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ORIGINAL ARTICLE

Limited role of free TDP-43 as a diagnostic tool in neurodegenerative diseases

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Abstract

TAR DNA-binding protein 43 (TDP-43) is one of the neuropathological hallmarks in amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD). It is present in patients' blood and cerebrospinal fluid (CSF); however, the source and clinical relevance of TDP-43 measurements in body fluids is uncertain. We investigated paired CSF and serum samples, blood lymphocytes, brain urea fractions and purified exosomes from CSF for TDP-43 by one- (1D), and two-dimensional (2D) Western immunoblotting (WB) and quantitative mass spectrometry (MRM) in patients with ALS, FTLD and non-neurodegenerative diseases. By means of 2D-WB we were able to demonstrate a similar isoform pattern of TDP-43 in lymphocytes, serum and CSF in contrast to that of brain urea fractions with TDP-43 pathology. We found that the TDP-43 CSF to blood concentration ratio is about 1:200. As a possible brain specific fraction we found TDP-43 in exosome preparations from CSF by immunoblot and MRM. We conclude that TDP-43 in CSF originates mainly from blood. Measurements of TDP-43 in CSF and blood are of minor importance as a diagnostic tool, but may be important for monitoring therapy effects of TDP-43 modifying drugs.

Key words: *TDP-43, frontotemporal lobar degeneration, amyotrophic lateral sclerosis, exosomes, cerebrospinal fluid, biomarker*

Introduction

The TAR-DNA binding protein TDP-43 is a nuclear protein with 414 amino acids and a molecular weight of 44.7 kDa, belonging to the group of heterogeneous ribonucleoproteins (hnRNP), which is involved in RNA and DNA metabolism. The function of TDP-43 is probably also regulated by protein interactions (1–3). Ubiquitinated TDP-43 was found to be aggregated in the cytoplasm of neurons and neuroglia in cases of amyotrophic lateral sclerosis (ALS) and in about half of the FTLD cases as a major neuropathological hallmark of these diseases (4), suggesting a common underlying pathogenesis (5,6). The relevance of TDP-43 measurement in blood and CSF remains a contentious issue. We could not find any relevant difference of TDP-43 levels in CSF in the disease groups ALS, FTLD and other neurological diseases (7). However, other studies have even reported elevated levels of TDP-43 in blood from ALS patients (8–11). Therefore, the source of TDP-43 in blood and CSF in particular deserves attention. We analysed paired CSF and serum samples of FTLD, ALS and controls for the presence and concentration of TDP-43 by means of immunoblotting in each pair of CSF serum samples. These CSF/blood ratios were compared with the CSF/blood ratios of proteins of similar molecular weight in the Felgenhauer blot according to their hydrodynamic radius in order to determine whether TDP-43 is mainly blood-derived (12,13). To investigate additional sources of TDP-43, we also looked at purified exosomes from CSF. TDP-43 was also investigated using 2D-immunoblotting and targeted mass spectrometry (14).

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Methods

Patients

The ALS group consisted of nine patients (seven males, two females). The mean (SD) age was 64 (4.2) years. The FTLD group consisted of four patients (two males, two females). The age of the patients at the time of CSF sampling was 66 (14.1) years. Diagnoses of ALS and FTLD were made according to the El Escorial criteria of Pradat and Bruneteau (15) and the recently revised consensus criteria for FTLD (16–19). The group of controls comprised eight patients (five males, three females) with an age of 62 (13.9) years. The final diagnoses of the patients were non-neurodegenerative diseases (for detailed diagnoses please see Supplementary material, which is only available in the online version of the journal. Please find this material with the following direct link to the article: http://www.informahealthcare.com/doi/abs/10.3109/21678421.2014.905606).

Collection and analysis of CSF samples were approved by the ethics committee. All individuals or their relatives gave written informed consent to their participation in the study. Routine CSF data were available for all samples. Additionally we investigated brain material from two ALS patients and two non-neurodegenerative cases. The human brain tissue originated from the Brain Bank of the Laboratory of Neuropathology at the University of Ulm (Germany). This Brain Bank collects brain tissue in accordance with German legal regulations. The project was approved by the ethics committee of the University of Ulm.

