Helicobacter pylori and faecal calprotectin in apparently healthy and HIV-infected Ugandan children

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Protect our children

Værn om våre barn

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
CD4	Cluster of differentiation 4
CI	Confidence interval
CT	Computer tomography
ELISA	Enzyme-linked immunosorbent assay
ESPGHAN	European Society of Paediatric Gastroenterology, Hepatology and Nutrition
FC	Faecal calprotectin
GI	Gastrointestinal
HAART	Highly active anti-retroviral therapy
HIV	Human immunodeficiency virus
H. pylori	Helicobacter pylori
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IDC	Infectious disease clinic
MRI	Magnetic resonance imaging
NASPGHAN	North American Society of Pediatric Gastroenterology, Hepatology and Nutrition
NEC	Necrotizing enterocolitis
NSAID	Non-steroidal anti inflammatory drug
OI	Opportunistic infections
OR	Odds ratio
PIDC	Paediatric infectious disease clinic
RAP	Recurrent abdominal pain
SD	Standard deviation
VLBW	Very low birth weight babies

Abstract

Gastrointestinal dysfunction, often presenting as diarrhoea, is one of the major causes of morbidity and mortality among children in low-income countries. It is estimated that 56% of child deaths were attributable to malnutrition's potentiating effects in children living in low-income countries. There are numerous gastrointestinal problems in children in low-income countries, in particularly in human immunodeficiency virus (HIV) infected children. The gastrointestinal tract is the largest immunological site of the body and HIV infection profoundly impacts on gut function and disease development. Most studies of the gastrointestinal function in patients in low-income countries have been performed among adults.

The objectives of this thesis are: 1) to examine the prevalence of *H. pylori* in apparently healthy urban Ugandan children and in HIV-infected, highly active anti-retroviral therapy (HAART) naïve children in the same geographical area, and 2) to examine if a faecal marker for gut inflammation, faecal calprotectin, can be used in children in a low-income country living in poor sanitary conditions, and if the marker can be used in an HIV-infected population.

Two surveys were conducted in urban Kampala, Uganda. The first was community-based, in children aged 0-12 years, and conducted by door-to-door visits in a neighbourhood characterised by slum-like living conditions. The second survey was hospital-based at the Department of Paediatrics and Child Health, Mulago National Referral Hospital, Kampala where all HIV-infected, HAART naïve children admitted were invited to participate. A questionnaire was used in both surveys to address questions about medical conditions and socio-economic factors. Faeces were examined for *H. pylori* by using a rapid monoclonal antigen test. Faecal calprotectin was analysed using the ELISA technique.

The overall prevalence of *H. pylori* antigen in apparently healthy urban Ugandan children aged 0-12 years was 44.3%. Early colonization was common with 28.7% in children younger than 1 year of age. There was a steady increase with age (1<3 years 46.0%, 3<6 years 51.7%, and 6<9 years 54.8%). Children living in permanent houses had a significantly lower colonization rate (38.5%) compared to those living in semi-permanent houses, 48.6%. HIV-infected children had a lower overall prevalence (22.5%). Age specific prevalence's were; 14.7% in infants 0<1 year, 30.9% among toddles 1<3 years, and 20.7% for children 3<12 years. HIV-infected children more seriously affected by their disease (lower CD4 cell percentage or WHO clinical stage II-IV) were less likely to be colonized with *H. pylori*.

Median faecal calprotectin concentrations in apparently healthy Ugandan children were comparable to those found in children living in high-income countries. They were 249mg/kg in infants 0<1 year (n=54), 75mg/kg among toddlers 1<4 years (n=89), and 28mg/kg for children 4<12 years (n=159). There was no significant difference in faecal calprotectin concentration when considering the education of female caretaker, wealth index, gender, habits of using mosquito nets, being colonized with *H. pylori* or having other pathogens in the stool. In the HIV-infected children, median faecal calprotectin concentrations were different from those in apparently healthy children. They were 208mg/kg in infants 0<1 year, 171mg/kg among toddlers 1<4 years, and 62mg/kg for

children 4<12 years. HIV-infected children more seriously affected by their disease (lower CD4 cell percentage) or diarrhoea at enrolment had a higher median faecal calprotectin concentration.

In conclusion, *H. pylori* colonization among apparently healthy urban Ugandan children is common at an early age and increases with age. The prevalence among HIV-infected children in the same geographical area is only about half. Faecal calprotectin is also a marker for gut inflammation that is well suited for use also in children in low-income countries, with the same cut-off values as suggested for children living in high-income countries. Faecal calprotectin can also be used as a tool also in an HIV-infected population for evaluation of gut inflammation. We found calprotectin to be higher in those HIV-infected children with more advanced disease, regardless of age.

Résumé in Norwegian

Gastrointestinal dysfunksjon, ofte presentert som diaré, er en av hovedårsakene til sykelighet og dødelighet blant barn i lavinntektsland. I en rapport fra Verdens Helseorganisasjon (WHO) fra 1995 er det anslått at feil-og underernæring er den underliggende årsaken til 56% av dødsfallene blant småbarn som lever i lavinntektsland. Disse funnene har senere blitt bekreftet. Mage-tarm kanalen er det største immunologiske organet i kroppen og humant immunsvikt virus (HIV) påvirker tarmfunksjonen. Det er mange gastrointestinale problemer hos barn i lavinntektsland, særlig hos HIV-positive barn. De fleste studier av gastrointestinal funksjon hos pasienter i lavinntektsland er blitt utført blant voksne.

Denne avhandlingen har to hovedmål: 1) å undersøke forekomsten av *H. pylori* hos tilsynelatende friske urbane ugandiske barn og hos HIV-smittede, highly active anti-retroviral therapy (HAART) naive barn i samme geografiske område, og 2) å undersøke om en fekal markør for tarm inflammasjon, fekalt kalprotektin, kan brukes hos barn i lavinntekts land som lever under dårlige sanitære forhold og om markøren kan brukes i en HIV-smittet populasjon.

For å kunne svare på disse spørsmålene, ble to studier gjennomført i urbane Kampala, Uganda. Den første studien ble gjennomført hos 0-12 år gamle barn i oktober / november 2007 ved dør-til-dør besøk i et nabolag preget av slumlignende levekår. Et spørreskjema ble brukt for å belyse spørsmål rundt medisinske forhold og sosio-økonomiske faktorer. Avføring ble undersøkt for *H. pylori* ved hjelp av en monoklonal antigen test. Fekalt kalprotektin ble analysert med ELISA teknikk. Den andre studien ble gjennomført mellom februar og oktober 2008 ved Barneavdelingen på Mulago National Referral Hospital i Kampala. Alle HIV-smittede, HAART naive barn som ble innlagt i denne perioden ble inviteres til å delta i studien. Et lignende spørreskjema som i den første studien og de samme metoder for påvisning av *H. pylori* og fekalt kalprotektin ble brukt.

Den generelle forekomsten av *H. pylori* antigen i tilsynelatende friske urbane ugandiske barn i alderen 0-12 år var 44,3%. Det var en signifikant økning med alder. Tidlig kolonisering var vanlig med en forekomst på 28,7% hos barn yngre enn 1 år. Barn som levde i permanente hus hadde en betydelig lavere kolonisering, 38,5%, enn de som bodde i semi-permanente hus, 48,6%. HIV smittede barn hadde en lavere generelle forekomst, 22,5%. Blant de HIV smittede barna var den høyeste forekomsten av *H. pylori* hos barn mellom 1-3 år. De eldste barna hadde en forekomst sammenliknbar med forekomsten hos de HIV smittede spedbarna. HIV-smittede barn med mer alvorlig sykdom (lavere prosent CD4-celler eller WHO klinisk stadium II-IV) hadde mindre sannsynlighet for å være kolonisert med *H. pylori*.

Konsentrasjonen av fekalt kalprotektin hos tilsynelatende friske ugandiske barn var sammenlignbar med nivåene som finnes hos friske barn i høyinntektsland. I de ulike aldersgruppene var median fekal kalprotektin konsentrasjon 249mg/kg hos barn 0<1 år (n=54), 75mg/kg hos barn 1<4 år (n = 89) og 28mg/kg hos barn 4<12 år (n = 159). Det var ingen signifikant forskjell i fekalt kalprotektin konsentrasjon og utdanning av kvinnelige

foresatt, familiens økonomiske situasjon, kjønn, vaner med å bruke myggnett, å være kolonisert med *H. pylori*, eller å ha påvist andre patogener i avføringen. Hos HIV-smittede barn var nivået av fekalt kalprotektin forskjellig fra det i tilsynelatende friske barn. Median fekalt kalprotectin konsentrasjon var 208 mg / kg hos barn i alderen 0< 1 år, 171mg/kg hos barn i alderen 1<4 år og 62mg/kg hos barn i alderen 4<12 år. HIV-smittede barn med mer alvorlig sykdom (lavere prosent CD4 celler) eller diaré ved innrullering i studien hadde høyere median fekalt kalprotektin konsentrasjon uavhengig av alder.

I denne avhandlingen konkluderes det med at forekomsten av *H. pylori* hos tilsynelatende friske urbane ugandiske barn er høy i en tidlig alder og øker med alderen. Forekomsten blant HIV-smittede barn i samme region er tilnærmet halvert sammenlignet med de friske barna. Fekalt kalprotektin er en markør for tarm inflammasjon som er godt egnet for bruk også hos barn som lever i lavinntektsland, og med samme referanse verdier som foreslått hos barn som lever i høyinntektsland. Fekalt kalprotektin kan brukes som et verktøy også i den HIV-infiserte populasjonen for evaluering av tarm inflammasjon. I vår studie var fekalt kalprotektin høyere hos de med mer avansert sykdom.

Articles in the thesis

Paper I

Hestvik E, Tylleskar T, Kaddu-Mulindwa DH, Ndeezi G, Grahnquist L, Olafsdottir E, Tumwine JK. *Helicobacter pylori* in apparently healthy children aged 0-12 years in urban Kampala, Uganda: a community-based cross sectional survey. BMC Gastroenterology 2010, June 16; 10:62

Paper II

Hestvik E, Tumwine J.K, Tylleskar T, Grahnquist L, Ndeezi G, Kaddu-Mulindwa DH, Aksnes L, Olafsdottir E. Faecal calprotectin concentrations in apparently healthy children aged 0-12 years in urban Kampala, Uganda: a community-based survey. BMC Pediatric 2011 February 2; 11(1):9

Paper III

Hestvik E, Tylleskar T, Ndeezi G, Grahnquist L, Olafsdottir E, Tumwine J.K, Kaddu-Mulindwa DH. Prevalence of *Helicobacter pylori* in HIV-infected, HAART naïve Ugandan children: a hospital-based survey. J Int AIDS Soc. 2011 Jun 30;14(1):34

Paper VI

Hestvik E, Olafsdottir E, Tumwine J.K, Tylleskar T, Ndeezi G, Kaddu-Mulindwa DH, Aksnes L, Grahnquist L. Faecal calprotectin in HIV-infected HAART naïve Ugandan children: a hospital-based survey. (Accepted J Pediatr Gastroenterol Nutr.)

1 Introduction

1.1 Gastrointestinal tract: function and dysfunction

The gastrointestinal (GI) tract consists of the upper and lower tract divided by the ligament of Treitz. The upper tract (mouth, throat, esophagus, stomach, and duodenum) is accessible or by endoscopy and an accurate diagnosis can be made. The lower tract (jejunum, ileum, colon, and rectum) is partly accessible by rectoscopy and colonoscopy, but the jejunum and most of the ileum are not. Diseases in the ileum and the jejunum must be diagnosed by markers and other modalities, such as ultrasound, computer tomography (CT) or magnetic resonance imaging (MRI). Children need general anaesthesia when endoscopy is required. In low-income countries including Uganda, endoscopy, CT, and MRI are rarely available and diagnosis of the GI tract must be made using other diagnostic tools.

