Molecular Genetics and Metabolism 105 (2012) 457–462

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Non-invasive evaluation of buccal respiratory chain enzyme dysfunction in mitochondrial disease: Comparison with studies in muscle biopsy

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ARTICLE INFO

Article history:
Received 19 September 2011
Received in revised form 21 November 2011
Accepted 21 November 2011
Available online 30 November 2011

Keywords:
Respiratory chain
Mitochondrial disease
Buccal swab

ABSTRACT

Making a diagnosis of mitochondrial disease (MD) is extremely challenging and often employs the analysis of respiratory complex (RC) activities in biopsied skeletal muscle. Given both the invasive nature and expense of biopsied-muscle based testing for mitochondrial defects, buccal swab enzyme analysis has been explored as an alternative approach to the more invasive muscle biopsy. Case studies have recently suggested that buccal swabs from patients can be used to accurately assess mitochondrial enzyme activities including RC I and RC IV using a dipstick methodology combined with spectrophotometric analysis. In this study, forty patients with suspected MD who have previously been found to have significant defects in either RC I or RC IV in skeletal muscle were assessed by buccal swab analysis and compared to enzyme values obtained with unaffected controls (n = 106) in the same age range. Buccal citrate synthase was used as an indicator of overall mitochondrial content, correlating well with overall buccal mitochondrial frataxin levels and was found to be elevated above control levels in 28% of the patients in this cohort. Of 26 cases with significant muscle RC I deficiency, 20 displayed significantly reduced levels of buccal RC I activity. All 7 of the patients with muscle RC IV deficiency showed significant buccal RC IV defect and 6 of the 7 patients with combined defects in muscle RC I and IV activity levels also exhibited analogous deficiencies in both buccal RC I and RC IV activities. In conclusion, the relatively high correlation (over 82%) of buccal and muscle RC deficiencies further supports the validity of this non-invasive approach as a potentially useful tool in the diagnosis of MD.

1. Introduction

Defects of the mitochondrial respiratory chain and oxidative phosphorylation (OXPHOS) are often found in a large and heterogeneous group of mitochondrial-based diseases (MD). The clinical presentation of these primarily neurological disorders ranges from isolated organ involvement including but not limited to muscle, brain, heart and eyes but are more commonly multisystemic. Some MDs are genetic disorders such as Leigh disease, mitochondrial encephalopathy, lactic acidosis, stroke-like episodes (MELAS), myoclonic epilepsy and ragged red fibers (MERRF), Kearns–Sayre syndrome (KSS) and Leber's hereditary optic neuropathy (LHON) which display specific clinical phenotypes often occurring as a result of specific pathogenic mutations in mitochondrial DNA (mtDNA) although nuclear DNA mutations have been increasingly identified [1–3]. However, severe mitochondrial dysfunction has also been reported in patients with seizures, muscle weakness, developmental delay, autism or hypotonia who don't necessarily fit easily into the above clinical categories and who show no observable genetic defect [4–6]. Definitive diagnosis of MD is therefore highly challenging involving a combination of clinical evaluation, metabolite measurement, histochemical and molecular genetic studies and an analysis of some or all of the 5 mitochondrial respiratory complex (RC) activities which function in electron transport and OXPHOS [1–3]. Biochemical evaluation in these patients, usually of biopsied skeletal muscle, often shows significant deficiencies in specific RC activities, most frequently in RC I and RC IV, although deficiencies in RC II, III and V have also been described [3,7].

Given both the invasive nature and expense of biopsied-muscle based testing for mitochondrial defects, enzyme testing of skeletal muscle biopsy poses more difficulties in children and infants.

While the ideal tissue for the evaluation of mitochondrial disorders is one that clinically expresses the disease, such as the brain, kidney, myocardium or skeletal muscle, these target tissues are often difficult to obtain in patients as well as in healthy control subjects, there has been increasing interest in alternative approaches. Studies have shown that a high proportion of patients with skeletal muscle RC deficiencies also displayed significant RC deficiency in lymphocytes [7] supporting the view that other cell-types can be reflective

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doi:10.1016/j.ymgme.2011.11.193

of muscle tissue with regard to RC enzyme activity evaluation. While findings from the analysis of cultured patient skin fibroblasts can be diagnostically informative [8–10], they often are not reflective of muscle RC defects [11,12] and the fibroblasts also may have an unstable phenotype depending on the culture conditions [13].

Therefore, our laboratory initiated a study as to whether buccal swab enzyme analysis might be used as an alternative approach to the more invasive muscle biopsy. Recent studies have shown that buccal swab analysis can be used to reliably detect pathogenic mtDNA mutations, suggestive that buccal analysis might in fact be reflective of the expression of mitochondrial dysfunction [14–16]. Studies in our laboratory have recently shown that buccal swabs from patients with MD can be used to accurately assess mitochondrial enzyme activities including RC I and IV using a sensitive dipstick methodology combined with spectrophotometric analysis [17,18]. In this study, we sought to further examine the utility of this non-invasive approach to evaluate specific mitochondrial dysfunction in a larger cohort of patients with diagnosed mitochondrial disease and demonstrated mitochondrial enzyme defects in biopsied skeletal muscle and to examine the correlation of buccal mitochondrial enzyme dysfunction in patients with previously diagnosed mitochondrial defects.

