Potential Utility of Plasma Fatty Acid Analysis in the Diagnosis of Cystic Fibrosis

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Background: An altered distribution of fatty acids in cells and tissues is found in patients with cystic fibrosis (CF). In this study, we assessed the potential role of plasma fatty acid analysis in the diagnosis of CF.

Methods: In this 2-part study, we first used gas chromatography–mass spectrometry to analyze fatty acids in plasma from 13 CF patients and 11 controls without CF. We then used the fatty acid distribution data to identify the fatty acids or multiple fatty acid calculations most effective in identifying CF patients. Part 2 of the study was a blinded analysis of 10 CF patients and 9 controls to directly test the effectiveness of the diagnostic parameters for CF identified from the plasma fatty acid analysis.

Results: In the nonblinded trial, the multiplication product of (18:2 n-6)/H11547/22:6 n-3 (each as percentage of total plasma fatty acid) was the most effective indicator for distinguishing patients with CF from controls (P = 0.0003). In part 2 (the blinded trial), this multiplication product was also the most effective indicator for distinguishing CF patients from controls (P = 0.0008).

Conclusions: The product of (18:2 n-6) × (22:6 n-3) is effective for distinguishing CF patients from persons without CF. This diagnostic marker may have value as an alternative to the sweat chloride test in selected patients being evaluated for CF.

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Altered plasma and tissue concentrations of fatty acids in cystic fibrosis (CF) patients have been well described (1–7). Changes in concentrations of selected essential fatty acids have been found in CF patients, notably decreases in the plasma and tissue concentrations of linoleic acid (18:2 n-6) and docosahexaenoic acid (DHA; 22:6 n-3). Increases in the concentration of eicosatrienoic acid (Mead acid; 20:3 n-9) have been identified in several studies (5–7). These alterations in fatty acid concentrations are significantly magnified in patients with severe variations in the CF transmembrane conductance regulator (CFTR) gene, suggesting an association between the basic defect and abnormal essential fatty acid metabolism in CF patients (3).

It is unclear how CFTR variation leads to the dysregulation of fatty acid biosynthesis, but these fatty acid abnormalities are clearly not attributable to pancreatic insufficiency and malabsorption in CF patients (5–6). The metabolism of essential fatty acids involves a metabolic sequence of specific enzymes that desaturate, elongate, or shorten the fatty acids. Although the same enzymes are involved in both the n-3 and n-6 pathways, there is no interconversion of products between the 2 pathways. Thus, these essential fatty acids compete for the same enzymes. A study with CF knockout mice demonstrated that arachidonic acid (20:4 n-6) concentrations are increased and DHA concentrations are decreased in tissues and organs most affected in CF (8). Interestingly, oral administration of DHA was responsible for reversing the phenotypic alterations of CF in these mice (8). Alterations in fatty acids similar to those found in CF knockout mice were shown to be present in CFTR-expressing tissues from human subjects with CF (1).
Because certain alterations in plasma fatty acid composition in CF are highly reproducible, fatty acid analysis may be useful as a diagnostic procedure for CF. The altered fatty acid distributions that we observed in our own studies with CF knockout mice and subsequently with CF patients prompted us to reexamine our database of CF patients to determine whether specific plasma fatty acid changes can be used to differentiate between CF patients and persons not suffering from CF.

The sweat chloride test is the gold standard screening test for CF, but this test has limitations. A subset of patients with CF show values for the sweat chloride test that are not diagnostic, and some CF patients demonstrate a sweat chloride value within reference intervals despite confirmed CF status (9–12). Another limitation for the sweat test is that false-positive values may occur in several clinical conditions readily distinguishable from CF (13). In rare cases, some homozygous CF patients have sweat chloride concentrations within reference intervals if a second ameliorating or neutralizing variation in the CFTR gene product, such as R553Q, is also present (13).

Nasal potential difference measurement is also used as a diagnostic procedure in the evaluation for CF (14, 15). An increased nasal potential difference is strong evidence for CF, but a value within reference intervals does not exclude the diagnosis (16). False-negative results may arise, especially in the presence of a nasal polyp (17).

