Huntington disease and other polyglutamine diseases : using CAG repeat variations to explain missing heritability

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Part 2.2

BIOENERGETICS IN FIBROBLASTS OF PATIENTS WITH HUNTINGTON DISEASE ARE ASSOCIATED WITH AGE AT ONSET

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ABSTRACT

Objective

We aimed to assess whether differences in energy metabolism in fibroblast cell lines derived from Huntington disease patients were associated with the age at onset independent of the CAG repeat number in the mutant allele.

Methods

For this case-control study, we selected nine pairs of Huntington disease patients matched for mutant CAG repeat size and sex, but with a difference of at least ten years in age at onset, using the Leiden Huntington disease database. From skin biopsies, we isolated fibroblasts in which we 1) quantified the ATP concentration before and after a hydrogen-peroxide challenge and 2) measured mitochondrial respiration and glycolysis in real-time, using the Seahorse XF Extracellular Flux Analyzer XF24.

Results

The ATP concentration in fibroblasts was significantly lower in Huntington disease patients with an earlier age at onset, independent of calendar age and disease duration. Maximal respiration, spare capacity, and respiration dependent on complex II activity, indices of mitochondrial respiration, were significantly lower in Huntington disease patients with an earlier age at onset, again independent of calendar age and disease duration.

Conclusion

A less efficient bioenergetics profile was found in fibroblast cells from Huntington disease patients with an earlier age at onset independent of mutant CAG repeat size. Thus, differences in bioenergetics could explain part of the residual variation in age at onset in Huntington disease.

Key words: Huntington disease, age at onset, bioenergetics status, fibroblasts

Huntington disease, a devastating neurodegenerative disorder is caused by an elongated CAG repeat sequence in exon 1 of the huntingtin gene (*HTT*).[1-3] The length of the CAG repeat sequence accounts for 50-70% of the variation in age at onset, leaving a substantial amount of unexplained variation which could be attributed to genetic as well as environmental modifiers.[4,5] In addition to progressive motor disturbances, neuropsychiatric symptoms and cognitive decline, Huntington disease patients and pre-manifest mutation carriers suffer from unintended weight loss.[6,7] Recently, we demonstrated that this weight loss was associated with a faster rate of disease progression independent of CAG repeat number.[8] Various evidence indeed indicates that disturbances in energy metabolism and mitochondrial defects play a role in Huntington disease pathology.[9-11] Furthermore, mitochondrial metabolism was found to be impaired in peripheral tissues of Huntington disease patients, including lymphoblastoid cell lines and skin fibroblasts.[12-15] In support of the association between mitochondrial defects and Huntington disease symptomology, Huntington disease characteristics emerged in humans after accidental exposure to 3-nitropropionic acid (3-NP), a mitochondrial toxin that selectively inhibits the activity of mitochondrial complex II.[16-19] However, to what extent differences in energy metabolism are associated with the onset of Huntington disease symptoms independent of the CAG repeat number is unknown. Therefore, the aim of our study was to investigate whether differences in energy metabolism were present in fibroblast cell lines from Huntington disease patients with identical CAG repeat sizes but a large difference in age at onset.

MATERIALS AND METHODS

Participants

From the Leiden Huntington disease database which contained data on 356 Huntington disease patients, we selected nine pairs of Huntington disease patients older than 18 years, matched for sex and CAG repeat length but with a large difference in age at onset, as defined by an expert neurologist (RACR) based on motor, psychiatric and/or cognitive symptoms. As a cut-off, we used a difference in age at onset of at least 10 years between each pair of patients. Aside from the age at onset derived from the date of clinical Huntington disease diagnosis, we also noted the age at onset as estimated by the rater based on patient information and the age at onset of different Huntington disease symptoms (**Table 1**). Exclusion criteria were presence of inflammatory diseases, an active infectious disease and the use of anti-inflammatory or immunosuppressive drugs (e.g. non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids) or anti-oxidants (e.g. vitamin C) in case temporary cessation of medication use (for about 7 times the drug's half-life) was not possible before sampling.

Standard protocol approvals, registrations, and patient consents

The study protocol was approved by the local ethics committee and written informed consent was obtained from all participants.

Table 1. Age at onset of Huntington disease symptoms.

Couple	CAG repeat number	Sex	Calendar age (year)	Disease duration (year)	Age at clinical diagnosis (year)	Difference in age at clinical diagnosis (year)	Age symptom onset estimated by rater (year)[a]	Difference in age at symptom onset estimated by rater (year)[a]	Symptom at onset
1	41	F	55.0	8.6	46.4	24.7	46.4	21.7	m
	41	F	73.8	2.8	71.1		68.1		m
2	41	M	58.2	4.1	54.1	10.1	50.1	-	m, p
	41	M	67.4	3.2	64.3		-		m
3	42	F	52.9	7.8	45.1	28.2	45.1	26.2	p
	42	F	78.9	5.6	73.3		71.3		m
4	42	F	42.3	6.4	35.9	18.0	34.9	14.0	m
	42	F	59.8	5.9	53.9		48.9		m, p
5	42	M	43.5	6.6	36.9	23.9	35.9	21.9	p
	42	M	61.8	1.0	60.8		57.8		m, p
6	42	M	43.0	20.8	22.3	27.1	20.3	28.1	p
	42	M	52.0	2.7	49.3		48.3		m, c
7	45	M	43.5	2.2	41.3	15.2	34.3	12.2	p
	45	M	58.1	1.6	56.5		46.5		m
8	46	F	39.3	3.9	35.4	11.6	34.4	9.6	m, p, c
	46	F	49.9	2.9	47.0		44.0		c
9	46	M	49.0	16.0	33.0	14.0	33.0	13.0	p, c
	46	M	51.9	4.9	47.0		46.0		m
Average (years):			54.5	5.9		19.2		18.3	

CAG =cytosine-adenine-guanine. F = female. M = male. m=motor symptoms. p=psychiatric symptoms. c=cognitive symptoms. [a]) a medical doctor estimates the time at which the symptoms of the patient started based on information provided by the patient and the patients family.

Sampling and cell culture

During a regular visit to our outpatient clinic, we obtained phenotypic data and a small skin sample (i.e. 3 mm diameter) from the upper thigh of each participant via a punch biopsy. Fibroblasts from the skin samples were cultured in Minimal Essential Medium (Gibco #10370-07) supplemented with 15% heat-inactivated Fetal Bovine Serum (Gibco #10270106), 1% Penicillin-Streptomycin (10,000 U/ml, Gibco #15140122) and 1% GlutaMAX supplement (Gibco #35050061) and stored in a humidified incubator at 37^0C with 5% CO_2. For the experiments described below, the fibroblasts were grown up to a maximum of 15 passages and harvested by trypsinization with Trypsin-EDTA (0.05%, Gibco #25300054) at 37^0C.

ATP concentration under oxidative stress

To assess whether bioenergetics differences in fibroblasts' response to oxidative stress between the matched pairs were present, we quantified the ATP concentration in the fibroblasts after subjecting them to 0.5 mM H_2O_2 for 0, 5, 10 and 15 minutes. In solid 96-well plates, we plated 12 replicates per cell line (i.e. three replicates per exposure period) with 30 000 cells in 100 µl regular culturing medium per well. Per plate, we included 14 blank wells (i.e. containing no cells) to which different ATP standard concentrations would later be added. Afterwards, we incubated the plate for a minimum of 2 hours in a humidified incubator at 37^0C with 5% CO_2 to allow the cells to attach.

