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Faecal Calprotectin Diagnostics

Focus on Primary Care
and Suspected Sources of Error

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Linköping 2023
To my family
ABSTRACT

Patients with gastrointestinal symptoms often present a diagnostic challenge for general practitioners. Faecal calprotectin (FC) is commonly used as a marker of intestinal inflammation and is useful for differentiating between inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS), as well as for the follow-up of patients with IBD and monitoring treatment response. However, several other causes of increased FC levels have been acknowledged, including intake of non-steroidal anti-inflammatory drugs or proton pump inhibitors and respiratory infections. Currently, there is insufficient knowledge about how these factors affect FC levels. It is crucial that physicians who use calprotectin as a diagnostic tool have the ability to conduct a sound evaluation of the test result to ensure accurate clinical decisions, and potentially avoid unnecessary referrals and invasive investigations.

The aim of this thesis was to investigate the contribution of FC in the diagnostics of gastrointestinal disease in primary care, its diagnostic value and accuracy as a predictor of gastrointestinal disease and the influence of different sources of error on calprotectin levels. In particular, the effects of oral diclofenac (a non-steroidal anti-inflammatory drug [NSAID]), omeprazole (a proton pump inhibitor [PPI]) and respiratory tract infection on FC levels are investigated. The normalization interval after cessation of diclofenac and omeprazole is assessed.

The first study is a retrospective analysis of data on all FC tests on adults conducted in primary care in Östergötland County in 2010. A higher proportion of patients with a positive FC result were diagnosed with IBD and organic gastrointestinal disease compared with those with a negative FC result. Predictors of IBD were positive FC, diarrhoea, rectal bleeding and male sex. Predictors of organic gastrointestinal disease were found to be positive FC, age >35 years, abnormal clinical findings and duration <3 months. FC had the highest sensitivity and negative predictive value compared with demographic factors, symptoms and duration. Intake of NSAIDs, PPIs and acetyl salicylic acid showed marginal effects on the diagnostic accuracy of FC for IBD and organic gastrointestinal disease. Among patients with a negative FC test, on whom no further investigations were performed, no missed diagnoses of IBD or organic gastrointestinal disease were detected at a 5-year follow-up.

The second study investigates the effect of diclofenac intake on FC levels. We found that short-term intake of oral diclofenac was associated with increased FC levels and that FC returned to normal within 2 weeks of cessation.

The third study reports on a randomized open-label clinical trial and investigates the effect of omeprazole, diclofenac and co-administration of these drugs on FC levels. The findings regarding diclofenac were consistent with those of the second study. Short-term intake of omeprazole alone or when co-administered with diclofenac was associated with increased FC levels. The normalization interval was 3 weeks after cessation.

The fourth study, a prospective cohort study, examines the effect of an acute respiratory tract infection on the FC level. Faecal and salivary calprotectin levels were not found to be increased during respiratory tract infections. This study did not confirm any correlation between calprotectin levels in saliva and faeces during infection.
In conclusion, FC reliably rules out IBD and contradicts the presence of other organic gastrointestinal diseases in patients with gastrointestinal symptoms attending primary care. Patients with a positive FC test together with other symptoms, such as diarrhoea, rectal bleeding, short duration or age $>$35 years should be prioritized for further investigations. Short-term intake of diclofenac, omeprazole, or their co-administration in healthy individuals is associated with increased FC levels. In patients with an increased FC level on diclofenac, it is sufficient to repeat the FC test 2 weeks after cessation. In patients on omeprazole alone or when co-administered with diclofenac, the FC test should be repeated 3 weeks after cessation. Acute respiratory tract infections were not found to be associated with increased faecal or salivary calprotectin levels.

Keywords calprotectin; inflammatory bowel disease; gastrointestinal disease; primary health care; non-steroidal anti-inflammatory agents; proton pump inhibitors; respiratory tract infection
Sammanfattning på svenska

Patienter med besvär från mag-tarmkanalen utgör en vanlig och diagnostiskt utmanande patientgrupp för allmänläkare. Fekalt kalprotektin (F-kalprotektin) är ett etablerat laboratorieprov för tarminflammation och används för att skilja mellan inflammatorisk tarmsjukdom (IBD) och funktionella mag-tarmsjukdomar. F-kalprotektin misstänks dock kunna öka även av andra orsaker, såsom intag av icke-steroida antiinflammatoriska läkemedel, magsyrahämmande läkemedel eller luftvägsinfektioner. För närvarande saknas tillräckliga kunskaper om i vilken utsträckning dessa faktorer påverkar nivåerna av kalprotektin. För allmänläkare som använder F-kalprotektin som ett diagnostiskt verktyg, är det viktigt att kunna göra en välgrundad värdering av utfallet, så att diagnostik och beslut om behov av vidare utredning kan optimeras.

Syftet med denna avhandling var att undersöka det diagnostiska värdet av F-kalprotektin vid utredning av mag-tarmsjukdomar i primärvården, samt hur nivån av kalprotektin påverkas av viktiga felkällor, såsom intag av vissa anti-inflammatoriska och magsyrahämnande läkemedel. Mer specifikt undersöktes hur oralt diklofenak (icke-steroid antiinflammatoriskt läkemedel), omeprazol (syrahämnande läkemedel) och pågående luftvägsinfektion påverkar nivåerna av F-kalprotektin. För diklofenak och omeprazol utvärderades också tiden för normalisering av F-kalprotektin efter utsättning av preparaten.


Delstudie 2 och 3 undersökte hur olika felkällor påverkar nivåerna av F-kalprotektin. Intag av läkemedlen diklofenak, omeprazol separat, eller i kombination med varandra under två veckor visade sig orsaka förhöjda nivåer av F-kalprotektin hos friska individer. Värdena återgick till det normala inom två veckor efter utsättning av diklofenak och inom tre veckor efter utsättning av omeprazol eller båda läkemedlen i kombination. Slutsatsen är att hos patienter som har ett förhöjt värde av F-kalprotektin och samtidigt infag av diklofenak eller omeprazol, bör testet upprepas minst 2 respektive 3 veckor efter utsättning för att garantera ett tillförlitligt testresultat.

Den fjärde delstudien undersökte om en akut luftvägsinfektion påverkar F-kalprotektin. Luftvägsinfektioner visade sig inte orsaka förhöjda nivåer av F-kalprotektin. Inget samband påvisades mellan kalprotektin i saliv och i avföring under pågående infektion.
LIST OF STUDIES

I Rendek Z, Falk M, Grodzinsky E, Kechagias S, Hjortswang H. Diagnostic value of faecal calprotectin in primary care patients with gastrointestinal symptoms: a retrospective Swedish cohort study. (Submitted)


LIST OF ABBREVIATIONS

ACE2  angiotensin-converting enzyme 2
ACL  acceptable change limit
ASA  acetyl salicylic acid
CD  Crohn’s disease
CI  confidence interval
COPD  chronic obstructive pulmonary disease
CRC  colorectal cancer
CV  coefficient of variation
ELISA  enzyme-linked immunosorbent assay
FC  faecal calprotectin
GI  gastrointestinal
IBD  inflammatory bowel disease
IBS  irritable bowel syndrome
IQR  interquartile range
IMP  investigational medicinal product
LLOQ  lower limit of quantification
LOD  limit of detection
NPV  negative predictive value
NSAID  non-steroidal anti-inflammatory drug
OGID  organic gastrointestinal disease
OR  odds ratio
PPI  proton pump inhibitor
PPV  positive predictive value
RTI  respiratory tract infection
TMPRSS2  transmembrane serine proteases 2
UC  ulcerative colitis
ULN  upper limit of normal
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INTRODUCTION

PATIENTS WITH GASTROINTESTINAL SYMPTOMS IN PRIMARY CARE

Approximately 8%–10% of primary care appointments involve patients with gastrointestinal (GI) symptoms (1, 2). The general practitioner (GP) has a central role in the early contact and management of these patients. Thus, GI symptoms are a common diagnostic challenge for GPs, who need to differentiate between functional and organic gastrointestinal diseases (OGIDs), such as inflammatory bowel disease (IBD) or GI tumours, to decide if further investigations and referrals to specialists are necessary.

Symptoms are often non-specific and may not allow a clear distinction to be made. Abdominal pain frequently leads to consultations in primary care and often poses a diagnostic and therapeutic dilemma and even leads to hospitalization in some cases. It is commonly observed that abdominal pain coexists with psychiatric disorders, such as depression or anxiety (3). About 75% of patients experiencing abdominal pain are handled in primary health care settings (4).

Other important symptoms include rectal bleeding, diarrhoea, constipation, change in bowel habits, or weight loss. However, these symptoms are also commonly present in benign conditions. Studies on primary care patients who were referred due to intestinal symptoms revealed an organic diagnosis in only 17%–37% of cases (5, 6). If there is suspicion of IBD, it is essential to perform a colonoscopy to ascertain the degree and distribution of inflammation and confirm the diagnosis. This procedure is invasive, requires bowel preparation and taking a day off work, and entails a risk of severe complications. In a study by Lasson et al. (7), the diagnostic yield of colonoscopy was found to be high only for symptoms of bleeding or diarrhoea, whereas in patients with other symptoms, the prevalence of significant findings was comparable with that observed in a screening population.

The rates of referrals for functional diseases vary considerably between GPs, and these patients tend to wait longer to receive referrals (8). Differentiating functional from OGID at an early stage could enhance the management of these patients and optimize referral decisions. The need to support GPs in this aspect has been advocated for a long time (9, 10).
GASTROINTESTINAL DISEASES OF IMPORTANCE

IRRITABLE BOWEL SYNDROME

Irritable bowel syndrome (IBS) is a common chronic functional GI disorder, with a worldwide prevalence of 4.1% and predominance among females and young adults (11). Most patients are diagnosed and managed in primary care. In a study by Thompson et al. (2), 30% of patients with GI complaints seen by GPs were diagnosed with IBS and 14% with other functional disorders.

IBS is thought to arise from a variety of factors, e.g. genetics, diet, stress, infection, and medicines such as antibiotics (12), however, the underlying pathogenesis remains uncertain. Possible underlying mechanisms include disturbances of the gut mucosal immune function, intestinal permeability, motility, microbial imbalance, altered brain-gut axis and visceral hypersensitivity (12). In many cases, these patients commonly experience other co-morbidities, such as somatic pain syndrome and psychiatric disorders (12).

Diagnosis is currently defined according to the internationally agreed Rome IV criteria (Box 1). In routine practice, IBS is commonly diagnosed by ruling out alternative organic conditions. According to Thompson et al. (2), almost 30% of primary care patients with IBS symptoms are referred to a specialist. Fear of cancer is common in these patients, and it remains in two-thirds of patients after seeing the doctor (2).

The long-term likelihood of developing an OGID is relatively low (2%–5%) (13). It has been shown that during a follow-up period of 2 years, approximately 12%–38% of patients experienced complete resolution of symptoms, 30%–50% experienced unchanged symptoms, and symptoms worsened in 2%–18% of patients (14).

A comprehensive approach by a trusted physician is essential for managing patients with IBS symptoms. Many of these patients can be managed effectively in primary care, given appropriate reassurance and education, often without the need for additional pharmacological treatment (14). The management includes information on general lifestyle, diet, and, based on the nature and severity of the symptoms, symptom-specific medication (15). For individuals who do not respond to pharmacological treatment, psychological interventions should be taken into consideration (15).

Box 1. Rome IV diagnostic criteria for irritable bowel syndrome (16).