2. Materials and methods

2.1. Patients

This study had the approval of Institutional Review Boards at St. Christopher's Hospital for Children and at Drexel University College of Medicine. All patients and control subjects provided written informed consent before participating in this study protocol.

Clinical and demographic information and buccal swabs were collected from 40 patients with suspected mitochondrial disease who had previously shown abnormal RC I and/or RC IV activity levels in biopsied skeletal muscle. Cheek swabs were also collected from healthy controls (n = 106) without any signs or symptoms of neurological disease in roughly the same age range. Patient samples were primarily drawn from the neurology clinic at St. Christopher's Hospital for Children, as well as from referring physicians.

2.2. Buccal sample preparation

Buccal cells were harvested using Catch-All Buccal Collection Swabs (Epicentre Biotechnologies) by firmly pressing a swab against the inner cheek while twirling for 30 s on each cheek. Typically, 3 swabs were employed for each subject (4 were used with infants). Each swab was clipped and then placed in a 1.5 ml microcentrifuge tube with 175 μl of ice cold extraction buffer A (MitoSciences) containing 1.5% lauryl maltoside, 100 mM of NaCl, 25 mM of HEPES pH 7.4, as well as a protease inhibitor cocktail (Sigma, P-8340) [19]. The swab-containing tubes were vortexed for 20 s and then microcentrifuged at 8500 ×*g* for 5 min to remove the extracted protein from the swabs. After incubation for 15 min on ice, sample extracts were cleared of insoluble material by centrifugation at 16,000 ×*g* for 15 min at 4 °C and the pooled protein extract concentrated using Amicon Ultra 3 k 0.5 ml centrifugal filters as suggested by the manufacturer. Duplicate aliquots were analyzed for protein concentration determination using the bicinchoninic acid method (Pierce). Samples were stored at −20 °C for up to 1 week prior to enzymatic analysis.

2.3. Enzyme assay protocols

MitoSciences dipstick assays were used to measure RC I [EC 1.6.5.3] activity levels in buccal cells [19]. Briefly, 50 μg of buccal cell protein in 25 μl of extraction buffer A was mixed with 25 μl blocking buffer and added to individual wells on a 96-well plate. In patient samples with less protein, up to 50 μl of buffer A extract and 50 μl of blocking buffer can be added per well. After samples were allowed to equilibrate, dipsticks were inserted into the well and the sample was allowed to wick up the membrane for 35 min, during which time RC I activity is allowed to be immunocaptured onto designated capture zones on the dipstick. After a 15 min incubation with a wash buffer (50 mM Tris–HCl, 150 mM NaCl, pH 7.4), the dipsticks were inserted into fresh wells containing 300 μl of substrate, including 0.3 mg/ml nitrotetrazolium blue (NBT) and 0.1 mg/ml β-NADH in reaction buffer (20 mM Tris–HCl pH 7.4) for 35 min. The dipsticks were subsequently rinsed in water (10 min) and then the RC I capture zones on the developed dipsticks which contained reduced, intensely colored NBT were quantified using a Hamamatsu immunochromato reader (MS1000 Dipstick reader). A standard curve using a range (5–100 μg) of total protein extracted from buccal cells was run to determine an appropriate concentration of sample to use within the linear working range of the assay. Raw mABS (milli-Absorbance) results were corrected for protein concentration and the data were expressed as percentages of the values obtained with control extracts run on the same assay. All assays were performed in duplicate.

Activity levels of buccal RC IV (also known as cytochrome *c* oxidase [EC 1.9.3.1] and citrate synthase [EC 4.1.3.7] (CS)) were assessed in duplicate using standard spectrophotometric procedures in 0.5 ml reaction volume [4]. Specific activities of RC I and RC IV were normalized relative to buccal CS activity levels, a commonly used gauge of overall mitochondrial content and were expressed as activity ratios (i.e. I/CS and IV/CS). The use of activity ratios as compared to absolute activities in mitochondrial enzyme assessment is well established [3,7,12] and provides a much narrower range of normal control values as compared to activities expressed on the basis of protein content of the sample, particularly critical for buccal analysis. Levels of buccal frataxin content were also determined using an immunocapture dipstick methodology as suggested by the manufacturers (MitoSciences, Inc.) [20] with 50 μg of each buccal extract used on each dipstick, well within the calculated linear range of response. Every enzyme evaluation session would include assays with at least 2 and more commonly 3 different control buccal samples as well as one skeletal muscle extract to insure limited variability and provide a measure of quality control for these tests.