We applied oxidative stress to the fibroblasts by replacing the regular culturing medium with medium containing 0.5 mM H_2O_2 and 5% Fetal Bovine Serum for the appointed stress periods.[20] To block the effect of H_2O_2 at the end of this period, we added catalase from bovine liver (50 U/ ml medium, SigmaAldrich #C9322-1G)[20] and replaced the 0.5 mM H_2O_2 medium with 250 µl per well of the regular culturing medium.

We quantified the ATP concentration of the fibroblasts per well with the Luminescent ATP detection Assay Kit (Abcam #ab113849) and added different concentrations of the ATP standard (i.e. 0, 10, 100, 1000, 10 000, 100 000 and 1000 000 nM) provided by the kit to the blank wells. Luminescence was assessed with Perkin Elmer Multimode Plate Reader, Victor X3. We quantified the ATP concentration per well by creating an ATP standard curve with a corresponding equation based on the luminescence per ATP standard. Using this standard curve equation, we determined the ATP concentration according to the average luminescence of the three wells per time point per cell line.

Mitochondrial respiration and glycolysis

Using the Seahorse XF Extracellular Flux Analyzer XF24, we could measure mitochondrial respiration and glycolysis simultaneously and in real-time in our fibroblast cell lines. Respiration was measured as oxygen consumption rate (OCR) and glycolysis was

measured as extracellular acidification rate (ECAR). In addition, the Seahorse allowed for the injection of four toxins during the experimental run and could monitor their effects over time. In succession, we injected the following toxins: 1 µM oligomycin (ATP-synthase inhibitor); 1 µM carbonyl, cyanide-4-(trifluoromethoxy) phenylhydrazone (or FCCP, oxidative phosphorylation uncoupler); 1M 3-nitropropionic acid (or 3-NP, complex II inhibitor) and 1µM antimycin A (complex III inhibitor). Fibroblasts were plated one day in advance of the experiment at 60 000 cells per well in a XF24 cell culture microplate. This density resulted in confluent cultures in which cell growth was blocked due to contact inhibition, avoiding potential biases because of different growth rates between fibroblast cell lines.[21] On the day of the experiment, the regular fibroblast medium was removed and the cells were washed twice with XF assay medium at 37 ^{0}C supplemented with 5 mM glucose and 1 mM sodium pyruvate, and medium was buffered at pH 7.4. Subsequently, 675 µl of the XF assay medium was added to each well and the cells were incubated for 60 minutes in a 37^{0}C incubator without CO_2 to allow the cells to equilibrate to the new medium.

During the experiment, four measurements were taken at baseline for both OCR and ECAR. Afterwards, three measurements were taken after every toxin injection. From these values, we calculated six OCR parameters and two ECAR parameters. Basal respiration was defined as the average OCR values at baseline. The average of the three OCR values after oligomycin injection was defined as the OCR due to proton leak. Maximal respiration was calculated by taking the average OCR of the three measurements after FCCP injection. The average OCR value after injection of 3-NP was defined as 'respiration after 3-NP injection' and the average respiration after antimycin A injection was defined as 'non-mitochondrial respiration'. From these OCR parameters, another three parameters were calculated: respiration dedicated to ATP production (defined as basal respiration minus proton leak), spare capacity (defined as maximal respiration minus basal respiration), and respiration dependent on complex II activity (defined as maximal respiration minus respiration after 3-NP injection). Basal glycolysis was defined as the average ECAR of the four baseline ECAR measurements and the increase in glycolysis after blocking ATP-synthase was calculated by subtracting the basal glycolysis from the average ECAR after FCCP injection. To assure differences in the absolute averages were not due to variations in basal respiration and glycolysis, we also calculated the mitochondrial measurements as percentages of basal respiration and glycolysis.

Statistical analysis

To account for both the correlation within matched pairs as well as the correlation due to serial measurements in time during each trial, we applied generalized linear mixed-effects models to analyze the results of the bioenergetics experiments. We set the calculated average ATP concentrations after oxidative stress of every cell line per time point as the target variable. Because of an exponential association between the ATP

concentrations and time, we used the natural logarithmic transform of the index variable as the target variable. Group (i.e. earlier age at onset vs. later age at onset), disease duration, calendar age at the time of biopsy and time of exposure to H_2O_2 were included as fixed effects. Furthermore, we included a random intercept for each patient pair as well as a random slope for time of exposure to H_2O_2 to adequately account for both the matching between each pair of subjects and the correlated measurements on each individual during the experiments. For mitochondrial respiration and glycolysis, we analyzed every calculated functional index separately. For each functional index, we defined the absolute OCR and ECAR or the relative OCR and ECAR as the target variables and constructed models as described above. For all models we used an unstructured random effect covariance matrix and robust estimations of covariance which result in consistent parameter estimates even if model assumptions are violated. All models were checked both graphically and analytically. All tests were two-tailed and the threshold for statistical significance (i.e. α) was set at 0.05. All analyses were performed in SPSS version 23.0 (IBM SPSS Statistics for Windows, IBM Corp).

RESULTS

Differences in age at onset

The age at onset in the 18 selected Huntington disease patients ranged between 22 and 73 years old and the average difference in age at onset between patient pairs was 19.2 years. The patient pairs had CAG repeat sequences between 41 and 46 repeats and five of the nine pairs were male. Furthermore, the symptoms at onset of disease varied per patient between motor, psychiatric and cognitive symptoms (**Table 1**).

ATP concentrations were lower in the skin fibroblasts of Huntington disease patients with an earlier age at onset

In all fibroblast cell lines, the ATP concentration decreased over the time exposed to 0.5 mM H_2O_2. The decrease was exponential (**Figure 1** and **Figure e-1**). Therefore, we used the natural logarithmic transform of the ATP concentration (lnATP) as the target variable in the analysis. We found that lnATP was significantly lower in patients with an earlier age at onset compared to the matched patients with a later age at onset (**Figure 1** and **Table 2**). This difference was present at baseline in seven of the nine couples and continued to be evident throughout the period the cells were subjected to oxidative stress in seven couples (**Figure e-1**). Since the difference in age at onset as estimated by the rater was missing in couple number two, causing the data of this couple to be less reliable (**Table 1**), we performed a sensitivity analysis by excluding these patients. Exclusion of these patients from the analysis did not materially alter our results (**Table 2**).

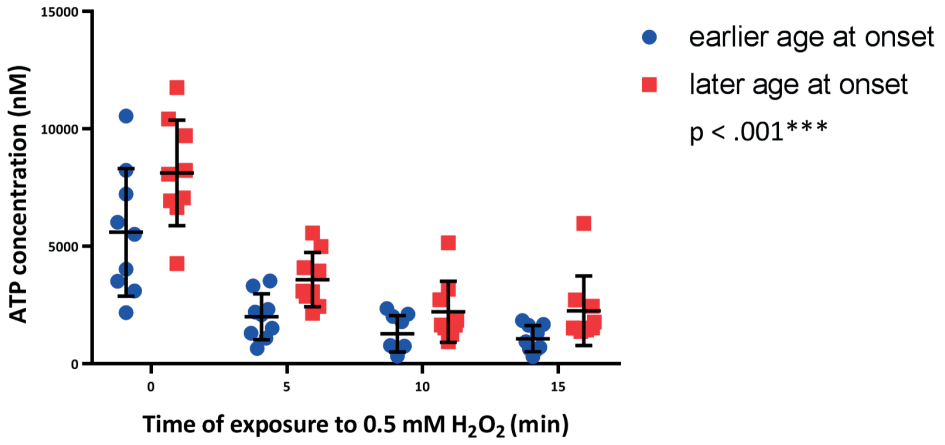


Figure 1. The average ATP concentration in skin fibroblasts of Huntington disease patients over time exposed to oxidative stress. In all fibroblast cell lines, the average ATP concentration decreased exponentially as the time exposed to oxidative stress increased. At every time point, the group of patients with an earlier age at onset had a lower ATP concentration compared to the group of patients with a later age at onset. p < .001*** indicates the significant effect of group (i.e. earlier or later age at onset) on the ATP concentration determined using linear mixed-effects models. Error bars indicate ± SD.