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<th>Recurrent abdominal pain, on average, at least 1 day per week in the last 3 months, associated with 2 or more of the following criteria:</th>
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<td>1. Related to defecation</td>
</tr>
<tr>
<td>2. Associated with a change in frequency of stool</td>
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<tr>
<td>3. Associated with a change in form (appearance) of stool</td>
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Criteria fulfilled for the last 3 months with symptom onset at least 6 months prior to diagnosis
INFLAMMATORY BOWEL DISEASE

IBD is an umbrella term that includes conditions associated with chronic inflammation of the GI tract and leads to intestinal damage and disability. The cause of IBD is unknown, although it is believed to develop in genetically susceptible individuals after an inadequate immune response to the intestinal flora (17). Crohn’s disease (CD) and ulcerative colitis (UC) are the principal forms. They are lifelong, severe conditions, with a heterogeneous clinical presentation, a wide spectrum of complications, and extraintestinal manifestations. They exhibit a progressive pattern, characterized by periods of relapse and remission.

IBD is more frequently diagnosed between the ages of 15 and 35 years (18), but it may occur at any age (19, 20). The incidence and prevalence of IBD have been increasing worldwide, with the highest reported prevalence in Europe (827 per 100,000 persons) and North America (568 per 100,000 persons) (21). In Sweden, about 60,000 patients are currently registered in the Swedish Inflammatory Bowel Disease Register (SWIBREG), a nationwide quality register for IBD (22, 23). Of these, about 1100 are children and almost 5800 patients were diagnosed at >60 years of age. The gender distribution is even (23). Although the incidence has been reported to be stable or decreasing in North America and Europe, it has been increasing in newly industrialized countries (24). The increasing trend does not seem to be caused only by genetic factors because these are stable over time, and there is a marginal effect of immigration (23); environmental factors, such as smoking habits, diet, disturbed microflora or antibiotics may play an important role (25).

CD can occur anywhere in the GI tract and affects the full thickness of the intestinal wall. The symptoms at the time of presentation can vary significantly, but the most commonly reported include chronic diarrhoea, abdominal pain, and weight loss. Chronic inflammation can lead to the development of intestinal complications, such as strictures, fistulas, and abscesses. Extraintestinal manifestations are also common and can involve almost any organ system (26), leading to conditions such as arthritis, erythema nodosum, primary sclerosing cholangitis, and episcleritis. CD manifests most commonly during late adolescence or early adulthood (18). The diagnosis is established through a combination of clinical presentation, assessment of the entire GI tract and histopathologic examination. Ileocolonoscopy is the current gold standard for the assessment of disease activity and allows direct visualization of the mucosa and biopsy for histologic evaluation (27). Almost half of patients are reported to require surgical intervention within 10 years, however, more than half of those who undergo surgery experience a recurrence of the condition (28). Nevertheless, a decline in the rates of surgical interventions has been reported (29), which may potentially be attributed to the impact of modern therapies that could have altered the natural course of the disease (30).

UC primarily affects the large intestine, usually starting in the rectum and extending proximally in the colon, and is limited to the mucosal and submucosal layers. The main symptom is diarrhoea, often accompanied by blood and a mucopurulent exudate. Abdominal pain or discomfort and rectal urgency are common, and during severe exacerbations, weight loss and fever
can be present. The extraintestinal manifestations closely resemble those of CD. UC may manifest at any age. However, it typically emerges between 15 and 40 years (31). The diagnosis is based on clinical presentation, endoscopic examination and histopathologic evaluation of biopsy samples. The risk of surgery has declined in recent decades, but nearly 15% of patients with UC require surgery within 10 years (32). Approximately 5%-10% of patients initially diagnosed with UC might have their diagnosis changed later to CD (33).

The prognosis for IBD is influenced by the age at onset, extent and distribution of disease, and lack of endoscopic healing while in clinical remission (28, 33). A delay in the diagnosis results in treatment deferral and increases the risk of disease progression. In a study by Vavricka et al. (34), the median diagnostic delay in patients with CD was reported to be 9 months and 4 months for patients with UC. Pharmacological therapy involves induction and maintenance treatment. Commonly used drugs are 5-aminosalicylic acid, corticosteroids, immunomodulatory drugs and biologic therapy. The treatment objective is to achieve deep remission, which involves mucosal healing on endoscopy because it has been associated with reduced intestinal damage, relapse rate and need for surgery (28).

Sometimes, the clinical manifestations cannot be classified definitively into either of the two recognized forms; in such cases, a less common entity, inflammatory bowel disease unclassified, is diagnosed. It occurs in about 10% of patients with IBD and is more commonly observed in pediatric patients (35). Another type of chronic inflammatory disorder is microscopic colitis, which includes lymphocytic and collagenous colitis. These share common characteristics, manifest with chronic non-bloody diarrhea, and are commonly found in middle-aged and elderly women. The colonic mucosa is either normal or almost normal during endoscopic evaluation and diagnosis relies on examinations of colon biopsy samples (36).

**Colorectal cancer**

Colorectal cancer (CRC) is the third most common cancer worldwide, and it ranks second in terms of mortality (37). Incidence rates have been reported to be increasing in countries in transition, whereas they have stabilized or decreased in countries with a high human development index. In the latter, mortality rates have decreased, but in many low- and middle-income countries, mortality rates have been increasing (38). The incidence has been increasing among younger age groups in several countries (37, 39). The introduction of screening programmes and exposure to protective factors are likely to have contributed to the decline or stabilization. GPs play an active role in the diagnostic process of most cancer patients. In Sweden, CRC has also been reported as the third most common cancer; more than 7000 new cases were reported in the Swedish Colorectal Cancer Registry in 2021 (40). Lifestyle factors, including poor diet, smoking, alcohol consumption, a lack of physical activity, and an increasing prevalence of obesity, play a significant role in the development of CRC (41). Cancer survival is highly dependent on the stage at diagnosis. International differences in the stage distribution at diagnosis may partially explain the differences in survival (42).
Calprotectin

In 1980, Fagerhol et al. (43) first described the quantification of a leukocyte-derived protein (L1) from blood samples of healthy donors and a small series of hospital patients. The authors reported increased L1 levels in patients with malignant diseases and septicemia, and they assumed a possible non-specificity but higher sensitivity of L1 than the erythrocyte sedimentation rate, as observed in these patients (43). L1 has been reported to account for about 5% of the total protein content and up to 60% of the cytosol protein fraction in neutrophil granulocytes (43, 44). It has been found in neutrophils, monocytes, reactive tissue macrophages, squamous mucosal epithelia, and reactive epidermis (44).

Around the same time, the cystic fibrosis antigen, calgranulin, MRP-8 and MRP-14 (migration inhibitory factor-related proteins) were recognized (44-47). In 1988, Andersson et al. (48) showed that the sequences of these proteins were identical and proposed the use of the terms L1 antigen, L1 light chain and L1 heavy chain. In 1990, Steinbakk et al. (49) proposed the name calprotectin to describe the antimicrobial effect and calcium-binding properties of this protein shown in vitro.

Calprotectin is a heterodimeric complex of 2 calcium-binding cytosolic proteins, S100A8 and S100A9, of the S100 family, which includes proteins with numerous intracellular and extracellular regulatory functions (50). It consists of 2 heavy chains and 1 light chain and has a molecular mass of 36.5 kDa (51). It is heat stable and resistant to proteolytic degradation in the presence of calcium (52, 53).

Calprotectin is released during neutrophil activation, cell death or endothelial adhesion of monocytes. It has an essential role in protective immunity during infection and inhibition of microbial growth. Steinbakk et al. (49) described its antibacterial and antifungal activity. Calprotectin acts as an inhibitor for matrix metalloproteinases (54) and microbial growth through chelation of zinc and manganese (55-57). The apoptosis-inducing activity of this protein has captured research interest, along with its potential involvement in tumorigenesis (58).

Initially, calprotectin was primarily analysed in the plasma of patients with rheumatoid arthritis (59, 60). However, it was later identified in various body fluids and tissues, proving to be a dependable indicator of neutrophil activation in various diseases, such as multiple sclerosis, sepsis, cystic fibrosis, urinary tract diseases, and cardiovascular inflammation (61-65).
FAECAL CALPROTECTIN IS A USEFUL MARKER OF INTESTINAL INFLAMMATION

In 1992, Roseth et al. (66) described a method for the extraction and quantification of calprotectin in stools. Since then, there has been increasing interest in this marker in a variety of GI conditions. Faecal calprotectin (FC) has been shown to be a useful non-invasive marker for assessing intestinal inflammation and helpful in distinguishing between patients with organic and non-organic intestinal disease (67). It is particularly useful for differentiating between IBD and functional GI disorders such as IBS (66, 68). FC has been shown to be cost-effective in selecting symptomatic patients for further investigation and identifying those with IBD (69, 70).

FAECAL CALPROTECTIN IN INFLAMMATORY BOWEL DISEASE

Various biochemical biomarkers have been evaluated in IBD, but FC has been shown to be the most accurate in the assessment of IBD activity (71). In IBD, FC levels correlate quantitatively with the endoscopic assessment of intestinal inflammation (72, 73). Although colonoscopy remains crucial in monitoring disease activity and treatment response, FC has gained increasing significance as a valuable tool in this context. It is also useful for follow-up and early detection of relapse in patients with IBD (74-76).

FC has also been examined for detecting subclinical inflammation in patients with IBD who also experience symptoms of IBS (77, 78). Several studies have demonstrated the utility of FC in predicting the clinical outcome and the potential for predicting the response to biological therapy in patients with IBD (79-81).

Recent meta-analyses (82, 83) have evaluated the applicability of FC as a non-invasive biomarker of mucosal healing in IBD, and the results were promising.

FAECAL CALPROTECTIN IN COLORECTAL CANCER

The presence of colonic inflammation is a major attribute of CRC and has an important role in disease progression and survival (84, 85). In 1992, Roseth et al. (66) described increased FC levels in patients with GI carcinomas. One year later, the same authors (86) confirmed increased FC concentrations in colorectal and gastric cancer and proposed this test for the detection of GI neoplasms. In accordance with these results, a recent systematic review and meta-analysis (87) has confirmed significantly higher FC levels in patients with CRC than in controls. FC had a high sensitivity, but low specificity, signifying that FC cannot be recommended for diagnosis or screening of CRC. This was supported by a meta-analysis by Ye et al. (88), which presented evidence indicating that FC is not advisable for the detection of CRC.

Blad et al. (89) confirmed increased pre-diagnostic FC levels in patients with CRC. Several studies have demonstrated a significant decrease in FC levels
after cancer resection (90, 91). However, in contrast, patients with adenomas showed no significant decrease in FC levels after polypectomy (92).

Regarding different tumour stages, Lehmann et al. (91) found significantly higher FC values in patients with T3 and T4 tumours. In contrast, other studies (90, 93) did not identify significant differences. No differences have been observed in the FC values concerning tumour localization, size, histologic grading or other tumour parameters (90, 91). In addition, FC levels were not found to be significantly influenced by size, location or number of adenomas (94, 95).

Turvill et al. (96) confirmed a high negative predictive value (NPV) for CRC and significant polyps (≥10 mm) in patients referred for suspected CRC. Subsequently, the same study group compared the diagnostic accuracy of FC and faecal haemoglobin in patients referred with suspected CRC and found that FC exhibited lower diagnostic accuracy than faecal haemoglobin (97).

**CALPROTECTIN IN RESPIRATORY DISEASES**

In recent times, there has been increasing interest in using calprotectin for assessing respiratory diseases. Calprotectin was found to be increased in bronchoalveolar lavage fluid, lung tissue, and serum in patients with pneumonia (98) and associated with transepithelial migration of neutrophils and macrophages to the alveoli (99, 100).

The protective role of calprotectin in type 2 allergic airway inflammation has been documented in asthma, with its role potentially varying depending on the inflammatory context (101, 102). Increased S100A9 sputum levels were detected in patients with severe asthma with neutrophil-dominant inflammation, compared with eosinophil-dominant or pauci-granulocytic groups (103).

