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IMMUNE RESPONSE TO *HELICOBACTER PYLORI* AND ITS ASSOCIATION WITH DYNAMICS OF CHRONIC GASTRITIS AND EPITHELIAL CELL TURNOVER IN ANTRUM AND CORPUS

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To my Mother and Father

This dissertation is dedicated to the 30th anniversary of co-operation between Estonian and Finnish gastroenterologist

Absence of evidence is not evidence of absence.

Renato Baserga

(The cell cycle. N Engl J Med 1981, 304: 453–459).

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications and on unpublished data:

I **Vorobjova T**, Kisand K, Haukanõmm A, Maaroos H-I, Wadström T, Uibo R. The prevalence of *Helicobacter pylori* antibodies in a population from southern Estonia. Eur J Gastroenterol Hepatol 1994, 6: 529–533.

II Uibo R, **Vorobjova T**, Metsküla K, Kisand K, Wadström T, Kivik T. Association of *Helicobacter pylori* and gastric autoimmunity: a population-based study. FEMS Immunol Med Microbiol 1995, 11: 65–68.

III **Vorobjova T**, Nilsson I, Kull K, Maaroos H-I, Covacci A, Wadström T, Uibo R. CagA protein seropositivity in a random sample of adult population and gastric cancer patients in Estonia. Eur J Gastroenterol Hepatol 1998; 10: 41–46.

IV **Vorobjova T**, Grünberg H, Oona M, Maaroos H-I, Nilsson I, Wadström T, Covacci A, Uibo R. Seropositivity to *Helicobacter pylori* and CagA protein in schoolchildren of different age living in urban and rural areas in southern Estonia. Eur J Gastroenterol Hepatol 2000, 12: 97–101.

V **Vorobjova T**, Maaroos H-I, Rägo T, Zimmermann A, Uibo R. *Helicobacter pylori (H. pylori)* in gastric mucosa of children with abdominal complaints. Immunohistochemistry detects antigen-reactive corpus mucosa cells. *Helicobacter* 1998, 3: 103–109.

VI Maaroos H-I, **Vorobjova T**, Sipponen P, Tammur R, Uibo R, Wadström T, Keevallik R, Villako K. An 18-year follow-up study of chronic gastritis and *Helicobacter pylori*: association of CagA positivity with development of atrophy and activity of gastritis. Scand J Gastroenterol 1999; 34: 864–869.

VII **Vorobjova T**, Faller G, Maaroos H-I, Sipponen P, Villako K, Uibo R, Kirchner T. Significant increase in antigastric autoantibodies in a long-term follow-up study of *H. pylori* gastritis. Virchows Arch 2000, 437: 37–45.

VIII **Vorobjova T**, Maaroos H-I, Sipponen P, Villako K, Uibo R. Apoptosis in different compartments of antrum and corpus mucosa in chronic *Helicobacter pylori* gastritis. An 18-year follow-up study. Scand J Gastroenterol 2001, 36: 136–143.

IX **Vorobjova T**, Ananieva O, Maaroos H-I, Sipponen P, Villako K, Utt M, Nilsson I, Wadström T, Uibo R. Seropositivity to *H. pylori* heat shock protein 60 is strongly associated with intensity of chronic inflammation, particularly in antrum mucosa: an extension of an 18-year follow-up study of chronic gastritis in Saaremaa, Estonia. FEMS Immunol Med Microbiol 2001; 30: 143–149.

X **Vorobjova T**, Hürlimann S, Zimmermann A, Uibo R, Halter F. Glandular proliferation and homeostasis of specific cells are differently affected in gastric antrum and corpus in *Helicobacter pylori* associated gastritis. Submitted for publication in Virchows Archiv.

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ABBREVIATIONS

ACAB	anticanalicular autoantibodies
AI	apoptotic index
APAAP	alkaline phosphatase anti-alkaline phosphatase method
BCIP	5-bromo-4-chloro-3-indol-phosphate
BSA	bovine serum albumin
cag A	cytotoxin-associated gene A
Cag A	cytotoxin-associated CagA protein, coded by *cag*A
CCUG	Culture Collection of University of Gothenburg, Sweden
CI	confidence interval
D-cells	somatostatin cells
DNA	deoxyribonucleic acid
dUTP	deoxyuridine triphosphate
ELISA	enzyme-linked immunosorbent assay
FC	foveolar cells
G-cells	gastrin cells
GP	glandular part
H+, K+ ATP-ase	H+, K+ adenosine triphosphatase
H. pylori	*Helicobacter pylori*
HSP60	heat shock protein 60
HMFG	human milk fat globule membranes
IgA,G, M	Immunoglobulin class A, G, M
IF	immunofluorescence
IL	interleukin
kb	kilobase
kD	kiloDalton
LI	labelling index
MALT	mucosa-associated lymphoid tissue
NAP	neutrophil activating protein
NBT	nitro blue tetrazolium
NCTC	National Collection of Type Cultures, London
OD	optical density
OR	odds ratio
PAI	pathogenesity island
PAP	peroxidase-antiperoxidase method
PCA	parietal cell antibodies
PCNA	Proliferating Cell Nuclear Antigen
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PZ	proliferating zone
RAA	relative antibody activity
SD	standard deviation

SDS-PAGE	Sodium-dodecyl-sulphate polyacryl-amide-gel-electrophoresis
TdT	terminal deoxynucleotidyl transferase
TFSS	type IV secretion system
TRIS	Tris (hydroxymethyl) aminomethane
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling
vac A	gene for vacuolating toxin
Vac A	vacuolating toxin

BACKGROUND

The long tradition of research into chronic gastritis in Estonia, initiated by Lauri Walk in the late 1930s and expanded further by a group of gastroenterologists from the University of Tartu under Prof. Kaljo Villako's guidance forms the background of present study. The long-term follow-up research of gastritis in a randomly selected population from Saaremaa Island undertaken by Prof. K. Villako in 1979, and conducted in close collaboration with a Finnish team under the aegis of Prof. Max Siurala since 1971, revealed that chronic gastritis is a very common disease among Estonians, with peculiarities of behaviour in the antrum and corpus mucosa.

Since the discovery of *H. pylori* in 1982, it become evident that enhanced gastric inflammatory response, development of gastric mucosa atrophy and increased risk for peptic ulcer and gastric cancer are associated with this gastric pathogen. Infection with *H. pylori* strains expressing cytotoxin-associated CagA protein constitutes factors particularly associated with the aforementioned diseases.

Based on the above mention considerations as well as on retrospective studies on the frequency of *H. pylori* in gastric biopsies from random samples of Estonian populations, peptic ulcer patients as well as hospitalized children (Maaroos *et al.* 1990, 1991a, 1991b), the present study was at first undertaken, as a cross-sectional investigation of seropositivity for *H. pylori* in two Estonian adult populations and in a population of schoolchildren, since serology is the most suitable indicator of infection rate in epidemiological studies. A large-scale epidemiological study of several immunologically mediated diseases organized by the Tartu University Department of Immunology, which started in 1990 in two Southern Estonian small towns, offered a good opportunity to fulfil the primary aim of the present study. A cross-sectional study of schoolchildren, carried out in 1993–1996 by the staff members of the Tartu University Department of Paediatrics, made it possible to determine the seroprevalence of *H. pylori* in nonhospitalized children.

The background of our seroepidemiological investigations includes also our meeting with Prof. Torkel Wadström during the Congress of Interlec 11 (Tartu-Tallinn, 1989). He and his colleagues from the University of Lund, particularly Ingrid Nilsson, introduced to us serological methods for determination of *H. pylori* and supplied us with *H. pylori* antigens.

The invaluable opportunity to use the samples of the 18-years follow-up study of chronic gastritis in Saaremaa, prompted us to investigate whether seropositivity for *H. pylori* antigens, such as acid glycine extracted cell surface proteins, CagA and heat shock protein 60 (HSP 60), which are highly immunogenic, is associated with increased recruitment of inflammatory cells as local immune response of the host to this pathogen and, specifically, with progression of atrophic gastritis in a direct long-term follow-up.

A considerable proportion of *H. pylori* infected patients develop autoimmune reactions against gastric epithelial cells. In particular, anticanalicular autoantibodies (ACAB), reacting against canalicular structures within human parietal cells, can be demonstrated in 30% of infected patients. Gastric H+, K+ — adenosine triphosphatase (H+, K+ ATP-ase) represents the major autoantigen of these autoantibodies. This particular type of antigastric autoimmunity can be associated with atrophic corpus gastritis (Negrini *et al.*1996, Faller *et al.* 1996, Claeys *et al.* 1998). Hence it was our aim to evaluate the significance of ACAB in development of gastric corpus mucosa atrophy, which represented an extension of an earlier research of parietal cell antibodies (PCA) in chronic gastritis patients and in a population sample during follow up in Estonia (Salupere 1968, Uibo *et al.* 1984a, 1989, 1991). This part of the study was performed under the kind supervision of Dr. Gerhard Faller and Prof. Thomas Kirchner from the University of Erlangen-Nürnberg, pioneers of the elaboration of the method for detection of anticanalicular autoantibodies.

There is evidence that *H. pylori* is associated with changes of epithelial cell turnover and increased apoptosis (programmed cell death) in the gastric epithelium, which can lead to development of atrophy of the gastric mucosa (Moss *et al.* 1996, Peek *et al.* 1997, Correa and Miller 1998, Steininger *et al.* 1998, von Herbay and Rudi 2000). However, in previous studies apoptosis was investigated only in one gastric area, without taking account of the fact that *H. pylori* colonization, as well as activity of gastritis and chronic inflammation can exert a different effect on apoptosis of epithelial cells in the antrum and corpus. Moreover, also some follow-up studies evaluated apoptosis only at the initial time point (Moss *et al.* 1999). Therefore, in the present study we attempted to examine gastric epithelial cell apoptosis both in the antrum and in corpus at the initial and end points of a long-term follow-up study. Also we tested the hypothesis that increased apoptosis may be responsible for progression of atrophy and is associated with higher degree of chronic inflammation, activity of gastritis and *H. pylori* colonization. Moreover, upregulation of apoptosis protectors, like bcl-2 oncoprotein, by presence of *H. pylori*, as well as enhancement of epithelial cell proliferation may supply cells with a survival advantage and hence play a role in carcinogenic pathway (Hockenberry *et al.* 1990, Lauwers *et al.* 1994). For a better understanding of the role of *H. pylori* and grade of gastritis in this aspect of cell turnover, as well as on the density of antral and corpus specific cells, an investigation of consecutive patients, undergoing gastroduodenoscopy in the University Hospital of Bern was undertaken under the kind supervision of Prof. Fred Halter and Prof. Arthur Zimmermann.

Taking into consideration the peculiarities of the behaviour of gastritis in the antrum and corpus (Stolte *et al.* 1990), we aimed also to analyse whether there exist dissimilarities between the antrum and corpus mucosa in immune response to different *H. pylori* antigens, in association with development of atrophy and in the effect of *H. pylori* on cellular turnover.

REVIEW OF LITERATURE

H. pylori is a gastric pathogen, which penetrates the gastric mucus layer, facilitated by its spiral shape and multiple flagella, and colonizes the lower part of the mucus layer of the stomach mucosa (Warren and Marshall 1983). Adhesion of *H. pylori* to gastric epithelial cells, involving the interactions between sialic acid-binding proteins of *H. pylori* with gastrointestinal mucins (Borén *et al.* 1993, Hirmo 2000) and Lewis blood group antigens (Klaamas *et al.* 1997, Kurtenkov *et al.* 1999, Guruge *et al.* 1998, Ilver *et al.* 1998), as well as the production of specific virulence factors, like urease and cytotoxins, allow for *H. pylori* to adapt excellently to the gastric environment and to contribute to mucosal damage, either directly or indirectly by mediating inflammatory response to *H. pylori.*

H. pylori infection is common world-wide and it is estimated that over one half of the world is infected with this organism (Mégraud *et al.* 1989, Taylor and Blaser 1991, Pounder 1995), whereas prevalence is much more common in developing countries, where most children are infected by 10 years of age (Taylor and Parsonnet 1995). Acquisition of *H. pylori* is a birth cohort-dependent phenomenon (Parsonnet *et al.* 1992, Banatvala et *al.* 1993, Cullen *et al.* 1993, Sipponen 1995, Sonnenberg 1995). It is established that most infections with *H. pylori* occur in childhood (Banatvala *et al.* 1993, Sipponen 1995) and that early acquisition of *H. pylori* results in more intense inflammation and early development of atrophic gastritis with higher risk of gastric cancer and gastric ulcer (Blaser *et al.* 1995, Sipponen *et al.* 1998).

There is evidence that *H pylori* causes acute and chronic inflammation in the stomach, the degree of inflammation varies from host to host and is dependent on pathogen strains (Covacci *et al.* 1999). *H. pylori* is recognized as a causative pathogen for gastritis and is strongly associated with peptic ulcers and gastric cancer (Maaroos *et al.* 1991a, 1994, Vorobjova *et al.* 1991, Parsonnet *et al.* 1991, Correa 1992, Cover and Blaser 1992a, Halter *et al.* 1992, Forman 1996, Kawaguchi *et al.* 1996, Sipponen *et al.* 1998, Danesh 1999, Sipponen and Marshall 2000). The disease associations are largely dependent on the phenotypic appearance of gastritis in the stomach (Sipponen 2001), on the *H. pylori* genotypes and also is determined by a complex interaction between host and bacterial factors (Ernst *et al.* 2000a, Jenks and Kusters 2000, Blaser and Berg 2001, Israel *et al.* 2001, Tham *et al.* 2001).

Virulence factors of *H. pylori* and pathogenesis of infection

The virulence factors as flagella and urease, required for colonization and survival in the human stomach, are produced by all *H pylori* strains (Covacci *et al.* 1999). Also, neutrophil activating protein (NAP) and vacuolating cytotoxin A (VacA), which act directly on the surrounding gastric mucosa, are expressed by all strains of *H. pylori.* NAP is involved in the recruitment of neutrophils to

the gastric mucosa and may contribute to inflammatory response (Evans *et al.* 1995).

Vacuolating cytotoxin A (VacA)

VacA is known as the major exotoxin, produced by *H. pylori*, which induces cytoplasmic vacuolation in eukaryotic cells (Leunk *et al.* 1988, Cover *et al.* 1990, Cover and Blaser, 1992b). VacA consists of 95 kDa monomers proteolytically cleaved into 37 kDa and 58 kDa fragments. One subunit is endowed with biological activity, while the other recognises the receptor on target cells and mediates adhesion (Telford *et al.* 1994). An important property of VacA is alteration of intracellular vesicular trafficking in eukaryotic cells leading to the formation of large vacuoles. Additionally, VacA causes changes in transepithelial electrical resistance in polarised monolayers and forms ion channels in lipid bilayers of cell membrane (Reyrat *et al.* 1999). The *vac*A gene is a mosaic that varies between strains, particularly in the region encoding its signal sequence (s) and its mid-region (m) (Atherton *et al.* 1995). The toxicity of *H. pylori* strains depends largely on presence of the cell binding domain which exists in two allele forms, m1 and m2, of the mid-region of the *vac*A gene, whereas only the m2 cytotoxin is able to induce vacuolisation in primary gastric cells and is found in populations that have a high prevalence of peptic ulcer and gastric cancer (Pagliaccia *et al.* 1998). Also, presence of antibodies to VacA is more frequently observed in patients with gastric adenocarcinoma (De Figueiredo *et al.* 1998, Grimley *et al.* 1999). A recent study of Sillakivi *et al.* (2001) in Estonia revealed that there are differences in the distribution of *vac*A s and m subtypes of *H. pylori* between Estonian and Russian patients with the above mentioned disease: namely, the s1a/m1 subtype was found predominantly in Estonian patients and the s1a/m2 subtype in Russian patients, which indicate the diversity of *H. pylori* genotypes among different nationalities in Estonia.

Pathogenesity island (PAI) and cytotoxin-associated gene A protein (CagA)

H. pylori strains are grouped into two families, type I and type II, based on the fact whether they express or do not express the vacuolating cytotoxin (VacA) and the CagA antigen (cytotoxin-associated gene A protein) (Xiang *et al.* 1993). CagA is an immunodominant antigen of *H. pylori* with molecular weight ranging from 128 kDa to 140 kDa (Covacci *et al.* 1993). There is evidence that patients with duodenal ulcers and gastric cancer are most often infected by type I strains (Covacci *et al.* 1993, Xiang *et al.* 1993). The CagA gene is present only in type I strains, while the *vac*A gene is present in both types and an active toxin is produced only by type I strains (Xiang *et al.* 1993).

Censini *et al.* (1996) have shown that the difference between type I and type II is restricted not only to the *cag*A and *vac*A genes but is due to presence in type I strains of a 40-kb pathogenesity island (PAI) that contains the *cag*A gene (Censini *et al.* 1996). Cag PAI induces epithelial cells to secrete IL-8, a mediator of inflammation, by activating nuclear factor kappa B (NFκB) complexes,

and induces remodelling of cell surface as well as pedestal formation (Censini *et al.* 1996, Segal *et al.* 1996). Cag-PAI encodes an ancient, contact-dependent secretion system, the so called type IV secretion system (TFSS) that serves to transfer a variety of multimolecular complexes across the bacterial membrane to the extracellular space or into other cells (Covacci *et al.* 1999, Christie *et al.* 2000). It has been demonstrated recently that via TFSS *H. pylori* translocates CagA protein directly into gastric epithelial cells where it is tyrosine-phosphorylated by host cell kinases (Segal *et al.* 1999, Asahi *et al.* 2000, Backert *et al.* 2000, Odenbreit *et al.* 2000, Stein *et al.* 2000), followed by a modification of host cell signal transduction pathways, resulting in actin reorganization and host cell morphological changes, such as elongation and spreading of host cells (Segal *et al.* 1999). CagA is necessary to induce a growth-factor–like phenotype (hummingbird) in host gastric cells and may play an important role in carcinogenesis associated with type I infection. Additionally, a second cellular phenotype, induced after attachment by *H. pylori* (stress fiber associated, SFA), is CagA independent and is produced by type I and type II *H. pylori* and may play a role in the pathogenesis of gastritis (Segal *et al.* 1999). Thus, the importance of CagA in the pathogenesis of peptic ulcer, gastritis and gastric cancer is associated with the fact that CagA is the first *H. pylori* protein shown to be translocated into the host and the one targeted to the host cell signal transduction pathway (Backert *et al.* 2000, Censini *et al.* 2001). Israel *et al.* (2001) showed that the ability of *H. pylori* strains to induce epithelial cell response related to inflammation depends on the presence of an intact *cag* PAI.

Heat shock protein (HSP) of H. pylori

In 1992, Dunn *et al.* identified a homologue of the chaperonin 60 family of heat shock proteins (HSP) in *H. pylori* and showed that this protein is immunogenic in individuals infected with *H. pylori*. HSPs are a highly conserved group of proteins found in all (prokaryotes as well eukaryotic) organisms (Craig *et al.* 1993). *H. pylori* synthesizes two HSPs: HSP A (GroES or HSP 10 homologue) and HSP B (GroEL or HSP 60 homologue) (Dunn *et al.* 1992, Macchia *et al.* 1993, Suerbaum *et al.* 1994). To protect itself against the host, the pathogen activates various evasion mechanisms including HSP synthesis (Zügel and Kaufmann 1999). On the other hand, several reports have shown that *H. pylori* HSP60 plays a role in induction of chronic mucosal inflammation, suggesting also the triggering role of HSP60 by an autoimmune response, which results in gastric atrophy (Engstrand *et al.* 1991, 1993, Kamiya *et al.* 1998). It has been shown that purified HSP60 of *H. pylori* is able to stimulate secretion of IL-8 from gastric epithelial cells and can be considered one of the virulence factors which plays an important role in mucosal inflammation (Kamiya *et al.* 1998).

Immunogenic cell surface associated proteins of *H. pylori* are summarised in Table 1. (according to Nilsson, 1998 with modifications):

MW (kDa)	Protein	References
120–140 (CagA)	Cytotoxin-associated gene A protein	Covacci *et al.*1993
37, 58, 95 (Vac A)	Vacuolating cytotoxin A	Telford *et al.* 1994
74 (BabA)	Adhesin (receptor) for the Lewis^b antigen	Ilver *et al.* 1998
26.5-30, 61.5	Urease subunits (A, B, E, F, G, H, I)	Hu and Mobley 1990
58 (HspB) 13 (HspA)	Heat shock-proteins, GroEL and GroES homologues	Dunn *et al.* 1992, Suerbaum *et al.* 1994
53 (FlaA) 54 (FlaB) 29 (HpaA)	Flagellin Flagellar sheath, outer membrane lipoprotein	Kostrzynska *et al.* 1991 O'Toole *et al.* 1995
31, 48–50, 67	Porins	Hancock and Exner 1997
29–31, 33	Immunogenic cell surface proteins with high specifity	Nilsson *et al.* 1997
26	Cell surface exposed protein, *H. pylori* specific	O'Toole *et al.* 1991
25	Lectin-like immunogenic adhesin	Oderda *et al.* 1997
19	Outer membrane protein, species specific	Drouet *et al.* 1991

Host response to *H. pylori*

In most bacterial infections, the host's immune defence is able to clear infection. This is not the case with *H. pylori* infection, which continues to persist despite strong mucosal and systemic antibody responses and local inflammatory responses to the pathogen (Figura 1996, Sommer *et al.* 1998).

Non-specific host response

Host defence against microbial infection is a complex of non-specific barriers and non-specific and specific immune responses. The first factors which respond to invasion of *H. pylori* include digestive enzymes, lysozyme, lactoferrin, pepsin and gastric acid. The mucus layer of the stomach is the final non-specific barrier against the bacteria. The glycoproteins of the mucus layer build a net containing lectin-like adhesins, which is necessary for prevention of bacterial penetration. However, the spiral shape of *H. pylori* and flagella allows *H. pylori* to penetrate through this glycoprotein gel and reach gastric epithelial cells (Andersen and Holck 1996).

