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THE EVALUATION OF CALPROTECTIN LEVELS IN THE STOOL OF PAEDIATRIC PATIENTS AND HEALTHY CHILDREN IN THE CZECH REPUBLIC

PhD Thesis

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Contents

List of Abbreviations

AG	Acute Gastritis
BAG	Bacterial Acute Gastroenteritis
CD	Crohrt's Disease
CP	Calprotectin
CRP	C-Reactive Protein
EHSG	European *Helicobacte*r Study Group
ESR	Erythrocyte Sedimentation Rate
FAP	Funcional Abominal Pain
GIT	Gastrointestinal Tract
HC	Healthy Controls
H. pylori	*Helicobacter pylori*
HP-NAP	H.pylori neutrophil activating protein (HP-NAP)
HpStAR	Detection of H.pylori stool antigen test (IDEIA HpStAR)
IBD	Inflammatory Bowel Disease
IC	Indeterminate Colitis
MIF	Macrophage Migration Inhibitory Factor
OD	Mean Optical Density
PMN	Polymorphonuclear Granulocytes
PCDAI	Paediatric Crohn's Disease Activity Index
RAP	Recurrent Abdominal Pain
ROIs	Reactive Oxidative Intermediates
TNF-a	Tumor Necrosis Factor- alfa
UBT	13C-Urea Breath Test
UC	Ulcerative Colitis
VAG	Viral Acute Gastroentertis

1 Introduction

1.1 Intestinal inflammation

Various types of organic diseases in the gastrointestinal tract (GIT) cause damage to the intestinal mucosal lining. Such damage may vary from increased permeability of the mucosa to GIT inflammation and ulcerations. The bowel content is rich in bacteria and other microorganisms (viruses, parasites) releasing substances which may be toxic or chemotactic to leukocytes, in particular polymorphonuclear granulocytes (PMN), to migrate into the gut lumen where they release their contents including antimicrobial substances such as calprotectin. This protein constitutes about 60% of total proteins of the PMN cytoplasm and can be reliably estimated in small, random stool samples even after storage for seven days at ambient temperature (1,2). The concentration of calprotectin in stools reflects the number of PMN migrating into the gut lumen (3,4). An easy approach in the diagnostic work-up of various intestinal disorders is the measurement of faecal parameters.

1.2 Calprotectin

S100A8 (MRP8) and S100A9 (MRP14) belong to the S100 family of calcium-binding proteins (5,6). They were initially purified using the monoclonal antibody 1C5, which was directed against the Macrophage Migration Inhibitory Factor (MIF). Although the proteins themselves did not exhibit any migration inhibitory properties they were called MIF-Related Protein (MRP) (7). Computer-based homology search revealed that MRP8 is identical to the sequence of the cystic fibrosis (CF) antigen with one exception. The sequence of MRP8 cDNA contains an additional G residue in position 292, which shifts the reading frame of the last 15 amino acids. MRP8 is also referred to by other names such as L1 ligh chain antigen, p8 and calgranulin A, and MRP14, correspondingly, as L1 heavy chain antigen, p14, and calgranulin B (8,9,10). The protein complex formed by the two S100 proteins is referred by S100A8/A9 or calprotectin.

The genes encoding the S100 family were found to be localised in a cluster on human chromosome 1q21. A new logical nomenclature for these genes has been proposed, which is based on the physical arrangement on the chromosome 1 q21 (11). According to this nomenclature, the MRP8 is referred to as S100A8, and the MRP14 as S100A9, respectively. Throughout this document, however, we shall refer to this complex as calprotectin.

The known main biological functions of calprotectin are shown in Table 1. Like other S100 proteins, the individual subunits are expressed in a tissue/cell-specific manner (12.) Their expression is restricted to a specific stage of myeloid differentiation, since both proteins are expressed in circulating neutrophils and monocytes, but not in normál tissue macrophages. They are absent in lymphocytes. In peripheral blood monocytes their expression is down-regulated during maturation to macrophages. The calprotectin heterocomplex is produced by PMNs, monocytes and squamous epithelial celis except those in normál skin (13,14,15). This small protein accounts for 5% of the total protein and 60% of the cytosol protein in neutrophils.

Table 1. Main biological functions of calprotectin

- Antimicrobial activity against
  - Bacteria
  - Fungi
- Growth-inhibitory activity against
  - Macrophages
  - Bone marrow celis
  - Lymphocytes
  - Fibroblasts
  - Tumor cell lineš
- Cytotoxic or apoptosis inducing activity in
  - Lymphocytes
  - Tumor cell lineš
  - Fibroblasts

After binding calcium it can resist degradation by leukocytic and bacterial enzymes (16). By competing with different enzymes for limited local amounts of zinc, calprotectin

may inhibit many zinc dependent enzymes (17) and thereby kill microorganisms or animal and human celis in culture (18,19). Different types of disease, for instance bacterial infections, rheumatoid arthritis or cancer can lead to activation of PMNs and increased levels of calprotectin in plasma, cerebrospinal fluid, synovial fluid or urine (20). It is of speciál importance that the concentrations of calprotectin in faeces is correlated with the number of PMNs migrating into the gut lumen and that it can be detected reliably even in small (less than one gram) random stool samples (21). Furthermore, organic diseases of the bowel are said to give a strong faecal calprotectin signál, i.e. elevations are often five to several thousand times the upper reference in healthy individuals, thus indicating intestinal inflammation (2,5,22,). The release of calprotectin is most likely a consequence of cell disruption and death (23). Functional disorders such as irritable bowel disease probably do not give increased faecal CS concentrations. Despite the evidence supporting the use of faecal calprotectin in children, its use is not widespread in paediatric gastroenterology paediatric practice. Further research is needed to ascertain whether any concems are well founded.

1.2.1 Calprotectin Assay Characteristics

In 1992, Roseth et al developed the first method for isolating and quantifying calprotectin in stool, an enzyme-linked immunosorbent assay (ELISA) using rabbit anti-calprotectin (2). Since then, an improved, commercially available, validated ELISA has been developed in which smailer stool samples (0.1 g) are extracted with 5 mL buffer in a closed tube. This assay exhibited very good correlation (r = 0.87) compared with 3-day excretion of 1111n labelled granulocytes (24). The newer assay measures calprotectin concentration in micrograms per gram rather than in milligrams per liter as in the originál assay. Data obtained with the new method can be extrapolated to those that would be achieved in the older procedure through the use of a conversion factor. Calprotectin is a remarkably stable protein in the presence of calcium and is resistant to proteolytic degradation in the stool. Stool samples can be kept for up to 5 days at room temperature

without appreciable loss of calprotectin, and spot samples of calprotectin in stool are as reliable as 24-h collections, with excellent correlation (r = 0.90). It is recommended that 20 g stool be provided in a nonsterile container and shipped overnight to the commercial laboratory. Studies have examined the intra-assay and interassay variability of both the newer and older calprotectin assays and have found them to be acceptable, with an intraassay variation of <5% and an interassay variation of 10% to 40%. However, they also have demonstrated that a subset of individuals can have labile calprotectin concentrations that have greater day-to-day variability and exceed the recommended cutoffs, thus making interpretation of a single calprotectin measurement more difficult (25). The improved ELISA uses polyclonal antibodies that recognize 6 epitopes of calprotectin, thereby reducing falsely low results. More importantly, the lower level of sensitivity of the assay is such that accurate values can be obtained for individuals with both normál and inflamed mucosa, potentially allowing for sensitive noninvasive detection of mucosal healing. Receiver operating characteristics (ROC) plot analysis has shown that a cutoff of 150 mg/g will distinguish between IBD and irritable bowel syndrome (IBS) with a sensitivity of 100% and a specificity of 97 (26).

Table 2 shows some factors that are able to influence the reliability of the results and must be kept in mind. Among these, it is important to note that stool samples collected from the baby's diaper could yield higher calprotectin levels than actually present, because water is absorbed inot the diaper (up to 30%) (27). Therefore infant's stools must be collected into a test tube directly at emission. Calprotectin levels can be overestimated in patients taking non-steroidal anti-inflammatory drugs (NSAIDS) or proton pump inhibitors, or in subjects presenting with nasal or menstrual bleeding (28).

Table 2. Potential factors influencing faecal calprotectin values determination.

- Age (< 12 months)
- Collection (diapers) and storage methods of stool samples
- Nasal and menstrual bleeding
- Drugs (proton pump inhibitors, non-steroidal anti-inflammatory drugs)

1.3 Reference values

Tables 3 through to 5 demonstrate established reference levels in several studies for different age groups. Conflicting data exists related to normál reference values and their use in paediatric subjects in clinical practice. There is far less experience in paediatrics compared with adult population and there are significant differences probably associated with the evolution of the child's organism. Furthermore, in paediatric patients, there is scant evidence related to the determination of normál calprotectin stool levels in particular age groups, biological variability, and of interest, in the environment of our geographical area. The application of adult values in children is not ideál, especially in certain age groups, particular those under 3 years of age (29). This problém has been addressed in only a small number of patients so far in the literature; newborns and breast fed infants have been noted to have high levels of calprotectin compared with older children though the reason for this is completely unknown (30).Two studies have shown that calprotectin values of below 300pg/g can be considered normál during the first year of life (31,32). The recommended cut-off values of < 50 pg/g stool have been cited in the literature as being appropriate for children aged 4 to 17 years (33) However, controversial views exist and further studies are needed to explore and to clarify normál reference faecal calprotectin values in children to be useful for clinical practice.

Table 3. Calprotectin concentrations in Healthy Adults

Reference	Year	Patients, n	Calprotectin Concentration pg/g	
			Medián	Range
Wassekk et al[34]	2004	27	9.3	7-63
Dolwain et al[35]	2004	26	10	2-43
Tibble et al[4]	2000	56	10*	-
Tibble et al[36]	1999	48	10*	-
Costa et al[37]	2003	34	11	
Corroccio et al[38]	2003	10	20	10-40
Thjodleifsson et al[39]	2003	163	20*	-
Summerton et al[40]	2002	28	23*	5-78*
Ton et al[41]	2000	59	26	4-262
Poullis et al[42]	2004	320	27	2-440
Husebye et al[43]	2001	19	30	-
Roseth et al[44]	1997	124	30*	-
Limburg et al[45]	2000	49	31	4-897
Roseth et al[46]	1999	9	58*	22-142*

* Converted to newer assay

Table 4. Faecal Calprotectin concentrations in Healthy Children

Reference	Year	Patients, *n*	Calprotectin Concentration pg/g	
			Medián	Range
Bunn et al[47]	2001	31	11*	–
Fageberg et al[48]	2003	117	14	-
Carroccio et al[49]	2003	10	15	10-40
Nissen et al[50]	2004	21	17	7-41
Berni Canani et al[51]	2004	76	28	1-113
Olafsdottir et al[52]	2002	24	40	-
Rugtveit et al[53]	2002	15	49	6-176

* Converted to newer assay.

Table 5. Faecal Calprotectin Concentrations in Healhty Infants

Reference	Year	Patients, *n*	Age	Calprotectin Concentration pg/g	
				Medián	Range
Nissen et al[54]	2004	11	1-2wk*	150	81-221
Campeotto et al[55]	2004	69	1wk	167	22-860
Nissen et al[56]	2004	16	1-2wk	235	172-2880
Rugtveit[57]	2002	20	6wk	264	48-2130
Rugtveit et al[58]	2002	20	3 mo	269	31-2100
Olafsdottir et al[52]	2002	27	6 wk	278	-

* Preterm infants.

1.4 Scope of Studied Diseases

1.4.1 Inflammatory Bowel Disease (IBD)

Collectively under the umbrella term of Inflammatory Bowel Diseases (IBD), these are chronic illnesses that affect predominantly the gastrointestinal tract (18). In clinical practice, the most common types of IBD are ulcerative colitis (UC) and Crohn's disease (CD). In most instances, these two disorders may be readily distinguished from each other pathologically, particularly when exhibiting classic histological features. However, some patients with IBD show overlapping pathological features of UC and CD, which makes definite distinction between these two disorders difficult and often results in an interim diagnosis of indeterminate colitis (IC).

Both diseases may appear from early childhood to late adulthood, and the diagnosis is often delayed due to vague symptoms or reluctancy to perform endoscopy and biopsy. Several standard markers are used to aid in the diagnosis of IBD and monitoring of IBD disease activity. These include albumin, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), acute-phase protein, and platelets; however, these markers are not specific for inflammation of the gastrointestinal tract and furthermore they are affected by several non-intestinal diseases (59,60). These blood tests may be negative in active IBD, particularly in ulcerative colitis, and increased in quiescent disease [61].

1.4.1.1 Calprotectin in IBD

Calprotectin has been investigated as a non-invasive marker of gut inflammation (2,23). Faecal concentrations have already been reported to correlate with invasive markers of gut inflammation such as 99Tc-labeled white cell scans and endoscopic and histological inflammation scores (24). And thus the stool test has been shown, with high probability to rule out non-inflammatory disorders on the one side (4), and contribute to an earlier diagnosis of IBD. It is suggested that the test can aid in the diagnosis of IBD relapse in patients who have been in remission (Tables 6 and 5), but further studies are

extremely important especially from a clinical point of view. Based on these premises, one of the aims of the present study was to investigate calprotectin levels in the paediatric IBD patients presenting at our clinic with a view to incorporate this test as a standard in the Czech Republic.

Table 6. Faecal Calprotectin Concentrations in Children with IBD

Reference	Year	Patients, n	Disease	Calprotectin Concentration pg/g	
				Medián	Range
Bunn et al[62]	2001	21	CD	70	4-299*
Carroccio et al[63]	2003	8	CD	260	160-350
Berni Canani et al[64]	2006	17	CD	305	175-850
Nissen et al[54]	2004	18	IBD	237	40-8575
Olafsdottir et al[65]	2002	17	IBD	293	
Brmner et al[66]	2005	43	IBD	337	22-1596
Bunn et al[67]	2001	16	UC	58*	3-1363
Bunn et al[68]	2001	9	UC	92*	19-1363
Berni Canani et al[69]	2006	10	UC	340	225-1100

* Converted to new assay.

Table 7. **Faecal Calprotectin Concentrations in Adults and Children with IBS**

Reference	Year	Patients, *n*	Age	Calprotectin Concentration |jg/g	
				Medián	Range
Dolwani et al[70]	2004	24	Adults	19	3-87
Tibble et al[71]	2002	339	Adults	20*	5-250
Tibble et al[72]	2000	159	Adults	20*	175-850
Wasssell et al[73]	2004	25	Adults	21	7-87
Costa et al[74]	2003	48	Adults	22	
Summerton et al[40]	2002	7	Adults	31*	10-160*
Carroccio et al[75]	2003	40	Adults	35	10-210
Carroccio et al	2003	15	Children	20	10-130

* Converted to new assay.

1.4.1.2 Paediatric IBD: The role of Tumour necrosis factor- a (TNF- a) and a Promotér Gene Polymorphism at Position - 308 G-+A

An understanding of the control of intestinal inflammation and disease activity is critical for evaluation of calprotectin concentartions in children with IBD.

As noted, the natural course of IBD is characterised by unpredictable exacerbations, remissions, and the development of serious complications, particularly in CD (76).

Paediatric IBD incidence rates have changed over the second half of the 20th century, with a steady, gradual increase for both UC and CD (77,78). A recent study also confirmed an increase in incidence of CD in children younger than 15 years in the Czech Republic (79). Though UC and CD share some genetic predisposing factors and immunological mechanisms, several lineš of evidence suggest they are genetically and otherwise fundamentally distinct disease processes (80,81).

Though our knowledge of the initiating events of IBD is incomplete, both major clinical forms appear to represent polygenic conditions with significant variability in pathophysiology and clinical symptoms. Susceptibility to polygenic disorders may be provoked by a combination of common genetic variants, including single nucleotide polymorphisms (SNPs) and immunological and environmental factors (82). These may increase the risk for developing of these disorders (83,84).

Cytokines are proteins encoded and often secreted by immunocompetent celis which influence immune activity within the cell or at a distance. Pro-inflammatory cytokines involved in IBD are part of a complex signalling network that is not completely understood. IBD is associated with increased tumour necrosis factor-alpha (TNF-a) secretion possibly leading to the initiation and propagation of the disease (85). In this cascade TNF-a is produced first, IL-1R. second, and IL-6 last. TNF-a and IL-1ÍI stimulate each other, and both stimulate IL-6. (86). TNF-a mediates increased epithelial antigen uptake in the ileum of CD patients (87) and induces T-helper1 cytokines important in the onset and progression of IBD (88).

The grade of inflammatory response is reflected at a systemic level. C-reactive protein (CRP), an acute phase protein, is expressed exclusively by the liver as part of the acute phase response upon stimulation by cytokines originating at the site of inflammation (89). Laboratory testing is an essential element in the establishment of IBD. A short half-life makes CRP a valuable marker in clinical practice to detect and follow up disease

activity in CD. In contrast and for reasons unknown, UC has a modest to absent CRP response despite active inflammation (90).

Although the cause of IBD is not entirely clear, abnormal immune responses based on genetics and environmental factors may play a role in its pathogenesis. IBD is characterised by unbalanced Th1 vs. Th2 responses. This leads to dysregulated secretion of both pro-inflammatory (TNF-a, IL-6, IL-1beta) and anti-inflammatory (IL-10) cytokines (91, 92, 93) in the lamina propria, and inflammatory cell infiltration in both CD and UC (94). TNF-a can potentiate production of interferon-") (95). Treatment of CD patients with anti-TNF-a antibodies (cA2) could inhibit production of TNF-a and interferon-T in mononuclear celis as well as improve the CD activity index and reduce intestinal inflammation (96).

Molecular genetics has increased our understanding of the strong genetic component in IBD disease susceptibility (97, 98), though little is known about the accountable genes. Cytokine genes are attractive candidate loci (99), but data regarding association studies in the paediatric population is largely unknown. To date, studies of polymorphisms in pro-inflammatory cytokines (IL-1, TNF-a, IL-6) or in regulátory cytokines (IL-2, IFNy, IL-10) have not yet shown reproducible parallels with disease phenotype in IBD adults (100).

A region on chromosome 6p21, IBD3, has been identified as an IBD-susceptibility locus in linkage studies (101, 102, 103). IBD3 encompasses the TNF gene, a strong positional and functional candidate for IBD. The TNF-a gene is located in the major histocompatibility complex region, and a large number of polymorphisms of its promotér have been described (104). The regulation of TNF expression is in part genetically determined because the polymorphisms -238, -308, -863, -857, and -1031 found in the promotér region are associated with increased TNF production (105). The polymorphisms at position -308 G-»A in the TNF-a promotér region (G allele, TNF*1, and A allele, TNF*2) are associated with inducible levels of TNF-a in vitro (106). A nucleotide change at -308 of

the human TNF-a gene promotér might influence transcriptional activation of the cytokine (107). A growing body of data supports the association of TNF-a polymorphism at position -308 with susceptibility and outcome of various autoimmune and infectious diseases (108). However, limited information is available on the TNF-a- G-*A polymorphism with the G to A transition at position -308 in the paediatric population with IBD. There are two relevant reports on the TNF-a- polymorphism in paediatric CD (109, 110). Confirmed data are not readily available relating to TNF-a 308 G->A polymorphism, disease activity, nor are objective biochemical markers of inflammation in IBD in children.

To address this issue, we conducted a study to determine: the association of TNF-a G-»A promotér SNP at position- 308 in subgroups of IBD stratified according to disease phenotype and in healthy controis, and the relationship between gene polymorphism, CRP levels and disease severity in a paediatric population.

1.4.2 Recurrent abdominal pain (RAP) and gastric *Helicobacter pylori* (*H.pylori*) infection

RAP is one of the most common symptom complexes in children. The association between H.pylori infection and RAP is still a clinical challenge for physicians because of the lack of biological markers for RAP and /or the lack of reliable and valid clinical measurements (111). Among the many open questions an exceptionally interesting one is to establish the precise mechanism underlying the RAP. In the light of the data in the current literature, it is rather difficult to conclude precisely the issue.

Patients with organic or functional abdominal disorders, irritable bowel syndrome (IBS), recurrent abdominal pain (RAP) may have similar symptoms, and clinical examination alone may not be sufficient to give a specific diagnosis. The underlying mechanisms of RAP are not known (112,113,114). No knowledge has been available regarding calprotectin levels in faeces in children with chronic abdominal pain.

Since further diagnostic procedures may be complex, expensive or expose the patient to pain, ionizing radiation or other risks, there is a need for a simple non-invasive, inexpensive and objective method which can help in selecting patients for additional examination, for instance endoscopy. The latter normally requires generál anaesthesia in children. Studies have shown that the test used in this study can serve this purpose (115,116). Since abdominal symptoms are common across the spectrum of ages of childhood, a negative calprotectin test can save many endoscopies and thereby also money. The evaluation of faecal calprotectin in children in this regard is truly and practically important.

H.pylori is currently recognised as a major etiologic factor in the development of chronic gastritis and peptic ulcer disease in adults and children (117). Overall, one-half of the worldwide population is infected with H.pylori (118), and its acquisition might occur predominantly during childhood (119). It is clear that H.pylori infection rarely resolves spontaneously.

Epidemiological studies have shown a higher *H.pylori* infection rate in children from developing countries compared with developed countries (120). However, studies conducted in asymptomatic children in Argentina reported seroprevalence rate of 15.7% (121). We conducted a study in the Czech Republic in children with GIT symptoms and found a 25.6% prevalence rate (122).

The infection usually persists indefinitely unless untreated with specific eradication therapy. If untreated, this chronic inflammation predisposes a subset of people to the development of gastric and duodenal ulcers and even gastric cancer, however most of the infected population remains asymptomatic (123). Hence, the development of disease depends, in part, on the virulence of the infecting strain, on the susceptibility of the host, and on environmental cofactors (124). However the association between H.pylori infection and gastroduodenal complaints in children is even less established. RAP is one of the

most common presentations to paediatricians, yet an organic aetiology can be found in only 10% cases. It is still under debate whether H.pylori infection in adults should be treated or not (125). The role of H.pylori in RAP in children also remains unsettled despite several investigations have been carried out in these patients (126).

Colonisation of gastric mucosa by these bacteria is strikingly associated with histological evidence of chronic gastritis (127). H.pylori infection stimulates the infiltration of the gastric mucosa with neutrophils, lymphocytes, plasma celis and macrophages. Although H.pylori is known to be a non-invasive micro-organism, it elicits gastric mucosal infiltration of inflammatory celis, especially neutrophils (128). Neutrophil infiltration is a variable feature of H.pylori associated chronic gastritis where neutrophils disappear rapidly after successful eradication treatment but also with chronicity with infections of longer duration. A prolongation of neutrophil lifespan could contribute to the pathogenesis of H.pylori infection. Recent studies continue to examine the activation of these celis and their impact on tissue injury. *H.pylori* neutrophil activating protein (HP-NAP) is a 150-kDa decamer protein that promotes adhesion to endothelial celis potentiated by TNF-alpha and interferon-gamma. Hence, HP-NAP stimulated chemotaxis of neutrophils and the subsequent production of reactive oxidative species (ROls) are probably critical to the progression of *H.pylori* - associated active inflammation and tissue damage. (129,130), Our group Sýkora et al previously observed that gastric corpus inflammation changes linked to *Helicobacter pylori* infection may accelerate gastric emptying of solids (131). Our study advances our understanding of the physiopathology of gastric motor function disorders in dyspeptic children. However, there is no literature dealing with calprotectin participation involving gastric inflammation in symptomatic children. Much research remains to be done in order to determine more definitely the role of calprotectin in the progression and the control of antral and fundic gastric inflammation

It is known that most cases of chronic gastritis result from infection with H.pylori and that some degrees of chronic gastric inflammation are invariable in infected subjects

(132). The ability to diagnose H.pylori infection non-invasively (by serology, C13-urea breath testing, and stool antigen test) means that subjects with H.pylori-associated chronic gastritis can now be identified without the need for endoscopy and biopsy. However, the severity and topography of gastritis varies considerably between individuals and only specific pattems of H.pylori-associated gastritis are linked with individua! gastroduodenal disease (133).

Furthermore, calprotectin is a reflection of the host-bacteria interaction, and their profile analysis could be used as an indicator of *H.pylori* pathogenic behaviour. There are no available data describing variations in stool calprotectin with *H.pylori* infection and histopathological features of H.pylori gastritis. Amongst children with gastritis, no correlation between stool calprotectin and inflammation and infection was observed. Hence, in symptomatic children the measurement of stool calprotectin may provide a non-invasive assessment of some aspects of H.pylori-associated gastritis and this has potential for use in clinical practice in screening for gastroduodenal conditions. In a patient with active dyspeptic symptoms who is known to have *H.pylori* infection, the use of additional stool biomarkers for assessing the topography and severity of gastritis may allow for better prediction of underlying diagnosis and assessing H.pylori pathogenic behaviour.

Abnormalities have been described in the faecal calprotectin of patients with IBD, but it is not known whether they are specific for IBD or to some extent common to other forms of GIT inflammation (134). The possible involvement in the alterations of gastric mucosa (gastric inflammation) associated with *H.pylori* infection remains undetermined. The gastric mucosa in children has been found to contain various proteins from the S100 protein group, S 100A8/A9, S100B, S100A4, S100A12 (135).These proteins have chemotactic properties related to inflammation. Even with large amounts of data on *H.pylori,* there is none on the intensity of calprotectin production in chronic gastritis caused by *H.pylori* infection. There have been no studies of the relations between *H.pylori*

status, the control of gastric pathology and stool calprotectin in paediatric symptomatic patients that have considered this application. The determination of this marker in correlation with the expression of H.pylori antigen in the stool would be a significant step in elucidating the activity of chronic gastritis in paediatric clinical setting.

Based on these premises, the present study was designed to investigate the role of faecal calprotectin in children with RAP and also with relation to *H.pylori* infection and gastric inflammation in symptomatic children.

