Levels of fecal S100A12 in normal children and children with Inflammatory Bowel disease

Degree Project in Medicine
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INTRODUCTION

Background
Inflammatory bowel disease (IBD) is the general name for a group of inflammatory gut conditions. The two most common types of IBD are Crohn’s disease (CD) and Ulcerative colitis (UC): some individuals with IBD are labeled as IBD Unclassified (IBDU).

CD and UC share several features; they are both chronic diseases characterized with inflammatory activity in the gastrointestinal wall and both can manifest with similar symptoms such as abdominal pain, diarrhea, bloody stools and weight loss. Their disease activity varies over time resulting in periods of disease remission interspersed with periods of disease relapses. As these conditions are incurable, they often require life-long treatments and regular follow-ups.

CD can be distinguished from UC by a number of particular features. In CD inflammation is most commonly located in the terminal ileum and cecum, but can be found in any part of the intestine, from mouth to rectum. On the other hand in UC the inflammation tends to start at the rectum and progresses continuously along the large bowel, only engaging the colon. Skip lesions, granulomas and transmural inflammation are particular features in CD, while the inflammation in UC is superficial. (1)

IBDU is the term used when the results of standard tests can not clearly differentiate between CD and UC. Patients with IBDU are often reclassified over time, most commonly to UC. (2,3)

IBD in children
IBD can develop at any age, but the most common of diagnosis is between 15 – 35 years. About 25% of individuals with IBD are diagnosed in childhood; before 20 years old. In childhood, the majority of patients present during the second decade of life, especially around the time of puberty. In children CD is about four times more common than UC and pre-pubertal boys appear to have a higher risk of developing CD. (3-6) Childhood onset IBD tends to progress early and more rapidly to extensive disease than in adults. (7) Relapses are also more frequent. There are histological differences in children compared to adults, for example gastritis a common feature in children with both UC and CD. Other important features unique to IBD in children are growth failure and delayed puberty.

The features of CD and UC lead to some variations in symptoms. Overall, the most common symptom in children with IBD is abdominal pain (4,8), whereas it in adults is rectal bleeding (UC) and diarrhea (CD). Extra intestinal symptoms appear in about 25-30 % of IBD patients at some stage of their disease course. (6)

Classic symptoms in children with CD are abdominal pain, weight loss and diarrhea. But many children present atypically and a range of other presenting features can be seen, sometimes in isolation. These include fever, nausea, anorexia, joint pain, erythema nodosum, delayed puberty, linear growth failure and depression. (9) Clinical findings include anemia, clubbing, perianal skin tags, fistula, abscess or aphthoid ulceration. (6)
The most common symptoms in children with UC are blood loss (hematochezia), diarrhea and abdominal pain. Weight loss and extra intestinal symptoms can be found but are less common than in CD. Clinical findings are more discrete than in CD, however lower abdominal discomfort and anemia can be found. Severe pain can be due to colonic dilatation (toxic megacolon). (9)

The variety of symptoms, especially when atypical, can cause diagnostic delays. Children with CD tend to have symptoms for a longer period of time before diagnosis, compared to children with UC (2). It is very important to ensure early diagnosis in children with IBD to avoid disease-related complications such as growth failure, delayed puberty, surgical interventions and adverse psychological impacts. (10,11) Diagnostic delay for more than six months is a risk factor for disease extension in children with UC. (12) Children with CD are more likely to get permanent growth failure (shortened stature) than children with UC. Growth failure can be due to several factors such as malnutrition, ongoing inflammation and medications (corticosteroids).

**Etiology and pathogenesis of IBD**

The etiology of IBD is still not completely understood, but it is likely that it is complex and multifactorial. Genetic predisposition, environmental factors, including the intestinal micro flora, and dysfunction of the immune response are interacting factors in the pathogenesis.

Twin studies have shown that the risk of developing CD is 50% if the other twin suffers from the disease, the same number is 15% for UC (your book about IBD). Several genes are associated with IBD, some are similar for CD and UC and some differs (37). Three different polymorphism of the NOD2 gene (CARD15), the most well studied gene in IBD, have been connected to CD (but not to UC) in Caucasians. Mutation of this gene is seen in about 40 % of CD patients, but also in 15% of healthy people. (39) A certain polymorphism, 3020insC, has been shown more common in childhood-onset CD. (13) There is disagreement in the literature whether these mutations cause a loss of function or a gain of function. NOD2 is coding for a cytosolic pattern recognition receptor (PRR) which is a receptor mainly expressed in mononuclear phagocytes, monocytes, epithelial- and panetcells. One function of PRR is to differ between foreign material and endogenous. NOD2 recognizes muramyl dipeptide (MDP) on bacterial cell walls and resulting in activation of the Nuclear Factor (NF)-κβ pathway. NF-κβ stimulates TNFα, IL-1β, IL-6 expression and other pro- inflammatory cytokines that attracts and activate effector cells of the innate and adaptive immune system. Among the effects of TNFα stimulation is expression of members of the S100 family. (14,15) M.G. Netea showed a loss of function of polymorphism 3020insC NOD2 in humans, via a decreased response to MDP by NOD2 resulting in low secretion of IL-1β. (16) The loss of function is suggested to be more like a loss of negative regulation of toll-like receptor (TLR) response to normal intestinal bacteria resulting in the increased inflammatory activity seen in IBD. Other studies have reported that the polymorphism Nod22939iC in mice causes an increase of NF-κB activity and IL-1β secretion, suggesting this might happen in the pathogenesis of IBD. (17,18)

