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# INFLAMMATORY BOWEL DISEASE:
## an immunogenetic study

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# INFLAMMATORY BOWEL DISEASE:
## an immunogenetic study

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# LIST OF ORIGINAL PUBLICATIONS

I. K. Kull, R. Salupere, R. Uibo, M. Ots, V. Salupere. Antineutrophil cytoplasmic antibodies in patients with inflammatory bowel disease. Prevalence and diagnostic role. Hepato-Gastroenterol 1998; 45: 2132–7.

II. K. Kull, R. Salupere, K. Metsküla, R. Uibo. Autoantikehade esinemissagedus ja tähendus põletikulise soolehaiguse korral. Eesti Arst 2000; 1:12–14, 16.

III. K. Kull, O. Uibo, R. Salupere, K. Metsküla, R. Uibo. High frequency of antigliadin antibodies and absence of antireticulin and antiendomysium antibodies in patients with ulcerative colitis. J Gastroenterol 1999; 34:61–5.

IV. K. Hirv, M. Seyfarth, R. Uibo, K. Kull, R. Salupere, U. Latza, L. Rink. Polymorphisms in tumour necrosis factor and adhesion molecule genes in patients with inflammatory bowel disease; associations with HLA-DR, -DQ alleles and subclinical markers. Scand J Gastroenterol 1999; 34:1025–32.

# ABBREVIATIONS

| | |
|---|---|
| 5-ASA | 5-aminosalicylic acid |
| AEM | antiendomysium antibodies |
| AGA | antigliadin antibodies |
| AIH | autoimmune hepatitis |
| AMA | antimitochondrial antibodies |
| ANA | antinuclear antibodies |
| ANCA | antineutrophil cytoplasmic antibodies |
| ANOVA | analysis of variance |
| ARA | antireticulin antibodies |
| BPI | bactericidal/permeability-increasing protein |
| BSA | bovine serum albumine |
| CD | Crohn's disease |
| EIU | enzyme immunosorbent units |
| ELISA | enzyme-linked immunosorbent assay |
| FITC | flourescein isothiocyanate |
| HLA | human leukocyte antigen |
| IBD | inflammatory bowel disease |
| IBS | irritable bowel syndrome |
| ICAM-1 | intercellular adhesion molecule |
| IgA | immunoglobulin A |
| IgG | immunoglobulin G |
| IIF | indirect immunofluorescence |
| IL-1RA | interleukin-1 receptor antagonist |
| LKMA | liver/kidney microsomal antibodies |
| MHC | major histocompatibility complex |
| OD | optical density |
| PBS | phosphate-buffered saline |
| PCA | parietal cell antibodies |
| PCR | polymerase chain reaction |
| PSC | primary sclerosing cholangitis |
| SD | standard deviation |
| SMA | smooth muscle antibodies |
| SSCP | single-strand conformation polymorphism |
| TMA | thyroid microsomal antibodies |
| TNF | tumour necrosis factor |
| UC | ulcerative colitis |

# 1. INTRODUCTION

Inflammatory bowel disease (IBD) encompasses two distinct disease entities, ulcerative colitis (UC) and Crohn's disease (CD). Despite extensive investigations over several decades, there is still no simple explanation formulated for the cause of these disorders. It is likely because of the pathogenesis of IBD, which is more complex than a single cause and effect relation, and probably represents an interaction between genetic predisposing factors, exogenous triggers, and modifying factors (1–4). The outcome of these interactions is a spontaneously relapsing and remitting inflammatory process in which tissue injury is mediated by the immune system (3, 5).

IBD is relatively common in most industrialised countries in the world. The incidence varies greatly geographically and ranges from about 1 to 10 per 100,000 per year for CD and from 5 to 18 for UC. The highest rates are reported in the Scandinavian countries and Scotland, followed by England and North America, whereas IBD seems to be less common in Central and Southern Europe and appears to be uncommon in developing countries (6, 7). The disease affects equally men and women of all ages, and mainly manifests during late adolescence or early adulthood with a peak onset between 15 and 30 years of age.

The basis for the present studies was an epidemiological investigation of the incidence and prevalence of UC and CD in Tartu County, Estonia. A retrospective study of Salupere R. *et al.* (8–10) for 1973–1993 revealed that IBD is not so common in Estonia as it is in other countries. The annual incidence of UC for this period was 1.5 cases per 100,000 and of CD 0.3 per 100,000. The reasons for such low incidence in Estonia are not clear.

Since there are substantial epidemiological differences in the incidence of IBD between Estonia and European countries, then only with the help of additional studies more data would be received about specific features of IBD in Estonia.

To ascertain an association between IBD and autoimmunity, the frequency and clinical value of antineutrophil cytoplasmic antibodies (ANCA) and several non-organ-specific antibodies in the patients with IBD are analysed in the first part of the study.

In the second part of the current study we examined the frequency of antigliadin antibodies (AGA), antireticulin antibodies (ARA), and antiendomysium antibodies (AEM) in the patients with UC and evaluated the correlations between these antibodies and various clinical features. Whereas the association of coeliac disease and UC has been reported by several authors (11–13). Furthermore, the association of IBD with known autoimmune diseases and the frequent occurrence of extraintestinal manifestations which share all the essential characteristics of their idiopathic counterparts and thus probably represent

autoimmune diseases as well, can be regarded as indirect evidence for the occurrence of autoimmune mechanisms in the context of IBD.

In the third part of the present study the role of several immunogenetic markers — the polymorphisms of HLA-DR and -DQ, tumour necrosis factor (TNF), E-selectin, L-selectin and intercellular adhesion molecule 1 (ICAM-1) have been investigated and the associations of these markers with the ANCA status in the patients with IBD was determined.

# 2. REVIEW OF LITERATURE

## 2.1. Definition, clinical features, course, therapy and outcome of ulcerative colitis

**Ulcerative colitis** is an inflammatory disease of the colonic mucosa of unknown aetiology. The disease is variable both in the extent and the severity of the involvement of the colon. The inflammation may be found in the rectum only (proctitis), or proximally up to the mid-transverse colon (left-sided colitis), or engaging the whole of the colon and rectum (extensive colitis or total colitis). The spectrum of severity ranges from mild inflammation to florid ulceration and haemorrhage extending into, and sometimes through, the full thickness of the colonic wall leading to perforation.

The most common signs of the UC relapse are hematochezia (visible blood in the stool) and diarrhoea. These symptoms are present in more than 80% of patients at the onset of their disease (14–16). Abdominal pain, rectal cramps and fever may occur at the time of diagnosis. More severely ill patients lose weight and develop anaemia (17). The onset of UC may be sudden with bloody diarrhoea appearing within a few days, but the usual mode of onset is more gradual with diarrhoea progressing from increased frequency of stool to frequent bloody liquid movements over a period of several weeks.

Approximately 30% of UC patients have the disease limited to the rectum, in about 40% the disease extends above the rectum but not beyond the hepatic flexure, and the remaining 30% develop total colitis at their first attack (14, 17, 18).

The course of UC is characterised by an intermittent course with remission and relapses in 90% of the patients (17, 19–21). A small minority never achieve satisfactory remission and continue with symptoms to greater or lesser degree.

One or more extraintestinal complications of the disease may occur during a life-long course. In a population-based study by Monsen *et al.* (22), the overall prevalence of extracolonic diagnoses was 21%, whereas 70% of these patients had extensive colitis. The most common extracolonic diagnoses were arthritis, ankylosing spondylitis, iritis, skin lesions (erythema nodosum, pyoderma gangrenosum) and hepatobiliary complications (primary sclerosing cholangitis).

Sulphasalazine was introduced for the treatment of UC in the early 40's (23). It was found later that the active moiety of sulphasalazine is the 5-aminosalicylic acid (5-ASA) (24). Several new types of 5-ASA or 5-ASA prodrugs are now available, although the exact mechanism(s) of their action remain unclear. Comparative trials of sulphasalazine and a variety of 5-ASA formulations have, as anticipated, demonstrated therapeutic equivalence but improved tolerance with 5-ASA (25). 5-ASA is effective at both the induction and maintenance of remission (26, 27). Corticosteroids were introduced in the mid 50's as the

treatment for severe attacks of UC (28) and are still the most commonly used agents for the treatment of moderate to severe inflammatory activity in UC (29).

Approximately 20% of the patients will undergo colectomy within 10 years, 30% within 25 years (21, 30), whereas the colectomy rate is highest during the first year after onset of disease and the main indication for early surgery is the failure of medical treatment. Later on the main indication for surgery is the cancer prophylaxis (31). As known, UC is associated with increased incidence of both colonic and extracolonic neoplasms (32, 33). The risk of developing cancer is closely related to the extent and duration of the disease, as well as colectomy also influences the risk for cancer. The cumulative risk of developing colonic cancer in two recent reports in patients with extensive colitis at 25 years was 3% and 10% (34, 35), respectively.

Advances in the diagnosis and management of the disease have substantially modified its natural history. When compared with the general population, Edwards and Truelove in their classic study (19) found a two-fold excess mortality in patients with UC. Langholz *et al.* (35), reporting 25 years later, showed the risk of death in the first year (<0.8%), reflecting a small number of patients with severe acute extensive colitis. After the first year, there was no significant difference in survival between patients with colitis and a background-matched population.

## 2.2. Definition, clinical features, course, therapy and outcome of Crohn's disease

**Crohn's disease** is a transmural chronic inflammation of unknown origin. It is usually segmental and may affect any part of the gastrointestinal tract from mouth to anus. It is, however, most commonly located to the ileocecal region.

The clinical features on the presentation are diverse and depend directly on the location of inflammation as well as the individual's reaction to that inflammation.

Approximately 40% of CD patients will present with ileocecal disease; the principal symptoms are diarrhoea, cramping abdominal pain, and low-grade fever. On a physical examination there is tenderness and often an abdominal mass in the right lower quadrant of the abdomen.

The disease limited to the small bowel is observed in approximately 30% of patients with CD. Small bowel extensive disease can lead to the loss of the effective absorptive surface with the resultant malabsorption syndrome and steatorrhoea. Weight loss and growth retardation in children may develop.

The exclusive colonic Crohn's disease is seen in approximately 25% of CD patients. Diarrhoea and hematochezia are the most common presenting features

of CD, with the volume of diarrhoea and the proportion of patients with bleeding in CD usually less than in UC.

Fistulae in the anorectal region or from the affected bowel segment to other organs or the skin may appear. Abscesses and other perianal lesions are also common.

Approximately 5% of patients with CD present with clinical findings that are not typical.

CD is a naturally remitting and relapsing disease and the patterns of the disease activity may vary from a patient to patient. It appears, however, that the course within the first two years after diagnosis indicates a close correlation to the course during the subsequent 5 years (36). As in UC, also in CD, extraintestinal manifestations such as arthritis, iritis, skin lesions and hepatic complications may occur.

Despite recent advances and the introduction of new drugs, there is still no cure for CD. Corticosteroids are still the most commonly used drugs for the treatment of active CD, however, recent studies indicate that they are of no benefit in maintaining the remission (37). 6-Mercaptopurine and azathioprine have been shown to be beneficial in reducing corticosteroid dependence, especially in patients with colonic disease and internal fistulas (38–40). Sulfasalazine and newer 5-ASA preparations appear to be beneficial in patients with mild to moderate CD, especially when the large bowel is involved (41, 42). Metronidazole has been recommended for the treatment of perianal Crohn's disease (43, 44). A novel class of therapeutic agents is emerging from the combined unravelling of the mechanisms, leading to inflammatory lesions and progressing in molecular engineering. Of which the anti-tumour necrosis factor (TNF)-α monoclonal antibodies have already showed definite activity against chronic active CD in controlled trials (45, 46) and being since late 1998 used in patients with fistulae or severe steroid-resistant CD.

The majority of patients with CD will sooner or later require surgical removal of the diseased bowel with the principal surgical indications being internal fistulization with abscess and small or large bowel obstruction (47). Surgery does not cure CD and reoperation rates are as high as 50% for all the sites of the disease and even higher for fistulous or perianal disease (48). The data about the risk of colonic cancer are still conflicting. In the four studies (49–52) the risk for cancer was not different from the expected compared to the background population. Two studies (53, 54), however, reported a significantly increased risk of colonic cancer.

Four population-based studies have been published to date mortality in CD (49, 51, 55–56). Ekbom *et al.* (56) showed modest excess in mortality, whereas the other studies did not find any significant difference in mortality in CD compared to the expected mortality of the background population. However, Munkholm *et al.* (51), analysing subgroups of patients, found a significantly increased mortality in patients diagnosed at the age of 20–29 within the first 5 years of the disease, and in a subgroup of patients with extensive both ileal

and jejunal small bowel disease. Many studies have pointed to CD as becoming progressively more dangerous with the duration of the disease (49, 51, 57).

## 2.3. Autoantibodies and autoantigens in patients with inflammatory bowel disease

### 2.3.1. Antineutrophil cytoplasmic antibodies

Antibodies to a variety of autoantigens have been sought as evidence for autoimmune mechanisms in patients with IBD.

Antineutrophil cytoplasmic antibodies (ANCA) were first described in a small group of patients with glomerulonephritis (58). In 1985 it was shown that the antibodies directed against cytoplasmic components of neutrophils occur in Wegener's granulomatosis and correlate directly with the disease course and pathogenesis (59, 60). The antibodies, when detected by indirect immunofluorescence (IIF) on ethanol-fixed neutrophils, produce a characteristic cytoplasmic fluorescence pattern (cANCA). The antigen recognised by cANCA proved to be a 29-kd serine protease from myeloid azurophilic granules, known as proteinase-3 (61, 62). During the routine screening of sera for the presence of ANCA, it became apparent that some sera produce a perinuclear to nuclear fluorescence pattern (pANCA) clearly different from the cANCA pattern and often directed against myeloperoxidase, another constituent of azurophilic granules (63).

More recently, ANCA have been reported to be present in 40–80% of the patients with UC and in 10–40% of the patients with CD (64–70). These ANCA usually produce an accentuated perinuclear ("atypical") staining pattern on ethanol-fixed neutrophils (71). The target antigens in IBD have not been identified fully. Various target antigens have been described, of which lactoferrin (66, 72) and bactericidal/permeability-increasing protein (BPI) (69, 73) are presently most prominent. Cathepsin G (74–76), lysozyme (77, 78), and β-glucuronidase (79) have also been reported as target antigens, although these results were not confirmed by others (66, 80). Recently, reactivity against catalase and α-enolase was observed by Roozendaal *et al.* (70). Several nuclear non-granulocyte-specific antigens have also been found to be recognised by ANCA positive serum samples from patients with UC (81). None of these antigens has been conclusively shown to be entirely responsible for the pANCA activity.

Certain aspects of the heterogeneity of the disease, particularly activity and extent at the precise time of venesection, are assessed in several studies. In most of the studies of UC the prevalence and titre of ANCA do not correlate with the disease activity or extent (64, 65, 69, 82), although some studies have suggested that pANCA may be associated with a more aggressive or more severe UC (83–85). Aitola and colleagues (86) have demonstrated reduction in the ANCA pre-

valence and ANCA titre, judged by immunofluorescence, in patients with UC who had undergone proctocolectomy. Subsequently, Lindgren *et al.* (85) have reported on 137 patients with UC, the majority in long term remission, that only 9% of the patients had circulating ANCA detectable. In CD, presence of pANCA seems to represent a distinct subpopulation of the patients who exhibit UC-like features (87, 88).

A related finding is that ANCA have been found in 15–20% of healthy unaffected relatives of the patients with UC (89, 90) and that it may be a marker of genetic susceptibility to UC. In addition to that, there is evidence to suggest that ANCA may be a marker of genetic heterogeneity within UC, because the linkage of HLA-DRB1*1502 alleles with ANCA-positive UC but not with ANCA-negative UC has been described (91). Also a different distribution of ICAM-1 alleles in ANCA-positive and ANCA-negative subgroups of IBD patients has been reported (92). Although results vary depending on the patient population because a significant difference in HLA-DRB1*1502 typing prevalence between ANCA-positive or -negative UC was not shown in separate studies from Pittsburgh (93) and Oxford (94). Recently, Satsangi *et al.* (88) have provided further evidence that HLA genes are involved in determing ANCA status. In UC HLA-DRB1*0301-DQB1*0201/TNF2 (HLA-DR3-DQ2/TNF2-positive patients) haplotype was associated with high frequency of ANCA in patients' serum, but again, in this population of UC, ANCA was not associated with HLA-DRB1*15 or HLA-DRB1*04 (serologically DR2 and DR4, respectively).

Although clearly pertinent to the present discussion, disease heterogeneity and ethnic differences alone would not explain the great discrepancies between the results of the many recent series in which ANCA prevalence has been measured in patients with UC and CD. Methodological differences may also be relevant. At present there is no consensus as to the "correct" assay for ANCA in UC. The techniques of alkaline phosphatase staining (95), immunofluorescence (67), and enzyme-linked immunosorbent assay (ELISA) (64) are all used in different centres. Differences in techniques also involve fixation (66) and dilution of serum. These are all likely to contribute to the present uncertainties.

The pathogenetic role of pANCA in the mucosal inflammation of IBD continues to be controversial. Supplementary evidence that inflammatory cells of the large bowel are important in ANCA production has recently been provided (96). These authors have been able to demonstrate spontaneous production of ANCA by the cells of the lamina propria of the colon of patients with UC. Spontaneous production was not demonstrated either by peripheral blood cells or by mesenteric lymph node cells. Although, the absence of pANCA in a significant fraction of patients with UC and the fact that pANCA can be found in some healthy individuals, indicates that they probably do not play a central role in the pathogenesis of UC, but may be indicative of an underlying immune disturbance (97, 98). In summary, humoral autoimmune phenomena are common in IBD, but their relevance to disease pathogenesis needs clarification.

### 2.3.2. Other autoantibodies including antinuclear antibodies

Circulating antibodies to colonic epithelial cells were first described in the sera of the patients with UC over thirty years ago (99). Since then there have been many reports on this subject, and the antibody has been further characterised as an immunoglobulin of the $IgG_1$ subclass binding to a 40 kDa protein expressed by colonic epithelium, that is significantly more often found in UC than CD (100). Because of the apparently restricted localisation of the 40 kDa antigen to the epithelium of the colon, skin, and biliary tract, it has been proposed that there may be a colonic autoantigen in UC, which would also explain some of the extraintestinal disorders associated with UC (101). Most recently, the 40 kDa antigen has been purified by Das *et al.* (102) and shown to be related to tropomyosin, an actin-binding cytoskeletal protein known to be localised in the apical cytoplasm and brush border of colonic enterocytes. There is evidence that tropomyosin-related antigens possess epitopes that may cross-react with bacterial proteins (103). Such cross-reactivity with colonic bacterial antigens could potentially induce a state of chronic immunopathology, characterised by immune complex deposition and leading to the activation of subepithelial macrophages through their Fc receptors. Whether these observations are simply epiphenomenal or whether colonic antibodies are really involved in the causation of UC remains to be established.

Other types of autoantibodies have been found to occur in IBD as, for example, antinuclear antibodies (ANA), smooth muscle antibodies (SMA), heat-shock protein antibodies, and antibodies against cytoskeletal proteins other than tropomyosin (104–107). Of which ANA and autoantigens targeted by ANA have revealed the most interest as valuable markers for the differential diagnosis in several systemic autoimmune diseases (108). Antibodies to the extractable nuclear antigens Sm (Smith), RNP, SS-A (Ro), SS-B (La), Scl-70 and Jo-1 have been described in few patients with IBD (109, 110).

There are isolated reports of autoantibodies to various components present in the intestinal wall, including intestinal, brush-border endothelial cells (111), and a trypsin-sensitive pancreatic antigen (112). However, none of them has been found to represent a specific marker for UC or CD and the mechanism of induction of these antibodies, and therefore the role in the pathogenesis of IBD remains obscure. Not all antibodies lack specificity. A novel antierythrocyte autoantibody appears to have some specificity for CD (113). Antibodies to oligomannosidic epitopes of the yeast *Saccharomyces cerevisiae* (ASCA) are increased in patients with CD but not in UC (114–116). A recent study by Quinton *et al.* (116) showed that the combined use of ASCA and pANCA could differentiate CD from UC and other colitides.

## 2.4. Extraintestinal manifestations of inflammatory bowel disease and associated diseases in patients with inflammatory bowel disease

IBD is associated with a variety of extraintestinal manifestations that may produce greater morbidity than the underlying intestinal disorder and may even be the initial presenting symptoms of IBD (117). About 30% of the patients with IBD have at least one extraintestinal manifestation. Extraintestinal manifestations may be divided into three categories (118), including (a) the complications directly related to IBD activity which usually respond to the therapy directed against bowel disease: peripheral arthritis, episcleritis, aphthous stomatitis, erythema nodosum, and pyoderma gangrenosum; (b) the disorders associated with IBD but running an independent course: ankylosing spondylitis, sacroiliitis, primary slerosing cholangitis; (c) the complications that relate directly to the disrupted bowel physiology: kindey stones, gallstones, amyloidosis, osteoporosis.

The pathogenic factors responsible for extraintestinal manifestations remain unclear. Some systemic disorders may be of immunologic origin and are likely related to the pathophysiology of the intestinal disease; others may be the sequelae of intestinal bacterial overgrowth or iatrogenic complications of the therapy used to control bowel inflammation (117).

A possible association between autoimmunity and UC was initially suggested by the case reports of the associations between UC and thyroid disease (119), pernicious anaemia (120), systemic lupus erythematosus (121), and autoimmune haemolytic anaemia (122). These observations were confirmed by Snook *et al.* (123), who demonstrated that the prevalence of a specified group of autoimmune disorders is three times greater than expected in subjects with UC; no association was found for CD. Monsen *et al.* (22), in a population-based study of 1274 patients with UC, determining the prevalence of extracolonic diagnosis, found that patients with extracolonic manifestations could be divided into two groups: activity-related and of autoimmune type. The overall prevalence of extracolonic diagnosis was 21%: 68 (25%) of these 271 patients with extracolonic diagnosis had more than one extracolonic diagnosis. Coexistence of several autoimmune diagnoses in patients with UC was common. The most common combinations were UC, Graves' disease (thyrotoxicosis), and spondylitis; UC, Graves' disease, and coeliac disease; UC, Graves' disease, and primary sclerosing cholangitis; UC, psoriasis, and chronic active hepatitis (22).

### 2.4.1. Associations of ulcerative colitis and coeliac disease

Coeliac disease is an immunologically mediated disorder caused by lifelong intolerance of the small-bowel mucosa to dietary cereals, mainly wheat, rye,

barley and oats (124). Ingestion of these cereal proteins results in villous atrophy, with crypt hyperplasia of the small-bowel mucosa, which recovers on a gluten-free diet. Coeliac disease may first develop either in childhood or in adult life with the clinical picture being very variable. Typical complaints are weight loss, fatigue, weakness and diarrhoea (125). In recent years it has become apparent that the patients may completely lack any of the symptoms described before and, therefore, the term "silent coeliac disease" has been introduced (126). Clinically silent coeliac disease is increasingly being recognised (127). Even among these patients it is desirable to detect coeliac disease, as treatment with a gluten-free diet can prevent later manifest symptoms of malabsorption and even avert the potential development of malignant diseases (128). Diseases frequently occurring concomitantly with coeliac disease constitute such a risk group of which insulin dependent diabetes mellitus, Sjögren's syndrome, and thyroid disease are the most frequent associations (12).

The first association of coeliac disease and UC was reported by Salem and Truelove over 30 years ago (13). Since then several other authors have described the association of coeliac disease and UC (123, 129–136), as well. Most of the reports are simply case reports and, therefore, the clinical significance of this association is not yet established.

A conclusive diagnosis of coeliac disease is based on the demonstration of villous atrophy and crypt hyperplasia in small-bowel biopsy specimens (137, 138). As endoscopy and the small-bowel biopsy are invasive procedures, they cannot be considered feasible for screening patients for coeliac disease, and therefore the desire for and the efforts to find suitable screening methods for replacement of the small-bowel biopsy, or for selecting patients who should further undergo small-bowel biopsy, have been great. Most suitable for the patients who might have silent coeliac disease are serologic tests. Three types of antibodies — antigliadin antibodies (AGA), antireticulin antibodies (ARA), and antiendomysium (AEM) antibodies have been extensively investigated and suggested as screening tests for coeliac disease (139–141).