2.4. Genetic analysis

Molecular screening of specific pathogenic point mutations and large-scale deletions in mtDNA was performed using a PCR/RFLP approach with DNA isolated using the QIAamp procedure (Qiagen) from the buccal sample extracts obtained from 20 patients of this cohort.

2.5. Statistical analysis

Data analysis was performed with difference in means compared using Student's *t*-test. The data assumed equal variance and were considered significantly different when $p<0.05$. Enzyme activity levels in patient samples were typically expressed as % of average control. Means were expressed as % of average control ± standard deviation (SD), and variability was expressed using the coefficient of variation (CV), the standard deviation expressed as percent of the mean.

3. Results

3.1. Demographic and clinical information

Demographic information for the 40 MD patients and the control subjects (n = 106) was similar. The age of MD patients ranged from 2 to 47 years, with a median of 14.3 years (SD, 12.2), with 22 females

and 18 males. Similarly, the age range of the control group ranged from 2 to 49 with a median range of 12.3 years (SD, 8.4) with 52 females and 58 males. In this group, there was no evidence in age-related changes in those enzyme activities assessed thus far (RC I and IV as well as CS), and our laboratory has begun testing both larger groups of infants (less than 2 years) and older adults to more rigorously ascertain significant age-related changes in mitochondrial function. No data concerning ethnicity or race was collected on these patients or control subjects.

As shown in Table 1, the clinical data collected from this cohort of patients with MD shows the expected broad range of phenotypic expression of mitochondrial disease with multiple systems affected. The most common phenotypes (in over 50% of the patients) included fatigue, muscle weakness, exercise intolerance and hypotonia. Also highly prevalent (between 30 and 45%) were GI abnormalities (most commonly presentation was motility issues), developmental delay and a variety of seizure-types. Other clinical manifestations present in this cohort included visual and hearing defects (23%), dysautonomia (15%), ataxia (15%), behavioral disorders including ASD (15%), cardiovascular abnormalities (10%), and brain abnormalities (7.5%). Data concerning patient lactic acid was available in only a limited segment of this cohort and is therefore not included in our analysis.

3.2. Genetic analysis

Limited genetic information was available from previous studies of this cohort. Using buccal DNA isolated from 20 patients, screening of common pathogenic point mutations and large-scale deletions revealed no evidence of 8 previously described mtDNA mutations in tRNAs (at nucleotides 611, 3243, 3251, 3260, 3271, 8296, 8344, 8356, 8363, 12147) or of 7 mutations in mtDNA-encoded complex I genes (at nucleotides 13153, 10158, 10191,11777, 11778, 12706 and 14459). Neither was there any evidence of common 5 kb and 7.4 kb deletions in these 20 patients.

These findings are consistent with the previously reported low yield of mtDNA in children with mitochondrial disease.

3.3. Assay reproducibility and linearity

Typically, buccal extracts from 3 swabs contained between 400 and 1000 μg of total cell protein, at a concentration of between 2 and 5 μg/μl, and up to 50 μl of each buccal cell sample was loaded into wells reliably for the RC I dipstick immunoassay. Standard curves generated using a range of buccal cell protein amounts from 0 to 100 μg of representative control samples showed that the RC I assay remained linear throughout the range of 100 μg ($R^2 = 0.995$) (data not shown). Therefore, 50 μg of total buccal swab protein from each subject was used in the RC I assays. This amount of protein is relatively small compared to the total amount of buccal protein collected per subject (mean ~ 785 μg), readily allowing the assessment of duplicate RC I assays as well as the evaluation of several other mitochondrial enzymes and frozen storage for additional subsequent studies.

Reproducibility of the assay was assessed by intra-assay, and interassay variability within the linear working range of buccal cell extracts collected from several patients and controls. The enzyme activity values for CS and activity ratios for RC I/CS and RC IV/CS were reproducible within the working range with reasonable interassay CVs (16.25% for CS, 14.5% for IV/CS and 12.5% for I/CS) for 7 subjects assessed at 3 different times. Assessment of intra-assay variability with 6 subjects, slightly lower CVs were obtained (6.3% for CS, 10.3% for IV/CS and 11.3% for I/CS). The increased variability seen between different collected samples from the same individual can be attributed to a combination of intra-assay variance and unknown biological factors. As noted previously, even with thorough rinsing, the presence of small, varying levels of non-buccal cell protein harvested along with buccal cells can alter protein concentrations measured. A disproportionate amount of this non-cellular protein in aliquots of swab-collected buccal material may have also contributed to the interassay variability. We also have found on several instances differences in the performance of biochemical reagents pivotal to the mitochondrial enzyme analysis including both the cytochrome *c* used in the RC IV assay and in the different batches of dipsticks obtained for RC I assay necessitating the rigorous use of several control samples with every evaluation.

