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Increased cytoplasmic mislocalization of TAR DNA binding protein 43 (TDP-43) in circulating lymphomonocytes of ALS patients recapitulates the major dysfunction featuring motor neurons in the disease.

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Monocytes of patients with amyotrophic lateral sclerosis linked to gene mutations display altered TDP-43 subcellular distribution

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Aims: Cytoplasmic accumulation of the nuclear protein transactive response DNA-binding protein 43 (TDP-43) is an early determinant of motor neuron degeneration in most amyotrophic lateral sclerosis (ALS) cases. We previously disclosed this accumulation in circulating lymphomonocytes (CLM) of ALS patients with mutant *TARDBP*, the TDP-43-coding gene, as well as of a healthy individual carrying the parental *TARDBP* mutation. Here, we investigate TDP-43 subcellular localization in CLM and in the constituent cells, lymphocytes and monocytes, of patients with various ALS-linked mutant genes. **Methods:** TDP-43 subcellular localization was analysed with western immunoblotting and immunocytofluorescence in CLM of healthy controls ($n = 10$), patients with mutant *TARDBP* ($n = 4$, 1 homozygous), *valosin-containing protein* (*VCP*; $n = 2$), fused in sarcoma/translocated in liposarcoma (*FUS*;

$n = 2$), *Cu/Zn superoxide dismutase 1* (*SOD1*; $n = 6$), *chromosome 9 open reading frame 72* (*C9ORF72*; $n = 4$), without mutations ($n = 5$) and neurologically unaffected subjects with mutant *TARDBP* ($n = 2$). **Results:** TDP-43 cytoplasmic accumulation was found ($P < 0.05$ vs. controls) in CLM of patients with mutant *TARDBP* or *VCP*, but not *FUS*, in line with TDP-43 subcellular localization described for motor neurons of corresponding groups. Accumulation also characterized CLM of the healthy individuals with mutant *TARDBP* and of some patients with mutant *SOD1* or *C9ORF72*. In 5 patients, belonging to categories described to carry TDP-43 mislocalization in motor neurons (3 *C9ORF72*, 1 *TARDBP* and 1 without mutations), TDP-43 cytoplasmic accumulation was not detected in CLM or in lymphocytes but was in monocytes. **Conclusions:** In ALS forms characterized by TDP-43 mislocalization in motor

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neurons, monocytes display this alteration, even when not manifest in CLM. Monocytes may be used to

support diagnosis, as well as to identify subjects at risk, of ALS and to develop/monitor targeted treatments.

Keywords: amyotrophic lateral sclerosis, gene mutations, monocytes, transactive response DNA-binding protein 43 subcellular localization

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal adult onset neurodegenerative disorder characterized by selective loss of motor neurons in the brain and spinal cord and is presently incurable [1,2]. In about 5–10% of cases, the disease has a family history [familial amyotrophic lateral sclerosis (fALS)], whereas in the majority of cases a familial background is not documented [sporadic amyotrophic lateral sclerosis (sALS)]. Mutations of more than thirty genes ([3] <http://alsod.iop.kcl.ac.uk>), usually inherited in an autosomal dominant pattern, have been found in about two-thirds of fALS and in some sALS cases [2]. Genetic and non-genetic forms are clinically indistinguishable [4,5]. ALS, besides being a multifunction and multifactorial disease, is a multisystem disease affecting not only motor neurons, but also other cells and tissues, such as glia as well as cells of the immune system and skeletal muscle [6–15].

A typical trait of ALS is the presence in the cytoplasm of motor neurons and glial cells of proteinaceous ubiquitin-immunoreactive aggregates, appearing as round or skein-like inclusions [16–18]. However, these inclusions are not specific for ALS, since they characterise the central nervous system (CNS) in other neurodegenerative diseases [19]. Transactive response DNA-binding protein 43 (TDP-43) is a ubiquitously expressed transcription factor localized mainly in the nucleus and minimally in the cytoplasm as it continuously shuttles between the two compartments [20,21]. In 2006, TDP-43 was discovered to be the main component of the cytoplasmic inclusions characterizing motor neurons of *post mortem* sALS cases [22–24], a feature frequently associated with its depletion in the nucleus [25,26]. However, our investigations revealed that TDP-43 may accumulate in the cytoplasm of motor neurons also if not sequestered inside inclusions [27]. This alteration is considered an early event in motor neuron degeneration [4,27–29]. The involvement of TDP-43 in the

pathogenesis of ALS has been strengthened by the discovery of several mutations in the TDP-43 coding gene, *TARDBP*, in some fALS and sALS cases [2]. These mutations predispose nuclear TDP-43 to redistribute to the cytoplasm and to form pathological aggregates [4,28,30–33].

Clearly, it is not possible to perform evaluations of TDP-43 subcellular distribution in the CNS *ante mortem*. Since TDP-43 is ubiquitously distributed, in a previous study [34] we investigated whether in ALS patients TDP-43 dysfunctions characterise cells outside the CNS. Our attention focused on circulating lymphomonocytes (CLM) since these cells are available on demand and without invasive procedures. We showed that TDP-43 cytoplasmic accumulation, along with nuclear decrement, was also a feature of CLM of ALS patients with mutant *TARDBP* and of about 50% of those without documented gene mutations. Of note, the same alteration was also revealed in CLM of a healthy young individual carrying the same *TARDBP* mutation of the parent affected by ALS.

Here, we explore whether, in CLM of ALS patients with other disease-linked mutant genes, TDP-43 subcellular localization reflects that described in motor neurons of corresponding cases. The mutant genes examined include *valosin-containing protein* (*VCP*), *fused in sarcoma/translocated in liposarcoma* (*FUS*), *Cu/Zn superoxide dismutase 1* (*SOD1*) and *chromosome 9 open reading frame 72* (*C9ORF72*). These genes, together with *TARDBP*, account for the majority of the ALS genetic forms [2]; furthermore, some of these mutations are associated with alterations of TDP-43 in motor neurons, whereas others are not [4,35–37]. We also examined the TDP-43 profile separately in lymphocytes and monocytes, the CLM constituent cells. Thus, besides the subcellular fractionation/sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)/western immunoblot (WB) approach previously applied [34], here we used an immunocytofluorescence (IF) assay, since the latter allows discrimination within

the CLM population between lymphocytes and monocytes.

Additionally, we had the opportunity to evaluate the TDP-43 profile in CLM of three individuals belonging to a family characterized by an interesting pedigree: one member was homozygous for the *TARDBP* mutation and was affected by a syndrome including ALS, whereas the other two were heterozygous for the mutation and were neurologically unaffected [38].

Materials and methods

Subjects

The demographic data of neurologically unaffected subjects (controls) recruited in this study are reported in Table 1. The demographic and clinical characteristics of the ALS patients considered in this study are summarized in Table 2. Since the previously examined controls, as well as patients with mutant *TARDBP* and patients without gene mutations [34] could no longer be recruited for IF assay, 10 new controls, as well as 3 new patients with mutant *TARDBP* and 5 new ALS patients without documented gene mutations were recruited. Therefore, the case series includes 10 controls, 6 individuals with mutant *TARDBP* (including 4 patients, 1 of whom was homozygous for the mutation, and 2 neurologically unaffected subjects), 2 patients with mutant *VCP*, 2 patients with mutant *FUS*, 6 patients with mutant *SOD1*, 4 patients with mutant *C9ORF72*, 5 patients without documented gene mutations and 1 patient with fALS and unknown gene

mutation. Hereafter, individuals are defined as *TARDBP*+, *VCP*+, *FUS*+, *SOD1*+ and *C9ORF72*+ according to the carried gene mutation.

The study design was approved by the institutional ethics committee. Patients and controls signed written informed consent.