GI symptoms are common in children all over the world. Different studies report that recurrent abdominal pain affects as many as 9-19% of school children, enough to interfere with normal daily activity [1, 2]. GI dysfunction, often presenting as diarrhoea, is one of the major causes of morbidity and mortality among children in low-income countries [3]. GI failure in children has an impact on illnesses associated with growth faltering [4], micronutrient deficiencies [5, 6], impaired neurodevelopment [7], and increased morbidity and mortality from other childhood diseases [8].

Diarrhoea accounts for 21% of all deaths in children under five years of age and causes 1.5-2.5 million deaths per year [9, 10]. Diarrhoea is categorised by the World Health Organisation (WHO) as acute and persistent diarrhoea [11]. Diarrhoea is defined as the passage of three or more loose or liquid stools per day, persistent diarrhoea lasting for more than 14 days. Between 5 and 18% of all diarrhoea episodes in low-income countries become persistent [12-14]. Persistent diarrhoea accounts for 50% of all days affected by diarrhoea [12]. Malnourished children and those with impaired immunity are more likely to develop persistent diarrhoea. Diarrhoea, in turn, tends to worsen their condition.

H. pylori is considered to be the major cause of recurrent chronic gastritis and duodenal ulcer disease in childhood. Some studies also suggest that gastric infection with *H. pylori* is associated with sub-optimal nutrition and retarded growth in childhood [15]. Studies in Africa have shown a significant increase in *H. pylori* prevalence with age [16, 17].

Faecal calprotectin is a marker for gastrointestinal inflammation and is frequently used to distinguish between irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) [18].

The GI tract is the largest immunological site of the body and human immunodeficiency virus (HIV) infection profoundly impacts on gut function and disease development [19, 20]. There are numerous GI problems in children in low-income countries and in particularly in HIV-infected children [20-22]. In low-income countries, it is estimated that diarrhoea may occur in as many as 80% of HIV-infected individuals [23].

1.2 HIV in Ugandan children

1.2.1 Epidemiology

Sub-Saharan Africa accounts for 67% of all people living with HIV, and carries the highest burden of the global HIV epidemic [24]. In Uganda, 940.000 people, including 130.000 children were living with HIV in 2007 [24]. About 1000 children are infected with HIV each day worldwide. At the end of December 2008, only 32% of HIV-infected children less than 15 years of age in eastern and southern Africa needing HAART were on therapy (http://www.who.int/hiv/topics/paediatric/data/en/index1.html). About 1.2 million children are orphaned in Uganda due to HIV/AIDS (http://www.unicef.org/infobycountry/uganda_statistics.html).

1.2.2 CD4 cell count and clinical staging in HIV-infected children

Children have a natural decline in CD4 (cluster of differentiation 4) cell count with age [25] and therefore it is more correct to report CD4 cell count in children as a percentage of the total lymphocyte count. The WHO therapy guideline, available at the time of the study [26], recommended starting HAART in children at different levels of CD4 percentage dependent on age, table 1. The newest WHO treatment guidelines recommend starting HAART in all HIV-infected children younger than two year of age, between 24 and 59 months of age with CD4 cell percentage ≤25, and in children older than 5 years of age with CD4 cell count ≤350 cells/mm^3 [27].

Table 1. CD4 cell criteria for severe HIV immunodeficiency

Immunological Marker [a]	Age-specific recommendation to initiate HAART [b]			
	≤ 11 months	12- 35 months	36 - 59 months	≥ 5 years
CD4 cell % [c]	< 25	< 20	< 15	< 15
CD4 cell count [c] (cell/mm3)	<1500	< 750	< 350	<200

(Copied from the WHO guideline [26])

[a] Immunological markers supplement clinical assessment and should therefore be used in combination with clinical staging. CD4 is preferably measured after stabilization of acute presenting conditions.

[b] HAART should be initiated by these cut-off levels, regardless of clinical stage; a drop of CD4 below these levels significantly increases the risk of disease progression and mortality.

[c] CD4 cell percentage is preferred for children aged <5 years.

In most settings where HIV treatment is provided today, virological testing and CD4 cell count diagnostic are available. Despite this, clinical staging is still an important tool for follow up after initiating treatment and also in some cases for treatment initiating. The WHO clinical staging system has 4 stages; 1 asymptomatic, 2 mild, 3 advanced, and 4 severe, for more detail, see appendix VI. All children should be classified according to this system, independent of virological testing and CD4 cell diagnostic. Many of the symptoms in the clinical staging system are due to opportunistic infections (OI). Children are identified as stage 3 when they have symptoms from disease in the GI tract, and regardless of CD4 cell count, initiating HAART is recommended.

1.2.3 Gastrointestinal aspects and HIV enteropathy

Worldwide, GI disease continues to account for a high proportion of presenting symptoms of HIV infection, especially in low-income countries [19, 20, 28-30]. Children with a low CD4 cell percentage are more at risk for acute and persistent diarrhoea [31]. Common conditions in children living in low-income countries are much more frequent in HIV-infected children with a much higher mortality and morbidity rate than non-HIV-infected children. In a study from Rwanda, the initial clinical sign that occurred most frequently was failure to thrive [32]. Children living with HIV without HAART treatment die due to other OI and GI failure [33].

Studies have shown that invasive investigations, demanding general anaesthesia in children, such as colonoscopy with intubation and biopsy of the terminal ileum together with endoscopy of the upper GI tract are necessary to identify GI opportunistic infections at high rates [29]. Numerous pathogens found in HIV-infected persons with diarrhoea are atypical and need highly sophisticated methods to be identified [23]. If identification is done, the treatment recommendations for many of the pathogens found are still pending [34] and if known, the drugs needed are often not available in low-income countries. Studies have shown that initiating HAART is one of the most effective treatment for many of the opportunistic infections [23].

Increased intestinal permeability in HIV-infected populations has been confirmed by several studies [35-37], and occurs in 40-60% of children with symptomatic HIV infection [38, 39]. This data comes from studies of children in high-income countries. Commonly among these studies, they have tested for carbohydrate malabsorption, and it has been concluded that there are increased small intestinal permeability. Sharpstone et al. [37] used a lactulose/L-rhamnose test to show that all AIDS infected participants in the study except from those being defined as “well”, had significantly increased intestinal permeability compare to healthy controls. Greenson et al. [40] found that adults with AIDS with and without diarrhoea have a significantly reduced villius/crypt ratio, villius height ratio and crypt depth compared to normal controls. Knox et al. [30] investigated 671 HIV-infected persons, 39% had diarrhoea, stool pathogens were identified in only 12% of these patients. Despite this, 48% of all patients had an abnormal D-xylose test, 22.5% had borderline or low serum B-12 levels, and 7.2% had a depressed albumin level. Especially the abnormal D-xylose test and depressed levels of albumin are pointing to a GI malabsorption in those HIV-infected. Increased malabsorption is often associated with increased permeability of the GI tract [37]. Gori et al. [41] investigated 53 healthy, asymptomatic, HAART naïve Italian adults and found faecal calprotectin to be higher than 50mg/ml in 27 subjects. They concluded that the GI tract in HIV-infected patients is impaired in the early phase of the disease, and that this impairment is associated with elevated levels of gut inflammatory markers.

Many factors contribute to GI dysfunction in HIV-infected persons [42]; frequent causes in low-income countries are different pathogens [23] or *Clostridium difficile* after protracted antibiotic treatment [42]. After endoscopy with biopsies, there are cases without an identified cause of a GI disorder; these are frequently referred to as HIV enteropathy. The exact pathogenesis of HIV enteropathy remains unclear, but has been attributed to dysegulation of local cytokine production and destruction of gut-associated lymphatic tissue (GALT), and HIV replication in residual GALT [43, 44]. It will be a combination of these

in most cases. HIV enteropathy can exist in HIV positive patients with refractory diarrhoea in a small percentage of the population, but OI must first be excluded [23]. Exclusion of OI has limitations as mentioned above. Over the time, since the identification of HIV, more pathogens have been identified and fewer changes in the GI tract are currently believed to be caused by HIV enteropathy [23].

1.3 Helicobacter pylori

1.3.1 Biology and origin

H. pylori is one of the most common causes of bacterial infection in human beings [45], with probably more than 50% of the world population being infected. It was first isolated and cultured by Warren and Marshall in 1983 [46], but spiral-shaped or curved bacilli were found in the human gastric mucosa already more than 100 years ago [47], with similar organisms being seen in the gastric mucosa of mammals as early as 1896 [48]. Initially it was termed Campylobacter-like, but was later assigned the name *Campylobacter pyloridis* because of its principal localization in the lower pyloric parts of the stomach. The name was changed to *H. pylori* in 1989, when taxonomic features of the bacterium shown that it was a genus of its own [49]. The role of *H. pylori* in duodenal and gastric ulcer disease was established and reported in a consensus statement in 1994 [50] as a result of the accelerating research on both microbiological aspects and pathogenic role of this newly discovered bacteria. In 1994, the International Agency for Research on Cancer (IARC) concluded, after numerous epidemiological studies investigating the link between *H. pylori,* gastric cancer and mucosa- associated lymphoid tissue (MALT)-lymphoma, that *H. pylori* is carcinogenic to humans and thus classified *H. pylori* as the first bacterial Group I carcinogen [51].

H. pylori is a Gram-negative, urease-, catalase- and oxidase positive bacterium that inhabits the mucous layer of the gastric mucosa of the stomach. The bacterium is about 3 micrometers long with a diameter of ~ 0.5 micrometer and has up to 6 sheeted polar flagella that are important for its mobility. The characteristic high urease activity is used by the bacteria to convert urea in the gastric juice to ammonia and bicarbonate. These products buffer the gastric acid and create a friendly microenvironment for *H. pylori* [52].

Only a small percentage of colonized individuals will express clinical manifestation. A proposed determinant for the outcome of colonization is the ability of the bacterium to attach to the gastric epithelium. Electron microscopy has confirmed the presence of the tight adherence of *H. pylori* to the gastric cell surface through formation of membrane attachment pedestals similar to those described with the enteropathogenic bacterium, *Escherichia coli* [53, 54].

Bacterial attachment is partially mediated by a number of adhesins and outer membrane proteins. Three Hop proteins have been implicated in the pathogenesis of *H. pylori* infection, BabA, OipA and SabA [55]. BabA mediates binding to fucosylated Lewis b (Le(b)) blood group antigens on host cells [56]. OipA may serve as an adhesin but it also promotes inflammation by increasing IL-8 expression [57]. SabA mediates binding to glycoconjugates containing sialic acid [58].

Functional differences exist between strains of *H. pylori* that may relate to virulence and tissue damage [59]. Vacuolating cytotoxin, vacA, is secreted by ~ 50% of *H. pylori* isolates in high-income countries. Infection with vacA positive strains is reported to be associated with gastric and duodenal peptic ulcerations, and gastric cancer [60, 61]. VacA behaves as a passive urea transporter that is potentially capable of increasing the permeability of the gastric epithelium to urea, thereby creating a favourable environment for *H. pylori* infectivity [62]. Virulence of VacA appears to depend upon the function of a tyrosine phosphatase receptor in gastric epithelial cells [62]. *H. pylori* strains with different VacA alleles have differing toxicity [63].

