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Whole and fractionated human platelet lysate biomaterials-based biotherapy induces strong neuroprotection in experimental models of amyotrophic lateral sclerosis

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease of motor neurons leading to death within 3 years and without a curative treatment. Neurotrophic growth factors (NTFs) are pivotal for cell survival. A reason for the lack of patient efficacy with single recombinant NTF brain infusion is likely to be due to the synergistic neuroprotective action of multiple NTFs on a diverse set of signaling pathways. Fractionated (protein size <50, <30, <10, <3 kDa) heat-treated human platelet lysate (HHPL) preparations were adapted for use in brain tissue with the aim of demonstrating therapeutic value in ALS models and further elucidation of the mechanisms of action. In neuronal culture all fractions induced Akt-dependent neuroprotection as well as a strong anti-apoptotic and anti-ferroptotic action. In the <3 kDa fraction anti-ferroptotic properties were shown to be GPX4 dependent highlighting a role for other platelet elements associated with NTFs. In the $SOD1^{G86R}$ mouse model, lifespan was strongly increased by intracerebroventricular delivery of HHPL or by intranasal administration of <3 kDa fraction. Our results suggest that the platelet lysate biomaterials are neuroprotective in ALS. Further studies would now validate theragnostic biomarker on its antiferroptotic action, for further clinical development.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is characterized by the degeneration of upper and lower motor neurons in the cerebral cortex, brainstem and spinal cord leading to a progressive, irreversible muscle paralysis. Death eventually occurs 3–5 years after diagnosis due to respiratory failure [1]. In most cases, ALS is sporadic with a multifactorial etiology modulated by both environment and genetic factors. In approximately 10% of ALS patients, a clear family history is present, and mutations identified since the 90's in more than 30 genes are known to be causative [2]. Mutations in proteins implicated in RNA metabolism and protein homeostasis increase the susceptibility of aggregated inclusions in the nucleus and cytoplasm of neurons [3]. Discovery of mutations in the gene *SOD1* [4] led to the generation of the first transgenic mouse models of ALS that overexpress various mutant forms of human SOD1 and their common use in ALS research has led to their motor dysfunction being well characterized.

An increasing number of publications suggest that ferroptosis may play a role in motor neuron death and thus have implications in ALS. Ferroptosis is a form of programmed cell death dependent on intracellular labile iron and characterized by the accumulation of lipid peroxides [5,6]. In ALS, increased levels of a biofluid marker of iron

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dyshomeostasis ferritin [7–10] is associated with shorter survival time [11–13] and central neuronal iron accumulation is associated with neurodegeneration in both ALS patients [14–18] and mouse models of ALS [19]. ALS patients also have enhanced levels of lipid peroxides in CSF and sera, as well as decreased GSH levels in motor cortex; all of which are consistent with ferroptosis [20–22]. Finally, spinal motor neurons appear to be particularly vulnerable to ferroptosis whereby the ablation of Gpx4 (a key player in inhibition of ferroptosis) in a mouse model recapitulate the cardinal features of ALS including rapid paralysis, severe muscle atrophy and motor neuron death [23].

To date, there is no curative treatment for ALS. Riluzole, as the only approved drug for the disease, induces only a modest effect on prolonging life in patients. Therefore, the development of new and effective treatments is of high priority. A range of neuroprotective and/or neuroregenerative approaches have been proposed for ALS patients, one of these axes being the exploration of neurotrophic growth factors (NTFs) as a biotherapy. NTFs regulate several physiological processes in the nervous system, including differentiation, cell survival, axonal outgrowth and synaptic function [24]. Based on promising results in animal models, clinical trials have been carried out on ALS patients through different strategies (reviewed in Ref. [25]). First the systemic injection of single recombinant NTF (rNTF) showed limited benefits and are likely to arise from several issues, most notably the delivery route and the requirement for rNTFs to cross the blood-brain-barrier (BBB) efficiently. Moreover, the lack of clinical efficacy with single NTF infusion may be due to an inability of one single NTF alone to induce the complex set of signaling pathways required to promote efficient neuroprotection or to limit its neuroprotective feature to the sub-population of responsive neurons [25]. More recently clinical trials using stem cell therapy were conducted based on their NTFs-rich secretome, but they showed limited efficacy due to the rapid cell destruction by the hostile environment and the limited capacity of transplanted cells to migrate to the area of interest [25]. In contrast, platelets constitute a natural source of physiologically balanced growth factors and have been a source biomaterial for regenerative medicine in tissue repair and wound healing [26,27]. We hypothesize that platelet NTFs (pNTFs), through their potent pleiotropic action, could have similar beneficial effect in ALS.

A tailor-made human platelet lysate (HPL) preparation, named Human Platelet Pellet Lysate (HPPL), was engineered for brain administration through removal of plasma and heat-treatment to deplete it of platelet fibrinogen, as well as thrombogenic and proteolytic factors [28]. Proteomics characterization of HPPL recently identified over 1000 proteins including NTFs, cytokines, anti-oxidative agents and anti-inflammatory factors. Moreover a GO-term analysis revealed the involvement of HPPL proteins in several biological processes, showing that platelet lysate composition complexity could exert synergistic functional and neuroprotective activities [29,30]. Moreover HPPL has been shown by us and others to protect against a toxic insult in cultured dopaminergic neurons as well as reduce dopaminergic neurodegeneration in a Parkinsonian mouse model that uses the neurotoxin MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) [28,31–33]. In cell-lines used to model Parkinson's disease (PD) and ALS (LUHMES and NSC-34 cell lines, respectively) HPPL has marked neuroprotective capabilities higher than pNTFs alone or combined, and this has been proposed to be mediated through impairing select cell death pathways (e.g. apoptosis and ferroptosis) as well as activating specific survival pathways (Akt and MEK) [34].

In the current work we aimed to evaluate the biotherapeutic potential of heat-treated human platelet lysate (HHPL) in ALS, and the role of pNTFs in this protection. To address this issue, various independent sub-fractions separated based on their molecular mass (<50, <30, <10 and < 3 kDa) were produced to remove pNTFs from HHPL. Our different products were tested in neurons cultures (i.e the non-oncogenic and differentiated human dopaminergic neuron cell line LUHMES for preliminary assessment, and primary motor neurons with defined conditions) and in the severe ALS $SOD1^{G86R}$ mice model. Results in LUHMES showed that all the fractions were neuroprotective against the ferroptotic inducer, with an Akt-dependent (like HHPL) and GPX4-dependent (unlike HHPL) effect of <3 kDa fraction. A deeper investigation into the protective effect of the <3 kDa fraction showed that it was less efficient than HHPL against apoptotic and pro-oxidative drugs in primary motor neurons and LUHMES cells. In the $SOD1^{G86R}$ mice model of ALS, treatment with HHPL through intracerebroventricular delivery increased lifespan and this was replicated when using only the <3 kDa fraction intranasally administered. Thus, our results suggest for the first time the high potential of neuroprotection of specific platelet lysate biomaterials in ALS biotherapy.

