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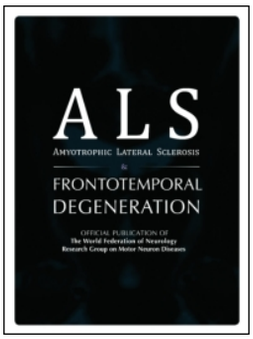

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ORIGINAL ARTICLE

TDP-43 plasma levels are higher in amyotrophic lateral sclerosis

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Abstract

Our objective was to investigate TDP-43 plasma levels in patients with amyotrophic lateral sclerosis (ALS). TDP-43 has been identified as a major component of protein inclusions in the brain of patients with ALS; mutations in the corresponding gene (*TARDBP*) have also been identified. Although increased TDP-43 levels have been reported in the cerebrospinal fluid, plasma levels have not yet been assessed in patients with ALS. TDP-43 levels were quantified by sandwich ELISA in plasma of 219 patients and 100 controls. In addition, we sequenced exon 6 of *TARDBP*, and performed longitudinal TDP-43 plasma measurements in a subset of patients. Results showed that TDP-43 plasma levels were significantly increased in patients with ALS ($p = 0.023$) and we found a positive correlation with age in patients and controls. Longitudinal measurements of TDP-43 plasma levels showed an increase in only one patient, with stable levels in five others. Three *TARDBP* variations were identified in the ALS group (1.7%), but the association with TDP-43 plasma levels was ambiguous. In conclusion, our data indicate that TDP-43 plasma levels may have potential as a marker for ALS. A genotype-phenotype relationship could not, however, be established in this cohort.

Key words: *Amyotrophic lateral sclerosis, TAR DNA binding protein of 43 kDa (TDP-43), ELISA, plasma, biomarker*

Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by loss of motor neurons from the brain and spinal cord. Patients suffer from progressive muscle weakness, eventually leading to respiratory failure and death, on average within three to five years (1). A subset of patients with ALS develops frontotemporal dementia (FTD) and vice versa; as such, they seem to be part of the same clinical and pathological spectrum (2,3).

A characteristic feature of degenerating motor neurons in ALS is the presence of cytoplasmic inclusions positive for ubiquitin. In 2006, the RNA-processing protein TAR DNA-binding protein 43 (TDP-43) was discovered to be the major component of these protein inclusions (4) and, therefore, characteristic for the sporadic cases and most of the familial cases of ALS. Proteins found in the minority of patients with TDP-43 negative inclusions are superoxide dismutase-1 (SOD1) and fused in sarcoma (FUS) (5). The mechanisms leading to TDP-43 accumulation and neurodegeneration have not yet been elucidated. Mutations in the TDP-43 gene, *TARDBP*, have been identified in a subset of patients with ALS and cerebral TDP-43 accumulation (6,7). These mutations account for 4% of all familial and 0–2% of sporadic ALS cases (6,8,9). Most mutations have been detected in exon 6 of *TARDBP* and affect the C-terminal region of TDP-43, which has been suggested to affect normal protein-protein interactions (6). TDP-43-positive protein inclusions have been detected in most cases of tau-negative FTD, providing the pathological substrate for the clinical overlap between ALS and FTD (5).

In patients with FTD, TDP-43 protein levels have been assessed in plasma by means of ELISA, showing increased levels in 46% of the patients with

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clinical FTD compared to 8% of the control subjects (10). Pathological studies have, however, been unable to correlate these plasma levels of TDP-43 with either the presence or the amount of TDP-43 protein in brain tissue (11). The significance of increased TDP-43 in plasma needs to be elucidated.

A biochemical marker for the diagnosis and prognosis of ALS is currently lacking, but is urgently needed to reduce diagnostic delay and to optimize patient care. The TDP-43 protein, as a key player in ALS pathogenesis, is an obvious candidate marker.

We hypothesized that a subset of ALS patients would show increased TDP-43 plasma levels, analogous to the findings in FTD patients. TDP-43 plasma levels were measured using ELISA and cross-sectionally compared between patients with ALS and control subjects. In addition, longitudinal measures were performed in a subset of patients. As mutations in *TARDBP* can cause ALS, we also screened ALS patients and control subjects for mutations in *TARDBP* and examined the association with plasma levels of TDP-43.

