

Parallel Secretion of Pancreatic Phospholipase A₂, Phospholipase A₁, Lipase, and Colipase in Children with Exocrine Pancreatic Dysfunction

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ABSTRACT

The cosecretion of pancreatic lipase and colipase are important in normal fat digestion. As adsorption of phosphatidylcholine to the lipid substrate interferes with lipase activity, hydrolysis to lysophosphatidylcholine with subsequent desorption is also essential for fat digestion. There are some data regarding the secretion of pancreatic phospholipases in normal adults but none in children or patients with pancreatic disease. In the present study, we aimed *a*) to develop an accurate fast assay method to measure phospholipase A₂ and *b*) to determine the secretion rate of pancreatic phospholipase A₂ and whether it is cosecreted with lipase and colipase in children with exocrine pancreatic dysfunction. Nine male patients aged 0.5 to 16 y (seven with cystic fibrosis, two with malabsorption) underwent pancreatic stimulation tests. Their colipase and lipase secretion rates were measured by titrimetric methods and phospholipase A₂ and A₁ by phosphorus magnetic resonance spectroscopy (³¹P NMR). It was found that the phospholipases, colipase, and lipase were absent in the two patients with pancreatic insufficiency. In patients with normal absorption, there were marked inter- and intrasubject variations of lipase, colipase, and phospholipase secretion rates that were consistent with the degree of exocrine pancreatic dysfunction. However, in the three 20-min stimulation periods of

the pancreatic function test, pancreatic phospholipase is cosecreted with lipase and colipase, and average colipase and phospholipase A₂ secretion rates follow a similar or parallel pattern. These findings are consistent with the important role of pancreatic phospholipases in intestinal phospholipid hydrolysis leading to the desorption of phospholipids from the lipid substrate and enhancing lipid hydrolysis and phospholipid absorption. (*Pediatr Res* 48: 735–740, 2000)

Abbreviations

CEH, carboxyl ester hydrolase
CF, cystic fibrosis
GPC, glycerophosphocholine
lysoPC, lysophosphatidylcholine
MCC, mucocutaneous candidiasis and polyendocrinopathy
NMR, nuclear magnetic resonance
PC, phosphatidylcholine
PLA₁, phospholipase A₁
PLA₂, phospholipase A₂
PI, pancreatic insufficient
PS, pancreatic sufficient

Exocrine pancreatic function has been well characterized in children with pancreatic disease with the use of quantitative pancreatic function tests (1–3). To determine the relationship between residual pancreatic function and fat absorption, sensitive titrimetric assays were developed for lipase and the lipase cofactor colipase (4) and their secretion rates compared with fecal fat excretions in children with CF, Shwachman syndrome, or controls (3, 5). These studies demonstrated that

patients with normal fat absorption had a wide range of enzyme/coenzyme secretion from less than 5% up to within the normal control range; and the threshold for lipolytic failure was near 1% of average control colipase values, *i.e.* patients with values below this level had fat malabsorption and above this level normal absorption. These observations led to the currently accepted definition that patients with malabsorption are PI and those with normal absorption are PS, indicating that they have either insufficient or sufficient endogenous enzyme output, respectively, to prevent malabsorption (6). The results also emphasized the important role of colipase *in vivo*, as, in a group of patients with seemingly adequate lipase output above the threshold level, fat malabsorption occurred because of inadequate cosecretion of colipase. This confirmed *in vitro*

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observations that lipase in the presence of supramicellar concentrations of bile salts is only active if colipase is present and remains inactive in the absence of colipase (4, 7, 8).

The exocrine pancreas also secretes another group of lipolytic enzymes, the phospholipid-hydrolyzing enzymes including PLA₁ (EC 3.1.1.32), PLA₂ (EC 3.1.1.4), and CEH (EC 3.1.1.1) (9). PLA₁ and PLA₂ are secreted in their zymogen form and activated by trypsin on entering the duodenum (10). In intraluminal digestion, PLA₂ is primarily responsible for hydrolyzing PC to 2-lysoPC. This reaction is important in triglyceride digestion as the amphipathic PC, in a manner similar to bile salts, will adsorb to the surface of the lipid droplets, preventing contact between the lipase-colipase complex and its lipid substrate (11). Hydrolysis of PC by PLA₂ will allow desorption of lysoPC, which is water soluble. The subsequent mucosal absorption of lysoPC is important in the generation of enterocyte phospholipids (12) and lipoproteins and, thus, chylomicron formation (13).