Reagents

ProteoJet Cytoplasmic and Nuclear Protein Extraction kit (K0311) and protein molecular markers (SM0671) were from Fermentas (Milan, Italy). Lymphoprep® (1114545) was from AXIS-SHIELD (Oslo, Norway). The RC DC protein content assay kit was from Bio Rad (Milan, Italy). MACS® Cell separation kit (130-050-201) was from Miltenyi Biotec (Bologna, Italy). TDP-43 monoclonal antibody, clone 2E2-D3 (H00023435-M01), directed to the epitope 205-222 of the human protein [39] was from Abnova Corporation (Taipei, Taiwan). Lamin B1 monoclonal antibody (33-2000) was from Zymed Laboratories (San Francisco, CA, USA), while β -tubulin polyclonal antibody (sc-9104), CD45 monoclonal antibody (sc-53666) and secondary antibodies conjugated to horseradish peroxidase (HRP) were from Santa Cruz Biotechnology (Heidelberg, Germany). Ubiquitin monoclonal antibody (MAB1510), secondary antibodies for immuno-fluorescence, goat anti-mouse Cy3- (AP124C) or Cy2-conjugated (AP124J), goat anti-rabbit Cy2-conjugated (AP132J), polyvinylidene difluoride membranes (PVDF; IPVH00010) and Luminata™ Forte Western HRP Substrate-enhanced chemiluminescence reagent (ECL; WBLUF0500) were from Millipore (Milan, Italy). Protease (P8340) and phosphatase (P2850) inhibitor cocktails, Hoechst 33258 fluorescent stain (861405) and all the other chemicals were high grade from Sigma-Aldrich (Milan, Italy).

Isolation of CLM

Peripheral venous blood was collected using EDTA pre-coated vials and immediately processed for CLM isolation as previously described [34]. Harvested cells were suspended in phosphate buffer saline (PBS) pH 7.2–7.4, counted manually, evaluated for vitality that, using the trypan blue exclusion test, was close to 95%, and finally divided into two parts. One was used for WB assay and the other for IF assay.

Table 1. Demographic data of the neurologically unaffected subjects recruited

Case	Sex	Age (years)
Ctrl 1	M	33
Ctrl 2	F	57
Ctrl 3	M	65
Ctrl 4	M	48
Ctrl 5	M	76
Ctrl 6	F	61
Ctrl 7	F	53
Ctrl 8	F	64
Ctrl 9	F	66
Ctrl 10	M	58
Mean		58
SD		10

Table 2. Demographic, clinical, genetic and pathological data of the patients recruited

Case	Sex	Age at onset (years)	Disease duration (months)*	Site of onset	Frontotemporal dementia	Familial/sporadic	Mutation
ALS 1	M	64	14	Spinal	No	sALS	—
ALS 2	M	47	8	Spinal	No	sALS	—
ALS 3	M	66	28	Spinal	No	sALS	—
ALS 4	M	68	38	Spinal	No	sALS	—
ALS 5	M	70	15	Bulbar	Yes	sALS	—
ALS 6	M	66	10	Bulbar	No	sALS	TDP-43 A382T
ALS 7	F	60	29	Spinal	No	fALS	TDP-43 A382T
ALS 8	M	54	19	Spinal	No	sALS	TDP-43 A382T
ALS 9	M	49	48	Bulbar	Yes	fALS	TDP-43 A382T [†]
ALS 10 [‡]	M	50	120	Spinal	No?	fALS	VCP R191Q
ALS 11 [§]	M	37	61, 76 [¶]	Spinal	No	fALS	VCP R191Q
ALS 12	F	48	32	Spinal	Yes	fALS	FUS R514S
ALS 13	M	40	12	Spinal	No	fALS	FUS R521G
ALS 14 ^{**}	M	27	70	Spinal	No	fALS	SOD1 L84F
ALS 15	F	58	13	Spinal	No	sALS	SOD1 D109Y
ALS 16	F	56	61, 67 [¶] , 98 ^{††}	Spinal	No	sALS	SOD1 D109Y
ALS 17	M	49	161	Spinal	No	sALS	SOD1 T137A
ALS 18	M	52	103	Spinal	No	fALS	SOD1 I113F
ALS 19	M	38	52	Spinal	No	fALS	SOD1 G93D
ALS 20	M	54	74 ^{‡‡}	Spinal	Yes	sALS	C9ORF72
ALS 21	M	50	14	Bulbar	Yes	sALS	C9ORF72
ALS 22	F	36	12	Spinal	No	fALS	C9ORF72
ALS 23	M	56	18	Bulbar	No	sALS	C9ORF72
ALS 24	F	57	30	Bulbar	No	fALS	Unknown ^{§§}

ALS, amyotrophic lateral sclerosis; fALS, familial ALS; sALS, sporadic ALS; *FUS*, fused in sarcoma/translocated in liposarcoma; *SOD1*, Cu/Zn superoxide dismutase 1; TDP-43, transactive response DNA-binding protein 43; *VCP*, valosin-containing protein; *C9ORF72*, chromosome 9 open reading frame 72.

*At the time at which the blood sample was taken.

[†]Homozygous for the mutation. Further clinical data are reported in Borghero *et al.* [38].

[‡]III:12 member of the genealogical tree ITALS#1 reported in Johnson *et al.* [35].

[§]IV:1 member of the genealogical tree ITALS#1 reported in Johnson *et al.* [35].

[¶]Disease duration when the second blood sample was taken.

^{**}This patient was recruited for the first time 62 months before and was reported as case S1 in De Marco *et al.* [34].

^{††}Disease duration when the third blood sample was taken.

^{‡‡}This patient was recruited for the first time about 4 years before and was reported as case ALS #15 in De Marco *et al.* [34]. At that time this case was considered without documented gene mutations.

^{§§}Negative for *SOD1*, *TARDBP*, *FUS* and *C9ORF72* mutations.

Subcellular fractionation and western immunoblot analysis

According to the previously described procedures [34], the CLM aliquot set for WB assay was divided into two parts: one was processed to obtain whole cell lysates and the other to obtain a cytoplasmic and a nuclear fraction. The latter was performed using ProteoJet Cytoplasmic and Nuclear Protein Extraction kit according to the manufacturer instructions, supplemented with protease (1% v/v) and phosphatase (2% v/v) inhibitor cocktails. All preparations were evaluated for protein amount using the RC-DC protein assay kit, frozen and stored at -70°C . Frozen samples were then subjected

to SDS-PAGE, and resolved proteins were electro-blotted onto PVDF membranes, as previously described [34]. Of note, the protein amount required for the visualization of TDP-43-immunoreactive band in the cytoplasmic compartment was at least threefold that required for the nuclear compartment. Membranes were blocked with 2% bovine serum albumin (BSA) in Tris-buffered saline added with 0.02% (v/v) Tween 20 (TBST buffer) and then exposed at 4°C to the TDP-43 antibody diluted (1:2000) in 2% BSA in TBST buffer. Probed blots were then incubated at 4°C with HRP-conjugated secondary antibodies in TBST buffer, washed and then incubated with ECL reagent. Immunostained bands were visualized, using Chemi-Box CCD gel imaging

system and Genesnap image acquisition software (Syngene, Cambridge, UK). Membranes were re-probed with antibodies against β -tubulin or lamin B1 to confirm equal protein loading of the cytoplasmic and nuclear fractions, respectively. The relative intensity of each protein band was quantified using GeneTools image analysis software (Syngene, UK).

In the previous study [34], mean values of immunoreactive TDP-43 in the cytoplasm and nucleus were evaluated separately for each group and compared. In this study as the case series was small, we expressed TDP-43 immunoreactive distribution as a ratio between normalized nuclear to cytoplasmic amounts for each case. To unify the interpretation of observations presently and previously acquired, the results referred to the previous study were here revised in terms of nuclear/cytoplasmic ratio (Table 3).

Immunocytofluorescence assay

CLM were fixed in 4% (w/v) paraformaldehyde, washed with PBS, permeabilized with PBS supplemented with 0.1% Triton X-100 (buffer A), blocked at

room temperature in 2% BSA in buffer A and finally incubated overnight at 4°C with TDP-43 antibody (1:250 in buffer A) and in some cases co-stained with ubiquitin antibody (1:250 in buffer A). Cells were then rinsed with buffer A and incubated at room temperature, in the dark, with secondary antibodies (1:1000 in buffer A). For nuclear staining, cells were exposed to Hoechst solution (1:2000 in PBS) at room temperature in the dark. Fluorescent images were acquired with inverted microscope Leica DMI 4000 B (Heidelberg, Germany) and with confocal microscope Leica TCS SP5 at defined λ_{ex} .