1.4.3 Gastroenteritis

While infectious gastroenteritis are the second most common disease in childhood worldwide, viruses are the most common frequent agents of infectious diarrhoea (136). During the past three decades, there has been a dramatic increase in the number of newly recognised etiological agents of gastroenteritis. Since 1970, more than 20 different micro-organisms - bacteria, parasites and viruses - have been recognised as etiological agents, and most cases of gastroenteritis are now presumed to have an infectious aetiology (137). Nevertheless, a specific pathogen is currently identified in only small proportion of cases. (138) Although numerous viruses have been identified in faecal samples, a causal relationship has been determined for relatively few (rotavirus, adenovirus, astrovirus, calicivirus). Rotavirus is the leading agent and may also have an inflammatory component (139). Limited inflammation is detected by histological studies. Rotavirus-induced diarrhoea is a multi-step and multi-factorial event, in which fluid secretion and cell damage are observed in sequence. Enteric infections exact a heavy toll on human populations, particularly among children (cholera, salmonella, shigella, campylobacter, yersinia, E. Coli, C.difficile). (140) The appreciation of the multifaceted aspects of the problém of bacterial infections is pivotal for full comprehension of that diarrhoeal disease pose to public health and for appropriate allocation of resources and efforts to tackle them. However, there are a series of obstacles that have historically

hampered this process, including non-standardised definition of disease and symptoms, failure to identify a causative agent in many, if not most, cases of disease, failure to report episodes to health authorities.

Acute symptoms in gastroenteritis occur most often in infants where it is most difficult at onset to decide whether the cause of diarrhoea is infectious or non-infectious in origin, and indeed if it is viral or bacterial (141).

Gastroenteritis of unknown aetiology (GUE) is a significant cause of mortality, even in highly developed countries (142). There is evidence that unknown agents affect human health, because population based surveillance has detected unexplained deaths that appeared to be caused by infections but were not associated with a known pathogen (143). Fatal GUE has often appeared to be infectious in origin, but death certificates provide insufficient information to determine whether the causative agents were known and if they had been food bourne. Both the accuracy of GUE reporting on death certificates and the aetiology of GUE merit further investigation.

Furthermore, from the prognostic and therapeutic point of view, it is important to know if the illness is caused by direct inflammatory or dietary cause. An easy approach in the diagnostic work-up of intestinal disorders is the measurement of faecal parameters. There is no conclusive data concerning faecal calprotectin values in children with acute gastroenteritis (AG) and its use in clinical practice. Croft NM, et al. reported 10-fold increase in IgM secretion compared with a smaller relative increase in IgA suggesting that this primary mucosal immunue response in acute diarhhoeal disease (146).However, data on changes of faecal calprotectin concentrations in young children is inconsistent. Kapel N et al. used tumor necrosis factor-a and faecal calprotectin as differential diagnostic markers for severe diarrheoa of small infants (144). A comprehensive assessment of the differences in faecal calprotectin values diversity in from both AGG children and healthy

children has not been carried out, to our knowledge, our study is the first. Hence further studies are warranted to deepen our knowledge.

1.4.4 Faecal calprotectin in children with chronic gatrointestinal symptoms

Faecal calprotectin is a new marker of intestinal inflammation. Data on faecal calprotectin in paediatric gastroenterology are still scarce. Children with organic or functional problems may have similar features and clinical examination is not, in most cases, enough to ascertain the specific diagnosis. Other methods are complex, time consuming, relatively expensive and unpleasant for the child. To solve this problém, the search for non-invasive biological markers which would be useful in clinical practice in various settings should be performed. Calprotectin has been studied in a variety of conditions, but only more recently it has received increasing attention in paediatric gastroenterology. Its functional aspects have been reviewed by Johne et al (145). Tibble et al found that faecal calprotectin may predict relaps of disease activity in patients with CD and UC (146) Calprotectin is also elevated in patients with coleractal cancer (147) and in non-steroidal anti-inflmamtory drug (NSDAI) - induced enteropathy (148).RAP is observed in 9 - 16 % of preschool and school children (149). It is important to distinguish between between organic and functional disoreders. Inestinal inflammation can be the cause or consequence of increased intestinal permeability. Some studies in children with RAP (150,151) have reported abnormal small bowel permeability and duodenitis. It is preferable to have a reasonable set of tests to select patients with organic diseases such as IBD that need more invasive procedures. A faecal calprotectin test has been promising in adults with IBD (152) and may constitue a valuable diagnostic tool in children with RAP There are few studies on faecal calprotectin in childhood. Therefore, further studies are needed to differenitiate between the organic causes of gastrointestinal symptoms and to determine whether this test could be a diagnostic tool in patients with abdominal pain.

2 Aims

As mentioned, while several studies have characterised the role of faecal calprotectin (S100A8/S100A9) values in clinical evaluation in adults, few studies have been conducted in children.

Confirmed data are not readily available relating to TNF-a 308 G-»A polymorphism, disease activity, nor are objective biochemical markers of inflammation in IBD in children. The analysis of TNF-a 308 A polymorphism in IBD subjects have provided essential clues for the pathophysiological mechanisms and identifying links for better defining and understanding the role of faecal calprotectin in IBD related complications and intestinal inflammation.

In the paediatric population the measurement of calprotectin in stool provides a non-invasive evaluation of some aspects of GIT disorders. The available data describing variations in calprotectin in stool in children are limited and somewhat controversial, especially in the Central European region.

Within the realms of primarily assessing clinical activity rather than on direct measures of inflammation with outcome data, the present research work aimed:

A. To assess normál reference age-stratified values of calprotectin in stool in the Czech paediatric population in samples of subjects aged Imonth to 15 years in our geographical region within the Czech Republic

B. To improve the diagnostics, follow-up and therapy of patients with IBD i**.e.**

a. To determine the association of TNF-a G-*A promotér SNP at position- 308 in subgroups of IBD stratified according to disease phenotype and in healthy controis, and the relationship between gene polymorphism, CRP levels and disease severity in a paediatric population.

b. **To establish for the first time in the Czech Republic, the faecal levels of calprotectin in normál children and in children with IBD (CD, UC),** to determine whether faecal calprotectin concentrations reflect disease severity, and to determine whether these levels change in response to treatment

C. **To explore the diagnostic potential and the pathogenic role of faecal calprotectin in symptomatic children with RAP** related to *H.pylori* infection in children.

D. **To investigate the inflammatory immune response during acute phase of diarrhoeal disease in infancy,** to explore for the first time faecal levels of calprotectin and its application in children with acute gastroenteritis

E. **To evaluate the efficacy and the utility of faecal calprotectin assay in consecutive subjects in different paediatric gastrointestinal diseases** comparing them with those obtained in healthy children

3 Material and methods

3.1 Study design and description of study

The current analysis is based on a subsequent prospective survey related to the faecal levels of calprotectin in the paediatric population with different GIT diseases as a biological marker of GIT inflammation to obtain baseline data from a sample of children with various GIT conditions and normál healthy controls. Each investigator was blinded to the results recorded by the other.

3.2 Study significance

The significance of this prospective study in determining faecal calprotectin in a paediatric population lies in optimising non-invasive, simple to use in clinical practice, reliable and inexpensive assessment of the inflammatory processes within the GIT, improving our knowledge of the pathophysiology and current diagnostics of various GIT disorders, assessing efficacy of therapy in clinical practice. Based on these results, the end effect is economically beneficial and optimises diagnosis and treatment of GIT disorders.

3.3 Patient populations

Clinical characteristics of each individual enrolled as well as their basic laboratory parameters have been taken. To obtain the most statistical power, sample size and power calculations have been taken into account before starting the study. The number of children enrolled was influenced by epidemiological factors and the frequency in which the investigated diseases present themselves. Individual groups of patients and subgroup analyses were performed on the basis of previously defined criteria as follows:

For this prospective trial of the faecal calprotectin values in childhood, all children were screened at the University Hospital (Department of Paediatrics, Department of Infectious Diseases) and the primary care setting. Criteria for entry into the study at onset were a clinical diagnosis of IBD (CD, UC), RAP *H.pylori* - negative and *H.pylori*-positive, AG, and apparently healthy children (for reference values) and age-matched analysis. All analyses (patients, controis) were performed on the one nationality (Czech).

The demographics of the study population as a whole are presented in Table 8.

Table 8. Demographics of the Study Population

Variables	Total (n)	Gender m/f (n)	Age mean ± SD (range)
Normál controis	41	21/20	4,6 ±4,2 (0,08-15)
1-12 months	13	6/7	6,3 ±3,3 (1-10)
1-6 years	12	7/5	3,0 ±1,7 (1-5)
6-15 years	16	8/8	9,1 ±2,6 (6-15)
IBD	57	35/22	14,0 ±4,3 (6-19)
Crohn's disease	37	21/16	14,0 ±3,1 (8-17)
Exacerbation	18	10/8	14,1 ±3,1 (8-17)
14 days after therapy	18	10/8	14,1 ±3,1 (8-17)
Remission	20	11/9	13,8 ±3,1 (8-17)
Ulcerative colitis	20	14/6	14,1 ±5,9 (6-19)
Exacerbation	9	7/2	13,5 ±3,6 (9-19)
14 days after therapy	9	7/2	13,5 ±3,6 (9-19)
Remission	12	8/4	14,5 ±7,0 (6-19)
RAP (symptomatic)	34	13/21	12,4 ±3,6 (4-16)
H.pylori positive	16	8/8	12,4 ±3,7 (4-16)
H.pylori negative	18	5/13	12,5 ±3,4 (4-16)
Acute gastroenteritis	40	24/16	1,8 ± 1,4 (0,25-7,75)
Bacterial	20	11/9	2,0 ±1.7 (0,5-7,75)
Viral	20	13/7	1.7 ±0,9 (0,5-3,75)

Legend

IBD Inflammatory Bowel Disease
RAP Recurrent Abdominal Pain

3.4 Healthy controis in the Calprotecin study

Healthy Controls (HC) Group comprised 41 healthy children without symptoms of chronic disease and symptoms of GIT disease, family history of GIT disease, without a history of allergies, or known medication use. Exclusion criteria also included menstruation and nosebleeds. Children from age groups 0 to 15 years were chosen from a population undergoing regular preventive check-ups at their generál practitioner or that were scheduled for minor surgical procedures and also children who had suffered minor falls with head injury. These were asked to provide a single stool sample. We categorized and enrolled healthy subjects into 3 groups by age, children ranged in age from neonates to 15 years (1-12 months, 1-6 years, 6-15 years). Parents agreed to the examination being performed and were provided with a letter as well as information by the paediatrician attending.

3.5 Inflammatory Bowel Disease

3.5.1 Diagnostic criteria for IBD

The definitive diagnosis of IBD (CD and UC) was based on a combination of clinical characteristics, laboratory assessment, ultrasonography, colonoscopy, histopathology, and CT scan. For upper gastrointestinal (Gl) tract disease, upper endoscopy, push-enteroscopy, CT-clysis or 99Tc-labeled white cell scanning (153,154) was utilized. Individuals whose IBD diagnosis was not confirmed were excluded. The total Paediatric Crohd's Disease Activity Index score (PCDAI) (155), and the Truelove index (156) were used to assess disease activity for CD and UC, respectively. All patients were screened for complications at follow-up management. UC patients were grouped according to extent of the lesion: proctitis, proctosigmoiditis, left-sided colitis (involvement limited to splenic flexure), and sub (-total) colitis. Furthermore, the numbers of recurrent exacerbations were specified. CD subjects were registered following the Vienna classification (158). This

included the anatomie location of CD involvement (only small bowel, only large bowel, small and large bowel, upper GI tract) and the phenotypic behaviour. The latter was defined as: stenosing when the luminal narrowing or bowel occlusion was demonstrated with pre-stenotic dilatation and/or obstructive symptoms; penetrating, defined as the occurrence of intra-abdominal or peri-anal fistulae, inflammatory masses and/or abscesses; or simply inflammatory without the previous eriteria. Furthermore, the need for surgical intervention was recorded. Extraintestinal manifestations included musculoskeletal, dermatological, ophthalmologic, thromboembolic, hepatobiliary and pancreatic involvement.

3.5.2 Calprotectin analysis in Inflammatory Bowel Disease

Children were enrolled prospectively at the time of their initial diagnosis of IBD and they were tested when admitted in a statě exacerbation. Children in a statě of remission were investigated when presenting for their periodical surveillance. Included in the study related to faecal calprotectin were 27 children (18 classified as CD, and 9 as UC). Patients with IBD are looked after by hospital specialists as outpatients or inpatients, with an open-access nurse specialist clinic. A database of patients was compiled following hospital outpatient visits, histology reports, and inpatient admissions. Children in this series were visiting consultant paediatric gastroenterologists atthe Department of Paediatrics, Division of Gastroenterology, all of whom gave permission for their patients to be included in this study. The diagnosis of IBD was based on the standard radiological, histological and endoscopic eriteria (the Porto eriteria) (159). These patients were age and sex matched with HC. IBD patients were maintained on various medical anti-inflammatory regimens (5-aminosalicylic acid, glucocorticoids, enteral nutrition, antibiotics, azathiprin, Infliximab). Basic demographic data, IBD treatment and sample collection, details of presenting features, and results of standard laboratory parameters and markers of inflammation (ESR, CRP, albumin, and platelets) were determined in all patients. Disease location was defined on the basis of radiological, endoscopic, and histological findings. Monitoring of

disease activity was calculated using the Paediatric Crohrt's disease activity index (PCDAI) in each patient on follow-up visits follow-up (157) and in children with UC using the Truelove index (161)

3.5.2 TNF-a G->A Polymorphism analysis in Inflammatory Bowel Disease

A total of 164 children were included in the study. 82 subjects with established diagnosis of IBD (46 boys, 36 girls; age range, 8-18 years) were recruited from two teaching hospital-based practices, (Department of Paediatrics, Division of Gastroenterology, Charles University Hospital, Pilsen and Hradec Králové). All of the patients were unrelated and belonged to the same ethnic group (Czech). A reference material consisted of 82 healthy individuals was also analysed. Normál controis consisted of subjects without apparent abnormal findings on medical examination, and were drawn from the same geographical area and sociál standing as the study group. All examined subjects were born in the Czech Republic. Demographic and clinical data were recorded. Investigators obtaining phenotype were unaware of genotype; conversely, genotype was analysed independently while blinded to clinical data.

3.6 RAP and *H.pylori* infection

At baseline, assessment of symptoms at the investigator's site was recorded related to the patient's GIT symptoms. Symptom score was based on the recommendations of European *Helicobacter* Study Group (EHSG) for evaluation of symptom score (severity, frequency, duration) in studies related to *H.pylori* infection in humans. To assess symptom characteristics in children with RAP in both groups, a questionnaire was filled by the gastroenterologist as described in our previously published study (19). A cohort of consecutive children with recurrent abdominal pain (RAP) who met the diagnostic criteria were included according to Apley's criteria (158). Apley's criteria were: more than three attacks of diffuse or localized abdominal pain during a period of more than 3 months affecting daily living activities of the child. After obtaining a detailed history and performing

a complete physical examination, clinical features of children with RAP was performed on all patients. Children with obvious organic pathology, elevated ESR and chronic IBD that could explain the RAP were excluded as a standard practice in studies concenring *H.pylori.* The cohort of children was examined with blood tests, stool test for parasites, microbes, viruses, urine test and ultrasonography of the abdomen. Children with RAP were subsequently divided into two subgroups, *H.pylori* infected and those not-infected. This group was at least partly formed of children investigated as part of an on-going epidemiological study of the prevalence of *H.pylori* infection in children in the Czech Republic conducted in our setting, supported by grant IGA from the Ministry of Health of the Czech Republic (7399-3/2003) (159). None of the subjects were previously treated for *H.pylori infection.* None of the subjects had received antibiotics, histamine - 2 receptor antagonists, or proton pump inhibitors in the four weeks before evaluation of *H.pylori* and faecal calprotectin had been performed.

3.7 Acute Gastroenteritis (AG)

All children had been referred for evaluation of symptoms and signs associated with acute gastroenteritis, and these studies were part of the routine clinical evaluation. In all children, the definitive diagnosis causing acute diarrhoea was based on complete analysis, conventionally accepted criteria, and infectious causes (clinical features, microbiology, virology, laboratory indicators - blood count, CRP, IL-6,AST, ALT, blood glucose, urea, creatinin, Na,K,CI, proteins and faecal calprotectin levels (162).

It is important to consider AG as a diagnosis *per exclusionem.* A few loose stools and vomiting may be the result of systemic infections such as pneumonia, septicaemia, urinary tract infection, and even meningitis. Exclusion criteria also included surgical conditions such as appendicitis, intussuception and Hirschspring's disease which may mislead the clinician.

Definitions for AG have varied between studies but usually required (frequency, consistency, duration). The consistency and frequency of bowel movements varies with a child's age and diet, and the definition of diarrhoea varies accordingly.

Frequency - it is normál for young infants to have eight to ten stools per day, though this varies individually. Older infants, toddlers, and children normally have one to two bowel movements per day. Diarrhoea has been defined as an increase in stool frequency to twice the usual number in infants.

Consistency - the consistency and colour of a child's stool normally changes with age, which highlights the importance of knowing what is normál for a child. Young infants' stools may be yellow, green, or brown, and may appear to contain seeds or small curds. All children's stools can vary as a result of their diet. The development of stools that are runny, watery, or contain mucus or blood is a significant change that was monitored and required medical attention.

Duration - A prolonged history of diarrhoea (one to four weeks or longer) is evaluated and treated differently and has not been included than an acute case of diarrhoea (lasting less than 2 weeks). AG was defined as the acute onset of watery or extremely loose stools with or without vomiting of 14 days of duration or less and without infection outside the gastrointestinal tract or other illness.

On admission details of severity and duration of diarrhoea and any treatment already received were noted. The degree of dehydration was assessed as absent, 2.5-5%, 5-10%, or 10 -15% (142). All dehydrated infants were first rehydrated with oral rehydratation solution or intravenous fluids before receiving their feeding regimen.

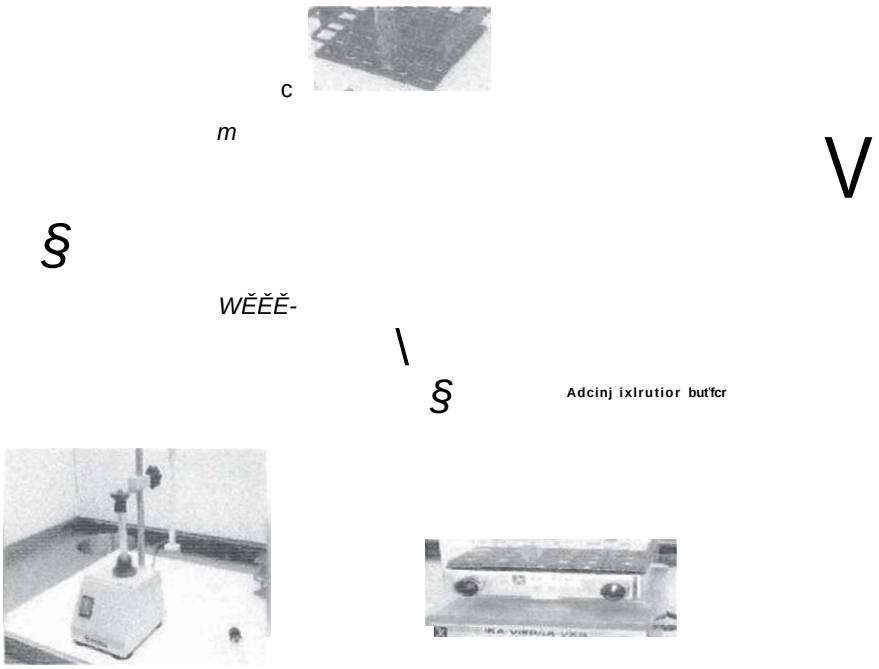
3.8 Data collection

All results of the clinical examination and laboratory findings were recorded on a structured data sheet and were entered into the centrál database

3.9 Calprotectin - specimen collection and preparation

1-5g of faecal material was collected (approximately one teaspoon full) and placed in a suitable container and delivered to the laboratory within 4 days. When put in a transport container it could be sent by ordinary mail without refrigeration. Samples were stored frozen, at -18°C or lower until required for running. Before extraction frozen samples were thawed at room temperature over night. Between 40 and 120mg of faeces was used within a screw cap tube and buffered with a weight/volume ratio of 1:50 before vortexing for 30 seconds and mixing (at approximately 1000rpm) for 30 minutes. 1-2 ml of the homogenate was centrifuged at 10.000g for 20 minutes at +4 °C for 20 minutes. The clear supernatant extract was then diluted and run on the ELISA. About 0.5 ml was transferred for assay or storage at +4 °C for several days or frozen for up to 12 months.

Figuře 1. Specimen collection and preparation



lollection and extraction of stool samples for the PhiCal test

3.10 Calprotectin Assay Procedure

The quality control guideiines set out by the manufacturer were adhered to specifically, and interchange of kit components from different lot numbers was avoided. The assay was carried out following the manual included with the kit. Incubation time and temperature, pipetting volumes of the components were defined by the producer and strictly adhered to. All samples and reagents were allowed to reach room temperature (20 - 25°C). The extracts were diluted 1:50 (20 pl sample + 980 pl dilution buffer) before running. Samples with very high concentrations were re-tested after further dilution, for instance 1:5. The manufacturers suggested plate layout was utilised whereby standards and controis were included in each run. Importantly, 50 pl of each standard, control and diluted sample were added in duplicate weils in rows according to the template before incubation at room temperature on a horizontál shaker for 45 minutes. The weils were then washed a total of 5 times and conjugate added before a repeat of the incubation and washing steps. After addition of substráte solution to each well the plate was incubated at room temperature for 20 to 30 minutes in the dark until the mean optical density (O.D.) value of the 1000ng/ml standard was about 2.0. O.D. values were read by means of an ELISA reader at 405 nm. Where a blank or 0 standard was used, it was necessary that its O.D. value was below 2.0. A maximum reading postponement of 24 hours at +4 °C was maintained.

3.11 Calprotectin evaluation

The concentration of calprotectin in stools was expressed as mg/kg as set by the test kit manufacturer. The calculation of concentrations in patient samples was performed by computer linked ELISA reader.

The functional operation carried out therein was as follows: The O.D was calculated of all duplicates. The log values of the standard concentrations were plotted against their OD to obtain a standard curve. The computer program used a Spině function, as

recommended by the manufacturer. The reading of the control was confirmed to be within the limits printed on the vial label. The values of the diluted samples were corrected for the dilutions and converted to mg/kg by multiplying by 2.5 and then presented as the now accepted p/g. Samples that had to be diluted further had the additional dilution factor entered into the calculation.

3.12 Calprotectin test - quality control

A new standard curve was included in each run and the control was included in each **run.**

3.13 Interpretation of Results of calprotectin

Threshold values supplied by the manufacturer as well as those accepted in the literatuře were as follows: <50(jg/g of stool was considred to be negative; 50 - 100 pg/g of stool was considered to be borderline; and >100 pg/g of stool considered to be positive. These values were then included for statistical analysis.

3.14 Precision

Table 9. Inter and Intra assay precision values

Interassay	N	mean	Cv [%]
Positive Séra	2(24)	0.89	5.1

Intraassay	N	mean	Cv [%]
Positive Sera	16	0.90	2.1

3.15 Clinical evaluation

Comparison with the reference method measured by the literature production method shows an agreement of 1^=0.976

3.16 Other Methods used for examination of studied patients

3.16.1 TNF-a G->A 308 Polymorphism analysis

For the determination of TNF-a G-+A promotér gene polymorphism at position -308, genomic DNA was extracted from peripheral blood leukocytes using the Qiagen Blood DNA Kit in accordance with the manufacturer's instructions. The TNF-a G-»A polymorphism was determined by polymerase chain reaction (PCR) with subsequent and respective restriction fragment length polymorphism (RFLP) (160). This method was developed to determine the G to A transition at position - 308. A 107 base pair (bp) region was amplified using PCR with both positive and negative controis included in each run. Amplification products were digested with Ncol. The TNF-a -308A allele remained undigested (107bp), while the wild type allele TNF-a -308G produced two fragments (87bp and 20bp). The DNA fragments were analysed on a 3% Metaphor agarose gel and visualized by ethidium-bromide staining. The 107 and 87 bp fragments are clearly visible on the plate in figuře 2.

Figuře 2. DNA fragments plated on metaphor agarose gel with ethidium bromide staining.

3.16.2 Biochemical laboratory assays

Routine blood chemistry was performed (blood count, IL-6, AST, ALT, urea, creatinine, ions, glycemia, CRP, urine analysis). CRP sérum levels were determined by immunoturbidimetric method using Olympus AU 2700 Analyser by K-assay set (Kamiya Biomedical Company). CRP was measured by nephelometry (340 nm). The normál range was considered 0-10 mg/l. CRP results were matched with disease activity and the promotér SNP at TNF-a -308.

3.16.3 Sample collection and detection of *H.pylori* stool antigen by the Amplified IDEIA HpStAR kit

All children who had laboratory testing done were prospectively screened for active H.pylori infection by means of monoclonal stool antigen testing. Previous work from our laboratory validated this test for evaluation of *H.pylori* infection in children in the Czech Republic (161). *H.pylori-spec\T\c* antigen was analysed by a commercially available validated enzyme immunoassay kit, the Amplified IDEIA HpStAR kit (DakoCytomation), currently marketed in the Czech Republic for stool antigen testing. All tests were performed at the local microbiological laboratory by the same laboratory staff during the study and under the same conditions, following the procedure provided by the manufacturer (DakoCytomation). It uses an immunoaffinity purified monoclonal anti-H.pylori antibody absorbed to microwells. A stool sample was collected from each subject, and faecal specimens were stored at - 70°C until analysed. Firstly, approximately 0.1g stool was emulsified in 500 ul sample diluent, vortexed and then centrifuged (5000 r.p.m for 5 min). Supernatant (50ul) was added to antibody-coated weils, along with 50ul peroxidase-conjugated anti-H.pylori antibody solution: this was incubated at for one hour at room temperature. The weils were washed with a washing buffer to remove unbound material, and a substráte was added before further 10 minutes incubation. Following the addition of 100 ul stop solution optical density (OD) was read by spectrophotometer and results were recorded as described for the HpSA kit. The test is qualitative, and samples

were assigned as either negative oř positive on the basis of OD450/OD630 double wavelength readings of < 0.150 and £ 0.150, respectively, in accordance with the manufacturer 's recommended cut-off values. All procedures with stool samples included appropriate safety measures to avoid risk of contamination of the specimen during collection, storage, and processing.

