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# Superoxide production is inversely related to complex I activity in inherited complex I deficiency

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**Running title:** Superoxide production in human CI deficiency

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**Abbreviations:** CI, complex I or NADH:ubiquinone oxidoreductase; CII, complex II or succinate dehydrogenase-ubiquinone oxidoreductase; CIII, complex III or ubiquinol:cytochrome c oxidoreductase; CIV, complex IV or cytochrome c oxidase; OXPHOS, oxidative phosphorylation; HET, hydroethidine;  $\Delta\psi$ , mitochondrial membrane potential.

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**ABSTRACT**

Deficiency of NADH:ubiquinone oxidoreductase or complex I (CI) is the most common cause of disorders of the oxidative phosphorylation system in humans. Using live cell imaging and blue-native electrophoresis we quantitatively compared superoxide production and CI amount and activity in cultured skin fibroblasts of 5 healthy control subjects and 21 children with inherited isolated CI deficiency. Thirteen children had a disease causing mutation in one of the nuclear-encoded CI subunits, whereas in the remainder the genetic cause of the disease is not yet established. Superoxide production was significantly increased in all but one of the patient cell lines. An inverse relationship with the amount and residual activity of CI was observed. In agreement with this finding, rotenone, a potent inhibitor of CI activity, dose-dependently increased superoxide production in healthy control cells. Also in this case an inverse relationship with the residual activity of CI was observed. In sharp contrast, however, rotenone did not decrease the amount of CI. The data presented show that superoxide production is increased in inherited CI deficiency and that this increase is primarily a consequence of the reduction in cellular CI activity and not of a further leakage of electrons from mutationally malformed complexes.

**Keywords:** fibroblast, mitochondria, hydroethidine, rotenone, fluorescence microscopy

## INTRODUCTION

The mitochondrial oxidative phosphorylation (OXPHOS) system consists of five multiprotein complexes: NADH:ubiquinone oxidoreductase (complex I or CI), succinate-dehydrogenase-ubiquinone oxidoreductase (CII), ubiquinol:cytochrome *c* oxidoreductase (CIII), cytochrome *c* oxidase (CIV) and F<sub>0</sub>F<sub>1</sub>-ATP synthase (CV) [1]. The first four complexes (CI-CIV), together with two electron carriers (coenzyme Q<sub>10</sub> and cytochrome *c*), constitute the respiratory chain that utilizes the energy of NADH and succinate, oxidized at CI and CII, respectively, to translocate protons from the mitochondrial matrix into the intermembrane space. The latter results in a negative potential ( $\Delta\psi$ ) across the inner mitochondrial membrane, which is crucial for the maintenance of mitochondrial integrity [2].

Dysfunction of the OXPHOS system is associated with a wide array of clinical manifestations, ranging from single lesions in Leber's hereditary optic neuropathy or maternally-inherited nonsyndromic deafness to more widespread lesions including myopathies, encephalomyopathies, cardiopathies, or complex multisystem syndromes [3-6]. Inherited disorders of the OXPHOS system are observed once every 10,000 live births and the most frequently occurring enzymatic deficiency is found in CI (EC 1.6.5.3), which catalyzes the transfer of electrons from NADH to coenzyme Q<sub>10</sub>. In human heart, this complex consists of at least 45 different subunits, together having a molecular weight close to 1 MDa [7]. With the aid of differential extraction techniques, CI can be subdivided into a flavoprotein (FP), iron-sulfur protein (IP) and hydrophobic protein (HP) fraction. The catalytic core of the complex comprises 14 evolutionary conserved subunits, 2 of which reside in the FP fraction, 5 in the IP fraction and 7 in the HP fraction [8]. In humans, these core subunits are encoded by the nuclear *NDUFV1* and *NDUFV2* genes, the nuclear *NDUFS1*, *NDUFS2*, *NDUFS3*, *NDUFS7* and *NDUFS8* genes and the mitochondrial *ND1-ND6*

and *ND4L* genes. Thus far, disease causing mutations have been identified in all 14 core subunits [1] and, as far as the nuclear genome is concerned, in genes encoding supernumerary subunits NDUFS4 [1] and NDUFS6 [9] and assembly factor B17.2L [10].

The pathophysiological mechanisms linking defects in CI genes to cellular dysfunction and disease are only partially understood. To obtain insight into these mechanisms, we use skin fibroblasts of patients with inherited isolated CI deficiency and study the biogenesis of CI and cytopathological consequences of its dysfunction. Native gel electrophoresis and in-gel activity assays revealed that mutations in CI subunits (NDUFS2, NDUFS4, NDUFS7 and NDUFS8) markedly reduced the expression of the fully assembled, catalytically active complex [11]. At the cell physiological level, this reduction was found to be associated with disturbed mitochondrial  $\text{Ca}^{2+}$  handling and ensuing ATP production during hormone stimulation [12,13].

Partial inhibition of CI activity by chronic treatment with rotenone significantly increased superoxide production and lipid peroxidation in cultured skin fibroblasts of healthy control subjects [14]. Similarly, the formation of superoxide-derived hydroxyl radicals and lipid peroxidation products was found to be increased in fibroblasts of three patients with a reduced rotenone-sensitive NADH-cytochrome *c* reductase (CI and CIII) activity [15]. At pathological concentrations, superoxide and its derived reactive oxygen species are hazardous for the cell. At physiological concentrations, however, they play important roles in a variety of signaling processes [16]. In fibroblasts of healthy control subjects we observed that the increase in superoxide production induced by chronic rotenone treatment was associated with a marked increase in mitochondrial length and degree of branching [14]. Moreover, in patient fibroblasts we found that this parameter was linearly correlated with the residual activity of CI [17]. Based on these observations, we hypothesized that the increase in mitochondrial length and degree of

branching constitutes an adaptive response to protect against the consequences of CI deficiency. In agreement with this idea, recent evidence showed that a more complex mitochondrial network, induced by manipulating mitochondrial fusion, protected against radical-induced mitochondrial depolarization possibly by allowing a more effective sharing of intra-mitochondrial antioxidants [18].

To obtain a quantitative understanding of the relationship between CI deficiency and superoxide production, we here compare superoxide production and amount and activity of CI between cultured skin fibroblasts of 5 healthy control subjects and 21 children with inherited isolated CI deficiency. The data presented show that superoxide production is increased in all patient cell lines but to a variable degree and that the extent of increase is inversely proportional to the amount and activity of CI.

## MATERIALS AND METHODS

**Patient skin fibroblasts** – Fibroblasts were derived from skin biopsies of 21 CI deficient children in the age range of 0-5 years, 2 age-matched healthy children and 5 healthy adults following informed consent and according to relevant institutional review boards [19]. **Table 1** shows that superoxide production did not differ between adult (CT1-CT5) and age-matched (CT6 and CT7) control cell lines. For 11 patients the biochemical analysis, mutations and clinical phenotypes were described before (P1, P2 [20]; P4-P6 [21]; P7, P8, P9, P11 [22]; P12 [23]), for 3 patients (P3, P10, P13) these data are not yet reported, whereas for the remaining 8 patients the mutations are hitherto unknown (P14-P21 [11]). Fibroblasts were cultured in medium 199 with Earle's salt supplemented with 10% (v/v) fetal calf serum, 100 IU/ml penicillin and 100 IU/ml streptomycin in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37° C. Measurements were performed within 5 passages after the start of the culture. The passage number at the onset of the culture is given in Table 1.

**Enzyme activity measurements** – Activities of NADH:ubiquinone oxidoreductase (CI; EC: 1.6.5.3) and cytochrome *c* oxidase (CIV; EC: 1.9.3.1) were determined in a mitochondrial enriched fraction of cultured fibroblasts as described previously [24]. The activity of CI was normalized against that of CIV and expressed as percentage of lowest control (110 mU/U CIV [19,24]).