Characterization of CLM population

Identification of the cellular species in CLM population was achieved as follows: (i) an aliquot of CLM was labelled with an antibody that binds the antigen CD45 (leucocyte common antigen), a membrane glycoprotein featuring hematopoietic cell lines; (ii) an additional aliquot was treated with MACS[®] Cell separation kit, according to the manufacturer's instructions. This procedure allowed collection of both CD14+ (monocytes/macrophages) and CD14- (lymphocytes) cells.

Table 3. Subcellular distribution of TDP-43 in CLM, as well as in lymphocytes and monocytes separately, evaluated with WB and/or immunocytofluorescence (IF) analysis

Controls	Nucleus*	Cytoplasm*	Ratio*	Lymphocytes [†]	Monocytes [‡]
Ctrl #1	1.1	0.7	1.6	n/a	n/a
Ctrl #2	1.07	0.68	1.6	n/a	n/a
Ctrl #3	0.91	1.3	0.71	n/a	n/a
Ctrl #4	0.94	1.2	0.81	n/a	n/a
Ctrl #5	1.0	1.0	1.0	n/a	n/a
Ctrl #6	0.88	1.4	0.63	n/a	n/a
Ctrl #7	1.2	0.80	1.4	n/a	n/a
Ctrl #8	1.0	0.82	1.2	n/a	n/a
Ctrl #9	1.1	1.0	1.1	n/a	n/a
Ctrl #10	1.1	0.99	1.1	n/a	n/a
Ctrl #11	0.89	1.3	0.68	n/a	n/a
Ctrl #12	1.0	0.89	1.2	n/a	n/a
Ctrl #13	0.98	1.1	0.89	n/a	n/a
Ctrl 1	0.98	1.0	0.98	—	2.9
Ctrl 2	1.1	0.83	1.3	—	2.9
Ctrl 3	1.2	0.75	1.6	—	4.5
Ctrl 4	0.75	0.95	0.79	—	3.0
Ctrl 5	1.1	0.98	1.1	—	3.6
Ctrl 6	0.40	0.85	0.47	+	2.1
Ctrl 7	1.1	1.1	1.0	—	3.6
Ctrl 8	1.8	0.92	2.0	—	4.0
Ctrl 9	0.90	1.1	0.82	—	3.4
Ctrl 10	n/a	n/a	n/a	—	3.3
Mean			1.1	Mean	3.3
SD			0.37	SD	0.67

Table 3. (Continued)

<i>No mutations</i>	<i>Nucleus</i>	<i>Cytoplasm</i>	<i>Ratio</i>	<i>Lymphocytes</i>	<i>Monocytes</i>
ALS #5	1.1	0.87	1.3	n/a	n/a
ALS #6	0.61	1.9	0.32	n/a	n/a
ALS #7	0.95	1.0	0.92	n/a	n/a
ALS #8	0.80	1.8	0.44	n/a	n/a
ALS #9	0.82	1.8	0.47	n/a	n/a
ALS #10	0.66	1.9	0.34	n/a	n/a
ALS #11	0.89	1.4	0.63	n/a	n/a
ALS #12	0.81	1.7	0.47	n/a	n/a
ALS #13	0.84	1.3	0.64	n/a	n/a
ALS #14	0.58	2.0	0.30	n/a	n/a
ALS #16	0.71	1.8	0.39	n/a	n/a
ALS 1	0.70	1.6	0.44	+	2.1
ALS 2	0.85	0.87	0.98	—	3.0
ALS 3	0.74	1.8	0.41	+	0.80
ALS 4	1.0	0.90	1.2	—	4.3
ALS 5	1.2	1.0	1.2	—	2.4
Mean			0.65	Mean	2.5
SD			0.35	SD	1.3
<i>TARDBP+</i>	<i>Nucleus</i>	<i>Cytoplasm</i>	<i>Ratio</i>	<i>Lymphocytes</i>	<i>Monocytes</i>
ALS #1	0.77	1.9	0.42	n/a	n/a
ALS #2	0.69	1.8	0.38	n/a	n/a
ALS #3	0.33	3.8	0.09	n/a	n/a
ALS #4	0.18	4.5	0.04	n/a	n/a
ALS 6	0.67	1.9	0.35	+	1.5
ALS 7	1.2	1.1	1.1	—	1.9
ALS 8	n/a	n/a	n/a	+	2.2
ALS 9 [§]	0.42	1.4	0.29	n/a	n/a
P 1 [¶]	0.58	1.3	0.45	n/a	n/a
P 2 [¶]	0.34	1.7	0.20	n/a	n/a
Mean			0.38	Mean	1.9
SD			0.35	SD	0.37
<i>VCP+</i>	<i>Nuclear</i>	<i>Cytoplasm</i>	<i>Ratio</i>	<i>Lymphocytes</i>	<i>Monocytes</i>
ALS 10	0.84	1.9	0.44	+	1.8
ALS 11	0.27	1.8	0.15	+	1.2
ALS 11**	0.32	1.6	0.20	+	1.5
Mean ^{††}			0.30	Mean ^{††}	1.5
SD			0.21	SD	0.45
<i>FUS+</i>	<i>Nucleus</i>	<i>Cytoplasm</i>	<i>Ratio</i>	<i>Lymphocytes</i>	<i>Monocytes</i>
ALS 12	1.0	0.59	1.7	—	4.2
ALS 13	1.1	0.72	1.5	—	3.0
Mean			1.6	Mean	3.6
SD			0.14	SD	0.85
<i>SOD1+</i>	<i>Nucleus</i>	<i>Cytoplasm</i>	<i>Ratio</i>	<i>Lymphocytes</i>	<i>Monocytes</i>
ALS 14/ALS S1	0.91	1.2	0.74	—	3.1
ALS 15	0.70	1.7	0.41	+	1.2
ALS 16	1.0	0.70	1.4	—	2.9
ALS 16**	1.2	0.80	1.5	—	3.0

Table 3. (Continued)

No mutations	Nucleus	Cytoplasm	Ratio	Lymphocytes	Monocytes
ALS 16 ^{**}	n/a	n/a	n/a	—	2.9
ALS 17	n/a	n/a	n/a	+	1.8
ALS 18	n/a	n/a	n/a	+	1.9
ALS 19	n/a	n/a	n/a	—	3.8
Mean ^{††}			0.85	Mean ^{††}	2.5
SD			0.50	SD	1.0
C9ORF72+	Nucleus	Cytoplasm	Ratio	Lymphocytes	Monocytes
ALS 20/ALS #15	1.1	0.98	1.1	n/a	n/a
ALS 20 ^{**}	0.90	1.3	0.69	— ^{§§}	2.0
ALS 21	0.53	1.5	0.35	+	1.2
ALS 22	0.86	0.90	0.96	—	1.4
ALS 23	0.36	0.58	0.62	— ^{§§}	1.3
Mean ^{††}			0.76	Mean	1.5
SD			0.34	SD	0.36
Unknown gene	Nucleus	Cytoplasm	Ratio	Lymphocytes	Monocytes
ALS 24	n/a	n/a	n/a	—	3.2

Cases and results reported in De Marco *et al.* [34] are shown in italics. Each value results from three independent experiments.

n/a, data not available; ALS, amyotrophic lateral sclerosis; CLM, circulating lymphomonocytes; *FUS*, fused in sarcoma/translocated in liposarcoma; *SOD1*, Cu/Zn superoxide dismutase 1; TDP-43, transactive response DNA-binding protein 43; *VCP*, valosin-containing protein; WB, western immunoblot; *C9ORF72*, chromosome 9 open reading frame 72.

*Values refer to the TDP-43 immunostaining intensity measured in CLM with WB analysis, normalized respect to lamin B1 (nucleus) and β -tubulin (cytoplasm) and expressed as a nuclear/cytoplasmic ratio.

[†]Distribution of TDP-43 in lymphocytes detected with IF assay: (—) TDP-43 uniformly distributed on the entire cell surface of most cells; (+) TDP-43 distributed in the perinuclear area of most cells.

[‡]Values refer to the TDP-43 immunostaining intensity measured with IF assay in the nucleus and cytoplasm of at least 10 monocytes and expressed as a nuclear/cytoplasmic ratio.

[§]Patient homozygous for the mutation.

[¶]ALS 9 patient's relative, who did not show any neurological sign of the disease.

^{**}Case tested for the second time after a defined lapse of time.

^{††}For patients tested more than once, only the values obtained in the first analysis are considered for the calculation of mean and standard deviation.