3.16.4 C13 - Urea Breath Test (UBT)

The procedures were done according to the manufacturer's instructions (Helicobacter test INFAI, Institut fur biomedizinische Analytik & NMR - Imaging GmbH, Germany) and a previously validated technique (162). The patient fasted overnight before test. After a fasting baseline sample breath collection, 75 mg of 13C urea was given to the patient together with citric acid. An additional breath sample was collected 30 minutes after the urea ingestion. Exhaled air samples were sent for mass spectrophotometry assay, and a value of 35%0 as a cut-off value.

3.16.5 Endoscopy, gastric biopsies, histology, culture and urease test

Upper GIT endoscopy was performed for RAP. At least six gastric specimens from the antrum and body mucosa were obtained in all children during endoscopy for histopathological evaluation (hematoxylin & eosin staining) and *H.pylori* assessment (Gramm, Warthin- Starry staining, rapid urease test, culturing). All biopsy samples were were examined by the two pathologists, experienced in digestive diseases who were blinded as to the clinical data (O.H., F.H.). Gastritis was graded by the Updated Sydney systém (163). Acute inflammation (presence of polymorphonuclears celis) and chronic inflammation (presence of lymphocytes and mononuclear celis) werr assessed on a scale (0, none, 1 mild, 2 moderate, 3 severe).

3.16.6 Determination of *H. pylori* infection status

In order to know the relation between faecal calprotectin profiles and whether active infection with H.pylori was present, the gold standard for positive infection, as suggested by the Maastricht Consensus Report 1997 (164) was used. The diagnosis of *H.pylori* status of all patients was assessed by combination of two non-invasive tests not requiring biopsy (employing multiple tests should increase the accuracy of diagnosis), including measurement 13C-urea breath test and monoclonal stool antigen test and direct invasive tests (bacteriology, histology, urease test).

3.16.7 Foilow-up management of IBD - the Paediatric Crohn's disease activity index (PCDAI)

Monitoring of disease activity was calculated using the PCDAI in each patient at follow-up. The PCDAI is a useful monitoring tool for disease activity calculated from clinical and laboratory markers (ESR, albumin, and haematocrit), but it is time-consuming to use in routine clinical practice and setting (160). A numerical score (0 -100) based on generál well-being, clinical symptoms and objective measurements of weight, height and clinical examination, as well as laboratory tests (ESR,CRP, CBC with differential, platelet count, sérum albumin) provide an objective means to follow disease activity and response to treatment. A score of 0-10 indicates inactive disease, 11-30 mild-to-moderate disease, and >30 moderate-to-severe disease.

Disease remission was defined as PCDAI score < 10. IBD patients were maintained on various medical regimens. Evaluation of disease activity was done at the time of stool collection. The clinicians were unaware of the faecal calprotectin levels when judging the disease activity. These patients were age and sex matched with healthy controls. All patients and controls were of Czech nationality.

3.17 Ethics

The research protocol of this prospective study was reviewed and approved by the Ethics Committee of the Charles University Hospital, Pilsen, and it conforms to the provisions of the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000). All parents or legal guardians of minors gave their consent prior to participation in the study and their anonymity was prepared.

3.18 Sample size and statistical analysis

Sample size and statistical analysis in SPSS (version 11, SPSS, Chicago, III) was used to analýze the data. The primary endpoint was to assess normál references of faecal calprotectin in the paediatric population. The secondary endpoints were to establish the faecal levels in children according to other gastrointestinal involvement. Based on 2- tailed testing with a = 0.05 and (3 = 0.20, a sample size of children in all groups was determined to be considered a clinically relevant and ensure equal numbers of children. Between and within assay variations were performed. All results are expressed as mean ± SD or as medián where appliacable. We used a majority of non-parametric rank tests. Comparisons among the diagnostic groups in terms of continuous measurements were made by the Kruskal-Wallis test (nonparametric ANOVA). The Kaplan-Meier method was used to compare the effect of treatment on GIT symptoms.Children enrolled in the study (controis, patients) were compared by using Student test or the Mann-Whitney test for normally distributed data and Wilcoxon's test for other data. Subgroups (percentages) in the populations were compared by means of Fischer's exact test. The Sperman and Kendall correlation coefficient were calculated to verify the correlation between individual faecal calprotectin concentrations and test values for the parameter study A level of $P < .05$ was considered statistically significant.

A receiver operating curve (ROC) analysis was used to assess the best cut-off for identifying the presence of organic disease or intestinal inflammation. ROC curves were

generated by plotting the relationship of the true positive cases (sensitivity) at avarious cut-off points during the test,. An area under the curve (AUC) of 1.0 is characteristic of an ideál tes, whereas 0.5 indicates a test of no diagnostic value.

3.18.1 Statistics TNF-a G-+A Polymorphism analysis

All data were entered into the centrál database. All values were presented as means with standard deviations (SD) and as proportions of the entire sample (%). Measurable data were compared using the t-test, Wilcoxon test, or Kruskal-Wallis test as appropriate. Subgroups (percentages) in the two populations were compared using chi-square test, or when appropriate, Fisher's exact test. Correlation coefficients were calculated by the Pearson formula. We calculated the odds ratios (ODs). For each OD, the 95% confidence intervals (Cis) and p-value were estimated. Multivariate logistic regression was carried out to determine which variables were independently associated with CD complications. Variables included TNF-a G-»A polymorphism, age, gender, disease duration, age at onset, age at inclusion, the PCDAI, extent of disease, number of previous flares, number of hospitalizations, bowel surgery, and CRP. Each variable was transformed for univariate and multivariate analyses. All factors that were at least marginally associated with CD complications were tested by multivariate logistic regression analysis. The SPSS software for Windows, Version 10, (SPSS lne Chicago, IL) was used to analyse the data. The level of significance was defined as $p < 0.05$ if not otherwise stated.

4 Results

Patients: Included in the study were a total of 306 children of which 142 were included in the calprotectin main study group and 164 in the IBD patient TNF-a analysis. The detailed demographics for the main study groups are presented in Table 8.

4.1 Calprotectin reference values

A total of 41 healthy children (21 male, 20 female) without a chronic disease, without a history of allergies and not on any regular medication were enrolled. Details of age and sex - standardised faecal calprotectin normál values in healthy children less than 15 years of age are demonstrated in (Table 10), Figures 3-11). All subjects were categorized into 3 groups by age A (0-12 months), B (1-6 years) C (6-15 years). Our clinical study provided the following values. Faecal calprotectin values ranged from (19.5 to 294pg/g) in healthy children as a whole without pathology, average faecal calprotectin irrespective of age of children was 69.0 pg/g. medián 45.25 (19.5-73.25. interquaratile range). As per recommendation from the manufacturer the following threshold values were supplied for adults: negative test when <50 pg/g, weakly positive test when between 50 and 100 pg/g, and strongly positive when >100 pg/g (13).

Figures 3 through to 11 demonstrate the results gained in the reference healthy age groups. Group A (0 to 1 year of age), (6boys, 7 girls), mean age ± SEM (6.3 ± 3.4 months), range (1 to10 months), average faecal calprotectin levels: 136 ± 85.0 pg/g, (range 19.5 to 294.0 pg/g), medián 142.75 (66.25-218, interquartile range), Group B (1 to 6 year of age), (7 boys and 5 girls), mean age ± SEM (3 ± 1.7years), range (1-4.5 years), average faecal calprotectin levels: average 51.2± 47 pg/g, (range 20.0 to132.0 pg/g), medián 45.25 (19.5-56.75 interquratile range). Group C (6 to 15 year of age), (8 boys, 8 girls), mean age 9.1± 2.6, average faecal calprotectin 29.9 ±16.6 pg/g, (range 19.5 to 59.5 pg/g), medián 19.5 (19.5-50.25 interquartile range). These values are presented in results Table 8, Figuře 3-11. A significant correlation between age and faecal calprotectin

concentration was found (Spearman's Coefficient = -0.5998 (p.001); Figuře 6 Significantly higher levels of calprotectin in feaces were found in infants compared to older children (the Mann- Whitney test). Group A vs B (p 0.05), Group A vs C (0.001), group B vs C (p=1.0). Boys and girls had similar calprotectin concentrations (p=0.98)

Ten children (77%) in the 1 month to 1 year group were found to have what could be considered overtly positive test results based on adult reference levels. Two of the children (15%) in the 1 to 6 year group were found to have overtly positive calprotectin levels. Two children (6%) in the 6 to 15 year group were found to have weakly positive calprotectin levels. No correlation was observed between leukocyte counts, neutrophils counts and lymphocytes counts and faecal calprotectin in healthy controls (the blood count was obtained as a part of routine examnination in children before minor surgical operations).

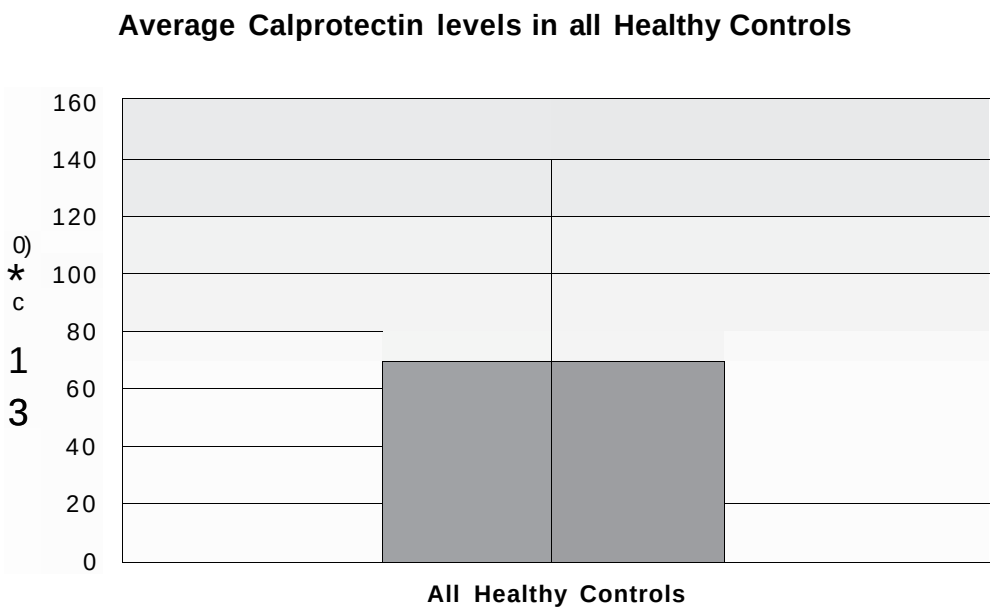
Table 10. Demographic characteristics of the study population combined with determined faecal calprotectin levels in pg/g, mean ± SD, (medián).

Variables	Total (n)	Gender m/f (n)	Age mean ± SD (range)	Calprotectin mean ± SD (median)pg/g
Normál controis	41*	21/20	4,6 ±4,2 (0,08-15)	69.8 ±71.9 (45.25)
1-12 months	13	6/7	6,3 ±3,3 (1-10)	136 ±85.0 (142.75)
1-6 years	12	7/5	3,0 ± 1,7 (1-5)	51.2 ±47.0 (45.25)
6-15 years	16	8/8	9,1 ±2,6 (6-15)	29.9 ± 16.6 (19.5)
IBD	57**	35/22	14,0 ±4,3 (6-19)	1100.0 ± 813.8 (1050.8)
Crohn's disease	37**	21/16	14,0 ±3,1 (8-17)	1156.5 ±786.6 (1172.3)
Exacerbation	18	10/8	14,1 ±3,1 (8-17)	2052.1 ±344.0 (2140.8)
14 days after therapy	18	10/8	14,1 ±3,1 (8-17)	1168.5 ±423.1 (1278.1)
Remission	18	10/8	13,8 ±3,1 (8-17)	318.8 ±210.7 (358.8)
Ulcerative colitis	20**	14/6	14,1 ±5,9 (6-19)	995.6 ±852.0 (592.4)
Exacerbation	9	7/2	13,5 ±3,6 (9-19)	2212.4 ±217.4 (2294.0)
14 days after therapy	9	7/2	13,5 ±3,6 (9-19)	1765.2 ±179.0 (1729.8)
Remission	12	8/4	14,5 ±7,0 (6-19)	333.5 ±232.5 (379.0)
RAP (symptomatic)	34	13/21	12,4 ±3,6 (3-17)	92.0 ±97.8 (67.8)
H.pylori positive	16	8/8	12,4 ±3,7 (3-17)	85.75 ± 82. (66.13)
H.pylori negative	18	5/13	12,5 ±3,4 (6-17)	97 ± 108,5 (67.75)
Acute gastroenteritis	40	24/16	1,8 ± 1,4 (0,25-7,75)	453.4 ± 500.6 (327.4)
Bacterial	20	11/9	2,0 ± 1.7 (0,5-7,75)	731.3 ±548.9 (555.4)
Viral	20	13/7	1.7 ±0,9 (0,5-3,75)	175.6 ±213.4 (71.0)

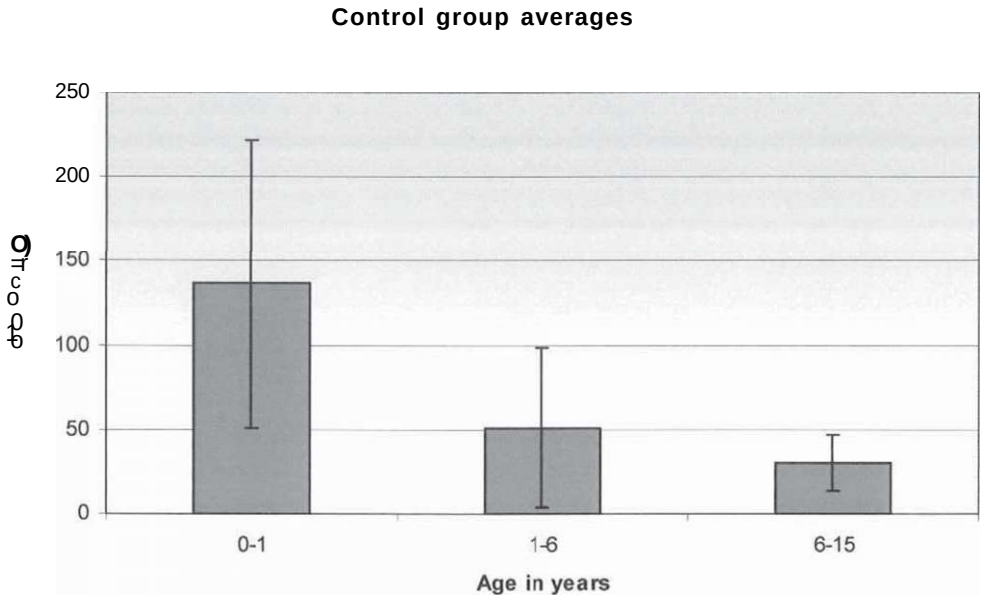
Legend

IBD Inflammatory Bowel Disease
RAP Recurrent Abdominal Pain
Depicting children enrolled only in the calptrotectin study group
Depciting children enrolled both into the calprotectin and TNFalpha study groups

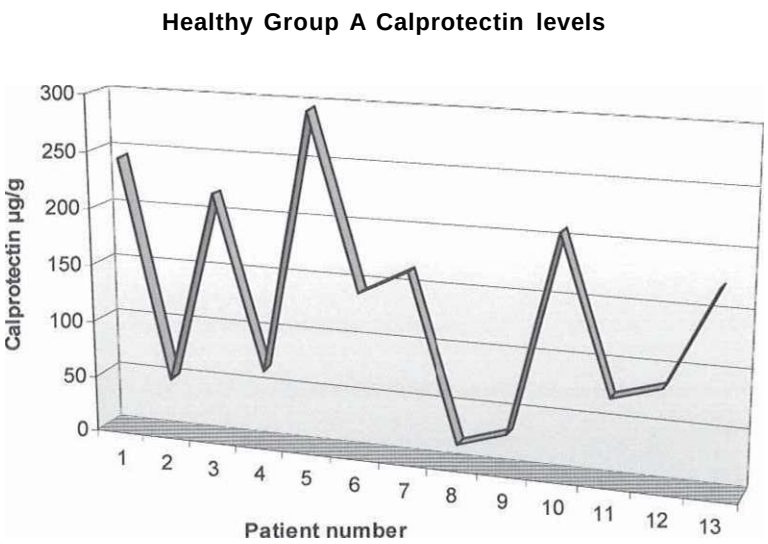
Figuře 3. Average faecal concentrations and range for the entire healthy controis group



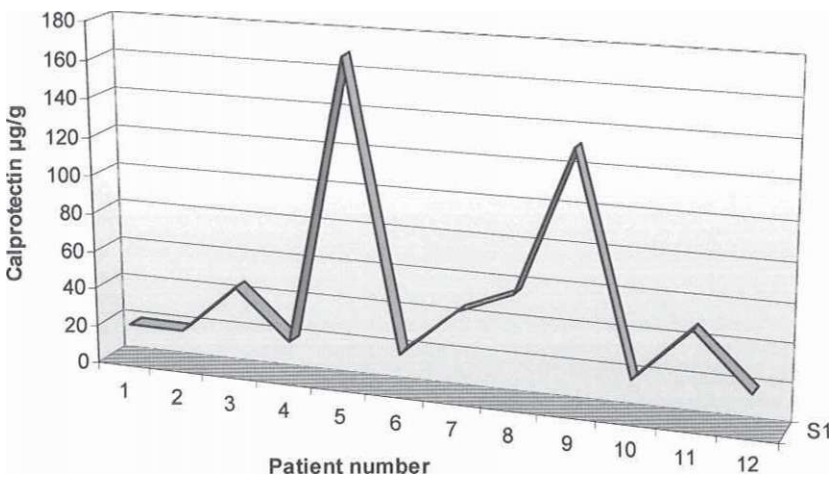
Figuře 4. Average faecal concentrations in three age groups of healthy children



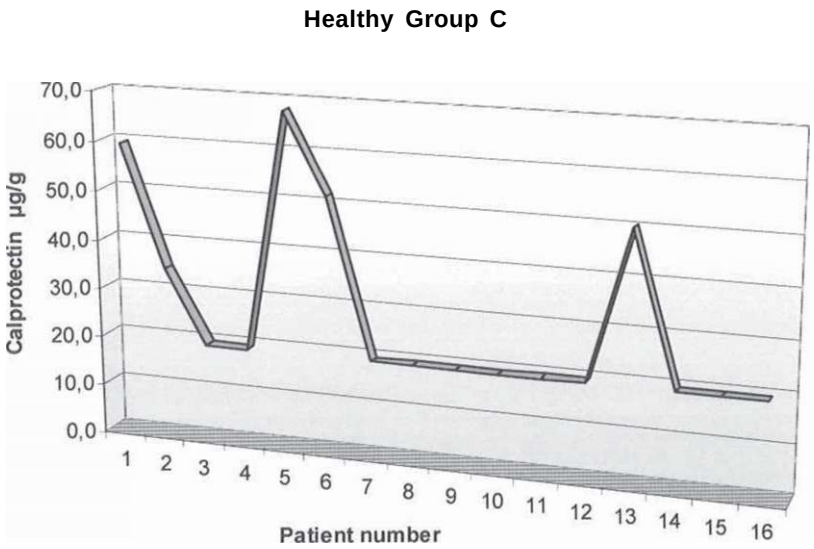
Figuře 5. Age and sex standardized normál faecal calprotectin levels in 13 healthy children aged up to year



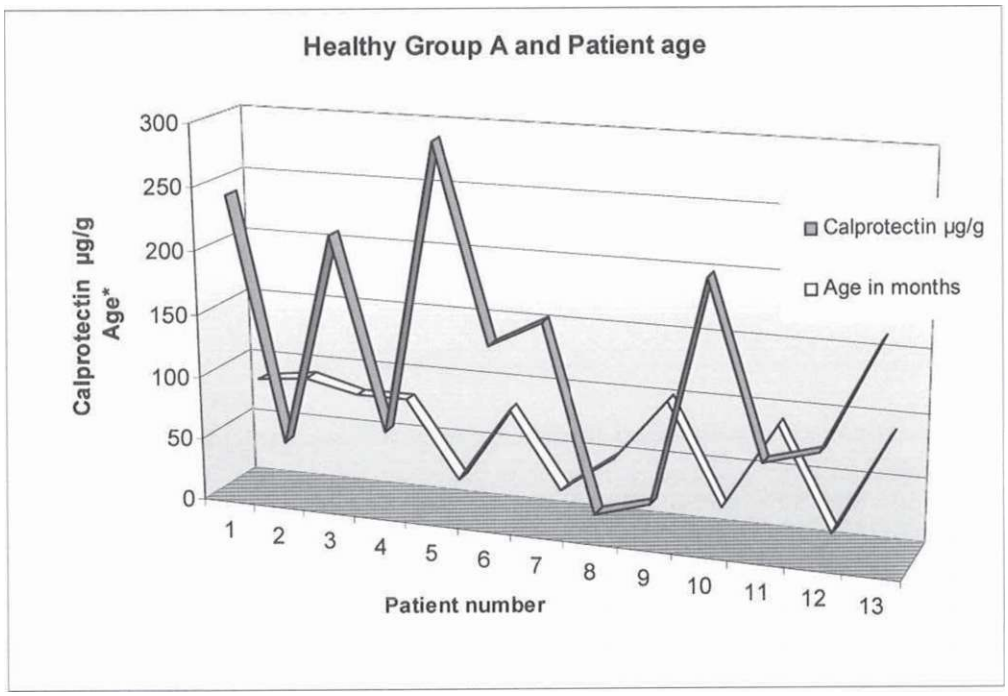
Figuře 6. Age and sex standardized normál faecal calprotectin levels in 13 healthy children aged 1 to 6 years



Figuře 7. Age and sex standardized normál faecal calprotectin levels in healthy children aged 6 to 15 years

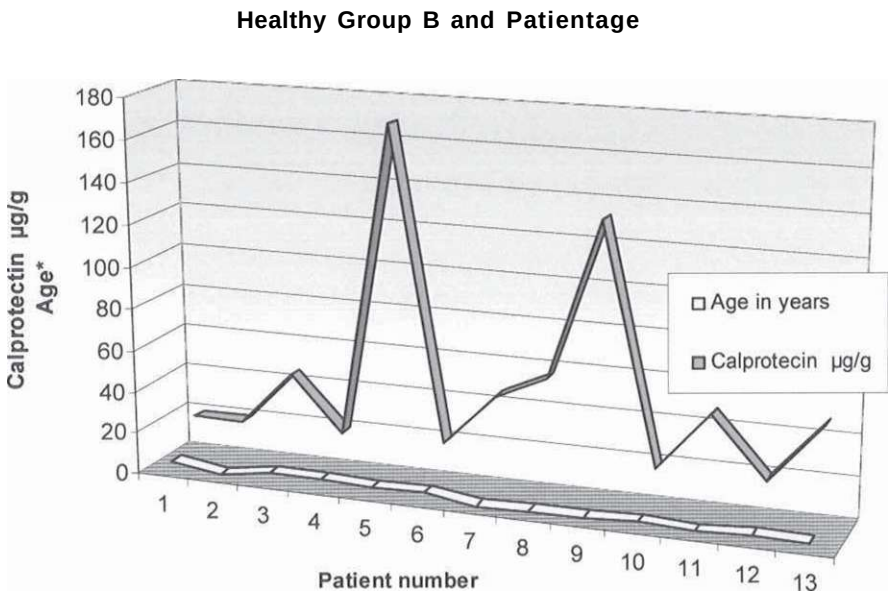


Figuře 8. Comparison of calprotectin levels of individual healthy children within the up to one year group



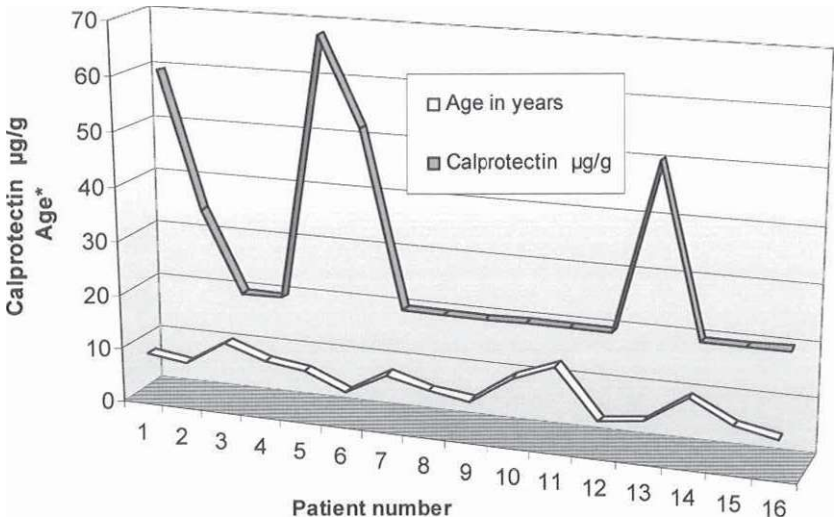
* The 100 point band denotes the age range of the groups up to 10 months

Figuře 9. Comparison of calprotectin levels of individual healthy children within the 1 to 6 years group



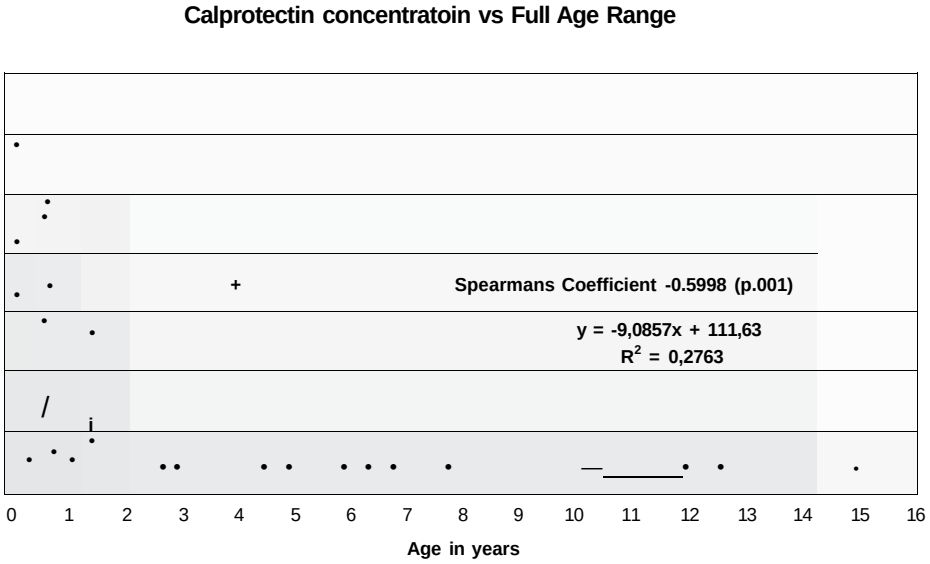
* The 20 point band denotes the age range of the groups up to 20 years

Figuře 10. Comparison of calprotectin levels of individual healthy children within the 6 to 15 years group



* The 10 point band denotes the age range of the groups around 10 years

Figuře 11. Faecal calprotectin concentration and age in 41 healthy children aged 1 month to 15 years



4.2 IBD Group Calprotectin Evaluation

Included in the study were children with IBD and normál control subjects. The IBD group as a whole, comprised 57 children (35 male, 22 female; mean age 14± SD 4.3, range 6 -19) was divided into two main groups designated as Crohn's disease and Ulcerative colitis. The overall calprotectin level for the IBD group as a whole expressed in pg/g as mean ± SD (medián) was 1100.0 ± 813.8 (1050.8).