The tests most widely used measure AGA of IgG and IgA-class, most commonly utilising an ELISA. Reports differ on the reliability of the tests (142), affected by the age of the patients and on the activity of the disease. Gliadin antibody tests are excellent in young children with active disease. The IgA-class antibody test has a sensitivity of approaching 100% (except in patients with IgA-deficiency) and its specificity is also high. The IgG-class test also has high sensitivity but lower specificity (143). In older children and adults, especially when they are clinically silent, results are less reliable. Sensitivity is only 50–80%. The reduction of the cut-off level for positivity leads to many false-positive results (144). The IgG-class test may be more sensitive and IgA-class more specific. These results were also confirmed by O. Uibo *et al.* (145), studying the prevalence of coeliac disease with screening tests among 1434 children and 1461 adults. According to their results the AGA test could be used for coeliac disease screening in childhood (of the 44 IgA AGA positive children in 33

coeliac disease was confirmed by small bowel biopsy); but in the case of adults there are some limitations (of the 52 IgA AGA positive persons from Karksi-Nuia population in 49 normal small bowel mucosa was revealed, 3 refused the biopsy). The significance of positive AGA in patients with normal small-bowel mucosa is obscure.

The determination of antibodies against reticulin has been reviewed quite recently. The detection of R1-type ARA by the IIF method, using rat kidney as an antigen, has been shown to be very sensitive and also specific for coeliac disease (146, 147). Furthermore, a positive ARA test is highly suggestive of latent coeliac disease in patients with normal mucosal architecture, and therefore these patients should not be lost to follow-up (148). The results of using this test in different centres are, however, extremely confusing, presumably due to differences in technical details (143).

Chorzelski *et al.* (149) described a new antibody directed against the membrane of smooth muscle bundles of primates. This endomysial antibody was present in patients with dermatitis herpetiformis and coeliac disease and has been found to have greater sensitivity and specificity than AGA in diagnosing coeliac disease (141, 150–153), and in the case of general screening AEM evaluation has also been used as the final step before intestinal biopsy (154, 155). These IgA-type AEM are detected by performing IIF on muscular sections of monkey oesophagus or umbilical cord (150, 152). Recently, tissue transglutaminase was identified as the autoantigen to endomysial autoantibody (156).

## 2.5. Genetic aspects of inflammatory bowel disease

Many studies have demonstrated increased prevalence of inflammatory bowel disease among the relatives of the patients with UC and CD (157, 158). Consistent trends are noticeable. First-degree relatives are at the greatest risk, particularly siblings, but more distant relatives also display increased disease prevalence. A positive family history is more common in the relatives of the patients with CD than UC.

In Oxford study (159) information was obtained from 433 adult patients with CD using postal questionnaire, review of case notes and personal interviews. In 78 families (18%), at least one first- or second-degree relative also had IBD. Siblings were most commonly affected. CD was more common than UC in the affected siblings. Assuming population prevalence of 100 per 100,000 for UC and 70 per 100,000 for CD the relative risks in siblings of the patients with CD, calculated from these data, were 16.6 for UC, 36.5 for CD and 24.7 for IBD, respectively.

These data complement the results of the Swedish twin study (160) which strongly implicated genetic susceptibility in the pathogenesis of UC and CD.

There is a significant increase in the concordance of IBD in monozygotic twins compared to that in dizygotic twins, especially with CD.

In addition, many groups have collected the clinical data from multiply affected families and studied the extent of disease heterogeneity and the influence of genetic and environmental factors on disease behaviour. The data from the studies in the U.S.A. (161) and France (162) have demonstrated a marked concordance in the members of the multiply affected families with CD for disease extent and behaviour. In a recently published Oxford study (163) of over 250 multiply affected families in affected parent-child pairs, parent and child were concordant for disease type in 75.3%, for extent in 63.6%, extra-intestinal manifestations in 70.1%, and smoking history in 85%. Of particular note in this study was the fact that the median age at the diagnosis in parents was significantly higher than in offsprings. In a study from Polito *et al.* (161) the children were not only significantly younger at the age of the onset of the disease, but were generally also suffering from a more extensive disease.

The explanation for this observation is uncertain. Polito *et al.* (161) have suggested that the observed age differences may represent genetic anticipation. This phenomenon, the tendency for successive generations to develop a disease of increasing severity and earlier onset, has been observed in monogenic disorders, particularly in neurodegenerative diseases. The molecular basis for this effect involves the progressive amplification of unstable triplet repeats of DNA (164). Whether this explanation is pertinent to complex disorders such as IBD, or whether the observations reflect ascertainment bias or any other variable (165), is still uncertain.

Although genetic predisposition seems relatively more important in CD than UC, no simple Mendelian mode of inheritance is pertinent to either condition. It is most likely that the IBD represent a heterogeneous group of the related polygenic disorders, sharing some but not all susceptibility loci (166). Clinical presentation may depend on the interaction between different genes, and also on allelic variation in individual genes.

### 2.5.1. Genes involved in susceptibility to inflammatory bowel disease

The genes of the human major histocompatibility complex (MHC), which plays a central role in the immune response, have gained a lot of interest. Although, the data concerning the importance of these genes in UC and CD have, until recently, remained confusing and inconsistent, except for those small subgroups of patients with concomitant ankylosing spondylitis (associated with HLA-B27) or primary sclerosing cholangitis (associated with HLA-DR3-DQ2 or HLA-DR2) (158). The results have been more consistent with UC than with CD. Allelic associations have been reported between HLA-DR2 (HLA-DRB1*1502) and UC in Japanese (167), Jewish (168) and recently in Dutch (169) patients. However, this has not been confirmed in British (94) and American (93)

patients. In CD, in two recent studies from Germany (170) and France (171), a positive association with the HLA-DRB1*07 allele, and a negative association with the HLA-DRB1*03 allele was observed. In sharp contrast, no evidence that class II genes contribute to CD susceptibility and disease phenotype was reported in a British study (94). These observations show the importance of ethnicity in studying the genetics of IBD.

A further controversy lies on the relationship between HLA genotype and disease behaviour in UC. Satsangi *et al.* (94) not only showed the linkage between UC and HLA-DRB1 genes in a group of affected sibling pairs but, in a large association study (175 UC patients, 173 CD patients), found a number of interesting associations between specific Class II genes and clinical phenotype. Thus, the HLA-DR3-DQ2 (HLA-DRB1*0301-DQB1*0201) haplotype was associated with extensive disease and was rarely present in distal colitis, especially in females. A further association was with the rare HLA-DR103 (HLA-DRB1*0103) allele which had a frequency of 3% in the control population and a frequency of 8.6% in UC. Further analysis showed that the frequency rose to 11% in patients with extensive disease or severe disease requiring surgery. This latter hypothesis was then tested in a separate group of 107 patients with UC that had undergone proctocolectomy and an ileo-anal pouch anastomosis (172). The frequency of HLA-DR103 was again 11.8% but rose to 24% in patients who had extraintestinal manifestations (mouth ulcers, arthritis, erythema nodosum, uveitis).

Cytokines have an important role in the initiation and amplification of the inflammatory response in the mucosa in IBD (3), and the genes involved in the regulation of the immune response or maintenance of mucosal integrity represent candidate genes in IBD. Of particular present interest are the genes encoding the naturally occurring antagonist to interleukin-1, interleukin-1 receptor antagonist (IL-1RA), and the gene encoding the pro-inflammatory cytokine tumour necrosis factor-α (TNFα). Nevertheless, the results of the studies investigating cytokine gene polymorphisms in patients with UC or CD have been very conflicting. Mansfield *et al.* (173) initially reported that a polymorphism in intron 2 of the IL-1RA gene on chromosome 2 was associated with UC, especially with extensive colitis. This observation has been confirmed in some of the studies only, thus, the association with UC is, at best, weak (174). Furthermore, the significance of the observation is not clear, since the polymorphism is in a non-coding part of the gene.

A similar confusion arises with regard to TNFα polymorphisms. The studies from Oxford (175) have not shown significant associations with bi-allelic polymorphisms in the promoter region of the gene, but a significant association between CD and the TNFα-308 allele 2 was found in the Dutch series (176). Positive effects in the treatment of the CD patients with anti-TNFα monoclonal antibodies also support the presumption that TNF is involved in the pathogenesis of IBD, but the detailed mechanism still remains unclear (46, 177).

Cell surface adhesion molecules represent a large family of the molecules essential for cell communication, activation, and homing. As IBD is characterised by the infiltration of the inflammatory cells derived from the circulation including monocytes, lymphocytes, and neutrophils, a fundamental role of adhesion molecules is expected. Aberrant expression of cell adhesion molecules in the areas of inflammation is confirmed by several reports. In active IBD, mucosal mononuclear phagocytes show a dramatic increase in expression of intercellular adhesion molecule 1 (ICAM-1) (178). In addition, increased circulating concentrations of the vascular cell adhesion molecule 1 (VCAM-1) and E-selectin are also found (179, 180). Recently, a different distribution of soluble 1 (ICAM-1) alleles in subgroups of IBD patients, stratified by ANCA status, has been reported (92).

However, these and other candidate genes remain under evaluation and require replication in independent data sets before final conclusions are possible.

# 3. AIMS OF THE STUDY

The overall aim of the present studies was to investigate the presence and distribution of different antibodies and genetic markers in the patients with IBD to improve our current understanding of their role in the aetiology and pathogenesis of IBD.

The specific objectives were:

1. to determine the prevalence and clinical value of ANCA in the patients with IBD (I, II);
2. to analyse the pattern of ANCA by IIF and to characterise the antigen specificity of ANCA by ELISA, using myeloperoxidase, proteinase-3, lactoferrin and cathepsin G as antigens (I);
3. to determine the prevalence and clinical value of several non-organ-specific antibodies in the patients with IBD and to analyse ANA containing sera by ELISA to the extractable nuclear antigens Sm (Smith), RNP, SS-A (Ro), SS-B (La), Scl-70 and Jo-1 (II);
4. to examine the frequency of AGA, ARA and AEM in the patients with UC and to evaluate the correlations between these antibodies and various clinical features in the patients with UC (III);
5. to investigate the polymorphisms of HLA-DR and -DQ, tumour necrosis factor (TNF), E-selectin, L-selectin and intercellular adhesion molecule 1 (ICAM-1) in the patients with IBD and to determine relationships between these markers and ANCA status (IV).

# 4. PART I: THE FREQUENCY AND CLINICAL VALUE OF DIFFERENT AUTOANTIBODIES IN PATIENTS WITH IBD (PAPERS I, II)

## 4.1. Patients

All the serum samples were obtained from the patients with UC or CD attending the Department of Internal Medicine, the University of Tartu, or the University Outpatient Department. In Paper I, 59 patients with UC and 17 with CD and in Paper II, 77 patients with UC and 31 with CD were studied. For UC, the diagnostic criteria were based on a history of bloody stools, typical colonoscopy findings, and morphological changes in biopsy specimens. The extent of colitis in the patients with UC was evaluated with colonoscopy and, in some cases, with barium enemas. Proctitis refers to mucosal inflammation found in the rectum only, the disease extending proximally up to the mid-transverse colon is classified as left-sided colitis and the disease engaging (extension beyond mid-transverse colon) the whole of the colon and rectum refers to extensive colitis. For CD, the diagnostic criteria worked out by Lennard-Jones were followed (181). Disease localisation was evaluated by small-bowel roentgenograms, barium enemas and colonoscopy. Disease location was defined: ileum (disease limited to the small bowel), colon (any colonic location between the caecum and rectum with no small bowel or upper gastrointestinal involvement), ileocolon (disease of the terminal ileum and any location between the caecum and rectum). The clinical characteristics of the studied patients are shown in Table 1.

**Table 1.** Clinical characteristics of the patients with inflammatory bowel disease (IBD) at the time of serum sampling.

| | Paper I | | Paper II | |
|---|---|---|---|---|
| **Diagnosis** | **UC (n=59)** | **CD (n=17)** | **UC (n=77)** | **CD (n=31)** |
| **Male/Female** | 28/31 | 13/4 | 40/37 | 23/8 |
| **Mean age (years)** | 44 | 27 | 43 | 36 |
| **Disease extent** | | | | |
| Proctitis, n (%) | 25 (42) | | 31 (40) | |
| Left-sided, n (%) | 13 (22) | | 23 (30) | |
| Extensive, n (%) | 21 (36) | | 23 (30) | |
| **Disease localisation** | | | | |
| Ileum, n (%) | | 3 (18) | | 7 (23) |
| Colon, n (%) | | 6 (35) | | 9 (29) |
| Ileum and colon, n (%) | | 8 (47) | | 15 (48) |
| **Duration of disease** | | | | |
| <5 years, n (%) | 21 (36) | 13 (76) | | |
| >5 years, n (%) | 38 (64) | 4 (24) | | |

UC – ulcerative colitis; CD – Crohn's disease

The control groups consisted of the patients with the irritable bowel syndrome (IBS) and healthy persons with no history of gastrointestinal or chronic disease. In paper I 25 patients with IBS (12 male, 13 female; mean age 38 years) and 86 healthy persons (31 male, 55 female; mean age 39 years); in paper II 32 patients with IBS (16 male, 16 female; mean age 38 years) and 76 healthy persons (30 male, 46 female; mean age 40 years) served as controls. The study was approved by the Ethics Committee of the University of Tartu.

## 4.2. Methods

10 ml of venous blood was taken from each subject and stored at –20°C until analysed. All tests were performed at the Department of Immunology, the University of Tartu, continuously controlled by UKEQAS (Sheffield, UK).

### 4.2.1. Indirect immunofluorescence assay (IIF)

The separation of peripheral blood neutrophils and the detection of ANCA by IIF were performed, with minor modifications, according to the method described by Wiik (182).

Briefly, neutrophils were separated from the peripheral blood of a healthy volunteer by Ficoll-Hypaque centrifugation and dextran sedimentation. The buoyant neutrophils were recovered and washed. Cellular suspensions were smeared on slides and fixed in 96% ethanol for 5 minutes, dried at room temperature and stored at –20°C until the time of the assay, but not more than for two weeks. The slides were incubated with coded patients or control sera (dilution 1:20) and stained with rabbit antihuman IgG-fluorescein isothiocyanate (FITC) conjugate (Dakopatts, Glostrup, Denmark). The slides were evaluated by fluorescence microscopy. Positive and negative controls were included for all assays. The serum samples giving a typical perinuclear (pANCA) or cytoplasmic (cANCA) staining reaction with the neutrophils were regarded as positive. pANCA are present when reactivity is seen only with the neutrophils and the monocytes or when present in a much higher titre than on, for example, lymphocytes. To ensure that ANCA in IBD indeed recognise cytoplasmic antigens, all sera for the presence of ANA on rat liver slices were screened.

IgG-type antinuclear (ANA), antimitochondrial (AMA), smooth muscle (SMA), antireticulin (ARA), liver/kidney microsomal (LKMA), parietal cell (PCA) and thyroid microsomal (TMA) antibodies were tested by IIF on unfixed 4 μm cryostat sections from a composite block of a mouse stomach and rat kidney and liver, as well as from a hyperplastic human (blood group 0) thyroid gland (183). Coded patients or control sera were diluted at 1:10 and 1:100 in

phosphate-buffered saline (PBS) and tested on the sections using rabbit anti-human IgG fluoresceinisothiocyanate (FITC) conjugate (Dako, Copenhagen, Denmark) at dilution 1:80 as a secondary antibody. All the sera containing ANA were further analysed on ethanol-fixed Hep2 (Immuno Concepts, Sacramento, USA) cells at the dilution of 1:40 in PBS.

### 4.2.2. Enzyme-linked immunosorbent assay (ELISA)

Commercially available kits based on enzyme immunoassay were used for the analysis of myeloperoxidase and proteinase-3 antibodies of IgG isotype (Wieslab AB, Lund, Sweden). Both kits were used according to the manufacturer's instructions. A serum dilution of 1:80 was used in both assays. Alkaline phosphatase-labeled anti-human immunoglobulin G was used as a second antibody. The reaction was visualised using p-nitrophenyl phosphate as a substrate, and read at 405 nm. Values exceeding the borderline (20 EU) were regarded as positive. The anti-lactoferrin and anti-cathepsin G ELISAs were performed as originally described (182, 184). Briefly, ELISA microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C, with 5 μg/ml human neutrophil lactoferrin (Sigma, St. Louis, Mo., USA), or 0.5 μg/ml cathepsin G (Calbiochem, La Jolla, Calif., USA) in carbonate buffer (pH 9.6), and in 50 mM sodium acetate with 150 mM NaCl (pH 5.5), respectively. Plates were saturated with 1% bovine serum albumin in PBS (PBS/BSA) or with 5% swine serum albumin in PBS (PBS/SSA), respectively. The controls and serum samples were diluted at 1:100 in PBS/BSA 1%/Tween 0.1% and incubated for one hour at room temperature. The bound IgG was detected using alkaline phosphatase-conjugated anti-human IgG (Dako, Glostrup, Denmark), followed by p-nitrophenyl phosphate as a substrate. Sera from 40 healthy persons were used as references to establish a base-line absorbance value and defined as two standard deviations above the mean value.

ANA containing sera were analysed by ELISA for secondary confirmatory testing of IgG antibodies to the extractable nuclear antigens Sm (Smith), RNP, SS-A (Ro), SS-B (La), Scl-70 and Jo-1 using the Immuno Concepts RELISA ENA multiparameter test system (Immuno Concepts, Sacramento, CA, USA).

### 4.2.3. Statistical analysis

The chi-square test was used to analyse differences between the groups, $p<0.05$ was considered to be significant. Comparing small numbers the Fisher's exact test was performed.

## 4.3. Results

### 4.3.1. Antineutrophil cytoplasmic antibodies in patients with inflammatory bowel disease

In paper I IgG ANCA were detected by IIF on ethanol-fixed granulocytes in 29 of the 59 (49%) patients with UC and in 4 of the 17 (24%) patients with CD ($p=0.06$). In the control group 4 of the 111 (4%) serum samples were positive to ANCA, 1 sera of the 25 patients with the irritable bowel syndrome, and 3 of the 86 healthy subjects sera ($p<0.001$ for UC compared with controls; $p<0.01$ for CD compared with controls). In paper II IgG ANCA were detected in 45 of the 77 (58%) patients with UC and in 6 of the 31 (19%) patients with CD ($p<0.05$ with Yates' correction). In the control group 2 of the 108 (2%) persons (1 patient with the irritable bowel syndrome, and 1 healthy person) were positive to ANCA ($p<0.001$ with Yates' correction for UC compared with the controls and for CD compared with the controls).

The predominant ANCA staining pattern seen by IIF in IBD is perinuclear (pANCA). The cytoplasmic staining (cANCA) was revealed in 6 patients with UC and 1 patient with CD in paper I and in 5 patients with UC and 1 patient with CD in paper II. One patient with UC had both, perinuclear and cytoplasmic staining (paper I, II). In the control groups only 3 of the 111 sera showed a weak perinuclear staining and 1 sera cytoplasmic staining in paper I, and in paper II only 2 of the 108 sera showed a weak perinuclear staining. The distribution of ANCA in the patients' and controls' sera is shown in Table 2.

**Table 2.** Distribution of the antineutrophil cytoplasmic antibodies (ANCA) in patients' and controls' sera detected by indirect immunofluorescence.

| | | N | IIF ANCA-positive n (%) | Immunofluorescence staining pattern | | |
|---|---|---|---|---|---|---|
| | | | | pANCA | cANCA | p+c ANCA |
| **Paper I** | UC | 59 | 29 (49) | 22 | 6 | 1 |
| | CD | 17 | 4 (24) | 3 | 1 | 0 |
| | Controls | 111 | 4 (4) | 3 | 1 | 0 |
| **Paper II** | UC | 77 | 45 (58) | 39 | 5 | 1 |
| | CD | 31 | 6 (19) | 5 | 1 | 0 |
| | Controls | 108 | 2 (2) | 2 | 0 | 0 |

UC – ulcerative colitis; CD – Crohn's disease

No correlation between the presence of ANCA and the extent or duration of IBD was found (Table 3).

**Table 3.** Relationship between the clinical features of inflammatory bowel disease (IBD) and the presence of antineutrophil cytoplasmic antibodies (ANCA).

| | Paper I | | Paper II | |
|---|---|---|---|---|
| | ANCA positive patients/total number of patients | | | |
| | UC | CD | UC | CD |
| | 29/59 | 4/17 | 45/77 | 6/31 |
| **Disease extent** | | | | |
| Proctitis | 11/25 | | 17/31 | |
| Left-sided | 7/13 | | 12/23 | |
| Extensive | 11/21 | | 16/23 | |
| **Disease localisation** | | | | |
| Ileum | | 0/3 | | 0/7 |
| Colon | | 0/6 | | 1/9 |
| Ileum and colon | | 4/8 | | 5/15 |
| **Disease duration** | | | | |
| <5 years | 14/21 | 3/13 | | |
| > years | 15/38 | 1/4 | | |

UC — ulcerative colitis; CD — Crohn's disease

To ensure that ANCA in IBD indeed recognise cytoplasmic antigens, all the sera for the presence of ANA on rat liver slices were screened. In paper I ANA (1:10) were detected in 5 UC patients and 1 patient with CD; all the patients were pANCA positive and without liver abnormalities. In the control group 1 sera was ANA positive. All the 7 ANA positive sera were retested on the Hep2 cell lines at the dilution of 1:40. Two female UC patients and one male CD patient were found to be ANA positive. ANA in all of them produced a homogeneous staining pattern of the nucleus. In paper II 8 out of the 45 pANCA positive UC patients had an additional nuclear staining on lymphocytes, in all cases confirmed to be due to ANA on the cryostat sections. In addition, 1 patient with CD showed a pure nuclear staining on both granulocytes and lymphocytes. This was shown to be due to the presence of ANA, by positive staining reactions on the cryostat sections.

To characterise the specificity of ANCA, sera were screened with ELISA using proteinase-3, myeloperoxidase, lactoferrin, and cathepsin G as antigens (paper I). Of the 59 UC and 17 CD sera, only 14 of the 76 (18%) sera were positive. The antibodies to proteinase-3 were detected in 5 UC and 1 CD sera, of which 3 UC and 1 CD sera had scored positive for cANCA. One UC serum scored positive for pANCA and 1 UC serum had both types of ANCA. The antibodies to myeloperoxidase were not detected. The anti-lactoferrin antibodies were detected in 6 UC and 1 CD patients' sera, of which 3 UC and 1 CD sera were pANCA positive, 1 UC serum had both types of ANCA, and 2 were negative on IIF. The antibodies to cathepsin G were detected in 3 UC and 2 CD patients' sera, of which 1 UC and 1 CD sera were pANCA positive, and 2 UC

and 1 CD sera were negative on IIF (Table 4). Three patients with UC and 1 with CD had positive ELISA for two different antigens.

**Table 4.** Number of the antineutrophil cytoplasmic antibodies (ANCA) positive sera detected by ELISA with different antigens (Paper I).

| | Ulcerative colitis (n=59) | | Crohn's disease (n=17) | |
|---|---|---|---|---|
| **Antigen** | **ELISA+** | **IIF+** | **ELISA+** | **IIF+** |
| Proteinase-3 | 5 | 5 | 1 | 1 |
| Myeloperoxidase | 0 | 0 | 0 | 0 |
| Lactoferrin | 6 | 4 | 1 | 1 |
| Cathepsin G | 3* | 1 | 2 | 1 |

* Fifty-six sera from the patients with ulcerative colitis were studied.

### 4.3.2. Other autoantibodies in the patients with inflammatory bowel disease

IgG ANA, SMA, TMA or/and PCA were detected by IIF in 19 of the 77 (25%) patients with UC. Eight of the 31 (26%) patients with CD had ANA or SMA. There was no difference in the frequency of autoantibodies between UC and CD ($p=1.0$ with Yates' correction). In the control group, 13 out of the 108 (12%) persons were positive for ANA, SMA, TMA or/and PCA ($p<0.05$ with Yates' correction for UC compared with controls; $p=0.1$ with Yates' correction for CD compared with controls).

Most of the UC and CD patients had these autoantibodies in low (1:10) titres. Only 4 patients with UC (2 with AIH and 2 females without liver abnormalities) and 1 male with CD (without liver abnormalities) had ANA at the serum dilution of 1:100, 1 female patient with UC had TMA and PCA at 1:100. In the control group 1 female had TMA and 1 male PCA in titre 1:100 positive. None of the 216 serum samples were positive for AMA, ARA or LKMA. The frequencies of different autoantibodies are summarised in Table 5.

All the 12 positive ANA sera were further analysed on ethanol-fixed HEp-2 cell preparations at the dilution of 1:40. Three out of 12, i.e. 2 female UC patients (1 with AIH and the other without liver abnormalities) and 1 male CD patient (without liver abnormalities), all 1:100 positive on the cryostat sections, were also found to be positive on Hep-2 cells (homogeneous pattern).

Out of the 11 ANA positive sera (1 healthy person's sera was not further available), screened by ELISA for Sm, RNP, SS-A, SS-B, Scl-70 and Jo-1 antibodies, reactivity to the Scl-70 antigen was seen in a 23-year old woman with long-standing extensive colitis and concurrent AIH. The medical records of our patient were carefully revised, but no signs of systemic sclerosis were found.

