Metabolic handling of $^{13}$C labelled tripalmitin in healthy controls and patients with cystic fibrosis

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Abstract

**Aim**—To examine the gastrointestinal handling and metabolic disposal of emulsified [$^{1-13}$C]palmitic acid esterified into a triglyceride in nine healthy children and seven patients with cystic fibrosis on enzyme replacement treatment.

**Methods**—After an overnight fast, each child was given 10 mg/kg body weight [1,1,1-$^{13}$C]tripalmitin with a standardised test meal of low natural $^{13}$C abundance. The total enrichment of $^{13}$C was measured using isotope ratio mass spectrometry in stool collected for a period of up to five days and in breath samples collected over a 24 hour period.

**Results**—The mean proportion of administered $^{13}$C label excreted in stool was 6% (range, 1–12.7%) in healthy children and 24.6% (range, 0–64%) in patients with cystic fibrosis. Healthy children excreted 31.3% of the administered label on their breath (range, 14.2–42.9%). Correcting the excretion of administered $^{13}$C label on the breath for differences in digestion and absorption in patients with cystic fibrosis increased the difference between individuals from 0–31.3% of administered dose (mean, 17.9%) to 0–49.1% of absorbed dose (mean, 23.2%) and was poorly related to the amount of $^{13}$C label in stool.

**Conclusion**—Measurements of breath $^{13}$CO$_2$ do not consistently reflect the gastrointestinal handling of emulsified $^{13}$C labelled tripalmitin because of differences in digestion and absorption in cystic fibrosis. Further studies need to examine whether “breath tests” alone can predict with confidence the gastrointestinal handling of other $^{13}$C labelled triglycerides and fatty acids.

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Keywords: fatty acids; absorption; metabolism; stable isotopes; cystic fibrosis

Measurements of total stool lipid made during gross balance studies provide a gross measure of the availability of dietary lipid in cystic fibrosis. Modern developments in mass spectrometry in combination with stable isotopes provide the opportunity to examine the functional capacity of the gastrointestinal tract to handle specific triglycerides found in the diet. Because palmitic acid is the predominant saturated fatty acid in the UK diet, previously we examined the absorptive capacity of the gut using $^{13}$C labelled palmitic acid in healthy children and patients with cystic fibrosis by measuring total excretion of the $^{13}$C label in stools. Despite raised total stool lipid losses, more of the $^{13}$C labelled palmitic acid was absorbed in this group of patients with cystic fibrosis compared with healthy children (94% v 76%, respectively), suggesting that there is not a specific malabsorptive defect of labelled palmitic acid in cystic fibrosis when ingested as the free acid. We also showed that palmitic acid was the major stool fatty acid in those stools with the highest $^{13}$C enrichment and that, primarily, the $^{13}$C label was restricted to the species consumed by the subjects (that is, palmitic acid).

Using different $^{13}$C labelled triglycerides and fatty acids, maldigestion and malabsorption have been assessed by measuring the excretion of label on the breath alone. Such studies have not directly determined the excretion of label in the stool, assuming that differences in the excretion of label on the breath are determined wholly by differences in digestion within the gastrointestinal tract. A further theoretical limitation of using breath tests is the implicit assumption that alterations in lipid malabsorption and/or malabsorption do not affect fatty acid oxidation. We are not aware of any studies in cystic fibrosis that have measured directly the extent of digestion and absorption in combination with “breath tests” using $^{13}$C labelled lipids. To address this issue, we have examined both the gastrointestinal handling and the subsequent metabolism of emulsified [1–$^{13}$C]palmitic acid esterified into a triglyceride in healthy children and patients with cystic fibrosis by measuring excretion of the label in stools and on the breath as $^{13}$CO$_2$.

Subjects and methods

Seven patients with cystic fibrosis (three boys and four girls) aged 4–11 years (mean, 8.0 years) from the cystic fibrosis clinic at Southampton University Hospitals NHS Trust were studied while on their normal habitual pancreatic enzyme replacement treatment. Patients with small bowel resection or other known factors that might limit absorption (for example Crohn’s disease) were excluded from the study. The cystic fibrosis patients were taking between 13 462 and 34 000 IU lipase/kg body weight/day (mean, 18 877); an enzyme dosage achieved by self-titrination against gastrointestinal symptoms and bowel habit. No attempt was made to alter or intervene with the management of the pancreatic enzyme replacement treatment. Nine healthy children (six boys and three girls) aged 5–8 years (mean, 7.2 years) from local schools also participated in the study. Informed consent was obtained from all of the subjects and the study protocol was...
approved by the Ethical Committee of Southampton and South West Hampshire Health Commission.