4.2.1 CD

The CD group comprised of 37 patients (21 male, 16 female; mean age 14.0 ± SD 3.1, range 8-17) which was subdivided into groups tested during exacerbation, 14 days after therapy and during a statě of remission as previously defined: 18 children of which 10 male, 8 female; mean age 14.1± SD 3.1, (range 8-17); 18 children of which 10 male, 8 female; mean age 14.1± SD 3.1; range 8-17; and 18 children of which 10 male, 8 female; mean age 13.8 ± SD 3,1; range 8-17, respectively. Although patients and control groups were not precisely sex and age matched, the age ranges in both groups were similar.

The calprotectin values in pg/g for the Crohn's disease group expressed as mean ± SD (medián) were 1156.5 ± 786.6 (1172.3) and the subgroups of exacerbation, 14 days after therapy and remission were 2052.1 ± 344.0 (2140.8); (1952.5-2270, interquratile range), 1168.5 ± 423.1 (1278.1); (873-1506, interquartile range), and 318.8 ± 210.7 (358.8) (68.75-534, interquartie range) respectively.

Calprotectin levels in CD patients with exacerbation significantly differed to those after 14 days of therapy: mean ± SD (medián) 2052.1 ± 344.0 (2140.8) and 1168.5 ± 423.1 (1278.1), ($p<0.01$). Additional measured parameters that differed significantly between these two groups were: mean ± SD (medián) CRP 61.0 ± 38.4 (44.5) versus 14.6 ± 16.6 (7.0) ($p<0.01$), PCDAI 35.2 + /- 10.2 (33.8) versus 14.5 ± 4.6 (13.8) ($p<0.01$) and ESR

45.3 ± 20.4 (44.5) versus 27.8 ± 16.8 (25.0) ($p<0.01$). Haemoglobin did not differ significantly ($p=0.48$) nor did albumin ($p=0.084$).

Calprotectin levels in CD patients with exacerbation significantly differed to those in remission: mean ± SD (medián) 2052.1 ± 344.0 (2140.8) and 318.8 ± 210.7 (358.8) ($p<0.001$) respectively. Additional measured parameters that differed significantly between these two groups were: mean ± SD (medián) CRP 61.0 ± 38.4 (44.5) versus 3.7 ± 27 (3.0) ($p<0.001$), PCDAI 35.2 + /- 10.2 (33.8) versus 1.4 ± 0.9 (1.0)($p<0.001$), ESR 45.3 ± 20.4 (44.5) versus 12.1 ± 5.9 (14.0) ($p<0.001$), Hb 114 ± 17.7 (115.5) versus 144.2 ± 10.9 (146.0) ($p<0.001$) and albumin 36.9 ± 6.1 (36.8) versus 47.4 ± 3.3 (48.5) ($p<0.001$).

Calprotetin levels in CD patients after 14 days of therapy significantly differed to those in remission: mean ± SD (medián) 1168.5 ± 423.1 (1278.1) and 318.8 ± 210.7 (358.8) ($p<0.001$) respectively. Additional measured parameters that differed significantly between these two groups were: mean ± SD (medián) PCDAI 14.5 ± 4.6 (13.8) versus 1.4 ± 0.9 (1.0) ($p<0.001$), ESR 27.8 ± 16.8 (25.0) versus 12.1 ± 5.9 (14.0) ($p<0.05$), Hb 107 ± 17.9 (110.5) versus 144.2 ± 10.9 (146.0) ($p<0.001$), Albumin 34.7 ± 2.3 (35.1) ($p<0.001$). The difference in CRP values between the two groups did not reach statistical significance. ($p=0.23$)

Further statistical evaluation demonstrated a significant correlation only between calprotectin concentrations during exacerbations and CRP levels ($r=0.6364$) ($p<0.05$). All remaining parameters for each of the two other groups and their additional studied parameters did not demonstrate significant correlations.

4.2.2 UC

The UC group comprised of 20 patients (14 male, 6 female; mean age 14.1 ± SD 5.9, range 6-19) which was subdivided into groups tested during exacerbation, 14 days after therapy and during a statě of remission as previously defined: 9 children of which 7

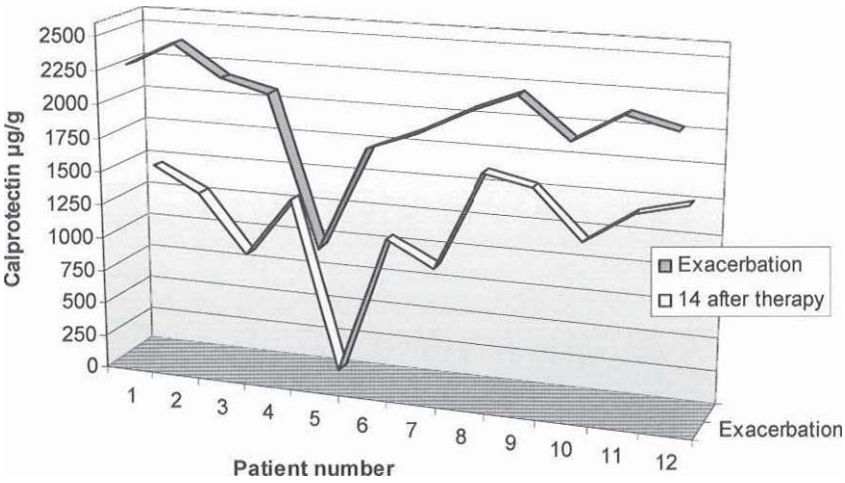
male;2 female, mean age 13.5± SD 3.6, range 9-19; 9 children of which 7 male;2 female mean age 13.5± SD 3.6; range 9-19; and 12 children of which 8 male, 4 female; mean age 14.5 ± SD 7.0; range 6-19, respectively. The calprotectin values in pg/g for the UC group mean ± SD (medián) were 995.6 ± 852.0 (592.4) and the subgroups of exacerbation, 14 days after therapy and remission were 2212.4 ± 217.4 (2294.0); (1852.5-2411.3, interquratile range), 1765.2 ± 179.0 (1729.8); (1554.7-2406.5, interquartile range) and 333.5 ± 232.5 (379.0) (54.5-508, interquartile range), respectively.Calprotectin levels in UC patients with exacerbation did not differ significantly to those after 14 days of therapy: mean ± SD (medián) 2212.4 ± 217.4 (2294.0) verus 1765.2 ± 179.0 (1729.8) (p=0. 12)

Calprotectin levels in UC patients with exacerbation differed significantly to those in remission: mean ± SD (medián) 2212.4 ± 217.4 (2294.0) versus 333.5 ± 232.5 (379.0) respectively ($p<0.001$). Additional measured parameters that differed significantly between these two groups were: mean ± SD (medián) CRP 78.8 ± 86.7 (46.0) versus 4.4 ± 5.6 (2.0) ($p<0.01$) and ESR 53.0 ± 31.7 (48.0) versus 15.5 ± 11.3 (11.6) (11.0) ($p<0.001$). None of the other additional measured parameters reached significance.

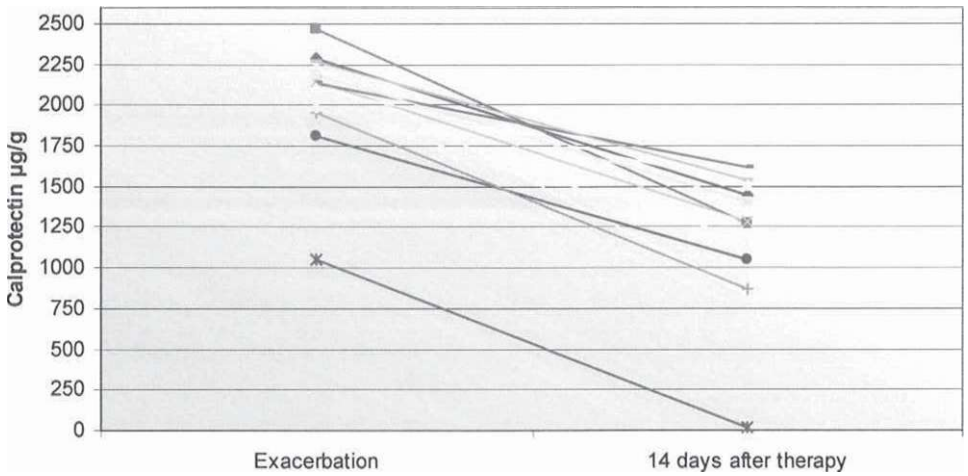
Calprotectin levels in UC patients after 14 days of therapy differed significantly to those in remission: mean ± SD (medián) 1765.2 ± 179.0 (1729.8) versus 333.5 ± 232.5 (379.0) ($p<0.001$). Additional measured parameters that differed significantly between these two groups were: mean ± SD (medián) CRP 23.3 ± 23.3 (14.5) versus 4.4 ± 5.6 (2.0)($p<0.001$) and ESR 28.5 ± 14.5 (29.5) versus 15.5 ±11.6 (11.0)($p<0.001$). None of the remaining additional measured parameters reached significance.

Diasese activity index as assessed by Truleove index correlated with calprotectin in excarebations and remissions in UC.

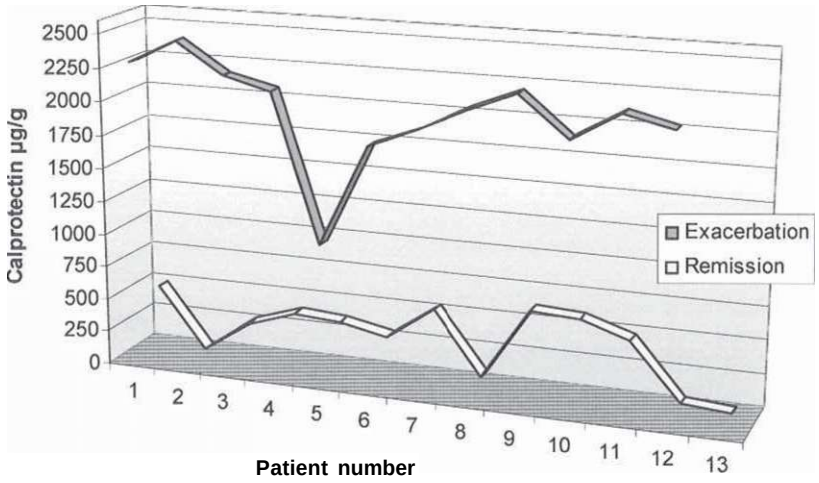
Figrue 12. Calprotectin concentrations in Crohn's Disease patients before and after therapy



Figuře 13. Calprotectin concentrations in Crohd's Disease patients before and after therapy



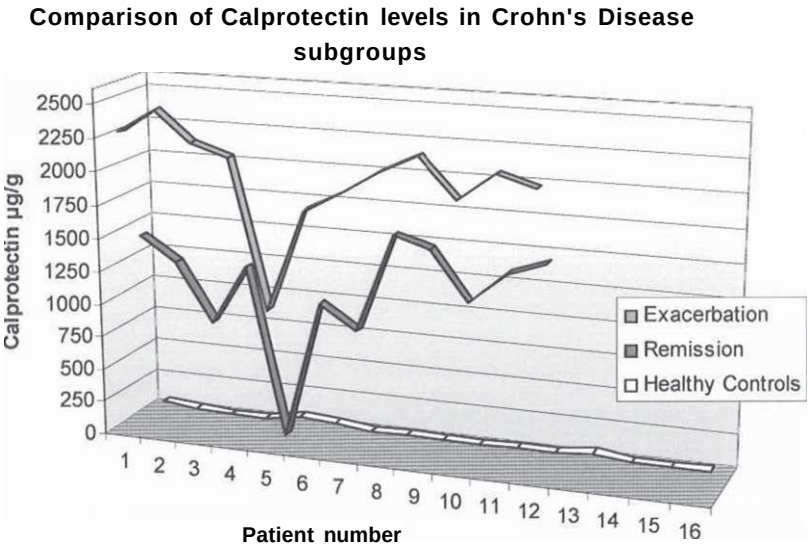
Figuře 14. Calprotectin concentrations in Crohn's Disease patients during exacerbation and remission



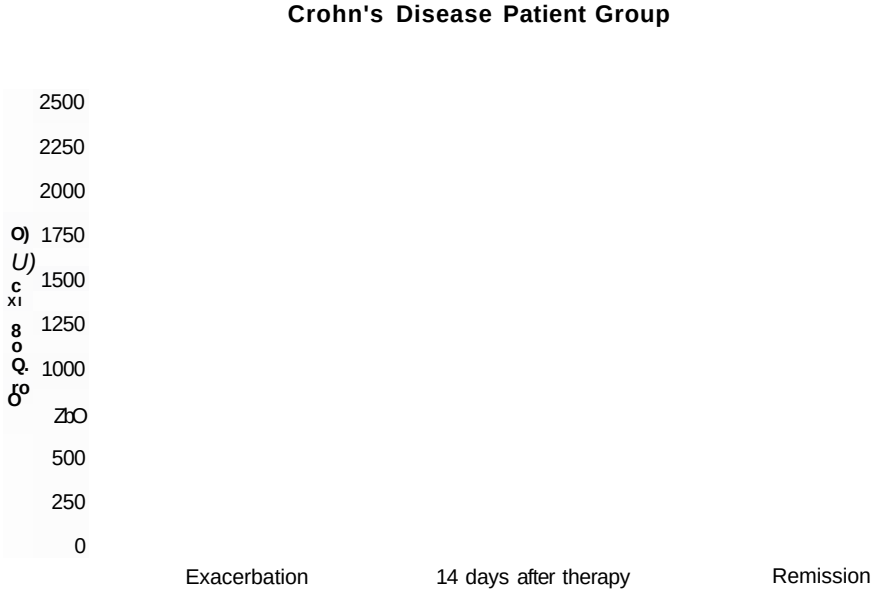
Figuře 15. Calprotectin concentrations in Crohn's Disease patients in remission and healthy controls

Crohn's Disease Remission patients and Healthy Controls

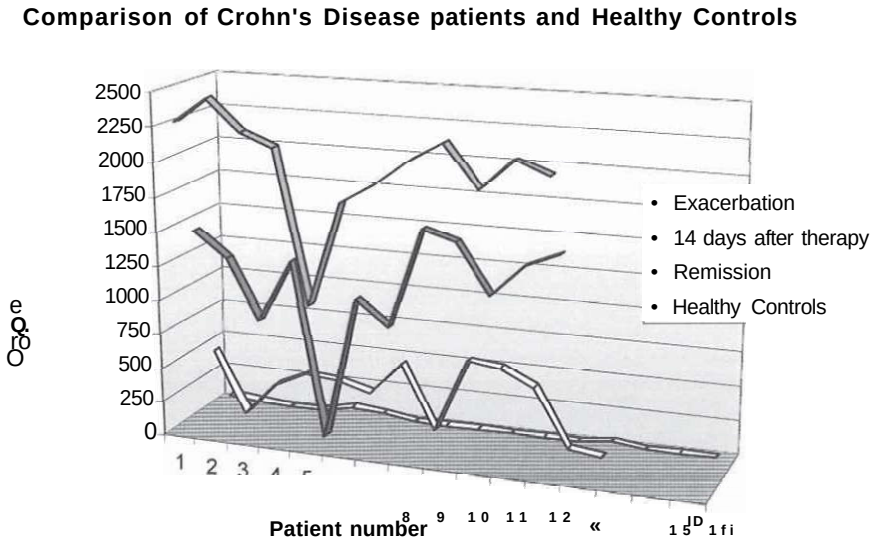
Figuře 16. Calprotectin concentrations in Crohn's Disease patients of the three defined subgroups



Figuře 17. Calprotectin concentrations in Crohn's Disease patients in the three defined subgroups

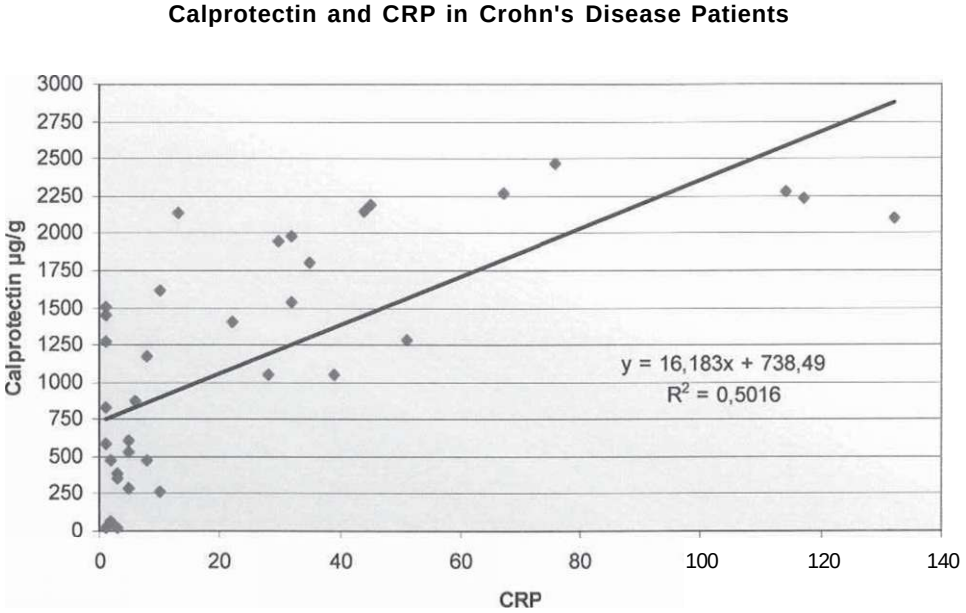


Figuře 18. Calprotectin concentrations in Crohn's Disease patients in the three defined subgroups and aged matched heatlhy controis

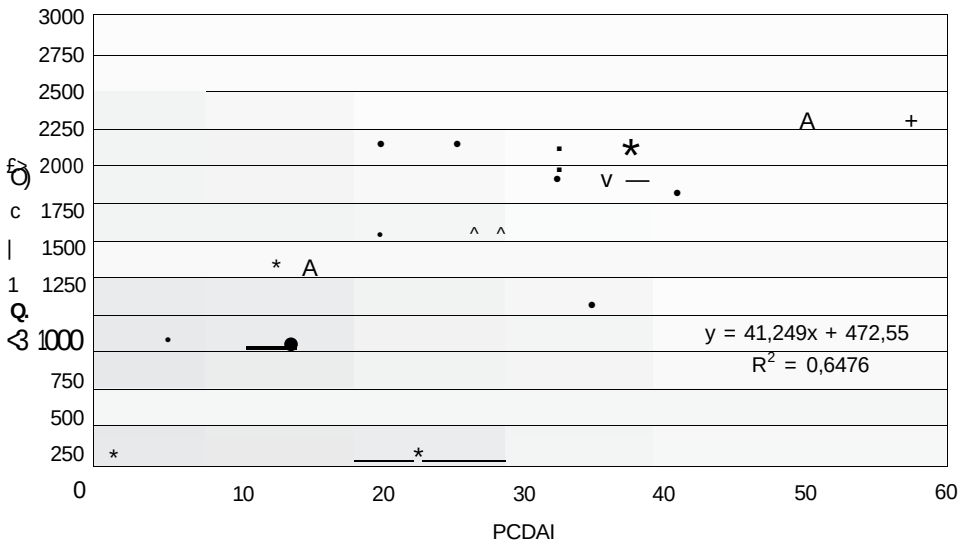


Figuře 19. Calprotectin concentrations in Crohn's Disease patients in the three defined subgroups and aged matched heatlhy controis

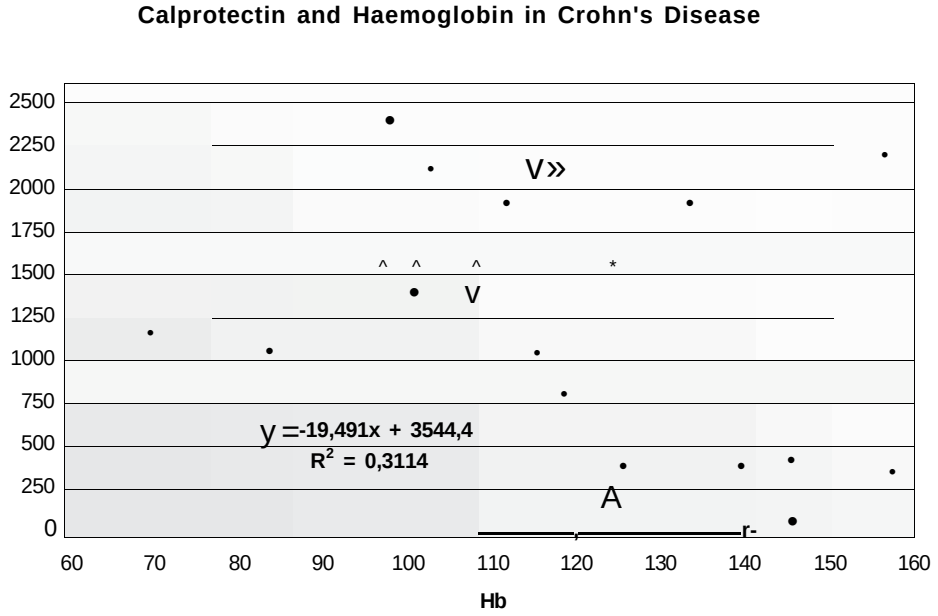
Figrue 20. Correlation between Calprotectin and CRP in Crohn's Disease patients as a whole



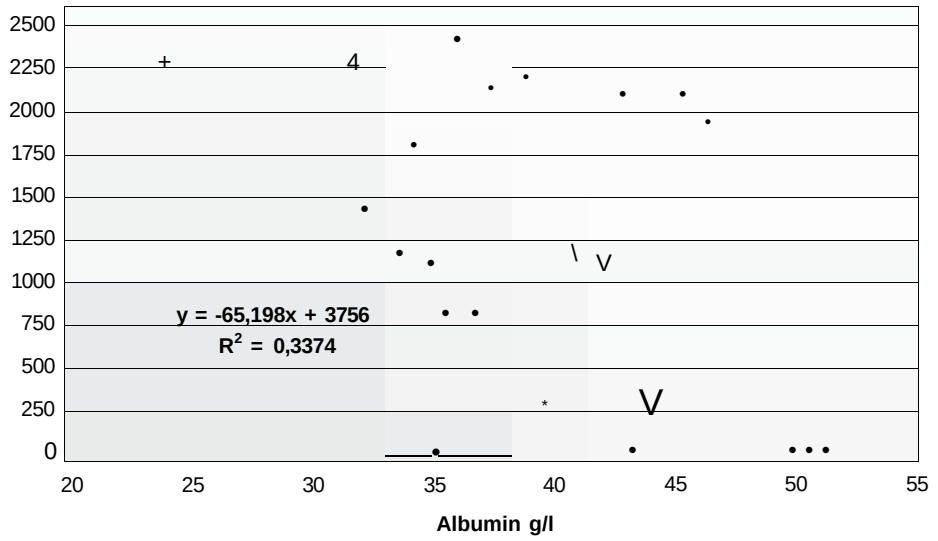
Figuře 21. Correlation between Calprotectin and PCDAI in Crohn's Disease patients as a whole



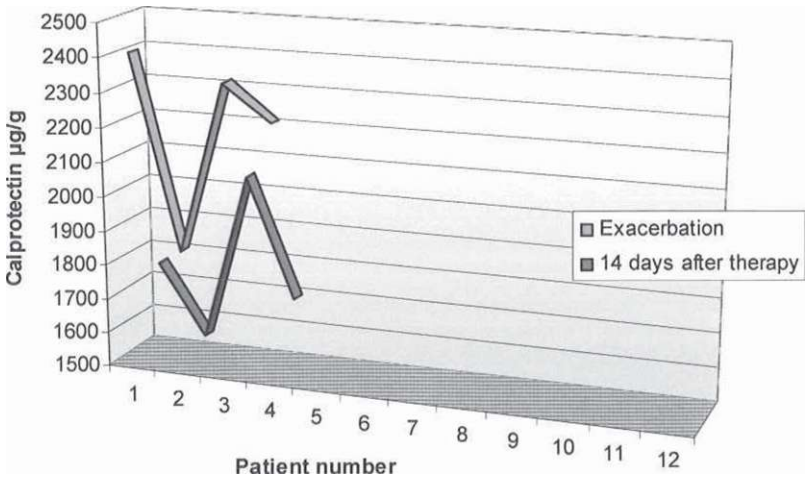
Figuře 22. Correiation between Calprotectin and Haemoglobin in Crohn's Disease patients as a whole