To ensure that CS content was related to overall buccal mitochondrial content, the levels of CS activity in each of 23 controls was correlated with the levels of mitochondrial frataxin, quantified using a frataxin-specific dipstick assay (MitoSciences, Inc.). The results as shown in Fig. 1 show a positive correlation ($R^2 = 0.926$) between the two indices of overall mitochondrial content in these samples.

3.4. Muscle biopsy data

Patients were included in this study based on their having had a significant deficiency in RC I and/or RC IV activity levels upon a

Table 1
Clinical presentation and findings in MD patients.

	Number of patients	Percent of patients
Muscle weakness	31	78
Fatigability/exercise intolerance	30	75
Hypotonia	20	50
Gastrointestinal dysfunction	16	40
Seizures	15	38
Developmental delay	15	38
Eye and hearing defects	11	27.5
Sensorineural hearing deficit	5	12.5
Ptosis	6	15
Ataxia	6	15
Dysautonomia	6	15
Autistic Spectrum Disorders (ASD)	5	12.5
Cardiovascular abnormality	4	10
Tachycardia	2	5
Conduction defects	1	2.5
Hypertrophic cardiomyopathy	1	2.5
Brain abnormalities	3	7.5

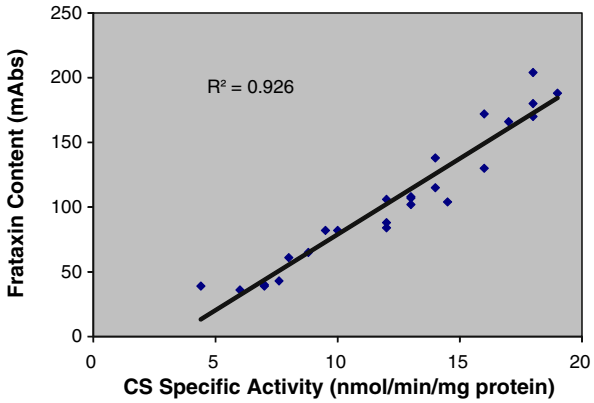

Fig. 1. Correlation of buccal citrate synthase activity and frataxin content. Twenty-five representative subjects from the control group were assessed for both buccal citrate synthase (CS) activity levels using spectrophotometric assessment and for buccal frataxin content using enzyme immunocapture ("dipstick"). An aliquot of the buccal extract of each subject containing 50 μg protein was assessed for CS and a second aliquot (50 μg buccal protein) was assessed for frataxin content as described in Materials and methods. As shown, the relationship between buccal CS specific activities (expressed as nmol acetyl coenzyme A produced/min/mg buccal protein) and buccal frataxin content (expressed as mAbs units) for these 25 control subjects shows a strong positive correlation ($R^2 = 0.9262$).

retrospective analysis of their skeletal muscle biopsies. The criteria used here for the determination of specific muscle RC enzyme dysfunction from these findings (which come from several different testing laboratories) utilized values 2 SD below the mean values (and well below the control range of values). The considerable variation in enzyme assay conditions and methods used by these different laboratories in our study of the muscle biopsy evaluations is a well-known concern in the mitochondrial diagnostic community [21,22] and limited the ability to pool the overall pre-existing muscle data for presentation here. Also while the extent of specific RC enzyme activity deficiencies present in the muscle biopsies in this cohort was often well below this level satisfying more rigorous criteria for inclusion as a significant deficiency, it was not that extensive in all cases. We used the same criteria (values at least 2 SD below the mean and below the overall control range) in defining the RC activity deficiencies found in the patients' buccal samples.

Analysis of this patient cohort's muscle enzyme values showed that isolated RC I activity deficiencies were the most prevalent defect (26 subjects or 65% of this cohort) while isolated RC IV activity deficiencies were present in 7 or 17.5%. Combined RC I and RC IV activity defects were noted in 7 subjects (17.5%).

3.5. Buccal enzyme data

The mean values ascertained for buccal activity ratios of RC I/CS and RC IV/CS as well as the CS activity values for our control subjects are shown in Table 2. The control buccal RC I/CS values ranged from 3.4 to 11.9 (with a mean value of 6.8 ± 2.1). The control buccal RC IV/CS values ranged from 0.16 to 0.6 (with a mean value of 0.31 ± 0.10). The control buccal CS activity values ranged from 4.4 to 22 (with a mean value of 12.7 ± 5.1).

Deficient buccal RC I/CS values at or below 2.5 (2 SD below the control mean of 6.8) were found in 26 patients in this cohort; 20 of these patients had isolated RC I/CS deficiencies with a mean RC I/CS value of 1.8 ± 0.5. Deficient buccal RC IV/CS values at or below 0.10 (2 SD below the control mean of 0.31) were found in 15 patients; 8 patients had isolated RC IV/CS deficiencies with a mean activity value of 0.08 ± 0.02. In addition, 7 patients exhibited significant deficiencies in both buccal RC IV/CS (mean 0.06 ± 0.02) and RC I/CS levels (mean 1.7 ± 0.6)

Table 2
Buccal enzyme values in controls and MD patients.

