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ORIGINAL COMMUNICATION

Mitochondrial dysfunction in blood cells from amyotrophic lateral sclerosis patients

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Abstract Mitochondrial dysfunction is implicated in amyotrophic lateral sclerosis, where the progressive degeneration of motor neurons results in muscle atrophy, paralysis and death. Abnormalities in both central nervous system and muscle mitochondria have previously been demonstrated in patient samples, indicating systemic disease. In this case–control study, venous blood samples were acquired from 24 amyotrophic lateral sclerosis patients and 21 age-matched controls. Platelets and peripheral blood mononuclear cells were isolated and mitochondrial oxygen consumption measured in intact and permeabilized cells with additions of mitochondrial substrates, inhibitors and titration of an uncoupler. Respiratory values were normalized to cell count and for two markers of cellular mitochondrial content, citrate synthase activity and mitochondrial DNA, respectively. Mitochondrial function was correlated with clinical staging of disease severity. Complex IV (cytochrome c-oxidase)-activity normalized to mitochondrial content was decreased in platelets from amyotrophic lateral sclerosis patients both when normalized to citrate synthase activity and mitochondrial DNA copy number. In mononuclear cells, complex IV-activity was decreased when normalized to citrate synthase activity. Mitochondrial content was increased in amyotrophic lateral sclerosis patient platelets. In mononuclear cells, complex I activity declined and mitochondrial content increased progressively with advancing disease stage. The findings are, however, based on small subsets of patients and need to be confirmed. We conclude that when normalized to mitochondria-specific content, complex IV-activity is reduced in blood cells from amyotrophic lateral sclerosis patients and that there is an apparent compensatory increase in cellular mitochondrial content. This supports systemic involvement in amyotrophic lateral sclerosis and suggests further study of mitochondrial function in blood cells as a future biomarker for the disease.

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Abbreviations

ALS	Amyotrophic lateral sclerosis
BSA	Bovine serum albumin
BZD	Benzodiazepines
CI	Complex I
CII	Complex II
CIV	Complex IV
CNS	Central nervous system
CS	Citrate synthase
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
ETS	Electron transport system
FCCP	Carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone
HEPES	4-(2-hydroxyethyl)-1-Piperazineethanesulfonic acid
mtDNA	Mitochondrial DNA
OXPHOS	Respiration associated with ATP synthesis by oxidative phosphorylation
PBMC	Peripheral blood mononuclear cell
PSMA	Progressive spinal muscular atrophy
SD	Standard deviation
SSRI	Selective serotonin re-uptake inhibitor
TMPD	Tetramethylphenylenediamine

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder affecting motor neurons, resulting in progressive muscle weakness and ultimately leading to respiratory failure and death in the majority of patients [14]. Mitochondrial abnormalities have been implicated in the pathogenesis of ALS, although the exact role of mitochondrial dysfunction is not known [29]. In human post mortem brain tissue, increase in mitochondrial electron transport system (ETS) complex I (CI) enzymatic activity per mitochondrial content has been reported [4, 6] and in post mortem spinal cord, reduction of cytochrome c-oxidase [ETS complex IV (CIV)]-activity has been shown in patients with ALS [11]. The evidence for mitochondrial dysfunction in ALS has been reviewed repeatedly, e.g., by Cozzoloni and Carri [7].

Systemic involvement has been suggested in ALS [1, 20, 28], and mitochondrial alterations have been reported in muscle, liver and blood cells from ALS patients [8, 9, 12, 17, 19, 28]. Here, we assess mitochondrial function in blood cells [platelets and peripheral blood mononuclear cells (PBMCs)] from ALS patients using high-resolution respirometry to elucidate whether previously reported mitochondrial perturbations in blood cells affect mitochondrial respiration. We measure mitochondrial oxygen utilization with live respiring mitochondria ex vivo in intact and permeabilized cells in order to evaluate the integrated mitochondrial respiration. This will provide further evidence of whether systemic mitochondrial dysfunction is present and detectable in ALS patients. Furthermore, we explore if measures of mitochondrial dysfunction in blood cells could serve as diagnostic or prognostic biomarkers for the disease.

Materials and methods

Patients

The study was approved by the regional ethical review board at Lund University (EPN no. 2011/89). The study population comprised patients diagnosed with motor neuron disease showing clinical phenotypes of upper and/or lower motor neuron affection, herein referred to as ALS [14]. Twenty-four patients (9 females and 15 males, age 64.9 ± 12.3 years) were included in the study between May 2011 and October 2012. Patients were recruited at the Neurology Clinic at Skåne University Hospital, Lund, Sweden. As controls, relatives (primarily spouses) of patients with chronic neurological disorders admitted to the same clinic were recruited. Written informed consent was acquired prior to inclusion. Demographic and clinical data are summarized in Table 1. Any prescription drugs or class of prescription drugs taken by 5 or more patients are listed, and the diagnostic certainty according to the El Escorial criteria is given [5]. The control group consisted of 21 individuals and was age matched (64.6 ± 11.6 years), but not gender matched (15 females and 6 males).

Staging

Patients were staged by an experienced specialist blinded to the experimental results at time of staging, based on criteria recently suggested by Roche et al. [23]. Stage 1, symptom onset (involvement of first region); stage 2A, diagnosis; stage 2B, involvement of a second region; stage 3, involvement of a third region; stage 4A, need for gastrostomy; stage 4B, need for respiratory support (non-invasive ventilation). Referral to surgery for gastrostomy at the time of blood sampling was considered as stage 4A. To allow for statistical analysis, subgroups A and B were pooled.