The above indicates an important physiologic function for PLA₂. However, although PLA₂ has been measured in duodenal juice in normal adult volunteers during a Lundh test meal (14), there is no information regarding pancreatic PLA₂ secretion rates in children or in patients with exocrine pancreatic disease (15). Previously, by using ³¹P NMR, we demonstrated that PLA₂ can be assayed in incubated duodenal fluid from PS patients by measuring the rate of 2-lysoPC formation from the hydrolysis of PC (16). In the present study, we have modified the assay by using PC in the duodenal fluid of PI patients, which lacks phospholipid-hydrolyzing enzymes as the substrate, and have assessed the secretion rate of PLA₂ in both PI and PS patients during pancreatic stimulation tests in relation to lipase and colipase secretion.

METHODS

Materials. PC and 2-lysoPC were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). D₂O (99.75%) was from the Australian Institute of Nuclear Science and Engineering (Lucas Heights, N.S.W., Australia). All other chemicals were analytical grade.

Patients. Nine male patients aged from 0.5 to 16 y underwent pancreatic stimulation tests: seven were PS and two were PI. Of the PS patients, five had CF (CF-PS), one MCC with persistent hypocalcemia, and one who was investigated for short stature with possible Shwachman syndrome and was ultimately shown to have normal pancreatic function. Both PI patients had CF (CF-PI).

Pancreatic stimulation tests. Pancreatic stimulation tests were performed as previously described (1–3). Briefly, after an overnight fast (except the children under age 2 y and the MCC patient who fasted for 4 h), a double-lumen catheter was inserted into the duodenum. One lumen for infusion had a single port located proximally near the ampulla of Vater, and the second aspiration lumen had multiple ports at least 5 cm distal to the ampulla and approximately 1 cm apart. A nonabsorbable marker solution [gentamicin 10 μg/mL in 5% mannitol (wt/vol)] was infused at a constant rate of 1.25 mL/min through the proximal port, and the mixture of marker and

pancreatic juice was aspirated by low-pressure suction from the distal ports. Aspirates were collected in four 20-min periods, one initial period without stimulation and three periods during which a continuous i.v. infusion of secretin and cholecystokinin was maintained as described previously (1, 6). The use of the marker perfusion solution enabled precise determination of the quantity of marker and, thus, pancreaticobiliary fluid that was not aspirated during the procedure (1, 6). All samples were collected on ice, and the fluid from each interval was aliquotted into smaller samples and stored immediately at –70°C.

Lipase and colipase assays. Lipase and colipase concentrations were measured, and their rates of secretion were calculated as previously described (3, 4). The titrimetric enzyme assays were carried out at 37°C with glycerol tributyrates as the lipid substrate. Colipase activity was determined in the presence of 4 mM sodium taurodeoxycholate, and lipase activity was determined by using a saturating concentration of porcine colipase. Pancreatic function was defined as sufficient if colipase output was greater than 1% of the mean normal level (120 U·kg⁻¹·h⁻¹) and insufficient if less than 1% of the mean normal level (3).

NMR experiments. Our previous work in measuring phospholipid concentration in samples of duodenal juice collected during pancreatic stimulation indicated variable concentrations of PC and lysoPC therein (16). To standardize the procedure for measuring PLA₂ and PLA₁, we used the pooled pancreaticobiliary juices from five CF-PI patients with absent PLA₂ and PLA₁ activities. Samples from pancreatic stimulation tests of patients with pancreatic sufficiency or insufficiency (1.2 mL) were added to the pooled pancreaticobiliary juice from CF-PI patients (1.3 mL) and transferred to 10-mm NMR tubes. The volume was brought up to 3 mL by adding 0.5 mL D₂O with 5 mM EDTA and an internal standard (trimethyl phosphate) to the NMR tube. The samples were then ready for incubation at 37°C and NMR measurements. Unless otherwise stated, all incubations were performed at pH 7.44. Standard samples were prepared by adding a known concentration of lysoPC in D₂O to samples of pancreaticobiliary secretions from PI-CF patients.

Quantitative analysis of the concentrations of phospholipids was performed as previously described (17–20).