**Table 5.** Frequencies of ANA, SMA, TMA and PCA in patients' and controls' sera detected by indirect immunofluorescence (Paper II).

| Titres of autoantibodies | No. of ANA, SMA, TMA or PCA positive patients | | |
|---|---|---|---|
| | UC (n=77) | CD (n=31) | Controls (n=108) |
| ANA 1:10 | 5 | 1 | 1 |
| 1:100 | 4 | 1 | – |
| SMA 1:10 | 5 | 6 | 8 |
| 1:100 | – | – | – |
| TMA 1:10 | 1 | – | – |
| 1:100 | 1 | – | 1 |
| PCA 1:10 | 2 | – | 2 |
| 1:100 | 1 | – | 1 |
| Total 1:10 | 13 (17%) | 7 (23%) | 11 (10%) |
| 1:100 | 6 (8%) | 1 (3%) | 2 (2%) |

ANA — antinuclear antibodies; SMA — smooth muscle antibodies; PCA — parietal cell antibodies; UC — ulcerative colitis; CD — Crohn's disease

## 4.4. Discussion

It is well accepted in rheumatology that ANA and ANCA have become useful tools not only for differential diagnosis but even for prognosis, early diagnosis and sometimes for monitoring disease activity. Disappointingly, in IBD most of the autoantibodies described lack sensitivity or specificity, and in this way, suggesting them to be of little pathogenic relevance. In recent years ANCA has been recognised in patients with IBD and have become a valuable laboratory aid in the diagnosis of IBD, although the antigen(s) responsible has not been identified yet.

The present studies showed that ANCA are often present in the ulcerative colitis patients in Estonia. ANCA were detected in 49–58% of the patients with UC and in 19–24% of the patients with CD (Paper I and Paper II). It has been suggested in the literature (185, 186) that ANCA may help differentiate UC from CD, as ANCA has been found in a low frequency in the patients with CD. The prevalence of ANCA in our patients with CD was rather high (24% and 19% in Paper I and Paper II, respectively), showing its limited role in differentiating the diseases. Therefore we do agree with the findings of Broekroelofs *et al.* (83), showing ANCA to be of limited or no value in distinguishing Crohn's disease from ulcerative colitis. At the same time, colonic involvement in ANCA positive patients with CD seems to be important as none of our CD patients with small bowel disease did have ANCA only. These results confirm the data described previously (64, 69, 88) and support the idea that pANCA positive CD patients may represent a distinct disease subgroup (87).

The predominant ANCA staining pattern observed in our studies was perinuclear that is being also affirmed by other authors in the case of IBD. However, it

is distinct from vasculitis-associated pANCA, and it is not generally reactive with myeloperoxidase (64, 67, 83). In contrast with cANCA, which have shown to serve a highly sensitive and specific marker for Wegener's granulomatosis (59, 187, 188), in IBD the pathogenetic role and clinical significance of pANCA is less favourably established. The problem is that ANCA are present only in 40–85% of the patients with UC (64–70), and most of the authors have failed to show neither the correlation between ANCA and disease activity nor ANCA and disease extent (64, 65, 69, 82), although some studies have suggested that pANCA may be associated with a more aggressive or more severe UC (83–85). No correlation between ANCA and the duration, or ANCA and the extent of IBD was found in our study.

The other problem is that test sera may contain other antibodies, including antinuclear antibodies which may result in a staining pattern not dissimilar to that of pANCA. Therefore, the screening of the sera with the non-specific staining patterns, and equivocally, ANCA positive for the presence of ANA, is useful until the antigen(s) responsible for ANCA are identified. And that is when it may be expected that the problems associated with the recognition of ANCA can be overcome. Fortunately, the number of IBD patients with concomitant ANA in our studies was not high and most of the patients had these antibodies in low titre (1:10) only. Therefore simultaneous screening of these sera for ANCA and ANA, using titration, is essential and this is how the problem in most of the cases can be solved.

Whereas the antigen specificity of ANCA in IBD is still uncertain, antibodies to several constituents of neutrophils including cathepsin G (74–76), lactoferrin (66, 72), β-glucuronidase (79), and recently bactericidal/permeability-increasing protein (69, 73) have been reported in some patients with IBD. None of these is being proved to be exclusively responsible for the pANCA activity. In the present study 18% of the serum samples were positive in the specific ELISA assays (Paper I). Though the immunofluorescence and ELISA results did not correspond in our study, we support the findings that other antigens than proteinase-3, myeloperoxidase, lactoferrin and cathepsin G can be the targets for ANCA in the patients with IBD and remain to be evaluated in further investigations.

We also studied the presence of ANA, AMA, SMA, ARA, LKMA, PCA and TMA by IIF. All ANA containing sera were further analysed by ELISA for 6 different antibodies against extractable nuclear antigens. The ANA, SMA, TMA and PCA detected in the patients with IBD were in general of low titre and frequency. Although 25% of the patients with UC and 26% of the patients with CD compared to 12% of the controls had these antibodies; only 8% of the UC patients, 3% of the CD patients and 2% of the controls had them at the dilution of 1:100. In contrast, Dalekos *et al.* (109) in their study found ANA in 41 out of the 80 (51%) patients with UC. Although they used HEp-2 cells (positive ANA titre ≥1:80) for the detection of ANA, it seems to us that the differences in the prevalence of ANA can not be explained by technical differences only. Indeed, in our studies 2 out of the 5 patients with ANA in titre 1:100 had concomitant

AIH. Therefore our results rather suggest that in our patients with IBD ANA in high titres are infrequently present. We suggest that different genetic and environmental backgrounds play role in the lower prevalence of ANA. Our results are also supported by Uibo *et al.* (189); comparing prevalence of different autoantibodies among young adults in Estonia and Sweden they found that the prevalence of ANA was significantly lower in Estonia (7% vs. 12%). Reactivity to the Scl-70 antigen was seen in a patient with long-standing extensive colitis and concurrent AIH. The antibodies to DNA topoisomerase I, also known as Scl-70, are generally associated with diffuse cutaneous systemic sclerosis (190) and have even been shown to be present for months or years before the clinical expression of the disease (191). No signs of systemic sclerosis were found in our patient.

It has been suggested that autoimmune mechanisms are more likely to be involved in the pathogenesis of UC than that of CD (192). The higher prevalence of associated autoimmune diseases and different autoantibodies in the case of UC supports the idea. Our study confirms that ANCA are more often present in the patients with UC than in the patients with CD. In the case of other autoantibodies the significant difference between UC and CD could not have been revealed. A fundamental question is the true relevance of autoantibodies to UC in particular and IBD in general. pANCA have yet to be proven to alter the neutrophil function, although the recent demonstration of pANCA-producing B-cell clones in UC mucosa leaves the door open to alternate roles (96). A related question remains to be asked, how many other autoantibodies exist in IBD and what is the range of their specificity. Humoral autoimmune phenomena are common in IBD, but their relevance to disease pathogenesis needs clarification, particularly because the simple occurrence of autoantibodies no longer indicates a pathological condition, but it is part of normal immune homeostasis (193). Further investigations on the molecular structure and function of target autoantigens of ANCA and other autoantibodies are extremely valuable and may significantly contribute to our understanding of the cellular processes taking place in IBD.

# 5. PART II: THE ASSOCIATIONS BETWEEN COELIAC DISEASE RELATED ANTIBODIES AND ULCERATIVE COLITIS (PAPER III)

## 5.1. Patients

A total of 103 persons were investigated in the present study. Fifty serum samples were obtained from the consecutive unselected patients with UC (25 males, 25 females, from 14 to 75 years of age, mean age 43 years) attending the Department of Internal Medicine, the University of Tartu or the University Outpatient Department. The diagnosis of UC was based on the conventional criteria described earlier. The UC patients were classified according to disease extent, disease activity and disease duration at the time the blood sample was obtained. The extent of colitis was evaluated in 43 cases in colonoscopy and in 7 cases in radiological investigation. Nineteen patients had proctitis, 12 left-sided colitis, and 19 extensive colitis. Disease activity was assessed by the criteria of Truelove and Witts (28). UC was characterised as mild, moderate, or severe based on equal weighting of the following six factors: diarrhoea, fever, tachycardia, anaemia, erythrocyte sedimentation rate, and physical examination. Twenty-two of the 50 UC patients were in clinical remission, 10 had mild disease, 14 moderate disease, and 4, severe disease. Twenty-two of 50 patients with UC were treated with a combination of systemic or topical steroids and sulfasalazine or 5-ASA. The other patients received no medication or sulfasalazine or 5-ASA only. Finally, duration of disease was considered: 21 patients had been suffering from UC less than for 5 years and 29 patients 5 years or more.

The control group consisted of 53 individuals, age- and sex-matched with the UC group (24 males, 29 females, from 16 to 79 years of age, mean age 43 years). Sixteen patients had the irritable bowel syndrome (IBS) (9 males, 7 females, from 16 to 62 years of age, mean age 40 years) and 37 were healthy individuals (15 males, 22 females, from 17 to 79 years of age, mean age 46 years) with no history of gastrointestinal or other chronic diseases. The study was approved by the Ethics Committee of the University of Tartu.

## 5.2. Methods

Ten ml of venous blood was taken from each subject and stored at $-20^0$C until analysed for IgA- and IgG-type AGA, IgG-type ARA and IgA-type AEM. All tests were performed at the Department of Immunology, the University of Tartu, continuously controlled by UKEQAS (Sheffield, UK).

### 5.2.1. The indirect immunofluorescence assay (IIF)

#### 5.2.1.1. Detection of the antireticulin antibodies

R1-type ARA were detected using a standard IIF method (194) with rat kidney, liver and mouse stomach mounted into a composite block as antigen substrates, and fluorescein isothiocyanate (FITC)-conjugated rabbit antihuman IgG (DAKO A/S, Glostrup, Denmark) as the secondary antibody. The sera were investigated at the dilutions of 1:10 and 1:100 in PBS, and were considered ARA positive if the characteristic R1-staining pattern was found in antigen substrates under the fluorescence microscope (Jenalumar SH 50; VEB Carl Zeiss, Jena, Germany [with ocular × 6.3 and objective × 25 and × 50]). The specific pattern included staining of the fibres around the glomeruli and tubuli and around the blood vessels in the kidney section; staining of the nodular fibres in the portal areas of the liver; and staining of the fibres between the gastric glands, in the connective tissue and in the muscular mucosa of the stomach. Known ARA-positive and ARA-negative sera were used as controls.

#### 5.2.1.2. Detection of the antiendomysium antibodies

IgA-type AEM were measured by IIF, using the cryostat sections from the human umbilical cord as antigen substrate (195). Tissue sections were incubated with serum diluted at 1:10 and 1:100 with PBS, and FITC-labelled rabbit antibody to human IgA (DAKO A/S, Glostrup, Denmark) was used as a secondary antibody. Under the fluorescence microscope (Jenalumar SH 50, ocular × 6.3, objective × 25 and × 50) the presence of a honeycomb brilliant green network pattern along the peritubular muscle layer of the vessels on umbilical cord was taken as positive. Known AEM-positive and AEM-negative sera were used as controls.

### 5.2.2. The enzyme-linked immunosorbent assay (ELISA)

#### 5.2.2.1. Detection of the antigliadin antibodies

AGA were determined by ELISA according to the method developed in the Department of Immunology, the University of Tartu. Briefly, wheat gliadin (Serva Diagnostica, Heidelberg, Germany) was dissolved in 70% ethanol (2 mg/ml), diluted at 1:100 in 0.05 M carbonate-bicarbonate buffer, and incubated for 20 hours at +4°C. After washing with phosphate-buffered saline (PBS) (pH 7.4), the plates were saturated with 5% normal sheep serum (NSS) in PBS for 2 hours at +37°C. Further, the test sera and known AGA-positive and -negative control sera, diluted at 1:100 in 1% NSS-PBS, were analysed in duplicate and incubated for 3 hours at +37°C. The plates were then washed again and

alkaline phosphatase-conjugated antihuman IgG or IgA immunoglobulins (DAKO A/S, Glostrup, Denmark) in 1% NSS-PBS were added to each well at a dilution suggested by the manufacturer. After incubation for 20 hours at +20°C, the plates were washed and p-nitrophenyl-phosphate (Sigma, St Louis, USA) in diethanolamine magnesium chloride buffer, pH 9.8, was added. The reaction was stopped after 30 minutes with 3M sodium hydroxide. The optical density (OD) of the reaction product was measured at 405 nm. The results were calculated in enzyme immunosorbent units (EIU). The sera from 37 healthy individuals were used as references to establish a baseline absorbance value. Readings above three SD of the mean value were considered positive. The cut-off level for the tests was ≥120 EIU for IgA-type AGA and ≥8 EIU for IgG-type AGA. The inter-assay coefficient of variation of the AGA test results was 10.6% and the intra-assay coefficient of variation was 5.7%.

### 5.2.3. Statistical analysis

The prevalence of AGA in various groups of the patients was compared by means of the chi-square test with Yates' correction. Analysis of variance (ANOVA) and the Student's *t*-test were used to correlate the titres of AGA with different clinical markers; $p<0.05$ was considered significant.

## 5.3. Results

### 5.3.1. Antireticulin antibodies in the patients with ulcerative colitis

All the studied 50 UC patients and 53 individuals of the control group were negative for IgG-type ARA.

### 5.3.2. Antiendomysium antibodies in the patients with ulcerative colitis

All the 49 patients with UC studied and the 52 controls studied were negative for IgA-type AEM (sera for 1 UC and 1 healthy individual, both AGA-negative, were not available for this examination).

### 5.3.3. Antigliadin antibodies in the patients with ulcerative colitis

Altogether 17 out of the 50 (34%) patients with UC had positive IgA- or/and IgG-type AGA test result, in 5 of them both types of AGA were detected. Of the 53 individuals in the control group, only 2 (4%) (both with IBS, males 24 and

28 years of age) had positive IgG-type AGA ($p<0.001$ for UC compared with controls). Figure 1 shows the distribution of IgA-and IgG-type AGA titres (EIU units) in the patients with UC and in the controls. There were significant differences in AGA titres between the UC patient group and controls. Median titres for IgA-type AGA were 76 EIU (range 0–219 EIU) in the patients with UC and 36 EIU (range 0-108) in the controls ($p<0.001$). For IgG-type AGA, the median titres were 5 EIU (range 0–50 EIU) in the UC patients and 2 EIU (range 0–16) in the controls ($p<0.01$).

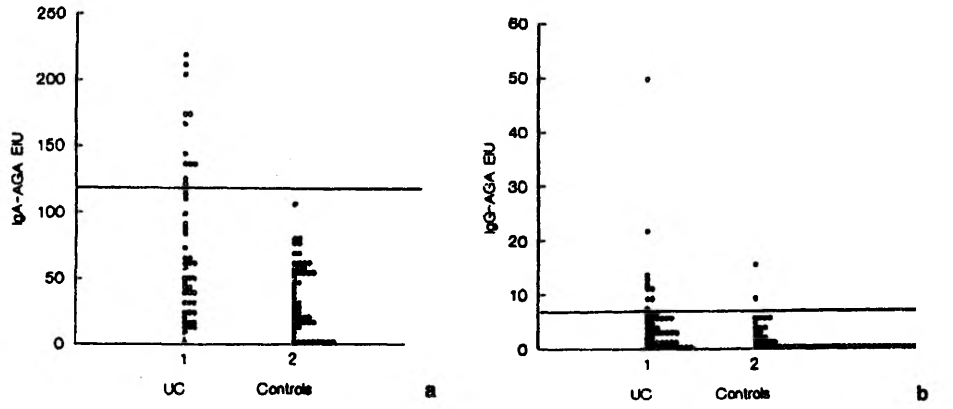


**Figure 1a,b.** Titres of IgA-type (**a**) and IgG-type (**b**) antigliadin antibodies (AGA) in the patients with ulcerative colitis (UC) and in the controls. Discrimination between negative and positive test results is represented by the *solid line*.

The relationships between the extent of UC and the presence of different types of AGA are shown in Table 6. No significant difference between the extent of the disease and the presence of positive or negative AGA test results was observed. But five of the patients with both AGA, IgA- and IgG-types, had all extensive colitis. No correlation was found between the extent of disease and titre of AGA.

**Table 6.** The relation between the extent of ulcerative colitis (UC) and the presence of different types of antigliadin antibodies (AGA).

| | No of AGA positive UC patients | | | |
|---|---|---|---|---|
| | **IgA AGA** | **IgG AGA** | **IgA + IgG AGA** | **Total** |
| **Disease extent** | | | | |
| Proctitis (n=19) | 6 | 2 | 0 | 8 |
| Left-sided (n=12) | 1 | 1 | 0 | 2 |
| Extensive (n=19) | 0 | 2 | 5 | 7 |

There was no significant correlation between the presence of AGA and disease activity, or duration of disease in the patients with UC (Table 7). In addition, these clinical markers were not related to the titre of AGA. No relationship was observed between the medication used and the presence of AGA (Table 7).

**Table 7.** The relationship between the presence of the antigliadin antibodies (AGA) and disease activity, disease duration and the medication used in the patients with ulcerative colitis (UC).

| | **No of AGA positive UC patients** |
|---|---|
| **Disease activity** | |
| Remission (n=22) | 8 |
| Mild (n=10) | 3 |
| Moderate (n=14) | 5 |
| Severe (n=4) | 1 |
| **Disease duration** | |
| < 5 years (n=21) | 10 |
| > 5 years (n=29) | 7 |
| **Medication** | |
| Steroids (n=22) | 6 |
| Others or none (n=28) | 11 |

## 5.4. Discussion

In 1965, Salem and Truelove (13) reported 12 patients with partial and 2 patients with subtotal villous atrophy among 60 patients with UC. The coexistence of coeliac disease with Crohn's disease and UC has thereafter been described in several case-reports (131, 135, 136). In 1987, Breen *et al.* (131) reviewed 42 rectal biopsies from a population of 438 patients with coeliac disease and, among them, found 3 patients with abnormal rectal biopsy having histopathologic and clinical features of UC. An epidemiological survey of IBD in first-degree relatives of 162 coeliac patients showed the relative risk of IBD to be five times greater for first-degree relatives than that for the general population (133). In this study six of the 861 relatives had IBD, five of whom had UC and one CD. Taken together, these reports suggested that coeliac disease and IBD coexist more commonly than would be expected by chance. On the other hand, Collin *et al.* (196) examined 335 coeliac patients and found only one patient with concomitant IBD in comparison with 7 patients among 335 age and sex matched controls. Therefore an important question has been raised — is the coexistence of IBD and coeliac disease true or coincidental?

The serum concentration of AGA has been used as a variable in screening for coeliac disease (197, 198). However, sensitivity and specificity levels are of around 80%, and the false-positive rate of AGA may be quite high (145, 153). The sensitivity and specificity of ARA and AEM are clearly greater than those of AGA in coeliac disease diagnosis (147, 150–153), but the testing of ARA and AEM is relatively expensive and time-consuming. Therefore, for coeliac disease screening a serial procedure using first AGA and then EMA analysis has been proposed. It is a procedure that may reduce the number of falsely positive tests and the number of small-bowel biopsy specimens (155).

In our study this problem was investigated by aiming to detect possible coeliac disease cases among the UC patients using all the three earlier described serological markers — AGA, ARA and AEM. AGA was found in 34% of the UC patients, but none of them had either IgG-type ARA or IgA-type AEM. Therefore, we can conclude that none of the UC patients we tested had coeliac disease, since ARA (140, 196) and AEM (141, 152, 153, 195, 199, 200) have been shown to be highly specific serological markers for coeliac disease.

However, there is another question left, why AGA, a type of antibodies frequently demonstrated in association with coeliac disease, are seen in 34% of the patients with UC in our study. Similar results were found also by Ferreira *et al.* (141) comparing the sensitivities and specificities of the AGA, ARA and AEM in the 117 patients with coeliac disease. The authors had in a disease control group a small number of patients with IBD (10 patients with UC and 21 patients with Crohn's disease). Positive AGA test was found in 10% of the patients with UC and in over 20% of the patients with Crohn's disease, while ARA and AEM were negative in all patients. In another study, done by Volta *et al.* (201), IgG AGA were present in 19% of the 37 patients with UC and in 27% of the 26 patients with Crohn's disease. Although these authors used IBD patients as disease controls for CD, their findings confirm our results.

In our study, we also found that the patients with extensive colitis only had both, IgA- and IgG-types of AGA. Based on the results gained we would suggest the following: as in the case of extensive colitis the gut mucosa is more extensively damaged, gut permeability may be increased. Therefore, we support the idea that the increased permeability to different food antigens of intestinal mucosa is also involved in the development of AGA in UC (202). But as none of the AGA positive UC patients did show any signs of malabsorption and ARA and AEM were negative, we considered endoscopy and small bowel biopsy not feasible for these patients.

In certain circumstances (e.g., in the presence of certain HLA haplotypes, such as HLA-DQ2) IBD may favour the onset of coeliac disease by altering intestine barrier function, thus allowing foreign antigens to breach the immune system (168, 203, 204). In approximately one-fourth of the patients reported, the diagnosis of IBD preceded that of coeliac disease (134). It is possible that some patients with latent coeliac disease have their disease diagnosed when an additional small bowel mucosal injury due to IBD overcomes their ability to

compensate coeliac disease. In these cases, positive serological screening tests, mainly ARA and AEM, may help in selecting patients in whom coeliac disease is suspected for small-bowel biopsy. To solve the question of whether the development of AGA arises from similar mechanisms in both coeliac disease and IBD, further comparative studies of AGA (e.g., immunoblot, enzyme-linked immunospot assays [ELISPOT]) in coeliac disease and IBD are needed.

Although we could not find any patients with possible coeliac disease in our study, and regardless of the aetiology of the connection between the two disorders, it is important that the clinicians would be aware of the association. Failure to recognise the possibility of coexistence of these two disorders can lead to clinical confusion and frustration, and can be detrimental to the patient. Any patient with an established diagnosis of coeliac disease who remains symptomatic or develops new symptoms despite adhering to a gluten-free diet should be evaluated for the presence of IBD. Similarly, if patients with IBD have atypical symptoms or fail to respond to therapy, evaluation for the presence of underlying coeliac disease is essential (134).

# 6. PART III: THE ASSOCIATIONS OF HLA CLASS II GENES AND OTHER CANDITADE GENES AND ANCA IN THE PATIENTS WITH INFLAMMATORY BOWEL DISEASE (PAPER IV)

## 6.1. Patients

A total of 53 unrelated patients with IBD were studied. Thirty-free serum samples were obtained from the patients with UC (16 male and 17 female, median age 37.3 years) and 20 from the patients with CD (15 male and 5 female, mean age 36.0 years) attending the Department of Internal Medicine, the University of Tartu, or the University Outpatient Department. The diagnosis of UC and CD was based on the conventional criteria described earlier. Seventy healthy blood donors served as controls. The distribution of age, sex and ethnicity were comparable between the patients and the controls. All the immunogenetic studies were carried out by Dr. Hirv at the Institute of Immunology and Transfusion Medicine (University of Lübeck School of Medicine, Lübeck, Germany). The study was approved by the Ethics Committee of the University of Tartu.

## 6.2. Methods

### 6.2.1. DNA extraction

Genomic DNA was extracted from 10 ml venous EDTA-blood or from peripheral blood leukocytes (controls) with QIAamp Blood Kit from QIAGEN according to the manufacturers' instructions.

### 6.2.2. HLA-DRB and -DQB genotyping

The HLA-DRB1 and HLA-DQB1 genotyping were performed by the INNO-LiPA DRB and DRQ key kit from Innogenetics. These HLA typing tests are based on the reverse hybridisation principle. Amplified biotinylated DNA material is chemically denatured, and the single strands are hybridised with specific oligonuclotic probes immobilised on membrane-based strips. With the exception of DQB1*0201–*0202, all other DQB1 alleles could be distinguished. Although the resolution at the allelic level is possible with the INNO-LiPA DRB key kit, only the discrimination at the HLA-DRB1*01, *15, *16, *03, *04, *11, *12, *13, *14, *07, *08, *09, *10 allelic-group level was considered, except for the HLA-DRB1*0103 allele, which could be distinguished from

other HLA-DRB1*01 alleles. HLA-DRB1*15 alleles were specifically typed with the Micro SSP™ Allele Specific Class II DNA Typing Kit (One Lambda Inc.).

### 6.2.3. Detection of the antineutrophil cytoplasmic antibodies

Separation of peripheral blood neutrophils and detection of ANCA by IIF was performed according to the standard method described earlier.

