# Biochemical parameters for the diagnosis of mitochondrial respiratory chain deficiency in humans, and their lack of age-related changes

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It is now widely acknowledged that a large number of human diseases originate from respiratory-chain dysfunctions. Because the molecular bases of these diseases are still poorly known, a biochemical approach has to be used in the screening procedures for the diagnoses of these conditions. Assessment of respiratory-chain function in human samples faces several problems: (i) the small size of available samples, (ii) the determination of discriminating parameters, and (iii) the interfering factors, such as age and physical activity. The present study focuses on isolated mitochondria prepared from a minute amount (100–200 mg) of skeletal-muscle biopsies from 201 patients between 0 and 65 years. Whereas 42 patients presented an isolated complex (C)I, CII, CIII or CIV deficiency, no respiratory-chain dysfunction or indirect evidence for a mitochondrial disorder could be attested in 159 of these patients. In this reference group, there was little correlation between enzyme activities and age, whatever the age class considered, 0–3 or 0–65 years of age. However, a confident handling of data points was largely hampered by the marked scattering of enzyme activities measured in the reference population. Activity ratios between the various respiratory-chain complexes presenting a much reduced scattering may be considered as diagnostic tools. As to the effect of age, no correlation with any of the enzyme-activity ratios could be shown. Use of age-matched controls for the diagnosis of respiratory-chain disorders may therefore be avoided, enzyme-activity ratios being highly discriminating and age-independent parameters.

## INTRODUCTION

The five multi-subunit complexes (CI–CV) of the mitochondrial respiratory chain (RC), co-enzyme $Q_{10}$ and cytochrome *c* catalyse the energy transduction from respiratory substrates to a proton-motive gradient further used for ATP synthesis. Deficiencies of the different RC complexes and co-enzyme $Q_{10}$ have been shown to cause numerous human diseases with a striking variety of presentation and evolution [1,2]. Although underlying mutations (deletions or point mutations of the mitochondrial DNA) have been identified in several of these diseases [2,3], their molecular bases remain largely unknown, particularly in childhood. The dual genetic origin of the RC-complex subunits, nuclear or mitochondrial, and the large number of genes interacting in the synthesis and assembly of the RC all probably account for the small proportion of elucidated cases. As a result, direct molecular screening for underlying mutations is not currently feasible, and a biochemical approach has to be used for the diagnosis of this condition.

Although part of the enzyme assays can be performed on frozen human samples or detergent-permeabilized cells, comprehensive information, including kinetic studies on enzyme parameters, cytochrome spectra, etc., is obtained only by studying isolated mitochondria [4,5]. In the last few years, the scaling-down of the investigation methods greatly extended the possibility of studying isolated mitochondria from human tissues [5,6]. For a long time, several grams of skeletal-muscle tissues were used to assess RC-enzyme activities in mitochondria isolated from fresh biopsies. Fortunately, equivalent information is now routinely obtained by using 100–200 mg of muscle tissue [5]. However, there are still problems encountered in these measurements, and no general agreement has been reached on parameters to be used so as to diagnose confidently RC-enzyme deficiencies in humans.

It is generally assumed that the mitochondria content in a cell closely depends on its requirement for oxidative metabolism [7]. In patients, and healthy individuals as well, this demand varies under the action of numerous, usually uncontrollable, interfering factors. In particular, physical training strongly affects the level of RC activity in human skeletal muscle [8,9]. Furthermore, an accumulation of mitochondria often happens in the RC-deficient tissues of adult patients. Accordingly, the occurrence of mitochondria-enriched ragged-red fibres is a hallmark for a RC deficiency in skeletal-muscle tissue [1]. As a result of these physiological and/or pathological variations, even the use of standardized biochemical methods does not reduce the extremely large ranges of RC-enzyme activities in the control populations, and the analysis of RC-enzyme residual activities is often unsuitable for discriminating between patients and controls [10]. Consequently, only patients presenting extremely low activity of one or more of the RC complexes in their tissues could be confidently diagnosed, if focusing on residual activity values only. In order to cope with this problem, we have previously suggested considering the activity ratios between the different components of the RC as well [10,11]. The rationale was that a balanced RC was required for an efficient, low-free-radical-generating, electron-transfer activity, and this may be reflected in the consistency of these ratios among individuals and among tissues for a given individual. Thus the detailed analysis of the RC-enzyme-activity ratios allowed the identification of several CII-defective patients and led to the consecutive determination of the first mutation of a nuclear gene encoding an RC-complex

Abbreviations used: CI, CII, CIII and CIV, the various complexes of the respiratory chain; RC, respiratory chain.

