BMJ Paediatrics Open

Fecal calprotectin and rectal histologic inflammatory markers in cystic fibrosis

Journal:	*BMJ Paediatrics Open*
Manuscript ID	bmjpo-2022-001422
Article Type:	Original research
Date Submitted by the Author:	18-Jan-2022
Complete List of Authors:	Roda, Juliana; Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Pediatric Gastroenterology and Nutrition Unit; Universidade de Coimbra Faculdade de Medicina, Clínica Universitária de Pediatria Maia, Carla; Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Pediatric Gastroenterology and Nutrition Unit Almeida, Susana; Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Pediatric Gastroenterology and Nutrition Unit Oliveira, Rui ; Centro Hospitalar e Universitário de Coimbra EPE, Pathology Department; Universidade de Coimbra Faculdade de Medicina, Coimbra Institute for Clinical and Biomedical Research (iCBR) Area of Environment Genetics and Oncobiology (CIMAGO) Ferreira, Ricardo; Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Pediatric Gastroenterology and Nutrition Unit Oliveira, Guiomar; Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Centro de Desenvolvimento da Criança e Centro de Investigação e Formação Clínica; Universidade de Coimbra Faculdade de Medicina, Clínica Universitária de Pediatria
Keywords:	Cystic Fibrosis, Gastroenterology

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Title: Fecal calprotectin and rectal histologic inflammatory markers in cystic fibrosis

Authors:

Corresponding author:

Juliana Roda

MD

Pediatric Gastroenterology and Nutrition Unit, Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Avenida Afonso Romão, 3000-602 Coimbra, Portugal

Telephone: +351 962748392

Email: juroda@hotmail.com

https://orcid.org/0000-0001-8990-779X

Carla Maia

MD

Pediatric Gastroenterology and Nutrition Unit, Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Email: carla.maia@chuc.min-saude.pt

Susana Almeida

MD

Pediatric Gastroenterology and Nutrition Unit, Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Email: susana.almeida.coimbra@gmail.com

Rui Caetano Oliveira

MD, PhD

Pathology Department, Centro Hospitalar e Universitário de Coimbra, Portugal; Biophysics Institute, Faculty of Medicine, University of Coimbra, 3000-548, Portugal

Coimbra Institute for Clinical and Biomedical Research (iCBR) Area of Environment Genetics and Oncobiology (CIMAGO), Faculty of Medicine, University of Coimbra, 3000-548, Coimbra, Portugal

Email: ruipedrocoliveira@hotmail.com

Ricardo Ferreira

MD

Pediatric Gastroenterology and Nutrition Unit, Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Email: ricardo.ferreira@chuc.min-saude.pt

Guiomar Oliveira

MD, PhD

Centro de Desenvolvimento da Criança e Centro de Investigação e Formação Clínica, Hospital Pediátrico – Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Clínica Universitária de Pediatria, Faculdade de Medicina da Universidade de Coimbra, Coimbra, Portugal

Email: guiomar@chuc.min-saude.pt

https://orcid.org/0000-0002-7049-1277

Word count: 2453 words

ABSTRACT

Objective: To analyze the association of fecal calprotectin with the genetic and clinical characteristics of pediatric patients with cystic fibrosis (PwCF). In a subset of these patients, we aimed to associate histologic inflammatory features of rectal mucosa to fecal calprotectin levels.

Methods: In a prospective study, fecal calprotectin levels were collected in all 23 PwCF attending our pediatric center, together with demographic and clinical data. Associations between fecal calprotectin and clinical features were determined. In 11 of these patients, endoscopic rectal biopsies were obtained and the association between fecal calprotectin and histologic inflammatory markers was analyzed.

Results: Median age of PwCF was 12 years, 19 had pancreatic insufficiency (PI) (19/23). Seventeen (17/23) had elevated fecal calprotectin, and the median value was 88 µg/g (IQR=178 µg/g). Higher fecal calprotectin levels were observed in the PI group (101 *vs* 30 µg/g, P=0.027). No significant correlation between elevated fecal calprotectin level and BMI z-score was found. Five patients (22%) reported abdominal pain, three (13%) complained of diarrhea and three (13%) had constipation, but these symptoms were not associated with elevated fecal calprotectin.

Unspecific focal rectal inflammation was found in four patients (4/11). An association between rectal mucosa inflammation and elevated fecal calprotectin was found (p=0.015).

Conclusions: In our PwCF, elevated fecal calprotectin was frequent, particularly if PI, and it was not related to gastrointestinal symptoms or malnutrition. Elevated fecal calprotectin was present in patients with histologic evidence of rectal inflammation. Fecal calprotectin may be a good indicator of asymptomatic rectal inflammation in PwCF.

Keywords: intestinal inflammation, rectal biopsies, pancreatic insufficiency

List of abbreviations:

BMI - Body mass index

CF - Cystic fibrosis

CFTR - Cystic Fibrosis Transmembrane conductance Regulator

IQR – Interquartile range

PERT - pancreatic enzyme replacement therapy

PI - Pancreatic insufficiency

PS - pancreatic sufficient

BACKGROUND

Cystic fibrosis (CF) is a severe autosomal recessive disease that results from mutations in a gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a chloride channel [1]. It is the most common life-limiting genetic disease among Caucasians, occurring in 1 of 2500 live births worldwide. In Portugal, a prevalence of 0.27 per 10,000 inhabitants and an incidence of 1 per 7500 newborns have been estimated [2, 3].

CF is also the most common cause of pancreatic insufficiency (PI) in children [4]. Historically, CF children died in infancy from severe malnutrition and later from respiratory failure, but due to improved clinical care, including pancreatic enzyme replacement therapy (PERT), currently, a majority of them reach adulthood [5]. Approximately 85% of CF patients have impaired digestion due to PI and despite adequate PERT, several CF patients still have malabsorption, growth impairment and gastrointestinal problems, including abdominal pain, steatorrhea, and altered motility [4, 5]. It has been suggested that digestive symptoms are attributable not only to PI but also to intestinal inflammation. However, the pathogenesis and nutritional implications of this finding remains unclear [5].

Studies, in both animals and humans, have reported evidence of intestinal inflammation in CF. In the mouse model of CF, the most common manifestation is intestinal obstruction resulting from inflammatory enteropathy, leading to perforation [6]. Furthermore, abnormal mucus accumulation in the intestines of murine models predisposes them to gut dysmotility, creating a niche for bacterial overgrowth and dysbiosis [7].

In humans, the presence of inflammatory biomarkers, including fecal calprotectin, eosinophil cationic protein, interleukin-1B and interleukin-8, was reported, suggesting that intestinal inflammation is a feature of CF [5, 8]. Videocapsule endoscopic studies elicited mucosal ulceration, erythema and mucosal breaks in the small bowel of CF patients, particularly those with PI [4]. Calprotectin is a neutrophil secretory product, and elevated fecal levels are well correlated with colonic inflammation in inflammatory bowel disease [9–11]. Dysbiosis may be

associated with intestinal inflammation as reflected by increased levels of fecal calprotectin that respond to antibiotic treatment [12]. CFTR modulators may potentially improve dysbiosis and inflammation, for example ivacaftor has been associated with a decrease in calprotectin levels[13].

However, to our knowledge, there are no studies characterizing intestinal histological inflammatory findings in CF patients and, particularly, studies analising the relationship between elevated fecal calprotectin and these histologic inflammatory findings.

The aim of the present study was to analyze, in a cohort of pediatric CF patients, the association between fecal calprotectin levels and genetic and clinical characteristics, including nutritional status and gastrointestinal symptoms. In a subset of this cohort, we also aimed to characterize histological inflammatory features of rectal mucosa and relate it to fecal calprotectin levels.

METHODS

This prospective study included children and adolescents aged 0 to 18 years followed in the Pediatric Unit of the Cystic Fibrosis Reference Centre of the Centro Hospitalar e Universitário de Coimbra, Portugal, in the year 2019. The criteria for the diagnosis of CF were: clinical characteristics compatible with CF, a positive sweat test and a genetic study with the identification of two disease-causing mutations, according to the latest consensus [14]. All patients willing to participate were included. Exclusion criteria included recent respiratory exacerbation/infection or ingestion of steroids or nonsteroidal anti-inflammatory drugs, both in the previous 4 weeks.

In all participants, demographic data, genotype and clinical data, including nutritional status and PERT medication, were collected at the time of the appointment where stool sample was obtained. Nutritional status was assessed according to the z-score of weight, height, and body mass index (BMI). Patients were asked whether they had experienced gastrointestinal

symptoms (abdominal pain, constipation or diarrhea) in the two weeks preceding the calprotectin measurement.

Exocrine pancreatic function was assessed using fecal elastase levels. PI was considered when the fecal elastase level was under 200 µg/g; above that, they were classified as pancreatic sufficient (PS).

Fecal calprotectin measurement

Stool samples were collected at home or at the CF Reference Centre. Calprotectin level in the fecal samples were measured using EliA Calprotectin® (reagents from Thermo Fisher Scientific®). Fecal calprotectin concentration was considered normal up to 50 µg/g stool, as was considered in previous studies with CF patients [4] and as it has been shown to be sensitive for detecting inflammation in children with inflammatory bowel disease [15].

Rectal biopsies

Rectal biopsies were obtained, from patients already enrolled in another study from our center, which aimed to test CFTR modulator responses in intestinal organoids from patients with rarer mutations (data not yet published; *UID/MULTI/04046/2019*). As a consequence, most of these patients had less common mutations. Time between fecal calprotectin measurement and rectal biopsies was a maximum of two days.

Rectal mucosa specimens (3-4 mm in diameter) were obtained from eleven patients, with or without sedation (depending on individuals will or collaboration) using a colonoscope and colon forceps (Endoflex®, diameter 2.8 mm). Samples were immediately stored in formalin.

One pathologist with experience in gastrointestinal pathology from the Pathology Department, Centro Hospitalar e Universitário de Coimbra, Portugal performed the histologic analysis. Samples were blinded to the clinical information and were analyzed under an optical

microscope (Nikon Eclipse 50i®). Pictures were taken with a Nikon-Digital Sight DS-Fi1® digital camera.

Since the diagnosis of CF is based mainly on bioelectrical/biochemical analyses, there are no defined standard histological classification score. Therefore, the evaluation took into consideration the density of mononucleated inflammatory population, on a semi quantitative approach: none, mild, moderate and severe.

Statistical Analysis

Statistical analyses were performed with SPSS software (v.19; SPSS Inc., Chicago, IL, USA), and a p value < 0.05 was considered statistically significant. Descriptive statistics were presented according to the normality of the data distribution using Shapiro-Wilk test. Spearman´s correlation coefficient, Mann-Whitney and Exact Fisher tests were used between patient groups to evaluate correlation, differences and associations, respectively.

Ethics Approval

The present study was performed in accordance with the Declaration of Helsinki and approved by the board of the Centro Hospitalar e Universitário de Coimbra (Portugal) after a favorable report by the Health Ethics Committee (Ref. CHUC-080-16). Informed consent was obtained by all participants aged over 16 years or by their parents or legal guardians if under 16 years old.

Patient involvement

Patients were not directly involved in setting the research question, the design or in the implementation of the project.

RESULTS

Fecal calprotectin was measured in all 23 PwCF followed in our center during one year. The median age was 12 years, aged from 2 months to 18 years old. Twelve were male. All patients with PI (19/23, 83%) were taking PERT. The F508del mutation was present in all patients in at least one allele. Fifteen (65%) patients were F508del homozygous, and the others carried one of the following mutations: R334W (n=3), 711+ 1G->T (n=2), and 2184insA (n=1), P5L (n=1) and a novel mutation, c.3321dup (n=1) (Table 1). Only two patients were taking CFTR modulator lumacaftor/ivacaftor (patient 15 and patient 20, presenting fecal calprotectin level of 347 and 142 µg/g, respectively). Sweat test of these patients did not improve with modulator drug.

Seventeen of the 23 patients (74%) had an elevated fecal calprotectin measurement, and the median value was 88 µg/g (IQR=178 µg/g). Most patients (16/19) with PI had elevated fecal calprotectin (84%). The PI group had higher fecal calprotectin levels then in the PS group (101 µg/g; IQR=234 vs 30 µg/g; IQR=53; p=0.0027).

A moderate positive correlation was found between fecal calprotectin and sweat test values (from the time of diagnosis) (r=0.46; p=0.029). No correlation was found between age and fecal calprotectin (r=-0.3; p=0.14). Also meconium ileus past history was not associated with elevated calprotectin (Table 2).