Table 1 Research subject characteristics

	Age (mean ± SD)	Males/females	Duration (mean ± SD)
ALS patients	65 ± 12	15/9	6 ± 8 Years
Control subjects	65 ± 11	6/15	

No.	Age	Gender	Duration	Medication	Symptoms (alive at follow-up[a])	El Escorial	Stage
1	68	Female	1½ years	Riluzole, BZD	Reduced muscle function left arm (onset), walks by herself	Probable	2B
2	82	Female	10 years	Riluzole, SSRI	Dysarthria (onset) and dysphagia, in wheelchair	Definite	3
3	79	Male	2 months	Riluzole	Dysphagia, lower limb weakness (onset), in wheelchair	Probable	2B
4	50	Male	10 years	Riluzole, SSRI	Onset was increased muscular tonus in lower limb, in wheelchair[a,c]	Definite[b]	4B
5	50	Female	9 years		Difficulties walking (onset), walks on crutches[a]	Possible	2B
6	40	Male	1½ years	Riluzole	Weakness in left foot (onset), Walks on crutches[a]	Possible	2B
7	56	Female	2 years	Riluzole	Pseudobulbar symptoms, onset was dysarthria[a]	Definite	3
8	81	Male	2 years	Riluzole	Anarthria (onset) and gastrostomy	Probable	4A
9	67	Male	1 years	Riluzole	Reduced muscle function in the upper limbs (onset)	Probable	2B
10	60	Male	1 years	Riluzole, BZD	Dysarthria, dysphagia and dementia. Walks with walker. Upper limb onset	Definite	3
11	54	Male	16 years	BZD	Spastic paraparesis, dysphonia (onset), slow progress. In wheelchair[a]	Possible	2B
12	69	Female	1 years	Riluzole	Upper limb weakness, dysarthria and dysphagia (onset). Gastrostomy[a]	Definite	4A
13	67	Male	4 years	Riluzole	Dysarthria, right side hemiplegia (onset). Walks by herself[a]	Definite	3
14	72	Male	37 years		Slow progress, tetraparesis (onset), wheelchair[a]	PSMA	2B
15	70	Female	13 years	BZD	Wheelchair, gastrostomy	Definite[b]	4A
16	53	Male	1 year	Riluzole	Weak left foot (onset), walks by himself, can manage daily life[a]	Probable	2B
17	78	Male	2 years	Riluzole, BZD	Bilateral lower limb weakness (onset)[a]	Probable	2B
18	73	Female	3 years	Riluzole	Left side hemiparesis (onset), walks with walker[a]	Possible	2B
19	51	Female	5 years	Riluzole, SSRI	Lower limb weakness (onset), walks on crutches[a]	PSMA	2A
20	59	Male	3 years	Riluzole, SSRI	Tetraplegia (bedridden), right side onset. Tracheostomy and gastrostomy[a]	Possible	4B
21	84	Female	5 years	Riluzole, BZD, SSRI	In wheelchair, anarthria (dysarthria onset symptom), gastrostomy	Definite	4A
22	74	Male	6 years	Riluzole	Bulbar symptoms, weak left arm (onset), needs help with daily life[a]	Definite	3
23	71	Male	2 years	Riluzole, SSRI	Mild dysarthria, weak right hand and arm (onset), wheelchair[a]	Definite	3
24	50	Male	4 years	Riluzole, BZD,	In wheelchair with ventilator and gastrostomy. Lower limb onset	Definite	4B

Clinical data at time of blood sampling. Subject no. 10 was excluded from the analysis of platelet data due to technical uncertainty of cell number in the sample, and in subject no. 4, 10, 11, 12, 14 and 16 there were insufficient PBMCs available for analysis

BZD benzodiazepines, *SSRI* selective serotonin re-uptake inhibitor, *PSMA* progressive spinal muscular atrophy

[a] Alive at follow-up

[b] Familial amyotrophic lateral sclerosis

[c] Known SOD1-mutation

Chemicals

Monopotassium phosphate was acquired from Merck KGaA (Darmstadt, Germany). All other chemicals were purchased from Sigma–Aldrich (St Louis, MO, USA).

Isolation of peripheral blood cells

From each patient or control, a total of approximately 20 ml of blood was drawn to K_2EDTA tubes (Vacutainer®, BD, Franklin Lakes, NJ, USA) via venous puncture. Platelets and PBMCs were isolated with consecutive centrifugation steps as previously described [26, 27].

High-resolution respirometry

Measurements were performed with an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) as previously reported [26, 27]. The oxygen concentration in the medium during an experiment was between 210 and 50 μM O_2. The intracellular-mimicking medium MiR05, containing sucrose 110 mM, HEPES 20 mM, taurine 20 mM, K-lactobionate 60 mM, $MgCl_2$ 3 mM, KH_2PO_4 10 mM, EGTA 0.5 mM, BSA 1 g/l, pH 7.1 [13] was used for all experiments with permeabilized cells. Experiments with intact cells were performed in the subject's own plasma.

For both platelets and PBMCs experiments were performed as previously published [26, 27] using two main protocols, one for intact and one for permeabilized cells. Cells were counted using an automated haemocytometer (SweLab Alfa, Boule Diagnostics, Stockholm, Sweden) and the cell count used in the experiments was 200×10^6 cells/ml for platelets and $3.5–5.0 \times 10^6$ cells/ml for PBMCs. The amount of digitonin used to permeabilize cells was 6 μg/1×10^6 cells for PBMCs and 1 μg/1×10^6 cells for platelets. Where material supply was insufficient for all analyses, measurements of permeabilized cells were prioritized.

Intact cells with endogenous substrates suspended in the patient's own plasma were allowed to stabilize at routine respiration ($ROUTINE_{plasma}$) and the proton leak over the inner mitochondrial membrane was measured by adding the ATP-synthase inhibitor oligomycin ($LEAK_{intact}$). By subsequent titration of the protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), the maximum flux through the ETS (ETS_{intact}) was achieved. The experiment was terminated by inhibition of CI and complex III, revealing non-mitochondrial oxygen consumption. Data were corrected for non-mitochondrial oxygen consumption, and control ratios ($ETS_{intact}/LEAK_{intact}$, $ROUTINE_{plasma}/LEAK_{intact}$ and $ETS_{intact}/ROUTINE_{plasma}$) were calculated.