**Quantification of superoxide production** – Fibroblasts were incubated in HEPES-Tris medium (132 mM NaCl, 4.2 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5.5 mM D-glucose and 10 mM HEPES, pH 7.4), containing 10 µM hydroethidine (HEt; Molecular Probes, Leiden, The

Netherlands) for 10 minutes at 37° C. HET is a lipophilic, uncharged compound that readily enters the cell where it reacts with superoxide to form two positively charged products, 2-hydroxyethidium and ethidium, both of which emit red fluorescence [25]. The reaction was stopped by thorough washing of the cells with PBS to remove excess HET. For quantitative analysis of the fluorescent HET oxidation products, coverslips were mounted in an incubation chamber placed on the stage of an inverted microscope (Axiovert 200 M, Carl Zeiss, Jena, Germany) equipped with a Zeiss 40x/1.3 NA fluor objective. The cells were excited at 490 nm using a monochromator (Polychrome IV, TILL Photonics, Gräfelfing, Germany). Fluorescence emission light was directed by a 525DRLP dichroic mirror (Omega Optical Inc., Brattleboro, VT, USA) through a 565ALP emission filter (Omega) onto a CoolSNAP HQ monochrome CCD-camera (Roper Scientific, Vianen, The Netherlands). Hardware was controlled with Metafluor 6.0 software (Universal Imaging Corporation, Downingtown, PA, USA). Routinely, 10 fields of view were analyzed per coverslip using an acquisition time of 100 milliseconds.

***Blue-native electrophoresis*** – Cultured skin fibroblasts were harvested by trypsinization, washed, and resuspended (approx.  $2 \cdot 10^6$  cells) in 100  $\mu$ l ice-cold PBS. For isolation of mitochondria, cells were incubated with 2 mg/ml digitonin (Biosciences Inc., La Jolla, CA, USA) in a final volume of 200  $\mu$ l for 10 min on ice. Next, 1 ml ice-cold PBS was added followed by centrifugation (5 min; 10,000g; 4° C). Mitochondrial pellets were washed twice with 1 ml ice-cold PBS and stored overnight (-20° C). Pellets were solubilized in 100  $\mu$ l ACBT buffer (Fluka, Steinheim, Germany) containing 1.5 M aminocaproic acid and 75 mM Bis-Tris/HCl (pH 7.0). To extract mitochondrial protein complexes, 20  $\mu$ l 10% (w/v)  $\beta$ -lauryl maltoside was added and the solution was incubated for 10 min on ice. After centrifugation (30 min; 10,000g; 4° C), 10  $\mu$ l of

blue native sample buffer (Biorad Laboratories, Hercules, CA, USA) was added to the supernatant. Blue native PAGE (BNP) and Western blotting, using monoclonal antibodies with human reactivity against the NDUFA9 (39-kDa) subunit of CI (Molecular Probes) and the 70-kDa subunit of CII (Molecular probes) at a dilution of 1:1000, was performed as described previously [26]. For quantitative analysis gels were loaded with exactly 40  $\mu$ g of mitochondrial protein. After Western blotting, luminescent signals were quantitatively analyzed by exposing illumination films to the blots for different periods of time (5-180 s). Non-saturating films were scanned using a G690 Imaging Densitometer (Biorad). From these scans, the integrated optical density of each band was determined and background corrected. The resulting numerical values were normalized to those obtained with control cells on the same blot.

**Data analysis** – Processing and analysis of fluorescence images and densitometer scans was performed with Image Pro Plus 5.1 (Media Cybernetics, San Diego, CA, USA) and Metamorph 6.1 (Universal Imaging Corporation, Downingtown, PA, USA). Numerical results were visualized using Origin Pro 7.5 (OriginLab Corp., Northampton, MA, USA) and presented as the mean  $\pm$  SEM. During linear regression analysis, the degree of bivariate correlation between sets of data was analyzed by calculating Pearson's R. This parameter expresses the proportion of total variation that is explained by the regression. If R is  $\pm 1$ , the total variation in the fit can be explained in terms of the regression curve [27]. For  $R > 0$  the correlation is positive, for  $R < 0$  the correlation is negative. Statistical differences between average values were determined using an independent two-population Student's *t*-test (Bonferroni corrected). P-values  $< 0.05$  were considered significant.

*Chemicals* – Culture materials were obtained from Invitrogen (Breda, The Netherlands), all other reagents were from Sigma-Aldrich (St. Louis, MO, USA).

ACCEPTED MANUSCRIPT

## RESULTS

*Quantitative measurement of superoxide production in human skin fibroblasts* – In this study we assessed superoxide production in living cells using hydroethidine (HEt), a membrane-permeable derivative of ethidium bromide that is specifically converted by superoxide into 2-hydroxyethidium and ethidium [25,28]. Both products are positively charged and emit red fluorescence when excited at 490 nm. Human skin fibroblasts were grown to ~70% confluence, treated with 10  $\mu$ M HEt for 10 min and thoroughly washed to remove non-oxidized HEt. Next, video-imaging microscopy was used for subcellular quantification of the fluorescent oxidation products formed during the 10-min incubation period with HEt. We reported before that the fluorescent products formed during the oxidation of HEt accumulated predominantly in the nucleus and a widespread network of tubular structures located within the cytosol [14]. **Figure 1A** shows that in both compartments the average fluorescence intensity remained stable for at least 200 s, indicating that non-oxidized HEt was effectively removed during the washing step and that its oxidation products did not translocate during the ensuing recording period. In addition, this result demonstrates that the fluorescent products were resistant to photo-bleaching. **Figure 1A** furthermore shows that dissipation of the mitochondrial membrane potential by FCCP (p-trifluoromethoxy carbonyl cyanide phenyl hydrazine; 1  $\mu$ M) leads to a rapid decrease in tubular fluorescence. This result identifies the tubular structures as active mitochondria in which positively charged HEt oxidation products are retained in a membrane potential-dependent manner. The FCCP-induced decrease in tubular fluorescence was mirrored by a concomitant increase in nuclear fluorescence, indicating the translocation of HEt oxidation products from the mitochondria to the nucleus. The results obtained with FCCP demonstrate that HEt oxidation

products can easily pass mitochondrial membranes. Therefore, it is not possible to make a statement concerning the exact site(s) of primary HEt oxidation.

***Chronic rotenone treatment dose-dependently increases superoxide production in human skin***

***fibroblasts*** – To mimic the pathological condition of chronic CI deficiency, control fibroblasts were cultured in the presence of different concentrations (0.1-5000 nM) of rotenone for 72 h [14]. At concentrations at or below 1 nM, this specific inhibitor of CI had no effect on the amount of fluorescent oxidation products that accumulated in the mitochondrial compartment during the 10-min incubation period with HEt (**Fig. 1B**, closed circles). At higher concentrations, however, the drug dose-dependently increased this amount. For the entire range of rotenone concentrations, mitochondrial and nuclear fluorescence were linearly correlated ( $R=0.98$ ,  $p<0.0001$ ). The increase in mitochondrial fluorescence was virtually mirrored by the decrease in residual CI activity (**Fig. 1B**, open circles), determined in a mitochondrial enriched fraction of cultured fibroblasts (data from [14]). Half-maximal concentrations were 110 nM and 20 nM, respectively. These findings show that in human skin fibroblasts drug-induced reduction in CI activity is accompanied by increasing production of cellular superoxide.

***Chronic rotenone increases fully assembled CI in human skin fibroblasts*** – We showed before

that chronic rotenone treatment (100 nM, 72 h) caused an increase in mitochondrial length and degree of branching [14]. Using blue-native polyacrylamide gel electrophoresis we here show that this treatment also caused a 1.3-fold increase ( $p<0.05$ ) in fully assembled CI (**Fig. 1C**). In contrast, this treatment did not alter the amount of CII.