Comparing the median BMI z-score in patients with normal and elevated fecal calprotectin, no significant difference was found (-0.58 kg/m^2; IQR= 2.8 vs 0.1 kg/m^2; IQR=1.08; p=0.09). No significant correlation between fecal calprotectin level and BMI z-score was found (r=0.19; p=0.36).

All 15 patients homozygous for the F508del mutation had elevated fecal calprotectin. These PwCF have a statistically significant higher fecal calprotectin then F508del heterozygous patients (110 µg/g; IQR=217 vs 40 µg/g; IQR=54, p=0.003).

Some CF patients reported digestive symptoms: five patients (22%) reported abdominal pain, three (13%) complained of diarrhea, and three (13%) had constipation (Table 1). Gastrointestinal symptoms were not associated with elevated fecal calprotectin (Table 2).

Histologic features

Rectal biopsies were obtained from 11 of the 23 PwCF. Eight patients had the rarer mutations R334W (n=3), 711+1G-T (n=2), P5L (n=1), c.3321dup (n=1) and 2184insA (n=1), and three patients were homozygous for the F508del mutation.

Unspecific mild focal inflammation was found in the three F508del homozygous patients, all of which had elevated fecal calprotectin (Table 1). Inflammatory features were characterized as small lymphocyte and plasma cell infiltrates distributed in a vaguely nodular pattern between colonic crypts (Figure 1 – B, C and D).

Mild to moderate focal inflammation composed of plasma cells and small lymphocytes in the mucosa was found in the patient carrying F508del in one allele and the new mutation c.3321dup in the other allele (Figure 1 – A). This patient had significantly elevated fecal calprotectin (341 µg/g) (patient 7; Table 1).

There was an association between elevated fecal calprotectin levels and the presence of inflammation in rectal biopsies (Table 3). Mean calprotectin was higher in PwCF with histological inflammatory alterations comparing with PwCF with normal histology (143 µg/g; IQR=249 vs 19 µg/g; IQR=30; p=0.024). None of the mucosal samples had architectural changes, which are a major sign of chronic inflammation.

DISCUSSION

Most patients from this study (78%) had an elevated fecal calprotectin level, some reaching a maximum level of more than 300 µg/g. This is consistent with previous studies and suggests the presence of intestinal inflammation in PwCF [4, 5, 16]. The pathophysiology of intestinal

inflammation may be explained by the same triad of obstruction by mucus accumulation, inflammation and infection that causes disease in the airways of PwCF [17]. The *CFTR* gene is strongly expressed all along the intestinal tract in a cephalad-caudal gradient, *CFTR* messenger RNA levels are highest in the duodenum and levels decrease distally along the small intestine to the large intestine [17]. This "CF enteropathy" may be an independent entity in the disease process or may be due to other factors. High doses of PERT can cause inflammation and fibrosing colonopathy[18]. Inspissated intestinal secretions, mucus accumulation, constipation, slow intestinal motility, the use of proton pump inhibitors and frequent courses of antibiotics are multiple risk factors for small bowel bacterial overgrowth in CF patients, which can cause inflammation, mucosal damage and aggravate maldigestion [16]. An unfavorable intestinal microbiome may also be a stimulus for inflammation [19], and one trial with probiotics supported this hypothesis, as the use of *Lactobacillus rhamnosus GG* reduced calprotectin concentrations in CF children [19].

In our study, lower fecal calprotectin levels were found in PS patients. Elevated fecal calprotectin only in PI patients has also been reported by Dhaliwal *et al* [5]. On the other hand, sixteen of nineteen patients with PI had elevated fecal calprotectin, and the difference in fecal calprotectin levels between the PI and PS groups was impressive (101 vs 30 µg/g). This means that either PI by itself or PERT may be responsible for intestinal inflammation in these patients[18]. However, there has been reported a lack of correlation between PERT and fecal calprotectin[20]. As reported by Dumoulin *et al.,* calprotectin is subject to proteolysis by tripsin activity, which is virtually absent in PI PwCF. As a result, calprotectin proteolysis is also reduced. Therefore, calprotectin levels detected in stools of PI patients may be higher, and this may not be exclusively attributed to intestinal inflammation[21]. Perhaps, in PI PwCF, the upper limit of the considered "normal" fecal calprotectin should be higher than the value considered for inflammatory bowel disease, as no association was found with digestive symptoms [21].

Another interesting finding was that PwCF F508del homozygous have significantly higher calprotectin levels in comparison to heterozygotes. This genotype may be associated with an increased risk of more sgnificant intestinal inflammation.

Overall, only a small number of patients complained of gastrointestinal symptoms, and no association between elevated calprotectin and digestive symptoms could be found. The same conclusion has been reported even in studies where digestive symptoms were much more frequent[20].

In contrast to previous studies [5], no relationship could be found between fecal calprotectin and nutritional status or growth parameters. However, interestingly, a positive correlation was found between fecal calprotectin and sweat test values, which may be indicative of the presence of significant intestinal inflammation in patients with a more severe phenotype.

Historically, distinct histological changes, which have been interpreted as signs of mucus hypersecretion, have been reported in light microscopic studies of large intestinal mucosa from CF patients. "Hypertrophic" or enlarged goblet cells and crypts distended by accumulated mucus were described, and these changes were considered useful in the diagnosis of CF by some authors [22]. However, to our knowledge, this is the first study to specifically look for histologic evidence of inflammation in CF patients and try to associate it to fecal calprotectin.

Four out of the eleven patients to whom rectal biopsies were performed had histologic inflammatory alterations. Interestingly, all the three patients homozygous for F508del mutation had histological signs of inflammation and this may be related to the severe phenotype associated to this common mutation.

Elevated calprotectin level was associated with histologic inflammation in the rectal mucosa (Table 3) and PwCF with rectal inflammation had a significantly higher calprotectin level. This allows us to conclude that fecal calprotectin may be a good indicator of rectal inflammation in PwCF. However, the clinical meaning of this finding remains to be explained, as this did not translate into more frequent gastrointestinal symptoms or influenced nutritional status.

We are aware that our study had several limitations: it is a small and unicentric study, and biopsies were performed on a subset of our patients and limited to the rectum. Larger multicenter studies with the aim of determining serial and longitudinal studies of calprotectin levels and biopsies of the upper and lower gastrointestinal tract may help to determine clinical relevance.

However, the finding of abnormal calprotectin levels and inflammatory alterations in the intestinal mucosa in the pediatric population raises questions about the early detection of CF enteropathy.

In conclusion, there is increasing evidence that intestinal inflammation is part of CF and is present early in life, particularly in childhood and adolescence. The additional contribution of low trypsin activity, chronic enzyme dosage, dysmotility, bacterial overgrowth, dysbiosis and other unidentified factors may play a role in its multifactorial cause. Fecal calprotectin seems to be an important noninvasive biomarker of intestinal inflammation in CF patients since a relationship with histologic evidence of rectal mucosa inflammation was found. Further and larger studies need to be performed to confirm and explain the mechanisms and clinical relevance of these findings.

Ethics approval and consent to participate: The present study was performed in accordance with the Declaration of Helsinki and approved by the board of the Centro Hospitalar e Universitário de Coimbra (Portugal) after a favorable report by the Health Ethics Committee (Ref. CHUC-080-16). Informed consent was obtained by all participants aged over 16 years or by their parents or legal guardians if under 16 years old.

Consent for publication: Informed consent for publication was obtained by all participants aged over 16 years or by their parents or legal guardians if under 16 years old.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

Funding: No funding was received for the present study.

Authors' contributions:

JR conceived and designed the study, collected, analyzed and interpreted the data and wrote the first draft of the manuscript.

CM interpreted the data and critically reviewed the manuscript

SA interpreted the data and critically reviewed the manuscript.

RCO prepared Figure 1 and analyzed and interpreted the data

RF analyzed and interpreted the data and critically reviewed the manuscript.

GO critically reviewed the manuscript

Acknowledgements: Not applicable

What is already known on this topic:

- There is increasing evidence of intestinal inflammation in cystic fibrosis (CF)
- Elevated fecal calprotectin has been found in CF patients

What this study adds:

- Focal inflammation in rectal biopsies was found in CF patients and it is associated to elevated fecal calprotectin
- Fecal calprotectin may be a good indicator of intestinal inflammation in CF

REFERENCES

1. Elborn JS. Cystic fibrosis. The Lancet. 2016.

2. Marcão A, Barreto C, Pereira L, Vaz LG, Cavaco J, Casimiro A, et al. Cystic fibrosis newborn screening in Portugal: PAP value in populations with stringent rules for genetic studies. Int J Neonatal Screen. 2018.

3. Farrell PM. The prevalence of cystic fibrosis in the European Union. J Cyst Fibros. 2008.

4. Werlin SL, Benuri-Silbiger I, Kerem E, Adler SN, Goldin E, Zimmerman J, et al. Evidence of intestinal inflammation in patients with cystic fibrosis. J Pediatr Gastroenterol Nutr. 2010.

5. Dhaliwal J, Leach S, Katz T, Nahidi L, Pang T, Lee JM, et al. Intestinal inflammation and impact on growth in children with cystic fibrosis. J Pediatr Gastroenterol Nutr. 2015.

6. Norkina O, Kaur S, Ziemer D, De Lisle RC. Inflammation of the cystic fibrosis mouse small intestine. Am J Physiol - Gastrointest Liver Physiol. 2004.

7. De Lisle RC. Altered transit and bacterial overgrowth in the cystic fibrosis mouse small intestine. Am J Physiol - Gastrointest Liver Physiol. 2007.

8. Bruzzese E, Callegari ML, Raia V, Viscovo S, Scotto R, Ferrari S, et al. Disrupted intestinal microbiota and intestinal inflammation in children with cystic fibrosis and its restoration with lactobacillus gg: A randomised clinical trial. PLoS One. 2014.

9. Summerton CB, Longlands MG, Wiener K, Shreeve DR. Faecal calprotectin: A marker of inflammation throughout the intestinal tract. Eur J Gastroenterol Hepatol. 2002.

10. Montalto M, Santoro L, Curigliano V, D'Onofrio F, Cammarota G, Panunzi S, et al. Faecal calprotectin concentrations in untreated coeliac patients. Scand J Gastroenterol. 2007.

11. García-Sánchez V, Iglesias-Flores E, González R, Gisbert JP, Gallardo-Valverde JM, González-Galilea Á, et al. Does fecal calprotectin predict relapse in patients with Crohn's disease and ulcerative colitis? J Crohn's Colitis. 2010.

12. Schnapp Z, Hartman C, Livnat G, Shteinberg M, Elenberg Y. Decreased Fecal Calprotectin Levels in Cystic Fibrosis Patients after Antibiotic Treatment for Respiratory Exacerbation. J

Pediatr Gastroenterol Nutr. 2019;68:282–4.

13. Stallings VA, Sainath N, Oberle M, Bertolaso C, Schall JI. Energy Balance and Mechanisms of Weight Gain with Ivacaftor Treatment of Cystic Fibrosis Gating Mutations. J Pediatr. 2018;201:229-237.e4. doi:10.1016/J.JPEDS.2018.05.018.

14. Farrell PM, White TB, Howenstine MS, Munck A, Parad RB, Rosenfeld M, et al. Diagnosis of Cystic Fibrosis in Screened Populations. J Pediatr. 2017.

15. Sands BE. Biomarkers of Inflammation in Inflammatory Bowel Disease. Gastroenterology. 2015.

16. Rumman N, Sultan M, El-Chammas K, Goh V, Salzman N, Quintero D, et al. Calprotectin in Cystic Fibrosis. BMC Pediatr. 2014.

17. De Lisle RC, Borowitz D. The cystic fibrosis intestine. Cold Spring Harb Perspect Med. 2013.

18. Brennan GT, Saif MW. Pancreatic Enzyme Replacement Therapy: A Concise Review.

19. Bruzzese E, Raia V, Gaudiello G, Polito G, Buccigrossi V, Formicola V, et al. Intestinal inflammation is a frequent feature of cystic fibrosis and is reduced by probiotic administration. Aliment Pharmacol Ther. 2004.

20. Rumman N, El-Chammas K, Goh V, Salzman N, Quintero D, Werlin S. Calprotectin in Cystic Fibrosis. 2014. doi:10.1186/1471-2431-14-133.