Permeabilized cells were assayed in MiR05 using a protocol with sequential addition of substrates, inhibitors, and titration of uncoupler. Specific mitochondrial CI-respiration was measured with CI-substrates (pyruvate, malate and glutamate) and ADP present, i.e., active oxidative phosphorylation ($OXPHOS_{CI}$). Convergent CI + CII-respiration associated with ATP synthesis ($OXPHOS_{CI+II}$) was then measured by addition of the CII-substrate succinate. Subsequently, the complex V-inhibitor oligomycin was added to induce $LEAK_{CI+II}$ (oxygen consumption due to proton leak over the inner mitochondrial membrane in presence of CI- and CII-substrates) and maximal flux through the electron transport system (ETS_{CI+II}) was achieved by titrating the uncoupling agent FCCP. Specific CII-dependent uncoupled respiration was then assessed through the addition of the CI-inhibitor rotenone (ETS_{CII}). CIV-activity was evaluated by addition of the artificial electron donor tetramethylphenylenediamine (TMPD) followed by the CIV inhibitor azide. Before evaluation of CIV, non-mitochondrial oxygen consumption was recorded as described for intact cells. All data were corrected for non-mitochondrial oxygen consumption, and uncoupling control ratio ($ETS_{CI+II}/LEAK_{CI+II}$) and phosphorylating control ratio ($OXPHOS_{CI+II}/LEAK_{CI+II}$) were calculated. For concentrations of toxins and substrates, please see methods in the two articles from 2013 by Sjövall et al. [26, 27].

Citrate synthase activity measurement

Measurements of citrate synthase (CS)-activity were performed with a commercially available assay kit (CS0720, Sigma–Aldrich, St Louis, MO, USA). After sonication (30 s for PBMCs and 2×60 s for platelets), samples were loaded into a 96-well plate in assay buffer with addition of 300 μM acetyl CoA and 100 μM 5,5-dithiobis-(2-nitrobenzoic acid). In a spectrophotometric plate reader set to 412 nm on a kinetic program with 1.5-min duration and 10-s interval, the absorbance of the baseline reaction was measured. Following this, 500 μM of oxaloacetate was added to each well and absorbance was measured. Calculations of CS-activity were then performed according to the manufacturer's instructions.

Mitochondrial DNA measurements

Quantification of cellular mitochondrial DNA (mtDNA) content was performed as previously reported [26, 27]. Frozen samples were thawed and diluted 500 times in a lysis buffer (10 mM TRIS–HCl, 1 mM EDTA, salmon sperm DNA 1 ng/μl, pH 8.0). 10 μl of this dilution was amplified in a 25 μl PCR reaction containing 1× Power SYBR® Green PCR Master Mix using an ABI Prism 7000 real-time PCR machine (Applied Biosystems Inc., Foster City, CA, USA) and 100 nM of primers directed to the human mitochondrial COX-1 gene (Eurofins MWG-operon, GmbH, Ebersberg, Germany). The threshold cycle (Ct) values were related to a standard curve using cloned PCR products.

Statistical analysis

All analyses were performed with SPSS (version 21 and 22, IBM, New York, USA) and all figures were generated using GraphPad PRISM (GraphPad Software version 5.01 and 6.0d, La Jolla, CA, USA). All values are presented as mean ± standard error of the mean, except for demographic data where standard deviation (SD) are used. Comparisons of respiratory values between ALS and the control group, and between survivors and non-survivors, were performed with unpaired Student's *t* test, not assuming equal distribution between groups. All ranges are 95 % confidence intervals of difference from the unpaired *t* tests. Comparisons between respiratory values at different disease stages were performed with Jonckheere–Terpstra's test, a non-parametric test for ordered alternatives, with post hoc correction for multiple comparisons between all pairs. Results were considered significant if $p < 0.05$. One data point, one of two CS replicates in subject no. 10, deviated more than 5 SD from the mean and was excluded as an outlier.

Results

Cellular mitochondrial content is increased in platelets of ALS patients

CS-activity in ALS patient platelets was significantly increased by 28 % (95 % confidence interval 11–50 %) compared to the control group and the mtDNA copy-number per cell was 38 % higher (7–68 %). Similarly, in PBMCs, ALS patients showed a 19 % higher CS-activity [(−8) to 45 %] and 6 % more mtDNA copies [(−21) to 33 %]; however, the differences in PBMCs were not significant (Fig. 1a–d; Table 2). For both cell types, CS and mtDNA correlated well (r^2 values ranging from 0.66 to 0.89), indicating the validity of both markers as a measure of mitochondrial content in peripheral blood cells (Fig. 2e, f).

Mitochondrial respiration dependent on complex IV is reduced in ALS

Data were first analyzed on a respiration per cell count basis (Table 2). In intact platelets $ROUTINE_{plasma}$ respiration was increased by 36 % (8–64 %) in ALS patients compared to control and ETS_{intact} respiration by 23 % (3–44 %). $LEAK_{intact}$, oxygen consumed to uphold mitochondrial inner membrane potential and compensate for endogenous proton escape, was close to zero for both groups. Analyses of respiration in intact PBMCs were

