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Fecal Calprotectin, Chitinase 3-Like-1, S100A12 and Osteoprotegerin as Markers of Disease Activity in Children with Crohn's Disease

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Abstract: Fecal calprotectin (FC), chitinase 3-like-1 protein (CHI3L1), S100A12 and osteoprotegerin (OPG) are biomarkers of intestinal inflammation. This cross-sectional study aimed to evaluate these biomarkers in a cohort of children with Crohn's disease (CD) and compare them with other measures of disease activity. Stool samples from children with CD were used to measure FC, CHI3L1, S100A12 and OPG by enzyme-linked immunosorbent assay. Serum inflammatory markers were measured and pediatric CD disease activity index (PCDAI) scores calculated. The simple endoscopic score for CD (SES-CD) was reported for a subgroup who underwent ileocolonoscopy corresponding with the stool samples. Sixty-five children were recruited. Children in clinical remission had lower FC and CHI3L1 levels than those with active disease (FC: 277 vs. 1648 µg/g, $p = 0.012$; CHI3L1: 23 vs. 227 ng/g, $p = 0.013$). FC levels differed between patients with clinically active or inactive isolated ileal CD. Although FC and CHI3L1 levels correlated strongly (r = 0.83), none of the fecal markers correlated well with serum markers. Only FC and OPG correlated with SES-CD scores (r = 0.57 and r = 0.48, respectively). In conclusion, FC correlated with both endoscopic and clinical disease activity and was the only biomarker that differentiated between active and inactive ileal CD. CHI3L1 also predicted clinical disease activity and correlated highly with FC. Further investigation of the role of CHI3L1 is required.

Keywords: inflammatory bowel diseases; children; Crohn's disease; fecal biomarker; calprotectin

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1. Introduction

Crohn's disease (CD) is one of two main subtypes of inflammatory bowel disease (IBD) [1]. Although the pathogenesis of IBD is not clearly understood, interactions between the intestinal microbiome and host immune responses in the setting of genetic risk are felt to be most important [2].

CD is generally characterized by a relapsing remitting pattern of GI tract inflammation. Disease monitoring is crucial in guiding treatment choices, assessing response to an intervention and for monitoring the maintenance of remission. Endoscopic assessment is considered to be the gold standard for diagnosis and assessment of intestinal inflammation in CD [3,4]. However, this is expensive, invasive and unpleasant, making it difficult to repeat regularly. Although serum biomarkers can be measured easily and cheaply to assess the presence of inflammation, they lack specificity [5]. Fecal biomarkers, on the other hand, can provide a more specific indication of intestinal inflammation, are relatively cheap to measure, are easy to obtain and changes may be detected earlier than serum markers [6].

Fecal calprotectin (FC) is the most studied fecal biomarker in the setting of IBD and is widely available in routine clinical practice. FC, predominantly derived from neutrophils, reliably reflects endoscopic findings of intestinal inflammation [7–9]. Although FC is more variable in children aged less than five years of age, a normal level in older children would be expected to be <50 µg/g [5]. Modest elevation of FC can be seen in other situations, such as gastrointestinal malignancy, nonsteroidal anti-inflammatory drug use or infectious gastroenteritis. FC assessment can also be limited by intrasample variability and variation over the course of a day [9,10]. In addition, FC measurement may be less reliable in individuals with disease involvement proximal to the colon [7,11,12].

Consequently, various other fecal biomarkers have been evaluated. S100A12, also known as calgranulin C or EN-RAGE, is another member of the S100 family of proteins [13]. S100A12 may be more specific in differentiating between IBD and functional GI disorders than FC, and also appears to vary less with age [5,14,15]. However, it may be less stable in stool than FC and also appears to be unhelpful in isolated ileal CD [16,17]. Osteoprotegerin (OPG) is a member of the tumor necrosis factor receptor superfamily and is involved in bone metabolism and tumorigenesis [18,19]. OPG contributes to inflammatory pathways and has been described as a fecal marker of inflammation in pediatric IBD [20,21]. More recently, Chitinase 3-Like-1 (CHI3L1) has been shown to be a useful biomarker of intestinal inflammation [22,23]. CHI3L1 is expressed by colonic epithelial cells, macrophages and neutrophils and is also upregulated in various solid tumors.

