Oxidized proportion of muscle coenzyme Q10 increases with age in healthy children

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BACKGROUND: Coenzyme Q10 (CoQ10) is synthesized in most human tissues, with high concentration in the skeletal muscle. CoQ10 functions in the mitochondrial respiratory chain and serves as a potent liphophilic antioxidant in membranes. CoQ10 deficiency impairs mitochondrial ATP synthesis and increases oxidative stress. It has been suggested that plasma CoQ10 status is not a robust proxy for the diagnosis of CoQ10 deficiency.

METHODS: We determined the concentration and redox-status of CoQ10 in plasma and muscle tissue from 140 healthy children (0.8–15.3 y) by high-performance liquid chromatography (HPLC) with electrochemical detection.

RESULTS: There was no correlation between CoQ10 concentration or redox status between plasma and muscle tissue. Lipid-related CoQ10 plasma concentrations showed a negative correlation with age (Spearman's, $P \leq 0.02$), but there was no significant age-related correlation for muscle concentration. In muscle tissue, we found a distinct shift in the redox status in favor of the oxidized proportion with increasing age (Spearman's, $P \leq 0.00001$). Reference values for muscle CoQ10 concentration (40.5 ± 12.2 pmol/mg wet tissue) and CoQ10 redox status (46.8 ± 6.8% oxidized within total) were established for healthy children.

CONCLUSION: The age-related redox shift in muscle tissue suggests changes in antioxidative defense during childhood. The reference values established here provide a necessary prerequisite for diagnosing early CoQ10 deficiency.

Coenzyme Q10 (CoQ10) is essential for electron transport in the mitochondrial respiratory chain as a carrier of electrons from complexes I and II to complex III. Coenzyme Q exists in reduced (ubihydroquinone) and oxidized (ubiquinone) forms in biological tissues (1,2), with the reduced form being one of the most potent endogenously synthesized lipophilic antioxidants (3). Redox status has been shown to be an important marker for oxidative stress associated with diseases such as neonatal hypoxia (4), hyperlipidemia (5), and Parkinson's disease (6). In addition to its central role in bioenergetics and its antioxidant functions, CoQ10 regulates mitochondrial permeability pores, activates uncoupling proteins, reduces lipid peroxidation, and facilitates pyrimidine biosynthesis (7,8). CoQ10 has also been identified as a modulator of gene expression (9–11). Lack of CoQ10 may thus impair several vital cellular functions including mitochondrial energy production, and may increase production of or susceptibility to reactive oxygen species.

CoQ10 deficiency has been correlated with clinical and genetically heterogeneous diseases, and has been found to be inherited as an autosomal recessive trait that begins predominantly in childhood (12,13). Primary deficiencies occur as a result of mutations in genes involved in ubiquinone biosynthesis, whereas secondary deficiencies include diseases caused by mutations in genes unrelated to ubiquinone biosynthesis (12,14,15). CoQ10 deficiency in muscle has been associated with disorders including severe infantile multisystemic disease (initially described by Rötig *et al.* (16) in siblings presented with neurological symptoms and nephropathy), encephalomyopathy (17), cerebellar ataxia (18,19), and isolated myopathy (20). Many of these disorders respond to CoQ10 supplementation, and are thus treatable (12,21–24). Even a small increase in CoQ10 concentration within mitochondrial membranes may lead to an increased respiratory rate (7). An early diagnosis of CoQ10 deficiency in childhood is therefore clinically important and may increase the benefits of CoQ10 supplementation.

Analyses of serum or plasma CoQ10 concentrations are frequently used to estimate the functional CoQ10 status of an organism. However, plasma CoQ10 concentrations are significantly influenced by dietary uptake (25) and lipoprotein transport capacity, since CoQ10 is known to bind to lipoproteins in circulation. Tissue CoQ10 levels mainly depend on *de novo* synthesis (21), as tissue uptake of CoQ is rather limited (26). Reduced biochemical activities of respiratory chain complexes, particularly complexes I and II, in muscle may suggest CoQ10 deficiency. However, direct measurement of CoQ10 levels in muscle tissue is generally considered to be the most reliable diagnostic test for defects in CoQ10 biosynthesis (12–14,27), and the results can assist clinicians to make appropriate treatment decisions for children with mitochondrial diseases.

