Establishing a Reference Interval For Measurement of Flux through the Mitochondrial Fatty Acid Oxidation Pathway in Cultured Skin Fibroblasts, Srinivas B. Narayani,1 Richard L. Boriack,3 Bette Messmer,3 and Michael J. Bennett1,2

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Mitochondrial fatty acid oxidation (FAO) represents a normal metabolic response to increased energy demands during periods of fasting, febrile illness, or muscular exertion. FAO is a complex pathway involving activation of free fatty acids to acyl-CoA species, transport into mitochondria, and cyclic oxidation to break the long-chain fatty acids into acetyl-CoA and a two-carbon chain acyl-CoA thiolase deficiencies, hypoglycemia, liver disease, sudden unexplained infant death, and hypoketotic hypoglycemia. FAO flux is typically measured by monitoring the conversion of radiolabeled fatty acids to carbon dioxide and water. Labeling can be with 14C with final analysis of the rate of production of 14CO2 or with 3H and analysis of production of 3H2O (2, 3). Palmitic acid (C16) is used to detect long-chain defects, including deficiencies in very long chain acyl-CoA dehydrogenase, long-chain 1-3-hydroxy acyl-CoA dehydrogenase, long-chain 3-keto acyl-CoA thiolase, carnitine/acylcarnitine translocase, carnitine palmitoyl transferase 1, and carnitine palmitoyl transferase 2. Myristic acid (C14) is used to detect defects in medium-chain FAO, such as medium-chain acyl-CoA dehydrogenase deficiency. We use tritiated fatty acids labeled in the (9,10) position. The assay will not detect defects in carnitine palmitoyl transferase 1B; short-chain FAO defects, including short-chain acyl-CoA dehydrogenase and short-chain 1-3-hydroxy acyl-CoA dehydrogenase; and short-chain-3-keto acyl-CoA thiolase deficiencies.

Historically, patient samples have been compared with batch controls, and a reference interval was not established for this assay. In the present study we have assigned a reference interval for the assay, using FAO flux results from triplicate assays performed on 1000 fibroblast samples. These samples were derived from individuals with clinical signs indicating impaired energy metabolism but with apparently normal FAO compared with data obtained from patients with well-characterized, genetically confirmed FAO defects. Assigning a reference interval is critical for a clinically reportable assay because it simplifies the interpretation of the results and provides a clear definition of disease vs nondisease.

The FAO flux assay consists of measurement of FAO flux in fibroblasts obtained from one T-25 flask for each patient, typically with fewer than eight passages of culture. Fibroblasts are trypsinized and resuspended in 12–36 mL of DMEM (cat. no. 11885-084; Gibco Life Technologies) containing 1 g/L glucose and supplemented with 100 mL/L fetal calf serum (cat. no. 26140-053; Gibco Life Technologies). From this suspension, 1 mL is transferred to 2 wells in each of five 24-well tissue culture plates. Three nondisease control fibroblast and two disease control fibroblast lines are simultaneously seeded in the same plate along with one to six additional patient lines. Cultures are placed in an incubator to allow the fibroblasts to attach and then grow for 48 h at 37 °C, in 5% CO2, 100% humidity (CO2 incubator; NUAIRE). The cells are then washed with phosphate-buffered saline (pH 6.9) and incubated with preincubation medium (Kreb's Ringer Bicarbonate Medium; cat. no. K-4002; Sigma) containing 0.5 g/L bovine serum albumin (fatty acid free, cat. no. A-6003; Sigma) in the assay plates or with inhibitor medium [preincubation medium + FAO inhibitors consisting of 0.45 mmol/L malonyl-CoA (cat. no. M-4263; Sigma), 0.02 mmol/L glybenclamide (cat. no. G-0639; Sigma), 0.79 mmol/L 3-mercaptopropionopric acid (cat. no. M-6750; Sigma), and 2.74 mmol/L glucose (cat. no. G-5767; Sigma) in a buffer containing 1.0 g/L bovine
After incubation, 50 μL of 1 mol/L NaOH is added to each well of this plate and the plate is incubated at 37 °C, 100% humidity for 2 h. After incubation, 50 μL of 1 mol/L HCl is added to neutralize the samples, and both wells from the same cell line are combined for protein measurement by the Lowry method (4). After preincubation, the medium is removed, and 200 μL of tritiated myristate or palmitate (incubation medium, 110 μmol/L fatty acid, 16.7 μCi/mL [3H]fatty acid, and 0.5 g/L bovine serum albumin in phosphate-buffered saline, pH 6.9) is added to each well of the assay plates and incubated. Blank plates are incubated with [3H]palmitate or [3H]myristate incubation medium containing the FAO inhibitors. This provides a blank reading for nonmitochondrial FAO, which is presumed to be mostly peroxisomal (5–8). The plates are returned to the incubator and incubated at 37 °C in 5% CO2, 100% humidity for 2–3 h, after which the incubation medium is transferred to columns containing ~2.9 mL of Dowex-1 ion-exchange resin (cat. no. 1X8-200; Sigma), which has been charged with 1.0 mol/L NaOH and washed with MilliQ water until the eluate is the same pH as the wash water. This binds the nonmetabolized fatty acids and allows the tritiated water produced by β-oxidation to pass through. The plates are washed with 300 μL of phosphate-buffered saline (pH 6.9), and the wash is passed through the columns, which are subsequently washed with 2 mL of water. The entire eluate is collected in a mini scintillation vial to which 4 mL of scintillation cocktail (Scintiverse II; cat. no. SX 12-4; Fisher Scientific) is added, and the vial is counted in a Beckmann LS 6500 liquid scintillation counter.

