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Review Article

Profiling *TREM2* expression in amyotrophic lateral sclerosis

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ABSTRACT

Background and Objectives: There is growing evidence of the contribution of neuroinflammation, and in particular microglia, in the pathogenesis of amyotrophic lateral sclerosis (ALS). *TREM2* gene plays a crucial role in shaping microglia in neurodegenerative conditions. To deepen the understanding of *TREM2* in ALS and investigate the performance of *TREM2* as a biomarker, we profiled *TREM2* expression levels in spinal cord, cerebrospinal fluid and blood of patients with sporadic ALS. We also wanted to investigate whether the combined measurement of sTREM2 in fluids could improve the diagnostic yield of total and phosphorylated TDP-43 levels.

Methods: We performed a case-control study to profile overall and transcript-specific *TREM2* mRNA levels by RT-qPCR and protein expression levels by Western-blot in *postmortem* specimens of spinal cord from ALS patients and controls. In parallel, we measured soluble TREM2 (sTREM2) protein levels and full length and phosphorylated TDP-43 (tTDP-43 and pTDP-43) by ELISA in CSF and serum from ALS patients vs healthy controls. Patients were prospectively recruited from an ALS unit of a tertiary hospital and fulfilled El Escorial revised criteria. After bivariate analysis, a logistic regression model was developed to identify adjusted estimates of the association of sTREM2 levels in CSF and serum with ALS status.

Results: Overall and transcript-specific *TREM2* mRNA were upregulated in the spinal cord of ALS patients ($n = 21$) compared to controls ($n = 19$). Similar changes were observed in TREM2 protein levels ($p < 0.01$) in spinal cord of ALS patients vs healthy controls. We also detected significantly higher sTREM2 levels in CSF (p-value < 0.01) of ALS patients ($n = 46$) vs controls ($n = 46$) and serum (p-value < 0.001) of ALS patients ($n = 100$) vs controls ($n = 100$). In a logistic regression model, both CSF and serum sTREM2 remained independently associated with ALS status with OR = 3.41 (CI 95 %=1.34–8.66) (p-value < 0.05) and OR = 3.38 (CI 95 %: 1.86–6.16) (p-value < 0.001), respectively. We also observed that pTDP-43 levels in CSF is an independent predictor of ALS (p-value < 0.05).

Conclusions: Our results support the role of *TREM2* in ALS pathophysiology and demonstrates that the three *TREM2* transcripts are deregulated in ALS in *postmortem* human specimens of spinal cord. We hypothesise about the possible influence of systemic-peripheral inflammation in the disease. Finally, we conclude that pTDP-43 levels in CSF could be a biomarker of ALS, and sTREM2 measurement in CSF and blood emerge as potential non-invasive biomarker in ALS.

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is a heterogeneous, progressive and devastating adult-onset neurodegenerative disease characterized by the degeneration of both upper and lower motor neurons in the spinal cord, brainstem and motor cortex. ALS is a heterogeneous disorder from clinical, genetic and pathological point of view, but the neuropathological hallmark of this disease, in most of cases, is the aberrant aggregation and accumulation of a mislocalized hyper-phosphorylated TDP-43 immunoreactive intracytoplasmic inclusions in motor neurons. So far, the measurement of TDP-43 as a biomarker of ALS in fluids is controversial and represents a challenge (Feneberg et al., 2021). The implementation of validated biomarkers in fluids is a major goal in neurodegenerative disorders, including ALS, which the diagnosis remains essentially clinical.

ALS pathophysiology is supposed to be multifactorial and neuroinflammation is one of the pathogenic processes presumably involved in the disease; however, its role has yet to be defined (Palotas et al., 2017; Geloso et al., 2017). Interestingly, a complex signaling network between central nervous system (CNS), resident immune cells (microglia) and peripheral immune cells, including monocytes and T cells has been reported (Brites et al., 2014; Town et al., 2005; Buckwalter et al., 2006; Dulken et al., 2019) and there is evidence that peripheral inflammation contributes to CNS inflammation in some neurodegenerative diseases (Webers et al., 2023; Chiot et al., 2020). However, nowadays, it is difficult to differentiate between microglia and peripheral infiltrating macrophages, and transcriptomics may help us to profile and distinguish between the two cell subtypes (Grassivaro et al., 2020).

Triggering receptor expressed on myeloid cells 2 (*TREM2*) is a member of the *TREM* transmembrane glycoprotein receptor family of the innate immune system, which is expressed in the membrane of myeloid cells, dendritic cells, osteoclasts and microglia (Jones et al., 2014; Krasemann et al., 2017). It is interesting to note that *TREM2* is not constitutively expressed (Natale et al., 2023). In turn, its expression is highly cell-and context-specific and the underlying molecular mechanisms are unknown (Jay et al., 2017). This receptor has an ectodomain in the cell surface that is cleaved by the metalloproteinase Adam10 to produce soluble TREM2 (sTREM2) (Thornton et al., 2017) which can be readily measured in different human fluids, such as blood or cerebrospinal fluid (CSF).

In the CNS, *TREM2* is involved in maintaining brain homeostasis in response to tissue damage and is expressed in the microglia promoting phagocytosis of apoptotic neurons, cellular detritus and misfolded proteins (Kleinberger et al., 2023). Therefore, it seems to play a protective role in the neuroinflammatory reaction. Indeed, the *TREM2* gene variant (rs75932628 (p.R47H)), associated with a loss-of-function, has been observed to increase the risk of AD (Jonsson et al., 2013; Guerreiro et al., 2012) and subsequent associations with Parkinson disease (PD) and frontotemporal dementia (FTD) have been reported (Zhou et al., 2019; Rayaprolu et al., 2013). Nevertheless, there are inconsistent results regarding its role in the pathophysiology of ALS (Siokas et al., 2021; Cady et al., 2014).

A recent study suggests that TDP-43 protein is a possible ligand for microglial *TREM2* and this interaction would have a neuroprotective effect in TDP-43-related neurodegenerative diseases as ALS, since *TREM2* deficiency impaired phagocytic clearance of pathological TDP-43 (Xie et al., 2021). During the development of neurodegenerative diseases, homeostatic microglia progressively acquire a unique transcriptional and functional signature that is responsible of evolution into disease-associated microglia (DAM). Also here, *TREM2* plays an essential role because microglia depend on *TREM2* expression for fully acquisition of a DAM profile (Krasemann et al., 2017; Ulland and Colonna, 2018; Deczkowska et al., 2018).

Levels of sTREM2 have also been analyzed in biological fluids as cerebrospinal fluid (CSF) and blood in neurodegenerative diseases, particularly in AD, and it has the potential to be used as a biomarker.

In this study, we aimed to deepen our understanding of *TREM2* expression alterations in ALS. To this end, we first profiled overall and transcript-specific *TREM2* mRNA and protein expression levels in *postmortem* specimens of spinal cord from ALS patients compared to controls. Once its differential expression was proven, CSF and peripheral blood sTREM2 protein levels were measured in living patients with ALS *versus* controls to explore its performance as candidate biomarkers.

2. Material and methods

2.1. Study design

A case-control study was designed to profile *TREM2* expression in the *postmortem* spinal cord from 21 ALS-TDP43 patients and 19 non-neurodegenerative control donors.

