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*BIOHIT™ Calprotectin ELISA: Assay Validation

Non-invasive Differential Diagnosis of Inflammatory Bowel Disease (IBD) and Irritable Bowel Syndrome (IBS) Using BIOHIT™ Calprotectin ELISA Test in Stool Samples

Jointly Executed by:

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*CALPROLAB™ Calprotectin ELISA (ALP) is a Trade Mark of CALPRO AS (Lysaker, Norway);
**Biohit Oyj (Helsinki, Finland) is a non-exclusive global distributor of BIOHIT™ Calprotectin ELISA test

Summary

Background: Gastroenterologists are often faced with the diagnostic difficulty in differentiating the patients with functional disorders, most notably **irritable bowel syndrome (IBS)** from those with clearly intestinal pathologies, in particular **inflammatory bowel disease (IBD)**. Many symptoms are common to both conditions, including abdominal pain, bloating, excessive flatus, and altered bowel habit, whereas other clinical features such as a predominance of diarrhoea and rectal bleeding will increase the likelihood of IBD. On the other hand, fulfilling the Rome criteria and having a normal full blood count, routine biochemical screening, ESR and CRP are reassuring indicators pointing to IBS.

Although symptoms are a surprisingly good guide to a correct diagnosis, most clinicians rely on laboratory tests to aid in the differential diagnosis. Because the clinical differentiation remains problematic, many patients in the IBS category are still investigated extensively with **invasive radiographic and endoscopic imaging** to make a diagnosis or exclusion. This has significant implications for both the health care costs and patient management, while exposing the patients to the inherent risks associated with invasive diagnostic procedures.

During the past 20 years, considerable expectations have been posed on **Calprotectin (Calgranulin, S100A8)** as a potential biomarker capable of differentiating IBD and IBS. Based on convincing clinical data, CALPRO AS (Oslo, Norway) developed the CALPROLAB™ Calprotectin ELISA (ALP) test for stool samples. The test is usually positive in IBD in contrast to IBS, where fecal output of Calprotectin remains normal. **Biohit Oyj** (Helsinki, Finland) has recently acquired global non-exclusive rights to market this test with the trade name **BIOHIT™ Calprotectin ELISA** for quantitative detection of Calprotectin concentration in fecal samples. Clinical validation studies are still needed in different settings.

Objective: To test the clinical performance of the **BIOHIT™ Calprotectin ELISA** in differential diagnosis of IBD and IBS as well as in monitoring the treatment efficacy and prediction of relapse of the former.

Study Design: The study setting consists of two distinct parts, **a clinical series** and a **screening trial.** The clinical series includes patients with prevalent IBD, representing both ulcerative colitis (UC) and Crohn's Disease (CD), as well as those with clinically confirmed or suspected IBS. Another cohort of hospital-referred subjects with non-specific gastro-intestinal symptoms will be screened by the Calprotectin test.

Methods: Study subjects (both genders) for the clinical cohort are enrolled among the consecutive adult patients with clinically confirmed IBD or IBS, (including those with suspicion of IBS), referred for endoscopic examination at Hospital X (City Y, Country Z). All patients are subjected to BIOHIT™ Calprotectin ELISA test for quantitative measurement of Calprotectin in the stool samples. In the clinical setting, all IBD- and IBS patients will be

subjected to colonoscopy, providing the histological confirmation to be used as the gold standard reference test. In the screening setting, only the patients testing positive with BIOHIT™ Calprotectin ELISA will be subjected to colonoscopy and biopsy confirmation. In addition, a random sample of 2% of Calprotectin ELISA-negative subjects will be invited for colonoscopy, to enable the statistical correction for verification bias. The cut-off values making distinction between IBD and IBS are derived from the ROC analysis, using the values with the best SE/SP balance. These established cut-off values will be tested in calculating the performance indicators (sensitivity, specificity, PPV, NPV and their 95%CI) of the test, separately for both study endpoints (IBD and IBS).

Specific Aims: The most important goal of this study is to assess the performance of the BIOHIT™ Calprotectin ELISA test in differentiating IBD and IBS. In addition to this primary study endpoint, this setting also provides information about the dynamic changes in Calprotectin levels as related to i) the activity of the disease (CDAI, UCDAI), ii) efficacy of the current treatment, and iii) prediction of disease relapse. This is accomplished by analysing Calprotectin concentrations in IBD patients with different disease activity and those on different medication.

Study execution and time table: The necessary preparations for the study execution at Hospital X will start immediately when the hospital has reached the agreement with Biohit Oyj. The study plan necessitates a review by the institutional review board (IRB, Ethical Committee) before permission to start. Given that the subjects in the study will be enrolled among consecutive patients with clinically diagnosed IBD or IBS, or among those reporting symptoms suggestive of these conditions, it is estimated that a cohort of 100 IBD patients (prevalent cases) and 100 IBS patients will be needed to calculate the reliable performance indicators for the BIOHIT Calprotectin ELISA test. To yield enough incident cases of IBD and IBS cases, at least 400 hospital-referred subjects need to be screened.

Impact of the study: Reliable distinction between IBS and IBD is rarely possible without invasive diagnostic procedures, which has significant implications in both the health care costs and patient management, while exposing the patients to the inherent risks associated with invasive diagnostic procedures. Devoid of these disadvantages, BIOHIT™ Calprotectin ELISA test is a non-invasive, quantitative method for i) reliable differentiation between IBS and IBD, ii) for monitoring the efficacy of therapy, as well as iii) for providing a quantitative pre-symptomatic predictor of imminent clinical relapse of IBD.

1.BACKGROUND

Gastroenterologists are often faced with the diagnostic difficulty in differentiating the patients with functional disorders, most notably **irritable bowel syndrome (IBS)** from those with clearly intestinal pathologies, in particular **inflammatory bowel disease (IBD)** (1,2). Many symptoms are common to both conditions, including abdominal pain, bloating, excessive flatus, and altered bowel habit, whereas other clinical features such as a predominance of diarrhea and rectal bleeding will increase the likelihood of inflammatory disease (3,4). Although symptoms are a surprisingly good guide to a correct diagnosis, most clinicians rely on laboratory tests to aid in the differential diagnosis.

Certainly, fulfilling the Rome criteria and having a normal full blood count, routine biochemical screening, ESR and CRP are reassuring indicators pointing to IBS. Consequently, many authors have recommended a straightforward approach to evaluate and treat the patients with IBS, based solely on the use of the Rome criteria as a means of cost-effective management (4-6). Despite this, the use of the Rome criteria has not been universal and is largely confined to use as entry criteria into research studies of patients with IBS (1,2).

