Mitochondrial and redox modifications in early stages of Huntington's disease

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ABSTRACT

Deficits in mitochondrial function and redox deregulation have been attributed to Huntington's disease (HD), a genetic neurodegenerative disorder largely affecting the striatum. However, whether these changes occur in early stages of the disease and can be detected *in vivo* is still unclear. In the present study, we analysed changes in mitochondrial function and production of reactive oxygen species (ROS) at early stages and with disease progression. Studies were performed *in vivo* in human brain by PET using [64Cu]-ATSM and *ex vivo* in human skin fibroblasts of premanifest and prodromal (Pre-M) and manifest HD carriers. *In vivo* brain [64Cu]-ATSM PET in YAC128 transgenic mouse and striatal and cortical isolated mitochondria were assessed at presymptomatic (3 month-old, mo) and symptomatic (6–12 mo) stages. Pre-M HD carriers exhibited enhanced whole-brain (with exception of caudate) [64Cu]-ATSM labelling, correlating with CAG repeat number. Fibroblasts from Pre-M showed enhanced basal and maximal respiration, proton leak and increased hydrogen peroxide (H_2O_2) levels, later progressing in manifest HD. Mitochondria from fibroblasts of Pre-M HD carriers also showed reduced circularity, while higher number of mitochondrial DNA copies correlated with maximal respiratory capacity. *In vivo* animal PET analysis showed increased accumulation of [64Cu]-ATSM in YAC128 mouse striatum. YAC128 mouse (at 3 months) striatal isolated mitochondria exhibited a rise in basal and maximal mitochondrial respiration and in ATP production, and increased complex II and III activities. YAC128 mouse striatal mitochondria also showed enhanced mitochondrial H_2O_2 levels and circularity, revealed by brain ultrastructure analysis, and defects in Ca^{2+} handling, supporting increased striatal susceptibility. Data demonstrate both human and mouse mitochondrial overactivity and altered morphology at early HD stages, facilitating redox unbalance, the latter progressing with manifest disease.

1. Introduction

Huntington's disease is an inherited neurodegenerative disorder

characterized by psychiatric, motor and cognitive symptoms, initially involving the striatum and later, other regions of the brain. Huntington's

disease platelet mitochondria) following a stress stimulus [18,19]. Evidences of redox deregulation were also found by us in STHdh^{Q111/Q111}

Abbreviations

BSA	Bovine serum albumin
CAG	Cytosine-Adenine-Guanine
EGTA	Ethylene glycol tetra-acetic acid
ETC	Electron transport chain
FCCP	Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone
FDR	False discovery rate
GPx	Glutathione peroxidase
GRed	Glutathione reductase
GSH	Glutathione, reduced form
GSSG	Glutathione, oxidized form
HD	Huntington's disease
H_2O_2	Hydrogen peroxide
HTT	Huntingtin
MCU	Mitochondrial calcium uniporter
mHTT	Mutant huntingtin
MIM	Mitochondrial inner membrane
mmp	Mitochondrial transmembrane potential
NaF	Sodium fluoride
NEM	*N*-ethylmaleimide
OCR	Oxygen consumption rate
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
STN	Subthalamic nucleus
SUV	Standardized uptake values
YAC	Yeast artificial chromosome

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disease is linked to a dynamic mutation (an expansion in CAG repeats) located in the exon 1 of the *HTT* gene which encodes for the huntingtin (HTT) protein [1]. Expression of mutant HTT (mHTT) has been associated with mitochondrial dysfunction, including reduced mitochondrial transmembrane potential, bioenergetic abnormalities, defects in Ca^{2+} handling, alterations in organelle morphology and neurite movement, and increased production of reactive oxygen species (ROS) [2–4] largely attributed to the symptomatic stages of Huntington's disease.

Evidence of mitochondrial bioenergetic dysfunction emerged from studies in post-mortem Huntington's disease striata demonstrating defects in activities of respiratory complexes (Cx) II, III and IV and aconitase [5–7]. Huntington's disease genetic models also showed mitochondrial defects, namely reduced levels and assembly of Cx II subunits, as observed in R6/1 and Htt171-82Q transgenic mice [8]. These studies supported that mitochondrial dysfunction could contribute to the hypometabolism and progressive atrophy of the caudate in Huntington's disease. No differences were observed in respiratory activity in YAC128 mouse brain synaptic and non-synaptic mitochondria and striatal neurons [9,10], or in isolated brain mitochondria and striatal neurons from R6/2 mice [11]. Conversely, we recently showed reduced respiratory profile and decreased mitochondrial membrane potential in YAC128 mouse cortico- and striatal neurons, and unexpected increased complexes activity in isolated striatal mitochondria from younger YAC128 mouse, at 3 mo [12]. In addition, we found reduced activity of complex I and citrate synthase in mitochondrial platelets from pre-manifest Huntington's disease carriers [13]. Results in skin fibroblasts from Huntington's disease patients also demonstrated a reduction in ATP levels, suggesting a deficit of mitochondrial oxidative metabolism [14,15]. A correlation between the age of onset and bioenergetic profile was reported in fibroblast cell lines, as patients with earlier disease onset displayed more severe mitochondrial defects, independent of CAG repeat number [14]. These data implicate imbalanced mitochondrial activity in Huntington's disease human peripheral cells.

Mitochondrial dysfunction and altered mitochondrial calcium accumulation are also interconnected. Previously, we observed increased Ca^{2+} loading capacity in brain mitochondria isolated from R6/2 and YAC128 mice [16], and reduced Ca^{2+} handling in YAC128 mouse striatal neurons following elevated cytosolic Ca^{2+} due to selective activation of *N*-methyl-ᴅ-aspartate receptors [17]. A close relationship between mitochondrial deregulation and excessive ROS levels, namely superoxide anion and hydrogen peroxide, was also previously demonstrated in Huntington's disease human cybrids (retaining Huntington's

striatal cells [20,21]. Other studies in Huntington's disease human skin fibroblasts and lymphoblasts showed increased ROS levels and upregulation of antioxidant enzymes (e.g. superoxide dismutase 2, glutathione reductase, catalase) [15,22,23]. We have also shown improvement in mitochondrial function and restoration to normal in affected mice when treated with a S1R agonist, pridopidine [12].

Despite several findings indicating deregulated mitochondrial function in Huntington's disease brain and peripheral cells, data is not totally consistent while *in vivo* mitochondrial related oxidative stress namely in living Huntington's disease patients requires further elucidation. The PET radioisotope [⁶⁴Cu]-ATSM ((64)Cu-labeled diacetyl-bis(N-(4)-methylthiosemicarbazone)) has been applied to visualize regional oxidative stress in patients with mitochondrial dysfunction-associated diseases [24–26]. [⁶⁴Cu]-ATSM accumulation occurs in electron rich environments, caused by mitochondrial impairment, as demonstrated in cells that are in overreduced states due to impaired mitochondrial electron transport chain (ETC) [27].

Therefore, to further elucidate the occurrence of mitochondrial dysfunction in Huntington's disease, in the present study we investigated redox changes linked to mitochondrial deregulation both in premanifest/prodromal *versus* manifest Huntington's disease patients and in YAC128 transgenic mouse model (at pre-/early-symptomatic to symptomatic stages) and its relationship with disease severity using [⁶⁴Cu]-ATSM. Furthermore, we correlated *in vivo* brain data with analysis of mitochondrial function, production of mitochondrial hydrogen peroxide (H_2O_2) and mitochondrial morphology in human fibroblasts (peripheral cells largely used in the study of Huntington's disease cytopathogenesis) and in isolated mitochondria derived from YAC128 mouse brain striatum and cortex at different stages of the disease to assess their relevance during disease progression.

2. Materials and methods

2.1. Human study

2.1.1. Participant's characterization

Patients and controls were selected according to clinical evaluation performed at Neurology consultation of *Centro Hospitalar e Universitário de Coimbra* (CHUC). Nine subjects were selected, six were mutant *HTT* gene carriers (Huntington's disease (HD) carriers) with ages ranging from 25 to 66 years-old, including two premanifest (pre-M1 and pre-M2), one prodromal (pre-M3) and three manifest (HD1-3), as defined by an expert neurologist, based on motor, psychiatric, and cognitive

symptoms [28], and three noncarriers/controls (C, CTR). Pre-M carriers had no signs or symptoms of Huntington's disease, while prodromal carriers had some symptoms but insufficient to make a definitive diagnosis of Huntington's disease. The age at onset was based on the date of clinical diagnosis, while the age at symptom onset was estimated on the information provided by the patient and the patient's family (Table 1). This study was performed after obtaining informed consent of all participants, according to the Declaration of Helsinki, and by following the guidelines of CHUC. The study was approved by the Ethic Committee of the Faculty of Medicine of the University of Coimbra (ref. CE-137/2015).

2.1.2. PET [^{64}Cu]-ATSM PET acquisition and processing

Preparation of [^{64}Cu]-ATSM was performed as described elsewhere [29]. Cyclotron produced [^{64}Cu]CuCl$_2$ solution was added to a reactor containing H2-ATSM in CH$_3$COONa 1 M. The mixture was purified in a Sep-Pak tC18 light. [^{64}Cu]-ATSM was obtained in a 10% ethanol saline sterile solution, with ≥99% of radiochemical purity and specific activity >200 GBq/μmol. A Philips Gemini GXL PET/CT scanner (Philips Medical Systems, Best, the Netherlands) was used to perform a dynamic 3-dimensional PET [^{64}Cu]-ATSM scan of the entire brain (90 slices, 2-mm slice sampling) and a low-dose brain CT scan, for attenuation correction. Antioxidant vitamin supplements were suspended 24 hprior to the scan.