NMR spectroscopy. ³¹P NMR spectra were recorded on a Bruker DRX (Avance) 400 spectrometer operating at 161.98 MHz in the Fourier transform mode with the probe temperature controlled to 37 ± 1°C. Broadband proton irradiation was applied to eliminate ¹H/³¹P NMR coupling. All measurements were performed on 3-mL samples in 10-mm NMR tubes with D₂O as the internal field/frequency lock. Samples were spun at 12 Hz. Rapid pulsing was used to acquire the spectra. Each spectrum was derived by Fourier transformation of 600 summed transients with a spectral accumulation time of approximately 5.5 min, and FID (free induction decay) were processed with 3-Hz line broadening. Other NMR parameters were as follows: transient acquisition time, 0.256 s; data size, 16 k; 50° pulse, duration 10 μs; intertransit delay, 300 ms.

The present study was approved by the Ethics Committee of the Royal Alexandra Hospital for Children, and parents provided written informed consent for pancreatic function studies.

RESULTS

³¹P NMR of pancreaticobiliary fluid. The ³¹P NMR spectrum obtained from a sample of biliary-pancreatic secretions from a CF-PS patient is shown in Figure 1A. The resonance at -0.06 ppm was assigned to PC and those at 0.25 and 0.42 ppm to its hydrolytic products, 1-lysoPC and 2-lysoPC, respectively. After incubation of the sample for 39 min at 37°C, there was substantial hydrolysis of PC and, thus, an increase in the two lysoPC peak intensities as shown in Figure 1B. The same experiment in a CF-PI patient is illustrated in Figure 1, C and D, demonstrating the preservation of the PC peak after even prolonged incubation (167 min) and absent lysoPC peaks, both observations indicating the lack of phospholipase activities.

The ³¹P NMR peak assignments in Figure 1 were confirmed by adding authentic phospholipids to the sample of biliary-pancreatic secretions and observing the corresponding increase in signal amplitude and intensity. The resonance for 1-lysoPC was assigned by subjecting a sample of biliary-pancreatic secretions to phospholipase B activity (16). Chemical shift assignments were referenced relative to external 85% orthophosphoric acid at 0.000 ppm.

PLA₂ and PLA₁ assays. NMR-based phospholipase assays were developed by subjecting the pooled pancreaticobiliary fluid from CF-PI patients (substrate) to phospholipase activities by the addition of pancreaticobiliary fluid from a subject under study (enzyme source). The samples were then incubated at 37°C, and sequential ³¹P NMR spectra were acquired. It was observed that the 2-lysoPC and 1-lysoPC concentrations progressively increased with the time. Under the assay conditions, the lysoPC was not further hydrolyzed to GPC due to the CEH activity. However, a small amount of GPC was produced after a prolonged incubation and only after complete hydrolysis of the substrate (PC). PLA₂ and PLA₁ activities were calculated from the initial slopes of 2-lysoPC and 1-lysoPC concentration increase, respectively (16, 17). The total phospholipase secretion was expressed as the activity (mmol·mL⁻¹·h⁻¹) times the total volume of duodenal aspirate during 1 h of stimulation

(three 20-min periods) per kilogram of body weight. Table 1 lists the secretion rate of PLA₂ and PLA₁ together with the secretions of lipase and colipase from patients with pancreatic sufficiency and compares them with samples from those patients who had pancreatic insufficiency. As shown in Table 1, the total secretion of PLA₂ and PLA₁ per kg/h was remarkably similar between patients in the CF-PS group, despite some variation of their lipase, colipase secretion rates and regardless of the patient's age. Substantially lower levels of PLA₂ and PLA₁ were seen in the MCC patient, possibly related to hypocalcemia (serum Ca²⁺ < 2 mmol/L) as calcium is required for PLA₂ and PLA₁ activity. Both PLA₂ and PLA₁ activities were absent from the two PI patients.

Parallel secretion of phospholipase, lipase, and colipase.