21. Dumoulin EN, Van Biervliet S, Delanghe JR. Trypsin is a Potential Confounder in Calprotectin Results. J Pediatr Gastroenterol Nutr. 2015;61:e19. doi:10.1097/MPG.0000000000000906.

22. Neutra MR, Trier JS. Rectal Mucosa in Cystic Fibrosis: Morphological features before and after short term organ culture. Gastroenterology. 1978.

Table 1. Genotype, body mass index, gastrointestinal symptoms, fecal calprotectin and rectal histology of patients with cystic fibrosis.

no.	Genotype	BMI z-score kg/m²	Pancreatic function	Abdominal pain	Diarrhea	Constipation	Calprotectin (µg/g)	Histology
1	F508del/R334W	-0,42	PS	yes	no	no	19	normal
2	F508del/R334W	1,18	PS	no	no	no	15	normal
3	F508del/R334W	0,94	PS	yes	no	yes	79	normal
4	F508del/711+1G-T	-0,74	PI	no	no	yes	9	normal
5	F508del/711+1G-T	-1,99	PI	no	no	no	19	normal
6	F508del/2184insA	-4,95	PI	yes	yes	no	45	normal
7	F508del/c.3321dup	-0,22	PI	no	no	no	341	inflammation
8	F508del/P5L	-0,3	PS	no	no	no	40	normal
9	F508del/F508del	-2,37	PI	no	no	no	223	inflammation
10	F508del/F508del	-2,6	PI	no	no	no	62	inflammation
11	F508del/F508del	-0,3	PI	yes	no	yes	63	inflammation
12	F508del/F508del	-0,64	PI	no	no	no	55	-
13	F508del/F508del	0,3	PI	no	no	no	90	-
14	F508del/F508del	0,1	PI	no	no	no	352	-
15	F508del/F508del	-0,27	PI	no	yes	no	347	-
16	F508del/F508del	-0,13	PI	no	no	no	116	-
17	F508del/F508del	0,1	PI	no	no	no	101	-
18	F508del/F508del	0,55	PI	yes	yes	no	104	-
19	F508del/F508del	1,42	PI	no	no	no	88	-
20	F508del/F508del	-1,16	PI	no	no	no	142	-
21	F508del/F508del	0,03	PI	no	no	no	55	-
22	F508del/F508del	1,1	PI	no	no	no	330	-
23	F508del/F508del	1,2	PI	no	no	no	289	-

BMI – Body mass index; m- months; PI – pancreatic insufficiency; PS-pancreatic sufficiency

Table 2. Association between gastrointestinal symptoms and meconium ileus past history and normal or elevated fecal calprotectin.

n=23	Normal calprotectin (n=6)		Elevated calprotectin (n=17)		
	yes	no	yes	no	p#
Abdominal pain	2	4	3	14	0.58
Diarrhea	0	6	3	14	1
Constipation	2	4	1	16	1
Meconium ileus	1	5	4	13	1

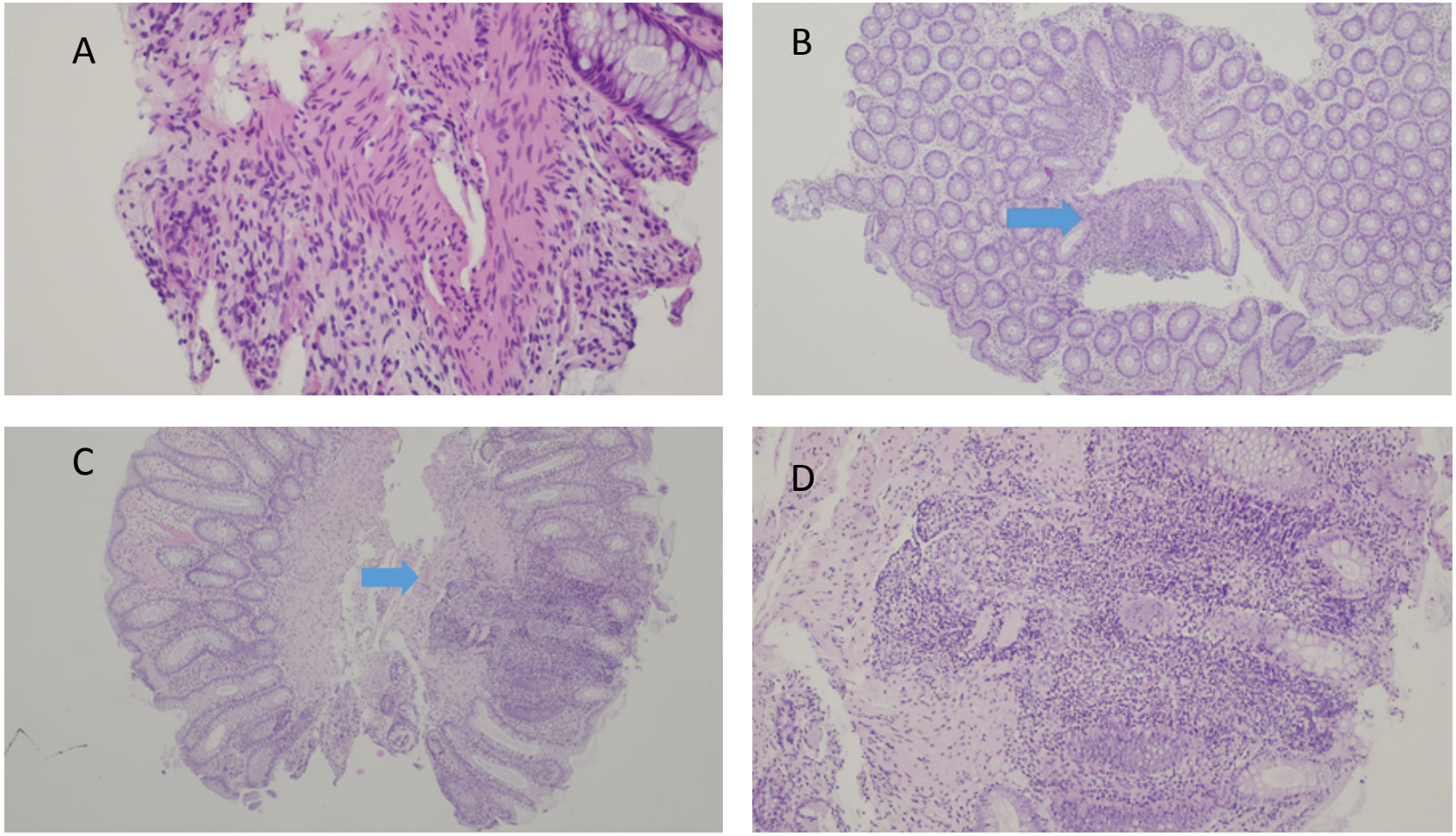
#Fisher exact test

Table 3. Comparison of histologic rectal inflammation presence between patients with normal or elevated fecal calprotectin.

(n=11)	Normal calprotectin n=6		Elevated calprotectin n=5		
	yes	no	yes	no	p#
Mucosal inflammation	0	6	4	1	0.015

#Fisher exact test

Figure 1 – A - Histologic features of patient 8 (F508del/c.3321dup) showing mild to moderate focal inflammation, composed of plasma cells and small lymphocytes, in the mucosa, H&E 200x. **B** - Histologic features of patient 9 (F508del/F508del) showing mild focal inflammation of mononucleated cells of the mucosa in a vaguely nodular pattern (blue arrow), H&E 40x; **C** - Histologic features of patient 10 (F508del/F508del) showing mild focal inflammation of the mucosa, with small lymphocytes and plasma cells, between colonic crypts (blue arrow), H&E 40x, highlighted in higher magnification **D,** H&E 200x.


A
B
C
D

BMJ Paediatrics Open

Fecal calprotectin and rectal histologic inflammatory markers in cystic fibrosis - single centre study

Journal:	*BMJ Paediatrics Open*
Manuscript ID	bmjpo-2022-001422.R1
Article Type:	Original research
Date Submitted by the Author:	15-Mar-2022
Complete List of Authors:	Roda, Juliana; Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Pediatric Gastroenterology and Nutrition Unit; Universidade de Coimbra Faculdade de Medicina, Clínica Universitária de Pediatria Maia, Carla; Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Pediatric Gastroenterology and Nutrition Unit Almeida, Susana; Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Pediatric Gastroenterology and Nutrition Unit Oliveira, Rui ; Centro Hospitalar e Universitário de Coimbra EPE, Pathology Department; Universidade de Coimbra Faculdade de Medicina, Coimbra Institute for Clinical and Biomedical Research (iCBR) Area of Environment Genetics and Oncobiology (CIMAGO) Ferreira, Ricardo; Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Pediatric Gastroenterology and Nutrition Unit Oliveira, Guiomar; Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Centro de Desenvolvimento da Criança e Centro de Investigação e Formação Clínica; Universidade de Coimbra Faculdade de Medicina, Clínica Universitária de Pediatria
Keywords:	Cystic Fibrosis, Gastroenterology

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Title: Fecal calprotectin and rectal histologic inflammatory markers in cystic fibrosis – a single centre study

Authors:

Corresponding author:

Juliana Roda

MD

Pediatric Gastroenterology and Nutrition Unit, Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Avenida Afonso Romão, 3000-602 Coimbra, Portugal

Telephone: +351 962748392

Email: juroda@hotmail.com

https://orcid.org/0000-0001-8990-779X

Carla Maia

MD

Pediatric Gastroenterology and Nutrition Unit, Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Email: carla.maia@chuc.min-saude.pt

Susana Almeida

MD

Pediatric Gastroenterology and Nutrition Unit, Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Email: susana.almeida.coimbra@gmail.com

Rui Caetano Oliveira

MD, PhD

Pathology Department, Centro Hospitalar e Universitário de Coimbra, Portugal; Biophysics Institute, Faculty of Medicine, University of Coimbra, 3000-548, Portugal

Coimbra Institute for Clinical and Biomedical Research (iCBR) Area of Environment Genetics and Oncobiology (CIMAGO), Faculty of Medicine, University of Coimbra, 3000-548, Coimbra, Portugal

Email: ruipedrocoliveira@hotmail.com

Ricardo Ferreira

MD

Pediatric Gastroenterology and Nutrition Unit, Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Email: ricardo.ferreira@chuc.min-saude.pt

Guiomar Oliveira

MD, PhD

Centro de Desenvolvimento da Criança e Centro de Investigação e Formação Clínica, Hospital Pediátrico – Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Clínica Universitária de Pediatria, Faculdade de Medicina da Universidade de Coimbra, Coimbra, Portugal

Email: guiomar@chuc.min-saude.pt

https://orcid.org/0000-0002-7049-1277

Word count: 2661 words

ABSTRACT

Objective: To analyze the association of fecal calprotectin with the genetic and clinical characteristics of pediatric patients with cystic fibrosis (PwCF). In a subset of these patients, we aimed to associate histologic inflammatory features of rectal mucosa to fecal calprotectin levels.

Methods: In a prospective study, fecal calprotectin levels were collected in all 23 PwCF attending our pediatric center, together with demographic and clinical data. Associations between fecal calprotectin and clinical features were determined. In 11 of these patients, endoscopic rectal biopsies were obtained and the association between fecal calprotectin and histologic inflammatory markers was analyzed. Statistical analyses included Spearman´s correlation coefficient, Mann-Whitney and Exact Fisher tests. Sensitivity and specificity was calculated.

Results: Median age of PwCF was 12 years, 19 had pancreatic insufficiency (PI) (19/23). Seventeen (17/23) had elevated fecal calprotectin, and the median value was 88 µg/g (IQR=178 µg/g). Higher fecal calprotectin levels were observed in the PI group (101 *vs* 30 µg/g, P=0.027). No significant correlation between elevated fecal calprotectin level and BMI z-score was found. Five patients (22%) reported abdominal pain, three (13%) complained of diarrhea and three (13%) had constipation, but these symptoms were not associated with elevated fecal calprotectin.

Unspecific focal rectal inflammation was found in four patients (4/11). An association between rectal mucosa inflammation and elevated fecal calprotectin was found (p=0.015). Sensitivity was 100% and specificity was 86%.

Conclusions: In our PwCF, elevated fecal calprotectin was frequent, particularly if PI, and it was not related to gastrointestinal symptoms or malnutrition. Elevated fecal calprotectin was

present in patients with histologic evidence of rectal inflammation. Fecal calprotectin may be an indicator of asymptomatic rectal inflammation in PwCF.