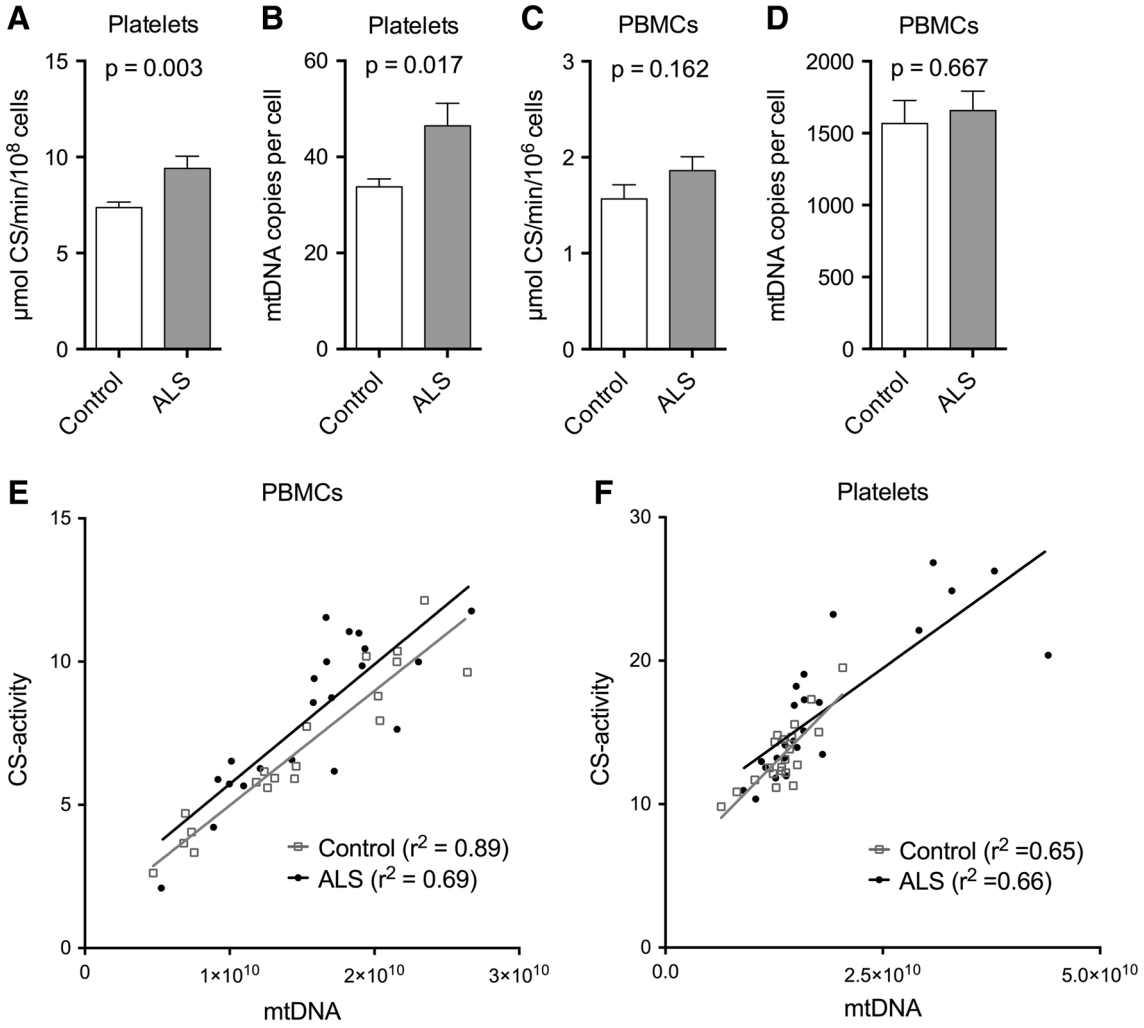

Fig. 1 Mitochondrial content in peripheral blood cells from ALS patients. Mitochondrial content per cell, measured using CS-activity (**a**) and mtDNA copies per cell (**b**), is significantly increased in platelets from ALS patients. In PBMCs no significant alteration could be demonstrated (**c** and **d**). For both PBMCs (**e**) and platelets (**f**), CS-activity and mtDNA correlate well, indicating the validity of these parameters as markers for mitochondrial content in peripheral blood cells. Two-tailed *p* values from unpaired Student's *t* test, not assuming equal distribution. *ALS* amyotrophic lateral sclerosis, *PBMC* peripheral blood mononuclear cells, *CS* citrate synthase, *mtDNA* mitochondrial DNA

excluded due to limited data (only 5 ALS and 3 control samples were analyzed due to insufficient amount of cells).

Next, we further characterized the nature of the altered mitochondrial function using permeabilized cells to be able to control the substrate supply to the mitochondria. Respiratory data were normalized to cell count (which is presented in Table 2) and mitochondrial content, respectively (CS and mtDNA) (presented in Fig. 2). Normalized to cell count, respiration was increased in $OXPHOS_{CI}$ [38 % (9–66 %)], ETS_{CII} [16 % (0–32 %)], $OXPHOS_{CI+II}$ [31 % (8–54 %)] and ETS_{CI+II} [19 % (1–36 %)] in permeabilized platelets of ALS patients. These differences were not found in PBMCs (Table 2).

When we analyzed data as oxygen consumed per mitochondrial content (by normalization for CS-activity and mtDNA) instead of per cell, mitochondrial CIV was significantly affected, but no other respiratory parameter. In platelets, the CIV-activity was significantly reduced in ALS patients both when normalized to CS-activity [24 % (5–42 %)] (Fig. 2b) and for mtDNA content [24 % (3–45 %)] (Fig. 2d). In PBMCs, the CIV-activity was significantly lower in ALS patients when normalized to CS [35 % (5–65 %)] (Fig. 2a), while when data were normalized to mtDNA (30 % [(−3) to 64 %]) the CIV-activity was reduced but did not reach statistical significance ($p = 0.075$) (Fig. 2c). There was no difference between the ALS and control group in any of the analyzed respiratory control ratios, either for intact or permeabilized cells (data not shown). The similar level of respiration in $OXPHOS_{CI+II}$ and ETS_{CI+II} states indicates that complex V is not a major rate limiting factor in platelets and PBMCs, which is in accordance with previous studies [27].

