Phosphatidylcholine and lysophosphatidylcholine excretion is increased in children with cystic fibrosis and is associated with plasma homocysteine, S-adenosylhomocysteine, and S-adenosylmethionine

Alice H Chen, Sheila M Innis, A George F Davidson, and S Jill James

ABSTRACT
Background: Hepatic steatosis and fat malabsorption are common in cystic fibrosis (CF). Choline deficiency results in decreased phosphatidylcholine synthesis through the cytidine diphosphocholine–choline pathway and hepatic steatosis and in increased synthesis of phosphatidylcholine from phosphatidylethanolamine using methyl groups from S-adenosylmethionine. The intestinal absorption of phosphatidylcholine in CF is unknown.

Objectives: The objective was to determine whether excretion of choline phosphoglyceride (phosphatidylcholine and lysophosphatidylcholine) is increased in CF and whether loss of fecal choline phosphoglyceride is associated with altered plasma methionine cycle metabolites.

Design: A cross-sectional study involved 53 children with CF and 18 control children without CF. Blood was collected from all participants. A subset of 18 children with CF and 8 control children provided 72-hr fecal samples and 5-day food records.

Results: Fat absorption was significantly lower (x ± SEM: 86.2 ± 1.6% and 94.1 ± 1.2%) and excretion of fecal fat (12.9 ± 1.7 and 3.9 ± 0.7 g/d), phospholipid (median: 130 and 47.7 mg/d), phosphatidylcholine (19.6 and 2.1 mg/d), and lysophosphatidylcholine (60.3 and 16.9 mg/d) was significantly higher in children with CF than in control children, respectively (P < 0.05). Choline phosphoglyceride excretion was positively correlated with plasma homocysteine and S-adenosylhomocysteine and inversely related with plasma methionine (P < 0.05).

Conclusions: Choline phosphoglyceride excretion is increased in children with CF and is associated with decreased plasma methionine and increased homocysteine and S-adenosylhomocysteine. These findings suggest choline depletion and an increased choline synthesis by S-adenosylmethionine–dependent methylation in CF, as well as a metabolic link between phosphatidylcholine metabolism and the methionine-homocysteine cycle in humans.

KEY WORDS Cystic fibrosis, steatorrhea, pancreatic insufficiency, phosphatidylcholine, lysophosphatidylcholine, fecal phospholipids

INTRODUCTION
Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutation in the CF transmembrane conductance regulator, an integral membrane protein that, when activated by cyclic AMP and protein kinase A, opens to form a channel to allow chloride ions to enter the epithelial cell (1). Impaired exocrine pancreatic function, which includes reduced secretion of lipase, colipase, phospholipase A2 (PLA2), and sodium bicarbonate, results in malabsorption of nutrients and is present in 85–90% of patients with CF (2–4). Despite pancreatic enzyme replacements, patients with CF continue to show fat malabsorption (5–7). Decreased bile salt concentrations, gastric acid hypersecretion, low intraluminal pH, and decreased mucosal absorption have been suggested to contribute to the decreased fat absorption in CF (6, 8–12).

Hepatic steatosis has been extensively described in CF (13, 14), although the incidence is uncertain because the presence of hepatic steatosis is not often diagnosed without clinically manifest problems. In addition to steatosis, significant progressive liver disease, which may include cirrhosis and fibrosis, was estimated to affect 17–37% of patients with CF (13, 14). The incidence of hepatic steatosis in children with CF as determined by a biopsy of liver tissue was estimated at 14–23% (14). Although several hypotheses, including carnitine and essential fatty acid deficiency, have been proposed (15–17), the cause of steatosis and its relation to the defective epithelial cell chloride ion transport in CF are unclear. Fatty infiltration of the liver, however, is a characteristic feature of choline deficiency (18, 19) and is believed to be due to the lack of de novo phosphatidylcholine synthesis that is required for secretion of triacylglycerol from the liver in apolipoprotein B (apo B)–containing VLDL (20). The principal pathway for phosphatidylcholine synthesis in humans

1 From the Department of Paediatrics and the Nutrition Research Program, University of British Columbia, Vancouver, Canada (AHC, SMI, and AGFD), and the Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock (SJJ).
2 Supported by a grant from the Hospital for Sick Children Foundation, Toronto, and by studentships from the Natural Sciences and Engineering Research Council of Canada and the Michael Smith Foundation for Health Research (to AC).
3 Address reprint requests to SM Innis, BC Research Institute for Children’s and Women’s Health, Department of Paediatrics, University of British Columbia, 950 West 28th Avenue, Vancouver, BC V5Z 4H4, Canada. E-mail: sinnis@interchange.ubc.ca.
Received May 20, 2004.
Accepted for publication November 2, 2004.

