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Association between faecal pH and fat absorption in children with cystic fibrosis on a controlled diet and enzyme supplements dose

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Joaquim Calvo-Lerma, Maria Roca-Llorens, Ana Andrés and Carmen Ribes-Koninckx designed the research. Mieke Boon, Barbara de Koning, Carla Colombo, Maria Garriga and Etna Masip collected the samples and the data. Maria Roca-Llorens and Joaquim Calvo-Lerma analysed the data. Victoria Fornés-Ferrer performed the statistical analyses. Joaquim Calvo-Lerma, Maria Roca-Llorens, Mieke Boon, Barbara De Koning, Carla Colombo and Jessie Hulst wrote the paper. Ana Andrés, Kris de Boeck, Jessie Hulst and Carmen Ribes-Koninckx revised and corrected the paper. All authors approved the final version of the article, including the authorship list. Joaquim Calvo-Lerma is the submission's guarantor (i.e. the person who takes responsibility for the integrity of the work as a whole, from inception to published article).

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Conflict of interests

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What is the key message?

- Faecal pH is a physiological parameter that has been correlated with fat absorption in children with cystic fibrosis

What does it add to the existing literature?

- Faecal pH, as a surrogate marker of intestinal pH, could explain why pancreatic enzyme replacement therapy is not effective for all the patients
- Use of proton pump inhibitors is associated to higher values of faecal pH

What is the impcat?

- Faecal pH could be used as a biomarker to routinely monitor the efficacy of pancreatic enzyme replacement therapy in the clinical practice
- Strategies to increasing intestinal pH in children with cystic fibrosis should be targeted in the clinical practice

ABSTRACT

Background: Despite treatment with pancreatic enzyme replacement therapy (PERT), patients with Cystic Fibrosis (CF) can still suffer from fat malabsorption. A cause could be low intestinal pH disabling PERT. The aim was assessing association between faecal pH (as intestinal pH surrogate) and coefficient of fat absorption (CFA). Additionally, faecal free fatty acids (FFA) were quantified to determine the amount of digested but unabsorbed fat.

Methods: In a 24h pilot study, CF patients followed a standardized diet with fixed PERT doses, corresponding to theoretical optimal doses (TOD) determined by an *in vitro* digestion model. Study variables were faecal pH, fat and FFA excretion, CFA and transit time. Linear mixed regression models were applied to explore associations.

Results: in 43 patients, median (1^{st}, 3^{rd} quartile) faecal pH and CFA were 6.1 (5.8, 6.4) and 90% (84,94) and they were positively associated ($p<0.001$). Inverse relationship was found between faecal pH and total fat excretion ($p<0.01$), and total FFA ($p=0.048$). Higher faecal pH was associated with longer intestinal transit time ($p=0.049$) and the use of proton pump inhibitors ($p=0.009$).

Conclusions: Although the clinical significance of faecal pH is not fully defined, its usefulness as a surrogate biomarker for intestinal pH should be further explored.

Word count: 3030

INTRODUCTION

Around 85% of paediatric patients with Cystic Fibrosis (CF) suffer from pancreatic insufficiency. The obstruction of the pancreatic duct precludes the release of pancreatic enzymes into the intestine, resulting in nutrient maldigestion and malabsorption [1].

Pancreatic Enzyme Replacement Therapy (PERT) consists in the exogenous administration of encapsulated pancreatic enzymes (amylase, protease and lipase) with every meal, and is the standard of care to treat pancreatic insufficiency, and thereby to help prevent malnutrition [1,2]. While digestion of carbohydrates and protein is generally satisfactory, digestion of fat can be compromised despite of PERT [3,4]. Pancreatic lipase requires an alkaline pH for optimal activity (it is inactive at pH lower than 5.5), which is altered in CF due to decreased secretion of sodium bicarbonate into the duodenum [4,5]. The complete process of fat digestion (fat hydrolysis or lipolysis) implies the breakdown of triglyceride molecules into free fatty acids (FFA) [6] and their absorption in the intestine [7,8]. If digestion is disturbed due to abnormally low pH and/or lack of pancreatic enzymes, triglycerides would neither be hydrolysed nor absorbed and would be excreted in the faeces as non-digested fat [6,9]. On the other hand, it is possible that fat is hydrolysed into FFA, but not absorbed due to bile acid deficiency resulting in lack of micelle formation [10].

