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**RESEARCH ARTICLE** | *Pancreatic Physiology/Pathophysiology*

# Metabolic markers of protein maldigestion after a $^{15}$N test meal in minipigs with pancreatic exocrine insufficiency

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[1]*UMR PNCA, AgroParisTech, INRA, Université Paris Saclay, Paris, France;* [2]*Institute for Animal Nutrition, University of Veterinary Medicine Hannover, Foundation, Hanover, Germany; and* [3]*Abbott Laboratories, Hannover, Germany*

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**Mary F, Moesseler A, Khodorova N, Foucault-Simonin A, Benamouzig R, Tomé D, Gregory PC, Gaudichon C.** Metabolic markers of protein maldigestion after a $^{15}$N test meal in minipigs with pancreatic exocrine insufficiency. *Am J Physiol Gastrointest Liver Physiol* 314: G223–G230, 2018. First published October 26, 2017; doi:10.1152/ajpgi.00218.2017.—The effect of pancreatic exocrine insufficiency (PEI) on protein malabsorption is little documented, partly due to methodological barriers. We aimed to validate biomarkers of protein malabsorption using a $^{15}$N test meal in a minipig model of PEI. Six pancreatic duct-ligated minipigs were used as a model of PEI and four nonoperated animals as a control. All animals were equipped with an ileocecal reentrant cannula. Minipigs were given a test meal containing [$^{15}$N]casein. The PEI animals repeated the test three times, in the absence of any pancreatic enzymes, or after pancreatic substitution at two levels [*A* or *B*: 7,500 or 75,000 (lipase) and 388 or 3881 (protease) FIP U]. Ileal chyme, urine, and blood were collected postprandially. Nitrogen and $^{15}$N were measured in digestive and metabolic pools. We obtained a gradient of ileal protein digestibility from 29 ± 11% in PEI to 89 ± 6% in the controls and a dose-dependent response of enzymes. Insulin and gastric inhibitory polypeptide secretions were decreased by PEI, an effect that was counteracted with the enzymes at *level B*. The total recovery of $^{15}$N in urinary urea and plasma proteins was 14 ± 5.1% in the control group and decreased to 5.5 ± 2.1% by PEI. It was dose dependently restored by the treatment. Both $^{15}$N recovery in plasma and urine were correlated to protein digestibility. We confirm that the $^{15}$N transfer in those pools is a sensitive marker of protein malabsorption. Nevertheless, an optimization of the test meal conditions would be necessary in the view of implementing a clinical test.

**NEW & NOTEWORTHY** We designed an intervention study to create a gradient of ileal protein digestibility in minipigs with pancreatic exocrine insufficiency and to validate reliable metabolic markers using a $^{15}$N oral meal test. $^{15}$N recovery in plasma proteins and to a higher extent in urine was sensitive to protein malabsorption. This test is minimally invasive and could be used to reveal protein malabsorption in patients.

bioavailability; biomarkers; dietary protein; malabsorption; stable isotopes

## INTRODUCTION

Pancreatic exocrine insufficiency (PEI) results in malabsorption of nutrients, especially fat (9, 22). The effect of PEI on protein absorption is less well documented, although it is known that protein digestion may be impaired due to the lack of pancreatic proteases (6, 15, 22). This question is nevertheless of clinical importance because protein maldigestion, combined with a chronic inflammatory status resulting from the disease, can cause lean body mass wasting and impaired immune status. Protein malabsorption seems to appear at a later point in time than fat malabsorption in chronic pancreatitis patients (33, 34), and results in an increase of fecal protein losses. Creatorrhea is diagnosed when there is daily fecal protein loss >2.5 g nitrogen (N) and can reach more than 5 g N in some patients suffering from PEI. However, little is known about these losses. Enzyme substitution has been shown to exert a marked effect on steatorrhea, but due to lack of data its impact on creatorrhea is less clear. Evenepoel et al. (12) reported a weaker oxidation of amino acids of either dietary or endogenous origin in patients than in healthy volunteers. It is not known if this low catabolic rate is a direct consequence of the poor availability of α-amino acid substrates or whether indirect factors such as impaired hormone secretion may disturb protein pathways. Trials using enzyme replacement therapy offer indirect information about protein maldigestion, but unfortunately, results vary widely. Van Hoozen et al. (35) found no significant effect of enzyme replacement therapy on apparent fecal protein digestibility over a 4-wk period but did observe an increase after 8 wk of treatment. In a randomized study, Withcomb et al. (36) reported a protein digestibility of 24% in the placebo group and 98% in the treated group, while Toskes et al. (31) reported values of 78% with placebo and 84% treatment. A study in pigs with experimentally induced PEI showed that ileal apparent digestibility was increased from 28 to 70% (16).

One of the main problems of these studies is that apparent digestibility, based on the measurement of total fecal N, is not a sensitive indicator for protein availability since protein absorption occurs in the small intestine. Colon fermentation of undigested amino acids can lead to the disappearance of dietary N, mainly in the form of ammonia that can be absorbed at the colon level. We found in patients with chronic pancreatitis that protein malabsorption was hardly detected by fecal tests but that metabolic markers using a $^{15}$N protein meal test could help diagnose malabsorption (1). We also observed a high variability of responses to enzyme therapy, depending on the severity of the PEI. However, we could not control the ileal protein digestibility because the use of intestinal tubes was not possible in those patients.

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The objective of our study was to trigger protein malabsorption at different levels using a minipig model of total pancreatic exocrine insufficiency (30) treated or not with pancreatic enzyme replacement therapy to confirm metabolic markers associated with protein digestion. For this purpose, we used a $^{15}N$ test meal in pancreatic duct-ligated minipigs, which were also equipped with an ileocecal reentrant canula.