Table 2. The association between the logarithmic transform of the average ATP concentration and earlier versus later age at onset in HD.

Analysis	Fixed effects	β-coefficient[a]	SE	t	p-value	95% CI	
All cases	Group (earlier)	-0.709	0.177	-4.00	<.001***	-1.063	-0.355
	Disease duration	0.016	0.025	0.66	.511	-0.033	0.066
	Calendar age	0.000	0.010	-0.04	.969	-0.021	0.020
	Time	-0.102	0.008	-13.01	<.001***	-0.118	-0.087
Sensitivity analysis[b]	Group (earlier)	-0.642	0.255	-2.52	.015*	-1.153	-0.132
	Disease duration	0.013	0.027	0.50	.619	-0.040	0.067
	Calendar age	0.000	0.011	0.03	.978	-0.021	0.021
	Time	-0.101	0.009	-11.56	<.001***	-0.119	-0.084

SE = standard errors. CI = confidence interval. [a]) This column indicates the change in logarithmic transform of the ATP concentration. [b]) The analysis excluding patient pair number two (see Table 1). *) p-value < .05. ***) p-value < .001.

Mitochondrial indices were lower in the skin fibroblasts of Huntington disease patients with an earlier age at onset

The average OCR and the ECAR per Huntington disease patient group during the experiment are presented in **Figure e-2**. Neither the absolute mitochondrial parameters nor the parameters expressed as percentage of basal respiration, differed significantly per group before correction for disease duration and calendar age

(**Figure e-3**). However, after correction, we found the absolute OCR averages to be significantly different between the two groups in four of the eight estimated parameters (**Table 3**). The average maximal respiration was lower in the group of Huntington disease patients with an earlier age at onset. All nine patients with an earlier age at onset had a lower maximal respiration compared to the matched patients with a later age at onset. Similarly, in eight of the nine couples, the spare capacity was lower in the earlier age at onset group. Furthermore, the respiration dedicated to ATP production (in six couples) and the respiration dependent on complex II activity (in eight couples) were significantly lower in the group of Huntington disease patients with an earlier age at onset. The average basal respiration was not markedly different between the two groups. Average OCR as a percentage of the basal respiration differed in three parameters between the groups, including maximal respiration (in eight couples), spare capacity (in eight couples) and respiration dependent on complex II activity (in nine couples). No difference in average respiration dedicated to ATP production was found when this variable was defined as a percentage of basal respiration. The glycolysis parameters did not differ between groups, although the difference in basal glycolysis showed a trend towards significance in which the group of Huntington disease patients with an earlier age at onset had a lower average basal ECAR. To illustrate these effects, we plotted the unadjusted values as well as the predicted values calculated using the model estimates after adjustment for disease duration and calendar age (**Figure 2** and **Figure e-3**). The effects did not change after excluding couple two in the sensitivity analysis (**Table 4**).

Interestingly, disease duration and calendar age were also significantly associated with several indices of mitochondrial bioenergetics. Longer disease duration was accompanied by lower absolute values of basal respiration, maximal respiration, respiration after 3-NP injection, respiration dependent on complex II activity and glycolysis after blocking ATP-synthase. Furthermore, patients with a higher calendar age had significantly lower levels of maximal respiration, respiration dedicated to ATP production, spare capacity, respiration dependent on complex II activity, basal glycolysis and glycolysis after blocking ATP-synthase. In addition, the parameters maximal respiration, spare capacity and respiration dependent on complex II activity were also significantly lower with a higher calendar age when expressed as percentages of the basal respiration (**Table 3**).

DISCUSSION

We found lower ATP concentrations in skin fibroblasts from Huntington disease patients with an earlier age at onset compared to those with a later age at onset, independent of CAG repeat number, sex, calendar age and disease duration. In addition, we demonstrated that the fibroblasts of Huntington disease patients with an earlier age at onset exhibited lower mitochondrial respiration indices, including lower maximal respiration, spare capacity, and respiration dependent on complex II activity in an absolute sense as well as relative to their basal respiration levels. Furthermore, we found that disease duration and

age at biopsy were also significantly associated with several parameters of mitochondrial bioenergetics. Although mitochondrial defects have been extensively documented before in Huntington disease, to our knowledge, we are the first to demonstrate that differences in bioenergetics were associated with the age at onset among Huntington disease patients, and thereby, could be an important target for future therapeutic interventions.

The fact that the ATP concentration was lower in Huntington disease patients with an earlier age at onset, suggests that the production of ATP in the cells of these patients may be impaired to a greater extent compared to their counterparts or that the cells of patients with a later age at onset carry a mechanism that protects their ATP metabolism. The presence of a potential problem with ATP production was supported by comparable differences in various indices of mitochondrial respiration. Maximal respiration and mitochondrial spare capacity are measures of the ability of mitochondria to react to increased energy demands and are critical for neuronal survival.[22-24] These measures were lower in patients with an earlier age at onset, suggesting that neurons in these patients could be more vulnerable to damage and death, thus causing Huntington disease symptoms to start at a younger age. Interestingly, Siddiqui et al. showed a correlation between decreased levels of spare capacity and increased reactive oxygen species (ROS) and mitochondrial DNA (mtDNA) lesions in mutant Huntington disease striatal immortalized neuronal cells.[15] Respiration dependent on complex II activity was also lower in Huntington disease patients with an earlier age at onset. Different studies showed that in postmortem samples of the striatum and cerebral cortex of Huntington disease patients, complex II of the mitochondrial electron transport chain displayed reduced activity which was associated with diminished expression of two complex II subunits.[25-29] The exact mechanism as to how the mutated huntingtin protein (HTT) causes the loss of complex II is unknown. Possible hypotheses include the direct association of mutant HTT with the mitochondrial membrane causing decreased import of subunits into the mitochondria, increased degradation or abnormal assembly.[13,29,30] Furthermore, mutant HTT has been shown to increase cellular oxidative stress which was associated with impaired activity of complex II in a yeast model of Huntington disease.[31-33] Our results here suggest that the reduced expression of complex II is more pronounced in patients with an earlier age at onset independent of CAG repeat length which could perhaps be due to a more potent association of mutant HTT with the mitochondrial membrane or a higher vulnerability to oxidative stress.

Candidate studies found that a single nucleotide polymorphism (SNP) in *PPARGC1A*, encoding the mitochondrial regulator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) affected the age at onset in three European Huntington disease cohorts, suggesting that our findings might have a genetic origin.[34,35] Moreover, in striata from Huntington disease patients and different rodent Huntington disease models, the expression of PGC-1α mRNA was reduced and upregulating this expression caused prevention of striatal neuronal atrophy, improvement of motor deficits and protection

Table 3. The association between the calculated mitochondrial stress test parameters and age at onset in Huntington disease.

