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Impaired Platelet Mitochondrial Activity in Alzheimer's Disease and Mild Cognitive Impairment.

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Abstract

Mitochondrial abnormalities are found in Alzheimer's disease (AD), but previous reports haven't examined at-risk groups. In subjects with AD, mild cognitive impairment (MCI), and nondemented aged controls, platelet and lymphocyte mitochondria were isolated and analyzed for Complexes I, III, and IV of the electron transport chain. Western blots were used to control for differential enrichment of samples. Results demonstrated significant declines in Complexes III and IV in AD, and a significant decline in Complex IV in MCI. This report confirms mitochondrial deficiencies in AD, extends them to MCI, and suggests they are present at the earliest symptomatic stages of disease.

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Keywords

cytochrome oxidase; cytochrome reductase; electron transport chain; Alzheimer's disease; mild cognitive impairment; at-risk population

1. Introduction

Complex IV is deficient in AD platelet mitochondria (Parker et al., 1990) suggesting that AD may involve mitochondrial sequelae and that components of AD are systemic and unlikely to result from central neurodegeneration and activity-dependent downregulation. Parker's original finding of reduced platelet Complex IV activity in AD has been replicated (Parker et al., 1994a; Bosetti et al., 2002; Cardoso et al., 2004a), yet the role of mitochondrial abnormalities remains largely undefined. Amyloid- β (A β), the protein that makes up the amyloid plaques in the brain of AD patients, has been shown to interact directly with mitochondria and inhibit Complex IV activity (Crouch et al., 2005) and induce oxidative stress (Lustbader et al., 2004).

Fluorodeoxyglucose (FDG) positron emission tomography (PET) studies of persons with Alzheimer's disease (AD) (Minoshima et al., 1994, 1995, 1997; Mielke et al., 1994; Reiman et al., 1996) and mild cognitive impairment (MCI) characteristically show preferential and progressive neocortical reductions in the cerebral metabolic rate for glucose. MCI subjects who ultimately convert to dementia show specific reductions in prefrontal cortex and progressive decrements in posterior cingulate (Drzezga et al., 2003, 2005). Postmortem examination of hypometabolic posterior cingulate cortex in AD brains revealed decrements in oxidative metabolism (Valla et al., 2001), specifically in cytochrome oxidase activity, a component of Complex IV of the mitochondrial electron transport chain (ETC). We have also found that cognitively normal young-adult and middle-aged carriers of the apolipoprotein E ϵ 4 allele (APOE4), a common AD susceptibility gene, have functional brain abnormalities in the same neocortical regions as patients with probable AD, prior to the development of cognitive symptoms (Reiman et al., 2001, 2004, 2005). These 'neurometabolic' alterations may be the earliest brain abnormalities yet found in living persons at-risk for late-onset AD.

Considering these findings of early metabolic declines in the brains of AD patients and groups at-risk for dementia, we sought to extend previous findings of reduced complex IV activity in AD and hypothesized that such deficiency might also be observed in MCI patients, many of whom have an early stage of AD. Finding a deficiency in MCI subjects would support the hypothesis that mitochondrial dysfunction is an early event in AD pathophysiology, occurring near the onset of cognitive symptoms, and support further research in even younger at-risk populations.

2. Materials and Methods

Subjects with possible (N=1) or probable (N=7) AD (NINCDS-ADRDA criteria; McKhann et al., 1984), amnesic MCI (Petersen et al., 1999), and nondemented aged controls 65 years of age and older, were recruited from the clinical core of the Arizona AD Center (AZ ADC). Patients were eligible for clinical core inclusion following clinical evaluation that included medical and family histories, a neurological exam, structural brain imaging and laboratory tests. Each then received a consensus diagnosis by AZ ADC neurologists and completed AZ ADC procedures including the CDR (Morris et al., 1993), a neuropsychological test battery (encompassing memory, language, spatial, executive, and behavioral domains), APOE genotyping, and annual reassessment. For this study, we correlated test scores from the Folstein Mini-Mental Status Exam (MMSE), the Memory subscore of the Mattis Dementia Rating Scale

(DRS), and the Total Learning and Percent Recall subscores of the Auditory-Verbal Learning Test (AVLT) with the results of mitochondrial assays. In addition to the general consent for the longitudinal studies from which subjects were recruited, all subjects (or legally authorized proxy) provided separate consent for the mitochondrial assays following IRB approval.