	N (%)	CS Mean ± SD Range	I/CS Mean ± SD Range	IV/CS Mean ± SD Range
Controls	106 (na)	12.7 ± 5.1 4.4–22	6.8 ± 2.0 3.4–11.9	0.31 ± 0.10 0.15–0.60
Patients with isolated defects in RC I	20 (50)	16.9 ± 9.9 6–40	1.8* ± 0.5 0.8–2.5	0.26 ± 0.07 0.15–0.39
Patients with isolated defects in RC IV	8 (20)	16.1 ± 8.5 6–33	6.0 ± 1.4 4.4–8.2	0.08* ± 0.02 0.02–0.1
Patients with combined defects in RC I + IV activities	7 (17.5)	25.6* ± 11.4 7. 6–54	1.7* ± 0.6 0.8–2.5	0.06* ± 0.02 0.02–0.1

N = number of subjects.
% = percentage of overall patient cohort.
CS = specific activity of citrate synthase (nmol/min/mg buccal extract protein).
I/CS = activity ratio of RC I relative to CS.
IV/CS = activity ratio of RC IV relative to CS.
na = not applicable.
SD = standard deviation.
* $p<0.05$ level of significance.

While none of the overall patient cohort displayed a significantly deficient buccal CS activity, 11 subjects (28% of the overall cohort) displayed CS activity values over 25 (>2 SD over the control mean value) suggestive of increased mitochondrial number in their buccal sample.

This finding also suggests that the evaluation of buccal CS activity levels may be useful as an additional informative biochemical marker of significant mitochondrial dysfunction in a significant proportion of MD cases. Interestingly, the group of 7 patients with combined RC I and IV activity defects also exhibited a higher mean CS activity value (25.6), suggestive of marked adaptive increases in mitochondrial content.

3.6. Correlation of muscle and buccal swab enzyme data

Among the 26 patients in this cohort who had previously displayed a significant isolated RC I activity deficiency in skeletal muscle, 20 (77%) harbored a significant buccal RC I/CS deficiency (see Fig. 2). Also shown in the figure, among the 7 patients with a significant isolated RC IV activity defect in skeletal muscle, all 7 (100%) displayed a marked buccal RC IV/CS deficiency. Of the 7 patients with combined RC I and IV defects in skeletal muscle, 6 (86%) had similar profiles in both buccal RC enzymes as depicted; 1 of this group of patients had marked evidence of RC IV defect but not of RC I deficiency. Finally, 1 of the 26 patients with isolated RC I deficiency in skeletal muscle also displayed a significant buccal RC IV deficiency. The overall correlation of buccal swab RC I and IV deficiency with skeletal muscle deficiencies in this cohort is relatively high with 82.5% of the patients showing the same RC defect in both tissues.

4. Discussion

In the current study, we have applied the non-invasive evaluation of buccal mitochondrial enzyme activities in a selected cohort of patients with suspected MD (satisfying modified Walker criteria) who had previously been shown to have significant activity deficiencies in either respiratory complex I or IV (or in both activities) in skeletal muscle. In the main, these data are supportive of the informative use of buccal enzyme analysis assessing RC I, RC IV and CS activities in

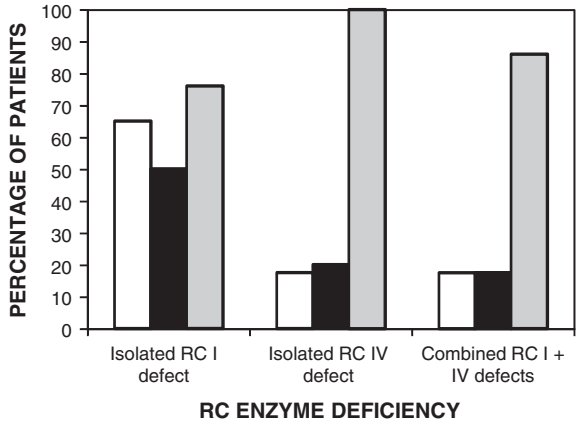

Fig. 2. Relationship between buccal and skeletal muscle respiratory complex activity deficiencies in a cohort of patients with suspected mitochondrial disease. In a cohort of 40 patients with suspected mitochondrial disease who had previously been shown to harbor significant deficiencies in either RC I and/or IV skeletal muscle enzymes, the percentage of patients with isolated RC I, isolated RC IV or combined I/IV muscle deficiencies is depicted (□). In the same patient cohort, the percentage of patients with isolated RC I, isolated RC IV or combined RC I/IV buccal activity deficiencies is depicted (■). In the sub-groups of patients with identified isolated RC I, isolated RC IV or combined RC I/IV skeletal muscle deficiencies, the percentages of patients with significant buccal deficiencies in the same enzymes are depicted (▩).

non-invasively screening patients suspected with MD to identify specific RC activity defects as well as increased CS activities.