Keywords: cystic fibrosis, gastroenterology

List of abbreviations:

BMI - Body mass index

CF - Cystic fibrosis

CFTR - Cystic Fibrosis Transmembrane conductance Regulator

IQR – Interquartile range

PERT - pancreatic enzyme replacement therapy

PI - Pancreatic insufficiency

PS - pancreatic sufficient

BACKGROUND

Cystic fibrosis (CF) is a severe autosomal recessive disease that results from mutations in a gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a chloride channel [1]. It is the most common life-limiting genetic disease among Caucasians, occurring in 1 of 2500 live births worldwide. In Portugal, a prevalence of 0.27 per 10,000 inhabitants and an incidence of 1 per 7500 newborns have been estimated [2, 3].

CF is also the most common cause of pancreatic insufficiency (PI) in children [4]. Historically, CF children died in infancy from severe malnutrition and later from respiratory failure, but due to improved clinical care, including pancreatic enzyme replacement therapy (PERT), currently, a majority of them reach adulthood [5]. Approximately 85% of CF patients have impaired digestion due to PI and despite adequate PERT, several CF patients still have malabsorption, growth impairment and gastrointestinal problems, including abdominal pain, steatorrhea, and altered motility [4, 5]. It has been suggested that digestive symptoms are attributable not only to PI but also to intestinal inflammation. However, the pathogenesis and nutritional implications of this finding remains unclear [5].

Studies, in both animals and humans, have reported evidence of intestinal inflammation in CF. In the mouse model of CF, the most common manifestation is intestinal obstruction resulting from inflammatory enteropathy, leading to perforation [6]. Furthermore, abnormal mucus accumulation in the intestines of murine models predisposes them to gut dysmotility, creating a niche for bacterial overgrowth and dysbiosis [7].

In humans, the presence of inflammatory biomarkers, including fecal calprotectin, eosinophil cationic protein, interleukin-1B and interleukin-8, was reported, suggesting that intestinal inflammation is a feature of CF [5, 8]. Videocapsule endoscopic studies elicited mucosal ulceration, erythema and mucosal breaks in the small bowel of CF patients, particularly those with PI [4]. Calprotectin is a neutrophil secretory product, and elevated fecal levels are well correlated with colonic inflammation in inflammatory bowel disease [9–11]. Dysbiosis may be

associated with intestinal inflammation as reflected by increased levels of fecal calprotectin that respond to antibiotic treatment [12]. CFTR modulators may potentially improve dysbiosis and inflammation, for example ivacaftor has been associated with a decrease in calprotectin levels[13].

However, to our knowledge, there are no studies characterizing intestinal histological inflammatory findings in CF patients and, particularly, studies analising the relationship between elevated fecal calprotectin and these histologic inflammatory findings.

The aim of the present study was to analyze, in a cohort of pediatric CF patients, the association between fecal calprotectin levels and genetic and clinical characteristics, including nutritional status and gastrointestinal symptoms. In a subset of this cohort, we also aimed to characterize histological inflammatory features of rectal mucosa and relate it to fecal calprotectin levels.

METHODS

This prospective study included children and adolescents aged 0 to 18 years followed in the Pediatric Unit of the Cystic Fibrosis Reference Centre of the Centro Hospitalar e Universitário de Coimbra, Portugal, in the year 2019. The criteria for the diagnosis of CF were: clinical characteristics compatible with CF, a positive sweat test and a genetic study with the identification of two disease-causing mutations, according to the latest consensus [14]. All patients willing to participate were included. Exclusion criteria included recent respiratory exacerbation/infection or ingestion of antibiotics, steroids or nonsteroidal anti-inflammatory drugs, both in the previous 4 weeks.

In all participants, demographic data, genotype and clinical data, including nutritional status and PERT medication, were collected at the time of the appointment where stool sample was obtained. Nutritional status was assessed according to the z-score of weight, height, and body mass index (BMI). Patients were asked whether they had experienced gastrointestinal

symptoms (abdominal pain, constipation or diarrhea) in the two weeks preceding the calprotectin measurement.

Exocrine pancreatic function was assessed using fecal elastase levels. PI was considered when the fecal elastase level was under 200 µg/g; above that, they were classified as pancreatic sufficient (PS).

Fecal calprotectin measurement

Stool samples were collected at home or at the CF Reference Centre. Calprotectin level in the fecal samples were measured using EliA Calprotectin® (reagents from Thermo Fisher Scientific®). Fecal calprotectin concentration was considered normal up to 50 µg/g stool, as was considered in previous studies with CF patients [4] and as it has been shown to be sensitive for detecting inflammation in children with inflammatory bowel disease [15].

Rectal biopsies

Rectal biopsies were obtained, from patients already enrolled in another study from our center, which aimed to test CFTR modulator responses in intestinal organoids from patients with rarer mutations (data not yet published; *UID/MULTI/04046/2019*). As a consequence, most of these patients had less common mutations. Time between fecal calprotectin measurement and rectal biopsies was a maximum of two days.

Rectal mucosa specimens (3-4 mm in diameter) were obtained from eleven patients, with or without sedation (depending on individuals will or collaboration) using a colonoscope and colon forceps (Endoflex®, diameter 2.8 mm). Samples were immediately stored in formalin.

One pathologist with experience in gastrointestinal pathology from the Pathology Department, Centro Hospitalar e Universitário de Coimbra, Portugal performed the histologic analysis. Samples were blinded to the clinical information and were analyzed under an optical

microscope (Nikon Eclipse 50i®). Pictures were taken with a Nikon-Digital Sight DS-Fi1® digital camera.

Since the diagnosis of CF is based mainly on bioelectrical/biochemical analyses, there is no defined standard histological classification score. Therefore, the evaluation took into consideration the density of mononucleated inflammatory population, on a semi quantitative approach: none, mild, moderate and severe.

Statistical Analysis

Statistical analyses were performed with SPSS software (v.19; SPSS Inc., Chicago, IL, USA), and a p value < 0.05 was considered statistically significant. Descriptive statistics were presented according to the normality of the data distribution using Shapiro-Wilk test. Spearman´s correlation coefficient, Mann-Whitney and Exact Fisher tests were used between patient groups to evaluate correlation, differences and associations, respectively. Sensitivity and specificity of calprotectin as a marker of rectal inflammation was calculated.

Ethics Approval

The present study was performed in accordance with the Declaration of Helsinki and approved by the board of the Centro Hospitalar e Universitário de Coimbra (Portugal) after a favorable report by the Health Ethics Committee (Ref. CHUC-080-16). Informed consent was obtained by all participants aged over 16 years or by their parents or legal guardians if under 16 years old.

Patient involvement

Patients were not directly involved in setting the research question, the design or in the implementation of the project.

RESULTS

Fecal calprotectin was measured in all 23 PwCF followed in our center during one year. The median age was 12 years, aged from 2 months to 17 years old. Twelve were male. All patients with PI (19/23, 83%) were taking PERT. The F508del mutation was present in all patients in at least one allele. Fifteen (65%) patients were F508del homozygous, and the others carried one of the following mutations: R334W (n=3), 711+ 1G->T (n=2), and 2184insA (n=1), P5L (n=1) and a novel mutation, c.3321dup (n=1) (Table 1). Only two patients were taking CFTR modulator lumacaftor/ivacaftor (patient 15 and patient 20, presenting fecal calprotectin level of 347 and 142 µg/g, respectively). Sweat test of these patients did not improve with modulator drug.

Seventeen of the 23 patients (74%) had an elevated fecal calprotectin measurement, and the median value was 88 µg/g (IQR=178 µg/g). Most patients (16/19) with PI had elevated fecal calprotectin (84%). The PI group had higher fecal calprotectin levels then in the PS group (101 µg/g; IQR=234 vs 30 µg/g; IQR=53; p=0.0027).

A moderate positive correlation was found between fecal calprotectin and sweat test values (from the time of diagnosis) (r=0.46; p=0.029). A weak negative correlation was found between age and fecal calprotectin (r=-0.3; p=0.14). Also meconium ileus past history was not associated with elevated calprotectin (Table 2).

Comparing the median BMI z-score in patients with normal and elevated fecal calprotectin, no significant difference was found (-0.58 kg/m^2; IQR= 2.8 vs 0.1 kg/m^2; IQR=1.08; p=0.09). No significant correlation between fecal calprotectin level and BMI z-score was found (r=0.19; p=0.36).

All 15 patients homozygous for the F508del mutation had elevated fecal calprotectin. These PwCF have a statistically significant higher fecal calprotectin then F508del heterozygous patients (110 µg/g; IQR=217 vs 40 µg/g; IQR=54, p=0.003).

Some CF patients reported digestive symptoms: five patients (22%) reported abdominal pain, three (13%) complained of diarrhea, and three (13%) had constipation (Table 1). Gastrointestinal symptoms were not associated with elevated fecal calprotectin (Table 2).

Histologic features

Rectal biopsies were obtained from 11 of the 23 PwCF. Eight patients had the rarer mutations R334W (n=3), 711+1G-T (n=2), P5L (n=1), c.3321dup (n=1) and 2184insA (n=1), and three patients were homozygous for the F508del mutation.

Unspecific mild focal inflammation was found in the three F508del homozygous patients, all of which had elevated fecal calprotectin (Table 1). Inflammatory features were characterized as small lymphocyte and plasma cell infiltrates distributed in a vaguely nodular pattern between colonic crypts (Figure 1 – B, C and D).

Mild to moderate focal inflammation composed of plasma cells and small lymphocytes in the mucosa was found in the patient carrying F508del in one allele and the new mutation c.3321dup in the other allele (Figure 1 – A). This patient had significantly elevated fecal calprotectin (341 µg/g) (patient 7; Table 1).

There was an association between elevated fecal calprotectin levels and the presence of inflammation in rectal biopsies (Table 3). In our study, sensitivity of calprotectin levels was 100% and specificity was 86%.

Median calprotectin was higher in PwCF with histological inflammatory alterations comparing with PwCF with normal histology (143 µg/g; IQR=249 vs 19 µg/g; IQR=30; p=0.024). None of the mucosal samples had architectural changes, which are a major sign of chronic inflammation.

DISCUSSION

Most patients from this study (78%) had an elevated fecal calprotectin level, some reaching a maximum level of more than 300 µg/g. This is consistent with previous studies and suggests the presence of intestinal inflammation in PwCF [4, 5, 16]. The pathophysiology of intestinal inflammation may be explained by the same triad of obstruction by mucus accumulation, inflammation and infection that causes disease in the airways of PwCF [17]. The *CFTR* gene is strongly expressed all along the intestinal tract in a cephalad-caudal gradient, *CFTR* messenger RNA levels are highest in the duodenum and levels decrease distally along the small intestine to the large intestine [17]. This "CF enteropathy" may be an independent entity in the disease process or may be due to other factors. High doses of PERT can cause inflammation and fibrosing colonopathy[18]. Inspissated intestinal secretions, mucus accumulation, constipation, slow intestinal motility, the use of proton pump inhibitors and frequent courses of antibiotics are multiple risk factors for small bowel bacterial overgrowth in CF patients, which can cause inflammation, mucosal damage and aggravate maldigestion [16]. An unfavorable intestinal microbiome may also be a stimulus for inflammation [19, 20]. One trial with probiotics supported this hypothesis, as the use of *Lactobacillus rhamnosus GG* reduced calprotectin concentrations in CF children [19]. Another study, found increased abundances of Staphylococcus, Streptococcus,and Veillonella dispar, along with decreased abundances of Bacteroides, Bifidobacterium adolescentis, and Faecalibacterium prausnitzii to be associated to intestinal inflammation in PwCF in similarity to changes found in patients with Crohn´s disease [20].

In our study, lower fecal calprotectin levels were found in PS patients. Elevated fecal calprotectin only in PI patients has also been reported by Dhaliwal *et al* [5]. On the other hand, sixteen of nineteen patients with PI had elevated fecal calprotectin, and the difference in fecal calprotectin levels between the PI and PS groups was impressive (101 vs 30 µg/g). This means that either PI by itself or PERT may be responsible for intestinal inflammation in these patients[21]. However, there has been reported a lack of correlation between PERT and fecal

calprotectin[22]. As reported by Dumoulin *et al.,* calprotectin is subject to proteolysis by tripsin activity, which is virtually absent in PI PwCF. As a result, calprotectin proteolysis is also reduced. Therefore, calprotectin levels detected in stools of PI patients may be higher, and this may not be exclusively attributed to intestinal inflammation[23]. Perhaps, in PI PwCF, the upper limit of the considered "normal" fecal calprotectin should be higher than the value considered for inflammatory bowel disease, as no association was found with digestive symptoms [23, 24]. Some recent studies suggest an upper limit of >50 μg/g or 250 μg/g and it remains unclear whether reference ranges that are useful in IBD are equally applicable in CF[20, 24]. Also, the pancreatic status is related to CFTR function and genotype and intestinal inflammation may be another manifestation of the multisystemic involvement of the disease and not only influenced by pancreatic function [25].