In parallel, a case-control study was planned to analyze sTREM2 levels in fluids (CSF and blood) as potential biomarkers in ALS. We conducted a first experimental study in a training cohort of 46 ALS patients and 46 controls to measure sTREM2 levels in CSF and blood. Next, a subsequent assessment of blood sTREM2 levels in an extended cohort of 100 cases and 100 controls was performed as validation cohort.

2.2. Spinal cord samples

Postmortem fresh-frozen cervical spinal cord (anterior horn) tissue samples of 21 ALS patients and 19 age- and gender-matched controls without neurodegenerative disease were provided by the Navarrabiomed Brain Bank, following the guidelines of Spanish legislation (García-Merino et al., 2015) on the research matter.

Patient selection was performed by expert neurologists using the following inclusion criteria: for ALS group were clinical and neuropathological diagnosis of ALS with TDP-43 deposition (Neumann et al., 2006); for control group age matched donors without neurodegenerative disease, recent vascular cerebral disease, infection or CNS injury. In addition to the mentioned inclusion criteria, the controls selection was limited by the unusual spinal cord collection among CNS control donors, which prevented gender-matching with cases.

2.3. Neuropathological examination and frozen tissue preservation

After brain and spinal cord autopsy, macroscopic examination and dissection across midline to separate both hemispheres were carefully performed. Brain stem was obtained including medulla oblongata and spinal cord in all cases. Half brain and spinal cord specimens were cryopreserved at −80 °C until further use. Left hemisphere was placed in 10 % formalin during 4 weeks. Formalin-fixed, paraffin-embedded tissue sections from each region of interest were sectioned at 5 μm and counterstained with haematoxylin-eosin for immunohistochemistry analysis with the anti-phospho TDP-43 monoclonal antibody (Cat# CAC-TIP-PTD-M01, clone11-9, phosphor Ser 409/410; 1:80,000) from Cosmo Bio (Hokkaido, Japan), mouse monoclonal antibody anti-human PHF-TAU (Cat# 90206, clone AT-8, 1:1000) from Innogenetics (Ghent, Belgium), mouse monoclonal anti Beta-amyloid (Cat# M 0672, clone 6F/3D, 1:200) from Agilent Dako (Santa Clara, CA, USA) and mouse monoclonal antibody against α-synuclein (Cat# NCL-L-ASYN, clone KM51, 1:50) from Leica Biosystems (Wetzlar, Germany) and were visualized using an automated slide immunostainer (Leica Bond Max, Leica Bond Max) with Bond Polymer Refine Detection (Leica Biosystems Newcastle ltd., Newcastle, UK). Luxol fast blue, rabbit polyclonal antibody anti-Iba1 (Cat# 019–19741; 1:2000) from Wako (Osaka-Japan) and mouse monoclonal prediluted antibody anti-CD68 (Cat# MAD-002097, clone KP-1, 1:1) from Master Diagnóstica (Andalucía, Spain) staining were included in brain and spinal cord sections for the study of myelin pathology and inflammatory infiltration. All ALS cases demonstrated upper and lower motor neuron degeneration accompanied by p-TDP43 neuronal inclusions.

2.4. CSF and blood samples

CSF and blood sTREM2 protein levels were assessed in 92 subjects (ALS n = 46; control n = 46). To validate blood results, sTREM2 protein levels were measured in an extended cohort of 200 living subjects (ALS n = 100; controls n = 100). This validation cohort consisted of 25 cases and 31 controls from the training cohort and, in addition, 75 ALS patients and 69 controls, which was intended to obtain a cohort strictly matched for age and gender. Patients were prospectively recruited from the Motor Neuron Diseases Clinic of two tertiary hospital from January 2014 to December 2020. Patients with definitive or probable ALS diagnosis according with the El Escorial revised criteria (Brooks et al., 2000) were included. In order to homogenize the sample set, we excluded patients with primary lateral sclerosis (PLS), primary muscular atrophy (PMA) and those with frontotemporal dementia (FTD). Controls were recruited from healthy relatives and volunteers matched for age and sex with the following features: no clinical manifestation of ALS or other neurodegenerative disease, no tumoral or systemic inflammatory disease and no active anti-inflammatory drugs intake. We included as CSF-controls subjects who underwent a spinal anesthesia in minor surgery and met the above criteria. The number of age- and sex-matched cases and controls during the sample collection period is what determined the sample size in this study. Accepting an alpha risk of 0.05 in a bilateral contrast with 100 subjects in the first group and 100 in the second, the power of the hypothesis contrast is 95 % to detect as statistically significant a difference of 500 pg/mL between the mean of both groups.

Samples of CSF were obtained by lumbar puncture. CSF samples were centrifuged at 2000×*g* for 10 min at 4 °C within 4 h after collection. Peripheral blood was collected by venipuncture and centrifuged at 3,500 rpm for 15 min at 4 °C in order to separate the serum. Serum and CSF supernatants were aliquoted into 1.5 mL polypropylene tubes and stored at −80 °C until further use.

2.5. Quantification of Iba1 and CD68 immunostaining in spinal cord

We performed an automated quantification of microglia/macrophages burden expressed as percentage of Iba1 and CD68 immunostaining área (Liu et al., 2019; Celarain et al., 2016), in spinal cord tissue in ALS patients vs controls. For this purpose, a blinded for clinical data quantitative image analysis was carried out using the scientific image analysis program ImageJ (Wayne Rasband, NIH, USA) (Collins, 2007).

In brief, a same Iba1 and CD68 immunostaining spinal cord field was scanned in all cases at 40× magnification (Fig. 1). Upon adjusting the threshold (min,max) for each molecular target, a binary conversion of the images on red channel was performed to quantify the percentage of immunoreactive area occupied by these targets. Imaging parameters and software settings were constant for all photomicrographs. (By adapting previously published protocols: https://imagej.nih.gov/ij/plugins/ihc-toolbox/index.html; https://imagej.nih.gov/ij/docs/exmapls/stained-sections/index.html; https://resources.finalsite.net/images/v1567623985/lsuhscshreveportedu/awsil3hxeld0mkym4bzl/immunohistochemistrystaining.pdf).

2.6. RNA isolation from frozen spinal cord samples

Total RNA was isolated from the anterior horn of spinal cord homogenates using RNeasy Lipid Tissue Mini kit (QIAGEN, Venlo, NED), following manufacturer's recommendations. Genomic DNA was removed with recombinant DNase (TURBO DNA-free™ Kit, Ambion, Inc., Austin, TX, USA). RNA integrity was checked by 1.25 % agarose gel electrophoresis under denaturing conditions. Concentration and purity of RNA were both evaluated with NanoDrop spectrophotometer. Only RNA samples showing a minimum quality index (260 nm/280 nm absorbance ratios between 1.8 and 2.2 and 260 nm/230 nm absorbance ratios higher than 1.8) were included in the study.

2.7. Reverse transcription and gene mRNA expression analysis by RT-qPCR

The RT-qPCR reactions were performed in triplicate for each sample and then repeated within independent cDNA sets to confirm the obtained results.