To confirm a diagnosis of CF, an increased nasal potential difference should be confirmed by measurement of plasma fatty acids. Table 2 shows the mean (SD) plasma fatty acids of CF patients and healthy controls. The ratios of fatty acids were compared using the Student’s t-test. The results indicate that certain fatty acid ratios are significantly different between CF patients and controls. The detailed statistical analysis is provided in the supplementary material.
difference must be demonstrated twice. The nasal potentia
difference measurement is a technically challenging proce-
dure (13).

These limitations of existing methods led us to pursue an alternative screening test involving plasma fatty acid
analysis to confirm the diagnosis of CF in patients with 
questionable sweat test results and positive clinical find-
ings for CF.

**Materials and Methods**

**Enrollment of Patients**

The nonblinded study was conducted from October 2000 to September 2003, and the blinded study was conducted from September 2004 to August 2005. CF patients attending the University of Massachusetts Medical Center and Beth Israel Deaconess Medical Center were included in the studies. Reference values were obtained from healthy controls recruited at both sites. Exclusion criteria for the controls included findings consistent with the presence of CF, the use of drugs that affect fatty acid metabolism, or a family history of CF. All study patients had pancreatic insufficiency and met the criteria for CF diagnosis (18). All study participants provided written informed consent before enrollment. Participants were randomly selected for part 2 (blinded) of the study. In the nonblinded and blinded studies, the CF patients, but not the controls, were encouraged to eat high-fat, high-energy diets and to visit a nutritionist regularly, in accordance with the guidelines of the Cystic Fibrosis Foundation (19). The nonblinded study included 13 patients with CF [mean (SD) age, 22.1 (7.5) years; range, 15–41 years] and 11 healthy controls [26.5 (7.2) years; range, 19–33 years]. The blinded study included 10 patients with CF [16.2 (5.6) years; range, 7–22 years] and 9

controls [46 (11.2) years; range, 30–62 years]; patients and controls were selected after we reviewed medical records.

**Plasma Fatty Acid Isolation and Methylation**

We obtained peripheral venous blood from nonfasting participants. Samples were collected in heparin-contain-
ing vacuum tubes and centrifuged at 45 g for 15 min at room temperature. Thereafter, the plasma was removed. Fatty acids from plasma were isolated and methylated according to Moser and Moser (20). The fatty acid methyl ester (FAME) mixture was analyzed by gas chromatography–mass spectrometry (GC-MS).

**GC-MS FAME Identification and Quantification**

GC-MS analysis was performed on a Hewlett-Packard Series II 5890 gas chromatograph coupled to an HP-5971 mass spectrometer (Agilent Technologies) equipped with a Supelcowax SP-10 capillary column (Supelco). The oven temperature was maintained at 150 °C for 2 min, ramped at 10 °C/min to 200 °C and held for 4 min, ramped again at 5 °C/min to 240 °C and held for 3 min, and then finally ramped to 270 °C at 10 °C/min and maintained for 5 min. The injector and detector were maintained at 260 °C and 280 °C, respectively. Carrier gas flow rate was maintained at a constant 0.8 mL/min throughout. Total ion monitor-
ing was performed, encompassing mass ranges from 50 to 550 atomic mass units. Peak identification was based on comparison of both retention time and mass spectra of the unknown peak to those of known standards within the GC-MS database library. A commercially available standard mixture of FAMEs (Nucheck) was used to calculate masses of fatty acids based on the response factor of 17:0 FAME (21).
Three samples from the control group were excluded from part 1 of the study because the fatty acid profile indicated significant loss of fatty acids in the processing of the samples. In the excluded samples, the normally prominent fatty acids had areas that were 3–10-fold below the area of the internal standard. Accepted samples were those in which the most prominent fatty acids were 3–6-fold greater in peak area than the internal standard. In part 2 of the study, all specimens were analyzed in a blinded fashion.

**Statistical Analysis**

Plasma fatty acid concentrations of CF patients and non-CF controls were compared. The unpaired Student *t*-test was used to evaluate differences between the means of the 2 groups. Differences were considered statistically significant at *P* ≤ 0.05.

**Results**

The demographic and genotypic characteristics of the CF patients who were evaluated with plasma fatty acid analysis are shown in Table 1. A genotype consistent with the diagnosis of CF obviated the need for sweat testing in some patients. One of the CF patients from the blinded trial had a genotype that was consistent with a negative sweat-chloride test value.