2. Material and methods

2.1. HHPL and fractions

2.1.1. Preparation of heat-treated human platelet lysate (HHPL) and fractions

The HPL ("MultiPL'30" containing 30% plasma and 70% platelet additive solution) was obtained from Macopharma (Mouvaux, France). It was obtained by pooling and processing platelet concentrates, obtained from whole blood collected from 240 volunteer non-remunerated donors by Etablissement Français du Sang (Lille, France) and stored at −80 °C. After thawing at 37 °C for 20 min, the lysate was incubated with 23 mM $CaCl_2$ and glass beads under gentle shaking for 2 h at room temperature [35]. This step allowed the conversion of fibrinogen into fibrin as well as activation of intact platelets and release of the content. The clot was removed by centrifugation (4600 g, 15 min, 22 °C). Then the platelet lysate supernatant was heated for 30 min at 56 °C, immediately cooled down to 22 °C, and centrifuged (1000 g, 15 min, 22 °C) to obtain the Heat-treated Human Platelet Lysate (HHPL). Fractionation of the HHPL into <50 kDa, <30 kDa, <10 kDa and <3 kDa sub-fractions was performed independently using Amicon® Ultra centrifugal filters (Merck®, Darmstadt, Germany) according to the manufacturer instructions, and the respective filtrates were aliquoted. HHPL and fractions were stored at −80 °C.

2.1.2. HHPL and fractions characterization

From at least three independent preparations of HHPL and the different fractions total protein content was determined by colorimetric detection of BCA assay kit (ThermoFisher Scientific, Waltham MA, USA) and quantification of cytokine, chemokine, and growth factors contents was performed by two Luminex Human Discovery Assays (R1D systems, Abingdon, UK) with a Luminex MAGPIX® system (Luminex, MV's-Hertogenbosch, The Netherlands). Cytokine, chemokine and growth factor levels were then normalized to total protein content for each sample.

2.2. LUHMES culture

2.2.1. Cell maintenance and differentiation

Lund human mesencephalic (LUHMES) cells were obtained from Pr. Leist (University of Konstanz, Germany) and cultured as described in Ref. [36]. Briefly, cells were maintained in proliferation medium (Advanced DMEM/F12, 1 × N_2 supplement, 2 mM L-glutamine and 40 ng/ml recombinant bFGF) in Nunclon™ cell culture flasks pre-coated with 50 μg/ml poly-L-ornithine and 1 μg/ml fibronectin, and were sub-cultured every 2–3 days. For differentiation, LUHMES cells were grown for 2 days in differentiation medium (advanced DMEM/F12, 1 × N_2 supplement, 2 mM L-glutamine, 1 mM cAMP, 1 μg/ml tetracycline and 2 ng/ml recombinant GDNF), before seeding in 96-well plate at 4 * 10^4 cells per well for an additional 3 days.

2.2.2. Cell treatment and viability assay

After 5 days of differentiation, LUHMES cells were treated with HHPL or fractions (5% vol/vol (v/v)) 1 h prior to a 24 h exposure to

1.25 μM erastin (Euromedex, Souffelweyersheim, France), 2 μM staurosporine (STS) or 5 μM menadione (Sigma Aldrich, St Louis MO, USA). The Akt inhibitor MK-2206 was added at 5 μM (Sigma Aldrich, St Louis MO, USA) 1 h before HHPL/<3 kDa treatment. Viability was measured by resazurin assay (Sigma Aldrich, St Louis MO, USA). The resazurin solution was added to the 96-well plate at a final concentration of 0.1 mg/ml. After 2 h incubation at 37 °C, the fluorescence signal was measured at the excitation/emission wavelength of 540/600 nm using a Mithras microplate reader. Fluorescence values were normalized to the percentage of untreated cells.

2.2.3. siRNA transfection

For transfection, solution A (0.2 μl RNAiMAX lipofectamine and 5 μl Opti-MEM™ per well (ThermoFisher Scientific, Waltham MA, USA)) and solution B (2 pmol of siRNA (Santa Cruz Biotechnology, Dallas TX, USA) and 5 μl Opti-MEM™ per well) were prepared. After 5 min incubation, both solutions were combined, incubated for 15 min and then transferred to 96-well plates. LUHMES differentiated for 2 days were then added in a volume of 90 μl differentiation medium at the density of 5 * 10^4 cells per well. After day 5 of differentiation, medium was exchanged and cells treated with compounds of interest for 24 h. The viability was evaluated by resazurin assay as previously described.

2.3. Primary motoneuron culture

2.3.1. Motoneuron isolation

Motoneuron cultures were purified from E12.5 spinal cord of *Hb9::GFP* or C57BL/6 embryos and isolated as described previously [37] using 5.2% iodixanol density gradient centrifugation combined with p75-based magnetic cell isolation (clone MLR2 of p75 monoclonal antibody (Millipore) and microbead-conjugated goat anti mouse IgG (Miltenyi Biotec)) [38]. Motoneurons were plated on poly-ornithine (3 μg/ml) and laminin (2 μg/ml) treated wells in the presence of a cocktail of neurotrophic factors (0.1 ng/ml glial-derived neurotrophic factor (GDNF), 1 ng/ml brain-derived neurotrophic factor (BDNF) and 10 ng/ml ciliary neurotrophic factor (CNTF)) in supplemented neurobasal medium, at a density of 2500 cells per well.

2.3.2. Cell treatment and survival assay

Motoneurons were treated day 1 (with erastin, STS or menadione) or 7 (glutamate) after seeding with HHPL or <3 kDa at 2.5% v/v, 1 h before drug exposure (10 μM erastin or 100 μM glutamate for 48 h, 1 μM STS or 4 μM menadione for 24 h). The number of surviving neurons was counted under fluorescence (*Hb9::GFP*) or light microscope using morphological criteria in 2 diameters of each well. To compare values from different experiments, survival values were expressed relative to the percentage of the control (neurotrophic factors only).

2.4. Animal experiments

2.4.1. Mice

All experiments were carried out in accordance with the Directive 2010/63/EU of the European Parliament and of the council on the protection of animals used for scientific, approved by the French regulatory framework on animal experimentation (CEEA75, APAFIS#2018022216546386) and followed the European ALS group's preclinical trial guidelines of 2010 [39]. FVB-Tg(Sod1*G86R)M1Jwg/J mice (JAX laboratories, Bar Harbor ME, USA) were group-housed (10 per cage) in a temperature-controlled room (22 ± 2 °C) with a 12/12-h light/dark cycle with food and water *ad-libitum*. Treatments were carried out from pre-symptomatic 75 day old mice until their death. In this model, the first symptoms of minor motor disturbances and weight loss are detected at around 90 days old. All experiments were performed in only males with littermate controls and all analyses were blinded.

2.4.2. Riluzole drug administration

Riluzole (Sigma Aldrich, St Louis MO, USA) was mixed in a defined diet and formulated in pellets. From 75 days of age, it was administrated at 44 mg/kg/day until death [40].

2.4.3. Intracerebroventricular (I.C.V) injection and intranasal administration (I.N)

Mice were anaesthetized using 5% of isoflurane along with oxygen (2L/min) at 60 days of age. Canula implantation was performed in the right lateral ventricle on mice placed in the stereotactic frame and kept under isoflurane anesthesia (1.5%) using a facemask throughout the surgery and continually monitoring body temperature. Mice were acclimated 1 week before the beginning of treatment. At the age of 75 days, mice received 4 μl of HHPL or vehicle at the rate of 0.5 μl/min by intermittent ICV, three times a week until death. For I.N administration, mice were treated with 20 μl of <3 kDa fraction or vehicle twice a day, and three times a week, from 75 days until death.