Complementary DNA (cDNA) was reverse transcribed from 1500 ng total RNA with SuperScript® III First-Strand Synthesis Reverse

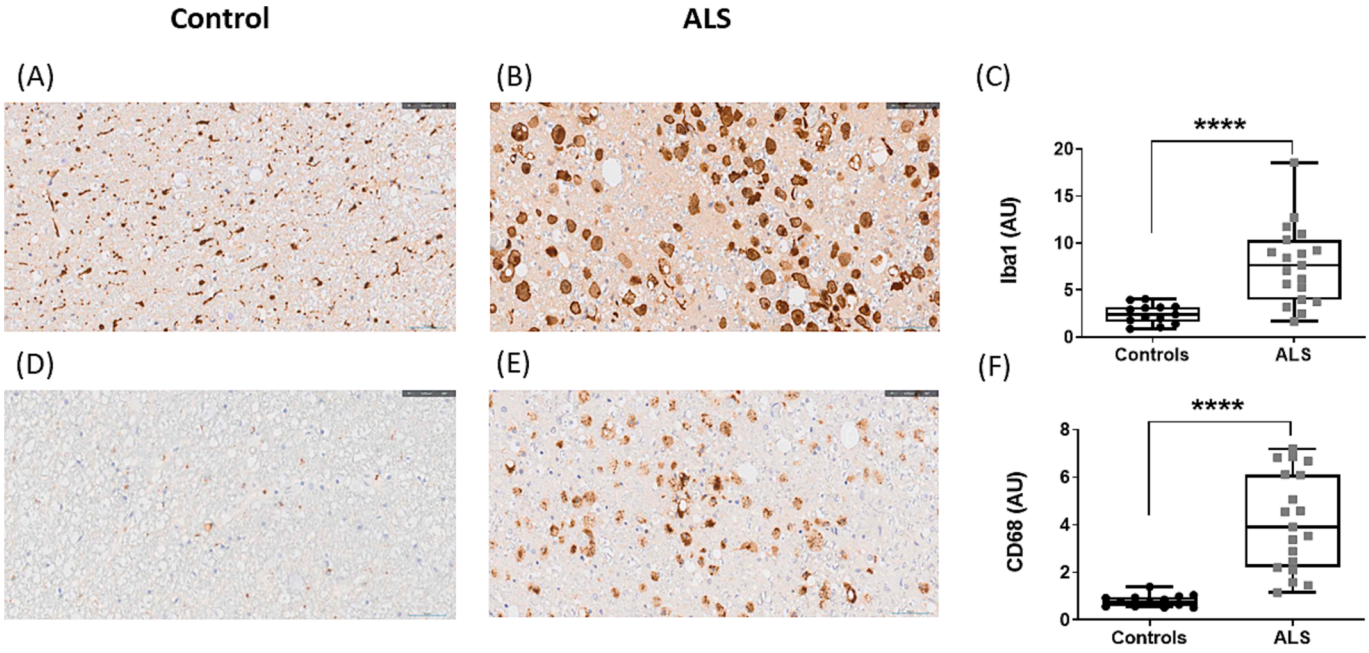

Fig. 1. Representative images for Iba1 immunostaining in (A) control and (B) ALS patient and for CD68 in (D) control and (E) ALS patient, in cervical spinal cord (lateral cord). Scale bar = 50 μm. The box plots represent a higher percentage of immunostained area in ALS patients compared to controls for (C) Iba1 and for (F) CD68 (n = 32). AU: arbitrary units. Data represent the mean value ± Standard Deviation (SD). ****p value < 0.0001.

Transcriptase (Invitrogen, Carlsbad, CA, USA) after priming with oligo-d (T) and random primers. Real time quantitative PCR (RT-qPCR) reactions were performed with Power SYBR Green PCR Master Mix (Invitrogen, Carlsbad, CA, USA) in a QuantStudio 12 K Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Sequences of primer pairs for *TREM2* were designed using Real Time PCR tool (IDT, Coralville, IA, USA) and are listed in Table 1. Relative expression levels of the selected genes in a particular sample were calculated as previously described (Livak and Schmittgen, 2001) (Livak 2001) and the geometric mean of *ACTB* and *GAPDH* genes was used as reference to normalize expression values.

2.8. TREM2 protein expression analysis by Western-blotting

Frozen spinal cord samples were lysed with 100 μL lysis buffer containing urea, thiourea, and DTT. After centrifugation at 35.000 rpm for 1 h at 15 °C, extracted proteins were quantified following the Bradford-Protein Assay (Bio-Rad, Hercules, CA, USA) by using a spectrophotometer.

Next, 20 μg of protein per sample were resolved in 4–20 % Criterion TGX stain-free gels (Bio-Rad) and electrophoretically transferred onto nitrocellulose membranes using a trans-blot Turbo transfer system (25 V, 7 min) (Bio-Rad). Equal loading of the gel was assessed by stain-free digitalization and Ponceau staining. Membranes were probed with mouse anti-human TREM2 primary antibody [MM0942-42E14] (Cat# ab201621; 1:750) from Abcam (Cambridge, UK) in 5 % nonfat milk and incubated with peroxidase-conjugated anti-mouse secondary antibody (Bio-Rad; 1:2000). Immunoblots were then visualized by exposure to an enhanced chemiluminescence ECL Select™ Western Blotting Detection Reagent (Amersham, GE Healthcare, Chicago, IL, USA) using a ChemidocMP Imaging System (Bio-Rad). Expression levels of TREM2 were standardized by the corresponding band intensity of β-actin (A5441, clone AC-15; 1:10000) from Sigma-Aldrich (St Louis, MO, USA).

2.9. sTREM2 measurement in CSF and serum by enzyme-linked immunosorbent assay (ELISA)

CSF and serum levels of sTREM2 in ALS patients and controls were determined by a commercial enzyme-linked immunoabsorbent assay (ELISA), the Human TREM2 DuoSet ELISA (Catalog number: DY1828-05; R&D systems, Minneapolis, MN, USA) according to manufacturer's instructions. Briefly, 96-well streptavidin plates were coated with TREM2 capture antibody (ref:844598, R&D) and incubated overnight. After three washes, plates were blocked with 1 % BSA in PBS for one hour. Then samples and TREM2 standards were added and incubated for two hours. Finally, the detection antibody (ref: 844599; R&D) was added and incubated for two hours. Recombinant human TREM2 standard (ref:844600) was used to construct the ELISA calibration curve. Absorbance readings were analyzed using an Epoch Microplate Spectrophotometer (BioTek Instrumentals, Inc., Winooski, VT, USA). All incubations were performed at room temperature. Samples were randomly distributed. Concentrations are expressed in pg/mL and the assay range was 46.9–3,000 pg/mL.

Four patients were excluded from the study because they showed results under the detection limit.

2.10. TDP-43 measurement in CSF and serum by enzyme-linked immunosorbent assay (ELISA)

Full-length TDP-43 (tTDP-43) and phosphorylated TDP-43 (pTDP-43) levels in CSF from the training cohort and serum from the validation cohort were assessed using commercially available ELISA kits (Human TDP-43, KE00005, Proteintech Group and pTDP-43 ELISA Kit, E9442h EIAab, respectively). According to the manufacturer, the assay is expected to detect full-length TDP-43, but the detection of pathological truncated forms of TDP-43 protein is not guaranteed.