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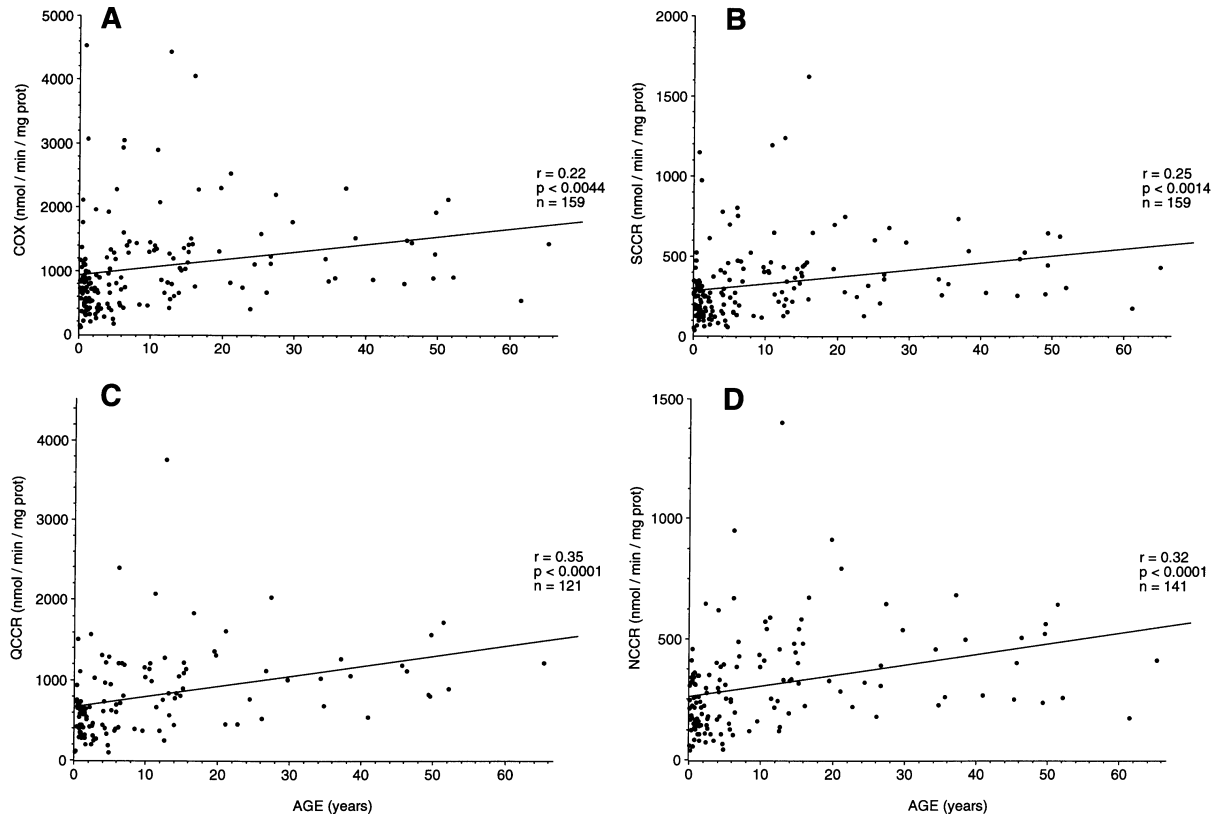