Another interesting finding was that PwCF F508del homozygous have significantly higher calprotectin levels in comparison to heterozygotes. This genotype may be associated with an increased risk of more significant intestinal inflammation.

Overall, only a small number of patients complained of gastrointestinal symptoms, and no association between elevated calprotectin and digestive symptoms could be found. The same conclusion has been reported even in studies where digestive symptoms were much more frequent[22].

In contrast to previous studies [5], no relationship could be found between fecal calprotectin and nutritional status or growth parameters. However, interestingly, a positive correlation was found between fecal calprotectin and sweat test values, which may be indicative of the presence of significant intestinal inflammation in patients with a more severe phenotype. The negative correlation found between fecal calprotectin and age is in line with the reported tendency towards lower values with increasing age reported in the literature, even though there are no well-established cut off levels for specific age ranges[26].

Historically, distinct histological changes, which have been interpreted as signs of mucus hypersecretion, have been reported in light microscopic studies of large intestinal mucosa from CF patients. "Hypertrophic" or enlarged goblet cells and crypts distended by accumulated mucus were described, and these changes were considered useful in the diagnosis of CF by some authors [27]. In this study we were specifically looking for histologic evidence of inflammation in CF patients and try to associate it to fecal calprotectin.

Four out of the eleven patients to whom rectal biopsies were performed had histologic inflammatory alterations. Interestingly, all the three patients homozygous for F508del mutation had histological signs of inflammation and this may be related to the severe phenotype associated to this common mutation.

Elevated calprotectin level was associated with histologic inflammation in the rectal mucosa (Table 3) and PwCF with rectal inflammation had a significantly higher calprotectin level. High sensitivity and specificity, allows us to conclude that fecal calprotectin may be a good indicator of rectal inflammation in PwCF. However, the clinical meaning of this finding remains to be explained, as this did not translate into more frequent gastrointestinal symptoms or influenced nutritional status. However, as the life expectancy of PwCF is increasing there has been reported an increased risk of gastrointestinal malignancies. Chronic intestinal inflammation is a risk factor for cancer development and this should probably be addressed early in life[24].

We are aware that our study had several limitations: it is a small and unicentric study, and biopsies were performed on a subset of our patients and limited to the rectum. Larger multicenter studies with the aim of determining serial and longitudinal studies of calprotectin levels and biopsies of the upper and lower gastrointestinal tract may help to determine clinical relevance.

However, the finding of abnormal calprotectin levels and inflammatory alterations in the intestinal mucosa in the pediatric population raises questions about the early detection of CF enteropathy.

In conclusion, there is increasing evidence that intestinal inflammation is part of CF and is present early in life, particularly in childhood and adolescence. The additional contribution of low trypsin activity, chronic enzyme dosage, dysmotility, bacterial overgrowth, dysbiosis and other unidentified factors may play a role in its multifactorial cause. Fecal calprotectin may be considered a noninvasive biomarker of intestinal inflammation in CF patients since a relationship with histologic evidence of rectal mucosa inflammation was found. Further and larger studies need to be performed to confirm and explain the mechanisms and clinical relevance of these findings.

Ethics approval and consent to participate: The present study was performed in accordance with the Declaration of Helsinki and approved by the board of the Centro Hospitalar e Universitário de Coimbra (Portugal) after a favorable report by the Health Ethics Committee (Ref. CHUC-080-16). Informed consent was obtained by all participants aged over 16 years or by their parents or legal guardians if under 16 years old.

Consent for publication: Informed consent for publication was obtained by all participants aged over 16 years or by their parents or legal guardians if under 16 years old.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

Funding: No funding was received for the present study.

Authors' contributions:

JR conceived and designed the study, collected, analyzed and interpreted the data and wrote the first draft of the manuscript.

CM interpreted the data and critically reviewed the manuscript

SA interpreted the data and critically reviewed the manuscript.

RCO prepared Figure 1 and analyzed and interpreted the data

RF analyzed and interpreted the data and critically reviewed the manuscript.

GO critically reviewed the manuscript

Acknowledgements: Not applicable

What is already known on this topic:

- There is increasing evidence of intestinal inflammation in cystic fibrosis (CF)
- Elevated fecal calprotectin has been found in CF patients

What this study adds:

- Focal inflammation in rectal biopsies was found in CF patients and it is associated to elevated fecal calprotectin
- Fecal calprotectin may be an indicator of intestinal inflammation in CF

REFERENCES

1. Elborn JS. Cystic fibrosis. The Lancet. 2016.

2. Marcão A, Barreto C, Pereira L, Vaz LG, Cavaco J, Casimiro A, et al. Cystic fibrosis newborn screening in Portugal: PAP value in populations with stringent rules for genetic studies. Int J Neonatal Screen. 2018.

3. Farrell PM. The prevalence of cystic fibrosis in the European Union. J Cyst Fibros. 2008.

4. Werlin SL, Benuri-Silbiger I, Kerem E, Adler SN, Goldin E, Zimmerman J, et al. Evidence of intestinal inflammation in patients with cystic fibrosis. J Pediatr Gastroenterol Nutr. 2010.

5. Dhaliwal J, Leach S, Katz T, Nahidi L, Pang T, Lee JM, et al. Intestinal inflammation and impact on growth in children with cystic fibrosis. J Pediatr Gastroenterol Nutr. 2015.

6. Norkina O, Kaur S, Ziemer D, De Lisle RC. Inflammation of the cystic fibrosis mouse small intestine. Am J Physiol - Gastrointest Liver Physiol. 2004.

7. De Lisle RC. Altered transit and bacterial overgrowth in the cystic fibrosis mouse small intestine. Am J Physiol - Gastrointest Liver Physiol. 2007.

8. Bruzzese E, Callegari ML, Raia V, Viscovo S, Scotto R, Ferrari S, et al. Disrupted intestinal microbiota and intestinal inflammation in children with cystic fibrosis and its restoration with lactobacillus gg: A randomised clinical trial. PLoS One. 2014.

9. Summerton CB, Longlands MG, Wiener K, Shreeve DR. Faecal calprotectin: A marker of inflammation throughout the intestinal tract. Eur J Gastroenterol Hepatol. 2002.

10. Montalto M, Santoro L, Curigliano V, D'Onofrio F, Cammarota G, Panunzi S, et al. Faecal calprotectin concentrations in untreated coeliac patients. Scand J Gastroenterol. 2007.

11. García-Sánchez V, Iglesias-Flores E, González R, Gisbert JP, Gallardo-Valverde JM, González-Galilea Á, et al. Does fecal calprotectin predict relapse in patients with Crohn's disease and ulcerative colitis? J Crohn's Colitis. 2010.

12. Schnapp Z, Hartman C, Livnat G, Shteinberg M, Elenberg Y. Decreased Fecal Calprotectin Levels in Cystic Fibrosis Patients after Antibiotic Treatment for Respiratory Exacerbation. J

Pediatr Gastroenterol Nutr. 2019;68:282–4.

13. Stallings VA, Sainath N, Oberle M, Bertolaso C, Schall JI. Energy Balance and Mechanisms of Weight Gain with Ivacaftor Treatment of Cystic Fibrosis Gating Mutations. J Pediatr. 2018;201:229-237.e4. doi:10.1016/J.JPEDS.2018.05.018.

14. Farrell PM, White TB, Howenstine MS, Munck A, Parad RB, Rosenfeld M, et al. Diagnosis of Cystic Fibrosis in Screened Populations. J Pediatr. 2017.

15. Sands BE. Biomarkers of Inflammation in Inflammatory Bowel Disease. Gastroenterology. 2015.

16. Rumman N, Sultan M, El-Chammas K, Goh V, Salzman N, Quintero D, et al. Calprotectin in Cystic Fibrosis. BMC Pediatr. 2014.

17. De Lisle RC, Borowitz D. The cystic fibrosis intestine. Cold Spring Harb Perspect Med. 2013.

18. Sankararaman S, Schindler T, Sferra TJ. Management of Exocrine Pancreatic Insufficiency in Children. Invit Rev Nutr Clin Pract. 2019;34:27–42.

19. Bruzzese E, Raia V, Gaudiello G, Polito G, Buccigrossi V, Formicola V, et al. Intestinal inflammation is a frequent feature of cystic fibrosis and is reduced by probiotic administration. Aliment Pharmacol Ther. 2004.

20. Enaud R, Hooks KB, Barre A, Barnetche T, Hubert C, Massot M, et al. 2019, 8, 645. J Clin Med. 2019;8:645. doi:10.3390/jcm8050645.

21. Brennan GT, Saif MW. Pancreatic Enzyme Replacement Therapy: A Concise Review.

22. Rumman N, El-Chammas K, Goh V, Salzman N, Quintero D, Werlin S. Calprotectin in Cystic Fibrosis. 2014. doi:10.1186/1471-2431-14-133.

23. Dumoulin EN, Van Biervliet S, Delanghe JR. Trypsin is a Potential Confounder in Calprotectin Results. J Pediatr Gastroenterol Nutr. 2015;61:e19. doi:10.1097/MPG.0000000000000906.

24. Stagi S, Tam RY, Van Dorst JM, Mckay I, Coffey M, Ooi CY. Clinical Medicine Intestinal Inflammation and Alterations in the Gut Microbiota in Cystic Fibrosis: A Review of the Current

Evidence, Pathophysiology and Future Directions. J Clin Med. 2022;2022:649.

25. Ahmed N, Corey M, Forstner G, Zielenski J, Tsui LC, Ellis L, et al. Molecular consequences of cystic fibrosis transmembrane regulator (CFTR) gene mutations in the exocrine pancreas. Gut. 2003;52:1159–64.

26. Koninckx CR, Donat E, Benninga MA, Broekaert IJ, Gottrand F, Kolho KL, et al. The Use of Fecal Calprotectin Testing in Paediatric Disorders: A Position Paper of the European Society for Paediatric Gastroenterology and Nutrition Gastroenterology Committee. J Pediatr Gastroenterol Nutr. 2021;72:617–40.

27. Neutra MR, Trier JS. Rectal Mucosa in Cystic Fibrosis: Morphological features before and after short term organ culture. Gastroenterology. 1978.

