High prevalence of NSAID enteropathy as shown by a simple faecal test

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Abstract

***Background*—The diagnosis of non-steroidal anti-inflammatory drug (NSAID) induced enteropathy is difficult, requiring enteroscopy or the use of four day faecal excretion of ^{111}In labelled white cells.**

***Aims*—To assess faecal calprotectin (a non-degraded neutrophil cytosolic protein) as a method for diagnosing NSAID enteropathy.**

***Methods*—Single stool faecal calprotectin concentrations were compared with the four day faecal excretion of ^{111}In labelled white cells in 47 patients taking NSAIDs. The prevalence and severity of NSAID enteropathy was assessed using this method in 312 patients (192 with rheumatoid arthritis, 65 with osteoarthritis, 55 with other conditions) taking 18 different NSAIDs.**

***Results*—The four day faecal excretion of ^{111}In white cells correlated significantly with faecal calprotectin concentrations. In the group of 312 patients on NSAIDs faecal calprotectin concentrations were significantly higher than in controls, the prevalence of NSAID enteropathy being 44%. The prevalence and severity of NSAID enteropathy was independent of the particular type or dose of NSAID being taken or other patient variables.**

***Conclusions*—Assay of faecal calprotectin provides a simple practical method for diagnosing NSAID enteropathy in man. Forty four per cent of patients receiving these drugs had NSAID induced enteropathy when assessed by this technique; 20% of these had comparable levels of inflammation to that previously reported in patients with inflammatory bowel disease.**

(*Gut* 1999;**45**:362–366)

Keywords: non-steroidal anti-inflammatory drug; calprotectin; enteropathy

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Accepted for publication
3 March 1999

Most non-steroidal anti-inflammatory drugs (NSAIDs) cause gastric damage in short term volunteer studies, ranging from erythema to ulcers. Cross sectional endoscopy studies in patients on long term NSAID treatment have shown gastric erosions in 20–40% and gastric ulcers in 10–25% which have the potential to perforate and bleed.[1 2] Perforations and bleeding ulcers associated with NSAIDs may cause 1200–3000 deaths annually in the UK,[2] and in the USA between 2600 and 10 000 deaths in patients with rheumatoid arthritis alone.[3]

NSAIDs also cause an enteropathy in 20–65% of patients receiving these drugs depending on the method used for diagnosis.[4–6] The clinical implications are that patients may bleed from the small intestine[7–10] and lose protein,[7] contributing to iron deficiency and hypoalbuminaemia respectively. Some patients develop intestinal strictures,[11 12] which may require surgery, and others develop small intestinal perforation.[5 13]

The diagnosis of NSAID enteropathy, however, is difficult, requiring either enteroscopy[4 14] which is invasive, may take up to 10 hours, is unpleasant for patients, and has inherent risks of intestinal perforation,[4] or the use of four day faecal excretion of indium-111 labelled white cells,[6 7] which involves exposure to radiation and is both expensive and demanding on patients.

Calprotectin is a calcium binding protein found in neutrophilic granulocytes, monocytes, and macrophages,[15] comprising up to 60% of the total cytosolic protein content of neutrophils.[16] Calprotectin resists metabolic degradation[16] and can be measured in faeces.[17] Its use as an objective and quantitative marker of intestinal inflammation has been suggested from studies in patients with inflammatory bowel disease where faecal calprotectin concentrations correlate significantly with histological and endoscopic assessment of disease activity in ulcerative colitis,[18] and with faecal α_1 antitrypsin levels and faecal excretion of ^{111}In labelled white cells in patients with Crohn's disease.[17 19]

The use of faecal calprotectin for the diagnosis of NSAID enteropathy in patients taking these drugs has not been studied, but if successful might lead to wider appreciation of the condition and appropriate treatment for those with severe inflammation and attendant complications.[20–22]

Patients and methods

AIMS

Our aims were: (*a*) to validate faecal calprotectin as a method for diagnosing NSAID enteropathy against the use of the faecal excretion of ^{111}In labelled white cells, which is currently the most frequently used technique to diagnose NSAID enteropathy; (*b*) to assess the day to day variation of faecal calprotectin in patients taking different NSAIDs, as a marker of method reproducibility; and (*c*) to assess the prevalence and severity of NSAID enteropathy

Abbreviations used in this paper: NSAID, non-steroidal anti-inflammatory drug; PPI, proton pump inhibitor.

in a cross sectional study of patients on NSAIDs using the calprotectin method.