As shown in Table 1, two age groups of patients with pancreatic sufficiency were studied, three less than and four more than 2 y of age. The three patients less than 2 y of age and the one patient with MCC underwent only a short fast of 4 h before their pancreatic stimulation tests; a more prolonged fast was considered inappropriate for the younger patients or the patient with MCC in view of his hypocalcemia. All other patients were fasted for more than 12 h. Figure 2A presents the mean secretion rates of PLA₂ and colipase of patients more than 2 y old (after 12 h fasting) during the three 20-min stimulation periods. As evident from Figure 2A, the secretion of PLA₂ and colipase was in parallel. The mean secretions of PLA₂ and colipase of patients less than 2 y old (nonfasting) during the stimulation periods are shown in Figure 2B. Although the basal values for PLA₂ and colipase secretions were considerably higher than their stimulated ones, their stimulated mean values were in parallel.

DISCUSSION

Our results demonstrate that CF-PI patients had no phospholipid-hydrolyzing enzymes including PLA₂ and PLA₁ activities in their duodenal juice even after prolonged incubation time (Fig. 1, C and D), consistent with their degree of exocrine pancreatic dysfunction as evident from their absent lipase and colipase secretion. In contrast, PS patients demonstrated substantial PLA₁ and PLA₂ activities by incubation of samples for 39 min as in Figure 1, A and B, as well as lipase and colipase secretions as listed in Table 1. In a previous study, normal values of colipase secretion among a group of normal young adult controls were more than 6500 U·kg⁻¹·h⁻¹, with an average value of 12,740 U·kg⁻¹·h⁻¹ (3). The values in the current study were consistent with values obtained previously in PS patients (2–4), noting that the one normal subject with a value of 5100 U·kg⁻¹·h⁻¹ was younger than previous normal controls. The patient with MCC had a lower output of colipase activity but comparable to one of the CF-PS patients (Table 1). He also had persistent hypocalcemia, a factor that may have impaired colipase activity in the titrimetric assay that was performed. The PLA₁ and PLA₂ values were consistent in the PS patients except for the lower value in the patient with MCC; again, an observation that could be accounted for by hypocalcemia, as PLA₁ and PLA₂ activity is calcium dependent. Both PLA₁ and PLA₂ did not appear to vary with age, with some of

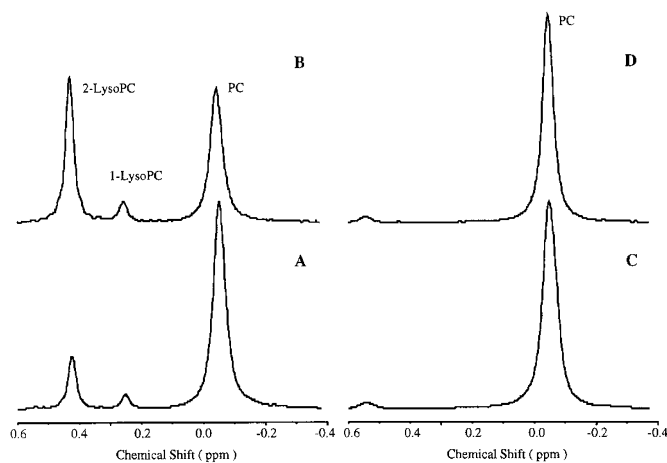


Figure 1. ³¹P NMR spectra of phospholipids in biliary-pancreatic secretions (duodenal juice) from a PS patient with CF (A), incubated at 37°C for 39 min (B), and from a PI patient with CF (C), incubated at 37°C for 167 min (D). NMR parameters are given in "Methods."

Table 1. PLA₂, PLA₁, lipase, and colipase secretion rates in CF patients with pancreatic sufficiency* and insufficiency

No.	Subject	Age (y)	PLA ₂ [mmol·h ⁻¹ ·kg ⁻¹ ·(total period) ⁻¹]**	PLA ₁	Lipase [U·kg ⁻¹ ·(total period) ⁻¹] × 10 ³	Colipase
1	Normal	1.3	18.7	1.7	7.5	5.1
2	MCC	11.7	7.5	0.5	4.9	3.4
3	CF-PS*	16.8	26.1	1.5	4.8	3.4
4	CF-PS	0.7	32.9	1.8	17.2	15.5
5	CF-PS	0.5	24.7	1.0	8.5	6.7
6	CF-PS	11.5	27.6	2.2	6.3	5.1
7	CF-PS	7	33.5	2.1	28.2	17.6
8	CF-PI	12.8	0	0	0	0
9	CF-PI	12.2	0	0	0	0

* Pancreatic function was defined as sufficient to prevent fat malabsorption if colipase output was greater than 1% of mean normal levels (120 U·kg⁻¹·h⁻¹).