PHOSPHATIDYLCHOLINE AND HOMOCYSTEINE IN CF

687

is the cytidine diphosphocholine (CDP) pathway that requires preformed coline (18, 21). In the alternative phosphatidyl-
ethanolamine-N-methyl transferase (PEMT) pathway, methyl
groups from methionine are transferred by S-adenosylmeth-
ionine (SAM) to phosphatidylethanolamine to form phosphati-
dylcholine (20). The other product of the PEMT pathway is 
S-adenosylhomocysteine (SAH), which is subsequently con-
verted to homocysteine. During choline deficiency, the liver 
increases the synthesis of phosphatidylcholine through the 
PEMT pathway (22). We recently showed that elevated plasma 
homeocysteine and SAH concentrations were inversely related to 
plasma phospholipid phosphatidylcholine in children with CF 
(23); this provided evidence of possible insufficient choline to 
support phosphatidylcholine synthesis through the CDP-choline 
pathway.

The average intake of phosphatidylcholine is \( \approx 1 \) g/d, and this amount represents \( \approx 90\% \) of the total intake of choline (24). Large amounts of phosphatidylcholine are also secreted into the intestine in bile. In normal persons, the enterohepatic pool of phosphatidylcholine is \( \approx 1 \) g, and this pool circulates 5–10 times/d with almost complete hydrolysis and reabsorption of phosphatidylcholine (25). The absorption of dietary and biliary phosphatidylcholine involves hydrolysis by pancreatic PLA\(_2\), which hydrolyzes phosphatidylcholine to lysophosphatidylcho-
line and an unesterified fatty acid (26), but this enzyme is inhib-
ited below \( \mathrm{pH} 5.8 \) (27), and secretion is impaired in patients with 
CF (4). The low intraluminal pH documented in many patients 
with CF (5, 12) could impair activity of PLA\(_2\) released from 
enteric-coated pancreatic enzymes, which together with pancre-
atic insufficiency could reduce phosphatidylcholine digestion 
and absorption. Our aim was to determine whether fecal choline 
phosphoglyceride excretion is increased in CF and whether it is 
related to the alteration in plasma methionine cycle metabolites.

SUBJECTS AND METHODS

This study involved 18 children with CF and 8 control children with no known health problems. All of the children with CF had 
pancreatic insufficiency and were taking enteric-coated pancreat-
ic enzyme replacements (500–2500 U lipase/kg · meal; co-

Fecal analysis

Fecal samples were frozen in preweighed containers immedi-
ately after collection and stored at \(-70 \) °C until analysis. For 
analysis, fecal samples were weighed and homogenized, and a 
portion was dried to constant weight to allow determination of 
fecal water content. Total lipids were extracted and quantified 
gravimetrically. Fecal phospholipids, including phosphatidyl-
choline, lysophosphatidylcholine, phosphatidylethanolamine, 
phosphatidylserine, phosphatidylinositol, and sphingomyelin, 
were separated, and we quantified them by using HPLC (Waters 
2690 Alliance, Milford, MA) with an evaporative light-
scattering detector (HPLC-ELSD, model 2000; Alltech, Guelph, 
Canada) (29, 30). Fecal energy was quantified by bomb calori-
metry (oxygen bomb calorimeter model 1341; Parr, Moline, IL). 
All of the analyses were completed within 12 wk of sample 
collection.