Non-specific immune response

Initial non-specific immune response to *H. pylori* suggests a strong neutrophilic component (Sobala *et al.* 1991). Severity of mucosal injury appears to be directly correlated with the extent of neutrophilic infiltration (Mai *et al.* 1992, Blaser 1992, Davies et al. 1994, Fiocca *et al.* 1994). *H. pylori* antigenic components, including the water-soluble *H. pylori* neutrophils activating protein (NAP) and the N-terminal end of the large subunit of *H. pylori* urease are chemotactic for neutrophils (Mai *et al.* 1992, Evans *et al.* 1995). Water extracts of *H. pylori* contains NAP which increase the surface expression of CD11b/CD18 on neutrophils, and facilitate and increase adhesion of neutrophils to endothelial cells via the intercellular adhesion molecule-1 (ICAM-1) (Yoshida *et al.* 1993). Attraction of inflammatory cells to the site of infection results in generation of reactive oxygen metabolites released by phagocytes, activated by *H. pylori,* which cause local mucosal damage, more expressed in case of cytotoxin-producing strains (Davies *et al.* 1994, Rautelin *et al.* 1996). When destruction of the gastric epithelium is progressing, the bacteria might be confronted with numerous phagocytes, which however, appears to be in-effective from the phagocytotic point of view. Recent observations suggests that *cag*-encoded factors through TFSS are translocated into host cells inducing anti-phagocytic response that may play an essential role in the immune escape of this persistent pathogen (Allen *et al.* 2000, Ramarao *et al.* 2000, Odenbreit *et al.* 2001).

Adherence of *H. pylori* to epithelial cells induces increased gene expression and secretion of neutrophil-activating and chemotactic chemokines such as IL-8 (Crabtree *et al.* 1994, Sharma *et al.* 1995, Crowe *et al.* 1995, Rieder *et al.* 2001). Expression of IL-8 in gastric epithelial cells is upregulated by the inflammatory cytokines TNFα and IL-1 following activation of the transcription nuclear factor κB (NF-κB) (Sharma *et al.* 1998), as well as by direct bacterial stimulation (Grabtree 1996a).

The ability of *H. pylori* to control IL-8 induction is associated with *cag*A genes in PAI, through tyrosine phosphorilation in host cell (Yakabi *et al.* 2000), with the *picB* gene (Tummuru *et al.* 1995) as well as with the *ice*A 1 (induced by contact with epithelium) gene (Peek *et al.* 1998) and is dependent on activation of protein kinase C (Kassai *et al.* 1999).

Cytokines, derived from inflammatory, stromal or epithelial cells, as well as expression of growth factors may modulate the growth and differentiation of the epithelial cells and contribute to gastric carcinogenesis (Sipponen *et al.* 1998). Cytotoxic *H. pylori* strains are stronger inducers of IL-8 expression and are associated with an increased antral infiltration of polymorphonuclear leukocytes (Cover *et al.* 1993, Crabtree *et al.* 1994, Sharma *et al.* 1995, Blaser 1996).

There are data providing some peculiarities of local immune response in *H. pylori*-infected children. Thus children demonstrate a chronic, macrophagic and monocytic inflammatory cell infiltrate and a lack of neutrophils as compared with the response observed in adults (Ashorn *et al.* 1995, Torres *et al.*

2000). In infancy and early childhood the stomach lacks a significant number of immunocompetent lymphocytes and plasma cells (Torres *et al.* 2000). Immaturity of immune system in early childhood may explain the dissimilarities between childhood and adult gastritis (Sipponen *et al.* 1996a, Meining *et al.* 1996).

H. pylori-specific immune response

H. pylori, a bacterium, harbouring highly immunogenic proteins like CagA, HSP60, flagella associated proteins as well as urease, releasing toxins and living close to epithelial surface, induces specific host immune response (Crabtree 1996a). Immunohistochemical studies have shown that in presence of *H. pylori,* T-cells (both CD4+ and CD8+ lymphocytes) increase in amount and can be located very close to the epithelium (Hatz *et al.* 1996, Bamford *et al.* 1998). During effector-specific immune response, different patterns of cytokine release are characteristic of certain functional T-helper (Th) cell subsets, Th1 and Th2 (Romagnani 1991, Del Prete 1998). There is evidence that during infection with *H. pylori,* the Th1 phenotype is dominating (Karttunen *et al.* 1995, D'Elios *et al.* 1997, Bamford *et al.* 1998, Sommer *et al.* 1998). Upon stimulation Th1 type cells produce proinflammatory cytokines (IFN-γ, TNF-α, IL-1, IL-2, IL-6, IL-8), whereas Th2 type cells produce predominantly anti-inflammatory cytokines (IL-4, IL-5, IL-10, IL-13, TNFβ, PGE) (Del Prete 1998, Bamford *et al.* 1998, Lindholm *et al.* 1998, Ernst 1999). In inflammatory response, associated with *H. pylori* infection, pro-inflammatory cytokines are predominantly induced and the balance of pro- and anti-inflammatory cytokines is inclined towards inflammation (Ernst 1999). It was suggested that during evolutionary adaptation to the stomach mucosa, *H. pylori* might have gained the ability to evoke "wrong" Th1 immune response, which may block the development of protective Th2 immune response and therefore induce chronic inflammation by production of IFN-γ (Sommer *et al* 1998).

Polarized Th1 type response to *H. pylori* may contribute to the pathogenesis of gastric disease (D'Elios *et al.* 1997). *H. pylori* is associated with a strong expression of class II transplantation antigens on the epithelial cells (Engstrand *et al.* 1989). The IFN-γ produced by stimulated Th1 cells increases the expression of class II MHC molecules, possible receptors for *H. pylori.* The IFN-γ increases attachment of *H. pylori* to gastric epithelial cells and induces apoptosis in infected cells expressing class II MHC molecules (Fan *et al.* 1998). It is suggested that VacA toxin interferes with protective immunity by a partial inhibion of MHC class II-dependent antigen presentation (Molinari *et al.* 1998).

Antibody response to H. pylori

Humoral response to *H. pylori* antigens results from antibody synthesis by the gastric B-cells supported by *H. pylori* antigen activated Th cells (D'Elios *et al.* 1998). Although secretory type IgA predominate in the gastric juice of

H. pylori-infected patients, majority of *H. pylori*-specific IgA are non-secretory (Birkholz *et al.* 1998). It was shown that the gastric mucosa of *H. pylori*-infected individuals contains large numbers of *H. pylori*-specific IgA-secreting cells (Ahlstedt *et al.* 1999). IgA plasmocytosis is associated with expression of the secretory component in the foveolar epithelial cells, which enables transepithelial IgA transport and a fine coating of *H pylori* with IgA immunoglobulins (Kirchner *et al.* 1990). In spite of local IgA response, immunological elimination of *H. pylori* from the stomach is inefficient (Borody *et al.* 1992, Blaser and Parsonnet 1994). For reaching the antigenic target, *H. pylori*, which remains on the luminal side of the epithelial barrier, IgA and IgM antibodies, produced by immunocytes, must be translocated through the epithelium. External transport of polymeric immunoglobulins into secretion to provide secretory IgA and IgM depends on the production of J (joining) chain by the mucosal immunocytes (Brandtzaeg and Korsrud 1984). In the study of Berstad *et al.* (1999) it was found that mucosal immunocytes show reduced J chain expression in case of chronic inflammation. Only J chain-positive IgA and IgM immunocytes can contribute to secretory immunity (Brandtzaeg 1995, Brandtzaeg *et al.* 1999). Thus, reduced J chain expression in gastritis may contribute to production of IgA as monomers instead of polymers (Berstad *et al.* 1999). Secretory immune system appears to be unable to eradicate *H. pylori* probably because of little or no secretory IgA antibodies elicited against this bacterium (Birkholz *et al.* 1998). On the other hand, the protective role of secretory IgA received with breast milk, against *H. pylori* colonization in early childhood (Thomas *et al.* 1993) or the result of active or passive IgA local vaccines in experimental animals are well known facts (Czin *et al.* 1993, Lee *et al.* 1995, Goto *et al.* 1999). Mucosal IgA response is important in blocking of bacterial adherence, in inhibition of the motility of *H. pylori* and in neutralization of toxins (Figura and Crabtree 1994, Crabtree 1996a). Crabtree *et al.* (1991) have shown that mucosal IgA recognition of the *H. pylori* CagA protein was associated with increased mucosal polymorph infiltration and epithelial surface degeneration. A recent study of Oksanen (2001) showed that presence of serum IgA antibodies to *H pylori* indicates higher risk for CagA-positive *H. pylori* infection and possibly more severe late sequelae of gastric diseases.

Serum antibodies to *H. pylori* antigens of classes IgG, IgA and less frequently IgM are detected in infected individuals (Kosunen *et al.* 1992) and the diagnostic significance of serum immune response, especially of the IgG class, is evident (Feldmann *et al.* 1995, Marchildon *et al.* 1996). Titres of serum antibodies to *H. pylori* fall with eradication of bacteria (Kosunen *et al.* 1992; Kato *et al.* 1999). In a study of Kosunen *et al.* (1992) it was shown that the high sensitivity (97%) of IgG antibody tests and the 50% decrease in IgG within 6 months after eradication of *H. pylori* infection have made IgG the most useful immunoglobulin class for follow-up of antimicrobial therapy in individual patients. IgA and IgM titres, initially elevated in 64% and 4% of patients, showed similar trends as IgG antibodies after eradication. In the study of Wang

et al. (1994), half patients became IgG negative at 6 months after treatment, and nearly all patients became IgG negative at 12 months. A long-term follow-up study of IgG and IgA in children after *H. pylori* eradication showed that approximately half patients with successful eradication remained IgG-seropositive and IgA-seropositive at 12 months' after treatment, whereas the decrease in the titre of the IgA class was significantly larger (Kato *et al.* 1999).

Systemic immune response to the highly immunogenic CagA protein (Cover *et al.* 1990, Sobala *et al.* 1991, Crabtree *et al.* 1991, Covacci *et al.* 1993) has revealed an association between the IgG antibodies to CagA and peptic ulcer, gastric epithelial degeneration, increased activity of gastritis, atrophy, intestinal metaplasia and gastric cancer (Figura *et al.* 1990, Cover *et al.* 1990, 1995, Crabtree *et al.* 1991, 1993, Kuipers *et al.* 1995, Beales *et al.* 1996, Ando *et al.* 2000, Oksanen *et al.* 2000, Vaucher *et al.* 2000). Systemic immune response to CagA in children was also found to be associated with duodenal ulcer, high score of chronic inflammation, increased activity of gastritis and high level of serum pepsinogen (Oderda *et al.* 1993, Korzon *et al.* 1999, Plebani *et al.* 1999, Lerro *et al.* 2000).

The high immunogenicity of HSP60 (HspB) of *H. pylori* was shown first by Dunn *et al.* in 1992. Several studies have supported the idea that *H. pylori* HSP60 is an immunodominant protein (Engstrand 1992, Yokota *et al.* 1994). HSP60 is located on the surface of the bacteria, which allows the host immune system to better recognize it (Dunn *et al.* 1992, 1997, Yamaguchi *et al.* 1996, Yunoki *et al.* 2000). Humoral immune response against *H. pylori* HSP60 is strongly induced in patients with *H. pylori* infection, and there exists a correlation between serological and mucosal inflammatory response to *H. pylori* (Macchia *et al.* 1993, Perez-Perez *et al.* 1994). Perez-Perez *et al.* (1994) suggest that specific serum IgG response to *H. pylori* HSP may be a marker of inflammation, or these antibodies can play a direct role in the pathogenesis of *H. pylori*-induced inflammation. Moreover, according to the study of Yunoki *et al.* (2000), decrease in inflammation in the gastric mucosa of successfully treated patients correlates with a decrease in the level of antibodies to HSP60. Serum IgA antibodies to HSP60 were found to be associated with *H. pylori* induced gastric atrophy (Barton *et al.* 1998). Macchia *et al.* (1993) discussed the issue why host immune system responds with high levels of antibodies against such a conserved protein like HSP, which should be very well tolerated by the host. Macchia *et al.* (1993) proposed two possibilities: 1). immune response is directed only against epitopes specific for *H. pylori* HSP60, and 2). immune response is directed against epitopes common of *H. pylori* HSP60 and its human homologue, which would mean a failure in host tolerance. It is also suggested that antibodies to *H. pylori* HSP60 could cross-react with their human counterpart playing a role in tissue damage that occurs in *H. pylori*-induced gastritis.

Summarizing this part of the review, concerning immune response to *H. pylori* cell surface proteins including highly immunogenic CagA and HSP60, it is evident that persons infected by *H. pylori* show strong local and systemic

immune response against the bacterial antigens, which is, however, insufficient for clearance of the bacterium and may in turn cause intensive active and chronic inflammation in the gastric mucosa. Cell-mediated response to *H. pylori* is Th1 response, which is normally induced against intracellular pathogens. In *H. pylori* infection, this type of cell-mediated immunity is not only ineffective but may be highly damaging owing to the induction of harmful cytokines, like IL-2, IFN-γ and TNF-α, which enhance inflammation.

Chronic *H. pylori* gastritis

H. pylori is the main cause of chronic gastritis. This gastric pathogen leads to acute and chronic inflammation of the gastric mucosa (Marchall and Warren 1984, Goodwin *et al.* 1986, Sipponen and Hyvärinen 1993). Initially non-atrophic gastritis develops into atrophic gastritis (Villako and Siurala 1981, Villako *et al.* 1982, 1995, Kekki *et al.* 1983, Siurala *et al.* 1985, Maaroos *et al.* 1990a, Sipponen *et al.* 1991). Without treatment *H. pylori* gastritis remains a chronic disease. Spontaneous improvement may occur in the antrum (in elderly persons) with progression of corpus atrophy (Sipponen and Hyvärinen 1993). The course and end-stage of gastritis may be influenced by peculiarities of *H. pylori* strains colonizing gastric mucosa and host response, as well as by presence or absence of autoimmunity or additional environmental factors (Sipponen *et al.* 1991, Blaser and Berg 2000, Jenks and Kusters 2000).

Classification of gastritis

In 1947, *Schindler* recognized acute and chronic gastritis on the basis of gastroscopy and intraoperative biopsy and introduced the terms "chronic superficial" and "chronic atrophic gastritis". This classification was modified in 1968 *by Siurala.* According to Siurala *et al.* (1968) normal mucosa (N): no inflammation, normal glands; chronic (superficial) gastritis without atrophy (S): chronic mononuclear inflammation in the lamina propria without loss of normal mucosal glands; chronic (atrophic) gastritis with slight, moderate or severe atrophy (A1, A2, A3 respectively): slight, moderate or severe (total) loss of normal glands, and of chronic mononuclear inflammation and growth of metaplastic glands of various extent (Sipponen *et al.* 1990a).

The classification of chronic gastritis introduced in 1972 by *Whitehead et al.* was morphological, describing mucosal type (antrum or corpus), grade of gastritis, and activity and presence of metaplasia. In 1973, *Strickland and Mackay* defined two topographical groups of advanced atrophic gastritis: Type A and Type B. Type A is chronic atrophic gastritis of the corpus with parietal cell antibodies (PCA) and elevated basal serum gastrin level, which leads to pernicious anaemia. Type B is antral atrophic gastritis without PCA, and serum gastrin level is within or below normal value. In 1975, *Glass and Pitchumoni* suggested the term "AB" gastritis to describe distribution of gastritis both in the antrum and corpus. The classification of A, B or AB types was proposed to indicate severe forms of atrophic gastritis, and it corresponds to atrophy-

induced changes in gastric function, like output of acid, pepsinogen and the intrinsic factor (in case of type A or AB), or secretion of gastrin (in case of type B) (Sipponen *et al.* 1996a). In 1987, *Kekki et al.* combined both topography and morphology in their classification of gastritis. According to this classification the antrum and corpus mucosa are documented separately and scored as follows: normal (grade 0) — no loss of glands and no round cell infiltration; chronic gastritis with no loss of glands, irrespective of the depth of inflammation, is termed "superficial gastritis" — slight, moderate or severe accumulation of round cells in the lamina propria (grades 0.5, 1.0, 1.5). Atrophy is graded as mild, moderate or severe loss of glands (grades 2, 3, 4). This grading shows an excellent correlation with different functional parameters (Kekki *et al.* 1987). The classifications of gastritis, described by Siurala, 1968, and Kekki, 1987, were used in longitudinal studies of chronic gastritis by Finnish and Estonian investigators (Siurala *et al.* 1968, Varis 1981, 1982, Sipponen *et al.* 1989, 1990a, 1994a, Villako et al. 1976, 1982, 1984, 1990, 1991, 1995, Maaroos *et al.* 1985, 1986, 1990a,b, 1991a,b, 1994).

In 1988, *Correa* and in 1990 *Yardley* classified chronic gastritis into three categories: diffuse antral gastritis (DAG), multifocal (metaplastic) atrophic gastritis (MAG) and corpus-limited atrophic gastritis of autoimmune origin. The disadvantage of this classification was that it does not take into account the fact that gastritis is a progressive disease, assuming that different forms reflect multiple aetiologic and pathogenic pathways (Prise 1991, Sipponen et al. 1996a). The classification of gastritis by *Wyatt and Dixon* (1988) maintains type A for autoimmune type of gastritis, while *H. pylori*-associated gastritis (bacterial) with its predominantly antral distribution is termed type B. Type C (chemical injury) is reflux gastritis or gastritis due to drugs or chemicals. The classification of *Stolte and Heilmann* (1989) is similar, but, in addition the authors regard type AB gastritis as combined autoimmune gastritis and gastritis caused by *H. pylori*. The disadvantage of this classification is non-recognition of the progressive nature of chronic gastritis, developing from superficial to atrophic.

The Sydney System

In 1990, the Working Party at the Scientific Committee of the 9th World Congress in Gastroenterology in Sydney proposed a new gastritis classification which emphasises the importance of combining topographical, morphological and etiological information (Price 1991), and which corresponds to the earlier classifications of gastritis, reflects impairments in function of the gastric mucosa and delineates the gastritis dissimilarly associated with different gastric disorders (Sipponen 1994b).

The Sydney System is divided into two parts, histological and endoscopic. This review focuses on the histological division.

The classification relies on a separate assessment of the antrum and corpus, while a minimum of two biopsies per site are recommended.

The Sydney System recognizes three morphological patterns of gastritis: acute gastritis, chronic gastritis and special forms. *Acute gastritis* is rare, usually a transient condition, in which acute granulocytic inflammation occurs without significant round cell inflammation. *Chronic gastritis* is defined as a disease in which lymphocytic and plasma cell inflammation is a predominant microscopic feature. *Special forms* contain a range of entities of lesions with established histopathological and clinical impacts, such as eosinophilic or lymphocytic gastritis.

Five items of gastritis-related histopathological features have been selected as the most important regarding the grading: *chronic inflammation* (lymphocytic/plasma cell inflammation in lamina propria); *activity* (acute granulocytic inflammation in lamina propria, gastric pits and surface epithelium); *atrophy* (loss of normal glands); *intestinal metaplasia* and density of *H. pylori*. These variables are to be graded as none, mild, moderate or severe.

No graded variables include lymphoid aggregates or follicles, degenerative and hyperplastic alterations of the epithelium, eosinophilic infiltration, *etc.* (Price 1991, Sipponen 1994b).

There exist three patterns of topographical distribution: *antral gastritis, corpus gastritis and pangastritis*. Marked dissimilarities in the grade of mucosal changes between the antrum and corpus are expressed with the terms "antral predominant" or "corpus predominant".

To indicate the aetiology of gastritis, such as *H. pylori*, autoimmune origin or certain gastric irritants, the Sydney System provided an aetiological classification limb (Price 1991; Sipponen 1994b).

Up dated Sydney System

In 1994, at the Houston gastritis workshop, the Sydney System was revised and updated. In general, the principles and grading of the Sydney System were only slightly modified, the grading being provided with a visual analogue scale. For optimal assessment, five biopsy specimens were recommended. Further, the terminology of the final classification was improved to emphasize the distinction between the atrophic and the nonatrophic stomach (Dixon *et al* 1996).

Chronic H. pylori gastritis — a cohort phenomenon

It is estimated that *H. pylori* is a birth cohort-dependent phenomenon (Parsonnet *et al.* 1992, Banatvala *et al.* 1993, Sipponen *et al.* 1994a, Sipponen 1995, 1997, Sipponen *et al.* 1996b, Roosendaal *et al.* 1997). Most *H. pylori* infections occur during childhood (under the age of 20) in every cohort (generation) (Banatvala *et al.* 1993, Sipponen *et al.* 1994a). Incidence of new cases of *H. pylori* infection in childhood has been high in cohorts, born at the beginning of the last

(Sipponen 1997). It is spectacular that after being high in childhood acquision rate slows down exponentially with age in all cohorts, and new infections occur quite rarely in adulthood (annual incidence 0.15–0.4%) (Cullen *et al.* 1993, Kuipers *et al.* 1993, Parsonnet 1995, Valle *et al.* 1996, Sipponen *et al.* 1996b, Sipponen 1997, Roosendaal *et al.* 1997). Prevalence of *H. pylori* infection varies between countries, being usually high in developing countries and low in developed countries (Mégraud *et al.* 1989, Pounder 1995, Mitchell 1999). Across child populations, *H. pylori* prevalence ranges from less than 10% to over 80% (Torres *et al.* 2000). This variation in the prevalence of *H. pylori* infection and *H. pylori* gastritis between different populations and countries can be explained by differences in the rate of *H. pylori* acquision in childhood, which is largely dependent on socio-economic conditions in childhood (Jones *et al.* 1987, Drumm *et al.* 1990, Mendall *et al.* 1992, Webb *et al.* 1994, Patel *et al.* 1994, Parsonnet 1995, Veldhuyzen 1995, Sipponen 1997, Miyaji *et al.* 2000). Low prevalence occurs in the USA, Canada and in Northern and Western Europe; high prevalence is noted in India, Africa, Latin America and Eastern Europe (Torres *et al.* 2000).