^{‡‡}Case tested for the third time after a defined lapse of time.

^{§§}TDP-43 cytoplasmic accumulation was appreciable in many cells, but not in the majority.

Quantitative analysis The analysis was carried out with ImageJ 1.48v software (National Institute of Health Bethesda, MD, USA; <http://imagej.nih.gov/ij/>) only on monocytes, since in lymphocytes the cytoplasm usually covers a minimal part of the entire cell surface, thus making hard to discriminate the cytoplasmic from the nuclear compartment.

For each slide, fields ($n = 5$) were selected and imaged in three channels corresponding to Hoechst, CD45 and TDP-43 staining. For the analysis, images were first converted to grayscale. Hoechst staining was used to identify the nuclear cell region of interest, and CD45 staining to identify the whole cell surface. Nuclear and

cytoplasmic TDP-43 staining intensities were quantified after background correction. In monocytes of each subject, the nuclear/cytoplasmic TDP-43 ratio derives from the mean value of the ratio between the TDP-43 intensity measured in the nucleus and that measured in the cytoplasm in at least 10 cells. The mean nuclear/cytoplasmic TDP-43 ratio of each group and the relative standard deviation were also reported (Table 3).

Statistical analysis

Data were analysed with the IBM software Statistical Package for Statistical Science (SPSS) version 22

(Armonk, NY, USA). Student's *t*-test was used to compare each group of patients carrying the same mutant gene with the control group or to compare two groups of patients. One-way ANOVA test with Tukey correction was used for comparisons among more than two groups of patients.

Results

Characterization of CLM

IF analysis was performed using the inverted microscope, as well as the confocal microscope. Immunolabelling with CD45 revealed that CLM population consisted of leucocytes with an intact plasma membrane in controls as well as in patients. Furthermore, CD45 and Hoechst staining disclosed that the population included two types of cells differing in size as well as in the nuclear shape (Figure 1a). Treatment of CLM

with MACS[®] Cell separation kit, followed by staining with Hoechst reagent, revealed that the bigger cells, usually characterized by a kidney or horseshoe-shaped nucleus, were monocytes (CD14⁺ cells) and the smaller ones were lymphocytes (CD14[−] cells) (Figure 1b).

Correlation between TDP-43 subcellular localization and gene mutations

In whole lysates and in nuclear and cytoplasmic fractions of CLM from all individuals, TDP-43 profile matched, in qualitative terms, that of controls and patients with mutant *TARDBP* or without gene mutations previously considered [34]. In detail, full length TDP-43 (~45 kDa) was a feature of whole lysates and nucleus, whereas the shortly truncated form at ~42 kDa only of cytoplasm. The TDP-43 immunoreactive band at ~38 kDa was detected in whole lysates and in the two subcellular compartments in all cases

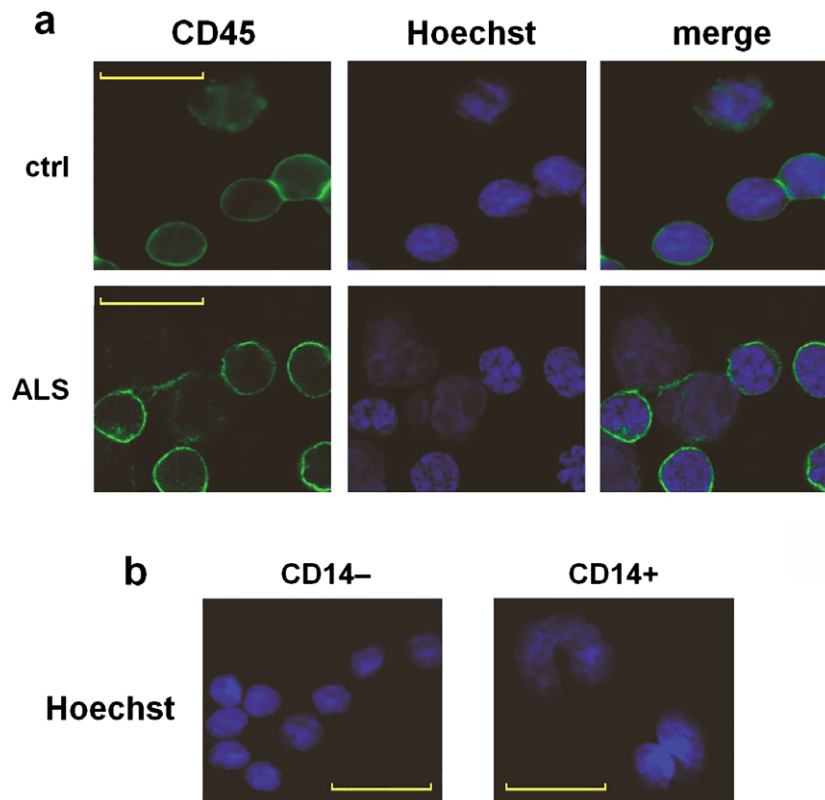


Figure 1. Types of cells included in circulating lymphomonocytes (CLM) population identified by immunofluorescence microscopy. (a) CLM of a control and a patient tested with an antibody directed to the CD45 antigen, selective for all white blood cells (leucocytes). (b) MACS[®] Cell separation kit allowed to identify, in CLM population, monocytes (CD14⁺) and lymphocytes (CD14[−]). The two isolated clones in a neurologically unaffected control were visualized following staining with Hoechst reagent. Green: CD45 antibody; blue: Hoechst reagent for nuclear staining. All images were taken at 100× magnification, and the scale bars represent 10 μm.

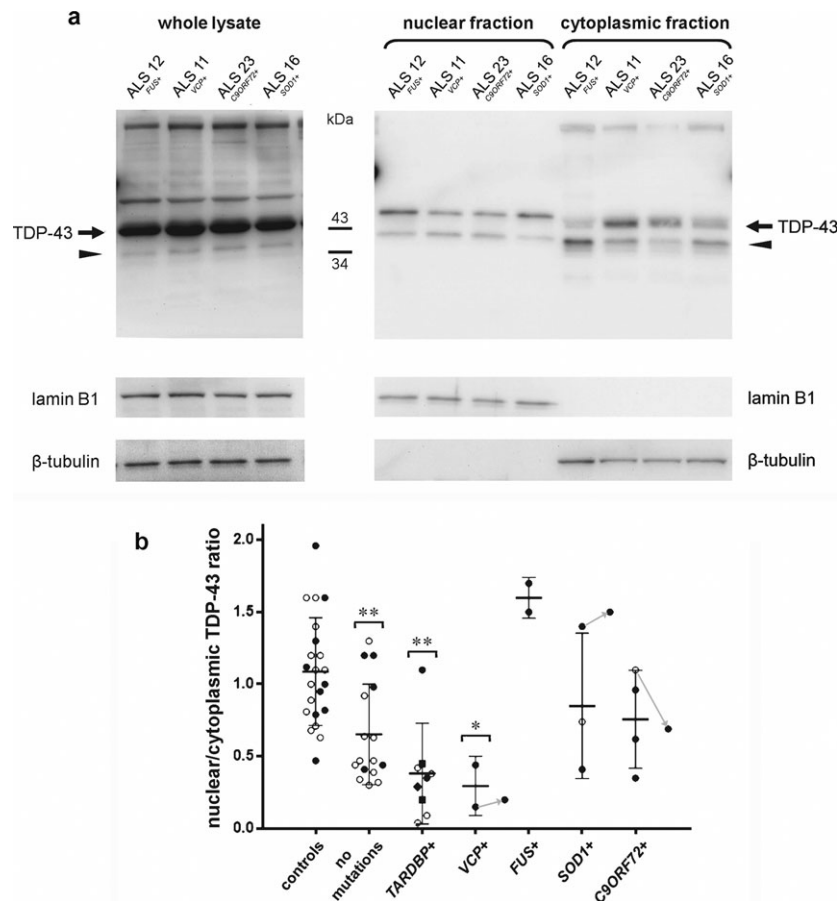


Figure 2. (a) Transactive response DNA-binding protein 43 (TDP-43) profile in western immunoblots of whole lysates and nuclear and cytoplasmic fractions from circulating lymphomonocytes (CLM) of patients with various mutant genes linked to amyotrophic lateral sclerosis (ALS); lamin B1 and β -tubulin were used as a loading control for the nuclear and cytoplasmic fractions, respectively. Arrowhead: 38 kDa protein band. (b) The scatter plot refers to the ratio between the nuclear and cytoplasmic TDP-43 amount in CLM of all individuals here examined, revealed with western immunoblot assay and normalized with lamin B1 and β -tubulin respectively (Table 3). Each point is the result of at least three independent experiments. The mean values, as well as the standard deviations, are reported for each group considered. Empty circles represent the nuclear/cytoplasmic TDP-43 ratio of the subjects analysed in the previous study [34]. The group of *TARDBP*⁺ includes a case homozygous for the mutation (diamond) and its neurologically unaffected relatives heterozygous for the mutation (square). Arrows: variation in the ratio in patients reconsidered after a definite lapse of time. * $P < 0.05$ vs. control group; ** $P < 0.01$ vs. control group.