### 6.2.4. TNF and the adhesion molecule polymorphism analysis

The detection of polymorphisms in the TNFα promoter at position –308 (TNF1/2) and in the TNFβ first intron (TNFB*1/2) with the help of PCR amplification and the subsequent digestion with the restriction enzyme *Nco*I were performed as described earlier (205, 206). The methods for identification of the adhesion molecule polymorphisms and primer sequences for the amplification of the investigated gene fragments are summarised in Table 8. Primer sequences for the amplification of the ICAM-1 Ig-like domain 3 and 5 were derived from the sequence described by Vora *et al.* (207). PCRs were performed by using 30 ng template DNA in a final reaction volume of 50 µl in a GeneAmp9600 (Perkin Elmer) thermal cycler. The amplification conditions were optimised by changing the annealing temperature and time or extension time for each of the investigated adhesion molecule gene region. The polymorphism in exon 6 of the ICAM-1 gene (Lys:AAG or Glu:GAG at codon 469), coding for Ig-like domain 5 was identified through the incubation of the amplified 110 bp ICAM-1 exon 6 gene fragments with the restriction enzyme *BstU*I. The presence of the *BstU*I restriction site (CG$^{\downarrow}$CG) resulted in 86 and 24 bp fragments. The fragments were analysed by electrophoresis on 4% NuSieve gels and by following staining of the gels in 0.1% ethidium bromide.

For the identification of the polymorphisms in all other amplified adhesion molecule gene regions, the single-strand conformation polymorphism (SSCP) method was used. Primer sequences to analyse E- and L-selectin polymorphisms were kindly provided by Dr. K. Wenzel (Charité, Berlin) (208). The fragments were denatured and run on non-denaturing 12.5% or 20% polyacrylamide gels (Homogeneous PhastGels) from Pharmacia Biotech. The conditions for the horizontal electrophoresis and silver staining were optimised for each gene fragment and performed semi-automatically with the PhastSystem (Pharmacia LKB Biotechnology AB). Figure 2 demonstrates the detection of the polymorphism in L-selectin EGF-like domain by PCR-SSCP analysis as an example of this method. To verify the SSCP results, the PCR products were sequenced.

**Table 8.** Outline of adhesion molecule polymorphism's analysis.

| Polymorphism | Method | Primer sequence (5′ to 3′) | PCR product |
|---|---|---|---|
| **ICAM-1 (CD54)** | | | |
| Ig-like domain 3 (R/G241) | PCR-SSCP | Forward GATTGAAGAAGCCAGCAG<br>Reverse GTCGTTGCCATAGGTGAC | 408 bp |
| Ig-like domain 5 (K/E469) | PCR-RFLP | Forward CCATCGGGGAATCAGTG<br>Reverse ACAGAGCACATTCACGGTC | 110 bp |
| **E-selectin (CD62E)** | | | |
| 5′-untranslated region (G/T98) | PCR-SSCP | Forward TAACTAGCTACCCACGATTTCC<br>Reverse ACTGACTTACCCAAAGTGAGAG | 149 bp |
| EGF-like domain (S/R128) | PCR-SSCP | Forward AGTAATAGTCCTCCTCATCATG<br>Reverse ACCATCTCAAGTGAAGAAAGAG | 186 bp |
| Membrane domain (L/F554) | PCR-SSCP | Forward CAATTCTTCCTCATGACCTTTC<br>Reverse CAAGACCATGACTTATCAATGAG | 226 bp |
| **L-selectin (CD62L)** | | | |
| Lectin-like domain (T/S49) | PCR-SSCP | Forward TGTAAGTCTGCATAGGTCACAC<br>Reverse TCAGTGAGAGATTTGTTGGTTC | 276 bp |
| EGF-like domain (F/L206) | PCR-SSCP | Forward CTTTGAGTACTAAAATGTAATCAC<br>Reverse CCTAAGAAGAAGCAAAGAAAGG | 198 bp |

PCR-RFLP — polymerase chain reaction–restriction fragment length polymorphism; PCR-SSCP — polymerase chain reaction–single-strand conformation polymorphism; EGF — epidermal growth factor

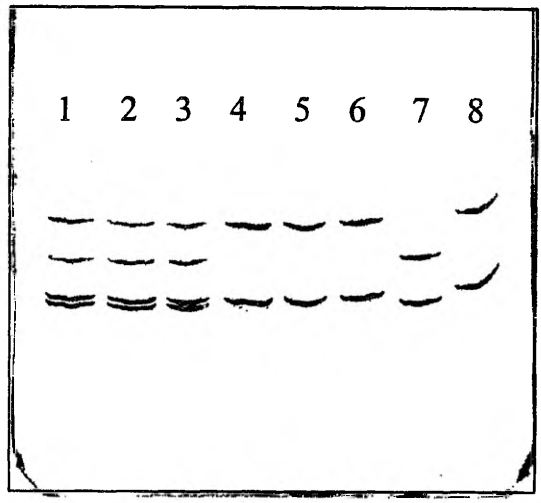


**Figure 2.** Representative single-strand conformation polymorphism (SSCP) analysis of the L-selectin epidermal growth factor (EGF)-like domain. An amplified 198-bp gene fragment was separated on PhastGel Homogeneous 20% gel. The amino acid polymorphism at codon 206 (F/L206) is caused by change from thymine to cytosine at nucleotide position 668, which results in a band shift in the electrophoretic analysis. Lanes 1, 2 and 3: heterozygous TC668. Lanes 4, 5, 6, and 8: homozygous TC668. Lane 7: homozygous CC668.

### 6.2.5. Statistical analysis

The patients with UC, grouped together, and the patients with CD, grouped together, were compared with the controls. Associations with subsets of the UC and CD patients, divided by ANCA status, were also evaluated. The statistical significance was tested by the chi-square test. The Fisher's exact test was used when appropriate (i.e., expected number <5). Significance was taken as $p<0.05$. By the correction of p values, the recommendations from Svejgaard and Ryder were taken into consideration (209). The correction factor for HLA-DRB1 and HLA-DQB1 alleles was 12 (extremely infrequent alleles, ranging from 0 to 2, were not included in the number of comparisons). In the subgroups of UC or CD, stratified by ANCA, the correction factor was 24. In the phenotypic combination analysis the p values were corrected by a factor of 48. The corrected p value is designated as $p_c$. The odds ratio (OR) as a measure of association was calculated using a Haldane's modification as suggested by Svejgaard and Ryder (209).

## 6.3. Results

### 6.3.1. Antineutrophil cytoplasmic antibodies in the patients with inflammatory bowel disease

In total, ANCA were detected by IIF in 27 of the 53 (50.9%) patients with IBD. Within UC and CD patients the presence of ANCA was different. Twenty-four of the 33 (72.7%) patients with UC and 3 of the 20 (15%) patients with CD were ANCA positive. The predominant ANCA staining pattern seen by IIF was perinuclear, only in one patient with UC cytoplasmic staining (cANCA) was revealed.

### 6.3.2. Adhesion molecule polymorphisms in the patients with inflammatory bowel disease

The observed allele frequencies of the investigated adhesion molecule polymorphisms in the control group and in the groups of patients suffering from UC and CD are shown in Table 9. There were no statistically significant differences in allele frequencies between the controls and the patients with UC or CD, or the patients considered as one group.

**Table 9.** Less common allele frequencies (%) of adhesion molecule genes in patients' and controls'.

| Polymorphism | Controls (n=70) | UC patients (n=33) | CD patients (n=20) |
|---|---|---|---|
| **ICAM-1** | | | |
| Ig-like domain 3 | 14.3 | 12.1 | 20.0 |
| Ig-like domain 5 | 42.1 | 48.5 | 52.5 |
| **E-selectin** | | | |
| 5´-untranslated region | 28.6 | 33.3 | 32.5 |
| EGF-like domain | 13.6 | 12.1 | 20.0 |
| Membrane domain | 3.6 | 7.6 | 2.5 |
| **L-selectin** | | | |
| Lectin-like domain | 4.3 | 3.0 | 5.0 |
| EGF-like domain | 22.1 | 18.2 | 15.0 |

ICAM — intercellular adhesion molecule; EGF — epidermal growth factor; UC — ulcerative colitis; CD — Crohn's disease

Also, after dividing the patients into subgroups according to ANCA status, no significant differences were observed. Increased frequencies of the uncommon R241 ICAM-1 allele in the ANCA-negative UC and ANCA-positive CD subgroups of the patients, described by Yang *et al.* (91, 92), were not found in our study.

### 6.3.3. Tumour necrosis factor polymorphisms in the patients with inflammatory bowel disease

Allele frequencies and carriage rates of the TNF1/2 polymorphism (in the TNFα promoter at position –308) and TNFB*1/2 polymorphism (in the TNFβ first intron) were similar in the patients with UC and CD as well as control individuals (Table 10). Subgroup analysis showed borderline significant differences in the frequency of the uncommon TNF2 allele in the ANCA-positive and ANCA-negative subgroups of the patients with UC (20.8% vs. 0.0%, respectively; p=0.051).

**Table 10.** Allele frequencies and carriage rates (%) of tumour necrosis factor (TNF) polymorphisms.

| Polymorphism | Allele frequencies | | Allele 2 carriage rate |
|---|---|---|---|
| | Allele 1 | Allele 2 | |
| **TNF1/2** | | | |
| Controls (n=70) | 83.6 | 16.4 | 30.0 |
| UC patients (n=33) | 84.8 | 15.2 | 24.2 |
| ANCA-pos. UC (n=24) | 79.2 | 20.8 | 33.3 |
| ANCA-neg. UC (n=9) | 100.0 | 0.0 | 0.0 |
| CD patients (n=20) | 80.0 | 20.0 | 40.0 |
| **TNFB*1/2** | | | |
| Controls (n=70) | 32.9 | 67.1 | 92.9 |
| UC patients (n=33) | 28.8 | 71.2 | 78.8 |
| ANCA-pos. UC (n=24) | 33.3 | 66.7 | 79.2 |
| ANCA-neg. UC (n=9) | 16.6 | 83.4 | 77.8 |
| CD patients (n=20) | 37.5 | 62.5 | 95.0 |

ANCA — antineutrophil cytoplasmic antibodies; UC — ulcerative colitis; CD — Crohn's disease

The TNFB*1 allele was in linkage disequilibrium with the TNF2 allele (p<0.00001). Therefore the frequency of the TNFB*1 allele showed similar

differences between the ANCA-positive and ANCA-negative UC patients, but did not reach statistical significance.

### 6.3.4. HLA class II genes in the patients with inflammatory bowel disease

The distribution of HLA-DRB1 alleles is shown in Table 11. The HLA-DRB1*15 allele frequency was increased in the patients with UC compared with the controls (28.8% vs. 15.7%; OR=2.16; p=0.028). By contrast, the HLA-DRB1*15 allele frequency was decreased in the patients with CD (2.5% vs. 15.7%; OR=2.20; p=0.027). There was only one carrier of this allele among the CD patients (5.0% vs.31.4% in the control group; OR=0.17; p=0.017). The apparently increased proportion of the CD patients carrying the HLA-DRB1*01 allele did not attain statistical significance compared with the controls (45.0% vs. 22.9%; p=0.051), possibly due to the small sample size. The HLA-DRB1*0103 allele was infrequent in our population.

**Table 11.** Frequencies (%) of HLA-DRB1 alleles in controls' and patients'.

| HLA-DRB1* | Controls (n=70) | UC patients (n=33) | CD patients (n=20) |
|---|---|---|---|
| *01† | 11.4 | 7.6 | 22.5 |
| *0103 | 0.0 | 1.5 | 0.0 |
| *03 | 15.7 | 9.1 | 17.5 |
| *04 | 5.7 | 4.5 | 5.0 |
| *07 | 11.4 | 12.1 | 5.0 |
| *08 | 5.7 | 4.5 | 7.5 |
| *09 | 0.0 | 1.5 | 5.0 |
| *10 | 0.7 | 0.0 | 0.0 |
| *11 | 14.3 | 9.1 | 20.0 |
| *12 | 2.9 | 6.1 | 2.5 |
| *13 | 11.4 | 10.6 | 7.5 |
| *14 | 0.7 | 1.5 | 2.5 |
| *15 | 15.7 | 28.8‡ | 2.5§ |
| *16 | 4.3 | 3.0 | 2.5 |

UC — ulcerative colitis; CD — Crohn's disease

† Other than HLA-DRB1*0103

‡ Compared with controls: P=0.028

§ Compared with controls: P=0.027

Dividing the patients by ANCA status, different distribution of the HLA-DRB1 alleles in the subgroups of the UC patients was identified. Increased frequency of the HLA-DRB1*15 alleles in the patients with UC could be reduced to the association of the HLA-DRB1*15 alleles with ANCA-positive UC (35.4% vs. 15.7% in the controls; OR=2.93; p=0.004). The ANCA-negative UC patients had approximately the same HLA-DR*15 allele frequency as the controls (11.1% vs. 15.7%). Interestingly, the HLA-DRB1*03 and HLA-DRB1*11 alleles have been found only in the UC patients with ANCA, but not among the ANCA-negative UC patients (Figure 3).

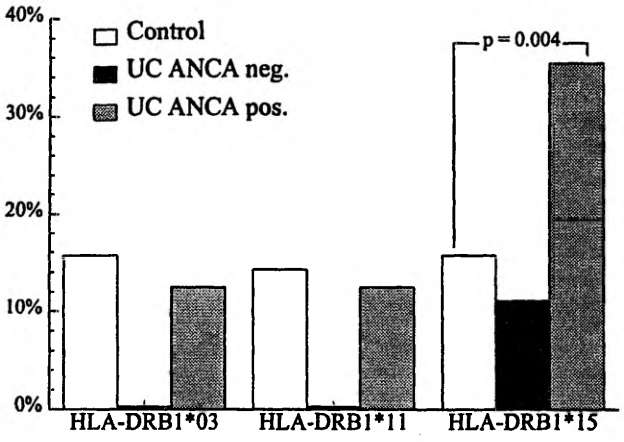


**Figure 3.** HLA-DRB1*03, *11 and *15 allele frequencies (%) in the control group and in the antineutrophil cytoplasmic antibody ANCA-negative and ANCA-positive subgroups of the ulcerative colitis (UC) patients.

To support the hypothesis that the HLA-DRB1*1502 allele may be responsible for the association of the HLA-DRB1*15 alleles with UC, and particularly with their ANCA-positive subgroup, a specific typing of the HLA-DRB1*15 alleles was performed. The HLA-DRB1*1501 and -DRB1*1502 alleles were both increased in the groups of the UC and ANCA-positive UC patients compared with the controls, but the HLA-DRB1*1502 allele was infrequent in the population studied (Figure 4), and it was not the factor explaining the association of the HLA-DRB1*15 alleles with UC and ANCA-positive UC.

As expected, the HLA-DR3-DQ2 haplotype was in strong linkage disequilibrium with the uncommon TNF2 allele ($p<0.00001$). In the UC patients the HLA-DR3-DQ2 haplotype was not found without the TNF2 allele (15% of HLA-DR3-DQ2 carriers in the control group). The HLA-DQB1 alleles were found to be associated with UC or CD only in relation to strong linkage disequilibrium with the HLA-DRB1 alleles, always with lower p values.

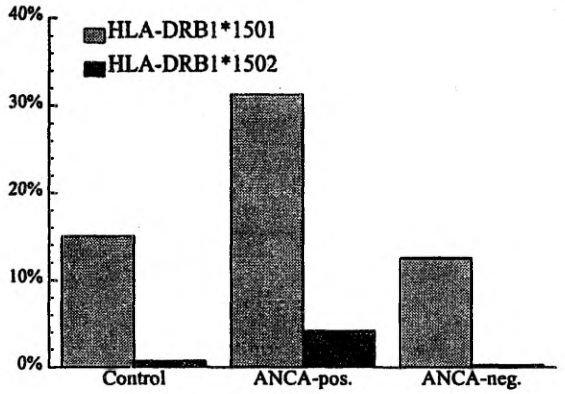


**Figure 4.** HLA-DRB1*1501 and DRB1*1502 allele frequencies (%) in the control group and in the antineutrophil cytoplasmic antibody ANCA-negative and ANCA-positive subgroups of the ulcerative colitis (UC) patients.

### 6.3.5. Associations between ANCA, HLA-DRB1*15 and TNF1/2 alleles in the patients with inflammatory bowel disease

Both the TNF2 and HLA-DRB1*15 alleles showed an increased frequency in the ANCA-positive subgroup of the UC patients. 87.5% of the ANCA-positive UC patients were carriers of at least one of the HLA-DRB1*15 or TNF2 alleles compared with 22.2% of the ANCA-negative UC patients (OR=18.97; $p<0.001$, $p_c=0.039$) and 51.4% of the controls (OR=5.81; $p=0.002$) (Figure 5).

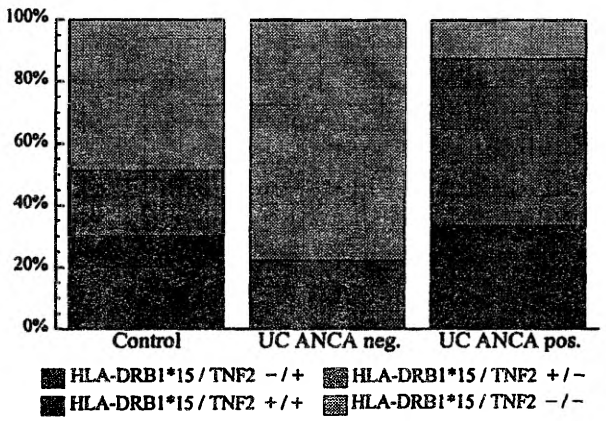


**Figure 5.** The proportion of HLA-DRB1*15/tumour necrosis factor (TNF) 2 phenotypic combinations in the control group and in the ulcerative colitis (UC) patients divided on the basis of the antineutrophil cytoplasmic antibody (ANCA) status.

## 6.4. Discussion

For the first time the distribution of the HLA class II and TNF alleles in Estonian patients with IBD was studied. The investigation of Estonian UC and CD patients is of particular interest because of the very low UC and CD incidence rates (1.5 and 0.3 per 100,000, respectively) (8–10) compared to other European populations (overall incidence about 1 to 10 per 100,000 for CD and from 5 to 18 for UC) (6, 7).

The results of this study provide further evidence of genetic heterogeneity within IBD, and also emphasise the importance of ethnic differences in disease susceptibility. Of particular note is the further evidence that HLA genes are involved in determing ANCA status. In the present study ANCA was positively associated with the HLA-DRB1*15 and TNF2 alleles in the patients with UC. However, the role of ANCA as a subclinical genetic marker is not fully established (88, 90, 93). Our data indicate that the HLA-DRB1*15 and TNF2 alleles themselves, or more likely in a strong linkage with some other gene loci, might be the genetic markers for the susceptibility to develop ANCA-positive UC. The role of these markers could vary in different ethnic groups because of a different linkage with the primary susceptibility genes.

TNFα facilitates inflammatory cell infiltration by promoting adhesion of neutrophils and lymphocytes to endothelial cells, and up-regulates the expression of HLA molecules in human colonic cell lines and small intestinal epithelial cells (210, 211). Although there is strong evidence to support the involvement of TNFα in the pathogenesis of IBD, a primary role of the TNF1/2 polymorphism in developing ANCA in UC patients or ANCA-positive UC is questionable. Recently Bouma *et al.* (212), studying the secretion of TNFα and TNFβ in relation to polymorphisms at positions –308 and –238 in the TNFα gene in 30 patients with IBD and 12 healthy controls, demonstrated that the individuals homozygous for the TNF1 allele (in the TNFα promoter at position –308) produced significantly less TNFα than the individuals homozygous for the TNF2 allele. However, the number of the patients with the homozygous TNF2 allele was too low to allow stratification for the disease. Also, the frequency of the uncommon TNFα –308 allele 2 has been found to be decreased in patients with UC compared with healthy controls (176). These studies suggest that a different genetic background may determine the height of the immune response, and might, therefore, be responsible for different disease manifestations and severity in IBD.

The association of the HLA-DRB1*15 alleles with ANCA-positive UC is not a generally accepted phenomenon. Studies in Japan and California (a mixed Jewish — non-Jewish population) have shown an association with the HLA-DRB1*1502 allele (167, 168, 213, 214). However, this has not been confirmed by Oxford (94) and Pittsburgh (93) studies, and almost certainly explained by the rarity of the DRB1*1502 allele in North European non-Jewish Caucasoids

(175). Recently, positive associations between the HLA-DRB1*15 allele and UC were also reported in Dutch (169) and Spanish Caucasian (215) patients. To test the hypothesis that the HLA-DRB1*1502 allele may be responsible for the association of the HLA-DRB1*15 alleles with UC, a specific typing of the HLA-DRB1*15 alleles was carried out. The present study reveal that the HLA-DRB1*1502 allele is infrequent in our population and the association of the HLA-DRB1*15 alleles with ANCA-positive UC could not be attributed to the HLA-DRB1*1502 allele. These observations underline the importance of ethnicity in studying the genetics of IBD.

Remarkable dissimilarities were found in the distribution of HLA-DR-DQ/TNF1/2 phenotypic combinations in the controls compared with the patients suffering from UC. There is a disagreement regarding the role of the well-known HLA-DR3-DQ2/TNF2 haplotype on the outcome of IBD. A protective effect of the HLA-DR3-DQ2 haplotype against IBD has been reported recently (215, 216). At the same time, the HLA-DRB1*0301-DQB1*0201/TNF2 (HLA-DR3-DQ2/TNF2-positive patients) haplotype has been demonstrated to be predictive of extensive disease in patients with UC (88, 94). In addition, a significant association between the carriage of the TNF2 allele and ANCA positivity in UC has been shown by two recent studies (88, 172) and in the present study. We suspect a different influence of the TNF2 allele and HLA-DR3-DQ2 haplotype on the outcome of IBD, as has been recently shown for fistulising CD (217).

The HLA-DRB1*0103 allele has been demonstrated to be predictive of extensive disease or severe disease requiring surgery, and may be associated with the presence of extra-intestinal manifestations (88, 94, 172). This allele was infrequent in our population and, therefore, no conclusions about the role of this allele in the pathogenesis of IBD could be made.

This is the first study investigating the possible involvement of E-selectin and L-selectin in addition to other adhesion molecule polymorphisms (ICAM-1), in the pathogenesis of IBD. The intercellular adhesion molecule 1 (ICAM-1) is one of the most important cell surface adhesion molecules and is expressed on the luminal surface of activated venular endothelial cells and macrophages during antigen presentation to T cells. We could not confirm the previous observation about the increased frequency of the uncommon ICAM-1 R241 allele among ANCA-negative UC and ANCA-positive CD patients (92). This discrepancy might be attributed to ethnic differences. In addition to the differences in the UC and CD incidence rates in Estonians, there was a markedly higher frequency of the less common R241 allele in our control group (14.3%) compared with the control group used by Yang *et al.* (6.4%) (92). The investigated polymorphisms in the gene regions encoding for E-selectin and L-selectin, seemed to have no influence on the aetiopathogenesis or outcome of IBD.

Statistical associations between HLA-DRB1 and TNFα polymorphisms and subgroups of IBD patients were observed. These polymorphisms might be directly associated with IBD or could be linked to certain susceptibility genes.

At least in some ethnic groups, ANCA appears to be a useful marker in combination with genetic markers such as the HLA-DRB1*15 alleles, by dividing IBD patients into more homogeneous subgroups with putative disparate pathogenic pathways. The HLA-DR3-DQ2 haplotype and the TNF2 allele, in strong linkage disequilibrium with each other, might have different influences on the outcome of IBD. Further studies with a larger number of patients and stratified for the disease are needed to test this hypothesis.

In conclusion, both UC and CD show considerable clinical and genetic heterogeneity, but available evidence suggests that chronic intestinal inflammation develops as a result of a complex interplay between shared susceptibility genes and environmental factors. The type of inflammation that develops probably depends on the possession of "disease-specific" genes; further genetic influence may determine the clinical behaviour of the disease. In addition to HLA and cytokine gene studies, genome-wide scanning in IBD has become the focus of intensive scientific interest. The regions on chromosomes 3, 7, 12 and 16 are known to contain a number of potential positional candidate genes (166). There is now a real hope that these studies will lead to an understanding of the basic pathophysiology of these complex disorders with clinical and therapeutic benefits.

# 7. CONCLUSIONS

1. ANCA were detected in 49–58% of the patients with UC, in 19–24% of the patients with CD and in 2–4% of the healthy controls. There are no significant differences in the prevalence of ANCA compared with the data observed in different European countries. In both UC and CD, there was no correlation between the presence of ANCA and the duration or extent of IBD. The presence of ANCA in a rather great amount of the patients with CD limits their diagnostic value in distinguishing between UC and CD.

2. The predominant ANCA staining pattern seen by IIF in IBD was perinuclear. In ELISA assays only 18% of the serum samples reacted positively to proteinase-3, myeloperoxidase, lactoferrin, and cathepsin G. Accordingly, other unknown antigens can be the targets for ANCA in the patients with IBD.

3. Of the non-organ-specific antibodies studied by IIF in titre 1:10 and 1:100, ANA, SMA, TMA and PCA were detected in the patients with IBD or healthy controls. In titre 1:100 8% of the UC patients, 3% of the CD patients and 2% of the controls had them. Statistically significantly higher prevalence of these autoantibodies in the UC patients, suggesting autoimmune mechanisms, could not have been revealed in our study, probably due to small study groups. All the ANA positive sera were screened by ELISA for Sm, RNP, SS-A, SS-B, Scl-70 and Jo-1 antibodies. The sera of the patient with extensive UC and AIH reacted positively to the Scl-70 antigen.