***Superoxide production is increased in patient skin fibroblasts*** – The amount of fluorescent oxidation products that accumulated in the mitochondrial compartment during the 10-min incubation with HEt did not significantly differ between fibroblasts of seven healthy control subjects (**Table 1**, CT1-CT7). Compared to control, however, mitochondrial accumulation of HEt oxidation products was significantly increased in all but two patient cell lines (P2 and P20). As for healthy control fibroblasts treated with various concentrations of rotenone, mitochondrial and nuclear fluorescence were linearly correlated for the whole cohort of patient cell lines ( $R=0.98$ ,  $p<0.0001$ ). Linear regression analysis furthermore revealed an inverse relationship between mitochondrial fluorescence and residual CI activity (**Fig. 2**,  $R=-0.83$ ,  $p<0.0001$ ). In all patient cell lines, acute treatment with 100 nM rotenone (during the 10-min incubation with HEt) caused a further increase in fluorescence intensity (**Table 1**), demonstrating the presence of a functional rotenone binding site.

***Fully assembled CI is decreased in patient skin fibroblasts*** – We reported before that the amount of fully assembled CI is markedly reduced in patient fibroblasts with mutations in nuclear-encoded CI subunits [11]. **Figure 3**, depicts assembly data of a subgroup of 13 patients, 7 with a known mutation (**Table 1**, P1-P5 and P12-P13) and 6 with a hitherto unknown mutation (**Table 1**, P14-P16 and P18-P20), and shows that in all but one (P20) of them the amount of fully assembled CI was lower than control. Except cell line P20, the cell lines of the other patients with a hitherto unknown mutation (open symbols) did not differ from those with a known mutation (closed symbols). Linear regression analysis revealed that for the whole cohort of patient cell lines this parameter was negatively related to the cell's superoxide production ( $R=-0.62$ ,  $p=0.017$ ) and positively to its residual CI activity ( $R=0.89$ ,  $p<0.0001$ ).

## DISCUSSION

Malfunction of the OXPHOS system causes a wide range of neuromuscular, cardiac, and endocrine disorders and, more recently, has also been implicated in age-related diseases and various forms of cancer [3,6,29]. In 40% of the cases of inherited OXPHOS disease, the deficiency is associated with an isolated (25%) or combined (15%) defect in CI, the first and largest complex of the OXPHOS system [19]. At present, however, there is only very limited information about the cytopathological effects of a disease-causing reduction in CI activity. Here, we quantitatively assessed superoxide production in living skin fibroblasts of a large cohort of 21 patients with inherited isolated CI deficiency, 13 of which with an established mutation in one of the nuclear-encoded CI subunits and 8 in which the disease causing mutation still has to be established, and demonstrate an inverse relationship with the amount and residual activity of this complex. The same inverse relationship between superoxide production and CI activity was observed in healthy fibroblasts treated with increasing concentrations of rotenone. In the latter case, however, the amount of CI increased rather than decreased, indicating that superoxide production can increase in the absence of any mutated subunit. Based on the latter finding we conclude that in isolated human CI deficiency increased cellular superoxide production is primarily a consequence of decreased cellular CI activity and not of further electron leakage due to the presence of a mutated subunit.

In healthy cells, the vast majority (>90%) of reactive oxygen species is produced as a consequence of oxidative phosphorylation [2,30]. Work on isolated mitochondrial preparations suggests that CI and CIII are the major contributors to superoxide production [2,30,31]. In addition, CII can contribute by donating electrons to CI via reverse electron flow [32,33]. As far as CI is concerned, single electron reduction of oxygen can occur at the FMN binding site, the

iron-sulfur clusters and/or the Q-binding site [32-37]. Evidence has been provided that superoxide produced by CI is released into the mitochondrial matrix, whereas that produced by CIII enters both the matrix and the intermembrane space [33]. It has been demonstrated that reverse electron flow can be effectively blocked by CI inhibitors, leading to a reduction in superoxide production. Here we show that in all patient cell lines acute addition of rotenone increased rather than decreased the amount of H<sub>2</sub>O<sub>2</sub> oxidation products, indicating that reverse electron flow is not the underlying cause of increased superoxide production in isolated human CI deficiency.

In addition to CI and CIII, the tricarboxylic acid cycle enzyme  $\alpha$ -ketoglutarate dehydrogenase can produce significant amounts of superoxide, especially under conditions that the NADH level in the matrix is increased [38]. Moreover, other organelles, such as the endoplasmic reticulum and peroxisomes, may contribute to the production of superoxide (reviewed in [39]). Because H<sub>2</sub>O<sub>2</sub> oxidation products can easily pass mitochondrial membranes, the method used here to measure cellular superoxide production does not allow to make a statement concerning the exact site(s) of H<sub>2</sub>O<sub>2</sub> oxidation. Whatever the case may be, the present finding that chronic rotenone decreased the activity of CI and increased the amount of H<sub>2</sub>O<sub>2</sub> oxidation products with virtually the same potency indicates that a reduction in mitochondrial CI activity is associated with an increase in cellular superoxide production. Importantly, because the inhibitor increased rather than decreased the amount of fully assembled CI, it can be concluded that the observed increase in superoxide production was not due to misassembly of the complex.

The same correlation between cellular superoxide production and residual CI activity was observed within our large cohort of CI deficient patient cell lines. In this case, however, the decrease in residual activity was quantitatively correlated with a decrease in fully assembled CI.

In agreement with our results, the NDUFS1-Q522K mutation has recently been published to be associated with a marked reduction in CI amount and activity and a significant increase in mitochondrial superoxide and derived reactive oxygen species [40]. Taken together, the present findings may provide first insight into the mechanism of superoxide production in human CI deficiency in that they suggest that decreasing numbers of active complexes produce increasing amounts of superoxide not because of the presence of a mutated subunit but as a consequence of a decrease in cellular CI activity. The alternative explanation that increasing numbers of partially assembled complexes are responsible for the observed increase in superoxide production is not supported by the present finding that chronic rotenone treatment increased rather than decreased the amount of fully assembled CI.

The cohort of patient cell lines used in this study included 13 cell lines with established mutations in nuclear-encoded CI subunits (see, **Table 1**). All 6 affected subunits are predicted to constitute part of the matrix-protruding arm of CI [41]. Five of them, NDUFV1 (51-kDa), NDUFS1 (75-kDa), NDUFS2 (49-kDa), NDUFS7 (PSST) and NDUFS8 (TYKY), belong to the group of 14 “central” subunits that are sufficient to perform all bioenergetic functions of the complex, whereas the remaining one, NDUFS4 (AQDQ), belongs to the large group of accessory proteins. Except for NDUFS2, the other 4 “central” subunits carry iron-sulfur clusters responsible for electron transport from NDUFV1, where NADH is oxidized, to NDUFS7, where ubiquinone is reduced. With the exception of 5 cell lines carrying a mutation in NDUFS4 (see below), 7 (P6 was not analyzed) of the other 8 cell lines displayed a fully assembled complex on a blue-native gel, the amount of which was decreased to a variable degree. Irrespective of the affected subunit, this amount was quantitatively correlated with the cell’s residual CI activity, indicating that primarily the expression of the fully assembled complex rather than its intrinsic

activity is altered. This conclusion is in agreement with our proposal that the increase in cellular superoxide production is not a direct consequence of further electron leakage from complexes containing a mutated subunit, but of the decrease in cellular CI activity. Importantly, when patient cells were acutely treated with rotenone, superoxide production was even further increased. This indicates that the rotenone-binding site, which is part of the coenzyme Q binding site [30,42], is intact in these CI deficient patients.