2.4.4. RNA extraction and RT-qPCR for acetylcholine receptor alpha (AchRα)

RNA was extracted with 'Extract-all' (ThermoFisher Scientific, Waltham MA, USA) from muscles of 100 day old mice and a reverse transcription (RT) reaction was performed from 1 μg total RNA using SuperScript® III Reverse Transcriptase. qPCR was performed using a LightCycler system with the LightCycler® FastStart DNA Master SYBR Green kit (Roche Applied Science, Penzberg, Germany). The threshold cycles (Ct) were determined for AchRα, and gene expression levels were calculated relative to the control gene TBP. Relative quantification analyses were performed by the LightCycler Software (Roche Applied Science, Penzberg, Germany).

2.4.5. Neurofilament light chain (NfL) quantification

NfL levels were determined using the R-PLEX Human Neurofilament L with a MESO QuickPlex SQ 120 instrument (Meso Scale Diagnostics, Rockville MD, USA) in sera from 100 day old mice I.N treated with vehicle or 3 kDa fraction.

2.5. Statistical analysis

In vitro experiments were performed at least three times independently and presented as means ± SEM. Where appropriate, the one-way or two-way analysis of variance (ANOVA) was performed with Tukey (one-way ANOVA) or Bonferroni (two-way ANOVA) post hoc test. For survival analysis, a log-rank test (Mantel-Cox) was used. qPCR and NfL quantification was by kruskal-Wallis testing and values compared to WT treated with vehicle (WT-veh) mice. A p value < 0.05 was considered significant.

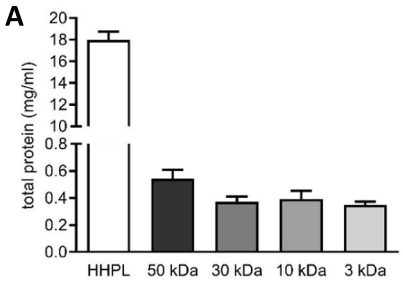
3. Results

3.1. Fractions of HHPL protect dopaminergic neurons against cell death

One axis of therapeutic research in neurodegenerative diseases (NDD) is the delivery of neurotrophic factors (NTFs) due to their properties in different neuronal processes. However the limited results obtained in clinical trials, especially in ALS, highlight the need of a synergic association of several NTFs to be more efficient [25]. We previously demonstrated that a similar type of HPL (HPPL) also heat-treated and fibrinogen-depleted but plasma-depleted and with lower total protein content [28,32], had higher protection compared to platelet recombinant NTFs in cell-based models of neurodegenerative diseases including human differentiated dopaminergic neurons (LUHMES) [28,34]. To better define the neuroprotective role of platelet NTFs in HHPL, fractions were prepared by centrifugal filtration to remove them based on a size-exclusion mechanism. Initial characterizations of growth factors and chemokines as well as other biochemical analysis were

performed on HPL [41] and HPPL [29,30]. In this study, to test our hypothesis, we focused the characterization of HHPL and its fractions on growth factors, cytokines and chemokines. As expected, total protein content was substantially altered after size fractionation with concentrations of ~18 g/L for HHPL, being reduced to ~0.54 g/L for <50 kDa and ~0.35 g/L for <10 kDa and <3 kDa (Fig. 1A). In accordance with the expected mass of the NTFs we observed a gradual partitioning of these proteins in each of the fractions. However, the absence of 30–40 kDa proteins (e.g. VEGF (Vascular Endothelial Growth Factor) or PDGF-BB (Platelet Derived Growth Factor-BB)) in <50 kDa fraction and 14–20 kDa proteins (e.g. IL6 (Interleukin-6) and BDNF (Brain Derived Neurotrophic Factor)) in <30 kDa may have been due to post-transcriptional modifications or their binding to other proteins in HHPL (Fig. 1A). A further observation was that traces of some proteins (e.g. PDGF-AA (Platelet Derived Growth Factor-AA), PF4 (Platelet Factor 4), EGF (Epidermal Growth Factor) or IL8 (Interleukin-8)) were still observed through to the <3 kDa fraction indicating that some proteins could have been degraded into smaller fragments or that the molecular weight cut off for the filters were not accurate.

We next tested the protection of HHPL and the four fractions on neurons cultures against a range of cell death insults. However, few *in vitro* study models of ALS (i.e non oncogenic and mature neurons culture) are available and primary motor neurons cultures may not be adapted for preliminary screening of neuroprotective properties of our products. Therefore we firstly test HHPL and the fractions on the non-oncogenic and differentiated human dopaminergic neuron cell line LUHMES, which was already used in our previous studies. LUHMES are particularly sensitive to ferroptosis, a specific iron dependent regulated



Molecular weight (kDa)	pg/mg of total protein	HHPL	under 50 kDa	under 30 kDa	under 10 kDa	under 3 kDa
60-70	**HGF**	1.2±0,2	ND	ND	ND	ND
44	**VEGF**	23.4±1,5	ND	ND	ND	ND
30.4	**GDNF**	0.2±0,03	ND	ND	ND	ND
29/14	**PDGF-AA**	399.3±68.7	136±79.6	10.8±7.7	62.4±31.3	8,8±6,6
28/12.3	**PDGF-BB**	485.4±105.8	62.6±48	ND	37.0±7.8	ND
20.9	**IL-6**	0.12±0,01	0.45±0.01	ND	ND	ND
18	**basic FGF**	ND	ND	ND	ND	ND
17.5	**TNF-a**	0.1±0,01	ND	ND	ND	ND
17	**acidic FGF**	ND	ND	ND	ND	ND
17	**IL1b**	0.2±0.02	ND	ND	ND	ND
14	**BDNF**	74.7±11.7	112.5±8.5	ND	ND	ND
8	**PF4/CXCL4**	13625±1138.9	10638.6±1962	7122.6±1188.1	6033.9±1964.9	9493.8±2726.7
8	**IL8/CXCL8**	44.2±38.3	47.4±11.8	13.3±2.8	33.5±23.4	83.0±51.4
6	**EGF**	202.5±12.5	739.5±133.8	191±90.7	68.2±38.1	206.8±83.2