The establishment of age-dependent cut-off values and standardized methods are prerequisites for the qualitatively reliable analyses of CoQ10 concentrations and redox states in muscle tissues. However, extensive interlaboratory variability in defining reference values for muscle CoQ10 content

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(14,15,18,19,23,28–31), a lack of external quality controls, and differences in reference units for CoQ10 concentrations mean that cut-off values from external laboratories cannot be adopted without reservation. In the present study, we analyzed the muscle and plasma CoQ10 content and redox status in 140 clinically healthy children. This study was designed to augment our knowledge of age-related changes in CoQ10 plasma and muscle statuses throughout childhood and to improve our understanding of the relationship between CoQ10 levels in plasma and muscle.

RESULTS

We sampled 140 abdominal internal oblique muscle biopsies over a 2-y period from otherwise healthy children who frequented the surgical ward of the children's hospital for inguinal hernia, undescended testis, and hydrocele testis. There was therefore a strong selection bias in favor of male patients (14 girls, 126 boys). However, we found no significant differences (Wilcoxon test, $P > 0.05$) between female ($n = 14$) and male ($n = 48$) probands in the same age range (3.5–8.3 y) in terms of CoQ10 concentration or redox status of CoQ10 in plasma or muscle tissue.

Table 1 summarizes the correlations between CoQ10 status in plasma and muscle tissue, cholesterol, and BMI. As expected, there was a positive correlation ($P \leq 0.0001$) between CoQ10 and cholesterol concentrations in the plasma. No correlation was found between the BMI and the plasma concentration of CoQ10 or cholesterol. However, plasma cholesterol increased with age ($P \leq 0.00001$), and although the overall plasma CoQ10 concentration (µmol/l) was not age-related, the lipid-related level of CoQ10 (µmol CoQ10/mol cholesterol) showed a negative correlation ($P \leq 0.02$) with age, such that older children had lower lipid-related CoQ10 plasma levels than younger children. There was no correlation between the plasma redox status of CoQ10 and age, or between the plasma CoQ10 redox status and CoQ10 concentration.

In accordance with these findings, there was no significant correlation between total CoQ10 concentration in muscle tissue and age, neither when concentrations were related to protein content nor to tissue weight; however there was a tendency toward a positive correlation between CoQ10 concentration (pmol/mg wet tissue) and age (Spearman's, $P \leq 0.052$) (**Figure 1a**). Interestingly, there was a distinct shift in redox status in favor of the oxidized proportion of muscle CoQ10 (Spearman's, $P \leq 0.00001$) in relation to age (**Figure 1b**), and a weak negative correlation (Spearman's, $P \leq 0.043$) between the oxidized proportion of CoQ10 and protein-related tissue CoQ10 concentrations.

The correlation of the CoQ10 concentration respectively the CoQ10 redox status between both compartments, plasma vs. muscle tissue, did not reach statistical significance ($P \leq 0.1$ resp. 0.14).

The CoQ10 states in plasma and muscle tissue from 140 children, categorized by 2-y increments, are shown in **Table 2**. Because of decreasing numbers of probands in the older age groups, children in their 9th to 16th years were combined into

Table 1. Correlation analysis between the CoQ10 status in plasma and muscle tissue, cholesterol, and BMI from 140 healthy children

Location	Parameter 1	Parameter 2	Spearman correlation of ranks, $P \leq$ (coefficient of correlation)
Plasma	CoQ10 concentration (µmol/l)	Cholesterol (mmol/l)	0.0001 (+0.33)
Plasma	CoQ10 concentration (µmol/l)	BMI	n.s.
Plasma	Cholesterol (mmol/l)	BMI	n.s.
Plasma	CoQ10 concentration (µmol/l)	Age (years)	n.s.
Plasma	Cholesterol (mmol/l)	Age (years)	0.00001 (+0.35)
Plasma	CoQ10/cholesterol (µmol/mol)	Age (years)	0.02 (−0.18)
Plasma	CoQ10 redox status (% oxidized in total)	Age (years)	n.s.
Plasma	CoQ10 redox status (% oxidized in total)	CoQ10 concentration (µmol/l)	n.s.
Muscle tissue	protein concentration (µg/sample)	Sample weight (mg wet tissue)	1E-12 (+0.55)
Muscle tissue	CoQ10 concentration (pmol/mg wet tissue)	CoQ10 concentration (pmol/mg protein)	0.00001 (+0.35)
Muscle tissue	CoQ10 concentration (pmol/mg protein)	Age (years)	n.s.
Muscle tissue	CoQ10 concentration (pmol/mg wet tissue)	Age (years)	n.s.
Muscle tissue	CoQ10 redox status (% oxidized in total)	Age (years)	0.00001 (+0.37)
Muscle tissue	CoQ10 redox status (% oxidized in total)	CoQ10 concentration (pmol/mg protein)	0.043 (−0.15)
Muscle tissue	CoQ10 redox status (% oxidized in total)	CoQ10 concentration (pmol/mg wet tissue)	n.s.
Plasma vs. tissue	CoQ10 concentration (µmol/l)	CoQ10 concentration (pmol/mg protein)	n.s.
Plasma vs. tissue	CoQ10 redox status (% oxidized in total)	CoQ10 redox status (% oxidized in total)	n.s.