2.11. Statistical data analysis

Statistical analysis was performed with SPSS 25.0 (IBM, Inc., USA). Before performing differential analysis, we checked out whether continuous variables showed a normal distribution, as per one-sample Kolgomorov-Smirnov test and the normal quantil-quantil (QQ) plots. Qualitative variables are expressed as a percentage for each group. Quantitative variables are shown as the mean (standard deviation (SD)), if normal distribution is followed, or the median (interquartile range) otherwise. A univariate general linear model adjusted by gender was used to study the proportions between the expression levels of *TREM2* mRNA variants in human spinal cord and between overall and transcript-specific *TREM2* mRNA levels in ALS *versus* control samples, for which the values of expression levels were transformed by using log10(X) to meet normality conditions. Comparison of sTREM2 protein levels between cases and controls was assessed by U Mann-Whitney test. Fold Change (FC), equivalent to the ratio of the mean relative expression in patients to the mean relative expression in controls, was used as a measure of the magnitude of change. Furthermore, for ELISA analysis, CSF and serum sTREM2 levels were correlated with clinical parameters using Pearson and Spearman correlations, as was the correlation between *TREM2* mRNA levels and percentage of immunostaining area in spinal cord tissue. A logistic regression model (ENTER method) was fit to assess the independent association of sTREM2 levels in CSF and serum with ALS status, using gender and age as covariates, and odds ratio (OR) was calculated. The diagnostic performance of CSF and serum sTREM2 levels was evaluated by receiver operating characteristic (ROC) curves. The areas under the curve (AUC) were calculated and optimum cut-off points of each matrix were selected based on their sensitivity and specificity. Significance level was set at p-value < 0.05. GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA, USA) was used to draw graphs.

3. Results

3.1. Profiling of TREM2 mRNA expression in human spinal cord

To investigate whether *TREM2* was differentially expressed in the spinal cord of ALS patients vs controls, we measured mRNA expression levels (overall and each transcript variant) by RT-qPCR (Fig. 2A) and analyzed the results, after adjusting for gender, in a cohort of

Table 1
RT-qPCR primers. The table shows the primer pairs used in the study. Amplified transcripts are identified by Ensembl Transcript ID.

Ensembl Transcript ID	Amplification	Amplicon Size	Tm	Forward Primer	Tm2	Reverse Primer	Exons
ENST00000338469.3; ENST00000373113.7; ENST00000373122.8	All isoforms: overall TREM2	116	61.0	CTGCTCATCTTACTCTTTGTCAC	62.3	CAGTGCTTCATGGAGTCATAGG	1_2
ENST00000338469.3	TREM2-201	245	60.0	AGCCATCACAGACGATACCC	60.0	TCTCAGCCCTGGAGATGC	3_4
ENST00000373113.7	TREM2-202	146	58.0	GCATCTCCAGGAGCCTCT	60.1	ATGTGTCCCTGGCTTCTGTC	3_4
ENST00000373122.8	TREM2-203	72	61.7	TTCGAGGATGCCCATGTG	62.0	GGAGAGACAAGAAGGCAGATG	3_4

Tm: Melting Temperature.

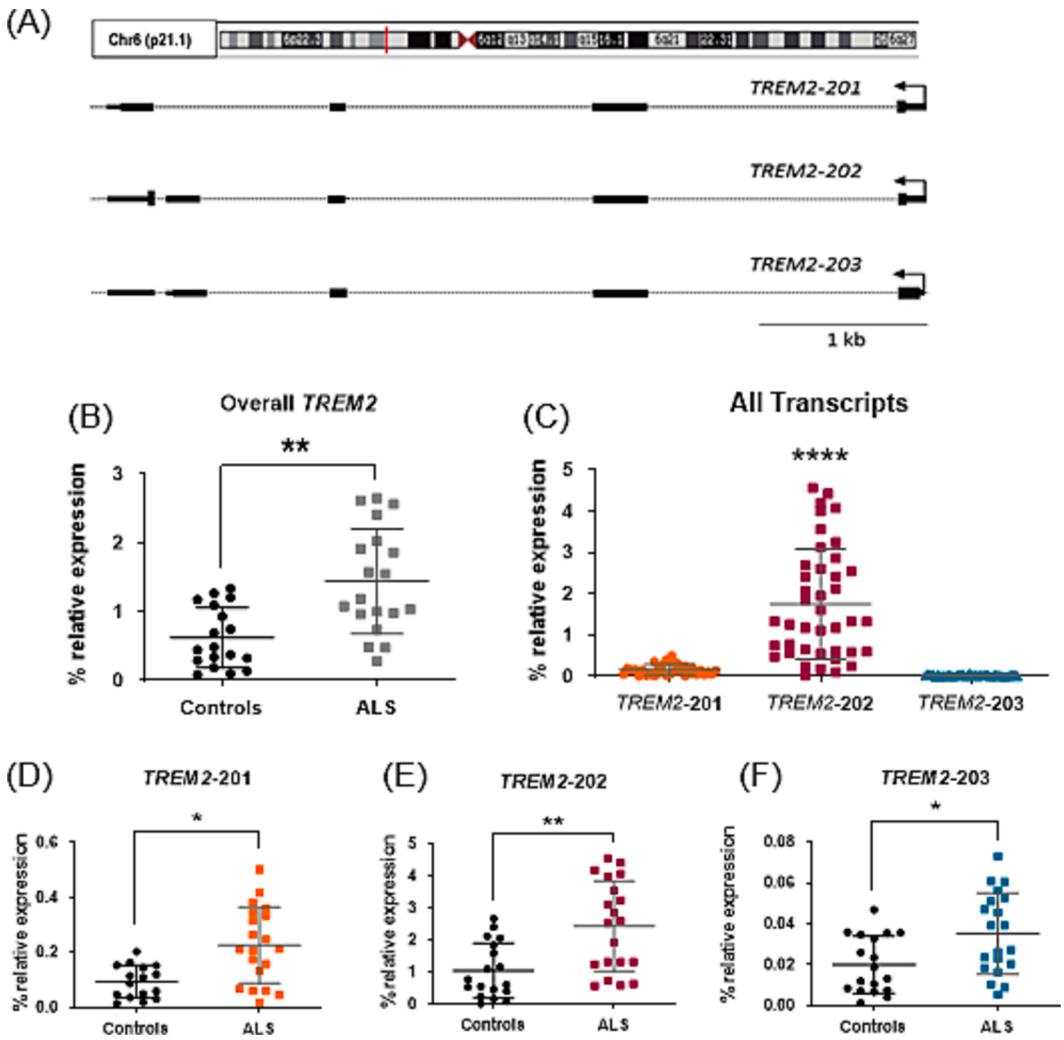

Fig. 2. *TREM2* mRNA expression profiling in human spinal cord in ALS. (A) The figure illustrates the maps of the 3 different *TREM2* transcript variants. Black boxes represent exons, and arrows represent transcription start sites. (B) Overall *TREM2* mRNA levels in the spinal cord in ALS samples (n = 21) were compared with controls (n = 19) as shown in the graph. (C) The dot-plot graph shows mRNA expression of the 3 *TREM2* variants in human spinal cord specimens. (D–F) The panels show mRNA expression levels in ALS patients when compared with controls for each of the *TREM2* transcript variant. Data represent the mean value ± SD. *p value < 0.05; **p value < 0.01; ***p value < 0.001; ****p value < 0.0001.