The primary aim of this study was to ascertain the relationship between clinical disease activity and levels of FC, CHI3L1, S100A12 and OPG in stool samples collected from a cohort of children with CD. Further aims were to determine the relationships of these markers to patient features (sex and age), disease location and standard inflammatory markers.

2. Methods

2.1. Patient Recruitment

This cross-sectional cohort study prospectively recruited children, aged 18 years or less, with existing or new CD in the South Island of New Zealand. Diagnosis of CD was based on upper and lower endoscopy, with histological and radiological criteria, as per the revised Porto criteria [3,24]. There were no specific exclusion criteria. Date of initial endoscopic assessment was used as the date of diagnosis. Patient demographics were recorded at enrolment, along with disease classification, anthropometric measurements, medical treatment and clinical disease activity. Endoscopic severity was documented utilizing the Simple Endoscopic Score for CD (SES-CD) only in the children recruited within one month of their endoscopic assessment [25].

2.2. Standard Serum Markers of Inflammation

The results of routinely measured serum inflammatory markers were recorded. These included C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), hematocrit (HCT), albumin and platelets. Standard laboratory cut-off values were used.

2.3. Clinical Disease Assessment

Disease activity was measured with the validated Pediatric CD activity index (PCDAI) [26]. Standard cut-off values were utilized: <10 for remission, 10–27.5 for mild disease, ≥27.5–37.5 for moderate disease and ≥37.5 for severe disease [27,28]. In addition, the modified PCDAI (modPCDAI) score was also calculated for each patient [29].

2.4. Fecal Biomarker Measurement

Patients were asked to collect stool samples at home. After collection, stools were initially stored at 4° Celsius prior to transport the same day to the laboratory, where they were stored at −80° Celsius until used for biomarker measurement.

Fecal extractions were conducted according to relevant enzyme-linked immunosorbent assay (ELISA) protocols and resulting supernatants were used immediately for immunoas-

says. In brief, stool was weighed and then extraction buffer added at set weight to buffer ratios. This was agitated on a vortex (VF2, IKA Labortechnik, Staufen, Germany) into a homogenous mixture. Further homogenizing took place on a suspension mixer (Gyrotory shaker model G2, New Brunswick Scientific Co, Edison, NJ, USA) for 30 min. Samples were then centrifuged at 13,000× *g* for 10 min and the resulting supernatant used in the ELISAs.

Commercial ELISA kits were utilized for FC (Bühlmann Laboratories, fCAL ELISA kit, EK-CAL2 192 test, Schönenbuch, Switzerland), CHI3L1 (R&D Systems, Human Chitinase 3-like-1 DuoSet ELISA, Minneapolis, MN, USA) and OPG (R&D Systems, Human Osteoprotegerin/TNFRSF11B DuoSet, Minneapolis, MN, USA) following manufacturers' protocols. S100A12 was measured by ELISA following a previously developed protocol [14].

2.5. Statistical Analysis

Standard descriptive statistics were used to summarize the data including medians and interquartile ranges for continuous variables and frequencies and percentages for categorical variables. The nonparametric Mann–Whitney U and Kruskall–Wallis tests were used for comparing biomarker levels between demographic and disease characteristic groups. Chi-square or Fisher's exact tests were employed for the comparisons of categorical measures. The Spearman's rank correlation coefficient was used for exploring the strength of the association between continuous measures. A correlation coefficient of 0.3–0.5 was considered as weak, 0.5–0.7 as moderate and >0.7 as strong. A two-tailed p-value < 0.05 was considered statistically significant. Statistical analysis was performed using SPSS (IBM SPSS version 26, Armonk, NY, USA) and figures were created utilizing GraphPad (GraphPad Prism version 9, San Diego, CA, USA).

2.6. Ethics

This project was approved by the New Zealand Health and Disability Ethics Committee (MEC/12/02/019). Informed consent was signed by patients or by their guardian if the patient was below the age of 16 years.

3. Results

3.1. Background Characteristics

A total of 65 children were enrolled: 20 (31%) were within one month of diagnosis (Table 1). A total of 70% of the patients were male and almost two-thirds (65%) were receiving at least one medical therapy at enrolment. The median PCDAI score for the group was 10 (IQR = 5–23, n = 65). Based upon PCDAI scores, most patients were in remission (40%) or had mild disease (43%) at enrolment. In addition, the median ModPCDAI score was 0 (IQR = 0–5, n = 35).

Table 1. Baseline characteristics of 65 children with Crohn's disease (CD).