Measurement	Output	Variable	β-coefficient[a]	SE	t	p-value	95% CI	
Basal respiration	OCR	Group (earlier)	-4.520	18.249	-0.25	.808	-43.661	34.621
		Disease duration	-1.799	0.650	0.02	.015	-3.193	-0.405
		Calendar age	0.194	0.566	0.74	.737	-1.020	1.409
Proton Leak	OCR	Group (earlier)	-14.298	11.319	-1.26	.227	-38.575	9.979
		Disease duration	0.143	0.472	0.30	.766	-0.869	1.156
		Calendar age	-0.343	0.435	-0.79	.443	-1.275	0.589
	%OCR	Group (earlier)	-6.784	8.820	-0.77	.455	-25.701	12.133
		Disease duration	0.807	0.475	1.70	.112	-0.213	1.826
		Calendar age	-0.174	0.378	-0.46	.652	-0.984	0.636
Maximal respiration	OCR	Group (earlier)	-54.176	17.454	-3.10	.008**	-91.610	-16.741
		Disease duration	-3.317	0.822	-4.04	.001**	-5.080	-1.554
		Calendar age	-2.118	0.818	-2.59	.021*	-3.872	-0.365
	%OCR	Group (earlier)	-46.730	20.325	-2.30	.037*	-90.321	-3.138
		Disease duration	-0.430	0.930	-0.46	.651	-2.426	1.565
		Calendar age	-2.577	0.949	-2.72	.017*	-4.613	-0.542
Respiration after 3-NP injection	OCR	Group (earlier)	-17.226	9.145	-1.88	.081	-36.839	2.387
		Disease duration	-1.277	0.421	-3.03	.009**	-2.180	-0.374
		Calendar age	-0.529	0.259	-2.05	.060	-1.084	0.025
	%OCR	Group (earlier)	-5.133	8.749	-0.59	.567	-23.898	13.632
		Disease duration	-0.527	0.459	-1.15	.269	-1.511	0.456
		Calendar age	-0.453	0.565	-0.80	.436	-1.664	0.758
Non-mitochondrial respiration	OCR	Group (earlier)	-12.355	9.515	-1.30	.215	-32.763	8.053
		Disease duration	0.699	0.434	1.61	.129	-0.231	1.629
		Calendar age	-0.139	0.386	-0.36	.723	-0.968	0.689

Table 3. (continued)

Measurement	Output	Variable	β-coefficient[a]	SE	t	p-value	95% CI	
	%OCR	Group (earlier)	-0.607	8.521	-0.07	.944	-18.881	17.668
		Disease duration	0.933	0.503	1.85	.085	-0.146	2.013
		Calendar age	0.292	0.344	0.85	.411	-0.446	1.030
Respiration dedicated to ATP production	OCR	Group (earlier)	-38.106	11.243	-3.39	.004**	-62.219	-13.992
		Disease duration	0.936	0.655	.1.43	.175	-0.468	2.341
		Calendar age	-1.818	0.450	-4.04	.001**	-2.783	-0.852
	%OCR	Group (earlier)	6.784	8.820	0.46	.455	-12.133	25.701
		Disease duration	-0.807	0.475	-1.70	.112	-1.826	0.213
		Calendar age	0.174	0.378	0.46	.652	-0.636	0.984
Spare capacity	OCR	Group (earlier)	-50.884	9.894	-5.14	<.001***	-72.104	-29.663
		Disease duration	-1.482	0.700	-2.12	.053	-2.984	0.019
		Calendar age	-2.387	0.685	-3.48	.004**	-3.856	-0.918
	%OCR	Group (earlier)	-46.730	20.325	-2.30	.037*	-90.321	-3.138
		Disease duration	-0.430	0.930	-0.46	.651	-2.426	1.565
		Calendar age	-2.577	0.949	-2.72	.017*	-4.613	-0.542
Respiration dependent on complex II activity	OCR	Group (earlier)	-36.950	13.397	-2.76	.015*	-65.685	-8.216
		Disease duration	-2.039	0.598	-3.41	.004**	-3.323	-0.756
		Calendar age	-1.589	0.597	-2.66	.019*	-2.868	-0.309
	%OCR	Group (earlier)	-31.122	12.292	-2.53	.024*	-57.486	-4.759
		Disease duration	-0.737	0.415	-1.78	.097	-1.627	0.153
		Calendar age	-1.683	0.520	-3.24	.006**	-2.797	-0.569
Basal glycolysis	ECAR	Group (earlier)	-5.404	2.763	-1.96	.071	-11.331	0.523
		Disease duration	-0.330	0.193	-1.71	.110	-0.745	0.085
		Calendar age	-0.447	0.129	-3.46	.004**	-0.724	-0.170

Table 3. (continued)

Measurement	Output	Variable	β-coefficient[a]	SE	t	p-value	95% CI	
Glycolysis after blocking ATP-synthase	ECAR	Group (earlier)	0.614	3.302	0.19	.855	-6.468	7.697
		Disease duration	-0.643	0.153	-4.20	.001**	-0.971	-0.314
		Calendar age	-0.187	0.079	-2.38	.032*	-0.356	-0.019
	%ECAR	Group (earlier)	48.604	25.707	1.891	.080	-6.532	103.739
		Disease duration	-0.010	2.250	-0.004	.997	-4.835	4.816
		Calendar age	4.988	2.202	2.266	.040*	0.267	9.710

SE = standard error. CI = confidence interval. OCR = oxygen consumption rate. ECAR = extracellular acidification rate. %OCR = OCR as a percentage of basal OCR. %ECAR = ECAR as a percentage of basal ECAR. [a]) This column indicates the change in OCR, ECAR, %OCR or %ECAR. *) p-value < .05. **) p-value < .01. ***) p-value < .001.

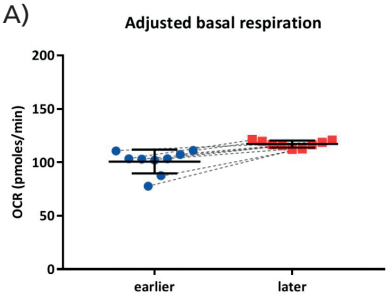
against mitochondrial dysfunction and cell death, implying that PGC-1α expression may modify mutant HTT induced mitochondrial toxicity. [36-38] Together with our results, these findings indicate that differences in PGC-1α expression and the consequential variations in bioenergetic profile may result in additional variation in age at onset in Huntington disease.

We found that as patients suffered from Huntington disease for a longer period of time, several indices of mitochondrial respiration were significantly lower independent of calendar age. This finding is in line with the fact that Huntington disease pathogenesis is known to involve mitochondrial deficits.[39,40] Therefore, a reasonable derivative is that these mitochondrial deficits become worse as the disease progresses. Mitochondrial function is also known to decline with age.[41] In accordance, we found that as the calendar age of patients increased several parameters of mitochondrial function decreased. The ageing process itself is unlikely to have accounted for our main finding (i.e. a better bioenergetics profile in late onset patients) given that patients with a later age at onset were older at the time of biopsy (**Table 1**).