Recent GWA (whole genome association) studies have resulted in new loci linked to IBD. Examples of new susceptibility genes for CD are ATGL 16 and IL23R (13), whose function is to
regulate the autophagy of intracellular bacteria, which is important for protecting from invading microorganisms and degrading damaged intracellular components. Mutations in these genes may lead to dysfunction of acute immune responses and acute reaction deficiency resulting in that specific microorganisms are not eradicated from the intestinal mucosa properly, contributing to the chronic inflammation and granuloma formation seen in CD. (32, 36, 39, 40) One particular variant/polymorphism of IL23R is linked with both CD and UC. (40) IL-23 is a cytokine which can stimulate a special T helper cell (Th17) to produce the pro-inflammatory IL-17. Activated Th17 mediates chronic inflammation in IBD. (13)

However, only a part of the pathogenesis can be explained by genetics, and genetic tests don’t have a role in current diagnostic processes. Among the environmental factors that are associated with IBD are smoking habits (smoking is a risk factor for CD, but is protective for UC) and appendectomy. A number of other possible contributing factors have been investigated such as low physical activity, stress, the diet, childhood gastrointestinal infections, and changes in the microbial flora of the intestine. (35)

Many types of bacteria have been studied in the context of IBD, but a single bacterium causing the immune response in IBD has not been found despite comprehensive efforts. One theory now is that the normal or an altered intestinal flora may trigger a dysfunctional immune response in the host. The dysfunction includes different steps; recognition of bacteria, immune response and autophagy. In this hypothesis, the impaired innate immune system doesn’t manage to handle the intestinal flora via an acute inflammatory reaction, resulting in some bacteria and components of bacteria left in the mucosa causing chronic inflammation. (39)

**The inflammatory activity**

Recent research has advanced our understanding of the inflammatory processes present in IBD. Traditionally the chronic inflammation was thought to be caused by hyperactive immune response, but the theory now is that an impaired acute innate immune response to microbial agents causes the persistent inflammation. (19)

Epithelial cells are important for the host’s defense against micro bacteria. And epithelial damage of the intestinal wall is central in the pathogenesis of IBD. This may manifest as atrophy of villus atrophy, ulceration, erosions or hyperplasia. It is not clear if inflammation causes damage to intestinal epithelia or if impaired epithelial cells trigger an inflammatory process.

When microorganisms manage to cross the first physical barrier, the innate immune system is the next defense towards foreign organisms. And the innate immune system plays a important role in the inflammatory process of IBD. (19) Neutrophils infiltrate the affected intestinal tissue early in the inflammatory response. They are attracted via cell surface receptors that sense chemoattractants on the cell-surface of activated epithelial cells, macrophages and mast cells. Exactly which antigens that activate epithelial cells and antigen presentation cells (APC) are not known. Activated neutrophils secrete pro-inflammatory and chemotactic cytokines, attracting more inflammatory cells and amplifying the inflammatory reaction. It’s been shown that S100A12 is highly up-regulated in the neutrophils that
infiltrate the affected mucosa and this is one of the secreted proteins of neutrophils (14,20), which in turn attracts more monocytes and mast cells.

The adaptive immune system is important for chronic inflammation. Intra luminal bacteria activate and stimulate epithelial cells, dendritic cells and macrophages to express cytokines. Antigen presenting cells present the antigen for T-cells in lymph nodes and peyers patches. Depending of type of cytokines the T-cells are exposed to, they differentiate into mature T-cells. INFγ and IL-12 induce Th1 cells, which are elevated in the mucosa of CD patients, producing more INFγ. While in UC the Th2 cells are induced by IL-5, producing elevated levels of IL-5 and not IL-12 or INFγ. IL-1, IL-8, IL-6 and TNFα are elevated in the inflamed tissue in both CD and UC (2). Recently a third Th-cell, Th17-cells been found play a important role in the inflammation in CD. IL-6 and TGFβ stimulate differentiation into IL-17 producing Th17-cells. IL-17 induces cytokine and chemokine production in endothelial cells, attracting neutrophils. In chronic inflammation monocytes accumulate and differentiate into macrophages.