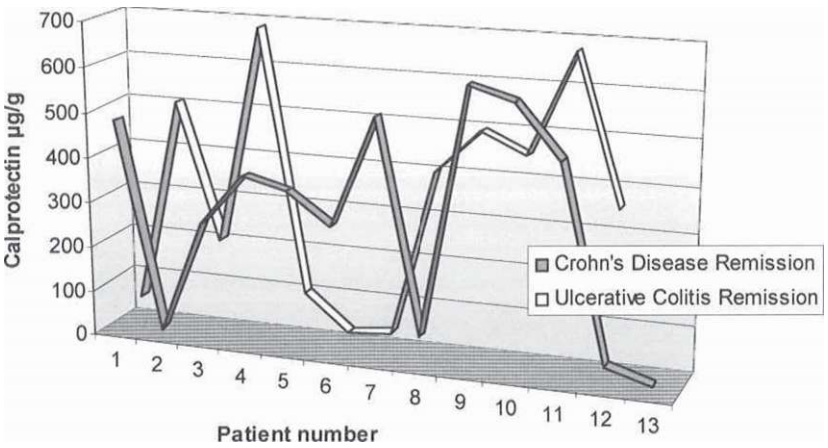
Figuře 23. Correiation between Calprotectin and albumin in Crohn's Disease patients as a whole



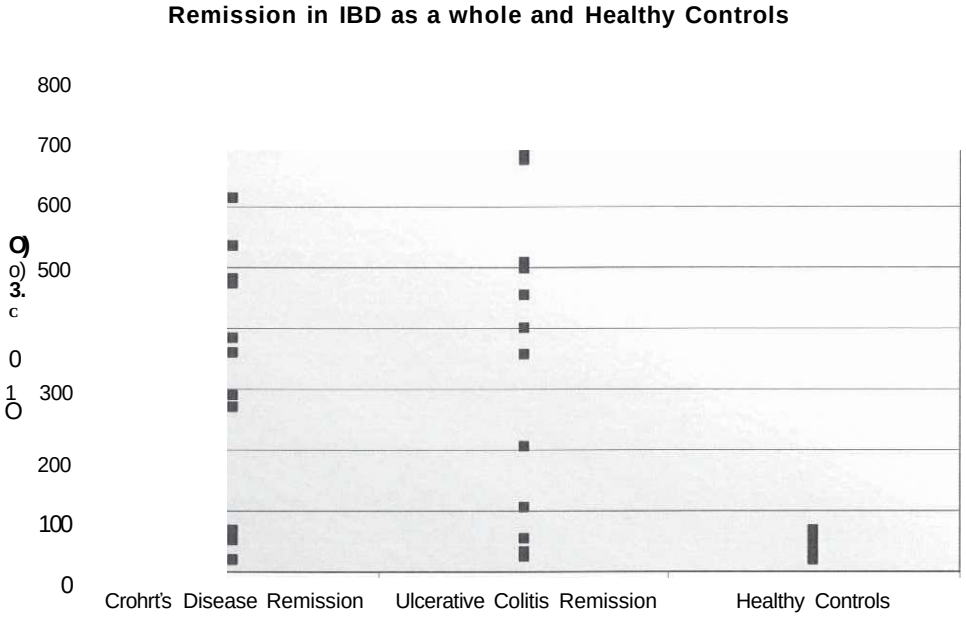
Figuře 24. Calprotectin concentrations in ulcerative colitis patients before and after 14 days of therapy



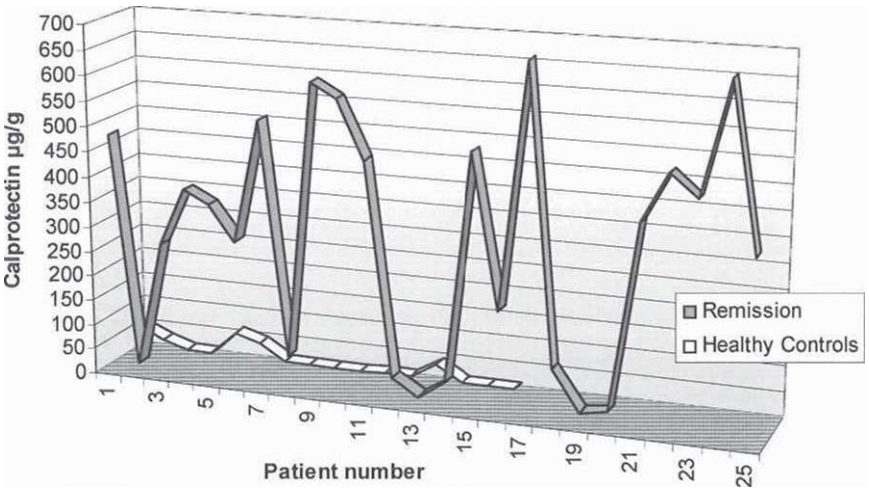
Figrure 25. Calprotectin levels in Crohn's disease and ulcerative colitis patients in remission



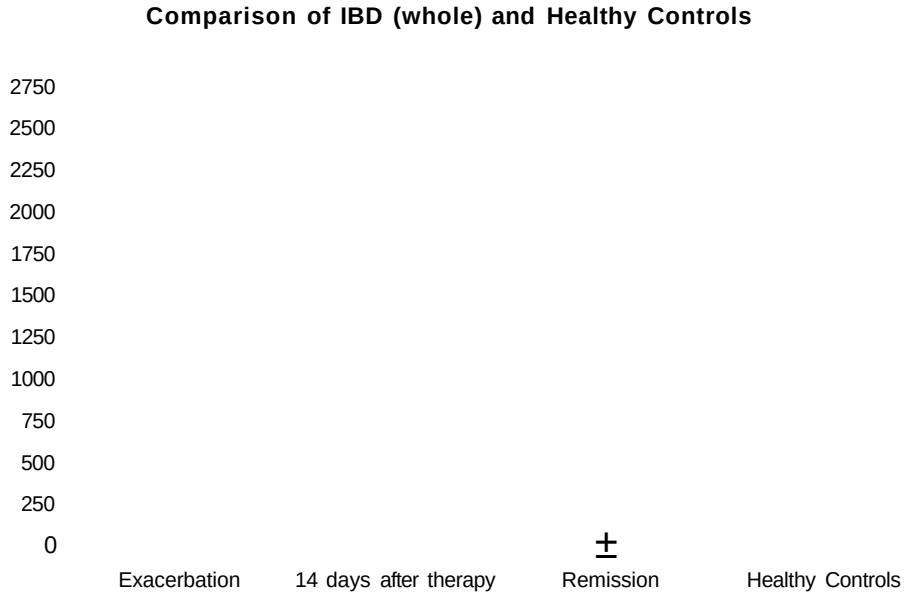
Figuře 26. Comparison of calprotectin levels in IBD patients as a whole in both subcategories and aged matched healthy



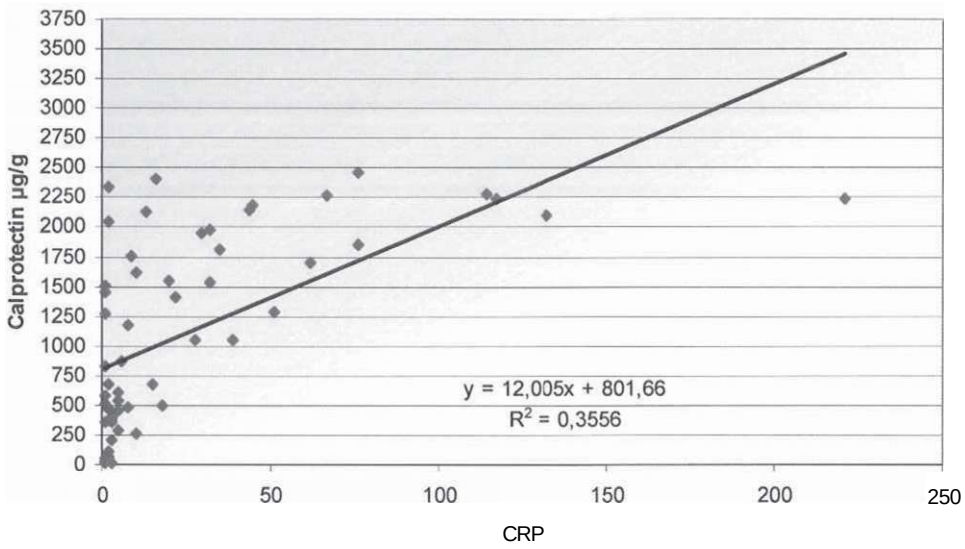
Figuře 27. Calprotectin concentrations in IBD patients as a whole compared with healthy controis



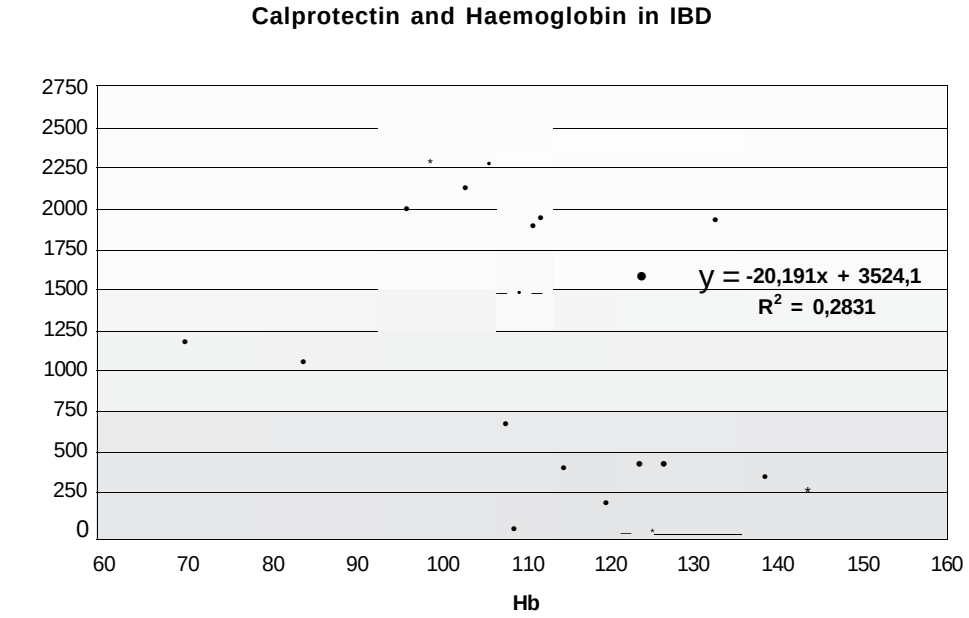
Figuře 28. Calprotectin concentrations in IBD patients as a whole compared with healthy controis



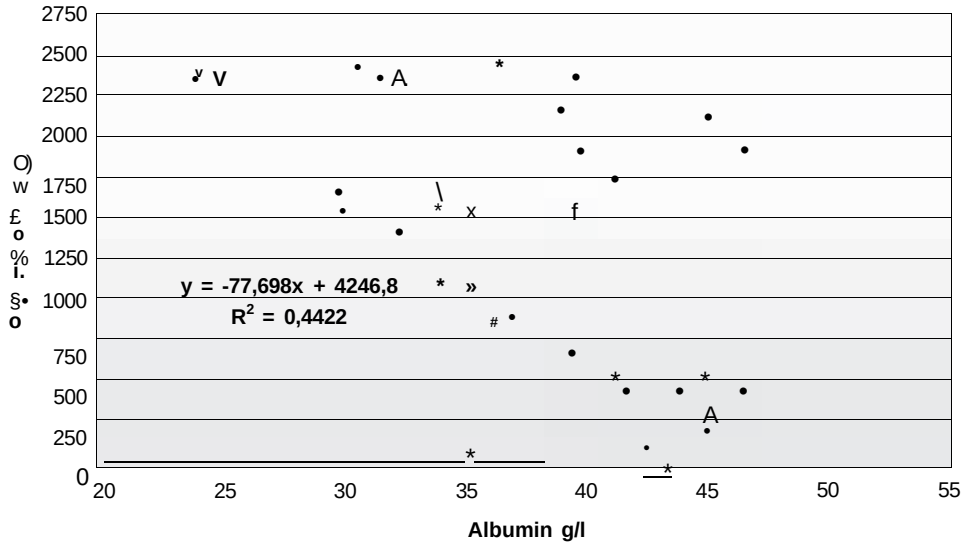
Figuře 29. Comparison of calprotectin levels and CRP in IBD patients taken as a whole



Figuře 30. Comparison of calprotectin levels and haemoglobin in IBD patients taken as a whole



Figuře 31. Comparison of calprotectin levels and albumin in IBD patients taken as a whole



4.3 Paediatric IBD and Cytokine Tumour Necrosis Factor-alpha (TNF-a) A Promotér Gene Polymorphism at Position-308 G-»A: Implications in UC and CD

4.3.1 Distribution of TNF-a-308 G-+A polymorphism in UC, CD patients, and healthy controis

Of the 164 subjects analysed, genotype results were available for all subjects. Overall, 8.5% (7/82) of patients had received a diagnosis before the age of 10 years. All others had the disease diagnosed after age of 10 years. Only 2.5% (2/82) patients had a positive family history of IBD. Of all the 82 IBD diagnoses in our study, 46 (56.1%) patients were diagnosed as having CD and 36 (43,9%) with UC. 22 patients with IBD (26,8 %) and 11 of the 82 controis (13,4 %) carried at least one copy of -308 A (GA, AA) compared with -308 GG patients. Of note, there were two (5.5 %) homozygous subjects for the -308A allele in UC group carrying two copies of -308 A (AA). One control (1.2%) was also homozygous for -308 A polymorphism. When all patients with IBD regardless of the disease phenotype were analysed together, significant differences were found comparing the distribution of the TNF-a 308 A polymorphism between IBD patients and control subjects ($p<0.05$). Comparison of the TNF-a 308 A polymorphism between the UC patients and the control group showed a statistically significant difference ($p<0.001$). The TNF-a 308 A polymorphism was not significantly over-represented in CD patients compared to healthy controis. Results of this analysis are detailed in Table 11.

Table 11. Frequency of TNF-a -308 A polymorphism of both CD and UC patients in comparison to that of healthy controis

	GG	GA	AA	**Total**
Controls, n (%)a	71(86,6)	10(12,2)	1(1.2)	82
Patients, n (%)a	60(73,2)	20 (24,4)	2 (2,4)	82
CD, n (%)	38(82,6)	8 (17,4)	0(0)	46
UC, n (%)	22 (61,1)	12 (33,3)	2 (5,6)	36

Legend
UC Ulcerative colitis
CD Crohn's disease
p < 0.05 for all comparisons

Table 12 shows demographic and clinical parameters in studied IBD patients.

Table 12. Demographic and clinical variables of analysed IBD patients

Characteristic variables	UC (n=36)	CD (n=46)
Mean age, yr (SD)	15.5(2.7)	15.3(2.8)
Sex (male : female)	20/16	25/21
Age at diagnosis, yrs (SD)	13.5(3)	12.7 (2.9)
Disease duration, yrs (SD)	2,1 (2,3)	2.6 (2.1)
Duration, percentage of total life, % (SD)	13(12,7)	17.0(13.2)
Average number of exacerbations, n (SD)	1.3(1.3)	1.6 (3)
Average number of IBD-related hospitalisations, n (SD)	2,4 (2)	3,2(2,1)
Extra-intestinal manifestations, n (%)	15(41,6)	13 (28,2)
Bowel surgery, n (%)	0(0)	9(19,5)

Legend
UC Ulcerative colitis
CD Crohn's disease
Data values are expressed as mean (SD)

4.3.1.1 Ulcerative colitis

At the time of diagnosis, clinical activity was mild in 58.3% (21/36) patients, and severe in 41.7% (15/36) patients. 41.7% (15/36) patients developed extra-GIT complications. Disease involved proctitis in 11.1%, proctosigmoiditis in 13.8%, left-sided colitis in 44.4%, and sub (-total) colitis in 30.5%. None of the UC patients underwent surgical intervention prior to inclusion into the study. 97.2% (35/36) patients were receiving treatment with 5-aminosalicylic acid (5-ASA), 33.3% (12/36) with steroids, 22.2% (8/36) with azathioprine, and 13.8% (5/36) with ursodeoxycholic acid. This therapy continued throughout the study course. No significant differences were found in sex, age, age at diagnosis, disease location, and extraintestinal complications with respect to TNF-a-308 G-»A polymorphism. A trend towards a higher frequency of left-sided colitis was observed in -308GG patients, compared to -308 GA and AA children, but these differences did not reach statistically significant level.

4.3.1.2 Crohrt's disease

No significant differences were found in age at diagnosis and sex in both CD and UC. 65.2% (30/46) demonstrated an inflammatory disease phenotype. 34.8% (16/46) CD patients had complex disease that was classified as either stricturing in 26.1% and penetrating in 13.1%. Among them, two patients had concurrent fibrostenosing and perforating complications. Disease was localized to the small bowel in 21.7%, combined small and large bowel in 67,4%, large bowel in 10.9%. Uppertract disease (with or without disease involvement elsewhere) was present in 10.9%. One patient with stenosing and penetrating behaviour had experienced a life-threatening thromboembolic event. 28.3% (13/46) patients had extraintestinal manifestations. 19.6% (9/46) patients had required resections of the involved bowel segments prior to inclusion. At inclusion, 97.8% (45/46) patients were given 5-ASA, 60.9% (28/46) steroids, 32.6% (15/46) azathioprine, 2.2% (1/46) methotrexate, and 13.1% (6/46) infliximab. All subjects with CD were given enteral nutrition.

4.3.2 Clinical features of CD related to strictures and penetrating complications

Comparative data of different clinical patterns between CD patients with and without complications Data are shown in Table 13 and Table 14. No significant differences in any of the background clinical features of CD were observed between patients with stenosing/penetrating behaviour and those without, except for disease duration (years) ($p<0.05$), disease duration as a percentage of total life (%) (0.01), and the number of hospitalizations due to CD ($p<0.01$), Table 13. Stenosing/penetrating complications were significantly associated with a higher PCDAI index ($p<0.001$). There were no significant differences between the location of disease and morphology in CD patients with complications as shown in Table 14.

Table 13. Comparison of clinical features between CD patients with and without complications

Characteristics of patients	Complications n = 16	Inflammatory n = 30	P-value
Sex, (male/female)	10/6	15/15	0.36
Age at onset, yr (SD)	11,9 (3,5)	12,8 (2,5)	0.37
Duration of CD, yr (SD)	3,5(1,2)	2,4 (1,2)	0.05
Duration of CD, (%) (SD)	24,4 (10,4)	14,4 (5)	0.01
PCDAI, mean (SD)	43,5 (15,4)	24,5(7,1)	0.001
CRP (SD) mg/l	81,4(53,3)	33,4(38,4)	0.001

Legend
PCDAI Paediatric Crohn's disease activity score
CD Crohn's disease
Data values are means ±SD

Table 14. Comparison between the iocation of disease and morphology in CD patients with and without complications

Disease location	Complications n = 16	Inflammatory n = 30	P-value
Small bowel, n (%)	2 (8.3)	8 (33,3)	0.1
Small and large bowel, n (%)	12(83,3)	19 (60)	0.09
Large bowel, n (%)	2 (8,3)	3 (6,7)	0.45
Upper GI tract, n (%)	2 (8,3)	3(13,3)	0.67

Legend
Upper GI tract upper gastrointestinal tract

4.3.3 Effect of TNF-a 308 G->A polymorphism on CD behaviour

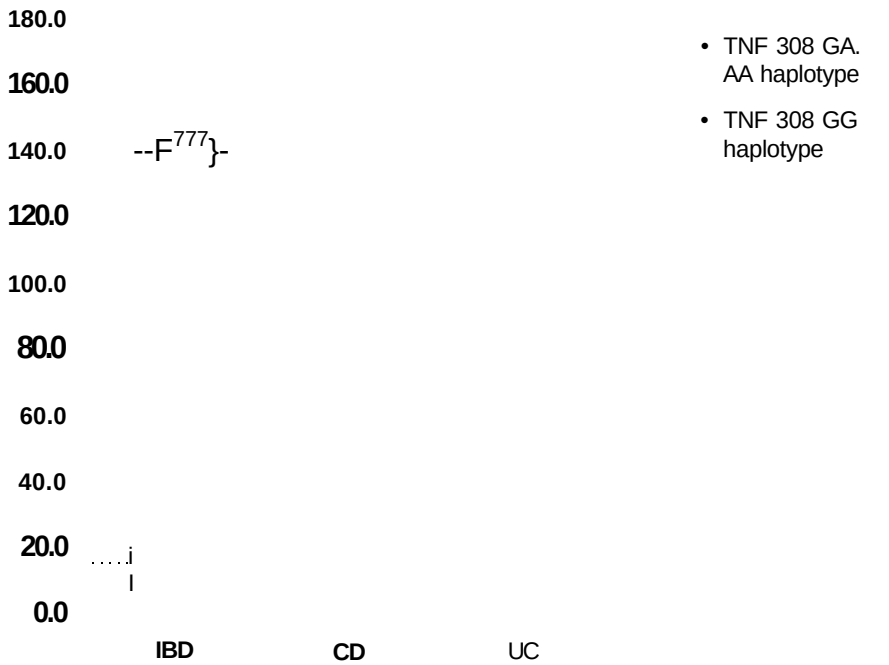
97.8% of newly diagnosed children with CD had simple inflammatory disease at the time of diagnosis. Of note, only one (2,2 %) already had stenotic behaviour at initial diagnosis. 16 of 46 (34.8%) patients were diagnosed as having stenotic and/or penetrating complications at inclusion based on the aforementioned defined criteria. All patients except one carrying TNF-a A polymorphism developed stenosing and penetrating disease, whereas only 9 of the 38 patients with normál GG allele developed complications of CD (21.6%). With regard to genotyping, stenosing/penetrating complications were associated with the TNF genotype. The allele frequencies of the TNF-a A polymorphism were significantly higher in CD patients with complications (stenosing/penetrating) as a whole than in the remaining CD children without complications ($p<0.001$). Moreover, in our CD patients a significant difference was seen in the occurrence of TNF-a A polymorphism

between CD patients predominantly with complications and normál control subjects (p<0.01).

4.3.4 Relationship between TNF-a 308 G-+A polymorphism, laboratory inflammatory activity (CRP) and disease activity of both CD and UC

Mean (±SD) CRP sérum levels (Figuře 32) were 74.9±54.9 mg/l vs. 35,9±40.1mg/l (p < 0.05), for IBD patients carrying at least one mutation -308 A, compared those without the polymorphism.

Figuře 32. Comparison of CRP sérum levels according to the presence of TNF-a -308 GA, AA or GG polymorphisms



Legend

IBD	the evaluation of all patients with inflammatory bowel disease regardless of the disease phenotype.
CD	Crohn's disease UC: ulcerative colitis

*** p< 0.05 ** p < 0.05 *** p < 0.05 Values with * are significantly different from each other. SD of each group is shown as a vertical bar.**

4.3.5 CRP and the PCDAI in CD

Mean (±SD) CRP sérum levels in CD patients were 50.1±49.3 mg/l. Sérum CRP levels were significantly higher in patients with CD intestinal inflammation carrying the -308A polymorphism (94.9 ± 62.8mg/l) compared to 308 GG children (40.7 ± 40.5 mg/l) ($p<0.05$). The PCDAI score was slightly but significantly increased in CD patients with TNF-a 308 A polymorphism compared to CD patients with the GG genotype, 40.4 ± 7.5 vs. 29.7 ± 12.6 ($p< 0.05$). Spearman's rank order correlation analysis clearly revealed a significant linear correlation between CRP and the PCDAI ($n=46$, $r=0.615$, $p< 0.001$). Significant differences in CRP sérum levels with respect to CD complications ($p< 0.001$) were observed, as shown in Table 32. However, our study did not prove a significant correlation between disease extent and both the PCDAI and CRP.

4.3.6 CRP and disease activity in UC

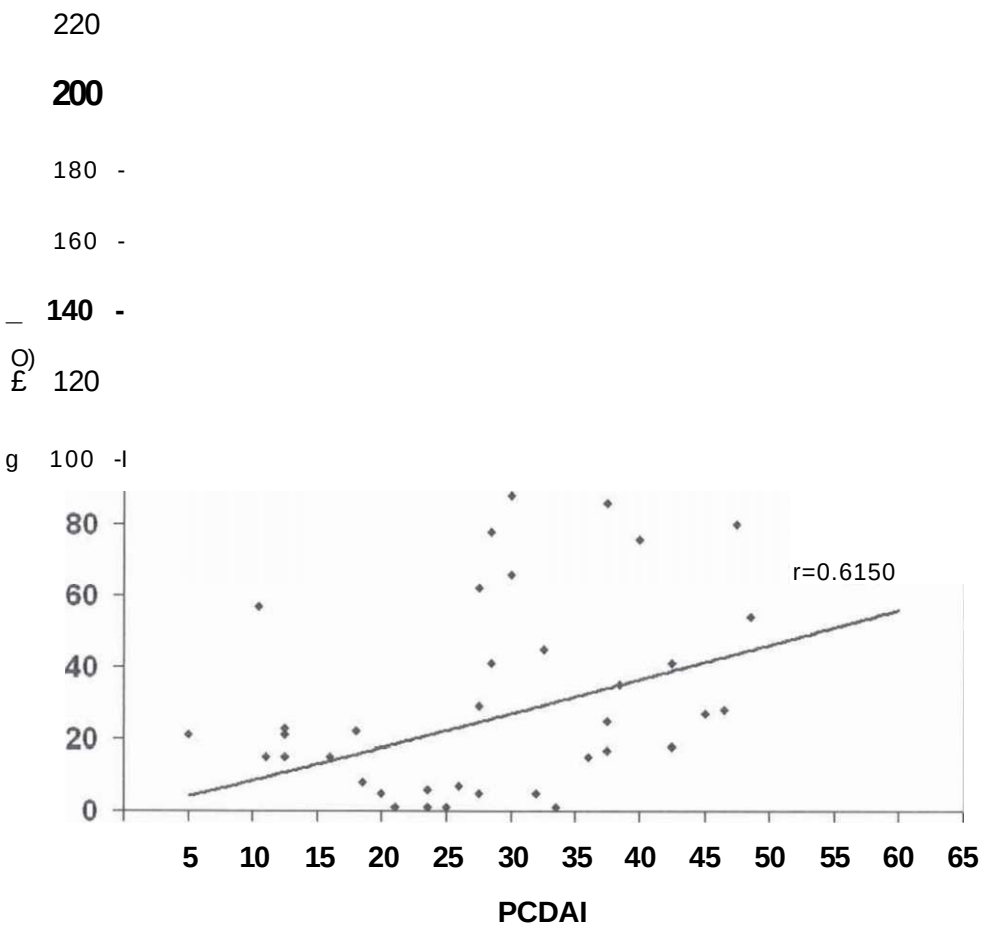
Mean (±SD) CRP sérum levels in the UC group were 43.2 ± 46.4 mg/l. Sérum CRP levels were significantly higher in patients carrying the -308 A polymorphism compared to those with - 308 GG (64.3 ± 42.2 mg/l and 26.4 ± 36.2 mg/l respectively) ($p<0.05$). CRP levels in UC children with mild activity (19.2 ± 27.4 mg/l) were significantly lower than in patients with severe UC activity (81.8 ± 45.1 mg/l) as expressed by the Truelove index ($p<0.001$). Additionally, a significant association was found between TNF-a 308 ***G-*A*** polymorphism and disease activity; mild 2/22 (9.1%), severe 12/14 (85.7%) ($p<0.001$). No significant differences were observed with regard to extent of disease, age and number of male patients.