Materials and methods

Subjects

Plasma samples of 219 patients with ALS were studied and compared with 100 age- and gender-matched healthy control samples. All subjects gave written informed consent, in line with the Declaration of Helsinki, as approved by the Medical Ethics Committee for Research in Humans of the University Medical Centre Utrecht, The Netherlands. Patients were recruited from a national referral centre for motor neuron disease, the University Medical Centre Utrecht, and diagnosed with possible, probable or definite ALS according to El Escorial criteria (12). The demographic and key clinical features of the participants are outlined in Table I; none of the patients fulfilled the clinical criteria for FTD (13).

Plasma was separated from whole-blood samples (10 ml blood with EDTA acting as anti-coagulant) by routine methods, and stored at −80°C until the assay.

Recombinant TDP-43 protein

The full open reading frame of human TDP-43 was obtained by PCR using an IMAGE clone as template and subcloned into the pET-46-EK/LIC system (Novagen, EMD chemicals, Darmstadt, Germany), introducing an N-terminal polyhistidine tag. The protein was expressed in E. coli BL21 (DE3) pLysS (Novagen), and purified using Ni-NTA agarose (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. The protein concentration was determined by BCA protein assay (Pierce, Thermo Scientific, Rockford, Illinois, USA) and the purified protein (estimated purity > 90%) was used as a standard in the ELISA.

TDP-43 ELISA

The ELISA for TDP-43 was performed essentially as described previously (10,14). In brief, a mouse monoclonal antibody directed against TDP-43 (clone 2E2-D3, Abnova, Taipei City, Taiwan) was coated overnight at 4°C in 0.1 *M* NaHCO3 buffer, pH 9.6, followed by blocking with 1% bovine serum albumin (BSA) in phosphate buffer solution (PBS) for 1 h at room temperature. Plasma and standard samples were incubated for 2 h at 37°C, followed by incubation with a rabbit polyclonal antibody directed against TDP-43 (Proteintech Group, Manchester, UK) for 1 h at 37°C. A goat anti-mouse horseradish peroxidase conjugated secondary antibody (Jackson Immunoresearch Laboratories, Suffolk, UK) was incubated for 1 h at 37°C, followed by detection of luminescence using SuperSignal ELISA Femto maximum sensitivity substrate (Pierce, Thermo Scientific) and a Lumistar Optima instrument (BMG Labtech, Ortenberg, Germany).

Sequencing exon 6 of TARDBP

A subset of ALS patients and control subjects available for sequencing (175 and 83 cases, respectively) were screened for mutations in exon 6 of *TARDBP* (6,7). Primers have been described previously (7). We used BigDye Terminator 3.1 sequencing kit (Applied Biosystems, Foster City, California, USA) and a DNA Analyzer 3730XL for sequencing. Data

Table I. Demographic and clinical characteristics of the patients and healthy subjects.

	Healthy control subjects ($n = 100$) Mean ± SD (range)	ALS patients ($n = 219$) Mean ± SD (range)
Age (years)	61.2 ± 10.4 (28–84)	62.6 ± 12.3 (24–89)
Male/Female	62/38	140/79
Site of onset (*n*)		
Bulbar		68 (32%)
Spinal		151 (68%)
Time to diagnosis (months)		12.0 ± 12.9 (2–131)
Disease duration (months)*		13.3 ± 17.4 (1–173)

*At time of sampling.

analysis was performed with PolyPhred and identified mutations were confirmed on genomic DNA (15). PMut was used to predict the impact of these mutations on the structure and function of TDP-43 (http://mmb2.pcb.ub.es:8080/PMut/).

Statistical analysis

All data were analysed using the R software package for statistical computing (http://www.R-project.org, R Version 2.12-0 GUI 1.35); TDP-43 plasma levels were tested for normality with the Kolmogorov-Smirnov test. As the data were skewed, normality requirements were not met and log transformation was performed. Subsequently, normality was reached and the difference between patients and healthy control subjects was assessed using a linear model, including covariates: age, gender and disease duration (at the time of sampling).

Results

TDP-43 concentration in plasma

TDP-43 levels were significantly increased in patients with ALS compared to healthy control subjects ($p = 0.023$) (Figure 1). In 28% of ALS patients, the absolute TDP-43 concentration was above the detection limit (0.31 µg/l; defined as two standard deviations above the mean background signal) versus 21% of the control subjects. It is important to note that whereas the highest absolute measure in control subjects was 3.76 µg/l, it was up to 10.85 µg/l in ALS patients. Specifically examining the subgroup of patients with measures above the highest of controls ($n = 8$) did not reveal a distinct clinical profile compared to the total group of patients (Table II). Survival analysis using a Cox regression model including high TDP-43 levels as a factor, besides other prognostic factors (age, site of onset and time to diagnosis) did not reveal a significant contribution of increased TDP-43 plasma levels (different cut-off values were explored). The distribution of measurements as shown in Figure 2 draws attention to a subgroup of relatively young patients with high TDP-43 plasma levels (encircled in graph); high measures in young patients might have more specificity for disease.