**Figure 1 RC-enzyme activities as a function of age in human skeletal-muscle mitochondria**

Regression plots for (**A**) cyanide-sensitive cytochrome *c* oxidase (COX), (**B**) antimycin-sensitive decylubiquinol:cytochrome *c* reductase (QCCR), (**C**) malonate-sensitive succinate:cytochrome *c* reductase (SCCR) and (**D**) rotenone-sensitive NADH:cytochrome *c* reductase (NCCR) with age. Enzyme activities were assayed as described previously [5]. prot, protein.

subunit [12]. CII deficiencies are generally very partial (ranging between 30 and 50% of control mean values) and may remain easily undetected by considering residual CII activity only. However, the maintenance of RC ratio values with age has been indirectly questioned. On the one hand, a differential decrease of RC-enzyme activities in elderly people has been reported [13–15] that would result in an alteration of the RC-enzyme-activity ratio values. However, other studies did not confirm such a differential decrease [16–18], and variations in the overall activity of the RC were shown to be associated with interfering variables (physical training and tobacco consumption) not related to age [16,17]. On the other hand, it has been recently claimed that the specific activity of the different RC complexes differentially increases in muscle tissue, concomitant with the muscular-mass increment of the first years of life [19,20].

This was an incentive to collect results obtained from more than 200 subjects with the aim both to strengthen the grounds for the use of RC ratios as diagnostic parameters and to examine the potential effects of age on the RC activities and their ratios, particularly in childhood.

## MATERIALS AND METHODS

### Samples

Biopsy samples from the deltoid muscle were obtained from 218 patients between 1 day and 66 years of age, among which 17 underwent orthopaedic surgery. The other patients (201/218) presented with clinical symptoms, a metabolic profile and/or a family history, suggestive of a mitochondrial disorder. A mitochondrial RC deficiency could be diagnosed in the skeletal muscle of 21% of the patients (42/201). Besides a few cases of clinically defined entities recognized as originating often from a mitochondrial dysfunction (myoclonic epilepsy with ragged red fibres, one case; Leigh syndrome, six cases), a large variety of clinical presentations was encountered in these patients, resulting from different organ involvement, isolated or in association. The prominent features of these various clinical presentations have been previously reported [2].

In 80% of the patients (159/201), the RC-enzyme activities were not different from control values measured in biopsies obtained during orthopaedic operations from 17 patients without metabolic diseases. None of these 159 patients had muscle morphological abnormalities. Major rearrangements of the mitochondrial DNA or point mutations commonly found in association with myoclonic epilepsy with ragged red fibres, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes, and neurogenic muscle weakness, ataxia and retinis pigmentosa were also excluded. Investigation of cultured skin fibroblasts and/or circulating blood lymphocytes of the patients did not reveal any RC deficiency. These 159 patients were therefore regarded as non-affected by a mitochondrial RC disease and were included in this study as the reference population.

### Biochemical and statistical analyses

Mitochondria were isolated from 100–200 mg of deltoid muscle biopsies as previously described [5]. Intactness of the mito-