## MATERIALS AND METHODS

*Animals.* The procedures used in this study were conducted in accordance with the German Animal Welfare Act and the European Council Directive of November 24, 1986 (86/609/EEC) and were approved by the Ethics Committee on Animal Welfare of the Hannover District Government. Ten female minipigs (Göttingen strain; Ellegaard) were selected from the pool of previously operated minipigs at Abbott Laboratories (Hanover, Germany). All animals were chronically equipped with an ileocecal reentrant cannula. Six of them had a ligation of the pancreatic duct to induce a complete PEI, and the four others served as controls. The body weight ranged from 30 to 40 kg at the beginning of the study.

*Protocol.* The PEI and control minipigs were subjected to digestive and metabolic investigations after the ingestion of a complex test meal. While the control animals underwent the test only once (without any enzyme treatment), the PEI animals repeated the test three times (with a "wash-out period" of at least 1 wk between the different trials), either in the absence of any pancreatic enzyme or after pancreatic substitution (Creon) given at two levels (7,500 and 75,000 FIP U lipase, and 388 and 3,881 FIP U protease, respectively), according to a cross-over design. Only one test was performed in the control minipigs without any pancreatic enzyme supplementation.

The experimental meal was composed of Calshake (Fresenius Kabi, Bad Homburg, Germany), skimmed milk, [$^{15}N$]casein as a marker of dietary protein, olive oil, fish meal, rice starch, cellulose, and warm tap water, as detailed in Table 1. Chromium oxide was added to the meal as a nonabsorbable marker. The meal was homogenized. After removal of a 100-g sample for analysis, the test meal amount was calculated to weigh 634.6 g. Its macronutrient composition is reported on Table 1. For the tests with enzyme supplementation, Creon (loose minimicrospheres) was mixed into the meal shortly before it was offered to the animals, and the whole meal was ingested within a few minutes.

The total amount of ileal chyme was continuously collected on ice for 8 h after the appearance of the meal marker in the ileum. Samples were pooled by 2-h periods and frozen (−20°C) immediately after a 2-h interval was completed. After the total weight was recorded, they were lyophilized for further determination of $Cr_2O_3$, N, $^{15}N$, fat, and starch. Total urine was collected during the same period, and blood was sampled hourly for 12 h through a temporarily implanted jugular vein catheter. The jugular vein catheter (Central Venous Catheterization Set with Blue Flex Tip CatheterArrow; Telefle, Morrisville, NC) was implanted the day before the test, using a short general anesthesia [Ketavet (ketamin 15 mg/kg body wt im) and Dormicum (midazolam 0.5 g/kg body wt im]. The catheter was fixed with two individual stiches and a bandage to the pigs′ neck. The jugular catheter was withdrawn at the end of the blood sampling or on the following day. Blood samples were immediately centrifuged at 2,000 *g* (4°C) for 10 min. The plasma was aliquoted and stored at −20°C for analysis of $^{15}N$ amino acids and plasma proteins, glucose, urea, or at −80°C after the addition of aprotinin for insulin and gastric inhibitory polypeptide (GIP) determination. Urine was weighed and stored at −20°C until analysis.

Table 1. *Detailed composition of the test meal*

| | Test Meal, g |
|---|---|
| Ingredients | |
| Skimmed milk | 209.2 |
| Rice starch | 129.5 |
| Calshake* | 79.4 |
| [$^{15}N$]casein | 40.6 |
| Olive oil | 25.8 |
| Fish meal | 11.2 |
| Cellulose† | 8.5 |
| $Cr_2O_3$ | 0.54 |
| Water | 129.5 |
| Total | 634 |
| Macronutrients | |
| Protein | 53.5 |
| Fat | 50.7 |
| Carbohydrates | 152 |

*From Fresenius Kab: nutrient content/100 g (information from the producer): protein: 4.4 g; fat 24.5 g; carbohydrates: 66.1 g; and fibers: <0.05 g. †Methylcellulose (MethoCel) from Dow Chemical (Midland, MI).

*Analyses.* Chromium oxide in the ileal chyme was measured by spectrophotometry at 365 nm according to the method described by Petry and Rapp (26). Crude fat content was determined by acid hydrolysis and petrol ether extraction using a filter bag technique in an extractor (ANKOM XT15; ANKOM Technology, Macedon, NY). Starch was analyzed polarimetrically after acid hydrolysis according to the method described by Naumann and Bassler (24).

Plasma glucose was assayed by a glucose oxidase method (Glucose RTU Kit; BioMérieux, Lyon, France). Urea concentrations were measured in plasma and urine by using a urease-glutamate dehydrogenase technique (Urea Kit; BioMérieux). Plasma insulin and GIP concentrations were simultaneously determined using an endocrine kit panel (Bio-Plex Pro Assay; Bio-Rad, CA) on a Bioplex 200 system (Bio-Rad). For isotopic determination, urinary urea and ammonia as well as plasma urea and free amino acids were isolated using a sodium and potassium form of a cation exchange resin (Bio-Rad Dowex AG50-X8, mesh 100–200; Interchim, Montluçon, France) as described previously (7).

Total N and $^{15}N$ enrichment of samples were determined by using isotope ratio mass spectrometry (Isoprime; GV Instrument, Manchester, UK) coupled with an elemental N analyzer (Vario L3; Elementar, Lyon, France), with atropine and glutamic acid as elemental and isotopic standards, respectively. Enrichment was expressed as atom percent excess (APE).