(Figure 2a). As previously observed, in quantitative terms, in CLM whole lysates TDP-43 did not differ significantly among the individuals considered (Figure 2a).

The analysis of TDP-43 subcellular localization with IF assay was performed in quantitative terms in monocytes and only in qualitative terms in lymphocytes (see Methods). In the latter, TDP-43 was considered not to accumulate in the cytoplasm, when protein immunolabelling was uniformly distributed on the entire cell surface of most cells, and instead to accumulate when the immunolabelling was prominently localized in the perinuclear area of most cells (Figures 3a, S1).

Controls, *TARDBP*⁺ individuals and patients without documented gene mutations

The mean nuclear/cytoplasmic TDP-43 ratio, obtained with WB assay (see Methods), in the *TARDBP*⁺ group was lower than that of controls ($P < 0.01$) (Figure 2b, Table 2). Thus, the results of the previous and present study confirmed TDP-43 accumulation in the cytoplasm of CLM of *TARDBP*⁺ patients. For each group, TDP-43 was considered to accumulate in the cytoplasm of CLM if the ratio mean value was significantly lower (at least $P < 0.05$) with respect to that of controls.

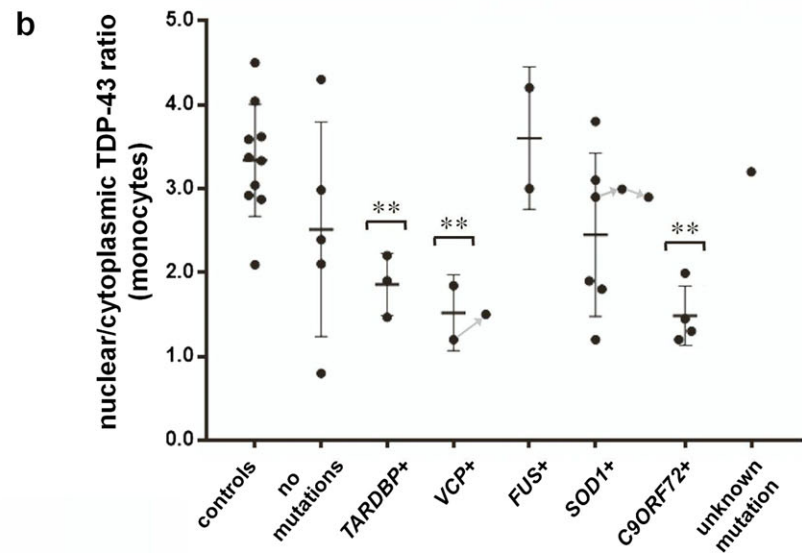
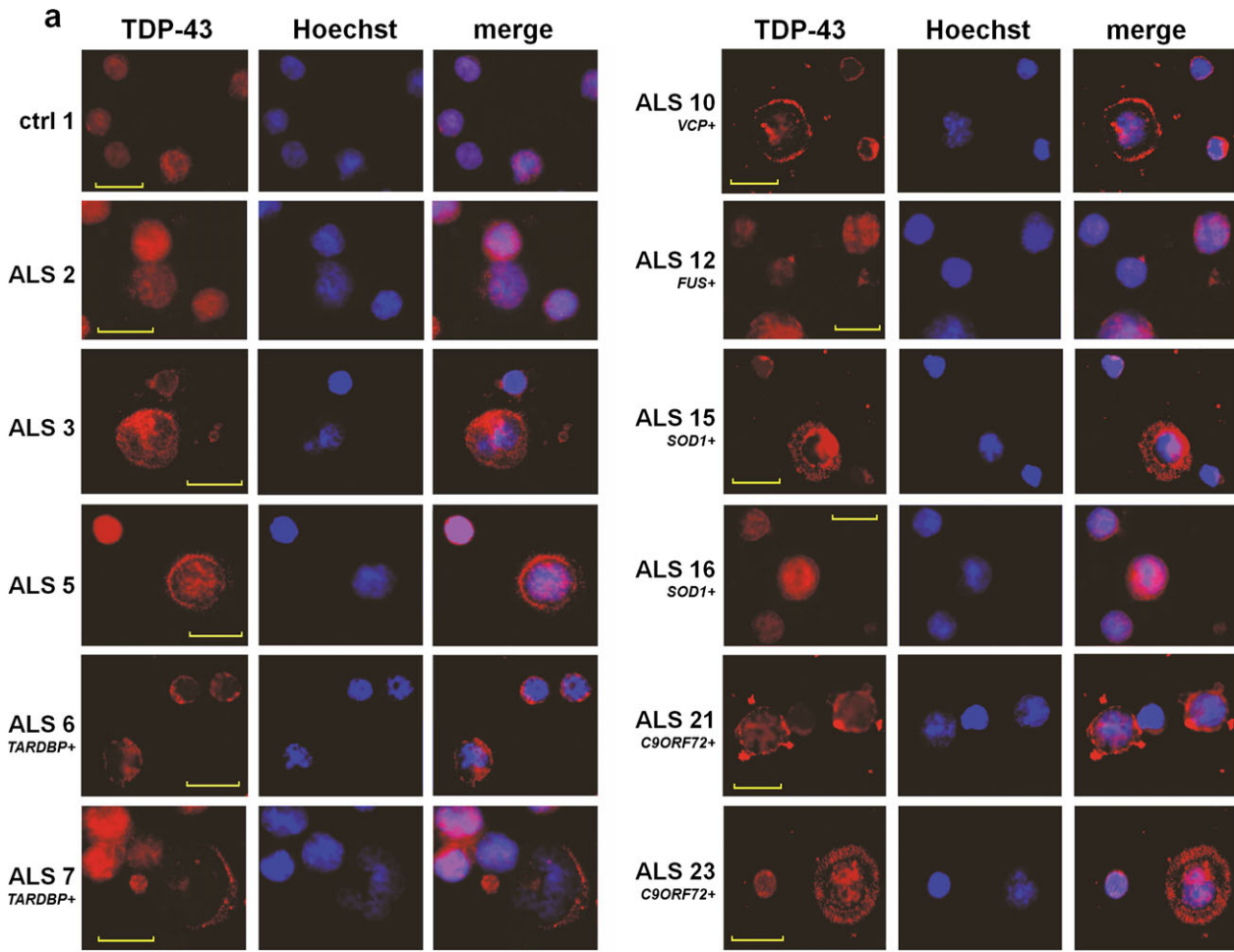


Figure 3. (a) Transactive response DNA-binding protein 43 (TDP-43) subcellular localization, detected by fluorescence microscopy, in circulating lymphomonocytes from neurologically unaffected controls, amyotrophic lateral sclerosis (ALS) patients without documented gene mutations, ALS patients with mutant *TARDBP*, *valosin-containing protein (VCP)*, *fused in sarcoma/translocated in liposarcoma (FUS)*, *Cu/Zn superoxide dismutase 1 (SOD1)* and *chromosome 9 open reading frame 72 (C9ORF72)*; red: TDP-43 antibody; blue: Hoechst reagent for nuclear staining. All images were taken at 100× magnification, and the scale bars represent 10 µm. The images are representative of at least four sections. (b) The scatter plot refers to the ratio between the nuclear and cytoplasmic intensities of TDP-43 in monocytes of all individuals here tested with immunocytofluorescence assay. Each point represents the mean nuclear/cytoplasmic TDP-43 ratio calculated on at least 10 monocytes of each individual (Table 3). The mean values, as well as the standard deviations, are reported for each group considered. Arrows: variation in the ratio in patients reconsidered after a defined lapse of time. ** $P < 0.01$ vs. control group.

