CME

# Intestinal Inflammation and Impact on Growth in Children With Cystic Fibrosis

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## ABSTRACT

**Objective:** The aim of the study was to evaluate and compare faecal markers of intestinal inflammation in children with cystic fibrosis (CF), and determine whether intestinal inflammation adversely affects the nutritional phenotype.

**Methods:** Faecal samples for markers of intestinal inflammation, calprotectin, S100A12, and osteoprotegerin, were collected from children with CF, healthy controls (HCs), and Crohn disease (CD). Associations between inflammatory markers and clinical and nutritional indices were determined in subjects with CF.

**Results:** Twenty-eight children with CF (mean [standard deviation (SD)] 8.4 [3.3] years old, 22 pancreatic insufficient [PI]), 47 HC, and 30 CD were recruited. Mean (SD) faecal calprotectin in CF (94.3 [100.6] mg/kg) was greater than HC (26.7 [15.4] mg/kg, $P < 0.0001$), but lower than CD (2133 [2781] mg/kg, $P = 0.0003$). Abnormal faecal calprotectin was found in subjects only with PI (17/22 (77%), $P = 0.001$). There was no difference in faecal mean (SD) S100A12 (0.8 [0.9] vs 1.5 [2.2] mg/kg, $P = 0.14$) and osteoprotegerin concentrations (72.7 [52.2] vs 62.5 [0.0] pg/mL, $P = 0.2$) between CF and HC. Patients with CD had significantly elevated S100A12 and osteoprotegerin compared with CF and HC. Faecal calprotectin inversely correlated with both weight ($r = -0.5$, $P = 0.003$) and height $z$ scores ($r = -0.6$, $P = 0.002$) in CF.

**Conclusions:** The pattern of intestinal inflammation in CF is unique and distinct from inflammatory bowel disease, with elevated faecal calprotectin but normal faecal S100A12 and osteoprotegerin concentrations. The severity of intestinal inflammation, based on faecal calprotectin, significantly correlates with poor growth.

**Key Words:** calprotectin, growth, gut inflammation, nutrition, osteoprotegerin, S100A12

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Children with cystic fibrosis (CF), historically, died in infancy and early childhood from severe malnutrition before the development of severe respiratory disease and failure (1). The majority of patients with CF are pancreatic insufficient (PI), resulting in maldigestion and malabsorption of nutrients. Advances in nutritional and pulmonary therapies have extended the life expectancy of patients with CF, which now exceed 40 years (2). Nutrient absorption and growth in children with CF today, however, may remain suboptimal despite high-calorie diets and adequate pancreatic enzyme replacement therapy (PERT). The reason for this is unknown but likely to be multifactorial; the altered intestinal milieu in CF has been implicated to play a role (3).

More recently, both animal and human studies have reported evidence of intestinal inflammation in CF. The pathogenesis and nutritional implications of this remain unclear. Dysfunction in the CF transmembrane conductance regulator (CFTR) protein results in the loss of transepithelial bicarbonate secretion from the pancreaticobiliary tree and intestinal mucosa, resulting in thick inspissated mucus and an acidic intestinal environment (4). Murine models of CF have abnormal mucus accumulation in the intestines (4–6), predisposing to gut dysmotility and creating a niche for microbial colonisation with up to 40-fold increase in bacterial load in the small intestine (5). Antibiotic treatment of CF mice with proven dysbiosis significantly reduced intestinal bacteria load and improved growth, but with no effect on wild-type mice (7). In addition, osmotic laxatives and *N*-acetylcysteine were reported to reduce both mucus accumulation and bacterial overgrowth in CF mice (8). In a previous report measuring intestinal inflammatory proteins from whole gut lavage of 21 children with PI CF, increased concentrations of a variety of inflammatory biomarkers such as eosinophil cationic protein, interleukin-1β, and interleukin-8 were noted compared with non-CF controls (9). Capsule endoscopy findings in patients with CF have further supported the evidence of intestinal inflammation with mucosal ulceration, erythema, and mucosal breaks observed in 20/28 (71%) of patients with PI. In this study, 18/21 (85%) of patients with PI also had elevated faecal calprotectin levels (10). Nevertheless, the majority of patients with CF do not experience the typical symptoms associated with intestinal inflammation such as those seen in inflammatory bowel disease (IBD) (eg, diarrhoea with blood and mucus).