Patients	**65**
Male	46 (70%)
Age at inclusion in years, median (IQR)	12 (9–15)
Age at diagnosis in years, median (IQR)	9 (6.5–12.5)
Disease duration in years, median (IQR)	1 (0–3.5)
New diagnosis of CD	32 (49%)
Disease location at diagnosis	
L1: Ileal	15 (23%)
L2: Colonic	16 (24%)
L3: Ileocolonic	32 (49%)
L4a/b: Proximal from ileum	31 (48%)

Table 1. *Cont.*

Patients		65
Crohn's disease behavior at diagnosis		
B1: Nonstricturing and nonpenetrating		61 (94%)
B2: Stricturing		2 (3%)
B3: Penetrating		2 (3%)
P: Perianal disease		9 (14%)
Medication at enrolment		
Exclusive enteral nutrition		9 (14%)
Aminosalicylates		19 (29%)
Antibiotics		5 (8%)
Corticosteroids		2 (3%)
Thiopurines/Methotrexate		21 (33%)
Biological		4 (6%)
No medication		23 (35%)
Disease activity at enrolment		
Remission (PCDAI < 10)		26 (40%)
Mild (PCDAI 10–27.5)		28 (43%)
Moderate (PCDAI $\geq$ 27.5–37.5)		6 (9%)
Severe (PCDAI $\geq$ 37.5)		5 (8%)
SES-CD at baseline		*n = 20*
Inactive (0–2)		2 (10%)
Mild (3–6)		2 (10%)
Moderate (7–15)		11 (55%)
Severe (>15)		5 (25%)
Serum markers (medians and IQR)		
ESR (in mm/h)	*n = 51*	12 (8–21)
CRP (in mg/L)	*n = 56*	3.0 (2.3–8.8)
Albumin (in g/L)	*n = 53*	41 (39–44)
Hematocrit (in L/L)	*n = 59*	0.38 (0.35–0.40)
Platelets ($\times 10^9$/L)	*n = 58*	356 (295–445)

CD = Crohn's disease, IQR = interquartile range, PCDAI = Pediatric Crohn's disease activity index, SES-CD = simple endoscopic score for Crohn's disease, ESR = erythrocyte sedimentation rate, CRP = C-reactive protein.

3.2. Fecal Biomarker Levels

CHI3L1 was measured in all 65 samples. Due to insufficient sample volume, fewer samples were available for analysis of FC (n = 58), S100A12 (n = 63) and OPG (n = 63).

Fecal biomarker levels did not vary according to sex or age at enrolment (Table 2). Children in clinical remission (PCDAI < 10 points) had significantly lower levels of FC (277 vs. 1648 µg/g; $p = 0.012$) and CHI3L1 (23 vs. 227 ng/g; $p = 0.013$) compared to patients with clinically active disease. S100A12 (13 vs. 58 µg/g, $p = 0.164$) and OPG (63 vs. 81 pg/mL, $p = 0.288$) did not differ between the children in remission and those with active disease.

When assessed according to the level of disease severity (remission, mild, moderate or severe), FC levels were elevated only in those with severe disease compared to those in remission (Figure 1). Fecal CHI3L1 levels were higher in those with moderate or severe disease compared to the group in remission. In contrast, OPG and S100A12 levels did not differentiate between grade of disease severity.