*Calculations.* The dietary N present in the samples was expressed in percentage of ingested N and calculated as follows:

$$\text{dietary N} = N_{\text{tot-mmol}} \times 100 \times \left(APE_{\text{sample}}/APE_{\text{meal}}\right)/\text{ingested N}$$

where $N_{\text{tot-mmol}}$ is the amount of total N in the sample, $APE_{\text{sample}}$ is the [$^{15}N$]-enrichment percent excess of the sample, and $APE_{\text{meal}}$ is the [$^{15}N$]-enrichment percent excess of the meal. [$^{15}N$]casein was assumed to be representative of total protein in the meal.

For ileal chyme, dietary N recovery was corrected for the recovery of chromium oxide, used as a nonabsorbable marker. Endogenous N was calculated as the difference between total and dietary N.

Real ileal digestibility (RID) was calculated as follows:

$$RID = (\text{N ingested} - \text{Nexo-ileal})/\text{N ingested} \times 100$$

where Nexo-ileal is the amount of dietary N recovered in ileal chyme.

For urinary urea, N pool size was calculated from urea measurement.

For plasma proteins, the blood volume was assessed as 6.5% of body weight (3).

*Statistics.* Results are expressed as means ± SD. The effect of the group (control, 0, 7,500, and 75,000 IU) was analyzed in a mixed model, using the MIXED PROCEDURE of SAS 9.1 (SAS Institute, Cary, NC).

For the measurement of digestive and urine losses, with only a single end point, the group was used as a repeated factor within

animals, according to the cross-over design in the PEI animals. For plasma kinetics (with 12 time points), the group, time, and their interaction were used as factors, and random effects for animal and animal × group are indicated. When main effects were significant, differences between groups were analyzed using the Bonferroni post hoc test. Differences were considered to be statistically significant at $P < 0.05$. The linear relationship between protein digestibility and $^{15}N$ recovery was analyzed by calculating the Pearson correlation coefficient and its associated $P$ value.

## RESULTS

*Ileal recovery of macronutrients.* Exogenous N recovery at the ileal level strongly (Fig. 1*A*) depended on the group ($P < 0.0001$). It was fivefold higher in untreated PEI pigs than in control pigs but decreased significantly with enzyme supplementation. With the dose of 75,000 IU, exogenous N recovery was not significantly different from the control. Endogenous losses during the 12 h following the meal varied between 91 to 220 mmol among groups, and there was no significant effect of the group. Nevertheless, a contrast analysis revealed a net trend for an increase of endogenous losses in the three-PEI group vs. the control ($P = 0.066$). Real ileal protein digestibility (Fig. 1*B*) was significantly different between treatment groups ($P < 0.0001$). It was 89 ± 6% in the control group and 29 ± 11% in untreated PEI minipigs. Treatment with pancreatic enzyme therapy dose dependently increased digestibility. The low dose of pancreatic enzymes increased the digestibility to 58 ± 14% and the high dose to 74 ± 14%, with no significant difference compared with the control for the highest dose. It must be noted that the variability was higher in PEI than control minipigs.

There was a strong effect of the treatment ($P < 0.0001$) on fat digestibility (Fig. 1*C*), which was 92.4 ± 4.6% in the control group. In untreated PEI minipigs, digestibility was only 17.7 ± 3.6% and increased to 44.1 ± 19.8% in the 7,500-IU group and to 59.2 ± 12.7% in the 75,000-IU group. In contrast,