Table 1. Genotype, body mass index, gastrointestinal symptoms, fecal calprotectin and rectal histology of patients with cystic fibrosis.

no.	Genotype	BMI z-score kg/m²	Pancreatic function	Abdominal pain	Diarrhea	Constipation	Calprotectin (µg/g)	Histology
1	F508del/R334W	-0,42	PS	yes	no	no	19	normal
2	F508del/R334W	1,18	PS	no	no	no	15	normal
3	F508del/R334W	0,94	PS	yes	no	yes	79	normal
4	F508del/711+1G-T	-0,74	PI	no	no	yes	9	normal
5	F508del/711+1G-T	-1,99	PI	no	no	no	19	normal
6	F508del/2184insA	-4,95	PI	yes	yes	no	45	normal
7	F508del/c.3321dup	-0,22	PI	no	no	no	341	inflammation
8	F508del/P5L	-0,3	PS	no	no	no	40	normal
9	F508del/F508del	-2,37	PI	no	no	no	223	inflammation
10	F508del/F508del	-2,6	PI	no	no	no	62	inflammation
11	F508del/F508del	-0,3	PI	yes	no	yes	63	inflammation
12	F508del/F508del	-0,64	PI	no	no	no	55	-
13	F508del/F508del	0,3	PI	no	no	no	90	-
14	F508del/F508del	0,1	PI	no	no	no	352	-
15	F508del/F508del	-0,27	PI	no	yes	no	347	-
16	F508del/F508del	-0,13	PI	no	no	no	116	-
17	F508del/F508del	0,1	PI	no	no	no	101	-
18	F508del/F508del	0,55	PI	yes	yes	no	104	-
19	F508del/F508del	1,42	PI	no	no	no	88	-
20	F508del/F508del	-1,16	PI	no	no	no	142	-
21	F508del/F508del	0,03	PI	no	no	no	55	-
22	F508del/F508del	1,1	PI	no	no	no	330	-
23	F508del/F508del	1,2	PI	no	no	no	289	-

BMI – Body mass index; m- months; PI – pancreatic insufficiency; PS-pancreatic sufficiency

Table 2. Association between gastrointestinal symptoms and meconium ileus past history and normal or elevated fecal calprotectin.

n=23	Normal calprotectin (n=6)		Elevated calprotectin (n=17)		
	yes	no	yes	no	p-value#
Abdominal pain	2	4	3	14	0.58
Diarrhea	0	6	3	14	1
Constipation	2	4	1	16	1
Meconium ileus	1	5	4	13	1

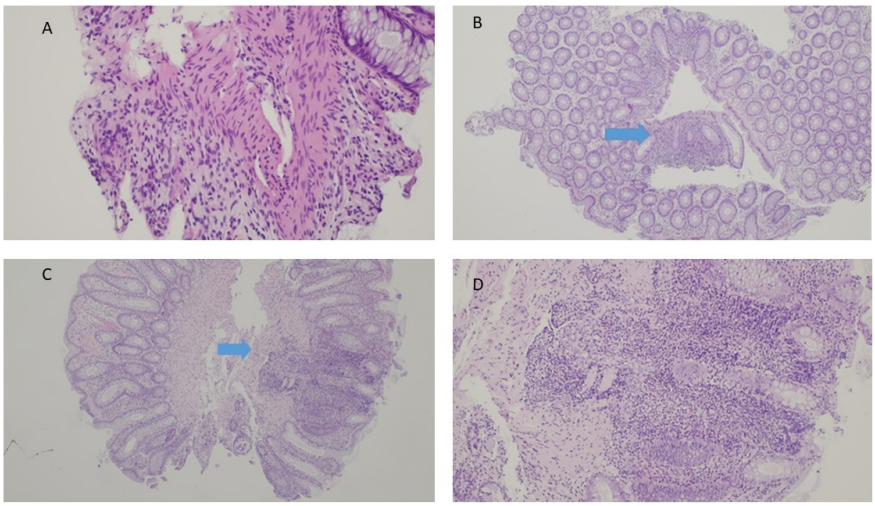
#Fisher exact test

Table 3. Comparison of histologic rectal inflammation presence between patients with normal or elevated fecal calprotectin.

(n=11)	Normal calprotectin n=6		Elevated calprotectin n=5	
	yes	no	yes	no
Mucosal inflammation	0	6	4	1

#Fisher exact test p-value=0.015

Figure 1 – A - Histologic features of patient 8 (F508del/c.3321dup) showing mild to moderate focal inflammation, composed of plasma cells and small lymphocytes, in the mucosa, H&E 200x. **B** - Histologic features of patient 9 (F508del/F508del) showing mild focal inflammation of mononucleated cells of the mucosa in a vaguely nodular pattern (blue arrow), H&E 40x; **C** - Histologic features of patient 10 (F508del/F508del) showing mild focal inflammation of the mucosa, with small lymphocytes and plasma cells, between colonic crypts (blue arrow), H&E 40x, highlighted in higher magnification **D,** H&E 200x.



338x190mm (96 x 96 DPI)

BMJ Paediatrics Open

Fecal calprotectin and rectal histologic inflammatory markers in cystic fibrosis - single centre study

Journal:	*BMJ Paediatrics Open*
Manuscript ID	bmjpo-2022-001422.R2
Article Type:	Original research
Date Submitted by the Author:	10-Apr-2022
Complete List of Authors:	Roda, Juliana; Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Pediatric Gastroenterology and Nutrition Unit; Universidade de Coimbra Faculdade de Medicina, Clínica Universitária de Pediatria Maia, Carla; Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Pediatric Gastroenterology and Nutrition Unit Almeida, Susana; Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Pediatric Gastroenterology and Nutrition Unit Oliveira, Rui ; Centro Hospitalar e Universitário de Coimbra EPE, Pathology Department; Universidade de Coimbra Faculdade de Medicina, Coimbra Institute for Clinical and Biomedical Research (iCBR) Area of Environment Genetics and Oncobiology (CIMAGO) Ferreira, Ricardo; Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Pediatric Gastroenterology and Nutrition Unit Oliveira, Guiomar; Centro Hospitalar e Universitario de Coimbra EPE Hospital Pediátrico de Coimbra, Centro de Desenvolvimento da Criança e Centro de Investigação e Formação Clínica; Universidade de Coimbra Faculdade de Medicina, Clínica Universitária de Pediatria
Keywords:	Cystic Fibrosis, Gastroenterology

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Title: Fecal calprotectin and rectal histologic inflammatory markers in cystic fibrosis – a single centre study

Authors:

Corresponding author:

Juliana Roda

MD

Pediatric Gastroenterology and Nutrition Unit, Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Avenida Afonso Romão, 3000-602 Coimbra, Portugal

Telephone: +351 962748392

Email: juroda@hotmail.com

https://orcid.org/0000-0001-8990-779X

Carla Maia

MD

Pediatric Gastroenterology and Nutrition Unit, Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Email: carla.maia@chuc.min-saude.pt

Susana Almeida

MD

Pediatric Gastroenterology and Nutrition Unit, Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Email: susana.almeida.coimbra@gmail.com

Rui Caetano Oliveira

MD, PhD

Pathology Department, Centro Hospitalar e Universitário de Coimbra, Portugal; Biophysics Institute, Faculty of Medicine, University of Coimbra, 3000-548, Portugal

Coimbra Institute for Clinical and Biomedical Research (iCBR) Area of Environment Genetics and Oncobiology (CIMAGO), Faculty of Medicine, University of Coimbra, 3000-548, Coimbra, Portugal

Email: ruipedrocoliveira@hotmail.com

Ricardo Ferreira

MD

Pediatric Gastroenterology and Nutrition Unit, Hospital Pediátrico - Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Email: ricardo.ferreira@chuc.min-saude.pt

Guiomar Oliveira

MD, PhD

Centro de Desenvolvimento da Criança e Centro de Investigação e Formação Clínica, Hospital Pediátrico – Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Clínica Universitária de Pediatria, Faculdade de Medicina da Universidade de Coimbra, Coimbra, Portugal

Email: guiomar@chuc.min-saude.pt

https://orcid.org/0000-0002-7049-1277

Word count: 2687 words

ABSTRACT

Objective: To analyze the association of fecal calprotectin with the genetic and clinical characteristics of pediatric patients with cystic fibrosis (PwCF). In a subset of these patients, we aimed to associate histologic inflammatory features of rectal mucosa to fecal calprotectin levels.

Methods: In a prospective study, fecal calprotectin levels were collected in all 23 PwCF attending our pediatric center, together with demographic and clinical data. Associations between fecal calprotectin and clinical features were determined. In 11 of these patients, endoscopic rectal biopsies were obtained and the association between fecal calprotectin and histologic inflammatory markers was analyzed. Statistical analyses included Spearman´s correlation coefficient, Mann-Whitney and Exact Fisher tests. Sensitivity and specificity was calculated.

Results: Median age of PwCF was 12 years, 19 had pancreatic insufficiency (PI) (19/23). Seventeen (17/23) had elevated fecal calprotectin, and the median value was 88 µg/g (IQR=178 µg/g). Higher fecal calprotectin levels were observed in the PI group (101 *vs* 30 µg/g, P=0.027). No significant correlation between elevated fecal calprotectin level and BMI z-score was found. Five patients (22%) reported abdominal pain, three (13%) complained of diarrhea and three (13%) had constipation, but these symptoms were not associated with elevated fecal calprotectin.

Unspecific focal rectal inflammation was found in four patients (4/11). An association between rectal mucosa inflammation and elevated fecal calprotectin was found (p=0.015). Sensitivity was 100% and specificity was 86%.

Conclusions: In our PwCF, elevated fecal calprotectin was frequent, particularly if PI, and it was not related to gastrointestinal symptoms or malnutrition. Elevated fecal calprotectin was

present in patients with histologic evidence of rectal inflammation. Fecal calprotectin may be an indicator of asymptomatic rectal inflammation in PwCF.

Keywords: cystic fibrosis, gastroenterology

What is already known on this topic:

- There is increasing evidence of intestinal inflammation in cystic fibrosis (CF)
- Elevated fecal calprotectin has been found in CF patients

What this study adds:

- Elevated fecal calprotectin is frequent, particularly if pancreatic insufficient patients with CF
- Focal inflammation in rectal biopsies was found in CF patients and it is associated to elevated fecal calprotectin

How this study might affect research, practice or policy: Fecal calprotectin may be used as an indicator of rectal inflammation in CF

List of abbreviations:

BMI - Body mass index

CF - Cystic fibrosis

CFTR - Cystic Fibrosis Transmembrane conductance Regulator

IQR – Interquartile range

PERT - pancreatic enzyme replacement therapy

PI - Pancreatic insufficiency

PS - pancreatic sufficient

INTRODUCTION

Cystic fibrosis (CF) is a severe autosomal recessive disease that results from mutations in a gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a chloride channel [1]. It is the most common life-limiting genetic disease among Caucasians, occurring in 1 of 2500 live births worldwide. In Portugal, a prevalence of 0.27 per 10,000 inhabitants and an incidence of 1 per 7500 newborns have been estimated [2, 3].

CF is also the most common cause of pancreatic insufficiency (PI) in children [4]. Historically, CF children died in infancy from severe malnutrition and later from respiratory failure, but due to improved clinical care, including pancreatic enzyme replacement therapy (PERT), currently, a majority of them reach adulthood [5]. Approximately 85% of CF patients have impaired digestion due to PI and despite adequate PERT, several CF patients still have malabsorption, growth impairment and gastrointestinal problems, including abdominal pain, steatorrhea, and altered motility [4, 5]. It has been suggested that digestive symptoms are attributable not only to PI but also to intestinal inflammation. However, the pathogenesis and nutritional implications of this finding remains unclear [5].

Studies, in both animals and humans, have reported evidence of intestinal inflammation in CF. In the mouse model of CF, the most common manifestation is intestinal obstruction resulting from inflammatory enteropathy, leading to perforation [6]. Furthermore, abnormal mucus accumulation in the intestines of murine models predisposes them to gut dysmotility, creating a niche for bacterial overgrowth and dysbiosis [7].

In humans, the presence of inflammatory biomarkers, including fecal calprotectin, eosinophil cationic protein, interleukin-1B and interleukin-8, was reported, suggesting that intestinal inflammation is a feature of CF [5, 8]. Videocapsule endoscopic studies elicited mucosal ulceration, erythema and mucosal breaks in the small bowel of CF patients, particularly those with PI [4]. Calprotectin is a neutrophil secretory product, and elevated fecal levels are well correlated with colonic inflammation in inflammatory bowel disease [9–11]. Dysbiosis may be

associated with intestinal inflammation as reflected by increased levels of fecal calprotectin that respond to antibiotic treatment [12]. CFTR modulators may potentially improve dysbiosis and inflammation, for example ivacaftor has been associated with a decrease in calprotectin levels[13].

However, to our knowledge, there are no studies characterizing intestinal histological inflammatory findings in CF patients and, particularly, studies analising the relationship between elevated fecal calprotectin and these histologic inflammatory findings.

The aim of the present study was to analyze, in a cohort of pediatric CF patients, the association between fecal calprotectin levels and genetic and clinical characteristics, including nutritional status and gastrointestinal symptoms. In a subset of this cohort, we also aimed to characterize histological inflammatory features of rectal mucosa and relate it to fecal calprotectin levels.

METHODS

This prospective study included children and adolescents aged 0 to 18 years followed in the Pediatric Unit of the Cystic Fibrosis Reference Centre of the Centro Hospitalar e Universitário de Coimbra, Portugal, in the year 2019. The criteria for the diagnosis of CF were: clinical characteristics compatible with CF, a positive sweat test and a genetic study with the identification of two disease-causing mutations, according to the latest consensus [14]. All patients willing to participate were included. Exclusion criteria included recent respiratory exacerbation/infection or ingestion of antibiotics, steroids or nonsteroidal anti-inflammatory drugs, both in the previous 4 weeks.

In all participants, demographic data, genotype and clinical data, including nutritional status and PERT medication, were collected at the time of the appointment where stool sample was obtained. Nutritional status was assessed according to the z-score of weight, height, and body mass index (BMI). Patients were asked whether they had experienced gastrointestinal

symptoms (abdominal pain, constipation or diarrhea) in the two weeks preceding the calprotectin measurement.