CagA protein is another putative virulence factor, encoded by the cagA gene (cytotoxin associated gene A) [64, 65]. This gene is present in ~ 60% of *H. pylori* isolates [66]. Although the function of CagA protein is unknown, the protein seems to be associated with peptic ulcer [67] and gastric cancer in Europe and North America [68, 69]. CagA -positive *H. pylori* strains are associated with interleukin-8 (IL-8) induction in gastric epithelium. Neutrophilic infiltration into the gastric epithelium, which is characteristic of *H. pylori* infection, may be due to the increased production of IL-8. CagA protein may therefore be related to gastric inflammation and gastroduodenal diseases [70]. Strains producing VacA and CagA cause more intense tissue inflammation and induce cytokine production [71-73].

1.3.2 Diagnostic modalities

The ^{13}Urea breath test [74, 75] or invasive methods, such as gastroscopy with biopsies and/or urease tests used to be the "gold standard" for detecting of *H. pylori*. The ^{13}Urea breath test is a very time- and personnel-consuming test, where much collaboration between investigator and patient is required. The patient have to stop taking acid-reducing medication 14 days in advance of the test, have to fast for 4-6 hours in advance of the test, then drink a juice before exhale into a bag often through a straw. This test has been reported to be done concurrently in young children and infants, but it is to some extend unsuitable for children. For the urease test and for culture of *H. pylori* a gastroscopy is required. To perform gastroscopy in children, general anaesthesia is required, and again this is time- and personnel-consuming, and invasive and unsuitable for children. The faecal monoclonal antigen test we used has high sensitivity, specificity, and accuracy in children, 91-96%, 95-96% and 94-96%, respectively [76, 77]. The faecal test can be performed in all age groups and gives rapid results without the need for sophisticated laboratory equipment. Acid-reducing medication can be continued. Common to all the tests mentioned above is the measurement of an ongoing *H. pylori* infection and *H. pylori* must be present at time of investigation.

Serological tests are also available, but in children they often show very low sensitivity and specificity [78-80]. A major drawback of serological tests is that they do not discriminate between current and past infections. A specific IgG may remain positive for years after the infection has been cleared. The joint European and North American guideline [81] does not recommend using serology in a clinical setting for children.

1.3.3 Epidemiology

H. pylori colonization is acquired early in life. The mode of transmission of *H. pylori* is not fully understood, and no certain environmental source has been identified [82]. In fact, it has been proposed that humans are the only reservoir of *H. pylori* [83]. Different routs of transmission are suggested, faecal–oral and oral-oral. Early colonization in children living in poor socio-economic conditions has been demonstrated, and several studies have shown a high prevalence of *H. pylori* among people in low-income countries [16, 84-86]. There is a substantial gap in prevalence in low- and high-income countries. In low-income countries, the prevalence of *H. pylori* infections often reaches 50% among 5-years old children, whereas in high-income countries the prevalence is low (<10%), table 2 and 3.

Table 2. Prevalence of *H. pylori* among asymptomatic children in sub-Saharan Africa

Population	Author	Comment [a]	Age (years)	Number [b]	% *H. pylori* +
Ethiopia	Lindkvist [87]	Serology	2-4	248	48
South-Africa	Pelser [84]	Serology	<2	104	13.5
			2-5	103	48.5
			5-10	104	84.2
Kenya	Langat [86]	Stool antigen	0-3	195	45.6
Tanzania	Mbulaiteye [88]	Serology	0-4	181	76
			5-9	180	93
			10-17	152	97
Cameron	Ndip[16]	Stool antigen	0<3	32	37.5
			3-6	106	50.0
			7-10	38	71.1
Nigeria	Holcombe [89]	Serology	<10	100	69
			10-19	43	91
Gambia	Sullivan [90]	Serology	1.5-3.3	136	27
			3.5-5	135	46

[a] Test method uses to identify *H. pylori* colonization
[b] Number of participants in the study

Table 3. Prevalence of *H. pylori* among asymptomatic children in other parts of the world

Population	Author	Comment [a]	Age (years)	Number [b]	% *H. pylori* +
Norway	Stray-Pedersen [91]	Stool antigen	0-7 days 7-30 days 1month-3 y	69 46 134	52 15 5
Sweden	Granstrom [92]	Serology	2 4 11	237 185 201	10.1 7.5 3.0
Germany	Grimm [93] Rotenbacher [94]	^{13}Urea breath Serology	7-20 6	540 475	9.4 5
Turkey	Abasiyanik [95]	Serology	1-9 10-19	33 56	42 55
Tunisia	K. Siai [96]	Serology	6-7	1055	51.4
Brazil	Kawakami [97]	^{13}Urea breath	0-1 2-5 6-10 11-20	86 273 313 529	12.8 19.4 28.8 46.3
Canada [c]	Segal [98]	^{13}Urea breath	5-18	214	7.1
Hong Kong [d]	Tam [99]	^{13}Urea breath	6-19	2480	13.1
India	Mishra [100]	Nested PCR in faeces	0-5 6-10 11-16	47 22 68	4.3 13.6 50.0

[a] Test method uses to identify *H. pylori* colonization
[b] Number of participants in the study
[c] Referred for upper endoscopy
[d] Chinese children residents in Hong Kong

1.3.4 Helicobacter pylori and HIV

There is little data on the prevalence in HIV-infected children, both in high- and low-income countries, table 4. To the best of our knowledge there is only one study from sub-Saharan Africa reporting the prevalence of *H. pylori* in HIV-infected children, and there are no studies performed in child populations showing differences in the prevalence of *H. pylori* according to CD4 cell count. The study, reporting the prevalence of *H. pylori* in HIV-infected African children, was designed to describe the findings in HIV-infected South-African children who underwent gastroscopy. They also reported the rates of *H. pylori* colonization, with only one out of 26 children was colonized [101]. Only two studies were done with the aim of assessing the prevalence of *H. pylori* in HIV-infected children [102, 103], none of them from endemic areas for HIV. A Belgian study [102] on 23 HIV-infected children of Central African origin, born in Belgium used a serology test to detect *H. pylori* colonization. They found none of the tested children to be colonized compared to 19.2% in a control population. An Italian study [103], using serology and ^{13}Urea breath tests in 45 perinatally HIV-infected children found prevalences of 17.7 and 20% respectively. In a review on the incidence of *H. pylori* in HIV-infected adults [104], diverging estimates of the prevalence of *H. pylori* were found. In a study from Argentina on adults [105], the authors

concluded that HIV-infected patients with *H. pylori* had a higher mean CD4 cell count than those without *H. pylori*, and a Zambian study [106] showed that HIV-infected adult patients with CD4 cell counts below 200cells/mm^3 were less likely to have positive *H. pylori* serology (odds ratio [OR] 0.29 [95% CI 0.09–0.93]). Many studies from sub-Saharan Africa have used serological tests [107-109], which have been shown to have a lower specificity in children.

Table 4. Prevalence of *H. pylori* among HIV-infected children

Population	Author	Diagnostic method(s)	Age (years)	Number [c]	% *H. pylori* +
South Africa	Cooke [101]	Endoscopy	0-12	26	3.8
Belgium [a]	Blecker [102]	Serology	1-15	23	0
Italy	Lionetti [103]	Serology + ^{13}Urea breath [b]	1-13	45	17.7 20.0

[a] Central African origin
[b] Serology and ^{13}Urea breath test were performed in all children
[c] Number of participants in the study

1.3.5 Clinical aspects

H. pylori is probably the major cause of recurrent chronic gastritis and duodenal ulcer disease in childhood. However, the association between *H. pylori* and recurrent abdominal pain (RAP) syndrome is still controversial. Until now, no association has been found between RAP and *H. pylori* infection [110]. A French group could not find any specific characteristics of symptoms in *H. pylori* infected children that had non-peptic dyspepsia [111]. Peptic ulcer disease is not particularly common among children, but is often caused by *H. pylori* [112]. Some studies also suggest that gastric infection with *H. pylori* is associated with sub-optimal nutrition and growth in childhood [15]. *H. pylori* has also been linked to iron deficiency anaemia [113, 114].

The connections between *H. pylori* infection and auto-immune thyroid disorder [115], and *H. pylori* infection and respiratory infection/acute otitis media [116], have been evaluated, but no significant association has been found. Furthermore, *H. pylori* infection is associated with cancer, but this is not often seen in children due to the long delay in developing the disease. *H. pylori* infections are associated with gastric cancer, MALT lymphoma and colon cancer, but the last one remains controversial.

1.3.6 Treatment of Helicobacter pylori in children

The first consensus report on treatment of *H. pylori* in European children [117] was published in 2002. The Canadian Helicobacter Study Group updated the Canadian consensus in 2005 [118]. Recently a joint evidence-based guideline [81] from the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) and the North American Society of Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) has been published. It aims to answer 4 questions: 1) Who should be tested? 2) What test should be used? 3) Who should be treated? and 4) What treatment regiments are most appropriate?. The guidelines stress that they only applies to children living in Europe and North America, and particularly not for low-income countries. There is no

general consensus on the treatment of children or for treating *H. pylori* in low-income countries with a high prevalence in children. A general treatment for all infected humans is not recommended. Only in *H. pylori* positive children with symptoms or GI ulcers demonstrated, is a tipple therapy is recommended. The combinations recommended in the joint guideline are a proton pump inhibitor (PPI) +Amoxicillin + Imidazole or Clarithromycin; alternatively Bismuth salts + Amoxicillin + Imidazole [81].

1.4 Faecal calprotectin

1.4.1 Biochemistry and origin of calprotectin

Calprotectin was discovered in 1979 by Fagerhol et al. [119] while searching for a simple assay for granulocyte turnover in vivo**.** Calprotectin is a calcium and zinc binding heterocomplex protein consisting of two heavy chains and one light one. It belongs to the S-100 protein family. The protein constitutes ~ 60% of the soluble proteins in the cytosol fraction of neutrophils [120]. In the early literature, it is also called MRP8/14 and L1 protein. Calprotectin has not been detected in B or T lymphocytes, erythrocytes, platelets or any non-myeloid cells of the intestines or pancreas [121, 122]. The protein is distributed in myelomonocytes, such as neutrophil granulocytes, monocytes and activated macrophages, and in submucosal epithelial cells [121, 122]. It is a putative protective protein that is remarkably resistant to degradation in vivo and in vitro in the presence of calcium, so faecal samples can be sent to the laboratory by mail. It is stable in faeces stored for 7 days at ambient temperature [123]. Calprotectin and its subunits appear to have regulatory functions in the inflammatory process, and various biological functions including antimicrobial and antiproliferative activity have been ascribed to the protein.

1.4.2 Faecal calprotectin analysis

A faecal calprotectin ELISA test has been available since 1994 [124]. Fagerberg et al. [125] have established a reference value for healthy Swedish children aged 4-17 years using an improved assay (Calprest®, Eurospital, Trieste, Italy) with greater sensitivity than previously available. Her conclusion was that the suggested cut-off level in adults, <50µg/g, can be used for children aged 4-17 years regardless of sex. In young infants, high faecal calprotectin concentrations are normal [126, 127]. In healthy pre-term babies the concentrations are comparable with those in healthy term babies [128, 129]. Studies diverge in their conclusions as to weather faecal calprotectin is higher in exclusively breast-feed children than in mix feed children [130, 131]. Faecal calprotectin decrease with age and in healthy children reaches stabile low levels as found by Fagerberg et al. at about 4 years of age.

1.4.3 Calprotectin in body fluids

Calprotectin is a normal constituent of human plasma. Normal levels of calprotectin in plasma are between 90 and 530µg/l in women and between 120 and 660µg/l in men [132]. Levels in plasma/serum increase in response to different types of inflammation or damage of tissue. Calprotectin is found in saliva and levels are different in parotid saliva and whole saliva [133]. The protein is found in cerebrospinal fluid in trace amounts [120]. High levels of calprotectin are found in synovial fluid in patients with rheumatoid arthritis, plasma levels reflecting disease activity [134].