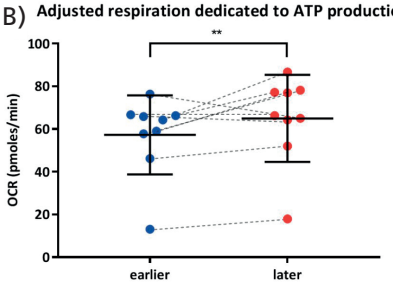
Our study had several limitations. First, although Huntington disease is primarily a neurological disease, we were able to find differences between Huntington disease patients with an early onset and late onset of symptoms in skin fibroblasts. The fact that we could acquire relevant results in these relatively easy to obtain cells is intriguing and supports the use of fibroblast cell models in the research of neurodegenerative diseases. However, these cells are not the primary cells of interest. Therefore, repeating similar experiments in a neuronal cell model, such as striatal neurons derived from induced pluripotent stem cells, is warranted. Second, establishing the age at onset in Huntington disease is subjective.[42] In order to achieve additional certainty concerning this age, we

Figure 2. Adjusted estimates of the functional indices calculated from the mitochondrial stress test per age group of symptom onset in Huntington disease. There was no difference in basal respiration between the two groups **(A)**. The absolute mitochondrial respiration dedicated to ATP production was significantly lower in the group of Huntington disease patients with an earlier age at onset **(B)**. Both the absolute maximal respiration and the maximal respiration as a percentage of the basal respiration were significantly lower in the group of Huntington disease patients with an earlier age at onset (**C** and **D**). The absolute spare capacity and the spare capacity as a percentage of the basal respiration were significantly lower in the group of Huntington disease patients with an earlier age at onset (**E** and **F**). The absolute respiration dependent on complex II activity and the respiration dependent on complex II activity as a percentage of the basal respiration were also significantly lower in the group of Huntington disease patients with an earlier age at onset (**G** and **H**). The basal glycolysis did not differ significantly between the two groups (**I**). OCR = oxygen consumption rate. ECAR = extracellular acidification rate. Adjusted estimates = values adjusted for disease duration and calendar age at the time of biopsy. ● = Huntington disease patients with an earlier age at onset. ■ = Huntington disease patients with a later age at onset. - - - - - indicate matched patients. Error bars indicate ± SD. *) p-value < .05. **) p-value < .01. ***) p-value < .001.

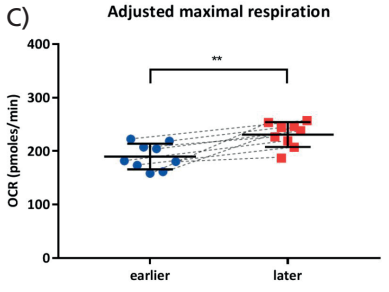
A)


Adjusted basal respiration
OCR (pmoles/min)
200
150
100
50
0
earlier
later

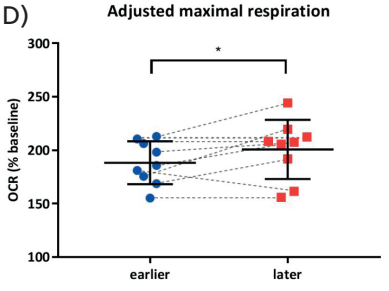
B)


Adjusted respiration dedicated to ATP production
**
OCR (pmoles/min)
100
80
60
40
20
0
earlier
later

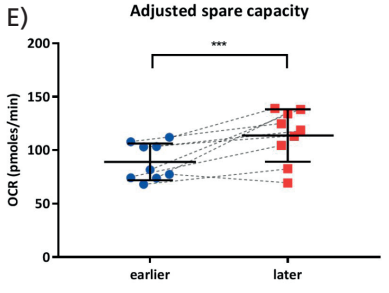
C)


Adjusted maximal respiration
**
OCR (pmoles/min)
400
300
200
100
0
earlier
later

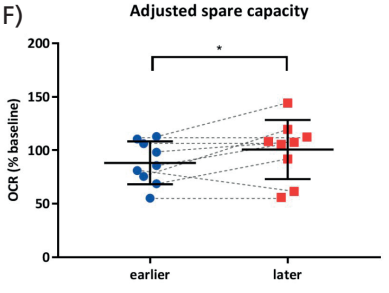
D)


Adjusted maximal respiration
*
OCR (% baseline)
300
250
200
150
100
earlier
later

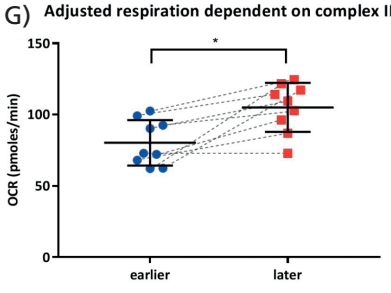
E)


Adjusted spare capacity
***
OCR (pmoles/min)
200
150
100
50
0
earlier
later

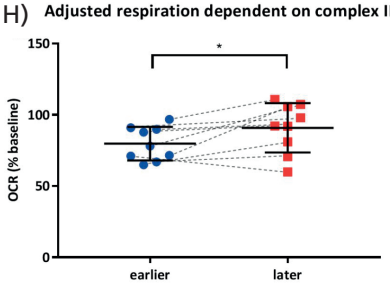
F)


Adjusted spare capacity
*
OCR (% baseline)
200
150
100
50
0
earlier
later

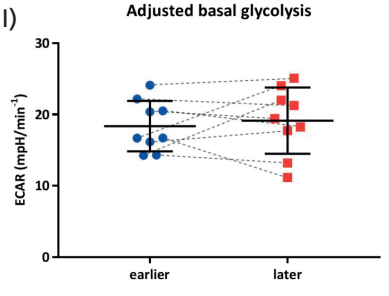
G)


Adjusted respiration dependent on complex II
*
OCR (pmoles/min)
150
100
50
0
earlier
later

H)


Adjusted respiration dependent on complex II
*
OCR (% baseline)
150
100
50
0
earlier
later

I)


Adjusted basal glycolysis
ECAR (mpH/min-1)
30
20
10
0
earlier
later

noted the age of Huntington disease diagnosis as well as the age estimated by the rater, which were analogous in eight of the nine couples. In addition, our results did not change after excluding the couple in which the rater estimated age at onset was missing for one patient, illustrating that our results are robust and reliable. Lastly, we performed several statistical analyses without applying a correction for multiple testing given our relatively small sample size as well as the fact that bioenergetics assessments are highly correlated and, thus, cannot be regarded as independent. Nevertheless, to obtain results with a higher level of confidence, future research investigating similar parameters should include a larger number of patients.

We demonstrated an association between age at onset and the bioenergetic profile in Huntington disease patients. Thus, differences in bioenergetics could explain part of the residual variation in age at onset among Huntington disease patients, while therapies aimed at enhancing mitochondrial function may delay disease onset. However, further research into the mechanisms mediating the association between bioenergetics and age at onset are needed to develop novel therapies aimed at delaying symptom onset and disease progression in Huntington disease.

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AUTHOR CONTRIBUTIONS

S.L.G. and N.A.A. contributed to the conception and design of the study, and to the acquisition and analysis of data; C.M. and W.M.C.v.R. offered crucial supervision during experiments; all authors contributed to drafting the text and preparing the figures.

CONFLICT OF INTEREST

Ms. Sarah L. Gardiner reports no disclosures. Dr. Chiara Milanese reports no disclosures. Ms. Merel W. Boogaard reports no disclosures. Dr. Ronald A.M. Buijsen reports no disclosures. Ms. Marye Hogenboom reports no disclosures. Professor Dr. Raymund A.C. Roos is advisor of UniQure. Dr. Pier G. Mastroberardino reports no disclosures. Dr. Willeke M.C. van Roon-Mom reports no disclosures. Dr. N. Ahmad Aziz reports no disclosures.

Table 4. Sensitivity analysis of the association between the mitochondrial stress test parameters and age at onset in Huntington disease.