After an overnight fast, the [1,1,1-\textsuperscript{13}C]tripalmitin was administered orally to the subjects at a dose of 10 mg/kg body weight (99 atom% excess; Masstrace, Woburn, USA) as part of a controlled standard test meal consisting of 120 g white bread, 100 g orange juice, and 10 g butter of low \textsuperscript{13}C abundance (~25.5‰). \textsuperscript{8} The patients with cystic fibrosis took the same amount of enzymes with the test meal that they would usually take with a snack meal and this ranged from 1458 to 6849 IU lipase/kg body weight/day (mean, 3750). The \textsuperscript{13}C labelled tripalmitin was given as a glucose-sucrose-casein emulsion, using a modification of Emken et al.,\textsuperscript{9} because emulsification before administration overcomes the problems associated with the physicochemical properties of the crystalline form of this label, which might otherwise result in poor digestion and absorption if given in a free, unemulsified form.\textsuperscript{10}

Breath samples were collected using breath collection bags (Quintron) before consuming the test meal to provide a measure of baseline \textsuperscript{13}C excretion on the breath, and were then collected at hourly intervals for a period of at least six hours and then after eight hours, 10 hours, and 24 hours. Specimen breath samples were transferred into evacuated gas sample containers (Exetainers; Isochem, Finchampstead, UK) for analysis in duplicate. All subjects were rested for the duration of the tests and were given constant supervision at the Clinical Nutrition and Metabolism Unit at Southampton General Hospital. No additional food or drink was permitted during the initial six hour period except for bottled mineral water. After six hours, the subjects ate a meal without any foods naturally enriched for \textsuperscript{13}C. Whole body CO\textsubscript{2} excretion was measured by indirect calorimetry (GEM; Europa Scientific Ltd, Crewe, UK) for a period of 15 minutes at the same time points as breath sampling over the first six hours to calculate the amount of label excreted each hour. It was not possible to measure CO\textsubscript{2} excretion at eight, 10, and 24 hours. The value for CO\textsubscript{2} excretion at six hours was used to calculate the amount of label excreted at eight and 10 hours. The proportion of label excreted on the breath at 24 hours was calculated using the baseline value for CO\textsubscript{2} excretion.

A stool sample was collected on the day before the labelled test meal to measure baseline \textsuperscript{13}C excretion. Thereafter, all stools passed were collected and processed individually for a period of up to five days. All the stool collections were carried out at home and monitored daily throughout the study period. All of the subjects and parents of children were cooperative, willing, and capable of following the details of the procedure. At no time was difficulty encountered nor was there any need for modification of the procedure to accommodate any of the subjects. All the stool collections were complete. The subjects were also instructed to avoid eating naturally \textsuperscript{13}C enriched foods (such as corn or maize based products and cane sugar) for at least two days before and during the stool collection period. None of the animal source protein foods eaten (such as eggs and meat) were naturally enriched with \textsuperscript{13}C.

**Breath and Stool Analyses**

The methodology for processing stools has been described previously.\textsuperscript{6} Enrichment of \textsuperscript{13}C in the stool and as \textsuperscript{13}CO\textsubscript{2} on the breath was analysed by continuous flow isotope ratio mass spectrometry (ANCA-NT GSL; Europa Scientific Ltd). Total stool lipid was extracted from stools by a modification of the method of Folch and colleagues.\textsuperscript{11} The \textsuperscript{13}C enrichment of samples of breath \textsuperscript{13}CO\textsubscript{2} and stool was expressed as the “per ml relative difference from the reference standard Pee Dee Belemnite (PDB)” as defined by Craig (\textsuperscript{13}C\textsubscript{PDB},‰).\textsuperscript{12} The proportion of administered \textsuperscript{13}C label in the stools was calculated according to the formula presented by Schoeller and colleagues.\textsuperscript{13} The apparent absorption of the \textsuperscript{13}C label was determined from the difference between the amount of label administered and that excreted in the stool.

The proportion of \textsuperscript{13}C label excreted on the breath as \textsuperscript{13}CO\textsubscript{2} was expressed as a percentage of absorbed \textsuperscript{13}C label each hour and as the cumulative percentage dose excreted over 24 hours, according to the formula presented by Watkins et al.\textsuperscript{4}

**Statistics**

The results are reported as mean (SD). Statistical comparisons between the data were performed using the unpaired Student’s \textit{t} test. Differences between means were considered significant when \(p < 0.05\). Associations between variables were tested by the Pearson product moment correlation coefficient (\(R\)).