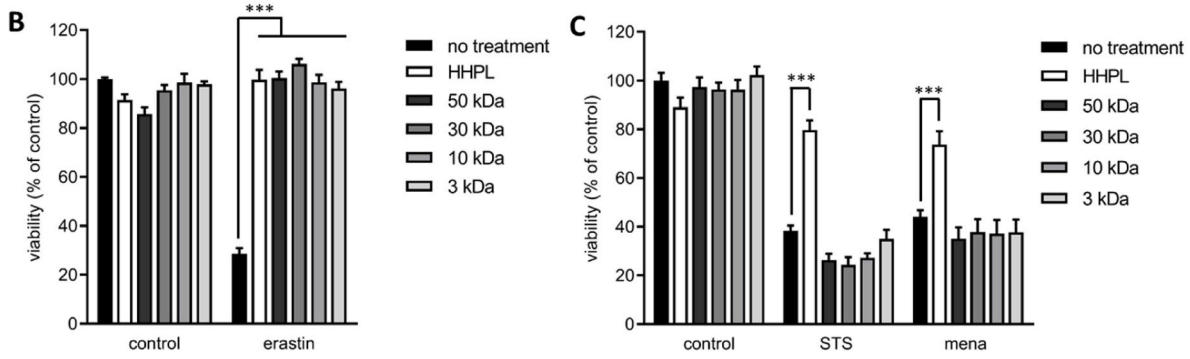


Fig. 1. Different fractions of HHPL protect LUHMES from cell death. (A) Concentrations of total protein and specific growth factors and cytokines were determined in HHPL and fractions <50 kDa, <30 kDa, <10 kDa and <3 kDa. (**B**) LUHMES were treated with HHPL or each fraction (5% v/v) 1 h before addition of erastin (1.25 μM) for further 24 h. (**C**) LUHMES were treated with HHPL or fractions (5% v/v) 1 h before addition of a pro-apoptotic drug (STS: staurosporine, 2 μM) or pro-oxidative drug (mena: menadione; 5 μM) for further 24 h. In B & C, cell viability was measured by resazurin assay. Values are means ± SEM from at least three independent experiments (***p < 0.001 two-way ANOVA with Bonferroni *post hoc* test). ND, not detectable.

cell death pathway, which can be specifically induced by the drug erastin [42]. Treatment with all fractions was comparable to HHPL in protecting cells from ferroptosis induced by erastin (Fig. 1B), despite the <3 kDa fraction containing almost no NTFs (Fig. 1A).

To determine the specificity of neuroprotection conferred by the different fractions, we compared the effect of HHPL and the different fractions against the canonical pro-apoptotic pathway induced by staurosporine (STS) and oxidative stress induced by menadione (Fig. 1C). HHPL was found to significantly protect LUHMES against cell death induced by STS or menadione. However, in contrast to the protective capacity of all fractions to erastin induced ferroptosis none were able to protect against an apoptotic stimulus or oxidative stress. These results indicated that the protection profile afforded by the different fractions differ according to the cell death mechanism and that while other forms of insult require components in HHPL greater than 50 kDa, ferroptosis can be blocked by products present in the <3 kDa fraction. As all the fractions provide similar neuroprotective properties, we next focused on comparing the smallest fraction and least concentrated in NTFs (i.e <3 kDa) to HHPL.

3.2. *Neuroprotective pathways elicited by HHPL and the <3 kDa fraction against ferroptosis differentially rely on GPX4*

We previously studied the involvement of the main signaling pathways involved in HPPL protection against different neurotoxins and showed that HPPL prevent ferroptosis predominantly through activating Akt [34]. When the <3 kDa fraction was used in the same conditions, we similarly observed that Akt inhibition blocked the protective capacity of this fraction against erastin-induced ferroptosis (Fig. 2A). Moreover, due to the pivotal role of GPX4 in protection against lipid peroxidation and subsequent ferroptosis [43], we tested whether the protection afforded by these two preparations was GPX4-dependent. GPX4 knockdown by siRNA in LUHMES was first confirmed by q-PCR (Fig. 2B). Intriguingly, HHPL protection appeared to be independent of GPX4 expression as knockdown of GPX4 in these cells had no effect, whereas the protection provided by <3 kDa on erastin induced ferroptosis did require GPX4 in these cells (Fig. 2C). This suggests that NTFs present in HHPL protect against ferroptosis through a GPX4 independent pathway whereas product within the <3 kDa fraction protect against ferroptosis through both an Akt and GPX4 dependent pathway.

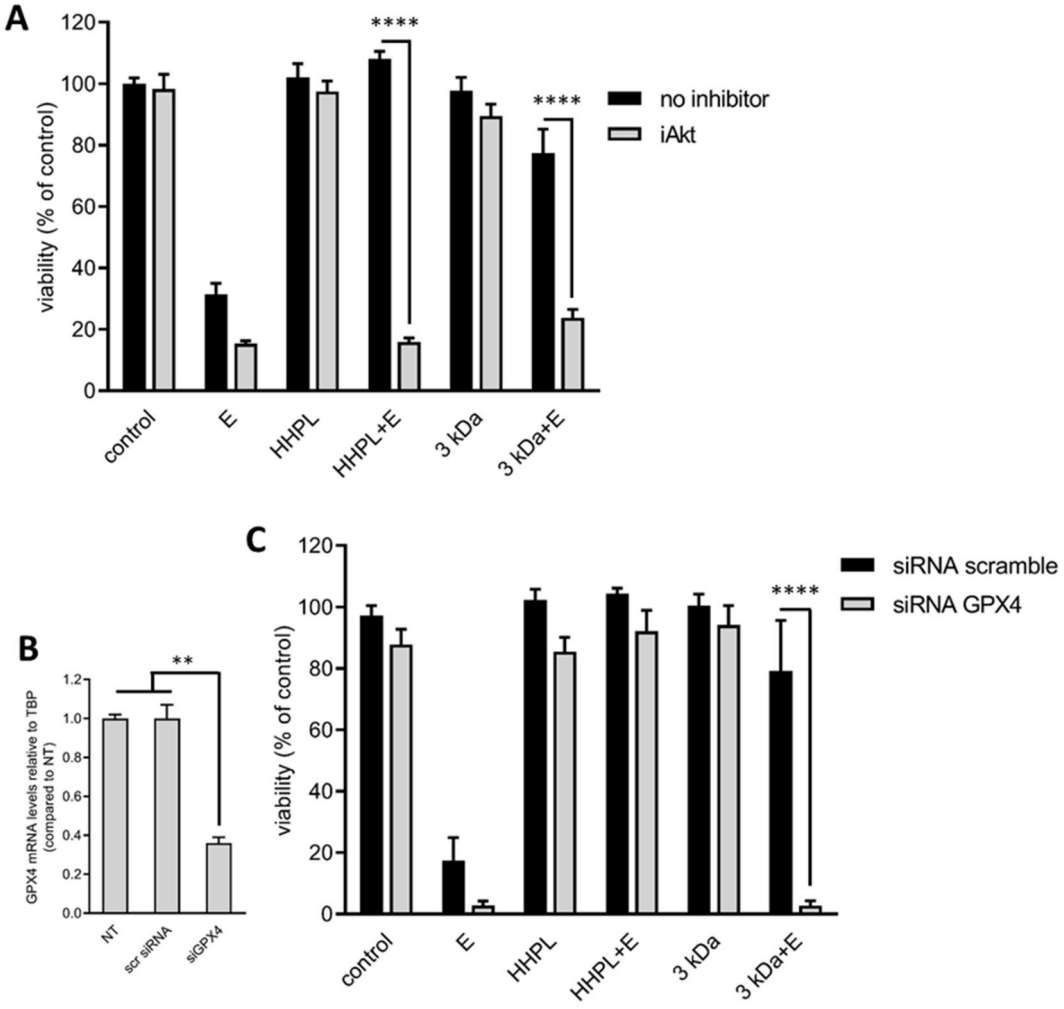


Fig. 2. HHPL and the <3 kDa fraction differently protect LUHMES cells from ferroptosis. (A) LUHMES were treated with HHPL or the <3 kDa fraction for 1 h before addition of erastin (E) for a further 24 h. The Akt inhibitor MK-2206 was added 1 h before treatments with HHPL or the <3 kDa fraction. (**B**) Knockdown of GPX4 was confirmed by qPCR and normalized to non-treated (NT) cells. (**C**) siRNA against GPX4 or a scramble sequence was added 3 days before treatment with HHPL or the <3 kDa fraction. In A & C, cell viability was measured by resazurin assay. Values are the mean ± SEM from at least three experiments (**p < 0.01 and ***p < 0,001 two-way ANOVA with Bonferroni *post hoc* test and one way ANOVA with Tukey *post hoc* test).