The concern among gastroenterologists is that some patients with organic intestinal disease (e.g. IBD) will be incorrectly diagnosed if excess reliance is placed upon these Rome criteria (3-6). Thus, it remains customary to exclude all organic diseases using invasive diagnostic investigations as objective evidence of non-existing significant intestinal pathology. Because the clinical differentiation remains problematic, many patients in the IBS category are still investigated extensively with invasive radiographic and endoscopic imaging to make a diagnosis of exclusion. This has significant implications for both the health care costs and patient management, while exposing the patients to the inherent risks associated with invasive diagnostic procedures. Thus, there is an urgent need for safe, inexpensive and cost-effective **non-invasive diagnostic tools** that would enable accurate distinction between IBS and IBD (1-6).

1.1.Epidemiology and diagnostic criteria of IBS and IBD

1.1.1.Irritable Bowel Syndrome (IBS)

The prevalence estimates for IBS indicate that 14%–24% of women and 14%–19% of men are affected by this condition in the UK and in the US (7). These estimates have generally been based on numbers of patients fulfilling the symptom-based diagnostic criteria that have been developed to identify patients with IBS (3,4). The criteria of Manning (8) and those of others (9-12) have been widely used in clinical research. Many symptoms are quite non-specific and common to both IBD and IBS, including abdominal pain, bloating, excessive flatus, and altered bowel habit, whereas other clinical features such as a predominance of diarrhea and rectal bleeding will increase the likelihood of inflammatory disease (3,4).

However, because there are no biological markers to define IBS, validation of such criteria has been cumbersome. There have been concerns regarding both the sensitivity and specificity of these criteria, which have ranged from 58% to 94% and 55% to 74%, respectively, in various studies (8,12,13) with their discriminate value possibly affected by the gender (14). As a result, a consensus definition and criteria were developed (the Rome I criteria), for IBS and other functional gastrointestinal disorders (15,16).

Some authors have recommended an approach to the evaluation and treatment of patients with IBS based on the use of the Rome criteria as a means of cost-effective management, avoiding the costly workup to sort through a confusing array of gastrointestinal symptoms (3,5,6). The stepwise assessment of the patients with suspected IBS, based on positive Rome criteria (6,7) includes a full blood count, erythrocyte sedimentation rate (ESR)/C-reactive protein (CRP), serum chemistry, thyroid function tests, and stool examination for parasites and ova to exclude organic diseases. However, as shown by Tolliver et al (17), these parameters have a disappointing diagnostic yield in patients meeting the Rome I criteria. Currently, the Rome I criteria are rarely used formally

in routine clinical practice, but their main usage is as an entry criteria for clinical studies as an attempt to standardize the characteristics of patient groups (1,2).

1.1.2.Inflammatory Bowel Disease (IBD)

1.1.2.1.Ulcerative colitis (UC)

The two most important clinical entities included among IBD are **ulcerative colitis (UC)** and **Crohn's disease (CD).** Ulcerative colitis (Colitis ulcerosa, UC) is a form colitis that includes characteristic ulcers, or open sores. The main symptom of active disease is usually constant diarrhea mixed with blood, of gradual onset. Clinically, UC shares much in common with CD, but the latter can affect the whole gastrointestinal tract while UC only attacks the large intestine. While UC can be cured by total colectomy, surgery for CD does not cure the disease, which can recur mostly at the site of the intestinal anastomosis.

UC is an intermittent disease, with periods of exacerbated symptoms, and periods that are relatively symptom-free. UC is a chronic inflammatory disease of the colon characterized by bloody diarrhea and abdominal pain. Although the symptoms can sometimes diminish on their own, the disease usually requires treatment to go into remission. UC has an incidence of 1-20/100,000 individuals, and prevalence of 8-246/100,000 (18). The disease is more prevalent in northern countries of the world, as well as in northern areas of individual countries or other regions. Rates tend to be higher in more affluent countries, which may indicate the increased prevalence is due to increased rates of diagnosis.

Although UC has no known cause, there is a presumed genetic component to susceptibility. The disease may be triggered in a susceptible person by environmental factors. Although dietary modification may reduce the discomfort of a person with the disease, UC is not thought to be caused by dietary factors. Despite recent advances in the understanding of the genetics, immune and inflammatory mechanisms, as well as potential environmental factors that contribute to the disease, an exact pathogenesis remains elusive. Hence, the treatment is aimed at modifying the pathogenic mechanisms involved,

mostly by using anti-inflammatory drugs such as mesalazine, corticosteroids, immunosuppressant agents, or biologics (18).

1.1.2.2.Crohn's disease (CD)

CD, also known as Crohn syndrome and regional enteritis, is a type of inflammatory bowel disease (IBD) that may affect any part of the gastrointestinal tract from mouth to anus, causing a wide variety of symptoms. CD affects between 400,000 and 600,000 people in the US. Prevalence estimates for Northern Europe have ranged from 27–48/100,000. CD disease tends to present initially in the teens and twenties, with another peak incidence in the fifties to seventies, although the disease can occur at any age. It primarily causes abdominal pain, diarrhea (which may be bloody if inflammation is severe), vomiting, or weight loss, but may also cause complications outside the gastrointestinal tract such as anemia, skin rashes, arthritis, inflammation of the eye, tiredness, and lack of concentration (1,2). CD is caused by interactions between environmental, immunological and bacterial factors in genetically susceptible individuals (19-21). This results in a chronic inflammatory disorder, in which the body's immune system attacks the gastrointestinal tract possibly directed at microbial antigens. While CD is an immune-related disease, it does not appear to be an autoimmune disease, but it may be an immune deficiency state (19-21).

1.2.Distinguishing between IBD and IBS

UC and CD are chronic inflammatory bowel disorders characterized clinically by periods of well-being interrupted by episodes of clinical disease activity. The presence of disease in UC and assessment of disease activity is not a major clinical problem and achievable by sigmoidoscopic visualization, with direct biopsy examination, because the disease is uniform and regularly originates distally in the colon. This is not the case for DC, however, because of its variable location and the patchy nature of the inflammation. Clinical and laboratory (ESR, CRP, α1 antitrypsin, neutrophil elastase, platelet counts) scores of disease activity have been devised for assessment of disease severity in these patients (22-24).

These are widely used and easily applied, but laboratory tests are at best non-specific, being abnormal in various non-intestinal diseases (1,2,4-6).

The most striking difference between IBS and IBD is that the former is non-inflammatory in nature. Therefore, one possibility is to measure surrogate **markers of intestinal inflammation** to differentiate between the two. Assessment of serologic markers (ESR, CRP) has in general been disappointing, probably because of their lack of sensitivity and specificity, because they are indirect measures of inflammation, and they can be affected by a number of non-intestinal diseases. The direct assay of feces for inflammatory markers has the potential to improve on the discriminant value of the serologic markers (1,2).

Calprotectin is a calcium-binding protein found in neutrophilic granulocytes, monocytes, and macrophages, comprising up to 60% of the total cytosolic protein content of neutrophils, resists metabolic degradation and can be measured in feces (25,26). Its use has been extensively validated, showing consistent abnormalities in patients with IBD, colorectal carcinoma (CRC), and non-steroidal enteropathy (27-33).