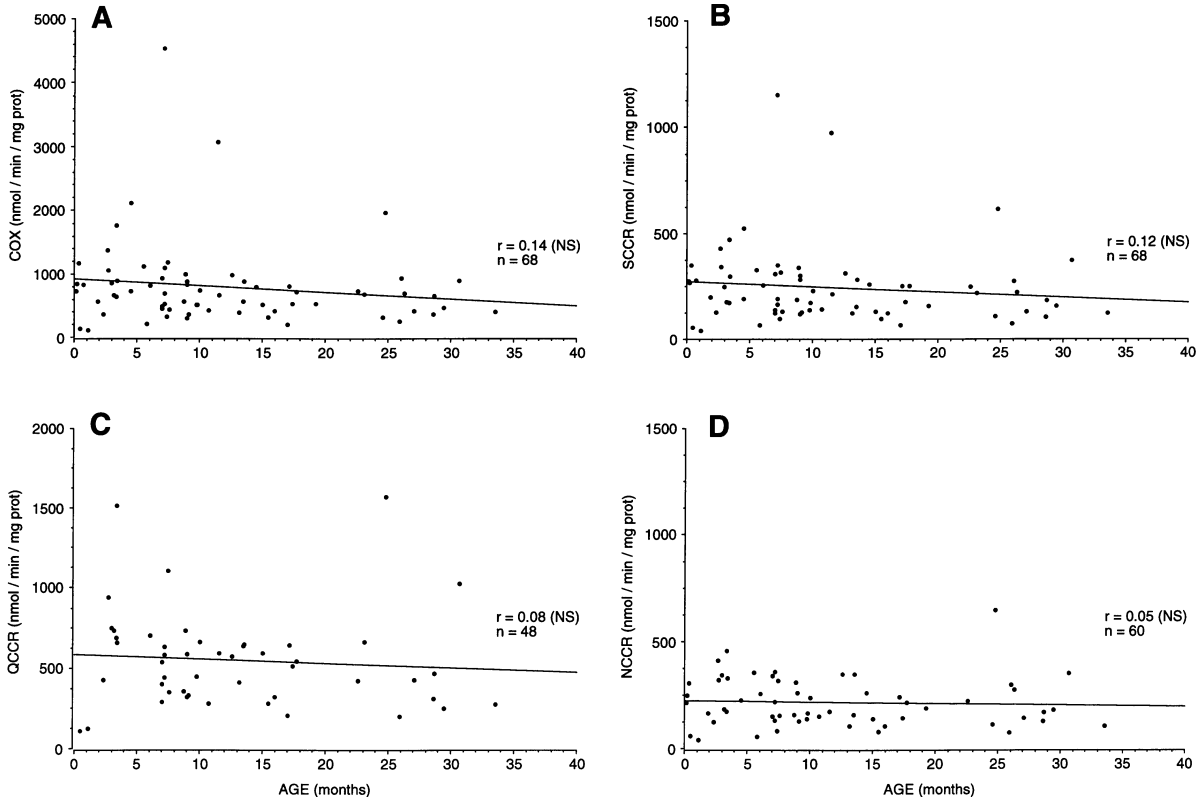


**Figure 2 RC-enzyme activities as a function of age in children (3 days–3 years old) skeletal-muscle mitochondria**

Regression plots for (**A**) cyanide-sensitive cytochrome *c* oxidase (COX), (**B**) antimycin-sensitive decylubiquinol:cytochrome *c* reductase (QCCR), (**C**) malonate-sensitive succinate:cytochrome *c* reductase (SCCR) and (**D**) rotenone-sensitive NADH:cytochrome *c* reductase (NCCR) for 3-day–3-year-old children. Enzyme activities were assayed as previously [5]. prot, protein; NS, not significant.