In chronic obstructive pulmonary disease (COPD), plasma calprotectin has been shown to be related to the neutrophil count and neutrophil to lymphocyte ratio and has been proposed as an independent marker of all-cause mortality in moderate to very severe COPD (104, 105). S100A9 was found to be increased in bronchoalveolar lavage fluid and serum during COPD exacerbations (106, 107). S100A9 signalling has also been reported to contribute to the progression of smoke-induced and age-related COPD in mice (108).

Several other respiratory diseases have also been shown to have an association with increased calprotectin in saliva, sputum, serum or faeces, e.g. respiratory exacerbation in cystic fibrosis (63), bronchiolitis obliterans (109), pulmonary tuberculosis (110) and pulmonary manifestations in Sjögren’s syndrome (111, 112).

Recently, evidence has emerged concerning COVID-19, including its impact on FC levels. However, there seem to be inconsistent findings on the correlation between FC levels and COVID-19. Some studies confirmed a significant association, possibly supporting the fact that increased FC levels may be linked to GI inflammation caused by SARS-CoV-2 infection (113-115). Diarrhoea is commonly found (2%-50%) in patients infected with SARS-CoV-2 (116). It has
been proposed that the virus interacts with angiotensin-converting enzyme 2 (ACE2) receptors in GI cells (116), with consequent inflammatory cell infiltration, thus resulting in the presence of calprotectin in the stool. Some authors found that FC levels correlate with the detection of SARS-CoV-2 in stools (117), whereas other authors did not observe any correlation (114, 118).

**OTHER FACTORS ASSOCIATED WITH INCREASED FAECAL CALPROTECTIN**

Several other factors and conditions may lead to increased FC levels. Increased FC concentrations have been reported in patients with, for example, bacterial gastroenteritis (119, 120), diverticulitis (121, 122), active coeliac disease (123, 124) or liver cirrhosis (125).

A recent study by Lundgren et al. (126) confirmed slightly increased FC levels in more than one-third of patients with a normal colonoscopy. This study has demonstrated an association with increasing age, which aligns with earlier investigations by Poullis et al. (127). Moreover, increased FC levels have been confirmed in children under the age of 4 years (128-130). In contrast, no influence on FC was observed from smoking, alcohol consumption or pregnancy (92, 95, 131).

The use of some common medications, such as non-steroidal anti-inflammatory drugs (NSAIDs), acetylsalicylic acid (ASA) or proton pump inhibitors (PPIs), has also been identified as a factor contributing to increased FC levels.

**NON-STEROIDAL ANTI-INFLAMMATORY DRUGS**

NSAIDs are one of the most commonly used medications worldwide. However, the frequency and severity of their GI side effects have been recognized for several years. Numerous studies have confirmed their damaging effects throughout the GI tract (132-134) and reported lesions in approximately 50%–71% of patients on NSAID therapy (135-137). These cover a wide spectrum, such as petechiae, reddened folds, red spots, denuded areas (loss of villi), mucosal breaks, erosions, ulcers, or strictures. The risk of GI complications has been reported to increase with age, a history of peptic ulcer, GI bleeding, use of anticoagulants, higher doses of NSAIDs, and concomitant use of corticosteroids (135, 138).

The pathogenesis of NSAID-induced small bowel injury is a complex and multi-stage process: (I) topical effects after direct exposure; (II) systemic effects after absorption; and (III) repetitive local effects after enterohepatic circulation (139, 140). The local effects manifest as damage to enterocytes by disrupting the membrane, interacting with phospholipids and uncoupling of mitochondrial oxidative phosphorylation, resulting in impaired function of the GI barrier and increased intestinal permeability. The systemic effect involves inhibition of cyclooxygenase 1 (COX-1) and COX-2. Decreased prostaglandin
production, activated by inhibition of COX-1, was shown to correlate with gastric and small bowel damage (141, 142). COX-2 selective inhibitors have been considered to be safer than traditional NSAIDs because COX-2 expression is low in the GI tract (143, 144). Prostaglandins contribute to mucosal defence in the stomach and their inhibition results in increased gastric acid secretion, decreased mucus and bicarbonate secretion, decreased cell proliferation and decreased mucosal blood flow (145). Further, NSAIDs induce inhibition of other mediators with protective functions, nitric oxide and hydrogen sulphide, which may also be linked to small bowel injury (146). Decreased mucosal prostaglandin production has been found to be less important in the pathogenesis of small bowel damage (141, 147), and it is probable that mechanisms of NSAID-induced GI damage other than inhibition of COX exist. Microvascular effects of NSAIDs exacerbate the inflammation, and these local and systemic pathogenetic mechanisms lead to the development of macroscopic lesions (as described above).

Nevertheless, no correlation has been confirmed between NSAID-induced mucosal injury and clinical symptoms (135). Symptoms may be subclinical and non-specific (148, 149) because of the analgesic effect of NSAIDs. Consequently, the frequency of intestinal damage might have been underestimated until the introduction of double-balloon enteroscopy and capsule endoscopy. Intake of NSAIDs has also been linked to early clinical relapse of quiescent IBD (150). Also, long-term use of ASA has been reported to be associated with small bowel injury (151, 152), but it appears to be less harmful in comparison with other NSAIDs (153).

Several studies have shown that treatment with NSAIDs may increase FC levels (132, 154). Increased FC levels were confirmed in 75%–95% of healthy individuals after only 2 weeks of NSAID treatment (155-158). However, increased FC levels were not found to be correlated with the results of capsule enteroscopy (155, 157). FC has been proposed as a non-invasive test for diagnosing NSAID enteropathy (132). However, as an individual test, it may lack specificity in making a diagnosis without accounting for other diagnostic factors, symptoms, and examinations.

**PROTON PUMP INHIBITORS**

PPIs are prescribed frequently because of their efficacy and good safety profile. They are used to prevent and treat gastric acid-related conditions, including gastroesophageal reflux disease, peptic ulcer, and in combination with antimicrobial agents, eradication of *Helicobacter pylori* infection, or functional dyspepsia. Also, there is a belief that they can effectively treat and prevent NSAID-associated gastroduodenal mucosal injury (159, 160).

In recent years, there has been increasing awareness about their adverse effects. They have the ability to directly target the proton pumps of naturally occurring bacteria and fungi or indirectly affect the microenvironment of the flora by altering the pH (161). These processes may lead to bacterial overgrowth in the small intestine, which has been found in PPI users (162-165), although the results in the literature have been inconsistent (164, 166). Studies have demonstrated that PPIs may compromise barrier function and induce a significant transmucosal leak, thus resulting in inflammation (167, 168). Thus,
the intestinal mucosa is more susceptible to further damage when exposed to other factors. PPIs were reported to induce significant shifts in enteric microbial populations and thereby exacerbate NSAID-induced intestinal injury (169).

Small intestinal mucosal damage has been reported in 26%–81% of healthy individuals taking NSAIDs co-administered with a PPI (160, 170). Furthermore, the results of some studies indicate an increased risk of flare-up in patients with IBD using PPIs (171, 172). Several studies have confirmed that PPIs are associated with increased FC levels (121, 126, 173). Therefore, it is crucial to make a careful assessment when interpreting an increased test result in clinical practice.

**RESPIRATORY TRACT INFECTIONS**

Several studies have indicated that upper respiratory tract infections could be a potential source of error in FC diagnostics (92, 154, 174, 175). Calprotectin is poorly degraded during its passage through the GI tract. The presence of neutrophilic granulocytes in swallowed saliva or sputum might lead to a positive FC result. However, there is currently no conclusive evidence to support this claim.

**FAECAL CALPROTECTIN TEST: SOME ASPECTS OF UNCERTAINTY**

It has been reported previously that calprotectin is stable in faeces for up to 7 days when stored at room temperature and samples can be sent by post (66, 176). Reliable estimates can be obtained in samples of only 5 g and a single faecal spot appears to be sufficient for determination of the calprotectin level (66, 86, 90). Lasson et al. (177) have verified the stability at room temperature over 3 days but found a significant decrease of 28% after 7 days in patients with active UC. Naismith et al. (178) previously reported good stability of faecal samples at 2°C–8°C for up to 10 days and of faecal extracts for 4 months at −20°C. In the study by Caenepeel et al. (179), freezing and long-term storage for 1.5 years yielded reliable FC measurements.

The average day-to-day variability in FC concentrations has been documented to be as high as 48%–54% (68, 180, 181), yet few variations were reported to have taken place in the interval between positive and negative values; the largest differences occurred with increasing concentrations of FC. In patients with CD, within-stool analysis showed a variation of 4%–19% (181). Lasson et al. (177) have demonstrated that calprotectin in faeces was distributed homogeneously, and there was a strong correlation in FC concentrations between 2 random samples. The authors reported great intra-individual variability of FC during a single day (coefficient of variation [CV], 52%; 95% confidence interval [CI], 4–178) and variability between 2 consecutive days (CV, 41%; 95% CI, 3–128) in patients with active UC. The variability was most
pronounced with high FC levels, and thus probably with a low clinical relevance, according to the authors. The presence of blood in the stool and FC concentrations were not found to be correlated. The authors suggested a standardized approach to reduce the variability, recommending sampling from the first bowel movement in the morning because the FC levels tended to increase with longer intervals between bowel movements. Burri (182) implied that day-to-day variability in FC concentration might be lower in patients with IBD in remission and greater in patients with active disease.

Various FC tests are currently available in different countries. These include fully quantitative laboratory-based technologies (most often enzyme-linked immunosorbent assay [ELISA]), fully quantitative rapid tests and semi-quantitative point-of-care tests. However, the methodological and technical differences between FC assays create a barrier to the comparability of laboratory results in clinical practice.

The lack of a universal cut-off is also a considerable limitation in the use of FC in primary care practices. The literature presents a wide range of cut-off values; the most common, recommended by manufacturers, is 50 µg/g. Waugh et al. (183) addressed an area of uncertainty concerning the optimal management of individuals with borderline results (50–150 µg/g), most of whom did not experience symptoms of IBD. In the United Kingdom, the York Faecal Calprotectin Care Pathway was introduced in 2016 to distinguish patients with IBS and IBD. Increasing the cut-off value from 50 µg/g to 100 µg/g was shown to lead to fewer referrals but no loss of sensitivity, with subsequent savings in health care costs (184). In a study on primary care patients with persistent GI symptoms, Pavlidis et al. (185) suggested increasing the cut-off value from 50 to 150 µg/g. This adjustment would slightly reduce the NPV from 98% to 97%, but substantially increase the positive predictive value (PPV) from 28% to 71%.

**RATIONALE FOR THE THESIS**

FC is commonly used as a diagnostic tool for patients experiencing GI symptoms. However, there is still only limited evidence on the accuracy of the FC test in primary care for the detection of IBD and OGID. Accuracy measures have mainly been assessed in selected populations in secondary care (9, 186, 187). Available evidence from primary care often comes from studies with a small sample size, heterogeneous nature, and different FC cut-offs. In addition, FC has been assessed principally as an individual test without considering other diagnostic factors (7, 68, 188). In many instances, the interpretation of the test result can pose challenges, necessitating consideration of potential sources of error.

Although several factors are thought to influence the FC level, there remains limited understanding regarding the extent and duration of their effects. Improved measures to achieve more accurate and efficient diagnostics and treatment decisions in primary care would help reduce unnecessary referrals and invasive investigations.
AIMS

The overall aim of this thesis was to investigate the contribution of FC in the diagnostics of GI disease in primary care, its diagnostic value as a predictor of IBD and OGID, and the influence of sources of error on FC levels.

SPECIFIC AIMS

Study I To determine the diagnostic accuracy of FC for IBD and OGID in primary care patients with gastrointestinal symptoms.
To examine the association of FC with demographic factors, symptoms and concomitant medical therapy.

Study II To investigate how oral diclofenac intake affects FC levels and assess how long it takes for an increased FC level to return to normal after cessation.