Table 2
Demographic and clinical data of all subjects

	ALS	Controls	p
Spinal cord donors			
N	21	19	
Age (years) (IQR)[1]	72 (59-81)	72 (63-82)	0.599
Gender (F/M)	13/8	6/13	<0.05
PMI[2] (h); median (range)	5(2-12.50)	5.75(2.45-16.30)	0.741
TREM2 variant p.R47H	0	1	
Training cohort			
N	46	46	
Age (years) (IQR)	63 (58-72)	59 (51-66)	<0.05
Gender (F/M)	17/29	13/33	0.374
ALS phenotype (B/S)[3]	13/33		NA
ALSFRS-R[4]median (range)	38 (18-47)		NA
Validation cohort			
N	100	100	
Age (years) (IQR)	69 (63-75)	69 (63-75)	0.856
Gender (F/M)	52/48	52/48	1
ALS phenotype (B/S)	33/67		NA
ALSFRS-R median (range)	38 (18-46)		NA

[1] Interquartile range;
[2] Postmortem interval;
[3] Bulbar/Spinal;
[4] ALS functional rating scale revised.

neuropathological characterized ALS patients (21 cases) and controls (19 cases). (Table 2).

We observed that overall *TREM2* mRNA levels were significantly increased by 2.40-fold in ALS patients vs controls (p-value < 0. 01) (Fig. 2B).

Next, we tested whether *TREM2* mRNA upregulation in spinal cord of ALS was due to elevation of a particular *TREM2* transcript variant. The shortest variant (ENST00000338469.3) is originated from a spliced transcript by skipping exon 4, which encodes the Trem2 protein transmembrane domain. Therefore, this variant lacking exon 4 is thought to encode the soluble TREM2 (sTREM2) protein (219aa). For purposes of this study, this variant was named *TREM2*-201, following the nomenclature of the Ensembl database (Zerbino et al., 2018). A second variant, referred to as *TREM2*-202 (ENST00000373113.7), encodes a larger protein (230aa) which is the canonical *TREM2* isoform. On the other hand, *TREM2*-203 (ENST00000373122.8) is a third transcript lacking exon 5 and part of exon 4, which encodes a shorter protein (222aa). In the spinal cord, we found that the transcript with the highest expression was the canonical *TREM2*-202 variant (p-value < 0.0001) (Fig. 2C) which also showed a differential expression between ALS patients and controls (fold-change = 3.30; p-value < 0.01) (Fig. 2E). In addition, *TREM2*-201 variant, which is supposed to encode sTREM2, was also

upregulated in the spinal cord of ALS patients compared to controls (fold-change = 2.11; p-value < 0.05) (Fig. 2D). Finally, the lowest expressed variant was *TREM2*-203, which was also increased in ALS patients vs controls (fold-change = 1.88; p-value < 0.05) (Fig. 2F).

3.2. Western-blot of TREM2 in the spinal cord

To explore whether increased expression of *TREM2* mRNA resulted in an increase in the levels of protein, a Western Blot analysis was performed. Protein extracts from frozen spinal cord samples that were included in the RT-qPCR experiment were obtained, and a monoclonal antibody which recognizes an epitope between residues 19–173 of TREM2 was used. Consistent with the results for *TREM2* mRNA expression, we observed that TREM2 protein expression levels were significantly higher in spinal cord from ALS patients as compared to controls (p-value < 0.01) (Fig. 3).

3.3. Correlation between TREM2 mRNA expression and CD68 and Iba1 immunostaining area.

In order to correlate *TREM2* mRNA expression with myeloid cells burden in CNS tissue, we performed a quantitative analysis (described in methods section) of the images provided by the Biobank (19 ALS patients and 13 controls), showing a statistically significant increase in Iba1 (p-value < 0.001) and CD68 (p-value < 0.001) immunolabelling in ALS spinal cord compared to controls (Fig. 1). Interestingly, we observed no significant correlation between *TREM2* mRNA levels and the percentage of area labelled by Iba1 and CD68 in spinal cord tissue of ALS patients (p-value = 0.469 and p-value = 0.301, respectively).

3.4. CSF and serum sTREM2 levels in the training cohort

The training cohort consisted of 46 patients and 46 controls for whom CSF and blood samples were available. A summary of demographic characteristics and clinical data of all individuals is shown in Table 2.

Regarding CSF analysis, we found significantly higher concentrations of sTREM2 in ALS patients (median = 790 pg/mL; IQR = 602–1,058) as compared with controls (median = 587 pg/mL; IQR = 355–941; p-value < 0.01) (Fig. 4A). Serum sTREM2 levels were also different between patients and controls. We found a significant difference in serum sTREM2 levels in ALS patients (median = 3,114 pg/mL; IQR = 1,955–5,432) compared to controls (median = 2,248 pg/mL; IQR = 1,437–3,419; p-value < 0.05) (Fig. 4B). Interestingly, there was a mild yet significant correlation between CSF and serum sTREM2 levels (r = 0.256, p-value < 0.05).

Next, logistic regression models were designed to identify adjusted estimates of the association of sTREM2 levels in CSF and serum with ALS status (control = 0; ALS = 1). Age and gender were included into the model in order to adjust for potentially confounding variables, since there were significant age differences between the control and ALS group (p-value < 0.05). As shown in Table 3, sTREM2 levels in CSF remained an independent predictor of ALS status after adjusting for age and gender with an odds ratio (OR) = 3.41 (CI95%=1.34–8.66; p-value < 0.05) as well as serum sTREM2 levels with an OR = 2.75 (CI95%= 1.10–6.84; p-value < 0.05) (Fig. 5).

To ascertain the performance of sTREM2 levels in CSF and serum for ALS diagnosis, ROC analysis was performed (Fig. 6A). For CSF, the AUC was 0.664 (CI = 0.549–0.779; p-value < 0.05) and the optimal cutoff point to differentiate between ALS patients and controls was 627.20 pg/mL (sensitivity = 0.72; specificity = 0.60). For serum, the AUC was 0.642 (CI = 0.525–0.758; p-value < 0.05) and the optimal cutoff point to differentiate between ALS patients and controls was 2,636.21 pg/mL (sensitivity = 0.59, specificity = 0.67).

We further tested whether these differences were still observed after stratifying ALS patients according to their phenotype bulbar or spinal (Figure S1.A in supplementary material). In a logistic regression model, sTREM2 levels in CSF remained an independent predictor of ALS status for spinal phenotype after adjusting for age and gender with an OR = 3.45 (CI95%=1.22–9.70); p-value < 0.05), but not for bulbar phenotype patients (p-value = 0.155) (Table S1 in supplementary material). AUC for CSF sTREM2 in the spinal phenotype patients was 0.667 (CI = 0.547–0.788; p < 0.05) and the optimal cutoff point to differentiate between ALS patients and controls was set at 595.95 pg/mL (sensitivity = 0.79, specificity = 0.56) (Figure S2.A).

We found no correlation between CSF or serum sTREM2 levels in training cohort and ALSFR-R (p-value = 0.679 and p-value = 0,723, respectively) neither survival (p-value = 0.546 and p-value = 0,160 respectively).