4. AGA was found in 34% of the UC patients and 4% of the controls. There was no correlation between the presence of AGA and the duration or extent of the disease, or disease activity and the medication used. The patients with extensive UC only had both of AGA, IgA- and IgG-types. Probably increased permeability in the case of extensive UC may favour dietary gliadin to break into the immune system and creation of these antibodies. None of the patients studied had coeliac disease, since ARA and AEM were negative.

5. The HLA-DRB1*15 allele frequency was increased in the Estonian patients with UC in comparison with the healthy controls. ANCA may be a useful marker by dividing IBD patients, as the TNF2 and HLA-DRB1*15 alleles were associated with the ANCA-positive UC subgroup. The HLA-DR3-DQ2 haplotype was in strong linkage disequilibrium with the uncommon TNF2 allele in the patients with IBD. We could not confirm the previous observation of increased frequency of the uncommon ICAM-1 R241 allele among the ANCA-negative UC and ANCA-positive CD patients. Studying polymorphisms in the gene regions encoding for E-selectin and L-selectin, no statistical differences in allele frequencies between the patients with IBD and healthy controls were found.

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# KOKKUVÕTE

## PÕLETIKULINE SOOLEHAIGUS
### Immuungeneetiline uuring

Haavandiline koliit ja Crohni tõbi on kroonilise kulu, selgusetu etioloogia ning korduvate ägenemistega kulgevad jäme- ja peensoolehaigused, kus peale soole tekivad haiguslikud muutused ka teistes elundites. Erialakirjanduses käsitletakse neid koos põletikulise soolehaigusena. Mõlema haiguse põhjused, tekkemehhanism, seos sooleväliste haigusnähtude ja mitmete kaasuvate autoimmuunsete haigustega on seniajani teadmata. Põletikulise soolehaiguse patogeneesis on oluline mitmete faktorite, nii geneetilise eelsoodumuse, eksogeense vallandava (*trigger*) faktori kui ka modifitseerivate faktorite koostoime, mille tulemusena käivitub põletikuprotsess ning immuunsüsteemi vahendusel tekib koekahjustus.

Põletikuline soolehaigus esineb arenenud riikides suhteliselt sageli. Esmashaigestumus on piirkonniti erinev, olles haavandilise koliidi puhul keskmiselt 5–18 juhtu ning Crohni tõve puhul 1–10 juhtu 100 000 inimese kohta aastas. Eestis 1973–1993 tehtud tagasivaatavast epidemioloogilisest uurimusest selgus, et haigestumus haavandilisse koliiti (1,5 haiget 100 000 elaniku kohta aastas) ja selle levimus (31,0 haiget 100 000 elaniku kohta) Eestis erines teiste maade omast üle kümne korra. Crohni tõve esinemine Eestis oli aga erakordselt tagasihoidlik (haigestumus 0,3; levimus 4,2), olles ligi 30 korda väiksem kui näiteks Skandinaaviamaades. Niivõrd madala haigestumuse põhjused ei ole teada ning ainult täiendavad epidemioloogilised, immunoloogilised, geneetilised ja mikrobioloogilised uuringud võimaldavad välja tuua põletikulise soolehaigusega seotud iseärasusi eestlastel.

Käesoleva töö eesmärgid:

1) uurida neutrofiilide tsütoplasma vastaste antikehade (ANCA) esinemissagedust ja kliinilist väärtust haavandilise koliidi ning Crohni tõve haigetel (I, II);
2) määrata ANCA immunofluorestsentsmuster ning iseloomustada ELISA-meetodil ANCA antigeenset struktuuri, kasutades antigeense substraadina müeloperoksüdaasi, proteinaas-3, laktoferiini ja katepsiin G (I);
3) määrata mitmete mittespetsiifiliste autoantikehade esinemissagedus ja kliiniline väärtus haavandilise koliidi ja Crohni tõve haigetel ning uurida ANA-positiivseid seerumeid ELISA-meetodil ekstraheeritavate tuumaantigeenide vastaste [Sm (Smith), RNP, SS-A (Ro), SS-B (La), Scl-70, Jo-1] antikehade suhtes (II);
4) uurida retikuliini (ARA), endomüüsiumi (AEM) ja gliadiini (AGA) antikehade esinemissagedust haavandilise koliidiga patsientide hulgas ja leida korrelatsioonid nimetatud antikehade ja erinevate kliiniliste näitajate vahel (III);
5) uurida polümorfisme HLA-DR ja -DQ, tuumori nekroosi faktori (TNF), E-selektiini, L-selektiini ja intratsellulaarse adhesiooni molekuli 1 (ICAM-1)

geenides põletikulise soolehaigusega patsientidel ning leida nende markerite ja ANCA vahelised seosed (IV).

## I. Erinevate autoantikehade esinemissagedus ja tähendus põletikulise soolehaiguse korral (artiklid I ja II)

Uuriti vastavalt 59 ja 77 haavandilise koliidi ning 17 ja 31 Crohni tõve patsienti (artiklid I, II). Kontrollgruppidesse kuulus vastavalt 111 ja 108 soole ärritussündroomiga patsienti või tervet isikut.

IgG ANCA määrati kaudsel immunofluorestsentsmeetodil (IIF), kasutades antigeenina etanoolis fikseeritud neutrofiile. IgG-tüüpi tuumavastased (ANA), mitokondritevastased (AMA), silelihaskoevastased (SMA), retikuliinivastased (ARA), maksa-neeru mikrosoomide vastased (LKMA), parietaalrakkudevastased (PCA) ja kilpnäärme mikrosoomide vastased (TMA) antikehad määrati samuti IIF-meetodil, kasutades antigeense substraadina erinevate kudede krüostaatlõike. Immunoensüümmeetodit (ELISA) kasutati müeloperoksüdaasi-, proteinaas-3, laktoferiini- ja katepsiin G vastaste antikehade ning ekstraheeritavate tuumaantigeenide vastaste [Sm, RNP, SS-A (Ro), SS-B (La), Scl-70, Jo-1] antikehade määramiseks.

IgG-tüüpi ANCA leiti 49–58%-l haavandilise koliidi ning 19–24%-l Crohni tõve haigetest (artikkel I, II). Kontrollrühmas oli ANCA statistiliselt oluliselt harvem, 2–4%-l. Põhiliselt esines pANCA. Nii haavandilise koliidi kui ka Crohni tõve korral puudus seos haiguse ulatuse ja kestuse ning ANCA esinemise vahel. Samas oli Crohni tõve haigetel ANCA vaid jämesoole haaratusega patsientide hulgas, mis viitab jämesoole osatähtsusele ANCA tekkes. Kirjanduses esinenud viidetele, et ANCA võib aidata eristada haavandilist koliiti Crohni tõvest, sest neid antikehi on vähestel Crohni tõve haigetel, me oma töös kinnitust ei leidnud. ANCA oli 19–24%-l Crohni tõve haigetest, seega võib järeldada, et ANCA kasutamine haavandilise koliidi ja Crohni tõve eristamisel on piiratud. Ainult 18%-l haavandilise koliidi ja Crohni tõve haigetest olid müeloperoksüdaasi-, proteinaas-3, laktoferiini- või katepsiin G vastased antikehad.

IgG-tüüpi ANA, SMA, TMA ja/või PCA leiti 25%-l haavandilise koliidi haigetel, 26%-l Crohni tõve haigetest olid ANA või SMA. Kontrollrühmas olid ANA, SMA, TMA ja/või PCA 12%-l. Enamikul haavandilise koliidi ja Crohni tõve haigetel esinesid need antikehad seerumi lahjenduses 1:10. Ainult kuuel (8%) haavandilise koliidi ja ühel (3%) Crohni tõve haigel ning kahel (2%) kontrollrühma kuuluval isikul oli neid antikehi lahjenduses 1:100.

Üheteistkümnest ANA-positiivsest seerumist, mida uuriti edasi ELISA-meetodil Sm, RNP, SS-A, SS-B, Scl-70 ja Jo-1 antikehade suhtes, reageeris üks positiivselt Scl-70 antigeenile. Scl-70 vastaseid antikehi leitakse sageli süsteemse sklerodermiaga haigetel. Antud juhul oli tegemist 23-aastase pankoliiti

põdeva naispatsiendiga, kellel kaasuva haigusena oli ka autoimmuunne hepatiit. Süsteemsele sklerodermiale viitavaid sümptomeid tal ei leitud.

## II. Retikuliini, endomüüsiumi ja gliadiini antikehade esinemissagedus ja tähendus haavandilise koliidi haigeil (artikkel III)

Retikuliini antikehade (ARA), endomüüsiumi antikehade (AEM) ja gliadiini antikehade (AGA) esinemist uuriti 50-l haavandilise koliidi haigel ning 53-l kontrollgrupi isikul.

ARA ja AEM määrati IIF-meetodil, AGA määrati ELISA-meetodil.

IgA- ja/või IgG-tüüpi AGA esines 34%-l haavandilise koliidi haigetest. Puudus korrelatsioon AGA esinemise ning haavandilise koliidi kestuse, ulatuse, haiguse aktiivsuse ja kasutatud ravimite vahel. Siiski, kõigil viiel patsiendil, kellel leiti nii IgA- kui ka IgG-tüüpi antikehad, esines pankoliit. Seetõttu võib väita, et mida ulatuslikum on soolekahjustus, seda tõenäolisem on antigeeni tungimine organismi ning ka vastavate antikehade produtseerimine immuunsüsteemi poolt. Ainult 2%-l kontrollgrupi haigetest esinesid gliadiini antikehad. Et ARA ja AEM olid kõikidel juhtudel negatiivsed, siis võib väita, et nimetatud uuringugruppides tsöliaakiat ei olnud.

## III. Polümorfismid HLA-DR ja -DQ, tuumori nekroosi faktori (TNF) ning adhesiooni molekuli geenides põletikulise soolehaigusega patsientidel; nende markerite ja ANCA seosed (artikkel IV)

Uuriti polümorfisme HLA-DR ja -DQ, tuumori nekroosi faktori (TNF), E-selektiini, L-selektiini ja intratsellulaarse adhesiooni molekuli 1 (ICAM-1) geenides 53-l põletikulise soolehaigusega patsiendil ja 70-l tervel isikul ning määrati nende markerite ja ANCA vahelised seosed.

HLA genotüpiseerimiseks kasutati pöördhübridisatsiooni meetodit ning järjestusspetsiifilisi praimereid. TNF-i ja adhesioonimolekulide polümorfismide uurimiseks kasutati polümeraasi ahelreaktsiooni (PCR) ning seejärel restriktsioonifragmentide pikkuse polümorfismi meetodit (RFLP) või üheahelalise DNA konformatsioonilise polümorfismi (SSCP) analüüsi.

HLA-DRB1*15 alleeli oli haavandilise koliidi haigetel statistiliselt oluliselt sagedamini, võrreldes kontrollgrupiga (28,8% vs. 15,7%; p=0.028). ANCA-positiivsetel haavandilise koliidi haigetel oli statistiliselt oluliselt sagedamini TNF2 ja HLA-DRB1*15 alleeli. 87,5% ANCA-positiivsetest haavandilise koliidi haigetest olid vähemalt ühe nimetatud alleeli kandjad, võrreldes 22,2% ANCA-negatiivsete haavandilise koliidi haigetega (p<0,001). HLA-DRB1*15 alleeli spetsiifiline tüpiseerimine näitas, et HLA-DRB1*1501 alleel seondub ANCA-positiivse haavandilise koliidi grupiga.

HLA-DR3-DQ2 haplotüüp oli tugevasti seotud harva esineva TNF2 alleeliga. Haavandilise koliidi haigetel oli HLA-DR3-DQ2 haplotüüp alati kombinatsioonis TNF2 alleeliga. Uuritud E-selektiini, L-selektiini ning ICAM-1 polümorfismide ja ANCA-positiivsete ning -negatiivsete haavandilise koliidi gruppide vahel statistiliselt olulisi erinevusi ei leitud.

## IV. Kokkuvõte

1. ANCA leiti 49–58%-l haavandilise koliidi, 19–24%-l Crohni tõve haigetest ning 2–4%-l kontrollrühmas. Kuigi haigestumus haavandilisse koliiti ja Crohni tõppe on Eestis madal, ei ole ANCA esinemises erinevusi teiste Euroopa maadega võrreldes. Nii haavandilise koliidi kui ka Crohni tõve korral puudus seos haiguse ulatuse ja kestuse ning ANCA esinemise vahel. ANCA kasutamine haavandilise koliidi ja Crohni tõve eristamisel on piiratud, sest neid antikehi oli arvestataval hulgal ka Crohni tõve haigetel.

2. Põhiliselt esines perinukleaarset tüüpi ANCA (pANCA). Muud antigeenid, mitte müeloperoksüdaas, proteinaas-3, laktoferiin ja katepsiin G, on ilmselt primaarseks ANCA-le, kuna ainult 18% haavandilise koliidi ja Crohni tõve haigetest reageeris ELISA-uuringutel eespool nimetatud antigeenidele positiivselt.

3. Muudest uuritud antikehadest olid haavandilise koliidi ja Crohni tõve haigetel ANA, SMA, TMA ja PCA. Üldiselt ilmnesid nimetatud antikehad madalas seerumi lahjenduses, vaid 8%-l haavandilise koliidi, 3%-l Crohni tõve haigetest ning 2%-l kontrollrühmas leiti neid seerumi lahjenduses 1:100. Statistiliselt tõepärast antikehade sagedasemat esinemist haavandilise koliidi haigetel, mis viitaks autoimmuunmehhanismide osatähtsusele, ei leitud ning tõenäoliselt oli see tingitud uuringugruppide väiksusest. Üheteistkümnest ANA-positiivsest seerumist, mida uuriti ELISA-meetodil Sm, RNP, SS-A, SS-B, Scl-70 ja Jo-1 antikehade suhtes, reageeris üks positiivselt Scl-70 antigeenile. Tegemist oli 23-aastase pankoliiti põdeva naispatsiendiga, kellel kaasuva haigusena oli ka autoimmuunne hepatiit.

4. AGA leiti 34%-l haavandilise koliidi haigetest ning 4%-l kontrollrühma isikutest. Puudus korrelatsioon AGA esinemise ning haavandilise koliidi kestuse, ulatuse, haiguse aktiivsuse ja kasutatud ravimite vahel. Mõlemat tüüpi AGA koosesinemist, IgA- ja IgG-tüüpi, leiti ainult pankoliidiga patsientide hulgas. Seetõttu võib väita, et mida ulatuslikum on soolekahjustus, seda tõenäolisem on antigeeni tungimine organismi ning ka vastavate antikehade produtseerimine immuunsüsteemi poolt. Et ARA ja AEM olid kõikidel juhtudel negatiivsed, siis võib väita, et nimetatud uuringugruppides tsöliaakiat ei olnud.

5. Haavandilise koliidi haigetel oli HLA-DRB1*15 alleeli statistiliselt oluliselt sagedamini kontrollrühma isikutega võrreldes. ANCA-positiivsetel haavandilise koliidi haigetel oli statistiliselt oluliselt sagedamini TNF2 ja HLA-DRB1*15 alleeli. Seetõttu võib ANCA kombinatsioonis geneetiliste markeritega osutuda oluliseks markeriks, mis võimaldab jagada põletikulise soolehaigusega patsiendid homogeensematesse alagruppidesse. Põletikulise soolehaigusega patsientide hulgas oli HLA-DR3-DQ2 haplotüüp tugevasti seotud harva esineva TNF2 alleeliga. Uuritud E-selektiini, L-selektiini ning ICAM-1 polümorfismide ja ANCA-positiivsete ning -negatiivsete haavandilise koliidi gruppide vahel statistiliselt olulisi erinevusi ei leitud.

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# PUBLICATIONS

**Table 2.** Relationship between presence of antigliadin antibodies (AGA) and disease activity, disease duration, and medication used in patients with ulcerative colitis (UC)

| | No of AGA-positive UC patients |
|---|---|
| Disease activity | |
| Remission ($n$ = 22) | 8 |
| Mild ($n$ = 10) | 3 |
| Moderate ($n$ = 14) | 5 |
| Severe ($n$ = 4) | 1 |
| Disease duration | |
| <5 years ($n$ = 21) | 10 |
| >5 years ($n$ = 29) | 7 |
| Medication | |
| Steroids ($n$ = 22) | 6 |
| Other or none ($n$ = 28) | 11 |

*Antiendomysium antibodies*

All 49 patients with UC studied and the 52 controls studied were negative for IgA-type AEM (sera for 1 UC patient and 1 healthy individual, both AGA-negative, were not available for this examination).

## Discussion

In 1965, Salem and Truelove[4] reported 12 patients with partial and 2 patients with subtotal villous atrophy among 60 patients with UC. The coexistence of CD with Crohn's disease and UC has since been described in several case reports.[6,12,13] In 1987, Breen et al.[7] reviewed 42 rectal biopsies from a population of 438 patients with CD and found 3 patients with abnormal rectal biopsy results who had histopathologic and clinical features of UC. An epidemiological survey of IBD in the first-degree relatives of 162 celiac patients showed the relative risk of IBD to be five times greater for first-degree relatives than for the general population.[10] Taken together, these reports suggested that CD and IBD coexist more commonly than would be expected by chance. On the other hand, Collin et al.[23] examined 335 celiac patients and found only 1 patient with concomitant IBD, in comparison with 7 patients among 335 age, and sex-matched controls. Therefore an important question has been raised—is the coexistence of IBD and CD true or coincidental?

In our study, we investigated this problem by aiming to detect possible CD cases among UC patients, using serological markers—AGA, ARA, and AEM. AGA was found in 34% of UC patients, but none of these had either IgG-type ARA or IgA-type AEM. Therefore, we can conclude that none of the UC patients we tested had CD, since ARA[17] and AEM[18,22,24-26] have been shown to be highly specific serological markers for CD.

However, another question remains; why AGA, the type of antibodies frequently demonstrated in association with CD, were seen in 34% of the patients with UC in our study. Results similar to ours were reported by Ferreira et al.,[18] who compared the sensitivities and specificities of AGA, ARA, and AEM in 117 patients with CD. In their disease control group, these authors had a small number of patients with IBD (10 patients with UC and 21 patients with CD). A positive AGA test result was found in 10% of the patients with UC and in over 20% of the patients with CD, while ARA and AEM were negative in all patients. In another study, by Volta et al.[27], IgG AGA were present in 19% of 37 patients with UC and in 27% of 26 patients with CD. Although these authors used IBD patients as disease controls for CD, their findings confirm our results.

In our study, we also found that patients with extensive colitis more frequently had IgA + IgG-types of AGA than patients with proctitis. But as none of the AGA-positive UC patients showed any signs of malabsorption, and as ARA and AEM were negative, we considered that endoscopy and small bowel biopsy were not indicated for these patients. Based on these results, we suggest that, as the gut mucosa is more extensively damaged in extensive colitis, gut permeability may be increased. Therefore, we support the idea that the increased permeability of the intestinal mucosa to different food antigens also involves the presence AGA in UC.[28]

In certain circumstances (e.g., in the presence of certain human leukocyte antigen haplotypes, such as HLA-DQ2), UC may favor the onset of CD by altering intestine barrier function, thus allowing foreign antigen to breach the immune system.[29-31] In approximately one-fourth of the patients exported, the diagnosis of IBD preceded that of CD.[11] In these patients, positive serological screening tests, mainly ARA and AEM, may help in selecting patients in whom CD is suspected for small-bowel biopsy. To resolve the question of whether the presence of AGA a rises from similar mechanisms in both CD and UC, further comparative studies of AGA (e.g., with immunoblot, enzyme-linked immunospot assays (ELISPOT), in CD and UC are needed.

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AEM-positive and AEM-negative sera were used as controls.

*Statistics*

The prevalence of AGA in various groups of patients was compared by means of the $\chi 2$ test with Yates' correction. Analysis of variance (ANOVA) and Student's *t*-test were used to correlate the titers fo AGA with different clinical markers; $P < 0.05$ was considered significant.

## Results

*Antigliadin antibodies*

In total, 17 of the 50 patients with UC (34%) had a positive IgA- or/and IgG-type AGA test result; in 5, both types of AGA were detected. Of the 53 individuals in the control group, only 2 (4%) (both with IBS; men, aged 24 and 28 years) were positive for IgG-type AGA ($P < 0.001$ for UC compared with controls). Figure 1 shows the distribution of IgA-and IgG-type AGA titers (EIU units) in patients with UC and in controls. There were significant differences in AGA titers between the UC patient group and controls. Median titers for IgA-type AGA were 76 EIU (range, 0–219 EIU) in patients with UC and 36 EIU (range, 0–108 EIU) in controls ($P < 0.001$). For IgG-type AGA, the median titers were 5 EIU (range, 0–50 EIU) in UC patients and 2 EIU (range, 0–16) in controls ($P < 0.01$).

The relations hips between the extent of UC and the presence of different types of AGA are shown in Table 1. No significant difference between the extent of the disease and positive or negative AGA test results was observed. But five of the patients with both AGA, IgA- and IgG-types, had extensive colitis. No correlation was found between the extent of disease and the titer of AGA.

There was no significant correlation between the presence of AGA and disease activity or duration of disease in the patients with UC (Table 2). In addition, these clinical markers were not related to the titer of AGA. No relationship was observed between the medication used and the presence of AGA (Table 2).

*Antireticulin antibodies*

All 50 UC patients and 53 controls were negative for IgG-type ARA.

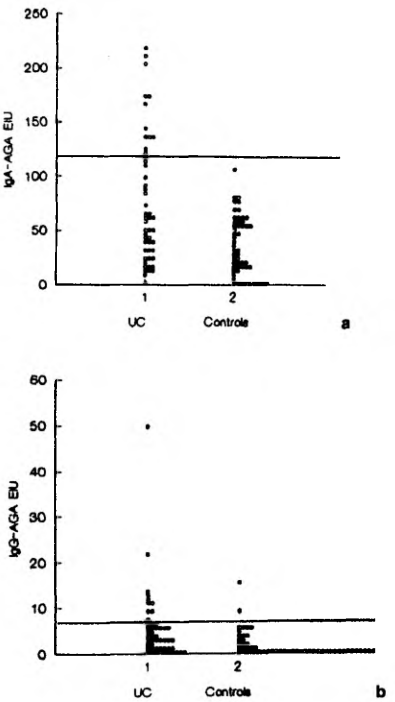


**Fig. 1a,b.** Titers of **a** IgA-type and **b** IgG-type antigliadin antibodies (*AGA*) in patients with ulcerative colitis (*UC*) and controls. Discrimination between negative and positive test results is represented by the *solid line*

**Table 1.** Relation between extent of ulcerative colitis (UC) and presence of different types of antigliadin antibodies (AGA)

| | No of AGA-positive UC patients | | | |
|---|---|---|---|---|
| | IgA AGA | IgG AGA | IgA + IgG AGA | Total |
| Disease extent | | | | |
| Proctitis ($n$ = 19) | 6 | 2 | 0 | 8 |
| Left-sided ($n$ = 12) | 1 | 1 | 0 | 2 |
| Extensive ($n$ = 19) | 0 | 2 | 5 | 7 |

patients with UC (25 men and 25 women, aged 14–75 years; mean, 43 years) attending the Department of Internal Medicine, University of Tartu, or the University Outpatient Department. The diagnosis of UC was based on conventional criteria recommended by Lennard-Jones.[19] UC patients were classified according to disease extent, disease activity, and disease duration at the time the blood sample was obtained. The extent of colitis was evaluated in 43 patients by colonoscopy, and in 7 by radiological investigation. Nineteen patients had proctitis, 12 left-sided colitis, and 19 extensive colitis. Disease activity was assessed by the criteria of Truelove and Witts.[20] UC was characterized as mild, moderate, or severe, based on equal weighting of the following six factors: diarrhea, fever, tachycardia, anemia, erythrocyte sedimentation rate, and physical examination. Twenty-two of the 50 UC patients were in clinical remission, 10 had mild disease, 14 moderate disease, and 4, severe disease. Twenty-two of the 50 patients with UC were treated with a combination of systemic or topical steroids and sulfasalazine or mesalazine. The other patients received no medication, or sulfasalazine or mesalazine only. Finally, duration of disease was considered: 21 patients had been suffering from UC less than 5 years and 29 patients for 5 years or more.

The control group consisted of 53 individuals age- and sex-matched with the UC group (24 men and 29 women; aged 16–79 years, mean, 43 years). Sixteen patients had irritable bowel syndrome (IBS) (9 men and 7 women; aged 16–62 years, mean, 40 years) and 37 were healthy individuals (15 men and 22 women; aged 17–79 years; mean, 46 years) with no history of gastrointestinal or other chronic diseases.

## Methods

Ten ml of venous blood was taken from each subject and stored at −20°C until analyzed for IgA- and IgG-type AGA, IgG-type ARA, and IgA-type AEM.