Tests of **intestinal permeability** (differential urinary excretion of lactulose/L-rhamnose) have been shown to be sensitive for the detection of inflammation in the small intestine, including celiac disease, CD, and intestinal infections (1,2). The principle of the differential urinary excretion of two orally administered test probes is that pre-mucosal and post-mucosal determinants of their excretion are equal. Hence, the ratio of lactulose/L-rhamnose becomes a specific index of intestinal permeability and has been proposed as a diagnostic screen for small bowel disease (34).

1.2.1.Intestinal function tests

Although not perfect, the above approach in diagnosis and management of the patients with IBD remains the daily practice in many clinics, and shown to perform reasonably well for most patients. Few practices are perfect, however, and there is room for improvement

also in the clinical management of IBD and IBS patients. Recently, an increasing interest has been focused on direct testing of intestinal function, with the potential to provide new, direct and different information. In addition, these intestinal function tests might be applicable for screening of intestinal disorders as well as providing prognostic and predictive information about the disease outcome. At present, three intestinal function tests are being explored, two of which (intestinal permeability test and white cell scans) already have a 20-year history. The third, i.e., direct assay of feces for inflammatory markers, is a more recent approach, and according to some authorities, has the greatest potential of these three (1,2,27-33).

1.2.1.1.Intestinal permeability testing

Intestinal permeability is assessed non-invasively in vivo by measuring urinary excretion of orally administered substances. The ideal permeability probe is water-soluble, non-toxic, non-degradable and not metabolised before, during or after permeating the intestine (35). Menzies was the first to introduce oligosaccharides as test substances for the non-invasive assessment of intestinal permeability, and later formulated the principle of differential urinary excretion of orally administered test substances (36). The differential principle advocates that a non-hydrolyzed disaccharide (i.e. lactulose) and a monosaccharide (L-rhamnose or mannitol) are ingested together. As the pre- and post-mucosal determinants of their excretion affects the two test substances equally and the differential 5 hour urinary excretion ratio (ratio of lactulose/L-rhamnose) is not affected by these variables the urinary excretion ratio becomes a specific measure of intestinal permeability. Tests of intestinal permeability were initially designed to allow reliable non-invasive detection of patients with untreated coeliac disease, but have since come to be viewed as synonymous with assessing intestinal barrier function.

In clinically active small bowel CD, the vast majority of patients (>95%) have an increase in the differential urinary excretion of ingested di-/monosaccharides (lactulose/L-rhamnose or mannitol) and half of those with Crohn's colitis are abnormal (35). The vast majority of

patients with UC have normal small intestinal permeability when assessed by these methods. However, tests of intestinal permeability have not found widespread application as screening tests to discriminate between patients with IBD and IBS (35). The reason for this is probably that the urinary sugar analysis is time consuming and demanding, and there is some concern that the tests lack specificity in a variety of small intestinal diseases (35).

1.2.1.2.White cell scans

Intense neutrophil recruitment to the intestinal mucosa is a feature common to inflammatory bowel diseases (1,2,35,37). When a patient's own radiolabelled neutrophils are re-injected they migrate to sites of acute inflammation as well as to the liver, spleen and bone marrow (38). Segal, Saverymuttu and Chadwick were instrumental in the introduction, validation and application of the ^{111}Indium white cell technique for use in gastroenterology (37-39). The technique visualizes inflamed segments of bowel and quantitates the degree of inflammatory activity.

A number of studies have established that abdominal scans are abnormal in virtually all patients with active IBD; their accuracy in localization of disease and distinguishing between actively inflamed and fibrous strictured disease has implications for treatment. It was suggested that the technique could be used to discriminate, with an accuracy approaching 100%, between patients with IBD and IBS at the first outpatient visit. In practice this suggestion was not followed up with relevant research, however (37-39).

When combined with measurement of the 4-day fecal excretion of labelled white cells for quantitation of the inflammatory activity, the technique becomes a powerful tool in both research and clinical work. The fecal excretion of the labelled white cells quantitate inflammation accurately and can be used to document therapeutic efficacy of various treatments in IBD (40-42). The method is not disease specific, however, resembling that of the permeability tests, but it is specific for intestinal inflammation. This is not a drawback

as it is a simple matter to distinguish between the inflammatory activity in patients with IBD and other enteropathies, since IBD patients have excretion values often an order of magnitude higher than the others (35).

The factors limiting a universal adoption of these white cell scans in diagnosis of IBD, include the following: 1) it requires expensive labeling facilities including labelling cabinets; 2) the labeling procedure is time consuming, taking more than 2 hours; 3) the cost of isotope and material is high; 4) the radiation dose is not trivial if abdominal scans are carried out; and 5) a complete 4-day fecal collection is also demanding and unpleasant for patients, occasionally requiring hospital admission.

1.2.1.3.Fecal biomarkers

The inflamed hyper-permeable mucosa of the patients with IBD is associated with increased protein loss into the bowel lumen (43). Studies using radiolabelled proteins have demonstrated that there is **fecal protein loss** in patients with active CD, and it may therefore be a useful marker of disease activity. Other studies have shown fecal α1-antitrypsin clearance to be a useful indicator of protein losing enteropathy, and that in patients with IBD, a 72-hour fecal clearance of α1-antitrypsin is a useful method for quantitating intestinal protein loss (44). Similarly, random fecal α1-antitrypsin levels have been shown to be as useful as more prolonged collection in measuring CD activity, and correlated with several other laboratory measures that have been proposed as indicators of CD activity (45).

Concerns about costs, radiation, and the need for prolonged fecal collections all worked against these techniques for routine use, although many remain very important for research studies (1,2,35). The idea then emerged that it might be possible to assay for cell proteins or substances that are specifically associated with a certain cell type and which would then provide information on a specific component of the inflammatory cascade (46). Concerned about bacterial degradation of markers they used a whole gut lavage

method involving ingestion of polyethylene-based purgatives (Kleenprep or GoLitely) for obtaining clear liquid fecal samples for analysis. The analysis took to various markers, such as immunoglobulins, neutrophils-specific elastase, and hemoglobin. Separate studies showed that CD could be identified with ease, and that the method had a greater sensitivity for CRC than the conventional fecal occult blood (FOB) technique. Ideally suited for research, the method has as yet not found wide application for routine screening purposes, possibly because of the drawback of patients needing to ingest large volumes of liquid (35,46). However, it is now clear that it is not necessary for the marker to be completely non-degraded, provided that the antibody (most of these assays are ELISA's or radio immunoassay) is directed at an epitope of the molecule which resists degradation. One such assay is available for lactoferrin (47). Lactoferrin is a relatively specific marker for neutrophils where it is present within the cytoplasmic granules.