This particular patient cohort displayed a higher prevalence of isolated RC I deficiency (50%) but significant levels of both isolated RC IV deficiencies (20%) and of combined RC I and IV defects (17.5%). While there are reports of the prevalence of RC I deficiencies in mitochondrial disorders [23] and in children with MD [24], others have found in selected large-scale studies either similar levels of RC I and IV defects [25] or even a prevalence of RC IV defects [26,27]. Most studies have also reported a significant frequency of cases with combined RC defects.

It is noteworthy that the agreement between the muscle biopsy enzyme activity findings and the buccal evaluation is somewhat higher for RC IV than for RC I activity deficiencies. The dipstick methodology used here for detecting RC I activity utilizes an electron acceptor that changes color upon reduction (e.g., nitrotetrazolium blue), which is primarily measuring the NADH dehydrogenase component of the RC I enzyme complex. In a recent review of mitochondrial diagnostic technologies, Rodenburg [3] noted that the spectrophotometric analysis of RC I activity which rather utilizes analogs of the natural substrate coenzyme Q offers an assessment of overall RC I activity which is more comprehensive and can detect RC I defects not limited to those in NADH dehydrogenase activity. This might be particularly relevant to those 6 subjects in who we found discordance between the RC defects in skeletal muscle, and not in buccal tissues. Other possible reasons for this discordance include but are not limited to: (a) change of patient status over time (i.e. a number of these RC defects were found in the patients between 5 and 10 years ago), (b) the possible (thus far unevaluated) impact of treatments used thus far (i.e. a number of these patients have been treated with various regimens of “mito cocktails” including Coenzyme Q10 and carnitine), and (c) differences in tissue specific phenotypic expression and heterogeneity (i.e. the particular tissue sampled may have more or less defective mitochondria). Moreover, buccal mitochondrial enzymes might also be subject to changes and enzyme-modulating variables which remain to be tested including diet and smoking.

At this time, the buccal enzyme analysis is limited to detecting RC I and IV activity defects. While these RC defects have been reported to be the most common defects reported in mitochondrial disorders, defects in RC II, III and V have also been reported. In fact, several studies have suggested that combined enzyme RC defects may be the more prevalent in mitochondrial disorders than isolated activity defects [28,29]. Without evaluating RC II, III and V, our study could not address this issue though showing combined RC I and IV defects with a significant prevalence (17.5%). Thus far, the use of buccal analysis in quantitatively evaluating RC II, III and V activity deficiencies and the reliable detection of RC I activity by spectrophotometric analysis have not yet been fully developed, likely as a function of the lower overall mitochondrial content present in buccal tissues. Moreover, spectrophotometric assays for RC I, III and V activities generally require higher amounts of extract protein as well as the need to perform parallel reactions in the presence and absence of specific inhibitors (e.g. rotenone, antimycin, oligomycin) to insure enzyme specificity, a difficult undertaking in buccal enzyme analysis considering the limited yield of buccal mitochondrial protein. Nevertheless, recent preliminary findings in our laboratory have shown that combined RC II and III and RC I and III activities appear to be more robust and reliably measured in our buccal extract preparations and increased numbers of controls are currently being evaluated to establish mean values and a usable reference range.

One particularly attractive advantage to the buccal swab approach lies in the straight-forward acquisition of a large healthy control population, who showed no evidence of neurological or mitochondrial defect. This is often not possible with other tissues such as skeletal muscle, heart or nerve which often come from patients with other defects which may impact the mitochondrial functionality of that tissue. A second important advantage is the ease of replicability which can also facilitate further studies of the effects of specific therapeutic options as well as following the clinical prognostic course of the mitochondrial disorders. Moreover, the simplicity and non-invasive nature of this testing also facilitate the screening of patients from underserved populations who may not be able to afford muscle biopsy analysis or who may be concerned about the use of anesthesia and surgical stresses on infants and children who might be more vulnerable to their effects. Furthermore, it is well-recognized that the interpretation of muscle mitochondrial enzyme activities measurements is not as straightforward as for many other metabolic enzyme disorders with a small margin between patient ranges and control ranges [30]. This has led to the concern that the potential risks of surgery and anesthesia may outweigh the benefits for a procedure that may provide inconclusive results.

It is unlikely that buccal enzyme testing will entirely replace the standard muscle biopsy analysis as the latter can provide a much broader and unparalleled view into the dysfunctional mitochondria. Muscle testing can take advantages of high resolution testing of either isolated muscle fibers or needle biopsies requiring very limited tissue, offers the availability of a broad range of mitochondrial functional testing as well as a larger spectrum of enzymatic assays and can incorporate the use of a wide variety of metabolic substrates and specific inhibitors to identify the specific defective locus in a number of metabolic pathways including in the respiratory chain, fatty acid beta oxidation, pyruvate dehydrogenase or in the Krebs cycle [31]. Moreover, buccal tissues are not primary targets of these diseases and while we have presented data showing that their evaluation does appear to be reflective of findings in muscle, it is presently recommended that their role in MD diagnosis be used as an adjunct and preliminary screening procedure prior to a more comprehensive testing of muscle mitochondrial enzyme function by spectrophotometry, polarimetry or respirometry whenever possible. In addition, several studies have come to conclusion that enzymatic assessment should be undertaken in alternate tissues particularly when muscle biopsy findings are inconclusive, a role that buccal analysis could well facilitate [22,32].