Epidemiological studies of chronic gastritis and *H. pylori* infection among Estonian populations (Kambja, Saaremaa, Karksi-Nuia) of different birth cohorts showed, however, that *H. pylori* prevalence in Estonia is high among persons born at the beginning and in the middle of the last century. In Estonia, the cohort specific prevalence of *H. pylori* infection is 70–90% for generations born at the beginning of 20th century and it remains high (60–80%) for those born after 1945 (Maaroos 1995). Studies of chronic gastritis in an Estonian population from Saaremaa in 1979 (Maaroos *et al.* 1990a) and in a Finnish outpatient series (Sipponen *et al.* 1994a) shows that the prevalence rates of *H. pylori* infection and gastritis in Estonia were similar to those in Finland (70–80%) for groups born at the beginning of the last century, while a difference was revealed for later birth cohorts born after 1945. *H. pylori* infection and gastritis frequency was higher in Estonia than in Finland, which means that acquisition rate of *H. pylori* in the childhood in the youngest birth cohort has remained high in Estonia. This is supported by studies of *H. pylori* prevalence in Estonian children with abdominal complaints, which established that 58% of children aged 4–15 years were *H. pylori* positive and 61% had *H. pylori* gastritis (Maaroos *et al.* 1991b, 1993), indicating that the rate of acquisition of *H. pylori* is still high in Estonian children.

Also, a three-year follow-up study of Estonian children from Saaremaa since birth (start in 1993), carried out by R.Tammur and published by Lindkvist (1999), demonstrated very high seroconversion rates during the first years of life: 27% in the first year, 25% in the second year and 12% in the third year. Cumulative incidence in the cohort (born 1993) for the three-year period was 59%. An important finding in the study of Tammur and Lindkvist (Lindkvist 1999) was that the peak age for infection in Estonia was under of 3 years, which is still very high in recent cohorts. Regarding the role of poor environmental

59%. An important finding in the study of Tammur and Lindkvist (Lindkvist 1999) was that the peak age for infection in Estonia was under of 3 years, which is still very high in recent cohorts. Regarding the role of poor environmental hygiene in acquision of *H. pylori* in childhood, it is noteworthy that a specific independent risk factor for infection other than age in Estonia was non-use of a toothbrush among small children. These results are consistent with the suggestion of an oral-oral transmission route for *H. pylori* (Lindkvist 1999).

Natural course of chronic gastritis

Chronic gastritis is local immune response to *H. pylori* infection (Sipponen *et al.* 1998). Chronic *H. pylori* gastritis results from an infection acquired in childhood and tends to persist at a quite constant prevalence level in the cohort for decades (Sipponen 1995). In the early stage, chronic gastritis appears as an immune response of the gastric mucosa against *H. pylori* in the form of mononuclear, round cell inflammation (immunocompetent lymphocytes and immunoglobulin secreting plasma cells) both in the antrum and corpus mucosa (Wyatt 1991, Price 1991, Sipponen 1997), with a predominance of inflammation in the antrum (Maaroos *et al.* 1990a, Stolte *et al.* 1990, Karttunen *et al.* 1991, Bayerdörffer *et al.* 1992, Sipponen *et al.* 1996a, Ahlstedt *et al.* 1999). A special feature of *H. pylori* gastritis is that it is practically always associated with acute inflammation (Sipponen *et al.* 1998). Polymorphonuclear neutrophil infiltration (indicating activity of gastritis) is associated with inflammatory process and its intensity depends upon differences in *H. pylori* strains (more pronounced in cytotoxic strains) (Crabtree 1996a) and upon dissimilarities in the host's reactions (Sipponen *et al.* 1996).

There exists a correlation between grade of mononuclear and acute infiltration and grade of *H. pylori* colonization of the gastric mucosa (Kekki *et al.* 1987, Kartunnen *et al.* 1991, Stolte *et al.* 1995), which in turn indicates that the pathogenesis of inflammation is the host's reaction to *H. pylori* itself or to specific bacterial mediators of mucosal inflammation (Blaser 1992, Crabtree 1996a, Sipponen *et al.* 1996a).

Activity of gastritis (acute polymorphonuclear inflammation) may contribute to the damage of the epithelium and mucosal glands due to neutrophil-related toxic free radicals and reactive oxygen metabolites from inflammatory cells (Davies *et al.* 1994, Fiocca *et al.* 1994, Rautelin *et al.* 1996), or due to transepithelial migration (Parkos *et al.* 1992). Neutrophil adherence to the endo-thelium may cause a leakage of protein as a result of microvascular damage (Kurose *et al.* 1994). Because neutrophilic inflammation is typically localized in neck areas of the mucosal glands, which is responsible for cell replication and renewal, the damaging properties of neutrophils may constitute a risk for loss of the glands (Fiocca *et al.* 1994).

Inflammatory, non-atrophic stage of chronic gastritis (non-atrophic gastritis) is known as "superficial gastritis" in the literature (Siurala *et al.* 1968, Whitehead *et al.* 1972, Kekki *et al.* 1987, Sipponen 1997). Gastritis progresses from

1985, Sipponen *et al.* 1991, Kawaguchi *et al.* 1996). In patients with advanced atrophic gastritis, chronic inflammation is milder with respect to degree (Sipponen *et al.* 1996a). An important observation based on a 6-year follow-up of a Saaremaa random sample and on a 32-year follow-up of a Finnish population was that atrophic gastritis developed only in persons who had a preceding *H. pylori* non-atrophic gastritis (Villako *et al.* 1991, Sipponen *et al.* 1996b, Valle *et al.* 1996).

Atrophy means loss of normal mucosal glands, and consequently, loss of normal physiological function of the gastric mucosa (in the corpus: reduction of acid output; in the antrum: a poor serum gastrin response to various stimuli) (Sipponen and Marchall 2000). In chronic *H. pylori* gastritis, atrophic changes appear first in the antrum, showing a gradual pylorocardial extension with time and increasing age (Kimura 1972, Maaroos *et al.* 1985). In some cases *H. pylori* gastritis may end in severe corpus-predominant atrophic gastritis or corpus-limited atrophy with healing of the antrum (Annibale *et al.* 1997, Sipponen and Marshall 2000). In parallel with progression of atrophic gastritis, the extent and severity of intestinal metaplasia increase with age (Siurala *et al.* 1985, Ihamäki *et al.* 1985). Intestinal metaplasia represents a transformation of the gastric epithelium and glands from secretory to absorptive (Sipponen *et al.* 1998). With the increasing grade and extent of atrophic gastritis, *H. pylori* infection tends to burn out and the colonization of the mucosa may be minimal (Sipponen 2001).

Atrophic gastritis can be limited to the antrum or corpus, or can be multifocal, affecting both to a varying extent and grade (Villako *et al.* 1976, Correa 1980, Siurala *et al.* 1985, Sipponen 2001). The common topographic phenotypes of *H. pylori* gastritis and atrophic gastritis are the following:

1. "Ulcer phenotype" of gastritis: predominance or restriction of *H. pylori*-related inflammation in the antrum, in association with the nonatrophic corpus mucosa. It is the most common phenotype, with an increased risk of peptic ulcer disease, particularly, duodenal ulcer.
2. "Corpus predominant gastritis": presence of atrophic gastritis in the corpus of the stomach, which indicates a low risk of peptic ulcer and reduction in the capacity of the patient to secrete acid.
3. Advanced "multifocal atrophic gastritis": the occurrence of advanced atrophic gastritis and intestinal metaplasia multifocally in the stomach, which indicates low acid secretion capacity and an increased risk of gastric neoplasias ("gastric cancer phenotype of gastritis").

Presence of normal and healthy gastric mucosa, which indicates an extremely low risk of both peptic ulcer disease or gastric cancer is a finding of high clinical relevance (Sipponen 2001).

The topographic phenotypes of *H. pylori* gastritis are similar risk conditions for gastric diseases all over the word. The existing geographical heterogeneity in the prevalence of the sequelae of *H. pylori* infection might be based on large differences in acquisition rate between populations but not only reflecting the differences in bacterial strains or host responses (Sipponen 2001).

Progression of atrophic gastritis from mild to severe is a time related phenomenon (Kekki *et al.* 1983, 1984, 1987, Sipponen *et al.* 1996a); several factors can influence progression of *H. pylori* gastritis into atrophic gastritis, such as differences in the cytotoxicity of *H. pylori*, genetic liability of the host to acquire atrophic gastritis, and in some cases also dietary habits and presence or absence of vitamins (Correa 1992, Kuipers et al. 1995, Sipponen and Marshall 2000). Long-term follow-up studies in Finland show that although more than half infected patients acquire atrophic gastritis during their lifetime, 20–30% of *H. pylori* infected subjects do not acquire atrophic gastritis (Ihamäki *et al.* 1985, Valle *et al.* 1996). Atrophic gastritis develops approximately 20 years after *H. pylori* acquisition and, thereafter, increases in prevalence rate with age. The age-specific prevalence of atrophic gastritis in general population is positively related to the incidence and prevalence rate of *H. pylori* infection in the population (Sipponen 2001).

Overview of the epidemiological studies of *H. pylori* gastritis in Estonian populations

The study of chronic gastritis has a long tradition in Estonia. Already in the 1930s roentgenological and endoscopic methods were introduced by L. Walk for defining atrophic gastritis, followed by endoscopic and roentgenological investigations of the stomach by K. Villako in the 1950s. V. Salupere introduced suction biopsy in Estonia in 1960 and a fiberoscopy in the 1970s, which opened wide possibilities for the morphological and immunological research of gastritis in different gastroduodenal diseases, conducted by V. Salupere as well as by H.-I. Maaroos and R. Uibo (Salupere 1965, 1966, 1978, 1991, Salupere *et al.* 1974, 1975, Maaroos *et al.* 1981, 1985, 1989, 1990a,b, 1991a,b, Uibo 1979, Uibo *et al.* 1984a,b, 1989, 1991).

Epidemiological studies of chronic gastritis among Estonians were carried out in two randomly selected adult populations, Kambja (1972, rural, age 15–65 years) and Kuressaare, Saaremaa (urban, initially 227 persons, age 15–69 years) with follow-up in 1979, 1985, 1991 and 1997 (Villako *et al.* 1976, 1982, 1990, 1991, 1995).

The study of chronic gastritis in Kambja showed that gastritis, either in the antrum or in the corpus, or simultaneously in both, occurred in 78%, while antral gastritis was more frequent than corpus gastritis (68% versus 45%). In case of pangastritis, changes in the antrum and corpus often reflected different stages of gastritis. It was established that prevalence of corpus and antrum gastritis increases with age (Villako *et al.* 1976).

An epidemiological study of a representative sample of urban population in Saaremaa, conducted in 1979, revealed the occurrence of chronic gastritis in the antrum in 64% and in the corpus in 62% of cases. The prevalence of atrophic antral and corpus gastritis was high: 38% and 37%, respectively (Villako *et al.* 1982). A follow-up study of behaviour of chronic gastritis in Saaremaa showed that the main trend of chronic gastritis was slow "one-step progression" in

severity of chronic inflammation and appearance of atrophy and intestinal metaplasia. Inflammation progressed particularly in the antrum in young age groups. Retrospective morphological studies on *H. pylori* showed that grade of chronic inflammation is well correlated with extent of *H. pylori* colonization in the antrum but not in the corpus (Villako *et al.* 1991, Maaroos *et al.* 1990a). Besides, there was revealed an evident intraindividual tendency for maintaining the grade of *H. pylori* colonization at a constant level during a 6-year follow-up. *H. pylori* colonization occurred concomitantly with the development of gastritis. The development of antrum atrophy was accompanied with a decreasing in and the eventual disappearance of *H. pylori* in the antrum. Normalization of the antral mucosa occurred with persistent corpus atrophy and the concomitant disappearance of *H. pylori* in both compartments of stomach mucosa. The grade of *H. pylori* increased with increase in severity of superficial gastritis and decreased with progression of atrophic changes. In the antrum, a life-long correlation was found between *H. pylori* colonization and inflammatory reaction. The host response ratio (ratio of chronic inflammation score to grade of *H. pylori* colonization) was significantly higher in the youngest age group (15–19 years) as compared with the other age groups as well as in subjects in whom the antral gastritis regressed during follow-up. Parietal cell antibodies developed in two subjects who were *H. pylori* positive at the first examination. These findings suggest that autoimmune mechanisms can be started in subjects who were previously exposed to *H. pylori*-related gastritis. A difference was established between the antral and corpus mucosa in their tendency to be associated with *H. pylori*: the severity of inflammation response and atrophy in the antrum are more specifically and strongly linked to changes in *H. pylori* colonization in the antrum compared with respective changes in the corpus (Kekki *et al.* 1991).

The re-examination of the same persons in 1991 (12 years later) showed that *H. pylori* gastritis is a rather stable, slowly changing disease. The prevalence of atrophic gastritis was three times higher in the corpus than in the antrum. Both progression as well as a significant improvement and complete healing of gastritis are possible but rare (healing rate 0.3% per year). In the antrum, low *H. pylori* colonization seems to predict improvement in chronic inflammation in the long term, suggesting that the antrum is immunologically more active than the corpus (Villako *et al.* 1995).

The data of the last observation (1997) will be presented further in this study (Papers VI–IX).

H. pylori and gastric autoimmunity

The initial division of chronic gastritis into Type A and Type B (Strickland and Mackay, 1973 and Wyatt and Dixon, 1988) indicated that Type A gastritis is characterized by autoimmune genesis and accompanies pernicious anaemia, whereas Type B is nonautoimmune (Strickland and Mackay 1973, Salupere 1978), predominantly *H. pylori*-associated gastritis (Whyat and Dixon 1988,

Strickland 1990). At present there is ample evidence that *H. pylori* too is responsible for induction of autoimmune processes in the gastric mucosa (Engstrand *et al.* 1991, Negrini *et al.* 1989, 1991, 1993, 1996, Varis *et al.* 1993, Ma *et al.* 1994, Faller *et al.* 1996, 1997, 1998, Appelmelk *et al.* 1996).

The main target of autoimmune reactions described in the stomach is parietal cells and its secretory product intrinsic factor (Taylor 1959, Irvine *et al.* 1962, Markson and Moor 1962). Among pernicious anaemia patients, parietal cell antibodies (PCA) have been found in 90% of patients with atrophic corpus gastritis (Irvine *et al.* 1962, Taylor *et al.* 1962, Strickland and Mackay 1973, Glass and Pitchumoni 1975). PCA have been found also in other gastritis forms, not associated with pernicious anaemia, which involve mainly the antrum (Schraier *et al.* 1983, Wyatt *et al.* 1992). Furthermore, there have been demonstrated immune reactions to antral mucosa (Salupere *et al.* 1972, Vandelli *et al.* 1979, Uibo *et al.* 1984b). Studies of Vandelli *et al.* (1979) and Uibo *et al.* (1984b) showed that in several antrum gastritis cases (6% of cases in a study of Vandelli and 16% of cases in a study of Uibo) there occur antibodies to gastrin cells; both researchers suggested that antral gastritis too can be associated with immunological abnormalities, in which the role of *H. pylori* has now been established (Jaskiewicz *et al.* 1993, Seifarth *et al.* 1996, Sommer *et al.* 1998).

Gastric H+, K+ ATPase as a major parietal cell autoantigen

The parietal cell autoantigen is located of cell surface membrane, covering microvilli on its free surface and lining the secretory canaliculi of the gastric parietal cells (Hoedemaeker and Ito 1970). Studies of biochemical and molecular cloning identified this autoantigen as the α-and β-subunits of gastric H+, K+ ATPase (Karlsson *et al.* 1988, Goldkorn *et al.* 1989, Toh *et al.* 1990). Membrane-bound gastric H+, K+ ATPase represents a proton pump responsible for acidification of the stomach lumen (Ganser and Forte 1973, Reuben *et al.* 1990). Gastric H+, K+ ATPase consists of two subunits, the transmembrane 100 kDa catalytic α-subunit and the 60–90 kDa glycoprotein β-subunit and it is localized in the intracellular membranes of gastric parietal cells (Karlsson *et al.* 1988, Toh *et al.* 1990, Ma *et al.* 1994). It has also been established that gastric H+, K+ ATPase serves as the causative autoantigen in the pathogenesis of experimental autoimmune gastritis (Scarff *et al.* 1997, Toh *et al.* 2000). Toh *et al.* (2000) proposed the following scenario for the genesis of the gastric lesion initiated by the β subunit of gastric H+, K+ ATPase: dendritic cells in the gastric mucosa become activated, capture and process gastric H+, K+ ATPase, released during the turnover of parietal cells, and migrate then to draining lymphnode. In T-cell areas of the lymphnode, dendritic cells present gastric H+, K+ ATPase peptide to naïve CD4+ CD25- T-cells. In the presence of naïve regulatory CD4+CD25+ T-cells, activation of naïve pathogenic CD4+ T-cells and IL-2 secretion is suppressed. In absence of regulatory T-cells, naïve pathogenic CD4+ T-cells become activated. Activated pathogenic CD4+ T-cells

acquire adhesion molecules that allow their migration to the stomach via efferent lymphatic and blood vessels of the gastric mucosa, where they infiltrate the mucosa in response to appropriate chemotactic stimuli. Antigen-specific CD4+ T-cells recruit other CD4+ T-cells, monocytes, CD8+ cells and B-cells through release of cytokines (Steinman 1996). Supported by activated CD4+ T cells, B-cells produce autoantibodies to gastric H+, K+ ATPase (Toh *et al.* 2000). Recent data of d'Elios *et al.* (2001) showed that H+ K+ ATPase is the target autoantigen of Th1 type cytotoxic T-cells in autoimmune gastritis. Activation of proton pump-specific Th1 cytotoxic/proapoptotic T-cells in the gastric mucosa can represent an effector mechanism for the target cell destruction in autoimmune gastritis.

It was shown in many studies that infection with *H. pylori* induces autoantibodies reactive with gastric parietal cell canaliculi and that autoantibodies to H+, K+ ATPase indicate a link between bacterial and classical autoimmune gastritis (Negrini *et al.* 1991, 1996, Ma *et al.* 1994, Faller *et al.* 1996, 1997, Appelmelk *et al.* 1996, 1998). Faller *et al.* (1997) showed a good correlation between *H. pylori* infection, antiluminar antibodies binding to the foveolar epithelium as well as anticanalicular autoantibodies and corpus mucosa atrophy with increased fasting gastrin levels (Faller *et al.* 1997). This relationship was supported by a significant reduction of anticanalicular autoantibodies after *H. pylori* eradication (Faller *et al.* 1997). Recently, it was shown that antigastric autoantibodies, reacting against canaliculi of human parietal cells, are not only detectable in the serum but are produced locally in the gastric mucosa of *H. pylori* infected patients (predominantly IgA type) (Faller *et al.* 2000).

Antigastric autoimmunity and molecular mimicry

Because most *H. pylori* strains contain human blood group antigens Lewis X and Lewis Y, which are expressed also on epithelial cells in the gastric mucosa (Sherburne and Taylor 1995, Aspinall *et al.* 1996, Simoons-Smit, 1996 *et al.*), it has been proposed that such molecular mimicry is responsible for formation of antigastric autoantibodies, particularly in animal models (Negrini *et al.* 1996, Aspinall *et al.* 1996, Appelmelk *et al.* 1996, 1997). In a study of Guruge *et al.* (1998) it was shown that if the host is colonized by a *H. pylori* strain that expresses adhesins, promoting attachment to epithelial receptors, and if that strain expresses also Lewis X surface antigens, mimicking host parietal cell structure then the course of infection leads to development of autoantibodies to parietal cells and, as a consequence, a tendency for atrophic gastritis with loss of parietal cells.

However, Faller *et al.* (1998) showed that human autoantibodies against the canaliculi of parietal cells could not be absorbed by Lewis X or Lewis Y positive *H. pylori* strains. Additionally, Clayes *et al.* (1998) demonstrated that 50% of anticanalicular autoantibodies react with the peptide part of the α or β subunit of gastric H+, K+, ATPase but not with a carbohydrate structure such as

the blood group antigen. These authors suggest that there exist some other pathogenic mechanisms leading to formation of antigastric autoantibodies.

Furthermore, heat shock proteins have been considered as the cause of antigenic mimicry between *H. pylori* and host structures, which gives evidence of a high degree of homology (Macchia *et al.* 1993). Engstrand *et al.* (1991) demonstrated that monoclonal antibodies, raised against HSP60 (GroEL) of Mycobacterium, cross-reacted exclusively with the HSP60 protein of *H. pylori* as well as with gastric epithelial cells in all *H. pylori* positive specimens. Engstrand *et al.* (1991) suggested that intraepithelial γ/δ T-cells may play a role in host defence against *H. pylori*, and that *H. pylori* may trigger autoimmune response to stress proteins expressed by gastric epithelial cells.

Negrini *et al.* (1996) reported an additional autoimmune target — the mucosal neck and chief cells of the gastric mucosa. The cross reaction between *H. pylori* and epithelial cells, observed by Negrini, was directed to the glandular epithelium of the stomach, in particular, to the regenerative mucosal neck cells. Also, a significant correlation of anticanalicular autoantibodies was found with presence of intraepithelial lymphocytes within the glandular epithelium, with periglandular lymphocytes and with corpus atrophy. In the concept of molecular mimicry introduced by Negrini, *H. pylori* presents common epitopes to the host which react with antibody response, recognizing the gastric mucosal epithelium and, together with other host and environmental factors, influences the tendency of chronic gastritis to progress into atrophic gastritis and gastric cancer (Negrini *et al.* 1996).