3.3. HHPL but not the <3 kDa fraction protects primary motor neurons against excitotoxic, apoptotic and oxidative insults

Our previous work has reported the protective capacity of HPPL in both dopaminergic neuronal LUHMES and the differentiated motoneuron-like cell line NSC-34 [34]. Considering the results obtained in LUHMES, we sought to test whether our two products of interest HHPL and the <3 kDa fraction could protect against ferroptosis, apoptosis, general oxidative stress and glutamate toxicity in primary motor neurons as an appropriate study model of ALS. The latter was added as glutamate-induced excitotoxicity plays an important role in motor neuron death in ALS [44,45]. First, we confirmed that at any dose (0.5%–5% v/v; data not shown) neither HHPL nor the <3 kDa fraction impaired primary motor neuron viability alone. The intermediary dose of 2.5% v/v was then selected for further protective study.

Interestingly, motor neurons were significantly less sensitive to erastin induced ferroptosis (approximately 30% of death) (Fig. 3A) than dopaminergic neurons (Figs. 1B and 2). Despite this reduced level of insult HHPL and <3 kDa fraction had no protective effect (Fig. 3A), contrary to what was observed on LUHMES (Fig. 1B). A similar level of toxicity was observed with glutamate (approximately 30% of motor neuron death) and only upon HPPL treatment, but not the 3 kDa fraction, was there a significant protection (Fig. 3B). Primary motor neurons were more sensitive to STS and menadione (approximately 60% of death) and again only HHPL treatment significantly protected motor neurons from these insults (Fig. 3C and D). Therefore, HHPL confers neuroprotective effect against both apoptosis and oxidative stress but not from erastin induced ferroptosis in primary motor neurons.

3.4. Chronic intracerebroventricular (I.C.V) delivery of HHPL increases the lifespan of $SOD1^{G86R}$ mice

HHPL is the formulation that most effectively protects primary motor neurons against the various cell death pathways that are described in the degenerative process leading to ALS. Our previous studies showed neuroprotection of human platelet lysate preparation in *in vivo* models of PD [28] and traumatic brain injury [29]. We therefore asked whether HHPL could be neuroprotective in an *in vivo* severe model of ALS, the $SOD1^{G86R}$ mice. In this model, body weight starts to decrease at around 80 days, the first motor symptoms appear at around 90 days and death at around 110 days [46]. Thus, due to the severity of the model and the speed of the disease progression, treatment was administrated at the well-defined stage of 75 days, on mice without motor symptoms but already showing non motor dysfunctions [47,48]. HHPL was chronically delivered (3 injections by I.C.V/week) to $SOD1^{G86R}$ mice from the pre-symptomatic age of 75 days to death and compared to oral delivery of riluzole (the only registered treatment available in ALS given to the highest dose reported in mice 44 mg/kg/day [40]) and sham treated controls (vehicle) (Fig. 4A). Since HHPL protein content was close to 18 g/L (Fig. 1), it required to be diluted to 1 g/L in PBS for I.C.V injections in order to be below the pathological threshold in the cerebro-spinal fluid (CSF) [49].

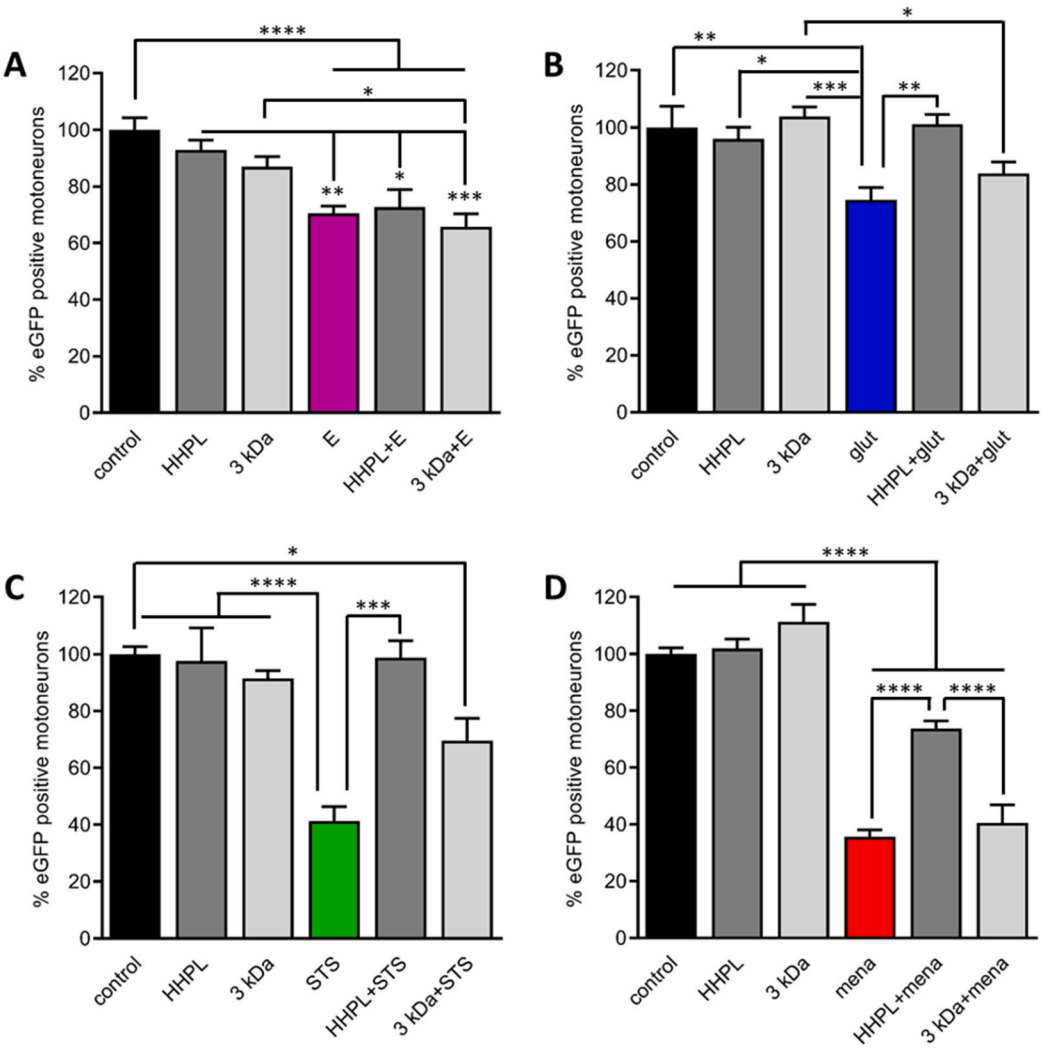


Fig. 3. HHPL and the <3 kDa fraction differently protect primary motor neurons. Day 1 (or day 7 for glutamate) after seeding, motoneurons were treated with HHPL or the <3 kDa fraction 1 h before adding (**A**) the pro-ferroptotic drug erastin (E, 20 μM for 48 h) or (**B**) glutamate (glut, 100 μM for 48 h), (**C**) the pro-apoptotic drug staurosporine (STS, 1 μM for 24 h) or (**D**) the pro-oxidative drug menadione (mena, 4 μM for 24 h). Cell viability was measured by directly counting the surviving motor neurons under fluorescence or phase contrast microscope using morphological criteria as described in the methods. Values are means ± SEM from at least three independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 One-way ANOVA with Tukey *post hoc* test).