All chemicals are from Sigma unless noted. Each subject provided 40 ml of blood collected via standard venipuncture into 8 ml Cell Preparation Tubes (Vacutainer) containing 0.1 M sodium citrate as an anticoagulant and a polyester gel and Ficoll Hypaque for blood separation. Processing yielded a platelet- and mononuclear cell-enriched plasma separated from erythrocytes and other cell types by the polyester gel. The plasma was diluted with phosphate-buffered saline (PBS, pH 7.2–7.35) and cycled through several centrifugation and wash steps at room temperature to pellet lymphocytes and platelets in turn. Residual erythrocytes in the lymphocyte prep were osmotically lysed using ammonium chloride (155 mM NH_4Cl [Fisher], 10 mM NaHCO_3 , 0.1 mM Na-EDTA in H_2O , pH 7.2–7.4). Final individual cell pellets were resuspended in isolation buffer (100 mM KCl, 50 mM Tris base, 5 mM MgCl_2 , 1 mM EGTA in H_2O , pH 8.0) in 5 ml polystyrene round-bottom tubes. Protease inhibitors (1 $\mu\text{l/ml}$ of 0.4 M PMSF in DMSO and 1 $\mu\text{l/ml}$ of 5 mg/ml leupeptin in H_2O) were added to prevent proteolytic damage. A portion of the intact platelet preparation was taken and stored at -80°C for additional analyses (e.g., mtDNA sequencing [Coon et al., 2006]). The tubes were then suspended in propylene glycol in the cup horn of a sonic dismembrator (Fisher Model 60) maintained at 4°C , and the cells were disrupted by brief sonication 0.5 cm above the cup horn. Considering that mitochondria appear to break down in AD (Hirai et al., 2001; Sergeant et al., 2003), special attention was paid to balancing mitochondrial yield against retention of a potentially fragile subpopulation of mitochondria in the patients, and thus, a number of initial patient samples were utilized to establish the appropriate cell disruption parameters (time and intensity), and only those within 2 steps (levels 3–6 of 20, for either 1 or 2 min) of the final platelet setting (level 4 of 20 for 1 min) were included in the final analysis. The overall mean time and intensity for platelet disruption was 1.5 min at level 4.6, with no significant differences between groups. Mean level for lymphocytes was 1.5 min at level 4.7, again with no significant differences. Debris and unlysed cells were subsequently pelleted with two centrifugations at 2000 RCF, followed by mitochondrial pelleting, repeated twice, at 10,000 RCF, all at 4°C . The resulting crude mitochondrial pellets, one for each cell type, were resuspended in 100 μl chilled buffer (0.25 M sucrose, 10 mM Tris base, 1 mM EGTA, 1 mM ATP in H_2O , pH 7.4), and stored at -80°C for the assays.

All subsequent procedures were identical for the platelet and lymphocyte preparations. Protein from the mitochondrial isolate was quantified using the BioRad DC kit (Pierce) according to manufacturer's instructions. Samples had to contain at least 40 μg of total protein to be included in the analysis; 2 AD and 2 control subjects did not have sufficient platelet protein yield and were excluded from further analyses. All assays were measured in a monochromator-equipped microplate reader (Molecular Devices Spectramax 190). The detailed methodology of the standardization and optimization of our microplate-based assays is presented in a manuscript in preparation. Final assay volume was 250 μl in a 96-well plate. Extinction coefficients for activity calculation were empirically determined for the instrument, and pathlength was measured at 0.85 cm. All activity results are in nmol substrate/min/mg total protein.

Complex I activity was determined by oxidation of NADH at 340 nm. 10 μg mitochondrial isolate protein were added to 0.1% deoxycholate and 5 mg/ml fatty acid (FA)-free BSA in H_2O to 200 μl and incubated for 1 min at 30°C . 50 μl of 5x assay buffer (50 mM Tris, pH 8.0, 240 μM KCN, 4 μM antimycin A, 800 μM NADH, 300 μM coenzyme Q1) were added and the change in absorbance of NADH was measured for 2 min. Subsequently, 2.5 μM rotenone (in EtOH) was added and the rate followed for 2 min. Activity was determined by subtracting the rotenone-inhibited rate from the initial rate.