Acknowledgments

We wish to sincerely thank all the MD patients, their relatives, and all other individuals who volunteered cheek swab samples for this study. This study was supported in part from funds awarded by the Creight Foundation and the St. Christopher's Hospital for Children PHEC Fund Grant awarded to M.J.G. and from the St. Christopher Foundation awarded to A.L. We very much regret the death of Professor Leon Salganicoff during this study.

References

[1] D.C. Wallace, Diseases of the mitochondrial DNA, Annu. Rev. Biochem. 61 (1992) 1175–1212.
[2] A. Munnich, P. Rustin, Clinical spectrum and diagnosis of mitochondrial disorders, Am. J. Med. Genet. 106 (2001) 4–17.
[3] R.J. Rodenburg, Biochemical diagnosis of mitochondrial disorders, J. Inherit. Metab. Dis. 34 (2011) 283–292.
[4] J. Marin-Garcia, R. Ananthakrishnan, M.J. Goldenthal, J. Filiano, H. Sarnat, Skeletal muscle mitochondrial defects in nonspecific neurological disorders, Pediatr. Neurol. 21 (1999) 538–542.
[5] D. Khurana, L. Salganicoff, J. Melvin, E. Hobdell, I. Valencia, H. Hardison, H. Marks, W. Grover, A. Legido, Epilepsy and respiratory chain defects in children with mitochondrial encephalopathies, Neuropediatrics 39 (2008) 8–13.
[6] J.J. Filiano, M.J. Goldenthal, C.H. Rhodes, J. Marin-Garcia, Mitochondrial dysfunction in patients with hypotonia, epilepsy, autism, and developmental delay: HEADD syndrome, J. Child Neurol. 17 (2002) 435–439.
[7] D. Chretien, P. Rustin, T.A. Bourgeron, A. Rotig, J.M. Saudubray, A. Munnich, Reference charts for respiratory chain activities in human tissues, Clin. Chim. Acta 228 (1994) 53–70.
[8] K.A. Kramer, D. Oglesbee, S.J. Hartman, J. Huey, B. Anderson, M.J. Magera, D. Matern, P. Rinaldo, B.H. Robinson, J.M. Cameron, S.H. Hahn, Automated spectrophotometric