2.FECAL CALPROTECTIN

Of all fecal markers assessed so far, the greatest experience has been gained with **Calprotectin** (27,28,48). It accounts for up to 50% of the neutrophilic cytosolic protein while being resistant to colonic bacterial degradation. It is easily measured in feces by a commercially available ELISA test. Calprotectin was first isolated from granulocytes by Fagerhol et al (49), and initially called as L1-protein (calgranulin), but subsequently re-named Calprotectin upon identification of its calcium-binding and antimicrobial properties (50). The protein is a hetero-complex protein consisting of two heavy (L1H) chains and one light (L1L) chain which are non-covalently linked (26,51).

Calprotectin is a 36kd calcium and zinc-binding protein, produced by PMNs, monocytes and squamous epithelial cells (except those in normal skin)(35). After binding with calcium, it can resist degradation by leukocytic and microbial enzymes. By competing with different enzymes for limited, local amounts of zinc, Calprotectin can inhibit many zinc-dependent enzymes and thereby kill micro-organisms or animal and human cells in culture (35). Different types of disease, for instance bacterial infections, rheumatoid arthritis and cancer,

lead to activation of PMNs and increased levels of Calprotectin in plasma, cerebrospinal fluid, synovial fluid, crevicular fluid, urine or other human materials. Thus, Calprotectin appears to play an important regulatory role in the inflammatory process, and it functions in both an antimicrobial and anti-proliferative capacity (52-56). It has both bactericidal and fungicidal properties with minimal inhibitory concentrations comparable to those of many antibiotics (50). It is released from the cells during cell activation or cell death. The C-terminal sequence of the L1H chain has been shown to be identical to the N-terminus of peptides known as neutrophil immobilizing factors (NIF)(56).

Interest in Calprotectin as a marker for inflammation in the gut followed the realization that ^{111}Indium-labelled granulocyte scans could be used to both visualize and quantitate the acute inflammation in the gut of patients with inflammatory bowel disease (37-40). These findings led to the idea that an increased **influx of granulocytes** into the intestinal mucosa in conditions of inflammation might give **increased levels of proteins** from such cells in feces. Other studies have demonstrated that eosinophilic granulocytes are the main cellular source of Calprotectin in the normal gut mucosa (57). However, relatively high levels of Calprotectin are found in the stools of normal individuals; about six times the plasma levels (which are about 0. 5mg/L). This is compatible with view suggesting that in normal individuals, most of the circulating neutrophils migrate through the mucosal membrane of the gut wall and thereby terminate their life-span (58). Subsequent lysis within the gut lumen and release of cytosolic Calprotectin thereby accounts for the median fecal levels of 2.0mg/L seen **in healthy controls** (1,2,28,32,33,35). The diagnostic use of fecal Calprotectin in a broad spectrum of intestinal diseases has been studied by a number of groups with remarkable agreement between the results (1,2).

It is of special importance that the concentration of Calprotectin in feces is closely correlated with the number of PMNs migrating into the gut lumen, and that it can be detected reliably even in small (less than one gram) random stool samples (1,2,35). Furthermore, organic diseases of the bowel give a strong Calprotectin signal, i.e., elevations

are regularly five to several thousand times higher than the upper reference of healthy individuals, thus being a reliable indicator of intestinal inflammation.

2.1.Calprotectin in IBD and IBS

It is almost possible to extrapolate all the findings obtained with the white cell fecal excretion technique to the Clprotectin method (37-40). Both techniques correlate with histopathological assessment of disease activity in UC, and there is a very good correlation between the 4-day fecal excretion of white cells and fecal Calprotectin concentrations (1,48); a correlation which is maintained when single stool Calprotectin concentrations are used as opposed to 1- or 4-day collections.

The fecal Calprotectin concentration has a narrow normal range with an upper limit of 10mg/L. As with the white cells, fecal Calprotectin has potential as a screening procedure to **differentiate between patients with IBD and IBS.** In addition, it may be useful for documenting a fall in intestinal inflammation in response to successful treatment of the IBD. Because Calprotectin concentration is rarely within the normal range in patients with IBD despite full clinical remission, it is therefore a highly sensitive method for detecting such patients irrespective of disease activity (1).

Since the method is so much simpler than the white cell technique, requiring only a single stool sample, extraction and an ELISA, it has a great potential as a screening tool to **distinguish between patients with IBD and IBS** in an outpatient setting. One study in over 200 patients showed that a cut-off of 30mg/L had a 100% sensitivity and 94% specificity for this purpose (1). Another recent study showed that this was also the case when over 600 unselected consecutive patients were studied. Indeed, a patient presenting with positive Rome criteria (i.e., IBS) and a normal fecal Clprotectin has virtually no chance of having IBD (2).

As a result of these studies, it is now possible to investigate such patients by radiology or

colonoscopy with considerable cost saving implications. It seems likely that the assay of fecal calprotectin will become an integral part of the assessment of **therapeutic efficacy** of the acute inflammation in future treatment trials in patients with IBD (1,2,,32,33,35). Apart from screening and assessing response to treatment, the fecal Calprotectin has a further major advantage over the white cell labeling technique in **predicting the relapse of IBD**. It has been shown that, in patients with clinically quiescent IBD (UC and CD), fecal Calprotectin values above 50mg/L may be used to predict clinical relapse of disease within a few months with over 80% sensitivity (59). Symptoms of IBD often appear to be the direct consequence of the inflammatory process itself and often vary dependent upon the location of the inflammation. Most patients with quiescent IBD have low-grade inflammation (37-40), and it is possible that symptomatic relapse occurs only when the inflammatory process reaches a critical intensity. Furthermore, as inflammation is a continuous process it may be that direct assessment of the level of inflammatory activity may provide a quantitative pre-symptomatic measure of imminent clinical relapse of the disease (1,2,59).

Taken together, IBD (UC and CD) may appear from early childhood to late adulthood and the diagnosis is often delayed due to vague and non-specific symptoms or reluctance to perform endoscopy and biopsy. Based on these convincing clinical data, CALPRO AS (Oslo, Norway) developed the **CALPROLAB™ Calprotectin ELISA (ALP)** test for detection of Calprotectin in stool samples. The test is intended to contribute to an earlier diagnosis of IBD, since the test is usually positive in active IBD, in contrast to functional disorders like IBS, which does not give increased fecal out of Calprotectin. As well known, however, patients with organic and functional intestinal disorders frequently have similar symptoms, and clinical examination alone is not sufficient to give a specific diagnosis. As discussed above, alternative diagnostic procedures are complex, expensive and may expose the patient to pain and other risks. A test for **fecal Calprotectin** is a simple, non-invasive, inexpensive and objective method that can help selecting the patients for additional examination, e.g. colonoscopy and biopsies.

3.BIOHIT Calprotectin ELISA (CALPROLAB™ Calprotectin ELISA ALP)

The BIOHIT™ Calprotectin ELISA is a quantitative method for determination of Calprotectin concentration in stool samples. The test is intended to 1) aid in identifying organic disease of the small intestine, large bowel or the stomach, to 2) determine the disease activity, and 3) to monitor the response to treatment of patients with UC or CD. Calprotectin determination itself is not limited to stool samples, but it has been validated for other body fluids, secretions and excretions, including serum, plasma, crevicular fluid and urine (35). This test has been validated for stool and plasma samples.