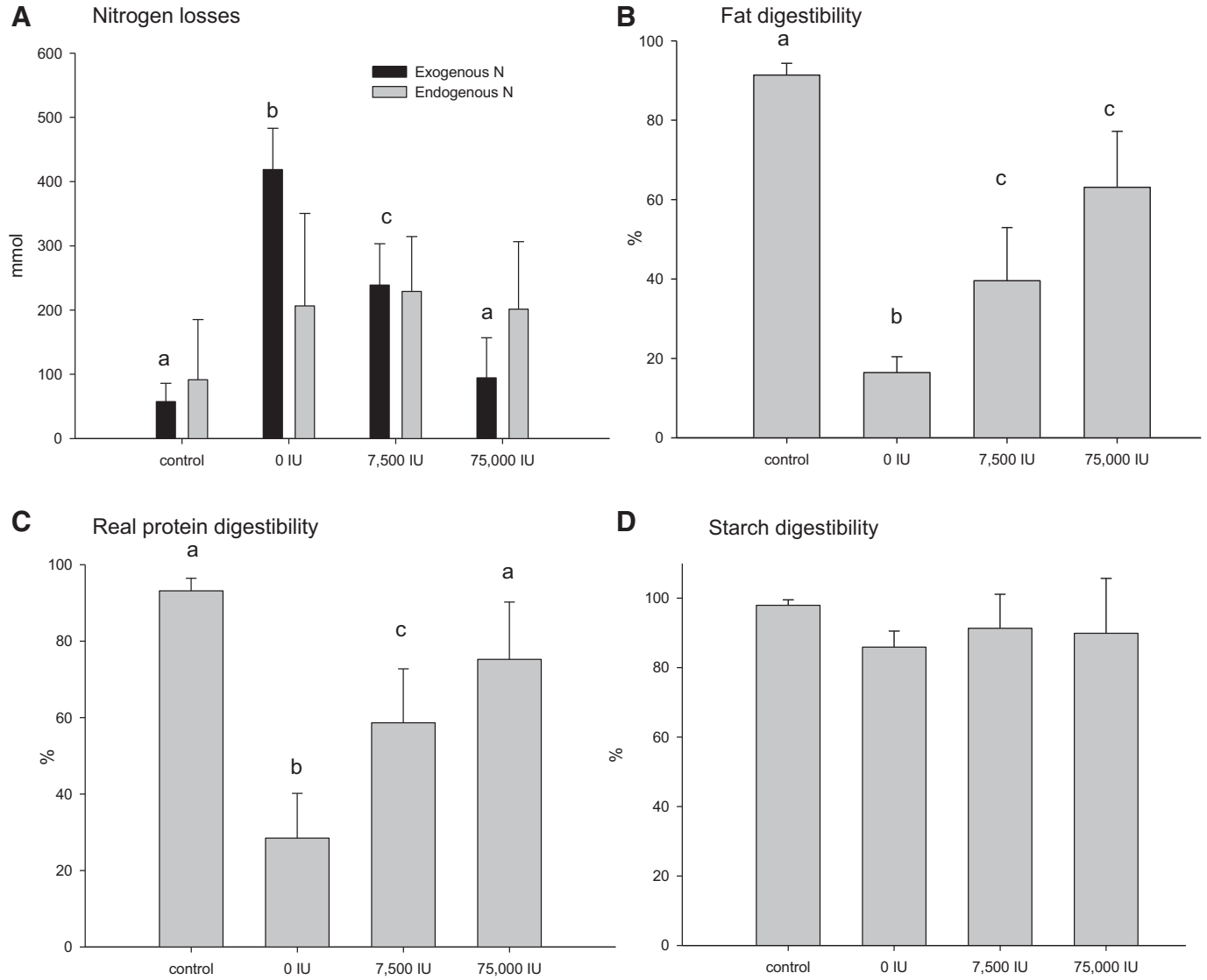


Fig. 1. Ileal N losses and nutrient digestibility after the ingestion of a $^{15}N$ mixed meal in control ($n = 4$) or pancreatic exocrine insufficiency (PEI) minipigs ($n = 6$) without (0 IU) or with enzyme substitution at 2 different levels (7,500 or 75,000 IU lipase and 388 or 3,881 IU protease). *A*: endogenous and exogenous N losses. *B*: real ileal protein digestibility. *C*: apparent ileal fat digestibility. *D*: ileal starch digestibility. Two values with different letters are statistically different ($P < 0.05$).

there was no effect of PEI and enzyme therapy on values of ileal starch digestibility.

*Plasma urea, glucose, and hormones.* Plasma urea varied significantly with time, and there was an interaction between time and treatment (Fig. 2*A*). Indeed, in the control group, urea increased after the meal, while there was no variation of plasma urea in the other groups. A time effect and an interaction between time and treatment were also observed for plasma glucose (Fig. 2*B*), due to a group effect at 1, 3, 4, and 10 h where glycemia was higher in the 7,500-IU group. For insulin (Fig. 3*A*), there was an interaction between time and group due to a higher concentration in the control group at 2, 5, and 6 h. The area under the curve (AUC) tended to be influenced by the group. There was a marked group effect on GIP concentrations (Fig. 3*B*) that were significantly lower in PEI (independently of the treatment) than in the control. The AUC of GIP was significantly lower in the 0- and 7,500-IU groups compared with the control, whereas it was intermediate in the 75,000-IU group and not different from the control group.

*Postprandial metabolism of dietary N.* The transfer of dietary N into plasma proteins was investigated for 12 h after meal ingestion (Fig. 4*A*). There was a strong effect of the group, time, and interaction among groups. The group effect was significant from 6 h after the meal until the end of the investigations. The $^{15}$N transfer was lower in the PEI group not treated with pancreatic enzymes and restored with both doses of enzymes, without any differences between either. At 12 h, the amount of dietary N incorporated in plasma proteins was 7.7 ± 1.3% of ingested N for controls and 4.9 ± 1.5% for the 0 IU group. With the doses of 7,500 and 75,000 IU, values were similar to the control.

The appearance of dietary N in plasma amino acids was investigated throughout the 12 h postprandial period (Fig. 4*B*). There was a trend for an interaction between time and group, indicating different kinetics of appearance depending on the group. In the 0-IU group, the appearance was slowed compared with the other groups. Nevertheless, the AUC did not differ among groups.

Total urinary excretion of N in urea (320 ± 300 to 395 ± 153 mmol) during the 12 h following the meal ingestion did not differ between groups (not shown). There was a trend for an effect of the group on $^{15}$N that was transferred to urinary urea ($P = 0.9$). The recovery of dietary N was 4.8 ± 2.5% in the control group, whereas it tended to be reduced in pigs with PEI (0.6 ± 0.4%) and tended to increase with treatment.

Finally, the total amount of dietary N recovered in plasma proteins and in urinary urea was 5.5 ± 2.1% in the 0-IU group, 9.2 ± 2.8% in the 7,500-IU group, 11.7 ± 3.2% in the 75,000-IU group, and 14 ± 5.1% in the control group, with a significant effect of the group ($P = 0.02$). The 0-IU group was significantly different from the control ($P = 0.02$) and tended to differ from the 7,500-IU group ($P = 0.06$).

A correlation analysis reveals that among the outcomes that were measured, the best Pearson coefficient was obtained for $^{15}$N recovery in urine (Fig. 5*A*). For plasma protein, the highest correlation was obtained 8 h after the meal (Fig. 5*B*). A similar correlation was also observed for the AUC of the $^{15}$N transfer to plasma amino acids (not shown), with $R = 0.47$ ($P = 0.02$). Finally, the pooled recovery in plasma proteins and urine (Fig. 5*C*) was also correlated to protein digestibility with $R = 0.59$ ($P = 0.007$).