Complex III activity was determined by reduction of cytochrome c at 550 nm. The electron donor was decylubiquinone (DB), reduced prior to the assay: a few grains of lithium borohydride (Aldrich) were added to 20 μ M stock DB, until clear, approx 4 μ l 12N HCl were added, reducing the pH to 1–2, and the clear product was stored at -80°C . For the assay, 10 μ g mitochondrial isolate protein were added to 0.1% deoxycholate and 10 mg/ml FA-free BSA in H_2O to 225 μ l and incubated at room temp for 1 min. 25 μ l of chilled 10x assay buffer (100 mM K^+ -phosphate, pH 7.55, 20 mM EDTA, 2.4 mM KCN, 40 μ M rotenone, 40 μ M cytochrome c, 800 μ M reduced DB) were added and change in absorbance of cytochrome c was measured for 2 min at 30°C . A no-protein control was run in parallel to provide background.

Complex IV activity was determined by reduction of cytochrome c at 550 nm. Cytochrome c was first reduced: 10 mM cyt c in 2.5 ml H_2O reduced with 1M ascorbic acid for 5 min and subsequently added to a PD-10 desalting column (Amersham) equilibrated with 20 mM K^+ -phosphate buffer, pH 7.0. Reduced cyt c was eluted from the column with 3.5 ml buffer, collected in equivalent fractions, and stored at -80°C . Reduction was verified >95% before each assay by comparing absorbance at 550 nm before and after adding dithionite. For the assay, 4 μ g mitochondrial isolate protein were added to pure H_2O to 225 μ l and incubated at 30°C for 1 min. 25 μ l of 10x assay buffer (200 mM K^+ -phosphate, pH 7.0, 0.7% reduced cyt c) were added and the change in absorbance of cytochrome c was measured for 2 min. Subsequently, 4 μ l of 1 mM KCN were added and the rate followed for 2 min to provide background.

The internal normalization of the activity results was to be accomplished by the ratio of complex I and IV to complex III, as recommended by Barrientos (2002), given that no significant difference had been found previously in platelet Complex III (Parker et al., 1994a). The unanticipated finding of a significant difference across groups in Complex III activity precluded normalizing subjects by that internal ratio. We did not feel that the commonly-used correction by matrix enzymes (e.g., citrate synthase) would be appropriate since we did not know the structural integrity of our mitochondrial preparation following sonication; we had used citrate synthase previously to assess the disruption parameters and had found a proportionally large signal in the supernatant of our mitochondrial pellets (which we lessened but did not eliminate by reducing disruption power; data not shown), indicating mitochondrial leakage. Subsequently, we opted to assess the relative purity of our mitochondrial preparations via SDS-PAGE where possible. 10 μ g mito isolate protein per lane were loaded into 10–20% 1.5 mm Tris-glycine 15-well minigels (Invitrogen) with Novex Tris-glycine SDS 2x sample buffer. Two lymphocyte preparations had sufficient samples for only 5 μ g per lane (1 AD, 1 control) and several other samples were exhausted prior to this unanticipated analysis. Gels were run in Novex mini-cell (Invitrogen) for 2 hours at constant 150V with Novex Tris-glycine SDS running buffer. Proteins were transferred to Immobilon P (Millipore) PVDF membrane, fully submerged in CAPS transfer buffer (10 mM 3-[cyclohexylamino]-1-propane sulfonic acid, pH 11 with NaOH, 10% methanol) using constant 150 mA for 2 hours. Membranes were blocked overnight in 5% Carnation dry milk in PBS at 4°C , and then probed with the Total OXPHOS Detection Kit (MS601; 1:1000 in 1% dry milk) available from Mitosciences (Eugene, OR), 2 hours at room temperature with gentle rocking. This five-antibody cocktail marks the 20 kDa subunit of Complex I (ND6), the 30 kDa iron-sulfur subunit of Complex II, the core 2 subunit of Complex III, COX II of Complex IV, and the F1 α subunit of ATP synthase. Goat anti-mouse alkaline phosphatase (AP)-conjugated secondary antibody (Santa Cruz Biotechnology; 1:10,000 in 1% dry milk) was used (2 hours room temperature with rocking), followed by application of an AP substrate detection kit (Biorad), according to manufacturer's instructions. Membranes were dried overnight then scanned on a Mustek A3 EP flatbed scanner along with an optical density (OD) step tablet for calibration. Membrane images were imported into Optimas (Media Cybernetics) image analysis software and average OD measures of each band were taken using a sampling window of constant size.