To prevent possible head movement during acquisition, patients' head was restrained with a soft elastic tape. PET scan was acquired in a group of seven subjects (two control participants and five Huntington's disease carriers, who had clinical conditions and accepted to perform the PET study) over a period of 60 min (24 frames: $4 \times 15s + 4 \times 30s + 3 \times 60s + 2 \times 120s + 5 \times 240s + 6 \times 480s$) and started immediately after the intravenous bolus injection of approximately 925 MBq of [^{64}Cu]-ATSM. PET data were reconstructed using a LOR-RAMLA algorithm, with attenuation and scatter correction. Standard uptake value (SUV) images were calculated offline (with in-house written Matlab® quantification software) for each participant. PET and MRI data were normalized to Montreal Neurological Institute (MNI) space, the same geometric transformation was used, after the PET scan had been rigidly co-registered with the correspondent anatomical MRI scan (using SPM toolbox with default parameters; standard MRI brain was used when subject's MRI were not available). An exploratory whole-brain and region-of-interest (ROI)-based analyses were performed. Data was exported for a set of distinct regions of interest including the cerebellum, basal ganglia and subregions (putamen, caudate and subthalamic nucleus) (regions known to be affected in Huntington's disease patients). The SUV image was calculated per subject using the vessels uptake values as reference. Vessels uptake were defined as the average of the max uptake values over the first 3 min of the dynamic acquisition. Individual time-activity curves for the different ROIs and the uptake sum

were determined during 10–26 min of acquisition.

2.2. In vivo animal study

2.2.1. Animal's characterization

YAC128 transgenic hemizygous (line HD53; 128 CAG repeats) and littermate non-transgenic wild-type (WT) mice (FVB/N background) males and females, with 3 (nWT = 12; nYAC = 12), 6 (nWT = 14; nYAC = 11), 9 (nWT = 16; nYAC = 16) and 12 (nWT = 5; nYAC = 12) months of age (mo) were used, divided throughout the different experiments. All animals were generated from our local colony, with breeding couples generously provided by Dr. Michael Hayden (University of British Columbia, Vancouver, Canada). All animals were genotyped by common procedures as described in "Supplemental Methods" section. YAC128 mice exhibited an age-dependent gain of body weight (Fig. S1A), as described previously [30,31]. All studies were carried out in accordance with the guidelines of the Institutional Animal Care and Use of Committee and the European Community directive (2010/63/EU) and protocols approved by the Faculty of Medicine, University of Coimbra (ORBEA_189_2018/11,042,018).

2.2.2. [^{64}Cu]-ATSM PET/MRI acquisitions

Animal PET acquisitions were performed by using the radiopharmaceutical [^{64}Cu]-ATSM in YAC128 and WT mice at 3, 6, 9 and 12 mo. In all PET scans a prototype of a high-acceptance small-animal PET based on resistive plate chambers (RPC-PET) was used [32]. A mean activity of 418 ± 85 kBq/g was injected. The PET acquisition lasted for 60 min post injection. Volumetric images were reconstructed using OSEM algorithm and cubic voxel of 0.5 mm width. Two structural volumetric MRI T2 images were acquired per mouse using a MRI scan with the objective to facilitate the registration (geometric alignment) of the functional volumetric PET images with the volumetric MRI images, and thus allowing the segmentation of the regions of interest. A fiducial marker that can be viewed both in the PET and MRI imaging was placed in the mice bed. MRI imaging was performed after PET without moving the mice from the bed. Mice were kept anesthetized by isoflurane (1–2%) with 100% O$_2$ with body temperature and respiration monitoring (SA Instruments SA, Stony Brook, USA). All MRI experiments were performed in a BioSpec 9.4T MRI scanner (Bruker Biospin, Ettlingen, Germany) with a volume head coil. High resolution morphological images were acquired with a 2D T2-weighted turbo RARE sequence and axial slice orientation. The sequence for fiducial marks had the following parameters: TR∕TE = 8372∕33 ms, FOV = 25.6 × 25.6 mm, acquisition matrix = 256 × 256, averages = 1, rare factor = 8, echo spacing = 11 ms, 80 axial continuous slices with 0.4 mm thick and acquisition time of 4min28s. The sequence for head mouse had the following parameters: TR∕TE = 4500∕33 ms, FOV = 25.6 × 25.6 mm, acquisition matrix = 256 × 256, averages = 8, rare factor = 8, echo

Table 1:
Clinical data of Huntington's disease carriers and patient's and control groups.

	Code	CAG repeat number	Sex	Calendar age, y	Disease duration, y	Age at clinical diagnosis, y	Age at symptom onset, y	Symptom at onset	Symptoms UHDR
Control	C1	23	F	25	n.a.	n.a.	n.a.	n.a.	n.a.
	C2	25	M	46	n.a.	n.a.	n.a.	n.a.	n.a.
	C3	23	M	47	n.a.	n.a.	n.a.	n.a.	n.a.
Premanifest & Prodromal/early HD	Pre-M1	46	M	25	n.a.	22	n.a.	n.a.	2
	Pre-M2	42	M	30	n.a.	29	n.a.	n.a.	n.a.
	Pre-M3	52	F	29	7	28	22	cognitive	24
Manifest HD	HD1	43	M	62	22	47	40	psychiatric	30
	HD2	42	F	66	19	54	47	psychiatric	33
	HD3	46	F	43	7	39	36	psychiatric	72

Abbreviations: F- female; M − male; n.a.- not applied; C- controls; pre-*M*- premanifest Huntington's disease carriers; HD- Manifest Huntington's disease patients.

spacing = 11 ms, 42 axial continuous slices with 0.4 mm thick and acquisition time of 19min12s.

2.2.3. [^{64}Cu]-ATSM uptake quantification

Based on the fiducial marker, the volumetric images obtained from the PET were manually registered by the volumetric MRI images using the 3D Slicer 4.4 software. The volumetric MRI images were manually segmented using the ITK-SNAP 2.2 software and the segmentation results applied to the registered volumetric PET images. The mean counts per mm^3 in each ROI were computed and normalized by the activity injected per gram. Only the counts between 20- and 60-min post injection were considered.

2.3. In vitro human and animal study

2.3.1. Cell culture

Fibroblasts from Huntington's disease carriers and controls were generated from a small skin sample (about 3 mm diameter) from the abdominal region via a punch biopsy, as described previously [33] (see "Supplemental Methods" for detailed description). Fibroblasts were cultured in DMEM medium (Gibco), supplemented with 15% FBS (Gibco) and 1% penicillin/streptomycin (Gibco) and maintained to a maximum of 15 passages.

2.3.2. Isolation of functional mitochondria

Mice were weighted and then sacrificed by cervical dislocation and decapitation. Brains were removed from the skull and washed once in PBS containing (in mM): 137 NaCl, 2.7 KCl, 1.8 KH$_2$PO$_4$, 10 Na$_2$H-PO$_4$·2H$_2$O, pH 7.4, followed by striatum and cortex dissection. Striatal and cortical mitochondrial-enriched fractions were then isolated using discontinuous Percoll density gradient centrifugation as previously described [34], and detailed in "Supplemental Methods".

2.3.3. Bioenergetic assay

The fibroblasts oxidative phosphorylation and glycolytic profile were obtained by measuring the oxygen consumption rate (OCR) and extracellular acidification ratio (ECAR) on a Seahorse XF24 or XF96 apparatus. OCR was also analysed in fresh striatal and cortical mitochondria isolated from YAC128 *versus* WT mice in coupling and uncoupling conditions by using a Seahorse XF24 apparatus (see "Supplemental Methods" for detailed description).

2.3.4. Measurement of cellular and mitochondrial hydrogen peroxide levels

Mitochondrial or cellular H$_2$O$_2$ levels were measured in fibroblasts using mitochondria peroxy yellow 1 (MitoPY1) (Sigma) or Amplex Red reagent and horseradish peroxidase from the Amplex® Red Catalase Assay Kit (Molecular Probes), respectively. H$_2$O$_2$ production by YAC128 and WT striatal and cortical mitochondria was measured by the Amplex® Red method. Detailed in "Supplemental Methods".

2.3.5. Transmission electron microscopy

For transmission electron microscopic (TEM) ultrastructural analyses fibroblasts were pelleted by centrifugation and striatum dissected out from 3 mo WT and YAC128 mice brain and processed as detailed in "Supplemental Methods".

2.3.6. Genomic DNA extraction and mitochondrial DNA copies

Genomic DNA was isolated from fibroblasts by means of high salt/ethanol precipitation. All DNA samples were considered pure when A260/A280 ratio was comprised between 1.8–2.0.

To quantify the average mitochondrial DNA (mtDNA) copy number of the fibroblasts the Absolute Human Mitochondrial DNA Copy Number Quantification qPCR Assay Kit (ScienCell Research Laboratories) was used according to manufacter protocol. Briefly, a mtDNA primer set recognizes and amplifies human mtDNA and another reference primer set recognizes and amplifies a 100 bp-long region on human chromosome 17 and serves as reference for data normalization. The reference genomic DNA sample with known mtDNA copy number serves as a reference for calculating the mtDNA copy number of target samples.

2.3.7. Mitochondrial Ca^{2+} handling capacity

Mitochondrial Ca^{2+} uptake was measured fluorimetrically using the Ca^{2+}-sensitive fluorescent dye Calcium Green-5N (150 nM; excitation and emission wavelengths of 506 nm and 532 nm, respectively) that exhibits an increase in fluorescence emission intensity upon binding to Ca^{2+} as described elsewhere [35] with minor modifications [34]; thus, a decrease in the Calcium Green fluorescence indicates the capacity of mitochondria to handle Ca^{2+}. Briefly, 5 µg of YAC128 and WT striatal or cortical isolated mitochondria was added to the incubation medium containing 125 mM KCl, 0.5 mM MgCl$_2$, 3 mM KH$_2$PO$_4$, 10 mM Hepes, 10 µM EGTA, supplemented with 3 mM pyruvate, 1 mM malate, 3 mM succinate, 3 mM glutamate, 0.1 mM ADP and 1 µM oligomycin, pH 7.4. After a basal fluorescence record, six pulses of 10 µM CaCl$_2$ were added every 4 min. Data are presented as traces of calcium handling capacity and plotted as extramitochondrial calcium (Extra mitoCa) for the third pulse of added calcium, as described in figure legend.