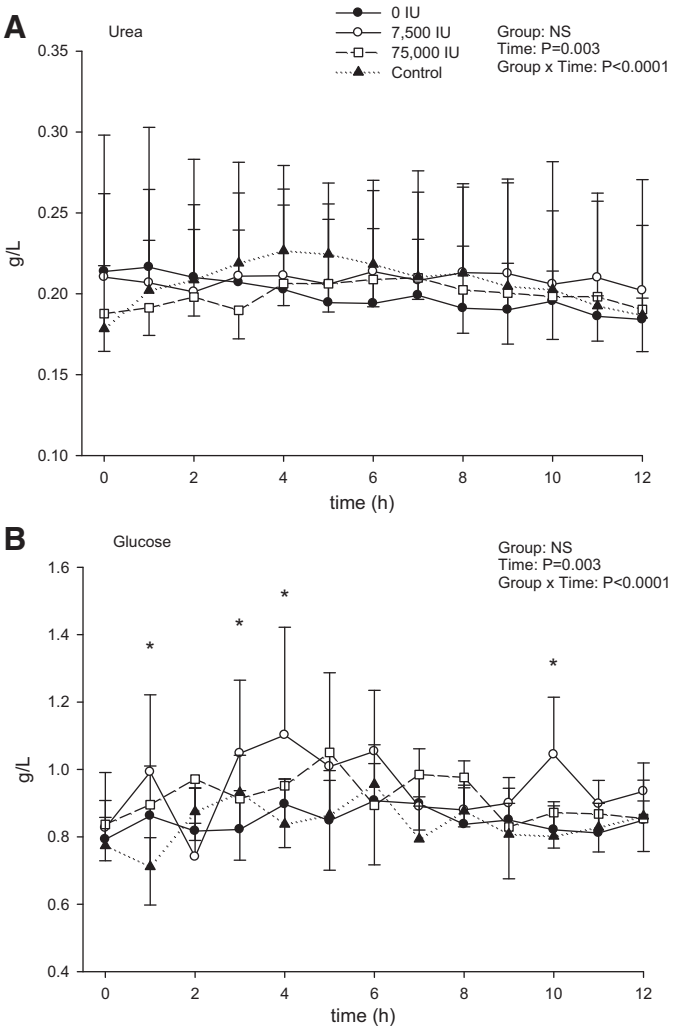


Fig. 2. Plasma urea (*A*) and glucose (*B*) after the ingestion of a $^{15}$N mixed meal in control ($n = 4$) or PEI minipigs ($n = 6$) without (0 IU) or with enzyme substitution at 2 different levels (7,500 or 75,000 IU lipase and 388 or 3,881 IU protease). *Significant effect of group at this time point.

## DISCUSSION

This study aimed to identify metabolic markers of protein malabsorption in pancreatic exocrine insufficiency. With the use of a PEI minipig model with a ligation of the pancreatic duct, pancreatic enzyme replacement therapy at different doses allowed us to obtain a gradient of protein malabsorption. We observed a positive correlation between ileal protein digestibility and the amount of $^{15}$N recovered in two metabolic pools, urinary urea and plasma proteins that we previously identified as sensitive markers in patients. However, an optimization of the methodological conditions is necessary to calibrate a test that could conveniently be used for clinical purpose.

We showed that the ligation of the pancreatic duct drastically reduced prececal absorption of dietary protein, from 89 to 29%. This value is consistent with those reported by Corring and Bourdon (8) and Kammlott et al. (16), as well as Mößeler and Kamphues (22) using the same model. This corresponds to a situation where the exocrine pancreatic secretion would be