PATIENTS

Recruited patients were from general rheumatology clinics at King's College Hospital and Bromley District General Hospital over a 12 month period. The male to female ratio represented the sex distribution of clinic attendance while the age criterion was 18–80 years. All patients were seen in the clinics for rheumatological conditions requiring treatment with an NSAID, and had been on a daily dose for at least one month.

Patients excluded were those with concomitant known intestinal or significant hepatic, respiratory, cardiovascular, or neurological disease, or those taking other medications which are associated with intestinal inflammation.[23] Data acquired on patients included the type of NSAID, length of regular usage, dose, disease for which NSAIDs were being used, other current medication, and other medical illnesses.

Forth eight healthy volunteers (15 men, 33 women; mean age 56 years, range 18–78) provided single stool samples to obtain a range of normality for faecal calprotectin.

As part of the validation procedure 47 patients taking NSAIDs were assessed for intestinal inflammation with four day faecal excretion of ^{111}In labelled white cells, which was compared with the faecal calprotectin concentration in the first stool sample (obtained on day 2 of the study) to assess the correlation between the two techniques.

A different group of 312 patients (102 men, 210 women; mean age 58 years, range 20–80), taking 18 different NSAIDs provided single stool samples for assessment of faecal calprotectin by ELISA; 192 had rheumatoid arthritis, 65 osteoarthritis, and 55 other rheumatological conditions such as fibromyalgia and non-specific lower back pain, not associated with gastrointestinal disease.[23] A total of 146 patients was using second line drugs (74 methotrexate, 32 sulphasalazine, 17 gold, nine azathioprine, eight penicillamine, six other), 30 were using prednisolone, and 60 were using a gastroprotective agent (12 proton pump inhibitors (PPI), 40 histamine H_2 receptor antagonists, and eight misoprostol). Table 1 details all medications used. Day to day variability in faecal calprotectin was assessed in 98 paired samples from these patients, taken on consecutive days. The study was approved by the King's Healthcare and Bromley Local Research Ethics Committees and all patients gave informed consent.

INDIUM-111 WHITE CELL LABELLING

Neutrophils were isolated by sedimentation and centrifugation and labelled with ^{111}In (30–300 μCi; 1–11 mBq) using tropolone as an ionophore as previously described,[23] and injected intravenously. The estimated radiation dose received was 0.85–8.5 milli Sieverts (effective dose equivalent) depending on the dose received. Complete individual stools were collected over a four day period after injection of the labelled cells and counted in a high resolution bulk sample counter along with standards for 20–60 seconds which allows measurement of 0.1–0.01% (low and high dose respectively) of the injected dose with a counting accuracy of ±4%. Control data from 21 normal subjects had been established previously (median 0.41%, 95% confidence interval (CI) 0.05 to 0.94%).

FAECAL CALPROTECTIN ASSESSMENT

Stool samples were frozen on receipt at −20°C. After thawing, 5 g aliquots were suspended in 10 ml of faecal extraction buffer (Tris buffered isotonic (150 mM) saline, with 10 mM $CaCl_2$ and 0.25 mM thiomersol as an antimicrobial agent, pH 8.4) and homogenised for 45 seconds at 20 000 rpm with an Ultra Turrax (Ika Werke, Germany) mechanical homogeniser. The homogenates were centrifuged for 15 minutes at 10 000 *g* at room temperature. The top halves of the supernatants were pipetted off, frozen, and stored at −20°C until quantitation by ELISA.