2.4. Additional methods

See *Supplementary Materials* for more comprehensive description of the mentioned methods and details on genotyping, measurement of glutathione levels and glutathione peroxidase and glutathione reductase activities.

2.5. Statistical analyses

Statistical computations were performed using GraphPad Prism version 7.0, GraphPad Software, La Jolla, CA, USA, and SPSS version 21.0 (IBM SPSS Statistics for Windows, IBM Corp). Statistical analysis was performed for individual subjects and among groups (controls, Pre-M and HD/manifest patients). For patients/controls data statistical calculations an One-Way ANOVA analysis was computed followed by a two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli to correct for multiple comparisons by controlling the False Discovery Rate. For fibroblasts experiments at least three independent assays were performed for each experimental condition. Statistical significance was analysed using parametric test, two-way ANOVA, followed by Bonferroni post-hoc test and non-parametric test Kruskal Wallis followed by Dunn's multiple comparison test. Correlations were done using the Spearman rank correlation coefficient (ρ). The power analysis approach to sample size determination was done using G power software on effect size (mean/standard deviation within groups) for 1–beta = 0.8 and alpha = 0.05. The results obtained from the animal's experiments are expressed as the mean ± SEM of the number of replicates, considering the number of animals indicated in the figure legends. Excluded outliers were defined at GraphPad Prism 8.0.2 analyses. Comparisons among multiple groups were performed by two-way ANOVA followed by Tukey's post-hoc test. Comparison between two populations, as described in figure legends was performed by nonparametric Mann Whitney *U* test. Significance was defined as p ≤ 0.05.

2.6. Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

3. Results

3.1. Clinical data

From the selected nine participants for this study, six were *HTT* gene mutation carriers, classified as premanifest and prodromal (Pre-M) and

manifest HD (Table 1). Pre-M carriers had no clinical motor signs or symptoms insufficient to make the diagnosis of Huntington's disease, based on standardized total motor score of Unified Huntington Disease Rating Scale (UHDRS) [36]. Huntington's disease carriers had CAG repeats ranging from 42 to 52; the prodromal patient exhibited the longest repeat expansion and an anticipated phenotypic onset, compared to age-matched Huntington's disease carriers. Manifest HD patients showed symptoms and signs of Huntington's disease. Furthermore, symptoms at onset varied from psychiatric (manifest HD patients) and cognitive (prodromal Huntington's disease carrier) symptoms.

3.2. Increased accumulation of [64Cu]-ATSM in premanifest Huntington's disease carriers

Whole brain and regional PET [64Cu]-ATSM retention in the SUV images was compared between groups (Huntington's disease carriers, Pre-M and HD, and controls) using statistical parametric mapping and ROI analysis. Increased accumulation of [64Cu]-ATSM was observed in the Pre-M Huntington's disease carriers, when compared to the control group in brain mask, cerebellum and subthalamic nucleus (STN) ($p < 0.05$). The exception was the caudate as the SUV decreased with disease severity ($p = ns$), suggesting neuronal degeneration (Fig. 1A–C). Spearman correlation analyses showed that [64Cu]-ATSM brain accumulation in whole brain and STN directly correlates with CAG repeat number ($\rho = 0.709$, $p = 0.074$; $\rho = 0.746$, $p = 0.05$, respectively) (Fig. 1D and E).

Enhanced [64Cu]-ATSM complex entrapment and labelling was observed in the majority of brain areas in Pre-M Huntington's disease carriers, which could be related with increased intracellular

overreductive state due to excessive release of electrons derived from modified mitochondrial respiratory chain, as previously described [24, 25,27,37].

3.3. Altered mitochondrial function and redox deregulation in premanifest/prodromal Huntington's disease carriers

To test whether [64Cu]-ATSM PET labelling could be related with modified mitochondrial function and enhanced production of ROS, potentially revealing an overreduced intracellular state, and while mitochondrial respiratory chain activity cannot be directly measured in human brains, we assessed the changes in mitochondrial function and ROS levels in human skin-derived fibroblasts obtained from the same controls and Huntington's disease mutation carriers (Pre-M and HD), detailed in Table 1.

Comparisons of oxygen consumption rates (OCR) between groups showed that basal and maximal respiration are enhanced in Pre-M *versus* controls, accompanied by increased H$^+$ leak ($p < 0.05$), as measured indirectly based on respiration measurements (Fig. 2A–D). By contrast, manifest HD patients demonstrated a general decrease in basal (*vs* controls, $p < 0.01$), maximal and spare respiration and H$^+$ leak (*vs* Pre-M, $p < 0.05$) (Fig. 2A–D). No significant differences in ATP production (Fig. 2A–D), ATP/ADP ratio or energy charge (Fig. S2) were found in Huntington's disease carriers, when compared to the control group. The observed increase in H$^+$ leak in Pre-M cells (above control conditions) with no accompanying rise in ATP synthesis is suggested to be responsible for the increase in respiration.

Interestingly, we observed a strong and statistically significant inverse correlation between individual disease duration, age of symptoms
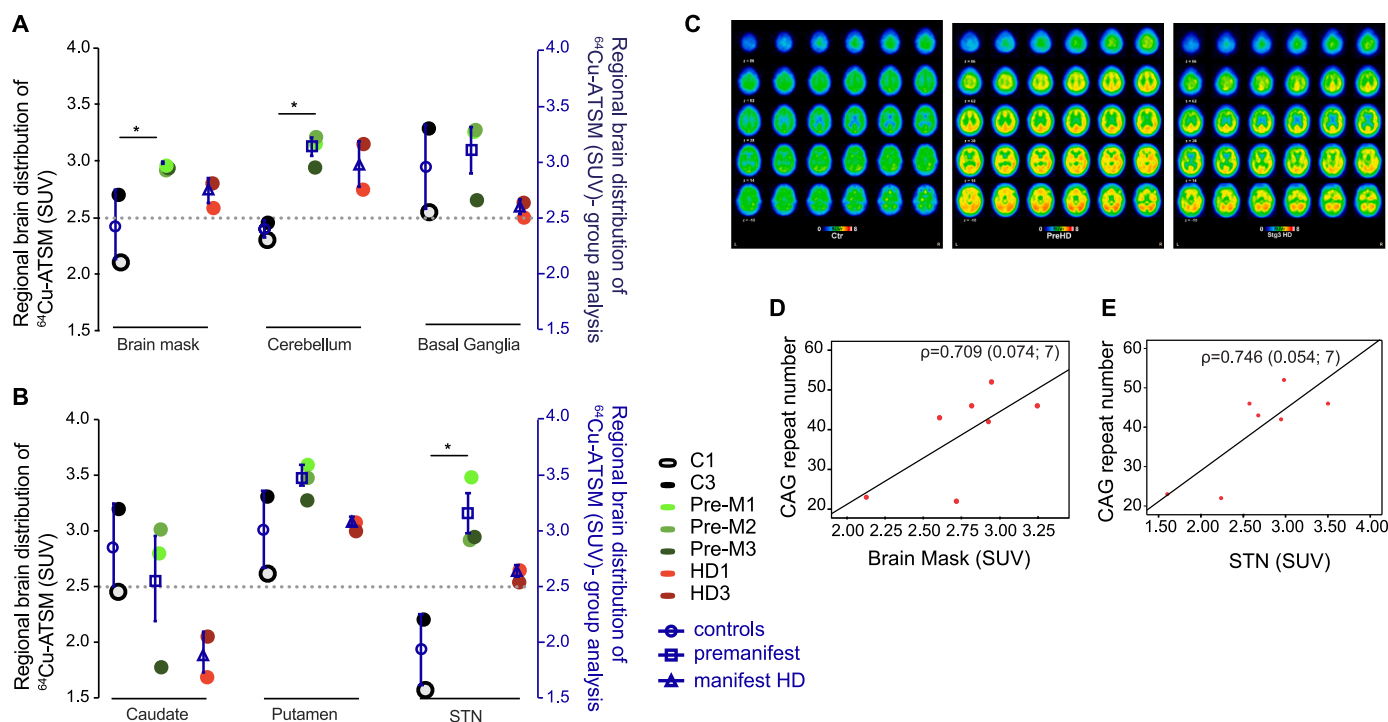