1.4.4 Faecal calprotectin and gastrointestinal disease

Increased faecal calprotectin is a marker for increased permeability of the GI tract [124]. In very low birth weight babies (VLBW) developing severe abdominal disease, for instance necrotizing enterocolitis (NEC), faecal calprotectin concentrations tend to increase more than in VLBW babies without NEC, and it may be a marker for early diagnosis [128, 129]. Faecal calprotectin levels are elevated in adults [123, 135] and children [126, 136, 137] with inflammatory bowel disease (IBD) and might be used to evaluate the degree of inflammation in these patients. Faecal calprotectin is used in paediatric clinics today as a "screening" for IBD in children transferred with recurrent abdominal pain or recurrent/prolonged diarrhoea. Faecal calprotectin may differentiate between functional abdominal pain and IBD in school-age children [138]. A significant correlation is found between calprotectin concentration in gut lavage fluid and intestinal permeability, suggesting that increased intestinal permeability in IBD might be a consequence of increased transepithelial migration of neutrophils [139]. Faecal calprotectin is also elevated in patients with GI cancer [140] and NSAID induced enteropathy [141]. In diagnosed colorectal cancer, faecal calprotectin concentrations above an upper reference limit were found in more than 90% of the patients in a Norwegian study [123]. Lymphonodular hyperplasia in the gut is common in infants and young children [142, 143], it occurs frequently in children with food protein-induced colitis [144], disrupts the normal mucosa [142], and increased levels of faecal calprotectin can occur. Faecal calprotectin concentrations have also been analysed in children with acute gastroenteritis [145, 146]. The concentration is elevated when compared to healthy children, but not as high as in IBD. In an Italian study of children aged 1-18 years, referred for gastrointestinal symptoms, faecal calprotectin was higher in those with acute gastroenteritis, but these children had a considerably lower mean age than 4 years [146]. In a study of 46 children, with bacterial GI infections, faecal calprotectin was not elevated regardless of the pathogen compared to 23 healthy controls [147]. It is known that the permeability of the gastrointestinal tract can be increased by gastroenteritis. This can lead to a higher concentration of faecal calprotectin due to higher levels of granulocytes in the lumen. Faecal calprotectin is a sensitive, but not disease-specific marker useful in detecting inflammation throughout the whole gastrointestinal tract in children.

1.4.5 Calprotectin and HIV/AIDS

To the best of our knowledge, there are no published articles on faecal calprotectin in children living with HIV/AIDS, except the work presented in this thesis. In 53 HIV-infected, HAART naïve Italian adults, faecal calprotectin has been found elevated in half of the patient [41]. Patients with HIV developing oral candidiasis, had significantly lower parotid calprotectin levels than those who did not [148-150]. Significantly elevated levels of calprotectin were found in the serum of 51 HIV-positive patients, both in asymptomatic patients and patients who had developed AIDS, compared with controls. The calprotectin level was not related to ongoing or recent opportunistic infection [151]. Calprotectin levels in cerebrospinal fluid have been studied in patient with HIV who developed symptoms of the CNS. Patients with opportunistic CNS-infections had levels above the reference limit, and patients with HIV-associated encephalopathy had levels within the reference range [152], but the sample size was low (15 patients).

2 Aim and objectives

2.1 Overall aim

The overall aim of this thesis was to assess the prevalence of *H. pylori* in Ugandan children, and to explore whether faecal calprotectin can be used in the clinical management of HIV in Ugandan children.

2.2 Specific objectives

The specific objectives were to:
a) Assess the prevalence and associated factors with *H. pylori* in apparently healthy children aged 0-12 years in Kampala, Uganda (Paper I).

b) Assess the prevalence and associated factors with *H. pylori* in HIV-infected, HAART naïve children aged 0-12 years in Kampala, Uganda (Paper II).

c) Establish reference values for faecal calprotectin concentrations in apparently healthy children aged 0-12 years in Kampala, Uganda (Paper III).

d) Determine faecal calprotectin concentrations in HIV-infected, HAART naïve children aged 0-12 years in Kampala, Uganda (Paper IV).

e) Study the correlation between the concentrations of faecal calprotectin and CD4 cell percentage in HIV-infected, HAART naïve children aged 0-12 years in Kampala, Uganda (Paper IV).

3 Methods

3.1 Study area and population

Uganda is a landlocked country in East-Africa; its neighbouring countries are Kenya, Sudan, Democratic Republic of the Congo, Rwanda and Tanzania, figure 1. Approximately 50% of the population is living below the international poverty line of US $ 1.25 per day, and the gross domestic product (GDP) per capita is ~ US $ 500. In 2008, the population of Uganda was estimated at 30 million people of whom ~ 1.5 million live in the capital city Kampala (www.ubos.org). Kampala has five divisions, one of them being Kawempe, figure 2. Kawempe division houses 22% of Kampala's population. Both surveys were carried out in Kawempe division.

Figure 1. Uganda with bordering countries

Figure 2. The 5 divisions of Kampala: Kawempe, Rubaga, Central, Nakawa and Makindye. The community-based survey was carried out in Kawempe

3.2 Design, sampling and data collection

The data in this thesis are based on two surveys conducted in 2007 and 2008;

1. Apparently healthy children
2. HIV-infected, HAART naïve children

Table 5. Connections between the two study populations and the test used inn all 4 papers

Two populations / Two tests	Apparently healthy Ugandan children	HIV-infected Ugandan children
Helicobacter pylori -faecal monoclonal antigen test	Paper I	Paper III
Faecal calprotectin -ELISA	Paper II	Paper IV

Both surveys were conducted in Kampala, Uganda. Both surveys included children aged 0-12 years, figure 3.

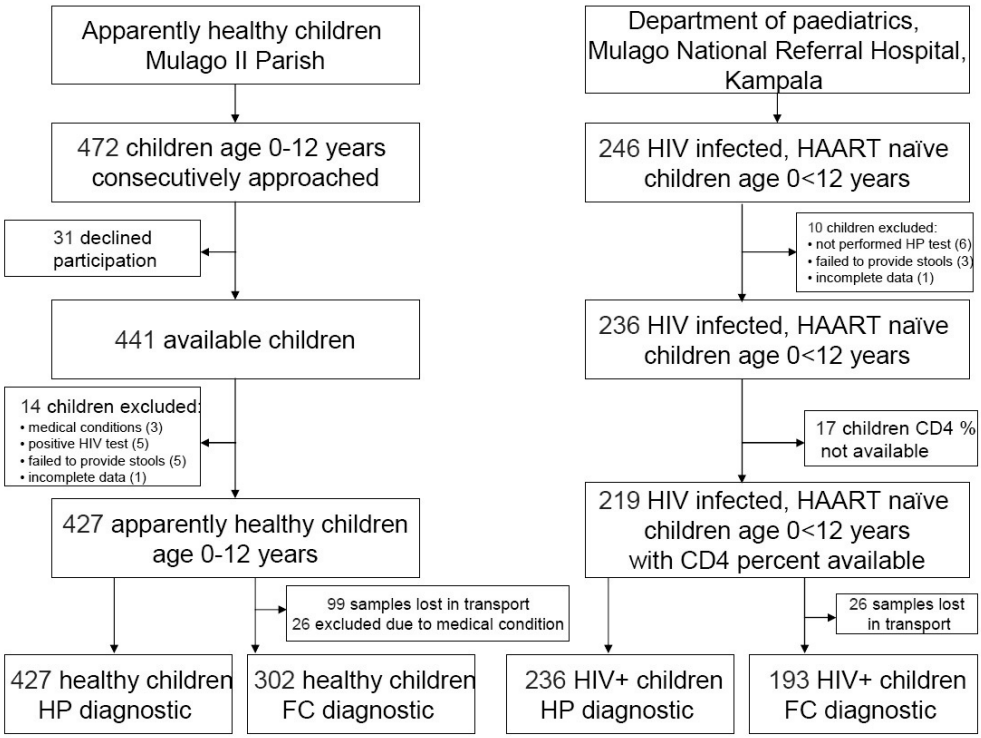

Figure 3. Enrolment and study profiles of both surveys

3.2.1 Apparently healthy children

A cross-sectional survey was conducted by door-to-door visits in October and November 2007. The study was carried out in all zones of Mulago II parish, one of the 22 parishes of Kawempe division, and one of 5 divisions in Kampala. The data collection was done within six weeks by eight data collectors. Elin Hestvik accompanied the data collectors in the field daily.

(Picture from www.maps.google.com)

Figure 4. Settlement of the Kawempe division

3.2.2 HIV-infected, HAART naïve children

This was a hospital-based survey. The hospital is situated within the Kawempe division and it is owned and run by the Ugandan government. It is the district hospital for people living in the area of Mulago Hill at Kawempe division, at the same time being the national referral hospital for the whole of Uganda. Participants were enrolled from the general paediatric medical wards, the acute care unit, the ward for malnutrition and the paediatric infectious diseases clinic (PIDC) at Department of Paediatrics, Mulago National Referral Hospital, Kampala between February and October 2008. The daily enrolment and investigations of participants were done by an Ugandan medical doctor employed for the project. The data collection was monitored by Dr. Grace Ndeezi on site and Elin Hestvik. For HIV-infected children the enrolment period was set at 9 months prior to the study, according to experience in the paediatric clinic and the number of children who had been newly diagnosed with HIV monthly. We had to accept a low number of HIV-infected, HAART naïve older children due to the natural history of HIV.

Figure 5. A general paediatric medical ward at Department of Paediatrics, Mulago National Referral Hospital, Kampala.

3.3 Statistical procedures

3.3.1 Sample size

OpenEpi (www.openepi.com) was used to calculate the sample sizes based on assessing a single proportion with a narrow confidence interval. In the survey of apparently healthy children we assumed a 50% prevalence of *H. pylori* colonization in the apparently healthy children, and in the HIV-infected children we assumed 30%. A 95% CI was used for the estimates. The sample sizes were calculated with the formula:

$$n = [DEFF*Np(1-p)]/ [(d2/Z^2_{1-\alpha/2}*(N-1)+p*(1-p)].$$

DEFF= design effect=1 (we did not had to adjust for cluster sampling)
N= total population size is large, assumed to 1million
p= prevalence, assumed prevalence of *H. pylori* was 50%
d= confidence limits (absolute precision) fixed to 5%
α= 0.05 by a CI 95% as we used
Z = Z value (e.g. 1.96 for 95% confidence level)

This gave us a sample size of 384 apparently healthy children and 323 HIV-infected children. Another 10% was added to allow for contingency in the survey of apparently healthy children, a total of 422.

3.3.2 Statistical procedures and data management

Data from the questionnaires and biochemistry were doubly entered using EpiData version 3.1 (www.epidata.dk). The data were exported to SPSS version 15.0 for paper I and version 17.0 for paper II-IV for statistical analysis. Data quality was ensured through careful selection and training of research assistants, supervision, field editing by use of the "check"

module at data entry, and double data entry and validation. The "checks" at data entry were limits set by the study team to ensure that it was impossible to enter clearly inaccurate information. A child could only be between 48 and 180 cm; it is impossible to enter other data and many answers could only be "yes" or "no". After entering all data twice in separate files, the 2 separate data files were validated by comparison and any non-matching data were checked manually against the original paper form.

To assess the prevalence of *H. pylori* and its association with other factors in paper I and III, binary logistic regression as well as multiple logistic regressions were performed. Findings are presented in odds ratios with their confidence intervals (CI).

In paper II and IV, faecal calprotectin concentrations were expected to have a skewed distribution; the median was used. Faecal calprotectin values in the different groups were compared by using the Mann-Whitney U test (for two different groups) and by the Kruskal-Wallis H test (for three or more groups). To control for diarrhoea as a confounder for a low CD4 cell percentage in the HIV-positive group, linear regression was used.