The important role TNFα plays in the inflammation of IBD has been emphasized by the beneficial effects of anti-TNFα infusions. Interestingly TNFα stimulate expression of S100A12. (21) Elevated levels of TNFα are seen in the inflamed intestinal mucosa in IBD. (22)

Increasing incidence and prevalence
The incidence of IBD has been increasing worldwide over the last decades in both children and adults. First the increase was mainly in the western world with traditional higher incidence but lately the incidence is also increasing in regions with traditionally low incidence, like the Eastern world. (6) It’s been speculated that this comes along with the introduction of the westernized lifestyle and socioeconomic development in the Eastern world. The incidence is higher in Caucasians and is lower near to the equator and becomes gradually higher further from the equator. A recent study revealed that Canterbury in New Zealand has one of the highest incidences of IBD in the world, with a higher incidence for CD (25, 2/100000) than UC. (22) This difference between the incidence in CD and UC agrees with other recent epidemiologic reports of IBD in both children and adults. (6) The prevalence of IBD is now 155/100000 for CD and 145/100000 for UC in Canterbury. (22) The increasing numbers of IBD patients also emphasise the need for easily accessible and inexpensive methods of assessing the inflammatory activity of IBD.

Diagnosis, diagnostic problems and disease monitoring in childhood IBD
When suspecting IBD in a child a comprehensive assessment should include history taking, clinical examination and multiple tests such as stool tests, blood tests, endoscopy and radiologic imaging. It is important to exclude other conditions in the differential diagnosis such as infectious gastroenteritis, celiac disease, cow’s milk allergy and other bowel diseases. The history taking includes documenting patterns of symptoms like abdominal pain, loss of appetite, fatigue, fever, stool consistency and number, blood in stool, nocturnal bowel motions, weight loss, extra intestinal symptoms, and also daily activity and family history. Blood tests include standard blood tests (CRP, ESR, complete blood count and albumin), minerals (calcium, magnesium) and vitamin B12. Stool tests include fecal cultures and more and more commonly analysis of fecal calprotectin as a marker of inflammation. Furthermore growth parameters need to be measured and pubertal development evaluated.
All children with suspected IBD should go through both upper and lower endoscopy with biopsies randomly taken, even in absence of endoscopic findings. Radiologic investigations, such as MRI, are also necessary for assessment of the small bowel. The diagnosis is finally based on standard criteria including endoscopic, histological and radiological findings. (23)

The endoscopy together with histology is to date the best method for visualizing the inflammatory activity inside the bowel, but it is invasive, expensive, time-consuming and inconvenient for patients and does not access the small bowel fully. Furthermore, subjective symptoms and clinical signs often correlate poorly with the endoscopic and histological findings in children. This makes the diagnostic process, as well as the monitoring of disease activity, more complicated. A better and safer non-invasive method of measuring the inflammatory activity of the intestine is therefore desired, in order to ease the diagnostic procedure and to reduce the numbers of unnecessary invasive endoscopies.

Disease monitoring in already established diagnosis is important with the aim of catching disease relapses in early stages and following the responses to treatment interventions. This is important to avoid short term and long term complications. Disease recurrences have good chances to be reversed by new immunosuppressant drugs. These should only be considered when inflammation is confirmed, but it is not practicable to complete endoscopy at each flare up for re-assessment of the intestinal inflammation.

**Current non-invasive markers**

Standard inflammatory blood tests are useful as guidelines of ongoing inflammation and are routinely utilised in disease management. A study showed that 94-98% of children with moderate to severe IBD have at least one abnormal blood result of ESR, hemoglobin level, platelet count and albumin level. Nonetheless children with IBD, especially with mild activity, may yield completely normal blood results. (24) Furthermore, these tests not sufficiently specific and sensitive for the gastrointestinal tract and IBD. (24)

Serologic markers also have been studied in the context of IBD diagnostics. Perinuclear anti-neutrophil cytoplasmic antibody (pANCA) and Anti-Saccharomyces cerevisiae antibody (ASCA) are two of the most well reported, but their specificity and especially the sensitivity for CD and UC are not sufficient to be used for routine analysis. (25-27) pANCA and ASCA may also be used in the differentiation between CD and UC, but unfortunately individuals with IBDU often don't develop these antibodies, which make the usefulness of their addition limited. (25) Recent studies have investigated serum antibodies towards other bacteria in patients with IBD and also the correlation between antibodies and different phenotypes of patients: these tests show more promise. (28,29)

Disease activity scores have been developed as instruments for the serial assessment of clinical disease activity. In pediatrics examples of these are Physician’s global assessment (PGA), Pediatric Crohn’s disease activity index (PCDAI)(30) and Pediatric Ulcerative Colitis activity index (PUCAI). (31) The PCDAI is based upon a combination of history, clinical findings and lab results (32), whilst the PUCAI is based on recent history and symptoms alone. (31) The correlation between these scores and other objective tests varies in different studies. (1,33-35)
Fecalmarkers

The inflammatory cells that infiltrate the affected bowel tissue secrete proteins and peptides that may leak out into the intestinal lumen. Several of them can be detected in the stool and have been studied as potential biomarkers of gut inflammation. Whether these can be appropriately sensitive and specific for IBD is still a question. Calprotectin is a member of the S100 family and is commonly accepted in clinical practice as fecal biomarker for screening for IBD. Other fecal markers that have been studied as markers of gut inflammation are Lactoferrin, Osteoprotegrin, α1-antitrypsin and M2PK. (33,36-38) S100A12 also a member of the S100 family was shown recently to be more sensitive and specific than calprotectin for IBD in children. (35,39)