Final assay results were calculated from a minimum of two reliable assays per ETC complex. Individual complex assay results either 3x greater or lesser than another measure of that complex from the same subject were deemed unreliable and not included in the calculation or count. Complex I assay was performed last and was omitted if insufficient sample existed to run at least two assays. Reported values are mean $\pm$ standard deviation. All comparative statistical tests are Student's 2-tailed t-tests, uncorrected for multiple comparisons.

3. Results

Demographic characteristics, clinical ratings, and neuropsychological test scores are shown in Table 1. Mean age (76.2 ± 7.5) and education (15.4 ± 3.7) did not differ between groups, although the ages of the MCI and AD groups were greater than that of controls. Differences between the groups on cognitive measures were commensurate with clinical diagnoses.

Platelet enzyme assays and the optical densities of the Western blot bands are shown in Table 2. In comparison with the aged normal controls, AD patients had significantly lower platelet Complex IV activity (with a mean reduction of 37%) and Complex III activity (with a mean reduction of 39%). Similarly, in comparison with the aged normal controls, MCI patients had significantly lower Complex IV activity (with a mean reduction of 45%) and a non-significant trend for lower Complex III activity (with a mean reduction of 23%; Table 2 and Figure 1). No significant differences were found between total protein levels isolated or in Complex I activity, although power in the Complex I analysis was reduced due to 3 unassessable samples in the control group. No significant differences were found for any of the same assays in lymphocytes ($P > 0.3$, data not shown).

Semi-quantitative assessment of the Western blots yielded one statistically significant result, although again, power was reduced by the exhaustion of a number of samples (see Table 2). In comparison with the aged normal controls, Complex IV subunit II (COXII) OD was significantly lower in the MCI group; however, this difference was no longer apparent when the ratio of each subunit to the Complex I ND6 subunit was calculated to normalize across these samples to correct for varying purity. No significant differences were found in the lymphocyte preparation (data not shown); although it suffered similar power issues, all means were similar across groups.

Pearson correlations were calculated between Complex III and Complex IV activities and the MMSE, the memory subscore of the DRS, and the total learning and percent recall subscores of the AVLT. Across all subjects, there were no significant correlations between activity and performance, nor were there any significant correlations when considering AD and MCI together ($P > 0.05$). However, in the AD group alone, Complex IV activity positively correlated with the DRS memory scale ($r = 0.72$, $P = 0.05$, uncorrected for multiple comparisons), and with MMSE score in the MCI group ($r = 0.88$, $P = 0.05$, uncorrected for multiple comparisons). None of the cognitive measures correlated significantly with Complex III activity.

4. Discussion

This study confirms previous reports finding abnormally low platelet ETC Complex IV (cytochrome oxidase) activity in patients with AD and it raises the possibility of abnormally low platelet ETC Complex III (cytochrome reductase) activity in patients with AD and in patients with MCI.

To our knowledge, this is the first report of Complex III deficiency in AD, although deficiencies in both Complexes III and IV has been reported in a transgenic mouse model of AD (Caspersen et al., 2005). The finding in patients with MCI raises the possibility of detecting similar findings even earlier (e.g., in younger, cognitively normal APOE $\epsilon 4$ carriers, before the onset of any

symptoms or AD pathology). The absence of lymphocyte deficiencies in the same mitochondrial measurements has been reported before (Molina et al., 1997), but not with simultaneous measurement of other cell types. It remains possible that negative findings could be due to the loss of a diminished mitochondrial population during the isolation procedure or other methodological reasons specific to the cell type. Alternatively, longer-lived cells, such as platelet-forming megakaryocytes or neurons, may be more vulnerable or have more opportunity to reach a threshold required to show such defects (Mazat et al., 2001).

The platelet activities of Complexes II–IV and citrate synthase were examined by Parker et al. (1994a), and they reported a significant reduction only in Complex IV in AD patients. Complex III was decreased slightly in their study, and other groups finding ETC Complex IV deficiency did not also examine Complex III (Bosetti et al., 2002; Cardoso et al., 2004a). The disparity between our results and Parker et al. could be ascribed to methodological differences in our assays, particularly in the selection of ubiquinol analogs: they cite usage of ubiquinol-2 (Q_2) while we employed decylubiquinol. Choice of analog can have significant effects on the background reactions in the assay (Krahenbuhl et al., 1994). Most other features of our analyses were similar as we drew from Parker and colleagues' published procedures during the establishment of our mitochondrial isolation.