With progressing disease stage, mitochondrial complex I-dependent respiration declines and mitochondrial content increases

Patients were grouped according to clinical disease stage [23] (see "Materials and methods") and key data on mitochondrial function were analyzed for significant continuous trends. In PBMCs, $OXPHOS_{CI}$ and $OXPHOS_{CI+II}$ normalized to CS both displayed a significant decline with progressing disease, each with a 43 % decline in respiration at stage 4 compared to controls, whereas patients in stage 2 of the disease only displayed a 2 and 8 % decrease, respectively (Fig. 3a, c). Mitochondrial content in PBMCs gradually increased with progressing disease stage, with a 61 % increase at stage 4 compared to controls (Fig. 3g). Further, CIV in PBMCs measured per cell was gradually increased from 62 % in stage 2–92 % of control in stage 4. No progressive change was shown for CIV when normalized to mitochondrial content (Fig. 3e). These findings could not be detected in platelets (Fig. 3b, d, f, g).

Non-survivors have lower complex I-linked respiration in PBMCs than survivors

Post hoc, patients were retrospectively grouped into survivors and non-survivors based on medical records at the end of study [follow-up time 13 ± 7 months (Mean ± SD)] and evaluated for differences in mitochondrial function with respect to survival. In PBMCs, $ROUTINE_{MiR05}$ respiration per cell was 21 % (1–41 %, 3.35 ± 0.16 versus 4.04 ± 0.26 pmol $O_2/s/10^6$ cells, $p = 0.043$) higher in non-survivors and, when investigated at the level of individual respiratory complexes, $OXPHOS_{CI}$ and $OXPHOS_{CI+II}$ was 22 % (3–42 %, 7.31 ± 0.47 versus 8.94 ± 0.48, $p = 0.027$) and 27 % (5–49 %, 11.39 ± 0.86 versus 14.47 ± 0.81, $p = 0.019$) higher, respectively, compared to survivors. Further, $LEAK_{CI+II}$ was 44 % (9–79 %, 1.50 ± 0.17 versus 2.16 ± 0.18, $p = 0.019$) higher in non-survivors (data not shown).

To control for influence of medication on mitochondrial function, ALS patients were grouped according to their prescribed medication for all drugs taken by 5 or more study subjects. For all parameters where a significant difference was found between the ALS and the control group in this study, the results for treated and non-treated patients in each drug class were compared (using non-parametric methods due to unequal and small groups). No significant differences were found (data not shown). Further, all significant differences between the ALS and the control group reported were reanalyzed excluding patients not meeting the revised El Escorial criteria for ALS [5], without any change in result or conclusion of the study (data not shown).

Discussion

In the current study we demonstrate increased mitochondrial content in ALS patient platelets, seen also in PBMCs with increasing disease stage. With this finding, it is imperative to normalize respiration to cellular mitochondrial content in order to be able to draw conclusions regarding the mitochondrial respiratory capacity of blood cells from ALS patients.

Using normalization, we provide evidence of mitochondrial dysfunction in ALS patients, a dysfunction related primarily to CIV of the electron transport system. We also report a progressive decline in mitochondrial CI-dependent respiration with advancing stage of the disease. These findings were detectable in peripheral blood cells suggesting that mitochondrial dysfunction affects cells beyond the CNS and muscles in ALS.

CIV, or cytochrome c oxidase, is the final electron receiver of the electron transport system in the mitochondrial

Table 2 Respiratory data and markers of mitochondrial content

	$ROUTINE_{plasma}$	$LEAK_{intact}$	ETS_{intact}	*n*
Intact platelets				
ALS patients	14.15 ± 1.28	0.65 ± 0.32	20.53 ± 1.50	22
Control	10.41 ± 0.57	−0.044 ± 0.15	16.65 ± 0.77	21
p value	0.012	0.056	0.029	

	$ROUTINE_{MiR05}$	$LEAK_{CI+II}$	$OXPHOS_{CI}$	$OXPHOS_{CI+II}$	ETS_{CI+II}	ETS_{CII}	CIV-activity	CS-activity	mtDNA	*n*
Permeabilized platelets										
ALS patients	10.79 ± 1.21	5.58 ± 0.45	26.58 ± 2.49	44.27 ± 3.51	40.54 ± 2.60	18.70 ± 1.08	36.11 ± 3.01	9.63 ± 0.63	47 ± 5.5	23
Control	8.27 ± 0.66	4.98 ± 0.20	19.32 ± 1.07	33.84 ± 1.58	34.20 ± 1.48	16.14 ± 0.61	39.55 ± 2.06	7.37 ± 0.29	34 ± 1.8	21
p value	0.076	0.234	0.012	0.011	0.042	0.049	0.353	0.003	0.017	
Permeabilized PBMCs										
ALS patients	3.65 ± 0.16	1.79 ± 0.14	8.04 ± 0.38	12.76 ± 0.69	10.22 ± 0.96	5.27 ± 0.56	6.57 ± 0.65	1.86 ± 0.15	1643 ± 142	18
Control	3.29 ± 0.14	1.94 ± 0.13	7.66 ± 0.30	12.57 ± 0.42	10.84 ± 0.55	5.37 ± 0.22	8.17 ± 0.51	1.57 ± 0.15	1568 ± 159	19
p value	0.108	0.467	0.437	0.815	0.577	0.869	0.064	0.108	0.667	

Data are presented adjusted for cell count; data normalized to mitochondrial content are presented in Fig. 2. $OXPHOS_{CI}$, $OXPHOS_{CI+II}$, ETS_{CII} and ETS_{CI+II} are increased per cell in platelets from ALS patients. Mitochondrial content (CS-activity and mtDNA) in platelets from ALS patients is significantly increased compared to control. All respiratory data are corrected for non-mitochondrial respiration and given as mean and SEM. Data are expressed in pmol O_2/s/100 × 10^6 cells from experiments in platelets and per 10^6 cells in PBMCs. Similarly, CS-activity is depicted as µmol CS/min/100 × 10^6 cells in platelets and per 10^6 cells in PBMCs. mtDNA is given as copies per cell. Two-tailed *p* values from Student's *t* test. Equal variance not assumed