Study III To investigate to what extent oral omeprazole, diclofenac or co-administration of these affects FC levels.
To assess how long it takes for an increased FC level to return to normal after cessation.

Study IV To investigate whether there is an increase in the FC value during an acute respiratory tract infection (RTI).
To investigate whether there is an increase in salivary calprotectin during an acute RTI and to what extent this affects the FC value.
METHODS

PARTICIPANTS AND ELIGIBILITY CRITERIA

Study I  1293 adult patients
Inclusion criterion: patients who underwent FC testing in 2010.
Exclusion criteria: patients aged <18 years; patients with FC tests not conducted in primary care; FC tests in patients occurring more than once; patients with missing medical records; and patients with a previously known diagnosis of IBD or GI cancer.

Study II  30 healthy volunteers
Inclusion criterion: normal levels of FC (upper limit of normal [ULN] reference value was 50 µg/g).
Exclusion criteria: NSAID or ASA intake in the past month; history of regular use of NSAIDs or ASA; intolerance to NSAIDs; pregnancy; alcohol misuse; and history of GI diseases or bleeding.

Study III  32 healthy volunteers
Inclusion criteria: age 18–64 years; healthy individuals; written informed consent form signed; health declaration form filled out and signed; normal FC test, normal blood count; negative Helicobacter pylori stool antigen test; and regular bowel movements.
Exclusion criteria: PPI, NSAID or ASA intake in the past month; history of regular PPI, NSAID or ASA use; intolerance to PPIs or NSAIDs; history of GI diseases or bleeding; conditions associated with bleeding disorders; asthma, cirrhosis, hepatic porphyria, liver failure, kidney failure, heart failure, ischaemic heart disease, peripheral arterial disease, cerebrovascular disease; pregnancy or lactation; desire to become pregnant during the study period; current alcohol misuse; participation in another clinical trial involving medicinal products within 30 days.
If the participants provided <50% of the stool samples during a study sequence or if they had <80% compliance with intake of the investigational medicinal product (IMP) in a study sequence, they were excluded from the study.

Study IV  100 patients
Inclusion criteria: age ≥18 years; patients with symptoms of acute RTI attending a primary care physician.
Exclusion criteria: intake of NSAID or ASA in the past 3 weeks; IBD (including microscopic colitis); and inability to make an informed decision to participate in the study (e.g. due to impaired cognitive ability or difficulties in understanding the Swedish language).
STUDY DESIGNS

Study I A retrospective population-based cohort study. Records on all FC tests analysed in 2010 in Östergötland County were retrieved from the Department of Clinical Chemistry, Center for Diagnostics, Linköping University Hospital. The data on sex, age, symptoms, symptom duration, concomitant medical therapy (NSAIDs, PPIs and ASA) and the final diagnosis registered by the treating physician were collected retrospectively in 2013. A 5-year follow-up was performed by reviewing the patients’ records for 2010–2015 pertaining to the ICD-10 diagnosis code groups C-D (Neoplasms) and K (Diseases of the digestive system), with inclusion of new GI findings.

Study II A phase 4, single-centre open study. Participants were recruited by advertising between September 2012 and May 2013. They took oral diclofenac 50 mg 3 times a day for 14 days. Stool samples were provided on day 0 (the day before the start of diclofenac intake), days 2, 4, 7 and 14 during diclofenac intake, and days 17, 21 and 28 after discontinuation. FC levels were then followed at 7-day intervals until normalization (i.e. 2 consecutive normal tests), but for at least 2 weeks after discontinuation.

Study III A phase 4, single-centre open study, with age-stratified block randomization into 2 cross-over groups. Participants were recruited by advertising between November 2017 and February 2022; 32 eligible participants completed the study. The participants were allocated to 2 groups 1:1 by stratified block randomization, based on age ≤40 years and ≥41 years. They received 20 mg oral omeprazole daily for 2 weeks in the first sequence, 50 mg oral diclofenac 3 times daily for 2 weeks in the second sequence and co-administration of these for 2 weeks in the third sequence, with washout periods in between. The washout period was estimated to be at least 3 weeks after cessation of IMP with a maximum of 4 months. The first and second sequences were randomized to achieve a different order of administration of IMPs in the 2 groups (Table 1) to rule out any potential speculation regarding the impact of the IMP in the preceding sequence on FC levels in the subsequent sequence, particularly if a more substantial increase in levels occurred in the latter. FC was measured on day 0 (the day before the start of drug administration), days 4, 7 and 14 during drug administration and days 21, 28 and 35 after cessation of the drug (±2 days). FC was then followed at 7-day intervals (±2 days) until normalization (i.e. 2 consecutive normal tests) in each sequence.

Table 1. Overview of administration of investigational medicinal products in Study III

<table>
<thead>
<tr>
<th></th>
<th>Sequence 1</th>
<th>Sequence 2</th>
<th>Sequence 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>omeprazole</td>
<td>diclofenac</td>
<td>omeprazole + diclofenac</td>
</tr>
<tr>
<td>Group 2</td>
<td>diclofenac</td>
<td>omeprazole</td>
<td>omeprazole + diclofenac</td>
</tr>
</tbody>
</table>
Study IV  A prospective cohort study. Patients were recruited at a visit to their primary care physician between March 2021 and January 2022; 100 eligible participants completed the study. Data on age, sex, symptoms, duration of symptoms and diagnosis registered on a case report form by the treating physician were collected. Patients provided 1 saliva and 2 stool samples for calprotectin tests (the first stool sample from the first bowel movement after the visit and the second on the next occasion, at least 24 h apart) during RTI and approximately 21 days later in a symptom-free period of at least 7 days. Baseline was defined as the state during acute RTI. Participants acted as their own controls with regard to salivary and stool calprotectin tests in the symptom-free period.
**FAECES SAMPLING**

**Study II**  
Stool samples were submitted in disposable plastic tubes with a screw cap and a spoon (Sarstedt faeces tube, Nürnberg, Germany) and filled to about 1/3. Samples were to be provided on the day of collection or stored in a refrigerator until delivery the next day. Upon arrival at the laboratory, the samples were stored in the refrigerator at +4°C until processing the following morning. Samples collected up to day 14 were analysed at the same time for each individual and samples collected from day 17 were analysed directly after they reached the laboratory.

**Study III**  
Samples were collected in the plastic tubes as for Study II and stored by the participants in the freezer at home until each sequence was completed, then collected for storage in a controlled freezer at −80°C for later analysis. Samples collected on day 28 and all subsequent samples were divided into 2 plastic tubes; 1 sample was frozen as described above and the other was analysed directly after it arrived at the laboratory to determine when the FC had normalized and thus allow termination of sampling in the particular sequence. Once sampling was completed for all participants, the frozen samples from each sequence were analysed at the same time for each individual to avoid interassay variation. In the case of an aberrant FC value (extremely high or different from that of the previously analysed non-frozen sample), the sample was re-measured.

**Study IV**  
Stool samples were submitted in plastic tubes as for Studies II and III. Patients took the samples at home, stored them in a refrigerator at +4°C and sent them by post the following day. Samples were then frozen and stored in a controlled freezer at −80°C pending analysis as soon as all samples were collected.

**SALIVA SAMPLING**

**Study IV**  
Unstimulated whole saliva was collected using SalivaBio Oral Swabs in disposable swab storage tubes with a small insert and snap cap, according to the manufacturer’s instructions (Salimetrics, State College, PA, USA) (189). The participants were instructed to fast for 1 h, rest for at least 30 min and rinse their mouth with water 10 min before sampling. Patients took the samples at home, stored them in a refrigerator at +4°C and sent them by post the following day. They were centrifuged at 2500 \( \times g \) for 10 min and the salivary supernatant was then frozen and stored in a controlled freezer at −80°C pending analysis when all samples were collected. Unstimulated whole saliva samples were collected from 18 apparently healthy donors (according to the description above). Samples were divided for storage at room temperature and at +4°C and assessed for calprotectin concentrations on days 0, 2, 4 and 6.
**FAECAL CALPROTECTIN ANALYSIS**

Study I  PreventID CalDetect (Preventis, Luxemburg) was used at the Department of Clinical Chemistry, Center for Diagnostics, Linköping University Hospital in 2010. This is a semi-quantitative immunochromatographic rapid test, with immunological lateral flow for the detection of human calprotectin via gold-conjugated anti-calprotectin antibodies. The results are expressed as an FC concentration of <15 mg/kg, 15–60 mg/kg or >60 mg/kg. The cut-off for positivity was \( \geq 15 \) mg/kg, according to the manufacturer (190).

Study II  FC was determined by the immunoenzymatic EliA Calprotectin assay on a Phadia 250 instrument (Thermo Fisher Scientific, Freiburg, Germany) at the Department of Clinical Chemistry, Laboratory Medicine, Ryhov County Hospital in Jönköping. The measuring range of the instrument is 15 to \( \geq 3000 \) µg/g. The test has a sensitivity of 97.7% and specificity of 89.8% for differentiation of IBD from IBS, intra-run variance of 2.8%–7.0%, and inter-run variance of 1.9%–7.3%, according to the manufacturer (191). A predetermined volume of 85 mg of stool was mixed with 4.2 mL of EliA Calprotectin Extraction Buffer. Samples were analysed 3 times per week, otherwise frozen at \(-20^\circ\)C for later analysis. The cut-off for positivity was set at 50 µg/g, according to the manufacturer.

Study III  FC was determined by DiaSorin Liaison XL assay, a fully automated chemiluminescent immunoassay for the quantitative determination of FC. The assays were conducted at the Department of Clinical Chemistry, Center for Diagnostics, Linköping University Hospital, in accordance with the manufacturer’s instructions (Diasorin Liaison XL, Revision D, 200/008-935 11/2013). The measuring range of the instrument is 0–800 µg/g. A lower limit of quantification (LLOQ) was set by the laboratory at <15 µg/g. Cut-off for positivity was 50 µg/g.

Study IV  ELISA calprotectin assay (BioV endor, Brno, Czech Republic) was used for the determination of FC. Stool samples were weighed (0.05–0.1 g) and mixed with an extraction buffer (BioVendor, Czech Republic) at a dilution factor of 50. Extracts were homogenized by vortexing and centrifuged at \( 3000 \times g \) for 5 min. The supernatant was diluted in reagent diluent 1:200. The assay procedure was conducted according to the manufacturer’s instructions (192). A microplate spectrophotometer (Multiskan Spectrum; Thermo Scientific, Waltham, MA, USA) was used to determine the absorbance, set at 450 nm with a reference wavelength of 630 nm and interpolated on a 4-parameter logistic curve. The detection range of the assay was 0.22–64 ng/mL. Measured concentrations were adjusted to micrograms of calprotectin per gram of faeces (detection range, 2.2–640 µg/g) with regard to the dilution factor. The LLOQ was set by the study group at <2 ng/mL (corresponding to 20 µg/g for faeces), due to overlap of the blank with standard point 1. The interassay CV was 14.7% at a mean concentration of 8.05 ng/mL and 14.9% at 20.3 ng/mL.
SAIVARY CALPROTECTIN ANALYSIS

Study IV  Calprotectin was determined with an ELISA calprotectin assay (BioVendor, Brno, Czech Republic). Several saliva samples were diluted 1:400 and 1:1000 in reagent diluent. The dilution of 1:400 provided results that exceeded the highest standard value. Therefore, the dilution of 1:1000 was used because it showed no interference with assay linearity and fit the range of the standard curve. The calprotectin assay was conducted according to the manufacturer's instructions (192). The absorbance was determined using a microplate spectrophotometer, according to the description for FC analysis in the present study. In samples with absorbance above the value of the highest standard, calprotectin concentration was determined by a second reading at 405 nm (this occurred in 11 saliva samples on 1 plate). Measured concentrations were adjusted to micrograms of calprotectin per millilitre of saliva (detection range 0.22–64 µg/mL) with regard to dilution factor. The LLOQ was set at <2 µg/mL, according to the description above. Calprotectin values above the upper standard point were set to 65 µg/mL.
**STATISTICAL ANALYSES**

Statistical analyses were performed using SPSS (IBM, Armonk, NY, USA; version 22.0 in Study II and version 27.0 in Studies I, III and IV) and GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA in Studies III and IV). P values <0.05 were considered statistically significant.