### *Antigliadin antibodies*

AGA were determined by enzyme-linked immunosorbent assay (ELISA) according to the method developed at the Department of Immunology, University of Tartu. Briefly, wheat gliadin (Serva Diagnostica, Heidelberg, Germany) was dissolved in 70% ethanol (2 mg/ml), diluted 1:100 in 0.05 M carbonate-bicarbonate buffer, and incubated for 20 h at +4°C. After being washed with phosphate-buffered saline (PBS) (pH 7.4), the plates were saturated with 5% normal sheep serum (NSS) in PBS for 2 h at +37°C. Further, the test sera and known AGA-positive and -negative control sera, diluted 1:100 in 1% NSS-PBS, were analyzed in duplicate and incubated for 3 h at +37°C. The plates were then washed again and alkaline phosphatase-conjugated antihuman IgG or IgA immunoglobulins (Dako, Glostrup, Denmark) in 1% NSS-PBS were added to each well at the dilution suggested by the manufacturer. After incubation for 20 h at +20°C, the plates were washed and *p*-nitrophenyl-phosphate (Sigma, St. Louis, MO, USA) in diethanolamine magnesium chloride buffer, pH 9.8, was added. The reaction was stopped after 30 min with 3M sodium hydroxide. The optical density (OD) of the reaction product was measured at 405 nm. The results were calculated in enzyme immunosorbent units (EIU). The sera from the 37 healthy individuals were used as references to establish a baseline absorbance value. Readings above three SD of the mean were considered positive. The cut-off level for tests was ≥120 EIU for IgA-type AGA and ≥8 EIU for IgG-type AGA. The inter-assay coefficient of variation of AGA test results was 10.6% and the intra-assay coefficient of variation was 5.7%.

### *Antireticulin antibodies*

R1-type ARA were detected using a standard indirect immunofluorescence (IIF) method[21] with rat kidney and liver and mouse stomach mounted into a composite block as antigen substrates, and fluorescein isothiocyanate (FITC)-conjugated rabbit antihuman IgG (Dako) as the secondary antibody. The sera were investigated at dilutions of 1:10 and 1:100 in PBS, and were considered ARA-positive if the characteristic R1-staining pattern was found in antigen substrates under a fluorescence microscope (Jenalumar SH 50; VEB Carl Zeiss, Jena, Germany [with ocular ×6.3 and objective ×25 and ×50]). The specific pattern included staining of the fibers around the glomeruli and tubuli and around the blood vessels in the kidney section; staining of the nodular fibers in the portal areas of the liver; and staining of the fibers between the gastric glands, in the connective tissue, and in the muscular mucosa of the stomach. Known ARA-positive and ARA-negative sera were used as controls.

### *Antiendomysium antibodies*

IgA-type AEM were measured by IIF, using the cryostat sections from the human umbilical cord as antigen substrate.[22] Tissue sections were incubated with serum diluted 1:10 and 1:100 with PBS, and FITC-labelled rabbit antibody to human IgA (Dako) was used as secondary antibody. Under the fluorescence microscope (Jenalumar SH 50; ocular ×6.3, objective ×25 and ×50) the presence of a honeycomb brilliant green network pattern along the peritubular muscle layer of vessels on umbilical cord was taken as positive. Known

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# High frequency of antigliadin antibodies and absence of antireticulin and antiendomysium antibodies in patients with ulcerative colitis

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**Abstract:** Several authors have described an association between celiac disease (CD) and ulcerative colitis (UC), but this has not yet been established. The aim of our study was to examine the frequency of antigliadin antibodies (AGA), antireticulin antibodies (ARA) and antiendomysium (AEM) antibodies in the sera of patients with UC ($n$ = 50), and to evaluate their correlation with clinical variables. Sixteen patients with irritable bowel syndrome (IBS) and 37 healthy individuals served as controls. An enzyme-linked immunosorbent assay was used for the detection of IgA- and IgG-type AGA. IgG-type ARA were determined by an indirect immunofluorescence assay (IIF) using rat kidney, liver, and stomach as antigen substrates. IgA-type AEM antibodies were measured by IIF, using cryostat sections from human umbilical cord. Seventeen of the 50 patients with UC (34%) were positive for IgA- or/and IgG-type AGA. There was no correlation between the presence of AGA and the duration or extent of the disease, or disease activity. However, 5 patients with both IgA- and IgG-types of AGA had extensive colitis. Only 2 controls (4%) were positive for IgG-AGA. ARA and AEM were not detected in any individuals studied. Since the ARA and AEM test results were negative, we conclude that none of the UC patients in this series had CD.

**Key words:** ulcerative colitis, celiac disease, antigliadin antibodies, antireticulin antibodies, antiendomysium antibodies

*Reprint requests to:* K. Kull
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## Introduction

The pathogenesis of ulcerative colitis (UC) is still unknown, although circumstantial evidence indicates that immune mechanisms may play an important role in mediating the gut lesion.[1] Over the years, many different diseases have been reported to occur concomitantly with inflammatory bowel disease (IBD).[2,3] The first association of celiac disease (CD) and UC was reported over 30 years ago by Salem and Truelove.[4] Since that publication, several authors have described an association of CD and UC,[5–13] although most of the reports are simply case reports; accordingly the clinical significance of this association is not yet established.

In CD, there is a permanent intolerance of the small-bowel mucosa to dietary cereals, mainly wheat, rye, barley, and oats. Ingestion of these cereal proteins results in villous atrophy, with crypt hyperplasia of the small-bowel mucosa, which recovers on a gluten-free diet. A conclusive diagnosis of CD is based on the demonstration of villous atrophy and crypt hyperplasia in small-bowel biopsy specimens.[14–15] As endoscopy and small bowel biopsy are invasive procedures, they cannot be considered feasible for screening patients for CD. Three types of antibodies—antigliadin antibodies (AGA), antireticulin antibodies (ARA), and antiendomysium (AEM) antibodies however have been extensively investigated and suggested as screening tests for CD.[16–18]

The aim of our study was to examine the frequency of AGA, ARA, and AEM in patients with UC and to evaluate the correlations between these antibodies and various clinical features in patients with UC.

## Patients

A total of 103 individuals were investigated. Fifty serum samples were obtained from 50 consecutive unselected

K. Kull, O. Uibo, R. Salupere, K. Metsküla, R. Uibo.
High frequency of antigliadin antibodies and absence of antireticulin and antiendomysium antibodies in patients with ulcerative colitis.
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# III

Tabel 3. **Seosed haavandilise koliidi ja Crohni tõve ulatuse ja ANCA esinemise vahel**

| Haigus | ANCA-positiivsed patsiendid/patsientide üldarv | |
|---|---|---|
| | haavandiline koliit (n = 45/77) | Crohni tõbi (n = 6/31) |
| Haiguse ulatus | | |
| proktiit | 17/31 | |
| vasakpoolne koliit | 12/23 | |
| pankoliit | 16/23 | |
| Haiguse lokalisatsioon | | |
| ileiit | | 0/7 |
| koliit | | 1/9 |
| ileokoliit | | 5/15 |


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**Summary**

**Autoantibody Profile of Inflammatory Bowel Disease**

To study an association between inflammatory bowel disease and autoimmunity the frequency and clinical value of antineutrophil cytoplasmic antibodies (ANCA) and several non-organ-specific antibodies were studied in sera from 77 patients with ulcerative colitis and in 31 patients with Crohn's disease. Thirty-two patients with the irritable bowel syndrome and 76 healthy persons served as controls. By indirect immunofluorescence - ANA, SMA, TMA or/and PCA were detected in 25 % of patients with ulcerative colitis, 26 % of patients with Crohn's disease and 12 % of healthy controls. ANCA were found in 58 % of patients with ulcerative colitis, 19 % of Crohn's disease and 2 % of controls. ANA containing sera were further analysed by enzyme-linked immunosorbent assay for Sm, RNP, SS-A, SS-B, Scl-70 and Jo-1 antibodies. One sera reacted positively to the Scl-70 antigen. Our results support the idea that autoimmune mechanisms are more likely to be involved in the pathogenesis of ulcerative colitis than Crohn's disease.



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milles on võrreldud erinevate antikehade esinemissagedust eestlaste ja rootslaste hulgas ning on leitud, et ANA esines eestlastel statistiliselt oluliselt harvem kui rootslastel (7% *versus* 12%).

Üllatuslikult leiti Scl-70 antikehad kaua kestnud pankoliidi ja kaasneva autoimmuunse hepatiidiga patsiendil. Antikehad topoisomeraas I vastu, tuntud ka Scl-70-na, on üldiselt seotud difuusse sklerodermiaga (5) ning võivad tekkida isegi kuid ja aastaid enne haiguse kliinilist avaldumist (7). Põletikulise soolehaiguse ja/või autoimmuunse hepatiidi korral neid varem kirjeldatud ei ole.

Haavandilist koliiti põdevatel haigetel leiti ANCA-d statistiliselt oluliselt sagedamini kui Crohni tõbe põdejatel (58% *versus* 19%). Enamikul juhtudel oli tegemist p-ANCA-ga. Puudus korrelatsioon haiguse ulatuse ja ANCA esinemise vahel. Kirjanduses on viiteid (3), et ANCA võib aidata eristada haavandilist koliiti Crohni tõvest, sest neid antikehi esineb vähestel Crohni tõbe põdejail.

Käesolevas töös leiti ANCA 19%-l Crohni tõbe põdejaist ning seega võib järeldada, et ANCA kasutamine haavandilise koliidi ersitamisel Crohni tõvest on piiratud. Samas aga võib ANCA kombinatsioonis geneetiliste markeritega osutuda subkliiniliseks haavandilise koliidiga seotud markeriks, sest on kirjeldatud positiivset seost HLA-DRB1*15 ja ANCA vahel (17). Crohni tõve juhtudel esines ANCA vaid jämesoole haaratusega patsientide hulgas. See tulemus viitab jämesoole osatähtsusele ANCA tekkes ning toetab E. A. Vasiliauskase ja kaasautorite (15) arvamust, et ANCA-positiivsed haiged võivad esindada teatud kindlat alarühma Crohni tõbe põdejate hulgas. Edasised uuringud ANCA antigeeni molekulaarse struktuuri ja funktsioonide osas on äärmiselt vajalikud selgitamaks nende antikehade patogeneetilist osatähtsust põletikulise soolehaiguse korral.

On arvatud, et autoimmuunmehhanismidel on olulisem osatähtsus haavandilise koliidi kui Crohni tõve patogeneesis (11). Kaasnevate autoimmuunhaiguste ja erinevate antikehade sagedam esinemine haavandilise koliidi haigeil toetavad seda ideed. See töö kinnitab, et ANCA esineb statistiliselt oluliselt sagedamini haavandilist koliiti kui Crohni tõbe põdejail. Kuigi Crohni tõve patogeneesis arvatakse olevat peamiseks häired T-raku aktivatsioonimehhanismide osas, on siiski võimalik, et teatud osal haigeist on haaratud ka antikehade vahendatud immuunmehhanismid.

Tabel 1. **ANA, SMA, TMA ja PCA esinemissagedus haavandilist koliiti (HK) ja Crohni tõbe (CT) põdejate ning kontrollrühma isikute vereseerumis**

| Antikehade | | ANA-, SMA-, TMA- või PCA-positiivsete patsientide arv | | |
|---|---|---|---|---|
| | tiiter | HK (n=77) | CT (n=31) | kontrollrühm (n=108) |
| ANA | 1:10 | 5 | 1 | 1 |
| | 1:100 | 4 | 1 | - |
| SMA | 1:10 | 5 | 6 | 8 |
| | 1:100 | - | - | - |
| TMA | 1:10 | 1 | - | - |
| | 1:100 | 1 | - | 1 |
| PCA | 1:10 | 2 | - | 2 |
| | 1:100 | 1 | - | 1 |
| Total | 1:10 | 13 (17%) | 7 (23%) | 11 (10%) |
| | 1:100 | 6 (8%) | 1 (3%) | 2 (2%) |

Tabel 2. **Neutrofiilide tsütoplasma antikehade (ANCA) esinemissagedus haavandilist koliiti (HK) ja Crohni tõbe (CT) põdejate ning kontrollrühma isikute hulgas**

| Patsient | Arv | IIF ANCA+ | Immunofluorestsentsmuster | | |
|---|---|---|---|---|---|
| | | n (%) | p-ANCA | c-ANCA | p+c-ANCA |
| Haavandiline koliit | 77 | 45 (58) | 39 | 5 | 1 |
| Crohni tõbi | 31 | 6 (19) | 5 | 1 | 0 |
| Kontrollrühm | 108 | 2 (2) | 2 | 0 | 0 |

antigeenide vastaste (Sm, RNP, SS-A (Ro), SS-B (La), Scl-70, Jo-1) antikehade suhtes, kasutades *Immuno Concepts* RELISA ENA multiparameetrilist testsüsteemi (*Immuno Concepts*, Sacramento, USA).

IgG ANCA määrati samuti IIF-meetodil (16), kasutades antigeenina etanoolis fikseeritud neutrofiile. Seerumid lahjendati (1:20) PBS-is, sekundaarse antikehana kasutati fluorestseiiniga konjugeeritud inimese IgG-vastast antiseerumit. Kõiki tulemusi hinnati fluorestsentsmikroskoobi (*Jenalumar*) abil.

Tulemuste statistilise usaldusväärsuse hindamiseks kasutati $\chi^2$-testi koos Yatesi korrektsiooniga. Erinevust rühmade vahel peeti statistiliselt oluliseks, kui $P<0,05$.

Töö on heaks kiitnud Tartu Ülikooli Inimuuringute Eetika Komisjon.

**Tulemused**

IgG-tüüpi ANA, SMA, TMA ja/või PCA leiti 19-l 77 haavandilist koliiti põdejast (25%). Kaheksal 31 (26%) Crohni tõbe põdejast esines ANA või SMA. Kontrollrühmas oli ANA, SMA, TMA ja/või PCA 13 isikul 108-st (12%) – tunduvalt harvemini kui haavandilist koliiti põdejail ($P<0,05$). Antikehade esinemissagedus kontrollrühmas ja Crohni tõbe põdejail ei olnud statistiliselt erinev ($P=0,1$). Enamikul haavandilist koliiti ja Crohni tõbe põdejail esinesid need antikehad lahjenduses 1:10. Ainult neljal haavandilise koliidi haigel (1 mees ja 1 naine samaaegse autoimmuunse hepatiidiga, 2 naist kaasnevate haigusteta) ja ühel Crohni tõvega meeshaigel (kaasnevate haigusteta) esines ANA tiitris 1:100, ühel haavandilise koliidiga naishaigel esinesid TMA ja PCA tiitris 1:100. Kontrollrühmas oli ühel naisel TMA ja ühel mehel PCA tiitris 1:100. AMA-d, ARA-d ega LKMA-d ei leitud ühestki 216 uuritud seerumist. Andmed autoantikehade esinemise kohta on toodud tabelis 1.

12 ANA-positiivsest seerumist, mida uuriti edasi etanoolis fikseeritud HEp-2-rakkudel, reageerisid kolm seerumit positiivselt (homogeenne muster). Kaks seerumit pärines haavandilise koliidiga naishaigeilt (üks autoimmuunse hepatiidiga ja üks kaasnevate haigusteta) ning üks Crohni tõvega meeshaigelt (kaasnevate haigusteta).

11 ANA-positiivset seerumit (ühe terve isiku seerum ei olnud enam kättesaadav) uuriti edasi ELISA abil Sm, RNP, SS-A, SS-B, Scl-70 ja Jo-1 antikehade suhtes. Üks seerum reageeris Scl-70 antigeeni suhtes positiivselt. Tegemist oli 23-aastase pankoliiti põdeva naispatsiendiga, kellel kaasneva haigusena oli ka autoimmuunne hepatiit.

IgG-tüüpi ANCA leiti 45-l 77 (58%) haavandilist koliiti ja kuuel 31 (19%) Crohni tõbe põdejast ($P<0,001$). Kontrollrühmas esines ANCA kahel isikul 108-st (2%): ühel soole ärritussündroomiga patsiendil ja ühel tervel isikul ($P<0,001$ haavandiline koliit võrreldes kontrollrühmaga, $P<0,01$ Crohni tõbi võrreldes kontrollrühmaga). Ülekaalus oli p-ANCA, ainult viiel haavandilist koliiti ja ühel Crohni tõbe põdejal oli c-ANCA. Ühel haavandilise koliidiga patsiendil esinesid mõlemat tüüpi antikehad (vt. tabel 2). Kaheksal 45-st p-ANCA-positiivsest haavandilist koliiti põdeva haige seerumist oli immunofluorestsentsreaktsioon ka lümfotsüütide tuumade osas, mis näitas ANA samaaegset olemasolu (kõikidel juhtudel kinnitati ANA olemasolu ka antigeensel krüostaatlõigul). Ühe Crohni tõve haige seerumis oli vaid nukleaarne helendus nii neutrofiilide kui ka lümfotsüütide osas ning krüostaatlõigul kinnitus ANA olemasolu.

Nii haavandilise koliidi kui ka Crohni tõve korral puudus seos haiguse ulatuse ja ANCA esinemise vahel (vt. tabel 3).

**Arutelu**

ANA, SMA, TMA ja PCA esinesid põletikulise soolehaigusega haigetel üldiselt harva ja madalas tiitris. Kuigi 25%-l haavandilist koliiti ja 26%-l Crohni tõbe põdejaist ning 12%-l kontrollrühma isikuist leiti eespool nimetatud antikehi, olid ainult 8%-l haavandilist koliiti, 3%-l Crohni tõbe põdejaist ja 2%-l kontrollrühma kuulujaist need tiitris 1:100. G. N. Dalekos ja kaasautorid (2) on aga vastupidiselt leidnud ANA 41-l 80 (51%-l) haavandilise koliidi haigest, kasutades ANA esmaseks määramiseks HEp-2 rakuliini (positiivne ANA tiiter >=80). Nii suuri erinevusi ei saa seletada üksnes metoodiliste iseärasustega, seda enam, et kahel viiest meie uuritud ANA 1:100 positiivse haavandilise koliidiga patsiendist oli kaasnevana autoimmuunne hepatiit. Seega võib arvata, et ANA-d (lahjenduses 1:100) tuleb põletikulise soolehaigusega patsientidel ette harva. Võimalik, et erinev geneetiline taust ja keskkonnatingimused tingivad ANA madalama esinemissageduse Eesti patsientide hulgas. Meie tulemusi toetab ka R. Uibo ja kaasautorite töö (14),

# Autoantikehade esinemissagedus ja tähendus põletikulise soolehaiguse korral

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**autoantikehad, autoimmuunsus, Crohni tõbi, haavandiline koliit**

Rohketele uuringutele vaatamata on põletikulise soolehaiguse (haavandilise koliidi ja Crohni tõve) etioloogia ja patogenees siiani lõplikult välja selgitamata. Siiski arvatakse, et immuunsüsteemil on põletikulise soolehaiguse patogeneesis keskne osa (1). Lisaks häiretele rakuliste immuunmehhanismide osas esinevad muutused ka humoraalsetes kaitsemehhanismides (4, 12) ning seetõttu on viimastel aastatel hakatud suurt tähelepanu pöörama autoantikehade uurimisele.

Neutrofiilide tsütoplasma antikehad (ANCA), mis on tähtsal kohal süsteemsete vaskuliitide ja glomerulonefriidi teatud vormide diagnoosimisel ja jälgimisel (6, 10), on tähtsad ka põletikulise soolehaiguse korral. Kaudse immunofluorestsentsmeetodi alusel eristatakse kahte tüüpi antikehi: p-ANCA, mille puhul reaktsioon lokaliseerub neutrofiilide tuumade ümber, ja c-ANCA, mille puhul helendus esineb neutrofiilide tsütoplasmas. Neid antikehi leitakse 50–80%-l haavandilist koliiti ja 10–30%-l Crohni tõbe põdeja vereseerumis (9). ANCA tähendus põletikulise soolehaiguse patogeneesis ei ole teada. Arvatakse, et tegemist on markeriga, mis väljendab häireid immuunsüsteemi regulatsioonimehhanismides.

Töö eesmärk on uurida haavandilist koliiti ja Crohni tõbe põdejail erinevate autoantikehade esinemissagedust ja kliinilist väärtust.

**Uurimismaterjal ja -metoodika**

Uurimisrühma kuulus 77 haavandilist koliiti (40 meest ja 37 naist, keskmine vanus 43 aastat, vahemik 14–75 aastat) ning 31 Crohni tõbe põdejat (23 meest ja 8 naist, keskmine vanus 36 aastat, vahemik 15–77 aastat). Haavandilise koliidi diagnoosimisel arvestati üldtunnustatud diagnostilisi kriteeriume: anamneesis veriroe, tüüpiline koloskoopialeid ja morfoloogilised muutused biopsiapreparaadis. 31 haavandilist koliiti põdejal oli proktiit (põletikulised muutused ainult rektumis), 23-l vasakpoolne koliit (muutused ulatusid kuni *colon transversum'i* keskosani) ja 23-l pankoliit (protsess ulatus kaugemale *colon transversum'i* keskosast). Kolektoomia oli eelnevalt tehtud 6 patsiendil. Crohni tõve diagnoosimisel kasutati Lennard-Jonesi kriteeriume (8) ning haiguse ulatuse määramiseks rakendati röntgenoloogilisi meetodeid ja koloskoopiat. Ileiit (muutused ainult peensooles) esines 7 Crohni tõve haigel, 9 patsiendil oli koliit (muutused koolonis *caecum'i* ja rektumi vahel ilma peensoole või seedetrakti ülaosa haaratuseta) ning 15-l oli ileokoliit (muutused terminaaliileumis ja koolonis ükskõik kus *caecum'i* ja rektumi vahel). Ühel ileokoliidiga patsiendil esinesid perianaalne lesioon ja fistul. Kuuel haigel oli eelnevalt tehtud ileotsekaalpiirkonna resektsioon, kahel hemikolektoomia, ühel kolektoomia koos ileostoomi rajamisega.

Kontrollrühma moodustasid 32 soole ärritussündroomiga patsienti (16 meest ja 16 naist, keskmine vanus 38 aastat, vahemik 16–70 aastat) ja 76 tervet isikut (30 meest ja 46 naist, keskmine vanus 40 aastat, vahemik 17–79 aastat).

Seerumid säilitati temperatuuril -20°C. IgG-tüüpi tuumavastased (ANA), mitokondritevastased (AMA), silelihaskoevastased (SMA), retikuliinivastased (ARA), maksa-neeru mikrosoomidevastased (LKMA), parietaalrakkudevastased (PCA) ja kilpnäärme mikrosoomidevastased (TMA) antikehad määrati kaudsel immunofluorestsentsmeetodil (IIF) (13), kasutades antigeense substraadina erinevate kudede krüostaatlõike. Seerumid lahjendati (lahjendus 1:10 ja 1:100) fosfaatpuhverlahuses (PBS), sekundaarse antikehana kasutati fluorestseiiniga konjugeeritud inimese IgG-vastast antiseerumit (*Dako*, Kopenhaagen, Taani). Kõik ANA-d sisaldavad seerumid testiti etanoolis fikseeritud HEp-2-rakkudel (*Immuno Concepts*, Sacramento, USA) lahjenduses 1:40 PBS-is ning immunoensüümimeetodil (ELISA) kuuel enam levinud ekstraheeritavate tuuma-

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of pANCA in healthy relatives of patients with UC may be explained by genetic heterogeneity (14,31), although some recent studies have not confirmed this (32,33). Associations between a positive pANCA result and HLA-DR2, and between a negative pANCA test result and HLA-DR4 have been found (34). However, an ensuing study has failed to show the present association between ANCA positivity and HLA-DR2 (35), and, therefore, the authors suggest that the conflicting results regarding an HLA-DR2 association with UC may be caused by ethnic variation in linkage disequilibrium between HLA-DR2 and the actual susceptibility genes for UC. An alternative or additional explanation would be heterogeneity within UC.

In Estonia, the incidence of UC, and especially CD, appears to be rare. The reasons for such a low incidence are not known, but the different genetic background, dietary habits, and unknown environmental conditions of the patients studied, could not be completely ruled out. At the same time, there seem to be no significant differences in the prevalence of ANCA compared with the data observed in different European countries (6-12). Therefore, we support the idea that ANCA positive IBD patients might represent a distinct subgroup within a heterogeneous disease.

In conclusion, the present study confirms that ANCA are common in IBD, and that patients from Estonia are not an exception. The presence of ANCA in a rather large amount of patients with CD patients limits their diagnostic value in distinguishing between UC and CD. Identification of the antigen(s) responsible for ANCA may facilitate the understanding of the underlying immunopathogenic mechanisms. Whether or not positive ANCA patients represent a subset of cases within a heterogeneous disease, requires the carrying out of further immunologic and genetic studies in Estonia and in other countries as well.