4.3.7 Multivariate analysis of independent risk factors of the development of CD complications

Univariate comparisons were made with chi-square and Fisher's exact test and multivariate analysis was performed using a multiple logistic regression model. Where comparisons could be made, this multiple analysis revealed that only TNF-a 308 A allele

distribution (OR: 12.9; CI, 1.18-140.81, p<0.001), and CRP sérum levels (OR: 1.020; CI, 1.00-1.04, p<0.001) were independently associated with the development of CD complications. No other clinical or analytical findings were predictive for the risk of development of CD complications. Validation of this logistic regression analysis was confirmed by examining the association of predicted probabilities and the observed mode of behaviour. The percent concordance was found to be 76.1%, thus demonstrating a high degree of predictability of mode of behaviour. None of the other clinical or analytical findings were found to be useful for the prediction of complications

Figuře 33. Relationship between C-reactive protein (CRP) sérum levels and the Paediatric Crohn's disease activity index (PCDAI).



Legend

r	Spearman's correiation coefficient (n=46, r=0.6150, p<0.001)
PCDAI	Paediatric Crohn's Disease Activity Index score
CRP	C- reactive protein

This part of the study has been published in *Journal of Paediatric Gastroenterology and Nutrition,* Cytokine Tumor necrosis factor-alpha A promotér Gene Polymorphism - 308 G^A and Paediatric Inflammatory Bowel Disease: Implications in ulcerative colitis and Crohn disease. 2006, 42: 479-487

4.4 Faecal calprotectin levels in children with RAP, *H.pylori* infection and healthy children

4.4.1 Demographics and clinical features

Thirty-four consecutive new RAP outpatients were finally included and studied, of whom 21 were male. In all cases, the clinical and laboratory follow-up confirmed these diagnoses.

16 patients with RAP were found positive for *H.pylori* infection *(HP+),* their mean age (years) (mean±SD) was 12.42 ± 3.87 years, (range 3-17) (medián 13.5), whereas the other 18 patients were H.py/o/7-negative (HP-), mean age was 12.47±3.54 years, range (6-17) (medián 13). The prevalence of *H.pylori* infection in symptomatic children with RAP is assumed to be in Czech Republic according to our recent study, Sýkora et al. 25.1 % (124). No significant differences were found with respect to age, gender, ethnic group and pláce of residence between infected and non-infected groups.

There were no differences in the mean ages of children or symptom clinical features between the two groups except for epigastric pain during meals, which was more frequent in infected children (25.6% vs. 3.8%) ($p<0.01$). No specific clinical symptoms were identified to predetermine *H.pylori* infection in RAP children. We reported that gastric *H.pylori* infection is associated with similar scores among all paediatric seen for RAP by specialist as compared to non-infected RAP children. There were no significant differences in faecal calprotectin levels with respect to sex.

4.4.2 Endosocopic and histologie evaluation

4.4.2.1 Endoscopy findings

A total of thirty-four children and adolescents were studied. Among the 16 *H.pylori* infected children, antral nodularity (60%) was the most common endoscopic findings, 1

had mild antral erythema, and the remaining 5 children (31%) had a normál gastric mucosa. Thirteen of the 18 (72%) *H.pylori* negative children had a normál gastric mucosa, one child had a pre-pyloric polyp, and four of the 18 (22%) had nodularity in the antrum. No mucosal ulceration was reported. No abnormalities were seen outside the stomach. Children with *H.pylori* infection had significantly higher gastric mucosa nodularity than *H.pylori* negative children ($p < 0.01$, chí2 test).

4.4.2.2 Histology findings

Histological evaluation showed gastritis and duodenitis in all children enrolled. There were no children with normál gastric muocasa. Histopathology (Sydney score) in the *H.pylori* negative gastritis group was significantly milder compared with *H.pylori* - associated antral and body gastritis, grade of gastritis (chronic inflammation) (antral mean score 1.58 ± 0.69 compared with 0.56 ± 0.51, body mean score 1.06 ± 0.71 compared with 0.06 ± 0.24; $p < 0.001$), All the values were significantly higher in *H.pylori* positive subjects compared *H.pylori* negative. No significant differences were observed in antral and body activity between both the groups (data not shown). Of the evaluated RAP patients, intestinal metaplasia and glandular atrophy was not observed.

4.4.3 The effect of *H.pylori* infection on faecal calprotectin biochemical assays

The 34 patients with RAP and upper GIT inflammation due to gastritis had a calprotectin level, which was different, 92.0 ± 97.8 (median67.8) from age matched healthy controis (29.9. ± 16.6) (medián 19.5) The level was higher than that for normál subjects ($p< 0.05$). In children with RAP faecal calprotectin level was 24.9% higher than in healthy controis.

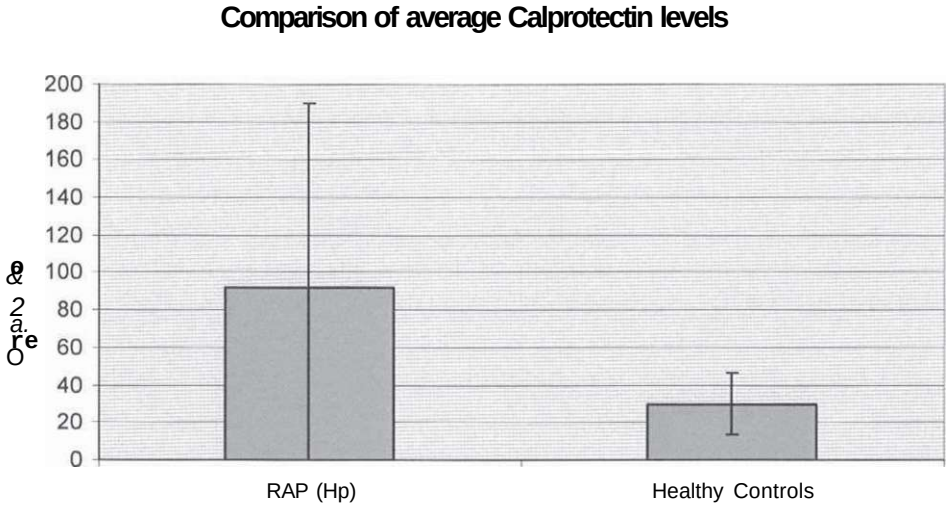
Of the 34 children examined, the mean faecal calprotectin levels were mean± SD (medián) 92.± 97.8 (medián 67.81) pg/g in the 16 *H.pylori* positive children, and 97.1 ±108.5 (67.8) pg/g in the H.pylori negative patients. The RAP group as a whole

differed significantly when compared with healthy control levels ($p<0.05$) though there was no significant difference between *H.pylori* positive patients and controis ($p=0.11$). There was only borderline significance between the *H. pylori* negative patients with chronic gastritis and controis ($p=0.069$). No significant differencese were observed between *H.pylori* positive and *H.pylori* negative RAP patients ($p=0.96$)

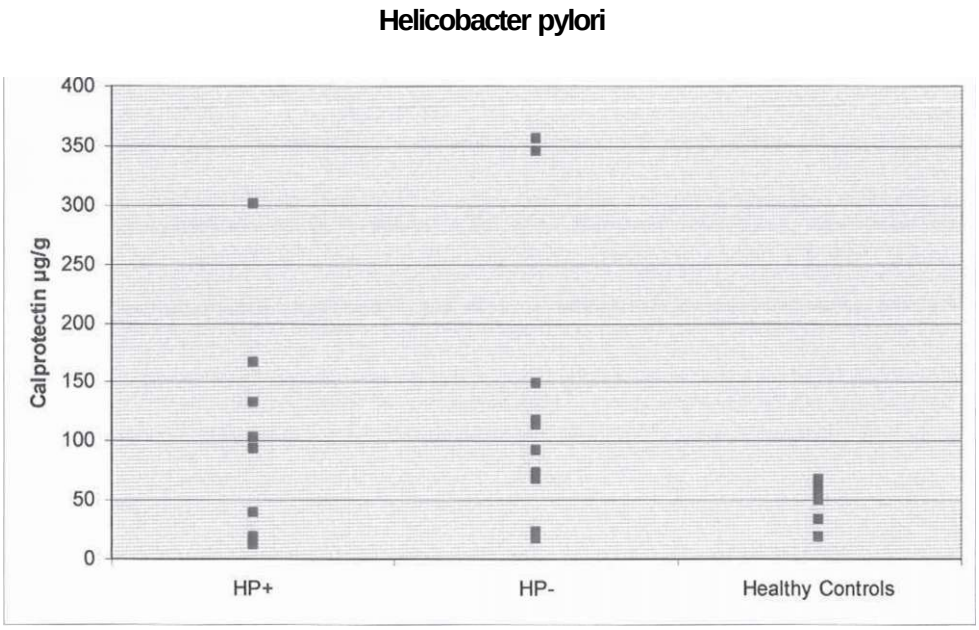
There were differences in the histological findings (grade of chronic inflammation), but not in activity), as mentioned above between the RAP *H.pylori* positive and the *H. pylori,* but no differences were observed in faecal calprotectin levels. The histological changes observed were consistently associated with infection ($p.<0.001$) and not with faecal calprotectin levels. There were no relationship between faecal calprotectin values in relation to histological diagnosis and topography of gastritis (antral, fundic).

The interactions of grade of antral and fundic gastritis and faecal calprotectin levels (Mann-Whitney tests) did not reach statistical significance ($p=0.15$). There was no relationship between clinical symptoms, score intensity and faecal calprotectin levels in both RAP groups. Because there were no relationship between histopathology and faecal calprotectin levels, in. These trends would not tend to support the hypothesis that chronic *H.pylori* infection may have a direct effect on faecal calprotectin levels and faecal calprotectin levels is not capable of .distinguishing between pattems of gastritis.

Figuře 34. Average calprotectin levels and ranges for recurrent abdominal pain patients and healthy controis



35. Spread of calprotectin values for Helicobacter pylori postive and negative patients and healthy controis



This part of the study has been published partly in *Acta Paediatrica* *Helicobacter heilmannii* gastroduodenal disease and clinical aspects in children with dyspeptic symptoms. *Acta Paediatrica* 2004, 93,pp. 707-709

4.5 Faecal calprotectin in AG subjects associated with gut pathogens

A total number of fourty consecutive children with newly diagnosed AG in a prospective manner at the time of definitive diagnosis were entered into the study. AG patients were divided into 2 groups depending on aetiology of AG, 20 with viral AG (VAG) (rotavirus, adenovirus.astrovirus) (medián age 14 months) and 20 with bacterial AG (BAG) *{Salmonella spp, Yersinina enterocolitica,* enteroapathogenic *E. Coli, Campylobacter, Shigella)* (medián age 22.5 months) was enrolled. In the BAG childrens group there were two older children. In the viral group there was one child over age. These three children were excluded from the finál study.

In all children, the definitive diagnosis causing acute diarrhoea was based on complete analysis (clinical features, microbiology, virology, laboratory indicators) and faecal calprotectin levels. Infants with undefined etiology of AG were excluded. No cause other than AG was found during evaluation to explain the symptoms in any of these infants. The data were compared between both groups and were also compared with those of matched controis.

One way analyses of variance or contingency table analysis, as appropriate, indicated no significant differences in admission characteristics between the groups (weight, duration of diarrhoea, no. of diarrhoeal stools/day). There was no significant difference in age at onset of diarrhoea between viral or bacterial AG. However, there was a tendency toward an earlier onset of digestive symptoms in children with viral AG (medián 14 months) compared to bacterial AG (medián 22.5 months), but did not reach statistical signficance.

4.5.1 Faecal calprotectin concentration analysis

In contrast with the onset of acute diarrhoea, faecal inflammatory parameters as assessed by calprtotectin concentrations were significantly different between the two groups. Faecal calprtotectin levels from patients at the time of diagnosis of AG were

evaluated in all paediatric subjects and were compared. Faecal calprtotectin values for children with BAG: mean±SD (medián) (731.3 ± 548.9 (555.4) were significantly higher compared to children with VAG (175.6 ± 213.4 (71.0) ($p<0.001$). Aditionally, we detected a marked difference between BAG and age matched controls ($p< 0.001$). Faecal calprtotectin values did not differ significantly between VAG and healthy controls ($p=0.95$). No differences were detected in faecal calprtotectin levels between female and male patients.

4.5.2 Biochemical assays

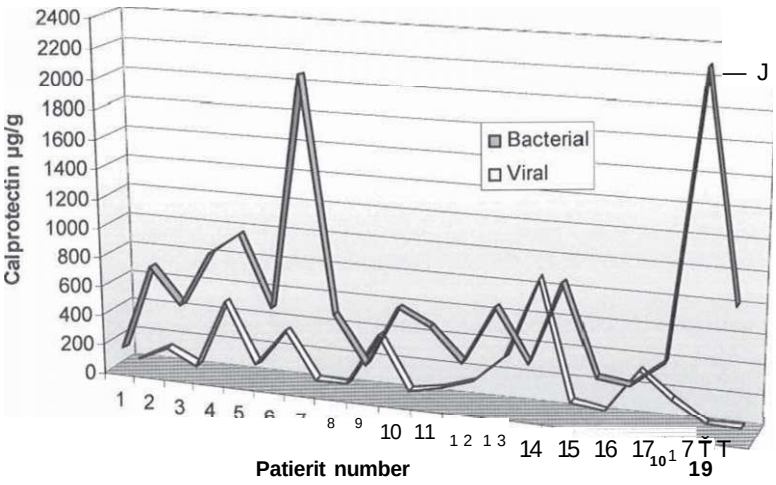
AG patients were segregated into 2 groups according to the aetiology and these data were compared between the two groups. ESR was significantly higher in children with BAG compared to VAG, (medián 23, 25.57 ± 18.21 vs medián 5.0, 8.407 ± 9.912 respectively) ($p< 0.001$). Peripheral leukocyte counts were significantly higher in BAG compared to VAG (medián 12.45, 13.45 ± 5.55 vs medián 9.30, 10.64 ± 6.2 respectively) ($p<0.05$). Early neutrophils (bands) were significantly higher in infants with BAG compared to subjects with viral infection (medián 10, 10.67± 11.09 vs. 0.09, 4.566 ± 7.74) ($p<0.05$) For CRP, they were (medián 42, 56.88 ± 54.91 in BAG vs. medián 1.5, 6.265 ± 7.688 in VAG children ($p< 0.001$). Finally, for potassium levels, they were significantly lower in children with VAG (medián 4.1, 4.047 ± 0.934) versus BAG (medián 4.55, 4.695 ± 0.748) ($p< 0.05$). Other markers of blood count and biochemical markers including IL-6 were also measured and collected at each time-point. These remaining parameters were not significantly different between the two groups.

4.5.3 Correiation between Calprotectin levels in feaces inflammatory and biochemical markers

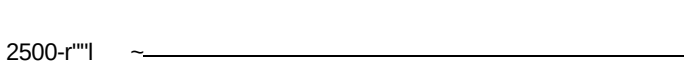
Faecal calprtotectin levels in the two groups were correlated with biochemical and inflammatory markers. In the BAG group, faecal calprtotectin levels negatively correlated with platelets ($r = - 0.4505$, $p< 0.05$), sodium ($r = -0.3686$, $p< 0.05$), and chloride ($r =$

0.4437, $p < 0.05$). In the VAG group, faecal calprtotectin values correlated with CRP ($r = 0.4926$, $p < 0.01$). Correlations between faecal calprtotectin concentrations and other biochemical and inflammatory markers within the two groups did not reach statistical significance.

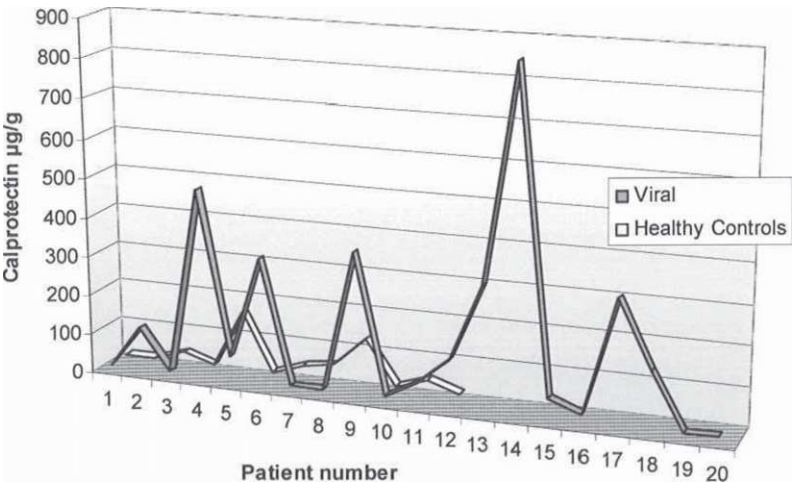
Figuře 36. Infectious diarrhoeas according to aetiology and their calprotectin levels



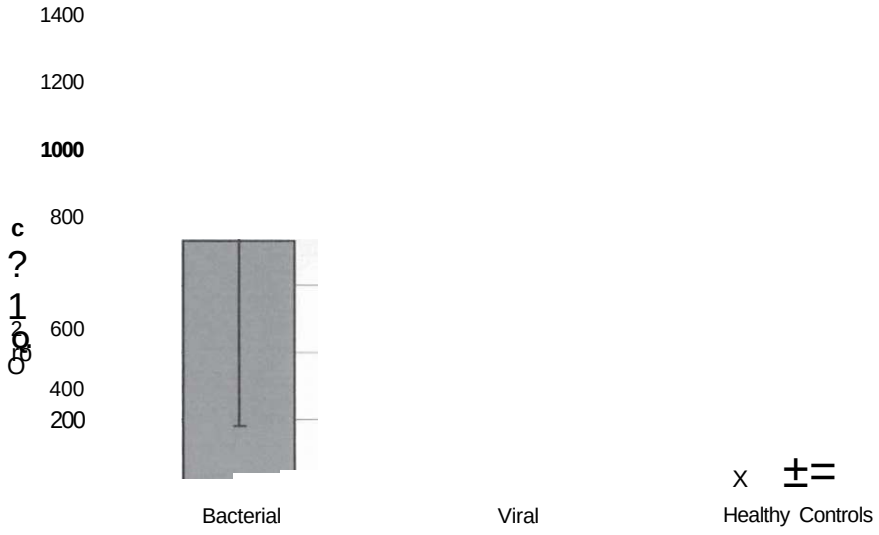
Figuře 37. Calprotectin levels in bacterial gastroenteritis and in healthy controls



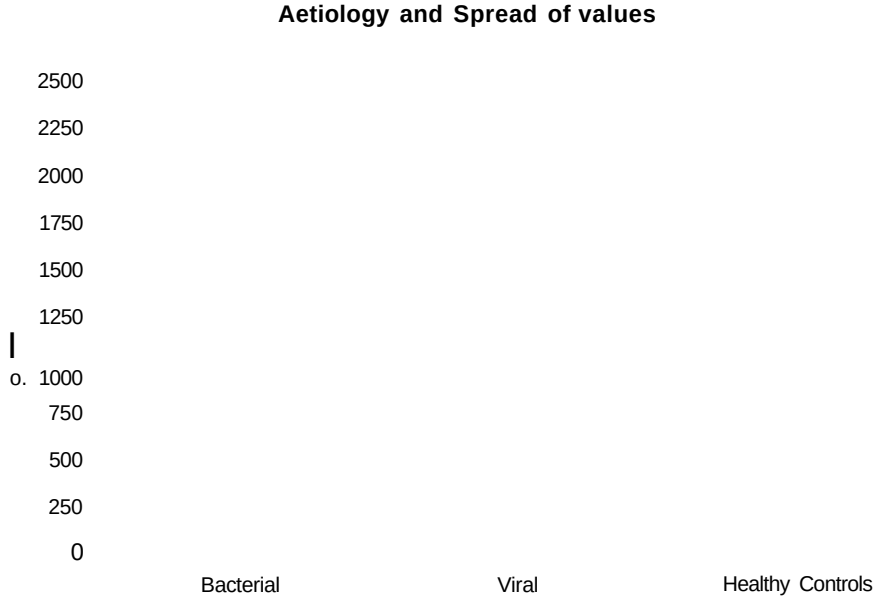
Figuře 38.Calprotectin levels in vrial gastroenteritis and in healthy controis



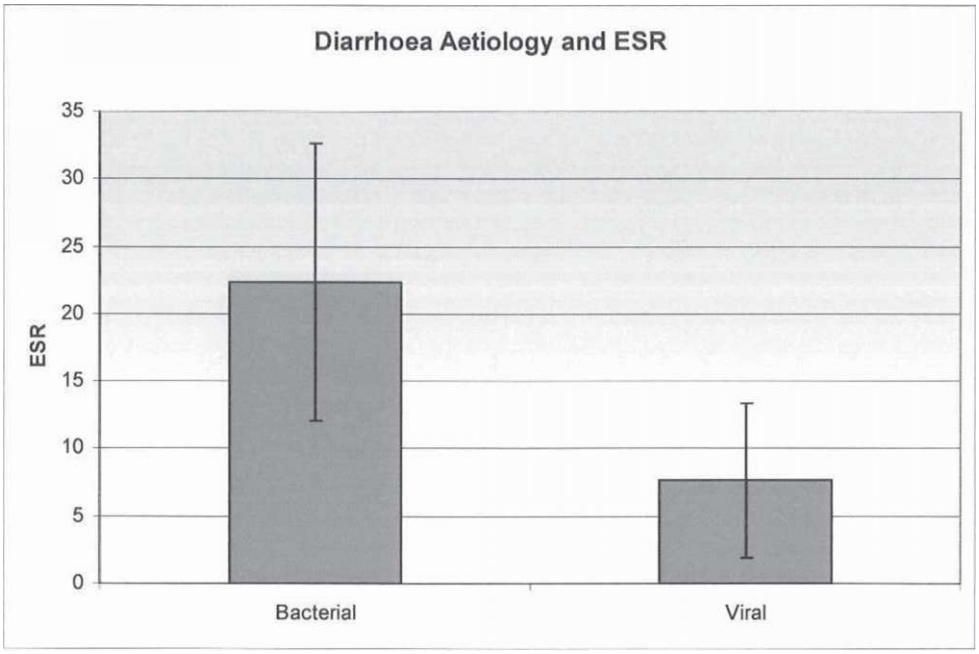
Figuře 39.Bacterial and viral aetiologies and healthy control calprotectin levels with ranges



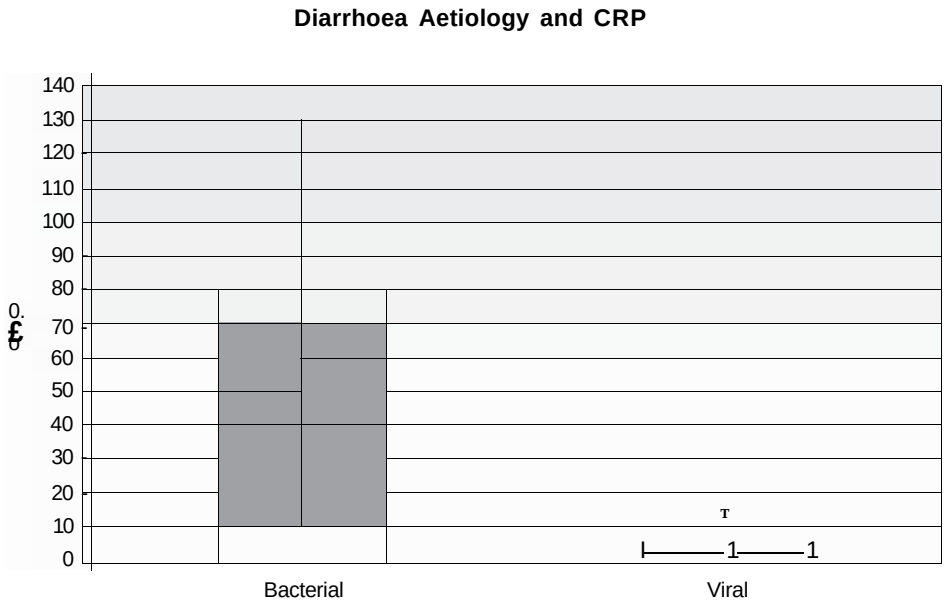
Figuře 40.Spread of calprotectin values for infectious aetiologies and healthy controls



