Fecal M2-Pyruvate Kinase (M2-PK): A Novel Marker of Intestinal Inflammation

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Background: Surrogate markers of bowel inflammation are increasingly being recognized as important, not only as markers of disease activity in inflammatory bowel disease (IBD) but also to differentiate irritable bowel syndrome (IBS) from IBD. The dimeric M2-isoform of pyruvate kinase (M2-PK) has been reported to be elevated in fecal specimens from colorectal cancer (CA) patients, but its role in IBD is unknown. This study investigated the usefulness of fecal M2-PK in cohorts of patients with IBD, IBS, and CA.

Methods: Stool samples were obtained for calprotectin and M2-PK measurements in patients with previously diagnosed IBD or new patients being investigated for lower gastrointestinal (GI) symptoms in a UK university hospital. Other investigations were performed as directed by the investigating physician and patients with known IBD were assessed for disease activity by a physician global assessment, Harvey–Bradshaw index (HBI), or endoscopic grading.

Results: Fecal M2-PK and calprotectin measurements were obtained for 148 patients: 50 with ulcerative colitis (UC); 31 with Crohn's disease (CD), 43 with irritable bowel syndrome/functional bowel disorders (IBS); 7 with colorectal CA, and 17 with miscellaneous conditions (excluded from the analysis). Median M2-PK values (U/mL) were significantly elevated in UC: 20.0 (95% confidence interval [CI] 5.4–69.0, $P < 0.0001$), CD: 24.3 (95% CI 6.4–44.0, $P < 0.0001$), and CA: 7.0 (95% CI 4.3–88.0, $P < 0.0006$) compared to IBS: 0.1 (95% CI 0.0–3.2). There was a strong linear correlation of M2-PK with calprotectin levels. A predetermined cutoff level of 3.7 U/mL for a normal M2-PK test produced a sensitivity, specificity, and positive predictive value (PPV) of 73%, 74%, and 89%, respectively, for organic disease. Furthermore, M2-PK levels were significantly elevated in active, compared to inactive, disease for CD (30 versus 0.55 U/mL, $P < 0.005$) and UC (40 versus 1.2 U/mL, $P = 0.006$), respectively.

Conclusions: Fecal M2-PK is elevated in IBD as well as in CA patients and is a sensitive and relatively specific marker for organic GI pathology, with a PPV of 89%. Furthermore, it appears to be a potentially valuable, noninvasive marker of disease activity in IBD.

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Key Words: M2-pyruvate kinase, calprotectin, inflammatory bowel disease, Crohn's disease, ulcerative colitis, colorectal cancer, irritable bowel syndrome

Differentiating between inflammatory bowel disease (IBD) and functional bowel disorders, such as irritable bowel syndrome (IBS), can often be difficult as they present with similar symptoms. The diagnosis of IBD typically necessitates invasive endoscopic procedures to visualize the mucosa and enable confirmatory histological specimens to be obtained. However, this may miss disease in the gastrointestinal (GI) tract, not directly visualized at endoscopy. Furthermore, in IBD accurate monitoring of disease activity may include repeated endoscopy, as symptoms correlate poorly with disease activity.[1] Noninvasive biomarkers in IBD are being increasingly recognized as important, both at the initial diagnosis and for monitoring disease activity. They also play a valuable role in differentiating organic GI disease from functional disorders by "examining" the entire GI tract.[2] Key characteristics of fecal biomarkers include stability in fecal samples and the existence of a sensitive and reliable assay.[3]

Pyruvate kinase (PK) is a key enzyme in the glycolytic pathway and is expressed by all cells.[4] Although a number of tissue-specific isoforms exist,[5,6] all rapidly dividing cells express the M2 type (termed M2-PK).[4] Normally a homotetramer, M2-PK is coexpressed as a dimer in cells undergoing rapid turnover and elevated levels can be reliably detected in serum and feces. This has been demonstrated in serum and fecal samples from patients with colorectal cancer[7] as well as in serum samples from those with pancreatic, gastric, renal, and lung cancers.[8] However, the role of M2-PK in GI inflammation is not known. Active IBD is accompanied by increased cell turnover and rapid division (the corollary being a return to normal cell turnover once inflammation has resolved).[9] Given the relationship of M2-PK to cell division, we postulated that the fecal concentrations of dimeric M2-PK would be elevated in patients with IBD. In this regard, M2-PK has shown promising results in a pilot study.[10] The aim of this study was to highlight the potential value of fecal,

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dimeric M2-PK as a surrogate marker of inflammation in patients with IBD, as well its value in differentiating these from patients with IBS.