Plasma analysis

Venous blood (2 × 7 mL) was drawn from each subject into 
tubes containing EDTA as anticoagulant within 3–4 h of the last 
meal (23). The plasma and red blood cells were separated by 
centrifugation at 2000 × g for 15 min at 4 °C and frozen at 
\(-70 \) °C within 20 min of blood collection. Plasma total lipids 
were extracted, and the phospholipids were separated and quan-
tified by HPLC-ELSD (23, 30). Plasma methionine, homocys-
teine, and their metabolites were analyzed by HPLC with 
reversed-phase ion pairing (31). Plasma apo B was quantified 
with the use of immunoturbidimetric reagents (Sigma Diagnos-
tic, St Louis, MO), and total and HDL cholesterol were deter-
mined with the use of colorimetric enzymatic reagents (Diag-
nostic Chemicals, Charlottetown, Canada).

Dietary analysis

All vitamin, mineral, and other nutritional supplements, in 
addition to foods and beverages, were recorded on the food 
records. The dietary records were entered into a nutrient analyses 
database (ESHA Food Processor Version 7.71; ESHA Research, 
Salem, OR), and the total energy fat, carbohydrate, and protein 
intakes (in g/d) were calculated. The total energy intake for each 
subject was calculated as the percentage of estimated energy 
requirement (EER) per day, according to age, sex, weight, and 
height and assuming a physical activity level in the low-active 
range (32). Fat absorption was calculated as (total fat intake 
g/d − fecal fat excretion g/d − total fat intake g/d) × 100%. Total 
choline intake and the intake of choline from phosphatidylcholine 
were estimated with the use of the US Department of Agri-
culture database on the choline content of common foods (33).

Statistical analysis

Data are presented as means ± SEMs. An independent two-
tailed \( t \) test for normally distributed data and the Mann-Whitney 
test for nonparametric data were used to compare the results for 
children with CF and the control subjects. Pearson’s correlation 
coefficients for normal data and Spearman’s rank correlation 
coefficients for nonparametric data were calculated to determine 
potential associations between fecal phospholipid excretion and
Fecal fat and energy content in children with cystic fibrosis (CF) and control subjects

<table>
<thead>
<tr>
<th></th>
<th>CF group</th>
<th>Control group</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 18)</td>
<td>(n = 8)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Fecal total fat (g/d)    | 12.9 ± 1.7  
(2.2–34.8)² ³ | 3.9 ± 0.7  
(2.0–7.2) |             |
| Fat absorption (% of fat intake) | 86.2 ± 1.6  
³ | 94.1 ± 1.2 |             |
| Fecal water content (% of fecal weight) | 69.0 ± 2.2 | 68.9 ± 2.3 |             |
| Fecal energy (kJ/d)      | 1130 ± 215   | 627 ± 146     |             |
| Fecal energy (kJ/g dry weight) | 24.2 ± 1.7 | 19.7 ± 1.4 |             |

² Significantly different from control group (independent two-tailed t tests); ³P = 0.003, ⁴P = 0.004.

Plasma homocysteine, methionine, and SAH. All statistical analyses were performed with the use of SPSS for Windows software (version 9.0.0; SPSS Inc, Chicago, IL). P values < 0.05 were considered significant.

RESULTS

In this study, we determined fecal fat and phospholipid excretion and the relation of fecal choline phosphoglyceride excretion to plasma methionine, SAH, and homocysteine in children with and without CF. The children with CF (n = 18) and the control children (n = 8) were 9.3 ± 1.4 and 10.0 ± 0.1 y old, respectively. The z scores for height-for-age in the CF and control groups were −0.47 ± 0.17 and 0.51 ± 0.24, respectively, and those for weight-for-age were −0.48 ± 0.16 and 0.24 ± 0.16, respectively (P > 0.05). Three of the 18 children with CF had meconium ileus as infants, and 5 were taking ursodeoxycholic acid for elevated liver enzymes or an abnormal finding on liver ultrasound scanning. None of the children had any gastrointestinal disease or resection or clinically significant liver disease.