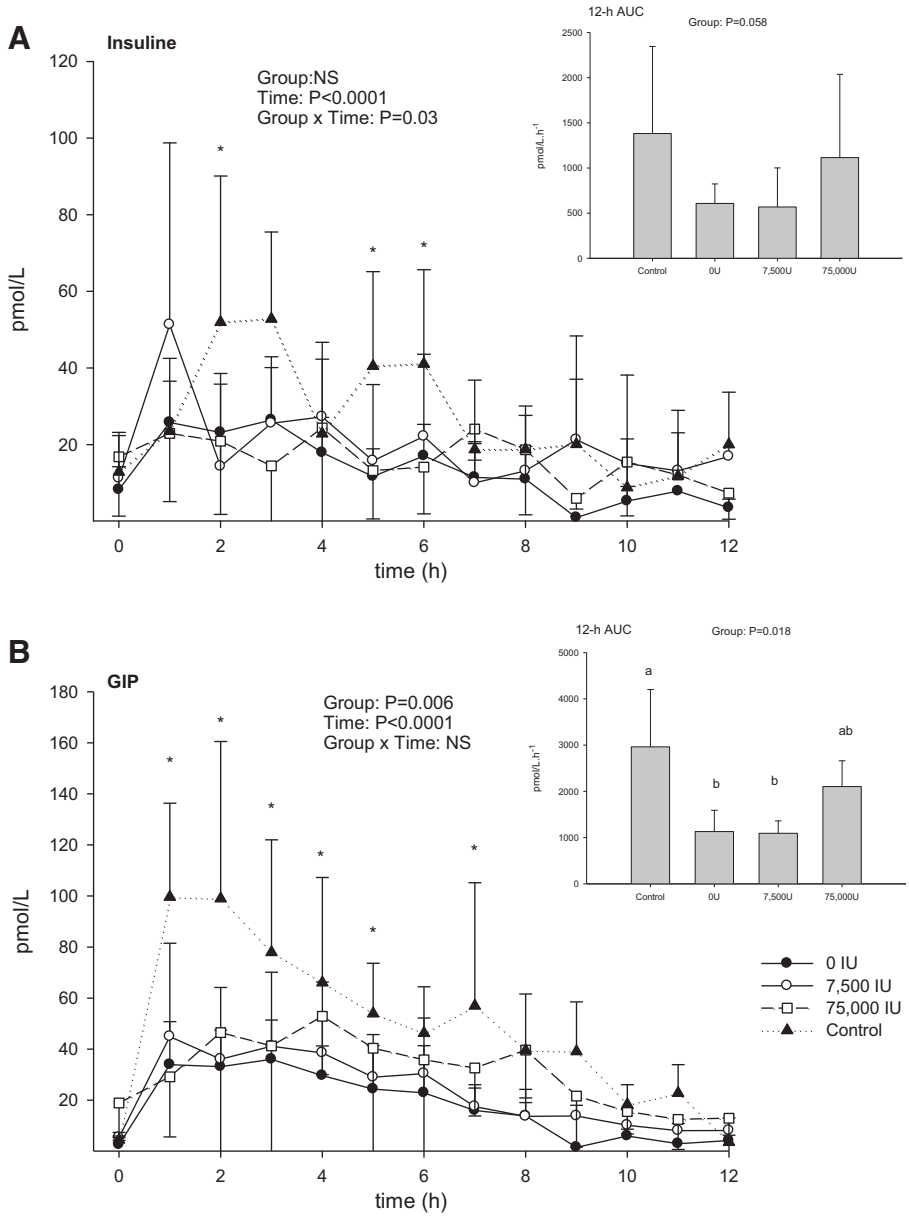


Fig. 3. Plasma insulin (*A*) and gastrointestinal peptide (GIP; *B*) and area under the curve (AUC) values after the ingestion of a mixed meal in control ($n = 4$) or PEI minipigs ($n = 6$) without (0 IU) or with enzyme substitution at 2 different levels (7,500 or 75,000 IU lipase and 388 or 3,881 IU protease). Two values with different letters are statistically different ($P < 0.05$). *Significant effect of group at this time point.

completely burned out. This indicates that the nonpancreatic enzymes, including pepsin and brush border enzymes, account for 29% of dietary protein digestion. The fact that total, but not endogenous, N losses were increased by PEI shows that the dietary protein fraction was specifically impacted. Indeed, we found a high inter-individual variability and we only observed a trend for an increase of endogenous losses in the pigs with experimentally induced PEI compared with the control, whatever the enzyme supplementation. However, in a previous study where endogenous losses were assessed using a protein-free diet, PEI significantly increased these losses (21). These discrepancies illustrate that excretion of total N is a less sensitive marker to assess protein malabsorption in PEI and provides fluctuating results (23, 31, 36). The absence of any effect of enzymes on endogenous losses may be due to resistant proteins to proteolysis, such as mucins, or to the presence of bacterial proteins to a higher extent in PEI pigs. In healthy humans, mucins and bacterial proteins represented 69 and 16% of total ileal proteins, respectively (19). Moreover, bacterial overgrowth has also been reported in humans with cystic fibrosis (13).

Some studies have reported a significant response to pancreatic enzymes on the basis of the apparent protein digestibility calculated from total N losses. It was the case in the work of Wooldridge et al. (37), in both cystic fibrosis and PEI patients. In the study of Van Hoozen (35), apparent digestibility was improved after 8 wk of enzyme therapy but not after 4 wk. In the work of Airinei et al. (1), the total losses of N in the absence of enzyme therapy were ~2 $g \cdot N^{-1} \cdot day^{-1}$, except for two subjects with a high steatorrhea (for which creatorrhea was 6 to 7 $g \cdot N^{-1} \cdot day^{-1}$). The work of Whitcomb et al. (36) also revealed a very high variability of apparent N digestibility. The use of $^{15}N$ to label dietary proteins is thus very useful to provide a sensitive evaluation of dietary protein malabsorption. A dual isotope method using a tracer dose of $^{15}N$ spirulina together with a deuterated amino acid in the meal has also been

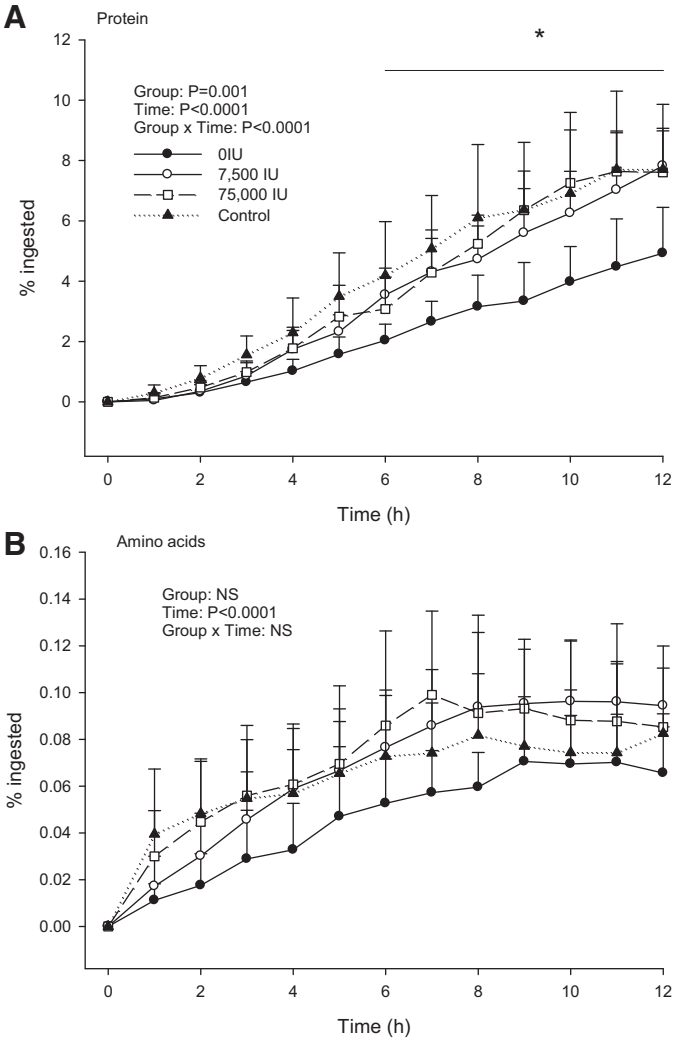


Fig. 4. Transfer of $^{15}N$ in plasma proteins (*A*) and free amino acids (*B*) after the ingestion of a mixed meal in control ($n = 4$) or PEI minipigs ($n = 6$) without (0 IU) or with enzyme substitution at 2 different levels (7,500 or 75,000 IU lipase and 388 or 3,881 IU protease). *Significant effect of group at this time point.