The faecal S100 proteins, calprotectin (S100A8 and S100A9 complex) and S100A12, and osteoprotegerin have been described as biomarkers of IBD, particularly Crohn disease (CD) (11,12). These faecal proteins have different modes of expression and the possible variation of expression in CF may provide further insight into the mechanisms of intestinal inflammation in this disease. Calprotectin has a broad expression pattern with expression and

release by granulocytes (neutrophils and eosinophils) and monocytes (macrophages and dendritic cells). In the intestine, calprotectin is mainly derived from neutrophils and eosinophils (13). S100A12 has a narrow expression and is expressed by neutrophils in the intestine (14,15). Osteoprotegerin is produced by a variety of cells including osteoblastic cells but is expressed in the intestine by B cells, macrophages, dendritic cells, and intestinal epithelial cells (16,17).

On the basis of the aforementioned observations, we hypothesise that the intestinal inflammation in CF is distinct from IBD but still adversely influences the clinical status in CF. We aimed to evaluate and compare faecal calprotectin, S100A12, and osteoprotegerin levels in CF, healthy controls (HCs), and CD, as disease controls. As a secondary aim, we assessed the association between faecal biomarker levels and the clinical phenotype in CF, with an emphasis on nutritional status.

# METHODS

## Study Population

The study population included children ages 0 to 18 years, from the CF clinic at Sydney Children's Hospital, diagnosed as having CF based on the United States Cystic Fibrosis Foundation consensus criteria (2). Children with gastroenteritis, on oral corticosteroids, probiotics and/or nonsteroidal inflammatory drugs in the preceding 2 weeks were excluded. Patients with a pulmonary exacerbation in the preceding 4 weeks were also excluded. Patients known to have coeliac disease were also excluded.

In all of the participants, demographic data; anthropometric data (height and weight $z$ scores); clinical data including PERT dosage, medication history, and self- or parent-reported gastrointestinal symptoms in the preceding month (abdominal pain, nausea, vomiting, constipation, bloating, and diarrhoea) were recorded at time of stool collection. Children with CF were weighed and measured at each clinic visit. Each child was weighed wearing light clothing only on the same calibrated digital scales to the nearest 0.1 kg. Height was measured to the nearest 0.1 cm using the same fixed, calibrated stadiometer. Age and sex-specific standard deviation scores ($z$ scores) for weight and height were calculated using the National Centre for Health Statistics 2000 growth data in EpiInfo (Centers for Disease Control and Prevention, Atlanta, GA). Dose of PERT (U lipase per kilogram per day) was determined from the medical records and cross-checked for accuracy by the CF clinic dietician. Lung function test was performed (in children 4 years of age or older) according to American Thoracic Society guidelines, and forced expiratory value in 1 second ($FEV_1$) percent predicted was used for analysis (18,19).

Faecal samples were collected and analysed for calprotectin, S100A12, and osteoprotegerin. Samples were collected at home before attendance at CF clinic and stored briefly in the home freezer before transported frozen to the laboratory, where samples were stored at $-80^{\circ}C$ until analysis.

As comparison, faecal samples from HC and patients with newly diagnosed CD were obtained. Healthy volunteers without CF, CD, or gastrointestinal complaints (eg, children from hospital staff or eye clinic) were prospectively recruited as HCs. A previously published cohort of patients with CD with measured stool calprotectin, S100A12, and osteoprotegerin was used (20,21). These children had confirmed CD based on radiological, histological, and endoscopic criteria (22). Samples from patients with CD were collected before their admission for diagnostic gastroscopy and colonoscopy and before the treatment was started. The study was approved by the South Eastern Sydney Area Health Service, human research ethics committee, Sydney, Australia.

## Stool Analysis

Calprotectin levels in the faecal extracts were measured using the PhiCal kit (Calpro, San Diego, CA) following the manufacturer's instructions. Faecal S100A12 and osteoprotegerin (R&D Systems, Minneapolis, MN) was assayed using a previously published protocol (11,12). Cut-offs of 50 mg/kg for calprotectin and 10 mg/kg for S100A12, which have shown to be highly sensitive and specific for detecting inflammation in children with IBD, were used (20). There is no established reference range for faecal osteoprotegerin levels in children.