n.s., not significant

a single subgroup. All infants in the first year of life were in the fourth quarter of this age category. Analysis according to this age classification was performed using analysis of variance, and confirmed the correlation analyses in **Table 1**. In plasma, only cholesterol concentration differed significantly among the age groups, with the lowest levels in younger children. In line with this, lipid-adjusted CoQ10 levels in plasma were highest in the youngest children. The youngest children also had the lowest oxidized proportion of CoQ10 in muscle tissue, and a marked increase in the oxidized proportion was found between the first and second years of life (39.7 ± 4.8 vs. 45.5 ± 5.5%).

Analysis of CoQ10 concentration was supplemented by analyzing the proportion of endogenous CoQ9 in muscle tissue. Endogenous CoQ9 was calculated in 11 of 140 muscle biopsies by comparing the area ratios of CoQ9 and CoQ10 within the HPLC chromatograms (**Figure 2**). The endogenous proportion of CoQ9 never exceeded 2.5% of the total CoQ (1.60 ± 0.64%).

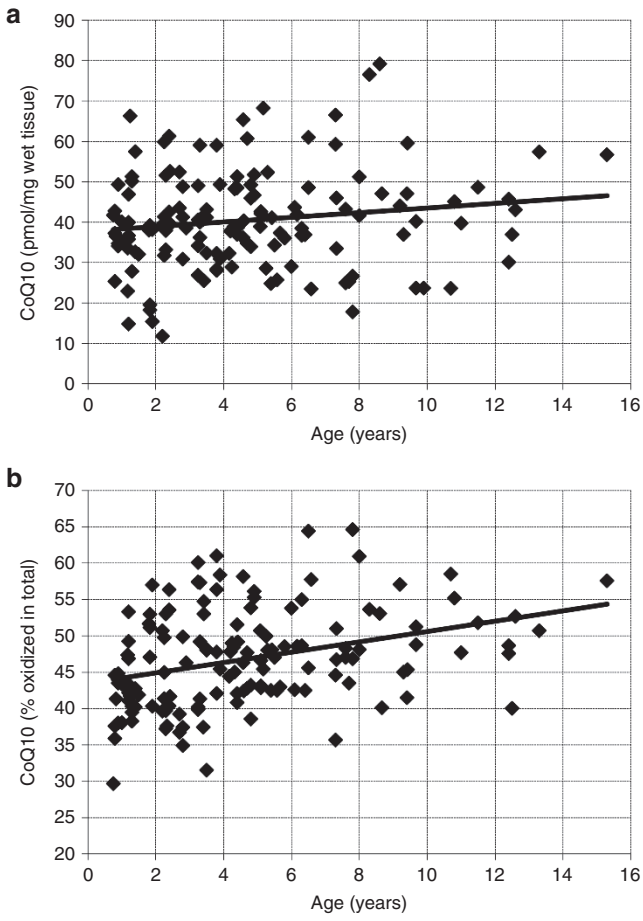

Figure 1. Correlation of age and CoQ10 concentration (**a**, Spearman's, $P \leq 0.052$) and redox status (**b**, Spearman's, $P \leq 0.00001$) in muscle tissue from 140 healthy children.

DISCUSSION

Reference values of CoQ10 status are needed to define threshold values for CoQ10 deficiencies. The present study therefore aimed to determine if there were age-related changes in CoQ10 concentration and redox status in muscle tissue during childhood, and to establish the relationship between tissue and plasma CoQ10 status.