S100 family and IBD

The S100 proteins are a family of 21 (well conserved) Ca-binding proteins. (2, 42) The name comes from their solubility in 100% ammonia sulfate. They are small proteins of about 10-20 kDa, with two different Ca binding EF hands. Binding with calcium leads to a conformation change of the EF hands resulting in intracellular Ca-signaling. Phosphorylation, cell proliferation, cell differentiation, protection from oxidative damage and anti-microbial and antifungal effects are some of the intra and extracellular properties of the various S100 proteins. (33) Three S100 proteins, the calgranulins; S100A8, S100A9 and S10012 have been associated with inflammatory bowel disease, and also with cancer and other inflammatory diseases like rheumatic arthritis (RA) and cystic fibrosis (CF). (40)

Calprotectin

Calprotectin is a heterocomplex of S100A8 and S100A9 found in neutrophils, epithelial cells and monocytes. In neutrophils it corresponds to 60% of the cytoplasmic proteins. Calprotectin is secreted from activated or damaged cells. (15)

Fecal calprotectin (FC) has previously been shown to be elevated in children with active IBD compared to children in remission and children without IBD. It is a indicator of inflammatory activity in the bowel but not entirely specific for IBD. (41) Faecal calprotectin can also be raised in other conditions of stress to the bowel such as colorectal carcinoma, gastric cancer, lactose intolerance, gastro enteritis, and NSAID treatment. (15) A number of studies have investigated the optimal cut off for best sensitivity and specificity. Several studies have shown that the negative predictive value is higher than the positive predictive value. Canani RB et al showed that at a cut off of 143 μg/g, FC was 100% sensitive for children in remission, with a negative predictive value of 100% and a positive predictive value of 67%. (42)

Higher levels of FC are seen in healthy infants, and inter individual variations of fecal calprotectin in infants are greater than in older children and adults. (43-45) The reason of this is not clear. One report showed more elevated levels in infants exclusively breastfed. (43) Another recent study showed an association of elevated FC levels in infants with establishment of gut bacteria. (45) After the age of 5, the levels are the same as in adults. (15)
S100A12
S100A12 (calgranulin C) is a cytosolic protein constitutively found in human neutrophils (16,21,46), but also induced in monocytes. (15,21) It is released by activated neutrophils or by damaged cells under stress as a damage-associated molecular pattern (DAMPs). (13) Neutrophils infiltrate the affected intestinal tissue early in the inflammatory process of IBD, and the released S100A12 spills over into the intestinal lumen, and can be detected in stool. (20)

Extracellular S100A12 has pro-inflammatory and chemotactic properties attracting other inflammatory cells such as monocytes and mast cells. It activates mast cells, stimulating release of cytokines, such as TNFα. (44) S100A12 probably binds to the receptor for advanced glycation endproducts (RAGE) (47), resulting in activating of the NF-κB signaling pathway, and subsequently expression of a number of cytokines including TNFα. (48) However a recent study couldn’t confirm this receptor binding, suggesting that this might not happen in human cells. (44)

Levels of S100A12 are raised in the serum and colonic mucosa in children with IBD. (49) Recent studies have shown that fecal S100A12 is elevated in stool of children with active IBD. (35,39) Sidler et al showed that fecal S100A12 levels at a cut off of 10mg/kg had sensitivity and specificity of 97% to distinguish between IBD and non-IBD. (39) Other studies have shown raised fecal S100A12 in adults compared to adults with irritable bowel syndrome (IBS). (50) Fecal S100A12 is stable in room temperature for 7 days, and is evenly distributed in the stool, which are desirable properties for a biomarker. (35)

Levels of S100A12 are also raised in other inflammatory conditions, such as in the serum of RA (51) and CF (52) patients.

In summary
The precise causes of the intestinal inflammation in IBD are not clear. IBD is a chronic disease with an increasing incidence in both children and adults worldwide. It is important to diagnose children with IBD with as little delay as possible because the appropriate management can reduce the risk of serious complications and surgical intervention. Children with IBD tend to progress rapidly and have more extensive disease than adults, but their symptoms do not always correlate with the ongoing mucosal inflammation. Endoscopic investigation together with histology is still the best way to evaluate the intestinal wall, but it comes along with number of drawbacks. An easy non-invasive method to assess the degree of inflammatory activity is needed to ease both the initial diagnostic procedure and subsequent disease monitoring in those with an established diagnosis. Such tools would enable doctors to prevent relapses with medical intervention and follow the response to such interventions closely. Different fecal biomarkers have shown promising results as markers of the gut inflammation, but there are still questions as to whether they are sufficiently sensitive and specific for IBD. We have looked at fecal S100A12 that in previous studies shown to be highly sensitive and specific for IBD in children compared to other fecal markers. (35,39) S100A12 belongs to the same superfamily as Calprotectin, which is at present a well known fecal biomarker. Reports have shown that healthy infants have higher levels of fecal calprotectin and that the levels vary greatly in the first period of life. We
wanted to investigate how levels of fecal S100A12 behave during early life in healthy children.