## Statistical Analysis

Descriptive statistics were presented according to the normality of the data distribution. Continuous data were presented as mean with standard deviation (SD) or median (range). Comparisons were made using unpaired $t$ test or Mann-Whitney test. Pearson correlation coefficient was used to evaluate for correlation. Categorical data were analysed using Fisher exact test. A $P$ value of $<0.05$ was used to indicate statistical significance. Statistical analysis was performed using the statistical software package GraphPad Prism version 6.0b (GraphPad, La Jolla, CA). A minimum of 20 subjects in each comparison group was required (calculated using mean faecal calprotectin of 25 and 70 mg/kg for the HC and CF groups, respectively, with an SD of 50) to provide 80% power and 5% significance level based on published faecal calprotectin measurements (10,20).

# RESULTS

## Patient Characteristics

A total of 28 patients with CF (16 [57.1%] boys) with mean (SD) age of 8.4 (3.3) years, 47 HCs (19 [40%] boys) with mean (SD) age of 5.3 (4.7) years and 30 patients with CD (18 [60%] boys) with mean (SD) age of 11.9 (3.3) years were recruited. Twenty-two (79%) patients with CF were PI and 6 (21%) pancreatic sufficient (PS). The genotype and clinical characteristics of PI and PS children are summarised in Table 1. Patients with PI had a lower mean (SD) weight $z$ score than those with PS ($-1.10$ [0.99] vs 0.21 [0.86], $P = 0.007$). There were no significant differences in the age, $FEV_1$ percent predicted, and height $z$ score between patients with PI and PS.

## Faecal Calprotectin

Mean (SD) faecal calprotectin levels were significantly greater in the CF cohort compared with HCs (94.3 [100.6] mg/kg vs 26.7 [15.4] mg/kg, $P < 0.0001$). Patients with CF had significantly lower levels of faecal calprotectin than the CD group (2133 [2781] mg/kg, $P = 0.0003$) (Fig. 1A).

A total of 17/22 (77%) of patients with PI had elevated levels (>50 mg/kg), whereas all of the 6 patients in the PS group (100%) had normal calprotectin levels ($P = 0.001$). Higher faecal calprotectin levels were observed in the PI group compared with the PS group (110.4 [108.3] vs 35.4 [9.4] mg/kg, $P = 0.008$) (Fig. 1B).

## Faecal S100A12

The mean (SD) faecal S100A12 concentrations between the CF cohort and HCs were not significantly different (0.8 [0.9] vs 1.5

TABLE 1. Patient demographics and clinical characteristics

| | PI (n = 22) | PS (n = 6) | P |
|---|---|---|---|
| Sex | Male = 14<br>Female = 8 | Male = 2<br>Female = 4 | 1.00 |
| Age, y | 7.84 (3.3) | 10.55 (2.4) | 0.07 |
| Genotype | DF508/DF508 = 18<br>DF508/W1282X = 1<br>DF508/R560T = 1<br>DF508/R334W = 1<br>DF508/621+1G>7 = 1 | DF508/R117H = 2<br>DF508/S945L = 1<br>DF508/- =3 | — |
| $FEV_1$ percent predicted | 94.0 (14.1) | 88.17 (8.68) | 0.36 |
| PERT dose (U lipase/kg per day) | 5823 (2872) | Nil | — |
| Weight $z$ score | −1.10 (0.99) | 0.21 (0.86) | 0.007 |
| Height $z$ score | −1.36 (1.20) | −0.53 (1.47) | 0.16 |

Continuous data presented as mean (standard deviation). $FEV_1$ = forced expiratory value in 1 second; PERT = pancreatic enzyme replacement therapy; PI = pancreatic insufficient; PS = pancreatic sufficient.

[2.2] mg/kg, $P = 0.1$). Subjects with CF had significantly lower levels compared with the CD group (114 [129] mg/kg, $P < 0.0001$) (Fig. 1C). Based on the S100A12 cut-off of >10 mg/kg, all the subjects with CF, both PI and PS, had normal S100A12 measurements.