Microtitre plates were coated by adding 200 μl of an IgG fraction of a rabbit anticalprotectin[15] diluted 1/2000 in phosphate buffered saline to each well. The plates were covered with mylar foil and kept at +4°C until use. Calprotectin standards[15 17] 3.75–60 mg/l, were prepared by diluting purified calprotectin in an assay buffer: Tris (50 mM) buffer containing 150 mM NaCl, 0.5 mM $MgCl_2$, 2.5 mM KCl, 0.25 mM thiomersol, 0.05% Tween 20, and 10 g/l bovine serum albumin pH 8.0. Before use the microtitre plates were washed four times in buffer, the assay buffer less the bovine serum albumin. The frozen faecal extracts were thawed and diluted 1/20 and

Table 1 Calprotectin concentrations and type of non-steroidal anti-inflammatory drug (NSAID) used

NSAID	No on NSAIDs	Age (y) (mean (range))	Calprotectin (mg/l) (median (range))	No using gastroprotective agents			No using	
				PPI	H_2 antagonists	Misoprostol	2nd line drug	Prednisolone
Diclofenac	116	56 (21–79)	8.0 (1.0–118.0)*	2	16	1	54	9
Naproxen	62	60 (30–80)	9.0 (1.0–60.0)*	3	9	3	33	11
Indomethacin	35	54 (28–77)	7.0 (1.0–60.0)*	5	3	2	21	3
Ibuprofen	35	56 (26–75)	7.0 (1.0–61.0)*	1	1	0	7	3
Diclofenac and misoprostol	27	60 (24–80)	8.0 (1.0–57.0)*	0	4	–	9	2
Meloxicam	7	63 (50–79)	4.5 (0.5–11.0)†	0	2	0	2	0
Etodolac	6	60 (37–74)	7.5 (0.5–15.5)†	0	1	1	5	0
Piroxicam	6	64 (43–77)	7.5 (5.0–18.0)†	1	0	0	4	1
Nabumetone	5	58 (46–77)	7.0 (4.0–16.0)†	0	0	0	4	0
Others	13	62 (46–71)	4.0 (0.5–41.4)†	0	4	1	7	1

*Significantly different from normals ($p<0.0001$, Mann-Whitney U test).
†Insufficient patient numbers for reliable statistical analysis.
PPI, proton pump inhibitor.

1/200 (and dilutions of 1/400–6400 when required) in the assay buffer. Standards and diluted samples (100 µl) were added to the plates (all done in duplicate) which were covered and incubated at room temperature for 45 minutes on a plate shaker with an agitating speed of 600/min. The wells were then washed four times with washing buffer. Alkaline phosphatase (100 µl) conjugated anticalprotectin (dilution 1/1600) was added to each well, the plate covered, and incubated at room temperature for 45 minutes on the plate shaker with an agitating speed of 600/min. Thereafter the wells were washed four times and 100 µl substrate solution was added (2 × 5 mg *p*-nitrophenol phosphate tablets dissolved in 10 ml substrate buffer which contained 10% diethanolamine with 0.5 mM $MgCl_2$, 0.25 mM thiomersal, pH adjusted to 9.6 with HCl). The optical density (405 nm; measured on Micro Tracer plate reader, Syva, Milton Keynes, UK) of the highest standard was monitored and the reaction stopped by adding 50 µl of a 1 M NaOH solution to each well when its optical density read between 1.2 and 1.5, similar to that previously described.[17]

STATISTICS

Spearman's correlation coefficient was used to assess the correlation between ^{111}In white cell labelling and faecal calprotectin concentration. Statistical comparison between controls and the NSAID treated group was performed using the Mann-Whitney U test. Wilcoxon matched pairs test, Altman Bland plot, Spearman's correlation, and mean coefficient of variation were calculated for the paired samples. Analysis of variance (ANOVA) and multiple regression analysis were used for assessing differences between subgroups of patients taking different NSAIDs, second line agents, gastroprotective agents, and prednisolone.