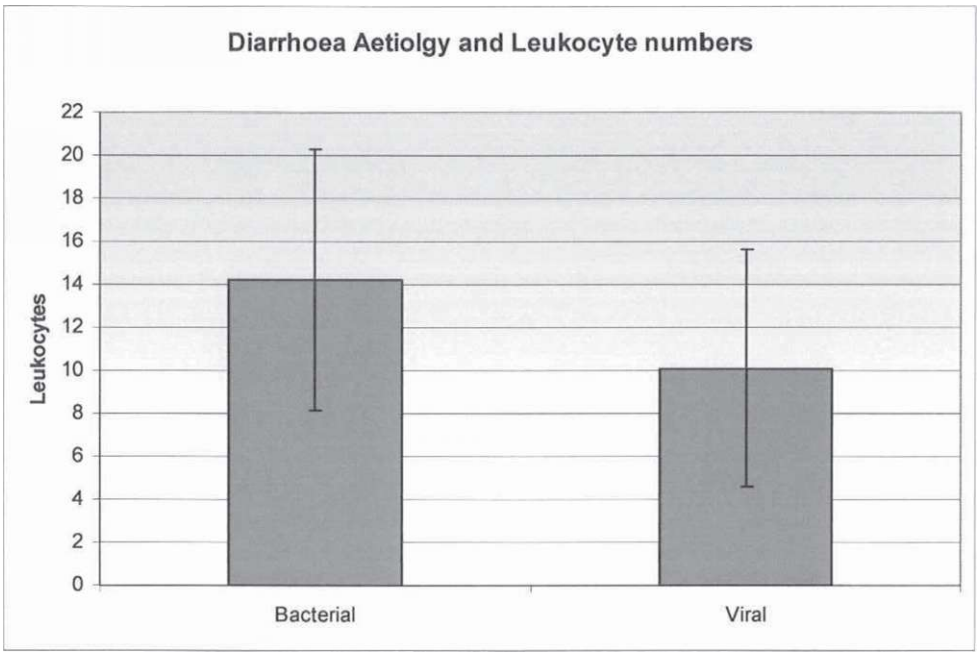
Figuře 41.Comparison of diarrhoeal aetiology and Erythrocyte Sedimentation Rate (ESR)



Figuře 42. Comparison of diarrhoeal aetiology and C-reactive protein (CRP)

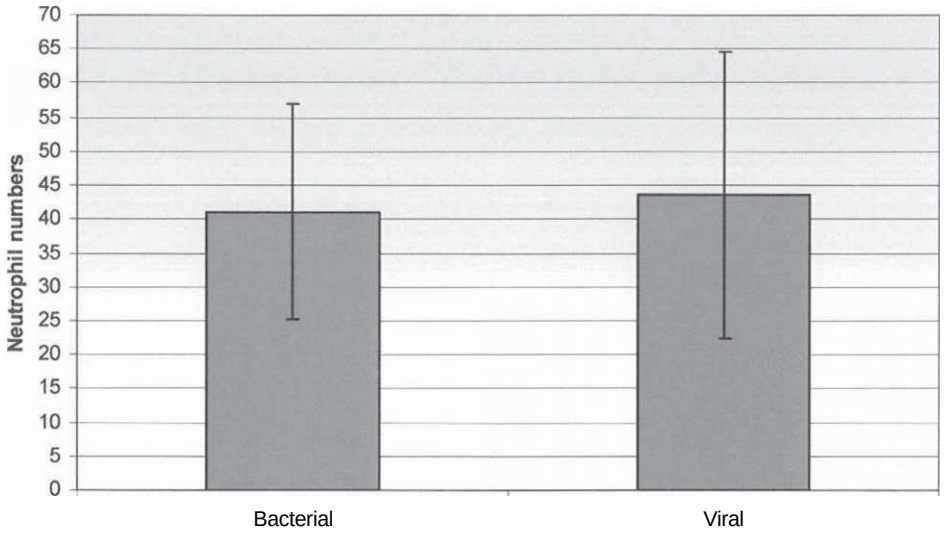


43. Comparison of diarrhoeal aetiology and leukocyte numbers)



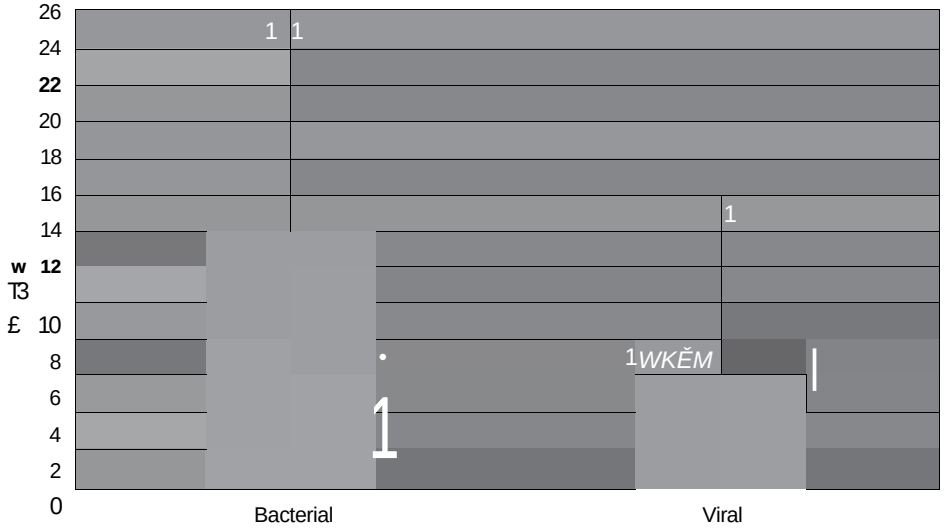
44. Comparison of diarrhoeal aetiology and neutrophil numbers

Diarrhoea Aetiology and Neutrophil numbers



45. Comparison of diarrhoeal aetiology and rods

Diarrhoea Aetiology and Rod indices



4.6 Faecal calprotectin concentrations in infants with AG, healthy infants, children with inflammatory bowel disease, children with RAP and healthy children

The mean values±SD, the medián and ranges of faecal calprotectin concentrations in patients with IBD, in patients with RAP, gastroenteritis and in the age matched healthy subjects that made up the control group are shown in Figuře 36.

Feacal calprotectin in infants with acute bacterial diarrheoa was different from that in healthy infants: mean ±SD (medián) 731.3 ± 548.9 (555.4) versus 136.0 ± 85.0 (142.75) p< 0.001). Those with acute viral gastroenteritis, however did not differ significantly: mean ±SD (medián) 175.6 ± 213.4 (71.0) (p=0.91). Faecal calprotectin levels were significantly higher in healthy infants than healthy children aged over one year of age mean ±SD (medián) 136 ±85 vs 51.2 ±47 (p <0.05). A significant correiation between age and faecal calprotectin concentration was found (Spearmaď's Coefficient = -0.5998, p. <001); Boys and girls had similar calprotectin concentrations.

Children with IBD had significantly higher calprotectin concentrations: mean ±SD (medián) 1100 ± 813.8 (1050.8) than children with RAP (*H.pylori* positive and negative) 92.0 ± 97.8 (67.8) (p<0.001) as well as healthy age matched controls: mean ± SD (medián), 29.9 ± 16.6 (19.5) (p<0.001)

Children with CD with exacerbation had significantly different levels versus RAP as a whole: mean ±SD (medián) 1156.5 ± 786.6 (1172.3) and 92.0 ± 97.8 (67.8) respectively (p<0.001). Children with CD after 14 days therapy had significantly different levels verusu RAP: mean ±SD (medián) 1168.5 ± 423.1 (1278.1) and 92.0 ± 97.8 (67.8) respectively (p<0.001). Children in remission with CD had less though still significant difference in levels versus RAP: mean ± SD (medián) 318.8 ± 210.7 (358.8) (p<0.01)

UC patients in exacerbation had signifacntly different levels compared with RAP: 2212.4 ± 217.4 (2294) and 92.0 ± 97.8 (67.8) respectively (p<0.001). UC patients after 14

days therapy had significantly different levels compared with RAP: mean ± SD (medián) 1765.2 ± 179.0 (1729.8) and 92.0 ± 97.8 (67.8) respectively ($p<0.001$). UC patients in remission had signifacntly different levels compared with RAP: mean ± SD (medián) 333.5 ± 232.5 (379.0) and 92.0 ± 97.8 (67.8) respectively ($p<0.01$)

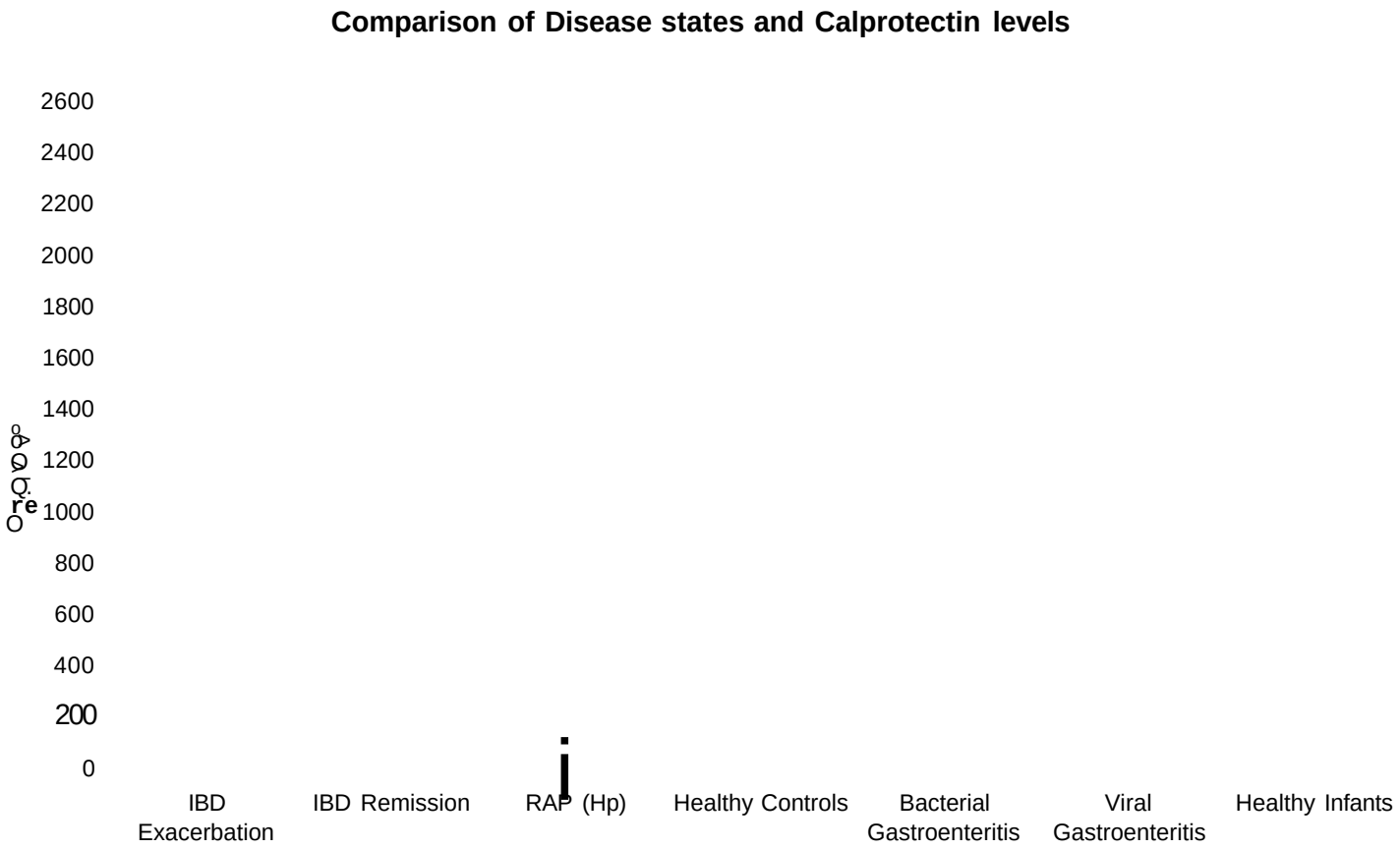
Children with RAP significantly differed in their calprotectin levels when compared with healthy controis: mean ± SD (medián) 92.0 ± 97.8 (67.8) and 29.9 ± 16.6 (19.5) respectively ($p<0.05$).

RAP *H.pylori* positive patients did not differ significantly when compared with controis: mean ± SD (medián) 85.5 ± 82.0 (99.1) and 29.9 ± 16.6 (19.5) ($p=0.11$).

RAP HP negative patients differed with borderline significance compared with controis: mean ± SD (medián) 97.1 ± 108.5 (67.8) respectively ($p=0.06$)

The receiver operating characteristic analysis was constructed with area under the curve calculated at 0.96. Faecl calprotectin was used to distinguish between children with IBD and normál healthy control subjects. At a cut-off value of 145 pg/g of faecal calprotectin a sensitivity of 95% and specificity of 82% were observed.

Figuře 46. A comparison of calprotectin levels in Disease states and healthy controis



5 Discussion

Although reports on the use of faecal calprotectin in children and adults appeared in the literatuře (19,20), few prospective studies with sample size and power calculations have been published to data in the paediatric population, a number of them with methodological limitations. Additionaly, faecal calprotectin data is missing in the environment of a centrál European country concerning paediatric gastroenterology practice. Hence, we provide with a prospective trial demonstrating that non-invasive evaluation of calprotectin can be effective and useful in terms of clinical use in children with GIT involvement and add new information on this issue in a paediatric population.

5.1 The Calprotectin test as a method for evaluation reference values in healthy children

To our best knowledge, this the first study related to normál reference faecal calprotectin values performed in a paediatric population in the Czech Republic, and one of the largest studies concenring normál values. We undertook age-stratified prospective study concenrning normál reference faecal calprotectin values in children. All potential factors influencing faecal calprotectin values determination were taken into account, as shown in Table 2.

Our primary endpoint was to establish normál reference values in children aged from 1 month to 15 years of age. Published evidence to support these approaches is limited, and not all studies have shown conclusive data. Because of great variations in study design and diagnostic eriteria, the evaluation of faecal calprotectin in the various studies cannot be compared directly with each other without taking specific points in mind.

Although there is a need for simple and non-invasive tests in paediatric care, there are only few studies on the use of faecal calprotectin as a diagnostic test in children. The improved faecal calprotectin assay has, as far as we know, been used just a few studies in infants and children. The originál ELISA method was first deseribed in 1992 by Roseth

et al. (2), and the results were provided in "per litre of faecal homogenate." They found that faecal calprotectin is stable up to 7 days in room temperature as the calcium-calprotectin complex is resistant to heat and proteolytic enzymes. Furthermore, they concluded that measurement of faecal calprotectin in a spot sample reflects the average daily excretion of calprotectin. Bunn et al. (13), who used the originál method for faecal calprotectin, showed that children with inflammatory bowel disease had faecal calprotectin concentrations that dosely correlated to macroscopic and histologicaly defined inflammation as well as to technetium 99-labeled white cell scanning score. The reference group in their study comprised 31 healthy children (medián age, 6.8 years; range, 1.5-15.3 years) and their medián faecal calprotectin concentration was 2.1 mg/L (range, 0.5-6.3 mg/L), which was below the cut-off (<10 mg/L) established for adults in the originál method (17).

The second study by Fagerberg et al., using the newly adapted test method found that in a group of 117 healthy children the overall medián faecal calprotectin concentration was 13.6 pg/g (95% confidence interval, 9.9-19.5 pg/g)(165). In their different age groups, 4 to 6 years, 7 to 10 years, 11 to 14 years, and 15 to 17 years, the medián calprotectin concentrations were 28.2, 13.5, 9.9, and 14.6pg/g, respectively. Of these children, 104 (89%) had a concentration <50 (jg/g.

During the first weeks and months of life, the composition of the intestinal microflora, which is involved in mucosal immunity maturation, depends on various factors, mainly the type of feeding (166). The high calprotectin concentrations found in the first weeks and months of life may be related to the unusual physiology of neonatal gut. A specific pattern of functioning in the initial weeks of life is characterised by, among other things, increased transmucosal leakage - a phenomenon that ends by the third trimester of life, in a process named "closure"(167). This leakage can be assessed by intestinal permeability studies (168) Indeed, data has indicated that faecal calprotectin concentrations decrease to adult values with a time scale similar to that of the "closure" process (29,31). Increased

permeability of the mucosal barriers may be associated with increased migration of granulocytes into the gut lumen, a phenomenon reflected by high faecal concentrations of calprotectin. As there is no accumulation of calprotectin-rich leucocytes in healthy mucosa in the first few months of life, the high calprotectin concentrations observed may reflect increased intestinal permeability related to transepithelial migration of neutrophils as observed in adults with inflammatory bowel disease (169). Furthermore, it has been suggested that increased intestinal permeability and intestinal inflammation are dosely interrelated, especially in children (170).

Interestingly, a preliminary study has been performed with the same methodology in preterm infants, which are known to have a more permeable intestine than term infants. In infants without any gastrointestinal symptoms, the concentration of calprotectin was very high: average (medián) 896 (572) mg/l which translates into 2240 (1430) ug/g. Furthermore, concentrations were even higher in infants suffering from gastrointestinal bleeding, an early stage of necrotising enterocolitis (1724 (1355) ug/g)(171). Similar results using a different assay were obtained by Carroll et al, with a significant difference between infants with necrotising enterocolitis and age matched controis (28).

Our data confirm that infants in the first few weeks and months of life have high calprotectin concentrations compared with referenced values for healthy adults and that the use of the calprotectin test for referencing levels in a statě of health, in the defined age group is speculative.

In the relatively newly adapted test method the use of dissociating agents in the extraction solution in conjunction with a higher dilution of the sample has resulted in the improved faecal ELISA calprotectin method (19). The extraction yield in samples with a calprotectin value <10 mg/L in the originál method was increased one- to six fold, whereas samples with high faecal calprotectin values showed a higher increase. This means that the separation between normál and pathologic values is better with the improved method.

The size of the sample is substantially reduced from 5 g to 50 to 100 mg, and the results are expressed as micrograms of faecal calprotectin per gram wet faeces. When the improved ELISA assay for faecal calprotectin was studied in 59 healthy adults, the medián faecal calprotectin concentration was 26 pg/g (range, 4-262 |jg/g) and the cut-off was suggested to be <50 pg/g for adults, but the ninety-fifth percentile was not mentioned (19). From the beginning, the commercial test was called PhiCal ELISA (Nycomed Pharma AS, Oslo, Norway) but now exists as Calprest and is manufactured by Eurospital SpA, Trieste, Italy. From Nycomed, the following threshold values were supplied for adults: negative test when <50 pg/g, weakly positive test when between 50 and 100 _g/g, and strongly positive when >100 pg/g (13). These threshold values seem to be applicable also in children aged from 4 to 17 years in the literature as well as our control group aged 6 to 15 years. X of the presumed healthy children in our study had a faecal calprotectin concentration higher than the reference value recommended for adults, none of these children had elevated levels on follow-up. For ethical reasons, we chose not to investigate the healthy children further. Children with apparent gastrointestinal symptoms such as abdominal pain or diarrhoea during the last month of the study were excluded. In this healthy population, latent disease could theoretically be present in some of the children and hence could influence the faecal calprotectin concentrations. The variation of calprotectin excretion in faeces may also be explained by normál biologie variability with day-to-day variation that previously has been deseribed in adults (22). Husebye et al. (25) noted that two populations emerged in adults with normál findings at colonoscopy: one group with remarkably low and stable faecal calprotectin values below a cut-off of 50pg/g and one group with labile values also beyond this limit. In our study, children with menstrual or nasal bleeding were excluded to avoid measuring calprotectin from neutrophils in blood that could have contaminated the faecal sample. It has been estimated that bleeding volume of at least 100mL daily may cause an elevated faecal calprotectin concentration (44). Children taking non-steroidal anti-inflammatory drugs also were excluded because this medication has been reported to induce enteropathy and

elevated faecal calprotectin excretion (65). Interestingly, it has been reported that medián faecal calprotectin concentrations in healthy children did not differ from children with upper respiratory tract infection or tonsillitis. The possible explanation given was that the children probably had non-purulent infections caused by a virus (35). Healthy children aged between 4 and 17 years apparently exhibit a similar pattern of faecal calprotectin excretion as in adults. In infants, however, the faecal calprotectin concentrations seem to be age dependent. Surprisingly high values have been found, especially in healthy infants 0 to 3 months of age, with a medián faecal calprotectin concentration of 265 pg/g (20,24). The explanation for this observation could well be the migration of neutrophils through the mucosal membrane during the development of oral tolerance and regulation of the microbial flora (98).

We suggest that studies on the feasibility of this test for investigation of gastrointestinal disorders in children could be based on the reference values found in this study.

5.2 Study in IBD patients

5.2.1 TNF- a polymorphism 308 G-> A

The present study demonstrated that there is a significant association between TNF-a 308 G->A polymorphism and IBD in our established paediatric population. An interesting observation was that TNF-a 308 G-»A polymorphism influences the IBD phenotype and may play an important role in IBD onset. Differences were clearly observed in the carriage rate for TNF-a 308 G-»A between UC and control-group children. The study of Koss et al showed that patients with distal colitis had a significantly higher occurrence of TNF-a 308 A allele compared to the control group (172). Sashio et al. who investigated UC and CD adult patients compared with controis also observed the same trend where TNF-a 308 G-*A and -238 G->A polymorphisms were found more frequently in UC patients (27).

However, our study has not proven any link between TNF-a 308 G-»A polymorphism and the extent of disease and other clinical variables in the UC group.

CD patients can be assigned into two distinct groups, as follows: firstly, a stenotic and/or penetrating disease, secondly, a subset of patients with an inflammatory phenotype (57). Inflammatory process of CD appears to destabilize over time and may evolve into strictures and penetrating complications. Characteristics of CD complications in our study were: no sex predominance, no relationship to therapy, but substantial occurrence of complications several years after initial diagnosis. Another relevant finding of our study was the demonstration of TNF-a 308G-»A polymorphism in the two clearly defined subgroups of CD patients with and without complications: low frequency of 308 G->A allelic variations in CD patients presenting with inflammatory disease, and a significantly higher frequency in those with evidence of complications. These findings may substantiate a common genetic predisposition underlying both processes. Kim et al reported in Korean patients that the TNF-a gene polymorphisms at positions -308 and -238 have influences on the susceptibility to CD or the behaviour of CD (173). Bouma et al showed that TNF-308 polymorphism did not confer protection against fistulising CD (174). Interestingly, as for CD our findings clearly demonstrated that TNF-a 308 G-*A gene polymorphism appears to have a greater effect on modifying the CD phenotype than overall susceptibility to CD. Levine at al made similar observations that the TNF-a 308 G-»A polymorphism was not significantly more frequent in paediatric onset of CD (52).

Despite extensive investigation, it remains incompletely understood that the above-mentioned designations are rooted in distinct pathogenetic mechanisms (175). The number of reference CD patients with complications in our study was not large enough to clearly define the relationship between TNF-a 308 G->A and patients with either stricturing or perforating behaviour. We therefore analysed all CD patients with complications as a whole group. Based on these findings, we came to the conclusion that paediatric CD patients presenting with complications may be a genetically distinct subgroup of CD. This

is an additional piece of evidence that may explain different pathogenetic mechanisms in CD phenotype. This hypothesis is in keeping with our observations that the occurrence of TNF-a 308 G-»A was higher in CD patients with complications than that in the normál controis. Louis et al reported that TNF gene polymorphism could be associated with certain CD phenotypes (176). González et al did not find a significant association between the distributions of the 308 G-»A polymorphism and the susceptibility of CD patients developing fistulae (177). Other studies related to TNF-a association with CD showed conflicting data (68,178). Several reasons could explain this discrepancy on the effect of the genotypic distribution on CD behaviour: genetic heterogeneity of the clinical populations examined, different definitions of CD phenotypes among different populations, and perhaps environmental factors may also contribute to CD behaviour. Furthermore, results can also be influenced by the ethnic differences in the frequency of TNF-a 308 G-»A polymorphism. Of note, our study sample did not include subjects from different ethnic backgrounds, thus preventing ethnic bias on TNF-a polymorphism. With the larger number of subjects to be attained, attempts should be made in the future not to miss certain clinical and pathogenetic associations. Further investigation among different ethnic populations is clearly warranted.

The promotér SNP at TNF-a -308 A was related to TNF-a production (179). Some patients with IBD have been found to have large amounts of TNF-a in the colonic mucosa and the stool, though relatively low levels in sérum (180). Vatay et al studied the relationship between TNF-a polymorphism and sérum CRP levels in CD and UC (78). They reported that CRP levels in the active phase were similar to those in the inactive phase when patients were homozygous carriers at position -308 GG. Mazlam et al showed, that TNF-a release correlated significantly with sérum CRP (181). It is notable that TNF-a also appears to be stimulated by CRP (182). Koss et al found in adults that increased TNF-a production in UC patients was not observed despite significantly higher occurrences of TNF-a 308 A polymorphism in patients compared to healthy controis

(41183). Van Heel et al reported that IBD is associated with a TNF polymorphism that can affect interplay between the OCT I and NF-kappa-B transcription factors (184). It is interesting to observe in our subjects that TNF-a 308 A polymorphism significantly correlated with sérum CRP in CD and UC. We have shown that the PCDAI-score points and CRP may be related to TNF-a -308 G-»A promotér gene polymorphism in CD patients. In our series, not only changes in CRP sérum levels but also disease activity, as reflected by the PCDAI score, were comparable with TNF-a -308 G-*A promotér gene polymorphism. Although a clear reason for the higher PCDAI activity, the Truelove index and CRP remains unclear, but it might be, in part, acounted for by - 308 A allele carriers. Our study supports the routine use of CRP and evaluation of disease activity markers in IBD. It has been suggested that adult patients who carry -308 A present with more intense inflammation than -308 G carriers, and that the polymorphism at -308 G-»A of TNF-a may play an essential role in modifying the production of TNF-a and the phenotypic expression of CD (46). Thus, it appears that a long period of sub-clinical inflammation unregulated by TNF-a may progress to fibrotic stenosis and/or fistulae. One explanation may lie in the potential role of the TNF-a 308A polymorphism that may favour and promote complications of CD by inducing more aggressive inflammatory activity at the mucosal level (45). This elucidation of the development of CD related complications might also be supported by our observations as the PCDAI and CRP were significantly higher in children with complications compared to children without such behaviour.