The confidence interval reported was set to 95%. All tests were 2-sided, p-value of 0.05 or less was considered significant. Age was reported in mean and years with standard deviation (SD). Principal component analysis was used, to explore the socio-economic status of the participants.

In paper III and IV CD4 cell percentage was classified as high or low with limits defined by age: 1) for children <12 months high if CD4 cell > 25%, 2) for 12<36 months high if CD4 cell > 20% and 3) for ≥36 months high if CD4 cell >15%. The limits chosen were consistent with the limits recommended for starting HAART according to the WHO guidelines available at the time of the study [26]. All children were clinically categorized using the WHO staging system [153] for HIV-infected children. For further details on the WHO staging see Appendix VI.

3.4 Helicobacter pylori antigen test

For the detection of *H. pylori* in both surveys, an antigen test called ImmunoCard STAT!HpSA was used. It is a rapid 5 minutes immunoassay based on a lateral flow chromatography technique using a monoclonal antibody for the qualitative detection of *H. pylori* antigens in human stool. Unlike serological tests, the detection of *H. pylori* antigens in the stool identify a current (= ongoing) infection using a truly non-invasive method. It can be used for diagnosing the infection, as well as for confirming eradication four weeks after the end of the therapy.

For both surveys, the stool specimen was collected in empty air-tight container and was stored in the fridge until the same afternoon or next morning before testing.

The manufacturer instructions were followed; the applicator stick of the diluent vial was used to transfer a small portion (5-6 mm in diameter) of stool specimen into the sample diluents, which was vortexed for 15 seconds. The tip of the vial was broken off, and four drops were dispensed into the round window at the lower end of the device before reading the result after five minutes. The results were interpreted as followed:
Negative: one BLUE line (control)
Positive: one BLUE line (control) and one PINK-RED line (test).
After every 20 tests, a positive control was run.

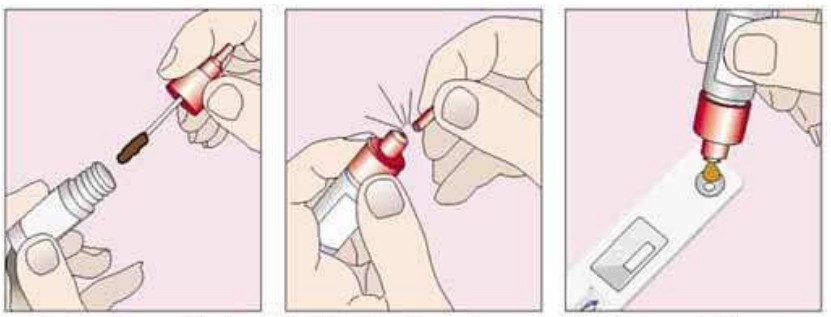

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Figure 6. Rapid method for *H. pylori* antigen detection

3.5 Faecal calprotectin measured by ELISA technique

For measuring faecal calprotectin concentrations in both surveys, we used the CALPROCalprotectin ELISA Test (ALP), a quantitative method for the determination of calprotectin in stool samples.

After taking stool from the air tight container for *H. pylori*, microscopy and culture, faeces were frozen within 48 hour after collection in Eppendorf tubes, taking ~ five gram at -80°C. At the end of both surveys, the faeces were transported frozen to Bergen, Norway on ice for the analysis, which was done in July 2008 (for apparently healthy children) and august 2010 (for HIV-infected children).

We followed the manufacturer instructions. Faeces were defrosted at room temperature, ~100 mg were placed in a screw-cap tube and diluted at the ratio1:50 with extraction buffer. The solution was vortexed for 30 minutes. 1-2 ml of the homogenate was transferred to an Eppendorf tube and centrifuged at 10.000g for 20 minutes. About 0.5 ml of the clear supernatant was transferred to a new tube and stored at +4°C until the next day. The extracts were diluted 1:50 before running. 50 µl of the 8 standards, the controls and the diluted samples were added to the ELISA plate (96 wells). The plate was covered with a sealing foil and incubated at room temperature for 45 minutes. It was washed 5 times and 50 µl of the conjugate was added to each well. The plate was again sealed and washed. 100 µl substrate solution was added to each well and thereafter incubated at room temperature for 20-30 minutes. 100 µl stop solution was added to each well. The optical density values were read by the means of an ELISA reader at 405 nm. The values of the diluted samples were corrected for the dilutions and converted to mg/kg by multiplying by 2.5.

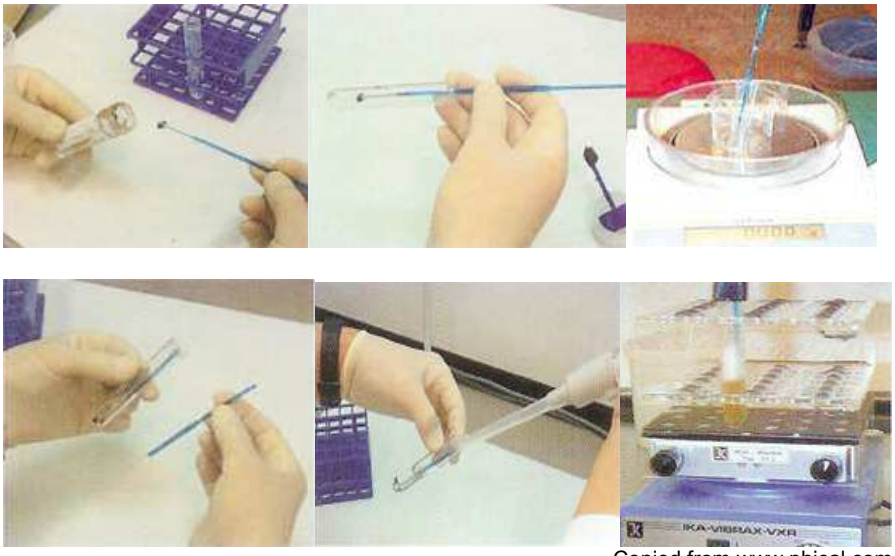

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Figure 7. ELISA techniques for determination of faecal calprotectin levels

3.6 Ethical considerations

The research protocols for the apparently healthy children and the HIV-infected children were approved by the Regional Committee for Medical and Health Research Ethics, West-Norway (REK-VEST) in Norway and Makerere University, Faculty of Medicine, Research and Ethics Committee in Uganda**.** Oral and written information about the study was given to the caretakers either in English or the local language. Informed consent was obtained from all the caretaker of the participants in the study. The data collectors were trained in ethical issues prior to the study.

HIV testing followed the Ugandan national guidelines [154] which closely follow the WHO guidelines. Children over 18 months were tested using a rapid blood test with a sensitivity rate > 98%. To confirm positive results, a second test with a different antigenic specificity was used. If there was discordance between the 2 tests, an ELISA test (tie-breaker) was used to make a final diagnosis. For children under 18 months of age, a polymerase chain reaction (PCR) test was used to give a reliable HIV diagnosis.

Children found to have symptoms of *H. pylori* and a positive antigen test, were offered a triple treatment of amoxicillin/claritromycin/omeprazole for 1 week free of charge. All participating children were offered a deworming treatment (if not treated in the last 6 months) after providing the faecal sample. The treatment for the children in the hospital was not influenced by their participation in the survey.

4 Summary of results

Paper I: "*Helicobacter pylori* in apparently healthy children aged 0-12 years in urban Kampala, Uganda: a community-based cross sectional survey"

The overall prevalence of *H. pylori* in the 427 children was 44.3% (189/427). Early colonization was common, 28.7%, in children younger than 1 year of age. The age specific rates were 46.0% in children aged 1<3 years, 51.7% in children aged 3<6 years, 54.8% in children aged 6<9 years, and 40.0% in children aged 9<12 years (Figure 2, Paper I). There was a significant difference in prevalence by gender; female 38.5% versus male 49.8%, and by type of housing; permanent house 38.5% versus semi-permanent house 48.6%. Congestive living and education level of the female caretaker showed a clear trend for a difference in prevalence. Factors independently associated with *H. pylori* colonization included; drugs taken in last three months, using a pit latrine, sources of drinking water, and wealth index (Table 2, Paper I).

The prevalence of *H. pylori* colonization among urban Ugandan children is high at an early age and increases with age. The impact of *H. pylori* colonization on children's health in Uganda needs to be further clarified.

Paper II: "Faecal calprotectin concentrations in apparently healthy children aged 0-12 years in urban Kampala, Uganda: a community-based cross sectional survey"

In the different age groups, the median faecal calprotectin concentrations were 249mg/kg in 0<1 year (n=54), 75mg/kg in 1<4 years (n=89) and 28mg/kg in 4<12 years (n=159) (Figure 2, Paper II). There was no significant difference in faecal calprotectin concentrations and education of the female caretaker, wealth index, gender, habits of using mosquito nets, being colonized with *H. pylori* or having other pathogens in the stool (Table 2, Paper II).

Concentrations of faecal calprotectin among healthy children, living in urban Ugandan, a low- income country, are comparable to those in healthy children living in high-income countries. In children older than 4 years, the faecal calprotectin concentration is low. In healthy infants, faecal calprotectin is high. The suggested cut-off concentrations in the literature can be used in apparently healthy Ugandan children. This finding also shows that healthy children living under poor circumstances do not have constant inflammation in the gut. We see an opportunity to use this relatively inexpensive test for further understanding and investigations of gut inflammation in children living in low-income countries.

Paper III: "Prevalence of *Helicobacter pylori* in HIV-infected, HAART naïve Ugandan children: a hospital-based survey"

236 children (121 female, 115 male) with a mean age of 2.9 ± 2.8 years were enrolled. The prevalence of *H. pylori* antigen was 22.5%. Age specific prevalence's were; 1) for 0<1 year 14.7%, 2) for 1<3 years 30.9%, 3) for 3<12 20.7% (Table 1, Paper III). Low CD4 cell percentage was significant associated with a lower prevalence of *H. pylori*; 12.5% versus 30.4% (Table 2 Paper III). Having HIV WHO stage I (37.5% *H. pylori* colonization) versus WHO stage II-VI (20.8% *H. pylori* colonization), was associated with a 2.3 fold higher prevalence of *H. pylori* (OR=2.3; 95% CI [0.9-5.6]. There was a trend for a difference in prevalence of *H. pylori* in children who had taken antibiotics for the last two weeks (21.6%) versus those who had not (35.7%). There was no significant difference in prevalence by gender, type of housing, congested living, education of female caretaker, drinking water sources or toilet facilities.

HIV-infected, HAART naïve urban Ugandan children had a lower prevalence of *H. pylori* colonization compared to healthy urban Ugandan children. Children with a low CD4 cell percentage and advanced clinical stage of HIV had an even lower risk of *H. pylori* colonization. Treatment with antibiotics due to co-morbidity with infectious diseases is a likely explanation of the relatively low prevalence.

Paper IV: "Faecal calprotectin concentrations in HIV-infected, HAART naïve Ugandan children: a hospital-based survey"

The median faecal calprotectin concentrations in the 193 HIV-infected children, aged 0-12 years in the hospital-based survey 208mg/kg (0<1 year), 171mg/kg (1< 4 years) and 62mg/kg (4< 12 years) (Figure 2, Paper IV). There was a significantly lower faecal calprotectin concentration in the oldest age group. Thirty four of 59 (57.6%) children older than 4 years of age had faecal calprotectin above 50 mg/ml, and 25 of 59 (42.4%) had faecal calprotectin above 100mg/ml. Children with a high CD4 cell percentage had a significantly lower median faecal calprotectin than those with a low CD4 cell percentage. Children older than 4 years with diarrhoea at enrolment had a significantly higher faecal calprotectin concentration than those without diarrhoea. There were no significant differences between concentration of faecal calprotectin and WHO stage, fever on examination, colonized by *H. pylori*, education of the female caretaker, drinking water or child's habit of using mosquito net.