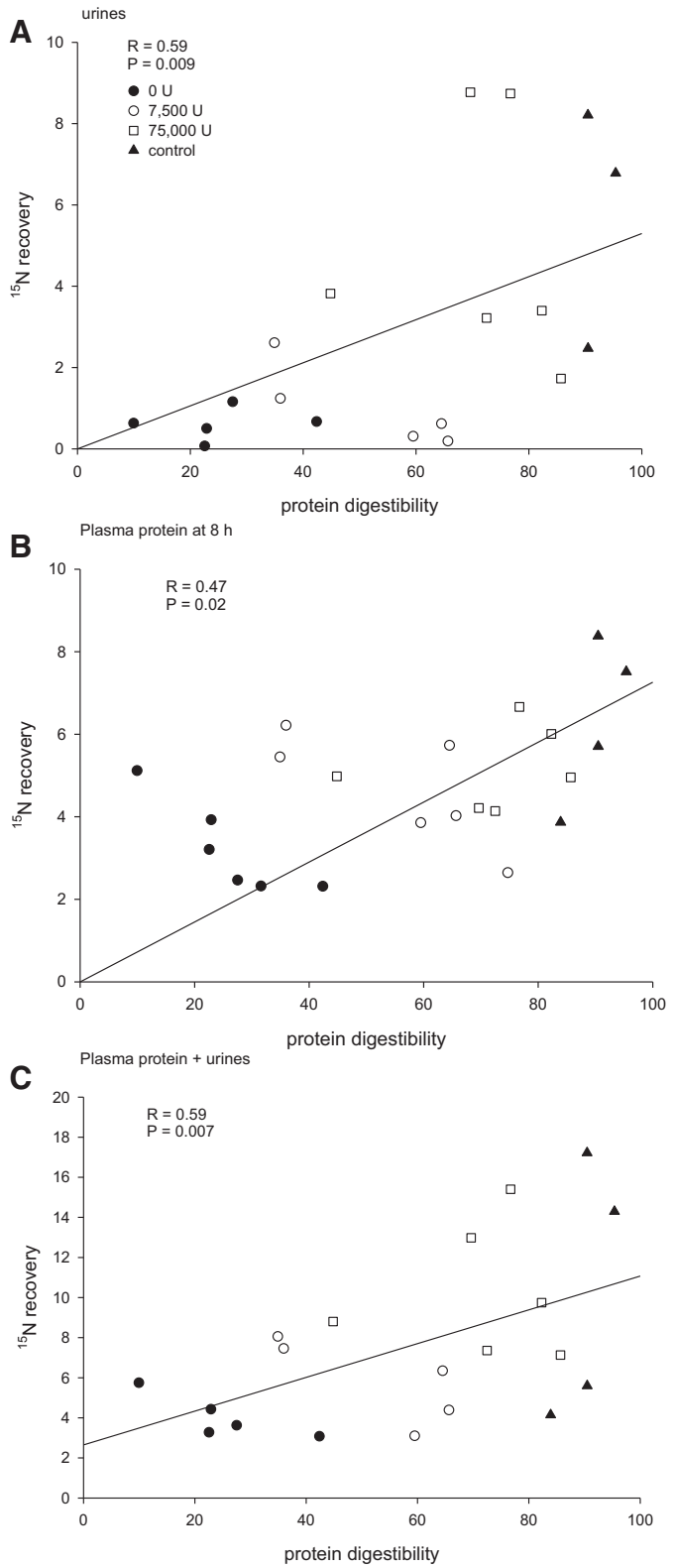


Fig. 5. Association bewteen real ileal protein digestibility and the $^{15}N$ recovery in urines (*A*), plasma proteins at 8 h (*B*), and urines + plasma proteins (*C*) after the ingestion of a $^{15}N$ mixed meal in control ($n = 4$) or PEI minipigs ($n = 6$) without (0 IU) or with enzyme substitution at 2 different levels (7,500 or 75,000 IU lipase and 388 or 3,881 IU protease). For urine, there were 3 missing values due to the absence of any urine sample.

reported to reveal a relative protein malabsorption in patients cystic fibrosis patients (11).

We showed a dose-dependent effect of pancreatic enzyme therapy, the dose of 7,500 IU being efficient but not high enough to restore the real ileal digestibility that reached 65% of the control group value. The dose of 75,000 IU increased protein digestibility to a level that was not statistically different from that of the control, although the mean value remained lower (74%) than in the control (89%), which was due to an outlier observation for which the digestibility was surprisingly low (44%). This value was nevertheless kept in the absence of any objective reason to remove it. Our present study showed an increase of real ileal protein digestibility variability in conditions of PEI, even under enzyme therapy. As previously reported in healthy volunteers, an alteration of protein digestibility is associated with an increased variance (7, 25). Fat digestibility was also drastically lowered by PEI since it fell to 20% and was dose dependently restored by enzyme therapy, a result that is consistent with other work (2, 32). In contrast, starch