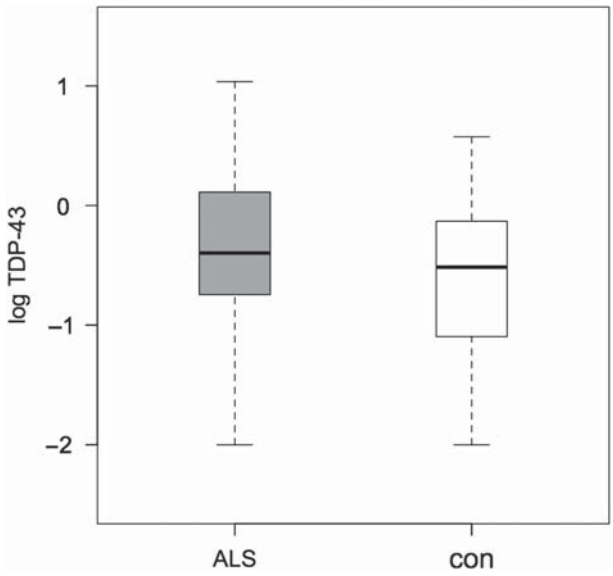

Figure 1. TDP-43 plasma levels in patients with ALS and healthy control subjects. TDP-43 protein levels were determined using ELISA in plasma of ALS patients and healthy controls (con). This box plot shows the log-transformed data indicating the median, quartiles, maximum, and minimum. TDP-43 plasma levels are significantly increased in patients with ALS compared to controls.

A linear model examining TDP-43 levels and age at sampling showed a significant positive correlation ($p = 0.029$, Figure 2), but no relation was found with other clinical variables such as gender ($p = 0.86$), disease duration ($p = 0.53$) and survival ($p = 0.55$; data not shown). If both disease status (as factor) and age at sampling were included in a linear model, the result was statistically significant (F-statistic, $p = 0.008$). Interaction between age at sampling and disease status was not significant.

Longitudinal measures

Samples, collected longitudinally at two to eight time-points, were available from six patients. In five patients, the TDP-43 plasma levels were relatively consistent in time. However, in one patient, four consecutive measurements showed a marked increase over time (Figure 3). This particular subject was a 61-years-old female patient with a bulbar onset who

Table II. Subgroup of patients with high TDP-43 measures.

	ALS; normal TDP-43 levels ($n = 211$) Mean ± SD (range)	ALS; high TDP-43 levels ($n = 8$) Mean ± SD (range)	
Age (years)	62.9 ± 12.1 (27–89)	55.6 ± 16.5 (24–72)	$p = 0.26$
Male/Female	135/76	5/3	$p = 0.60$
Site of onset (*n*)			
Bulbar	66 (31%)	2 (25%)	$p = 0.52$
Spinal	145 (69%)	6 (75%)	
Time to diagnosis (months)	12.1 ± 13.1 (2–131)	9.6 ± 5.6 (2–21)	$p = 0.56$
Disease duration (months)*	13.4 ± 17.7 (1–173)	11.0 ± 6.5 (2–21)	$p = 0.37$

*At time of sampling.