We found no age-related differences in redox status or total CoQ10 plasma concentrations in 140 healthy children (0.8–15.3 y). In the circulation, CoQ10 is associated with lipoproteins. We accordingly found a clear positive correlation between CoQ10 and cholesterol levels in plasma. Cholesterol concentrations increase with age in children, independent of BMI. Accordingly, CoQ10 concentration adjusted to lipid concentration revealed a negative correlation with age. The current results are in line with those of Miles *et al.* (32), who found that the redox statuses and total plasma CoQ10 concentrations were similar in younger (0.2–7.6 y) and older children (10.4–17.4 y), though younger children had higher mean lipid-adjusted total CoQ10 concentrations. Likewise, we previously showed a distinct age-dependent decrease in lipid-adjusted CoQ10 plasma concentrations in infants and children (33). Infants in the first to the fourth months of life had higher oxidized proportions of CoQ10 (median 19 vs. 9% in older infants in the first year of life). In the current study, all participating infants were older

Table 2. CoQ10 status in plasma and muscle tissue from 140 children categorized by 2-y increments

	Total	Year of life				
	1st-16th	1st-2nd	3rd-4th	5th-6th	7th-8th	9th-16th
	n = 140	*n* = 30	*n* = 38	*n* = 41	*n* = 14	*n* = 17
Plasma						
CoQ10 (μmol/l)	0.865 ± 0.248	0.902 ± 0.270	0.805 ± 0.218	0.867 ± 0.281	0.909 ± 0.232	0.870 ± 0.200
CoQ10 (% oxidized in total)	7.45 ± 1.18	7.59 ± 1.2	7.47 ± 1.29	7.31 ± 1.08	7.44 ± 1.59	7.47 ± 0.70
Cholesterol (mmol/l)	4.09 ± 0.65	3.86 ± 0.64[a]	3.92 ± 0.64[a]	4.10 ± 0.63[a,b]	4.32 ± 0.41[b,c]	4.64 ± 0.55[c]
CoQ10/cholesterol (μmol/mol)	214 ± 60	236 ± 61[b]	207 ± 54[a]	215 ± 67[a,b]	210 ± 50[a,b]	191 ± 54[a]
Muscle tissue						
CoQ10 (pmol/mg wet tissue)	40.5 ± 12.2	36.4 ± 11.9[a]	40.4 ± 10.7[a,b]	41.5 ± 10.4[a,b]	45.6 ± 19.4[b]	41.3 ± 11.4[a,b]
CoQ10 (pmol/mg protein)	832 ± 386	803 ± 381	777 ± 283	921 ± 489	902 ± 371	740 ± 305
CoQ10 (% ox in total)	46.8 ± 6.8	43.5 ± 5.9[a]	46.3 ± 8.0[a,b]	47.8 ± 5.5[b]	48.9 ± 7.6[b]	50.0 ± 5.5[b]

Shown are mean values ± SDM; data with different superscripts (a, b, and c) are statistically different ANOVA LSD $P \leq 0.05$.