The ratios of the two neurologically unaffected relatives heterozygous for the mutation (P1, P2) were both below the ratios of 90% of controls; in addition, in one, the ratio was below, whereas in the other it was above, the ratio of the homozygous ALS-affected relative (Figure 2b, Table 3).

IF analysis revealed that the mean nuclear/cytoplasmic ratio value in monocytes of the control group was 3.3 (range = 2.1–4.5). Thus, in monocytes the average amount of TDP-43 in the nucleus is about threefold that in the cytoplasm. Only the subject with the lowest ratio in monocytes showed TDP-43 cytoplasmic accumulation in lymphocytes.

In the *TARDBP*+ group, the mean nuclear/cytoplasmic TDP-43 ratio in monocytes was lower than that of controls ($P < 0.01$). Furthermore, the ratios of all the *TARDBP*+ patients were below those in 90% of controls (Figure 3, Table 3). Thus, TDP-43 cytoplasmic accumulation in monocytes was considered to be a feature of all patients belonging to this group. In two patients (ALS 6, ALS 8) TDP-43 cytoplasmic accumulation was found also in lymphocytes. Instead, in another patient (ALS 7), TDP-43 cytoplasmic accumulation was not disclosed in lymphocytes nor in CLM (in the latter, the nuclear/cytoplasmic ratio obtained with WB analysis was close to the mean value of the control group) (Figures 2b, 3b, Table 3).

WB analysis showed that, in patients without documented gene mutations, TDP-43 accumulated in the cytoplasm of CLM ($P < 0.01$ vs. control group). However, the TDP-43 ratios of some of these patients were above those of one-third of controls and even above the mean value of controls (Figure 2b, Table 3). Three of these patients were analysed with IF assay. In one (ALS 5), TDP-43 accumulation was not found in lymphocytes and instead disclosed in monocytes, since the nuclear/cytoplasmic TDP-43 ratio was below that of 90% of controls (Figure 3, Table 3).

VCP+ patients

WB assay disclosed that TDP-43 accumulated in the cytoplasm of CLM of these patients ($P < 0.05$ vs. control group). Accumulation of TDP-43 was disclosed also in monocytes (IF assay), since the mean ratio in this group was significantly lower than that of controls ($P < 0.01$) and the ratios of the two VCP+ patients were both below those of 90% of controls. TDP-43 cytoplasmic accumulation characterized also lymphocytes of these patients. One (ALS 11) was reprobated 15 months later and accumulation in both lymphocytes and monocytes (as well as in CLM) was confirmed (Figure 3, Table 3).

FUS+ patients

In two patients, TDP-43 ratios calculated in CLM (WB assay) as well as in monocytes (IF assay) were not significantly different respect to those in the control group. Furthermore, in both, TDP-43 cytoplasmic accumulation was not found in lymphocytes (Figure 3, Table 3).

SOD1+ patients

In these patients, the mean TDP-43 ratio in CLM (WB assay) was not significantly different respect to that of controls (Figure 2, Table 3). However, the TDP-43 ratios in monocytes (IF assay) in three patients of this group (ALS 15, ALS 17, ALS 18) were below those in 90% of controls and thus monocytes of these patients were considered to accumulate TDP-43 in the cytoplasm. In these three cases, TDP-43 cytoplasmic accumulation was revealed also in lymphocytes (Figures 2, 3, Table 3). Instead, in three other patients (ALS 14, ALS 16, ALS 19), TDP-43 cytoplasmic accumulation was not detected in monocytes (the ratios were not below those found in 90% of controls) nor in lymphocytes. Cytoplasmic accumulation was not found when one of these cases (ALS 16) was recruited 7 and 31 months later (Figures 2, 3, Table 3).

C9ORF72+ patients

In these patients, the mean TDP-43 ratio in CLM (WB assay) was not significantly different with respect to that of controls (Figure 2, Table 3). By contrast, IF analysis disclosed TDP-43 cytoplasmic accumulation in monocytes of all patients of this group ($P < 0.01$ vs. controls and all the ratio values were below those disclosed in 90% of controls; Figure 3, Table 3). In one patient (ALS 21), in whom the ratio in CLM (WB assay) was below that of all controls, TDP-43 cytoplasmic accumulation was detected also in lymphocytes. Instead, in one patient (ALS 22), TDP-43 cytoplasmic accumulation was not found in lymphocytes. In two patients (ALS 23, ALS 20), distribution was judged to be intermediate because TDP-43 accumulation was appreciable in many cells, but not in the majority (Table 3). One of these patients (ALS 20) had been tested previously [34] and included in the group of patients without gene mutations (reported as ALS #15) because C9ORF72 mutations had not yet been linked to ALS. At that time, the nuclear/cytoplasmic TDP-43 ratio of CLM (WB assay) coincided with the mean ratio of the control group. However, when this patient was retested in this study (52 months after the first evaluation), the ratio was below that of 87% of controls (Figure 2b, Table 3).

fALS patient with unknown gene mutation

In a fALS patient with unknown gene mutation (ALS 24), TDP-43 accumulation was detected neither in lymphocytes nor in monocytes, since the TDP-43 ratio (IF assay) was close to the mean value of controls (Figure 3b, Table 3).

Correlation between TDP-43 subcellular localization in CLM and clinical as well as demographic parameters

In ALS patients, no significant difference was found when the nuclear/cytoplasmic TDP-43 ratio was correlated with gender, familiarity for the disease, age at disease onset, age at blood sample collection or disease

duration. The ratio was significantly lower ($P < 0.05$) in patients with bulbar respect to spinal disease onset (Figure 4).

Evaluation of ubiquitin immunoreactivity

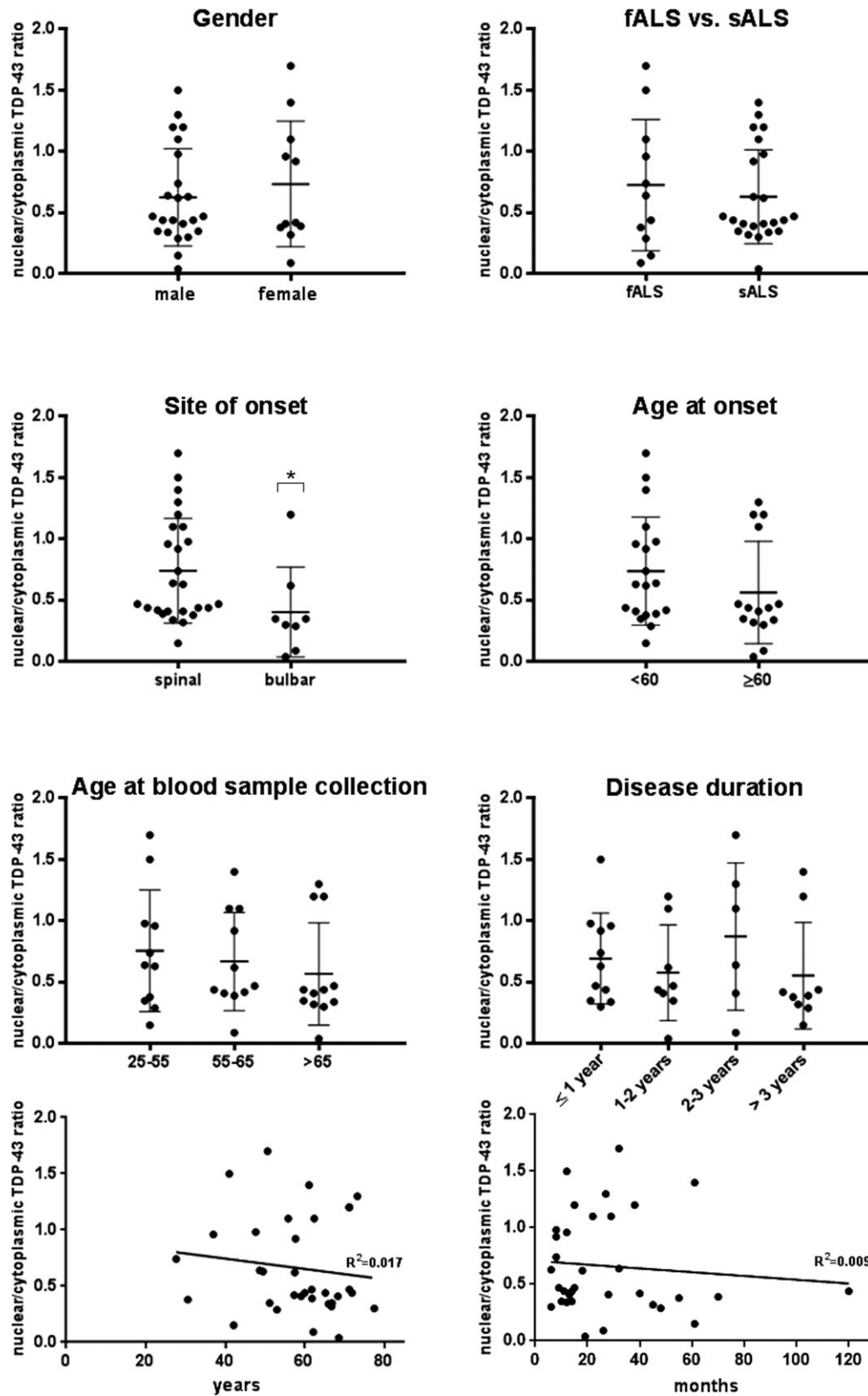
In CLM of controls and patients ubiquitin immunoreactivity was diffusely distributed. Furthermore, double-labelling of TDP-43 and ubiquitin did not disclose ubiquitin-positive aggregates, even in those cells in which cytoplasmic accumulation of TDP-43 was manifest (Figure 5).