3.5. Serum sTREM2 levels in the validation cohort

In order to assess if sTREM2 could be useful as a non-invasive blood-derived biomarker, we aimed to validate sTREM2 levels in serum of an extended cohort comprised of 100 ALS patients and 100 controls. We found statistically significant higher levels of sTREM2 in the serum of ALS patients (median = 3,679 pg/mL; IQR = 2,692–4,778) when compared with controls (median = 2,811 pg/mL; IQR = 1,844–3,802) (p-value < 0.001) (Fig. 4C).

In a logistic regression model, serum sTREM2 remained independently associated with ALS status with the following OR = 3.383 (CI 95 %= 1.858–6.160) (p-value < 0.001). A forest plot is shown in Fig. 5 to illustrate the associations of sTREM2 levels in CSF and blood with ALS status. In addition, AUC for serum sTREM2 in the validation cohort was

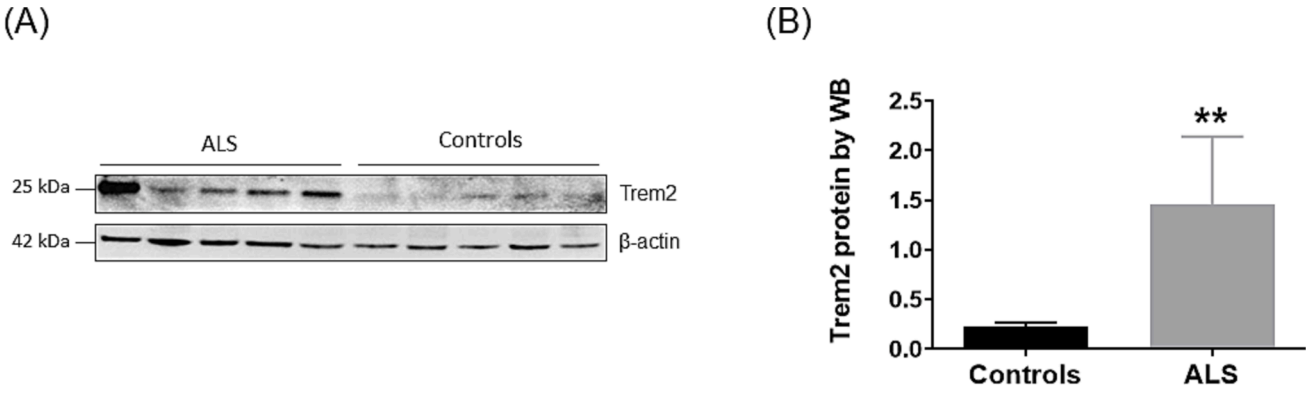

Fig. 3. TREM2 protein expression is increased in spinal cord from ALS patients by western blot analysis. (A) Spinal cord samples from controls and ALS patients were loaded as labeled on top of lanes. β-actin expression is shown as reference control. (B) The bar chart represents the quantitative measurement of TREM2 relative to β-actin protein expression (n = 10). Data represent the mean value ± Standard Error of the Mean (SEM). **p value < 0.01.

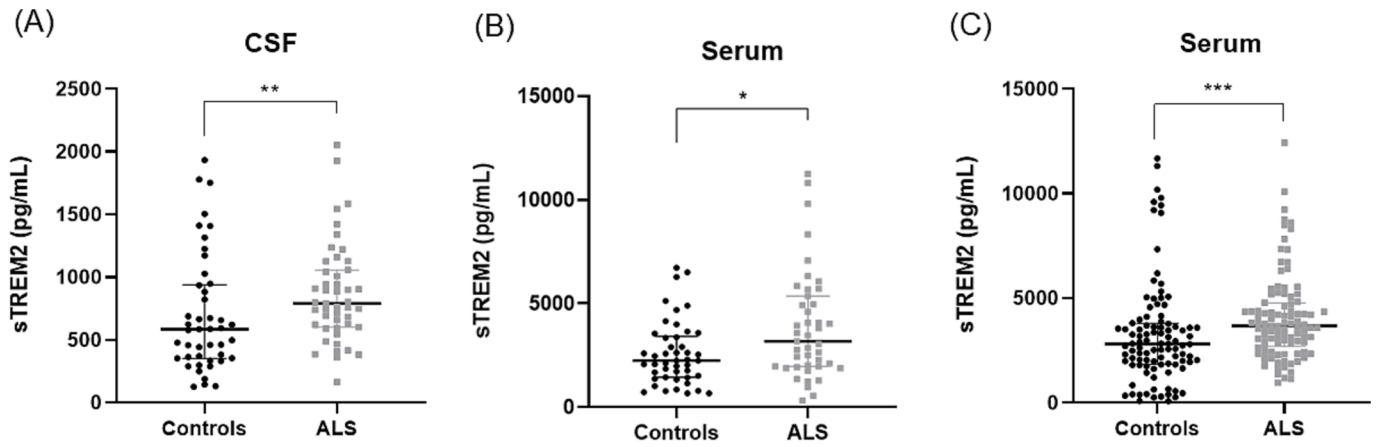

Fig. 4. Strem2 concentrations obtained in (a) csf (n = 92) and (B) serum (n = 88) from controls and ALS patients in the training cohort. sTREM2 concentrations in (C) serum from controls and ALS patients in the validation cohort (n = 200). Data are expressed as median and interquartile range. *p value < 0.05; **p value < 0.01; ***p value < 0.001.

Table 3
Adjusted logistic regression model to predict ALS status in CSF and serum from the training cohort and serum from the validation cohort.

	Variable	B	Wald	P value	OR	95 %CI lower	95 % CI upper
CSF	**sTREM2**	1.225	6.615	0.010*	3.406	1.339	8.665
	Gender (female)	0.032	2.172	0.141	1.032	0.990	1.077
	Age	0.454	0.850	0.357	1.575	0.600	4.134
	Constant	−2.738	4.539	0.033	0.065		
Serum	**sTREM2**	1.010	4.707	0.030*	2.747	1.103	6.843
	Gender (female)	0.153	0.096	0.756	1.165	0.044	3.055
	Age	0.033	2.249	0.134	1.033	0.990	1.078
	Constant	−2.590	3.945	0.047	0.075		
Serum (validation)	**sTREM2**	1.219	15.890	0.000***	3.383	1.858	6.160
	Constant	-0.751	9.591	0.002	0.472		

ALS status (control = 0; ALS = 1) was considered as the dependent variables and sTREM2 levels as covariate. Gender an age were included as covariates in the logistic regression model for the training cohort. B regression coefficient, OR odds ratio, *p value < 0.05; **p value < 0.01; ***p value < 0.001.