Regarding the 5 patient cell lines carrying a mutation in *NDUFS4*, they all showed a rotenone-sensitive CI activity when assayed in a mitochondrial enriched fraction. On the other hand, all 5 cell lines showed only an inactive subcomplex on a blue-native gel. Previous work revealed the complete absence of any *NDUFS4* in patient cell lines P7 and P11 [43]. Together with the information that the other 3 patient cell lines of the present study are either homozygous for a premature stop codon (P8 and P9) or carry a premature stop codon and a frameshift (P10) and, therefore, do not express any *NDUFS4*, these results indicate that in the absence of *NDUFS4* catalytically active complexes are formed, which are, however, relatively unstable and fall apart during blue-native electrophoresis. Analysis of the amount of subcomplex as a measure of the amount of catalytically active complex in the mitochondrial enriched fraction then shows that also in these patient cells significantly less catalytically active complex is formed. In contrast to the present study, it was recently shown that fibroblasts of a patient with a nonsense mutation in the first exon of the *NDUFS4* gene (*NDUFS4*-W16X) produced normal amounts of superoxide [40]. Similarly, the present study demonstrates that superoxide production can be increased (P1) or normal (P2) in fibroblasts of two CI deficient brothers. Both cell lines showed the same decrease in fully assembled CI and residual CI activity. These findings indicate that additional factors, such as detoxifying enzymes and nonenzymatic antioxidants, play a role in determining

net superoxide production. The involvement of such factors is also evident from the large variation in superoxide production at any given residual CI activity (**Fig. 2**).

Finally, our results partially agree with earlier work showing that superoxide generation was increased in 5 CI deficient patients, whereas it was not different from control or even lower than control in another 8 patients [44]. Moreover, in contrast to the latter study, we found no correlation between the rate of superoxide production and clinical phenotype (depicted in **Table 1**). At present, we have no explanation for these discrepancies. Major differences are the use of living fibroblasts rather than frozen-thawed mitochondria and the use of HEt instead of the chemiluminescent probe lucigenin. Both studies had only two clinical phenotypes in common, Leigh's Disease (LD) and Fatal Infantile Lactic Acidosis (FILA). In both studies, the LD patients showed an increase in superoxide, whereas the two FILA patients investigated in the earlier study showed either a decrease or no change in superoxide production. Our study did not include patients displaying Cardiomyopathy with Cataracts and Hepatomegaly with Renal Tubulopathy, shown in the previous study to have a decreased and normal rate of superoxide production, respectively. Obviously, evaluation of these latter clinical phenotypes is required to be confident that under all conditions of isolated CI deficiency a decrease in CI activity is associated with an increase in cellular superoxide production.

In accordance with the above findings, antioxidants were found to improve OXPHOS function in superoxide dismutase 2 null mice [45] and fibroblasts of Parkinson's disease patients [46]. Furthermore, chronic antioxidant treatment was shown to increase CV activity and ATP synthesis in cybrids containing the mtDNA of patients with the T8993G mtDNA mutation associated with impaired oxidative phosphorylation in NARP (neuropathy, ataxia and retinitis pigmentosa) and MILS (maternally inherited Leigh's syndrome) [47]. Likewise, chronic oral

administration of high concentrations of vitamin E was found to prevent the loss of mitochondrial function and reduce protein and lipid oxidation in brain and liver of aging mice [48]. These beneficial effects were paralleled by an increased lifespan, better neurological performance and higher exploratory activity. Regarding human CI deficiency, patients have been found to respond differentially to antioxidant treatment (e.g. ref. [49]).

## REFERENCES

1. J.A.M. Smeitink, L.W.P.J. van den Heuvel, S. DiMauro, The genetics and pathology of oxidative phosphorylation, *Nature Rev. Genet.* 2 (2001) 342-352.
2. M.R. Duchon, Mitochondria in health and disease: perspectives on a new mitochondrial biology, *Mol. Aspects Med.* 25 (2004) 365-451.
3. E.A. Shoubridge, Nuclear genetic defects of oxidative phosphorylation, *Hum. Mol. Genet.* 10 (2001) 2277-2284.
4. L.W.P.J. van den Heuvel, J.A.M. Smeitink, The oxidative phosphorylation (OXPHOS) system: nuclear genes and human genetic diseases, *Bioessays* 23 (2001) 518-25.
5. S. DiMauro, E.A. Schon, Mitochondrial respiratory-chain diseases, *New Engl. J. Med.* 348 (2003) 2656-2668.
6. M. Zeviani, A. Spinazzola, V. Carelli, Nuclear genes in mitochondrial disorders, *Curr. Op. Genet. Devel.* 13 (2003) 262-270.
7. J. Carroll, I.M. Fearnley, J.M. Skehel, R.J. Shannon, J. Hirst, J.E. Walker, Bovine complex I is a component of 45 different subunits, *J. Biol. Chem.* 281 (2006) 32724-32727.
8. T. Friedrich, B. Böttcher, The gross structure of the respiratory complex I: a lego system, *Biochim. Biophys. Acta* 1608 (2004) 1-9.
9. D.M. Kirby, R. Salemi, C. Sugiana, A. Ohtake, L. Parry, K.M. Bell, E.P. Kirk, A. Boneh, R.W. Taylor, H.H.M. Dahl, M.T. Ryan, D.R. Thorburn, NDUFS6 mutations are a novel cause of lethal neonatal mitochondrial complex I deficiency, *J. Clin. Invest.* 114 (2004) 837-845.

10. I. Ogilvie, N.G. Kennaway, E.A. Shoubridge, A molecular chaperone for mitochondrial complex I assembly is mutated in a progressive encephalopathy. *J. Clin. Invest.* 115 (2005) 2784-2792.
11. C. Ugalde, R.J. Janssen, L.W.P.J. van den Heuvel, J.A.M. Smeitink, L.G.J. Nijtmans, Differences in assembly or stability of complex I and other mitochondrial OXPHOS complexes in inherited complex I deficiency, *Hum. Mol. Genet.* 13 (2004) 659-667.
12. H.J. Visch, G.A. Rutter, W.J.H. Koopman, J.B. Koenderink, S. Verkaart, T. de Groot, A. Varadi, K.J. Mitchell, L.W.P.J. van den Heuvel, J.A.M. Smeitink, P.H.G.M. Willems, Inhibition of mitochondrial  $\text{Na}^+$ - $\text{Ca}^{2+}$  exchange restores agonist-induced ATP production and  $\text{Ca}^{2+}$  handling in human complex I deficiency, *J. Biol. Chem.* 279 (2004) 40328-40336.
13. H.J. Visch, W.J.H. Koopman, A. Leusink, S.E. van Emst-de Vries, L.W.P.J. van den Heuvel, P.H.G.M. Willems, J.A.M. Smeitink, Decreased agonist-stimulated mitochondrial ATP production caused by a pathological reduction in endoplasmic reticulum calcium content in human complex I deficiency, *Biochim. Biophys. Acta* 1762 (2005) 115-123.
14. W.J.H. Koopman, S. Verkaart, H.J. Visch, F.H. van der Westhuizen, M.P. Murphy, L.W.P.J. van den Heuvel, J.A.M. Smeitink, P.H.G.M. Willems, Inhibition of complex I of the electron transport chain causes  $\text{O}_2^{\cdot-}$ -mediated mitochondrial outgrowth, *Am. J. Physiol. Cell. Physiol.* 288 (2005) C1440-C1450.
15. X. Luo, S. Pitkänen, S. Kassovska-Bratinova, B.H. Robinson, D.C. Lehotay, Excessive formation of hydroxyl radicals and aldehydic lipid peroxidation products in cultured skin fibroblasts from patients with complex I deficiency, *J. Clin. Invest.* 99 (1997) 2877-2882.
16. W. Dröge, Free radicals in the physiological control of cell function, *Physiol. Rev.* 82 (2001) 47-95.