**Results**

In healthy children, 6.0% (3.7%) of the administered \textsuperscript{13}C label was excreted in the stool (range, 1–12.7%) (table 1). In contrast, patients with cystic fibrosis excreted more \textsuperscript{13}C label in the stool: 24.6% (22.3%) of the administered \textsuperscript{13}C label was found in the stool, although there was a wide range (range, 0–64%; \(p > 0.05\); table 1). Usually, the stools with the greatest \textsuperscript{13}C enrichment were found on the day after the test meal and enrichment returned to baseline values by day 3 or 4. In patients with cystic fibrosis, excretion of total lipid in the stool was four times greater than that seen in healthy children (13.2 g/day (3.1) v 2.95 g/day (1.0); \(p < 0.001\)). Analysing both groups together, there was only a weak association between the amount of \textsuperscript{13}C label and the total lipid in the stool (\(R = 0.53\); \(p > 0.09\)).

In both groups, excretion of the label on the breath as \textsuperscript{13}CO\textsubscript{2} peaked between one and six hours after the test meal, returning to baseline values in almost all subjects by 24 hours. In one of the healthy children and one cystic fibrosis patient, \textsuperscript{13}C enrichment on the breath was only 2 SD units greater by 24 hours than that observed at baseline. In healthy children, the proportion of administered \textsuperscript{13}C label excreted on the breath was 31.3% (10.6%) (range,
Table 1  Excretion of $^{13}$C in stool and on breath as $^{13}$CO$_2$ in healthy children and patients with cystic fibrosis

<table>
<thead>
<tr>
<th>Subject</th>
<th>Stool $^{13}$C (% adm. dose)</th>
<th>Breath $^{13}$CO$_2$ (% adm. dose)</th>
<th>Breath $^{13}$CO$_2$ (% absorbed dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy children</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>6.6</td>
<td>31.4</td>
<td>33.7</td>
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<td>2</td>
<td>8.1</td>
<td>14.2</td>
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</tr>
<tr>
<td>Mean (SD)</td>
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<td>31.3 (10.6)</td>
<td>33.1 (10.9)</td>
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<td>Cystic fibrosis</td>
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<tr>
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<td>64.4</td>
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<td>36.5</td>
<td>31.3</td>
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<td>4.2</td>
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</tr>
<tr>
<td>Mean (SD)</td>
<td>24.6 (22.3)</td>
<td>17.9 (9.5)</td>
<td>23.2 (15.2)</td>
</tr>
<tr>
<td>Significance</td>
<td>p &lt; 0.05</td>
<td>p &lt; 0.05</td>
<td>p &gt; 0.05</td>
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</table>

14.2–42.9%), whereas patients with cystic fibrosis excreted significantly less $^{13}$C label on the breath (17.9% (9.5%); range, 0–31.3%; p < 0.05). In healthy children, the proportion of $^{13}$C label excreted on the breath was not altered if expressed directly as $^{13}$C label administered rather than as label made available through digestion and absorption (33.1% (10.9%); range 15.5–43.3%; table 1). However, correcting excretion of the label on the breath for differences in digestion and absorption in patients with cystic fibrosis increased the differences seen between individuals (23.2% (15.2%) of absorbed label; range, 0–49.1%; p > 0.05). There was no association between the amount of $^{13}$C label in the stool and proportion of absorbed $^{13}$C label excreted on the breath (R = −0.15; p > 0.75).

Discussion

Our study aimed to examine both the gastrointestinal handling and metabolism of emulsified [1-$^{13}$C]palmitic acid esterified into a triglyceride in healthy children and patients with cystic fibrosis by measuring excretion of the label in the stool and on the breath as $^{13}$CO$_2$. This is the first time that $^{13}$C labelled tripalmitin, orally administered as an emulsion, has been used to measure the digestion and absorption of dietary triglycerides in healthy children and patients with cystic fibrosis on habitual pancreatic enzyme replacement treatment by measuring directly the excretion of $^{13}$C label in the stool. In healthy children, only a small proportion of the $^{13}$C label was excreted in the stool, which is consistent with total stool lipid losses within the normal range of < 5 g/day. Almost a quarter of the $^{13}$C label appeared in stools from patients with cystic fibrosis, with as much as 64% in one child, and there were substantially greater amounts of lipid in the stool when compared with healthy children. The differences seen in excretion of the $^{13}$C label in the stool between the patients with cystic fibrosis could be a result of poor digestion of the labelled triglyceride, which limits subsequent absorption. Alternatively, or in addition, the labelled products of digestion could have been malabsorbed because of a failure in the absorptive capacity of the gastrointestinal tract. These observations are in contrast to those shown previously by our group using [1-$^{13}$C]palmitic acid presented as the free acid, where more of the label was absorbed in patients with cystic fibrosis compared with healthy children. It might be that these differences reflect the physicochemical properties of the crystalline form of saturated fatty acids, which appear to favour enhanced absorption in patients with cystic fibrosis compared with healthy subjects when the fatty acid is given as the free acid.