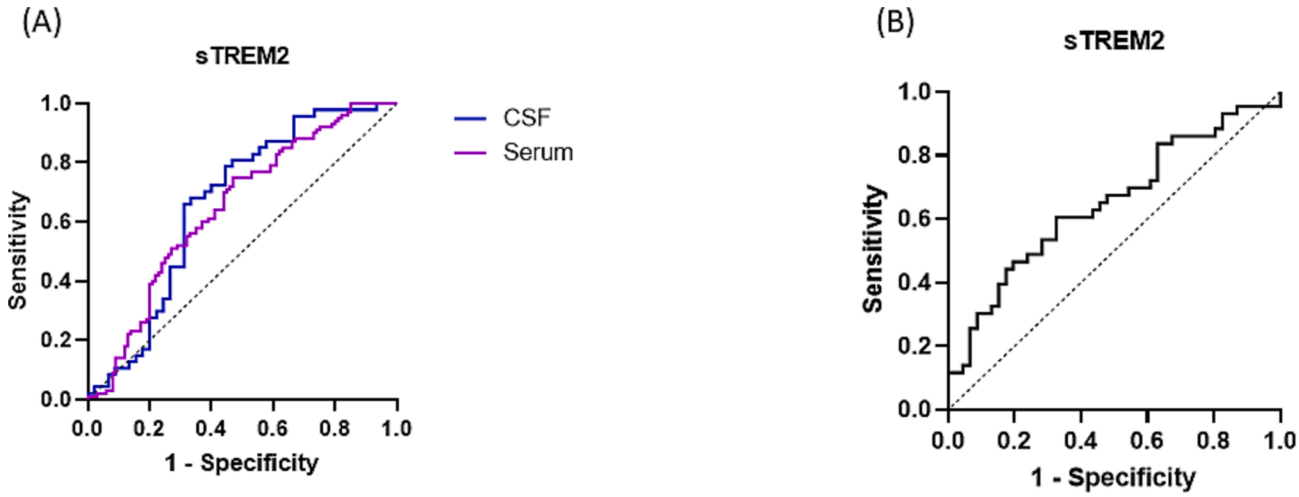

Fig. 5. (A) ROC curve analysis of CSF and serum sTREM2 in discriminating ALS patients from controls in a training cohort. (B) ROC curve analysis of serum sTREM2 in ALS patients from controls in a validation cohort.

0.648 (CI 95 %=0.571–0.724; p < 0.001) and the optimal cutoff point to differentiate between ALS patients and controls was set at 2,834.81 pg/mL (sensitivity = 0.75, specificity = 0.53) (Fig. 6B).

When performing analysis separately according to ALS phenotypes we found that sTREM2 levels were higher in both spinal and bulbar compared to controls (p-value < 0.01 for both comparisons) (Figure S1. B). Serum sTREM2 remained independently associated with ALS status in both, spinal (OR = 3.070) and bulbar (OR = 4.188) (Table S2). For spinal phenotype, the AUC was 0.633 (CI 95 %=0.549–0.717; p-value < 0.01) and the optimal cutoff point to differentiate between ALS patients and controls was 2848.19 pg/mL (sensitivity = 0.73; specificity = 0.53). For bulbar phenotype, the AUC was 0.678 (CI 95 % =0.577–0.778; p-value < 0.05) and the optimal cutoff point to differentiate between ALS patients and controls was 2834.81 pg/mL (sensitivity = 0.79, specificity

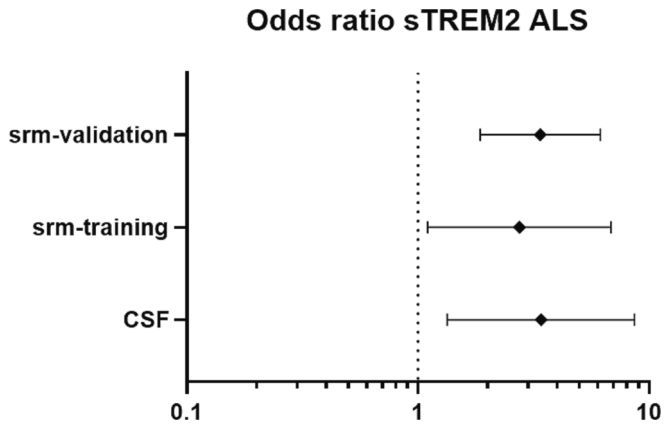

Fig. 6. Forest plot for the association of sTREM2 protein levels with ALS status, in both cohorts. *(srm: serum)*.

= 0.47). (Figure S2.B).

We found no correlation between serum sTREM2 levels in the validation cohort and ALSFR-R (p-value = 0.357) neither survival (p-value = 0.908).

3.6. Measurement of tTDP-43 and pTDP-43 levels in CSF and serum.

TDP-43 role is central to ALS pathology and a potential specific biomarker of disease. We have analyzed full-length TDP-43 (tTDP-43) and phosphorylated TDP-43 levels (pTDP-43) in CSF from the training cohort and serum from the validation cohort by ELISA. Contrary to expectations, we observed statistically significant higher levels in controls *versus* patients for tTDP-43 in both CSF and serum (Table S3). Regarding pTDP-43, we observed statistically significant higher levels in CSF from ALS patients (median = 4.39 pg/mL; IQR = 3.66–5.14) compared to controls (median = 3.81 pg/mL; IQR = 3.31–4.24; $p < 0.01$). pTDP-43 levels were undetectable in serum samples from both ALS patients and controls (Table S3).

In a logistic regression model, pTDP-43 levels in CSF remained an independent predictor of ALS status after adjusting for age and gender with an OR = 1.69 (CI95%=1.01–2.82; p-value < 0.05) (Table S4). When adding pTDP-43 levels to the sTREM2 model, the OR for sTREM2 was slightly increased (OR = 4.75; CI95%= 1.56–14.28; p-value < 0.01) (Table S5).

4. Discussion

There is increasing evidence of *TREM2* playing a crucial role in neurodegeneration, especially in AD pathology but also in ALS. These insights suggest that different neurodegenerative diseases may share common patterns of deregulation in innate immune molecular pathways, making *TREM2* an interesting candidate biomarker and therapeutic target for these diseases. The aim of this study was to characterize *TREM2* expression changes in ALS and further explore its usefulness as a candidate biomarker for ALS. Our findings suggest that changes in *TREM2* gene expression are consistent across all known TREM2 transcripts and extent to the protein level in the spinal cord of ALS patients. In addition, we have observed a significant rise of CSF and serum sTREM2 protein levels in ALS, possibly as a biomarker of inflammatory activity.

First, we have observed a significant upregulation of *TREM2* (both mRNA expression and protein level) in the spinal cord from ALS patients comparing with non-neurodegenerative control donors. *TREM2*-dependent microglial function appears to play a protective role in neurodegenerative disorders (Ulland and Colonna, 2018; Deczkowska et al., 2018; Angel et al., 2020; Schlepckow et al., 2020) so that upregulation in *postmortem* spinal cord could be interpreted as a need for elevated levels of *TREM2* in order to perform its function of phagocytosis or end-stage debris removal or, alternatively, could suggest a dysregulation of protective *TREM2* DAM signaling. Furthermore, we have not observed a correlation between *TREM2* mRNA expression levels and an estimation of the number of activated microglia and/or infiltrating peripheral macrophages in the ALS spinal cord samples, quantified by percentage of the area immunostained by Iba1 and CD68. This result would support, as do other studies,(Dols-Icardo et al., 2020) that *TREM2* marks a microglial functional state and is part of the transcriptional signature that defines activated microglia in ALS (DAM phenotype), making it a potential therapeutic target.