One limitation of this study is the failure to thoroughly control for protein levels of the affected enzyme complexes. Our original intent to use Complex III activity as an internal control would have been effective (Barrientos, 2002); Complex I could be used, but it tends to be the most difficult complex to assay and results were not available for every subject. Our Western data, as well as previous literature reports (Parker et al., 1990, 1994a; Parker and Parks, 1995), support the contention that Complex IV is catalytically abnormal in AD while protein levels remain normal; it remains unproven what effect that would have on Complex III, if Complex III itself were not also abnormal but subsequently downregulated, or vice versa. While Parker and Parks (1995) characterized a specific catalytic abnormality in Complex IV, the same type of analysis has not been performed on Complex III.

There were only 2 modest correlations between Complex IV function and our selected measures of cognitive impairment, and the lack of significant correlations across the combined AD and MCI groups indicates those may have been spurious. Considering that we measured mitochondrial activity in platelets rather than brain, such deficiencies may not correlate with neurological failure. However, similar deficits in ETC Complex IV function have been reported in postmortem AD cortex (Kish et al., 1992; Mutisya et al., 1994; Parker et al., 1994b; Chagnon et al., 1995; Gonzalez-Lima et al., 1997; Valla et al., 2001; Bosetti et al., 2002). The mitochondrial threshold effect may also preclude strong correlations between enzyme activity and cognitive impairment (Mazat et al., 2001).

The precise role of mitochondrial deficiencies in AD and their etiologies remain largely undefined. It is possible that mitochondrial changes are an early event in AD pathophysiology, playing a role in the etiology of the disease, alone, or as a co-factor synergistically interacting with early amyloid (Lustbader et al., 2004; Crouch et al., 2005; Caspersen et al., 2005) and/or tau alterations (Mandelkow et al., 2003). Platelets produce considerable amyloid precursor protein (APP; Di Luca et al., 2000) and possess β -secretase activity, so platelet preparations would not be immune from potential A β interactions (Padovani et al., 2002). The pathologies may simply induce catalytic changes in the enzymes, an idea supported by energy metabolism changes in transgenic mouse models of AD (Dodart et al., 1999; Reiman et al., 2000; Strazielle et al., 2003; Reddy et al., 2004; Caspersen et al., 2005; Valla et al., 2006). Conversely, energy inhibition can alter APP processing in cultured cells (Gabuzda et al., 1994), in mouse models of amyloid overexpression, increasing β -secretase activity and amyloidogenicity (Velliquette et al., 2005), and in rats, exacerbating A β peptide toxicity, particularly in synapses (Arias et al.,

2002). Also, mitochondrial involvement in AD etiology is supported by studies using a cybrid cell model, which utilizes mtDNA-depleted cultured cells re-populated with AD patient or control mitochondria. This model has been shown to recapitulate many AD features, including depressed ETC activity, increased reactive oxygen species production, reduced mitochondrial membrane potential, abnormal mitochondrial morphology, increased basal Ca^{2+} level, increased cytosolic cytochrome c, increased caspase-3 activity, and increased β -amyloid secretion, increased intracellular $\text{A}\beta$, and the development of Congo red-positive deposits in vitro (Sheehan et al., 1997; Swerdlow et al., 1997; Cassarino et al., 1998; Khan et al., 2000; Trimmer et al., 2000, 2004; Cardoso et al., 2004b).

In conclusion, this study has verified the hypothesis that mitochondrial deficits in Complex IV of the ETC can be found in subjects with MCI, a common precursor to AD. Furthermore, we describe a previously unreported deficit in Complex III in AD patients, and a trend toward the same in MCI. These findings leave open the possibility that mitochondrial dysfunction is occurring *prior to* symptom onset, perhaps in conjunction with other pathological changes occurring many years before the disease manifests and suggest the need to extend these studies even earlier to cognitively normal persons at differential genetic risk for AD. Further research is underway to determine the precise interplay and temporal relationship between mitochondrial changes and neuropathology and to find mitochondrial targets for intervention that may slow or stop the progression of this debilitating disease.

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Figure 1.

Platelet mitochondrial Complex IV and Complex III activities in nmol cyt c / min / mg total mitochondrial isolate protein. Significant ($p < 0.05$) decrements in Complex IV activity were found in AD and MCI patients; significant decrements in Complex III were found in AD patients only.