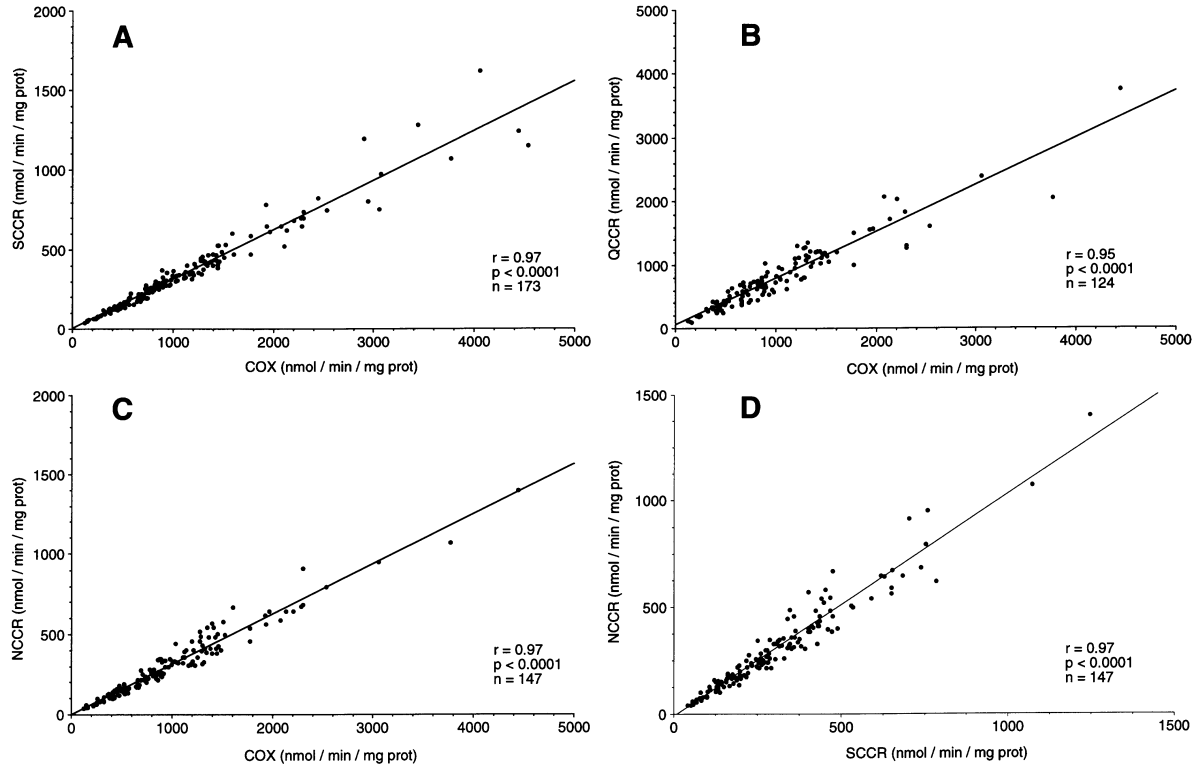


**Figure 3 Correlations between RC-enzyme activities in human skeletal-muscle mitochondria**

Regression lines for correlation between (**A**) malonate-sensitive succinate:cytochrome *c* reductase (SCCR) and cyanide-sensitive cytochrome *c* oxidase (COX), (**B**) antimycin-sensitive decylubiquinol:cytochrome *c* reductase (QCCR) and COX; (**C**) NADH:cytochrome *c* reductase (NCCR) and COX, and (**D**) NCCR and SCCR. Experimental conditions were as described previously [5]. prot, protein.

chondria obtained by this procedure was always higher than 90%, as estimated by the measurement of the permeability of the outer membrane to cytochrome *c* [5]. Accordingly, respiratory control values associated with succinate oxidation were always higher than 4.0. Although the yield of mitochondria extraction was roughly similar among experiments (about 40% of total mitochondrial activities was pelleted in the mitochondria-enriched fraction), the specific activities of mitochondrial enzymes in the mitochondrial pellet were generally slightly decreased (i.e. more contamination) when using a very small sample biopsy (100 mg). Therefore, enzymes were systematically assayed on both initial homogenates and mitochondria-enriched fractions, with quite similar trends observed for changes of enzyme activities as a function of age in both types of fraction. Consequently, only data obtained from the study of the mitochondria-enriched fractions are presented here.

KCN-sensitive cytochrome *c* oxidase (CIV; EC 1.9.3.1), antimycin-sensitive decylubiquinol:cytochrome *c* reductase (CIII; EC 1.10.2.2), rotenone-sensitive NADH:cytochrome *c* reductase (CI + CIII) and malonate-sensitive succinate:cytochrome *c* reductase (CII + CIII) were spectrophotometrically measured according to standard procedures [5]. Correlations between age and mitochondrial enzyme activities, or between various enzyme activities, were studied by simple linear-regression analyses [21].

## RESULTS

The RC-enzyme activities in mitochondria isolated from 159 deltoid-muscle biopsies from individuals between 0 and 66 years of age were very poorly correlated with age (reference population; Figure 1), as indicated by the low $r^2$ values ($0.048 < r^2 < 0.12$) [21]. Incidentally, both the lowest and the highest values of RC activities were observed among the young individuals, casting additional doubts on the meaning of the correlation between age and RC activity level and suggesting the implication of other variables. Finally, no significant changes in RC activities could be observed in the group of 48 individuals between 0 and 3 years of age (Figure 2).