17. W.J.H. Koopman, H.J. Visch, S. Verkaart, L.W.P.J. van den Heuvel, J.A.M. Smeitink, P.H.G.M. Willems, Mitochondrial network complexity and pathological decrease in complex I activity are tightly correlated in isolated human complex I deficiency, *Am. J. Physiol. Cell. Physiol.* 289 (2005) C881-C890.
18. M. Neuspiel, R. Zunino, S. Gangaraju, P. Rippstein, H. McBride, Activated mitofusin 2 signals mitochondrial fusion, interferes with Bax activation, and reduces susceptibility to radical induced depolarization, *J. Biol. Chem.* 280 (2005) 25060-25070.
19. J.A.M. Smeitink, R. Sengers, F. Trijbels, L.W.P.J. van den Heuvel, Human NADH:ubiquinone oxidoreductase, *J. Bioenerg. Biomembr.* 33 (2001) 259-66.
20. M. Schuelke, J.A.M. Smeitink, E. Mariman, J. Loeffen, B. Plecko, F. Trijbels, S. Stockler-Ipsiroglu, L.W.P.J. van den Heuvel, Mutant NDUFV1 subunit of mitochondrial complex I causes leukodystrophy and myoclonic epilepsy, *Nat. Genet.* 21 (1999) 260-261.
21. J. Loeffen, O. Elpeleg, J.A.M. Smeitink, R. Smeets, S. Stockler-Ipsiroglu, H. Mandel, R. Sengers, F. Trijbels, L.W.P.J. van den Heuvel, Mutations in the complex I NDUFV2 gene of patients with cardiomyopathy and encephalomyopathy, *Ann. Neurol.* 49 (2001) 195-201.
22. S.M. Budde, L.W.P.J. van den Heuvel, R.J. Smeets, D. Skladal, J.A. Mayr, C. Boelen, V. Petruzzella, S. Papa, J.A.M. Smeitink, Clinical heterogeneity in patients with mutations in the NDUFV4 gene of mitochondrial complex I, *J. Inherit. Metab. Dis.* 26 (2003) 813-815.
23. R.H. Triepels, L.W.P.J. van den Heuvel, J.L. Loeffen, C.A. Buskens, R.J. Smeets, M.E. Rubio Gozalbo, S.M. Budde, E.C. Mariman, F.A. Wijburg, P.G. Barth, J.M. Trijbels, J.A.M. Smeitink, Leigh syndrome associated with a mutation in the NDUFV7 (PSST) nuclear encoded subunit of complex I, *Ann. Neurol.* 45 (1999) 787-790.

24. A.J. Janssen, J.A.M. Smeitink, L.W.P.J. van den Heuvel, L.W.P.J., Some practical aspects of providing a diagnostic service for respiratory chain defects, *Ann. Clin. Biochem.* 40 (2003) 3-8.
25. H. Zhao, J. Joseph, H.M. Fales, E.A. Sokoloski, R.L. Levine, J. Vasquez-Vivar, B. Kalyanaraman, Detection and characterization of the product of hydroethidine and intracellular superoxide by HPLC and limitations of fluorescence, *Proc. Natl. Acad. Sci. USA* 102 (2005) 5727-5732.
26. L.G.J. Nijtmans, N.S. Henderson, I.J. Holt, Blue Native electrophoresis to study mitochondrial and other protein complexes, *Methods* 26 (2002) 327-334.
27. A.J.C. de Groof, J.A. Fransen, R.J. Errington, P.H.G.M. Willems, B. Wieringa, W.J.H. Koopman, The creatine kinase system is essential for optimal refill of the sarcoplasmic reticulum  $\text{Ca}^{2+}$  store in skeletal muscle, *J. Biol. Chem.* 277 (2002) 5275-5284.
28. H. Zhao, S. Kalivendi, H. Zhang, J. Joseph, K. Nithipatikom, J. Vasquez-Vivar, B. Kalyanaraman, Superoxide reacts with hydroethidine but forms a fluorescent product that is distinctly different from ethidium: potential implications in intracellular fluorescence detection of superoxide, *Free Radic. Biol. Med.* 34 (2003) 1359-1368.
29. D.C. Wallace, A Mitochondrial Paradigm of Metabolic and Degenerative Diseases, Aging, and Cancer: A Dawn for Evolutionary Medicine, *Annu. Rev. Genet.* 39 (2005) 359-407.
30. R.S. Balaban, S. Nemoto, T. Finkel, Mitochondria, oxidants, and aging, *Cell* 120 (2005) 483-495.
31. S. Raha, B.H. Robinson, Mitochondria, oxygen free radicals, and apoptosis, *Am. J. Med. Genet.* 106 (2001) 62-70.

32. Y. Liu, G. Fiskum, D. Schubert, Generation of reactive oxygen species by the mitochondrial electron transport chain, *J. Neurochem.* 80 (2002) 780-787.
33. A.J. Lambert, M.D. Brand, Inhibitors of the quinone-binding site allow rapid superoxide production from mitochondrial NADH:ubiquinone oxidoreductase (Complex I), *J. Biol. Chem.* 279 (2004) 39414-39420.
34. M.L. Genova, B. Ventura, G. Giuliano, C. Bovina, G. Formiggini, G. Parenti-Castelli, G. Lenaz, The site of production of superoxide radical in mitochondrial Complex I is not a bound ubisemiquinone but presumably iron-sulfur cluster N2, *FEBS Lett.* 505 (2001) 364-368.
35. Y.R. Chen, C.L. Chen, L. Zhang, K.B. Green-Church, J.L. Zweier, Superoxide generation from mitochondrial NADH dehydrogenase induces self-inactivation with specific protein radical formation, *J. Biol. Chem.* 280 (2005) 37339-37348.
36. S.T. Ohnishi, T. Ohnishi, S. Muranaka, H. Fujita, H. Kimura, K. Uemurak, K. Yoshida, K. Utsumi, A possible site of superoxide generation in the complex I segment of rat heart mitochondria, *J. Bioenerg Biomembr.* 37 (2005) 1-15.
37. A. Bacsi, M. Woodberry, W. Widger, J. Papaconstantinou, S. Mitrac, J.W. Peterson, I. Boldogh, Localization of superoxide anion production to mitochondrial electron transport chain in 3-NPA-treated cells. *Mitochondrion* 6 (2006) 235-244.
38. L. Tretter, V. Adam-Vizi, Generation of reactive oxygen species in the reaction catalyzed by  $\alpha$ -ketoglutarate dehydrogenase, *J. Neurosci.* (2004) 7771-7778.
39. L.C. Hool, Reactive oxygen species in cardiac signaling: from mitochondria to plasma membrane ion channels, *Clin. Exper. Pharmacol. Physiol.* 33 (2006) 146-151.