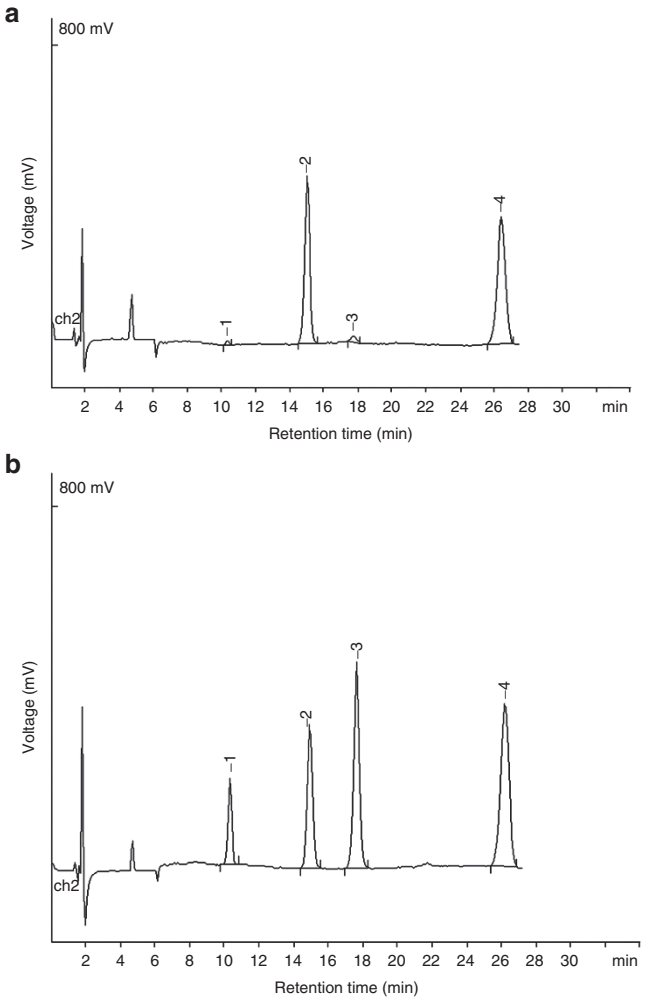

Figure 2. HPLC-chromatograms of muscle-homogenates without (**a**) and with (**b**) the internal standard Coenzyme Q9. 1 = ubihydroquinone-9, 2 = ubihydroquinone-10, 3 = ubiquinone-9, 4 = ubiquinone-10.

than 0.75 y, and no age-related changes in plasma redox status were found. Hara *et al.* (4) found decreased total plasma CoQ10 concentrations in 5-d-old neonates associated with an increased oxidized proportion (25–31% of total CoQ10). These results suggest that changes in endogenous antioxidative defense occur during the first weeks or months of life.

CoQ10 in the circulation is significantly influenced by dietary uptake and by levels of ubiquinone-containing lipoproteins (25,28), while tissue CoQ10 levels depend mainly on *de novo* synthesis (21,26). Physical activity may affect CoQ10 muscle tissue levels, given that mean muscle levels were doubled in young endurance-trained athletes compared with subjects with normal activity (28). Muscle endurance exercise training in rats also increased CoQ concentrations, probably via an increase in mitochondrial mass (34). These results suggest that plasma and muscle tissue concentrations of CoQ10 are unlikely to be correlated. Although the uptake of external CoQ is limited in healthy subjects, CoQ supplementation may re-establish mitochondrial and other functions in situations of severe CoQ10 deficiency (34). Reports comparing plasma and tissue CoQ10 concentrations are rare. Duncan *et al.* (30) found no correlation between plasma and muscle CoQ10, while Miles *et al.* (35) found correlations between basal plasma CoQ10 levels and activities of mitochondrial respiratory chain complexes II and I+III in children with recurrent food intolerance and allergies. In the current study, we found no significant correlations between CoQ10 concentrations or redox statuses in plasma versus muscle tissue in children.

Coenzyme Q is synthesized ubiquitously in cells. Decreased tissue CoQ10 levels have been reported in several diseases, including cardiomyopathies, degenerative muscle diseases, mitochondrial encephalomyopathy, and cerebellar ataxia (1,13,18,19,34). Muscle CoQ10 deficiency is associated with wide variations in age, myofibre pathology, and CoQ10 content, while a link between CoQ10 deficiency and dysfunctions of the electron chain I+III and/or II+III complex are frequently observed (14). Tissue levels of CoQ10 have also been reported to be elevated in certain pathological conditions, such as cancer or diabetes (8,34), which are associated with oxidative stress and damage caused by reactive oxygen species. Increased oxidative stress has been suggested to induce an adaptive response by raising antioxidant concentrations (36). In addition to its concentration, information on the redox status of CoQ10 in tissues may allow insights into the cellular bioenergetic status, and reveal potential exposure to oxidative stress. In its reduced form, CoQ10 is an important antioxidant and free-radical scavenger. A shift toward oxidized CoQ10 is likely to indicate increased oxidative stress (4–6), whereas a shift toward reduced CoQ10 may be regarded as an endogenous compensatory response toward an increased demand for antioxidant capacity. Miles *et al.* (29) found a decrease in total CoQ10 combined with a shift in the redox status in favor of the reduced form in skeletal muscle biopsies from children with muscular dystrophy. The authors suggested that this shift may be a result of a compensatory response of the body's antioxidant defense. CoQ10 tissue concentrations and redox statuses vary among different organs and tissues (37,38) in relation to their different bioenergetic and antioxidative requirements. In human tissues, the highest oxidation status was observed in brain and lung (77 and 76%, respectively), compared with about 40% in skeletal muscle (37).