The energy intake of 142.0 ± 3.5% of the EER for the children with CF was significantly (P = 0.001) higher than the energy intake of 106.2 ± 4.7% EER for the control group. Although fat intake was significantly higher in the children with CF (96.7 ± 6.8 g/d) than in the control children (70.1 ± 6.7 g/d), the intakes of choline (335 ± 33 and 268 ± 25 mg/d) and choline from phosphatidylcholine (124 ± 16 and 116 ± 22 mg/d) by the children with and without CF, respectively, did not differ significantly. Of the total energy intake in children with CF, fat contributed 35 ± 1.6%, carbohydrate contributed 50 ± 1.7%, and protein contributed 14 ± 0.7% of total energy, whereas, in the control children, fat contributed 30 ± 1.6%, carbohydrate contributed 56 ± 1.5%, and protein contributed 14 ± 0.6% of total energy.

The children with CF absorbed a significantly lower proportion of their dietary fat intake (86.2 ± 1.6%) than did the control children (94.1 ± 1.2%; Table 1). Although the mean fecal energy excretion was approximately twice as much in children with CF as in the control children, the difference was not significant, possibly because of the wide interindividual variability in fecal energy excretion (Table 1). Fecal fat and energy content were significantly associated (r = 0.89, P < 0.0001). No significant difference was observed in fecal water content between the children with CF and the control children.

Fecal total phospholipid, phosphatidylcholine, and lysophosphatidylcholine excretions were significantly higher in the children with CF than in the control children (Table 2). The lower limit of the HPLC-ESLD linear range for phospholipid was 50 mg/g dry stool. Of the 8 children in the control group, 2 had fecal phosphatidylcholine excretion < 0.5 mg/d; the range of phosphatidylcholine excretion was 0.86–140.9 mg/d in the children with CF and < 0.5–8.8 mg/d in the control children. Lysophosphatidylcholine represented ≈90% of the fcelal choline phosphoglycerides excreted by the control children, but, in the children with CF, it represented ≈75% and phosphatidylcholine represented ≈25% of the choline phosphoglycerides excreted (Table 2). Fecal phospholipid excretion was significantly associated with total fat excretion (Figure 1). Fat absorption (as %) was also inversely associated with total fat (r = −0.86, P < 0.001), total phospholipid (r = −0.82, P < 0.001), phosphatidylcholine (r = −0.75, P < 0.001), lysophosphatidylcholine (r = −0.78, P < 0.001), and phosphatidylethanolamine (r = −0.49, P = 0.011).
TABLE 3
Plasma thiols and phospholipids in children with cystic fibrosis (CF) and control children

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>CF group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 8)</td>
<td>(n = 18)</td>
</tr>
<tr>
<td>Homocysteine (μmol/L)</td>
<td>9.2 ± 0.4 τ</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>Methionine (μmol/L)</td>
<td>19.0 ± 0.9 τ</td>
<td>30.5 ± 2.6</td>
</tr>
<tr>
<td>S-adenosylhomocysteine (μmol/L)</td>
<td>32.6 ± 2.0 τ</td>
<td>20.3 ± 1.4</td>
</tr>
<tr>
<td>S-adenosylmethionine (μmol/L)</td>
<td>74.7 ± 3.4</td>
<td>84.5 ± 6.4</td>
</tr>
<tr>
<td>Total phospholipid (μmol/L)</td>
<td>732 ± 76.7</td>
<td>747 ± 110</td>
</tr>
<tr>
<td>Phosphatidylethanolamine (%)</td>
<td>10.2 ± 1.4 τ</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>Phosphatidylcholine (%)</td>
<td>76.7 ± 2.1</td>
<td>82.3 ± 1.8</td>
</tr>
<tr>
<td>Sphingomyelin (%)</td>
<td>9.1 ± 0.9</td>
<td>11.0 ± 1.4</td>
</tr>
<tr>
<td>Lysophosphatidylcholine (%)</td>
<td>3.6 ± 0.3</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>Phosphatidylcholine:phosphatidylethanolamine</td>
<td>13.0 ± 3.2 τ</td>
<td>24.8 ± 3.5</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>158 ± 10.4</td>
<td>154 ± 7.6</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>42.1 ± 2.2</td>
<td>44.5 ± 4.2</td>
</tr>
<tr>
<td>Apolipoprotein B (mg/dL)</td>
<td>46.9 ± 1.7 τ</td>
<td>54.4 ± 3.2</td>
</tr>
</tbody>
</table>

† All values are x ± SEM.
τ Significantly different from control group (independent two-tailed t tests): τP < 0.001, τP = 0.001, τP = 0.03.