Results

VALIDATION OF FAECAL CALPROTECTIN AS A MARKER OF NSAID ENTEROPATHY

The median calprotectin concentration in the 48 normal controls was 2.0 mg/l (range 0.2–10.9 mg/l).

The four day faecal excretion of ^{111}In white cells (median 1.85%, range 0.05–4.89%; normal <1%, 95% CI 0.05 to 0.94%) in the 47 patients on NSAIDs differed significantly from controls (p<0.0001) and correlated significantly (r=0.83, p<0.0001, Spearman) with the faecal calprotectin concentrations (median 11.0 mg/l, range 1.0–89 mg/l; reference range <8.9 mg/l) in patients on NSAIDs. Of the 47 patients studied using both techniques, 70% had evidence of intestinal inflammation as assessed by the indium white cells and 53% as assessed by the calprotectin method. Only one patient with an elevated faecal calprotectin had a normal four day faecal excretion of ^{111}In white cells.

There was no significant difference (p>0.1, Wilcoxon matched pairs sign rank) between calprotectin concentrations in the paired samples (bias 1.54; 95% CI −4.94 to 1.85 by Altman Bland plot; r=0.8, Spearman). The mean coefficient of variation for all paired samples was 42%. However, if we use a reference range of normality as the 95th centile of the normal control samples we obtain an upper reference limit for faecal calprotectin of 8.9 mg/l. The mean coefficients of variation for paired samples were 63% and 27% where either of the two calprotectin concentrations was less or greater than 8.9 mg/l respectively.

PREVALENCE AND SEVERITY OF NSAID ENTEROPATHY USING FAECAL CALPROTECTIN

Figure 1 shows the calprotectin concentrations from the 312 patients on NSAIDs (median 7.3 mg/l, 95% CI 6.5 to 8.0) which differed significantly (p<0.0001, Mann-Whitney U test) from the 48 controls (median 2.0 mg/l, 95% CI 1.6 to 5.0). There was no significant difference (p=0.07, Mann-Whitney) in faecal calprotectin levels between the 312 patients studied and the 47 patients studied using both the calprotectin (median 11 mg/l, 95% CI 6 to 20) and ^{111}In white cell excretion technique.

There was no significant difference (p>0.1, ANOVA) in faecal calprotectin concentration in patients receiving different types or doses of NSAIDs or between those patients using or not using a gastroprotective agent (including those taking misoprostol as part of a combined formulation) in addition to their NSAID (table 1). Furthermore, multiple linear regression analysis (table 2) did not show any significant effect (p>0.05) of age, sex, type of NSAID used, diagnosis, or treatment with second line agent, gastroprotective agent, or prednisolone on the level of faecal calprotectin when all variables were taken into account.

Discussion

The gastrointestinal side effects of NSAIDs are a major health issue responsible for an estimated 30 000 hospital admissions and

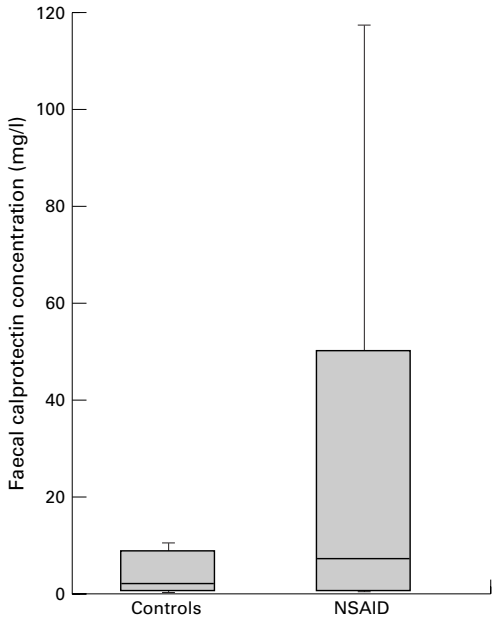

Figure 1 Box and whisker diagram showing median faecal calprotectin concentration (horizontal line), 5th and 95th centiles (box), and range (whiskers) in controls (n=48) and patients taking non-steroidal anti-inflammatory drugs (NSAIDs) (n=312).