Discussion

Sequestration of TDP-43 inside ubiquitin-immunoreactive inclusions in motor neurons is the hallmark of most ALS cases [22–24]. This feature, detectable only at autopsy, is preceded by accumulation of TDP-43 in the cytoplasm along with depletion in the nucleus [4,26–28,30]. Previously [34], we reported TDP-43 accumulation in the cytoplasm of CLM of ALS patients with mutant *TARDBP* and of about half of patients without a documented genetic background for the disease. Here, we evaluate if, in ALS patients with other disease-linked mutant genes besides *TARDBP*, TDP-43 subcellular localization in CLM reproduces that found in motor neurons of corresponding cases. In addition, we analyse the TDP-43 profile in lymphocytes and monocytes separately, the two cell types constituting CLM.

In motor neurons of ALS-affected cases, mutant *TARDBP* is considered to be a determinant of TDP-43 mislocalization [4,28,30–33]. Our previous and present observations show that, in ALS-affected patients, mutations of this gene are associated with TDP-43 cytoplasmic accumulation also in CLM, as well as in lymphocytes and monocytes. Interestingly, in one of these patients the accumulation was restricted to monocytes. Cytoplasmic accumulation of TDP-43 is a feature also of CLM of subjects carrying mutant *TARDBP*, even in the absence of signs of the disease. In fact, the accumulation in CLM was observed previously

Figure 4. Correlation between transactive response DNA-binding protein 43 (TDP-43) subcellular distribution in circulating lymphomonocytes and various clinical parameters in amyotrophic lateral sclerosis (ALS)-affected patients. The parameters include: gender, familiarity for the disease, site of onset, age at blood sample collection and disease duration at blood sample collection. For age at blood sample collection and disease duration, the correlation is reported by dividing patients according to age or disease duration ranges as well as in terms of linear regression between time and TDP-43 ratio. * $P < 0.05$ vs. the other group.



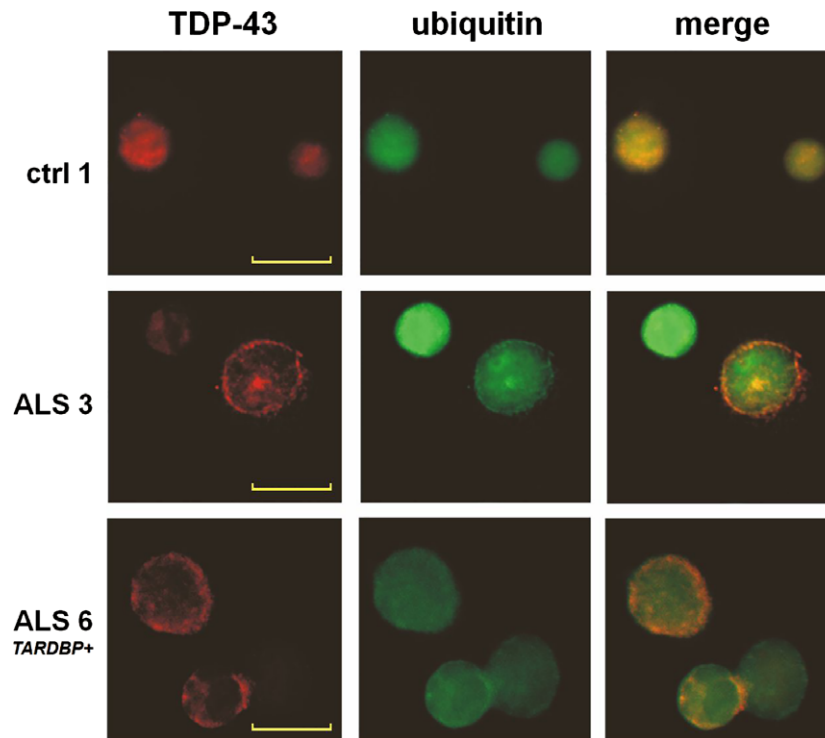


Figure 5. Double immunolabelling of circulating lymphomonocytes (CLM) with transactive response DNA-binding protein 43 (TDP-43) and ubiquitin in CLM from a neurologically unaffected control and of two amyotrophic lateral sclerosis (ALS) patients. Ctrl 1: neurologically unaffected control; ALS 3: patient without gene mutations; ALS 6: patient with mutant *TARDBP*. Red: TDP-43 antibody; green: ubiquitin antibody. All images were taken at 100 \times magnification, and the scale bars represent 10 μ m. The images are representative of at least four sections.

in one [34], and here in two neurologically unaffected individuals carrying the familial *TARDBP* mutation. The accumulation was not more severe in a relative of the latter two individuals, who was ALS-affected and homozygous for the mutation. It is conceivable that the accumulation, besides being unrelated to the presence of perceptible signs of disease, is also unrelated to the amount of mutated protein in the cell.

Mutations of *VCP* are associated with TDP-43 alterations in *post mortem* nervous tissue as well as in biopsied muscle of various neurodegenerative diseases, in murine motor neurons and in cell lineages [35,40–48]. In agreement with these observations, in the ALS patients with mutant *VCP* tested in this study, TDP-43 was revealed to accumulate in cytoplasm of CLM, as well as in that of lymphocytes and monocytes evaluated separately; this feature was confirmed in one of these patients when re-probed about 1 year later.

In motor neurons of ALS cases with mutant *FUS* the encoded protein is sequestered inside cytoplasmic ubiquitinated inclusions but TDP-43 is not a component

of these inclusions [4,5,49–53]. Furthermore, the protein complexes that control nuclear/cytoplasmic trafficking of *FUS* differ with respect to those controlling TDP-43 [54,55]. Accordingly, in patients with mutant *FUS* enrolled in this study TDP-43 did not accumulate in the cytoplasm of lymphocytes or monocytes.

Alterations of TDP-43 are described to be absent in *post mortem* motor neurons of ALS cases with *SOD1* mutations [4,23,53,56]. However, some exceptions have been disclosed [57–60] and evidence supports the existence of a link between TDP-43 and *SOD1* in the pathogenesis of ALS [61–63]. Here, in the group of patients carrying *SOD1* mutations, TDP-43 subcellular localization in CLM was heterogeneous. In fact, in some cases TDP-43 cytoplasmic accumulation was observed in both monocytes and lymphocytes whereas in others neither in monocytes nor in lymphocytes. At present, the absence of a comparison between CLM and motor neurons from the same subject makes it impossible to establish whether there is a correspondence between TDP-43 localization in CLM and motor neurons in this

category of patients. TDP-43 alterations have been discovered in nervous tissue of cases with SOD1 mutations involving Cys¹¹¹, Ile¹¹² or Ile¹¹³ [57–59]. Of note, TDP-43 cytoplasmic accumulation was detected in CLM of one patient carrying a SOD1 mutation involving Ile¹¹³.