ALS amyotrophic lateral sclerosis, *CS* citrate synthase, *OXPHOS* respiration associated with ATP synthesis by oxidative phosphorylation, *ETS* respiration associated with maximal protonophore stimulated flux through the electron transport system, *LEAK* idle respiration without ATP-synthase activity, *CI* complex I, *CII* complex II, *CIV* complex IV, *mtDNA* mitochondrial DNA, *n* number of patients, *PBMC* peripheral blood mononuclear cells, *ROUTINE* endogenous respiration in plasma or MiR05 medium, *SEM* standard error of mean

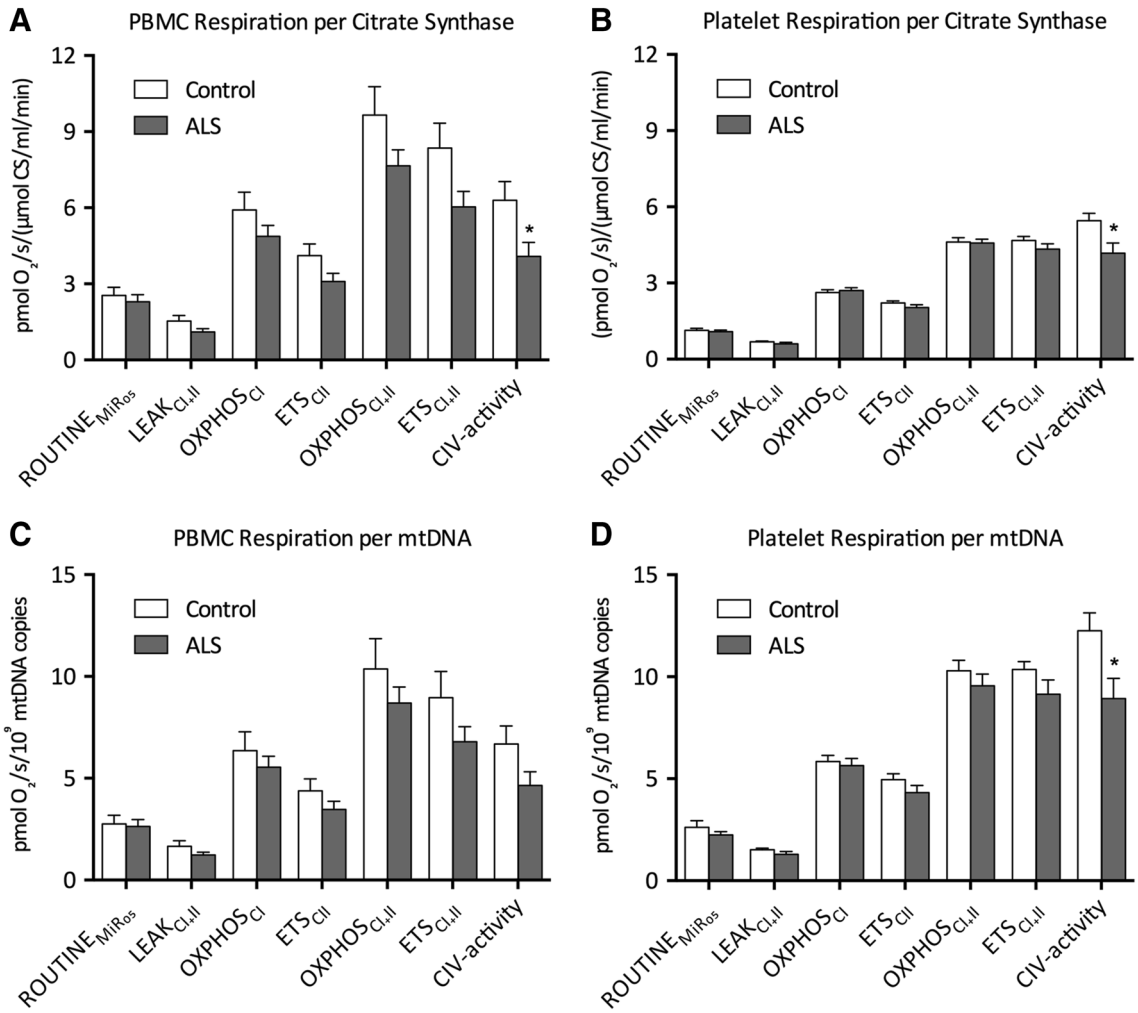

Fig. 2 Respiration of platelets and peripheral blood cells from ALS patients normalized to mitochondrial content. Respiratory activity was normalized to mtDNA and CS-activity, respectively. In platelets, CIV-dependent respiration was decreased both when normalized to CS-activity (**b**) and mtDNA (**d**), while in PBMCs CIV-activity was reduced when normalized to CS-activity (**a**) but not significantly so when normalized to mtDNA (**c**). Data are presented as mean and standard error. * $p \leq 0.05$ using unpaired Student's *t* test with two-tailed *p* values without assuming equal distribution. *ALS* amyotrophic lateral sclerosis, *CI* complex I, *CII* complex II, *CIV* complex IV, *CS* citrate synthase, *ETS* respiration associated with maximal protonophore stimulated flux through the electron transport system, *LEAK* idle respiration without ATP-synthase activity, *mtDNA* mitochondrial DNA, *OXPHOS* respiration associated with ATP synthesis by oxidative phosphorylation, *PBMC* peripheral blood mononuclear cells, *ROUTINE* endogenous respiration in MiR05 medium

inner membrane and is in turn donating electrons to oxygen, in the process pumping electrons over the mitochondrial inner membrane to build up the electrochemical gradient that powers ATP synthesis at complex V. A reduction of CIV respiration was present in both platelets and PBMCs, cells from different hematopoietic origins. Previously, mitochondrial CIV deficiency has been reported both in post mortem CNS tissue [2, 31] and muscle fibers [8, 30] from ALS patients. Here we show that CIV deficiency extends beyond the previously described tissues and is also detectable in peripheral blood cells in ALS.