Any conclusion that could be drawn from such analyses is, however, largely hindered by the huge scattering of values observed within each class of age for all the measured enzyme activities. Therefore additional parameters to detect potential changes in enzyme activities with age better were examined. We have previously suggested the use of the ratios between RC-enzyme activities as a tool to detect confidently even partial RC deficiency in human samples [10]. The statistical relationships between several of these activities were examined (Figure 3). Highly significant correlations ($0.90 < r^2 < 0.94$) were observed whatever the considered pair of RC enzyme activities. The striking alignments of data points contrasted with the high scattering of absolute values measured in the same mitochondrial preparations (compare Figures 1 and 3). The close linear relationship between RC activities further supported our previous claim that the analysis of these ratios actually provided a tool to detect confidently specific changes in any of the RC-enzyme activities. We next compared the values of the ratios measured in skeletal-muscle mitochondria of patients presenting either CI, CII, CIII or CIV deficiency with those measured in the reference population (Figure 4). An unequivocal discrimination between patient and reference values was found when considering for each type of enzyme deficiency the appropriate enzyme-activity ratios. Thus cytochrome *c* oxidase/NADH:cytochrome *c* reductase activity ratios measured in CI-, CIII- or CIV-deficient mitochondria markedly differed from reference values, whereas values measured for CII-deficient mitochondria were included in the reference values (Figure 4A). Similarly, cytochrome *c* oxidase/succinate:cytochrome *c* reductase ratios measured in CII-, CIII- and CIV-deficient mitochondria similarly differed from reference values, whereas values measured in CI-deficient patients were included in the reference values (Figure 4B).

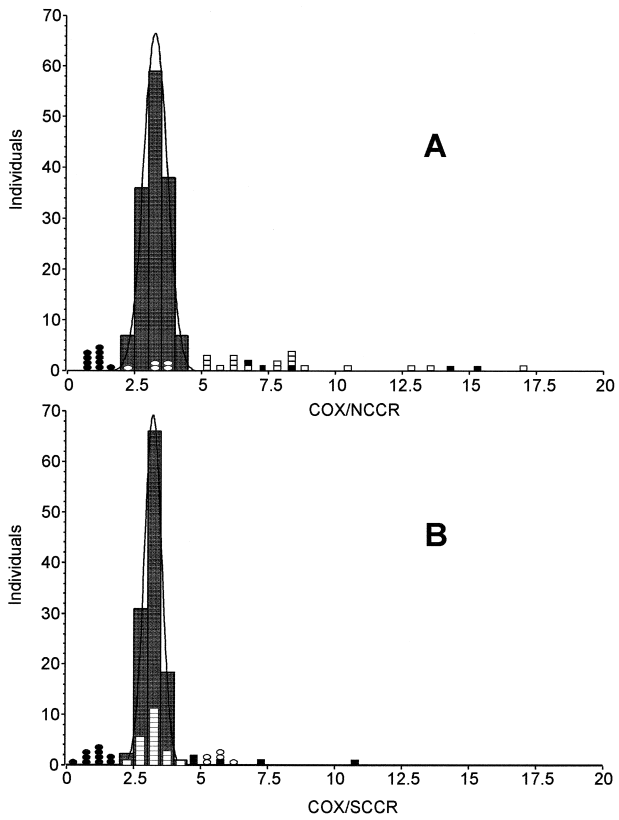


**Figure 4 Comparison of cyanide-sensitive cytochrome *c* oxidase (COX)/NADH:cytochrome *c* reductase (NCCR) and COX/malonate-sensitive succinate:cytochrome *c* reductase (SCCR) activity ratios measured in skeletal-muscle mitochondria of patients presenting CI, CII, CIII or CIV deficiency and of the reference population**

The normal distributions of (**A**) COX/NCCR and (**B**) COX/SCCR activity ratios in the reference population were compared with the values measured in patients presenting a RC deficiency. □, CI deficiency; ○, CII deficiency; ■, CIII deficiency; ●, CIV deficiency. RC-complex deficiencies were all established by the concurrent deficiencies of the individually measured complex activities and of the concerned grouped assays. Experimental conditions were as described previously [5].