## Faecal Osteoprotegerin

There was no significant difference in mean (SD) faecal osteoprotegerin concentrations between the patients with CF and HCs (72.7 [52.2] vs 62.5 [0.0] pg/mL, $P = 0.2$). In the CD cohort,

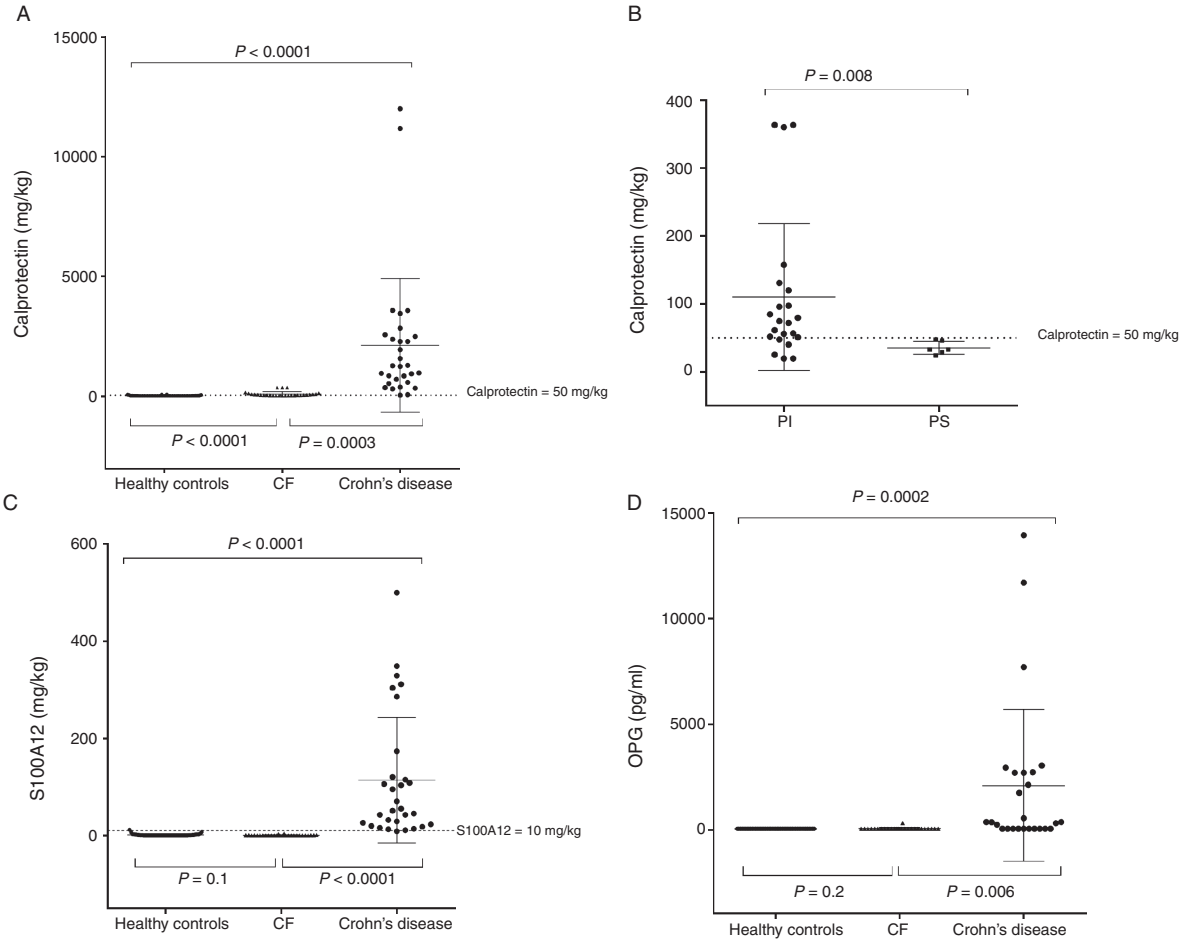


**FIGURE 1.** Comparison of faecal inflammatory markers: (A) faecal calprotectin levels in CF, HCs and CD; (B) faecal calprotectin levels in PI and PS CF subjects; (C) fecal S100A12 concentrations in CF, healthy controls and CD; and (D): faecal osteoprotegerin (OPG) levels in CF, control and CD. CD = Crohn disease; CF = cystic fibrosis; HC = healthy control; PI = pancreatic insufficient; PS = pancreatic sufficient.

osteoprotegerin levels were significantly greater when compared with the CF cohort (2088 [3614] pg/mL, $P = 0.006$) (Fig. 1D).