Western immunoblotting (WB)

Samples were stored at –80°C until analysis. CSF samples were first precipitated with acetone. SDS-PAGE was performed with 12% polyacrylamide gels (7). WBs were probed with antibodies against different TDP-43 epitopes (N-terminus, C-terminus, and aa 205–222), against calnexin, GP Ib-V-IX and flotillin-1. As standards, human Jurkat cells and murine neuroblastoma (N2A) cells were used (for antibody details and cell lysates see Supplementary material, which is only available in the online version of the journal. Please find this material with the following direct link to the article: http://www.informahealthcare.com/doi/abs/10.3109/21678421.2014.905606).

Purification of exosomes

Exosomes were prepared as described previously (20) with some modifications: 10 ml fresh native CSF from ALS, FTLD and control patients was centrifuged for 10 min at 3500 *g*, supernatant was gathered and supplied to the second step of centrifugation at 4500 *g* for 10 min. Further steps of centrifugation followed for 10 min at 4500 *g*, 30 min at 10,000 *g* and 60 min at 100,000 *g*. After each step the resulting cell pellet was dissolved in 20 µl sample buffer and boiled for 5 min. After the last step at 100,000 *g*, the CSF supernatant was removed and the pellet was washed with PBS and centrifuged for 1 h at 100,000 *g*. To prove the quality of the exosome preparation by WB, we used primary mouse antibody against flotillin-1, a protein enriched within exosomes (21). To check purity, membranes were stripped (see Supplementary material). As a negative control, an antibody against calnexin – an endoplasmic reticulum resident protein – was used to exclude microsomal contamination in the purified exosome pellets and against glycoprotein Ib-V-IX – a thrombocyte-receptor protein – to rule out serum contamination, since the majority of exosomes in serum are derived from thrombocytes (22, 24, 25).

Transmission electron microscopy

The morphology of exosomes was controlled after centrifugation and fixation by transmission electron microscopy (TEM) (Zeiss EM 10) (23). Negative stained samples were prepared according to the standard protocol for thin-section TEM (see Supplementary material to be found online at http://www.informahealthcare.com/doi/abs/10.3109/21678421.2014.905606).

CSF/serum concentration of TDP-43

In order to estimate whether TDP-43 is brain- or blood-derived, we compared the TDP-43 CSF/blood ratio with the CSF/blood ratio of proteins of similar molecular weight and known hydrodynamic properties according to Felgenhauer blot (1974). Using this approach, it is possible to determine whether the protein is merely blood-derived or has an additional intrathecal fraction (12).

2D-polyacrylamide gel electrophoresis (PAGE)

2D-PAGE was carried out as described previously (26). Samples were resuspended in 100 µl lysis buffer. Isoelectric focusing was performed on 7-cm strips with isoelectric points (pI) of 4–7 and 3–10 followed by SDS-PAGE and WB (26,27). Brain urea fractions were prepared from frontal cortex of one ALS patient with TDP-43 positive pathology and one control according to Neumann et al., with minor changes (for the sequential extraction protocol please see the see Supplementary material to be found online at http://www.informahealthcare.com/doi/abs/10.3109/21678421.2014.905606) (4).

Quantitative mass spectrometry

For mass spectrometry analysis, exosomes were purified from 1 ml of native CSF from eight ALS, seven FTLD and seven control patients. Exosome pellets and recombinant flotillin-1 (Novus Biologicals, Germany) were resuspended in 100 µl of 50-mM ammonium bicarbonate and proteins denatured by adding 8 mM

DTT and incubation at RT for 1 h. Alkylation was performed with 50 mM iodoacetamide at RT for 1 h. Excess iodoacetamide was inactivated by adding DTT. Digestion was carried out with 1 µg Trypsin Gold (Promega, Mannheim, Germany) overnight at 37°C. Peptides were evaporated to dryness and resuspended in 30 µl of 0.5% acetic acid. For MS analysis 10 µl of the peptide mixture was used. As standards, synthetic TDP-43 peptides and recombinant digested flotillin-1 were mixed and serially diluted to create a standard curve. Peptide separation was carried out on an Ultimate 3000-nano-HPLC (Thermo Scientific, Dreieich, Germany) on a 15-cm PepMax cC18 column (Thermo Scientific) using a gradient of 20–80% acetonitrile within 10 min at a flow of 300 nl/min. MS analysis was performed on a QExactive mass spectrometer (Thermo Scientific) in targeted-SIM mode (t-sim). For each protein two independent peptides were chosen for quantification. For flotillin-1, peptides corresponding to amino acids 92–106 and 197–217, and for TDP-43, peptides corresponding to amino acids 84–95 and 182–189, were used as their doubly charged ions (27). The amount of TDP-43 and flotillin-1 was measured in ng per pellet. Flotillin-1 was used as an indirect marker for the number of exosomes contained in each pellet, while TDP-43 was set in relation to the amount of flotillin-1. For statistical analysis please see Supplementary material to be found online at http://www.informahealthcare.com/doi/abs/10.3109/21678421.2014.905606.