**Study I** Data are presented as numbers, percentages, or median, range and interquartile range (IQR). Fisher’s exact test or Pearson’s chi-squared test was used for nominal data to compare proportions. The associations of clinical variables with IBD and OGID were analysed using binary logistic regression. Variables with a statistically significant association were further assessed for accuracy (sensitivity, specificity, NPV and PPV) as predictors of IBD and OGID.

**Study II** Data are presented with descriptive statistics (number, percentage or median, IQR). Wilcoxon’s signed-rank test was used to compare FC levels between baseline (day 0) and different measurement occasions during the study period. Observations below the limit of detection (LOD) or quantification were regarded as intervals. A response below the LOD was set to half the LOD.

**Study III** Data are expressed using descriptive statistics (numbers, percentages; median, range, IQR; means ± standard error of the mean). Observations below the LLOQ were set to half the LLOQ. Statistical analysis of the primary end point (change in the FC level during and after drug administration compared with baseline) was carried out using Wilcoxon’s signed-rank test. FC values were compared with baseline (day 0) for each measurement point in each sequence. A secondary outcome was the number of participants with an FC level over the ULN. The median test was used to compare FC values between the groups and age strata.

**Study IV** Categorical variables are expressed as numbers and percentages, and continuous variables as medians and IQRs. Responses below LLOQ were set to 1.5 ng/mL (corresponding to 15 µg/g for faeces and 1.5 µg/mL for saliva). Proportions for categorical variables were compared using Fisher’s exact test and medians for continuous variables using the Mann-Whitney U test. Wilcoxon’s signed-rank test was used to analyse the differences in salivary calprotectin levels and mean values of FC levels during RTI and in a symptom-free period. Correlations between calprotectin levels in saliva and faeces were calculated using Spearman’s correlation. For analysis of saliva stability, the percentage deviation in concentration from baseline (day 0) was determined for each measurement point using the formula: deviation% = 100 × (measured value – baseline value)/baseline value. The percentage deviation was compared with the acceptable change limit (ACL). The ACL for interpreting a measured difference is based on the analytical imprecision (CV), using the formula $ACL = 1.96 \times \sqrt{2} \times CV$ (193, 194). Percentage deviations were considered significant if the value of the deviation was above the percentage ACL.
ETHICAL APPROVAL

All study protocols were approved by the Regional Ethical Review Board in Linköping with the following registration numbers:

I  Dnr 2011/467-31 and Dnr 2016/456-32
II Dnr 2012/29-31
III Dnr 2016/528-31 and Dnr 2018/523-32
IV Dnr 2020/03381

Written informed consent was obtained from all participants in Studies II, III and IV.

The Swedish Medical Products Agency granted permission to carry out Study III, and it was performed in accordance with the Good Clinical Practice protocol, the Declaration of Helsinki and current rules and regulations. The study was monitored by Forum Östergötland, an independent regional support unit for clinical trials. This clinical trial was registered in EudraCT (2015-003903-5), protocol code number DOC2016.

The Biobanks Act (2002:297) was applied to samples taken in Studies III and IV. All samples were destroyed immediately after analysis.
RESULTS

CHARACTERISTICS OF THE STUDY POPULATIONS

Study I 1293 patients were included (median age, 43 years; range, 18–93 years); 715 patients with a negative FC test (median age, 37 years; range, 18–93 years) and 578 with a positive FC test (median age, 52 years; range, 18–92 years), see Table 2.

Of these patients, 3% were consuming NSAIDs (n=41; median age, 53 years; range, 19–87 years), 12% were taking PPIs (n=154; median age, 54 years; range, 18–91 years), 5% were taking ASA (n=70; median age, 71.5 years; range, 35–93 years) and 5% were taking a combination of these (n=60; median age, 67 years; range, 18–90 years).

Table 2. Overview of the demographics, symptoms, duration and medications in Study I

<table>
<thead>
<tr>
<th></th>
<th>&lt;15 mg/kg n (%)</th>
<th>≥15 mg/kg n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>232 (32.4)</td>
<td>212 (36.7)</td>
</tr>
<tr>
<td>Female</td>
<td>483 (67.7)</td>
<td>366 (63.3)</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;35 years</td>
<td>309 (43.2)</td>
<td>158 (27.3)</td>
</tr>
<tr>
<td>35–60 years</td>
<td>264 (36.9)</td>
<td>200 (34.6)</td>
</tr>
<tr>
<td>&gt;60 years</td>
<td>142 (19.9)</td>
<td>220 (38.1)</td>
</tr>
<tr>
<td>Symptoms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdominal pain or discomfort</td>
<td>461 (64.5)</td>
<td>371 (64.2)</td>
</tr>
<tr>
<td>Gases /flatulence/bloating</td>
<td>205 (28.7)</td>
<td>153 (25.6)</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>270 (37.8)</td>
<td>271 (46.9)</td>
</tr>
<tr>
<td>Stool consistency fluctuations</td>
<td>146 (20.4)</td>
<td>145 (23.9)</td>
</tr>
<tr>
<td>Constipation</td>
<td>57 (8.0)</td>
<td>44 (7.6)</td>
</tr>
<tr>
<td>Altered stool consistency or form</td>
<td>69 (9.7)</td>
<td>56 (9.7)</td>
</tr>
<tr>
<td>Nausea</td>
<td>64 (9.0)</td>
<td>20 (3.5)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>30 (4.2)</td>
<td>21 (3.6)</td>
</tr>
<tr>
<td>Rectal bleeding</td>
<td>166 (23.2)</td>
<td>164 (28.4)</td>
</tr>
<tr>
<td>Weight loss</td>
<td>97 (13.6)</td>
<td>93 (16.1)</td>
</tr>
<tr>
<td>Abnormal clinical findings</td>
<td>16 (2.4)</td>
<td>22 (3.7)</td>
</tr>
<tr>
<td>Family history of GI cancer or IBD</td>
<td>67 (9.4)</td>
<td>49 (8.5)</td>
</tr>
<tr>
<td>Duration</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;3 months</td>
<td>168 (23.5)</td>
<td>166 (23.9)</td>
</tr>
<tr>
<td>&gt;3 months</td>
<td>439 (61.4)</td>
<td>288 (49.8)</td>
</tr>
<tr>
<td>Unclear</td>
<td>108 (15.1)</td>
<td>94 (16.3)</td>
</tr>
<tr>
<td>Medications</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NSAID</td>
<td>13 (1.8)</td>
<td>28 (4.8)</td>
</tr>
<tr>
<td>PPI</td>
<td>48 (6.7)</td>
<td>106 (18.3)</td>
</tr>
<tr>
<td>ASA</td>
<td>28 (3.9)</td>
<td>42 (7.3)</td>
</tr>
</tbody>
</table>

Each patient could have reported more than 1 symptom. GI, gastrointestinal; IBD, inflammatory bowel disease; NSAID, non-steroidal anti-inflammatory drug; PPI, proton pump inhibitor; ASA, acetylsalicylic acid.
Study II 30 healthy volunteers were included in one participant group that received the same intervention (17 men and 13 women; median age, 27 years; range, 20–63 years).

Study III 32 healthy volunteers were included (17 men and 15 women; median age, 33 years; range, 19–57 years). Participants were randomly allocated to Group 1 (n=18; median age, 34.5 years; range, 22–57 years; n=11 ≤40 years and n=7 ≥41 years) and Group 2 (n=14; median age, 31 years; range, 19–57 years; n=9 ≤40 years and n=5 ≥41 years).

Study IV 100 patients were included (median age, 39 years; range, 18–76 years). Patients were divided into 2 groups according to the diagnosis: viral infection (n=92) and bacterial infection (n=8). A subgroup of patients with COVID-19 (n=11) in the viral infection group was also examined (Table 3). No statistically significant difference was identified between the viral and bacterial groups with regard to age, sex, symptoms or symptom duration ($p>0.05$).

Table 3. Overview of the demographics, symptoms and symptom duration in Study IV

<table>
<thead>
<tr>
<th></th>
<th>Viral infections</th>
<th>Bacterial infections</th>
<th>COVID-19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
</tr>
<tr>
<td>Sex</td>
<td>Female 67 (72.8)</td>
<td>Male 6 (75.0)</td>
<td>Male 8 (72.7)</td>
</tr>
<tr>
<td>Symptoms</td>
<td>Fever 33 (35.9)</td>
<td>Runny nose 75 (81.5)</td>
<td>Sore throat 62 (67.4)</td>
</tr>
<tr>
<td></td>
<td>Sore throat 62 (67.4)</td>
<td>Runny nose 75 (81.5)</td>
<td>Sore throat 62 (67.4)</td>
</tr>
<tr>
<td></td>
<td>Productive cough 24 (26.1)</td>
<td>Dry cough 49 (53.3)</td>
<td></td>
</tr>
<tr>
<td>Duration</td>
<td>&lt;3 days 37 (40.2)</td>
<td>1 (12.5)</td>
<td>5 (45.5)</td>
</tr>
<tr>
<td></td>
<td>4–7 days 37 (40.2)</td>
<td>4 (50.0)</td>
<td>6 (54.5)</td>
</tr>
<tr>
<td></td>
<td>1–2 weeks 13 (14.1)</td>
<td>2 (25.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td></td>
<td>2–3 weeks 3 (3.3)</td>
<td>1 (12.5)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td></td>
<td>&gt;3 weeks 2 (2.2)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

Each patient could have reported more than 1 symptom. Values are given as number (%), unless otherwise indicated. IQR, interquartile range.
POSITIVE VS NEGATIVE FC TEST

Study I  The likelihood of a positive FC was higher in the presence of diarrhoea (odds ratio [OR], 1.56; 95% CI, 1.20–2.01) and age >60 years (OR, 2.59; 95% CI, 1.87–3.58), but was lower in the presence of nausea (OR, 0.35; 95% CI, 0.19–0.64) and duration >3 months (OR, 0.59; 95% CI, 0.45–0.77). Rectal bleeding and age 35–60 years were found to have significant associations in the univariate analysis (p<0.05), but not in a binary logistic regression.

Patients on NSAIDs and PPIs had a higher likelihood of a positive FC (OR, 2.72; 95% CI, 1.32–5.62 and OR, 2.92; 95% CI, 1.93–4.41, respectively). The use of ASA was found to be significant only in the univariate analysis.

FC TEST OUTCOME

Study I  OGID (including IBD) was found in 39.6% of patients with positive FC compared with 9.7% of patients with negative FC. IBD was diagnosed in 8.8% of the patients with positive FC and OGID (other GI inflammation, GI infections and GI tumours) in 30.8%, compared with 0.1% and 9.5%, respectively, of the patients with negative FC. Functional GI disorders were found to a greater extent in patients with negative FC, 33.6%, compared with 22.7% of patients with positive FC (Figure 1).

![Figure 1](image.png)

*Figure 1.* Overview of the final diagnoses in Study I. FC, faecal calprotectin; IBD, inflammatory bowel disease; GI, gastrointestinal; FGIDs, functional gastrointestinal disorders.
**Prediction models of IBD and OGID**

**Study I**

Significant predictors of IBD were found to be positive FC (OR, 82.82; 95% CI, 11.15–615.27), diarrhoea (OR, 2.77; 95% CI, 1.26–6.10) and rectal bleeding (OR, 10.55; 95% CI, 5.02–22.15). The likelihood of having IBD was lower in females (OR, 0.41; 95% CI, 0.20–0.84) and patients with stool consistency fluctuations (OR, 0.25; 95% CI, 0.07–0.92). Family history of IBD or GI cancer, duration and intake of NSAIDs were found to be significant only in the univariate analysis. Abdominal pain was experienced more in patients with negative FC \( (p<0.05) \) (Figure 2).