Discussion
To our knowledge, these studies provide the first quantitative demonstration of increased fecal choline phosphoglyceride (phosphatidylcholine and lysophosphatidylcholine) excretion in CF. Our study also provides new data to show that increased choline phosphoglyceride excretion is associated with elevated plasma homocysteine in humans. Together with our recent report showing that plasma homocysteine and SAH are positively associated with plasma phosphatidylethanolamine and inversely related to plasma phosphatidylcholine (23), our work suggests that hepatic phosphatidylcholine synthesis by PEMT is interrelated with the transfer of methyl groups from SAM to SAH in a manner that affects plasma homocysteine in humans. Consistent with this suggestion, recent studies showed an 50% lower plasma homocysteine concentration in PEMT−/− mice than in wild-type mice (34), which suggested that the rate of PEMT methylation of phosphatidylethanolamine to form phosphatidylcholine is a determinant of plasma homocysteine in this species. It is well known that, despite pancreatic enzyme replacement therapy, dietary fat absorption remains lower in patients with CF than in persons without CF (8, 35, 36). In our study, children with CF taking pancreatic enzymes absorbed 86% of their dietary fat intake, whereas control children absorbed 94% of their dietary fat intake. Our results are similar to those of Murphy et al (35) and Burdge et al (36), which showed that 86% and 78% of dietary fat, respectively, was absorbed in children with CF. To the best of our knowledge, our work is the first to describe the amount and type of phospholipids excreted in CF. Standard methods for the extraction of fecal lipids do not quantitatively recover phospholipids (29). To address this situation, we recently developed methods for quantitative extraction, followed by separation and quantification of fecal phospholipids with the use of HPLC-ELSD (29). The significant relations between fecal fat and phospholipid excretion in the children in the current study suggest that fat malabsorption is accompanied by loss of phospholipids, as well as dietary energy. Whether malabsorption of choline phospholipids occurs in other disorders that involve impaired pancreatic, biliary, or intestinal absorptive function is not known. The high amounts of lysophosphatidylcholine excreted by the control children and the children with CF in our study were unexpected, although we were unaware of similar data on the composition of fecal phospholipids. Fecal lysophosphatidylcholine could originate from dietary and biliary phosphatidylcholine,

TABLE 4
Associations between plasma thiols and fecal phospholipid excretion

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Total phospholipid</th>
<th>Phosphatidylcholine</th>
<th>Lysophosphatidylcholine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methionine</td>
<td>r = −0.49, P = 0.013</td>
<td>r = −0.59, P = 0.002</td>
<td>r = −0.60, P = 0.001</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>r = 0.64, P = 0.001</td>
<td>r = 0.76, P &lt; 0.001</td>
<td>r = 0.58, P = 0.002</td>
</tr>
<tr>
<td>SAH</td>
<td>r = 0.52, P = 0.008</td>
<td>r = 0.64, P = 0.001</td>
<td>—</td>
</tr>
</tbody>
</table>

r = Pearson’s correlation coefficient; r = Spearman’s rank correlation coefficient for fecal phospholipids; SAH, S-adenosylhomocysteine.
hydrolyzed lower in the intestine beyond the site of lysophosphatidylcholine absorption or from the activity of colonic microflora. In addition to impaired PLA₂ secretion from the pancreas, low activity of PLA₁ from enteric-coated enzyme supplements resulting from limited bicarbonate secretion, slow dissolution of enteric pancreatic enzymes in the upper intestine, or both could explain an increased excretion of lysophosphatidylcholine. However, lysophosphatidylcholine was also the principal choline phosphoglyceride excreted by the children without CF. Lecithinase-positive bacteria are present in colonic microflora, and the abundance of these organisms was reported to be sensitive to dietary variables (37, 38). Whether the types and amounts of phosphoglycerides present in the colon have any physiologic relevance to intestinal function is unknown.