Invasion of human gastric mucosa by *H. pylori*

Considering the interaction between *H. pylori* and host immune response and gastric tissue damage, there arises the question about the possible entry of *H. pylori* to the human gastric epithelium. *H. pylori* has been considered to colonize the gastric mucosa exclusively extracellularly (Blaser 1997). However, there are data confirming an intracellular location of *H. pylori* (Bode *et al.* 1988, Andersen and Holck 1990, Evans and Graham 1992, Noach *et al.* 1994, Engstrand *et al.* 1997, Ko *et al.* 1999, Su *et al.* 1999, Björkholm *et al.* 2000, Petersen *et al.* 2000). Björkholm *et al.* (2000) show that *H. pylori* can invade epithelial cells actively in culture, while production of vacuolating cytotoxin increases the ability to invade Hep-2 cells. The authors suppose that invasion of the gastric epithelium contributes to the bacterium's ability to establish persistent infection that evades mucosal defence and sometimes also antimicrobial therapy in case antibiotics with extracellular activity are used. Su *et al.* (1999) found that invasion of cultured cells by *H. pylori* occurs via an integrin-mediated pathway and entry is higher for type I (cytotoxin) strains.

H. pylori and induction of apoptosis and proliferation of gastric epithelial cells

Cell proliferation and apoptosis (programmed cell death) are essential events involved in the cellular turnover of the gastric tissue (Hall *et al.* 1994). Normal mucosa integrity is maintained when the rate of cell loss by apoptosis is well balanced by the rate of production of new cells by epithelial proliferation (Kerr *et al.* 1972, Suzuki and Ishii 2000). Abnormalities of cell turnover may contribute to atrophy when cell loss is excessive compared with proliferation, and to neoplasia in an opposite case (Thompson 1995, Que and Gores 1996, Moss *et al.* 1996, Jones *et al.* 1997, Suzuki and Ishii 2000).

Programmed cell death (apoptosis)

The term apoptosis (αποπτωσις- falling off) and its description were introduced by Kerr in 1972 to denote controlled cell deletion, which appears to play a complementary role but opposite to that of mitosis in the regulation of animal cell populations. Cell death can occur by necrosis or by apoptosis.

Distinction between apoptosis and necrosis

The key morphological features of apoptosis, originally described by Kerr *et al.* (1972), included shrinkage and blebbibg of the cytoplasm; preservation of the structure of cellular organelles, including the mitochondria; condensation and margination of chromatin. In contrast, necrotic cells first increase their cellular water content and thus their volume; nuclei lose the typical chromatin structure which is seen as irregular clumping; cell membrane ruptures, discharging cellular contents into the environment (Studzinski 1999). Apoptosis affects cells one at a time. At tissue level, apoptosis produces little or no inflammation, since shrunken portions of the cell are engulfed by the neighbouring cells, especially macrophages, rather than being released into the extracellular fluid. In contrast, in necrosis, cellular contents are released into the extracellular fluid and have an irritant effect on the nearby cells, causing inflammation. The apoptotic mechanism of cell death is fundamental to the normal development of tissues and organisms, whereas cell death by necrosis is usually accidental and therefore does not have such significance (Studzinski 1999).

One of the most extensively studied biochemical events in apoptosis is chromatin fragmentation by endonuclease activation. DNA double-strand cleavage occurs in the linker regions between nucleosomes, producing DNA fragments. In contrast, necrosis is accompanied by random DNA breakdown. Presence of 5′-phosphate (5'-P) and 3′-hydroxyl (3'-OH) DNA termini in apoptotic cells is an important biochemical feature of apoptotic DNA fragments. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL) method, which is commonly used to detect apoptotic cells, is based on presence of 3′-OH termini (Gavrieli *et al.* 1992).

Factors involved in induction and regulation of apoptosis

Initiating signals for apoptotic cascades can be either extracellular or intracellular. Apoptosis is dependent upon a balance between a series of inducer and inhibitory factors (Majno and Joris 1995). Activation of cysteine proteases that are present in the cytoplasm as zymogens and that cleave protein chains after aspartic acid residues (caspases) is the most important event characterizing the process of apoptosis (Blajeski and Kaufmann 1999). Caspase-3 is regarded as one of central executioner molecules that is activated in many cell types after exposure to apoptotic stimuli and that is responsible for cleaving various proteins and thereby disabling important cellular processes (Nicholson and Thornberry, 1997). Release of three apoptotic protease-activating factors (including cytochrome C) from mitochondria may play a key role in the activation of caspase-3 (Zhou and Savesen 1997). Mitochondrial caspase-3 can be activated by numerous proapoptotic stimuli, including *H. pylori* (Mancini *et al.* 1998, Slomiani *et al.* 1999, Pothoff *et al.* 2000). The cellular target for caspases-6 and -7 is actin, which can induce cytoskeletal changes and, as a consequence, cell condensation (Studzinski 1999).

Apoptosis can be regulated by several mechanisms. Bcl-2 family proteins play critical roles in the regulation of apoptosis (Reed 1996). Bcl-2 was initially discovered as an overexpressed protein in human B-cell lymphomas (Pegoraro *et al.* 1984). Bcl-2 protein is membrane-associated and is localized in a nuclear envelope, in the endoplasmic reticulum and on the inner aspect of mitochondrial membrane (Hockenbery *et al.* 1990). Overexpression of bcl-2 protects many cell types against apoptosis in response to viral infection, hypoxia, and chemotherapeutic agents (Reed 1996). A number of bcl-2 family members such as Bcl-2, Bcl-X_l, Bcl-w, Mcl-1, A1/bfl-1 serve to inhibit apoptosis, whereas Bax, Bik, Bak Bad, Bid, Bcl-X_s, Mrk promote apoptosis (Reed 1996, Granville *et al.* 1998). Changes in the levels or bioactivities of these proteins are associated with a variety of physiological processes where cell death occurs, including fetal development, haematopoietic and immune cell differentiation. However, pathological alterations in expression of bcl-2 family proteins have been documented in cancer, autoimmunity, immunodeficiency and other diseases (Thomson 1995, Krajewska *et al.* 1996, Reed 1999).

Apoptosis can be induced by the interaction of the receptor molecule Fas with Fas ligand (Fas-L). Fas (CD95) is a glycosylated transmembrane receptor belonging to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor family which, by binding to Fas ligand, induces apoptosis through activation of the caspase cascade (Nagata and Goldstein 1995).

Induction of apoptosis by H. pylori

There is ample evidence of induction of apoptosis of gastric epithelial cells as well as gastric T-cells by *H. pylori* (Moss *et al.* 1996, Chen *et al.* 1997, Wagner *et al.* 1997, Anti *et al.* 1998, Konturek *et al.* 1999, Jones *et al.* 1999, von Herbay and Rudi 2000, Jang and Kim 2000, Yoshimura *et al.* 2000).

Several mechanisms have been proposed regarding how *H. pylori* can influence apoptosis of epithelial cells. Adherence of *H. pylori* to epithelial cells seems to be important by induction of apoptosis, because the latter can be prevented by a physical barrier separating *H. pylori* from epithelial cells (Chen *et al.* 1997).

Fas-mediated signalling

The role of Fas-mediated signalling in apoptosis of gastric epithelial cells in response to *H. pylori* infection is also indicated (Wagner *et al.* 1997, Rudi *et al.* 1998, Jones *et al.* 1999, von Herbay and Rudi 2000, Koyama *et al.* 2000). Jones *et al.* (1999) suggested that during infection with *H. pylori*, gastric epithelial cells, exhibiting enhanced Fas receptor expression, could be eliminated by infiltrating lymphocytes that express the Fas ligand. In a study of Rudi *et al.* (1998), a markedly elevated number of apoptotic cells were identified in the surface epithelium, antral pyloric glands, and lamina propria in 83% of biopsies from patients with *H. pylori* gastritis. These authors suggest that *H. pylori*-associated chronic gastritis involves apoptosis of gastric epithelial cells by upregulation of CD95 (Fas) expression and increase in CD95L (FasL) expression in both lymphocytes of the lamina propria and gastric epithelial cells, and therefore apoptosis might occur also by suicide mediated by FasL-Fas interaction among epithelial cells. Moreover, these authors found that upregulation of CD95 receptor expression and induction of apoptosis was achieved with cytotoxic *H. pylori* strains. Koyama *et al.* (2000) suggested a possible apoptotic depletion of invading mucosal lymphocytes, which expressed the Fas receptor and its ligand. The role of Fas signalling in gastric injury was shown in animal models of autoimmune gastritis, where enhanced Fas expression was detected on gastric parietal cells, which correlates topographically with induction of apoptosis of these cells (Nishio *et al.* 1996). This indicates that one mechanism by which autoimmune-mediated target cell destruction may be affected is Fas-FasL interactions. It is noteworthy that autoantibodies, directed against gastric parietal H+, K+ ATPase detected in sera from *H. pylori* infected patients, correlates with presence of gastric atrophy and, moreover, with apoptotic cell loss (Clayeas *et al.* 1998, Steininger *et al.* 1998). In a recent study of Randlkofer *et al.* (2000) it was shown that *H. pylori* and inflammatory cytokine TNF-α induce apoptosis in isolated parietal cells, presumably stimulating the effector caspase-3, which might contribute to development of atrophic gastritis. Also other cytokines, produced by Th1 cells (IFN-γ, IL-2, IL-1), can potentiate *H. pylori*-induced apoptosis in gastric epithelial cells (Wagner *et al.* 1997, Fan *et al.* 1998, Rudi *et al.* 1998). These cytokines may induce sensitisation of epithelial cells to Fas-mediated apoptosis (Houghton *et al.* 1999).

H. pylori and the bcl-2 family

Regulation of apoptosis is a complex process that includes also apoptosis-related proteins such as the bcl-2 family. Konturek *et al.* (1999) demonstrated that *H. pylori* induces apoptosis in the gastric epithelium due to an upregulation of proapoptotic Bax and a down regulation of antiapoptotic bcl-2 proteins. Bax mRNA and protein expression was higher in the antrum than in the corpus, which was associated with greater inflammatory changes observed in the antrum. Konturek *et al.* (1999) suggested a possible role of proinflammatory cytokines in induction of apoptosis.

There is also evidence that *H. pylori* induces apoptosis of T-cells. Ernst *et al.* (2000b) showed that *H. pylori* induced apoptosis in Fas-bearing T-cells through induction of FasL expression. Since this effect was linked to products encoded by *cag* PAI, the authors proposed that this mechanism can lead to persistent infection with cytotoxic strains favoured by a negative selection of T-cells encountering specific *H. pylori* antigens.

Apoptosis at different states of gastric mucosa

As a consequence of *H. pylori* infection, apoptotic cells, are localized mainly in epithelial cells in the upper foveolae. Increase in apoptosis may be accompanied by epithelial proliferation in the middle portions of gastric pits (proliferating zone) (Suzuki and Ishii 2000).

Apoptotic cells are rare in the generative cell zone of the normal mucosa. With progression of atrophic gastritis, the generative cell zone shifts downward and there occur a relatively large number of apoptotic cells. In the intestinalized glands, both apoptotic and proliferative cells are present in deeper gland portions corresponding to the generative zone. The frequency of apoptotic cells per crypt is higher in incomplete than in complete metaplasia, implying greater underlying DNA damage in the former and are presumably more frequently eliminated by apoptosis to avoid cell transformation (Ishida *et al.* 1996).

Only a few studies have dealt with association of atrophic alterations, developing in the course of time, with apoptosis in epithelial cells. Moss *et al.* (1999), who evaluated the apoptosis to cell proliferation ratio for the antrum epithelial cells in a 31-year follow-up study of a cohort of Finnish patients, found that patients who developed atrophy later, had initially mildly increased gastric epithelial cell proliferation and apoptosis, and assumed increased cellular turnover in the atrophy group. However, the ratio of apoptosis to proliferation was not a determinant risk for development of atrophy decades later. At the same time, some authors have proposed that atrophic gastritis is the result of sustained large-scale apoptosis of the gastric mucosa (Mannick *et al.* 1996, Correa and Miller 1998). Apoptosis of the neck region mucosal glands is critical, as this region is the site of epithelial replication and regeneration of mucosal glands and surface epithelia. Depletion of mucosal glands may be caused by the failure to replace gland cells steadily over time via apoptosis — a consequence of persistent inflammation (Correa and Miller 1998).

Another aspect, upregulation of apoptosis protectors, such as bcl-2 oncoprotein, and consequent decrease in apoptosis with enhanced cell proliferation, raises the problem of malignant transformation. The bcl-2 confers survival advantage on cells harbouring this oncogene (Hockenberry *et al.* 1990). The bcl-2 plays an important role in many continuously proliferative epithelia preventing apoptosis in regenerative compartments (Xia and Talley 2001). An aberrant expression of bcl-2 was demonstrated in patients with chronic atrophic gastritis with intestinal metaplasia as well as with gastric epithelial dysplasia (Lauwers *et al.* 1994, Krajewska *et al.* 1996, Mao-Kendler *et al.* 1999). The bcl-2 is often topographically restricted to the long-lived or proliferating cell zone (Hockenberry *et al.* 1991), evidently to protect the renewal potential of the mucosa (Lauwers *et al.* 1994). According to Lauwers *et al.* (1994), the cellular components of premalignant lesions of the stomach, like intestinal metaplasia and gastric epithelial dysplasia, might have a prolonged life span through an aberrant expression of bcl-2 protein. These authors also suggested that bcl-2 is associated with cellular immaturity, which is in concordance with the finding that gastric preneoplastic lesions are composed of immature cells (Eastwood 1977, Lipkin *et al.* 1985). Thus, highly proliferative immature neoplastic cells would have a growth advantage because of their prolonged life span resulting from an alteration of apoptosis.

H. pylori and epithelial cell proliferation in the gastric mucosa

The gastric mucosal proliferative zone is located at the base of the gastric pits. The major flow of cells proceeds upward, towards the foveolar zone and the lumen. This migration takes approximately one week and ends in apoptosis. There occurs also a downward migration, from the proliferative zone towards the base of the gastric glands, which appears over 2–3 months (Moss 1998).

For examination of the numbers of proliferating cells in tissues, a variety of different techniques have been used, such as measurement of the number of cells incorporating tritiated thymidine or bromodeoxyuridine (BrdU) in their nucleic acids, or detection by immunohistochemical methods with antibodies against endogenous cell cycle-associated antigens such as the proliferating cell nuclear antigen (PCNA) and Ki-67 (Hart-Hansen *et al.* 1979, Lipkin *et al.* 1985, Hall *et al.* 1990, Brenes *et al.* 1993, Filipe *et al.* 1993, Cahill *et al.* 1994, Lynch *et al.* 1994, Pich *et al.* 1994, Fraser *et al.* 1994, Chow *et al.* 1995, Havard *et al.* 1996, Yamaguchi *et al.* 2000). The proliferating cell nuclear antigen (PCNA) is a 36 kDa nuclear protein involved in DNA synthesis and related to the S-phase and expressed also during the late G1 and early G2 cell cycle phases (Takasaki *et al.* 1981). The Ki-67 is a nuclear antigen expressed in all phases of the proliferating cell cycle except for G0 (Gerdes *et al.* 1984).

Many investigators have demonstrated increased cell proliferation in gastric glands, associated with *H. pylori* infection (Brenes *et al.* 1993, Fraser *et al.* 1994, Cahill *et al.* 1994, Lynch *et al.* 1995, Bechi *et al.* 1996, Baldini *et al.* 1999, Hishi *et al.* 1999, Nardone *et al.* 1999, Szaleczky *et al.* 2000). *H. pylori-*

infected patients display an overall increase in epithelial cell proliferation within the gastric foveolae, with an upward shift of proliferating cells towards the surface (Anti *et al.* 1998), which is thought to be caused by an increase in the mucosal content of ammonia, known to be a strong stimulus of cell proliferation (Tsujii *et al.* 1993).

Increase in gastric epithelial cell proliferation has been observed mostly *in vivo* in response to increase in cell injury and death (Fan *et al.* 1996, Wagner *et al.* 1997, Shirin *et al.* 1999). However, *in vitro* studies have shown inhibition of cell growth by direct exposure of *H. pylori* (Knipp *et al.* 1996, Shirin *et al.* 1999). Shirin *et al.* (1999) and Ahmed *et al.* (2000) showed that *H. pylori* can arrest gastric cells in the G0/G1 phase of the cell cycle. It was suggested that cyclin-dependent kinase inhibitors play a role in the G1 cell cycle arrest caused by *H. pylori* and its involvement in changing regulatory proteins p53, p21 and cyclin E in the cell cycle (Ahmed *et al.* 2000). It was surmised that one possible explanation for the discrepancy between *in vivo* and *in vitro* studies may be that while *in vivo* studies on patients, infected by *H. pylori,* reveal of the effect of persistent *H. pylori* infection, then *in vitro* studies as well as *in vivo* experimental studies reveal a short-term *H. pylori* mediated effect (Ricci *et al.* 1996). Elevated cell proliferation rate in patients with *H. pylori* infection might be related to *H. pylori* induced inflammation rather than to the direct effect of the pathogen (Tracz *et al.* 1995).

A study of Fan *et al.* (1996) indicated that proliferation of the gastric epithelial cell line can be stimulated directly by *H. pylori* and indirectly by *H. pylori* induced cytokine. Also, a study of Anti *et al.* (1998) found a correlation between cell proliferation indices and polymorphonuclear and mononuclear cell densities in the antrum mucosa. In a study of cell kinetics at different stages of progression from normal mucosa through chronic active gastritis, gastric atrophy and intestinal metaplasia to gastric cancer, Cahill *et al.* (1996) established increased epithelial cell proliferation in all stages, with the highest index in gastric cancer. This study showed also increased epithelial cell proliferation associated with *H. pylori.*

The literature has been inconsistent regarding the fact whether CagA positive strains can stimulate more gastric cell proliferation compared with CagA negative strains. There are data that unlike CagA-negative strains, CagA-positive strains induce higher rates of epithelial cell proliferation without increased apoptosis, which indicates the disbalance between cell growth and death and accounts for the association of cytotoxic *H. pylori* strains with gastric cancer (Peek *et al.* 1997, Sipponen *et al.* 1998). In some studies CagA-positive and CagA-negative strains did not display any significant differences in stimulating cell cycle arrest in *vitro* studies (Ahmed *et al.* 2000). In a recent study of Moss *et al.* (2001) increased proliferation in both the antrum and corpus in *H. pylori* positive patients was not related to *H. pylori* CagA status. According to a study of Ricci *et al.* (1996), VacA specifically inhibited cell proliferation, whereas CagA exerted no effect on cell proliferation.

H. pylori and antral gastrin- and somatostatin-cell density

Gastrin is a hormone regulating gastric acid secretion and growth of the gastrointestinal epithelium, which is also implicated in the genesis of peptic ulcer in conjunction with *H. pylori* infection. The secretion and expression of gastrin are under the paracrine control of somatostatin, which is produced by somatostatin (D-cells) having in close contact with gastrin-producing G-cells. D-cells contain also neuronal nitric oxide synthetase and appear to regulate apoptosis of G-cells by paracrine release of nitric oxide. Both G- and D-cells are derived from a common multihormonal precursor cell present in the regenerative (isthmus) region of the gastric units (Larsson 2000).

In 1967, E. Solcia (University of Pavia) described a cell (G-cell) in the antral mucosa, which he identified as the probable site of gastrin secretion (Solcia *et al.* 1967). In 1968, McGuigan demonstrated, using direct immunofluorescence method (McGuigan 1968), that these cells contained gastrin (Modlin 1995).

H. pylori infection is associated with increased serum gastrin concentration which normalizes after eradication of the bacteria (Chittajallu *et al.* 1991, Katelaris *et al.* 1993, Queiroz *et al.* 1993, Annibale *et al.* 2000). Several studies established that *H. pylori* gastritis is associated with selective reduction and suppression of D-cells, which results in reduced inhibition of G-cell secretion by somatostatin and, as a consequence, enhanced level of gastrin (Moss *et al.* 1992, Ødum *et al.* 1994, Kaneko *et al.* 1992, Calam 1998, Tham *et al.* 1998, Kamada *et al.* 1998, Park *et al.* 1999). G-cell density does not seem to be affected by presence of *H. pylori* (Moss *et al.* 1992, Kamada *et al.* 1998). In contrast, there has been described a reduction in antral G-cells and gastrin secretion with increasing atrophic gastritis (Asnaes and Johansen 1975, Creutzfeld *et al.* 1976, Crivelli *et al.* 1977, Stave *et al.* 1978, Marotta *et al.* 1990, Sipponen *et al.* 1990b). Several data point also to the role of proinflammatory cytokines as well as the influence of lymphocyte infiltration on gastrin biosynthesis in G-cells (Konturek *et al.* 1999b) and a correlation of hypergastrinemia with antrum chronic inflammation (Kaneko *et al.* 1992).

Graham *et al.* (1993) proposed that increased gastrin response in *H. pylori* infection is dependent more on the functional activity of G- and D-cells rather than on G- or D-cell mass, which is in consistent with the data of Tahara and Ito (1983) that serum gastrin level does not reflect directly the number of G-cells in the antrum.

In summary, the outcome of colonization of the gastric mucosa with *H. pylori* depends on many interactions between host factors, bacterial factors and various environmental influences that modulate host response, including immune response to this pathogen. Chronic *H. pylori* gastritis resulting from acquision of the gastric pathogen in childhood, appears to be local immune response to

this pathogen and, on the other hand as consequence of direct damaging properties of *H. pylori* or indirect, mediating by inflammatory response.

The purpose of our study was to evaluate some associations between host immune response to different *H. pylori* antigens and development of atrophic gastritis, to analyse its peculiarities for the antrum and corpus mucosa and to determine the influence of *H. pylori* on epithelial cell turnover in the stomach mucosa.