digestion appeared to be slightly impacted by PEI, without reaching statistical significance. Several studies have been performed with minipigs, most showing that ileal starch digestion is moderately lower in PEI than in controls, dependent on the type and amount of starch fed (14). In patients with pancreatitis or pancreatic cancer, starch maldigestion was detected using a hydrogen breath test (18). In our work, variability of starch digestibility was also much higher in PEI minipigs, reflecting a disturbance in starch digestion. We also observed a marked effect of PEI on GIP and to a lesser extent on insulin. It has been reported in patients with pancreatitis and glucose intolerance that GIP and insulin secretions after oral fat and glucose were improved by the supplementation with pancreatin (10). More recently, Knop et al. (17) also observed better glucagon-like peptide-1 and GIP responses to a mixed meal with pancreatic enzyme substitution than without in pancreatitis patients with PEI. This effect is probably due to the improved assimilation of nutrients, especially fat and carbohydrates, which are responsible for GIP secretion (5). In our study, the effect of pancreatic enzyme treatment was unclear since we only observed a trend for an increased AUC of GIP with the highest dose of enzymes. Nevertheless, this lack of clear effect is concordant with the partial recovery of fat absorption with this dose.

Our study aimed to identify reliable markers of protein digestibility. Indeed, in humans, access to the small intestine is very invasive and nearly impossible in patients. Moreover, the fecal losses of N are not a sensitive marker, as mentioned above. The objective was to quantify the amount of $^{15}N$ in the different metabolic pools that are easily accessible. To be determined, both the $^{15}N$ enrichment and the pool size must be determined. We thus quantified this transfer of dietary N in urinary urea and plasma proteins, two pools that we previously found to be good predictors of malabsorption in patients (1). Accordingly, the transfer of dietary N into plasma proteins was significantly influenced by the group, with the incorporation being the lowest in the PEI pigs not treated with pancreatic enzymes. However, we did not observe any difference between the control group and the PEI pigs treated with pancreatic enzyme replacement therapy, no matter of enzyme dose. This absence of discrimination between these groups can be attributed to the size of the test meal containing 53 g protein, an amount that is more than twofold what we gave in pancreatitis patients, thus minimizing the impact of protein malabsorption. Indeed, considering the true digestibility that we obtained, the amount of protein absorbed in the 7,500 IU group was 30 g, an amount large enough to saturate anabolic capacities. Accordingly, we previously showed that increasing the amount of protein in the meal above the requirements did not lead to a higher incorporation of dietary N in plasma proteins (20).

The amount of dietary N transferred to urine only tended to be influenced by the group. We could notice a dispersion of the values, due to the fact that in pigs the variable collection of urine is a methodological barrier to see systematic differences with a low number of animals. As an example, one of the four control animals did not void during collection period of 12 h. In animal models, collection of urine should be standardized using, for instance, an intravenous saline infusion (27) to accurately determine urinary N loss. In humans, however, the sampling procedure is easy and accurate. Besides the methodological limits linked to urine sampling, the fact that in some supplemented PEI pigs $^{15}N$ recovery in urine was very low while digestibility was improved by enzymes may be due to N sparing. Indeed, as digestive losses are important in those animals, ammonia produced by bacterial fermentation in the colon must be partly absorbed and reincorporated in circulating amino acids. When the amino acid absorption and subsequently urea production acutely increase in the presence of enzyme therapy, metabolic pathways are still optimized to save nitrogen via urea salvage (4, 29), thus increasing urea hydrolysis and ammonia return to the nitrogen pool. It has been reported that ~80% of this reabsorption was channeled to amino acid synthesis whereas only a minor part was excreted as urea (28). In our study, a prior adaptation to enzyme therapy treatment during several days should have permitted a lowering of this phenomenon. Despite these methodological limits, the $^{15}N$ transfer in urine could be identified as the best marker of protein maldigestion, as revealed by its significant correlation with real protein digestibility. Among the 11 observations for which protein digestibility was $<70\%$, the recovery of urine was $<2\%$ of ingested N for 9 of them. On the eight observations for which protein digestibility was $>70\%$, the recovery of urine was under 2% of ingested N in only one of them. Thus, if the threshold of 2% of $^{15}N$ recovery in urines was taken as an indicator of malabsorption, this would lead to 2/11 false negative and 1/8 false-positive results. However, it would be necessary to translate such an approach in human subjects to determine this threshold value.

In conclusion, our study shows that using a single $^{15}N$ test meal, the postprandial recovery of $^{15}N$ both in urinary urea as well as in plasma proteins at 8 h is a suitable marker of protein malabsorption. Additionally, plasma GIP and insulin appear as markers of fat and starch absorption. However, the methodological conditions, in particular regarding the test meal, should be refined to be able to implement a clinical test. Such a test should be performed using a limited amount of protein (20 g) ensuring not to saturate plasma protein anabolism. It would be of clinical importance to calibrate this test to be able to diagnose protein malabsorption and thus prevent protein malnutrition in patients suffering from PEI and other pathologies associated with malabsorption.

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## DISCLOSURES

P. C. Gregory was an employee of Abbott Laboratories during the experiment. No conflicts of interest, financial or otherwise, are declared by the other authors.

## AUTHOR CONTRIBUTIONS

A.M., R.B., D.T., P.C.G., and C.G. conceived and designed research; F.M., A.M., N.K., A.F.-S., P.C.G., and C.G. performed experiments; F.M., A.M., N.K., A.F.-S., and C.G. analyzed data; F.M., A.M., N.K., D.T., P.C.G., and C.G. interpreted results of experiments; C.G. prepared figures; C.G. drafted manuscript; F.M., A.M., N.K., R.B., D.T., P.C.G., and C.G. edited and revised manuscript; F.M., A.M., N.K., A.F.-S., R.B., D.T., P.C.G., and C.G. approved final version of manuscript.

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