Furthermore, we found that mitochondrial content per cell measured either as mtDNA copies per cell or activity of the matrix enzyme CS was increased in platelets from ALS patients. It is conceivable that the increase in mitochondrial content and the detected increase in mitochondrial respiration per cell are due to allostatic compensation for the inadequacy of the mitochondria to comply with cellular energy demands, possibly due to decreased CIV function. Mitochondria have recently been proposed as a key mediator of cellular damage due to allostatic load in chronic systemic stress responses [22], and similar mechanisms are plausible to play a role in progressing neurodegeneration. At this stage it is, however, uncertain what the biological implications of reduced CIV-activity and increased mitochondrial content are. This and the causative mechanisms of these alterations warrant further investigation.

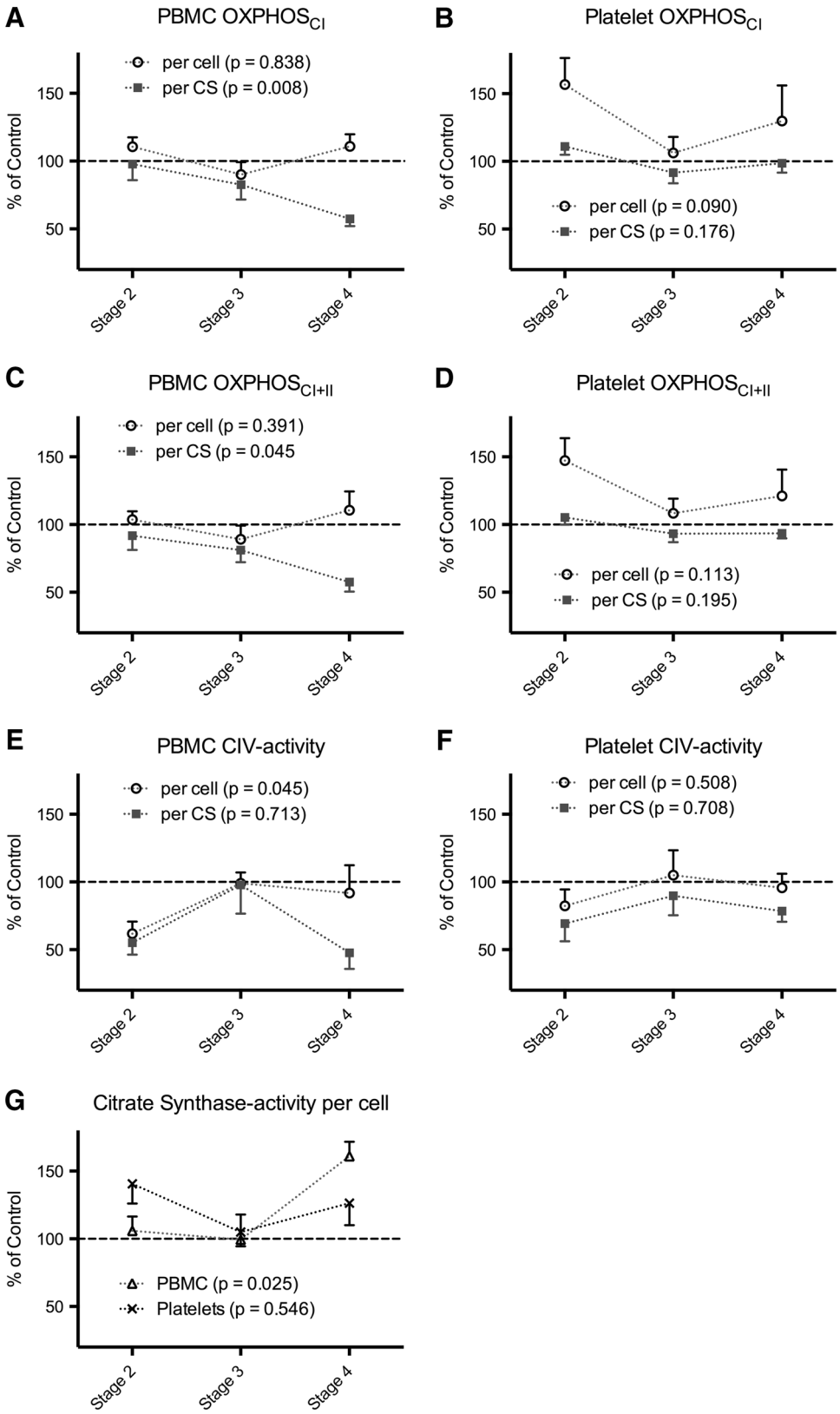

Fig. 3 Mitochondrial function and ALS disease stage. With progressing ALS disease stage, respiration coupled to ATP synthesis in PBMCs using convergent CI- and CII-linked substrate oxidation is gradually reduced (**c**), as is CI-linked respiration (**a**) when normalized to mitochondrial content. CIV-activity measured per cell displays an increasing trend with advancing stage in PBMCs, while no progression can be detected when normalized to mitochondrial content (**e**). Mitochondrial content, measured as CS-activity, is significantly increased with advancing ALS disease stage in PBMCs (**g**). In platelets, no significant trends could be demonstrated (**b**, **d**, **f**, **g**). All data are presented as mean and SEM, and expressed as percent of control. The zero-hypothesis that the distribution of data was equal across all ALS disease stage was rejected at p values <0.05 using the non-parametric Jonckheere–Terpstra test for ordered alternatives, with post hoc test of all pairwise comparisons. The number of subjects in each group (ALS Stage 2, 3 and 4, respectively) was 8, 5 and 5 for PBMCs and 11, 5 and 7 for platelets. *PBMC* peripheral blood mononuclear cells, *CS* citrate synthase, *OXPHOS* respiration associated with ATP synthesis by oxidative phosphorylation, *CI* complex I, *CII* complex II, *CIV* complex IV