AIMS OF THE STUDY

1. To determine the seroprevalence of *H. pylori* infection and seropositivity for different *H. pylori* antigens in Estonian adult populations, in a non-selected group of schoolchildren of different age and residence and in a consecutive group of gastric cancer patients.

2. To assess the prevalence of *H. pylori* in gastric biopsies of children with abdominal complaints in case of different morphological and topographical types of gastritis, and to compare the efficacy of *H pylori* detection by Giemsa staining and by immunohistochemical method, as well as to examine the reactivity of antibodies to *H. pylori* towards epithelial cells in the antrum and corpus mucosa.

3. To establish the association of immune response to different *H. pylori* antigens, like CagA, *H. pylori* HSP 60 and acid glycine extracted cell surface proteins of *H. pylori* as well as presence of anticanalicular autoantibodies (ACAB) and parietal cell autoantibodies (PCA) as a sign of autoimmune reaction of *H. pylori* infected persons to host gastric epithelial cells, with the course of *H. pylori* gastritis, particularly development of atrophy in the antrum and corpus mucosa in adult population during 18 years of follow-up.

4. To examine the apoptosis of gastric epithelial cells both in the antrum and corpus mucosa at the initial and end time points of a 18-year follow-up study and to test the hypothesis that increased apoptosis may be associated with development of atrophy.

5. To evaluate the extent of apoptosis of gastric epithelial cells as well as immune response to different *H. pylori* antigens as depending on grade of chronic inflammation, activity of gastritis, intestinal metaplasia and grade of *H. pylori* colonization.

6. To assess the effect of *H. pylori* on epithelial cell proliferation, on expression of the apoptosis protector oncoprotein bcl-2 in the epithelial cells and in interstitial lymphocytes and on the density of specific antral (gastrin and somatostatin cells) and corpus (β H+, K+ ATP-ase immunoreactive) mucosa cells.

STUDY POPULATIONS

1. Population study from Karksi-Nuia and Abja-Paluoja, Southern Estonia (Papers I and II)

This study was part of a large epidemiological investigation of several immunologically mediated diseases, begun in 1990 in two small towns in Southern Estonia. All inhabitants from Karksi-Nuia, aged 15–95 years, and the inhabitants from Abja-Paluoja, aged 50–91 years, were invited to participate in the study. Response rate was 84% in Karksi-Nuia and 77% in Abja-Paluoja. Altogether 1461 persons (637 men, median age 41 years; 824 women, median age 42 years) from Karksi-Nuia and 497 persons (189 men, median age 60 years; 308 women, median age 62 years) from Abja-Paluoja were studied.

Sixty-four persons (28 male, 36 female) from Karksi-Nuia were invited 6 months later for further studies and gastroduodenoscopy due to elevated serum IgA or IgG anti-gliadin antibody levels to find out cases of coeliac disease (Uibo *et al* 1993). In all of them also two biopsy specimens were taken from the gastric corpus and antral mucosa.

The results obtained from the two towns were analysed separately since the persons participating in the study in Abja-Paluoja were older.

Serum samples were taken from all participants and were analysed for IgG antibodies to acid glycine-extracted cell surface proteins of *H. pylori* and for parietal cell antibodies (PCA).

2. Representative sample of the Karksi-Nuia adult population (Paper III)

A total of 199 subjects (86 male, 113 female, median age 42.4 years) formed a representative sample of adult population from the South Estonian town of Karksi-Nuia. The subjects were selected from the 1461 persons (637 male, 824 female, median age 42.3 years) who had participated in the large seroepidemiological study in 1990 and donated blood. The relative proportion of males/females of different age was calculated on the original material of 1461 samples, and this proportion was extrapolated to a set of 200 samples. According to these proportions, serum samples from each age/sex group of the whole material were randomly selected by the computer. In the present study, 199 sera out of the 200 samples were available for the determination of *H. pylori* and CagA status.

3. Group of patients with gastric carcinoma (Paper III)

Forty-five consecutive patients (22 male, 23 female, median age 64.5 years) with gastric adenocarcinoma, operated on and diagnosed pathohistologically in the Clinic of Oncology, University of Tartu, and recruited during two periods, in 1986–1987 and in 1995–1996, were studied. Gastric cancer patients came from Tartu and from South Estonian counties including Karksi-Nuia. Adenocarcinoma was localised in the antrum in 14 cases, in the corpus in 17 cases,

and both in the antrum and corpus in 14 cases. Gastric carcinomas were classified histologically according to Laurén (Laurén, 1965). The intestinal type adenocarcinoma was diagnosed in 18 cases, the diffuse type in 9 cases and the indeterminate type in 14 cases; 4 cases were inoperable (histological type not known).

The sera of these persons were collected before gastric surgery and were stored at –20°C until assayed and analysed for IgG antibodies to acid glycine extracted cell surface proteins of *H. pylori* and anti-CagA IgG.

In order to compare the presence of IgG antibodies to CagA, using ELISA and immunoblot, 141 sera which included all 45 sera from gastric cancer patients, 52 sera from a representative population sample with borderline absorbance values in CagA ELISA, 9 sera from gastric and duodenal ulcer patients (all *H. pylori* positive in ELISA with the use of the glycine extracted cell surface antigen) and 35 sera from children with abdominal complaints, treated in Tartu Children's Hospital (10 *H. pylori* positive cases and 25 *H. pylori* negative cases both in ELISA and morphologically) were consequently analysed by immunoblot. Additionally, a group of age and sex matched controls (45 sera from the serum bank of the Department of Immunology, University of Tartu) were studied in immunoblot assay, using a commercial Helico Blot 2.0 kit, for comparison with gastric cancer patients.

4. Group of schoolchildren of different age living in urban and rural areas in Southern Estonia (Paper IV)

A cross-sectional study of schoolchildren was carried out from autumn 1993 to spring 1996 in five Southern Estonian counties. Secondary schools were selected proceeding from the consideration that they should be of similar size in each county, which yielded a total of 20 schools. Within a particular school, children were selected randomly from the school roll, and every third 9-, 12- and 15- year-old child was afforded an opportunity to participate. The overall response rate of 78% yielded a total of 1074 children who agreed to participate in the study. However, 56 of these children were excluded for the following reasons: absence from school on the day of the study (16 children), chronic disease (diabetes, asthma, etc.: 7 children), reluctance to undergo blood sampling despite a prior agreement (13 children), inadequately completed questionnaires (20 children). The investigated children were ethnic Estonians.

Serum samples were tested and analysed from a total of 421 children (190 boys, 231 girls). The distribution of the studied 421 schoolchildren according to age, gender and place of residence is presented in Paper IV, Table I.

Serum samples were collected and stored at –20°C until assayed for IgG antibodies to acid glycine extracted cell surface proteins of *H. pylori* and to CagA protein.

5. Group of children with abdominal complaints (Paper V)

One hundred and twelve children (41 boys and 71 girls; age range 1–16 years, median age 12 years) with abdominal complaints, defined according to Apley's criteria (Apley and Naish 1958) and endoscoped in Tartu Children's Hospital in the period 1990–1991, were studied.

In this group the prevalence of *H. pylori* in gastric antrum and corpus biopsy specimens with different grades of gastritis was evaluated using Giemsa staining and immunohistochemistry. In order to compare the results of the study based on the children's mucosa respective data for adults, 38 biopsies (20 from the antrum and 18 from the corpus mucosa) taken from 23 consecutive patients with peptic ulcer (16 with gastric and 7 with duodenal ulcer; mean age 52.7±9.0), endoscoped in the Department of Gastroenterology in Tartu University Hospital, were studied.

6. Adult sample from the island of Saaremaa in a 18 year follow-up study of chronic gastritis (Papers VI–IX)

Seventy persons (31 male, 39 female, median age 57.5 years) of an adult sample of 304 from Saaremaa Island, most of whom had previously been investigated by endoscopy and biopsy in 1979, 1985 and 1991, were re-investigated by endoscopy and biopsy in 1997. Of the initial 304 subjects, 227 were randomly selected and 77 wished to be investigated due to an abdominal complaint (Villako *et al.* 1982).

During the 18-year follow-up, of the original 304 subjects, 39 had died by 1997 (gastric carcinoma — 2, coronary heart disease and heart failure — 17, malignancies other than gastric causes or leukaemia — 11, cerebral insult — 6, other causes — 3). The other subjects were lost because of high age, severe illness, change of residence, failure of communication or refusal. Gastroduodenal endoscopy was performed at the beginning of the study and subsequently at 6, 12 and 18 years. The results of the 6- and 12-year follow-up studies of the course of gastritis are described elsewhere (Villako *et al.* 1991, 1995). The course of gastritis is evaluated as the difference between the first and the last follow-up examinations over 18 years.

In paper VI, the state of gastric mucosa and the presence of *H. pylori* in histological sections were assessed in 66 subjects, and a serologic evaluation of IgG antibodies to *H. pylori* and to the CagA protein of *H. pylori* was performed on 70 sera.

In paper VII, anticanalicular autoantibodies (ACAB) were evaluated in the sera of 62 persons (30 men, 32 women, mean age 57.6±11.4). The inclusion criteria for 62 out of 68 persons for assessment of ACAB were: seropositivity for *H. pylori* in ELISA, presence of *H. pylori* in antrum and/or corpus biopsy specimens, assessment of antrum and corpus biopsy specimens by two pathologists.

Additionally, antrum and corpus biopsies and serum samples for 37 out of the 62 persons (mean age 43.7 ± 10.6, 21 males, 16 females) were available

from 1985, i.e. from the time 12 years earlier. Serum samples from both points of time were stored at –20°C and were then screened for ACAB and classical PCA.

In paper VIII, apoptotic index (AI) was evaluated in 68 persons (31 men, 37 women, median age 39 in 1979), whose antrum and corpus biopsy specimens were available for both time points, 1979 and 1997. Apoptotic index (AI) was evaluated in the samples of 1979 for 52 antrum and 64 corpus biopsy specimens and in the samples of 1997 for 58 antrum and 66 corpus biopsy specimens. The dynamics of AI between the two time points, 1979 and 1997, was evaluated in the antrum biopsies of 49 persons and in the corpus biopsies of 64 persons.

In paper IX, a serological evaluation of IgG antibodies to *H. pylori* HSP60 was performed on 68 sera collected in 1997. In these persons the state of gastric antrum and corpus mucosa was evaluated in 1979 and 1997. Additionally, IgG antibodies to *H. pylori* HSP60 were tested in a 12-year follow-up in 52 persons whose serum and histological data were available for 1985 and 1997.

7. Group of 59 patients investigated by routine endoscopy in the Department of Gastroenterology of the University Hospital, Bern (Paper X)

A total of 59 patients (34 men, median age 47), endoscoped in the Department of Gastroenterology of University Hospital, Bern, in 1993 (retrospective study — 39 persons) and in January-February 1994 (prospective study — 20 persons), were studied. The group of patients included 20 persons with peptic ulcer (gastric ulcer — 4 cases; duodenal ulcer — 16 cases), 18 with erosion in the antrum or/and corpus mucosa and duodenitis, 5 with hiatus hernia, one with reflux oesophagitis, one with gastric corpus adenocarcinoma, one with a tumour of the liver and pancreas, one with colon carcinoma, and one with liver cirrhosis. In 11 patients no pathological gastroduodenoscopic findings were established.

In 40 patients, biopsies were sampled both from the antrum and corpus, in 16 patients only from the antrum, and in 3 patients only from the corpus.

In this group of patients gastric epithelial cell proliferation was studied, and staining for bcl-2, and for gastrin and somatostatin cells as well as for β H+, K+ ATPase reactive parietal cells was performed.

METHODS

Serologic evaluation of IgG antibodies to *H. pylori*

Enzyme-linked immunosorbent assay (ELISA) for evaluation of IgG antibodies to the acid glycine-extracted cell surface proteins of H. pylori

Seropositivity to *H. pylori* cell surface proteins (strains NCTC 11637 or CCUG 1784) was evaluated using ELISA. Acid glycine (pH 2.2)-extracted cell surface proteins (0.5 μg per well) were used for the coating of microtitre plates (NUNC, Roskilde, Denmark). The studied sera were diluted to 1:800; the second antibody was alkaline phosphatase-labelled anti-human IgG (DAKO, Glostrup, Denmark) which was diluted to 1:500. The results were expressed by corrected mean absorbance values as a percentage of reference standards (human gammaglobulin, Pharmacia & UpJohn, Stockholm, Sweden). The cut-off value for seropositivity was set to a relative antibody activity (RAA) (Lelwala-Guruge *et al.* 1992) of 25. The sensitivity of the test was 98%, specificity 82%.

ELISA for evaluation of IgG antibodies to the 128-kDa cytotoxin-associated (CagA) protein

Anti-CagA IgG were detected by ELISA using the recombinant fragment of the CagA antigen His-17/12 of HP CCUG 17874 strain following the methodology, described by Xiang *et al.* (1993), with modifications. In our study the recombinant antigen was diluted in 0.1 M carbonate buffer of pH 9.6 to a final concentration of 1.25 μg/ml. Maxisorp Immunoplates (NUNC, Roskilde, Denmark) were coated with 100 μl/well in duplicate and incubated for 16h at 4°C, washed three times with PBS (pH 7.4) containing 0.05% Tween 20. The wells were saturated with 200 μl/well of 1% bovine serum albumin (BSA) in PBS — Tween at 37°C for 2h. After washing the plates three times, 100 μl of each serum sample diluted to 1:300 in 1% BSA — PBS, were added to the wells in triplicate (two wells with the antigen and one without the antigen) and incubated at 37°C for 2h. The plates were then washed three times, and 100 μl/well of alkaline phosphatase-conjugated anti-human IgG (DAKO, Glostrup, Denmark), diluted to 1:1000 in 1% BSA-PBS, were added and incubated at 37°C for 1.5h. After three washes, 100 μl/well of a substrate solution, containing p-nitrophenyl phosphate (Sigma Chemical Co, St. Louis, MO, USA) in diethanolamine-$MgCl_2$ buffer, were added to each well and incubated at 37°C for 30 min. Each plate contained a positive and a negative control serum. Absorption was read at 405 nm. The absorption value derived from the mean value of absorption in two wells with the antigen minus the absorption value in well without antigen was taken as the absorbance value of the studied sera. Instead of the cut-off of 0.250 OD used by Xiang *et al.* 1993, the absorbance value of 0.300 (A 405 nm) was set as the cut-off point in our study, based on the calculation of ELISA results, derived from the mean absorbance value of 25 *H. pylori* negative persons (histologically and serologi-

cally) plus two standard deviations, by using square root transformation (Brown *et al.* 1989). Interassay variation was 11.5%.

ELISA for evaluation of IgG antibodies to the H. pylori heat shock protein 60
For determination of antibodies to *H.pylori* HSP 60, LabSyst (Labsystems, Finland) 96 well plates were coated with *H. pylori* HSP 60 at a concentration of 1 μg/ml (0,1 μg/well) for 16 h at 4°C. The HSP 60 used was purified from the supernatant of sonicated *H. pylori* cells by gel filtration on Superose 12® and by ion exchange chromatography on MonoQ® at pH 6.7. The purity of the antigen was tested by immunoblot using the serum of *H. pylori* infected patients and was found to be approximately 97% homogeneous. After 1.5 h of blocking with 2% bovine serum albumin in PBS, the plates were washed four times with PBS-0.05% Tween 20 (Sigma, St. Louis, USA). Serum samples were diluted to 1:800 in PBS-0.02% Tween and incubated for 1.5 h at 37°C. Alkaline phosphatase conjugated rabbit anti-human IgG (DAKO, Glostrup, Denmark) was used as the secondary antibody (dilution 1:500, incubation for 1h at 37°C). Bound antibodies were visualized by addition of a substrate solution containing 1 mg/ml *p*-nitrophenylphosphatase (Sigma Chemical Co, St. Louis, MO USA) in diethanolamine buffer, pH 9.8. Optical density was read at 405 and 492 nm after 50 min of incubation.

The results were expressed as corrected mean absorbance values as a percentage of the value for a reference standard (human gammaglobulin, Pharmacia & UpJohn, Stockholm, Sweden) as relative antibody activity (RAA). The cut-off value for seropositivity to *H. pylori* HSP 60 (RAA=24) was based on the results obtained from the testing of 28 sera previously known to be negative for antibodies against the *H. pylori* glycine extracted cell surface protein (mean absorbance value of 28 *H. pylori* negative persons plus two standard deviations). The sensitivity of the test was 69% and specificity 83%.

Immunoblot for evaluation of IgG antibodies to H. pylori
Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Protean II Cell Vertical Electrophoresis equipment (Biorad, Richmond, CA). Glycine extracted proteins from seven pooled antigen batches of HP strain NCTC 11637 were separated in a gradient gel (5–20%) with a 5% stacking gel (acrylamide/bis 29:1, Biorad, Richmond, CA) and were used for protein separation. The antigen (140 μg/gel) was diluted four times in a sample buffer (0.5M TRIS-HCL pH 6.8, 0.5% bromphenol blue, 8% glycerol, 4% SDS, 4% 2-mercaptoethanol) and heated at 95°C for 3 min. After cooling, the proteins were loaded on the gel and separated for 16h at 80 V. Molecular weight standards (Promega, Scandinavian Diagnostic Service, Falkenberg, Sweden), including proteins ranging from 14.3 to 97.4 kD in size, were treated similarly.

The proteins were transferred electrophoretically to the Immobilon PVDF membrane (0.45 μm, Millipore Intertech, Bedford, MA, USA) using a semi-dry

electro-blotter equipment (Ancos, Vig, Denmark) during 1.5h at a constant current of 0.8 mA/cm^2.

The membrane was saturated by incubation for 2×15 min. in blocking buffers I and II (from M. Rucheton, Orstom Laboratories, Montpellier, France) (Rucheton *et al.* 1992). Saturated membranes were rinsed once for 10 min. in a washing buffer and cut into strips. The strips were overlaid with sera diluted to 1:100 in a washing buffer under gentle agitation for 16 h at 4°C. The strips were then rinsed 3x5 min. and incubated for 2h at 4°C with horseradish-peroxidase labelled anti-human IgG antibodies (DAKO, Glostrup, Denmark) diluted to 1:600. After repeated rinsing, bound antibodies were detected by reaction in a 50 mM sodium acetate buffer (pH 5.0) containing 0.04% 3-amino-9-ethylcarbazole (Sigma Chemical Co, St. Louis, MO, USA) and 0.015% H_2O_2. The intensity of the bands obtained with patient sera was compared to that of the strip incubated with human gammaglobulin (KABI/ Pharmacia, Stockholm, Sweden) included as a calibrator in each analysis.

In analysis, special attention was paid to presence of antibodies to the cytotoxin-associated high molecular protein band of 128 kD.

Immunoblot using a Helico Blot 2.0 kit

IgG response to different *H. pylori* proteins in the gastric cancer group and in age-sex matched non-cancer controls was analysed using a commercial kit Helico Blot 2.0 (courtesy of Genelabs Diagnostics, Singapore), following the manufacturers' recommendations. Patients' sera (dilution 1:100) were incubated on commercial strips for 1 h at room temperature, followed by incubation with goat anti-human IgG conjugated with alkaline phosphatase, dilution 1:1000, for 1 h at room temperature. Finally, the strips were incubated with a substrate solution (5-bromo-4-chloro-3-indoyl-phosphate (BCIP) and nitro blue tetrazolium (NBT) for 15 min. The intensity of the bands obtained with patients' and control sera was compared with that of the commercial control. The sample was considered positive in case it was found reactive with any one band of 116 kD, 89 kD, 35 kD, or with any two bands of 30 kD, 26.5 kD, 19.5 kD.

Histopathological evaluation of state of gastric mucosa and *H. pylori* colonization

In 68 persons from a group of adult persons from Saaremaa, formalin-fixed, paraffin-embedded gastric biopsy specimens from the antrum (3 specimens) and corpus mucosa (6 specimens), taken in 1979 and 1997 and in 52 persons taken also in 1985, were stained with haematoxylin and eosin as well as with a modified Giemsa stain. The state of the gastric mucosa and presence of *H. pylori* in histological sections were assessed in accordance with the updated Sydney system (Dixon *et al.* 1996) and scored from 0 (no changes) through 1 (mild) and 2 (moderate) to 3 (severe changes). The course of gastritis was evaluated as the difference between the first (1979) and the last (1997) follow-up examination over 18 years.

In 120 children with abdominal complaints, endoscoped in Tartu Children's Hospital, and in 59 patients, endoscoped at the Department of Gastroenterology of the University Hospital, Bern, presence of *H. pylori* and the state of the gastric mucosa were examined in haematoxylin and eosin-stained sections and were graded as follows: normal, i.e. no loss of glands and no chronic inflammation (score 0); superficial gastritis, i.e. non-atrophic gastritis in three stages: mild, moderate or severe (score numbers 0.5, 1.0 and 1.5 correspondingly); atrophic gastritis in three stages: mild, moderate, severe (score numbers 2.0, 3.0 and 4.0 correspondingly).

Immunohistochemical methods

Evaluation of H. pylori on histological sections

Formalin-fixed, paraffin-embedded biopsy specimens were studied using peroxidase-antiperoxidase (PAP) method. After deparaffinisation, 5 μm tissue sections were pre-treated with 0.1% trypsin (Spofa, Prague, Chech Republic) 15 min. at 37° C and with hydrogen peroxide in methanol for 30 min. at 20°C to prevent non-specific reactions. As primary antibodies, rabbit anti-*H. pylori* polyclonal antibodies (anti-*H. pylori* IgG; DAKO, Glostrup, Denmark) were used (dilution 1:50; incubation overnight at 4°C). Sections from the same biopsy specimens, incubated without the primary antibody, served as negative controls. As the secondary antibody, swine-anti- rabbit IgG (DAKO, Glostrup, Denmark) was used (dilution 1:100; incubation 1 h at 20°C), followed by incubation with the peroxidase antiperoxidase rabbit complex (DAKO, Glostrup, Denmark; dilution 1:500; incubation 1 h at 20°C). Binding of specific antibodies was visualized using 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co, St. Louis, MO, USA). The sections were counterstained with haematoxylin and examined by one investigator (T.V.) blinded to the results of gastritis grading and the results of *H. pylori* positivity based on Giemsa preparations. The entire surface of all sections present on the slide was examined. Staining was considered positive if brown-stained organisms of a typical shape were seen on the luminal surface of the corpus or antrum glands (Fig. 1). Additionally, we assessed the immunostaining of antral or corpus epithelial cells with anti-*H. pylori* IgG (Fig. 2).