Fig. 1. **[64Cu]-ATSM brain accumulation in premanifest and manifest Huntington's disease patients and correlation with CAG repeats.** [64Cu]-ATSM brain PET imaging of Huntington's disease carriers and controls was evaluated in cerebellum and basal ganglia **(A)** and basal ganglia subregions: caudate, putamen and subthalamic nucleus (STN) **(B)** of controls (C), premanifest (Pre-M) and manifest Huntington's disease patient's (HD) brains, as described in Methods section. **(C)** Representative axial slices of PET imaging of the three groups analysed (left to right): controls, premanifest and manifest Huntington's disease carriers; PET color scale indicates the uptake of the [64Cu]-ATSM normalized for cerebral blood vessels SUVs. Correlation between CAG repeat number and normalized SUV of [64Cu]-ATSM uptake from total brain (brain mask) **(D)** and uptake from STN **(E)**. Regional brain distribution of [64Cu]-ATSM **(A)(B)** was calculated as the sum of three time-points SUVs between 14 and 22 min acquisition time; mean ± SEM values of the groups are plotted on the secondary Y axis (blue). **(D)(E)** Correlation was performed using the Spearman correlation coefficient ρ (sig; n). SUV - standardized uptake values. Statistical analysis: One-Way ANOVA corrected for multiple comparisons by false discovery rate (FDR) using two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli. *$p < 0.05$ (controls vs premanifest). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
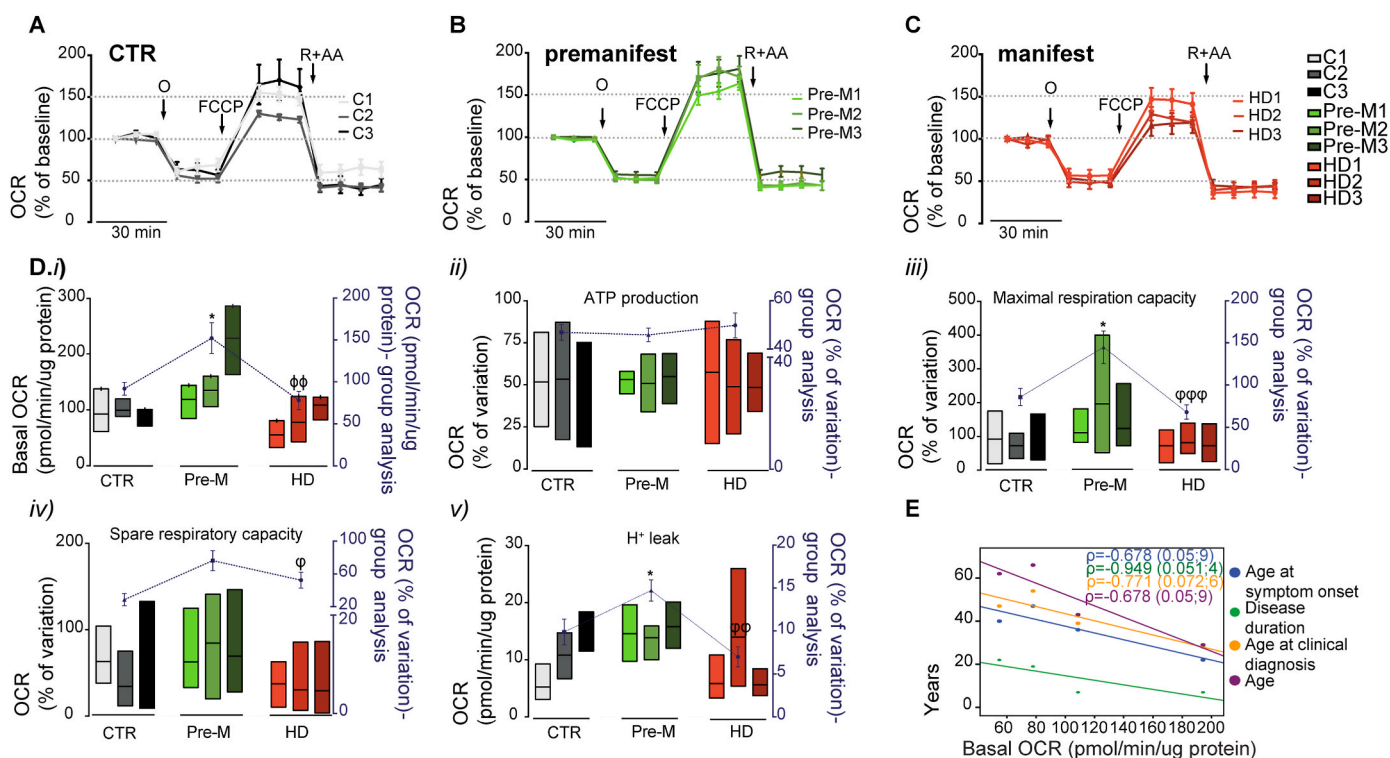
Fig. 2. Oxygen consumption rate (OCR) in skin fibroblasts from premanifest and manifest Huntington's disease carriers and controls. (A–C) OCR of cells treated with the different compounds. The mitochondrial inhibitors were sequentially injected into different ports of the Seahorse XF24 analyzer and the final concentrations of each were: 1 μM oligomycin (O), 0.3 μM FCCP (F), 1 μM rotenone and 1 μM antimycin A (R + A). **(D)** (i) Levels of basal OCR; (ii) Oxygen consumed for ATP generation through the complex V; (iii) Maximal respiration capacity; (iv) Spare respiratory capacity; (v) Component of OCR representing passive H$^+$ leakage across the mitochondrial inner membrane were calculated as described in Methods section; individual data are presented as floating bars (min, max) with mean lines shown at least three independent experiments; group analysis represents the mean ± SEM values (controls (C, CTR), premanifest (Pre-M) and manifest HD patients) and are plotted on the secondary Y axis (blue). **(E)** Correlation was performed using the Spearman correlation coefficient ρ (sig; n). Statistical analysis: One-Way ANOVA – post hoc Bonferroni's multiple comparisons test. *p < 0.05 (controls vs premanifest); ^{φφ}p<0.01 (controls *vs* HD manifest) and ^{φ}p<0.05, ^{φφ}p<0.01, ^{φφφ}p<0.001 (premanifest *vs* HD manifest). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

onset and chronological age with basal OCR. Longer disease duration (ρ = −0.949, p = 0.05), more advanced age at symptom onset (ρ = −0.678, p = 0.05) and at clinical diagnosis (ρ = −0.770, p = 0.07) and older age (ρ = −0.678, p = 0.05), the later corresponding to two Huntington's disease manifest patients, was accompanied by lower absolute values of basal respiration (Fig. 2E).

We also analysed glycolytic parameters by assessing extracellular acidification rates (ECAR) in human skin fibroblasts derived from the same individuals. Data showed no major differences between groups, except for the glycolytic capacity of manifest HD patients that had lower absolute values, when compared to controls (p < 0.05) and to Pre-M Huntington's disease carriers (p < 0.01) (Fig. S3).

Because mitochondrial dysfunction is largely associated with increased of ROS production, potentially triggering an overreduced state associated with [^{64}Cu]-ATSM accumulation, the relative levels of mitochondrial and cellular-derived H$_2$O$_2$ were measured in human skin fibroblasts using MitoPY1 and Amplex Red, respectively (Fig. 3). We found significantly higher levels of cellular H$_2$O$_2$ in fibroblasts derived from Pre-M carriers, which were further elevated with disease progression (Fig. 3A). Interestingly, a significant rise in basal levels of mitochondrial H$_2$O$_2$ (mito-H$_2$O$_2$) was observed in more advanced stages of Huntington's disease (Fig. 3B). Following complex III inhibition with myxothiazol (3 μM), the levels of mito-H$_2$O$_2$ increased with disease severity, being significantly higher in cells derived from manifest HD patients *versus* controls (p < 0.05) (Fig. 3C). Correlation analysis showed augmented cellular H$_2$O$_2$ levels in Huntington's disease carriers with longer CAG repeat number (ρ = 0.769, p = 0.05), and a lower uptake of [^{64}Cu]-ATSM in the caudate was associated with increased cellular H$_2$O$_2$

levels (ρ = −0.714, p = 0.071) (Fig. 3D and E).

These data indicate early increased mitochondrial respiration in Pre-M carriers that is not accompanied by ATP production, but rather promotes redox dysregulation through enhanced cellular ROS levels, which is further enhanced in cells from manifest HD.

3.4. Premanifest and prodromal Huntington's disease carriers show decreased circularity and increased mitochondrial DNA copies correlated to higher [^{64}Cu]-ATSM uptake