In fact, previous research confirmed that small bowel pH in CF is positively associated with the efficacy of PERT. Lipid digestion was evaluated in several studies within MyCyFAPP Project [11]; first, an *in vitro* study determined the theoretical optimal doses (TOD) of enzymes needed for maximum lipolysis from different food products, in the CF-specific intestinal environment [9]. The TODs were next evaluated in an *in vivo* pilot study: all patients adhered to the same standard 24h diet and used

the same dose of PERT (the TOD), to identify patient specific factors that influence lipolysis [12]. This resulted in a median CFA of 90% [12] suggesting that adjustment of PERT on TOD was accurate enough for adequate dietary fat digestion and absorption. However, a subgroup of the study population could not reach the 90% clinical target, and none of the studied patient characteristics so far could explain this result. Therefore, we hypothesized that a low small intestine pH could have caused insufficient PERT activity, leading to fat maldigestion and malabsorption, as previously reported in a small series of patients with CF [6]. In addition, we hypothesised that assessing FFA excretion could be an indicator of the efficacy of the administrated PERT dose, as its presence in faeces would indicate digestion was effective, but further absorption was impaired.

To test this hypothesis, the faecal samples collected in MyCyFAPP study were further assessed for pH and FFA, in addition to fat excretion quantification. Given the impossibility to assess intestinal pH in this series of patients, faecal pH was explored as a surrogate marker.

Therefore, the objective of the present study was to further investigate the association between CFA and faecal pH. Secondarily we aimed to explore total faecal FFA concentration as an additional biological indicator of the efficacy of the fat digestion process in patients with controlled dietary fat and PERT intake.

PATIENTS, MATERIALS, AND METHODS

Subjects and study design

Study patients were regularly followed at 5 European CF Centres (Madrid, Valencia, Milan, Leuven and Rotterdam), with a confirmed CF diagnosis, pancreatic insufficiency, age between 2 and 18 years in stable gastrointestinal and respiratory

conditions since at least 2 weeks before signing the informed consent. Acute infections, use of antibiotics, severe cholestasis and changes in the usual treatment during the 2 weeks before enrolment were considered exclusion criteria. Details about inclusion and exclusion criteria, and the study design are explained in detail in a previous publication by our group [12]. The Ethics Committees of all the participating centres approved the study protocol.

Patients were instructed to adhere to a standardised 24h test diet with corresponding fixed PERT doses (based on the TODs, obtained during the *in vitro* studies) [9]. In this way, the influence of patient-specific characteristics on fat digestion could be evaluated. The diet consisted of five meals, starting with an afternoon snack (bread toasted bread with butter and jam) and dinner (ham and cheese sandwich) on the first day, and breakfast (milk with breakfast cereal), morning snack (yoghurt and orange juice) and lunch (pizza and dairy desert) on the second day. Portions could be adjusted according to age and individual preferences. PERT doses were strictly instructed per each meal, corresponding to the TOD. Encapsulated dyes (E-120, red carmine and E-132, indigo blue) were ingested at start and end of the 24h fixed diet in order to identify the faeces specifically pertaining to the study period [12].

Clinical parameters obtained were age, gender, genotype, PPI use, transit time, total faecal fat (and the subsequent calculated coefficient of fat absorption), total faecal FFA and faecal pH.

Faecal analyses

The faecal samples were frozen (-20 to -80 ºC) and shipped from all the participating centres to the central lab in Instituto de Investigación Sanitaria La Fe

155 (Valencia, Spain) for analysis. Since there is no well described or generally accepted
156 standardised method for homogenisation of faecal samples in the literature [13-16], the
157 homogenisation of samples was carried out carefully after thawing at room
158 temperature. The samples that were not dyed were discarded before
159 homogenisation. Faeces were mixed with 750 W stirrers (Braun MQ735) until
160 complete homogenisation was obtained (approx. 5 minutes per sample, depending
161 on the consistency and volume).

162 *Total faecal fat*

163 Total faecal fat (which does not differentiate between triglycerides and free
164 fatty acids) was quantified on the homogenised faecal samples (10 g) with infrared
165 spectroscopy, the gold standard to analyse fat in faeces [1]. Random selected
166 samples (n=10) were used to evaluate the developed homogenisation protocol.