Predictors of OGID were positive FC (OR, 3.75; 95% CI, 2.62–5.35), higher age (35–60 years: OR, 3.24; 95% CI, 1.96–5.35; >60 years: OR, 9.07; 95% CI, 5.55–14.84) and abnormal findings in abdominal physical examination (OR, 3.14; 95% CI, 1.15–8.56). The likelihood was lower in patients with duration >3 months (OR, 0.35; 95% CI, 0.38–0.77). Sex, gases, diarrhoea, stool consistency fluctuations and intake of PPIs or ASA were found to be significant only in the univariate analysis.

Predictors of functional disorders were abdominal pain (OR, 1.58; 95% CI, 1.14–2.19), gases (OR, 1.67; 95% CI, 1.24–2.26), diarrhoea (OR, 2.68; 95% CI, 1.97–3.64), stool consistency fluctuations (OR, 2.50; 95% CI, 1.76–3.55) and duration >3 months (OR, 2.14; 95% CI, 1.54–2.97). The likelihood of FGIDs was lower with higher age (35–60 years: OR, 0.49; 95% CI, 0.36–0.67; >60 years: OR, 0.27; 95% CI, 0.18–0.41),rectal bleeding (OR, 0.59; 95% CI, 0.42–0.82) and positive FC (OR, 0.70; 95% CI, 0.52–0.94).

![Figure 2](Image)

*Figure 2.* Forest plots showing the univariate and binary logistic regression analyses for (A) inflammatory bowel disease (IBD) and (B) organic gastrointestinal disease (OGID). Binary logistic regression was limited to variables that showed a \( p \) value <0.10 in the univariate analysis (*). OR, odds ratio; CI, confidence interval; GI, gastrointestinal; FC, faecal calprotectin; NSAID, non-steroidal anti-inflammatory drug; PPI, proton pump inhibitor; ASA, acetyl salicylic acid.
DIAGNOSTIC ACCURACY OF FC

Study I  FC alone showed the highest sensitivity and NPV for IBD and OGID. In combination with other predictors, specificity and PPV increased but sensitivity was lower (Table 4).

Abdominal pain was found to be non-specific and when it was added to the accuracy calculations for IBD, it decreased the sensitivity and PPV, and increased the specificity only marginally.

Altering the cut-off value from 15 mg/kg to 60 mg/kg increased the specificity and PPV by 10% and 4%, respectively, and decreased the sensitivity by 2%–10%; NPV remained essentially unchanged. NSAID, PPI and ASA intake showed only a minor increase in the sensitivity and PPV and a decrease in the specificity, and no significant effect on NPV.

### Table 4. Diagnostic accuracy of FC and other significant variables for inflammatory bowel disease (IBD) and organic gastrointestinal disease (OGID; excluding IBD) in Study I

<table>
<thead>
<tr>
<th></th>
<th>No. of patients</th>
<th>Sensitivity, % (95% CI)</th>
<th>Specificity, % (95% CI)</th>
<th>PPV, % (95% CI)</th>
<th>NPV, % (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IBD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FC</td>
<td>51</td>
<td>98.1 (88.4–99.9)</td>
<td>57.5 (44.7–60.3)</td>
<td>8.8 (5.7–11.5)</td>
<td>99.9 (99.1–100.0)</td>
</tr>
<tr>
<td>Male</td>
<td>28</td>
<td>53.8 (39.6–67.5)</td>
<td>66.5 (61.8–69.4)</td>
<td>6.3 (4.3–9.4)</td>
<td>97.4 (95.8–98.3)</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>40</td>
<td>76.9 (62.8–87.0)</td>
<td>59.6 (56.8–62.4)</td>
<td>7.4 (5.4–10.0)</td>
<td>98.4 (97.1–99.3)</td>
</tr>
<tr>
<td>Rectal bleeding</td>
<td>40</td>
<td>76.9 (62.8–87.0)</td>
<td>76.6 (74.2–78.6)</td>
<td>12.4 (8.4–16.3)</td>
<td>98.8 (97.8–99.3)</td>
</tr>
<tr>
<td>FC + diarrhoea</td>
<td>39</td>
<td>75.0 (60.8–85.3)</td>
<td>81.3 (79.0–83.4)</td>
<td>14.4 (10.5–19.3)</td>
<td>98.7 (97.8–99.3)</td>
</tr>
<tr>
<td>FC + rectal bleeding</td>
<td>39</td>
<td>75.0 (60.8–85.3)</td>
<td>89.9 (88.1–91.5)</td>
<td>23.7 (17.6–31.2)</td>
<td>98.8 (98.0–99.4)</td>
</tr>
<tr>
<td>Diarrhoea + rectal bleeding</td>
<td>31</td>
<td>59.6 (45.1–72.7)</td>
<td>91.2 (89.5–92.7)</td>
<td>22.1 (15.8–30.6)</td>
<td>98.2 (97.2–98.3)</td>
</tr>
<tr>
<td>FC + diarrhoea + rectal bleeding</td>
<td>30</td>
<td>57.7 (43.9–71.6)</td>
<td>93.5 (91.4–95.6)</td>
<td>34.9 (29.1–41.6)</td>
<td>98.2 (97.2–98.8)</td>
</tr>
<tr>
<td><strong>OGID</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FC</td>
<td>178</td>
<td>72.4 (66.2–77.8)</td>
<td>61.8 (58.8–64.7)</td>
<td>36.8 (31.1–43.8)</td>
<td>99.9 (98.0–99.5)</td>
</tr>
<tr>
<td>Age 35–60 years</td>
<td>80</td>
<td>32.5 (26.8–38.8)</td>
<td>64.5 (61.5–67.4)</td>
<td>17.7 (14.4–21.6)</td>
<td>80.3 (77.4–83.2)</td>
</tr>
<tr>
<td>Age &gt;60 years</td>
<td>140</td>
<td>56.9 (50.5–63.1)</td>
<td>77.1 (74.4–79.6)</td>
<td>36.8 (33.0–40.6)</td>
<td>88.4 (86.1–90.4)</td>
</tr>
<tr>
<td>Abnormal clinical findings</td>
<td>12</td>
<td>4.9 (3.0–8.8)</td>
<td>98.5 (97.4–99.3)</td>
<td>42.4 (34.0–50.8)</td>
<td>81.5 (79.2–83.8)</td>
</tr>
<tr>
<td>Duration &lt;3 months</td>
<td>90</td>
<td>38.6 (30.6–46.0)</td>
<td>76.0 (73.9–78.6)</td>
<td>26.4 (21.9–31.5)</td>
<td>83.6 (81.4–85.9)</td>
</tr>
<tr>
<td>FC + age 35–60 years</td>
<td>56</td>
<td>22.8 (17.8–28.6)</td>
<td>87.8 (83.6–91.7)</td>
<td>30.4 (24.7–37.7)</td>
<td>82.9 (80.5–85.1)</td>
</tr>
<tr>
<td>FC + age &gt;60 years</td>
<td>109</td>
<td>44.3 (38.0–50.8)</td>
<td>86.8 (84.9–88.6)</td>
<td>43.8 (57.5–60.2)</td>
<td>86.9 (84.6–88.8)</td>
</tr>
<tr>
<td>FC + age 35–60 years + duration &lt;3 months</td>
<td>28</td>
<td>14.4 (7.8–21.7)</td>
<td>96.4 (94.7–97.3)</td>
<td>49.6 (40.1–59.0)</td>
<td>82.2 (79.0–85.3)</td>
</tr>
<tr>
<td>FC + age &gt;60 years + duration &lt;3 months</td>
<td>34</td>
<td>13.8 (9.0–19.8)</td>
<td>96.8 (93.4–97.7)</td>
<td>50.0 (42.7–57.3)</td>
<td>82.7 (80.4–84.7)</td>
</tr>
</tbody>
</table>

PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval; FC, faecal calprotectin; IBD, inflammatory bowel disease; GI, gastrointestinal.

FIVE-YEAR FOLLOW-UP REVEALED NO CASES OF MISSED IBD AND OGID

Study I  New GI diagnoses were identified in 78 patients. OGID (including IBD) was diagnosed in 4.0 valid percent of patients with a positive FC test compared with 3.1 valid percent of patients with a negative FC test. Three patients (0.6 valid percent) with a positive FC test, but no patient with a negative FC test, developed IBD.
DICLOFENAC INTAKE WAS ASSOCIATED WITH INCREASED FC LEVELS

Study II
In Study II, the FC level increased above the ULN in 27% of the participants (median, 76 µg/g; range, 60–958 µg/g) during IMP intake, corresponding to 8.3% of the measurements. FC levels were on average higher during IMP intake than at baseline ($p=0.003$). No significant differences in FC levels were found between baseline and different measurement points after cessation. FC returned to normal within 2 weeks of cessation.

Study III
In Study III, the FC level increased above the ULN in 39% of the participants (median, 70.8 µg/g; range 50.2–1080 µg/g) during the period of IMP intake, corresponding to 22 valid percent of the measurements. FC levels were significantly higher for the total cohort during the period of diclofenac intake compared with baseline ($p<0.05$), but not after cessation (Table 5). FC returned to normal within 2 weeks of cessation.

OMEPRAZOLE INTAKE, ALONE OR IN COMBINATION WITH DICLOFENAC, WAS ASSOCIATED WITH INCREASED FC LEVELS

Study III
During omeprazole intake, the FC level exceeded the ULN in 53% of the participants (median, 85.3 µg/g; range, 51.1–249 µg/g), corresponding to 36 valid percent of the measurements. FC levels were significantly higher during the period of omeprazole intake compared with baseline ($p<0.05$), but not after cessation. FC returned to normal within 3 weeks of cessation.

When omeprazole was co-administered with diclofenac, the FC level increased above the ULN in 69% of the participants (median, 101.5 µg/g; range, 51.5–532 µg/g), corresponding to 51 valid percent of the measurements. FC levels were significantly higher during IMP intake than at baseline ($p<0.05$), and after cessation in Group 1 and the total cohort even on day 21. FC returned to normal within 3 weeks of cessation (Figure 3).

Table 5. Median differences (each measurement point compared with baseline) in faecal calprotectin levels in Study II and III

<table>
<thead>
<tr>
<th>Day</th>
<th>Study II</th>
<th>Study III</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DICLOFENAC</td>
<td>OMEPRAZOLE</td>
</tr>
<tr>
<td></td>
<td>Total cohort</td>
<td>Group 1</td>
</tr>
<tr>
<td>4</td>
<td>0.01 (1.96)</td>
<td>0.01 (2.93)</td>
</tr>
<tr>
<td>7</td>
<td>0.01 (1.96)</td>
<td>0.00 (1.81)</td>
</tr>
<tr>
<td>14</td>
<td>0.00 (1.42)</td>
<td>0.00 (1.00)</td>
</tr>
<tr>
<td>28</td>
<td>0.00 (1.42)</td>
<td>0.00 (1.00)</td>
</tr>
<tr>
<td>35</td>
<td>0.00 (1.00)</td>
<td>0.00 (1.00)</td>
</tr>
</tbody>
</table>

Values are given as median (interquartile range).
Figure 3. Comparison of FC levels in the different investigational medicinal product (IMP) sequences in Studies II and III. Data are presented as (A) the median and (B) the mean. Days 4, 7, 14, during IMP intake; days 21, 28, 35, after cessation.