40. A. Iuso, S. Scacco, C. Piccoli, F. Bellomo, V. Petruzzella, R. Trentadue, M. Minuto, M. Ripoli, N. Capitano, M. Zeviani, S. Papa, Dysfunctions of cellular oxidative metabolism in patients with mutations in the NDUFS1 and NDUFS4 genes of complex I. *J. Biol. Chem.* 281 (2006) 10374-10380.
41. U. Brandt, Energy Converting NADH:Quinone Oxidoreductase (Complex I), *Annu. Rev. Biochem.* 75 (2006) 69-92.
42. J.G. Okun, P. Lümmer, U. Brandt, Three classes of inhibitors share a common binding domain in mitochondrial complex I (NADH:ubiquinone oxidoreductase), *J. Biol. Chem.* 274 (1999) 2625-2630.
43. S. Scacco, V. Petruzzella, S. Budde, R. Vergari, R. Tamborra, D. Panelli, L.W.P.J. van den Heuvel, J.A.M. Smeitink, S. Papa, Pathological mutations of the human NDUFS4 gene of the 18-kDa (AQDQ) subunit of complex I affect the expression of the protein and the assembly and function of the complex, *J. Biol. Chem.* 278 (2003) 44161-74416.
44. S. Pitkänen, B.H. Robinson, Mitochondrial complex I deficiency leads to increased production of superoxide radicals and induction of superoxide dismutase, *J. Clin. Invest.* 98 (1996) 345-351.
45. D. Hinerfeld, M.D. Traini, R.P. Weinberger, B. Cochran, S.R. Doctrow, J. Harry, S. Melov, Endogenous mitochondrial oxidative stress: neurodegeneration, proteomic analysis, specific respiratory chain defects, and efficacious antioxidant therapy in superoxide dismutase 2 null mice, *J. Neurochem.* 88 (2004) 657-667.
46. K. Winkler-Stuck, F.R. Wiedemann, C.W. Wallesch, W.S. Kunz, Effect of coenzyme Q10 on the mitochondrial function of skin fibroblasts from Parkinson patients, *J. Neurol. Sci.* 220 (2004) 41-48.

47. M. Mattiazzi, C. Vijayvergiya, C.D. Gajewski, D.C. DeVivo, G. Lenaz, M. Wiedmann, G. Manfredi, The mtDNA T8993G (NARP) mutation results in an impairment of oxidative phosphorylation that can be improved by antioxidants, *Hum. Mol. Genet.* 13 (2004) 869-879.
48. A. Navarro, C. Gomez, M.J. Sanchez-Pino, H. Gonzalez, M.J. Bandez, A.D. Boveris, A. Boveris, Vitamin E at high doses improves survival, neurological performance and brain mitochondrial function in aging male mice. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 289 (2005) R1392-R1399.
49. J. Panetta, L.J. Smith, A. Boneh, Effect of high-dose vitamins, coenzyme Q and high-fat diet in paediatric patients with mitochondrial diseases. *J. Inherit. Metab. Dis.* 27 (2004) 487-498.

**FIGURE LEGENDS**

**Figure 1: Effect of chronic rotenone treatment on subcellular superoxide production, residual CI activity and cellular CI expression in human skin fibroblasts** – (A) FCCP-induced redistribution of HEt oxidation products between mitochondria and nucleus. Fibroblasts (CT1, **Table 1**) were incubated with 10  $\mu$ M hydroethidine (HEt) for 10 min at 37° C, thoroughly washed and visualized using fluorescence microscopy. FCCP (1  $\mu$ M) was added at the designated time (arrow). Fluorescence signals were obtained from the indicated compartments and background corrected. (B) Dose-dependency of the effect of chronic (72 h) rotenone treatment on the accumulation of HEt oxidation products in the indicated compartment (closed symbols) and the residual CI activity (open symbols). Fibroblasts were pretreated with vehicle or the indicated concentration of rotenone (closed symbols) for 72 h. Residual CI activity (right y-axis) and fluorescence intensity (left y-axis) are expressed as percentage of vehicle-treated control. The data presented are the average  $\pm$  SEM of 3 independent measurements. (C) Effect of chronic rotenone treatment (100 nM, 72 h) on CI expression. Mitochondrial enriched fractions of vehicle- and rotenone-treated cells were subjected to blue native electrophoresis and Western blotting. Intensities of the CI and CII bands were determined by densitometry and expressed as percentage of vehicle-treated control. The data presented are the average  $\pm$  SEM of three independent experiments. \*, significantly different from control.

**Figure 2: Inverse relationship between superoxide production and residual CI activity in fibroblasts of patients with isolated CI deficiency** – Measurement of superoxide production was performed as described in the caption to **figure 1**. Fluorescence intensity in the indicated

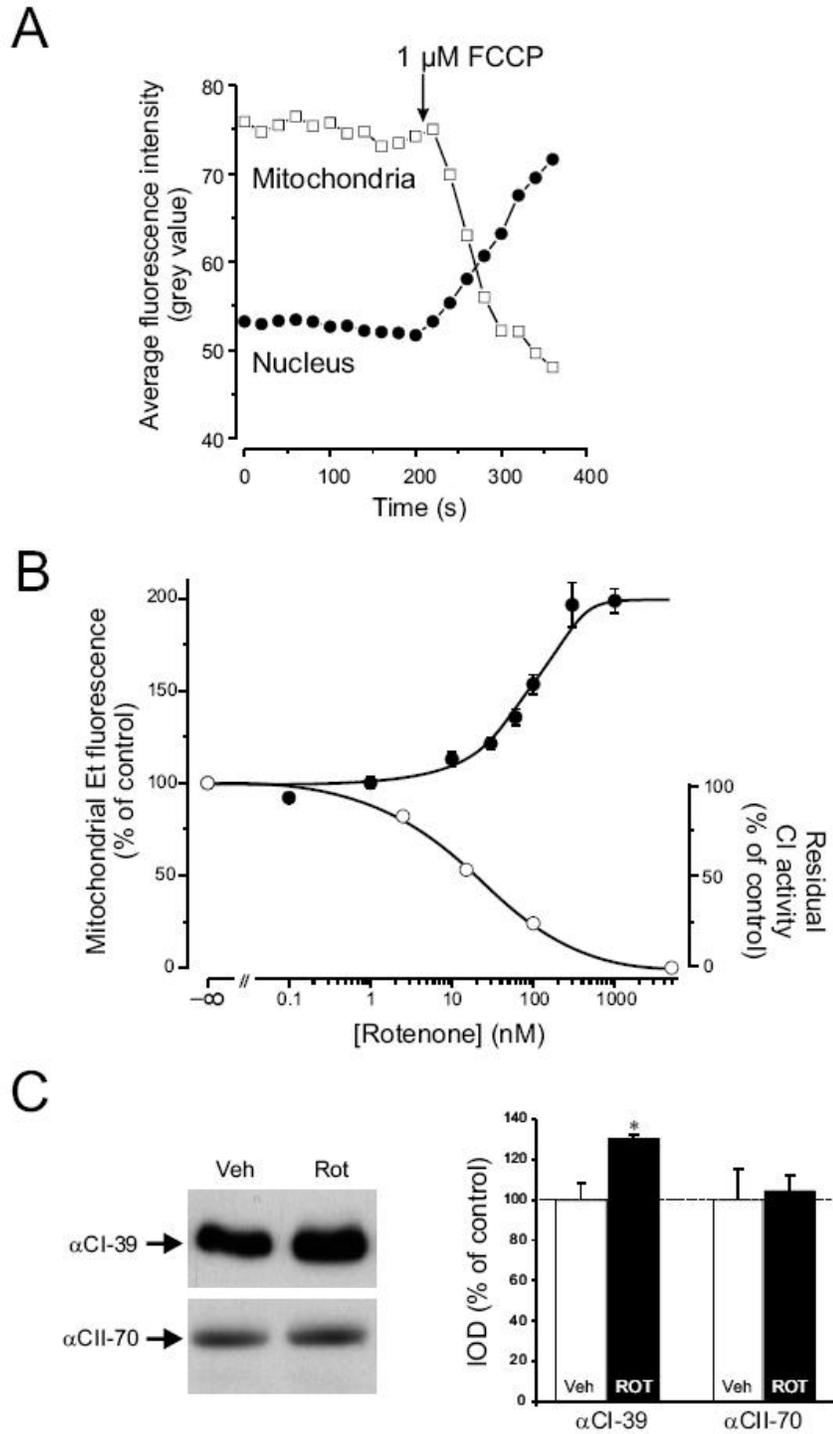
compartment (left y-axis) is expressed as percentage of vehicle-treated control (CT). Mean values, number of cells analyzed and patient numbers are depicted in **Table 1**. Closed and open symbols represent patient cell lines with a known (13) and hitherto unknown (8) mutation, respectively. Linear regression analysis reveals an inverse correlation between superoxide production and residual CI activity for the whole cohort of patient cell lines.