Because mitochondrial dysfunction has been linked to changes in mitochondrial morphology, we analysed the morphometric parameters of mitochondria that were individually traced from TEM images obtained in human skin fibroblasts (Fig. 4A). We measured the major mitochondrial shape factors, namely area, circularity (an index of sphericity), Feret's diameter, perimeter, form factor, and aspect ratio (Fig. 4B). Mitochondria from Pre-M Huntington's disease carriers show decreased circularity, are more elongated (higher Feret's diameter), have more complex and branched networks and larger aspect ratios (length/width ratio) when compared with the control or manifest HD patients (Fig. 4A and B; Suppl. Table 1) (p < 0.05). Decreased mitochondrial circularity in Pre-M fibroblasts may indicate that mitochondria are less fragmented. However, slight increased circularity was observed in fibroblasts from patients with manifest HD, when compared to the controls and Pre-M carriers. Fibroblasts from Pre-M Huntington's disease carriers showed a higher absolute number of mtDNA copies, although non-significant when compared to CTR (371,460 ± 59,452 vs 216,717 ± 43,034), whilst manifest HD patients showed reduced
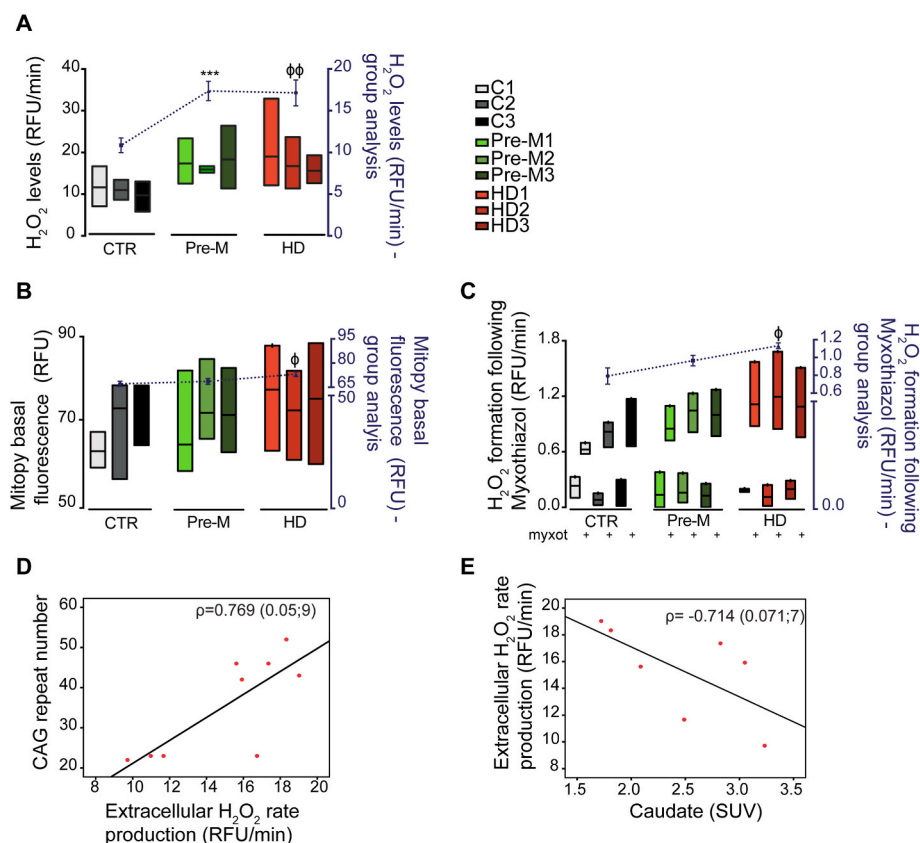
Fig. 3. Cellular and mitochondrial ROS levels in skin fibroblasts from Huntington's disease patient's and controls - correlation with CAG repeats and [64Cu] ATSM uptake in caudate. Cellular-derived **(A)** and basal mitochondrial **(B)** H_2O_2 production were measured as described in Methods section and was plotted as bars graph. **(C)** Time-dependent H_2O_2 production in the mitochondria after stimulation with complex III inhibitor-myxothiazol (3 μM). Individual data are presented as floating bars (min, max) with mean lines shown at least 3 independent experiments; group analysis represents the mean ± SEM values (controls (C, CTR), pre-symptomatic (Pre-M) and manifest HD patients) and are plotted on the secondary Y axis (blue). **(D)(E)** Correlation was performed using the Spearman correlation coefficient ρ (sig; n). Statistical analysis: non-parametric Kruskal-Wallis test followed by Dunn's Multiple Comparison Test. ***$p < 0.001$ (controls *vs* premanifest); and [φ]$p<0.05$, [φφ]$p<0.01$ (controls *vs* HDs). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

mtDNA copy number, when compared to cells from Pre-M (147,490 ± 28,476 vs 371,460 ± 59,452, $p < 0.05$) (Fig. 4C). Furthermore, we observed that human skin fibroblasts with higher number of mitochondrial DNA copies have significantly greater spare (ρ = 0.867, $p = 0.01$) and maximal respiratory capacity (ρ = 0.783, $p = 0.05$). In addition, patients with longer disease duration have a lower number of mtDNA copies (ρ = −0.949, $p = 0.041$). Interestingly, enhanced mtDNA copy number was positively correlated with augmented [64Cu]-ATSM uptake in whole brain (ρ = 0.786, $p = 0.036$), cerebellum (ρ = 0.679, $p = 0.094$), basal ganglia (ρ = 0.75, $p = 0.052$) and putamen (ρ = 0.857, $p = 0.014$). These data may suggest that Pre-M Huntington's disease carriers showing higher number of mtDNA copies in peripheral cells also have higher accumulation of [64Cu]-ATSM in selected brain areas.

3.5. Enhanced [64Cu]-ATSM brain accumulation in YAC128 mouse brain

In order to verify whether changes in [64Cu]-ATSM brain accumulation correlate with mitochondrial changes in affected areas in Huntington's disease brain, we further analysed *in vivo* [64Cu]-ATSM accumulation by PET and studied mitochondrial fractions isolated from the striatum and cortex of YAC128 mice along aging.

In vivo age-dependent analysis of intracellular overreductive status was also evaluated in YAC128 *versus* wild-type mice over the progression of the disease, at 3 (pre-symptomatic), 6 (early-symptomatic) and 9 and 12 (symptomatic) months of age (mo), through the analysis of [64Cu]-ATSM accumulation in the striatum (Fig. 5A and B) and frontal cortex (Fig. 5A,C). Statistical analysis by two-way ANOVA revealed a significant genotype (F(1,35) = 12.07; $p = 0.0014$) and age-dependent (F(3,35) = 36.67; $p < 0.0001$) [64Cu]-ATSM accumulation in the striatum (Fig. 5B). *In vivo* data obtained in the cortex also demonstrated an age-dependent effect (F(3,38) = 12.61; $p < 0.0001$) and a slight, but significant, genotype effect (F(1,38) = 4.192; $p = 0.0476$) in [64Cu]-

ATSM accumulation (Fig. 5Ci). In addition, when [64Cu]-ATSM accumulation in YAC128 mouse striatum or cortex were replotted in relation to the respective WT mice, a significant increase in the radioligand accumulation was observed in the striatum of 6 mo ($p < 0.05$) and a trend at 12 mo ($p = 0.0571$) (Fig. 5Bii), but not in the cortex (Fig. 5Cii).

3.6. Increased oxygen consumption rate, complexes II and III activities and oxidative status in striatal mitochondria derived from pre-symptomatic YAC128 mice

The respiratory activity in striatal and cortical isolated mitochondrial-enriched fractions derived from YAC128 and WT mouse brain striatum and cortex was analysed by determining OCR and complexes activities (Fig. 6 and S4). Notably, both wild-type HTT and mHTT were shown to associate with cortical- and striatal-enriched isolated mitochondria derived from WT and YAC128 mice, respectively (Fig. S1C).

Coupling experiments performed under Cx II feeding (in the presence of succinate) and Cx I inhibition (with rotenone) in striatal mitochondria obtained from 3 mo YAC128 mice showed increased basal and maximal respiration and ATP production, but unchanged H^+ leak, (Fig. 6Ai), when compared to WT mice. In order to further explain these changes in OCR, we analysed the electron flow through mitochondrial respiratory chain, to evaluate Cx I-IV activities under conditions of direct mitochondrial feeding (pyruvate, to generate acetyl-CoA) plus malate (to activate the TCA cycle) in the FCCP-induced uncoupled state.