MATERIALS AND METHODS

Patients attending the gastroenterology outpatient clinic of a South London teaching hospital with new-onset lower GI symptoms or with previously diagnosed IBD were prospectively enrolled in the study. Patients with symptoms of dyspepsia or gastroesophageal reflux disease were excluded. All patients underwent clinical evaluation, including history (including medication) and full physical examination before providing a fecal sample for calprotectin and M2-PK assays, as well as undergoing a lactulose/L-rhamnose intestinal permeability test[2,11] where appropriate. Patients presenting with diarrhea also provided 3 stool samples for microscopy (ova, cysts, and parasites) and culture (for *E. coli, Salmonella, Shigella*, and *Campylobacter* species). Further investigations, including endoscopic examinations, were instigated by the attending gastroenterologists as clinically indicated. For the purposes of this analysis, patients were subdivided into the following diagnostic categories; ulcerative colitis (UC), Crohn's disease (CD), irritable bowel syndrome/functional bowel disorder (IBS), and colorectal cancer (CA). In a subanalysis of IBD patients, disease activity was assessed by a combination of physician global assessment, Harvey–Bradshaw index (HBI), and/or endoscopic grading[12] where available. In the functional bowel disorder subgroup, the vast majority of patients had IBS, although the ROME II[13] criteria were not always applied. However, in all these patients all clinically indicated endoscopic investigations were normal. For simplicity, this group is referred to as IBS herein. All specimens were analyzed using previously established and validated methods (see below) and were performed in a single, accredited clinical biochemistry laboratory.

Measurement of Fecal Calprotectin and M2-PK

Patients provided a single stool sample for analysis, submitted within 48 hours. For calprotectin, handling of stool and measurement of calprotectin by enzyme-linked immunosorbent assay (ELISA) have been previously described.[11] A similar method was used to analyze M2-PK with a commercially available ELISA kit (ScheboTech, Giessen, Germany), which has no crossreactivity to other forms of pyruvate kinase.[14] This assay has an intratest and intertest variability coefficient of 4.5% and 6.1%, respectively. Stool samples for M2-PK are stable at room temperature (21°C) for 3 days or for up to 1 year at −20°C.[15]

Statistical Analysis

Fecal calprotectin and M2-PK results were tested for normal distribution using the Shapiro–Wilk test. Kruskal–Wallis ANOVA was used to detect differences between the medians of calprotectin and M2-PK in the UC, CA, CD, and IBS groups. Where differences were detected, pairwise comparisons were made using the Mann–Whitney test. Correlation between calprotectin and M2-PK concentrations, and M2-PK concentrations and age, were sought using Pearson correlation. Statistical analysis was carried out using Analyse-It (Leeds, UK). *P*-values less than 0.05 were considered statistically significant. Graphs were produced with GraphPad Prism v. 4 (San Diego, CA).

Ethical Considerations

This study was approved by the King's College Local Research Ethics Committee.

RESULTS

Of the 148 patients evaluated, 50 had biopsy-proven UC and 31 had biopsy-proven CD. In all, 43 had normal investigations and were diagnosed as having functional bowel disorders / IBS after full assessment. Seven were newly diagnosed with colorectal CA at endoscopy. The main diagnostic groups are summarized in Table 1. In the 43 patients with IBS, used as the 'control' group for subsequent analyses, M2-PK levels did not show any significant variation with age.

The remaining 17 patients had the following diagnoses: 1 had pouchitis (not included in UC category); 2 with adenomatous polyps; 3 diverticular disease; 2 with coeliac disease with subtotal villous atrophy on biopsy; 5 with duodenal ulcer, duodenitis, or gastritis; 1 melanosis coli; 2 infective diarrhea; and 1 anal CA. They were not included in the statistical analyses because the numbers in each group were small. In all, 35 patients did not undergo lower GI endoscopic examination as part of their investigations. Of these, 30 had previously endoscopically diagnosed inflammatory bowel disease, 1 patient was found to have duodenal ulceration to explain upper GI symptoms and iron deficiency anemia, 1 patient was found to have anal carcinoma, and 1 rectal carcinoma at rigid sigmoidoscopy.