HIV-infected children older than 4 years had a higher median concentration of faecal calprotectin than apparently healthy children in the same geographical area. They had a higher average faecal calprotectin concentration than the recommended cut-of. Children with more advanced disease had higher faecal calprotectin concentrations regardless of age. More research is needed to see whether faecal calprotectin can be a marker for GI dysfunction in HIV-infected children. We hypothesise that a new tool for investigation of gut engagement in HIV-infected children is available.

5 Discussion

5.1 Methodological issues

The strengths and limitations of the two cross-sectional surveys building the foundation of this thesis and how they appear in the four papers are summarized in table 6. We have defined 3 main categories where strengths and limitations are potential; 1) Relating to precision, 2) Relating to internal validity, and 3) Relating to external validity. The details are discussed in-depth in the following.

5.1.1 Study design

The four papers in this thesis are based on two cross-sectional surveys; one population-based and one hospital-based. A cross-sectional study gives information on a community at just one point in time, and is so suitable for measuring the prevalence of, but not the incidence of a disease. Another limitation of a cross-sectional study is that it cannot assess causal relationships, only associations. The outcome of a cross-sectional study depends on the sampling method used. The aim, of paper I and III, was to assess the prevalence of *H. pylori* in apparently healthy and HIV-infected children. We also wanted the healthy population to reflect the children coming to the hospital and therefore we chose to carry out the community-based survey in the neighbouring slum areas of the hospital. This selection should be representative of an urban low-income population of Kampala. The aim of paper II was to assess whether cut-off values for faecal calprotectin used in children in high-income countries were also valid in children in low-income countries. As we found faecal calprotectin in apparently healthy children within the recommended cut-off value, we set out to explore whether it could be a useful method in HIV-infected children, paper IV. We used a cross-sectional design to perform the baseline study both in the apparently healthy children and the HIV-infected children since surveys are often used by finding baseline values.

Both surveys were conducted in an urban area of the biggest city in Uganda. Some of the children in the hospital-based survey came from a more rural area after longer travelling, but most children were from urban Kampala. We do not have a rural population to compare with, which limited our findings in term of the generalization to the rural areas of the country and also rural areas in other sub-Saharan African countries. We have also studied only hospitalized HIV-infected children, which also limits the generalization to non-hospitalized HIV-infected children.

Table 6. Methodological characteristics: Strengths and limitations of the studies

Methodological characteristics	Paper I	Paper II	Paper III	Paper IV
Relating to precision				
Sample size	Adequate	Adequate	Reduced	Adequate
Precision of the estimates of major findings	High Narrow CI's	High Narrow CI's	High Narrow CI's	High Narrow CI's
Laboratory tests used [a] -Sensitivity -Specificity -Accuracy	High High High	High Low High	High High High	High Low High
Feasibility of the tests [a]	Excellent	Good	Excellent	Good
Relating to internal validity				
Selection of the participants: -Inclusion -Refusals -"Drop-outs" -Potential for selection bias	Consecutive door-to-door Few Few Small	Consecutive door-to-door Few 99 samples lost Small	Consecutive admitted children Few Few Small	Consecutive admitted children Few Few Small
Potential for recall bias	Limited	Limited	Limited	Limited
Efforts to reduce information bias	Training and pilot prior to study, PI in field daily	Training and pilot prior to study PI in field daily	Regular data quality controls	Regular data quality controls
Efforts to reduce random error	Daily data entry Double data entry Data cleaning Field editing by "Checks" [b] Use of statistics	Daily data entry Double data entry Data cleaning Field editing by "Checks" [b] Use of statistics	Early data entry Double data entry Data cleaning Field editing by "Checks" [b] Use of statistics	Early data entry Double data entry Data cleaning Field editing by "Checks" [b] Use of statistics"
Efforts to reduce Confounding	Multiple logistic regression	Children with diarrhoea and nose bleeding were excluded	Multiple logistic regression	Linear logistic regression
Relating to external validity				
Study design	Cross-sectional	Cross-sectional	Cross-sectional	Cross-sectional
Generalizability	Urban children	Urban children	Urban children Hospitalized HAART naïve	Urban children Hospitalized HAART naïve

[a] *H. pylori* monoclonal antigen test and ELISA faecal calprotectin
[b] "Checks" is explained in chapter 3.2.2
FC Faecal calprotectin
PI Principal Investigator
CI Confidence Interval

5.1.2. Limitations by comparing the results of the two surveys

Two surveys were conducted in two different populations, apparently healthy children and HIV-infected children. Comparing the results of the two surveys have limitations, but the two studies also have similarities, making a comparison reasonable. Both study populations had children aged 0-12 years, similar gender distributions. The same antigen test and test for detection of faecal calprotectin was used, and both studies were performed in urban areas. Sanitation conditions did not differ much in the two populations. The limitations in comparing them were that one was a community-based with home visits and the other was a hospital-based. In the community-based study, 39% of the participants had taken antibiotics in the last 3 months versus 74% in the hospital-based study.

5.1.3 The sample size

A sample size calculation was done prior to both studies. Sample size calculations were based on reported prevalences of *H. pylori* in children and adults in other sub-Saharan countries. The spread of reported prevalences is large, table 2, and therefore we decided to use 50% prevalence for the sample size calculation, which maximises the sample size and minimises the risk of having a too small sample. In HIV-infected children, there are few reports on the prevalence of *H. pylori*, table 4, but according the literature a lower prevalence than in the apparently healthy children. Open-Epi data was used (http://www.openepi.com) to calculate the sample size needed in the apparently healthy children, assuming a 50% prevalence and using a 95% confidence interval. We calculated the sample size for the whole study population, but did not calculate the power needed for analysis of subgroups in advance of the studies. In paper I, we were able to enrol the sample calculated in advance. In the HIV-infected group, paper III, a 30% prevalence and a 95% confidence interval were assumed, we would have needed 323 children. The study was of 236 HIV-infected children. If a prevalence of 22% had been assumed in advance, the sample size calculation would have given us a sample size of 264 children.

For faecal calprotectin, no power calculation was done prior to the studies. We based our assumptions on the literature where no studies with higher participant numbers than those needed for *H. pylori* calculations were found. It was reasonable to use the median, instead of the mean, in paper II and IV because of the expected skewed distribution of faecal calprotectin.

5.1.4 Validity

The results of any research are only as trustworthy as the data upon which they are based. Data may be affected by the study participants, instruments used, people's memories and biological variation. As a result, no epidemiological study will ever be perfect except to minimise errors as far as possible, and then assess the practical effects of any unavoidable error. The validity of an epidemiological study depends on the study design, the study conducted and data analysis. The validity of a test or an instrument is based on its ability to measure what it is intended to measure. The validity of a questionnaire is based on how

close the information collected is to the truth. Validity of a study can suffer due to bias. Validity of a study can be internal and external validity [155, 156].

Internal validity
Internal validity of a study says something about how it provides an unbiased estimate of what it claims to estimate. The usual threats to internal validity are 'selection bias', 'information bias', confounding and chance, which needs to be kept to a minimum either through the study design or the data analysis. In the following paragraphs we will discuss the internal validity of the two surveys.

Selection bias
A selection bias is an error in selecting the study participants. Ideally, to assess the *H. Pylori* prevalence in a population, all subjects in that population should be studied, but this is too time-and money-consuming.

In paper I and II we did a consecutive enrolment in the study conducting door-to-door-visits, omitting homes with no caretaker at home. This could potentially lead to a selection bias where we maybe have selected the poorest families being at home due to unemployment. In addition, we carried out data collection on Saturdays to try to minimise this. Daily assessment of non-participation revealed on average one to three homes out of ~30 homes with no identifiable caretaker, i.e. less than 10%. We therefore consider the risk for an important selection bias to be small.

In paper III and IV the enrolment was consecutive in the paediatric department; all children admitted who met the inclusion criteria were invited to participate in the study. Remarkably few refusals were reported, and therefore we consider our sample to be representative of children attending the hospital. For paper II, we lost 99 samples, and in paper IV, we lost 26 samples in the transport. By comparing the subjects whose samples were lost with the main sample, by age, sex, socio-demographic factors and medical history, they do not differ essentially from those where faecal calprotectin was analysed. If we had used all the samples, the 95% confidence interval calculated in both papers would have been narrower. In paper IV, if more accurate data on faecal calprotectin had been available, maybe a connection to the WHO staging could have been found, and maybe the difficult explainable sex-difference would have been eradicated. We consider our findings of higher faecal calprotectin in children with more advanced disease, are valid as we had a large sample size (193).

Information bias
An information bias, also referred to as a measurement bias, have several aspects; 1 instrumental errors, 2 underlying variability, 3 respondent errors, 4 observer errors and 5 data-processing errors. Effort must be made to reduce the information bias in a study; high degrees of information bias reduce its precision.

Ad 1 and 2: The faecal monoclonal antigen test we used for detecting *H. pylori* in paper I and III, has a high sensitivity, specificity, and accuracy in children, 91-96, 95-96 and 94-96%, respectively [76, 77]. The test is very easy to use and read. A standard positive control test was performed after every 20 tests, all being verified as positive. The test used for

detecting the levels of faecal calprotectin in paper II and IV, is widely used in the clinic, and a good reproducibility was shown by our group in paper II. The technician who helped in performing the test did this as a part of her routine work. We found support in the literature that faeces could be frozen before testing [157].

The questionnaires used were not influencing the prevalence of *H. pylori* or the levels of faecal calprotectin measured, but they may have been less precise in assessing socio-economic status. We tried to minimize this error by creating a wealth index using principle component analysis (PCA). We combined 12-15 different assets in the households for the PCA.

Ad 3: We asked questions that required recall: "Has the child had diarrhoea at any time within the last two weeks?" or "Has the child taken any antibiotics within the last three months?" These questions were followed up by linked questions such as "Has your child seen a doctor in the last two weeks/three months?" The data collectors cross-checked the answers if there were inconsistencies in the answers. They tried to achieve consistency without telling the responders what they were seeking. Some questions were prone to eliciting socially desirable answers, for instance the question "Is your child using a mosquito net regularly?". The fact that only 33% responded "yes" to this question shows that social desirability did not gravely bias our studies. For other questions like the status of the sanitation facilities, we requested the data collectors to check these.

Ad 4: To minimize the observer errors, we had a one-week training session prior to the study about the healthy children, and we had a two-day pilot training which was evaluated in advance of the study. Errors that occurred were corrected in the study. To minimize the observer errors concerning the HIV-infected children, E. Hestvik went trough the questionnaires regularly and discussed this with G. Ndeezi, who again had discussions with the data collectors.

Ad 5: To reduce the errors in the data processing, we used logical coding such as "0" for No and "1" for Yes. Data was entered twice, once from the data collector, and once from E. Hestvik which were validated. We used "check" module for the data entering. Even if effort was made to minimize the measurement errors, it is unlikely that all errors were identified.