**Effect of age strata and order of IMPS**

Study III  
Age stratification (≤40 respectively ≥41 years) or a different order of the IMPS did not influence FC levels.
FAECAL AND SALIVARY CALPROTECTIN WERE NOT INCREASED DURING RESPIRATORY TRACT INFECTION

Study IV  Comparison of FC levels during an RTI and in a symptom-free period revealed no significant difference for the total study cohort or the respective groups. Both FC values (during RTI and in the symptom-free period) were under the LLOQ in most of the patients in the viral infection group (77.2%) and all patients in the bacterial and COVID-19 groups (Figure 4).

Regarding salivary calprotectin, the levels during RTI and in the symptom-free period were not significantly different for the total study cohort, the viral group or the bacterial group. However, the levels during infection were lower in the COVID-19 subgroup in comparison with the symptom-free period ($p=0.028$), or the levels in healthy donors ($p=0.004$), but not the levels in the viral cohort ($p=0.096$). Levels in the symptom-free period for patients with COVID-19 were not significantly different from those in the viral group ($p=0.357$) or the healthy cohort ($p=0.912$). Calprotectin levels in saliva and faeces during RTI were not found to be correlated ($\rho=-0.122$, $p=0.228$).

Figure 4. Faecal and salivary calprotectin levels in Study IV in the total study cohort (A) and the diagnosis groups (B, C) during respiratory tract infection and in a symptom-free period.
STABILITY OF SALIVARY CALPROTECTIN

Study IV  Table 6 shows the statistical analysis for salivary calprotectin in 18 healthy donors. The median calprotectin level at baseline was 43.32 µg/mL (range, 9.79–65.00 µg/mL). The ACL was determined to be 38.81% with a CV of 14%. In general, the decrease was greater in samples stored at +4°C. After 6 days, 12 samples (66.7%) were not significantly affected when stored both at room temperature and +4°C. After 6 days of storage, calprotectin levels exhibited a mean decrease of 14.72% at room temperature and 19.96% at +4°C (Figure 5).

Table 6. Stability of salivary calprotectin in Study IV

<table>
<thead>
<tr>
<th>Baseline value µg/mL</th>
<th>2 days</th>
<th>4°C</th>
<th>4 days</th>
<th>+4°C</th>
<th>6 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.36</td>
<td>−14.78</td>
<td>−21.75</td>
<td>−13.06</td>
<td>−21.52</td>
<td>−26.40</td>
</tr>
<tr>
<td>65.00</td>
<td>−4.37</td>
<td>−21.75</td>
<td>−4.49</td>
<td>−21.52</td>
<td>−26.40</td>
</tr>
<tr>
<td>23.42</td>
<td>−18.36</td>
<td>−68.91</td>
<td>−59.09</td>
<td>−77.07</td>
<td>−67.93</td>
</tr>
<tr>
<td>52.26</td>
<td>−0.63</td>
<td>−36.43</td>
<td>−50.94</td>
<td>−53.46</td>
<td>−47.95</td>
</tr>
<tr>
<td>62.61</td>
<td>−7.04</td>
<td>−17.09</td>
<td>−22.30</td>
<td>−36.22</td>
<td>−3.82</td>
</tr>
<tr>
<td>15.36</td>
<td>−7.58</td>
<td>−13.27</td>
<td>−4.99</td>
<td>−47.74</td>
<td>−109.05</td>
</tr>
<tr>
<td>59.43</td>
<td>11.24</td>
<td>−47.99</td>
<td>11.24</td>
<td>−45.13</td>
<td>−74.69</td>
</tr>
<tr>
<td>9.79</td>
<td>−38.82</td>
<td>−15.32</td>
<td>−32.99</td>
<td>−31.05</td>
<td>−71.20</td>
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<td>65.00</td>
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<td>−23.31</td>
</tr>
<tr>
<td>65.00</td>
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<td>0.00</td>
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<tr>
<td>41.12</td>
<td>−36.72</td>
<td>−51.26</td>
<td>−64.74</td>
<td>−57.08</td>
<td>34.19</td>
</tr>
<tr>
<td>44.87</td>
<td>−12.03</td>
<td>−46.80</td>
<td>−4.21</td>
<td>−56.16</td>
<td>−17.83</td>
</tr>
<tr>
<td>41.76</td>
<td>−34.84</td>
<td>−40.97</td>
<td>−19.80</td>
<td>−1.70</td>
<td>−20.32</td>
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<td>15.65</td>
<td>36.10</td>
<td>−20.58</td>
<td>125.94</td>
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<td>57.20</td>
<td>−12.15</td>
<td>−22.13</td>
<td>−20.62</td>
<td>−32.19</td>
<td>−31.23</td>
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<tr>
<td>36.96</td>
<td>3.41</td>
<td>1.43</td>
<td>2.38</td>
<td>30.00</td>
<td>26.27</td>
</tr>
<tr>
<td>26.30</td>
<td>−5.93</td>
<td>−19.20</td>
<td>−16.24</td>
<td>40.72</td>
<td>−20.91</td>
</tr>
</tbody>
</table>

Values are given as percentage differences, unless otherwise indicated.
RT, room temperature; –, omitted test sample.

Figure 5. Scatter plot comparison between salivary calprotectin levels (µg/mL) of day 0 and days 2, 4 and 6 in Study IV.
DISCUSSION

This thesis, together with the 4 studies on which it is based, evaluates the contribution and the diagnostic value of FC for IBD and OGID in primary care, and the influence of sources of error in FC diagnostics. This thesis hopefully provides some new insights into FC diagnostics in the context of primary care and the impact of diclofenac, omeprazole and RTI on its assessment. The main findings of the studies are summarized and discussed below.

OUTCOME OF POSITIVE AND NEGATIVE FC TESTS

The proportion of patients with a positive FC result diagnosed with IBD (8.8%) in Study I was similar to findings of 4.8%–10% in previous reports (195, 196). The proportion of patients with a positive FC result diagnosed with OGID (39.6%) was also in line with previous findings of 13%–37% (5, 6, 196-198).

In patients with a negative FC result, IBD was diagnosed in 0.1% of patients, which also agrees with previous reports on negative test results (0.0%–0.4%) (195, 199). Of the patients with a negative FC result, 9.7% were diagnosed with OGID in our study. This is a higher number compared with 3% and 3.6% described in previous studies by Pavlidis et al. (185) and Turvill (5), respectively, possibly caused by the selection of the patient populations in these studies, i.e. primary care patients aged 18–45 years with suspected IBS in the study by Pavlidis et al. and the secondary care setting with only referred patients in the study by Turvill.

PREDICTION MODELS OF IBD AND OGID

Study I examined associations between calprotectin and combinations of demographic factors and symptoms, allowing a positive diagnosis of IBD or OGID. IBD was found to be associated with a positive FC test, male sex, diarrhoea and rectal bleeding. Diarrhoea is a common symptom associated with IBD and can be present in nearly 80% of cases (200, 201). Further common attributes are rectal bleeding and a positive family history of IBD (202-205), although family history was found to be non-significant in the binary logistic regression in the present study. Family history was combined for IBD and cancer, which could have influenced the result. We have also found a significant association between IBD and male sex. A recent Swedish nationwide cohort study (206) found a higher overall incidence of IBD for males, however, the data in the SWIBREG registry suggest an even sex distribution (23). Other studies by Sjöberg et al. (207, 208) from Uppsala found no sex differences for CD or UC. Shah et al. (209) reported a higher risk
for CD for females after puberty, a similar incidence of UC for males and females <45 years and a higher risk for males >45 years in western countries. In Asia–Pacific countries, they reported a higher risk of incident CD for males until age 50 years as well as a male predominance of UC until age 65 years (210).

The National Institute for Health and Care Excellence (NICE) guidelines (187) recommend FC testing in those aged 18–60 years, with lower GI symptoms, and no suspicion of CRC. Younger age has generally been accepted in the diagnosis of IBD. However, age was not statistically significant in our study and showed a degree of variability (CD: median, 41 years; range, 18–65 years; UC: median, 38 years; range, 18–76 years; IBD UNS: n=1; 18 years). In our study, 1 patient diagnosed with CD and 9 with UC were >60 years old, i.e. 19% of all patients with IBD. Recently, the number of patients with elderly-onset IBD has been reported to be increasing as a result of an ageing population and the increasing incidence of IBD (23, 211).

Similar to the present study, diarrhoea and rectal bleeding were more common in organic conditions (7, 185). On the contrary, abdominal pain was found to be non-specific for IBD and OGID, which is in line with findings by Pavlidis et al. (185) and Walker et al. (197), who reported abdominal pain to be more common in non-organic disease.

In the present study, functional disorders showed a negative association with a positive FC test and higher age; on the other hand, no alarm symptoms and duration >3 months showed a positive association. This is also in agreement with the NICE guideline on IBS (15).

**Diagnostic accuracy of FC and different cut-offs**

FC had the highest sensitivity and NPV among all other variables studied. In the diagnosis of IBD, FC yielded a sensitivity of 98.1%, specificity of 57.5%, PPV of 8.8%, and NPV of 99.9% at the cut-off of ≥15 mg/kg. The specificity and PPV were substantially lower than the results by Otten et al. (212) who reported a specificity of 94.5% and PPV of 82.1% for the same CalDetect test in 114 patients referred for endoscopy. The variance in these test accuracy measures is most likely related to the different population selection (5, 185, 213, 214).

By increasing the cut-off to 60 mg/kg, in the study by Otten et al., the specificity increased to 97.8%, but the sensitivity decreased to 60.6%. Consequently, there would be a substantial loss of IBD diagnoses. In the study by Vestergaard et al. (215), similarly, raising the cut-off from 15 to 60 mg/kg resulted in an increase in the specificity from 70% to 100% and a decrease in the sensitivity from 96% to 66%. In our study, correspondingly, the specificity increased and the sensitivity decreased, but to a considerably smaller extent. An additional association of FC with age 35–60 years and male sex was found when the cut-off was increased, but the other associations and ORs increased only marginally.

A systematic review by Waugh et al. (183) included 7 studies on adults on distinguishing between IBD and IBS and found heterogeneous data on the
accuracy of different FC tests at a cut-off of 50 µg/g; sensitivities ranged between 83% and 100%, specificities from 60% to 100%, PPV from 24% to 100% and NPV from 75% to 100%. However, all these studies were conducted in a secondary care setting. Data on FC testing in primary care are still scarce. A recent British retrospective cohort study (216) with 5970 patients in the primary care setting found a specificity of 61.5% and PPV of 8.1% at an IBD prevalence of 3.5% and cut-off of 50 µg/g, supporting our results.

Intake of NSAIDs and PPIs marginally influenced the accuracy measures of FC; the sensitivity and PPV increased, and the specificity decreased. Notwithstanding the relatively limited number of these patients, these results offer valuable insights into the effect of these drugs on FC.

**Effect of Diclofenac and Omeprazole Intake on FC levels**

Studies II and III examined how short-term oral diclofenac intake affects FC levels and the normalization interval after cessation.

We found increased FC levels above the ULN in 27% and 39% of the participants, respectively. Previous studies have shown increased FC concentrations in patients on various NSAIDs (132, 154). Studies on the short-term intake of diclofenac by Maiden et al. and Thornjóðleifsson et al. (155, 156) showed increased FC levels in 75% and 95% of the participants and confirmed small intestinal injuries in 68% and 70% by capsule endoscopy. These higher numbers may be due to co-administration of omeprazole in both studies (for gastroprotection, according to the authors), as discussed in the next sections. Despite these findings, the likelihood of a positive FC test induced by diclofenac seems to be low. Most of the measurements during diclofenac intake were under the ULN in our studies (93% in Study II and 78% in Study III).