Fecal M2-PK and Calprotectin in UC, CD, IBS, and CA

The individual values of M2-PK and calprotectin are shown in Figure 1A,B and the median values are summarized

TABLE 1. Age and Gender in the Main Diagnostic Groups

Diagnosis	*n*	M:F Ratio	Age (yrs)	*P* for age* (vs. IBS)
CA	7	3:4	74 (63.06–75.55)	0.0005
CD	31	11:20	35 (31.61–44.64)	0.05
UC	50	20:30	47 (36.77–57.78)	0.48
IBS	43	16:27	43 (35.38–55.43)	—
Total	131	50:81	44 (35–57)	—

*2-tailed *P*-value from Mann–Whitney U test; Median age (and interquartile range).

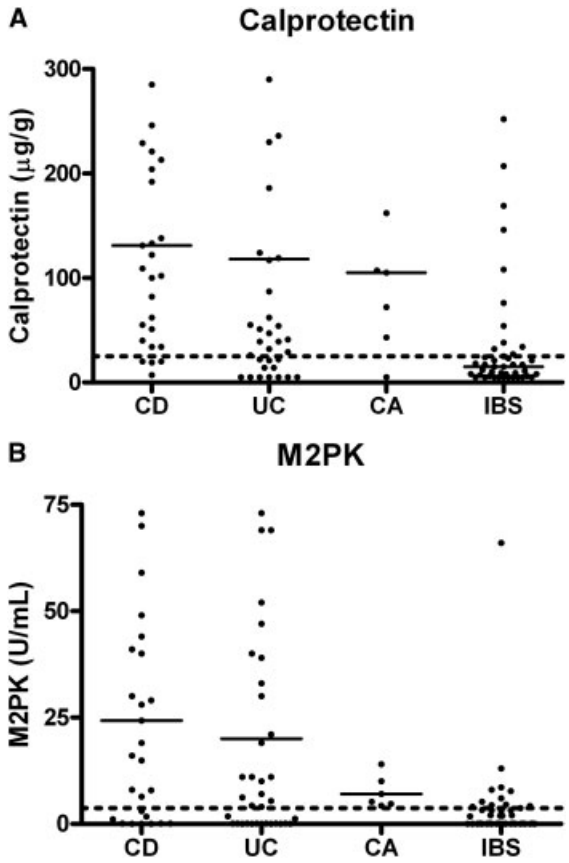

FIGURE 1. M2-PK and calprotectin by disease group. Dotted line indicates cutoff value for 'normal' test. Vertical axis truncated at 75 U/mL for M2-PK and 300 μg/g for calprotectin, for clarity.

in Table 2A. Kruskal–Wallis ANOVA was positive for both M2-PK and calprotectin between the 4 diagnostic groups, indicating a significant difference between the groups. Pairwise comparisons between the groups using Mann–Whitney tests showed that the median values of both M2-PK and calprotectin were highly significantly elevated in UC, CD, and CA patients compared to IBS (Table 2A). Although M2-PK and calprotectin concentrations in UC and CD patients were higher than the CA group, these differences were not statistically significant.

Relationship of Fecal M2-PK and Calprotectin to IBD Activity

A subgroup analysis of IBD patients was performed, where disease activity was assessed by either physician global assessment, HBI, and/or endoscopic grading. HBI >4 was taken to indicate active disease and a combination of physician global assessment and HBI or endoscopic grading were used where appropriate. There were striking differences between the fecal markers in patients with active versus inactive disease (Fig. 2A,B). Mann–Whitney pairwise comparisons showed significant differences in both the M2-PK and calprotectin concentrations between the active and inactive groups for both UC and CD (Table 2B).

M2-PK and Calprotectin as Markers of Organic GI Disease (IBD and CA)

Using a receiver operating characteristic analysis, sensitivities and specificities were determined for organic disease. Using a 'normal' cutoff of 3.7 U/mL for M2-PK and 25 μg/g for calprotectin, the results are summarized in Table 3. Fecal M2-PK had a sensitivity of 73%, specificity of 74%, PPV of 89%, and a negative predictive value (NPV) of 57% for organic GI diseases. Corresponding values for fecal calprotectin were 80%, 74%, 87%, and 65%, respectively. Similar results were obtained when the analyses were performed for IBD alone (data not shown), as the inclusion of the small number of colorectal CA patients did not markedly alter the results. Furthermore, fecal calprotectin and M2-PK were highly significantly correlated (Fig. 3; $r = 0.62$, $P < 0.0001$).