## Correlation Between Faecal Calprotectin and Growth Parameters

There were significant correlations between faecal calprotectin levels and both weight $z$ scores ($r = -0.5$, $r^2 = 0.3$, $P = 0.003$) and height $z$ scores ($r = -0.6$, $r^2 = 0.4$, $P = 0.002$) in patients with CF. These correlations remained significant whether or not subjects with PS CF were included. The correlation between faecal calprotectin levels and weight and height $z$ scores in subjects with PI were $r = -0.5$ ($r^2 = 0.3$, $P = 0.01$) and $r = -0.6$ ($r^2 = 0.4$, $P = 0.002$), respectively. Because faecal S100A12 and osteoprotegerin levels were not elevated in the CF cohort, no correlation analyses were performed for these markers.

## Correlation Between Faecal Calprotectin and Clinical Parameters

There was no correlation between faecal calprotectin levels and $FEV_1$ percent predicted ($r = 0.04$, $P = 0.8$). Furthermore, there was no relationship between faecal calprotectin and PERT dosage ($r = 0.4$, $P = 0.08$).

## Correlation Between Faecal Calprotectin and Gastrointestinal Symptoms

The majority of CF subjects did not report any symptoms. Seventeen (61%) patients with CF had elevated faecal calprotectin levels, whereas 11 (39%) had levels within the normal range (<50 mg/kg). None of the gastrointestinal symptoms were found to be associated with faecal calprotectin levels (Table 2).

# DISCUSSION

In this study, we report the gradation of severity of intestinal inflammation, based on faecal calprotectin, from HC, CF, to CD. The majority of patients with CF PI in this study, and not PS, showed increased markers of intestinal inflammation that is distinct from the intestinal inflammation seen in patients with CD, with biomarkers S100A12 and osteoprotegerin not elevated in CF. Furthermore, we found an association with faecal calprotectin and poor growth.

In this study, the degree of inflammation in CF was significantly less than that observed in CD, which may reflect the lack of overt gastrointestinal symptoms observed in the CF group. Furthermore, the intestinal inflammation in CF appears to be different from the typical inflammatory processes and mechanisms seen in IBD, in light of the normal faecal S100A12 and osteoprotegerin concentrations in children with CF. In the intestinal tract, calprotectin is expressed by neutrophils and eosinophils, whereas S100A12 is predominately expressed by neutrophils, and osteoprotegerin by a range of cells including B cells, macrophages, dendritic cells, and epithelial cells (13,14,16,23). Thus, the finding of elevated calprotectin levels but normal levels of S100A12 and osteoprotegerin proteins may suggest that gut inflammation in CF is predominately a non-neutrophilic process. The mechanisms and source of intestinal inflammation in CF warrants further investigation.

The majority of patients with PI (77%) in our study, as similarly reported by Werlin et al (10), had elevated calprotectin levels. The pathogenesis of intestinal inflammation in CF is unknown and the role and impact of CFTR dysfunction on the development of intestinal inflammation remains unclear. Because we found no evidence of intestinal inflammation in patients with PS but was predominately observed in patients with PI, we speculate a possible link between intestinal inflammation and severity of CFTR dysfunction.

There are multiple plausible factors responsible for intestinal inflammation in CF, with a complex interplay between these factors likely. Intestinal dysbiosis has been suggested as a key player (24). Altered patterns of intestinal microbiota in patients with CF has been described in various studies (24–28), with administration of probiotics resulting in partial restoration of intestinal microbiota (with increases in *Bacteroides* and *Faecalibacterium prausnitzii* species) and reduction in intestinal inflammation (24). Intestinal dysbiosis has also been similarly described in other inflammatory conditions affecting the gut, such as CD and ulcerative colitis (29).