Exocrine pancreatic function was assessed using fecal elastase levels. PI was considered when the fecal elastase level was under 200 µg/g; above that, they were classified as pancreatic sufficient (PS).

Fecal calprotectin measurement

Stool samples were collected at home or at the CF Reference Centre. Calprotectin level in the fecal samples were measured using EliA Calprotectin® (reagents from Thermo Fisher Scientific®). Fecal calprotectin concentration was considered normal up to 50 µg/g stool, as was considered in previous studies with CF patients [4] and as it has been shown to be sensitive for detecting inflammation in children with inflammatory bowel disease [15].

Rectal biopsies

Rectal biopsies were obtained, from patients already enrolled in another study from our center, which aimed to test CFTR modulator responses in intestinal organoids from patients with rarer mutations (data not yet published; *UID/MULTI/04046/2019*). As a consequence, most of these patients had less common mutations. Time between fecal calprotectin measurement and rectal biopsies was a maximum of two days.

Rectal mucosa specimens (3-4 mm in diameter) were obtained from eleven patients, with or without sedation (depending on individuals will or collaboration) using a colonoscope and colon forceps (Endoflex®, diameter 2.8 mm). Samples were immediately stored in formalin.

One pathologist with experience in gastrointestinal pathology from the Pathology Department, Centro Hospitalar e Universitário de Coimbra, Portugal performed the histologic analysis. Samples were blinded to the clinical information and were analyzed under an optical

microscope (Nikon Eclipse 50i®). Pictures were taken with a Nikon-Digital Sight DS-Fi1® digital camera.

Since the diagnosis of CF is based mainly on bioelectrical/biochemical analyses, there is no defined standard histological classification score. Therefore, the evaluation took into consideration the density of mononucleated inflammatory population, on a semi quantitative approach: none, mild, moderate and severe.

Statistical Analysis

Statistical analyses were performed with SPSS software (v.19; SPSS Inc., Chicago, IL, USA), and a p value < 0.05 was considered statistically significant. Descriptive statistics were presented according to the normality of the data distribution using Shapiro-Wilk test. Spearman´s correlation coefficient, Mann-Whitney and Exact Fisher tests were used between patient groups to evaluate correlation, differences and associations, respectively. Sensitivity and specificity of calprotectin as a marker of rectal inflammation was calculated.

Ethics Approval

The present study was performed in accordance with the Declaration of Helsinki and approved by the board of the Centro Hospitalar e Universitário de Coimbra (Portugal) after a favorable report by the Health Ethics Committee (Ref. CHUC-080-16). Informed consent was obtained by all participants aged over 16 years or by their parents or legal guardians if under 16 years old.

Patient involvement

Patients were not directly involved in setting the research question, the design or in the implementation of the project.

RESULTS

Fecal calprotectin was measured in all 23 PwCF followed in our center during one year. The median age was 12 years, aged from 2 months to 17 years old. Twelve were male. All patients with PI (19/23, 83%) were taking PERT. The F508del mutation was present in all patients in at least one allele. Fifteen (65%) patients were F508del homozygous, and the others carried one of the following mutations: R334W (n=3), 711+ 1G->T (n=2), and 2184insA (n=1), P5L (n=1) and a novel mutation, c.3321dup (n=1) (Table 1). Only two patients were taking CFTR modulator lumacaftor/ivacaftor (patient 15 and patient 20, presenting fecal calprotectin level of 347 and 142 µg/g, respectively). Sweat test of these patients did not improve with modulator drug.

Seventeen of the 23 patients (74%) had an elevated fecal calprotectin measurement, and the median value was 88 µg/g (IQR=178 µg/g). Most patients (16/19) with PI had elevated fecal calprotectin (84%). The PI group had higher fecal calprotectin levels then in the PS group (101 µg/g; IQR=234 vs 30 µg/g; IQR=53; p=0.0027).

A moderate positive correlation was found between fecal calprotectin and sweat test values (from the time of diagnosis) (r=0.46; p=0.029). A weak negative correlation was found between age and fecal calprotectin (r=-0.3; p=0.14). Also meconium ileus past history was not associated with elevated calprotectin (Table 2).

Comparing the median BMI z-score in patients with normal and elevated fecal calprotectin, no significant difference was found (-0.58 kg/m^2; IQR= 2.8 vs 0.1 kg/m^2; IQR=1.08; p=0.09). No significant correlation between fecal calprotectin level and BMI z-score was found (r=0.19; p=0.36).

All 15 patients homozygous for the F508del mutation had elevated fecal calprotectin. These PwCF have a statistically significant higher fecal calprotectin then F508del heterozygous patients (110 µg/g; IQR=217 vs 40 µg/g; IQR=54, p=0.003).

Some CF patients reported digestive symptoms: five patients (22%) reported abdominal pain, three (13%) complained of diarrhea, and three (13%) had constipation (Table 1). Gastrointestinal symptoms were not associated with elevated fecal calprotectin (Table 2).

Histologic features

Rectal biopsies were obtained from 11 of the 23 PwCF. Eight patients had the rarer mutations R334W (n=3), 711+1G-T (n=2), P5L (n=1), c.3321dup (n=1) and 2184insA (n=1), and three patients were homozygous for the F508del mutation.

Unspecific mild focal inflammation was found in the three F508del homozygous patients, all of which had elevated fecal calprotectin (Table 1). Inflammatory features were characterized as small lymphocyte and plasma cell infiltrates distributed in a vaguely nodular pattern between colonic crypts (Figure 1 – B, C and D).

Mild to moderate focal inflammation composed of plasma cells and small lymphocytes in the mucosa was found in the patient carrying F508del in one allele and the new mutation c.3321dup in the other allele (Figure 1 – A). This patient had significantly elevated fecal calprotectin (341 µg/g) (patient 7; Table 1).

There was an association between elevated fecal calprotectin levels and the presence of inflammation in rectal biopsies (Table 3). In our study, sensitivity of calprotectin levels was 100% and specificity was 86%.

Median calprotectin was higher in PwCF with histological inflammatory alterations comparing with PwCF with normal histology (143 µg/g; IQR=249 vs 19 µg/g; IQR=30; p=0.024). None of the mucosal samples had architectural changes, which are a major sign of chronic inflammation.

DISCUSSION

Most patients from this study (78%) had an elevated fecal calprotectin level, some reaching a maximum level of more than 300 µg/g. This is consistent with previous studies and suggests the presence of intestinal inflammation in PwCF [4, 5, 16]. The pathophysiology of intestinal inflammation may be explained by the same triad of obstruction by mucus accumulation, inflammation and infection that causes disease in the airways of PwCF [17]. The *CFTR* gene is strongly expressed all along the intestinal tract in a cephalad-caudal gradient, *CFTR* messenger RNA levels are highest in the duodenum and levels decrease distally along the small intestine to the large intestine [17]. This "CF enteropathy" may be an independent entity in the disease process or may be due to other factors. High doses of PERT can cause inflammation and fibrosing colonopathy[18]. Inspissated intestinal secretions, mucus accumulation, constipation, slow intestinal motility, the use of proton pump inhibitors and frequent courses of antibiotics are multiple risk factors for small bowel bacterial overgrowth in CF patients, which can cause inflammation, mucosal damage and aggravate maldigestion [16]. An unfavorable intestinal microbiome may also be a stimulus for inflammation [19, 20]. One trial with probiotics supported this hypothesis, as the use of *Lactobacillus rhamnosus GG* reduced calprotectin concentrations in CF children [19]. Another study, found increased abundances of Staphylococcus, Streptococcus,and Veillonella dispar, along with decreased abundances of Bacteroides, Bifidobacterium adolescentis, and Faecalibacterium prausnitzii to be associated to intestinal inflammation in PwCF in similarity to changes found in patients with Crohn´s disease [20].

In our study, lower fecal calprotectin levels were found in PS patients. Elevated fecal calprotectin only in PI patients has also been reported by Dhaliwal *et al* [5]. On the other hand, sixteen of nineteen patients with PI had elevated fecal calprotectin, and the difference in fecal calprotectin levels between the PI and PS groups was impressive (101 vs 30 µg/g). This means that either PI by itself or PERT may be responsible for intestinal inflammation in these patients[21]. However, there has been reported a lack of correlation between PERT and fecal

calprotectin[22]. As reported by Dumoulin *et al.,* calprotectin is subject to proteolysis by tripsin activity, which is virtually absent in PI PwCF. As a result, calprotectin proteolysis is also reduced. Therefore, calprotectin levels detected in stools of PI patients may be higher, and this may not be exclusively attributed to intestinal inflammation[23]. Perhaps, in PI PwCF, the upper limit of the considered "normal" fecal calprotectin should be higher than the value considered for inflammatory bowel disease, as no association was found with digestive symptoms [23, 24]. Some recent studies suggest an upper limit of >50 μg/g or 250 μg/g and it remains unclear whether reference ranges that are useful in IBD are equally applicable in CF[20, 24]. Also, the pancreatic status is related to CFTR function and genotype and intestinal inflammation may be another manifestation of the multisystemic involvement of the disease and not only influenced by pancreatic function [25].

Another interesting finding was that PwCF F508del homozygous have significantly higher calprotectin levels in comparison to heterozygotes. This genotype may be associated with an increased risk of more significant intestinal inflammation.

Overall, only a small number of patients complained of gastrointestinal symptoms, and no association between elevated calprotectin and digestive symptoms could be found. The same conclusion has been reported even in studies where digestive symptoms were much more frequent[22].

In contrast to previous studies [5], no relationship could be found between fecal calprotectin and nutritional status or growth parameters. However, interestingly, a positive correlation was found between fecal calprotectin and sweat test values, which may be indicative of the presence of significant intestinal inflammation in patients with a more severe phenotype. The negative correlation found between fecal calprotectin and age is in line with the reported tendency towards lower values with increasing age in healthy individuals, even though there are no well-established cut off levels for specific age ranges[26]. However, it is in contrast with

some studies that found an increase in calprotectin with age in patients with CF, particularly in those with severe disease[27–29].

Historically, distinct histological changes, which have been interpreted as signs of mucus hypersecretion, have been reported in light microscopic studies of large intestinal mucosa from CF patients. "Hypertrophic" or enlarged goblet cells and crypts distended by accumulated mucus were described, and these changes were considered useful in the diagnosis of CF by some authors [30]. In this study we were specifically looking for histologic evidence of inflammation in CF patients and try to associate it to fecal calprotectin.

Four out of the eleven patients to whom rectal biopsies were performed had histologic inflammatory alterations. Interestingly, all the three patients homozygous for F508del mutation had histological signs of inflammation and this may be related to the severe phenotype associated to this common mutation.

Elevated calprotectin level was associated with histologic inflammation in the rectal mucosa (Table 3) and PwCF with rectal inflammation had a significantly higher calprotectin level. High sensitivity and specificity, allows us to conclude that fecal calprotectin may be a good indicator of rectal inflammation in PwCF. However, the clinical meaning of this finding remains to be explained, as this did not translate into more frequent gastrointestinal symptoms or influenced nutritional status. However, as the life expectancy of PwCF is increasing there has been reported an increased risk of gastrointestinal malignancies. Chronic intestinal inflammation is a risk factor for cancer development and this should probably be addressed early in life[24].

We are aware that our study had several limitations: it is a small and unicentric study, and biopsies were performed on a subset of our patients and limited to the rectum. Larger multicenter studies with the aim of determining serial and longitudinal studies of calprotectin levels and biopsies of the upper and lower gastrointestinal tract may help to determine clinical relevance.

However, the finding of abnormal calprotectin levels and inflammatory alterations in the intestinal mucosa in the pediatric population raises questions about the early detection of CF enteropathy.

In conclusion, there is increasing evidence that intestinal inflammation is part of CF and is present early in life, particularly in childhood and adolescence. The additional contribution of low trypsin activity, chronic enzyme dosage, dysmotility, bacterial overgrowth, dysbiosis and other unidentified factors may play a role in its multifactorial cause. Fecal calprotectin may be considered a noninvasive biomarker of intestinal inflammation in CF patients since a relationship with histologic evidence of rectal mucosa inflammation was found. Further and larger studies need to be performed to confirm and explain the mechanisms and clinical relevance of these findings.

Funding: This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests: The authors declare that they have no competing interests.