The rate of FAO is calculated by the following formula:

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\text{Sample CPM} - \text{Inhibited CPM} \times \frac{\text{Total CPM/fatty acid concentration}}{\text{time, in min} \times \text{mg protein/well}} = x \text{ pmol} \cdot \text{min}^{-1} \cdot \text{(mg protein)}^{-1}
\]

The oxidation rates are compared between nondisease and disease controls. Oxidation rates that are consistently <70% of the nondisease controls in triplicate analyses are flagged as low.

In the present study, we used the FAO values from nondisease control fibroblasts over a period of 10 years to determine reference intervals, which are shown in Fig. 1, A and B. We calculated the central 95% intervals with the EP Evaluator 5 series, using the nonparametric method (NCCLS C28-A) and transformed parametric data. Palmitic and myristic acid oxidation showed nongaussian distribution of data within the 95% confidence interval (Fig. 1, A and B). In the transformed parametric method, the scale was changed to fit the data to a gaussian

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**Fig. 1.** Distribution of palmitate (A) and myristate (B) oxidation in 1000 patient samples, and distribution of the FAO flux results of patients with mutationaly confirmed FAO defects (C).

(A), mean (SD) palmitate concentration, 46 (29) pmol · min−1 · (mg protein)−1 (n = 1000); median, 38 pmol · min−1 · (mg protein)−1. True gaussian distribution is superimposed for comparison with original data. (B), mean (SD) 27 (19) pmol · min−1 · (mg protein)−1 (n = 1000); median, 23 pmol · min−1 · (mg protein)−1. True gaussian distribution is superimposed for comparison with original data. (C), the lower horizontal line of the scatter plot represents 70% of mean normal activity and our arbitrarily defined cutoff value. The upper horizontal line represents 70–80% residual activity and equivelocal results. The numbers of confirmed patients are shown in parentheses below the x axis (see Table 2 in the online Data Supplement). •, palmitate (mean, □); □, myristate (mean, □). CPT1, carnitine palmitoyltransferase 1 deficiency; CPT2, carnitine palmitoyltransferase 2 deficiency; GA2, glutaric acidemia type 2; LCHAD, long-chain ω3-hydroxy acyl-CoA dehydrogenase deficiency; MCAD, medium-chain acyl-CoA dehydrogenase deficiency; SCHAD, short-chain ω3-hydroxy acyl-CoA dehydrogenase deficiency; TFP, trifunctional protein defect; VLCAO, very long chain acyl-CoA dehydrogenase deficiency.
distribution, and the mean (±2 SD) was computed and then converted to the original units. True gaussian distributions are shown superimposed in panels A and B of Fig. 1 for comparison with the original data. Palmitic acid had a mean oxidation rate of 46 pmol min⁻¹ (mg fibroblast protein⁻¹) (range, 15–122 pmol min⁻¹ mg⁻¹) and a median of 38 pmol min⁻¹ mg⁻¹ with a SD of 29 pmol min⁻¹ mg⁻¹. Myristic acid had a mean (range) oxidation rate of 27 (15–85) pmol min⁻¹ (mg protein)⁻¹ and median of 23 pmol min⁻¹ mg⁻¹ with a SD of 18 pmol min⁻¹ mg⁻¹. FAO flux is considered to be abnormal in our laboratory when a patient’s value is <70% of the control value on repeated analysis. We calculated the lower and upper limits of the mean values and 90% confidence intervals, which are shown in Table 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue3/. The cutoffs at which there were deficiencies in FAO (70% of the mean of the lower reference limit) were 16 pmol min⁻¹ (mg protein)⁻¹ for palmitate and 11 pmol min⁻¹ (mg protein)⁻¹ for myristate. These limits would be 11 pmol min⁻¹ (mg protein)⁻¹ for palmitic acid and 8 pmol min⁻¹ (mg protein)⁻¹ for myristic acid. However, to increase the specificity and sensitivity, we decided to use 10 pmol min⁻¹ (mg protein)⁻¹ as the cutoff for both, giving palmitate and myristate oxidation rates with 88% and 90% sensitivity, respectively, for detecting deficiency. Patients who exhibit values between 10 and 15 pmol min⁻¹ (mg protein)⁻¹ (Table 2 in the online Data Supplement) fall into an equivocal zone where interpretation would be unclear and may require an additional analytical technique, such as measurement of metabolite accumulation by tandem mass spectrometry (1, 9, 10).

Data from patients with several well-characterized FAO defects are shown in Fig. 1C. The lower horizontal line represents the chosen cutoff as a percentage of the normal FAO rate, and the upper line indicates the zone in which the results are equivocal. This scatter plot shows that patients with <70% of the normal oxidation rate are clearly identified as abnormal. However, the scattered data points in the range of 70–80% of normal are without a clear indication of a deficiency. These data show a small overlap with the lower end of the reference interval but indicate that most patients would be identified by this reference interval. Several patients with FAO values in the lower end of the reference interval had mutations with high residual activity, which would predict higher flux. For example, the case of carnitine palmitoyltransferase 1 deficiency with the highest residual flux is the patient who was reported to be homozygous for the mild P479L mutation (11) and adult-onset disease.

The major advantage of the FAO flux assay is that it is the only assay that can test the true flux through the FAO pathway, whereas the alternative method using tandem mass spectrometry can accurately verify the exact site of an enzyme defect, making the two approaches complementary. However, in our experience, many patients in whom enzyme activities are within the reference interval have abnormal flux. These patients are currently characterized as having FAO defects of unknown etiology and may have defects of a nonenzymatic nature. The FAO flux assay is the only assay that can diagnose these patients at present.

**References**


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Lysosomal enzymes are crucial for the degradation of numerous macromolecular substrates. Deficiencies of many of the known lysosomal enzyme activities have been associated with different clinical disorders, collectively termed the lysosomal storage diseases (1–4). Accurate measurement of lysosomal enzyme activities, there-