Eleven different plasma fatty acid markers for differentiating CF patients from controls, including (18:2 n-6) × (22:6 n-3), were tested in the nonblinded trial (Table 2). There were significant differences between CF patients and controls for the multiplication product of (18:2 n-6) × (22:6 n-3; *P* = 0.0003), the ratio of (22:5 n-6):(22:6 n-3; *P* = 0.001), and 22:6 n-3 alone (*P* = 0.007). The multiplication product of (18:2 n-6) × (22:6 n-3; each as percentage of total plasma fatty acid) most effectively distinguished patients with CF from controls (Fig. 1).

In the blinded trial, we tested 11 different plasma fatty acid markers (Table 2). Again, the multiplication product of (18:2 n-6) × (22:6 n-3) was the most statistically significant parameter in distinguishing CF patients from controls (*P* = 0.0008; Fig. 1).

The multiplication product of (18:2 n-6) × (22:6 n-3) in the nonblinded trial of our study showed sensitivity, specificity, positive predictive value, and negative predictive value of 92%, 91%, 92%, and 91%, respectively, for a cutoff of 40 arbitrary units compared with 100%, 56%, 71%, and 100% for the blinded trial with the same cutoff of 40 arbitrary units. Combined data from the blinded and nonblinded trials showed values for sensitivity, specificity, positive predictive value, and negative predictive value of 96%, 75%, 81%, and 94%, respectively, for the same cutoff of 40 arbitrary units. In addition, there were no technical limitations associated with sample collection. The sensitivity of the assay for detection of CF was very high, and false positives were definitively identified as CF or non-CF by genetic testing.

**Discussion**

Our data demonstrate that a multiplication product of plasma (18:2 n-6) × (22:6 n-3) can be used to differentiate CF patients and non-CF controls in the majority of cases. This multiplication product was the most effective parameter in measuring plasma fatty acid status between CF patients and controls, and as a diagnostic marker, it provided a higher level of statistical significance than any other mathematical operations or clinical markers tested.

In a study by Benabdeslam et al. (22), plasma phospholipid fatty acid analysis was performed with fasting blood samples collected from 65 CF patients and 39 controls, whereas in our study, both the blinded and nonblinded trials were performed with samples collected from nonfasting CF patients and controls, a procedural difference that may slightly alter plasma fatty acid composition. Other investigators have shown, however, that it is unlikely that total plasma fatty acid composition is significantly altered by a fasting period (23). The data in Fig. 2 show 3 paired comparisons between a CF group and a control group, including comparison of data from total plasma fatty acid analysis in CF patients and controls using our data and data from the study by Benabdeslam et al. (22). The (18:2 n-6) × (22:6 n-3) value separates CF patients from non-CF controls in all 3 paired comparisons. The absolute values for (18:2 n-6) × (22:6 n-3) are very different, however, especially for the control groups. In the current study, we used total plasma fatty acids because samples do not require additional processing to isolate phospholipids from total fatty acids. Thus, our method simplifies sample preparation for clinical use. In the 2 studies involving total fatty acids, although the control groups were markedly different, the CF groups were very similar. The control group for the blinded trial showed a lower DHA concentration than the control group for the nonblinded trial (Fig. 2), the major difference between the 2 control groups. This finding may be

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**Table 3. Diagnostic sensitivity and specificity of sweat chloride test and of (18:2 n-6) × (22:6 n-3).**

<table>
<thead>
<tr>
<th>Diagnostic Tests</th>
<th>Technical Failure Rate</th>
<th>Diagnostic Sensitivity</th>
<th>Diagnostic Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweat test, % [Mastella et al. (24)]</td>
<td>3.6</td>
<td>91</td>
<td>100</td>
</tr>
<tr>
<td>Sweat test, % [Warwick et al. (25)]</td>
<td>14</td>
<td>93</td>
<td>99</td>
</tr>
<tr>
<td>Nonblinded analysis, % (18:2 n-6) × (22:6 n-3)</td>
<td>Negligible</td>
<td>92</td>
<td>91</td>
</tr>
<tr>
<td>Blinded analysis, % (18:2 n-6) × (22:6 n-3)</td>
<td>Negligible</td>
<td>100</td>
<td>56</td>
</tr>
<tr>
<td>Blinded + nonblinded analysis, % (18:2 n-6) × (22:6 n-3)</td>
<td>Negligible</td>
<td>96</td>
<td>75</td>
</tr>
</tbody>
</table>