167 *Coefficient of fat absorption (CFA)*

168 The CFA was calculated as the percentage of grams of fat excreted in the
169 collected faeces relative to the grams of fat in the test diet [12-17]. This parameter is
170 considered equivalent to the % lipolysis extent used in the *in vitro* setting. Transit
171 time was calculated as the time between the ingestion of the colour markers and the
172 moment the first dyed-stool appeared.

173 *Free fatty acids in faeces*

174 Aliquots from the homogenised faecal samples (5 g) were lyophilised and
175 esterified. Then the FFA profile was characterised by gas chromatography-mass
176 spectrometry (GC-MS) technique [18].

177 *Faecal pH measurement*

178 Aliquots from the homogenised samples (5 g) were thawed at room
179 temperature and then mixed with 10 ml of deionized water and homogenized 1-2 min

using, firstly, a vortex and then using a horizontal shaker for 10 min (200 rpm). Faecal pH was measured by directly inserting the glass electrode of the Accumet AE150 pH Benchtop-Meter (Fisher Scientific) into the homogenized faeces. Measurements were taken twice per sample. In addition, in a random selection of 20 samples, pH was measured at three moments over a period of 24 h and repeated 1 month later to assess possible changes in the measurement over time.

Statistical analysis

A pilot study was conducted, provided that there was not an estimated effect or association to refer to given the lack of bibliography about the association between CFA and faecal pH. Therefore, an estimation of the statistical power was not required. Preparatory studies are designed to test the performance characteristics and capabilities of measures, procedures and operational strategies and or provide the means to evaluate aspects of novel approaches [19,20]. Recommended sample sizes for pilot study range between 10-30 participants for pilot studies [21].

A beta regression model was used to study the effect of the faecal pH on the CFA. Logarithmic transformation of faecal fat in stools and transit time was performed to approach a normal distribution with normalised extreme values. The association between faecal pH with the total amount of fat in stools (g) was studied by a univariate linear regression model, and the effect of the faecal pH on total FFA (mg/g faeces), and the effect of FFA on CFA, by means of a linear regression robust model). Finally, a linear regression multivariate robust model was established to study the association of age, transit time and use of PPI on faecal pH.

Analyses were carried out using the R software (version 3.5.1) and the libraries betareg (version 3.1-0), clickR (version 0.3.64) and MASS (version 7.3-49). A p value <0.05 was considered statistically significant.

RESULTS

Patients characteristics and descriptive results

Forty-three children with CF were included. The study population characteristics were previously described by Calvo-Lerma et al. and are summarized in table 1 (2019) [12].

Association of faecal pH with CFA, and free fatty acid excretion

As shown in **Table 1**, the median total amount of fat in faeces was 8.4 (4.8, 12.3) g resulting in a median CFA of 90% with a small range between 1st and 3rd Q (84, 94%). The total amount of free fatty acids was 2.3 (1.6, 3.6) mg/g of faeces. The median pH of the samples was 6.08 (5.78, 6.38), with a minimum pH of 5.26 and a maximum of 6.85.

Reproducibility of the faecal pH measurement was high, with a mean standard deviation of 0.02 when measuring the pH at three different points of the homogenized sample. Changes in the pH measurement along a 24 h period were not detected. Test results were highly reproducible (CV <15%) between two different aliquots of the same homogenised sample.

The faecal pH was significantly associated with the CFA ($p<0.001$, $R^2 = 0.42$) (**Figure 1A**), with higher pH values relating to higher CFA. This result is confirmed by the significant inverse relationship between the pH and the total amount of fat in faeces (**Figure 1B**), as the diet was fixed ($p<0.001$, $R^2 = 0.38$). This finding was also

reinforced by the inverse association between faecal pH and the total amount of FFA (**Figure 1C**) ($p = 0.048$, $R^2 = 0.35$): the lower the pH the higher the total amount of fat and FFA in faeces.

When considering patients adhered to PPI therapy separately, median faecal pH values was 6.34 (6.09, 6.72), and in patients without following this therapy the value was found to be 5.91 (5.57, 6.31), the difference being statistically significant ($p<0.01$) (**Figure 2**).

Fecal pH was positively and significantly associated with transit time ($p = 0.049$ and with the use of PPI ($p = 0.009$) in a multivariable analysis (**Table 2**). In contrast, neither the age of the patient nor any of the other study variables showed any association with faecal pH values.