Results

TDP-43 immunoblotting

In WB a 45-kDa band was detected by N-terminal TDP-43 antibody in CSF and serum from patients and controls (Con) (Figure 1a). In addition, we found an approximately 50-kDa and 55-kDa band of higher intensity. The same band was found in purified albumin and IgG (see Supplementary Figure 1, which is only available in the online version of the journal. Please find this material with the following direct link to the article: see Supplementary material to be found online at http://www.informahealthcare.com/doi/abs/10.3109/21678421.2014.905606.

In order to exclude that the 45-kDa band in CSF detected by polyclonal antibodies is unspecific and unrelated to TDP-43, we subjected CSF immunoblots to immunodetection with polyclonal antibodies specific for the C-terminus of TDP-43. The 45-kDa band in CSF was recognized by both antibodies. In nearly all immunoblots, similar signal intensities for TDP-43 in serum and CSF could only be observed as long as the serum volume was 200 times less than the CSF volume, independently of patient diagnosis. According to the estimated hydrodynamic radius, such a serum/CSF relation is observed for proteins of similar molecular weight, which are known to be blood-derived. As shown in Figure 1a, the WB signal intensity obtained with 1 µl serum (lane 2) is almost

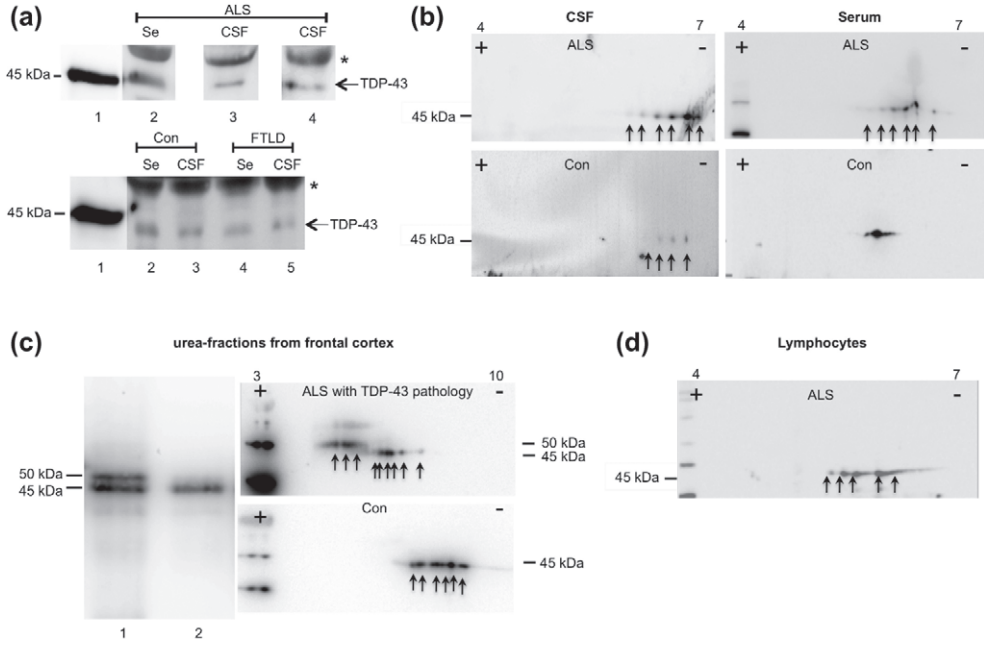

Figure 1. TDP-43 immune reactivity of ALS, FTLD patients and non-neurodegenerative controls (Con) by means of 1D- and 2D-immunoblot. (a) WB with TDP-43 polyclonal antibody of serum and CSF (precipitated with acetone). Upper lanes: (1) N2A cell lysate, (2) 1 µl serum ALS, (3) 200 µl CSF ALS, (4) 200 µl CSF ALS. Lower lanes: (1) N2A cell lysate, (2) 1 µl serum Con, (3) 200 µl CSF Con, (4) 1 µl serum FTLD, (5) 200 µl CSF FTLD. Arrow: TDP-43 reactive band at 45 kDa. *Unspecific cross-reaction at about 55 kDa. (b) 2D-PAGE of CSF and serum pI 4 to 7: TDP-43 reaction at 45 kDa. Left panel: 40 µl CSF ALS, 40 µl CSF Con. Right panel: 1 µl serum ALS, 1 µl serum Con. (c) Brain urea fractions with monoclonal TDP-43 antibody: WB (1) frontal cortex of an ALS patient with TDP-43 positive neuropathology: TDP-43 reaction at 45 