**Figure 3: Inverse relationship between superoxide production and fully assembled CI in fibroblasts of patients with isolated CI deficiency** – Measurement of superoxide production and analysis of fully assembled CI was performed as described in the caption to **figure 1**. The Western blot shows the decrease in fully assembled CI in one of the patient cells lines (P4). Linear regression analysis reveals an inverse correlation between superoxide production and amount of fully assembled CI for 7 patient cell lines with a known (black symbols) and 6 with a hitherto unknown (open symbols) mutation. Assembly data are from 9 (CT), 1 (P5, P14, P15, P16, P18, P19, P20), 2 (P1, P2, P3, P13), 4 (P4) and 5 (P12) independent experiments. Mean fluorescence intensities ( $\pm$  SEM), number of cells analyzed and patient numbers are depicted in **Table 1**.

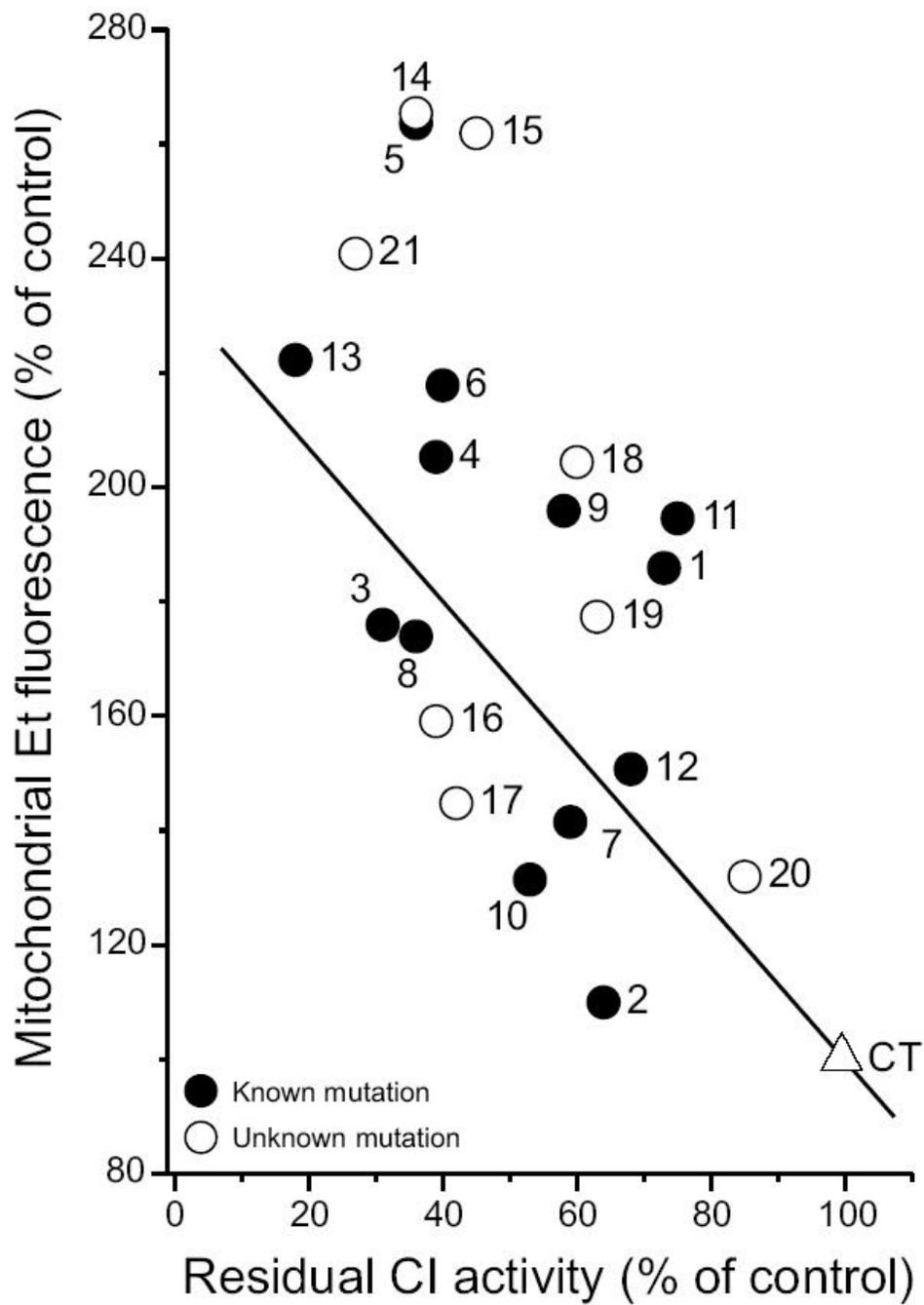
**Table 1. Characteristics of the complex I deficient fibroblast cell lines**

CELL LINE <sup>5</sup>	AFFECTED SUBUNIT AND MUTATION <sup>4</sup>	CLINICAL PHENOTYPE <sup>6a</sup>	PN <sup>6b</sup>	CI ACT <sup>2</sup>	SUPEROXIDE PRODUCTION <sup>4</sup>			
					Nucleus		Mitochondria	
					Untreated	Rotenone	Untreated	Rotenone
CT1 (#5120) <sup>1</sup>	None	n.a.	17	113	100±1 (n=526)	237±3 (n=516)	100±1	172±2
CT2 (#5119) <sup>1</sup>	None	n.a.	17	105	112±3 (n=89)	237±8 (n=89)	102±2	156±6
CT3 (#5118) <sup>1</sup>	None	n.a.	21	103	102±2 (n=126)	229±5 (n=127)	98±3	172±5
CT4 (#4996) <sup>1</sup>	None	n.a.	10*	n.d.	112±3 (n=95)	283±9 (n=89)	100±3	182±5
CT5 (#223V) <sup>1</sup>	None	n.a.	8*	103	112±3 (n=94)	286±6 (n=90)	99±3	175±4
CT6 (#5117) <sup>1</sup>	None	n.a.	15	223	99±4 (n=92)	n.d.	92±3	n.d.
CT7 (#5503) <sup>1</sup>	None	n.a.	6*	20	91±3 (n=98)	n.d.	93±3	n.d.
P1 (#5171)	NDUFV1-R59X/T423M	MLM/LL	18	75	214±7 (n=84)	405±10 (n=87)	186±6	281±7
P2 (#5866)	NDUFV1-R59X/T423M	MLM/LL	18	64	117±4 (n=61)	244±8 (n=58)	110±3	167±8
P3 (#6173)	NDUFS1-D618N/R577X	L/LD	12*	31	207±8 (n=83)	271±8 (n=71)	176±4	205±6
P4 (#5170)	NDUFS2-R228Q	HCEM	11*	39	229±7 (n=66)	331±12 (n=69)	205±8	243±8
P5 (#5067)	NDUFS2-P229Q	HCEM	10*	36	321±10 (n=100)	528±15 (n=108)	264±9	356±10
P6 (#4605)	NDUFS2-S413P	HCEM	15*	40	301±21 (n=101)	378±18 (n=47)	218±13	272±17
P7 (#5077)	NDUFS4-W97X	L/LL	22*	89	170±4 (n=100)	339±11 (n=95)	142±4	232±7
P8 (#5260)	NDUFS4-R106X	L/LL	12	36	196±10 (n=64)	293±8 (n=68)	174±8	203±6
P9 (#4827)	NDUFS4-R106X	L/LL	7*	58	238±8 (n=93)	398±13 (n=93)	196±6	252±9
P10 (#5737)	NDUFS4-VPEEH167/VEKSIstop	L/LL	12	53	150±4 (n=92)	228±6 (n=87)	131±4	166±5
P11 (#4608)	NDUFS4-K158E	L/LL	18*	75	207±5 (n=106)	377±9 (n=102)	195±6	270±7
P12 (#5175)	NDUFS7-V122M	L/LL	15*	68	161±4 (n=94)	322±9 (n=95)	151±5	209±7
P13 (#6603)	NDUFS8-R04C	L/LL	15	18	263±7 (n=147)	375±9 (n=126)	222±7	267±8
P14 (#4611)	Unknown	L/LL	14*	36	395±27 (n=36)	581±39 (n=40)	265±19	330±28
P15 (#5409)	Unknown	FILA	6*	45	265±11 (n=104)	425±22 (n=65)	262±11	340±20
P16 (#4591)	Unknown	UEM	6*	39	189±5 (n=79)	377±15 (n=60)	189±5	234±11
P17 (#4584)	Unknown	L/LL	6*	42	176±6 (n=60)	286±11 (n=56)	145±7	192±8
P18 (#4590)	Unknown	FILA	8*	60	217±7 (n=70)	401±13 (n=59)	204±7	315±15
P19 (#4606)	Unknown	FILA	6*	63	197±9 (n=42)	302±18 (n=45)	177±10	237±14
P20 (#4617)	Unknown	L/LL	3*	85	127±5 (n=56)	217±6 (n=64)	131±6	174±7
P21 (#5671)	Unknown	L/LL	10	27	331±18 (n=38)	402±22 (n=34)	241±15	282±16