Evaluation of anticanalicular autoantibodies (ACAB)
in serum against canalicular structures within human parietal cells

ACAB, reacting against canalicular structures within human parietal cells, were detected using immunohistochemical method. Heterologous formalin-fixed and paraffin-embedded gastric corpus mucosa, without pathological alterations and not expressing blood groups A and B, was incubated overnight at 20°C with sera diluted to 1:100 in the RPMI 1640 medium (Biochrom, Berlin, Germany). As the secondary antibody, alkaline phosphatase conjugated rabbit anti-human IgG (DAKO, Hamburg, Germany), diluted to 1:10 in the RPMI 1640 medium,

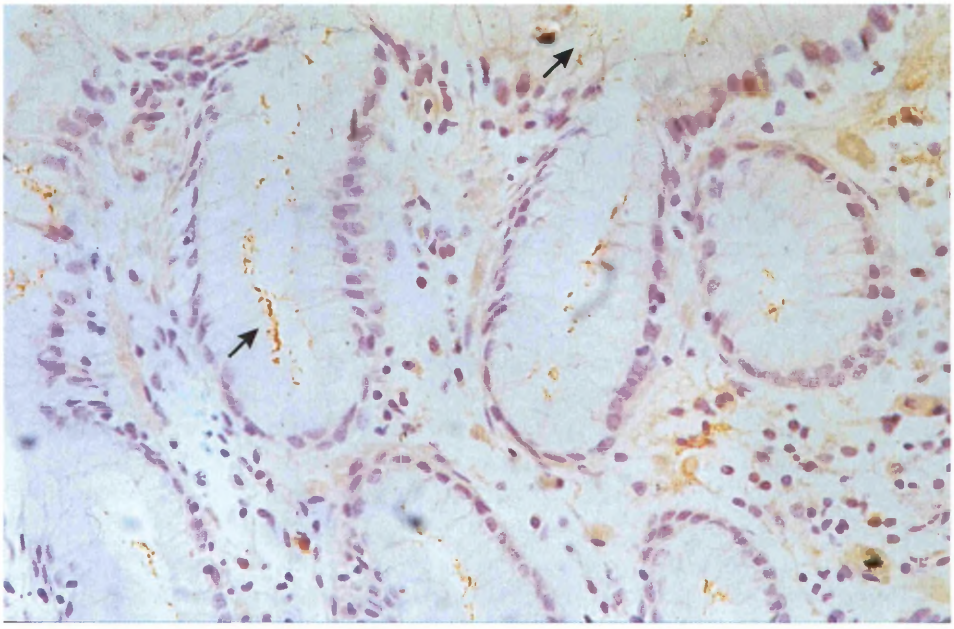


Figure 1. Positive staining for *H. pylori* on the surface of the foveolar epithelium cells of the corpus mucosa (arrow). Peroxidase-antiperoxidase method, using anti-*H. pylori* polyclonal antibodies (DAKO); × 40 objective, × 10 eyepiece.

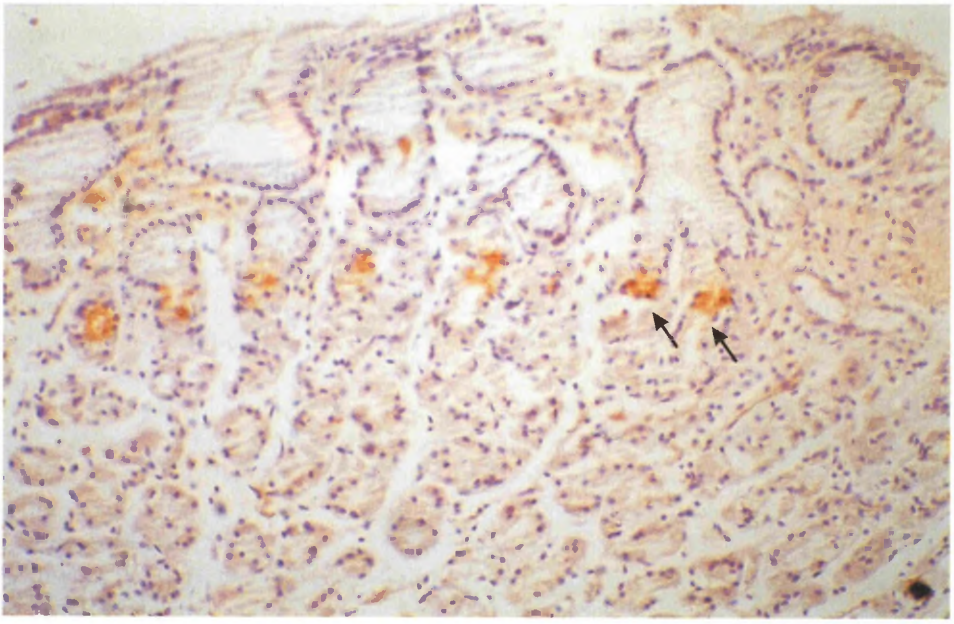


Figure 2. Positive staining with anti-*H. pylori* polyclonal antibodies (DAKO) localized in the cytoplasma of corpus mucosa cells in the neck part of the glands (arrow). Peroxidase-antiperoxidase method; × 20 objective, × 10 eyepiece.

was used (incubation at 20°C for 90 min.). After washing, positive reaction was induced with fast red. The sections were counterstained with haematoxylin, mounted with Aquatex and examined by light microscopy, without the knowledge of respective patient data or histological alterations. One positive control serum was included in each immunohistochemical staining session, and negative controls were performed omitting the human serum. Intensive red immunohistochemical staining, visible at the canaliculi of parietal cells in the corpus mucosa, was taken as positive reaction (Fig. 3, Fig. 4 and Paper VII, Fig 2.).

Indirect immunofluorescence method for evaluation of PCA
Classical IgG-type PCA was examined using indirect immunofluorescence method (Fig. 5). Unfixed cryostat sections from the mouse stomach fundal region was used as the antigen. Rat kidney and liver sections were studied as controls. The dilution of the studied sera was 1:10 and 1:100. FITC-conjugated sheep anti-human IgG was used as the secondary antibody. Every set of tests contained a known PCA positive and a PCA negative serum.

Evaluation of apoptosis using terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling (TUNEL) method
Apoptosis was evaluated with the use of TUNEL (terminal deoxynucleotidyl-transferase mediated dUTP-biotin nick end labelling) histochemistry method (Gavrieli *et al.* 1992). After deparaffinisation and rehydration, the sections were digested with proteinase K (Sigma, St Lois, MO) for 30 min. at 20°C (20 μg/ml in TRIS-HCL). After washing with PBS (pH 7.4) a TUNEL reaction was performed using the In Situ Death Detection Fluorescein Kit (Boehringer Mannheim, Germany). The principle of the test is the following: DNA strand breaks, generated during apoptosis, were identified by labelling with fluorescein dUTP (digoxigenin-11-deoxyuridine triphosphate) using terminal deoxynucleotidyl transferase (TdT). By an enzymatic reaction (incubation for 40 min. at 37°C) with TdT, fluorescein labelled dUTP was specifically transferred to the 3'-OH ends of DNA. After washing, with PBS (pH 7.4) and treatment with BSA (bovine serum albumin, 1 mg/ml in PBS) for 10 min., the sections were subsequently incubated with monoclonal mouse antifluorescein antibodies (DAKO, Glostrup, Denmark, diluted 1:10, incubation for 30 min. at 20°C). After washing the sections were incubated with rabbit anti mouse biotinylated monoclonal antibodies (DAKO, Glostrup, Denmark, diluted 1:50, for 30 min. at 20°C), followed by incubation with the Strept AB Complex (streptavidin complexed with biotinylated alkaline phosphatase; DAKO, Glostrup, Denmark) and fast red as a substrate. Counterstaining was done with haematoxylin. Apoptotic cells with incorporated dUTP showed a distinct bright red signal in the nucleus (Fig. 6 and in Paper VII, Fig. 1). For negative controls TdT was omitted. The corpus gastric mucosa with lymphofollicules, containing apoptotic lymphocytes, was used as positive control.

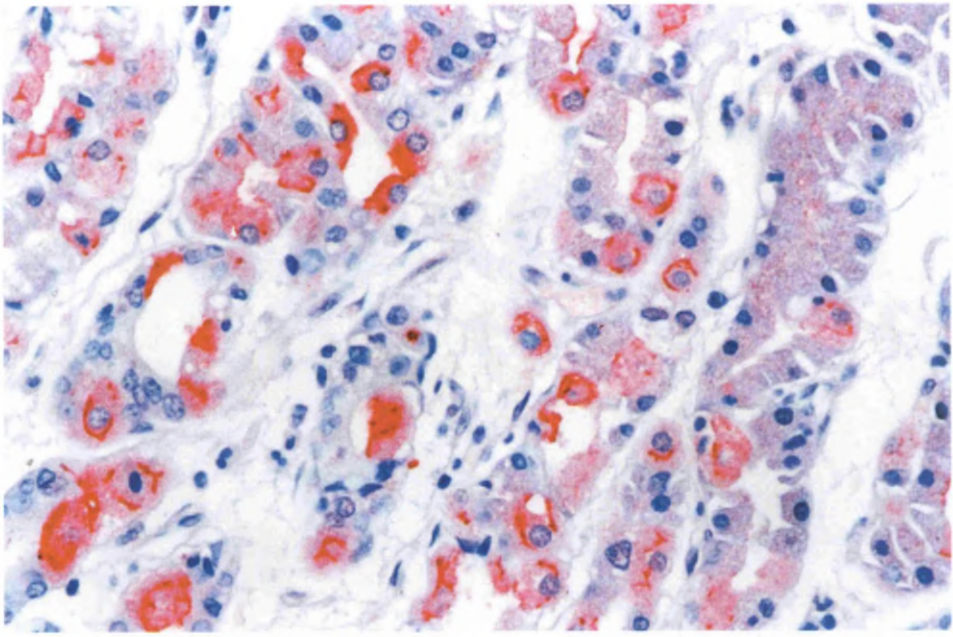


Figure 3. In situ binding pattern of anticanalicular autoantibodies reacting against the canalicular membranes within the human parietal cells of the gastric corpus mucosa; × 40 objective, × 10 eyepiece.

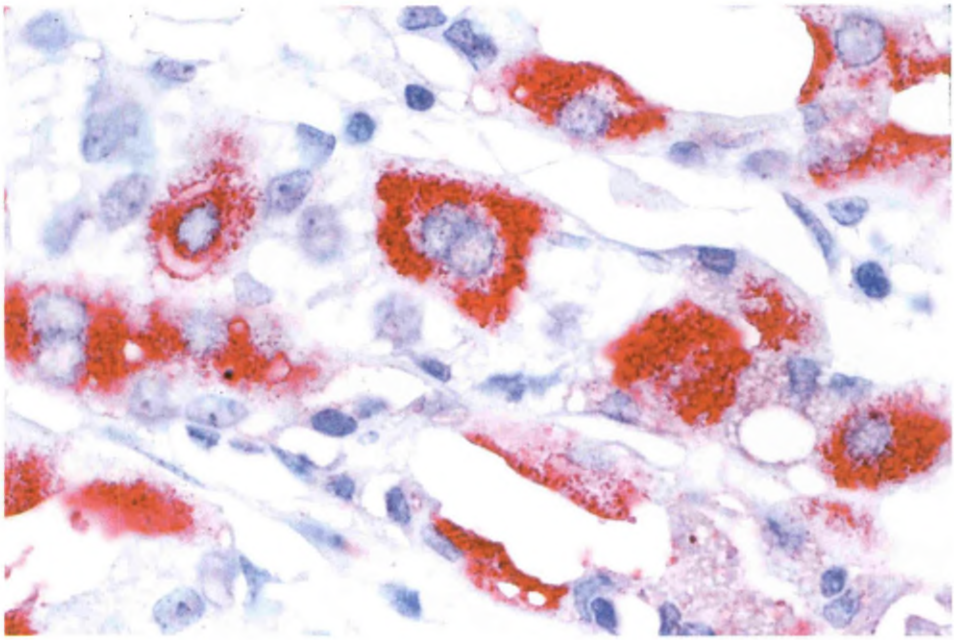


Figure 4. Intensive staining for anticanalicular autoantibodies throughout the cytoplasma of parietal cells in PCA positive cases (× 100 objective, × 10 eyepiece).

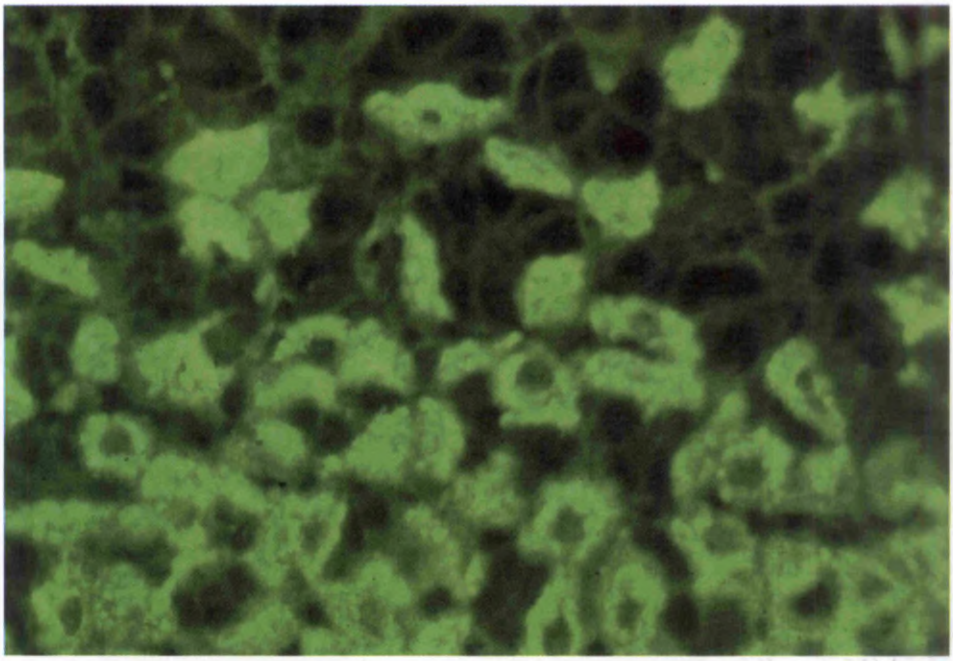


Figure 5. Parietal cell autoantibodies reacting by indirect immunofluorescence test (× 100 objective, × 10 eyepiece).

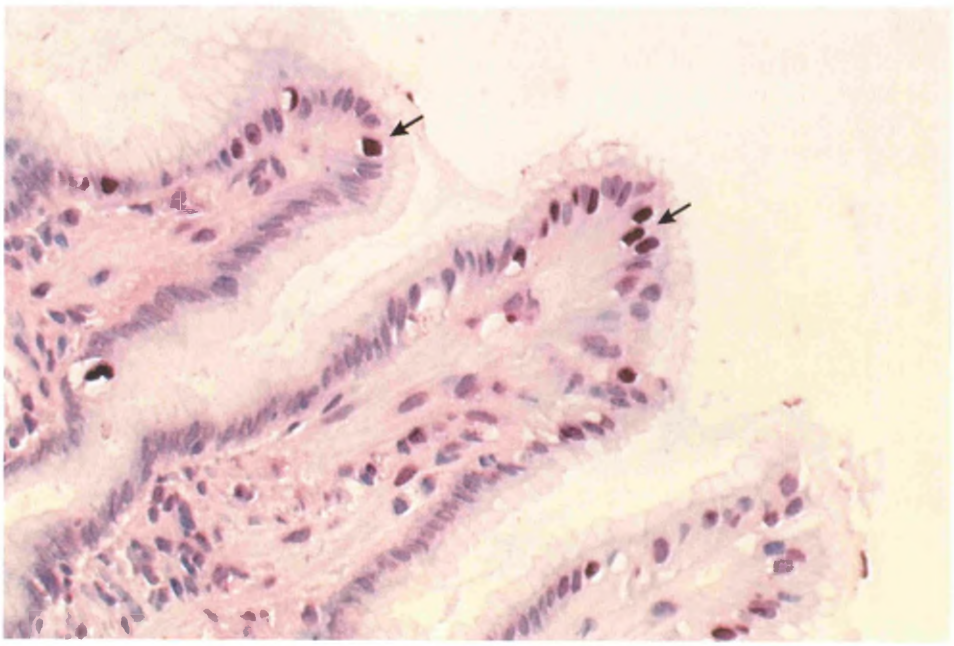


Figure 6. Apoptotic cells in the foveolar cell compartment of the antrum mucosa (arrow, × 40 objective, × 10 eyepiece). TUNEL histochemistry.

Morphometry: The rate of apoptotic cells in the antrum and corpus mucosa was quantified by counting, in triplicate, 200 cells of the foveolar cells (FC) compartment, 200 cells in the proliferating zone (PZ) (neck part) (only for well oriented glands) and 200 cells of the glandular part (GP) of the section, using a light microscope with magnification ×400. The mean value of positively labelled nuclei per 200 cells, calculated in three different section areas separately for FC, PZ and GP, were expressed as apoptotic index (AI). Intra-assay variation for AI was 11%. Counting was done by one observer (T.V.) who was not aware of morphological data or the year of sampling (done in mixed manner).

Evaluation of gastric epithelial cell proliferation, apoptosis protector bcl-2 protein and immunostaining for gastrin and, somatostatin cells and β-H+, K+ ATP-ase in the canaliculi of parietal cells using Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP) method and Avidin-Biotin method

Gastric epithelial cell proliferation was assessed by employing the Proliferating Cell Nuclear Antigen (PCNA) and Ki-67 (MIB 1) labelling. PCNA immunostaining was performed using alkaline phosphatase anti-alkaline phosphatase (APAAP) procedure. After deparaffinisation and pre-treatment with 20% acetic acid (15 sec, 4°C) and 3% BSA (Merck) in TRIS-NaCl buffer (20 min., 20°C), tissue sections were incubated overnight (4°C) with a primary mouse monoclonal antibody against PCNA (PCNA PC10, DAKO, Glostrup, Denmark, dilution 1:50). Rabbit anti-mouse immunoglobulin (DAKO, Glostrup, Denmark, dilution 1:30) was used as the secondary antibody (incubation 30 min, at 20°C). The APAAP mouse monoclonal antibody complex (DAKO, Glostrup, Denmark, dilution 1:50) was then applied for 60 min. To enhance the intensity of final staining, these two steps were repeated. New fuchsine (Merck)-naphthol AS-Bi phosphate (Sigma, St. Louis, USA), in a TRIS-HCl buffer, pH 8.7, containing 2.1 mg/ml levamisole (Sigma, St. Louis, USA), was used as the alkaline phosphatase substrate for 20 min. at room temperature. The reaction was stopped by rinsing the sections in cold water. The sections were counterstained with haematoxylin (Merck) and mounted with Aquatex (Merck).

Immunostaining for Ki-67, bcl-2 oncoprotein as well as for gastrin, somatostatin, and β- H+, K+ ATPase immunoreactive cells was performed using avidin-biotin procedure. After deparaffinisation and rehydration, the sections were treated in a microwave oven in a 10 mM citrate buffer, pH 6.0, once at 190 W for 5 min. and twice at 140 W for 5 min. before staining for Ki-67, bcl-2 and gastrin cells. Further, the plastic jar was allowed to cool for 15 min. at room temperature and was then washed for 10 min. in a TRIS-NaCl buffer, pH 7.5.

As primary antibodies, monoclonal anti Ki-67 (MIB-1, Dianova, Hamburg, dilution 1:50), the anti-human bcl-2 oncoprotein (clone 124, DAKO, Glostrup, Denmark, dilution 1:50), the anti- β subunit of H+, K+ ATP-ase (AB 2611 kindly donated by Dr. J.G. Forte, Berkeley, USA; dilution 1:200), polyclonal

anti-gastrin (DAKO, Glostrup, Denmark; dilution 1:300) and anti-somatostatin (DAKO, Clostrup, Denmark; dilution 1:1000) were used. Anti- β-H+, K+ ATPase and anti-somatostatin antibodies were applied on tissue sections without microwave pre-treatment. All primary antibodies were diluted in a TRIS-NaCl buffer with 0.1% casein and 0.1% NaN_3 (pH 7.5). The preparations were incubated with primary antibodies for 60 min. at 20°C. Subsequently, the sections were incubated with rabbit anti-mouse biotinylated immunoglobulins (DAKO, Glostrup, Denmark) for monoclonal primary antibodies, or with swine anti-rabbit immunoglobulins biotinylated immunoglobulins (DAKO, Glostrup, Denmark) for polyclonal primary antibodies at dilution 1:200 for 45 min. The Avidin-Biotin Complex (DAKO, Glostrup, Denmark) was applied for 45 min. at room temperature. Alkaline phosphatase reaction was developed for 30 min. in the New Fuchsine substrate solution. The specimens were then washed in tap water, counterstained with haematoxylin and mounted with Aquatex (Merck). Negative controls were performed by replacing the primary antibodies by the TRIS-NaCl buffer.

A typical Ki-67 labelling pattern in the PZ and FC compartments of the corpus mucosa is shown in Fig. 7. Bcl-2 positive staining of interstitial lymphocytes in the antrum mucosa is shown in Fig. 8. Staining of G-cells and D-cells is presented in Figs. 9 and 10.