3.1.Test principle

The BIOHIT™ Calprotectin ELISA is based upon preparation of an extract of feces using our patented Fecal Extraction Buffer (35). The level of Calprotectin is determined by testing the extract in an enzyme-linked immunoassay (ELISA) specific for Calprotectin. In the ELISA, samples and standards are incubated in separate micro-titer wells coated with monoclonal antibodies which bind Calprotectin. After incubation and washing of the wells, bound Calprotectin is allowed to react with enzyme-labelled, immunoaffinity-purified Calprotectin-specific antibodies. After this reaction, the amount of enzyme bound in the microtiter wells is proportional to the amount of Calprotectin in the sample or standard, which is determined by incubation with a substrate for the enzyme giving a colored product. The color intensity is determined by absorbance using an ELISA plate reader, and is proportional with the concentration of Calprotectin in the standards and in the samples. The assay is calibrated using Calprotectin purified from leukocyte extract.

4.STUDY DESIGN

The present clinical trial is testing the performance of BIOHIT™ Calprotectin ELISA in detection of fecal Calprotectin levels in patients with different intestinal disorders (both organic and functional), using colonoscopy and biopsies as the gold standard. The primary study endpoints include 1) the assessment of cut-off values for fecal Calprotectin concentration that enables the most accurate distinction (SE/SP balance) between IBS and

IBD, as well as 2) to calculate the performance indicators for the Calprotectin test in diagnosing (with these cut-off values) these two study endpoints (IBS, IBD), and 3) to establish the value of Calprotectin ELISA test in screening of the patients with non-specific gastro-intestinal symptoms to detect incident cases of IBD and IBS.

4.1.Aims of the study

The most important goal of this study is to assess the overall performance of BIOHIT™ Calprotectin ELISA in differential diagnosis of IBS and IBD as well as in the follow-up of treatment efficacy and prediction of relapse in the latter. This goal is reached through the following specific aims.

1. To establish the cut-off values for BIOHIT Calprotectin ELISA test in patients with IBD (UC & CD) and IBS that most accurately distinguish between these two conditions, by calculating the AUC (area under ROC curve), showing the best sensitivity/specificity balance.

2. Sensitivity (SE), specificity (SP), negative predictive value (NPV), positive predictive value (PPV) and area under ROC curve (AUC) for the established cut-off values of BIOHIT Calprotectin ELISA in diagnosis of IBD and IBS in the screening setting.

3. By monitoring the IBD patients with prevalent ulcerative colitis or Crohn's disease to assess the dynamic changes in Calprotectin levels as related to i) the activity of the disease (CDAI, UCDAI), and ii) efficacy of the current treatment.

4.2.Patients

This clinical study is conducted in collaboration between Biohit Oyj (Helsinki, Finland) and Hospital X (City Y, Country Z)(hereafter called "the Partners"). The study is performed exclusively in Hospital X, supervised by a steering committee consisting of members from

both Partners. The study setting includes two separate parts, **a clinical series** and a **screening trial**.

4.2.1.Clinical series

The clinical series includes patients with prevalent IBD, representing both UC and CD as well as those who have been clinically confirmed IBS.

4.2.1.1.IBD patients

The two most important entities included in IBD are ulcerative colitis and Crohn's disease. Both are known to be associated with elevated fecal concentration of Calprotectin, and included in the present study with approximately equal numbers and with similar gender distribution.

Crohn's disease: A diagnosis of Crohn's disease (CD) is made based on standard criteria from a combination of radiological, endoscopic, and histopathological investigations as well as the clinical history (60-62). The eligible patients shall have an active CD (defined as a Harvey-Bradshaw score of 5 or more, which corresponds to a modified **Crohn's disease activity index (CDAI)** score of over 150 (61,62), or an <4-point increase in the Harvey-Bradshaw disease activity index from baseline in patients who have undergone intestinal surgery. Patients with serious coexisting cardiovascular, pulmonary, hepatic, renal, or musculoskeletal disease, severe immune deficiency, malignancy, misusing alcohol, or receiving non-steroidal anti-inflammatory drugs should be excluded from study, because these conditions may be associated with intestinal inflammation in their own right. Also excluded should be pregnant women or those at risk of pregnancy and patients with complicated (fistulae, abscesses, or symptomatic intestinal strictures requiring surgery) CD.

Ulcerative colitis: Ulcerative colitis (UC) can be considered as an immune-mediated disorder that develops in genetically predisposed individuals because of dysregulated immune responses against intraluminal antigens (63). Diagnosis of UC is based on medical

history and clinical valuation and then confirmed by laboratory, radiologic, endoscopic, histologic and serological findings. Before making the diagnosis, infective, ischemic and irradiation causes of colitis must be excluded. Lennard-Jones (64), suggested the following criteria for the diagnosis of UC: Continuous mucosal inflammation without granulomata, always involving the rectum and extending continuously in a various degree to a part or the whole colon. There are several definitions or criteria used to classify patients with UC (65,66). Patients with UC can be classified according to disease extend, disease severity, age of onset, extra-intestinal manifestations and genetic markers (67). During the recent years, the use of **UCDAI (Ulcerative Colitis disease activity index)** has gained increasing popularity as a standardized method of classifying disease activity (63,68).

To be eligible, the diagnosis of UC should be based on the presence of i) bloody diarrhoea with negative stool cultures and ii) endoscopic evidence of diffuse, continuous mucosal inflammation involving the rectum and extending to a point more proximal in the colon. Inclusion criteria: 1) male and female patients aged more than 18 years; 2) diagnosis of UC established by previous colonoscopy, with consistent histology and clinical course; 3) UC involving at least the recto-sigmoid region; 4) activity confirmed by colonoscopy at the beginning of the study; 5) Mild-to-moderate relapsing UC, defined as a UCDAI score ranging from three to eight; 6) Symptoms (relapsing episodes) for less than 4 weeks before study entry; 7) A minimum endoscopic score of three on the UCDAI at screening (mucosal appearance); 8) Use of oral 5-ASA at least 4 weeks before study entry at a stable dose (mesalazine at least 1.6 g/day or balsalazide at least 4.5 g/day) and/or use of azathioprine (at least 1.5 mg/kg / day) or 6-mercaptopurine (at least 1 mg/kg/day) at least 3 months before study entry at a stable dose.

4.2.1.2.IBS patients

These subjects are being enrolled among the consecutive patients referred to examinations at the outpatient department of Gastroenterology, Hospital X. Eligible are patients with i) previously established IBS (prevalent cases) as well as those with ii) **clinical symptoms**

suggestive of IBS that has not responded to therapy instituted by the primary care physicians, being of sufficient severity for further consultation and investigation to exclude organic pathology (incident cases).