Authors' contributions:

JR conceived and designed the study, collected, analyzed and interpreted the data and wrote the first draft of the manuscript.

CM interpreted the data and critically reviewed the manuscript

SA interpreted the data and critically reviewed the manuscript.

RCO prepared Figure 1 and analyzed and interpreted the data

RF analyzed and interpreted the data and critically reviewed the manuscript.

GO critically reviewed the manuscript

Acknowledgements: Not applicable

Ethics approval and consent to participate: The present study was performed in accordance with the Declaration of Helsinki and approved by the board of the Centro Hospitalar e Universitário de Coimbra (Portugal) after a favorable report by the Health Ethics Committee (Ref. CHUC-080-16). Informed consent was obtained by all participants aged over 16 years or by their parents or legal guardians if under 16 years old.

Consent for publication: Informed consent for publication was obtained by all participants aged over 16 years or by their parents or legal guardians if under 16 years old.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on request.

REFERENCES

1. Elborn JS. Cystic fibrosis. The Lancet. 2016.

2. Marcão A, Barreto C, Pereira L, Vaz LG, Cavaco J, Casimiro A, et al. Cystic fibrosis newborn screening in Portugal: PAP value in populations with stringent rules for genetic studies. Int J Neonatal Screen. 2018.

3. Farrell PM. The prevalence of cystic fibrosis in the European Union. J Cyst Fibros. 2008.

4. Werlin SL, Benuri-Silbiger I, Kerem E, Adler SN, Goldin E, Zimmerman J, et al. Evidence of intestinal inflammation in patients with cystic fibrosis. J Pediatr Gastroenterol Nutr. 2010.

5. Dhaliwal J, Leach S, Katz T, Nahidi L, Pang T, Lee JM, et al. Intestinal inflammation and impact on growth in children with cystic fibrosis. J Pediatr Gastroenterol Nutr. 2015.

6. Norkina O, Kaur S, Ziemer D, De Lisle RC. Inflammation of the cystic fibrosis mouse small intestine. Am J Physiol - Gastrointest Liver Physiol. 2004.

7. De Lisle RC. Altered transit and bacterial overgrowth in the cystic fibrosis mouse small intestine. Am J Physiol - Gastrointest Liver Physiol. 2007.

8. Bruzzese E, Callegari ML, Raia V, Viscovo S, Scotto R, Ferrari S, et al. Disrupted intestinal microbiota and intestinal inflammation in children with cystic fibrosis and its restoration with lactobacillus gg: A randomised clinical trial. PLoS One. 2014.

9. Summerton CB, Longlands MG, Wiener K, Shreeve DR. Faecal calprotectin: A marker of inflammation throughout the intestinal tract. Eur J Gastroenterol Hepatol. 2002.

10. Montalto M, Santoro L, Curigliano V, D'Onofrio F, Cammarota G, Panunzi S, et al. Faecal calprotectin concentrations in untreated coeliac patients. Scand J Gastroenterol. 2007.

11. García-Sánchez V, Iglesias-Flores E, González R, Gisbert JP, Gallardo-Valverde JM, González-Galilea Á, et al. Does fecal calprotectin predict relapse in patients with Crohn's disease and ulcerative colitis? J Crohn's Colitis. 2010.

12. Schnapp Z, Hartman C, Livnat G, Shteinberg M, Elenberg Y. Decreased Fecal Calprotectin Levels in Cystic Fibrosis Patients after Antibiotic Treatment for Respiratory Exacerbation. J

Pediatr Gastroenterol Nutr. 2019;68:282–4.

13. Stallings VA, Sainath N, Oberle M, Bertolaso C, Schall JI. Energy Balance and Mechanisms of Weight Gain with Ivacaftor Treatment of Cystic Fibrosis Gating Mutations. J Pediatr. 2018;201:229-237.e4. doi:10.1016/J.JPEDS.2018.05.018.

14. Farrell PM, White TB, Howenstine MS, Munck A, Parad RB, Rosenfeld M, et al. Diagnosis of Cystic Fibrosis in Screened Populations. J Pediatr. 2017.

15. Sands BE. Biomarkers of Inflammation in Inflammatory Bowel Disease. Gastroenterology. 2015.

16. Rumman N, Sultan M, El-Chammas K, Goh V, Salzman N, Quintero D, et al. Calprotectin in Cystic Fibrosis. BMC Pediatr. 2014.

17. De Lisle RC, Borowitz D. The cystic fibrosis intestine. Cold Spring Harb Perspect Med. 2013.

18. Sankararaman S, Schindler T, Sferra TJ. Management of Exocrine Pancreatic Insufficiency in Children. Invit Rev Nutr Clin Pract. 2019;34:27–42.

19. Bruzzese E, Raia V, Gaudiello G, Polito G, Buccigrossi V, Formicola V, et al. Intestinal inflammation is a frequent feature of cystic fibrosis and is reduced by probiotic administration. Aliment Pharmacol Ther. 2004.

20. Enaud R, Hooks KB, Barre A, Barnetche T, Hubert C, Massot M, et al. 2019, 8, 645. J Clin Med. 2019;8:645. doi:10.3390/jcm8050645.

21. Brennan GT, Saif MW. Pancreatic Enzyme Replacement Therapy: A Concise Review.

22. Rumman N, El-Chammas K, Goh V, Salzman N, Quintero D, Werlin S. Calprotectin in Cystic Fibrosis. 2014. doi:10.1186/1471-2431-14-133.

23. Dumoulin EN, Van Biervliet S, Delanghe JR. Trypsin is a Potential Confounder in Calprotectin Results. J Pediatr Gastroenterol Nutr. 2015;61:e19. doi:10.1097/MPG.0000000000000906.

24. Stagi S, Tam RY, Van Dorst JM, Mckay I, Coffey M, Ooi CY. Clinical Medicine Intestinal Inflammation and Alterations in the Gut Microbiota in Cystic Fibrosis: A Review of the Current

Evidence, Pathophysiology and Future Directions. J Clin Med. 2022;2022:649.

25. Ahmed N, Corey M, Forstner G, Zielenski J, Tsui LC, Ellis L, et al. Molecular consequences of cystic fibrosis transmembrane regulator (CFTR) gene mutations in the exocrine pancreas. Gut. 2003;52:1159–64.

26. Koninckx CR, Donat E, Benninga MA, Broekaert IJ, Gottrand F, Kolho KL, et al. The Use of Fecal Calprotectin Testing in Paediatric Disorders: A Position Paper of the European Society for Paediatric Gastroenterology and Nutrition Gastroenterology Committee. J Pediatr Gastroenterol Nutr. 2021;72:617–40.

27. Parisi GF, Papale M, Rotolo N, Aloisio D, Tardino L, Scuderi MG, et al. Severe disease in Cystic Fibrosis and fecal calprotectin levels. Immunobiology. 2017;222:582–6.

28. Więcek S, Woś H, Kordys-Darmolińska B, Sankiewicz-Szkółka M, Grzybowska-Chlebowczyk U. The concentration of calprotectin in the stools of children with diagnosed cystic fibrosis. Gastroenterol Rev. 2017;12:38–43.

29. Garg M, Leach ST, Coffey MJ, Katz T, Strachan R, Pang T, et al. Age-dependent variation of fecal calprotectin in cystic fibrosis and healthy children. J Cyst Fibros. 2017;16:631–6. doi:10.1016/j.jcf.2017.03.010.

30. Neutra MR, Trier JS. Rectal Mucosa in Cystic Fibrosis: Morphological features before and after short term organ culture. Gastroenterology. 1978.

Table 1. Genotype, body mass index, gastrointestinal symptoms, fecal calprotectin and rectal histology of patients with cystic fibrosis.

no.	Genotype	BMI z-score kg/m²	Pancreatic function	Abdominal pain	Diarrhea	Constipation	Calprotectin (µg/g)	Histology
1	F508del/R334W	-0,42	PS	yes	no	no	19	normal
2	F508del/R334W	1,18	PS	no	no	no	15	normal
3	F508del/R334W	0,94	PS	yes	no	yes	79	normal
4	F508del/711+1G-T	-0,74	PI	no	no	yes	9	normal
5	F508del/711+1G-T	-1,99	PI	no	no	no	19	normal
6	F508del/2184insA	-4,95	PI	yes	yes	no	45	normal
7	F508del/c.3321dup	-0,22	PI	no	no	no	341	inflammation
8	F508del/P5L	-0,3	PS	no	no	no	40	normal
9	F508del/F508del	-2,37	PI	no	no	no	223	inflammation
10	F508del/F508del	-2,6	PI	no	no	no	62	inflammation
11	F508del/F508del	-0,3	PI	yes	no	yes	63	inflammation
12	F508del/F508del	-0,64	PI	no	no	no	55	-
13	F508del/F508del	0,3	PI	no	no	no	90	-
14	F508del/F508del	0,1	PI	no	no	no	352	-
15	F508del/F508del	-0,27	PI	no	yes	no	347	-
16	F508del/F508del	-0,13	PI	no	no	no	116	-
17	F508del/F508del	0,1	PI	no	no	no	101	-
18	F508del/F508del	0,55	PI	yes	yes	no	104	-
19	F508del/F508del	1,42	PI	no	no	no	88	-
20	F508del/F508del	-1,16	PI	no	no	no	142	-
21	F508del/F508del	0,03	PI	no	no	no	55	-
22	F508del/F508del	1,1	PI	no	no	no	330	-
23	F508del/F508del	1,2	PI	no	no	no	289	-

BMI – Body mass index; m- months; PI – pancreatic insufficiency; PS-pancreatic sufficiency

Table 2. Association between gastrointestinal symptoms and meconium ileus past history and normal or elevated fecal calprotectin.

n=23	Normal calprotectin (n=6)		Elevated calprotectin (n=17)		
	yes	no	yes	no	p-value#
Abdominal pain	2	4	3	14	0.58
Diarrhea	0	6	3	14	1
Constipation	2	4	1	16	1
Meconium ileus	1	5	4	13	1

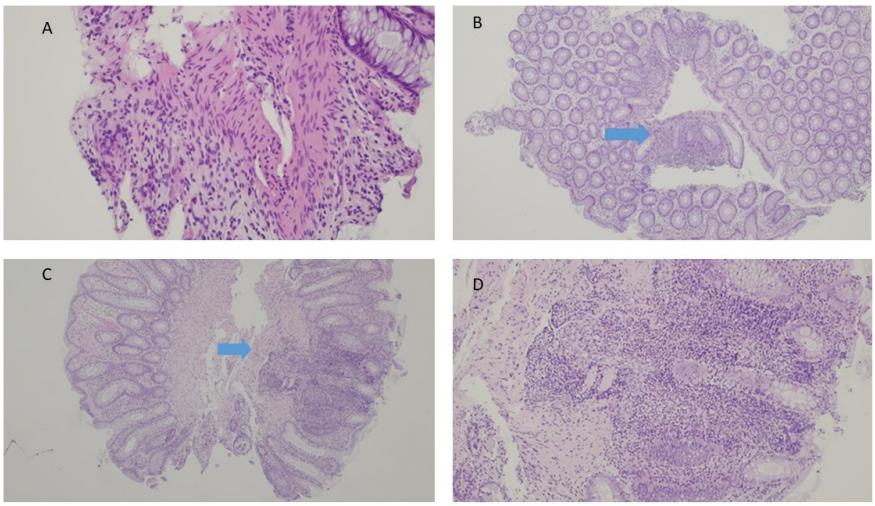
#Fisher exact test

Table 3. Comparison of histologic rectal inflammation presence between patients with normal or elevated fecal calprotectin.

(n=11)	Normal calprotectin n=6		Elevated calprotectin n=5	
	yes	no	yes	no
Mucosal inflammation	0	6	4	1

#Fisher exact test p-value=0.015

Figure 1 – A - Histologic features of patient 8 (F508del/c.3321dup) showing mild to moderate focal inflammation, composed of plasma cells and small lymphocytes, in the mucosa, H&E 200x. **B** - Histologic features of patient 9 (F508del/F508del) showing mild focal inflammation of mononucleated cells of the mucosa in a vaguely nodular pattern (blue arrow), H&E 40x; **C** - Histologic features of patient 10 (F508del/F508del) showing mild focal inflammation of the mucosa, with small lymphocytes and plasma cells, between colonic crypts (blue arrow), H&E 40x, highlighted in higher magnification **D,** H&E 200x.



338x190mm (96 x 96 DPI)