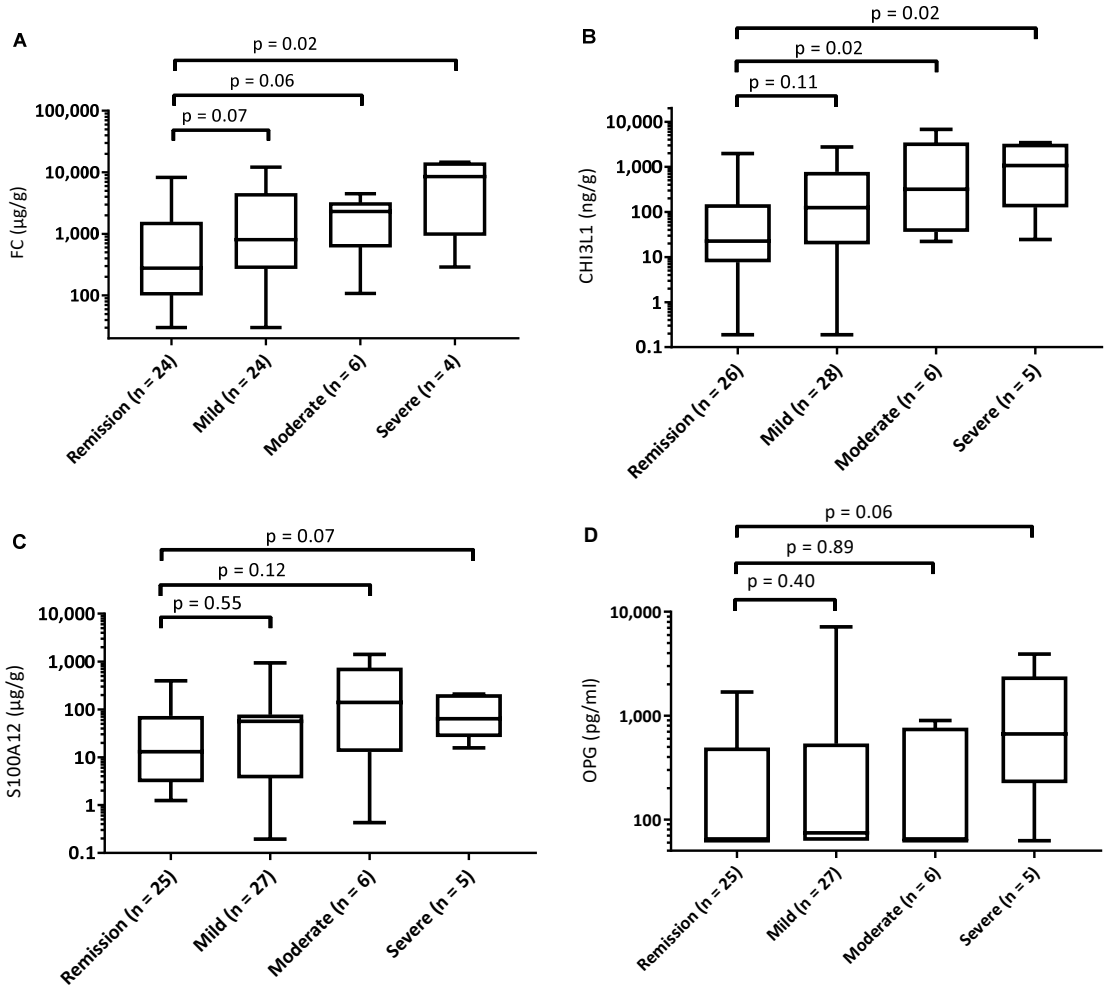

Figure 1. Boxplots showing biomarker levels for patients with Crohn's disease according to clinical disease severity group. (**A**): fecal calprotectin, (**B**): fecal chitinase 3-like-1 protein, (**C**): fecal S100A12, (**D**): fecal osteoprotegerin. PCDAI remission: <10, mild: 10–27.5, moderate: 27.5–37.5, severe: 37.5–100.

Table 2. Levels of fecal biomarkers in children with Crohn's disease.

	FC µg/g	**(IQR)**	***n***	**CHI3L1 ng/g**	**(IQR)**	***n***	**S100A12 µg/g**	**(IQR)**	***n***	**OPG pg/mL**	**(IQR)**	***n***
Overall levels	**784**	(180–2878)	*58*	**83**	(17–637)	*65*	**31**	(4–88)	*63*	**69**	(63–577)	*63*
Gender												
Female	**1344**	(131–2857)	*17*	**26**	(8–706)	*19*	**40**	(8–98)	*19*	**74**	(63–219)	*19*
Male	**779**	(230–3640)	*41*	**89**	(17–621)	*46*	**26**	(3–78)	*44*	**66**	(63–824)	*44*
	p = 0.878			*p = 0.713*			*p = 0.472*			*p = 0.434*		
Age at inclusion												
≤6 years	**1952**	(495–2841)	*7*	**155**	(39–1696)	*9*	**65**	(60–84)	*9*	**219**	(63–2024)	*9*
7–12 years	**456**	(98–1679)	*27*	**40**	(6–400)	*28*	**9**	(2–69)	*28*	**63**	(63–577)	*28*
≥13 years	**1420**	(166–4616)	*24*	**99**	(17–759)	*28*	**26**	(7–116)	*26*	**81**	(63–867)	*26*
	p = 0.357			*p = 0.372*			*p = 0.074*			*p = 0.447*		
Crohn's disease location at diagnosis												
L1: Ileal	**294**	(76–494)	*15*	**17**	(6–36)	*16*	**7**	(2–24)	*15*	**63**	(63–63)	*15*
L2: Colonic	**1629**	(196–4346)	*13*	**194**	(24–1027)	*16*	**66**	(6–85)	*16*	**71**	(63–1014)	*16*
L3: Ileocolonic	**1416**	(371–4572)	*29*	**226**	(29–996)	*32*	**40**	(7–119)	*31*	**143**	(63–856)	*31*
	p = 0.025			*p = 0.005*			*p = 0.033*			*p = 0.021*		