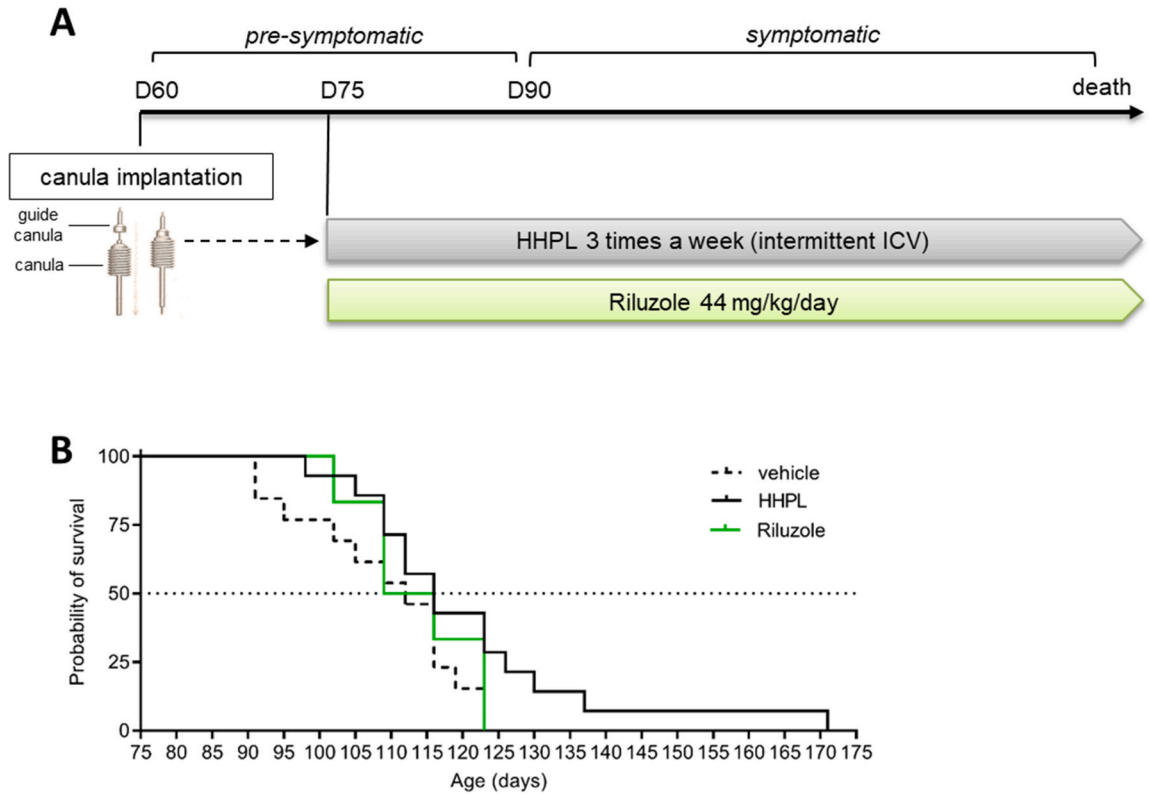


Fig. 4. I.C.V administration of HHPL significantly increases the lifespan of SOD1^{G86R} mice. **(**A) Protocol for chronic I.C.V delivery of HHPL compared to oral delivery of riluzole in SOD1^{G86R} mice. (**B**) The survival curves of SOD1^{G86R} males treated from day 75 to death either with administration of HHPL, Riluzole or vehicle. (veh: vehicle) (vehicle n = 13, HHPL n = 14, Riluzole n = 7, log-rank test: $p < 0.01$ or $p = 0.05$ without the 171 days old mouse).

In SOD1^{G86R} mice, after drug administration started at day 75, the disease onset was similarly delayed between HHPL and riluzole (7 and 11 days respectively). But the lifespan of ALS mice was significantly increased with HHPL, with an important survival of 48 days for one mouse and still a significant increase of 14 days not taking this mouse into account, whereas no change was observed with riluzole treatment (Fig. 4B).

3.5. *Intranasal injection of the <3 kDa fraction increases the survival of SOD1^{G86R} mice*

Despite the absence of NTFs, the <3 kDa fraction was neuroprotective against a ferroptotic inducer in LUHMES (Fig. 1) but no protection was observed in primary motor neurons (Fig. 3), suggesting a possible cell-type dependent specificity of the efficiency of this fraction (e.g on other neuronal and/or non-neuronal cell types). Therefore we wanted to compare the SOD1^{G86R} treatment with the <3 kDa fraction (without NTFs) to the pleiotropic action of NTFs contained in HHPL. Moreover the lower molecular weight and total protein content of the <3 kDa fraction makes this therapy more conducive to blood brain barrier penetrance and led us to evaluate non-invasive intranasal (I.N) administration rather than I.C.V. Similar to I.C.V treatment of HHPL, chronic I.N of 20 μl of the <3 kDa fraction was performed on SOD1^{G86R} mice six times a week from pre-symptomatic age of 75 days to death. When SOD1^{G86R} mice were I.N-treated with the <3 kDa fraction no modification of the disease onset was observed. However, we did observe a significant 10-day extension of survival of mice that received the <3 kDa fraction (Fig. 5A). Therefore, these results revealed a strong impact of the <3 kDa fraction on disease progression despite its lack of protection on primary cultured motor neuron (Fig. 3).

To further characterize the benefits of the <3 kDa fraction on the lifespan of SOD1^{G86R} mice, we evaluated biomarkers to neuronal damage previously reported for ALS. Acetylcholine receptor alpha subunits (AChRα) expression is known to be up-regulated in muscle as a consequence of denervation [50,51] and is a previously reported marker in ALS models [52,53]. The serum level of light chain neurofilament (NfL) also represents the most promising wet biomarker of axonal destruction in ALS [22,54,55].

Taking samples from the gastrocnemius, tibialis and soleus muscles of 100 day old mice, levels of AChRα mRNA were similar in wildtype (WT) with or without the <3 kDa fraction I.N treatment (Fig. 5B). These levels were increased in all 3 muscles of transgenic SOD1^{G86R} mice, with significance being reached in the tibialis and soleus muscle, but treatment with the <3 kDa fraction only reduced levels of AChRα back to WT levels in the tibialis and soleus muscle (Fig. 5B). For serum NfL level, a rise in untreated SOD1^{G86R} mice was reduced back towards WT littermate levels when treated with the <3 kDa fraction (Fig. 5C).

