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# Faecal leukocyte esterase activity is an alternative biomarker in inflammatory bowel disease

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## Abstract

**Background:** Leukocyte cytosolic proteins (e.g., calprotectin) are emerging biomarkers for inflammatory bowel disease. Leukocyte aryl esterase activity has been commonly used for sensitive detection of leukocytes in human body fluids such as urine. Urine test strip results are generally reported in categories. As automated strip readers allow quantitative data to be reported, sensitive quantitative detection of leukocytes in body fluids has become possible. Here, we explored the use of leukocyte esterase as a potential alternative faecal biomarker for inflammatory bowel disease.

**Methods:** We evaluated leukocyte esterase activity in faecal extracts and compared Cobas u 411 (Roche) quantitative reflectance data with calprotectin concentration for 107 routine samples. Stability of leukocyte esterase for trypsin digestion was carried out by adding trypsin to the extract. Incubation occurred at 37 °C for 24 h or 48 h.

**Results:** Reproducibility of the reflectance signal was good (within-run imprecision: 6.1%; between-run imprecision: 6.2%). Results were linear in the range  $10^3$ – $10^6$  WBC/100 mg faeces. The lower limit of detection was 4 WBC/ $\mu$ L and the lower limit of quantification was 5 WBC/ $\mu$ L. Stability of LE activity in stool and faecal matrix was good. An adequate correlation was obtained between leukocyte esterase activity and the faecal calprotectin concentration:

$\log(y)=4.28+0.29\log(x)$ . In vitro experiments monitored the digestion of leukocyte esterase and faecal calprotectin. Leukocyte esterase activity was significantly less affected by trypsin activity than calprotectin immunoreactivity.

**Conclusions:** Quantitative leukocyte esterase activity of faecal extracts provides information about the leukocyte count in the gut lumen. Leukocyte esterase is a promising and affordable alternative biomarker for monitoring inflammatory bowel disease.

**Keywords:** calprotectin; faeces; inflammatory bowel disease; leukocyte esterase; trypsin.

## Introduction

Crohn's disease (CD) and ulcerative colitis, collectively known as inflammatory bowel disease (IBD), are chronic illnesses of the gastrointestinal tract characterised by a recruitment of leukocytes in the intestinal wall [1, 2]. In IBD, activated leukocytes (mostly polymorphonuclear cells) infiltrate the mucosa and appear in faeces due to shedding in the intestinal lumen [3, 4]. Faeces is in direct contact with this inflamed intestinal mucosa, thus making stool is an obvious place for biomarker discovery in IBD [5]. High faecal neutrophil levels have been detected in IBD and may be used as markers of disease activity [6, 7].

At the time being, the two most commonly used faecal biomarkers for IBD are calprotectin and lactoferrin [8]. Calprotectin is a 36 kDa protein, accounting for 60% of soluble proteins in the cytosol of neutrophils. Calprotectin is a marker of local inflammation in which the concentration reflects the neutrophil cell infiltrate in the intestinal mucosa [9, 10]. An increased value of faecal calprotectin leads to the identification of patients with suspicion of IBD and in need of an endoscopy [9, 11]. Lactoferrin is an iron-binding glycoprotein expressed by activated neutrophils. It is released by the injured tissue during inflammation. Other faecal neutrophil-derived proteins (e.g., lysozyme, myeloperoxidase, granulocyte elastase, S100A12) have been investigated in IBD with less success [8]. Calprotectin, lactoferrin and other proteins are produced in significant

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amounts by inflammatory cells and these biomarkers correlate well with colorectal and intestinal inflammation [12, 13]. The faecal excretion of Indium 111-labelled leukocytes is still the golden standard faecal marker of inflammation. Unfortunately, neutrophil counting in faecal specimens is hampered by pre-analytical issues: the sample should be examined within a few hours of its collection [7].

Leukocyte esterases (LE) are cytoplasmatic enzymes present in polymorphonuclear cells. LE activity has been used for decades to detect the presence of white blood cells (WBC) in human body fluids (e.g., urine, peritoneal fluid). These esterases cleave a derivatised pyrazole amino acid ester to liberate derivatised hydroxy pyrazole. This pyrazole can react with a diazonium salt to produce a purple colour, which allows a simple and affordable quantification [14]. In 1993, Brouwer proposed semiquantitative LE analysis of faecal extracts for the monitoring of IBD. A strong correlation between faecal lysozyme concentrations and faecal LE activity was found [15]. Modern automated urine test strip analysers offer the possibility to obtain reflectance readings meaning that test results no longer need to be expressed semiquantitatively in an ordinal scale. The reflectance value (%) is inversely related to the concentration of the analyte in the sample. Access to the instrument's raw data theoretically allows a higher analytical sensitivity and a better precision, as also shown by Penders et al. [16].