Potential age-related changes in the values of these discriminating ratios were also sought. Values were plotted as a function of age (Figure 5). None of the activity ratios was found to vary between 0 and 66 years of age. As shown above (Figure 3), none of the RC activities changed with age in the population between 0 and 3 years of age. Accordingly, none of the activity ratios varied with age in this group (results not shown).

## DISCUSSION

The potential relationships between age and RC-enzyme activities in human skeletal muscle is a quite controversial issue that might determine our ability to diagnose confidently patients with an RC deficiency. There are advocates that a significant decrease in RC-enzyme activity occurs with age [13–15,22–24]. Depending

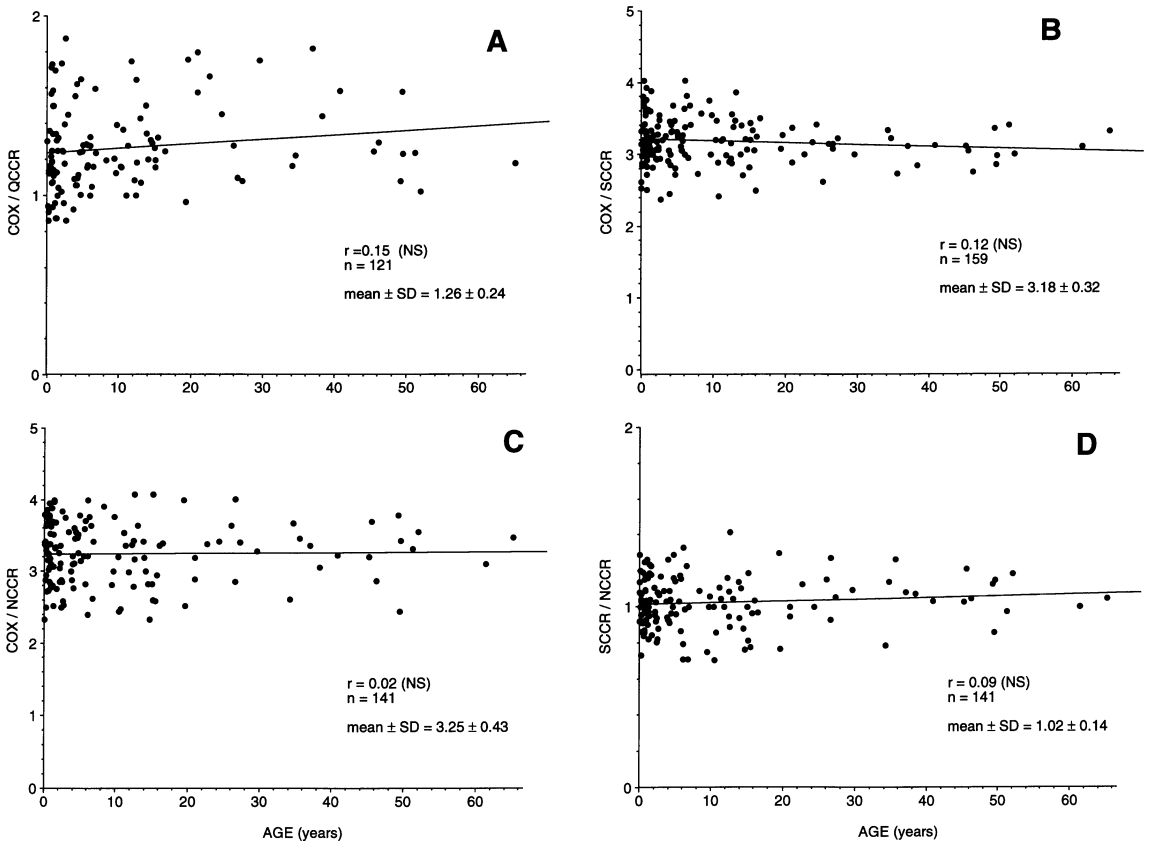


**Figure 5 RC-enzyme-activity ratios as a function of age in human skeletal-muscle mitochondria**

Regression plots of (**A**) cyanide-sensitive cytochrome *c* oxidase (COX)/antimycin-sensitive decylubiquinol:cytochrome *c* reductase (QCCR), (**B**) COX/malonate-sensitive succinate:cytochrome *c* reductase (SCCR), (**C**) COX/NADH:cytochrome *c* reductase (NCCR) and (**D**) SCCR/NCCR activity ratios with age (0–66 years). Experimental conditions were as described previously [5].