A hexanucleotide repeat within a non-coding region of the *C9ORF72* is the most common mutation found in ALS [2,37,64,65]. Cytoplasmic ubiquitinated inclusions immunoreactive for TDP-43 are a feature of *post mortem* motor neurons of ALS cases with mutant *C9ORF72* [37,66–68]. However, neuronal aggregates negative for TDP-43, but enriched in other proteins (such as p62), are considered pathognomonic for this group of patients [36,69–71]. Furthermore, TDP-43 dysfunctions may or may not characterize cell lineages with mutant *C9ORF72* [72,73]. Herein, of the mutant *C9ORF72* patients recruited, cytoplasmic accumulation of TDP-43 was manifest in monocytes in all and also in lymphocytes only in one. In one of the patients, the nuclear/cytoplasmic TDP-43 ratio had decreased when re-tested 4 years after the first evaluation (when the ratio was in the control range) [34]. Therefore, in this lapse of time, TDP-43 cytoplasmic accumulation must have involved ever more monocytes and possibly lymphocytes (many lymphocytes, although not most, showed perinuclear TDP-43 accumulation, see Results). This outcome suggests that, in CLM of patients with mutant *C9ORF72*, TDP-43 cytoplasmic accumulation progresses over time. In this respect, there is evidence that mutant *C9ORF72* affects TDP-43 indirectly and evolves over time in motor neurons [65,71]. Possibly, the follow up of the disease in these patients benefits from the evaluation of TDP-43 subcellular distribution in monocytes and lymphocytes.

In a fALS patient with unknown genetic background, TDP-43 cytoplasmic accumulation was not observed either in lymphocytes or in monocytes. While awaiting the genetic identification of this case, this outcome suggests that the mutated gene is not associated with TDP-43 proteinopathy (i.e. *FUS*-like) or concurs to its development over time (as suspected for mutant *C9ORF72*).

No correlation was found between TDP-43 subcellular distribution and gender, familial disease, age at disease onset, age at blood sample collection and disease duration. Instead, significantly greater cytoplasmic accumulation was found in CLM of patients with bulbar rather

than spinal site of disease onset. However, this result needs to be confirmed on a larger case series.

This study disclosed that in all cases in which WB assay reveals cytoplasmic accumulation of TDP-43 in CLM, IF assay detects this accumulation in both lymphocytes and monocytes. Instead, in cases in which WB analysis did not reveal TDP-43 cytoplasmic accumulation in CLM, IF assay disclosed that there was not always concordance between lymphocytes and monocytes. In fact, in one patient with mutant *TARDBP* and three patients with mutant *C9ORF72* (see above) as well as in one patient without documented gene mutations, TDP-43 cytoplasmic accumulation was detected in monocytes but not in lymphocytes. These patients can all be expected to have, or to develop over time, TDP-43 mislocalization in motor neurons. Instead, accumulation in lymphocytes and not in monocytes was never observed. Thus, in all those cases in which cytoplasmic accumulation of TDP-43 was detected with WB assay, the alteration must have characterized both cell types. TDP-43 cytoplasmic accumulation is not detected with WB assay when it is a feature of only monocytes, likely because monocytes constitute only 10–20% of CLM population and WB assay does not discriminate between lymphocytes and monocytes. Thus, from the clinical point of view, the analysis of TDP-43 subcellular localization in monocytes is more sensitive than that in CLM in identifying patients who have mislocalized TDP-43 in their motor neurons. We do not know why lymphocytes sometimes do not manifest the TDP-43 alteration featuring motor neurons when instead monocytes do. It would not appear to be a question of severity of disease since TDP-43 cytoplasmic accumulation was disclosed in CLM (i.e. both lymphocytes and monocytes) of neurologically unaffected individuals carrying mutant *TARDBP*. However, we can conclude that monocytes, more reliably than lymphocytes, reflect the molecular background that in motor neurons is associated with their degeneration. In this regard, ALS is a multisystem disease in which monocytes are described to play a role in the pathogenesis [9]. In fact, recruitment of inflammatory monocytes in the CNS appears to be related to disease progression [74] and activated monocytes/macrophages have been found in blood samples from sALS patients [75]. Furthermore, activation of microglia, a cell type which shares with monocytes a common embryonic hematopoietic precursor [76,77] and in particular

conditions can even derive from monocytes [78], is associated with TDP-43 proteinopathy in the brain of ALS cases [10].

In motor neurons of ALS patients, sequestration of TDP-43 inside cytoplasmic ubiquitinated inclusions is associated with cell degeneration [22–24,79]. Furthermore, accumulation of TDP-43 in the cytoplasm precedes the formation of ubiquitinated inclusions [27] and is considered an early step in ALS pathogenesis [4,27–29]. Here, accumulation of TDP-43 in the cytoplasm of CLM is not associated with cell degeneration since plasma membrane integrity and nuclear shape are preserved, and occurs in the absence of ubiquitinated inclusions. Furthermore, cytoplasmic accumulation of TDP-43 in CLM is rarely associated with complete nuclear depletion, as instead occurs in motor neurons [25,26,32]. Additionally, TDP-43 fragments characterizing inclusions in motor neurons [24,80–82] have not been detected in CLM of ALS patients, and fragments of the size seen in CLM (~42 kDa) have not been observed in motor neurons [34]. Possibly, the mechanisms underlying TDP-43 fragmentation and ubiquitination in motor neurons differ with respect to those in CLM. Alternatively, CLM, which have a much shorter life span than motor neurons, might not live long enough for ubiquitin-immunoreactive inclusions to develop.

In conclusion, in patients with various ALS-linked mutant genes, there appears to be a close parallel between TDP-43 distribution in CLM and that in motor neurons, whether altered or not. Interestingly, mislocalization of TDP-43 occurs in motor neurons, in CLM and even more in monocytes, irrespective of whether the protein is wild type or not. This suggests that TDP-43 localization in monocytes and motor neurons is regulated by similar mechanisms and that stimuli impairing TDP-43 localization in motor neurons also do so in monocytes. In this view, monocytes may be used to support the diagnosis of ALS, as well as to identify subjects at risk of disease. Additionally, monocytes may be exploited to investigate the mechanisms involved in the initial stages of ALS, in the hope to find treatments that prevent, or at least delay, motor neuron death.

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Conflict of interest

Dr. Restagno received research support from the Italian Ministry of Health (Ricerca Finalizzata) and Regione Piemonte (Ricerca Finalizzata). Dr. Calvo received research support from the Italian Ministry of Health (Ricerca Finalizzata). Dr. Chiò received research support from the Italian Ministry of Health (Ricerca Finalizzata), Regione Piemonte (Ricerca Finalizzata), University of Torino, Federazione Italiana Giuoco Calcio, Fondazione Vialli e Mauro ONLUS and the European Commission (Health Seventh Framework Program). Dr. Chiò serves on the editorial advisory board of Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration and on the scientific advisory boards for Biogen Idec and Cytokinetics. The other authors declare that they have no conflicts of interest.

Author contributions

Study conception and design: MT Rinaudo, G De Marco, A Chiò, MT Giordana. *Patient characterization and sample collection:* A Chiò, A Calvo, J Mandrioli, C Caponnetto, G Borghero, G Restagno, U Manera, A Canosa, C Moglia, N Fini. *Acquisition of data:* G De Marco, A Lomartire, A Risso, E De Luca. *Analysis and interpretation of data:* MT Rinaudo, G De Marco, A Lomartire, C Tarella, M Mostert. *Drafting of manuscript:* MT Rinaudo, G De Marco, A Lomartire, M Mostert. *Critical revision:* A Chiò, MT Giordana, A Calvo, J Mandrioli. MT Rinaudo, G De Marco and A Lomartire had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. All authors have approved the submitted version of the paper.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Transactive response DNA-binding protein 43 (TDP-43) subcellular localization, detected by

confocal microscopy, in circulating lymphomonocytes (CLM) from a neurologically unaffected control and three amyotrophic lateral sclerosis (ALS) patients, one with *TARDBP* mutation and the other two without documented gene mutations.

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