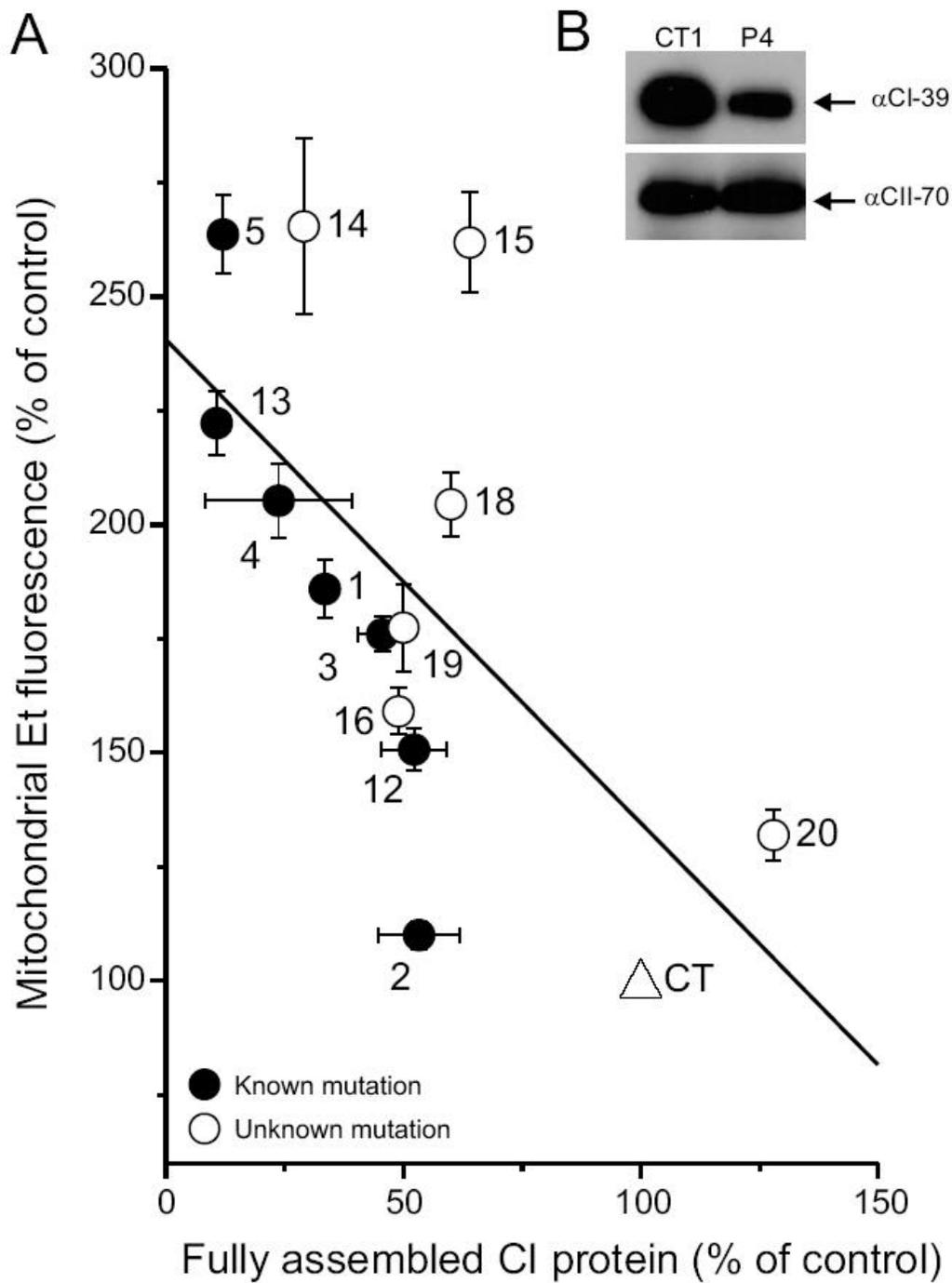
<sup>5</sup>Numbers indicate the designation of the cell lines within the Nijmegen Centre for Mitochondrial Disorders (NCMD). CT and P indicate control and patient cell lines, respectively. <sup>4</sup>Human subunit designation corresponds to the following nomenclature in *Bos taurus*: NDUFV1 (51-kDa), NDUFS1 (75-kDa), NDUFS2 (49-kDa), NDUFS4 (18-kDa), NDUFS7 (PSST), NDUFS8 (TYKY). Mutations are given at the protein level. <sup>6a</sup>FILA: Fatal infantile lactic acidosis, HCEM: Hypertrophic cardiomyopathy and encephalomyopathy, L/LD: Leigh syndrome and leukodystrophy, L/LL: Leigh syndrome and Leigh-like syndrome, MLM: Macrocephaly, leukodystrophy and myoclonic epilepsy, UEM: Unspecified encephalomyopathy. <sup>6b</sup>PN: passage number, an asterisk indicates the passage number after arrival at the Nijmegen Center for Mitochondrial Disorders. <sup>2</sup>CI activity is measured in mitochondrial enriched fractions and expressed as % of lowest control. <sup>4</sup>Mean (+ SEM) nuclear and mitochondrial fluorescence intensity expressed as percentage of untreated control #5120 (CT1) for the number of cells between brackets. Each cell line was tested on at least two days. Values in bold are below lowest control value (CI activity) or significantly different ( $p < 0.05$ ) from highest control (CT2; superoxide production). Rotenone indicates acute treatment with 100 nM rotenone during HET incubation (30 min, 37° C). <sup>1</sup>Cells obtained from healthy adults. <sup>2</sup>Cells obtained from healthy age-matched children. Abbreviations: n.a., not appropriate, n.d., not determined.



Verkaart et al., Fig. 1



Verkaart et al., Fig. 2



Verkaart et al., Fig. 3