Confounding
Confounding is a variable that might influence the results without being the component measured. Typical confounders are age, sex and factors linked to each other. Confounding can be understood as a triangle:

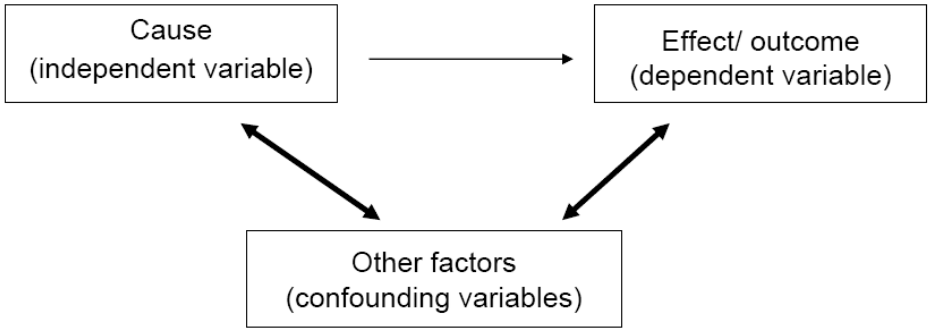

Figure 8. Confounding in a cross-sectional survey

We have tried to control for confounding by using multiple logistic regression and adjustments for several factors (shown in table 2 in paper I and table 2 in paper III). After adjustment in the apparently healthy children, we still found a higher prevalence of *H. pylori* in older children. In paper III, we still found a lower prevalence in those with more advanced disease. The unadjusted and adjusted ORs are not significantly different from each other. In paper II, we did not control for confounding by statistical analysis, but all children with nose bleeding or diarrhoea within last two weeks before enrolment were excluded from the analysis. We do not consider that any important confounding factors are attributable in this paper. In paper IV, we controlled in all children and in the subgroup of children older than 4 years whether diarrhoea at enrolment was not a factor on its own but a confounder for a low CD4 cell percentage (data not shown). From linear regression analysis there was no significant change in the median faecal calprotectin with 95% CI after adjusting for diarrhoea. We also adjusted for all factors shown in table 3 in paper IV by linear regression (data not shown), but there were no significant changes in median faecal calprotectin with 95% CI.

Chance
If the results presented in this thesis are not explained by the abovementioned factors, can they be the result of chance? In order to assess this, all results are reported with statistical indicators of the possibility of chance, mainly the 95% confidence intervals. With the rather high numbers of children studied, our confidence intervals are narrow, which means it is unlikely that the results are due to chance.

External validity
Can the results be generalised to a larger population than the one we have studied? The results from paper I and II are found in apparently healthy children, living under slum-like conditions with overall poor sanitation conditions in urban areas. We have to be careful using results generalized to other parts of Uganda or East-Africa, where living conditions may be quite different. In paper III and IV the children were hospitalized and probably

sicker than the average HIV-infected child. They had probably received more treatment for opportunistic infections compared to the average HIV-infected child, several were in clinical WHO stage IV. Today the paediatric HAART treatment is being scaled up in Uganda and other sub-Saharan countries, and we will need to conduct other studies to assess the effect of this implementation on the prevalence of *H. pylori*. The effect on the GI tract has to be evaluated in the light of increased accessibility to HAART.

In paper I, we have found an overall prevalence of *H. pylori* of 44.3%, increasing with age, associated with poor living standard and male gender. Our findings are supported by the literature, but it is expected that the exact prevalence will show variations from place to place. In paper III, we have shown that the prevalence of *H. pylori* in HIV-infected children is about half than in apparently healthy children. Several studies have highlighted the low prevalence of *H. pylori* in their findings. Our study adds to a growing body of evidence that HIV-infected children are less frequently infected with *H. pylori*.

Surprisingly few studies have focused on faecal calprotectin in populations in low-income countries, only one report being found [158]. More research is needed before any of the results in those two papers can be generalized.

5.2 Discussion of the findings

5.2.1 Prevalence of *Helicobacter pylori*

The prevalence of *H. pylori* has been studied extensively since its discovery in 1983 [46], and many surveys have been conducted in low-, middle- and high-income countries. Comparing studies, however, is not easy as the study settings, the diagnostic methods, age of participants and the criteria for inclusion and exclusion differ. Very few studies have been carried out focusing on children, particularly apparently healthy children and no studies have looked at urban children specifically. In HIV-infected children, living in high endemic regions both for HIV and *H. pylori,* no larger studies have been conducted. Commonly, there is a mix of participants with and without different HAART regimes [101]. Many studies are conducted in countries with a low prevalence of HIV and good treatment options for HIV [102, 103]. One sub-Saharan study was performed with children reporting to have gastrointestinal complications [101]. As far as we know, no prevalence studies of *H. pylori* in Ugandan children had been carried out before our surveys.

We found an overall prevalence of 44.3% in the apparently healthy population, with an increasing prevalence with age. In the HIV-infected children, the prevalence was 22.5% with the highest prevalence among children aged 1-3 years. A comparison of the prevalences in the surveys is shown in Figure 2, Paper III. The prevalence found in apparently healthy children compares well with the range of prevalences reported from other sub-Saharan African countries [15, 16, 84-86, 88, 107-109].

In HIV-infected children, the literature is much more limited, and a comparison is more complicated. To the best of our knowledge, only three papers have reported the prevalence of *H. pylori* in HIV-infected children [101-103], and none in exclusively HAART naïve children. Despite the difference found in prevalence rates of *H. pylori* among HIV-infected children and adults, many studies point to a lower prevalence in the HIV-infected population compared to non-HIV-infected populations, but the findings are not universal. The controversies are well documented in table 1 in the review by Romanelli et al. 2007 [104]. Our survey is one of the largest studies carried out in an HIV-infected population. In contrast, almost all studies show that the prevalence of *H. pylori* in HIV-infected children is lower, especially in areas with a high prevalence of HIV and *H. pylori*. We found that *H. pylori* colonization was significantly higher in children aged 1-3 years compared to children younger than 1 year of age. This is comparable to data from apparently healthy children from the same region [159], but it has not been described previously among HIV-infected children in this age group. In our survey, the prevalences in all age groups are lower than those in apparently healthy children. We speculate that the colonization rates among the HIV-infected are the same as in apparently healthy children, but the use of antibiotics in the HIV-infected children is high, which is enough to decrease the prevalence. Seventy-four percent of the children had taken antibiotics within the last 3 months and 95% of the survey participants were on antibiotics at enrolment or had taken antibiotics within the preceding two weeks, which might explain the lower colonization rates. The use of antibiotics against opportunistic infections in HIV-infected populations is the most hypothesised explanation for lower colonization rates [104, 160, 161]. The difference between HIV-infected children

not treated or treated with antibiotics is not significant mainly due to the low numbers of untreated subjects.

5.2.2 Factors associated with *Helicobacter pylori* colonization

Several studies have shown a high prevalence of *H. pylori* among people in low-income countries [16, 84-86]. Well recognized risk factors include crowded living conditions in childhood, a large number of siblings and unclean water [86, 162]. A meta-analysis of 10 studies conducted over the last 20 years [163] failed to find a difference in prevalence of *H. pylori* according to gender. In apparently healthy children in our survey, the factors found to be significantly associated with *H. pylori* colonization were male gender and semi-permanent housing. Those living in semi-permanent housing might represent a lower socio-economic status than those living in permanent houses (table 2 Paper I). Congested living with more than 4 people in the household was associated with an increase in *H. pylori* prevalence, but it was not significant. We did not found that the prevalence of *H. pylori* colonization was increased with decreasing socio-economic status of the family as found in other studies from the same region [16, 86, 164]. A possible explanation is the small socio-economic differences in our study population; the study was conducted in one parish only and within an area characterized by informal settlements, congested living, lack of proper sanitation conditions, low education level among adults and small variation in income per family. Almost 90% of the participants in our survey got drinking water from a public tap; Plan International® has provided the parish with tap water at subsidized prices.

In HIV-infected populations, it has been shown that the prevalence of *H. pylori* is lower when CD4 cell count is low [101, 105]. In a study from Argentina of adults [105], the authors conclude that HIV-infected patients with *H. pylori* had a higher mean CD4 cell count than those without *H. pylori*, and a Zambian study also of adults [106] showed that HIV-infected adult patients with CD4 cell counts below 200cells/mm^3 were less likely to have positive *H. pylori* serology. Unfortunately, none of these studies have reported a CD4 cell count in percentage in children. We identified a statistically significantly lower colonization rate of *H. pylori* in HIV-infected children who had a low CD4 cell percentage (table 2 Paper III). In the HIV-infected children, we could not find the same associations as found in the non-HIV-infected children. There was no significant difference in prevalence by sex, type of housing, congested living, education of female caretaker, drinking water sources, toilet facilities, reported abdominal pain or wealth index. A possible explanation for the lack of such association, as described in the non-HIV-infected children [16, 86, 159, 164], is that the impact of the CD4 cell percentage is strong and is independent of the above mentioned factors.

5.2.3 Concentrations of faecal calprotectin

Calprotectin was discovered in 1979 and soon came into use to distinguish IBD from other GI disorders. With time, other conditions causing elevated levels of faecal calprotectin were found. Prior to this thesis, all research on calprotectin was conducted in high-income countries. The prevalence of diseases in the GI tract in high-income countries is different compared with sub-Saharan Africa with lack of studies on prevalence of for instance IBD

and celiac disease, but with a high burden of infectious diseases in the GI tract. In 2000, a new method for detecting faecal calprotectin was established [157] that was more accurate, easier to perform and required smaller amounts of faeces. Normal values for faecal calprotectin have been established in children and adults, table 7. The upper cut-off point is still under debate, which upper limits provide the best sensitivity and accuracy? Several studies have also looked at levels of faecal calprotectin, by upper and lower GI infections, but most of these studies had a limited number of infections, different stages of infections or GI infections have been seen as one without closer information about the type of infection. No conclusions on concentrations in different infections can be drawn from the existing literature.

Table 7. Faecal calprotectin concentrations in apparently healthy children and adults

Author	Age	Number of participants	Assay	Median FC (mg/kg)	95% CI
Fagerberg [125]	4-17 years	117	New	13.6	9.9-19.5
Olafsdottir [126]	1-13 years	24	New	40[a]	28[b]
Berni Canani [146]	1-18 years	76	New	28	3-95.3[c]
Bremner [145]	5-14 years	7	New	<15.6	<15.6-39[d]
Nissen [165]	1-15 years	21	New	17	7-41[d]
Carroccio [166]	1-10 years	10	New	15	10-40
Bunn [137]	1.5-15.3 years	31	Old	10.5[e]	2.5-31.5[e]
Carroccio [166]	adults	10	New	20	10-40
Tøn [157]	adults	59	New	26	20-36
Tibble [167]	adults	96	Old	11.5[e]	8-25[e]
Røseth [140]	adults	53	Old	15[e]	35[e, f]
Hestvik [168]	0-1 year	54	New	249	180-403
	1-4 years	89	New	75	53-119
	4-12 years	159	New	28	25-35

[a] mean value
[b] standard deviation
[c] 5th and 95th percentile
[d] range
[e] original data in mg/L, multiplied with factor 5 to get mg/kg [157]
[f] Røseth choose 10mg/l as the upper limit, no lower limit reported
[ ] reference number
CI confidence interval
FC faecal calprotectin

In our survey on apparently healthy children, all those who reported having had diarrhoea or had had nasal bleeding within the last two weeks before the study, were excluded, but we did not exclude those with detected GI infections. The median faecal calprotectin concentration in children older than 4 years of age was 28mg/kg, and within the cut-off level suggested for that test. Based on this, we concluded that cut-off limits used in children living in high-income countries could be used in children, and most likely also in adults in low-income countries. Those children were healthy, but they were colonized with more pathogens than children in high-income countries. Studies have shown that adults with

Giardia intestinalis have comparable levels [169]. Colonization with *H. pylori* can cause changes in the gastric mucosa [170], but upper-GI disorders have shown little increase in faecal calprotectin levels [171]. A study from Uganda on children and adults infected with *Schistosomiasis*, could not find increased levels of faecal calprotectin in those infected compared to those not infected [158]. By identifying elevated levels of faecal calprotectin in children living in low-income countries, the follow-up might have to be different from that in children living in high-income countries due to the known differences in the underlying causes of GI disorders. Initially, GI pathogens have to be eliminated as the cause of the increased concentrations, and only thereafter more rare conditions causing increased GI permeability like IBD, celiac disease and cancer should be searched for.