analysis of mitochondrial respiratory chain complex enzyme activities in cultured skin fibroblasts, Clin. Chem. 51 (2005) 2110–2116.
[9] J.L. Loeffen, J.A. Smeitink, J.M. Trijbels, A.J. Janssen, R.H. Triepels, R.C. Sengers, L.P. van den Heuvel, Isolated complex I deficiency in children: clinical, biochemical and genetic aspects, Hum. Mutat. 15 (2000) 123–134.
[10] E. Fernández-Vizarra, V. Tiranti, M. Zeviani, Assembly of the oxidative phosphorylation system in humans: what we have learned by studying its defects, Biochim. Biophys. Acta 1793 (2009) 200–211.
[11] L.P. van den Heuvel, J.A. Smeitink, R.J. Rodenburg, Biochemical examination of fibroblasts in the diagnosis and research of oxidative phosphorylation (OXPHOS) defects, Mitochondrion 4 (2004) 395–401.
[12] D.R. Thorburn, C.W. Chow, D.M. Kirby, Respiratory chain enzyme analysis in muscle and liver, Mitochondrion 4 (2004) 363–375.
[13] J.M. Cameron, V. Levandovskiy, N. MacKay, B.H. Robinson, Respiratory chain analysis of skin fibroblasts in mitochondrial disease, Mitochondrion 4 (2004) 387–394.
[14] H. Narbonne, D. Perucca-Lostanlen, C. Desnuelle, B. Vialettes, A. Saunières, V. Paquis-Flucklinger, Searching for A3243G mitochondrial DNA mutation in buccal mucosa in order to improve the screening of patients with mitochondrial diabetes, Eur. J. Endocrinol. 145 (2001) 541–542.
[15] J. Uusimaa, J.S. Moilanen, L. Vainionpää, P. Tapanainen, P. Lindholm, M. Nuutinen, T. Löppönen, E. Mäki-Torkko, H. Rantala, K. Majamaa, Prevalence, segregation, and phenotype of the mitochondrial DNA 3243A>G mutation in children, Ann. Neurol. 62 (2007) 278–287.
[16] P.F. Chinnery, P.J. Zwijnenburg, M. Walker, N. Howell, R.W. Taylor, R.N. Lightowlers, L. Bindoff, D.M. Turnbull, Nonrandom tissue distribution of mutant mtDNA, Am. J. Med. Genet. 85 (1999) 498–501.
[17] H. Ezugha, M. Goldenthal, I. Valencia, C.E. Anderson, A. Legido, H. Marks, 5q14.3 deletion manifesting as mitochondrial disease and autism: case report, J. Child Neurol. 25 (2010) 1232–1235.
[18] W.R. Yorns, I. Valencia, A. Jayaraman, S. Sheth, A. Legido, M.J. Goldenthal, Buccal swab analysis of mitochondrial enzyme deficiency and DNA defects in a child with suspected MERRF, J Child Neurol (2011) Nov 22. [Epub ahead of print].
[19] J.H. Willis, R.A. Capaldi, M. Huigsloot, R.J. Rodenburg, J. Smeitink, M.F. Marusich, Isolated deficiencies of OXPHOS complexes I and IV are identified accurately and quickly by simple enzyme activity immunocapture assays, Biochim. Biophys. Acta 1787 (2009) 533–538.
[20] J.H. Willis, G. Isaya, O. Gakh, R.A. Capaldi, M.F. Marusich, Lateral-flow immunoassay for the frataxin protein in Friedreich's ataxia patients and carriers, Mol. Genet. Metab. 94 (2008) 491–497.
[21] F.N. Gellerich, J.A. Mayr, S. Reuter, W. Sperl, S. Zierz, The problem of interlab variation in methods for mitochondrial disease diagnosis: enzymatic measurement of respiratory chain complexes, Mitochondrion 4 (2004) 427–439.
[22] F.P. Bernier, A. Boneh, X. Dennett, C.W. Chow, M.A. Cleary, D.R. Thorburn, Diagnostic criteria for respiratory chain disorders in adults and children, Neurology 59 (2002) 1406–1411.
[23] J.A. Smeitink, L.P. van den Heuvel, S. DiMauro, The genetics and pathology of oxidative phosphorylation, Nat. Rev. Genet. 2 (2001) 342–352.
[24] F. Scaglia, J.A. Towbin, W.J. Craigen, J.W. Belmont, E.O. Smith, S.R. Neish, S.M. Ware, J.V. Hunter, S.D. Fernbach, G.D. Vladutiu, L.J. Wong, H. Vogel, Clinical spectrum, morbidity, and mortality in 113 pediatric patients with mitochondrial disease, Pediatrics 114 (2004) 925–931.
[25] J. Uusimaa, A.M. Remes, H. Rantala, L. Vainionpää, R. Herva, K. Vuopala, M. Nuutinen, K. Majamaa, I.E. Hassinen, Childhood encephalopathies and myopathies: a prospective study in a defined population to assess the frequency of mitochondrial disorders, Pediatrics 105 (2000) 598–603.
[26] D. Skladal, C. Sudmeier, V. Konstantopoulou, S. Stöckler-Ipsiroglu, B. Plecko-Startinig, G. Bernert, J. Zeman, W. Sperl, The clinical spectrum of mitochondrial disease in 75 pediatric patients, Clin. Pediatr. 42 (2003) 703–710.
[27] A. Munnich, A. Rötig, D. Chretien, V. Cormier, T. Bourgeron, J.P. Bonnefont, J.M. Saudubray, P. Rustin, Clinical presentation of mitochondrial disorders in childhood, J. Inherit. Metab. Dis. 19 (1996) 521–527.
[28] M.E. Rubio-Gozalbo, K.P. Dijkman, L.P. van den Heuvel, R.C. Sengers, U. Wendel, Clinical differences in patients with mitochondriocytopathies due to nuclear versus mitochondrial DNA mutations, Hum. Mutat. 15 (2000) 522–532.
[29] Y.Y. Ma, X.L. Zhang, T.F. Wu, Y.P. Liu, Q. Wang, Y. Zhang, J.Q. Song, Y.J. Wang, Y.L. Yang, Analysis of the mitochondrial complex I–V enzyme activities of peripheral leukocytes in oxidative phosphorylation disorders, J. Child Neurol. 26 (2011) 974–979.
[30] P. Rustin, D. Chretien, T. Bourgeron, B. Gérard, A. Rötig, J.M. Saudubray, A. Munnich, Biochemical and molecular investigations in respiratory chain deficiencies, Clin. Chim. Acta. 228 (1994) 35–51.
[31] M.A. Puchowicz, M.E. Varnes, B.H. Cohen, N.R. Friedman, D.S. Kerr, C.L. Hoppel, Oxidative phosphorylation analysis: assessing the integrated functional activity of human skeletal muscle mitochondria—case studies, Mitochondrion 4 (2004) 377–385.
[32] D. Oglesbee, D. Freedenberg, K.A. Kramer, B.D. Anderson, S.H. Hahn, Normal muscle respiratory chain enzymes can complicate mitochondrial disease diagnosis, Pediatr. Neurol. 35 (2006) 289–292.