The present data confirm that TNF-a -308 G-»A polymorphism may participate in defining the biological basis of IBD in children. Indeed, our preliminary observations and the strength of associations clarified the association of the polymorphism with the UC group as well as the CD patients with complications. These results may contribute to the explanation of disease heterogeneity. However, it is reasonable to speculate that the effect of the 308 A polymorphism in the TNF-a promotér is directly or indirectly mediated by the increase in TNF-a production. Whether the TNF-a 308 A polymorphism is directly

involved in regulating cytokine production, or serves merely as a marker in the linkage disequilibrium, should be further investigated.

In summary, we conclude though the promotér SNP at TNF-a 308 may not necessarily dictate IBD initiation, the available evidence suggests that TNF-a 308 A polymorphism may be associated with subsets of IBD patients after stratification for the disease phenotype. A higher incidence of this polymorphism was found in UC patients compared to those with controis and CD, where TNF-a 308 A polymorphism may play a role in modifying the CD phenotype. TNF-a 308 A polymorphism may participate in determining disease activity as well as more intense inflammatory activity in both forms of IBD, and may modify the progression of chronic digestive tract inflammation. Future research will be necessary regarding IBD-genes, and the potency of biological therapy (chimeric anti-TNF antibody). The analysis of TNF-a 308 A polymorphism (5.2) involved in IBD paediatric patients have provided essential clues for the pathophysiological mechanisms and identifying links for better defining and undesrtasnidng the role of faecal calprotectin in IBD related complications and intestinal inflammation.

5.2.2 Calprotectin in IBD patients

The aim of the present study was also to address the association between changes in calprotectin level in a population of IBD patients.

It is important to recognise that the high values of faecal calprotectin seen in our IBD groupis not automatically indicative of IBD and other conditions may also induce high levels (185,4). Therefore, colonoscopy is needed to confirm the diagnosis.Traditionally the disease activity has been evaluated by clinical indices like the P/CDAI or by the Harvey-Bradshaw "simple index" (186). However, the use of more sophisticated methods, for instance the use of isotope labelled white celis, has shown that such indices tend to be inaccurate (20). The quantitation of calprotectin in simple extracts of small (0.1g) stool samples is an attractive alternative to the previous methods (9).

Our study suggests that the assessment of faecal calprotectin is a valuable tool in screening forthe presence of IBD in children with suspicious symptoms. In our series, the calprotectin levels of children in exacerbation were consistently high (>1000|jg/g) and indeed higher than those previously demonstrated by other studies (Table 4). Studies in adults with Crohn's disease suggest that there are higher levels present in those patients likely to relapse, although there is only a weak correiation between disease activity scores and faecal calprotectin (4). It is also interesting that in our study, the PCDAI, as an accepted index of disease activity, correlated with CD during exacerbation but not after therapy or in remission. Importantly in the UC group the disease activity correlated with calprotectin in exacerbation and in remission. Another studies also reported observations that in adults with UC there is a stronger association with disease activity (187,24). These observations lead us to believe that there may be a difference in the inflammatory process that need more study or indeed that the role of calprotectin in assessing disease activity in children with CD remains unclear, and longitudinal studies examingin this are necessary.

5.2.2.1 Response to Treatment in IBD

Most clinicians now rely on clinical symptoms to evaluate a patient's response to treatment because follow-up endoscopy to document improvement is invasive and cannot be performed frequently. Because faecal calprotectin concentration has been shown to correlate with endoscopic and histologie inflammation in IBD, it follows that it would be a useful marker with which to follow response to treatment. A transient deerease in faecal calprotectin concentrations in 2 patients with IBD after treatment with infliximab, has been demonstrated, corresponding to an improvement in clinical disease activity (188). These patients, however, did not undergo endoscopy with biopsies, so confirmation of histologicaly improvement was not obtained. Seriál faecal calprotectin concentrations in a single patient with UC over an 18-week period.have also been studied (189) Faecal calprotectin concentration deereased with treatment of UC and corresponded with clinical, endoscopic, and histologicaly improvement. In the same study, the authors performed

colonoscopies on 45 patients with IBD in clinical remission with normál faecal calprotectin concentrations. Thirty-eight of these patients had complete, histologically proven mucosal healing; the remaining 8 had evidence of only mild colonic lamina propria inflammation. Faecal calprotectin concentrations during prior active disease were available for 18 of these patients, and all were significantly elevated. A more recent study declared that faecal calprotectin remains high during glucocorticoid therapy in children with IBD (190). In active disease treated with glucocorticoids, levels declined in line with the clinical improvement but seldom fell within the normál range. None of our children reached normál levels on achieving remission which suggests ongoing inflammation in a clinically silent disease. Clearly, however, larger prospective studies with histological confirmation of disease activity both before and after treatment are needed to evaluate the role of faecal calprotectin in this setting. Hence, it would be feasible to incorporate this test as a standard in the Czech Republic in the care of children with IBD.

5.2.2.2 IBD and Subclinical Intestinal Inflammation

Because of its correlation with intestinal inflammation and ease of administration, the faecal calprotectin test has been examined in apparently healthy first-degree relatives of patients with CD to assess the possible presence of subclinical intestinal inflammation in this population. It has been reported that 49% of first-degree relatives of patients with CD had elevated faecal calprotectin concentrations, suggesting its use as a subclinical biomarker of IBD (131). In contrast, only 13% of spouses of CD patients had elevated levels, implicating genetic rather than environmental factors as the cause of the increased levels. However, because only 5% to 10% of family members of patients with CD develop clinical IBD, not all patients with subclinical intestinal inflammation will progress to clinically apparent IBD. This subset of patients with elevated faecal calprotectin concentrations deserves further study, and the use of faecal calprotectin in the detection of subclinical intestinal inflammation may help to elucidate predisposing factors to IBD.

5.2.2.3 Faecal Calprotectin Relative to Other Biomarkers in IBD patients

It is important to note that multiple faecal biomarkers have been evaluated with respect to screening patients for evidence of IBD. A detailed discussion of these is beyond the scope of this disertation. Recent anaylsis regarding established and emerging biomarkers for IBDhave been published, including erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), anti-Saccharomyces cerevisiae antibody/perinuclear anti-neutrophil cytoplasmic antipody (ASCA/pANCA), and a variety of cytokines, chemokines, and cytokine receptors (61). The authors concluded that none of these assays were yet sensitive or specific enough to replace standard clinical evaluations. For example, although several studies using faecal tumor necrosis factor-a have shown promising results, large interindividual variations in both sérum and faecal levels have limited its widespread applicability.Interestingly, the most promising class of faecal biomarkers for IBD to date is the neutrophil products. Lactoferrin is an iron-binding glycoprotein that is a major component of the secondary granules of neutrophils. PMNelastase is a neutral proteinase that is released by activation of neutrophils. Lysozyme also is a neutrophil-derived enzyme that mediates degradation of bacterial cell weils. Myeloperoxidase is another neutrophil product that is released from the primary granules. By studying all available data in the literatuře, calprotectin compares favourably with other faecal biomarkers that have been evaluated for IBD.

Our data show that calprotectin level correlates with CRP in IBD, consistent with findings of other studies (4)

In summary, the data from this study support the association between faecal calprotectin and IBD in children and support the use of facel calprotectin to guide a diagnostic journey in a child with chronic GI symptoms. IBD is likely where faecal calprotectin is >145pg/g of the stool in the absence of intestinal infection though it is important to remember that these levels are found in children non-IBD enteropathy, as well as some first-degree relatives of patients with IBD. Levels between 100 to 200pg/g

are less specific for IBD, but are unlikely in children without organic GIT disease. In both IBD subgroups we found well above upper limit calprotectin levels in patients in clinical remission compared with healthy subjects.

5.3 Faecal calprotectin concentrations, RAP, and *H.pylori* infection in childhood

RAP is a popular descriptor that has evolved from the seminal definition by Apley of intermittent abdominal pain in children between the ages of 4 to 16 years which persists for longer than 3 months and affects normál activity (166). Although the focal point of the disorder is abdominal pain, patients exhibit a broad range of symptoms. No standard diagnostic criteria have evolved from Apleys's originál description. Most physicians continue to dichotomize RAP aetiologies into "organic" and "nonorganic". This approach is clearly inadequate, especially since the term nonorganic may imply a psychological aetiology. RAP aetiologies are better classified into five broad categories: anatomie, infectious, noninfectious inflammatory, biochemical, or functional. Expression of pain from all causes of RAP may be influenced by both physical and psychosocial factors.

FAP comprises a group of disorders that includes paroxysmal periumbilical abdominal pain, nonulcer dyspepsia, and irritable bowel syndrome (IBS). Although it is usually possible to classify a patient into a specific group, it is unclear whether the symptoms in the distinct groups have different underlying pathophysiologic mechanisms. Since the exact aetiology and pathogenesis of the pain are unknown, and no specific diagnostic markers exist for any group, FAP is too often perceived as a diagnosis of exclusion.

RAP that affects normál acitivity has been reported to occur in 10% to 15% of school-aged children between 4 and 14 years (191). Importantly, another 15% of school aged children may have paroxysmal abdominal pain lasting in excess of 3 months which either does not affect activity, or is not brought to the attentionof a physician (192). There may be a slight predominance toward girls in children with RAP, but data are sparse. Our study

also demonstrated predominance towards female but this did not reach statistical significance.

Prevalence figures for FAP are unkown. Functional abdominal pain is by far the most common aetiology of RAP in children.

H.pylori is a very important gastroduodenal pathogen and is considered to be the major cause of chronic gastritis and duodenal ulcer in childhood. However, the association between gastric infection with H.pylori and RAP is still controversial because of the lack of biological markers for RAP. There are several controversial reports on *H.pylori* infection and GIT symptoms, especially RAP, in children. Prevalence rates of *H.pylori* infection from 0-81% have been reported in children with RAP (193). Some reports showed an association between RAP and *H.pylori* infection in children (116), whereas others did not (194). Recently, we studied a large cohort of symptomatic children for the presence of *H.pylori* infection and found a prevalence rate of 25.1% (126).

Possible involvement of calprotectin associated with *H.pylori* infection and alterations of gastric mucosa in *H.pylori* infected children remains inconclusive. Moreover, there is no data on the differences between upper GIT inflammation and intestinal inflammation related to faecal calprotectin values in children. In children a non-invasive test is especially appreciated.

Due to lack of informmation about the values of faecal calprotectin for RAP, *H.pylori* and upper GIT inflammation in our paediatric population, we conducted this prospective clinical study. Our study was focused on the evaluation of faecal calprotectin values in symptomatic children with RAP, *H.pylori* infection and gastric inflammation.

Faecal calprotectin has been reported in groups of infants with infantile colic and in children suffering from RAP (195), and data from children highly suspected to be suffering from IBD have been published (13). However, no previous data on the usefulness of

faecal calprotectin in consecutive children with RAP related to *H.pylori* infection have been published to data. Faecal calprotectin may differentiate between RAP without identified organic disease and IBD in school-aged children (59).

Our findings showed that upper GIT disorder (gastrititis) showed little diference in calprotectin levels, but these levels were all higher than calprotectin level of normál age matched subjects. The level of calprotectin in patients with upper-GIT inflammation suggests that the response to gastritis is not producing significantly more calprotectin than a diffuse inflammation in the other conditions. However, in our series, as noted previously, active IBD compared to RAP is a condition that produces the greatest rise in faecal calprotectin.

The present study was also designed to investigate the role of faecal calprotectin in gastric inflammation in symptomatic children. We found that 34 patients with RAP and upper - GIT inflammation due to gastritis had a calprotectin level higher than that for normál age matched controis. The available data describing variations in faecal calprotectin values with H.pylori infections and gastritis are limited and somewhat controversial in adults. Summerton et al showed in adults that a calprotectin level was not significantly different in patients with gastritis compared to normál patients (40).

We showed in this report that faecal calprotectin values in our patient population with RAP were not different between H.pylori infected and non-infected children using H.pylori as dependent variable. Faecal calprotectin values are not able to discriminate between infected and non-infected symptomatic subjects with RAP.AIthough the faecal calprotectin level was higher in children with RAP *H.pylori* negative compared to controis, it was not statistically significantly different ($p= 0.069$).

Of interest, there were differences in activity of chronic gastritis, but on the other hand, faecal calprotectin levels probably do not seem to be a valuable additional stool biomarker for assessing the topography and severity of gastritis to allow for better

prediction of underlying diagnosis and *H.pylori* pathogenic behaviour. It might be reasonable to speculate that calprotectin is not critical to the progression of *H.pylori* associated inflammation and tissue damage. Our previously published study (135), and in our present study we demonstrated on gastric biopsies from children with RAP chronic lymphocytic infiltration but we did not find any inflammation with polymorphonuclear leukocytes in our group. The grade of gastritis in the HP+ve group was notably higher than the HP-ve group; however histologie analysis demonstrated that neutrophil infiltration was low in both of them. Chronic lymphocytic infiltration was the main findings in both groups. This was also reflected in the low and similar levels of calprotectin in both of these groups. Neutrophil infiltration is a variable feature of *H.pylori* associated chronic gastritis and neutrophils disappear rapidly after successful eradication treatment or with the longer duration of untreated infection.

The fact that our symptomatic RAP patients had significantly raised calprotectin levels raises the issue of whether or not many persons labelled as having a functnional disorder would not benefit from further and close examination. One of the most important results of our study is that faecal calptrotectin concetrations may be a marker for RAP in clinical practice. Faecal calprotectin levels were 24.9% higher compared to normál controis. Since diserimination between RAP and other subjects is important for determining diagnostic process and treatment, this response is considered to be a useful and valuable diagnostic marker for RAP in clinical practice.

The mechanisms and the exact clinical relevance of this response were not determined in our study, being beyond the scope of this docment, and do warrant further study. It has been reported that children with RAP have shown abnormal small bowel permeability and duodenitis (4). We can speculate that one possible mechanism accounting for these results is increased levels of calprotectin indicating increased intestinal permebality in children with RAP. In this study, our data also demonstrated duodenitis in RAP subjects. Further in vivo study is necessary to elucidate the effect of

calprotectin in the intestine under RAP. However, it is unclear how the level of calprotectin in patients with upper-GIT inflammation responses to *H.pylori* related and unrelated gastrodueonal ulceration in children.

In conclusion, in the present study, we could not demonstrate the differences in faecal calprotectin concentrations between *H.pylori* infected and non-infected. However, one important finding of our study is significant difference in faecal calprotectin levels between RAP and healthy controls in our paediatric population. It is therefore possible, theoretically at least, that faecal calprotectin changes could be involved in some aspects of the RAP and symptomatology of patients with RAP.

Further confirmation of our results will be essential to evaluate the best diagnostic and therapeutic regimens for control of children with RAP in our region indicating that other alternative strategies to investiage other underlying conditions in children with RAP should be studied.

5.4 Faecal calprotectin analysis in Gatroenteritis

AG is a generál term referring to inflammation or infection of the GIT. Identifying gastrointestinal disease or inflammatory causes of intestinal symptoms is crucial to clinical decisions and therapeutic strategies (196). This is particularly the case in infants, as they are the most severly affected by this disease. Several studies have proposed leukocyte - derived protein assay to identify inflammatory vs functional causes of diarrheoa (15,149).

To our best knowledge, there have been no coherent studies definig the faecal calprotectin levels in children with acute gastroenteritis especially in the young age group < 2 years of age related to origin of infection. Only one study with a small number of patients could be found that dedicated the study of calprotectin in relation to severe diarrheoa of small infants (197). The search for non-invasive diagnostic modalities for infection and inflammatory disorders in children has continued to capture the interest of

investigators. In the past years, various diagnostic tools were examined but results were inconclusive, especially in young children.

A previous study has demonstrated a 10-fold increase in IgM secretion compared with a smaller relative increase in IgA in acute diarrheoeal disease as a primary mucosal response (146). However data on changes of faecal calprotectin concentrations is inconsistent. The present study provides the first detailed examination of virus and bacteria induced response of faecal calprotectin values in children during the acute phase of diarrhoeal disease in infants. Interestingly our study sheds light on the pathophysiological mechanisms that delineate bacterial and viral AG. Further studies are needed to elucidate the processes involved.

We observed an influence of bacterial AG on faecal calprotectin levels in this study of faecal calprotectin values in children with AG, trends for associations between the high levels of faecal calprotectin and bacterial infection were observed. Faecal calprotectin concentration is increased in patients with BAG when compared to those with VAG. Faecal calprotectin levels did not rise significantly in VAG and hence may be a good marker to predict bacterial infections in acute diarrhoea disease.. Among our patients with acute gastroenteritis, calprotectin was low in the patients with viral infection. In comparison to the age-matched, healthy children, there is a significant difference between bacterial infections and controis, on the contrary no significant difference was observed between viral infection and healthy controis.

Faecal calprotectin levels have been found to correlate with platelets, sodium and chloride in a group of BAG but not with CRP, on the other hand in children with viral acute diarrhoea I disease faecal calprotectin significantly correlated with CRP but not with other markers.

The findings from the study support the potential importance of the utility of faecal calprotectin levels in children with acute GE under 2 years of age.

Faecal calprotectin has been shown to consistently differentiate IBD from irritable bowel syndrome because if has excellent negative predictive value in ruling out IBD in undiagnosed, symptomatic patients (65).

Faecal calprotectin also may be useful in determining whether clinical symptoms in patients with known IBD are caused by disease flares or non-inflammatory complications/underlying irritable bowel syndrome and in providing objective evidence of response to treatment. Although more studies are needed to define fully the role of faecal calprotectin, convincing studies and growing clinical experience point to an expanded role in the diagnosis and management of IBD.

Interestingly one study has found that in children infected with Trichuris trichiura dysentery there is no increase in granulocyte cell numbers in the lamina propria of colonic biopsies compared with healthy controis. There is, however, a significant increase in the proportion that stains for calprotectin (using MAC387). The authors suggest that for immunohistochemistry studies calprotectin may be a marker of granulocyte activation (198).

In conclusion, faecal calprotectin is promising marker. Assessment of faecal calprotectin values in children with acute gastroenteritis is important both in clinical practice and in clinical trials. The faecal calprotectin enzyme-linked immunosorbent assay is a simple test with excellent potential use in small children. Faecal calprotectin can be used to select optimal diagnostic methods and therapeutic modalities

5.5 Faecal calprotectin concentrations in infants with gastroenteritis, healthy infants, children with IBD, children with RAP and healthy children

Faecal calprotectin is a new marker of intestinal inflammation. Data on faecal calprotectin in paediatric gastroenterology are still scarce. Children with organic or functional problems may have similar features and clinical examination is not, in most

cases, enough to ascertain the specific diagnosis. Other methods are complex, time consuming, relatively expensive and unpleasant for the child. To solve this problém, the search for non-invasive biological markers which would be useful in clinical practice in various settings should be performed.

Among the many open questions an exceptionally interesting one is the precise mechanism underlying the RAP. In the light of the data in the literatuře, it is rather difficult to address precisely the issue.

Our study clearly showed that faecal calprotectin may be an additional marker differentiating between RAP and organic disorders (inflammatory processes). This obvious difference is, however, not possible to elucidate in routine clinical practice. The determination of calprotectin levels in stool in a paediatric population in from this point of view is practically important. An increase in faecal calprotectin concentration was observed in all disease characterised by gastrointestinal inflammation, and the active inflammatory bowel disease patients showed the higher faecal calprotectin values. All children affected by RAP showed low values compared to IBD but significantly higher compared to healthy subjects. However the fact that our symptomatic RAP patients had significantly raised calprotectin levels raises the issue of whether or not many persons labelled as having a functional disorder would not benefit from further and close examination. Calprotectin is a useful, but not disease specific marker in the differential diagnosis to easily detect inflammation throughout the whole GIT tract. Calprotectin may distinguish patients with an organic disease characterised by intestinal mucosa inflammation from healthy subjects and form patients with disorders RAP. Consistent with the findings of others (13), this present study found significantly increased faecal calprotectin concentrations in children with IBD compared with healthy children. In adults with IBD, Roseth et al found faecal calprotectin to be a useful measure of disease activity (2). We calculated an optimised calprotectin cut-off value of 145pg/g (revealed by the receiver operating analysis) to distinguish patients with IBD from healthy subjects. Raised

faecal calprotectin should prompt further assessment in children with chronic GIT symptoms, since an organic disorder is likely.

In children a non-invasive method in clinical practice is especially appreciated. According to our results faecal calprotectin measurement may be a useful, non-invasive marker for distinguishing between RAP and IBD in school-aged children also related to disease activity. Based on our results the test may not be a reliable marker for diagnosis IBD in infants - at that age a high faecal calprotectin level is normál. The high calprotectin level in faeces in infants is a valid observation. The significance reduction with age may indicate a maturational process in the intestinal mucosa.

In conclusion, the current study demonstrated that the faecal calprotectin test is a promising, non-invasive test for differentiating between RAP and IBD in school-aged children. In young infants high faecal calprotectin levels are normál.

6 Conclusions

A. Results of our faecal calprotectin study are encouraging for early and routine use of a faecal calprotectin assay in clinical practice in the Czech Republic. This test is non-invasive, cost effective, simple and objective and can be used repeatedly. **The faecal calprotectin enzyme-linked immunosorbent assay is a simple test with excellent potential use in children.**

B. We established reference values for faecal calprotectin in healthy children aged between 1 month and 15 years in our geographical region in the Czech Republic. **A significant correlation between age and faecal calprotectin concentration was found. Significantly higher levels of calprotectin in faeces were found in infants compared to older children or adults.** So we have confirmed in our study that an accurate definition of normál faecal calprotectin ranges in subjects aged less than 12 months is advocated before it can be routinely used in infants.

C. **Faecal calprotectin is a useful marker of intestinal inflammation in children with IBD in clinical practice.** We have confirmed that children with active IBD defined by standard accepted criteria have significantly higher calprotectin values in stool than healthy children. Our results demonstrated the relationship between exacerbations, response to medical treatment, during the statě of remission and faecal calprotectin concentrations. This study demonstrates the usefulness of faecal calprotectin for non-invasive detection of intestinal inflammation in children with GIT symptoms suggestive of IBD. We have found that faecal levels of calprotectin significantly correlate with disease activity both in UC and in CD, with a more pronounced association found in UC. This leads us to suggest that the inflammatory processes UC and CD are different .The subset of patients with elevated faecal calprotectin concentrations with subclinical intestinal inflammation deserves warrants and further study. As calprotectin is a representative of

intestinal inflammation in the bowel in IBD patients, it raises the question whether endoscopic examination could potentially be avoided. **Our clinical experience points to an expanded role in the diagnosis and management of IBD.**

D. **Although not necessarily dictating IBD initiation, the TNF-> 308 A polymorphism may play a role in modifying the CD phenotype.** The polymorphism may influence disease activity as well as more intense inflammatory activity in both forms of IBD and may modify the progression of chronic digestive tract inflammation.

E. **Our study proved for the first time that children with RAP and upper gastrointestinal inflammation (gastritis) had significantly elevated calprotectin levels compared to healthy children.** We observed no significant differences of faecal calprotectin values between subgroups of RAP children with chronic gastritis *H. pylori* positive and negative. *H. pylori* positive and negative subgroup of RAP children did not differ significantly from each other and healthy children; however the RAP group as whole did have significantly higher calprotectin level than controis. This has important implications for clinical practice concerning urging the clinicians into search for underlying conditions in children with RAP. It is therefore possible, theoretically at least, that faecal calprotectin changes could be involved in some aspects of the RAP and symptomatology of patients with RAP.

F. **Our study for the first time demonstrated significantly elevated calprotectin levels in young children with acute gastroenteritis, where children with bacterial infections had higher level compared with children with acute diarrhoea I disease caused by viral infection.** We did not observe significantly different levels of faecal calprotectin in viral infected group compared with healthy children. This adds new information on the pathophysiological mechanisms of

acute gastroenteritis in a paediatric population. **The measurement of faecal calprotectin may provide new tools for the quick assessment and therapeutic stratégy in children with acute gastroenteritis.**

G. **Calprotectin is emerging as useful neutrophil marker in a variety of GIT conditions, calprotectin is a sensitive, but not disease specific marker to easily detect inflammation throughout the whole GIT tract.** It may help in identifying an organic disease characterised by intestinal inflammation and in the differential diagnosis of RAP. The faecal calprotectin test is a promising, non-invasive test for differentiating between RAP and IBD in school aged children.

H. **We calculated an optimised calprotectin cut-off value of 145pg/g (revealed by the receiver operating analysis) to distinguish patients with IBD from healthy subjects.** Raised faecal calprotectin should prompt further assessment in children with chronic GIT symptoms, since an organic disorder is likely.

I. **Calprotectin as a marker of GIT inflammation, and as a tool for diagnosing organic disease or excluding functional disorders, is useful only when considered on the background of patient's age and the clinical presentation.** In young infants high faecal calprotectin levels are normál.

Key words: faecal calprotectin, children, normál references, healthy children, inflammatory bowel disease, TNF-a 308 A polymorphism, *H.pylori,* recurrent abdominal pain, acute gastroenteritis, clinical application

7 Appendix of Figures and Tables

7.1 Tables

7.2 Figures

8 Author's pubiications

8.1 Chapters in Scientific Monographies

1. Varvařovská J, Štětina R, Sýkora J , Rušavý Z, Laciqová S, Racek J and Siala K. Frontiers in Antioxidants Research: Impact of Oxidative Stress on Diabetes Mellitus and Inflammatory Bowel Diseases, pp. 133-200, Editor: H.V. Panglossi, (Nova Publishers, New York, 2007, ISBN 1 -600021-273-5)

2. Varvařovská J, Štětina R, Sýkora J , Rušavý Z, Lacigová S, Racek J and Siala K. Leading Edge Antioxidant Research: Impact of Oxidative Stress on Diabetes Mellitus and Inflammatory Bowel Diseases, Editor: H.V. Panglossi, (Nova Publishers, New York, 2007, ISBN 1 -600021-273-5) (In Press)

8.2 Journals

1. Siala K. Sýkora J , Hes O. et al. Helicobacter heilmannii Reinfection in a Helicobacter pylori Negative Adolescent: A 4-year Follow-up. Journal of Clinical Gastroenterology. 41(2): pp. 221-222, 2007. **(IF 2.311)**

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