4. Discussion

Knowledge on the molecular basis of ALS has progressed rapidly over the last few years, though such discoveries have yet to translate into new therapeutics. Since its approval in 1996, riluzole is still the only drug currently available but has modest efficacy in increasing patient survival [56]. To match such a fast progressive disease, all possible innovative therapies that are presently theorized must be tested. As well as more conventional routes for drug design, this also includes gene therapy against familial forms of the disease [57], biotherapies using glial cells from non-disease patients and delivering neurotrophic factors to degenerating spinal tissue to alleviate overall neuron dysfunction by stimulating endogenous CNS repair [25,58]. Neurotrophin release from glial cells is one of the main mechanisms by which motor neuron survival is regulated. Their deficiency could induce motor neuron death in ALS patients [59] and thus replenishing neurotrophins to the diseased

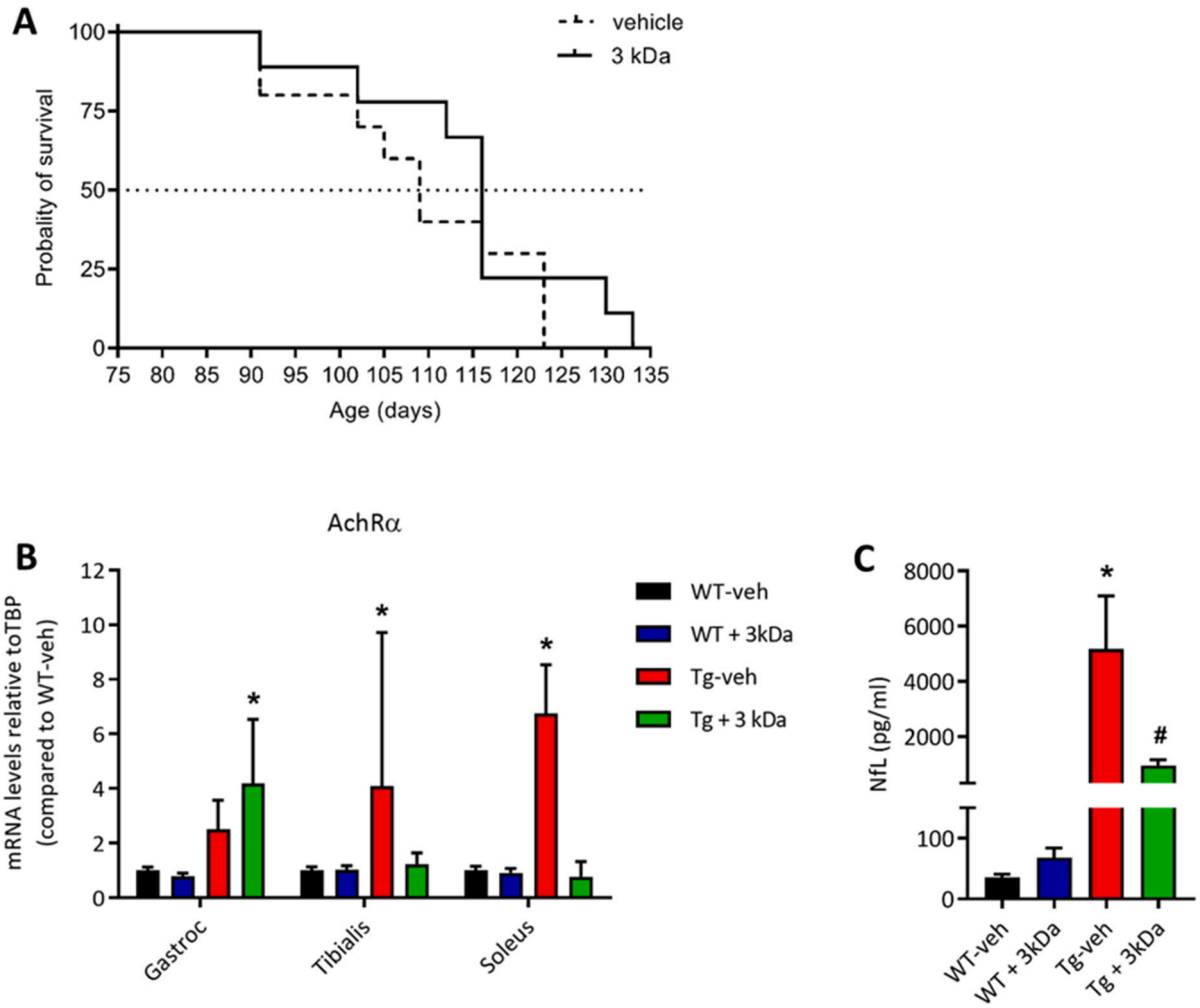


Fig. 5. Intranasal administration of the <3 kDa fraction significantly increases the lifespan of SOD1^{G86R} mice and rescues neuronal damage. (**A**) The survival curves of SOD1^{G86R} mice chronically treated from 75 days to death by I.N administration of the <3 kDa fraction (6 doses/week) (vehicle n = 10, 3 kDa n = 9, log-rank test: $p < 0.05$). (**B**) mRNA levels of acetylcholine receptor AChRα was measured in the gastrocnemuis (gastroc), tibialis and soleus muscles of wildtype (WT) and transgenic (Tg) SOD1^{G86R} mice treated with vehicle (veh) or <3 kDa fraction. Values were compared to WT-vehicle (veh) mice (Kruskal– Wallis test *$p < 0.05$ vs. other conditions; Tg + 3 kDa was not significantly increased as compared with control conditions for tibialis and soleus muscles but not for Gastroc). (**C**) Serum level of light chain neurofilament (NfL) from WT and Tg mice with or without treatment (Kruskal– Wallis test *$p < 0.001$ vs. other conditions; Tg + 3 kDa was significantly reduced as compared with Tg-veh #$p < 0.01$). In B & C values are means±standard error of the mean from at least three experiments.

CNS is a strategy that we have embarked upon. However, in contrast to previous approaches, we believe that a physiologically balanced mixture of naturally sourced growth factors present in platelets is a more efficient CNS therapy than using individual growth factors [25,26].

In the α-granule storage pool of platelets there is an abundant source of physiologically balanced growth factors (e.g., PDGF, VEGF, IGF-1, EGF, BDNF or TGFβ) as well as adhesion molecules and secreted chemokines all of which have anti-inflammatory properties of interest in ALS therapy. Besides, platelets are rich in other biomolecules such as serotonin, dopamine, histamine, Ca^{2+}, ADP, ATP, etc [60]. To utilize the neuroprotective potential of platelets, we developed a heated purified platelet lysate preparation to deplete unwanted factors such as fibrinogen, thrombogenicity and proteolytic activities. The neuroprotective and anti-inflammatory properties of this preparation have already been demonstrated in models of Parkinson's disease [28,33]. The pleiotropic and strong actions of a platelet lysate biotherapy result necessarily from the nature of the synergic physiological healing system contained in platelets. Our most recent studies attempted to characterize the proteome of platelet lysates for (neuro-)regenerative medicine [29]. Such proteomics exploration identified over 1200 proteins, including growth factors, cytokines, antioxidants, and proteins known to exert neuroprotective activity. Using unbiased proteomic approaches, this platelet lysate used in a mouse TBI model reversed several pathways related to transport, post-synaptic density, mitochondria or lipid metabolism [29]. One approach to narrow down the identification of some of the active compounds in the platelet lysate is to carry out fractionation steps, as we did using a 3 kDa size-exclusion procedure. Although the precise content of all neuroprotective factors present in our heated purified platelet lysate preparation is partially characterized, its use in biotherapy should be pursued to treat severe neurological disease in lack of treatment, such as ALS.