We also wanted to quantify the expression levels of the three *TREM2* transcripts in ALS vs control spinal cord, to determine whether there was a specific-transcript *TREM2* expression profile. Differential expression levels of these *TREM2* isoforms have been analysed in Alzheimer's disease (AD) and progressive supranuclear palsy (PSP) but, to the best of our knowledge, not yet in ALS.

We thus found a significant overexpression of the three *TREM2* transcripts in the ALS group, especially for *TREM*-201 that encode the soluble protein (sTREM2) and the canonical transcript (*TREM*-202). These results show a similar pattern to that previously reported in AD brain cortex (Jay et al., 2017; Del-Aguila et al., 2023; Chih Jin et al., 2023) and in PSP substantia nigra (De et al., 2020) suggesting that the expression pattern of full length and spliced isoforms of *TREM2* may be shared by several neurodegenerative disorders. Importantly, gene expression changes in spinal cord extended to the protein level, so we next investigated the potential of sTREM2 protein levels measurement as ALS biomarker in biological fluids such as CSF or blood. Our results showed a statistically significant elevation of CSF sTREM2 levels in ALS patients vs controls. This association remained statistically significant after adjusting for age and gender in a logistic regression model for the spinal phenotype. The lack of association with the bulbar phenotype could be explained by the low representativeness of this phenotype in the sample studied. Previous studies have shown increased levels of sTREM2 in the CSF of AD patients, especially in the early stages of the disease (Suárez-Calvet et al., 2023; Domingo Gispert et al., 2023; Henjum et al., 2016; Piccio et al., 2016). Regarding ALS, significant elevated levels of sTREM2 in CSF have also been previously described, which seems to predict survival when measured in late stages of disease (Cooper-Knock et al., 2023).

CSF is the logical source to study biomarkers in neurodegenerative diseases due to their close interaction with the CNS; however, its collection by lumbar puncture is a relatively invasive diagnostic method and not strictly necessary or readily available for clinical use in ALS. For this reason, we focused our interest on the potential usefulness of sTREM2 levels measure as blood biomarker. Evaluation of sTREM2 in blood has been performed in other neurodegenerative diseases, for instance it has been observed elevated blood sTREM2 levels and upregulation of *TREM2* mRNA and protein levels in peripheral blood of AD subjects (Kleinberger et al., 2023; Hu et al., 2014; Bekris et al., 2018; Mori et al., 2015; Ashton et al., 2023).

However, to our knowledge, there are no previous studies that support the usefulness of sTREM2 measurement in blood as a non-invasive biomarker in ALS.

We have also observed a statistically significant elevation of sTREM2 in the serum of ALS patients compared to controls. These results were validated in an extended cohort of patients and controls (validation cohort). Thus, serum sTREM2 levels above 2,834.81 pg/mL were independently associated with ALS status in a logistic regression model, for the total sample and for both phenotypes (bulbar and spinal). On the whole, the predictive capacity of both CSF and serum TREM2 levels were moderate. Therefore, we can conclude that sTREM2 may be a biomarker of inflammatory activity in ALS instead of a diagnostic biomarker. sTREM2 predictive role in ALS would be probably improved in association with other blood biomarkers reflecting immune alterations, neurodegeneration or other deregulated pathways in the disease,

for which further studies are needed. In this sense, we wanted to explore whether the predictive value of sTREM2 levels in fluids was modified in a combined analysis with TDP-43 levels, although the pathological role of sTREM2 in TDP-43-mediated diseases remains unknown. Despite cytosolic neuronal inclusions of TDP-43 are considered one of the main pathological markers of ALS, its determination in CSF and blood has shown a limited value as a diagnostic tool, showing high variability in the results of different studies (Feneberg et al., 2021; Ren et al., 2021; Molinuevo et al., 1932). Our results revealed that pTDP43 levels in CSF is an independent predictor of ALS and increased the predictive value of sTREM2 in a combined logistic regression model. However, it is necessary to explore the correlation between these two biological aspects of ALS disease, and validate our experimental observations regarding TDP-43 results in other cohorts.

We would also like to emphasize that *TREM2* is also expressed in peripheral immune cells and there is a growing evidence that systemic inflammation has a significant role to play in progression of neurodegenerative diseases (Cunningham, 2023) and on the peripheral monocyte-macrophage infiltration of CNS during neurodegeneration (Chiot et al., 2020). Even, peripheral immune cells could have a central role as drivers of inflammation in ALS onset and progression and blocking the immune cells infiltration in CNS could be a therapeutic target (Garofalo et al., 2022). It seems that during neuroinflammation CNS-infiltrating macrophages, but not peripheral myeloid cells, acquire microglial markers, indicating that the CNS environment in neurodegeneration could have an impact on phenotypic plasticity of the myeloid cells (Grassivaro et al., 2020).

We thus hypothesize that the increase in blood sTREM2 levels could also be a reflection of peripheral inflammatory cellular activity in ALS patients. Further studies will be needed to determine whether this peripheral inflammation would be reactive to primary CNS inflammation, i.e., a sort of recruitment call to enhance phagocytic capacity in the neurodegeneration niche for subsequent phenotypic reconversion (Grassivaro et al., 2020), or whether it could actually mark a primary or parallel systemic inflammation with secondary CNS infiltration. In this regard, several studies advocating a persistent inflammatory state in the gut mucosa and its possible contribution to diseases such as AD, PD, ALS and multiple sclerosis (MS) are noteworthy as well as the hypothesis that endotoxin causes or contributes to neurodegeneration (Kowalski and Mulak, 2019; Quigley, 1910; Brown, 2019). It is remarkable that there is increasing evidence that ALS pathology involves extra-neuronal biological systems(Zufiría et al., 2016) and the list of environmental factors associated with ALS is growing (Riancho et al., 2018).

In any case, our results may suggest that blood sTREM2 levels in ALS could reflect an inflammatory state and may be useful as biomarker in future therapeutic interventions against neuroinflammation, despite its role is not completely understood.

5. Limitations

Probably-one of the limitations of our study is the absence of serial measurements of sTREM2 in blood, to assess whether sTREM2 levels vary throughout the course of the disease, which could highlight the influence of inflammatory activity at different stages of the disease as well as assess its impact as a prognostic biomarker.

6. Conclusion

In conclusion, our results support the role of *TREM2* in ALS pathophysiology and demonstrates that the three *TREM2* transcripts are deregulated in ALS in *postmortem* human specimens of spinal cord. We still need to further investigate its correlation with specific markers of microglia *versus* CNS-infiltrating blood-monocyte-derived peripheral macrophages. The results open up a new line of research on the contribution of systemic-peripheral inflammation to the overexpression of sTREM2 in blood. We describe for the first time a significant elevation of sTREM2 levels in peripheral blood in ALS, so sTREM2 levels emerge as a potential non-invasive biomarker for inflammation in this disease.

7. Ethical approvals and consent to participate

The Navarra and Sant Pau Ethics Research Committees approved this study (project 61/2014 in Navarra and project 20/016 in Sant Pau hospital) and written informed consent was obtained from all subjects, or next of kin before brain donation. Procedures were in accordance with Helsinki Declaration of 1975 as revised in 2000.

8. Availability of data and materials

Anonymized data are available upon reasonable request.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbi.2023.01.013.

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