kDa and 50 kDa (2) control cortex with TDP-43 reaction at 45 kDa. 2D-PAGE pI 3–10: urea fraction of the frontal cortex of an ALS patient with TDP-43 positive neuropathology. TDP-43 reaction at 45 kDa and 50 kDa. Control cortex TDP-43 reaction at 45 kDa. (d) 2D-PAGE pI 4–7: lymphocyte whole cell lysate from blood of an ALS patient with TDP-43 reaction at 45 kDa.

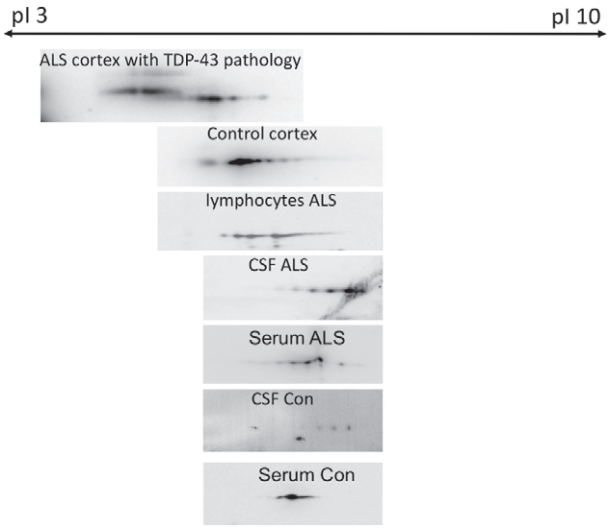

Figure 2. PIs and morphology of TDP-43 reactivity in 2D-PAGE. Top down: Urea fraction of the frontal cortex of an ALS patient with TDP-43 positive neuropathology: pI 4–6, 2–3 spots at 50 kDa, six spots at 45 kDa. Urea fraction of control cortex: pI 5–6, six spots at 45 kDa. Whole cell lysate of lymphocytes: pI 5–6, five spots at 45 kDa. 40 µl CSF ALS: pI 5.5–7, six spots at 45 kDa. 1 µl serum ALS: pI 5.5–6.5, six spots, 40 µl CSF Con: pI 6–7, 3–4 spots at 45 kDa much weaker than in ALS. 1 µl serum Con: pI 5.5–6.5, four spots at 45 kDa.

identical to the signal intensity of 200 µl CSF (lanes 3 and 4). The bottom row shows the representative WB of TDP-43 from 1 µl serum and CSF of 200 µl from a non-neurodegenerative control patient (lanes 2 and 3) and a patient with FTLD (lanes 4 and 5).

TDP-43 in the CSF and serum of an ALS patient and control was also characterized by means of 2D-PAGE applying N-terminal antibody (Figure 1b). Here a positive immunoreaction for N-terminal TDP-43 at 45 kDa was observed in both samples. We observed a six-spot pattern in the CSF and serum of ALS (Figure 1b, right). In the control CSF and serum we only observed a four-spot pattern, with a weaker reaction in CSF than in ALS.

In addition to CSF and serum we analysed brain urea fractions for TDP-43. In the WB of the brain urea fraction of an ALS patient with TDP-43 positive pathology, a double band at about 45 kDa and at 50 kDa was detected in contrast to a single TDP-43 band at 45 kDa of the control (Figure 1c). We did not detect a pathological 20-kDa band. In the 2D-PAGE of the pathological ALS-TDP brain we also observed an additional three-spot pattern at about 50 kDa shifted to a more acidic pI than the six spots at 45 kDa. In the control cortex only six spots at 45 kDa were detected.