on the study, experimental assay conditions and the number of subjects included, from 25–30% ($n = 34$) [13] to up to 70% ($n = 63$) [14] of the CIV activity measured in mitochondria from 20-year-old individuals might be lost in 60-year-old individuals. A dramatic and specific loss of 50% of the CIV activity was even reported to take place between 4 and 20 years ($n = 43$) [22]. Accordingly, cumulative decreases might then lead to an impressive, life-threatening, 15% residual CIV activity at the age of 60. Another study reported on a specific loss of CI activity, without significant alteration of CIV activity ($n = 31$) [23]. However, in good agreement with the present report, other studies failed to disclose convincing correlations between changes in RC activities and age in human skeletal muscle {($n = 132$) [16], ($n = 51$), [17] and ($n = 14$) [18]}. These studies also established that any potential decline in mitochondrial oxidation with age should rather be ascribed to reduced physical activity [16,17], and/or to the detrimental effect of tobacco consumption, tested among many potential confusing variables [17].

Few studies have dealt with potential age-related evolution of RC activities in neonates, infants or children. One study reported a statistically significant and considerable increase (from 5- to 10-fold) in the activity of all the RC, including CII and CIV, between neonates ($n = 4$) and adults ($n = 20$) [19]. A second study reported a low (although significant) correlation between age and CII + CIII activity ($r^2 = 0.18$; $n = 23$) and between age and CI + CIII ($r^2 = 0.20$; $n = 23$) in the first 3 years of life, but without increase of CII and CIV [20]. The present data obtained on a larger group of children of the same age (0–3 years; $n = 48$) did not confirm age-related changes of the RC-complex specific activities in mitochondria isolated from the skeletal muscle.

The marked scattering of RC specific activities in human skeletal muscle, which obviously hampers the handling of data, may well account for the discrepancies between these statistically significant, although contradictory, results. Noticeably, such a marked scattering of RC specific values as that reported here for a diseased population was quite similarly observed in healthy-people control populations [14,16,22]. This scattering might also mask an actual correlation of RC activities with age. However, even a detailed examination of additional parameters routinely used in the context of the screening procedure for the RC deficiency (i.e. enzyme-activity ratios) failed to reveal a significant variation of any RC activity along with age. It therefore appears safe to conclude the lack of age-related changes of these highly discriminating parameters for RC activities during both the first years of life and old age. As a direct practical consequence, it appears meaningless to use age-matched controls in the biochemical diagnosis of mitochondrial disease in human when using these parameters. Noticeably, the measurement of non-varying RC activities with age does not rule out a potential impairment of RC function in a cell sub-population during a decrement of the muscle mass. It has been hypothesized that an RC impairment might act as a signal for apoptosis (and/or necrosis) [18]. In keeping with this, two recent studies report on the role of mitochondrial cytochrome *c* release as an early event

triggering the apoptotic cascade [24,25]. As a result, RC-deficient cells may be eliminated, as they appear in senescing tissues. The non-varying RC enzyme activities actually measured in tissues may correspond to the activities of respiratory-competent cells.

A second aspect of the present study deals with parameters to be used to diagnose RC deficiencies in humans. The use of residual activity rates is obviously quite constrained, in view of the huge scattering of control values. Null values are indeed often included in the 2 S.D. from the mean values of the reference population [11]. Why RC activity differs so strongly among individuals remains an open question. Beside methodological problems, numerous physiological interfering factors may be advocated (physical activity, smoking, nutrition, etc.). In this context, the above data noticeably extended, and statistically strengthened, our previous observation that RC-enzyme-activity ratios constitute remarkably consistent features allowing detection of even partial defects of the RC [10,11].

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