In agreement with results obtained in Fig. 6Ai, striatal mitochondria obtained from 3 mo YAC128 mice exhibited a significant increase in Cx II and III activities, but unaltered activities in Cx I and IV (Fig. 6Aii), when compared with WT mouse mitochondria. No significant changes in mitochondrial-coupled respiration parameters or Cx I-IV activities were observed in cortical mitochondria (at 3, 6, 9 and 12 mo) (Fig. 6 Bi and Bii; Fig. S4Bi and S4Bii) or striatal mitochondria obtained from older
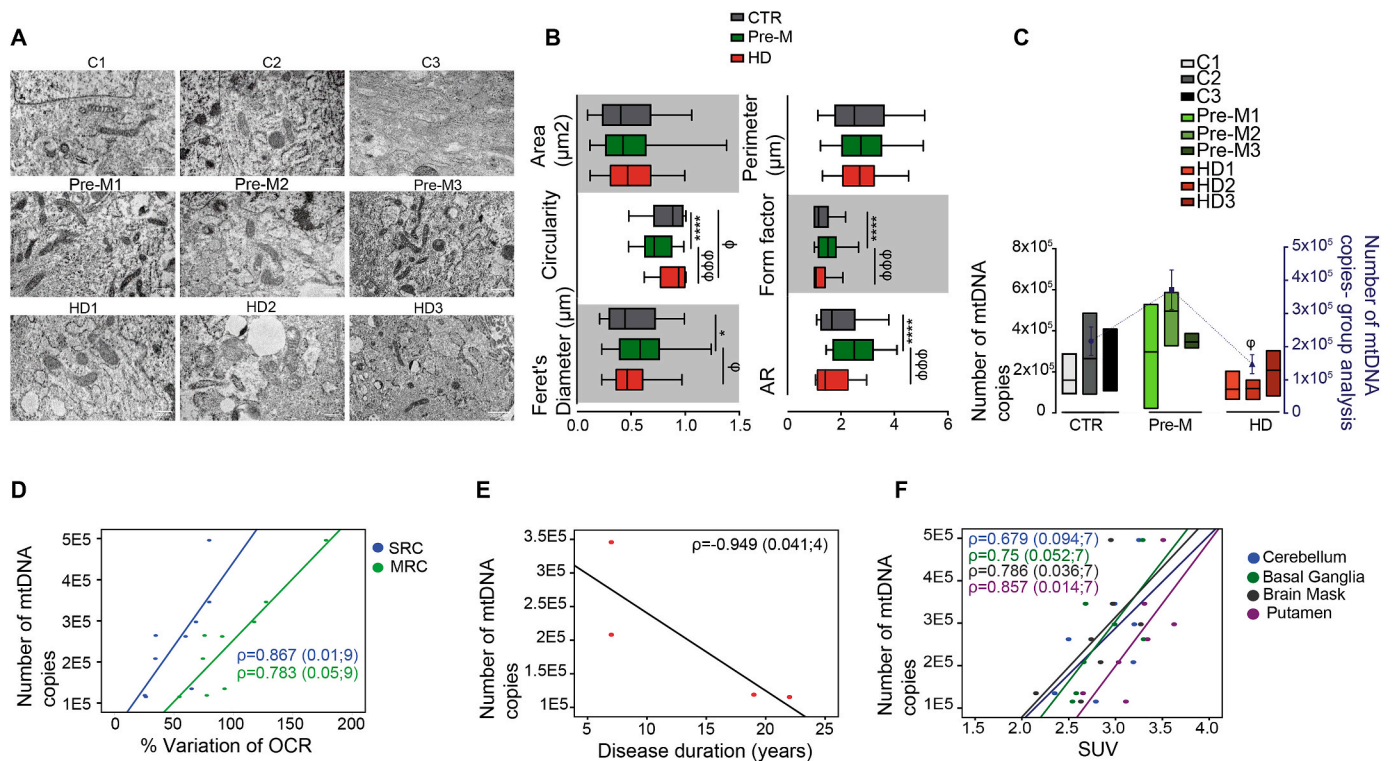
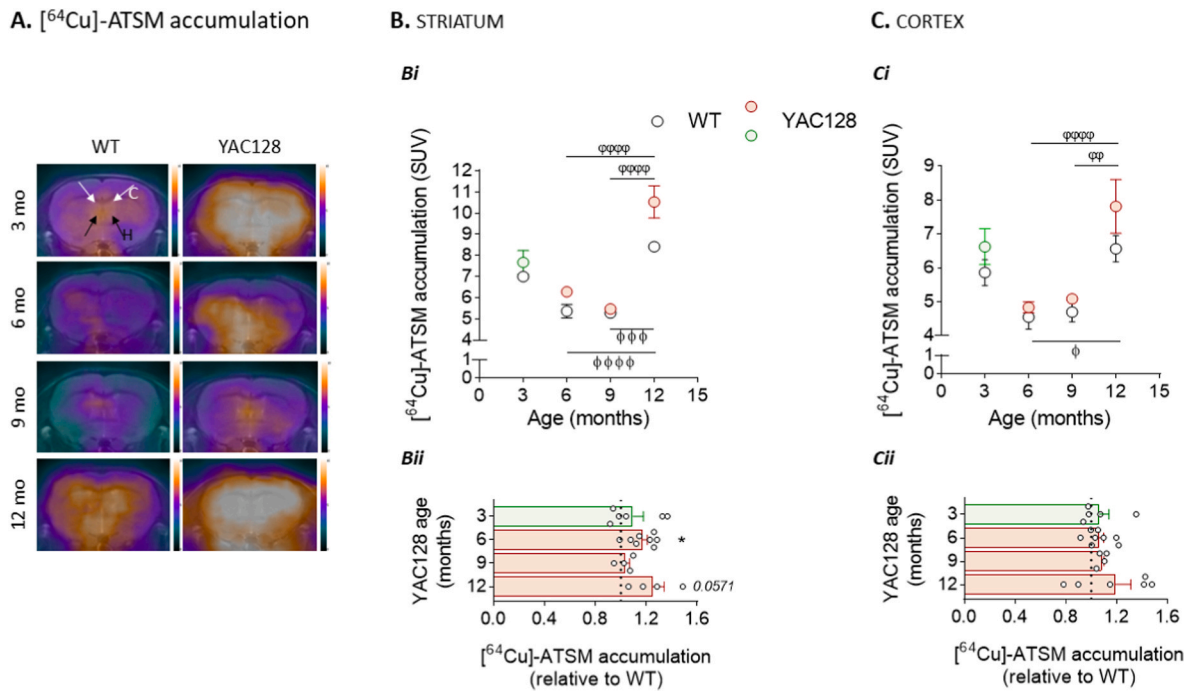**Fig. 5. Age-dependent [64Cu]-ATSM brain accumulation in YAC128 and WT mouse striatum and cortex.** [64Cu]-ATSM PET and MRI overlapped axial images of brain YAC128 and WT mice where PET color scale indicates the uptake (number of counts per mm^3) of the [64Cu]-ATSM normalized by the injected activity, mass of the mouse and sensibility of the PET equipment. White arrows point to frontal cortex and black arrows to striatum (**A**). [64Cu]-ATSM accumulation was plotted for striatum (**B**) and cortex (**C**) at 3, 6, 9 and 12 mo YAC128 vs WT mice and in relation to WT for each age, for striatum (***Bii***) and cortex (***Cii***). Results are presented in relation to the unit (dashed line) representing WT mouse values. Data are the mean ± SEM of acquisitions from 4 to 8 mice per group. Statistical analysis: φp<0.05, φφφp<0.001, φφφφp<0.0001, when compared between ages in WT mitochondria and φφp<0.01, φφφφp<0.0001 when compared between ages in YAC128 mitochondria by Two-way ANOVA followed by Sidak's multiple comparisons test. *p < 0.05 when compared with 6 mo WT mitochondria by nonparametric Mann-Whitney *U* test. SUV - standardized uptake values. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

derived from pre-symptomatic YAC128 mice, exhibiting lower Ca^{2+} thresholds after repeated Ca^{2+} loads. Ca^{2+} taken up by WT and YAC128 striatal-derived mitochondria was shown to be released through a FCCP-induced "releasable pool" (Fig. 7B*ii*) and prevented by pre-incubation with the mitochondrial Ca^{2+} uniporter (MCU) inhibitor, Ru360 (Fig. 7B*ii*). This indicates that Ca^{2+} retention occurred through the MCU in polarized mitochondria. Similar results were observed in cortical mitochondria (data not shown).

In order to investigate YAC128 mouse striatal mitochondrial morphology, electron micrographs obtained by TEM of 3 mo YAC128 and WT mouse striatum were assessed using quantitative measures for mitochondrial morphology (Fig. 7C). Over 154 individual mitochondria were assessed for area, perimeter, aspect ratio and circularity (Fig. 7D). YAC128 mouse striatal mitochondria showed significantly increased area and perimeter, along with decreased aspect ratio and increased circularity, when compared to WT mitochondria.

These observations suggest deregulated YAC128 mouse striatal mitochondria at presymptomatic disease stage.

4. Discussion

This work is the first to demonstrate higher accumulation of [64Cu]-ATSM in premanifest and prodromal (Pre-M) Huntington's disease human carriers and in an pre-symptomatic animal model in different brain regions. However, in human caudate, a region reported to be affected in the prodromal phase and showing progressive atrophy along the course of the disease due to neuronal loss [38–40], a progressive reduction in the mean SUV was observed. Our results also show that fibroblasts from Pre-M carriers and striatal mitochondria derived from pre-symptomatic YAC128 mice exhibit increased mitochondrial H_2O_2 levels, which is unrelated to decreased antioxidant levels or activity, but rather with an apparently compensatory enhanced activity of

mitochondrial respiratory chain and altered organelle morphology.

Classically, disease burden is defined based on CAG repeat length and age. In this study we show a correlation between imaging of the whole brain and particularly the STN and the number of CAG repeats. The STN is a key component of the basal ganglia and several reports, in animal models, argue that STN dysfunction and neuronal loss precede cortico-striatal abnormalities in Huntington's disease, which might contribute to motor impairment, and explain the greater accumulation of [64Cu]-ATSM in Pre-M individuals [42,43].