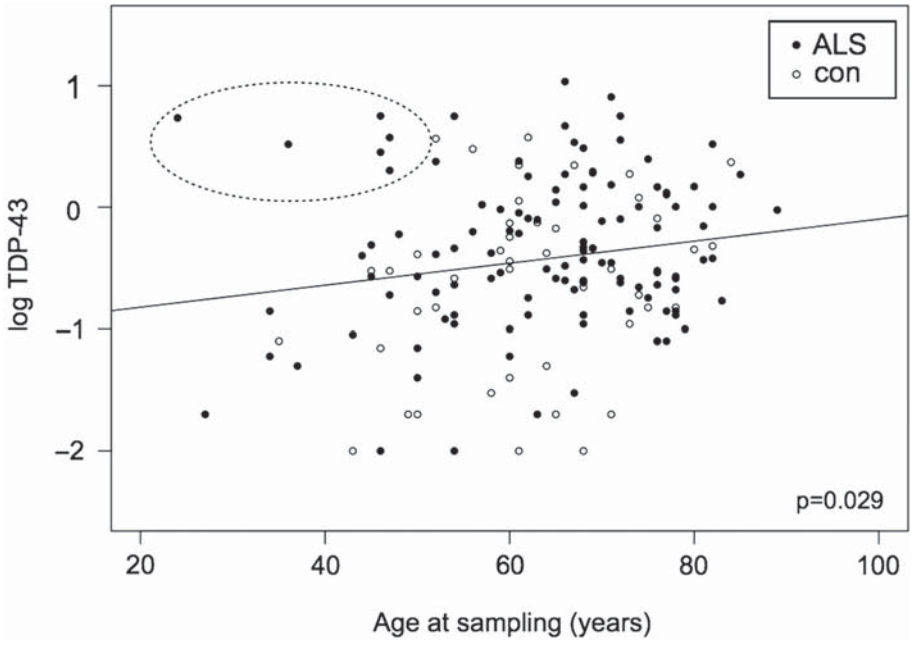

Figure 2. Correlation between TDP-43 plasma levels and age. The log-transformed TDP-43 plasma levels were plotted against the age at sampling in patients with ALS and control subjects (con). It is noticeable that a small number of relatively young patients have increased TDP-43 levels (encircled in plot).

died a few months after the last measurement. Other subjects who died during a similar follow-up did not show an increase in TDP-43 level. There was one other subject with bulbar onset, but in this case TDP-43 plasma levels were not detectable.

TDP-43 genotyping

In the patient group, we found three subjects (1.7%) with a *TARDBP* variation. In two of these, it was a non-synonymous variation in exon 6 of *TARDBP*, and, based on the PMut analysis, most probably pathogenic. One patient probably had a silent variation. The clinical characteristics of patients with *TARDBP* variants are presented in Table III. The two patients with a pathogenic variation had a measurable TDP-43 concentration in plasma: 0.47 and 1.01 µg/l, respectively.

Discussion

In this study, we investigated whether the TDP-43 plasma level is a suitable biomarker for ALS, especially since nearly all ALS patients, apart from

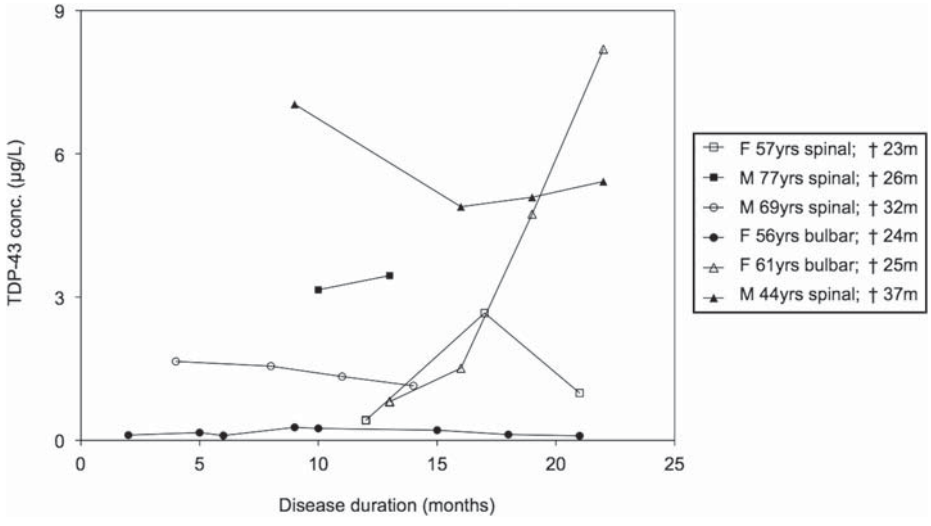

Figure 3. Longitudinal TDP-43 plasma measurements. In six patients we performed two to eight longitudinal measurements. This graph displays the TDP-43 plasma levels measured at different time-points during the disease course. All patients were deceased at the time of analysing the data. The survival is indicated per subject in the legend. In five out of six patients these measurements are relatively stable over time; one patient (F 61, bulbar) had a rising plasma TDP-43 level. Abbreviations. Legend (gender (F = female, M = male); age at onset (years); site of onset (spinal/bulbar); survival (months)); TDP-43 conc.: TDP-43 plasma concentrations.