Trypsin (EC 3.4.21.4) is an endopeptidase produced in abundant amounts by the human pancreas and found in the intestinal lumen where it hydrolyses several proteins. Following release of trypsinogen, the enzyme reaches its highest activity in the proximal part of the duodenum. During transit, activity of proteases decreases [17, 18]. As calprotectin contains 14 residues which may act as potential cleavage sites for trypsin, (partial) digestion of calprotectin by trypsin can be postulated [19]. Digestion of LE by trypsin has not yet been described.

In this study, we first wanted to explore the possibilities of faecal LE activity as an alternative biomarker for IBD, which is of major clinical importance. Additionally, we wanted to evaluate the proteolytic effect of trypsin on the faecal LE concentration.

## Materials and methods

### Extract preparation

Faecal extracts were prepared with the faecal sample preparation kit (Roche diagnostics, Mannheim, Germany) according to the manufacturer's instruction. After adding a standardised amount of

faeces (100 mg) to a tube, 5 mL of EliA Calprotectin Extraction Buffer (Thermo Scientific, Uppsala, Sweden) was added and the sample was vortexed. After centrifugation (at 1200 g for 10 min) the supernatant was used as a faecal extract in our experiments. 107 routine faecal samples from outpatients consulting the Department of Gastro-Enterology were used (some patients provided more than one sample with several weeks between every sample). All samples were completely processed within 2–4 h after arrival. The use of these samples was approved by the Ethical Committee.

### Method evaluation

Test strip analysis was carried out using Combur Test M strips on the Cobas u 411 analyser (Roche diagnostics, Mannheim, Germany). The strips include reagent pads for ordinal scale reporting of relative density, pH, LE, nitrite, protein, glucose, ketones, urobilinogen, bilirubin, and haemoglobin/myoglobin. The intensity of the reaction colour of the test pad is detected by measuring the percentage of light reflected from the surface of the test pad. The reflectance value, expressed as a percentage is inversely related to the concentration of the analyte in the sample. The following equation by Penders et al. was used to estimate the number of WBC in faecal extract:  $y=83.7-15.4\log(x)$  where  $y$  is the reflectance in% and  $x$  the number of WBC ( $10^6$  cells/L) [16]. First an ideal dilution was chosen for experiments and 20  $\mu$ L was pipetted onto the LE reagent pad. Next, within-run imprecision, between-run imprecision, linearity, stability, limit of detection, and limit of quantification were assessed for LE activity in faecal extracts. LE activity was compared to the calprotectin levels in the faecal extract and regression analysis was performed. Calprotectin was measured on the Phadia ImmunoCAP 250 analyser (Thermo Scientific, Uppsala, Sweden) according to the manufacturer's instructions. Results were expressed in mg/kg. The limit of detection of the method is 15 mg/kg [20].

### Proteolysis by trypsin

Trypsin activity was determined using  $N\alpha$ -Benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA, Sigma Aldrich, St. Louis, MI, USA) as a substrate for trypsin (Sigma Aldrich) in a colorimetric method. The amount of 4-nitroaniline formed was subsequently measured by the increase in absorbance at 405 nm (25 °C). A triethanolamine solution (TEA 0.2 mol/L, Sigma Aldrich;  $CaCl_2$  0.02 mol/L, Sarstedt, Nümbrecht, Germany) adjusted to pH 7.8 was used as buffer. Results were expressed in BAPNA units (U/L). To evaluate the stability of LE activity, trypsin digestion was carried out *in vitro* by adding trypsin (Sigma Aldrich) solution (1 mg/mL, 1mM HCl). Incubation was performed at 37 °C. Samples were analysed for calprotectin and LE activity after 24 h and 48 h of incubation.