**Aims of this study**
The specific aims of this study were to investigate patterns of fecal levels of S100A12 in healthy children. We looked at differences in age groups and between genders. This was a first part of a larger study with overall aim to establish S100A12 as a non-invasive marker in the management of IBD in children. In this study we also compared fecal levels of S100A12 in children with IBD with levels in healthy control children. Furthermore we looked at association of fecal S100A12 with disease activity scores and standard inflammatory blood tests.
MATERIAL AND METHODS

Patients with IBD and control group
Children with established diagnosis of IBD were recruited prospectively at the time of their normal outpatient clinic appointment or at the time of colonoscopy. Inclusion criterion was age between 1 month and 18 years. Background information collected from each patient included; details about their current age, age at diagnosis, past and current medications, symptoms and if any complications or extra intestinal manifestations were present. Growth parameters were recorded. The results of standard blood tests (ESR, CRP, albumin and Hematocrit) were noted. Physicians Global Assessment (PGA) and relevant current disease activity scores; Pediatric Crohn disease activity index (PCDAI) or Pediatric ulcerative colitis activity index (PUCAI) were calculated according to baseline information. (30,31) All patients were asked to provide an initial stool sample.

A healthy control group of children were enrolled from outpatient clinics, pediatric wards or from families of hospital staff. Children older than 1 month and younger than 18 years were included. Exclusion criteria were acute infectious gastroenteritis or gastrointestinal symptoms such as diarrhea, vomiting and abdominal pain. Children with known rheumatological conditions or Cystic fibrosis were excluded. Ongoing treatment with nonsteroidal anti-inflammatory agents (NSAID) or corticosteroids were also an exclusion criterion. Weight, height and information about current medications and other diagnosis were collected. Each child was asked to provide one stool sample.

All patients were recruited during an 8 week period. They were residents in Canterbury, New Zealand. All data collected were stored in a secure electronic database.

Ethics
The study was approved by the Upper South Regional Ethics Committee. Informed consent was obtained from each patient or one of his or her caregivers.

Calculation of Disease Activity Scores
The Pediatric Crohn’s Activity Index (PCDAI) (30) is calculated from history of symptoms, physical examination, laboratory parameters (ESR, Albumin and Hematocrit) and growth parameters. The score ranges from 0-100, <15 encompasses disease remission.

Physicians global assessment (PGA) is three step score (0-2), where 0= none, 1 = mild, and 2= severe disease. PGA is based on the physicians’ overall subjective impression of the patient’s state.

Pediatric Ulcerative Colitis Activity index (PUCAI) is calculated from history of recent symptoms and stool habits. The score range from 0-85. A score <10 suggests inactive disease. (31)
Sample Collection and Preparation

Stool samples were collected on the ward or at home by parents. After collection samples were first stored in fridge at 4 °C for maximum 7 days prior to aliquoting into 4 eppendorf tubes. They were then placed in storage at -20°C until required for analysis.

Feecal extraction from samples

Approximately 100 mg of frozen stool was taken from one of the stored Eppendorf tubes per sample and measured to an exact weight. Faecal Extraction Buffer (Containing: 0.25 M Tris, 0.25 citric acid, 2.5 M urea, 0.025 urea, 1.25% bovine serum albumin, 0.05% sodium acid) was added at the ratio of 49µl buffer per mg of stool to produce a dilution factor of 1:50. Sample dilutions were vortexed for 30sec and then more vigorously shaken for 30 minutes on a shaker table for homogenization. 1.0 ml of homogenized faeces was then transferred to an eppendorf tube and centrifuged for 5 minutes at 13000 g. After centrifugation 0.7 ml of clear supernatant was transferred to a new clean Eppendorf tube. The samples were stored at 2-8 °C over night prior the assay analysis. At one stage some sample extractions were frozen down to -20 °C for long-term storage before analysis. Those frozen samples were thawed slowly at 2–8° C for 12 hours, and then warmed to room temperature on the day of analysis.

Measurement of S100A12 levels with Enzyme-linked Immunosorbent Assay (ELISA)