Demographic characteristics, clinical ratings, and neuropsychological test scores (mean $\pm$ standard deviation where appropriate). Statistical tests are Students 2-tailed t-tests, uncorrected for multiple comparisons, with calculated P values shown. Years since onset indicates years since estimated initial onset of symptoms, based on subject/informant's reported age at symptom onset. Some test scores were not available for all subjects; sample N for each test is shown in parenthesis.

Table 1

	Controls N = 7	MCI N = 5	AD N = 8 (1 poss, 7 prob)	P: Ctl v. MCI	P: Ctl v. AD
Gender	6 F, 1 M	4 F, 1 M	4 F, 4 M		
Age (years)	72.6 $\pm$ 5.7	78.1 $\pm$ 9.4	78.2 $\pm$ 7.2	0.229	0.124
Range	65.2 – 80.6	67.2 – 89.3	69.0 – 90.7		
Education (years)	15.8 $\pm$ 5.2	15.0 $\pm$ 2.4	15.3 $\pm$ 3.3	0.762	0.813
APOE4 carriers	3	1 (1 unknown)	5		
Years since onset		4.3 $\pm$ 0.8	5.3 $\pm$ 2.4		
Range		3.0 – 5.2	2.0 – 9.4		
Folstein MMSE	29.7 $\pm$ 0.5 (6)	26.4 $\pm$ 1.5 (5)	17.3 $\pm$ 6.9 (7)	0.0008	0.0009
Mattis DRS-Memory	24.0 $\pm$ 1.1 (6)	15.8 $\pm$ 2.4 (5)	9.5 $\pm$ 3.9 (8)	0.00003	0.000001
AVLT-Total learning	47.6 $\pm$ 8.2 (7)	28.6 $\pm$ 2.7 (5)	19.0 $\pm$ 5.0 (5)	0.0006	0.00004
AVLT-Percent recall	82.7 $\pm$ 22.4 (7)	13.2 $\pm$ 8.2 (5)	12.8 $\pm$ 21.7 (5)	0.00006	0.0003

Platelet enzyme assays (nmol/min/mg) and SDS-PAGE analysis (OD). Statistical tests are Students 2-tailed t-tests, uncorrected for multiple comparisons, but with calculated P values shown. All subjects yielded Complex IV and III assay data; 1 MCI subject and 3 controls did not yield Complex I assay data. SDS-PAGE data for some bands were not available for some subjects; sample N for each is shown in parenthesis. A high outlier in the AD group skewed the SDS-PAGE OD means upward but was ameliorated by calculating the internal expression ratios of III and IV to I.

Table 2

	Controls	MCI	AD	P: Ctl v. MCI	P: Ctl v. AD
Assays:					
Complex IV	114.4 ± 30.0	63.1 ± 38.6	72.0 ± 29.1	0.026	0.016
Complex III	131.1 ± 28.4	101.0 ± 31.2	80.2 ± 24.4	0.112	0.002
Complex I	50.2 ± 24.2	58.0 ± 29.2	49.8 ± 17.4	0.682	0.975
Protein (µg/µl)	1.4 ± 0.3	1.3 ± 0.5	1.4 ± 0.2	0.684	0.908
SDS-PAGE:					
Complex IV	0.156 ± 0.035 (5)	0.077 ± 0.046 (3)	0.171 ± 0.124 (6)	0.031	0.798
Complex III	0.050 ± 0.033 (5)	0.022 ± 0.016 (3)	0.056 ± 0.053 (5)	0.312	0.848
Complex II	0.029 ± 0.022 (5)	0.022 (1)	0.075 ± 0.144 (6)	0.779	0.507
Complex I	0.094 ± 0.070 (5)	0.051 ± 0.050 (3)	0.105 ± 0.109 (6)	0.397	0.849
ATPase (V)	0.280 ± 0.061 (5)	0.202 ± 0.087 (3)	0.264 ± 0.106 (6)	0.183	0.777
Ratio IV:I	2.41 ± 1.54 (5)	1.96 ± 1.16 (3)	2.26 ± 1.29 (6)	0.683	0.867
Ratio III:I	0.75 ± 0.87 (5)	0.38 ± 0.11 (2)	0.67 ± 0.61 (5)	0.601	0.878