### Statistics

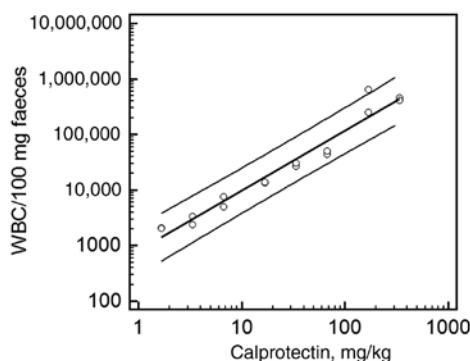
All calculations were made in Excel 2010 and MedCalc (version 11.6.1.0, Mariakerke, Belgium). Results were given as median and inter-quartile ranges (IQR). Comparisons between conditions were made using a Wilcoxon test. A value of  $p<0.05$  was considered

statistically significant. Regression analysis was performed. The lower limit of detection was calculated as the mean value plus 3 standard deviations (SD) for a blank sample. The lower limit of quantification was calculated as the mean value plus 6 SD for a blank sample.

## Results

### Method evaluation

Pilot experiments showed that a 1:10 dilution of faecal extract (prepared according to the manufacturer's instruction) in saline was ideal for test strip analysis and for reflectance readings. 20  $\mu$ L was pipetted onto the reagent pad for reflectance measurements. Reproducibility of the reflectance signal was good for LE activity in faecal extract. A within-run imprecision of 6.1% was calculated after analysing a routine sample 15 times. Consequently, a between-run imprecision of 6.2% was calculated after analysing the same sample on 15 different days. The sample contained a mean number of white blood cells of  $47 \times 10^3/100$  mg faeces. Results were linear in the range  $10^3$ – $10^6$  WBC/100 mg faeces (corresponding to faecal calprotectin concentration ranges from  $\pm 1$  to  $\pm 1000$  mg/kg). Results are shown in Figure 1. Calprotectin levels shown are theoretically as a serial dilution was performed. The lower limit of detection for leukocytes was 4 WBC/ $\mu$ L and the lower limit of quantification was 5 WBC/ $\mu$ L (respectively corresponding to 2019 en 2688 WBC/100 mg faeces). Data of the method evaluation are summarised in Table 1. To evaluate the stability of LE activity, 14 samples were processed and faecal matrix was placed at room temperature



**Figure 1:** Linearity between calprotectin levels (x, mg/kg) and leukocyte esterase (LE) activity expressed as the number of white blood cells in 100 mg faeces (y, WBC/100 mg faeces). Results were linear in the range  $10^3$ – $10^6$  WBC/100 mg faeces (corresponding to calprotectin concentration ranges from  $\pm 1$  to  $\pm 1000$  mg/kg). WBC, white blood cells.

**Table 1:** Method evaluation of leukocyte esterase (LE) activity in faecal extracts. Within-run imprecision ( $C_{wr}$ ), between-run imprecision ( $C_{br}$ ), limit of detection (LOD), limit of quantification (LOQ) were assessed. Regression analysis was performed for LE activity (y) and calprotectin levels (x) in faecal extracts for 46 samples out of 107.

Performance characteristics of LE activity in faecal extracts	Results
$C_{wr}$	6.1%
$C_{br}$	6.2%
LOD	4 WBC/ $\mu$ L 2019 WBC/100 mg faeces
LOQ	5 WBC/ $\mu$ L 2688 WBC/100 mg faeces
Linearity	$10^3$ – $10^6$ WBC/100 mg faeces
Regression analysis	$\log(y) = 4.28 + 0.29 \log(x)$ $r = 0.27$

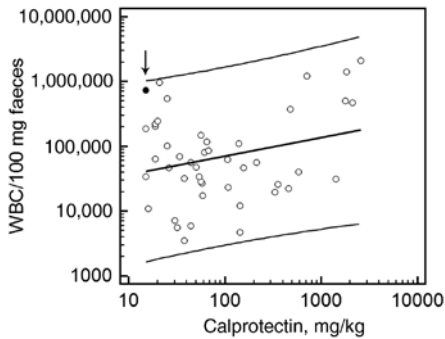
$C_{wr}$ , within-run imprecision;  $C_{br}$ , between-run imprecision; LE, leukocyte esterase; LOD, limit of detection; LOQ, limit of quantification; r, correlation coefficient; WBC, white blood cells.

for 24 h. A mean decline of 1.52% was seen after 24 h. Next, four stool samples were extracted again after 24 h while kept at 4  $^{\circ}$ C, a difference of respectively 1.6%, 3.3%, 0.0% and 0.9% was seen. As mentioned above, LE activity between-run imprecision was evaluated over 15 different days. These 15 aliquots were frozen at  $-20$   $^{\circ}$ C and between-run imprecision is therefore a measurement for stability at  $-20$   $^{\circ}$ C.