Similar to the ALS CSF sample, we observed a 45-kDa five-spot pattern in whole cell lysates of blood lymphocytes with a slight shift to a more acidic pI (Figure 1d). Regarding pI range of all samples, the brain urea fractions are shifted to a more acidic pI, whereby this effect is most obvious in the cortex with TDP-43 pathology, while pIs in CSF are between 5.5 and 7 (Figure 2).

TDP-43 in exosomes

We examined exosomes in CSF of patients and controls to determine whether there is an additional fraction of TDP-43 that is not blood-derived. TDP-43 could be identified by WB in purified exosomes irrespective of diagnostic groups (Figure 3). Purity of the exosomal fraction was proved by flotillin-1 reactivity. Additionally, we investigated human Jurkat cells as a preparation control. TDP-43 and flotillin-1 could be detected in the purified exosome fractions (Figure 3b). Immunoreactivity for glycoprotein Ib-V-IX and calnexin were negative in the

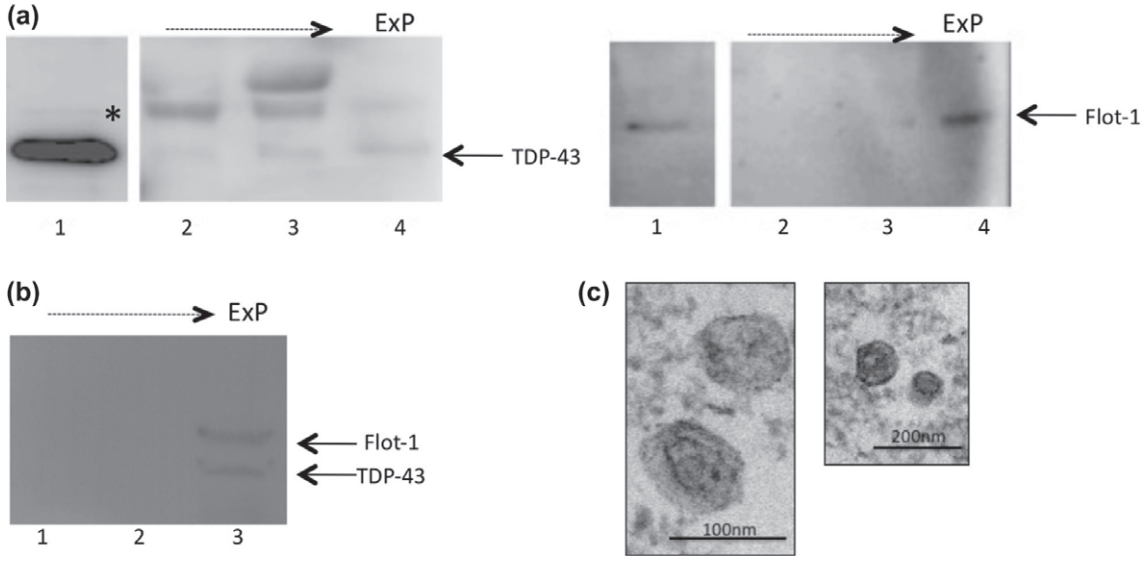

Figure 3. TDP-43 immune reactivity at 45 kDa in exosomes from 10 ml native CSF (b). (a) Exosome preparation from an ALS patient. Left: (1) Jurkat cell lysate, (2) Pellet after twice 4500 *g*, (3) pellet after 10,000 *g*, (4) purified exosome pellet (ExP). Arrow TDP-43 at 45 kDa. *unspecific cross-reaction at about 55 kDa. Right: membrane after stripping: Arrow: flotillin-1 immune reactivity at 48 kDa in exosome pellet (ExP). (b) Stripped membrane from CSF exosome preparation from a FTLD patient. (1) Pellet after twice 4500 *g*; (2) pellets after 10,000 *g*; (3) purified exosome pellet (ExP). Arrows: TDP-43 immune reactivity at 45 kDa and flotillin-1 immune reactivity at 48 kDa. (c) Characterization of CSF exosomes after ultracentrifugation by TEM.