Table 2. *Cont.*

	FC µg/g	(IQR)	*n*	CHI3L1 ng/g	(IQR)	*n*	S100A12 µg/g	(IQR)	*n*	OPG pg/mL	(IQR)	*n*
L1: ileal	**294**	(76–494)	*15*	**17**	(6–36)	*16*	**7**	(2–24)	*15*	**63**	(63–63)	*15*
L2/L3: (ileo)colonic	**1523**	(245–4527)	*42*	**226**	(27–996)	*48*	**56**	(7–116)	*47*	**94**	(63–857)	*47*
	p = 0.007			*p = 0.001*			*p = 0.009*			*p = 0.007*		
*Clinical disease activity **												
Remission	**277**	(100–1576)	*24*	**23**	(8–149)	*26*	**13**	(3–73)	*25*	**63**	(63–495)	*25*
Active disease	**1648**	(343–4527)	*34*	**227**	(27–1109)	*39*	**58**	(6–123)	*38*	**81**	(63–758)	*38*
	p = 0.012			*p = 0.013*			*p = 0.164*			*p = 0.288*		
*Clinical disease activity for patients with L1 ileal disease **												
Remission	**98**	(30–294)	*7*	***15***	*(6–24)*	*8*	**3**	(2–10)	*7*	**63**	(63–63)	*7*
Active disease	**475**	(256–2028)	*8*	**22**	*(2–232)*	*8*	**16**	(2–63)	*8*	**63**	(63–128)	*8*
	p = 0.014			*p = 0.645*			*p = 0.336*			*p = 0.867*		
SES-CD												
Inactive (0–2)	**98**		*1*	**43**	(2–43)	*2*	**1**		*1*	**63**		*1*
Mild (3–6)	**30**		*1*	**1717**	(14–1717)	*2*	**107**	(10–107)	*2*	**364**	(63–364)	*2*
Moderate (7–15)	**1952**	(630–3640)	*9*	**155**	(27–776)	*11*	**60**	(12–116)	*10*	**63**	(63–225)	*10*
Severe (>15)	**4482**	(2841–4482)	*3*	**776**	(125–2549)	*5*	**210**	(81–732)	*5*	**898**	(399–3439)	*5*
	p = 0.068			*p = 0.456*			*p = 0.120*			*p = 0.058*		

* remission: pediatric Crohn's disease activity index < 10 points. Values reported in medians with interquartile range. *p*-values calculated with Mann–Whitney U test for comparing 2 groups and Kruskall–Wallis for comparing > 2 groups. n= number of fecal samples, FC = fecal calprotectin, CHI3L1 = fecal chitinase 3-like-1 protein, S100A12 = fecal S100A12, OPG = fecal osteoprotegerin, SES-CD = simple endoscopic score for Crohn's disease.

Overall, the patients with isolated ileal (L1) CD had lower fecal biomarker levels than patients with any colonic involvement (L2 and L3 together) (Table 2). In addition, CHI3L1 and S100A12 (but not FC and OPG) levels differed between the L1 and L2 subgroups (Figure 2). Upon evaluating the subgroup with isolated ileal location (L1), the children in remission had lower FC levels than those with active disease (81 vs. 475 µg/g, $p = 0.014$). However, CHI3L1, S100A12 and OPG levels did not differ between these two subgroups (Table 2), although the small sample sizes limit the power for these comparisons.

