Furthermore, neuropsychological assessment and magnetic resonance neuroimaging was performed as reported previously (Marksteiner et al., submitted). EDTA blood (10 ml) was collected during normal routine clinical treatments and processed within 90 minutes. Peripheral blood mononuclear cells (PBMC) were separated from whole blood on a continuous Biocoll density gradient (1.077 g/ml) after centrifugation (400xg, 30 min). PBMC were visible as a white stratum between plasma phase and Biocoll-Paque. The interphase with the PBMC was carefully removed, washed in 50 ml PBS, and the pellet was immediately frozen at  $-80^{\circ}\text{C}$  until use. The pellet was dissolved in 400  $\mu\text{l}$  PBS + protease inhibitor cocktail (Sigma), sonicated on ice (30 sec, 125W/cm<sup>2</sup>, 140  $\mu\text{m}$  amplitude, 100%), centrifuged 10 min 13 000xg at 4 $^{\circ}\text{C}$ , and the supernatant used for the ELISA. Total protein was determined by Bradford assay using bovine serum albumine as a standard. GSK-3 $\beta$  was determined by a commercial enzyme-linked immunoassay (ELISA) from TiterZyme (catNo. 900-144). The ELISA detects GSK-3 $\beta$  with 100% but with less than 0.01% crossreactivity to GSK-3 $\alpha$ . The standard curve was linear up to 5 ng/ml and had a sensitivity of 74 pg/ml. Briefly, PBMC lysates were diluted 1+1 with assay puffer, then applied to the pre-coated wells, incubated for 1hr

at room, washed and then the capture antibody was added. After incubation for 1 hr at room, wells were washed, the conjugate was added, wells incubated for 30 min at room, washed and then the substrate was added and the color measured in an ELISA reader at 450 nm for 30 min. Analysis was determined in duplicates and correlated to a standard curve. Statistical analysis was performed by one Way ANOVA with a subsequent Fisher-PLSD posthoc test where  $p < 0.05$  was significant.

The patients' characteristics are shown in table 1. AD patients were significantly older (table 1). The MMSE score was not statistically different between control subjects, patients with MCI and with depression, but significantly lower in AD patients (table 1). GSK-3 $\beta$  levels were significantly decreased in MCI patients, but not in patients with depression. The numerical reduction of GSK-3 in AD was not statistically significant (table 1).

GSK-3 has been subsequently shown to function in a wide range of cellular processes, including differentiation, growth, motility and apoptosis (Forde and Dale, 2007; Manoukian and Woodgett, 2002). GSK-3 involves several different signalling pathways, such as e.g. the Akt, Zak-1, Wnt, RCN or glucocorticoid receptor pathways (Forde and Dale, 2007; Manoukian and Woodgett, 2002). Aberrant regulation of GSK-3 has been implicated in several human pathologies. including Alzheimer's disease, non-insulin-dependent diabetes mellitus or cancer (Forde and Dale, 2007). The functional role of GSK-3 in PBMCs has to be elucidated. So far, only one paper has reported Tau expression in human blood lymphocytes at very low levels in healthy subjects (Kvetnoy et al., 2000). The same group reported enhanced Tau levels in blood lymphocytes of AD patients (Kvetnoy et al., 2000). While Hye et al. (2005) showed enhanced expression of GSK3- $\alpha\beta$  in white blood cells, the present data indicate a decrease of GSK-3 $\beta$  in PBMC of MCI patients as measured by quantitative ELISA. We can not offer an explanation for these contradictory results. Some of the differences may be explained by differences in the methodological procedures. Interestingly, Castri et al. (2007) reported that the enzyme phosphatidylinositol-3-kinase (PI3K) is reduced in PBMC in AD patients. This enzyme is directly linked to GSK-3, because the insulin-induced PI3K controls GSK-3 activity. Thus it may be possible that both enzymes, GSK-3 and PI3K are regulated the same way in PBMC in dementia. In conclusion, further experiments are

necessary to clarify whether GSK-3 is a suitable biological marker for MCI or AD.

This study was supported by the Austrian Science Funds (L429-B05).

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**Table 1: Glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) levels in peripheral blood mononuclear cells**

<u>Group</u>	<u>Age</u>	<u>n</u>	<u>MMSE score</u>	<u>GSK-3<math>\beta</math> (ng/mg protein)</u>
Control	68 $\pm$ 3	12	28.4 $\pm$ 0.3	14.3 $\pm$ 2.2
MCI	71 $\pm$ 1 ns	57	27.0 $\pm$ 0.4 ns	9.0 $\pm$ 0.5 *
AD	76 $\pm$ 1 *	44	18.6 $\pm$ 1 ***	10.2 $\pm$ 0.9 ns
Depression	68 $\pm$ 2 ns	25	28.0 $\pm$ 0.2 ns	11.0 $\pm$ 1.1 ns

PBMC were isolated from EDTA blood of healthy subjects, patients with mild cognitive impairment (MCI) or with Alzheimer's disease (AD) or with depression. The Mini Mental score (MMSE) was significantly lower in AD patients. Values are given as mean $\pm$ SEM; n = number of subjects; Statistical analysis was performed by one way ANOVA with a subsequent Fisher PLSD posthoc test (\* p<0.05, \*\*\* p<0.001; ns not significant).

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