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positive sera were retested on Hep2 cell lines at a dilution of 1:40. Two female UC patients and 1 male CD patient were found to be ANA positive. ANA in all of them produced a homogeneous staining pattern of the nucleus.

Of the 59 UC and 17 CD sera screened with ELISA, using proteinase-3, myeloperoxidase, lactoferrin, and cathepsin G as antigens, only 14 of 76 (18%) sera were positive. Antibodies to proteinase-3 were detected in 5 UC and 1 CD sera, of which 3 UC and 1 CD sera scored positive for cANCA. One UC serum scored positive for pANCA and 1 UC serum had both types of ANCA. Antibodies to myeloperoxidase were not detected. Anti-lactoferrin antibodies were detected in 6 UC and 1 CD patients' sera, of which 3 UC and 1 CD sera were pANCA positive, 1 UC serum had both types of ANCA, and 2 were negative on IIF. Antibodies to cathepsin G were detected in 3 UC and 2 CD patients' sera, of which 1 UC and 1 CD sera were pANCA positive, and 2 UC and 1 CD sera were negative on IIF (**Table 4**). Three patients with UC and 1 with CD had positive ELISA for two different antigens.

**TABLE 4** Number of ANCA Positive Sera Detected by ELISA with Different Antigens

| | Ulcerative colitis (n=59) | | Crohn's disease (n=17) | |
|---|---|---|---|---|
| Antigen | ELISA+ | IIF+ | ELISA+ | IIF+ |
| Proteinase-3 | 5 | 5 | 1 | 1 |
| Myeloperoxidase | 0 | 0 | 0 | 0 |
| Lactoferrin | 6 | 4 | 1 | 1 |
| cathepsin G | 3* | 1 | 2 | 1 |

* Fifty-six sera from patients with UC were studied.

## DISCUSSION

The present study showed that ANCA are quite often present in ulcerative colitis patients in Estonia. ANCA were detected in 49% of the patients with UC and in 24% of the patients with CD. The presence of ANCA in a rather great amount of patients with Crohn's disease limits their diagnostic value in distinguishing between UC and CD. Therefore, we do agree with the findings of Broekroelofs et al (10), showing ANCA to be of limited or no value in distinguishing Crohn's disease from ulcerative colitis.

The predominant ANCA staining pattern observed in our study was perinuclear, which is also affirmed by other authors in the case of IBD. However, it is distinct from vasculitis-associated pANCA, and it is not generally reactive with myeloperoxidase (4-5,22). In contrast to cANCA, which have shown to serve as highly sensitive and specific markers for Wegener's granulomatosis (3,23,24), in IBD the pathogenetic role and clinical significance of pANCA is less favorably established. The problem is that ANCA are present only in 40-85% of the patients with UC (4-14), and most of the authors have failed to show either the correlation between ANCA and disease activity or ANCA and disease extent (4-6,12,25). No correlation between ANCA and the duration or ANCA and the extent of IBD was found in our study either.

The other problem is that test sera may contain other antibodies, including antinuclear antibodies, which may result in a staining pattern not dissimilar to that of pANCA. Therefore, the screening of the sera with non-specific staining patterns and, equivocally, ANCA positive for the presence of ANA, is useful until the antigen(s) responsible for ANCA are identified. When this is realized it may be expected that the problems associated with the recognition of ANCA can be overcome.

Whereas the antigen specificity of ANCA in IBD is still uncertain, antibodies to several constituents of neutrophils, including cathepsin G (26,27), lactoferrin (28), β-glucuronidase (29) and, recently, bactericidal/permeability-increasing protein (30) have been reported in some patients with IBD. None of these have been proven to be exclusively responsible for pANCA activity. In the present study, 18% of the serum samples were positive in specific ELISA assays. Though immunofluorescence and ELISA results did not correspond in our study, we support the findings that antigens other than proteinase-3, myeloperoxidase, lactoferrin and cathepsin G can be the targets for ANCA in Estonian patients with IBD, and remain to be evaluated in further investigations.

Since it now is believed that ANCA probably do not contribute to tissue injury in IBD, the finding

TABLE 2 Distribution of Antineutrophil Cytoplasmic Antibodies in Patient and Control Sera Detected by Indirect Immunofluorescence

| | n | IIF ANCA+ n (%) | Immunofluorescence staining pattern perinuclear | cytoplasmic | perinuclear and cytoplasmic |
|---|---|---|---|---|---|
| UC | 59 | 29 (49) | 22 | 6 | 1 |
| CD | 17 | 4 (24) | 3 | 1 | 0 |
| Controls | 111 | 4 (4) | 3 | 1 | 0 |

myeloperoxidase and proteinase-3 antibodies of IgG isotype (Wieslab AB, Lund, Sweden). Both kits were used according to the manufacturer's instructions. A serum dilution of 1:80 was used in both assays. Alkaline phosphatase-labeled anti-human immunoglobulin G was used as a second antibody. The reaction was visualized using p-nitrophenyl phosphate as a substrate, and read at 405nm. Values exceeding the borderline (20 EU) were regarded as positive.

The anti-lactoferrin and the anti-cathepsin G ELISAs were performed as originally described (20,21). Briefly, ELISA microtiter plates (Nunc, Roskilde, Denmark) were coated overnight, at 4°C, with 5µg/ml human neutrophil lactoferrin (Sigma, St. Louis, Mo., USA), or 0.5µg/ml cathepsin G (Calbiochem, La Jolla, Calif., USA) in carbonate buffer (pH 9.6), and in 50mM sodium acetate with 150mM NaCl (pH 5.5), respectively. Plates were saturated with 1% bovine serum albumin in PBS (PBS/BSA), or with 5% swine serum albumin in PBS (PBS/SSA), respectively. Controls and serum samples were diluted 1:100 in PBS/BSA 1%/Tween 0,1% and incubated for 1 hour at room temperature. The bound IgG was detected using alkaline phosphatase-conjugated anti-human IgG (Dako, Glostrup, Denmark), followed by p-nitrophenyl phosphate as a substrate.

Sera from 40 healthy persons were used as references to establish a base-line absorbance value, and defined as two standard deviations above the mean.

### Statistical Analysis

Statistical analysis of the data using the $\chi 2$ test was performed. $P<0.05$ was considered to be significant. For comparing small numbers, Fisher's exact test was performed.

## RESULTS

IgG ANCA were detected by IIF on ethanol-fixed granulocytes in 29 of 59 (49%) patients with UC and in 4 of 17 (24%) patients with CD ($p=0.06$). In the control group, serum samples from 1 of 25 patients with irritable bowel syndrome, and 3 of 86 healthy subjects were positive for ANCA ($p<0.001$ for UC compared to controls; $p<0.01$ for CD compared to controls) **(Table 2)**.

TABLE 3 Relationship between Clinical Features of IBD and the Presence of ANCA

| | ANCA positive patients/ total number of patients Ulcerative colitis 29/59 | Crohn's disease 4/17 |
|---|---|---|
| **Disease extent** | | |
| Proctitis | 11/25 | |
| Left-sided colitis | 7/13 | |
| Extensive colitis | 11/21 | |
| **Disease localization** | | |
| Ileum | | 0/3 |
| Colon | | 0/6 |
| Ileum and colon | | 4/8 |
| **Duration of disease** | | |
| <5 years | 14/21 | 3/13 |
| >5 years | 15/38 | 1/4 |

The predominant ANCA staining pattern seen by IIF in IBD was perinuclear (pANCA). Twenty-two of 29 positive patients with UC had pANCA, compared to 3 of 4 patients with CD. In 6 patients with UC and 1 patient with CD, cytoplasmic staining (cANCA) was revealed. One patient with UC had both perinuclear and cytoplasmic staining **(Table 2)**. In the controls, only 3 of 111 serum samples showed a weak perinuclear staining and 1 serum sample showed cytoplasmic staining.

In both UC and CD, there was no correlation between the presence of ANCA and the duration or extent of IBD **(Table 3)**.

To ensure that ANCA in IBD indeed recognize cytoplasmic antigens, we screened all sera for the presence of ANA on rat liver slices. ANA (1:10) were detected in 5 UC patients and in 1 patient with CD; all patients were pANCA positive and without liver abnormalities. In the controls, 1 serum sample was ANA positive. All 7 ANA

the incidence of UC and CD varies in different countries (17,18), Estonia having one of the lowest rates among them. No published data on the epidemiology of IBD were available from the neighboring states of Estonia (Latvia and Lithuania) nor from Eastern European countries.

Since there are substantial epidemiological differences in the incidence of IBD between Estonia and European countries, only additional studies would yield more data about specific features of IBD in Estonia. The aim of our study is to: determine the prevalence and pattern of ANCA in Estonian patients with IBD; compare our results with the results from other countries; analyze the presence of ANCA in connection with the duration and extent of IBD; and, characterize the antigen specificity by enzyme-linked immunosorbent assay (ELISA), using myeloperoxidase, proteinase-3, lactoferrin and cathepsin G as antigens.

## METHODOLOGY

### Study Population and Serum Samples

Sera from a total of 187 patients were analyzed in the present study. Fifty-nine sera were obtained from the patients with UC and 17 from the patients with CD. For UC, the diagnostic criteria were based on a history of bloody stools, typical colonoscopy findings, and morphological changes in biopsy specimens. The extent of colitis in the patients with UC was evaluated with colonoscopy, and, in some cases, with barium enemas. For CD, the diagnostic criteria were followed according to the Lennard-Jones scoring table (19), and disease localization was evaluated by small-bowel roentgenograms, barium enemas, and colonoscopy. The duration of the disease was also considered. The clinical characteristics of the patients are shown in **Table 1**.

Twenty-five patients with irritable bowel syndrome (12 male, 13 female; mean age 38 years) and 86 healthy persons (31 male, 55 female; mean age 39 years) with no history of gastrointestinal or chronic disease served as controls. All sera were stored at -20°C until assayed.

TABLE 1 Clinical Characteristics of Patients with Inflammatory Bowel Disease at the Time of Serum Sampling

| Diagnosis | Ulcerative colitis (n=59) | Crohn's disease (n=17) |
|---|---|---|
| Male/Female | 28/31 | 13/4 |
| Mean age (years) | 44 | 27 |
| Disease extent | | |
| Proctitis, n (%) | 25 (42) | |
| Left-sided, n (%) | 13 (22) | |
| Extensive, n (%) | 21 (36) | |
| Disease localization | | |
| Ileum, n (%) | | 3 (18) |
| Colon, n (%) | | 6 (35) |
| Ileum and colon, n (%) | | 8 (47) |
| Duration of disease | | |
| <5 years, n (%) | 21 (36) | 13 (76) |
| >5 years, n (%) | 38 (64) | 4 (24) |

### Indirect Immunofluorescence Assay (IIF)

The separation of peripheral blood neutrophils and the detection of ANCA by IIF according to the method described by Wiik (20) was performed with minor modifications.

Briefly, neutrophils were separated from the peripheral blood of a healthy volunteer by Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) centrifugation and dextran (BDH Chemicals Ltd, Poole, England) sedimentation. The buoyant neutrophils were recovered and washed. Cellular suspensions were smeared on slides and fixed in 96% ethanol for 5 minutes, dried at room temperature and stored at -20°C until the time of the assay, but not longer than 2 weeks. The slides (coded patients or control sera; dilution 1:20) were incubated and stained with rabbit antihuman IgG - fluorescein isothiocyanate (FITC) conjugate (Dakopatts, Glostrup, Denmark).

The slides were evaluated by fluorescence microscopy. Positive and negative controls were included for all assays. Serum samples giving a typical perinuclear (pANCA) or cytoplasmic (cANCA) staining reaction with the neutrophils were regarded as positive.

Antinuclear antibodies (ANA) were detected by IIF on rat liver slices of 4µm at a dilution of 1:10 and on ethanol-fixed HEp2 (Immuno Concepts, Sacramento, USA) cells at a dilution of 1:40 in phosphate buffered saline solution (PBS).

### Enzyme-linked Immunosorbent Assay

Commercially available kits based on enzyme immunoassay were used for the analysis of

Original Paper

# Antineutrophil Cytoplasmic Antibodies in Estonian Patients with Inflammatory Bowel Disease. Prevalence and Diagnostic Role

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**KEY WORDS:** Antineutrophil cytoplasmic antibodies; Inflammatory bowel disease

**ABBREVIATIONS:** Antineutrophil Cytoplasmic Antibodies (ANCA); Inflammatory Bowel Disease (IBD); Ulcerative Colitis (UC); Crohn's Disease (CD); Indirect Immunofluorescence Assay (IIF); Enzyme-linked Immunosorbent Assay (ELISA)

## ABSTRACT

**BACKGROUND/AIMS:** In Estonia, the incidence of ulcerative colitis and especially Crohn's disease appears to be rare. Antineutrophil cytoplasmic antibodies (ANCA) are frequently found in ulcerative colitis but less frequently in Crohn's disease, their pathophysiological significance is still unclear.

**METHODOLOGY:** Fifty-nine serum samples from patients with ulcerative colitis, 17 with Crohn's disease, 25 with irritable bowel syndrome, and 86 healthy persons were studied. Sera were analyzed for the presence of ANCA by indirect immunofluorescence, and enzyme-linked immunosorbent assay for specific ANCA using different antigens was performed.

**RESULTS:** ANCA were detected in 29 of 59 (49%) patients with ulcerative colitis, 4 of 17 (24%) patients with Crohn's disease, and in 4 of 111 (4%) controls. The immunofluorescence staining was mostly perinuclear (pANCA). There was no correlation between ANCA and the duration or extent of the inflammatory bowel disease. In specific enzyme-linked immunosorbent assays, only 14 sera elicited binding above the normal range.

**CONCLUSIONS:** Although the prevalence of ulcerative colitis and Crohn's disease in Estonia is much lower than in European countries, there seem to be no differences in the presence of ANCA.

## INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory disorders of unknown origin. In either case of UC or CD, autoantibodies as well as associations with disorders of autoimmune origin can be found. Although the role of autoantibodies and the underlying pathogenesis in inflammatory bowel disease (IBD) remain unclear, it is generally believed that immune mechanisms are involved (1).

Antineutrophil cytoplasmic antibodies (ANCA) were first described in patients with glomerulonephritis (2). In 1985, it was shown that the antibodies directed against cytoplasmic components of neutrophils occur in Wegener's granulomatosis and serve as sensitive and specific markers for active disease (3). More recently, serum immunoglobulin G (IgG) ANCA have been reported to be present in 40-85% of the patients with UC and in 4-40% of the patients with CD (4-14). By indirect immunofluorescence in IBD, the antigen localizes to the perinuclear area (pANCA) and is distinct from the cytoplasmic granular pattern (cANCA) seen characteristically in Wegener's granulomatosis. The antigen(s) to which those antibodies are reactive in IBD are not clearly defined yet.

The basis for the present study is an epidemiological investigation of the incidence and prevalence of UC and CD in Tartu County, Estonia. A retrospective study for the years 1973-1993 revealed that IBD is not so commonly found in Estonia as it is in other countries, and that the reasons for such a low incidence in Estonia are not clear. The annual incidence of UC is 1.5 cases per 100,000, and of CD 0.27 per 100,000 (15,16). Studies of the epidemiology of IBD indicate that

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# IV

K. Hirv, M. Seyfarth, R. Uibo, K. Kull, R. Salupere, U. Latza, L. Rink. Polymorphisms in tumour necrosis factor and adhesion molecule genes in patients with inflammatory bowel disease; associations with HLA-DR, -DQ alleles and subclinical markers. Scand J Gastroenterol 1999; 34:1025–32.

ORIGINAL ARTICLE

# Polymorphisms in Tumour Necrosis Factor and Adhesion Molecule Genes in Patients with Inflammatory Bowel Disease: Associations with HLA-DR and -DQ Alleles and Subclinical Markers

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Hirv K, Seyfarth M, Uibo R, Kull K, Salupere R, Latza U, Rink L. Polymorphisms in tumour necrosis factor and adhesion molecule genes in patients with inflammatory bowel disease: associations with HLA-DR and -DQ alleles and subclinical markers. Scand J Gastroenterol 1999;34:1025–1032.

**Background:** When investigating susceptibility to inflammatory bowel disease (IBD), a multifactorial disorder with a genetic predisposition, polymorphisms of molecules with immunoregulatory function are of potential interest. This is the first time that the polymorphisms of HLA-DR and -DQ, tumour necrosis factor (TNF), E-selectin (CD62E), L-selectin (CD62L), and intercellular adhesion molecule 1 (ICAM-1, CD54) were determined in Estonians, a population with a low IBD incidence rate, and their occurrence investigated in subgroups of a total of 53 IBD patients. **Methods:** The reverse hybridization principle and sequence specific primers were used for HLA genotyping. To analyse the TNF and adhesion molecule polymorphisms, the polymerase chain reaction with subsequent restriction fragment length polymorphism or single-strand conformation polymorphism method was used. **Results:** In the subgroup of antineutrophil cytoplasmic antibody (ANCA)-positive ulcerative colitis (UC) patients we found a higher frequency of the TNF2 (20.8% versus 0.0% in ANCA-negative UC patients, $P = 0.051$) and HLA-DRB1*15 allele (35.4% versus 15.7% in controls; $P = 0.004$). Of ANCA-positive UC patients 87.5% were carriers of one of these alleles (22.2% among ANCA-negative UC patients ($P < 0.001$, $P_c = 0.039$) and 51.4% among controls ($P = 0.002$). Specific typing of HLA-DRB1*15 alleles showed that the HLA-DRB1*1501 allele was responsible for the HLA-DRB1*15 association with ANCA-positive UC. Associations of ICAM-1, E-selectin, or L-selectin polymorphisms with IBD were not found. **Conclusions:** TNF2 and HLA-DRB1*15 alleles were associated with ANCA-positive UC in the investigated population. ANCA might be a useful marker, at least in some ethnic groups, for dividing IBD patients into genetically more homogeneous subgroups.

**Key words:** Adhesion molecules; antineutrophil cytoplasmic antibody; HLA class-II genes; inflammatory bowel disease; polymorphism; tumour necrosis factor

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Genetic predisposition, environmental factors and infectious agents are discussed as being in causal association with inflammatory bowel disease (IBD)—Crohn disease (CD) and ulcerative colitis (UC) (1–4). Despite extensive investigations, the aetiopathogenesis of IBD remains enigmatic. This may be partly due to the heterogeneity of the disease.

Persistent infiltration of the colonic mucosa with inflammatory cells and failure to down-regulate the inflammatory processes and other alterations of the immune system, mediating tissue damage, have been identified in patients with IBD in numerous studies. Accordingly, polymorphisms of genes involved in initiation and regulation of the immune response may be responsible for susceptibility to IBD. Polymorphisms of the major histocompatibility complex (MHC) class-II genes are at the centre of interest in most studies investigating diseases with immunoregulatory abnormalities. With regard to IBD, significant positive associations have repeatedly been found between HLA-DRB1*15 and UC, especially with the antineutrophil cytoplasmic antibody (ANCA)-positive subgroup of patients (5–7). In patients with CD a positive association with HLA-DRB3*0301-DRB1*1302 and HLA-DR1-DQw5 haplotypes has been observed (8–10). Recently, in studies from Germany and France, some other interesting associations between CD and MHC class-II alleles have been found: positive association with HLA-DRB1*07 and negative association with HLA-DRB1*03 (11, 12). Nevertheless, many conflicting

results exist, which may partly be explained by the differences in the investigated ethnic groups and clinical forms of the diseases. This shows that the aetiopathogenesis of IBD cannot be elucidated only by restriction to the MHC class-II alleles. Other genes in the MHC locus, like tumour necrosis factor (TNF) genes, are of potential interest. TNF-$\alpha$ and TNF-$\beta$ are proinflammatory cytokines that have been suspected of being involved in the pathogenesis of many human diseases (13). Many TNF-gene polymorphisms have been described in the last few years, some of which are probably correlated with differences in TNF-$\alpha$ or TNF-$\beta$ secretion (14). Because of the strong linkage disequilibrium between TNF and MHC class-II alleles (15), speculations could be made concerning the primary role of the TNF gene polymorphisms in the aetiopathogenesis of MHC class-II-associated diseases. Furthermore, positive effects in the treatment of IBD with anti-TNF-$\alpha$ monoclonal antibodies support the assumption that TNF is involved in the pathogenesis of IBD, but the detailed mechanism remains unclear (16, 17).

IBD is characterized by a persistent infiltration of the colonic mucosa by the inflammatory cells. The upregulation of the adhesion molecules is one of the physiologic requirements for the entry of these cells into the tissue (18, 19). Recently, the genetic heterogeneity of intercellular adhesion molecule (ICAM)-1 (CD54) in patients with IBD has been investigated. There was an increased frequency of the less common allele in the functionally important domain of ICAM-1 in the subgroups of patients with UC and CD, stratified by ANCA status (20). This supports the role of adhesion molecules in the aetiopathogenesis of IBD and suggests the existence of heterogeneity within IBD.

Because of the assumption that IBD is only in part genetically determined, the degree of importance of genetic markers for heterogeneous subgroups of IBD may vary. After dividing the UC and CD patients into more homogeneous subgroups, the genetic associations may become visible (21, 22). Apart from the discussions about the primary or secondary role of ANCAs in the pathogenesis of IBD, the presence of ANCAs is one of the suitable candidates for dividing the UC and CD patients into genetically homogeneous subgroups. ANCAs were found in 27%–83% of patients with UC and in 0%–25% of patients with CD (23). Although still under discussion, the frequent presence of ANCAs in healthy relatives of IBD patients and associations with developing pouchitis and persistence of ANCAs after colectomy indicate that the presence of ANCAs may represent an underlying genetically determined disturbance of immunoregulatory function (24–26). The presumable association of HLA-DRB1*15 alleles with ANCA-positive UC but not with ANCA-negative UC and a different distribution of ICAM-1 alleles in subgroups of IBD patients, divided by the ANCA status, support this hypothesis (6, 20).

The aim of this study was to investigate the distribution of polymorphisms of HLA class-II and TNF molecules in a novel population of IBD patients. In addition, we wanted to study the possible involvement of ICAM-1 and, for the first time, E-selectin (CD62E) and L-selectin (CD62L) polymorphisms in the pathogenesis and outcome of IBD. The usefulness of ANCAs for dividing IBD patients into more homogeneous subgroups with different genetic background was verified.

## Subjects and Methods

### *Patients and controls*

A total of 53 unrelated Estonian IBD patients, 33 with UC (16 male; median age, 37.3 years) and 20 with CD (15 male; median age, 36.0 years), were studied. For UC the diagnosis was based on a history of bloody stools, typical findings at colonoscopy, and morphologic changes in biopsy specimens.

Table I. Outline of adhesion molecule polymorphisms analysis

| Polymorphism | Method | Primer sequence (5′ to 3′) | PCR product |
|---|---|---|---|
| *ICAM-1 (CD54)* | | | |
| Ig-like domain 3 (R/G241) | PCR-SSCP | Forward GATTGAAGAAGCCAGCAG<br>Reverse GTCGTTGCCATAGGTGAC | 408 bp |
| Ig-like domain 5 (K/E469) | PCR-RFLP | Forward CCATCGGGGAATCAGTG<br>Reverse ACAGAGCACATTCACGGTC | 110 bp |
| *E-selectin (CD62E)* | | | |
| 5′-untranslated region (G/T98) | PCR-SSCP | Forward TAACTAGCTACCCACGATTTCC<br>Reverse ACTGACTTACCCAAAGTGAGAG | 149 bp |
| EGF-like domain (S/R128) | PCR-SSCP | Forward AGTAATAGTCCTCCTCATCATG<br>Reverse ACCATCTCAAGTGAAGAAAGAG | 186 bp |
| Membrane domain (L/F554) | PCR-SSCP | Forward CAATTCTTCCTCATGACCTTTC<br>Reverse CAAGACCATGACTTATCAATGAG | 226 bp |
| *L-selectin (CD62L)* | | | |
| Lectin-like domain (T/S49) | PCR-SSCP | Forward TGTAAGTCTGCATAGGTCACAC<br>Reverse TCAGTGAGAGATTTGTTGGTTC | 276 bp |
| EGF-like domain (F/L206) | PCR-SSCP | Forward CTTTGAGTACTAAAATGTAATCAC<br>Reverse CCTAAGAAGAAGCAAAGAAAGG | 198 bp |

Primers for amplification of the intercellular adhesion molecule (ICAM)-1 Ig-like domains 3 and 5 as described by Vora et al. (31). Primer sequences to analyse E- and L-selectin polymorphisms were kindly provided by Dr. K. Wenzel (Charité, Berlin) (32).
PCR-SSCP = polymerase chain reaction–single-strand confirmation polymorphism; EGF = epidermal growth factor.

For CD the diagnostic criteria were studied in accordance with the Lennard–Jones scoring table (27), and disease localization was evaluated by small-bowel roentgenograms, barium enemas, and colonoscopy. Seventy healthy Estonian blood donors served as controls. The distribution of age, sex, and ethnicity were comparable in the patients and controls. The study was approved by the Ethics Committee of the University of Tartu.