** The phospholipase secretion rate was expressed as the activity (mmol·mL⁻¹·h⁻¹) times the total volume of duodenal aspirate during 1 h of stimulation (three 20-min periods) per kilogram of body weight. The phospholipase activities were calculated from sequential ³¹P NMR spectra of pancreaticobiliary secretions during the course of incubation at 37°C.

the younger CF-PS patients who were less than 12 mo old having secretion levels comparable to older children or adolescents and higher than the one normal patient in the study.

In the two age groups, depending on the duration of fasting, the pattern of PLA₂ secretion was very similar to colipase secretion (*cf.* Fig. 2, A and B), thus indicating the occurrence of parallel secretion of PLA₂ and colipase, a known marker of pancreatic lipolysis. The cosecretion of PLA₂ with other pancreatic enzymes or coenzymes could have been predicted from other studies demonstrating parallel pancreatic cosecretion of lipase and colipase (3) and also of nonlipolytic enzymes, proteases, and amylase (21, 22). This observation is also consistent with the role of phospholipases in intraluminal phospholipid and triglyceride digestion and the subsequent role of phospholipids in enterocyte lipid transport. In the small intestine, amphipathic biliary and dietary phospholipids, mainly PC, are adsorbed to ingested triglyceride and can impair the action of the pancreatic lipase-colipase complex during triglyceride digestion (11). PLA₂, by hydrolyzing PC, results in desorption of phospholipids from the triglyceride substrate and, thus, facilitates normal lipolysis. The major product of PC digestion, lysoPC, is water soluble and readily absorbed by enterocytes. It is a major source of enterocyte phospholipids for the generation of lipoproteins, and chylomicron formation as shown in the multidrug resistant P-glycoprotein knockout mice (23), for transport of triglyceride from the cell to lymph and for replenishment of membrane phospholipids used in exocytosis (12, 13, 24, 25). Lack of cosecretion of PLA₂ could substantially impair these processes. Unhydrolyzed phospholipids substantially impair absorption of fatty acids and cholesterol (26) and interfere with bile salt binding to the bile salt receptors in the terminal ileum, impairing its absorption and increasing fecal bile salt losses (27). The cosecretion of pancreatic phospholipase with other components of the lipolytic system is, thus, rational from a physiologic point of view, noting that whereas its major role is hydrolyzing phospholipids in the intestine, by so doing it is an adjunctive enzyme to triglyceride hydrolysis.

The variation in the pattern of PLA₂ and colipase secretion dependent on the duration of fasting was not investigated further in the current study. The high basal levels before pancreatic stimulation of those with a short duration of fasting

(Fig. 2B) could reflect prior release at the time of formula or liquid meals and, thus, lack of stimulation at the time of i.v. stimulation of the pancreas. This could be explained by the considerable variation in gastric emptying times between individual children and the recognized slow intestinal transit times described in CF-PS patients (28). Consequently, pancreatic stimulation could be delayed after a test meal, thus accounting for the late or persistent appearance of the lipolytic enzymes. This phenomenon requires further investigation.

In the present study, we did not assess the pancreatic secretion of other phospholipid-hydrolyzing enzymes including the CEH responsible for intraluminal hydrolysis of lysoPC (9). This enzyme is of interest as it appears identical to the bile salt-stimulated lipase in human milk (29). The presence of this enzyme in the intestine from either the pancreas or human milk indicates that a certain proportion of lysoPC remains unabsorbed in both normal breast-fed infants and older children/adults. The physiologic reason for this incomplete absorption of lysoPC remains unclear, but, of interest, lysoPC has a known toxic effect on epithelial cells (30). Although in normal subjects the lysoPC could be hydrolyzed in the presence of CEH, the low activity of CEH in oral enzyme therapy given to PI subjects (31) could allow toxic levels of lysoPC to accumulate in the intestinal lumen and account for some of the intestinal disease seen in CF subjects. Further work is required to assess the pancreatic secretion and activity of CEH in CF and other patients with exocrine pancreatic dysfunction.