A previous study has shown that doubling the dose of enzymes could increase $^{13}$C recovery on the breath after oral administration of a mixed triglyceride, which is assumed to reflect a reduction in excretion of the label in the stool. In our study, the habitual dose of enzymes was taken with the test meal, a dose that might not have been optimal for this particular meal, and this could have contributed to poor digestion of the labelled tripalmitin. Owing to concern over the adverse effects of high doses of pancreatic enzyme on the large intestine, any increase in the intake of pancreatic enzymes would be supported by further studies examining the nature of species bearing the $^{13}$C label to discriminate between maldigestion and malabsorption. The appearance of the label in the stool predominantly as a triglyceride would indicate a failure of digestion (impaired lipase activity arising from pancreatic insufficiency, or solubilisation, or poor pancreatic enzyme replacement treatment management), while appearance of the label as free fatty acid, 2-monoacylglycerol, or as soaps would indicate a failure in absorption of hydrolysed triglyceride.

Our study revealed that $^{13}$C labelled tripalmitin was not oxidised to the same extent in both groups, whether it was expressed as a proportion of the $^{13}$C label administered or the proportion of that absorbed. This could be examined in two ways. First, the value of characterising the extent of digestion and absorption in cystic fibrosis using emulsified $^{13}$C labelled tripalmitin in breath tests needs to be considered. While patients with cystic fibrosis excreted more $^{13}$C label in their stools, recovery of the label on their breath was lower than in healthy children, although there was some overlap between the two groups. For example, in patient 1 (table 1), although excretion of the $^{13}$C label in the stool was raised, no label was detected in the breath; other patients not only had raised stool losses but also a greater recovery of the label on the breath, comparable to healthy children (patients 3 and 5; table 1). This suggests that in some individuals emulsified $^{13}$C labelled tripalmitin does not sufficiently reflect the processes involved in the digestion and absorption of dietary long chain saturated triglycerides. However, it should be noted that measurements of $^{13}$C label in the stool have not been reported for other $^{13}$C labelled mixed triglycerides and medium chain triglycerides used in breath tests. The other consideration is the extent of recovery of the...
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absorbed label. In both groups, there was a large variability between subjects in the proportion of absorbed $^{13}$C label recovered on the breath. Having corrected for differences in digestion and absorption, it is not clear what other factors might account for this observation. The metabolic competence of an individual influenced by either genetic, nutritional, or lifestyle factors would be important determinants of the partitioning and subsequent oxidation of $^{13}$C labelled fatty acids. Other factors such as physical activity might influence the extent of breath $^{13}$CO$_2$ recovery, particularly when performed throughout the postprandial period, although it is unlikely that they contributed much towards the differences between the subjects in our study. All subjects were measured under similar resting conditions and avoided eating and drinking for at least 12 hours before label administration and for six hours during the period of the breath test. The subjects also avoided any foods naturally enriched for $^{13}$C over the period of the study.

Previous studies have shown that not all of the CO$_2$ generated from the label would have been excreted as breath $^{13}$CO$_2$. A proportion of the $^{13}$CO$_2$ formed from the oxidation of labelled substrate is not excreted directly on the breath but might be retained in the body pools or excreted via the urine, skin, or stool.

Previous studies have either presented uncorrected data or have used correction factors to predict more accurately the extent of oxidation of labelled substrates. Precisely how much of the $^{13}$CO$_2$ is retained in the body pools remains unresolved, with estimates ranging from 10% to 50% in normal individuals. Because there is no specific information on this issue in patients with cystic fibrosis, we have not attempted to correct the oxidation data. It is possible that the $^{13}$C labelled products of maldigestion and malabsorption provide a substrate for colonic bacterial metabolism. However, little is known about the extent to which fermentation of fatty acids by colonic bacteria influences measurements of $^{13}$C recovery on the breath. Fermentation of these labelled products might result in the excretion of the label as flatus gases (such as methane or carbon dioxide), which would result in an underestimation of oxidation. Alternatively, any such $^{13}$C labelled carbon dioxide from fermentation might be absorbed across the colonic mucosa and could contribute to the amount of $^{13}$CO$_2$ excreted on the breath. The potential contribution made by colonic bacterial fermentation of $^{13}$C labelled lipids to differences in the recovery of $^{13}$C on the breath requires further study.

In conclusion, after oral administration of emulsified $^{13}$C tripalmitin, patients with cystic fibrosis excreted more of the $^{13}$C label in the stool when compared with healthy children, although a wide difference in excretion was observed between these patients. This suggests that in cystic fibrosis there are differences in the functional capacity of the gastrointestinal tract to handle saturated fatty acids when esterified into triglycerides, despite pancreatic enzyme replacement therapy. It should not be assumed that excretion of the label on the breath reflects the gastrointestinal handling of emulsified $^{13}$C labelled tripalmitin. Further studies are needed to examine whether breath tests alone can predict with confidence the extent of digestion and absorption in cystic fibrosis of other $^{13}$C labelled triglycerides and fatty acids.

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