The current study found no age-related changes in total CoQ10 concentrations in muscle tissue from healthy children; however, there was a distinct increase in the oxidized proportion of CoQ10 with age (average 40% in the first year of life up to 50% in the 9th to 16th years). It is possible that older children may be more exposed or more susceptible to oxidative stress, or younger children may have a better enzymatic reduction capacity of CoQ10. A marked increase in oxidative status occurred between the first and second years of life, when infants learn to walk. Motor development associated with an increased energy demand may mirror early signs of increased free-radical production in mitochondrial energy metabolism. Furthermore, children with low total muscle CoQ10 concentrations tended to have higher oxidized proportions, independent of age, which may reflect increased consumption of antioxidative capacity under conditions of decreased antioxidant availability.

Table 3. Comparison of different studies regarding the concentration of CoQ10 in human skeletal muscle

	Age	Number	Muscle coenzyme Q10	
Reference	Years	*n*	pmol/mg fresh tissue	pmol/mg protein
Laaksonen *et al.* (28)	(32–51)	15	69.5[a]	nd
Musumeci *et al.* (18)	Not specified	30	29.0 ± 4.1[a]	nd
Lamberti *et al.* (19)	Not specified	121	32.0 ± 5.1[a]	nd
Duncan *et al.* (30)	24.5 ± 3.9 (0.5–59)	26	nd	241 ± 95 (140–580)
Pastore *et al.* (31)	Pediatric controls	40	18.3 ± 5.5 (10.2–27.5)	nd
Miles *et al.* (29)	5.7 ± 4.6 (1.3–18)	21	nd	142.32 ± 35.02
Miles *et al.* (14)	7.0 ± 5.2	20	nd	215 ± 49
Lopez *et al.* (15)	Not specified	185	37.2 ± 7.8[a]	nd
Artuch *et al.* (23)	Not specified	Not specified	nd	(157–488)
This study	4.7 ± 3.2 (0.75–15.3)	140	40.5 ± 12.2 (11.7–79.2)	832 ± 386 (331–2464)

Shown are mean values ± SDM; the range is presented in brackets.
[a](1 µg/ g corresponds to 1.158 pmol/mg).
nd, not determined.

There are large interlaboratory differences in reference values for human skeletal muscle CoQ10 concentrations (**Table 3**). This may be attributable to variations in specimen types. In our study, all biopsies were taken from small muscle samples (12–30 mg) from the abdominal internal oblique muscles, while other studies used quadriceps samples, or the muscles were not specified. The CoQ10 content of tissues may depend on bioenergetic requirements, and differences in the number or size of CoQ10-rich mitochondria may modify the CoQ10 tissue content (8,39). Interlaboratory differences may also be caused by variations in the homogenization or extraction methods, or in sampling procedures. In the current study, specimens were frozen on dry ice immediately after biopsy, and the internal standards were added directly to the frozen specimens before homogenization to avoid artificial upoxidation during tissue processing (38). Interlaboratory comparisons are complicated by the use of different reference values for CoQ10 tissue concentrations, which may be derived directly from the mass of the wet tissue sample, or by analysis of the tissue protein content. Based on wet tissue weight, the current muscle CoQ10 concentrations were in good agreement with other findings (**Table 3**); however, when related to protein content, the muscle CoQ10 range exceeded those reported in other studies. This might be an inherent problem of the samples. Although it is unlikely that a high fat content could explain the low protein content of the muscle samples, given that the biopsies were very small, a high connective tissue content could have a significant impact on the protein assay. However, there were good correlations between the protein concentration in the muscle tissue and the wet sample weight, and between the CoQ10 concentrations related to tissue weight and to protein weight.

Given these discrepancies, the adjustment of CoQ10 concentration to wet tissue weight may provide a more reliable index for comparing the present data with data from other laboratories. Furthermore, interlaboratory variability and the lack of an external quality control mean that good internal quality control is essential. In the absence of human tissue, swine tissue may be used to establish a tissue pool for internal quality control (38).