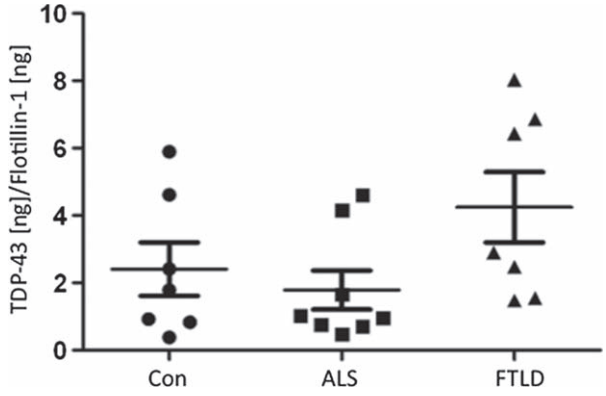

Figure 4. Results of mass spectrometry analysis of purified exosome pellets from 1 ml CSF from controls (Con, •), ALS- (■), and FTLD-patients (▲). Shown are mean and SD content of TDP-43 (ng) in relation to flotillin-1 (ng) per exosome pellet.

exosome pellets. TEM revealed that the CSF exosome preparations contained vesicles with a mean size of about 100 nm in diameter and of a morphology similar to exosomes, as previously described by Thery (29) and Raposo (30) (Figure 3c).

The TDP-43 content relative to flotillin-1 was determined in exosomes by mass spectrometry analysis. The mean content of TDP-43 (ng) in relation to flotillin-1 (ng) did not vary significantly between the diagnostic groups ($p = 0.08$) (Figure 4).

Discussion

In our study, we detected TDP-43 by means of 1D- and 2D-WB. In 1D-WB, a 45-kDa band was shown with different TDP-43 antibodies. Besides the 45-kDa band, additional bands at 50 and 55 kDa were shown as known, due to unspecific reactions (7). In 1D-WB, TDP-43 has a 200 times higher concentration in the serum than in the CSF, regardless of the patients' diagnosis. In 2D-PAGE, we found a similar spot pattern in the CSF and serum of the ALS patient (six spots), but also in those of the control patient (four spots). Post-translational modifications of TDP-43 demonstrated by 2D-PAGE are similar in CSF, blood and blood lymphocytes of ALS. In the brain urea fraction with TDP-43 pathology there is an additional higher spot pattern at about 50 kDa shifted to a more acidic pI. This may represent phosphorylated TDP-43 (4). A pathological TDP-43 fragment was not detectable by this preparation (4). As this pathological alteration is not seen in CSF, we assume that most of the CSF TDP-43 is not the pathologically altered TDP-43. Regarding its concentration ratio CSF/serum in 1D-WB, we assume that most of the TDP-43 measured in CSF is blood-derived. As 2D-WB is a quite sophisticated method, it is not applicable for the measurement of serial concentrations of TDP-43.

After two studies recently reported pathological TDP-43 in lymphocytes from ALS patients, we investigated TDP-43 in lymphocytes of ALS blood by 2D-PAGE and found isoform patterns similar to CSF and serum (31,32). This might be a further objective for TDP-43 investigations. As there is emerging evidence that exosomes may play a role in several neurodegenerative disorders (23,33,34), we investigated purified exosomes from CSF for the presence of TDP-43. According to their function as vesicles, which are secreted into the extracellular environment (35), exosomes might carry an intrathecal fraction of TDP-43 in CSF (36). We were able to detect TDP-43 in exosomes from CSF that have their origin in the brain, since there was no positive reaction for blood-derived exosomes (thrombocyte markers). Regarding future investigations of TDP-43 in exosomes as an intrathecal fraction, it might be interesting to look for post-translational modifications similar to the brain urea fractions. To date, 2D-PAGE of exosomes has not been possible due to the limited amount of this material. Using a refined technique of exosome preparation, we were able to measure TDP-43 in relation to flotillin-1 by means of quantitative mass spectrometry. We did not find significant differences between the FTLD group and the ALS group. We therefore suggest that it is necessary to refine MRM techniques and to look for pathologically altered TDP-43. Another interesting aspect will be to measure TDP-43 in serum and CSF by MRM. We conclude that most TDP-43 in CSF is blood-derived and appears to be of minor importance as a diagnostic tool, at least with the current methods. For future investigations, a promising approach would be to take a closer look at TDP-43 found in exosomes, which may be brain-derived and therefore better reflect underlying neuropathology.

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Supplementary material available online

Supplementary Figure 1 to be found online at http://www.informahealthcare.com/doi/abs/10.3109/21678421.2014.905606