Table 2 Multiple linear regression analysis of variables affecting calprotectin concentrations in 312 patients taking non-steroidal anti-inflammatory drugs (NSAIDs)

Variable	*Standard error*	*95% confidence interval*	*p Value*
Age	0.07	−0.21 to 0.07	0.34
Sex	2.04	−2.38 to 5.67	0.42
Type of NSAID used	0.53	−2.04 to 0.08	0.07
Diagnosis	0.73	−0.75 to 2.13	0.35
Treatment with second line drug	0.62	−1.94 to 0.53	0.26
Treatment with gastroprotective agent	1.40	−1.91 to 3.67	0.54
Treatment with prednisolone	3.25	−6.83 to 5.97	0.90

Second line drugs used included sulfasalazine, methotrexate, gold, penicillamine, azathioprine, hydroxyurea, cyclophosphamide, and chlorambucil.
Gastroprotective agents used included histamine H_2 receptor antagonists, proton pump inhibitors, and misoprostol, as shown in table 1.

1200–3000 premature deaths in the UK annually.[2] Lesions in the gastroduodenum can readily be diagnosed and followed up by upper gastrointestinal endoscopy. Diagnosis of NSAID induced damage to the small intestine has however remained problematic as ^{111}In white cell studies and/or enteroscopy[4 7 14 24 25] are time consuming, expensive, uncomfortable for patients, and not widely available. Small bowel permeability studies show a high prevalence of increased intestinal permeability induced by NSAIDs,[26 27] but this is not a direct measure of intestinal inflammation. The defining feature of NSAID enteropathy is the increased influx of neutrophils to the intestinal mucosa[6 7 23] and subsequent excretion into the bowel lumen. Measurement of faecal calprotectin, a stable granulocyte cytosolic protein,[17] also exploits this increased influx of granulocytes and seems to provide a simple method for the diagnosis of NSAID induced enteropathy.

Its significant correlation with the four day faecal excretion of ^{111}In labelled white cells seen in our study further validates the use of faecal calprotectin as a marker of intestinal inflammation induced by NSAID usage.[28] The degree of agreement between the two techniques in identifying the abnormal results was in general acceptable. Seventy per cent of subjects were abnormal by the ^{111}In white cell method, in agreement with comparable previous studies,[25 26] while 52% were abnormal by the calprotectin method. Only one patient with an elevated faecal calprotectin had a normal four day faecal excretion of ^{111}In white cells.

The issue of whether the increase in faecal neutrophil excretion may be due to NSAID induced gastropathy has been addressed in a previous study by Hayllar *et al*.[21] These investigators showed, in patients taking NSAIDs, no significant difference in the four day faecal excretion of ^{111}In labelled white cells between those with normal antral biopsy samples and those with chronic active gastritis as assessed both histologically and endoscopically. In view of the correlation between faecal calprotectin excretion and four day faecal excretion of ^{111}In labelled leucocytes it is unlikely that NSAID induced gastropathy is contributing significantly to the elevated levels of faecal calprotectin seen in the 312 patients taking NSAIDs.