There are species differences in the number of isoprenoid units in the side chain of coenzyme Q. The most prevalent form in humans is CoQ10 (1). In the current study, endogenous CoQ9 in human skeletal muscle tissue did not exceed 2.5%; however, internal CoQ9 status in muscle tissue was calculated by comparing the area ratios of CoQ9 and CoQ10 peaks in the chromatograms, and was not based on an exact determination of concentration. The calibration lines (peak area vs. concentration) of CoQ9 and CoQ10 may differ slightly. An proportion of endogenous CoQ9 <2.5% is marginal, and in line with the findings of Miles *et al.* (29) who found an internal CoQ9 status of 2.79 ± 0.76 compared with 142.32 ± 35.02 CoQ10 (nmol/g protein) in muscle tissues from a control reference group of 21 children, equivalent to a mean endogenous proportion of 1.9% CoQ9.

In conclusion, the establishment of reference values for muscle CoQ10 in healthy children is a precondition for the early diagnosis of CoQ10 deficiency. An age-related redox shift in CoQ10 in muscle tissue in favor of the oxidized proportion suggests changes in antioxidative defense during childhood.

METHODS

This study was approved by the Human Ethics Committee of the Medical Faculty, University Witten-Herdecke (35/2011). Muscle specimens (12–30 mg) were collected from biopsies from 140 healthy children (mean age 4.7 y, range 0.8–15.3 y) between July 2012 and June 2014. These children attended the children's hospital for the following medical interventions: inguinal hernia (n = 40; 26 boys and 14 girls), undescended testis (n = 77), and hydrocele testis (n = 23). All biopsies were taken in the context of the routine operation, with no additional procedures. All patients and/or guardians gave their informed consent. Body height and weight were recorded to calculate BMI. Biopsy specimens from the abdominal internal oblique muscle (musculus obliquus internus abdominis) were immediately frozen on dry ice during the surgical procedure, and then stored at −84 °C prior to homogenization and analysis of CoQ10. For measurement of CoQ10 in plasma, a 500 µl heparinized blood sample was taken during surgery, and a 100-µl plasma aliquot was stored at −84 °C within 20 min. An additional 10 µl plasma aliquot was stored at −84 °C for analysis of cholesterol, using the CHOD-PAP-method (Human, Wiesbaden, Germany).

The total CoQ10 content and redox status were analyzed by HPLC with electrochemical detection and internal standardization, as described previously for plasma (40) and tissues (38). Specimens of frozen muscle tissues (12–30 mg) were homogenized by bead milling (oscillation frequency, 50 HZ, 5 min) using a TissueLyser LT (Qiagen, Hilden, Germany). The internal standards 270 pmol ubihydroquinone-9 and 370 pmol ubiquinone-9 in 50 µl ethanol were added to the homogenization medium containing 1,300 µl of 2-propanol. After homogenization, the tubes were centrifuged (10,000 × g for 5 min at 4 °C) and 150 µl saline was added to 350 µl of the supernatant and stored at −84 °C for further use. For CoQ10 extraction, 500 µl of hexane was added to the samples, which were mixed for 2 min and then centrifuged at 1,000 × g

for 5 min at 4 °C. The separated hexane phase was evaporated to dryness under an argon stream and redissolved in 60 µl of reagent grade alcohol (methanol/ethanol/propanol, 100/95/5, v/v/v) for injection into the HPLC system. All tissue samples were analyzed in duplicate. For quantitative protein analysis (38), tissue residues of muscle homogenates were dried under argon to remove residual propanol, diluted with 1 ml saline solution, and analyzed using a Micro Lowry Total Protein Kit (Onishi & Barr modifications) according to the manufacturer's instructions (Sigma-Aldrich, Taufkirchen, Germany).

Eleven of the 140 muscle biopsies included more than the 12–30 µg required for tissue CoQ10 analysis. The excess samples of 13–30 µg tissue were uses for analysis of endogenous CoQ9. Muscle tissues were homogenized as described above, without addition of internal standards. The endogenous proportion of CoQ9 was calculated by comparing the area ratios of CoQ9 and CoQ10 in the HPLC chromatograms.

Data are expressed as mean ± SD. Differences between two groups were analyzed using Wilcoxon's test. Differences between age groups were analyzed by one-way ANOVA (least significant difference). Correlations between parameters were tested using Spearman's rank correlation. The significance level was set at $P \leq 0.05$ for all tests.

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