These patients are identified by fulfilling the symptom-based diagnostic criteria that have been developed to identify patients with IBS (3,4). Many symptoms are quite non-specific and common to both IBD and IBS, including abdominal pain, bloating, excessive flatus, and altered bowel habit, whereas other clinical features such as a predominance of diarrhea and rectal bleeding will increase the likelihood of inflammatory disease (3,4). The consensus definition and criteria were developed (**the Rome II criteria**), for IBS and other functional gastrointestinal disorders are regarded helpful (15,16). The stepwise assessment of the patients with suspected IBS, based on positive Rome criteria (6,7) includes a full blood count, erythrocyte sedimentation rate (ESR)/C-reactive protein (CRP), serum chemistry, thyroid function tests, and stool examination for parasites and ova to exclude organic diseases. Indeed, the main usage of Rome II criteria is to standardize the characteristics of the patient groups enrolled in clinical studies (1,2).

While sorting out the true IBS patients, it is advisable to exclude all those who are referred for investigation due to esophageal reflux, symptoms clearly associated with gastro-esophageal pathology, or functional or isolated dyspepsia (defined as a combination of symptoms related to food with localization in the upper abdomen, including epigastric pain or discomfort, bloating, belching, and nausea without symptoms of colicky abdominal pain or alteration in bowel habit at time of referral). However, patients with dyspepsia in addition to intestinal symptoms should be enrolled into the study. Patients with a previously known diagnosis of IBD, colorectal carcinoma, and serious cardiopulmonary, hepatic, renal, neurologic, and psychiatric disease should also be excluded

The final diagnosis of the irritable bowel syndrome (IBS) should be made on the basis of no significant hematological or biochemical abnormality, normal radiology

and/or normal endoscopy with no specific biopsy-confirmed pathology.

4.2.2.Screening trial

Apart from a clinical series consisting of patients with prevalent IBD and clinically confirmed (prevalent or incident) IBS, the present study setting also involves a screening component where BIOHIT Colprotectin ELISA is used to examine the subjects attending the clinic for examination due to non-specific gastro-intestinal symptoms.

4.2.2.1.Subjects with non-specific gastro-intestinal symptoms

The screening setting involves systematic examination of fecal Calprotectin levels using Biohit Calprotectin ELISA test in all subjects referred for further examination due to non-specific abdominal symptoms. Automatically excluded are all patients with previously diagnosed IBD or IBS. The eligible patients are **adults** aging 18 years or older, in whom IBD or IBS has not been previously diagnosed, i.e., who represent potential **incident cases of either IBD or IBS.** Typically, these patients complain different symptoms that are quite non-specific and can be common to both IBD and IBS, including abdominal pain, bloating, excessive flatus, and altered bowel habit. Some other clinical features, e.g. a predominance of diarrhea and rectal bleeding are more suggestive of IBD (3,4).

Patient enrollment in the screening trial is taking place **in a single step**. The potentially eligible patients are identified (by the clinical staff) among the endoscopy-referral adults, who have **not been previously diagnosed** as having IBD or IBS. At this stage, every patient will be asked for their willingness to take part in the study and sign a written consent to participate. The following patients should be considered non-eligible: 1) those who refuse to sign written consent; 2) those who refuse to deliver fecal sample; 3) those who refuse colonoscopy (the cold standard for diagnosis).

The total cohort to be screened by BIOHIT Calprotectin ELISA is estimated to be at least **400 subjects** (adults, both genders), to reach a cohort of 50 patients enriched with enough

IBS and IBD cases that are needed to calculate the performance indicators for the test.

4.2.3.Patient preparation

Proper conduction of and reliable results from the BIOHIT™ Calprotectin ELISA test does not necessitate any particular preparatory measures of the patient. The patients can take the stool sample at home, following the instructions given to each study subject at the enrolment.

4.3.Methods

In this study, all patients provide a single stool sample for fecal calprotectin assay. In addition, pending on the discretion of the clinic (Hospital X), the patients can be subjected to other examinations used to confirm/exclude the diagnosis of IBD. Such examinations might include 1) the lactulose/L-rhamnose small intestinal permeability test, 2) ^{111}Indium-labellel leucocyte counting, and 3) additional blood tests used in routine diagnoses and follow-up. These tests are not mandatory to reach the objectives of the present study protocol, however, designed to assess the clinical performance of BIOHIT™ Calprotectin ELISA test, and because of this, these are not described in this Section (4.3. Methods).

4.3.1.Sample collection for BIOHIT™ Calprotectin ELISA

Since Calprotectin is very stable in stools, patients can collect small fecal samples at home. The patients are instructed to collect 1–5g (approximately one teaspoonful), place it in a suitable clean container and deliver it to the laboratory as soon as possible but within four days. When put in a container approved for transport, it can be sent by ordinary mail, i.e., no refrigeration is needed. Exposure to temperatures above 30°C should be avoided.

Samples can also be stored frozen, at -20°C or lower, until delivery or mailing. Frozen samples must be thawed and equilibrated to room temperature before extraction and testing. Avoid freezing and thawing more than once. Note that freezing fecal samples can result in increased Calprotectin levels, most likely due to release from granulocytes.

4.3.2.Sample processing

Note: Before starting the extraction, the stool sample should be homogenized well using for example a spatula, before the small amount for extraction is taken out. For extraction, two optional methods are applicable from the manufacturer, but other validated methods and devices can be used as well.

4.3.2.1. Extraction using the fecal extraction device

Use the small spatula provided to fill the beaker. Alternatively, an inoculation loop can be used. The beaker will take about 100 mg sample. Grains and seeds should be avoided. Also avoid trapping air bubbles. Wipe off excess sample and push the cap tightly into the cone of the extraction tube lower end. Fill the extraction tube with 4.9 ml extraction buffer and attach the top cap. Vortex the assembled device vigorously for about three minutes to disrupt large particles. If necessary, further vortexing may be performed so that only solid particles remain. Allow particles to settle by leaving the tube on the bench for a couple of minutes and pipette carefully from the top of the tube. No centrifugation is necessary, but a short centrifugation can be performed if a particle-free solution is required. The extract, which represents a 1:50 dilution (weight:volume) of the stool sample, is now ready for dilution and testing. For storage, transfer about 0.5 ml to a new tube. Extracts can be stored at 2–8°C for at least five days or frozen below -20°C for up to six months (69).

4.3.2.2. Extraction using the weighing method without extraction device

If the extraction device is not feasible, this method can be used equally well. First, weigh (tare) an empty screw cap tube with an inoculation loop. Take out approx. 100 mg (between 40 and 120 mg) feces by means of the inoculation loop and place it into the screw cap tube. Avoid taking out solid, undigested material like fibres and seeds. Weigh tube and loop with feces which will give the net feces weight. Break or cut off the top half of the loop handle and leave the bottom part inside the tube. Add extraction buffer to a weight: volume ratio 1:50, for instance 4.9 ml buffer to 100 mg feces. Close the tube. Mix vigorously for 30 seconds by means of a vortex mixer.