Table III. Clinical characteristics of patients with *TARDBP* variants. PMut was used to predict the impact of the identified variants on the function of TDP-43. The p.G295C and p.N352S variants were predicted to be pathogenic.

Variant	PMut	Group	Gender	Age at onset	Site of onset	Survival (months)	TDP-43 plasma level (µg/l)
p.A315A	Silent	SALS	M	54	Cervical	>48*	0.00
p.G295C	Pathogenic	FALS	F	80	Bulbar	43	1.01
p.N352S	Pathogenic	SALS	M	53	Cervical	180	0.47

Abbreviations. M: male; F: female; SALS: sporadic ALS.
*Alive at the time of data-analysis.

those with FUS or SOD1 mutations, exhibit TDP-43 protein inclusions as a pathological entity in the brain. We showed that plasma TDP-43 levels were significantly increased in patients with ALS, compared to healthy control subjects. Furthermore, TDP-43 plasma levels correlated positively with age. In addition, we identified *TARDBP* variants in three ALS patients, but these could not be associated with TDP-43 protein levels in plasma due to the small number. In longitudinally performed measurements, the levels appeared to be consistent over time with the exception of one subject who showed a marked increase with disease progression.

With the development of disease-modifying therapies, an in vivo assessment of the type and extent of neuropathological changes in the brain (e.g. by means of a blood test) is of increasing importance and would facilitate diagnosis and drug discovery. Our finding of significantly increased TDP-43 levels in plasma might be related to TDP-43 accumulation in the brain and is supported by a positive relationship with age. Previous studies have found raised plasma TDP-43 levels in FTD patients (10) as well as in patients with inclusion body myositis (IBM), an important mimic of ALS (16). FTD patients had detectable TDP-43 levels in 46% of cases, which is a higher number than in our study (28% of ALS patients) (10). This could be due to minor technical differences in assay performance, more extensive protein accumulation in the brains of patients with FTD compared to ALS, or differences in the pathogenesis yet unidentified. A study in patients with FTD that focused on the correlation between TDP-43 plasma levels and neuropathological changes could not confirm a positive correlation (11). As similar studies in ALS are currently lacking, the pathological correlate of elevated TDP-43 in plasma of patients with ALS remains to be established.

Previous studies in ALS have shown increased TDP-43 levels in cerebrospinal fluid (CSF) (14,17,18). One of these studies suggests that patients with a short disease duration (of up to 10 months) have higher CSF levels of TDP-43 (14) compared to those with longer disease duration. Another study suggested a relationship between increased TDP-43 levels and longer survival (18). In the present study we could not confirm a relationship between plasma TDP-43 and disease duration or survival. We did, however, observe an association of plasma TDP-43 with age.

Longitudinal measurements performed in a small subset of patients revealed stable levels over time in all but one patient. This particular subject did not have any specific characteristics compared to the other patients. The increase in TDP-43 protein levels was not a sign of impending death, since other patients also died within a similar follow-up period and did not show increasing plasma TDP-43 levels. The stable plasma levels found in the other five patients argues against TDP-43 in plasma as a biomarker of the total TDP-43 load in the central nervous system and is rather suggestive for increased TDP-43 as a risk factor for ALS. Nevertheless, this finding stresses the importance of including longitudinal analyses on a larger scale in future (international) studies.

Mutational analysis of *TARDBP* revealed three variants (1.7%), most probably pathogenic in two. This percentage is in accordance with previous studies on *TARDBP* gene mutations in sporadic ALS patients (6–9). The numbers in this cohort are too small to establish a genotype-phenotype relationship as reported in studies on progranulin. Mutations in the progranulin gene (*GRN*) cause FTD and were found to be related to plasma levels of progranulin protein (19).

We conclude from the present study that patients with ALS, as a group, have significantly raised plasma levels of TDP-43 compared to healthy controls, and that this finding could possibly be related to the cerebral accumulation of this protein. We could not assess a potential correlation between TDP-43 plasma levels and the three *TARDBP* variants due to the small numbers. Future studies should include pathological assessments to study a potential correlation between TDP-43 plasma levels and cerebral protein accumulation, as well as quantification of the pathological, phosphorylated form of TDP-43 in plasma. Finally, the specificity of TDP-43 levels for ALS as opposed to clinical mimics (1), e.g. cervical stenosis or multifocal motor neuropathy, needs to be established.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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