Next, in our method evaluation we compared faecal calprotectin values and LE activity for 107 samples. After excluding samples with values below 15 mg/kg (limit of quantification for calprotectin), regression analysis was performed for 46 samples. A fair agreement was obtained between LE activity and faecal calprotectin concentration:  $\log(y) = 4.28 + 0.29 \log(x)$  with a correlation coefficient (r) of 0.27. LE activity is expressed as the number of white blood cells in 100 mg/faeces [16]. Figure 2 depicts the correlation between LE activity and calprotectin concentration in faecal extracts. The arrow shows a case where proximal lesions were confirmed by endoscopy.

### Proteolysis by trypsin

In vitro-experiments monitored the digestion of LE and faecal calprotectin in 10 samples. The same conditions as in our previous publication were used [19]. After adding 500  $\mu$ L of trypsin solution (1 mg/mL) to 500  $\mu$ L 1:100 diluted calprotectin in faecal matrix, proteolysis was monitored for 24 h and 48 h. The median trypsin activity after adding trypsin was 57.3 U/L (IQR: 55.9–65.9 U/L).

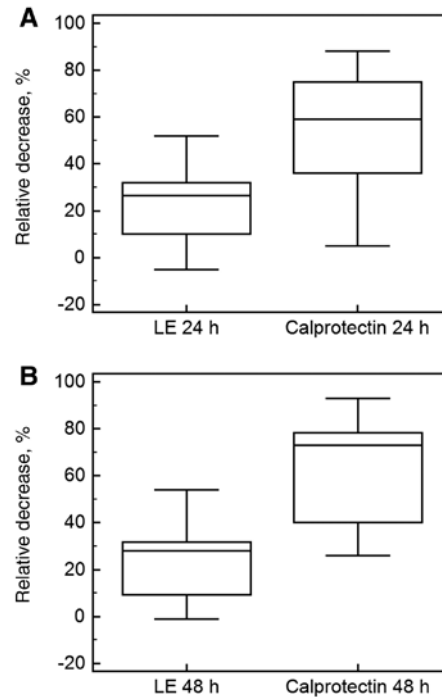


**Figure 2:** Regression analysis between calprotectin levels ( $x$ , mg/kg) and leukocyte esterase (LE) activity expressed as the number of white blood cells in 100 mg faeces ( $y$ , WBC/100 mg faeces). The following regression equation was obtained:  $\log(y) = 4.28 + 0.29 \log(x)$  ( $r = 0.27$ ). The arrow depicts a confirmed case of IBD with proximal lesions. WBC, white blood cells.

Analogue findings for calprotectin levels were found, trypsin digestion led to loss of immunoreactivity and analyte stability. A median relative decrease of 59.0% (IQR: 37.3%–74.3%) was seen after 24 h of incubation, and a median absolute decrease of 63.5% (IQR: 32.8%–77.3%) after 48 h of incubation. Nevertheless LE activity was less affected by trypsin activity than calprotectin immunoreactivity. After trypsin treatment, a median relative decrease of 26.5% (IQR: 13.5%–31.3%) was seen after 24 h of incubation, and a median absolute decrease of 28.5% (IQR: 12.8%–32.5%) after 48 h of incubation. Differences between decreases of LE activity and calprotectin levels after 24 h and 48 h of incubation were evaluated using a Wilcoxon test. Decreases were statistically significant after 24 h and 48 h of incubation (for both  $p = 0.01$ ). Results are shown in Figure 3.

## Discussion

Analysis of the test strip reflectance data allows a quantification of the analyte over a wide activity range. Using a hyperbolic calibration curve, reflectance data can be converted into quantitative WBC counts [16]. As urine test strips are inexpensive, as compared to immunochemical calprotectin testing, this could improve screening for IBD. It offers an improved, fast, reliable method that is easy to handle and usable in primary care laboratories. In our study, LE activity could be quantified in routine faecal extracts. Reproducibility of the obtained results was good (both within- and between-run imprecision were below 10%), which is guaranteed by the constant



**Figure 3:** Distribution of relative decreases of leukocyte esterase (LE) activity and calprotectin after adding a median trypsin concentration of 57.3 U/L (IQR: 55.9–65.9 U/L) ( $n = 10$ ) and incubation for 24 h (A) or 48 h (B) at 37 °C. Differences are expressed as compared to the baseline reflectance levels (baseline = before adding trypsin). A statistically significant difference between LE activity and faecal calprotectin was found ( $p = 0.01$ ). h, hours; LE, leukocyte esterase activity.

reaction temperature. Furthermore, the buffered extraction medium used for sample preparation adds to create an optimal environment for measuring the enzyme activity. As a result of the procedure for applying the faecal extract to the test pads on the reflectance reader, improper dipping is not a problem, neither is confusion about sample contamination caused by dipping the strip in the tube, which potentially leads to interferences.