DISCUSSION

Previous studies have shown fecal M2-PK to be elevated in GI CA (including colorectal CA) but its role in IBD is unknown. This is the first study to demonstrate that elevated concentrations of M2-PK are found in fecal samples of IBD patients, as well as those with colorectal CA, in keeping with the fact that the dimeric M2-PK is elevated in rapidly dividing cells. Although the number of patients with colorectal CA in this study was small, all had elevated fecal M2-PK concentrations. However, the true potential of fecal M2-PK as a screening or diagnostic test for colorectal CA has yet to

TABLE 2A. Fecal Marker Values in the Main Diagnostic Groups

	n	M2-PK (U/mL)	*P*-value	Calprotectin (μg/g)	*P*-value
IBS	43	0.1 (0.0–3.2)	—	15 (8–21)	—
UC	50	20.0 (5.4–69.0)	<0.0001	118 (41–385)	<0.0001
CD	31	24.3 (6.4–44.0)	<0.0001	131 (62–221)	<0.0001
CA	7	7.0 (4.3–88.0)	0.0006	105 (5–530)	0.015

Values are medians (and interquartile range). Mann–Whitney test for pairwise comparisons.
The *P*-value is for difference between the diagnostic group compared to IBS.

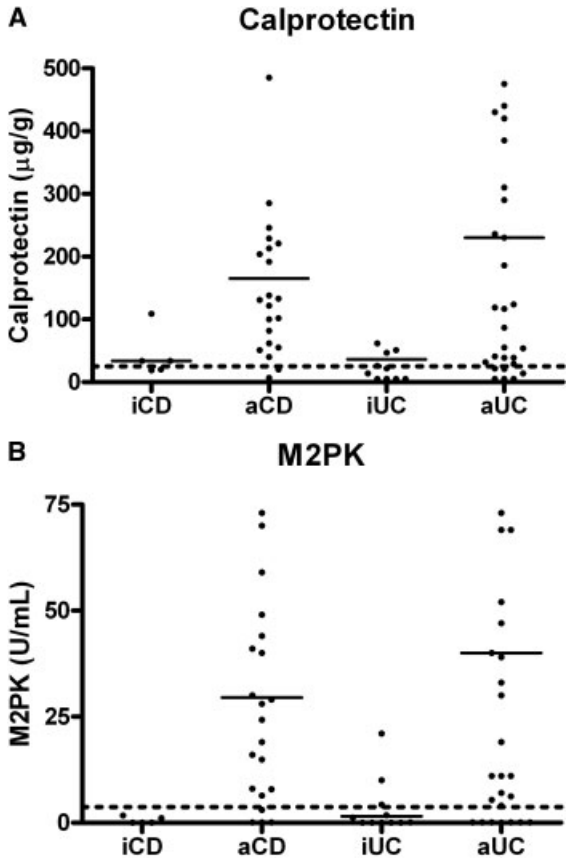

FIGURE 2. M2-PK and calprotectin and disease activity. Dotted line indicates cutoff value for 'normal' test. Vertical axis truncated at 75 U/mL for M2-PK and 500 μg/g for calprotectin, for clarity. (Prefix 'i' = inactive and 'a' = active disease.)

be fully evaluated in a large population setting, but its lack of specificity may preclude this. Therefore, a more appropriate use of fecal M2-PK may be to differentiate between organic and functional disease, as it is not specific for colorectal CA.

M2-PK concentrations were significantly higher in IBD patients than those who had IBS and, as with calprotectin,[2] M2-PK is able to differentiate between patients with IBD or IBS, and there was a strong linear correlation between the 2 markers. The relationship of calprotectin to GI inflammation has been validated in previous studies.[16–18] Fecal lactoferrin, another marker of GI inflammation, appears to have a broadly comparable sensitivity and slightly higher specificity of 78% and 90%, respectively, for detecting IBD.[16] However, these differences may be due to differing methodologies and patient selection.

The role of M2-PK and calprotectin as markers of disease activity in IBD is more interesting. Although there were no significant differences in M2-PK values between patients with CD and UC, there were significant differences in both M2-PK and calprotectin concentrations between active and inactive disease for CD and UC. There appeared to be a greater difference in M2-PK values, compared to calprotectin, between the active and inactive disease states. Disease activity was assessed by different methods in the study, which could have led to some bias, but these results are strongly suggestive that fecal M2-PK concentration may be an accurate noninvasive marker of disease activity in IBD. We are currently investigating this further, particularly the relationship between fecal M2-PK to endoscopic and histological assessments of disease activity. Furthermore, whether M2-PK can be used to predict relapse in asymptomatic IBD patients, as has been shown with calprotectin,[19] has yet to be tested.