There is a significant correlation and no statistical difference in the levels of faecal calprotectin in stools obtained on consecutive days, and the Altman Bland plot shows no significant bias between the two days. The day to day coefficient of variation of faecal calprotectin concentrations of 27% and 63% above and below the normal range is acceptable for a faecal marker[29] and is almost certainly due, in part, to the slightly uneven distribution of calprotectin in faeces.[17]

When the 312 patients taking NSAIDs were studied using the calprotectin method, using the 95th centile (8.9 mg/l) for faecal calprotectin from our normal controls as a reference value, 44% had evidence of intestinal inflammation. This is somewhat lower than the reported prevalence (55–75%)[5 21 23] where ^{111}In white cell labelling techniques were used. It may be that the calprotectin method is less sensitive than the ^{111}In white cell technique and only identifies those with the more severe enteropathy. However, with the increasing awareness of NSAID induced toxicity, patients may now be more reluctant than before to take a regular full therapeutic dose of NSAIDs, tailoring their NSAID intake according to clinical symptoms. A prevalence of 44%, however, accords well with enteroscopic studies showing erosions and ulcers in about the same proportion of patients (40%), which is still somewhat higher than in postmortem studies (20%)[5] where autolysis may have obscured some pathology.

Subgroup analysis did not show any significant difference ($p>0.05$; ANOVA/multiple regression analysis) in the levels of faecal calprotectin in relation to the possible effects of age, sex, dose of NSAID, or type of NSAID used, which conforms to results using ^{111}In labelled white cells.[23] In particular patients taking Arthrotec, a combination of diclofenac and misoprostol, had the same prevalence and severity of intestinal inflammation as those not receiving prostaglandins. Of six patients taking meloxicam (a putative selective cyclooxygenase-2 inhibitor) none had elevated levels of calprotectin, and of five patients taking nabumetone (a non-acidic pro-NSAID which does not increase intestinal permeability[30 31]), only one had an elevated level of calprotectin (16 mg/l). However, neither group had sufficient patient numbers to allow reliable statistical analysis.

The calprotectin levels in our 312 patients taking NSAIDs are comparable to those in a previous study using calprotectin to assess mucosal inflammation induced by short term NSAID ingestion in normal controls.[28] In contrast all of our patients had an underlying rheumatological diagnosis and had been on NSAIDs for a mean treatment period of four years. The similarity in calprotectin values in patients and volunteers on NSAIDs implies, as suggested in other studies,[6 7] that the underlying rheumatological diagnosis (apart possibly for patients with spondylarthropathy[32]) does not contribute significantly to the intestinal inflammation seen in patients taking NSAIDs. Furthermore, it suggests that adaptation to the damage does not occur in the small intestine as has been suggested to be the case in the stomach.[33]

The median faecal calprotectin level of 7.3 mg/l seen in our NSAID treated group, which

suggests a low grade enteropathy, is considerably less than the median values seen in patients with active ulcerative colitis and Crohn's disease (30–60 mg/l).[17 18] This agrees with the findings of Teahon and Bjarnason,[24] who found median four day faecal excretion of ^{111}In labelled white cells to be elevated 20-fold in patients with active ulcerative colitis, 17-fold in patients with Crohn's disease, and twofold in patients with NSAID enteropathy. Of the 44% of patients with faecal calprotectin levels greater than 8.9 mg/l, 20% had levels comparable (>40 mg/l) with those seen in patients with active inflammatory bowel disease.[17] Subgroup analysis did not reveal this group to differ from the remainder with regard to patient characteristics in terms of disease type, age, or type/dose of NSAID being used. It has been suggested that patients with the highest inflammatory activity are those at highest risk of complications (bleeding and protein loss) and hence most likely to be considered for treatment.[21 22]

In conclusion, measurement of faecal calprotectin seems to be a simple non-invasive means of identifying patients with NSAID enteropathy. The technique provides similar information to the four day faecal excretion of ^{111}In labelled white cells but has the obvious advantages of avoiding the need for four day faecal collections and exposure to radiation.

Dr J A Tibble is funded by a research fellowship from the NHS Executive South Thames Regional Office. Dr G Sigthorsson was supported by a grant from the Helga Jonsdottir and Sigurlidi Kristjansson Memorial Fund, Iceland.

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