Measurement[a]	Output	Variable	β-coefficient[b]	SE	t	p-value	95% CI	
Maximal respiration	OCR	Group (earlier)	-56.810	24.570	-2.31	.039*	-110.343	-3.277
		Disease duration	-2.938	1.079	-2.72	.018*	-5.288	-0.588
		Calendar age	-2.815	0.568	-4.96	<.001***	-4.053	-1.577
	%OCR	Group (earlier)	-56.330	24.401	-2.31	.040*	-109.495	-3.164
		Disease duration	0.018	0.965	0.02	.985	-2.084	2.120
		Calendar age	-2.903	1.061	-2.74	.018*	-5.215	-0.591
Respiration dedicated to ATP production	OCR	Group (earlier)	-31.600	9.310	-3.39	.005**	-51.884	-11.315
		Disease duration	0.619	0.506	1.22	.245	-0.483	1.721
		Calendar age	-1.684	0.396	-4.25	.001**	-2.547	-0.821
	%OCR	Group (earlier)	15.130	7.065	2.14	.053	-0.263	30.523
		Disease duration	-1.186	0.328	-3.61	.004**	-1.901	-0.471
		Calendar age	0.482	0.312	1.55	.148	-0.197	1.162
Spare capacity	OCR	Group (earlier)	-56.929	11.504	-4.95	<.001***	-81.995	-31.863
		Disease duration	-1.066	0.619	-1.72	.111	-2.414	0.283
		Calendar age	-2.825	0.556	-5.08	<.001***	-4.037	-1.613
	%OCR	Group (earlier)	-56.330	24.401	-2.31	.040*	-109.495	-3.164
		Disease duration	0.018	0.965	0.02	.985	-2.084	2.120
		Calendar age	-2.903	1.061	-2.74	.018*	-5.215	-0.591
Respiration dependent on complex II activity	OCR	Group (earlier)	-39.637	17.613	-2.25	.044*	-78.012	-1.261
		Disease duration	-1.773	0.790	-2.24	.045*	-3.495	-0.051
		Calendar age	-2.015	0.483	-4.17	.001**	-3.067	-0.962
	%OCR	Group (earlier)	-35.371	14.785	-2.39	.034*	-67.584	-3.157
		Disease duration	-0.522	0.465	-1.12	.284	-1.535	0.492
		Calendar age	-1.866	0.559	-3.34	.006**	-3.083	-0.649

SE = standard error. CI = confidence interval. OCR = oxygen consumption rate. ECAR = extracellular acidification rate. %OCR = OCR as a percentage of basal OCR. %ECAR = ECAR as a percentage of basal ECAR. [a]) Analyses performed with exclusion of couple number two. [b]) This column indicates the change in OCR, ECAR, %OCR or %ECAR. *) p-value < .05. **) p-value < .01. ***) p-value < .001.

REFERENCES

1. Bruyn G. Huntington's chorea: historical, clinical and laboratory synopsis. In: Vinken P, Bruyn G, eds. Handbook of clinical neurology. Amsterdam: Elsevier; 1968: 298-378.
2. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group. *Cell* 1993; 72(6): 971-83.
3. Langbehn DR, Hayden MR, Paulsen JS. CAG-repeat length and the age of onset in Huntington disease (HD): a review and validation study of statistical approaches. *American journal of medical genetics Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics* 2010; 153b(2): 397-408.
4. Wexler NS, Lorimer J, Porter J, et al. Venezuelan kindreds reveal that genetic and environmental factors modulate Huntington's disease age of onset. *Proceedings of the National Academy of Sciences of the United States of America* 2004; 101(10): 3498-503.
5. Gusella JF, MacDonald ME, Lee JM. Genetic modifiers of Huntington's disease. *Movement disorders : official journal of the Movement Disorder Society* 2014; 29(11): 1359-65.
6. Aziz NA, van der Burg JM, Landwehrmeyer GB, Brundin P, Stijnen T, Roos RA. Weight loss in Huntington disease increases with higher CAG repeat number. *Neurology* 2008; 71(19): 1506-13.
7. Mochel F, Charles P, Seguin F, et al. Early energy deficit in Huntington disease: identification of a plasma biomarker traceable during disease progression. *PloS one* 2007; 2(7): e647.
8. van der Burg JMM, Gardiner SL, Ludolph AC, Landwehrmeyer GB, Roos RAC, Aziz NA. Body weight is a robust predictor of clinical progression in Huntington disease. *Annals of neurology* 2017; 82(3): 479-83.
9. Feigin A, Leenders KL, Moeller JR, et al. Metabolic network abnormalities in early Huntington's disease: an [(18)F]FDG PET study. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine* 2001; 42(11): 1591-5.
10. Ciarmiello A, Cannella M, Lastoria S, et al. Brain white-matter volume loss and glucose hypometabolism precede the clinical symptoms of Huntington's disease. *Journal of nuclear medicine : official publication, Society of Nuclear Medicine* 2006; 47(2): 215-22.
11. Koroshetz WJ, Jenkins BG, Rosen BR, Beal MF. Energy metabolism defects in Huntington's disease and effects of coenzyme Q10. *Annals of neurology* 1997; 41(2): 160-5.
12. Sawa A, Wiegand GW, Cooper J, et al. Increased apoptosis of Huntington disease lymphoblasts associated with repeat length-dependent mitochondrial depolarization. *Nature medicine* 1999; 5(10): 1194-8.
13. Panov AV, Gutekunst CA, Leavitt BR, et al. Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. *Nature neuroscience* 2002; 5(8): 731-6.
14. Seong IS, Ivanova E, Lee JM, et al. HD CAG repeat implicates a dominant property of huntingtin in mitochondrial energy metabolism. *Human molecular genetics* 2005; 14(19): 2871-80.
15. Siddiqui A, Rivera-Sanchez S, Castro Mdel R, et al. Mitochondrial DNA damage is associated with reduced mitochondrial bioenergetics in Huntington's disease. *Free radical biology & medicine* 2012; 53(7): 1478-88.
16. He F, Zhang S, Qian F, Zhang C. Delayed dystonia with striatal CT lucencies induced by a mycotoxin (3-nitropropionic acid). *Neurology* 1995; 45(12): 2178-83.
17. Ludolph AC, He F, Spencer PS, Hammerstad J, Sabri M. 3-Nitropropionic acid-exogenous animal neurotoxin and possible human striatal toxin. *The Canadian journal of neurological sciences Le journal canadien des sciences neurologiques* 1991; 18(4): 492-8.

18. Brouillet E, Jenkins BG, Hyman BT, et al. Age-dependent vulnerability of the striatum to the mitochondrial toxin 3-nitropropionic acid. *Journal of neurochemistry* 1993; 60(1): 356-9.

19. Brouillet E, Conde F, Beal MF, Hantraye P. Replicating Huntington's disease phenotype in experimental animals. *Progress in neurobiology* 1999; 59(5): 427-68.

20. Mocali A, Caldini R, Chevanne M, Paoletti F. Induction, effects, and quantification of sublethal oxidative stress by hydrogen peroxide on cultured human fibroblasts. *Experimental cell research* 1995; 216(2): 388-95.

21. Ambrosi G, Ghezzi C, Sepe S, et al. Bioenergetic and proteolytic defects in fibroblasts from patients with sporadic Parkinson's disease. *Biochimica et biophysica acta* 2014; 1842(9): 1385-94.

22. Kumar MJ, Nicholls DG, Andersen JK. Oxidative alpha-ketoglutarate dehydrogenase inhibition via subtle elevations in monoamine oxidase B levels results in loss of spare respiratory capacity: implications for Parkinson's disease. *The Journal of biological chemistry* 2003; 278(47): 46432-9.

23. Vesce S, Jekabsons MB, Johnson-Cadwell LI, Nicholls DG. Acute glutathione depletion restricts mitochondrial ATP export in cerebellar granule neurons. *The Journal of biological chemistry* 2005; 280(46): 38720-8.