Then continue the mixing on a shaker (at approx. 1000 rpm) for 30±5 minutes with the loop inside the tube as an agitator. Allow a couple of minutes on the bench for particles to settle and pipette carefully from the top of the tube. No centrifugation is necessary, but a short centrifugation can be performed if a particle-free solution is required. The extract, which represents a 1:50 dilution (weight:volume) of the stool sample, is now ready for dilution and testing. For storage, transfer about 0.5 ml to a new tube. Extracts can be stored at 2–8°C for at least five days or frozen below -20°C for up to six months.

4.3.3.ELISA testing

Before performing the assay, read the test protocol carefully, because the result reliability depends on strict adherence to the test protocol as described. Prior to initiating the ELISA assay, a Plate Layout for all standards, samples and controls should be carefully established, using for example the sheet supplied in the kit. Select the required number of microtiter strips. Unused strips should be removed from the frame and immediately re-sealed in the aluminium foil pouch along with the desiccant supplied. Store at 2–8°C.

A 1:100 dilution of feces extracts is recommended. This dilution will give sample results between 25 mg/kg (LoQ) and 2500 mg/kg in feces. Extracts with higher Calprotectin values can be diluted more (>1:100) and re-tested if a value is required. Extracts with low Calprotectin values can be diluted less (1:50). The adjusted dilution factor must be taken into account when converting from ng/ml to mg/kg (see Section 4.3.4.)

The test should proceed as follows: 1) Dilute feces extract samples 1:100 (e.g. 10 µl sample + 990 µl Sample Dilution Buffer) and mix well by vortexing. 2) Add 100 µl of each standard, control and diluted sample in duplicate wells; see recommended plate layout in Instruction leaflet. 3) Cover the plate with a sealing foil and incubate at room temperature for 40±5 min on a horizontal plate shaker (approximately 500 – 700 rpm). 4) At the end of the incubation time, remove the liquid and wash the wells by adding 300 µl Washing Solution to each well. Remove as much liquid as possible and repeat until a total of three washings

have been performed. If a plate washer is used, check that all aspirating and filling probes are unblocked to ensure efficient washing of all wells. After the final wash, invert the plate and tap the well openings thoroughly on absorbent tissue to remove any remaining Washing Solution. 5) Mix the content of the Enzyme Conjugate vial gently prior to use (do not shake). Add 100 µl of conjugate to each well, preferably using a repetitive or multichannel pipette. 6) Cover the plate with sealing foil and incubate at room temperature for 40±5 min on a horizontal plate shaker (approximately 500 – 700 rpm). 7) Repeat the washing steps as described above, three times with 300 µL Washing Solution per well. 8) Add 100 µl Enzyme Substrate Solution to each well, preferably using a repetitive or multichannel pipette. 9) Incubate the plate at room temperature (without shaking) for 20 – 30 minutes, protected from light. 10) Optional: Add 100 µL 1M NaOH stop solution to each well if a fixed incubation period is required. Finally 11) read the optical density (OD) values at 405 nm using an ELISA reader.

4.3.3.1.Interpretation of the BIOHIT™ Calprotectin ELISA results

A new standard curve must be included in each run. Similarly, the positive controls should be included in each run. The value of the controls should be within the limits printed on the vial labels. As a guide, the OD value of Standard F (500 ng/ml) should be ≥1.8 and the OD value of Standard A (0 ng/ml) should be ≤0.25.

Calculation of Calprotectin concentration in the fecal samples should proceed as follows: 1) Calculate the mean OD values of all duplicate wells (standards and samples). 2) Plot the value of each standard concentration (ng/ml) on the x axis against its mean OD value on the y axis to obtain a standard curve. A 4-parameter curve fit function is recommended. If a logarithmic x axis is required, a value of 0.001 ng/ml must be used for standard A (0 ng/mL). 3) Use the calibration curve to determine the Calprotectin concentration in the diluted samples (ng/ml) based on their OD values. 4) Multiply the Calprotectin concentration (ng/ml) in the diluted fecal extracts by 5 in order to convert to mg/kg Calprotectin in the original stool sample.

This factor corrects for the total dilution of 1:5000 (1:50 during the extraction procedure and the following 1:100 dilution of the extracts) and converts the value from ng/ml to mg/kg. For example, if a diluted extract sample has a value of 100 ng/ml, the concentration in the original stool specimen is 100x5=500 mg/kg. Note: If the extracts have been diluted more (or less) than the recommended 1:100, the additional dilution factor must be entered into the calculation (69).

4.3.4.Colonoscopy

In the clinical setting, all IBD- and IBS patients examined with the BIOHIT Calprotectin ELISA test will be subjected to **colonoscopy**, providing the histological confirmation to be used as the gold standard in calculating the performance indicators for the test. In the case of completely normal colonoscopy, however, biopsies will not be taken, and in such a case, normal colonoscopy is used as the gold standard indicating a negative result regarding the study endpoint (IBD), but confirmatory to IBS endpoint.

In the screening setting where subjects with non-specific gastro-intestinal symptoms are being examined with the Calprotectin ELISA, only the patients testing clearly positive will be subjected to colonoscopy and biopsy confirmation. In addition, a random sample of 2% of Calprotectin ELISA-negative subjects will be invited for colonoscopy, to enable the statistical correction for verification bias.

The colonoscopy will be done according to the usual practice, and in each patient, a record will be made of all findings as a regular colonoscopy report. In classifying the colonoscopy findings for colorectal tumors, the **Paris classification** should be used. According to this classification, all superficial colorectal lesions (=lesions with no invasion) are called type 0 neoplastic lesions, with different variants. These include polypoid (Ip and Is), non-polypoid (IIa, IIb, and IIc), non-polypoid and excavated (III)(p=pedunculated; s=sessile; IIa=slightly elevated; IIb=flat; IIc=slightly depressed; III=excavated or ulcer).

In this study, however, the main focus is to confirm or exclude the diagnosis of IBD, and its correct classification as either UC or CD. In most cases, but not always, it is possible to make distinction between CD and UC on the basis of their colonoscopic appearance, because the pattern of these two forms of IBD in the intestinal tract is quite distinct. UC tends to be continuous throughout the inflamed areas. In many cases, UC begins in the rectum or sigmoid colon, and spreads up through the colon as the disease progresses. In CD, however, the inflammation may occur in patches in 1 or more organs in the digestive system. For instance, a diseased section of the colon may appear between two healthy sections.

In a colon affected by CD, the intestinal wall may be thickened and, because of the intermittent pattern of diseased and healthy tissue, may have a "cobblestone" appearance. In UC, the colon wall is not that thick and shows continuous inflammation with no patches of healthy tissue within the diseased section. Granulomas are composed of inflammatory (epitheloid) cells that become lumped together to form a lesion. Granulomas are present in CD, but practically lacking in UC lesions. Therefore, when they are found in tissue samples taken from an inflamed section, they are an excellent indicator that CD is the correct diagnosis. In UC, the mucous membrane of the large intestine is ulcerated. These ulcers do not extend beyond this inner lining, wherever, in contrast to CD, in which the ulceration is deeper and may extend into all the layers of the bowel wall (full-thickness lesion).