In addition, the association between CFA and FFA excretion was studied (**Figure 2**). Low CFA being associated with high amount of faecal FFA ($p<0.001$) suggests that lipolysis is occurring, but the FFAs were not being absorbed. ~~Low amounts of FFA in faeces were associated with higher values of CFA ($p<0.001$), suggesting that triglyceride molecules were properly hydrolysed, resulting in FFA release and absorption. In contrast, low absorption of fat was associated with high amount of excreted FFA.~~

DISCUSSION

In this study we tried to further explore the mechanisms of fat absorption in CF. We have therefore characterised total fat and FFA excretion and pH in faecal samples of children with CF adhering to a standardised diet and using fixed doses of PERT, based on a CF specific *in vitro* digestion model. Both faecal pH and FFA were

used as an investigational tool. Faecal collection was very accurate being performed between colorimetric dyes, and this enabled also to measure transit time.

First, we found that faecal pH was positively associated with CFA, and was inversely related with total FFA. In our previous study we concluded that when using the *in vitro* predicted optimal dose of enzymes (TOD), CFA was not associated with any of the assessed patients' characteristics (genotype, nutritional status, lung function, age) with the only exception of transit time [12]. We concluded that other patients' characteristics could have had an impact on CFA, particularly intestinal pH [12]. Thus, the present findings on faecal pH, could explain why some patients did not reach the 90% target CFA, supporting the hypothesis of the present work: the association between faecal pH and CFA suggests that the TOD cannot achieve satisfactory levels of fat digestion in patients with a faecal pH lower than the pH range for lipase activity. Secondly, we found that faecal FFA is associated with CFA.

Higher CFA was related to lower FFA excretion, suggesting that in patients with satisfactory fat digestion, the absorption of delivered FFA must have been efficient, as low amounts were present in faeces. Thus, this finding also suggests that when the *in vitro* predicted dose of PERT was prescribed, fat was not only well digested but also well absorbed. Conversely, some patients with low CFA tended to have high FFA excretion, pointing at possible further malabsorption problems. However, none of these patients characteristics could explain this finding.

A previous study by our group has shown the strong influence of the intestinal pH on lipolysis under simulated *in vitro* gastrointestinal digestion [9,22,23]. One of the few studies found in the literature showed that patients on tube feeding (n = 18) with low intestinal pH profiles were unable to reach satisfactory levels of fat digestion and absorption, despite high PERT doses [6]. Compared to this study, our sample size

was larger (n = 43), patients were on a normal diet (rather than being on enteral nutrition), and faecal pH was measured as a surrogate of intestinal pH (given the impossibility to assess it because of ethical restrictions and need for invasive techniques). Robinson et al. (1990), in contrast, performed a direct measurement of the intestinal pH. Both studies, however have reached the same finding of the association between intestinal pH and fat absorption. Also, the proportion of patients with a very low intestinal pH (<5.8) was similar in both studies (around 20%). Complementarily, other digestion agents, such as bile salts concentration, have proved to exert a relevant role in fat digestion and absorption. Malabsorption of bile acids occur in patients with CF, and abnormal biliary secretion or intraluminal acidic precipitation of bile acids could contribute to steatorrhea (REF). In addition, the study of the impact of reduced bile salts concentration in the context of in vitro digestion study of several foods, has repeatedly been associated with decreased extents of lipolysis (REF).

Possible determinants for faecal pH values were explored, finding that PPI use and longer transit time were associated with higher pH values. Studies evaluating the pH profile along the gastrointestinal tract in a healthy population by using the wireless motility capsule and radiotelemetry device support the main assumption that faecal pH may be considered as a surrogate for intestinal pH, as pH in the duodenum (lipid digestion) and rectum (before faecal deposition) were equivalent [10,24,25]. In case of CF, concretely, pH along the first hour of small intestine digestion ranges between 5 and 7 [24,26], in agreement with the pH range found in the faecal samples of our patients. Overall, the mean intestinal pH reported by Aburb et al. (2018) for healthy subjects was 6.2 and, in our cohort, median 6.02 was obtained. Despite this difference could appear to be small, a decrease of 0.2 in pH in terms of

lipase enzyme activity can imply a substantial reduction of fat digestion (REF). In addition, this difference in pH becomes higher when only considering those patients not following the PPI therapy, which would be reflected in lower fat digestion.