Our studies are aimed at elucidating whether decreased availability of preformed choline, possibly involving phosphatidylcholine malabsorption, may contribute to the high prevalence of hepatic steatosis among patients with CF (13, 14). Assessment of hepatic triacylglycerol accumulation involving the biopsy of liver tissue was not ethically acceptable. In addition, no measures of choline status were made. In this regard, plasma-free choline does not appear to be a sensitive measure of choline status because plasma-free choline concentrations are low, ranging from 7 to 20 μmol/L in normal adult men, a range that overlaps the mean plasma concentration of 7.5 μmol/L found in adults fed a diet deficient in choline (39). Therefore, we sought evidence of phosphatidylcholine malabsorption through measures of fecal choline phosphoglyceride excretion and used a novel approach, based on the intersection of the methionine-homocysteine pathway with phospholipid metabolism at the methylation of phosphatidylcholine, to form phosphatidylethanolamine. This process requires the involvement of PEMT-mediated synthesis of phosphatidylcholine, and this result is in the generation of SAH that is subsequently hydrolyzed to homocysteine (23). Hepatic triacylglycerol accumulation is a characteristic feature of choline deficiency (18, 19), which is explained by the requirement for phosphatidylcholine biosynthesis through the CDP-choline pathway for secretion of triacylglycerol in apo B–containing VLDL (20, 21). Plasma apo B was lower in the children in our study with CF than in the control children, which is similar to findings reported in previous studies with patients with CF (40, 41) and is consistent with reduced hepatic VLDL secretion. Our results show that children with CF excrete more phosphatidylcholine and lysophosphatidylcholine than do children without CF, and this finding is not explained by a higher intake of phosphatidylcholine or choline. The children with CF excreted 50–200 mg lysophosphatidylcholine + phosphatidylcholine/d, whereas the control children excreted 6–70 mg, but the dietary intake of choline from phosphatidylcholine was 124 and 116 mg/d in the 2 groups of children, respectively. Our results raise the question of whether chronic malabsorption of lysophosphatidylcholine and phosphatidylcholine in CF results in decreased availability of preformed choline to support the CDP-choline pathway, thus resulting in an increased demand to generate phosphatidylcholine through the PEMT pathway. The significant positive relations between choline phosphoglyceride excretion and plasma SAH and homocysteine and the inverse relation with plasma methionine support a hypothesis that the elevated homocysteine and SAH concentrations found in children with CF are related to the increased synthesis of phosphatidylcholine by PEMT. However, increased PEMT pathway activity could also result from an increased endogenous phosphatidylcholine requirement. In this regard, Ulane et al (42) reported an increased uptake of choline by ex vivo platelets taken from patients with CF, although others have reported that mutations of the CF transmembrane conductance regulator do not alter phosphatidylcholine or lysophosphatidylcholine uptake (43). Alternatively, elevation of SAH for some reason unrelated to phosphatidylcholine absorption could result in the inhibition of methyltransferase reactions, including PEMT (44), and thus decrease the ratio of plasma phosphatidylcholine to phosphatidylethanolamine.

In summary, children with CF excrete greater amounts of choline phosphoglycerides than do children without CF. The excretion of choline phosphoglycerides is positively associated with plasma homocysteine and inversely associated with plasma methionine. These novel findings suggest potential hepatic choline deficiency in children with CF. Our work also provides new information to suggest an important functional interdependence between phospholipid metabolism and the methionine-homocysteine cycle in humans. We postulate that altered phospholipid metabolism could be relevant to some of the complications associated with CF, such as hepatic steatosis. Further studies are needed to address the importance of the PEMT cycle in phosphatidylcholine metabolism in humans and the interrelation of this pathway with homocysteine.

We thank the parents and children who participated in this study, RA Dyer and JD King for technical assistance, and the staff at the British Columbia Children’s Hospital for facilitating this study.

AC enrolled subjects, collected and analyzed fecal and plasma samples, and provided data analysis and interpretation. SI was the principal investigator in grant funding and in study concept and design, data analysis, and interpretation. AGFD was the clinician-scientist responsible for patient selection, enrollment, and collection of clinical information. SJJ provided analyses of plasma thiols. None of the authors had any personal or financial conflicts of interest.

REFERENCES