24. Yadava N, Nicholls DG. Spare respiratory capacity rather than oxidative stress regulates glutamate excitotoxicity after partial respiratory inhibition of mitochondrial complex I with rotenone. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 2007; 27(27): 7310-7.

25. Gu M, Gash MT, Mann VM, Javoy-Agid F, Cooper JM, Schapira AH. Mitochondrial defect in Huntington's disease caudate nucleus. *Annals of neurology* 1996; 39(3): 385-9.

26. Brennan WA, Jr., Bird ED, Aprille JR. Regional mitochondrial respiratory activity in Huntington's disease brain. *Journal of neurochemistry* 1985; 44(6): 1948-50.

27. Browne SE, Bowling AC, MacGarvey U, et al. Oxidative damage and metabolic dysfunction in Huntington's disease: selective vulnerability of the basal ganglia. *Annals of neurology* 1997; 41(5): 646-53.

28. Tabrizi SJ, Cleeter MW, Xuereb J, Taanman JW, Cooper JM, Schapira AH. Biochemical abnormalities and excitotoxicity in Huntington's disease brain. *Annals of neurology* 1999; 45(1): 25-32.

29. Benchoua A, Trioulier Y, Zala D, et al. Involvement of mitochondrial complex II defects in neuronal death produced by N-terminus fragment of mutated huntingtin. *Molecular biology of the cell* 2006; 17(4): 1652-63.

30. Choo YS, Johnson GV, MacDonald M, Detloff PJ, Lesort M. Mutant huntingtin directly increases susceptibility of mitochondria to the calcium-induced permeability transition and cytochrome c release. *Human molecular genetics* 2004; 13(14): 1407-20.

31. Browne SE, Ferrante RJ, Beal MF. Oxidative stress in Huntington's disease. *Brain pathology (Zurich, Switzerland)* 1999; 9(1): 147-63.

32. Bogdanov MB, Andreassen OA, Dedeoglu A, Ferrante RJ, Beal MF. Increased oxidative damage to DNA in a transgenic mouse model of Huntington's disease. *Journal of neurochemistry* 2001; 79(6): 1246-9.

33. Solans A, Zambrano A, Rodriguez M, Barrientos A. Cytotoxicity of a mutant huntingtin fragment in yeast involves early alterations in mitochondrial OXPHOS complexes II and III. *Human molecular genetics* 2006; 15(20): 3063-81.

34. Che HV, Metzger S, Portal E, Deyle C, Riess O, Nguyen HP. Localization of sequence variations in PGC-1alpha influence their modifying effect in Huntington disease. *Molecular neurodegeneration* 2011; 6(1): 1.

35. Taherzadeh-Fard E, Saft C, Andrich J, Wieczorek S, Arning L. PGC-1alpha as modifier of onset age in Huntington disease. *Molecular neurodegeneration* 2009; 4: 10.

36. Cui L, Jeong H, Borovecki F, Parkhurst CN, Tanese N, Krainc D. Transcriptional

repression of PGC-1alpha by mutant huntingtin leads to mitochondrial dysfunction and neurodegeneration. *Cell* 2006; 127(1): 59-69.

37. Chaturvedi RK, Calingasan NY, Yang L, Hennessey T, Johri A, Beal MF. Impairment of PGC-1alpha expression, neuropathology and hepatic steatosis in a transgenic mouse model of Huntington's disease following chronic energy deprivation. *Human molecular genetics* 2010; 19(16): 3190-205.
38. Hathorn T, Snyder-Keller A, Messer A. Nicotinamide improves motor deficits and upregulates PGC-1alpha and BDNF gene expression in a mouse model of Huntington's disease. *Neurobiology of disease* 2011; 41(1): 43-50.
39. Bossy-Wetzel E, Petrilli A, Knott AB. Mutant huntingtin and mitochondrial dysfunction. *Trends in neurosciences* 2008; 31(12): 609-16.
40. Chen CM. Mitochondrial dysfunction, metabolic deficits, and increased oxidative stress in Huntington's disease. *Chang Gung medical journal* 2011; 34(2): 135-52.
41. Bratic A, Larsson NG. The role of mitochondria in aging. *The Journal of clinical investigation* 2013; 123(3): 951-7.
42. Orth M, Schwenke C. Age-at-onset in Huntington disease. *PLoS currents* 2011; 3: Rrn1258.

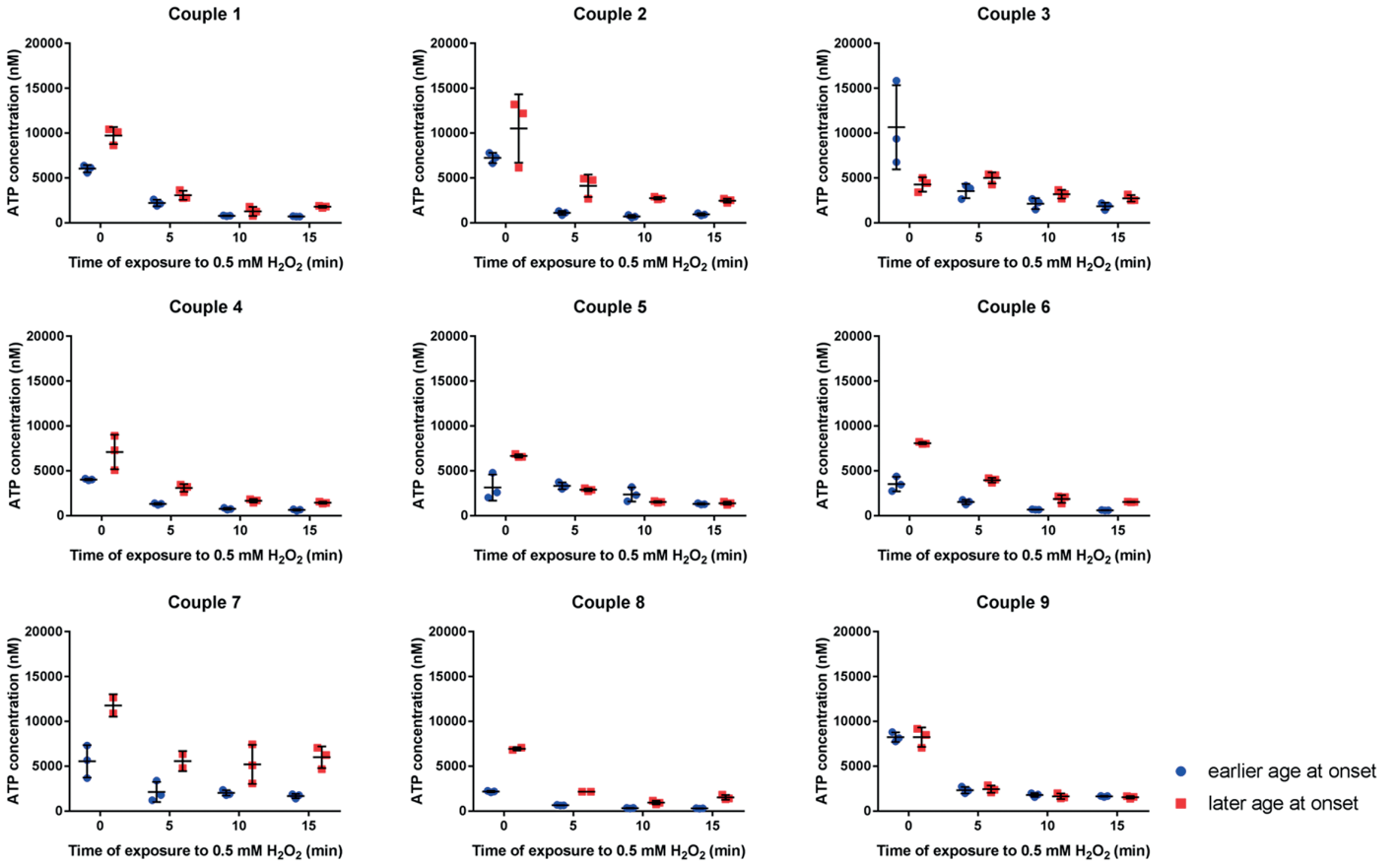

Figure e-1. The ATP concentration per couple in skin fibroblast of HD patients over time exposed to oxidative stress. In all fibroblast cell lines, the ATP concentration decreased over time exposed to oxidative stress and in most couples, the patient with the earlier age at onset showed lower ATP levels over the time.