To the best of our knowledge, our study of the concentrations of faecal calprotectin in an HIV-infected, HAART naïve population is the first to be conducted in children. Only one study in Italian adults has been published before, and they found that faecal calprotectin was higher than 50 mg/ml in 27 of 53 HIV-infected, HAART naïve adult patients [41]. This is comparable to our findings in children, 34 of 59 had levels higher than 50mg/ml. In the study children, we did not exclude those with diarrhoea, and nasal bleeding was not reported in any of the children. The two populations had different exclusion criteria and a direct comparison is difficult, but in spite of this we found more than two-fold increase in the median faecal calprotectin concentration in children older than 4 years of age, 62mg/kg and a median concentration over the recommended cut-off level for the test. We do know that persons presenting with advanced HIV disease have involvement of the GI tract in their diseases: it is reasonable that the faecal calprotectin increased with more advanced disease. This is an indicator for an ongoing inflammation of the gut in the HIV-infected children. They have an impaired gut mucosa with increased permeability, which again can lead to loss of micronutrients, proteins and a damaged gut/blood barrier and an increased chance of bacteraemia.

5.2.4 Concentrations of faecal calprotectin in children younger than 4 years of age

In children younger than 4 years of age, there are no established reference values. Several studies have shown that the concentrations decrease with age [126, 127] which agree with our decrease (table 1, Paper II). The concentrations found in the apparently healthy children are comparable to those reported elsewhere [126, 165]. In the HIV-infected children, the concentrations in the youngest children were high and comparable with those in non-HIV-infected children. This might be explained by a naturally high permeability of the gut in healthy infants. In the HIV-infected toddlers, aged 1-4 years, the median faecal calprotectin was 2.3 fold higher than in the apparently healthy children, and in the HIV-infected children, aged 4-12 years a 2.5 fold increased levels was found compared to non-infected children. In healthy toddlers, the permeability of the gut is decreasing and the levels of faecal concentration are decreasing, but in HIV-infected children, the gut is repeatedly exposed to different pathogens, repeatedly treated with antibiotics due to opportunistic infection, and some might have developed an HIV enteropathy. Those factors could explain the elevated levels of faecal calprotectin in this age group. A comparison of all age groups of non-HIV and HIV-infected children is shown in figure 9.

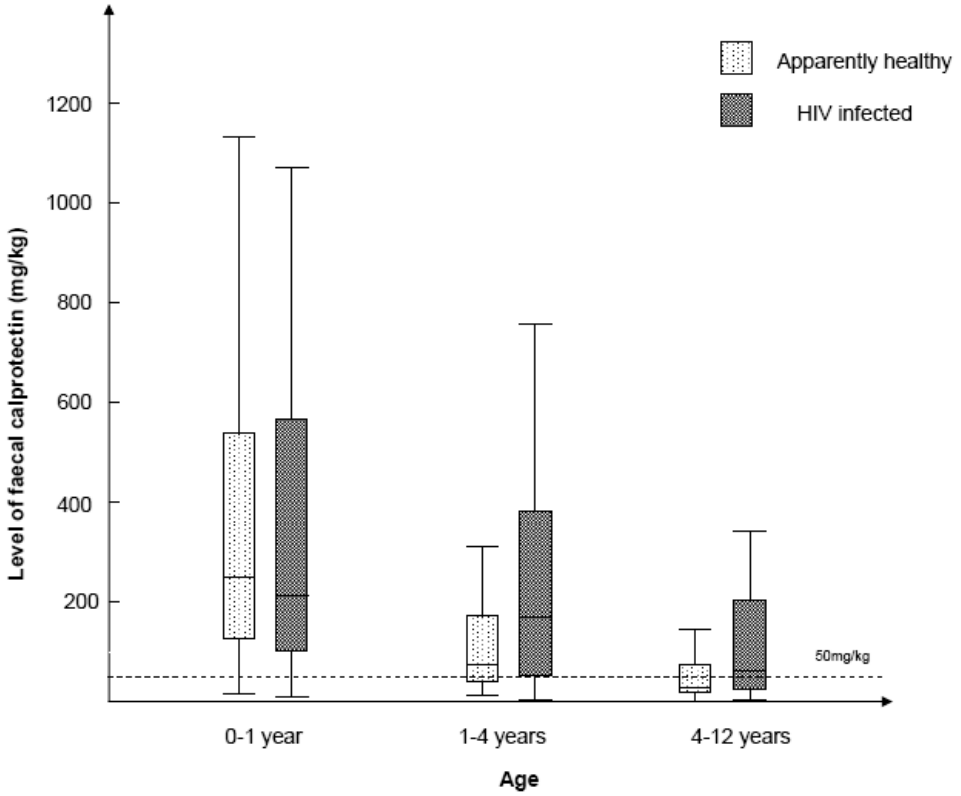

Figure 9. Faecal calprotectin in apparently healthy and HIV-infected children by age

5.2.5 Factors associated with increased concentrations of faecal calprotectin

Increased concentrations of faecal calprotectin are caused by an increase of neutrophils in the gut and the two main factors shown are increased gut permeability and bleeding into the GI-tract. Gut inflammation will lead to increased permeability. It is estimated that a blood loss of about 100ml/day would be required to bring the level above the upper reference limit [140]. In our surveys, we have excluded those with nose bleeding, which can cause a blood loss of 100ml/day or more. None of the girls included had started having menstrual bleeding. Røseth [140] argued that GI haemorrhage due to GI cancer is an unlikely source of increased faecal calprotectin. The increase of faecal calprotectin in our HIV-infected populations is most likely due to gut inflammation and increased permeability. In paper II, we have shown that two common pathogens, *H. pylori* and *Giardia intestinalis*, were not causing increased levels of faecal calprotectin. An Ugandan study of children and adults with *Schistosomiasis*, increased levels of faecal calprotectin were not found [172]. Only 8.2% of the children in the apparently healthy population had other pathogens than *H. pylori* and *Giardia intestinalis* found by microscopy or faecal culture; and even those infected had a median faecal calprotectin below the recommended cut-off value. We have also shown that faecal calprotectin levels are independent of education of the female caretaker, health behaviour and wealth index. In the apparently healthy population, we did not check for

viruses or crypto-and microsporidia infections. But if infected, as long as they were asymptomatic, they were unlikely to have an increased permeability of their gut.

In the HIV-infected children, faecal calprotectin was detectable in all children regardless of age, clinical staging of HIV and CD4 cell percentage. This shows that immunosuppressed people are capable of producing calprotectin and that they have variability in their concentrations of faecal calprotectin similar to immunocompetent subjects. We therefore conclude that faecal calprotectin can be used in HIV-infected persons. HIV-infected children with ongoing diarrhoea had significantly higher levels of faecal calprotectin than those without diarrhoea. The higher median concentration of faecal calprotectin in those with ongoing diarrhoea can be explained by increased gut permeability caused by pathogens, but also by HIV enteropathy [23]. Only in three children older than 4 years of age were pathogens others than *H. pylori* detected, one with *E.coli*, one with hook worm and one with *Giardia intestinalis*.

In our survey of HIV-infected children the faecal calprotectin concentrations increased as the CD4 cell percentage decreased (table 2 Paper IV). This is reflecting that children with more advanced disease have increased inflammation of the gut. This shows that faecal calprotectin is a useful marker in an HIV-infected population. Faecal calprotectin is mainly found in the neutrophils and is not influenced by the decrease in lymphocytes seen in HIV-infected persons. More research is needed to determine whether faecal calprotectin can be a marker for GI dysfunction in HIV-infected children. We hypothesise that we have a new tool for investigation of gut engagement in HIV-infected children.

6 Research challenges

H. pylori is classified as a group I carcinogen [51], and is associated with a 1-2% lifetime risk of gastric cancer [55]. At Mulago Hospital where our second survey took place and where many of the children encountered in the first survey sought medical advice, there was no established method for detecting of *H. pylori* colonization. Gastroscopy was not available on a regular basis. Gastric cancer in adults frequently went undiagnosed.

In the joint European and North-American guideline [81], the recommendation is not to test asymptomatic children for *H. pylori*. Testing for *H. pylori* in asymptomatic children can be considered if a first degree relative has gastric cancer or in children with refractory iron deficiency anaemia. To treat *H. pylori* infections is only recommended if a child has clinical symptoms or if a gastric ulcer is detected by gastroscopy with or without a positive test of *H. pylori*. Several studies of children have indicated that *H. pylori* can play a role in growth failure [15, 173] and iron deficiency [174], and both these problems are common in Ugandan children [175, 176]. Due to the known high prevalence of *H. pylori* in Uganda and sub-Saharan Africa, diagnostic tools for detection need to be considered, and more research on treatment strategies according to resistance problems should be initiated. Guidelines for management of *H. pylori* infection in endemic low-income countries are urgently required.

Faecal calprotectin is a “new tool” in gastroenterology in Uganda. We know that many children suffer from acute and persistent diarrhoea in Uganda. For those suffering from acute diarrhoea, fluid treatment is necessary, and good guidelines are available. For those with persistent diarrhoea, all types of pathogens must be detected and eventually treated. Here we have found a lack of good guidelines for work-up and treatment. In many cases, there will be no identifiable pathogen causing the diarrhoea. They may suffer from other conditions like IBD, celiac disease, food allergy/intolerance, etc. Due to the lack of opportunities to perform gastroscopy, faecal calprotectin might here be a tool to distinguish between different conditions, helping to choose a treatment strategy and follow up treatment response. The burden of disease of persistent diarrhoea is remarkable. Effort must be made to find good methods to monitor and treat the condition; faecal calprotectin can be a tool to follow up the degree of gut inflammation in children. In several medical centres in Uganda, the equipment needed to perform ELISA is available, and due to the quality of faecal calprotectin being stable at ambient temperature for a longer time, faeces can be sent by regular mail to centres for testing. The role of faecal calprotectin in HIV-infected children needs more research, but our findings show that faecal calprotectin is a tool to be used in the diagnosis and follow up of HIV-infected children. Can repeated individual faecal calprotectin measurements be used as a risk marker for developing GI failure in HIV-infected children and adults?

7 Conclusions

The surveys presented in this thesis show that colonization with *H. pylori* is common, even in apparently healthy young children in Uganda (44.3%). It is increasing with age and is associated with poor socio-economic status and boys are more at risk than girls (Paper I).

HIV-infected children had a lower prevalence of *H. pylori* (22.5%) than apparently healthy children in the same geographical area. Children with more advanced disease had a lower prevalence; this might be due to frequent use of antibiotics for opportunistic infections (Paper II). In other studies, it is suggested that *H. pylori* causes growth failure and micronutrient deficiencies, but no such studies have been carried out in Uganda. The impact of *H. pylori* on children's health in Uganda [177] needs to be further clarified.

Cut-off limits for faecal calprotectin, suggested used in children living in high-income countries, can also be used in Ugandan children (Paper III). We have shown that pathogens inhabiting the upper gastrointestinal tract have limited influence on the faecal calprotectin. There is an opportunity to use this relatively inexpensive test for further understanding and investigations of gut inflammation in children living in low-income countries.

Faecal calprotectin can be measured in HIV-infected children, and we suggest that it can be used as a tool for evaluating gut inflammation. In our survey, we found it to be higher in those with more advanced disease (Paper IV). The diagnostic value of faecal calprotectin in HIV-infected children, and its use in follow up the gut function in those infected children, need to be further clarified.

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