In this article, we proposed to explore the therapeutic potential of heat-treated human platelet lysate (named HHPL) and its independent fractions under 50, 30, 10 and 3 kDa in ALS study models. In dopaminergic neurons, HHPL as well as all of its fractions protected against ferroptosis, whereas only HHPL protected against apoptosis and general oxidative stress (Fig. 1). Thus, the removal of some NTFs appears to alter the protection profile afforded by HHPL, supporting the therapeutic approach of using a mixture of NTFs instead of the single NTFs that have previously been clinically tested. Our results also indicate that HHPL components greater than 50 kDa are necessary to protect against apoptosis and oxidative insults, while other factors <3 kDa are necessary

for specific protection from ferroptosis. Moreover, since protection against ferroptosis by the <3 kDa fraction was both Akt and GPX4 dependent, this implies that NTFs present in HHPL are not implicate or able to compensate for a loss of GPX4 activity. There are a multitude of molecules under 3 kDa in platelets, some of which have been reported to have neuroprotective properties [61]. In order to determine specific ferroptotic wet and dry biomarkers for monitoring the efficiency of treatment, it would be important to investigate in the future whether platelet antioxidants associated with glutathione, a cofactor of GPX4 activity, is key in the <3 kDa fraction having neuroprotective properties. These observations may also have implications in other neurodegenerative diseases that also have several features in common with the initiation of ferroptotic cell death [6,22,62,63].

Intriguingly, while our results on primary motor neurons were comparable with dopaminergic neurons for an HPPL neuroprotective response to apoptosis and general oxidative stress this was not the case with ferroptosis. Motor neurons were less responsive to erastin-induced ferroptosis and neither HHPL nor the <3 kDa fraction were protective (Figs. 1 and 3). This suggests that dopaminergic neurons could be more sensitive to a ferroptotic insult and that a component in the <3 kDa fraction of platelets is important for a protective pathway specific to these neurons.

Based on promising cell-based results, *in vivo* experiments using two very different administration routes were carried out in a severe ALS mouse model [46]. First, the use chronically delivering HHPL by I.C.V was used to mitigate the complexity of getting multiple high molecular weight components from HHPL across the BBB by bypassing it via direct injection into the CNS. An I.C.V route is less than ideal as it remains very invasive and can only be clinically proposed in severe neurodegenerative diseases such as the more aggressive forms of ALS that are defined by a rapid progressive phenotype. However, a benefit of I.C.V for HPPL is the enhanced efficacy from focusing the treatment directly to the brain and reducing additional on-target side effects that may occur in the periphery. The second administration route chosen, I.N administration of the 'lighter' preparation (<3 kDa fraction), represents a less invasive route of delivery which is obviously more practical and compatible with chronic treatments targeting the CNS [64]. In our study, HHPL delivery by intermittent I.C.V in $SOD1^{G86R}$ mice delayed the disease onset by 7 days and the global survival was increased by 14 days (Fig. 4), which constitutes a very strong effect in this severe SOD1 mice model. In comparison I.N administration of the <3 kDa fraction showed no modification of the disease onset but a 10-days significant lifespan extension (Fig. 5). In contrast, no change was observed with riluzole treatment. Whilst the protective effects of the <3 kDa fraction initially seem surprising in light of the absence of protection by the same fraction on motor neuron cultures, it may suggest that molecules present in the <3 kDa fraction target other neuronal and non-neuronal cell types *in vivo*, that contribute to disease progression [65]. Similar mechanism could also be implicated with HHPL in addition to the confirmed direct protective properties observed in motoneuron culture, but this will require further investigation.

The comparison of these two platelet biotherapies allows us to draw some important conclusions. First of all, NTFs as well as other smaller molecular sized components in HHPL have a strong effect on disease progression in a severe ALS model. This improvement in survival rate was larger than many other previously published reports using the same model [66,67]. Secondly, despite the weak effect the <3 kDa fraction had on select cell pathways in primary cultured motor neuron, there was a clear beneficial impact of this fraction on disease progression that was comparable to HHPL and riluzole. The <3 kDa fraction of platelet lysate will mostly contain small peptides, amines, nucleotides *etc*. and among them will be several anti-inflammatory peptides and cytokines [68,69]. It is well known that chronic inflammation plays an important role in ALS [70] and the anti-inflammatory activity as well as immunomodulatory capacity of the <3 kDa fraction when administered I.N may attenuate a neuroinflammatory component of the disease to extend longevity. Finally, studies are planned to validate key specific mechanisms that are engaged (e.g. Akt, specific ferroptotic markers such as iron metabolism and lipidic stress markers) as well as theragnostic biomarkers (e.g. NfL) and these should assist the clinical development of platelet lysate in early ALS patients.

Author's contribution

FG, TB, JCD and DD conceived the experimental design, discussed the results ans wrote the manuscript. KT discussed the experimental design with the mice and performed the in vivo experiments. MD prepared platelet lysates and did LUHMES experiments. FG performed LUHMES and primary moror neurons experiments. PG did the experiments on primary motor neurons. CR discussed the experimental design with the primary motor neurons, discussed the results and provided comments on the manuscript. AJ provided technical assistance for LUHMES cells and primary motor neuron culture. GG did the dosages on platelet lysates and sera of mice. CM and VBD provided comments on the manuscript. JD discussed the results, provided comments on the manuscript, and corrected english grammar and spelling.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Financial support (author : David Devos). - the SATT Nord (TTO for Lille University, CHU of Lille and Inserm). - I-Site Université Lille Nord Europe. - the Fondation Credit Agricole. - ARSLA charity (Association pour la Recherche sur la Sclerose Laterale Amyotrophique) for their financial support. Non financial support (author : David Devos). - Macopharma society for providing batches of platelet lysate. Three academic patents concern the current paper. - EP16305332.5 (23/03/2016): "Preparation of platelet pellet lysate and its use for treating neurological disorders " (inventor : DEVOS David, BURNOUF Thierry, DEVEDJIAN Jean-christophe, CHOU Ming-Li. Licensee : SATT Nord (TTO for Lille University, CHU of Lille and Inserm)). - EP17305739.9 (16/06/2017) : process for preparing a pooled human platelet lysate, pooled human platelet lysate and its use fir treating neurological disorders. (inventor : DEVOS David, BURNOUF Thierry, DEVEDJIAN Jean-christophe, CHOU Ming-Li, GOUEL Flore. Licensee : SATT Nord (TTO for Lille University, CHU of Lille and Inserm)). - EP17306284.5 (27/09/2017) : Process for preparing a platelet lysate fraction, platelet lysate fraction and its use for treating disorders of the central nervous system. (inventor : DEVOS David, BURNOUF Thierry, DEVEDJIAN Jean-christophe, CHOU Ming-Li, GOUEL Flore. Licensee : SATT Nord (TTO for Lille University, CHU of Lille and Inserm)). E.M. is a founding equity shareholder of Capricor Therapeutics. No other authors declare that they have no known competing financial interests.

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