When assessing the function of mitochondria in tissue or cells, it is crucial to discriminate between functional mitochondrial activity per cell (or mg tissue or protein) and activity per mitochondrial content. Several measures of mitochondrial respiration were increased in ALS patient cells per cell (Table 2), both in intact and in permeabilized

cells. At the level of mitochondria, however, the measure of mitochondrial function that was significantly altered was the activity of CIV, which was decreased. Hence, the alterations seen at cell level can be attributed to the increased mitochondrial content in the cells. The two independently measured markers of mitochondrial content used in this study, mtDNA copies per cell and CS-activity, correlated well.

Platelets have previously been proposed as markers for global mitochondrial function in other neurodegenerative disorders [3, 16, 18, 21, 24] and a few previous studies analyzing peripheral blood cells in ALS patients are relevant to the present findings. One study reported altered Ca^{2+} metabolism, but no ETS complex alterations in ALS patient lymphocytes [9], and the other study suggested a decrease in enzymatic CI-activity per mg of protein in the same cell type. In the latter study the investigators also saw a correlation between disease duration and reduced enzymatic CI-activity [12]. In cybrids generated from ALS patients' platelets, altered mitochondrial ultrastructure and decreased enzymatic activity of CI per mg have been shown [28]. These findings could not be confirmed in this study, but a relation between disease stage and CI-activity was found (see below). Altered mitochondrial membrane potential in platelets from ALS patients has also been reported [25], which further strengthens the notion of mitochondrial dysfunction in blood cells from ALS patients. Recently, reduced expression of mtDNA encoded genes has been reported in PBMCs from ALS subjects, and a corresponding alteration in post mortem spinal cord tissue was seen [17].

When patients were grouped according to clinical disease stage as described above, a significant progressive decrease in CI-associated respiratory parameters (normalized to mitochondrial content) was seen in PBMCs with advancing disease stage, suggesting a progressive alteration in mitochondrial function with the progression of the disease. Interestingly, this recently suggested staging [23] is solely based on hallmark clinical events for the individual patient, without any biomedical markers or biometric data. The progressing mitochondrial impairment with higher staging seen in the current study suggests biomedical validity to this staging. However, it should be noted that no change in CI-activity per mitochondrial content was observed when the total group of 24 was considered. Albeit different at group level, no changes in CIV function at the level of mitochondria could be shown with progressing disease stage, which argues for caution when interpreting the results. However, CIV function in ALS patients remained lower than in controls for all disease stages indifferent of normalization to mitochondrial content or cells. Echaniz-Laguna et al. [10] and colleagues investigated temporal alterations in muscle mitochondrial function in ALS patients using repeated biopsies, and showed increased CI-activity per dry weight from first to second sampling. No correction for mitochondrial content was made, and thus it can be postulated that this increase is due to increase in mitochondrial content, similar to our findings in platelets with progressing disease stage. In the current study, no correlation between any measure of mitochondrial function and disease duration could be shown.

In contrast to analyzing enzymatic activities of isolated respiratory complexes, we used ex vivo measurements of integrated mitochondrial activity of living respiring cells either suspended in their normal physiological environment (patients' own blood plasma) or permeabilized cells, in a buffer mimicking intracellular conditions, to enable for control of substrate access. By supplying the mitochondria with either CI- or CII-substrates or both, the role of individual complexes of the integrated mitochondrial function is analyzed. This approach has several advantages compared to measurements of the enzymatic activity of individual complexes, where the physiological context and function of the mitochondria are disrupted.

There are differences in cellular metabolism and mitochondrial function between platelets and PBMCs, differences that are reflected in the findings of this study. We show reduced CIV activity normalized to mitochondrial content in ALS patient blood cells in both platelets and PBMCs but in PBMCs only statistically significant for one of the two markers used. Under physiological conditions, there are lower levels of the catalytic subunit of CIV in platelets than in PBMCs, and thus platelets could provide a more sensitive marker for CIV dysfunction [15]. Further, platelet mitochondria habitually operate at a higher percentage of their maximum capacity relative to PBMCs, and a failure to comply with energy requirements would possibly result in a signal of mitochondrial biogenesis affecting platelets as they are produced and released to the circulation [15]. Platelets and lymphocytes also differ due to their hematopoietic origin, and through the lack of nuclei in platelets. PBMCs have all transcription mechanisms intact and can respond to external stimuli and stressors affecting metabolism by more elaborate means.

We conclude that mitochondrial content is increased and mitochondrial CIV-function/mitochondria is impaired in peripheral blood cells of ALS patients, further supporting the proposition that ALS is a systemic disease affecting mitochondria in cells outside the CNS rather than being a strictly neurodegenerative disorder [1, 20].

Further investigations in larger cohorts are warranted to elucidate the pathophysiological significance of the observed changes in mitochondrial content and function in ALS and to evaluate the potential of mitochondrial function as a future candidate biomarker for ALS.

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Conflicts of interest Johannes Ehinger, Magnus J. Hansson and Eskil Elmér own shares in NeuroVive Pharmaceutical AB, a public company active in the field of mitochondrial medicine. Saori Morota, Johannes Ehinger, Magnus J. Hansson and Eskil Elmér received salary support from NeuroVive Pharmaceutical AB during parts of the study. Gesine Paul has no conflict of interest to disclose.

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