The NMR assay method for determining pancreatic PLA₂ and PLA₁ secretion in this study represented an extension of a method that was introduced in our previous work (16). In the previous study, phospholipase activities were measured using the pancreaticobiliary fluid as both substrate and enzyme source, whereas, in the present study, the source of the PC substrate was the pooled pancreaticobiliary juice from CF-PI patients. Use of the pooled human fluid in this study had the advantage of 1) satisfying all the necessary physiologic requirements for phospholipase activity including appropriate bile salt concentrations and 2) introducing additional PC to phospholipase exposure, thus avoiding product inhibition. We had also previously observed in PS subjects that PLA₂ activity was remarkably fast and that a considerable amount of the endogenous PC had already been hydrolyzed to lysoPC during

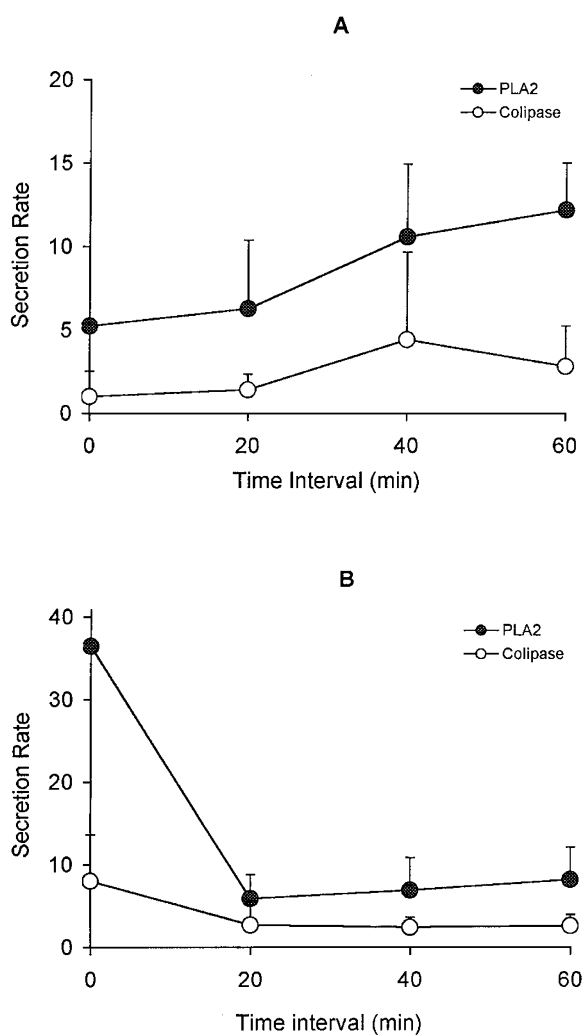


Figure 2. Mean \pm SD basal and stimulated values for PLA₂ and colipase secretion (see Table 1 for comments on the secretion units) in PS patients more than 2 y old after 12 h fasting (A) and in PS patients less than 2 y old after 4 h fasting (B).

the sample collection. The current assay system with the additional PC, thus, also circumvented this problem.

A variety of methods have been used for the determination of PLA₂ and PLA₁ activities including titrimetric, radiochemical, immunologic, and HPLC (32–37). These methods in general are multistep, tedious, and time consuming. The classic titrimetric methods are insensitive (32), nonspecific, and require individual phospholipase purification (32, 33). Radiochemical procedures are sensitive but suffer from the disadvantages of being discontinuous and involve radioactive handling that is undesirable (32). Immunologic procedures are also sensitive but can only be used for the determination of the quantities of lipolytic enzymes. Other methods including HPLC usually involve sample extraction using organic solvent mixture. Selective loss of lysoPC, the product of PLA₂ and PLA₁ activities, is one of the great disadvantages of widely used extraction procedures (20, 30, 38). The NMR assay method for measuring PLA₂ and PLA₁ used in this study, on the other hand, is convenient, one-step, and nondestructive and does not require extraction of phospholipids. It is, therefore, possible to

monitor both PLA₂ and PLA₁ activities simultaneously in a complex mixture of body fluids such as duodenal juice (16).

In conclusion, we have developed a sensitive and noninvasive assay method for measuring phospholipase activity by using NMR spectroscopy. During pancreatic stimulation tests, CF-PI patients had absent PLA₂ and PLA₁ activities. In contrast, those with pancreatic sufficiency had pancreatic lipase/colipase secretions up to and within the normal range, with parallel phospholipase secretions.

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