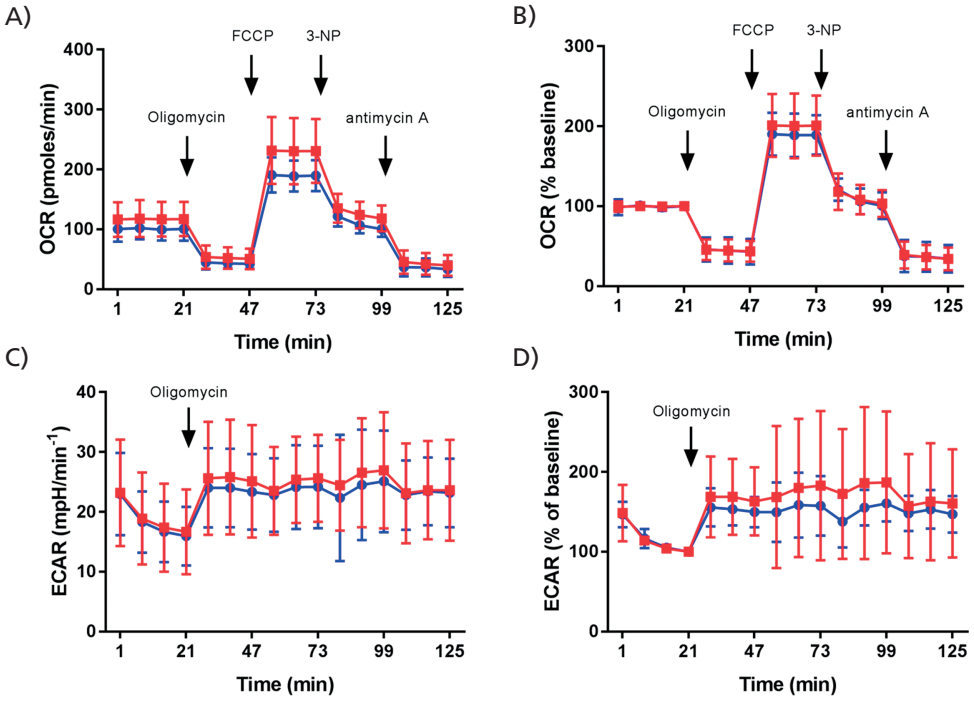

Figure e-2. Average oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) during the mitochondrial stress test. A. The average OCR during the mitochondrial stress test seems lower in the group of HD patients with an earlier age at onset. **B.** The average OCR as a percentage of the baseline consumption does not seem to differ much between groups. **C.** The average ECAR during the mitochondrial stress test shows ample variation across the cell lines and the average ECAR as a percentage of the baseline displays similar results (**D**). ● = HD patients with an earlier age at onset. ■ = HD patients with a later age at onset.

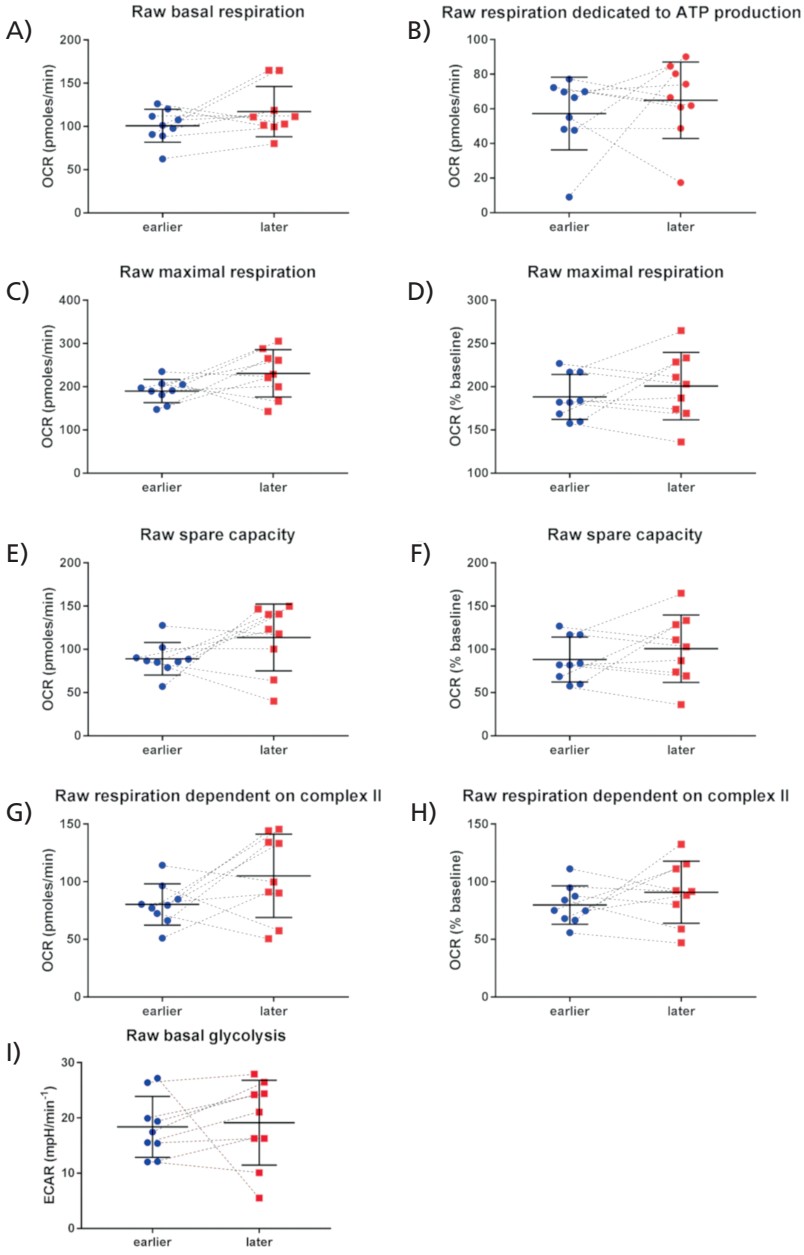

Figure e-3. Raw data of the functional indices calculated from the mitochondrial stress test per age group of symptom onset in Huntington disease (HD). When the results are not corrected for disease duration and calendar age, the differences in absolute and relative oxygen consumption rates (OCR) and extra cellular acidification rate (ECAR) are not significantly different between the group of HD patients with an earlier age at onset compared to the group of HD patients with a later age at onset. OCR = oxygen consumption rate. ECAR = extracellular acidification rate. raw data = the unadjusted values of the experiments. ● = HD patients with an earlier age at onset. ■ = HD patients with a later age at onset. - - - - - indicate matched patients. Error bars indicate ± SD.