Fecal M2-PK and calprotectin represent useful noninvasive tests for organic GI disease with sensitivities of 73% and 80%, respectively, and a similar specificity of 74%. The PPVs of M2-PK and calprotectin for organic GI disease are 89% and 87%, respectively. Although calprotectin has a slightly higher sensitivity in differentiating organic from nonorganic pathology, both suffer from a relative lack of specificity and low NPVs of a normal M2-PK and calprotectin are 57% and 65%, respectively. The relatively low NPV may be explained by patients with inactive IBD in this cohort, as all discordant cases (organic pathology with normal M2-PK or calprotectin) had histologically quiescent IBD. This observa-

TABLE 2B. Fecal Marker Values in Active vs. Inactive UC and Crohn's Disease

	n	M2-PK (U/mL)	*P*-value	Calprotectin(μg/g)	*P*-value
UC (total)	50	20.0 (5.4–69.0)		118 (41–385)	
Active UC	37	40 (6.2–87)	0.006	230 (99–555)	<0.05
Inactive UC	13	1.2 (0–239)		26 (5–1090)	
CD (total)	31	24.3 (6.4–44.0)		131 (62–221)	
Active CD	26	30 (8–70)	<0.005	192 (100–285)	<0.0005
Inactive CD	5	0.55 (0–1.7)		27 (19–109)	

Values are medians (and interquartile range). Mann–Whitney test for pairwise comparisons.
The *P*-value is for difference between active and inactive disease.

TABLE 3. Sensitivity, Specificity, PPV, and NPV for Organic GI Disease

	Sensitivity	Specificity	PPV	NPV	Likelihood Ratio
M2-PK >3.7 U/mL	73%	74%	89%	57%	4.57
Calprotectin >25 μg/g	80%	74%	87%	65%	13.97

tion further supports the role of M2-PK as a noninvasive marker of disease activity in IBD.

By using a combination of clinical factors, such as age, symptoms (especially those fulfilling ROME II criteria), and noninvasive fecal markers, a significant proportion of patients with inactive IBD or IBS may avoid further invasive endoscopic investigations, reserving endoscopy only for those with elevated fecal M2-PK or calprotectin concentrations. This strategy is likely to have beneficial resource and cost implications for endoscopy services, as up to 40% of new gastroenterology referrals are for patients with suspected IBS.[20] At current UK rates, the cost of a single M2-PK or calprotectin measurement are approximately equivalent at £40 (US $75), comparing very favorably with the cost for colonoscopy of £550 (US $1045).

Reliable markers of disease activity in IBD are important to guide therapy, as symptoms correlate poorly with disease activity. Also increasingly, the aim of IBD therapy is to achieve mucosal healing, in order to reduce later complications and surgery rates. Fecal markers show promise in this area as they are noninvasive, surrogate markers of inflammation and may predict active disease, as well as mucosal healing. There is no perfect marker but in future a combination of markers and/or clinical evaluation will be used to direct therapy. This study demonstrates that dimeric fecal M2-PK has the potential to be an important noninvasive marker of disease activity in IBD.

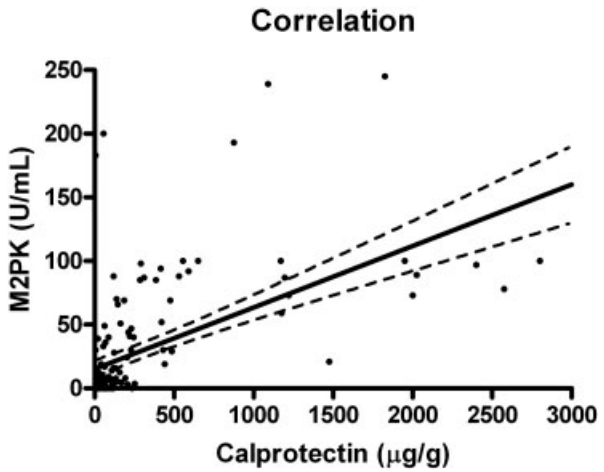

FIGURE 3. Correlation of M2-PK to calprotectin. Dotted lines indicate upper and lower limits of 95% confidence interval.

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