Delayed small intestinal transit (30), thick inspissated mucus (4), alterations in the bicarbonate buffering capacity in the proximal small bowel (31), and regular repeated course of antibiotics (29,32) are likely important factors in the development of dysbiosis. These factors may, however, also independently be responsible for gut inflammation, although the exact mechanism(s) remains unclear. There is evidence of increased intestinal permeability in children with PI unrelated to the cause of the pancreatic disease and independent from CFTR dysfunction (33). The link between a ''leaky'' gut epithelium and inflammation in patients with PI, however, remains unknown. Impaired bacterial killing in the airways of the CF pig, owing to reduced surface pH, leading to an inflammatory response may also be present in the gut (34). Altered eicosanoid metabolism has also been suggested as another possible factor, with an imbalance in essential fatty acid profile favouring a proinflammatory state observed in the small intestines of CF mice (35).

A relation between severity of inflammation with weight and height was observed in this study. These findings may indicate a potential to improve nutritional outcomes in CF through treatment of intestinal inflammation. Poor growth is common in CF (36),

TABLE 2. Relation between gastrointestinal symptoms and calprotectin

| | Calprotectin >50 mg/kg (n = 17) | | Calprotectin <50 mg/kg (n = 11) | | |
|---|---|---|---|---|---|
| | No | Yes | No | Yes | *P* |
| Abdominal pain in the last month | 12 | 5 | 9 | 2 | 0.5 |
| Nausea in the last month | 15 | 2 | 11 | 0 | 0.2 |
| Vomiting in the last month | 15 | 2 | 11 | 0 | 0.2 |
| Constipation in the last month | 13 | 4 | 11 | 0 | 0.08 |
| Abdominal bloating in the last month | 12 | 1 | 8 | 0 | 1.00 |
| Diarrhoea in the last month | 17 | 0 | 11 | 0 | — |

primarily because of exocrine pancreatic insufficiency and increased energy expenditure. Independent of these factors, we speculate that patients with PI CF have an additional degree of gut inflammation, contributing further to the malabsoprtive state. The growth effect of intestinal inflammation in CF may be associated with intestinal dysbiosis. CF mice with dysbiosis were reported to gain weight with reduction in intestinal bacterial load after antibiotic treatment (7). A recent trial evaluating Ivacaftor, a CFTR-potentiator drug (Vertex Pharmaceuticals, Cambridge, MA), in paediatric patients with a *G551D-CFTR* mutation, reported improvements in lung function, but also an unexpectedly significant improvement in weight gain (average gain of 2.8 kg relative to placebo), likely independent of lung function (37). There is preliminary evidence that Ivacaftor treats the CFTR dysfunction in the intestinal tract and has been shown to increase proximal intestinal pH in patients with CF (38). Increasing the intestinal pH may address the acidic intestinal milieu in CF, which is believed to contribute to the underlying dysbiosis and gut inflammation.

No association was found between calprotectin and PERT dose. High doses of PERT in patients with CF have been strongly correlated with the development of fibrosing colonopathy (39). This study was not designed to specifically determine the effects of PERT on gut inflammation.

There are several limitations worthy of discussion. Concurrent collection and analysis of airway and blood samples for calprotectin levels were not undertaken to determine whether calprotectin measured in faeces was derived from ingested sputum (and if stool calprotectin levels correlated with airway and/or serum levels). Calprotectin has been measured in respiratory tract secretions in patients with CF, and elevation in levels associated with infective pulmonary exacerbations (40). This is, however, unlikely to be the case in this study, in view of the normal faecal S100A12 protein concentrations. If ingestion of inflamed airway secretions was responsible for the ''false-positive'' faecal calprotectin results, elevations in faecal S100A12 would also be expected (41). Furthermore, subjects with pulmonary exacerbations requiring intravenous or nebulised antibiotics were excluded. The sample size was relatively small because of the prospective nature of this study. Future studies using multivariate analysis are needed to account for multiple growth confounders. The phenotypic effects of intestinal inflammation, namely poor growth, observed in this study require validation in a larger multicentre study.

In conclusion, the intestinal inflammation in CF was distinct from other forms of intestinal inflammation such as IBD, with patients with CF having raised faecal calprotectin but normal S100A12 and osteoprotegerin levels, and was correlated with adverse growth in children.

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