4.3.5.Biopsy protocols

In the cases with no objective findings in colonoscopy, no biopsies will be taken. In positive colonoscopy, however, all polyps and tumors will be biopsies (or removed if small enough), following the current practice. Most importantly, biopsy confirmation will be made for all inflammatory lesions implicating IBD of either type. The biopsy sites as well as accurate description of the biopsied lesions (IBD, polyp, carcinoma), should be given in the colonoscopy report. The biopsy site should be given at the level of the exact anatomic location (caecum, ascendens, transversum, descendens, sigma, recto-sigmoid, rectum),

although the final evaluation will be done collectively only for the dichotomized variable (IBD or non-IBD).

4.3.5.1.Preparation of the microscopy slides

The biopsies from formalin bottles/tubes are embedded in paraffin using the routine procedures at the Pathology Laboratory of Hospital X. The blocks are cut into 4-µ-sections, and stained with hematoxylin eosin (HE) and PAS and Ab-PAS for routine diagnosis.

4.3.5.2.Interpretation of the biopsies

All colonoscopy biopsies are examined by the expert pathologists at Hospital X, among the daily routine samples. While the diagnosis of IBD is confirmed using the commonly accepted histological criteria, the disease should be classified as either UC or CD. Normal mucosa without evidence on IBD, associated with the clinical symptoms consistent with irritable bowel syndrome can be regarded as a positive indicator of IBS in this study.

Although of secondary importance for the present study, all neoplastic lesions should be accurately reported using the standard WHO classification of colorectal neoplasia. In addition to their size, all polyps must be classified as hyperplastic polyps or adenomas. All adenomas are further classified according to their histological growth pattern as tubular, tubule-villous, villous and serrate adenomas. In the new WHO classification, several sub-categories of the latter are used, including micro-vacuolar hyperplastic polyp, goblet cell rich hyperplastic polyp, sessile serrate adenoma, traditional serrate adenoma. A composite entity know as advanced neoplasia (AN) contains adenocarcinoma, adenomas 10mm or greater in diameter, adenomas with high-grade dysplasia, and those with ≥25% of villous histology. Because of this, an estimate on the grade of dysplasia should be given of all diagnosed adenomas.

4.3.6.Statistical analyses

All statistical analyses will be performed using the SPSS 22.0.0.1. for Windows (IBM, NY,

USA) and STATA/SE 13.1 software (STATA Corp., Texas, USA). The descriptive statistics will be calculated according to routine procedures. BIOHIT Calprotectin ELISA is a quantitative test, and the cut-off values making distinction between IBD and IBS can be derived using the AUC (Area under ROC curve) analysis, and picking up the values giving the best SE/SP balance. These established cut-off values will be tested in calculating the performance indicators (sensitivity, specificity, positive predictive value, PPV, negative predictive value, NPV and their 95%CI) of the test, separately for both study endpoints (IBD and IBS), confirmed by colonoscopic biopsies. The STATA/SE software and the *diagti* algorithm introduced by Seed et al. (2001) will be used, that also calculates the AUC for each study endpoint (70). The same approach will be used to confirm the diagnosis of IBD and IBS in the screening setting. In the screening setting, these crude (non-corrected) performance indicators must be subjected to correction for verification bias, which is done by a commonly used method described by Reichenheim et al. (71), and of which an algorithm (*validesi*) is available is STATA. In this procedure, the 95%CIs are derived by the parametric bootstrap method, with the simulation of 10,000 replications.

5.ETHICAL ISSUES

The study plan necessitates a review by the institutional review board (IRB, Ethical Committee) before permission to start. The study design and its execution do not involve any significant ethical issues except those in other clinical studies of similar type. The study is conducted in accordance with the Declaration of Helsinki.

Study subjects are enrolled among consecutive outpatients with previously diagnosed IBD or IBS (i.e., prevalent cases) at the outpatient Department of Endoscopy, Hospital X, to complete their routine clinic al workup. Also eligible are patients attending the hospital due clinical symptoms suggestive of IBS that has not responded to therapy instituted within the primary health care, but being of sufficient severity for further consultation and investigation to exclude organic pathology (incident IBS cases). The third arm includes Calprotectin testing of the subjects referred for further examination due to non-specific

abdominal symptoms. Among these individuals, potential incident cases of both IBD and IBS can be anticipated.

Thus, all study subjects represent regular outpatients who have clinically established IBD or IBS, or those who have symptoms compatible with IBS, and who are scheduled for confirmatory clinical examinations. The same applied to patients referred for additional examinations due to non-specific gastrointestinal symptoms. Addition of BIOHIT Calprotectin ELISA test in stool samples among the other clinical diagnostic tests in these individuals is clinically justified and bears no ethical issues. All patients must sign the informed consent for their participation. When all results are available, the patients will be informed about the results, following the usual hospital practices. This includes an explanation of the meaning of these test results, and the appropriate measures for further conduct, including the institution of new or adjustment of the existing therapies.

6.TIME TABLE

The necessary preparations for the study execution at Hospital X will start immediately when the hospital has reached an agreement with Biohit Oyj to conduct the study. Given that the subjects in the study will be enrolled among consecutive patients with clinically diagnosed IBD or IBS, or among those reporting symptoms suggestive of these conditions, attending the Outpatient Department of Endoscopy, Hospital X, it is estimated that a cohort of 100 IBD patents and 100 IBS patients will be needed to calculate the reliable performance indicators for the BIOHIT™ Calprotectin ELISA test. Of interest is the power of the test in making distinction between IBD and IBS. By monitoring the IBD patients with prevalent UC or CD, it is also possible to assess the dynamic changes in Calprotectin levels as related to i) the activity of the disease (CDAI, UCDAI), and ii) efficacy of the current treatment. In the screening setting of hospital-referred individuals with non-specific gastrointestinal symptoms, we evaluate the power of BIOHIT™ Calprotectin ELISA test in diagnosis of the patients with incident IBD or IBS. To yield enough cases of IBD and IBS, we estimate that at least **400 subjects** (adults, both genders) need to be screened.

Because of the test characteristics (quick test), the laboratory arm of this study is completed in parallel with the patient enrollment and performed endoscopies. There will be some delay (of days) due to the biopsy examination at the Department of Pathology, until the results of each subjects are available. Accordingly, the full database of the patients will be ready for statistical analysis almost on real-time after completion of the enrollment and Calprotectin testing.

7.PROJECTED COSTS TO BE COVERED by Biohit Oyj

This section will be completed as soon as an agreement has been reached in the cost estimates for the (eventually modified) study protocol.

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