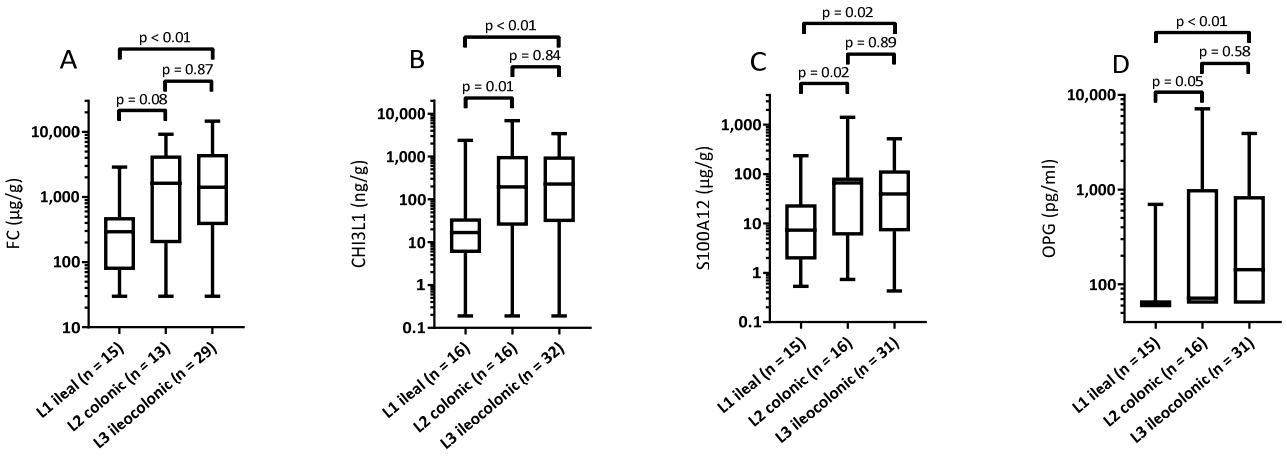

Figure 2. Fecal biomarker levels according to disease location in children with Crohn's disease. Box plot with median, minimum, maximum 25th and 75th percentiles for levels of (**A**) calprotectin; (**B**) chitinase 3-like-1; (**C**) S100A12; and (**D**) osteoprotegerin.

3.3. Correlations between Fecal Biomarkers and Other Markers

There were weak to strong correlations between the individual fecal biomarkers (Table 3). The strongest correlation was between FC and CHI3L1 and the weakest correlation was between OPG and S100A12.

Table 3. Spearman's rank correlation coefficients in children with Crohn's disease.

	FC	CHI3L1	S100A12	OPG
CHI3L1	0.83			
S100A12	0.62	0.66		
OPG	0.68	0.60	0.48	
CRP	ns	0.30 *	ns	ns
ESR	0.40	0.54	ns	0.40
Albumin	ns	ns	ns	ns
Platelets	0.45	0.60	ns	0.46
Haematocrit	ns	ns	ns	ns
PCDAI	0.40	0.38	ns	ns
modPCDAI	0.42 *	0.45	0.36 *	ns
SES-CD	0.57 *	ns	ns	0.48 *

* indicates $p < 0.05$: all others $p < 0.005$. FC = fecal calprotectin, CHI3L1 = fecal chitinase 3-like-1 protein, S100A12 = fecal S100A12, OPG = fecal osteoprotegerin, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, HCT = hematocrit, PCDAI = pediatric Crohn's disease activity index, modPCDAI = modified PCDAI, ns = not significant.

Overall, the fecal biomarkers correlated poorly with serum markers. CHI3L1 correlated with ESR, platelets and CRP values, while FC and OPG correlated only with ESR and platelet count. Albumin and HCT values did not correlate with any fecal marker. Only FC and CHI3L1 showed weak correlations with both the PCDAI and the modPCDAI scores. S100A12 levels correlated poorly with the modPCDAI but not with the PCDAI. Only FC (r = 0.57) and OPG (r = 0.48) showed a correlation with the SES-CD scores in the 20 children who had undergone ileocolonoscopy in the preceding month (Table 3).

4. Discussion

The current study is the first to assess and compare the utility of four fecal biomarkers, namely FC, CHI3L1, S100A12 and OPG, in a cohort of children with CD. FC and CHI3L1 were higher in patients with clinically active disease than in those in clinical remission. Each of the four biomarkers appeared to be lower in the subset with isolated ileal disease compared to those with any colonic involvement. Within the children with isolated ileal disease, only FC levels differed according to disease activity. FC and OPG correlated with endoscopic severity in the subgroup who had a concurrent ileocolonoscopy.