*Cutoff value for fatty acid analyses is ≤40 arbitrary units*
attributable to lower fish consumption or fish oil supplementation in the blinded trial than the nonblinded trial control groups, both of which were randomly selected. Because total fatty acid values were very similar in both CF populations, a value ≤40 could be used as a clinical cutoff for CF; therefore, patients with a value ≤40 should undergo follow-up genetic studies. In the blinded and nonblinded trials, use of a cutoff of 40 would have resulted in a genetic study for 4 controls and 1 control, respectively. The rationale for this approach is analogous to the protocol for the HIV ELISA screening test, which is followed up by a Western blot test for confirmation, with the goal of 100% sensitivity in the screening study.

To determine whether plasma fatty acid analysis can help in the evaluation of CF patients, we compared the sensitivity, specificity, positive predictive value, and negative predictive value of the sweat chloride test reported in 2 different published studies (24, 25) with the most favorable diagnostic fatty acid marker in our study (Table 3). The technical failure rate of plasma fatty acid analysis as a diagnostic test for CF is negligible because blood samples are readily collected.

The Gibson–Cooke Sweat Test (GCST) is the standard technique used in the diagnosis of CF. Two previous published studies (24, 25) compared the results of the GCST technique with results obtained using different

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Fig. 3. A diagnostic algorithm illustrating a suggested evaluation of patients with CF, including fatty acid analysis as a complementary and/or alternative test to the sweat chloride test in patients with CF (28).

* Sweat test results must be >80 to be abnormal in infants. ** Sweat test results must be <40 to be normal in infants.
assays for sweat chloride collection and measurement. The 1st study (24) used a cutoff value of 70 mmol/L to differentiate between intermediate and abnormal sweat chloride test results rather than the cutoff value of 60 mmol/L used by the Cystic Fibrosis Foundation (26). In the Mastella et al. study (24), 3.6% of the samples did not contain enough sweat to perform a sweat chloride analysis. Among the CF patients, sweat chloride concentrations were outside the reference interval in 91.2%, within the reference interval in 1%, and borderline in 7.8%. Among healthy controls, 4% had borderline sweat chloride concentrations (24). In this study (24), the sensitivity and the specificity for the GCST were 91% and 100%, respectively. In the 2nd study (25) the GCST could not be performed on 15% of the CF patients because these patients failed to produce enough sweat for analysis. The sensitivity and the specificity values for the GCST were 93% and 99%, respectively, for this study (25).

Despite the high sensitivity and specificity of the sweat chloride gold standard diagnostic screening test, practical difficulties limit the performance of the sweat test, particularly in infants younger than 4 weeks (27). In cases in which sweat testing is technically not possible or is clinically misleading, plasma fatty acid analysis as a screening test for CF may be useful, as proposed in Fig. 3.

Although the sensitivity values of plasma fatty acid analysis support its utility as a diagnostic test for patients with CF, this analysis requires a relatively sophisticated assay involving gas chromatography to generate the required fatty acid profile. Because the fatty acids of interest are predominant in the plasma, it is likely that the fatty acid analysis can be performed with a gas chromatograph with a standard flame ionization detector and may not require a mass spectrometer, as was used in our study. We reported plasma fatty acid concentrations as percent total fatty acids because mole percent or gram percent data are much simpler to obtain than are amounts of the individual fatty acids in micrograms of fatty acid per milliliter of plasma. When actual amounts in mass are required, meticulous attention to fatty acid recovery for the individual fatty acids is necessary. With variable loss of fatty acids between specimens, the mole or gram percentage of total fatty acids stays the same. The simplicity of using mole percentages is important for fatty acid analyses performed in a clinical laboratory.

In summary, fatty acid analysis is not a substitute for sweat testing, but it may be a useful test for CF when the sweat chloride test does not provide a definitive answer at the screening level. Our findings demonstrate that the sweat chloride test does not provide a definitive answer at sweat testing, but it may be a useful test for CF when the GCST could not be performed. In cases in which sweat testing is technically not possible or is clinically misleading, plasma fatty acid analysis as a screening test for CF may be useful, as proposed in Fig. 3.

References