Staining with the monoclonal antibodies directed against β- H+, K+ ATPase in parietal cell canaliculi is presented in Fig. 11.

In our earlier study (Vorobjova *et al.* 1989), which was undertaken to evaluate the expression of the epithelial membrane antigen in the antrum and corpus mucosa of patients with different states of gastric mucosa (normal, chronic gastritis, gastric ulcer and gastric cancer), using monoclonal antibodies to human milk fat globule membranes (HMFG) by peroxidase-antiperoxidase (PAP) method, we revealed a dark brown staining in the cytoplasm of the parietal cells. Positive staining, evaluated using HMFG monoclonal antibodies, corresponded to the staining of parietal cell canaliculi (cf. Fig. 11, use of monoclonal antibodies to β H+, K+ATPase and Fig. 12a, for 1989, use of monoclonal antibodies to HMFG). Regrettably, in 1989 when we published our paper we failed to interpret these results properly, nor did we pay attention to this fact. As early as 1979, Heyderman *et al.* indicated that monoclonal antibodies directed against HMFG membrane proteins cross-reacted with gastric mucosal cells. Later, in 1995, Walker *et al.* published in J Clin Pathol the study "Identification of parietal cells in gastric body mucosa with HMFG-2 monoclonal antibody". The authors used the mentioned antibodies and, additionally, double staining with monoclonal antibodies, directed against an epitope on the α subunit of H+, K+ ATPase, and recommended to use HMFG monoclonal antibodies as highly specific markers of human gastric parietal cells.

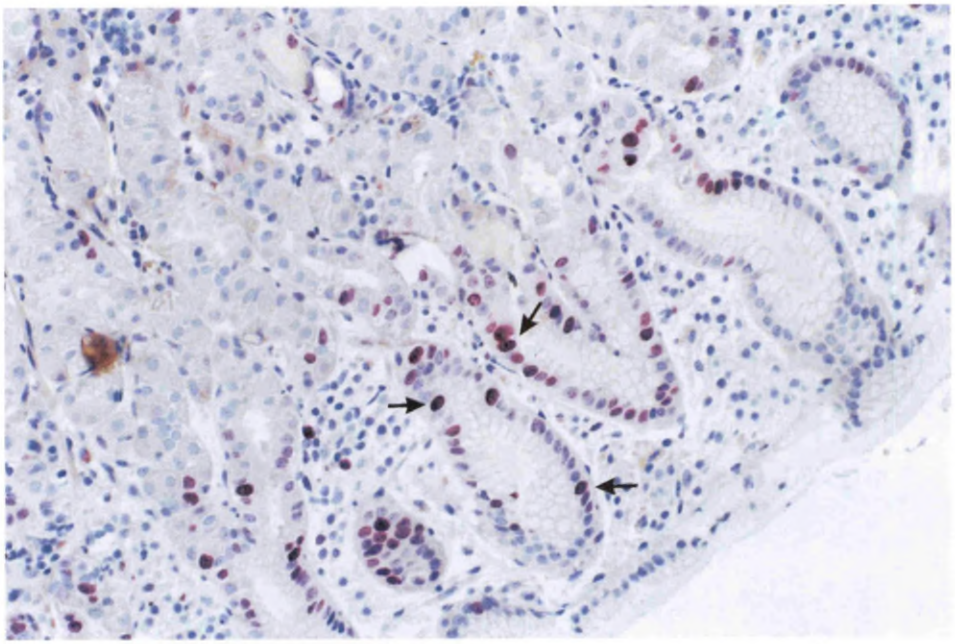


Figure 7. Ki-67 labelling pattern in the proliferating zone and in the foveolar cell compartment of the corpus mucosa (intensive red staining, arrow; × 20 objective, × 10 eyepiece). Avidin-Biotin method.

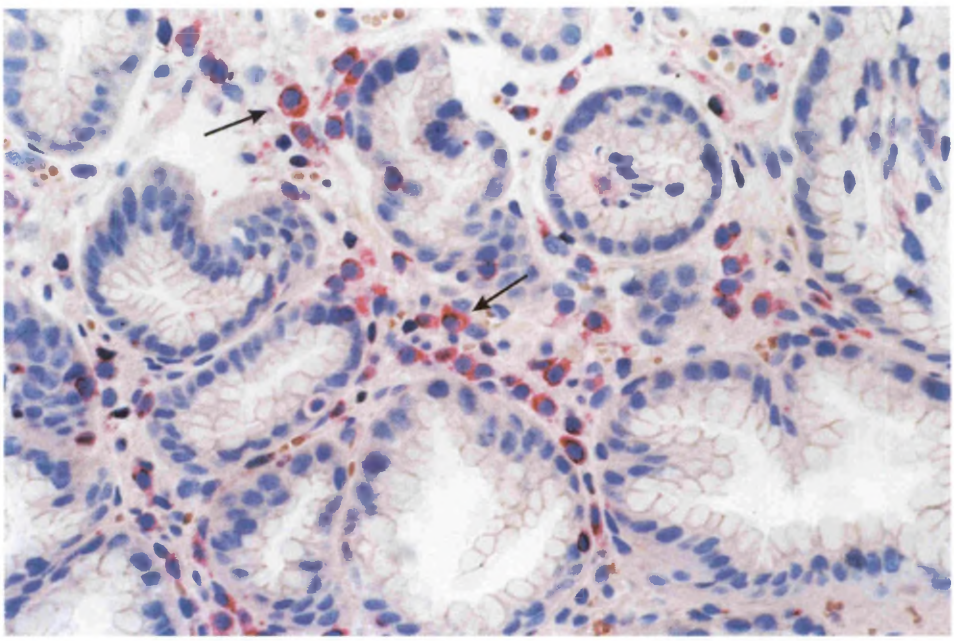


Figure 8. Bcl-2 positive staining for interstitial lymphocytes in the antrum mucosa (intensive red staining, arrow; × 40 objective, × 10 eyepiece). Avidin-Biotin method.

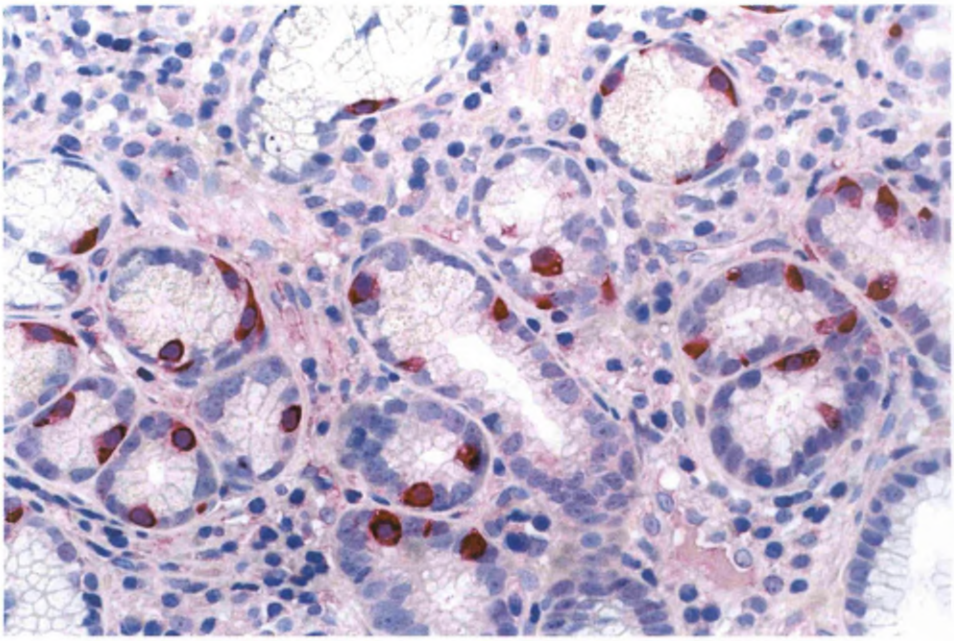


Figure 9. Positive staining for gastrin cells in the glandular part of the antrum mucosa (intensive red staining; × 40 objective, × 10 eyepiece). Avidin-Biotin method.

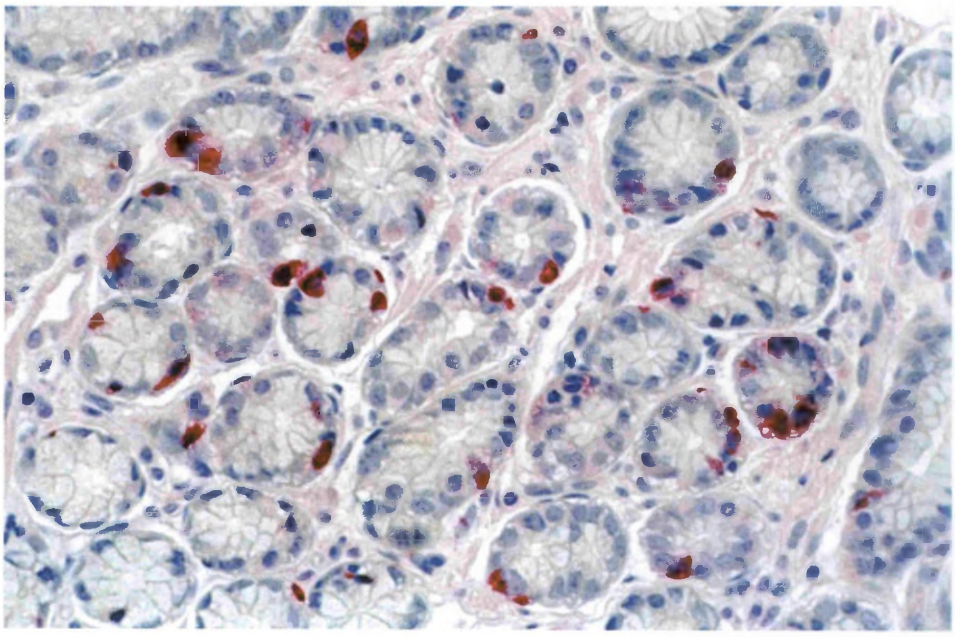


Figure 10. Positive staining for somatostatin cells in the glandular part of the antrum mucosa (× 40 objective, × 10 eyepiece). Avidin-Biotin method.

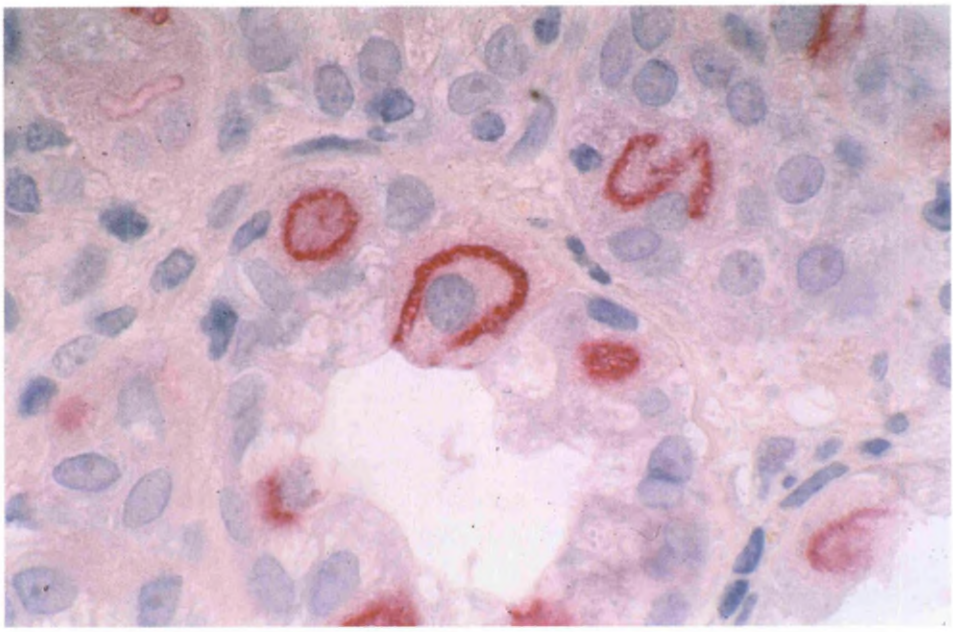


Figure 11. Positive staining for β-H+, K+ ATP-ase in the canaliculi of parietal cells (intensive red staining, × 100 objective, × 10 eyepiece). Avidin-Biotin method.

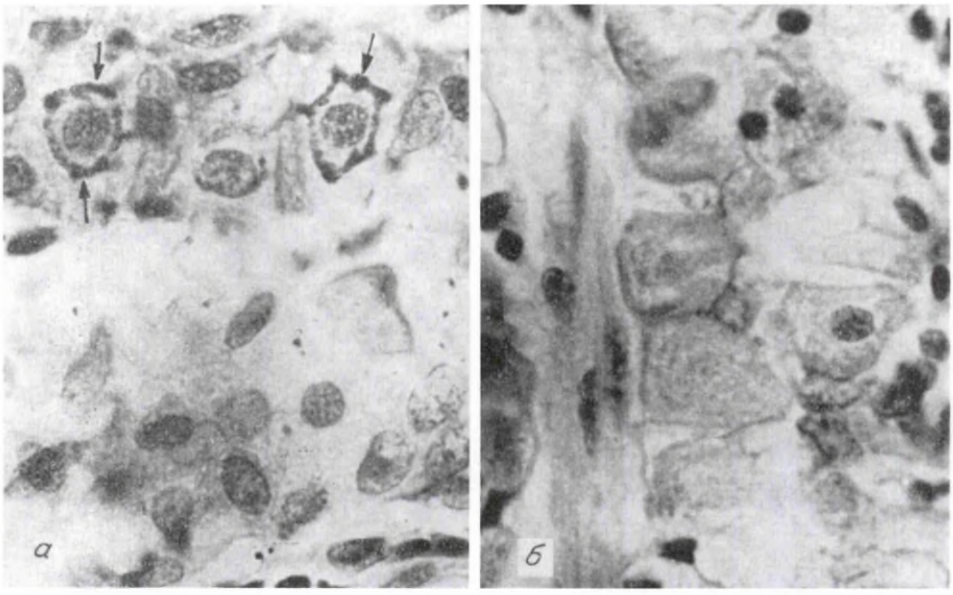


Figure 12a. Positive staining for parietal cell canaliculi using monoclonal antibodies to HMFG (reproduced by a permission from Arch Pathol (Vorobjova *et al.* 1989)).

Figure 12b. Negative staining for parietal cell canaliculi (reproduced by a permission from Arch Pathol (Vorobjova *et al.* 1989)).

Evaluation of immunostained sections.
The sections were examined using an objective x40 and an eyepiece x10. Antral and corpus mucosa glands were divided into the foveolar cells, the proliferating zone (neck-isthmus) and the glandular part. In each of them five fields were examined.

Only bright red stained nuclei were considered positive either for PCNA or for Ki-67. PCNA and Ki-67 labelling index (LI) were defined as the proportion of bright red stained nuclei in relation to 100 nuclei counted per field. Bcl-2 positive staining for interstitial lymphocytes was scored as 0–5. Bcl-2 staining of epithelial cells in the antrum and corpus mucosa was considered positive (red staining of the cytoplasm) or negative.

The densities of G, D and β- H+, K+ ATP-ase positive cells were expressed as the mean values of cells counted in five fields of each gland part. The slides were analysed by one investigator (T.V.) who was not aware of the data of histological alterations. Intraobserver variability was 12.6±7.0%.

The persons studied and the methods employed are summarised in Table 2.

Statistical methods
Differences in *H.pylori* status and in the prevalence of antibodies to the acid glycine extracted cell surface proteins of *H. pylori* and to CagA between different groups were tested by the chi-square test. Odds ratios (OR) with 95% confidence intervals (CI) were estimated using the Mantel-Haenzel statistics with the Exact software. Effects were considered to be statistically significant if the 95% CI did not include the value 1.0. Differences in the mean values of OD as the expression of the level of anti-CagA IgG were calculated by Student's t-test.

Association of grade of activity of gastritis, chronic inflammation, atrophy, intestinal metaplasia and damage of surface epithelial cells with CagA positivity was evaluated using multiple logistic linear regression analysis by the SAS package; p value <0.05 was considered statistically significant.

Statistical differences between the ACAB-positive group and ACAB-negative group were evaluated by chi-square and the McNemar tests using Statgraphics and SPSS statistical software. Odds ratios (OR) with 95% confidence intervals (CI) were estimated using the Mantel-Haenzel statistics with the Exact software. Differences were considered statistically significant if 95% CI did not include the value 1.0. Differences in the mean values of age were calculated by the Mann-Whitney test (Statgraphics software). The κ-value for measuring agreement between histological assessments was calculated using the SPSS statistical software. P values < 0.05 were considered significant.

The Wilcoxon signed rank test served for evaluation of the differences between the apoptotic index (AI) of the same persons in the samples of 1979 and 1997, and the Mann-Whitney unpaired nonparametric test served for analysis of AI for two groups according to different grades of atrophy, chronic infiltration, activity of gastritis and *H. pylori* colonization. Differences

Table 2. Persons studied and methods employed.

Studied Groups	Number of persons	Age (median)	Male/ Female	Study performed	Method
1. Population study					
Karksi-Nuia	1461	42	637/824	*H. pylori* status, PCA	ELISA (IgG to surface proteins of *H. pylori*), Indirect immunofluorescence method
Abja-Paluoja	497	61	189/308	*H. pylori* status	ELISA (IgG to surface proteins of *H. pylori*)
2. Representative sample of Karksi-Nuia	199	42	86/113	*H. pylori* status, CagA status	ELISA, Imunoblot (IgG to surface proteins of *H. pylori*) ELISA (IgG to recombinant fragment of CagA)
3. Patients with gastric adenocarcinoma	45	64.5	22/23	*H. pylori* status CagA status	ELISA, Imunoblot (IgG to surface proteins of *H. pylori*) ELISA (IgG to recombinant fragment of CagA)
4. Schoolchildren	421	9, 12, 15	190/231	*H. pylori* status CagA status	ELISA (IgG to surface proteins of *H. pylori*) ELISA (IgG to recombinant fragment of CagA)
5. Children with abdominal complaints	112	12	41/71	*H. pylori* status	Morphological (Giemsa staining), Immunohistochemical (anti-*H. pylori* antibodies)
6. Adult sample from Saaremaa Island					
Sample of 1997	70	57.5	31/39	*H. pylori* status (n=70), CagA status (n=70), *H. pylori* HSP60 (n=68) ACAB (n=62), PCA (n=62), Apoptotic index (n=68)	ELISA (IgG to surface proteins of *H. pylori*) ELISA (IgG to recombinant fragment of CagA) ELISA (IgG to *H. pylori* HSP60) Immunohistochemical Indirect immunofluorescence method TUNEL
Samples of 1979 and 1997	68	39 in 1979	31/37	*H.pylori* status, Apoptotic index	Morphological (Giemsa staining) TUNEL
Samples of 1985 and 1997	52	45 in 1985	26/26	*H. pylori* status, *H. pylori* HSP60	Morphological (Giemsa staining), ELISA (IgG to surface proteins of *H. pylori*), ELISA (IgG to *H. pylori* HSP60)
	37	44 in 1985	21/16	*H. pylori* status, HSP 60 ACAB	TUNEL
7. Patients investigated by routine endoscopy (University Hospital, Bern)	59	47	34/25	*H. pylori* status, Staining for PCNA, Ki-67, bcl-2, G-, D- cells, β H+K+ATPase	Morphological APAAP, Avidin-Biotin immunohistochemistry

in the number of persons with different grades of *H. pylori* colonization at two time points of observation were checked by the chi-square test. The effect of different variables on the expected values of AI and on the difference between the mean values of AI for different compartments of antrum and corpus mucosa sections in the samples of 1979 and 1997 was evaluated by multifactorial dispersion analysis by SAS/LAB with the SAS package. P values < 0.05 were considered significant.

Differences in the number of cases positive to *H. pylori* HSP 60 between the groups were tested using chi-square test. Differences in the mean values of RAA as the expression of the anti-*H. pylori* HSP 60 IgG level were calculated with the Student t test. Odds ratios (OR) with 95% confidence intervals (CI) were estimated using the Mantel-Haenzel statistics with the Exact software. The differences were considered statistically significant when 95% CI did not include the value 1.0. Correlation between the RAA values of the cell surface proteins of *H. pylori* as well as the RAA values of *H. pylori* HSP 60 antibodies and grade of *H. pylori* colonization, chronic inflammation, atrophy and activity of gastritis, as well as their interrelationships were evaluated, using the Spearman rank and Pearson correlation tests (Statistika, Statsoft 99) and multifactorial dispersion analysis with the SAS package. P-values < 0.05 were considered significant.

The values of the labelling index of PCNA, Ki-67, bcl-2, gastrin, somatostatin and β-H+, K+ ATP-ase positive cells were indicated as mean ± SD. Statistical analysis was done with Student's t-test, chi-square test, Spearman rank correlation analysis (Statgraphics software) as well as with the use of multiple regression analysis (Statistika, Statsoft 99). P-values < 0.05 were considered significant.

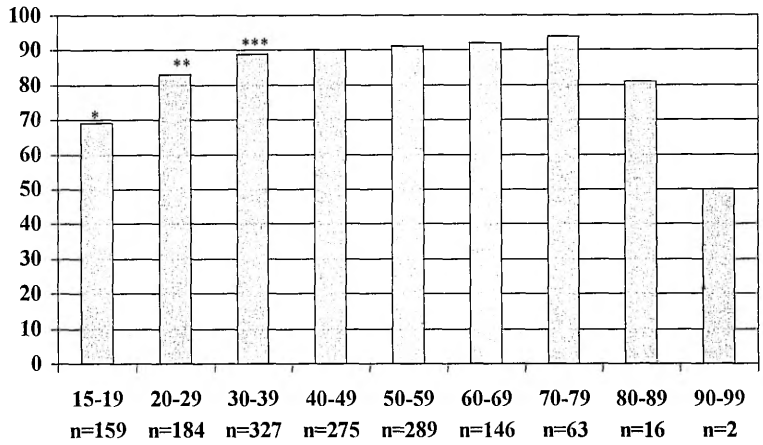
Ethics

This study was approved by the Committees of Ethics of the University of Tartu and of the University of Bern, Switzerland.