Levels of S100A12 were measured by an Enzyme-linked Immunosorbent Assay (ELISA) method used in previous studies of fecal S100A12 in children (35). 96 well ELISA plates (Maxisorp, Nunc) were used. Each plate was coated with 100 µl coating antibody (0.5 µg/ml of Rabbit polyclonal S100A12, from Abcam) in carbonate buffer (0.1M Na₂CO₃ and 0.1M NaHCO₃ adjusted to pH 9.6.) and incubated at 4°C overnight. Plates were washed twice with wash solution (phosphate-buffered saline, 0.05% Tween 20) and 100µl/well blocking buffer (wash solution with 1% skim milk powder) was added for 2 hour’s incubation in room temperature. The washing procedure was then repeated. Fecal extractions diluted in blocking buffer to three dilutions (1:2, 1:20, 1:200) were added (100µl/well). Dilutions of recombinant S100A12 (Recombinant Human EN-RAGE/S100A12, R&D Systems) was added (100µl/well) in order to create a standard curve. Plates were incubated for 1 hour in room temperature followed by three more times of washing with wash solution. A secondary antibody (Goat anti-Human EN-RAGE polyclonal antibody, Biotin Conjugate, R&D Systems) was added and plates incubated for 1 hour in room temperature. Three times washing procedure was repeated followed by incubation with streptavidin-horseradish peroxidase (Strep-HRP, BD Biosciences) for 30 minutes in room temperature. Plates were washed three times more before TMB substrate was added (100µl/well). The color reaction developed instantly and a stop solution (2M H₂SO₄, 50µl/well) was added after approximately 2 minutes. Plates were read within 15 minutes after stopping the reaction in an ELISA plate reader (Spectramax 190, Molecular Devices) using softmax Pro V5.3.
Statistical Analysis
GraphPad Prism 5 for Windows was used for the statistical analysis and for creating graphs. Levels of S100A12 in different groups were compared with Mann-Whitney test and ANOVA. Spearman’s correlation test was used to investigate correlations between levels of fecal S100A12 and PCDAI, ESR, CRP, Albumin, Platelets and Hematocrit values. Significance was accepted at values of P<0.05.
RESULTS

Patients
Ten children with IBD diagnosis were included: 7 with CD, and 3 with IBDU. The IBD diagnosis was previously established according standard criteria. Included in the control group were 38 children: 22 children from hospital staff and 16 children recruited from pediatric wards or from outpatient clinics with symptoms but not restricted by study exclusion criteria. The age range in the IBD group was between 3.3–17.0 years, and in the control group between 0.16-13.83 years (significant difference of age between groups). Although not matched directly, the age ranges overlapped.

TABLE 1. Demographics of study population.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Total, N</th>
<th>Mean±SD, Age(range), Y</th>
<th>Male Sex, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Children</td>
<td>38</td>
<td>3.2 ± 3.5 (0.16 - 13.83)</td>
<td>60</td>
</tr>
<tr>
<td>- From Families of hospital staff</td>
<td>22</td>
<td>4.2 ± 4.0 (0.33-13.83)</td>
<td>54</td>
</tr>
<tr>
<td>- From Ward and outpatient clinics</td>
<td>16</td>
<td>1.8 ± 2.3 (0.16-7.42)</td>
<td>69</td>
</tr>
<tr>
<td>IBD</td>
<td>10</td>
<td>12.2± 4.1 (3.3 - 17.0)</td>
<td>40</td>
</tr>
<tr>
<td>- CD</td>
<td>7</td>
<td>12.4 ± 2.6 (8.2 - 15.4)</td>
<td>42</td>
</tr>
<tr>
<td>- IBDU</td>
<td>3</td>
<td>11.7 ± 7.3</td>
<td>67</td>
</tr>
</tbody>
</table>

Patterns of Fecal S100A12 within Healthy children
Control subjects were divided into three age groups; <1, 1-5, and >5 years of age. There was a pattern of higher levels of S100A12 in the group with lowest age (Figure 1.). The differences was small but significant, with highest values in the group with children <1 year (median 1.6, range 0.4- 25, n= 13), slightly lower values in the 1-5 years group (median 0.6, range 0.4-4.6, n= 16), and the lowest values in the >5 years group (median 0.4, range 0.4-3.1, n=9; P=0.011, Fig. 2.). Intra variations within the groups are also greater in the <1 year group than the other two groups. There were more children in the youngest age group that were recruited from the ward and a higher number of children from families of hospital staff in the other two age groups. But there was not a significant difference of S100A12 levels when comparing subgroups of controls enrolled from ward or outpatient clinics with children enrolled from families of hospital staff in any of the age groups (P=NS for each age group). There was no significant difference in fecal S100A12 levels between males (60%) and females (40%), (P=0.22).
Fecal levels of S100A12 in control subjects arranged by age into 3 groups; <1, 1-5, and >5 years. There was a significant difference between the three groups, with highest median in the <1 group (median 1.6, range 0.4-25, n=13), slightly lower median in the 1-5 years group (median 0.6, range 0.4-4.6, n=16), and the lowest median in the >5 years group (median 0.4, range 0.4-3.1, n=9, P=0.011).

**Fecal Analysis**

Fecal levels of S100A12 were significantly higher in children with IBD (median 46.0 mg/kg, range 1.2-99.3 mg/kg; n=10) than in control subjects (median 0.7 mg/kg, range 0.4-25.0 mg/kg; n=38; P <0.0001; Fig.2). In the control group only 2 subjects had a level over 10 mg/kg which in previous studies is defined as the upper limit for normal values. No significant difference was found between male patients and female patients within the IBD group (Data not shown).

**FIGURE 1.** Fecal levels of S100A12 in control subjects arranged by age into 3 groups; <1, 1-5, and >5 years. There was a significant difference between the three groups, with highest median in the <1 group (median 1.6, range 0.4-25, n=13), slightly lower median in the 1-5 years group (median 0.6, range 0.4-4.6, n=16), and the lowest median in the >5 years group (median 0.4, range 0.4-3.1, n=9, P=0.011).