When faecal extracts were subjected to trypsin degradation for prolonged incubation times (24–48 h) at 37 °C, LE proved to be more resistant against proteolysis than calprotectin. Human calprotectin shows 14 potential cleavages sites for trypsin and previous experiments showed a statistically significant breakdown of calprotectin [19]. As trypsin shows a limited stability, our findings do not imply major pre-analytical precautions for LE or calprotectin following sampling [21]. These results are in agreement with earlier data where the half-life of LE in faecal extracts was measured to be 2–3 days. In contrast, the half-life of lysozyme in faecal extracts varied between 0.5 h and 3.2 h [15].



In this study, we further compared the LE activity quantitatively assayed on the Cobas u 411 automated strip reader with the calprotectin levels to evaluate the possible diagnostic value of faecal LE. As expected, calprotectin and LE showed an adequate correlation, as they are both markers of the number of neutrophils which have migrated through the mucosal membrane of the gut wall. In contrast to WBC count, LE is not affected by subsequent cell lyses within the gut lumen. Furthermore, dilution experiments of faecal extracts demonstrated that LE was a more sensitive biomarker than calprotectin. Test strip measurements had excellent lower limits of detection and quantification. Results were also linear in the range  $10^3$ – $10^6$  WBC/100 mg faeces (corresponding to faecal calprotectin concentration ranges from  $\pm 1$  to  $\pm 1000$  mg/kg). In a previous study correlating test strip data with urinary flow results,  $r$  was  $-0.69$  between the flow cytometric WBC count and the LE reaction. The presence of esterase inhibitors in faeces and increased protein concentrations might negatively affect test results for LE [22].

The turnaround time for LE was short enough (2–4 h) not to affect the readings [23]. It is known that time intervals can influence results of automated test strip analysis, especially for leukocyte ratings. Froom et al. found that that urine testing for red and white blood cells with a dipstick is precise but unstable if testing is delayed for 24 h [24]. As the technique is not time-consuming and well known in the laboratory, the preparation of faecal matrix is only a minor addition to the workload. When samples cannot be analysed immediately, aliquots of faecal matrix can be placed at room temperature. Evaluation of stability showed that LE activity remains stable when faecal matrix is stored at room temperature (mean decline of 1.52%) or at  $-20$  °C (between-run imprecision of 6.2%). Preliminary data showed that LE activity remains stable during 24 h when stool is kept at 4 °C. This may be an important feature, as samples are not always brought immediately to the laboratory.

Interpretation of calprotectin values in proximal bowel disease is proven to be difficult [25, 26]. Sipponen et al. showed a low sensitivity for calprotectin in small bowel CD. Despite active CD in the small bowel, faecal calprotectin values were low [27]. Possible explanations could be the proteolytic effect of the large amounts of trypsin released by the pancreas into the lumen of the duodenum [19]. As our experiments show that LE activity is less influenced by trypsin, it could be an alternative biomarker. Also in patients with cystic fibrosis (CF) where faecal calprotectin values are higher, possibly explained by pancreatic insufficiency, LE activity could be a valuable alternative [28]. The correlation coefficient found is

rather low, which can be explained by the fact that low faecal calprotectin levels can correlate with high levels of LE activity, when proximal lesions are present. The arrow in Figure 2 depicts such a patient, where proximal lesions were confirmed by endoscopy. Summarised, LE activity cannot simply replace calprotectin, further prospective studies will be needed to assess sensitivity and specificity of the test.

In conclusion, quantitative test strip analysis provides reliable data on faecal WBC counts. LE reflectance data are promising for monitoring IBD. The faecal esterase allows non-invasive assessment of the intestinal inflammation. In comparison with immunochemical assays of specific leukocyte proteins, the LE test is about two orders of magnitude cheaper. The required technology needed for quantification is within reach of present day point of care testing. Further clinical studies will be required to define the exact role of faecal LE and the specific clinical applications in different settings of IBD patients and other patient populations. Potential clinical applications might be patients with proximal bowel disease, detecting active IBD, predicting recurrence of disease after surgery or monitoring the effects of medical therapy [8].

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