Although the copper uptake mechanism is still controversial, high levels of [64Cu]-ATSM retention appear to be indicative of an over-reductive state [27]. Colombié and co-authors postulated that [64Cu]-ATSM uptake is linked to redox potential and ROS, potentially constituting a promising marker of intracellular overreduced states for disorders with mitochondrial dysfunction [41]. Nevertheless, the effectiveness of [64Cu]-ATSM for hypoxia mapping has been demonstrated in several *in vitro* and *in vivo* pre-clinical models [37,41]. In the present study the overreduced microenvironment found in Pre-M Huntington's disease carriers was correlated to an overall increase in basal and maximal mitochondrial respiration in skin fibroblasts. Nevertheless, ATP synthesis remained unaffected, probably due an increase in H^+ leak. The glycolysis parameters did not differ among the three groups, except for the glycolytic capacity that was decreased in manifest HD only, suggesting a secondary process to disease progression. In skin fibroblasts, we also found lower values of basal OCR in Huntington's disease patients correlating with earlier age of clinical diagnosis and age at onset, with longer disease duration and increased age. Additionally, a lower mitochondrial network fragmentation occurred, concomitantly with a tendency for upregulation in mtDNA copy number in peripheral cells from Pre-M Huntington's disease carriers. Importantly, the higher mtDNA copy number observed in Pre-M carriers correlates with enhanced mitochondrial respiration and increased accumulation of
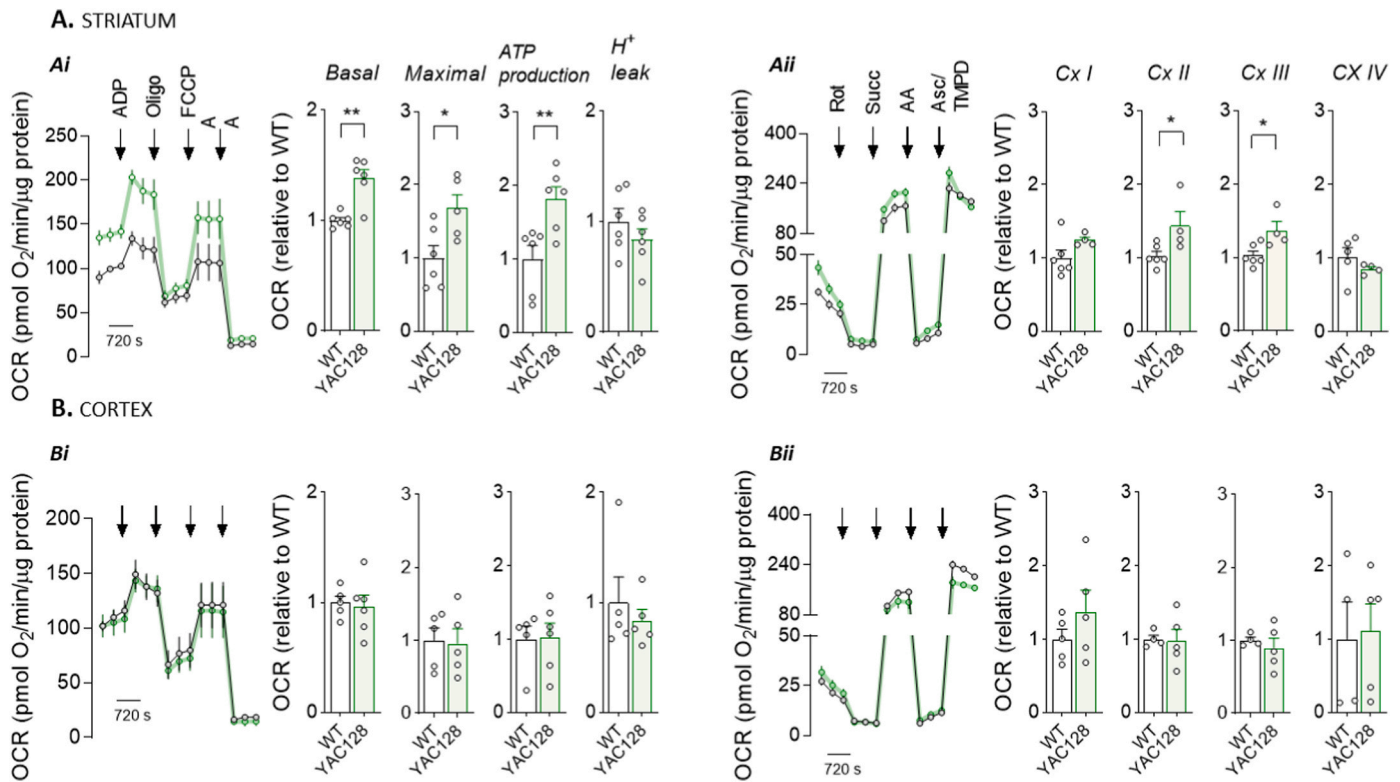
Fig. 6. Oxygen consumption rates (OCR) in 3 mo YAC128 mouse striatal and cortical mitochondria. Mitochondrial respiration was measured using the Seahorse flux analyzer in 3 mo YAC128 and WT striatal **(A)** and cortical **(B)** isolated mitochondria. Levels of respiratory coupling (**A***i* **and B***i;* for striatal and cortical mitochondria, respectively) were analysed in MAS containing 10 mM succinate plus 2 μM rotenone under sequentially injection of mitochondrial inhibitors and substrates (final concentration: 4 mM ADP; 2.5 μg/mL oligomycin; 4 μM FCCP and 4 μM antimycin A) as shown in representative traces and basal respiration and maximal respiration, ATP production and H$^+$ calculated as described in Methods section. The activity of mitochondrial respiratory chain complexes (**A***ii* **and B***ii;* for striatal and cortical mitochondria, respectively) was analysed in uncoupling conditions performed in MAS containing 4 μM FCCP, 10 mM pyruvate and 2 mM malate. Mitochondrial inhibitors and substrates were sequentially injected (final concentration: 2 μM rotenone, 10 mM succinate, 4 μM antimycin A and 10 mM ascorbate/ 100 μM TMPD) as shown in the representative traces and Cx I-IV activities calculated as described in Methods section. Data are the mean ± SEM of experiments performed in independent mitochondrial preparations obtained from 3 to 7 mice from each genotype, run in duplicates or triplicates. Statistical analysis: *$p < 0.05$, **$p < 0.01$ when compared with WT mitochondria, by nonparametric Mann-Whitney *U* test.

[^{64}Cu]-ATSM, the later most probably a reflex of altered redox status.

Strikingly, many of the hallmarks of aging itself are similar to the mechanisms involved in HD pathology, which may have accounted for by some of our findings. Most of the processes described in our study decline with aging, although in HD patients they seem to be exacerbated. The role of chronological age at onset and disease duration are actively under investigation to understand the link between aging and neurodegeneration. Notably, and as would be expected, the manifest HD group has a higher chronological age and longer disease duration associated to worse mitochondrial deficits, compared to controls or Pre-M carriers, suggesting a synergistic interplay between aging and degeneration. Indeed, brain aerobic glycolysis and mitochondrial function decrease during normal human aging [44,45]. Nevertheless, a recent study showed that, although the age of HD patients can be an important factor, disease duration seems to be more relevant since HD patients suffering for longer periods of time show more severe deficits in several indices of mitochondrial respiration, independently of their calendar age [14].

In YAC128 mice, increased [^{64}Cu]-ATSM retention was mostly evident in the striatum of 6 and 12 mo and less manifest in the cortex. Interestingly, striatal isolated mitochondria (but not cortical mitochondria) derived from pre-symptomatic (3 mo) YAC128 mice exhibited not only increased levels of H$_2$O$_2$, but also enhanced activity of complexes II and III, linked to increased basal and maximal OCR and ATP production. Increased complexes activity and H$_2$O$_2$ levels were recently observed by us in isolated YAC128 mouse striatal mitochondria at 3 mo [12] and in R6/2 mouse striatal synaptic mitochondria [46]. The

increase in mitochondrial ATP generation in striatal mitochondria contrasts with human fibroblasts showing unchanged ATP levels, probably due to H$^+$ leakage. In addition, striatal mitochondria from pre-symptomatic YAC128 mice showed defects in Ca^{2+} handling and altered mitochondrial morphology, suggesting deregulated mitochondrial function despite the increase in mitochondrial respiratory activity, which precedes the increase in [^{64}Cu]-ATSM retention, at 6 mo.

Altered striatal mitochondrial function observed in this study implicates enhanced susceptibility of this brain region, consistently with previous reports showing a highly susceptibility of the striatum to impaired mitochondrial oxidative phosphorylation [47] and age-dependent striatal volumetric changes defined through longitudinal structural imaging in the YAC128 mouse model [48]. Unchanged OCR in YAC128 mouse mitochondria at 6–12 mo may be accounted for by alternative metabolic profile and/or decreased striatal energy demand, as demonstrated by enhanced levels of creatine and phosphocreatine, determined by spectroscopy (1H-MRS) [48].

Several studies have established a link between [^{64}Cu]-ATSM accumulation, mitochondrial dysfunction, impairment of mitochondrial respiratory chain, due to hypoxia, and related oxidative stress [26,27, 49], suggesting that dysfunctional mitochondrial electron transport chain causes an overreduced state due to ROS generation, as detailed in this work. Furthermore, in a patient with MELAS (mitochondrial encephalopathy, lactic acidosis, and stroke-like episode), caused by a mitochondrial mutation, [^{62}Cu]-ATSM accumulation reflected changes in regional oxidative stress [26]. Increased ROS production and mitochondrial dysfunction were also described in Parkinson's disease and
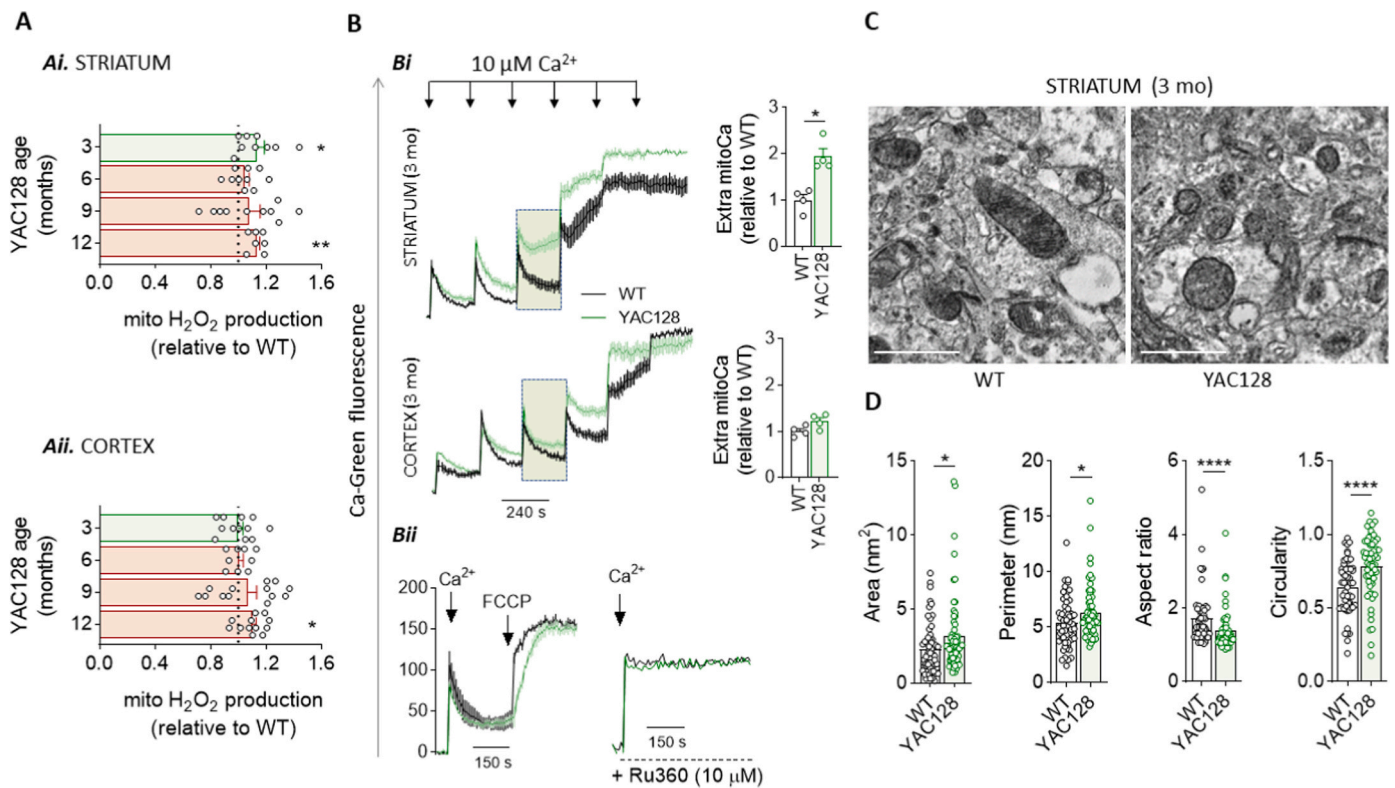
Fig. 7. Mitochondrial ROS levels, Ca^{2+} handling, and mitochondrial ultrastructure in YAC128 mouse striatal and cortical mitochondria.
H_2O_2 production was measured in functional isolated mitochondria **(A)** derived from 3, 6, 9 and 12 mo YAC128 and WT mice striatum **(Ai)** and cortex **(Aii)** using the Amplex Red assay as described in Methods section. The capacity to handle Ca^{2+} was evaluated in striatal and cortical mitochondria obtained from 3 mo YAC128 and WT mice **(B)** by using Calcium Green-5N fluorescent probe. Pulses of 10 μM Ca^{2+} were applied to raise intramitochondrial Ca^{2+} concentrations until the Ca^{2+} retention capacity was reached as shown by the upward deflection of the traces **(Bi)**. The Ca^{2+} handling capacity for striatal and cortical mitochondria at the third Ca^{2+} pulse is detailed in the dashed green window and presented as extra-mitochondrial Ca^{2+} (area under the curve) **(Bi, inset)**. The effect of the mitochondrial uncoupler FCCP and the pharmacological inhibition of the mitochondrial calcium uniporter (MCU; Ru360) are also depicted **(Bii)**. Electron micrographs were obtained from 3 mo YAC128 and WT mice striatum and mitochondrial morphology presented for area, perimeter, aspect ratio and circularity as evaluated by Fiji-imageJ software **(C)**. Over 62 individual mitochondria were assessed in independent preparations from 6 independent TEM images from WT and YAC128 mice (scale bar: 1000 nm). Data are the mean ± SEM of experiments performed in independent mitochondrial preparations obtained from 3 to 16 mice from each genotype run in triplicates to quadruplicates. In **(A)** results are presented in relation to the unit (dashed line) representing the WT mouse values. Statistical analysis: *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$ when compared with WT mitochondria, by nonparametric Mann-Whitney U test. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