**FIGURE 2.** Fecal levels of S100A12 in children with IBD (n=10) and in the control group of healthy children (n=38). Fecal S100A12 was elevated in children with IBD (P <0.0001).
Correlation of Fecal S100A12 with PCDAI, PGA and Inflammatory markers

PCDAI was calculated for each patient with IBD, their scores varied between 0 and 20. Seven children had a PCDAI <15, which was used as definition of clinical remission. There was no significant correlation between PCDAI and fecal S100A12 (Data not shown).

PGA was evaluated for each IBD patient. Three patients had PGA=0, which is interpreted as no disease activity. An association between fecal S100A12 and PGA could not be detected (Data not shown).

Fecal S100A12 was compared with standard inflammatory blood tests; CRP, ESR, platelets, Albumin and Hematocrit. There were no significant differences between levels of fecal S100A12 and any of those blood tests. But a trend pointing towards a correlation between fecal S100A12 and CRP was observed (P=0.14, r=0.51, Fig. 3). The group of 7 CD patients was looked at to see if there was a correlation between fecal S100A12 and CRP and ESR. No significant correlation was observed but there was a trend towards an association of S100A12 with both CRP (P=0.66, r=0.74, Fig. 4) and ESR (P=0.17, r=0.6, Fig.5)

**FIGURE 3.** Fecal levels of S100A12 compared with serum levels of CRP in 10 IBD patients. There is a trend towards a correlation, but it is not significant.
**Figure 4.** A trend towards association between levels of fecal S100A12 and serum CRP was observed in children with CD (n=7, $P=0.66$).

**Figure 5.** A trend towards association between levels of fecal S100A12 and serum ESR in children with CD (n=7, $P=0.17$).
DISCUSSION

This study shows that levels of fecal S100A12 are low in healthy children and in children who suffer from non-gastrointestinal diseases. The current study also identified that levels of fecal S100A12 varied slightly with age in healthy young children. S100A12 levels were greatest in children younger than 1 year compared to older children. This study also agrees with previous work that fecal levels of S100A12 are greater in children with IBD than normal children without IBD. (35,39) Interestingly, in current study there were no correlations between fecal S100A12 and PCDAI or between fecal S100A12 and any of the standard inflammatory blood tests.

It is very important to be able to monitor the extent of intestinal inflammation in children with IBD, because appropriate management can then be modified to prevent serious complications. To date, endoscopic and histological investigations are the gold standards for assessing inflammation in the bowel. Current non-invasive markers have not yet proven to have sufficient sensitivity and specificity, therefore better non-invasive methods are needed. Fecal S100A12 is one of the new non-invasive biomarkers that have been studied recently. In previous studies it has shown to have the potential to be a sensitive and specific biomarker of inflammation in children with IBD. (1,35) S100A12 is a Ca binding protein which is a member in the same family, the S100-family, as Calprotectin (S100A8/S100A9) and is also mainly produced by neutrophils.

Fecal Calprotectin (FC) has in several previous reports shown to be elevated in healthy children in the first period of life, then dropping to lower levels in older children and adults. This study observes similar patterns of fecal S100A12 in normal young children without IBD. One study reports that fecal calprotectin is higher in children between 2-9 years than children higher older than 10. (44) Another recent study showed that levels can be high in healthy infants and that wide inter individual variations of FC is normal in infants. In that study the levels of FC were lower in infants whose mothers had received antibiotics before or at delivery, showing a link between excretion of FC and colonization of gut bacteria. (45) They also suggest that a great inter individual variation in healthy infants are normal. Another theory is that levels of FC vary with different diet. One single study has shown that exclusively breastfed infants had higher levels of FC (43), but other reports have not repeated that result. It is possible that the slightly higher levels of fecal S100A12 in first year of life can be explained by same basis as the elevated FC in infants. S100A12 is more restricted to neutrophils, monocytes and mastcells than calprotectin which can be induced in neutrophils, epithelial cells, macrophages and keratinocytes. And reports have shown that S100A12 and calprotectin are independent of each other, suggesting there are stimulated of different factors.