Our findings also showed that patients taking PPI had higher faecal pH. Treatment with PPI reduces gastric acid secretion and leads to less acidic stomach environment during digestion [27]. As a consequence, the gastric contents enter the duodenum with a more alkaline pH than the usual, thus modifying the intestinal conditions towards a normal environment, despite the limited pancreatic bicarbonate secretion in patients with CF [10,18].

The strengths of this study include the accuracy of faecal collection using colorimetric markers. The samples are of unique value since for the first time to our knowledge, they represent the product of the digestion and absorption of the same diet and the same PERT doses followed by a cohort of 43 patients with CF. It was therefore possible to assess differences in CFA only in terms of individual patients' characteristics, avoiding confounding factors related to the diet or the variability in PERT. Another strength relies on the GC-MS technique used, the most accurate and state of the art method to quantify free fatty acids [28].

On the other hand, the main limitation of the present study is the assumption that faecal pH reflects the pH of the small intestine. In the colon, several reactions occur, including fermentation of unabsorbed or undigested nutrients (carbohydrates, protein) by some specific bacteria conforming the microbiota. This results in the production of short chain fatty acids, which may reduce the luminal pH. However, even if the study subjects could have different microbiota profiles, the production of short chain fatty acids is highly dependent on the pattern of food intake [29], and all of our patients had the same standardised meals. Thus, our positive findings support

328 the inclusion of microbiota assessment in future series. Another factor possibly
329 modifying the pH in the colon is related to carbohydrates malabsorption [30], which
330 however was unlikely to be present in our patients since normally it is associated to
331 liquid stools [31] (present in only one of our patients [12]). In light of the significant
332 association between faecal pH, total faecal fat and free fatty acids and CFA, along
333 with the comparable range of pH as in previous series, we suggest this indirect
334 measurement could reflect at least in the intestinal conditions. These findings
335 support conducting a future study to validate the relationship between intestinal pH
336 and faecal pH in these population, so faecal pH could be considered as a non-
337 invasive biomarker of the intestinal pH and provide insight on the mechanism of
338 malabsorption.

339 The strong association and moderate correlation between faecal pH and CFA
340 reinforces the previous data obtained by our research group, indicating that
341 strategies aimed at increasing intestinal pH may result in better fat absorption. Thus,
342 our results support the use of PPI therapy in addition to PERT in patients with low
343 CFA but already taking high doses of PERT, as previously concluded by Robinson et
344 al. (1990) [6]. However, the clinical value of using faecal pH to guide management
345 requires further evaluation. It should be always kept in mind that long-term PPI
346 treatment may lead to complications by suppressing the natural barrier of the gastric
347 acid against pathogenic bacteria [32]. It has been associated with an increase number
348 of pulmonary exacerbation and hospitalisation [33].

349 Another clinical application, although with possible limitations, would be
350 measuring pH in faeces as a complementary qualitative method to indirectly assess
351 fat digestion and absorption. It is a simple, non-invasive and fast technique that only

requires a pH-metre, a common and not expensive equipment. This technique could possibly complement the 24h faeces collection.

In conclusion, faecal pH is suggested as a possible factor explaining differences found in the CFA in a cohort of patients with CF that followed the same diet and had the same doses of PERT according to an *in vitro* evidence-based method. Strategies to increase the intestinal pH in CF are encouraged in order to enhance lipid digestion, being aware of the potential side effects. In addition, the use faecal pH as surrogate for fat malabsorption could be a fast and easy technique, which with pertinent additional validation, could be used in the clinical practice.

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457

FIGURE LEGENDS

Figure 1. Faecal pH values are (A) positively associated with the coefficient of fat absorption (CFA); (B) negatively associated with the faecal fat excreted; and (C) negatively associated with the free fatty acid (FFA) excretion. *95% CI, confidence interval of the estimated effect.*

Figure 2. Patients adhered to therapy with proton pump inhibitors (PPI) (n=14) obtained significantly higher median faecal pH than those not following this therapy (n=28).

~~**Figure 2.**~~ **Figure 3.** Free fatty acid (FFA) excretion is negatively associated with the coefficient of fat absorption (CFA). *95% CI, confidence interval of the estimated effect.*