amyotrophic lateral sclerosis patients, as evaluated by PET using [^{62}Cu]-ATSM and further associated with disease severity in both diseases [24,25,50].

In Huntington's disease, mHTT is widely expressed in all body cells which highlights the importance of energy metabolism studies in peripheral cells. A previous study stressed that fibroblasts from healthy donors and Huntington's disease patients exhibited different profiles if stratified according to motor function, psychiatric and functional capacity. Fibroblasts obtained from manifest Huntington's disease patients displayed compromised cell growth and reduced ATP production, accompanied by decreased mitochondrial activity, which was indicative of respiratory chain defects [15]. Of interest, the age at onset seems to be a crucial element together with CAG expansion to define the bioenergetic profile of fibroblasts of Huntington's disease patients [14].

Similarly, our data suggests that Huntington's disease patients symptomatic for longer periods of time have highly compromised mitochondrial function. In these cells, a slight increase in mitochondrial ROS was reported, despite a significant elevation in antioxidant enzymes (SOD2 and GR) [15], while other authors described a decrease in catalase activity in Huntington's disease fibroblasts [22]. Impaired antioxidant defenses, namely a decrease in GPx and SOD1 activities, were also observed in erythrocytes from Huntington's disease patients [51]. Previous studies performed in our lab showed increased mitochondrial-driven ROS in Huntington's disease human cybrids [18]

and mouse striatal cells expressing full-length mHTT [20,21,52,53]. An increase in ROS was also found in the brain striatum and cortex of symptomatic R6/1 [54], R6/2 mouse models [55,56] and in YAC128 embryonic fibroblasts and fibroblasts derived from Huntington's disease patients [57]. In YAC128 mouse striatal and cortical brain mitochondria enhanced levels of H_2O_2 did not account for by changes in antioxidant profile.

Mitochondrial function has a significant impact on organelle morphology, defined by a regulated balance between fission and fusion events. Loss of bioenergetic capacity results in an inability to maintain the mitochondrial network organization [58]. In our study, skin fibroblast mitochondria from Pre-M Huntington's disease carriers became larger, whereas circularity decreased, when compared to healthy controls and manifest patients. These ultrastructural characteristics suggest a promotion of mitochondrial fusion events that might be required to maximize oxidative phosphorylation. Conversely, striatal mitochondria from pre-symptomatic YAC128 mice, retaining mHTT interaction, exhibited increased circularity, as revealed by ultrastructure analysis. These results might be determined by striatal mitochondria–specific vulnerability in Huntington's disease. Accordingly, fragmented mitochondria were previously reported in many Huntington's disease cell models, including peripheral cells (e.g. Ref. [59]). Mitochondrial fragmentation was previously observed in rat cortical neurons expressing mHTT and in fibroblasts from manifest Huntington's disease patients

and in YAC128 Huntington's disease mouse model, before the appearance of neurological deficits and mHTT aggregates [60]; in striatum of YAC128 mice, an increase in the number of small mitochondria and decreased cristae surface area were also detected [60].

Decreased ability to take up Ca^{2+} through the MCU was further observed in striatal mitochondrial-enriched fractions from pre-symptomatic YAC128 mice, when compared to cortical mitochondria, again underlying a highly dysfunctional organelle derived from the striatum. Defects on mitochondrial Ca^{2+} handling were previously demonstrated in lymphoblast mitochondria from Huntington's disease patients and brain mitochondria from YAC128 mice [61], isolated mitochondria from transgenic Huntington's disease rats with 51 CAG repeats [62] and in mutant mouse striatal cell line [63,64]. Concordantly, in striatal mitochondria obtained from rat brain, lower calcium levels consistently evoked a permeability transition more easily than in cortical mitochondria [65]. Conversely, an increase in Ca^{2+} loading capacity in forebrain (brain minus the cerebellum)-derived mitochondria from R6/2 and YAC128 mice, but not in organelle isolated from Hdh150 knock-in mice, was observed [16]. More recently, a large Ca^{2+} uptake capacity in synaptic and nonsynaptic mitochondria isolated from 2 to 12 mo YAC128 mice brain, when compared with YAC18 or FVB/NJ mice [35] was reported. Indeed, changes in mitochondrial Ca^{2+} handling have not been consistent in Huntington's disease models, particularly when studying isolated brain mitochondria, which might be related with different methodologies of isolating the organelle and/or assessing ion changes, as well as the isolated brain tissue.

The number of mtDNA copies has a crucial role in cellular metabolism and bioenergetics. The mechanisms responsible for regulation of mtDNA copy number in Huntington's disease pathology are still under discussion. Our study reveals that Pre-M Huntington's disease carriers harbor a higher number of mtDNA copies; interestingly, a strong and significant correlation was observed with enhanced mitochondrial bioenergetics and [^{64}Cu]-ATSM uptake. Contradictory conclusions may be dependent on the cell type, as shown by a significantly higher mtDNA/nDNA copy number in leukocytes, but lower in fibroblasts from manifest Huntington's disease patients [66]. Moreover, mtDNA copy number was increased before disease onset in leukocytes of Huntington's disease mutation carriers and declined after disease onset [67].

Of relevance, this study provides novel and important data suggesting a compensatory mechanism in premanifest and prodromal Huntington's disease carriers and pre-symptomatic Huntington's disease mice model to overcome the early insults, particularly the underlying oxidative stress. Compensatory mechanisms at early stages of age-related brain diseases, as determined in human and mouse, have been described elsewhere [68,69]. Reports of upregulation in the expression of mitochondrial-encoded genes in blood samples of mild cognitively impaired patients, compared to Alzheimer's disease patients and healthy controls, suggested a compensatory-like mechanism at the early stages of disease [70,71].

Overall, this study provides novel data regarding *in vivo* PET analysis showing accumulation of [^{64}Cu]-ATSM that correlates with major redox changes and mitochondrial deregulation at very early stages in Huntington's disease.Increased oxygen consumption rates concomitantly with enhanced ROS levels at early/presymptomatic Huntington's disease stages (as observed in both human and mouse mHTT carriers) reinforce the hypothesis that mitochondrial and redox deregulation contribute to Huntington's disease pathogenesis. Thus, apparently compensatory mitochondrial changes occurring in Huntington's disease presymptomatic stages might constitute a biomarker of therapeutic response, as previously tested by us in pridopidine-treated YAC128 mice [12].

The major limitation of the current study is the small number of participants. Further studies with a larger number of participants and at different disease stages are necessary to validate the human data. Nonetheless, the overlapping results obtained in mitochondrial function and oxidative stress in pre-symptomatic stages in YAC128 mice supports the changes reported in Huntington's disease carriers. Moreover, data in mouse striatum reinforces an increased susceptibility of this brain region in Huntington's disease. Thus, potential benefits of early therapeutic interventions based on mitochondrial-targeted compounds aiming to regularize mitochondrial function and reduce ROS generation, particularly in the striatum, are anticipated to help to slowdown Huntington's disease progression.

Declaration of competing interest

The authors report no competing interests.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102424.

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