An important finding in current study was that the inter individual variations of S100A12 were less pronounced than previously observed for levels of FC in healthy infants, where levels of FC could be much higher than the cut-off for normal range. Nevertheless the reasonably consistent low levels in normal controls may give S100A12 better potential to be a reliable biomarker of gut inflammation than FC in young children.
In current study there were more children recruited from the ward in the youngest age group and a higher number of children from families of hospital staff in the other two age groups. But if the groups were divided into subgroups of completely healthy children and children recruited from ward or outpatient clinics there were not any significant differences between the subgroups in any of the age groups. All controls except from two subjects were lower than previous defined cutoff 10mg/kg for IBD. There is a possibility that sick children recruited from the ward would have higher risk to have subclinical gastrointestinal conditions resulting in elevated fecal S100A12. The control subjects with elevated levels had recently suffered acute respiratory diseases. One had been treated with antibiotic and had diarrhea because of that, the sample was collected when the child was according to parents better again. However a number of other control children had also recently gone through respiratory diseases without elevated levels of fecal S100A12. In the subgroup of completely healthy children (not recruited from ward or outpatient clinics), no control subject had a level over 10 mg/kg. Elevated intestinal permeability could be caused by other reasons than IBD, which might lead to elevated number of infiltrating neutrophils. Sidler et al investigated levels in children attending endoscopy because of varies gastrointestinal symptoms, their only patient without IBD with elevated levels of fecal S100A12 had noninfectious chronic diarrhea. (39) The patterns of fecal A12 in other gastrointestinal conditions needs to be defined in further studies. In the current study levels of fecal S100A12 was significantly higher in children with established IBD than the control subjects. This agrees with the results of previous studies that showed that fecal S100A12 is elevated at the time of diagnosis of IBD. (35,39) In this study the IBD patients had known diagnosis of IBD and were enrolled prospectively at the time of routine follow-up. Many of them were asymptomatic with minimal disease activity, as judged by PCDAI scores. Despite this, these children still had elevated levels of fecal S100A12. This could indicate that subclinical inflammation was still present even in absence of symptoms. Previous reports have pointed toward that symptoms and disease activity indices do not always indicate the extent of inflammation. (53) S100A12 may be a much more sensitive marker of inflammation than serum tests or symptoms. Furthermore, the elevated levels in children in the current study could indicate the risk of future relapse. Further studies on S100A12 need to be done to investigate its potential as a useful biomarker to follow the disease activity.

It has already been shown that fecal S10012 is stable in room temperature for 7 days and evenly distributed in the stool, which are good properties for a fecal marker. Furthermore only a small amount of stool, around 100mg of stool is needed for analysis. S100A12 has shown to be more specific to IBD in children than FC. One report showed sensitivity and specificity 97% with fecal S100A12, and specific 67% with FC (39). FC is more and more used both in hospital and health centers as a screening tool for IBD. But FC has shown to be more reliable in adults than in children as a marker to differentiate IBD from functional disorder. Assay of fecal S100A12 is not measured in routine laboratories in New Zealand, and is still a relatively expensive test. FC elevated in a number of other conditions, S100A12 is shown to be elevated in bacterial enteritis but not viral gastroenteritis in adults (50). Fecal S100A12 did not correlate with standard inflammatory test (CRP, ESR, hematocrit, albumin and platelets). This emphasizes the deficiencies of current inflammatory blood tests as markers of the ongoing inflammation in IBD. Furthermore, those results are consistent
with findings of Sidler and colleagues (39), where no correlation between fecal S100A12 and any standard blood tests was seen in a larger group of children. In contrast, de Jong et al (35) had previously shown that S100A12 levels related to CRP levels. In adults one study has found a correlation between fecal S100A12 and CRP and ESR in patients with CD. (50) Further studies with larger numbers of children with IBD may be required to understand this fully.

Limitations of this study were the short period of time for enrollment of patients. Since IBD patients were recruited when coming to their ordinary outpatient clinic, the number that came during this period was limited. The short period of time also resulted in that only one sample per child was collected, and it was not possible to investigate if the levels of S100A12 could predict the disease course. The small number of patients may also limit correlations. Furthermore no endoscopic assessment at the time of measurement was obtained, so it was not possible to correlate S100A12 to the inflammation according to gold standards.

Another drawback was the age difference between the control group and the IBD group. And the control group included some conditions that may have influenced results. The study did not include a non-IBD gastrointestinal disease group, such as Celiac disease an important differential diagnosis of IBD.

**Conclusion**

This study showed that completely healthy children had low levels of fecal S100A12 with small inter-individual variations. This can make it a more reliable biomarker in young children compared to FC, which varies a lot in normal infants. Further, studies on other inflammatory conditions in the stomach need to be investigated to evaluate the sensitivity and specificity of fecal S100A12. For example FC sometimes is elevated in celiac patients, which is one important differential diagnosis to IBD.

This study agreed with previous results that levels of fecal S100A12 were significant elevated in children with established IBD compared to normal children. The levels of fecal S100A12 were elevated even in the children with IBD which at the moment were in quiescent phase clinically with few symptoms. Further studies are now needed to follow levels of S100A12 over time to investigate if individual variations could predict disease relapses. Future studies should also investigate if levels of fecal S100A12 alter after medical interventions and if the biomarker could be used to evaluate the effect of the medication.

To date, no non-invasive biomarker has proven to have sufficient reliability as a biomarker of gut inflammation in children with IBD. Fecal S100A12 has good potential to be a useful biomarker, but further studies are needed.
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REFERENCES:


28. Schoepfer AM, Schaffer T, Mueller S, Flogerzi B, Vassella E, Seibold-Schmid B, m.fl. Phenotypic associations of Crohn’s disease with antibodies to flagellins A4-Fla2 and


49. Leach ST, Yang Z, Messina I, Song C, Geczy CL, Cunningham AM, m.fl. Serum and mucosal S100 proteins, calprotectin (S100A8/S100A9) and S100A12, are elevated at diagnosis in children with inflammatory bowel disease. Scand. J. Gastroenterol. 2007 Nov;42(11):1321-1331.