Study III also examined the short-term effect of omeprazole. Previous research has provided evidence on the association between PPIs and increased FC levels. In 2003, Poullis et al. (173) demonstrated a significantly increased FC level in patients using PPIs compared with those who did not and proposed discontinuing PPIs before testing to increase the specificity of FC for detecting OGIDs. In a recent study on 36 patients on long-term PPI therapy (average 63 months; mainly pantoprazole and esomeprazole), Horvath et al. (217) found increased FC levels over the ULN in 83% of the patients at baseline. However, most of the available studies reported on PPIs in combination with NSAIDs (155, 157, 218). In our study, the effect of omeprazole was greater (with regard to the number of participants and the number of measurements above the ULN) and of longer duration than that of diclofenac. In accordance with these results, a previous study on patients referred for colonoscopy by Lundgren et al. (126) demonstrated a stronger association between PPI use and increased FC levels (FC >50 mg/g) than that of NSAIDs or ASA. This is in line with findings in Study I, which found that PPI use had a stronger association with a positive FC result compared with NSAIDs or ASA.
In Study III, the co-administration of omeprazole and diclofenac showed the most pronounced effect on FC levels (with regard to the number of participants and the number of measurements above the ULN) compared with the administration of these drugs alone. Other studies on diclofenac co-administered with omeprazole (155, 156) have reported this effect in an even higher proportion of participants. Further support on increased FC concentration in healthy individuals on diclofenac and omeprazole has been provided by Kuramoto et al. (160). In addition, 68%–81% of the participants in these studies had mucosal injuries confirmed by capsule endoscopy. A study by Goldstein et al. (170) showed a significantly higher association with small bowel mucosal injuries for naproxen co-administered with omeprazole (55% of the participants) compared with celecoxib (16%) or placebo (7%). The same study group found higher FC levels in healthy participants receiving ibuprofen and omeprazole compared with celecoxib (157). However, no correlation was found between FC levels and the results of capsule enteroscopy in the studies by Goldstein et al. (157) or Maiden et al. (155). Wallace et al. (169) further supported these findings by providing evidence of significant shifts in enteric microbial populations in rats caused by PPIs and thus exacerbation of NSAID-induced intestinal damage.

**Effect of Respiratory Tract Infection on Calprotectin**

Questions have been raised on whether respiratory diseases can cause a positive FC test result (92, 174, 175). In respiratory diseases, increased calprotectin levels in saliva or sputum may be indicative of underlying mechanisms involved in inflammation or immune system activation within the respiratory system. Calprotectin is a stable protein with low degradation during passage through the GI tract. Swallowed saliva in a case of an RTI with the presence of neutrophilic granulocytes was suspected to give an increased calprotectin level, still measurable in faeces.

In Study IV, FC levels were found to be generally very low during RTIs, and in most participants, they were below the LLOQ. In all patients, there were no concurrent GI symptoms at the time of inclusion, which might be a possible explanation for the lack of positive FC levels. Increased FC levels were found in only a few participants; at the most 203 µg/g during an RTI and 168 µg/g in the symptom-free period. Other causes of increased FC are not known because these individuals did not report any other problems. However, if the patients had IBD, distinguishing whether a positive outcome was because of IBD or an RTI would be difficult.

Three of the participants had asthma and 1 had COPD. In all these patients, FC levels during RTI were below the LLOQ except in 1 patient with asthma who had a test result of 95 µg/g during infection and 168 µg/g in the symptom-free period. Due to the small number of these patients in the present study, it is not possible to draw any conclusions about the effect of asthma and COPD on FC.

With regard to COVID-19, some studies have observed correlation between faecal SARS-CoV-2-RNA load and FC (117), but others have not (114, 118). In
In our study, FC values were under the LLOQ in all 11 patients with COVID-19, possibly because they had no GI symptoms at inclusion. Recent studies have confirmed significantly higher FC values in patients with COVID-19 with GI symptoms and suggest that FC concentrations can be correlated to the severity of disease (219), but other studies do not support these findings (118, 220).

There is a lack of evidence on salivary calprotectin in RTIs. In the present study, salivary calprotectin values were slightly higher on average than FC values during RTI but were not found to be correlated. Studies on pulmonary diseases have found increased calprotectin levels in saliva and sputum (109, 112), however, this could not be confirmed in the present study, possibly because only a few of the patients had underlying chronic pulmonary disease.

Although the sample size was small in the bacterial and COVID-19 cohorts, the results did not indicate increased salivary levels in these groups. In COVID-19, however, the levels were found to be significantly lower during the infection compared with the symptom-free period (observed in 6 of 11 patients). Also, they were lower compared with the levels in healthy individuals. A similar trend was also seen in comparison with the viral cohort, although this was non-significant. The levels in the symptom-free period did not differ between the groups, therefore it is tempting to speculate that the infection could be a cause of this effect.

In the literature, the salivary glands have been reported to be a potential target for SARS-CoV-2 infection due to expression of the ACE2/TMPRSS2 receptor in salivary gland epithelial cells (221, 222). Two recent Chinese studies have demonstrated a long duration of the viral load of SARS-CoV-2 in saliva (11 and ≥20 days) (223, 224). A recent study by Santos et al. (225) has demonstrated altered salivary immune defences in patients with COVID-19. They found decreased levels of salivary lactoferrin and IgA in patients with acute COVID-19 compared with healthy controls and recovery post-COVID-19 of at least 2 months, suggesting a loss of antiviral protection against the virus and impairment of the immunoprotective mechanisms of the mucosal barrier in these patients. The question remains on whether this could also be applicable in the present study.

In Study IV, it was determined that the patients would serve as their own controls in a symptom-free period after an RTI. These individuals still had other potential underlying conditions or risk factors, providing an exact match. Including healthy individuals as a control group would likely allow comparison of the calprotectin levels with those of the patients. However, that would require the sample size to be increased and associated time costs. Furthermore, the control group would need to be matched in terms of sex and age.

Regarding the stability of salivary calprotectin, the value decreased substantially in samples stored at +4°C, therefore it might be more appropriate to store them at room temperature. Majster et al. (226) showed increased concentrations when samples were stored at +4°C for 3 days, however, the process of saliva sampling and handling was different. In clinical practice, the test result should be assessed in conjunction with the patient’s medical history and symptoms. In addition, the test can be repeated if the result is ambiguous.
CALPROTECTIN ASSAYS

In each study (I–IV), FC was determined by a different laboratory calprotectin assay, which makes comparison of the data across the studies difficult. In Studies I–III, FC was determined using the method offered by the laboratory at the time of the study. A different assay had to be used for Study IV, because of the unavailability of salivary calprotectin assay in the laboratory. Because the current research was ongoing for a longer period of time, it was not possible to use the same assays over time.
LIMITATIONS

Several limitations need to be acknowledged. The data in Study I are based on patients’ medical records. There is a risk that the symptoms were not recorded correctly, or some may not have been included in the records. However, this seems to reflect the actual circumstances of daily practice. The 5-year follow-up was therefore helpful in identifying the patients whose symptoms were due to disease but initially went undiagnosed. Furthermore, selection bias may have been introduced, resulting in a higher proportion of women in the total study cohort and hence a reduced likelihood of having IBD and OGID diagnoses in women. This is probably because the eligibility criteria did not include demographics or symptoms, but only an FC test. There is also potential for review bias because the data collectors were not blinded to the FC test results. FC results were included in the patients’ records so blinding was not possible. However, the results of investigations were recorded by the examining physician and therefore should not have affected the data collector’s interpretation of the index test. The semi-quantitative FC analysis used in this study is also a limitation; it has since then been replaced by fully quantitative laboratory-based analyses. Point-of-care rapid tests are still in use in other countries, mostly in an outpatient setting (9, 187, 227) and have been shown to compare well with ELISA across a range of FC levels (212, 215, 228, 229).

In Study III, the study protocol had to be simplified because we encountered difficulties recruiting participants, probably due to the large number of tests and long study duration. Therefore, we excluded the initial sequence without IMP and blood count testing after each sequence. As a result, we lack a sequence with natural intra-individual day-to-day FC variability.

A number of limitations need to be considered regarding Study IV. The ELISA assay was not validated for saliva samples by the manufacturer. However, this assay was used previously for saliva with a positive outcome (226). We encountered some difficulties when conducting the assay. The standard point corresponding to 1 ng/mL was often equal to or overlapped with the blank. A standard point corresponding to 2 ng/mL was therefore determined as the LLOQ with a concentration of 2 µg/mL for saliva and 20 µg/g for faeces. The total interassay CV was 14.8%, which was higher than the interassay CV for serum (4.1%–4.3%) presented by the manufacturer (192). This could possibly be explained by the large number of manual steps involved. We also had no knowledge of the patients’ oral health and its possible effect on our results. Furthermore, the small sample size in the bacterial infection group and COVID-19 subgroup adds further caution regarding the generalizability of the results for these diagnoses.

Stability analysis showed that calprotectin was stable in saliva at 6 days in most of the samples when stored at room temperature and +4°C. These results are promising, however, it is essential to consider the measurement uncertainty of the analysis. Notwithstanding the limitations, the findings will hopefully serve as a base for future studies on this topic.
CONCLUSIONS

The major findings of this thesis are:

- A negative FC test has a high NPV and effectively rules out IBD and contradicts the presence of other organic GI diseases in primary care patients with GI symptoms.
- Patients with a positive FC result together with diarrhoea, rectal bleeding, short symptom duration or age >35 years should be prioritized for further investigations.
- Combining FC with other significant predictors tends to increase the specificity and PPV and decrease the sensitivity in the prediction of IBD and organic GI disease.
- Intake of NSAIDs, PPIs and ASA may affect the diagnostic accuracy of FC test for IBD and organic GI diseases.
- Short-term intake of diclofenac, omeprazole, or their co-administration is associated with increased FC levels.
- The influence of diclofenac on FC may persist for up to 2 weeks after cessation. Therefore, patients on diclofenac with a positive FC test should undergo a repeat FC test 2 weeks after cessation.
- The influence of omeprazole alone, or when co-administered with diclofenac, may persist for up to 3 weeks after cessation. In patients with an increased FC level on these medications, it would therefore be reasonable to repeat the FC test at least 3 weeks after cessation.
- In primary care patients with acute respiratory tract infection, faecal and salivary calprotectin levels were not found to be increased. These findings suggest that respiratory tract infections are unlikely to be a source of error when interpreting an increased FC test result. These findings are promising, and warrant further research.
FUTURE PERSPECTIVES

Most of the evidence on FC comes from secondary care settings. It may not be correct to transfer evidence between settings, and evidence on FC tests in primary care is still scarce in the literature. Prospective studies with a representative primary care population are needed to determine the utility of FC testing in primary care.

Data on salivary calprotectin in patients with acute RTI and COVID-19 are limited in the literature, and further studies should be undertaken to explore our findings further. In addition, future studies are needed to evaluate the role of FC as a potential biomarker in patients with severe asthma, COPD, or pneumonia.

Access to point-of-care calprotectin testing would probably facilitate the diagnostics of patients with GI symptoms in primary health care and improve the differentiation between IBD and functional GI diseases. This could save laboratory resources in secondary care settings and help identify which patients need further examinations and referrals. Such testing is currently not available in Sweden and would need further evaluations to assess the reliability and diagnostic accuracy.

Current variation in the commercial assays for calprotectin hinders comparability of laboratory results in clinical practice, as well as accurate comparison between studies. Standardization would enable collaboration between laboratories, prevent problems with the interpretation of test results, and ensure comparable and reliable results from research findings.

The future is bound to be dynamic for laboratory calprotectin assays. New assays continue to be developed at a high rate. Patients can now perform a quantitative test at home, utilizing technology within smartphones. This allows patients to monitor IBD regularly and will probably lead to better patient adherence. The use of home tests is likely to expand. Future studies to evaluate this method of testing are necessary.
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Studies

The studies associated with this thesis have been removed for copyright reasons. For more details about these see:

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Faecal Calprotectin Diagnostics

Focus on Primary Care and Suspected Sources of Error

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