Assessing disease activity in children with CD is crucial in guiding treatment, assessing response to an intervention, monitoring for maintenance of remission and predicting disease course. Valid and reliable markers are especially important in establishing if an intervention has enabled the achievement of therapeutic targets, including mucosal healing [30]. The results from the current study indicate that fecal biomarkers can provide a reliable indication of current disease activity, when compared to clinical disease activity scores and serum markers. Interestingly, in this regard the levels of FC, CHI3L1 and S100A12 correlated more strongly with the modPCDAI scores than PCDAI scores, reflecting the restricted focus of this index upon just serum markers of inflammation [29].

The four fecal biomarkers showed weak-to-good correlation with each other. Although expressed by different cell types, FC and CHI3L1 showed the strongest correlation, which was numerically higher than reported in one previous study involving children with IBD [22,31,32].

Previous studies have shown contradictory results about the value of fecal biomarker levels in ileal CD without colonic involvement [7,11,12]. In the current study, fecal biomarker levels were lower in the children with isolated ileal CD compared to those with colonic or ileocolonic disease regardless of disease activity. Further, only FC levels were different between patients with quiescent or active isolated ileal disease. Together these findings suggest that only FC might be useful for ileal CD. This may not reflect the location specifically: rather, it may reflect the product of severity and disease extent.

The current study did not demonstrate any clear relationship between age and fecal biomarker levels for any of the four markers of interest. Some previous reports have shown

variable and generally higher levels of FC in preschool children [10,33–35]. S100A12 has previously appeared to be less affected by age than FC [14]. In contrast, OPG and CHI3L1 have not yet been assessed in younger children.

The results arising from the current report indicated that fecal CHI3L1 measurement provided a useful assessment of disease activity in the group of children with CD. This 40 kDA protein (also known as YKL40), that binds chitin without any catalytic activity, is elevated in serum and airways secretions in individuals with cystic fibrosis (CF) [36]. A recent report also indicated elevated levels of fecal CHI3L1 in children with CF [37]. In that report, which included children from New Zealand and Australia, the levels in the children with CF were lower than the levels seen in the children in the current study, but greater than the levels detected in healthy children.

Vind et al. [38] also showed elevated levels of serum CHI3L1 in individuals with UC and CD compared to control subjects. Serum levels differentiated disease activity only in the subjects with UC in that study. Subsequently, two studies have evaluated fecal levels of CHI3L1 in the setting of IBD. Both these reports demonstrated a significant relationship between levels of the protein and endoscopic disease activity [31,39]. In addition to a correlation between endoscopic severity in the group with CD, Buisson et al. [39] showed that fecal CHI3L1 values also correlated with CD activity index scores, CRP results and FC in 54 adults with CD. Furthermore, Aomatsu et al. [31] demonstrated a relationship between fecal CHI3L1 levels and the SES-CD and PCDAI scores, CRP levels and ESR results in 87 children with CD.

In contrast, the current study did not find any association between CHI3L1 and endoscopic severity for those patients who underwent ileocolonoscopy corresponding with the fecal stool sample. However, the correlation with CHI3L1 and clinical disease activity was higher than that reported in the earlier study of children with CD [31].

This prospective study enabled the collection of stool samples from 65 children with well-defined CD. Furthermore, clinical assessments and standard inflammatory markers were recorded to match the time of stool-sample collections. However, endoscopic scores were not available for the entire cohort and no histologic scores were available. Furthermore, disease location was defined as location at diagnosis, which may not reflect subsequent disease extension. Although the total cohort included 65 children, subgroup analyses were limited due to smaller sizes.

5. Conclusions

In conclusion, the current study has demonstrated a correlation between specific fecal biomarkers and disease activity in children with CD, with fecal biomarker levels appearing lower in children with isolated ileal disease. Overall, FC performed well in these analyses: FC differentiated disease activity in patients with isolated ileal disease, correlated with endoscopic severity and correlated with clinical disease activity. Interestingly, CHI3L1, a less-studied biomarker of disease activity in CD, correlated highly with FC and showed correlation with clinical disease activity. While further prospective studies with larger cohorts of patients should focus further on the utility of CHI3L1 in comparison to other markers and on the additive value of biomarker combinations, at present the results arising do not yet support the use of CHI3L1, S100A12 or OPG instead of FC in clinical practice.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the New Zealand Health and Disability Ethics Committee (MEC/12/02/019).

Informed Consent Statement: Written informed consent was obtained from all subjects included in the study.

Data Availability Statement: Further data available from the authors on request.

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