*DNA extraction*

Genomic DNA was extracted from 10 ml venous ethylenediaminetetraacetic acid (EDTA) blood or from peripheral blood leukocytes (controls) with the QIAamp Blood Kit from Qiagen in accordance with the manufacturers' procedure.

*HLA-DRB and -DQB genotyping*

The HLA-DRB1 and HLA-DQB1 genotyping was performed by INNO-LiPA DRB and DQB key kit from Innogenetics. These HLA typing tests are based on the reverse hybridization principle. Amplified biotinylated DNA material is chemically denatured, and the single strands are hybridized with specific oligonucleotide probes immobilized on membrane-based strips. With the exception of DQB1*0201–*0202, all other DQB1 alleles could be distinguished. Although resolution at the allelic level is possible with the INNO-LiPA DRB key kit, only discrimination at the HLA-DRB1*01, *15, *16, *03, *04, *11, *12, *13, *14, *07, *08, *09, and *10 allelic-group level was considered, except for the HLA-DRB1*0103 allele, which could be distinguished from other HLA-DRB1*01 alleles. HLA-DRB1*15 alleles were specifically typed with Micro SSP Allele Specific Class-II DNA Typing Kit (One Lambda Inc.).

*Detection of ANCAs*

The separation of peripheral blood neutrophils and detection of ANCAs by indirect immunofluorescence assay were performed in accordance with the method described by Wiik et al. (28), with minor modifications.

*TNF and adhesion molecule polymorphism analysis*

The detection of polymorphisms in the TNF$\alpha$ promoter at position -308 (TNF1/2) and in the TNF$\beta$ first intron (TNFB*1/2) with the help of PCR amplification and subsequent digestion with restriction enzyme *NcoI* were performed as described (29, 30). Methods for identification of the adhesion molecule polymorphisms and primer sequences for amplification of the investigated gene fragments are summarized in Table I. PCRs were performed by using 30-ng template DNA in a final reaction volume of 50 µl in GeneAmp9600 (Perkin Elmer) thermal cycler. The amplification conditions were optimized by changing the annealing temperature and time or extension time for each investigated adhesion molecule gene region. The polymorphism in exon 6 of the ICAM-1 gene (Lys:AAG or Glu:GAG at codon 469), coding for Ig-like domain 5, was identified through incubation of the amplified 110-bp ICAM-1 exon 6 gene fragments with the restriction enzyme *BstUI*. The presence of the *BstUI* restriction site (CG$^{\downarrow}$CG) resulted in 86 and 24 bp fragments. Fragments were analysed by electrophoresis on 4% NuSieve agarose gels and by subsequent staining of the gels in 0.1% ethidium bromide.

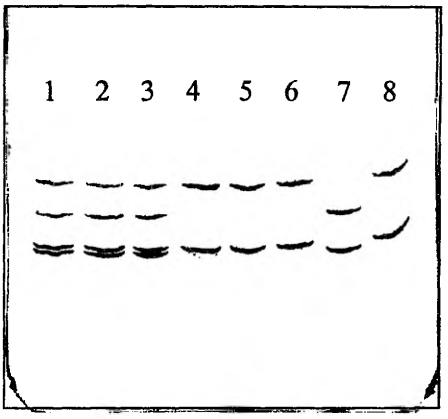


Fig. 1. Representative single-strand conformation polymorphism (SSCP) analysis of the L-selectin epidermal growth factor (EGF)-like domain. An amplified 198-bp gene fragment was separated on PhastGel Homogeneous 20% gel. The amino acid polymorphism at codon 206 (F/L206) is caused by change from thymine to cytosine at nucleotide position 668, which results in a band shift in the electrophoretic analysis. Lanes 1, 2, and 3: heterozygous TC668. Lanes 4, 5, 6, and 8: homozygous TT668. Lane 7: homozygous CC668.

For the identification of the polymorphisms in all other amplified adhesion molecule gene regions, a single-strand conformation polymorphism (SSCP) method was used. The fragments were denatured and run on non-denaturing 12.5% or 20% polyacrylamide gels (Homogeneous PhastGels) from Pharmacia Biotech. The conditions for the horizontal electrophoresis and silver staining were optimized for each gene fragment and were performed semi-automatically with the PhastSystem (Pharmacia LKB Biotechnology AB). Fig. 1 shows the detection of the polymorphism in the L-selectin epidermal growth factor (EGF)-like domain by PCR-SSCP analysis as an example of this method. To verify the SSCP results, the PCR products were sequenced.

*Statistical analysis*

Patients with UC, grouped together, and patients with CD, grouped together, were compared with controls. Associations with subsets of UC and CD patients, divided on the basis of ANCA status, were also evaluated. The statistical significance was tested by the chi-square test. The Fisher exact test was used when appropriate (that is, expected number $< 5$). Significance was taken as $P < 0.05$. By the correction of $P$

Table II. Less common allele frequencies (percentage) of adhesion molecule genes in patients and controls

| Polymorphism | Controls ($n = 70$) | UC patients ($n = 33$) | CD patients ($n = 20$) |
|---|---|---|---|
| *ICAM-1* | | | |
| Ig-like domain 3 | 14.3 | 12.1 | 20.0 |
| Ig-like domain 5 | 42.1 | 48.5 | 52.5 |
| *E-selectin* | | | |
| 5'-untranslated region | 28.6 | 33.3 | 32.5 |
| EGF-like domain | 13.6 | 12.1 | 20.0 |
| Membrane domain | 3.6 | 7.6 | 2.5 |
| *L-selectin* | | | |
| Lectin-like domain | 4.3 | 3.0 | 5.0 |
| EGF-like domain | 22.1 | 18.2 | 15.0 |

ICAM = intercellular adhesion molecule; EGF = epidermal growth factor; UC = ulcerative colitis; CD = Crohn disease.

values, the recommendations of Svejgaard & Ryder (33) were taken into consideration. The correction factor for HLA-DRB1 and HLA-DQB1 alleles was 12 (extremely infrequent alleles, ranging from 0 to 2, were not included in the number of comparisons). In the subgroups of UC or CD, stratified by ANCA, the correction factor was 24. In the phenotypic combination analysis the $P$ values were corrected by a factor of 48. A corrected $P$ value is designated $P_c$. The odds ratio (OR) as a measure of association was calculated using a Haldane modification as suggested by Svejgaard & Ryder (33).

## Results

### *ANCA*

ANCAs were detected in 27 of 53 patients (50.9%). Within UC and CD patients the occurrence of ANCAs differed. ANCA positivity rate in the 33 UC patients was 72.7% (69.7% perinuclear staining pattern and 3.0% cytoplasmic staining pattern). Only 3 of 20 CD patients were ANCA-positive (15.0%), all of them with a perinuclear staining pattern.

Table III. Allele frequencies and carriage rates (both in percentages) of tumor necrosis factor (TNF) polymorphisms

| Polymorphism | Allele frequencies | | Allele 2 carriage rate |
|---|---|---|---|
| | Allele 1 | Allele 2 | |
| *TNF1/2* | | | |
| Controls ($n = 70$) | 83.6 | 16.4 | 30.0 |
| UC patients ($n = 33$) | 84.8 | 15.2 | 24.2 |
| ANCA-pos. UC ($n = 24$) | 79.2 | 20.8 | 33.3 |
| ANCA-neg. UC ($n = 9$) | 100.0 | 0.0 | 0.0 |
| CD patients ($n = 20$) | 80.0 | 20.0 | 40.0 |
| *TNFB*1/2* | | | |
| Controls ($n = 70$) | 32.9 | 67.1 | 92.9 |
| UC patients ($n = 33$) | 28.8 | 71.2 | 78.8 |
| ANCA-pos. UC ($n = 24$) | 33.3 | 66.7 | 79.2 |
| ANCA-neg. UC ($n = 9$) | 16.6 | 83.4 | 77.8 |
| CD patients ($n = 20$) | 37.5 | 62.5 | 95.0 |

ANCA = antineutrophil cytoplasmic antibody; CD = Crohn disease; UC = ulcerative colitis.

Table IV. Frequencies (%) of HLA-DRB1 alleles in controls and patients

| HLA-DRB1* | Controls ($n = 70$) | UC patients ($n = 33$) | CD patients ($n = 20$) |
|---|---|---|---|
| *01† | 11.4 | 7.6 | 22.5 |
| *0103 | 0.0 | 1.5 | 0.0 |
| *03 | 15.7 | 9.1 | 17.5 |
| *04 | 5.7 | 4.5 | 5.0 |
| *07 | 11.4 | 12.1 | 5.0 |
| *08 | 5.7 | 4.5 | 7.5 |
| *09 | 0.0 | 1.5 | 5.0 |
| *10 | 0.7 | 0.0 | 0.0 |
| *11 | 14.3 | 9.1 | 20.0 |
| *12 | 2.9 | 6.1 | 2.5 |
| *13 | 11.4 | 10.6 | 7.5 |
| *14 | 0.7 | 1.5 | 2.5 |
| *15 | 15.7 | 28.8‡ | 2.5§ |
| *16 | 4.3 | 3.0 | 2.5 |

UC = ulcerative colitis; CD = Crohn disease.
† Other than HLA-DRB1*0103.
‡ Compared with controls: $P = 0.028$.
§ Compared with controls: $P = 0.027$.

### *Adhesion molecule polymorphisms*

The observed allele frequencies of investigated adhesion molecule polymorphisms in the control group and in the groups of patients with UC or CD are shown in Table II. There were no statistically significant differences in allele frequencies between controls and patients with UC or CD or the patients considered as one group.

Also after dividing the patients into subgroups on the basis of ANCA status, no significant differences were observed. Increased frequencies of the uncommon R241 ICAM-1 allele in the ANCA-negative UC and ANCA-positive CD subgroups of patients, described by Yang et al. (6, 20), were not found in our study.

### *TNF polymorphisms*

Allele frequencies and carrier rates of the TNF1/2 polymorphism (in the TNFα promoter at position -308) and TNFB*1/2 polymorphism (in the TNFβ first intron) were similar in patients with UC and CD and control individuals (Table III). Subgroup analysis showed borderline significant differences in the frequency of the uncommon TNF2 allele in the ANCA-positive and ANCA-negative subgroups of patients with UC (20.8% versus 0.0%, respectively; $P = 0.051$). This fact gained more importance with regard to other factors associated with ANCA positivity, as discussed later.

The TNFB*1 allele was in linkage disequilibrium with the TNF2 allele ($P < 0.00001$). Therefore, the frequency of the TNFB*1 allele tended to show similar differences between ANCA-positive and ANCA-negative UC patients, but this trend did not reach statistical significance.

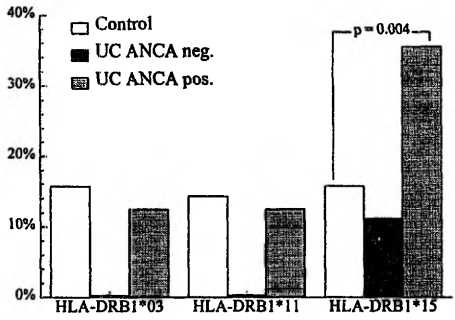


Fig. 2. HLA-DRB1*03, *11, and *15 allele frequencies (percentage) in the control group and in the antineutrophil cytoplasmic antibody (ANCA)-negative and ANCA-positive subgroups of ulcerative colitis (UC) patients.

*HLA class-II genes*

The distribution of HLA-DRB1 alleles is shown in Table IV. HLA-DRB1*15 allele frequency was increased in UC patients compared with controls (28.8% versus 15.7%; OR = 2.16; $P = 0.028$). By contrast, the HLA-DRB1*15 allele frequency was decreased in patients with CD (2.5% versus 15.7%; OR = 0.20; $P = 0.027$). There was only one carrier of this allele among CD patients (5.0% versus 31.4% in the control group; OR = 0.17; $P = 0.017$). The apparently increased proportion of CD patients carrying the HLA-DRB1*01 allele did not attain statistical significance compared with controls (45.0% versus 22.9%; $P = 0.051$), possibly due to the small sample size. The HLA-DRB1*0103 allele was infrequent in our population, so no conclusions about the role of this allele in the pathogenesis of IBD, as suspected in some studies, could be drawn (34).

When patients were stratified by ANCA status, a different distribution of the HLA-DRB1 alleles in subgroups of UC patients was identified. Increased frequency of HLA-DRB1*15 alleles in UC patients could be reduced to the association of HLA-DRB1*15 alleles with ANCA-positive UC (35.4% versus 15.7% in controls; OR = 2.93; $P = 0.004$). ANCA-negative UC patients had approximately the same HLA-DR*15 allele frequency as controls (11.1% versus 15.7%). Interestingly, HLA-DRB1*03 and HLA-DRB1*11 alleles were found only in UC patients with ANCAs and not among ANCA-negative UC patients (Fig. 2).

To support the hypothesis that the HLA-DRB1*1502 allele may be responsible for the association of HLA-DRB1*15 alleles with UC, and particularly with their ANCA-positive subgroup, specific typing of HLA-DRB1*15 alleles was performed. HLA-DRB1*1501 and -DRB1*1502 alleles were both increased in the groups of UC and ANCA-positive UC patients compared with controls, but the HLA-DRB1*1502 allele was infrequent in the population studied (Fig. 3) and was not the factor explaining the association of HLA-DRB1*15 alleles with UC and ANCA-positive UC.

As expected, the HLA-DR3-DQ2 haplotype was in strong linkage disequilibrium with the uncommon TNF2 allele ($P < 0.00001$). HLA-DR-DQ/TNF1/2 phenotypic combination analysis showed an interesting pattern in the distribution of possible phenotypes. In the group of UC patients the HLA-DR3-DQ2 haplotype was not found without the TNF2 allele (15.0% of HLA-DR3-DQ2 carriers in the control group). And more often the TNF2 allele carriers were presented without the HLA-DR3-DQ2 haplotype in TNF2-positive UC patients (37.5% versus 19.0% in the control group).

HLA-DQB1 alleles were found to be associated with UC or CD only in relation to strong linkage disequilibrium with HLA-DRB1 alleles, always with lower $P$ values.

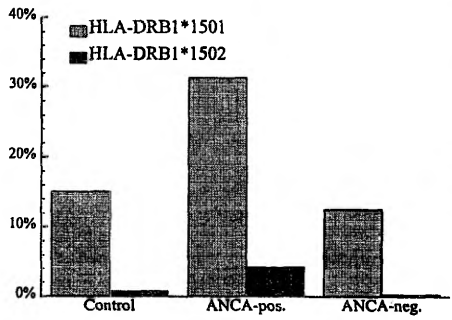


Fig. 3. HLA-DRB1*1501 and DRB1*1502 allele frequencies (percentage) in the control group and in the antineutrophil cytoplasmic antibody (ANCA)-negative and ANCA-positive subgroups of ulcerative colitis (UC) patients.

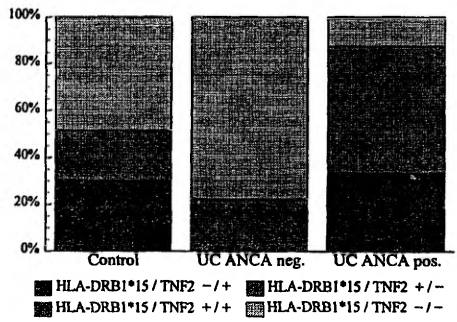


Fig. 4. The proportion of HLA-DRB1*15/tumour necrosis factor (TNF)2 phenotypic combinations in the control group and in ulcerative colitis (UC) patients divided on the basis of antineutrophil cytoplasmic antibody (ANCA) status.

*ANCA, HLA-DR, and TNF1/2 triangle*

Both TNF2 and HLA-DRB1*15 alleles showed an increased frequency in the ANCA-positive subgroup of UC patients. The distribution of four phenotypic combinations of the HLA-DRB1*15 and TNF2 alleles was very different in the control group and in the UC patient groups divided by ANCA status (Fig. 4); 87.5% of the ANCA-positive UC patients were carriers of at least one of the HLA-DRB1*15 or TNF2 alleles, compared with 22.2% of the ANCA-negative UC patients (OR = 18.97; $P < 0.001$, $P_c = 0.039$), and 51.4% of the controls (OR = 5.81; $P = 0.002$). To further investigate the associations of these two factors with UC and its ANCA-positive subgroup, various two-by-two tables were generated, involving stratification of each of the two factors against the other. This analysis of the four phenotypic combinations showed a significant association of HLA-DRB1*15 with UC (OR = 3.29; $P = 0.015$) and ANCA-positive UC (OR = 8.59; $P < 0.001$, $P_c = 0.018$) in TNF2-negatives. The TNF2 allele was associated with ANCA-positive UC in HLA-DRB1*15-negatives (OR = 5.10; $P = 0.027$).

## Discussion

The distribution of the MHC class-II and TNF alleles in a novel subpopulation of IBD patients was studied. The investigation of Estonian UC and CD patients is of particular interest because of the very low UC and CD incidence rates (1.5 and 0.28 per 100,000, respectively) in this country as compared with other European populations (overall incidence of UC and CD, 10.4 and 5.6 per 100,000, respectively) (35–37).

We observed associations of the investigated MHC class-II alleles and TNF1/2 alleles with subgroups of patients. Both the TNF2 and HLA-DRB1*15 alleles were associated with ANCA positivity in patients with UC. The usefulness of ANCA as a marker of genetic heterogeneity in ulcerative colitis is still being discussed (38–40). Our data indicate that TNF2 and HLA-DRB1*15 alleles themselves or, more likely, in strong linkage with some other gene loci, might be genetic markers for the susceptibility to develop ANCA-positive UC. The role of these markers could vary in different ethnic groups because of the different linkages with the primary susceptibility genes. Although there is strong evidence to support the involvement of TNFα in the pathogenesis of IBD, a primary role of the TNF1/2 polymorphism in developing ANCA in UC patients or ANCA-positive UC is questionable. The TNF1/2 polymorphism was not responsible for striking differences in the capacity of cells to produce TNFα, as shown by Bouma et al. (14). However, studies with larger sample size and with different cell stimulants must be performed to draw final conclusions.

The association of HLA-DRB1*15 alleles with ANCA-positive UC is not a generally accepted phenomenon. Opposing findings might be attributed to the variability in HLA-DRB1*15 allelic frequencies in different ethnic groups. HLA-DRB1*1502, the commonest allele in Japanese and Jewish populations, is supposed to be responsible for the HLA-DRB1*15 association with UC (5, 34, 41). However, the HLA-DRB1*15 allele frequency was also increased in Dutch and Spanish white UC patients (7, 42). To test this hypothesis, specific typing of HLA-DRB1*15 alleles was carried out. There was a low frequency of the DRB1*1502 allele in our population, and the association of HLA-DRB1*15 alleles with ANCA-positive UC could not be attributed to the HLA-DRB1*1502 allele. In view of this fact and the conflicting results concerning HLA-DRB1*15 association with ANCA-positive UC, a linkage between HLA-DRB1*15 and susceptibility genes for ANCA-positive UC, at least in some ethnic groups, is a plausible explanation (39).

Remarkable dissimilarities were found in the distribution of HLA-DR-DQ/TNF1/2 phenotypic combinations in controls compared with patients with UC. There is some disagreement with regard to the role of the well-known HLA-DR3-DQ2-TNF2 haplotype in the outcome of IBD. On the one hand, a protective effect of the HLA-DR3-DQ2 haplotype against IBD has recently been reported (42, 43). On the other hand, an expected similar negative correlation between the TNF2 allele and IBD has not been observed (44, 45). Moreover, the association of the TNF2 allele with ANCA-positive UC has been shown by others and in this study (40, 46). We suspect a different influence of the TNF2 allele and HLA-DR3-DQ2 haplotype on the outcome of IBD, as has recently been shown for fistulizing CD (47). The analysis of the HLA-DR3-DQ2/TNF2 phenotypic combinations showed an increased frequency of the −/+ phenotypic combination and decreased frequency of the +/− combination in UC patients. The number of phenotypically informative individuals was very small because of the strong linkage disequilibrium between HLA-DR-DQ and TNF1/2. Given the population size of Estonians (1.5 millions) and the low incidence of IBD, it is very difficult to obtain statistically significant results in this population. A study with sufficient power would require multicentric collaboration or selection of patients with a special genetic background.

This is the first study investigating the possible involvement of E-selectin and L-selectin, in addition to other adhesion molecule polymorphisms (ICAM-1), in the pathogenesis of IBD. We could not confirm the previously observed increased frequency of the uncommon ICAM-1 R241 allele among ANCA-negative UC and ANCA-positive CD patients (6). This discrepancy might be attributed to ethnic differences. In addition to the differences in the UC and CD incidence rates in the Estonian population, there was a markedly higher frequency of the less common R241 allele in our control group (14.3%) than in the control group used by Yang et al. (6.4%) (6). Investigated polymorphisms in the gene regions encoding for E-selectin and L-selectin seemed to have no influence on the aetiopathogenesis or outcome of IBD.

In conclusion, we observed statistical associations between

HLA-DRB1 and TNFα polymorphisms and subgroups of IBD patients. Statistical associations provide no evidence of a pathogenic role but suggest an involvement in the pathogenesis. These polymorphisms might be directly associated with IBD or could be linked to certain susceptibility genes. At least in some ethnic groups, ANCA appears to be a useful marker in combination with genetic markers such as HLA-DRB1*15 alleles, by dividing IBD patients into more homogeneous subgroups with putative disparate pathogenic pathways. The HLA-DR3-DQ2 haplotype and the TNF2 allele, in strong linkage disequilibrium with each other, might have different influences on the outcome of IBD. Further studies with more patients are needed to test this hypothesis.

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# CURRICULUM VITAE

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### Education

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| 1987 | Tartu Secondary School No.10 |
| 1993 | M.D., University of Tartu, therapy |
| 1993–1994 | Internship, Tartu Policlinic |
| 1994–1999 | Postgraduate student in gastroenterology, Department of Internal Medicine, University of Tartu |
| 1999– | Resident in gastroenterology, Department of Internal Medicine, University of Tartu |

### Special courses

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| 1996 | 4th Postgraduate Course of Hepatobiliary Diseases, University of Ljubljana, Slovenia; |
| 1998 | Training at the Department of Gastroenterology, University of Lübeck School of Medicine,Lübeck, Germany (six weeks); |
| 2000 | 3rd Scandinavian Course on Inflammatory Bowel Diseases, Örebro, Sweden |

### Scientific work

Main study areas: immonologic aspects of ulcerative colitis and Crohn's disease, autoantibodies in inflammatory bowel disease and coeliac disease. Fifteen scientific publications, 5 presentations at international meetings.

Member of the Estonian Society of Gastroenterology and Estonian Junior Doctors' Association.

## Honours

1995 4th United European Gastroenterology Week, Berlin, Germany (poster, Young Clinician Prize)
1997 Estonian National Student Research Competition, 2nd prize
1997 Liisa Kolumbus Foundation Scholarship
1998 11th World Congress of Gastroenterology, Vienna, Austria (Young Clinician Prize)
1999 Ants and Maria Silvere and Sigfried Pant Memorial Foundation Scholarship

# CURRICULUM VITAE

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### Haridus

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| 1987 | Tartu 10. Keskkool |
| 1993 | Tartu Ülikooli arstiteaduskond, raviosakond |
| 1993–1994 | Tartu Polikliinik, internatuur |
| 1994–1999 | Tartu Ülikool, doktorantuur (gastroenteroloogia) |
| 1999– | Tartu Ülikool, residentuur (gastroenteroloogia) |

### Erialane enesetäiendus

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|---|---|
| 1996 | *4th Postgraduate Course of Hepatobiliary Diseases*, Ljubljana Ülikool, Sloveenia; |
| 1998 | Täiendus Lübecki Meditsiiniülikooli gastroenteroloogia osakonnas Saksamaal (1,5 kuud); |
| 2000 | *3rd Scandinavian Course on Inflammatory Bowel Diseases*, Örebro, Rootsi |

### Teadustegevus

Peamised uurimisvaldkonnad: haavandilise koliidi ja Crohni tõve immunoloogilised aspektid, autoantikehad põletikulise soolehaiguse ja tsöliaakia korral.

Teaduspublikatsioonide üldarv on 15, viis ettekannet rahvusvahelistel konverentsidel.

Eesti Gastroenteroloogide Seltsi liige; Eesti Nooremarstide Ühenduse liige.

## Tunnustused

1995 4th United European Gastroenterology Week, Berliin, Saksamaa (*Young Clinician Prize*);
1997 Eesti üliõpilaste teadustööde riiklik konkurs, II koht;
1997 Sihtasutus Liisa Kolumbus Mälestusfond, stipendium;
1998 11th World Congress of Gastroenterology, Viin, Austria (*Young Clinician Prize*);
1999 Ants ja Maria Silvere ning Sigfried Panti mälestusstipendium

# DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

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