RESULTS

1. Seroprevalence of *H. pylori* in a population from Southern Estonia (Papers I and II)

IgG antibodies to acid glycine extracted cell surface proteins of the *H.pylori* strain NCTC 11637 were detected in 1271 out of 1461 (87%) persons studied in Karksi-Nuia. The prevalence rate of antibodies increased significantly from 69% in the age group of 15–19 years to 83% in the age group of 20–29 years (p=0.002), and to 89% in the age group of 30–39 years (p=0.04). There was no significant difference between any of the older age groups (p>0.05) (Paper I, Table 1) and Fig 13.



** > * p=0.002; *** > ** p=0.04

Figure 13. Seroprevalence of antibodies to the acid glycine extracted cell surface proteins of *H. pylori* in different age groups in the Karksi-Nuia population.

In Abja-Paluoja, 444 out of 497 (89%) persons had IgG antibodies to *H. pylori* acid glycine extracted cell surface proteins. There was no significant difference in the prevalence of *H.pylori* antibodies in different age groups (Paper I, Table 2).

A comparison of the results of serological and histological *H. pylori* determinations, and the state of the gastric mucosa in 64 persons of Karksi-Nuia, subjected to endoscopy, is presented in Table 3 (Paper I). There was good agreement between the histological and serological findings for *H. pylori*. The sensitivity of the ELISA test for the NCTC 11637 strain antigen of *H. pylori* was 98% (51/52), specificity was 82% (9/11). The positive predictive value was 96%. The false positive rate for the NCTC 11637 antigen was 18%, the false negative rate 2%.

We found statistically significant correlations between RAA values in ELISA and grade of *H. pylori* colonization evaluated histologically as the sum of grades for the antrum and corpus (r=0.389; p=0.002). *H. pylori* grade in the antrum correlated significantly with the scores of mucosa changes in the antrum (r=0.482; p=0.0001). *H. pylori* grades for the corpus correlated with the scores of mucosa changes in the corpus (r=0.310; p=0.015). No significant correlation was found between RAA values in ELISA and severity of gastritis (total score of antral and corpus gastritis) (r=0.220; p=0.08).

PCA were detected in 36 (2.5%) of the 1461 persons studied. PCA were found in 26 out of 1271 (2%) *H. pylori* positive, and in 10 out of 190 (5.2%) *H. pylori* negative persons (p=0.007). OR for association between PCA and *H. pylori* positivity was 0.37 (95% CI 0.18–0.78). However, there was a significant age-dependent increase in PCA positivity among the *H. pylori* infected persons but not among the *H. pylori* negative persons (Paper II, Table 1). Thus, in the age group of 30–49 years, 1.1% of *H. pylori* positive persons had PCA in comparison with 3.8% and 5.5% in the following age groups (p=0.008). In *H. pylori* negative persons, the prevalence of PCA among three subsequent age groups was not significantly different (9.4%, 8.3% and 1.5% for the age groups of 30–49, 50–69 and >70 years, respectively; p=0.03).

2. CagA protein seropositivity in a random sample of adult population and gastric cancer patients in Estonia (Paper III)

The prevalence of IgG antibodies to acid glycine extracted cell-surface *H. pylori* proteins was 85% (169/199) in a random adult population sample and 91% (41/45) in gastric cancer patients (Paper III, Table 1), which was not significantly different (p=0.39). Anti-CagA IgG were present in 63% (126/199) of the population sample, while the highest prevalence of CagA antibodies was found in the 20–29 age group: 19/25 (76%) in all studied persons and 18/22 (82%) in persons defined as *H. pylori* positive.

In gastric cancer patients prevalence of anti-CagA IgG was significantly higher, 87% (39/45; p=0.004). *H. pylori* positive gastric cancer patients showed anti-CagA IgG reactivity in 90% (37/41) of cases versus 71% for CagA positive persons in the *H. pylori* positive population sample (120/169; p=0.01). The significant association between CagA positivity and gastric cancer was confirmed (OR=3.76; 95% CI 1.59–8.91).

IgG response to different *H. pylori* proteins, evaluated using a Helico Blot 2. 0 kit in 45 gastric cancer patients and in 45 age and sex matched controls, is presented in Table 3:

Group	No	116 kD	89 kD	35 kD	30 kD	26.5 kD	19.5 kD
Gastric cancer patients	45	43/45	25/45	8/45	17/45	30/45	9/45
Controls	45	25/45	12/45	21/45	31/45	31/45	12/45

Seropositivity to 116 kDa and 89 kDa proteins, associated with pathogenic CagA and VacA proteins of *H. pylori,* was significantly more frequent in the group of gastric cancer patients than in age and sex matched non-gastric cancer controls (p=0.00003, p=0.005 correspondingly). Simultaneous seropositivity to CagA (116 kDa) and VacA proteins (89 kDa) in the gastric cancer group (25/45) was also significantly more frequent than for controls (9/45) (p=0.0005). IgG response to any protein of 116, 89, 35, 30, 26.5 and 19.5 kDa or their combinations in case of different localisation, type, differentiation or stage of gastric cancer was not significant. Nor did we observe a significant difference between the histological type or localisation of cancer and anti-CagA positivity in ELISA.

The difference in the prevalence of anti-CagA IgG between the corresponding age groups in the population sample and in gastric cancer patients (Paper III, Table 1) was not significant (p>0.05 for groups 30–39 to 70 and >70 years). However, when only the subjects aged 50 years and older from the population and from among gastric cancer patients were taken into account, a significant difference was revealed in the prevalence of anti-CagA IgG between the cancer group and the population sample defined as *H. pylori* positive (31/35 and 41/62, correspondingly; p=0.02; OR=3.96; 95% CI 1.24–12.5).

A comparison of the occurrence of IgG antibodies to a recombinant fragment of CagA antigen, evaluated by using ELISA, and to CagA protein of acid glycine extracted cell-surface proteins of *H. pylori*, evaluated by using immunoblot, both of which were performed on 141 sera, is presented in Table 2 (Paper III). ELISA and immunoblot showed an agreement of results in 113/141 (80%) of cases.

3. Seropositivity for *H. pylori* and for the CagA protein in schoolchildren of different age from urban and rural areas in Southern Estonia (Paper IV)

The prevalence of IgG antibodies to acid glycine extracted cell-surface *H.pylori* proteins in schoolchildren was 56% (235/421), to CagA, 28% (118/421). Out of 235 *H.pylori* positive schoolchildren, 109 were CagA positive (46%). Nine children negative to the cell surface protein in *H.pylori*-ELISA were positive in CagA-ELISA. *H.pylori* and CagA seropositivity in ELISA in schoolchildren of various age from different places of residence in Southern Estonia is presented in Table 2 (Paper IV). There was no significant difference in *H.pylori* and CagA positivity between girls and boys. Schoolchildren aged 9, 12 and 15 years revealed no significant difference either in *H.pylori* status or CagA positivity.

Schoolchildren living in a rural area (including both villages and farms) had a significantly higher *H.pylori* prevalence rate (118/181; 65%) as well as CagA positivity (64/181; 35%) compared with those living in towns, 117/240 (49%) and 54/240 (22%), respectively (p=0.001 for *H.pylori* prevalence rate and p=0.005 for CagA prevalence rate). The odds ratio (OR) for the association between *H.pylori* infection and place of residence in rural areas was 1.96 (95%

CI 1.32–2.92). We found also a significant association between CagA positivity and place of residence in rural areas: OR=1.88 (95% CI 1.22–2.88).

4. *H. pylori* in gastric mucosa of children with abdominal complaints: superiority of *H. pylori* detection by Giemsa staining over specific antibody staining. Detection of antigen-reactive corpus mucosa cells by immunohistochemistry (Paper V)

Gastritis of any degree and localisation was found in 84/112 (75%) children with abdominal complaints. The most frequent topographical and morphological type of gastritis was gastritis without atrophy localised simultaneously in the antrum and corpus mucosa (47/103 or 45.6% cases studied). Gastritis without atrophy restricted to the antrum was seen in 30/103 (29%) cases and gastritis without atrophy restricted to the corpus, in 3/103 (3%) cases. The prevalence of different states of gastric mucosa and of *H. pylori* positivity, detected using two different methods, is presented in Table 1 (Paper V).

H. pylori was detected by Giemsa staining in 83/112 (74%) of children. Immunohistochemical assessment of *H. pylori,* using anti-*H. pylori* IgG, resulted in a significantly lower *H. pylori* yield (55/112 or 49%; p=0.001). A comparison of positive staining for *H. pylori,* using the Giemsa method, and anti-*H. pylori* IgG in antrum and corpus biopsy specimens for different morphological types of gastric mucosa change is presented in Table 2 (Paper V).

In 12 out of 108 (11%) corpus mucosa specimens a positive staining with anti-*H. pylori* IgG was localised in the cytoplasma of corpus epithelial cells in the neck part of the glands (Fig. 2). This staining was diffuse and affected mainly the apical part of the cells. No *H. pylori* bacilli were identified in the cytoplasma of the mucus neck cells. All control sections (incubated without primary antibodies) were negative for such staining (Fig. 1 and 2, Paper V). In 8 out of these 12 cases, the corpus mucosa was normal, and gastritis without atrophy was found in 4 out of 12 cases. *H.pylori* were detected in Giemsa-stained sections in 2 out of these12 cases.

The state of the gastric mucosa of adult controls was the following: gastritis without atrophy in the antrum was established in 9/20 and atrophic antrum gastritis was established in 11/20 cases. In corpus mucosa specimens normal mucosa was identified in 2/16, non-atrophic gastritis in 7/18 and atrophic gastritis in 9/18 cases. Antrum biopsy specimens were Giemsa-positive in 14/20 cases and immunostain-positive in 10/20 cases. Corpus biopsy specimens were Giemsa-positive in 12/18 cases and immunostain-positive in 9/18 cases. In adult biopsy specimens, Giemsa staining revealed *H. pylori* in 26/38 (68%) cases and immunohistochemical method revealed it in 19/38 (50%) cases (p=0.08). In adult mucosa sections, immunostaining in the mucus neck cells was established neither in the corpus nor in the antrum.

5. Association of CagA positivity with development of atrophy and activity of gastritis (Paper VI)

Histological observation

Histological data and *H. pylori* status in the antrum and corpus mucosa for 66 samples studied for histology and CagA status in 1997 are presented in Table 1 (Paper VI). The course of atrophy in the antrum and corpus mucosa over 18 years is presented in Table 2 (Paper VI). Between two observations, in 1979 and 1997, 7/64 (11%) subjects developed atrophy in the antrum, while 22/64 (35%) developed atrophy in the corpus (p=0.001). Development of atrophy in the corpus but not in the antrum was significantly associated with high activity of gastritis both in the initial and late period of observation (p=0.0003) (Paper VI, Table 3).

Serological data

The prevalence of IgG antibodies to acid glycine extracted cell surface proteins of *H. pylori* in 1997 was 94% (66/70). Of *H. pylori* positive persons, 71% (47/66) were CagA positive.

The odds ratios (OR) for the association of grade of activity of gastritis, inflammation, atrophy, intestinal metaplasia or epithelial cell damage with CagA positivity in 1997 are presented in Table 4 (Paper VI). Logistic linear regression analysis showed that the strongest effect on CagA positivity was exerted by activity of gastritis in the corpus (p=0.0001) followed by activity of gastritis in the antrum mucosa (p=0.04). However, CagA positivity was significantly associated both with presence of atrophy in the antrum and corpus (p=0.04, p=0.002, respectively). In the corpus mucosa, the effect of atrophy on level of anti-CagA antibodies was also significant (p=0.0007). CagA positivity was associated with presence of intestinal metaplasia in the antrum but not in the corpus.

Course of gastritis during follow-up and CagA status

There was an association between CagA positivity in 1997 and progression of atrophy in the antrum and corpus mucosa during follow-up from 1979 to 1997. Among 42 persons who had no atrophy in the antrum mucosa in 1979 and in 1997, 24 (57%) were CagA positive, whereas among 9 in whom atrophy developed during follow-up, 8 (89%) were CagA positive. In the corpus mucosa, persons who did not develop atrophy during follow-up were CagA positive in 13 out of 25 (52%) cases. On the other hand, among 26 persons who developed atrophy, or in whom it progressed 23 (88%) were CagA positive (p=0.01; OR=7.07 (1.80–27.7). The intensity of immune response to CagA (mean value of optical density (OD) of IgG antibodies to CagA) was significantly higher among persons who developed atrophy in the corpus (OD=0.982±0.508) compared with those who did not develop it (OD=0.476±0.438; p=0.003). Also, development of atrophy in the antrum was associated with higher response to the CagA protein (OD=0.985±0.577 versus 0.601±0.532 in those who did not developed atrophy; p=0.05).

Persons with high grades of chronic inflammation in the corpus during 18 years of observation had significantly higher immune response to CagA (1.352±0.433) compared with those in whom chronic inflammation remained mild during the follow-up period (0.201±0.026; p=0.0005).

6. Significant increase in antigastric autoantibodies (ACAB) in a long-term follow-up study of *H. pylori* gastritis (Paper VII)

Prevalence of ACAB

The prevalence of ACAB in the follow-up group (samples of 1985 and 1997) as well in 62 persons investigated in 1997 are presented in Tables 1 and 3 (Paper VII).

ACAB were found in 29 of the 62 (47%) subjects investigated in 1997. In the follow-up group, ACAB were detected in 8/37 (22%) in 1985 and in 17/37 (46%) in 1997. All eight patients, positive for ACAB in 1985, remained positive for these autoantibodies, whereas nine of the 29 patients who were negative in 1985 developed ACAB during the follow-up period. The increase in ACAB during this period was statistically significant (p=0.004; McNemar test).

Prevalence of PCA

Among the 62 serum samples examined in 1997, 3/62 were positive for classical PCA (Fig. 5). All three persons were positive for ACAB also in 1997. In the follow-up sample, all 37 persons who had been followed up from 1985 to 1997, were negative for classical PCA in 1985, while 2/37 became PCA positive in 1997. The latter were ACAB+ also in 1985 and 1997. All PCA+ persons revealed highly intensive staining for ACAB throughout the cytoplasma (Fig. 4.).

Course of gastritis and ACAB

In samples for 62 persons examined in 1997 a significant association was found between ACAB and corpus mucosa atrophy (grades 1–3). Of 30 persons with atrophy of the corpus mucosa (grades 1–3), 19 (63%) had ACAB, while autoantibodies were found in only ten of 32 (31%) subjects without atrophy (p=0.01; OR=3.8; 95% CI 1.4–10.6).

The relationship between ACAB and development of corpus atrophy of different grades is shown in Table 4 (Paper VII) separately for 37 persons who were followed up for 12 years. In summary, although the prevalence of atrophy was higher in the subset of infected persons with ACAB than in the subset without these autoantibodies, no statistically significant association between ACAB and corpus mucosa atrophy could be established either at the beginning of the study or at the end of the follow-up period.

Intestinal metaplasia, chronic inflammation, activity of gastritis, H. pylori colonization and ACAB

In the sample of 1997 (n=62) a significant association was noted between presence of intestinal metaplasia in the corpus (grades 1–2) and ACAB (8

ACAB+ out of 8 cases with intestinal metaplasia grades 1–2 versus 21 ACAB+ out of 54 cases without intestinal metaplasia; p=0.004).

There was no significant association between grade of activity of gastritis and presence or absence of ACAB at any time of observation. A tendency of the prevalence of ACAB to increase during 12 years follow-up was observed among persons with a moderate grade of chronic inflammation.

In the sample of 1997 the number of persons with *H. pylori* colonisation grade 3 increased significantly: from 7/37 specimens in the antrum in 1985 to 20/37 in 1997 (p=0.003); from 5/37 specimens in corpus in 1985 to 17/37 in 1997 (p=0.005). However, development of ACAB in these persons was not statistically significant (p=0.11 for antrum specimens with *H. pylori* colonization grade 3; p=0.42 for corpus mucosa specimens with *H. pylori* colonization grade 3).

7. Apoptosis in different compartments of the antrum and corpus mucosa in chronic *H. pylori* gastritis. An 18-year follow-up study (Paper VIII)

Gastric mucosa alterations

Grading of chronic inflammation, activity of gastritis, atrophy, intestinal metaplasia and *H. pylori* colonization in the antrum and corpus mucosa samples from 68 persons studied in 1979 and 1997 are presented in Table 1 (Paper VIII). Additionally, the grading of the parameters of the Sydney classification for chronic gastritis, affecting the antrum and the corpus separately or simultaneously, are presented in Table 4. Our finding was that activity of gastritis, chronic inflammation, presence of intestinal metaplasia and *H. pylori* colonization affected in most cases both parts of the stomach simultaneously, whereas atrophic alterations in 1997 were localised predominantly in the corpus mucosa (20/68 versus 5/68 for the antrum, and 12/68 for both). Moreover, the prevalence of isolated atrophic corpus gastritis increased significantly during 18 years of follow-up (20/68 versus 9/68; p=0.02).

Gastric mucosa alterations and age

The results of the comparison of the mean age of the studied persons regarding absence or presence of atrophy, chronic inflammation, activity of gastritis, intestinal metaplasia and *H. pylori* colonization in the antrum and corpus mucosa are presented in Table 5. In 1997, persons who had atrophic alterations in the antrum mucosa (grades 1–3) were older (64.0±9.7) than those without atrophy (55.2±11.0; p=0.004). The age difference was not observed for corpus atrophy in 1997, whereas at the beginning of the study persons who had atrophy in the corpus already in 1979 were older (46.6±10.2) than those without atrophy (36.7±10.5; p=0.002; r=0.45; p=0.0001). This was not valid for the antrum in 1979. However, persons who developed atrophy in the antrum 18 years later were older in 1979 than those who did not develop atrophy (45.4±7.5 versus 36.9±10.8;p=0.03).

Table 4. Grading of activity of gastritis, chronic inflammation, atrophy,intestinal metaplasia and *H. pylori* colonization by different topographical localisations in the antrum and corpus mucosa in 1979 and 1997.

Grade	1979					1997				
	Activity of gastritis	Chronic inflamm	Atrophy	Intestinal meta-plasia	*H. pylori*	Activity of gastritis	Chronic inflamm.	Atrophy	Intestinal meta-plasia	*H. pylori*
Isolated ANTRUM										
1	4	2	6	7	4	12	0	5	6	0
2	4	7	1	1	3	1	0	0	1	0
3	2	0	0	0	0	0	0	0	0	0
Total	**10**	**9**	**7**	**8**	**7**	**13**	**0**	**5**	**7**	**0**
Isolated CORPUS										
1	5	1	6	3	1	3	1	12	3	2
2	2	3	2	0	2	1	0	6	1	0
3	1	1	1	0	3	0	1	2	0	1
Total	**8**	**5**	**9***	**3**	**6**	**4**	**2**	**20****	**4**	**3**
Simultaneously present (grades 1–3) in ANTRUM and CORPUS	**27**	**35**	**8**	**2**	**46**	**39**	**61**	**12**	**6**	**59**
Simultaneously absent from ANTRUM and CORPUS	**23**	**19**	**44**	**55**	**9**	**12**	**5**	**31**	**51**	**6**
Total	68	68	68	68	68	68	68	68	68	68

Significant difference: ** > * p=0.02.

Table 5. Comparison of the mean age of the studied persons from Saaremaa by absence or presense of atrophy, chronic inflammation, activity of gastritis, intestinal metaplasia and *H. pylori* colonization in the antrum and corpus mucosa as well by development or non-development of atrophy.

Status of gastric mucosa	Year of study	ANTRUM			CORPUS		
		n	Mean age	p value	n	Mean age	p value
No atrophy	1997	51	55.2±11.0		36	55.6±11.9	
Atrophy (1–3)	1997	17	64.0±9.7	**0.004**	32	59.6±10.4	0.18
No atrophy	1979	53	38.4±10.8		51	36.7±10.5	
Atrophy (1–3)	1979	15	42.9±12.7	0.17	17	46.6±10.2	**0.002**
No development of atrophy	1997	44	36.9±10.8		28	35.1±10.2	
Development of atrophy	1997	9	45.4±7.50	**0.03**	23	38.8±10.6	0.21
No chronic inflammation	1997	7	60.0±11.6		5	60.6±6.9	
Chronic inflammation (1–3)	1997	61	57.1±11.3	0.52	63	57.1±11.6	0.51
No activity of gastritis in	1997	16	59.2±10.6		25	56.8±12.0	
Activity of gastritis (1–3)	1997	52	56.8±11.5	0.47	43	57.5±11.2	0.82
No intestinal metaplasia	1997	55	55.6±11.2		58	56.1±11.1	
Intestinal metaplasia (1–2)	1997	13	64.7±8.7	**0.008**	10	64.7±9.6	**0.02**
No *H. pylori* colonization	1997	9	59.5±12.0		6	54.8±11.1	
***H. pylori* colonization (1–3)**	1997	59	57.0±11.2	0.55	62	57.6±11.4	0.56

Course of gastritis and dynamics of AI

The course of atrophy in the antrum and corpus mucosa during 18 years of observation is presented in Table 2 (Paper VIII). During the follow-up period, development of atrophy in the antrum mucosa was established in 9/68 persons and in the corpus in 23/68 persons.

The dynamics of AI in three compartments of the studied sections of samples from persons without and with development of atrophy in the antrum mucosa are presented in Fig. 2; the dynamics of AI in the corpus is shown in Fig. 3 (Paper VIII).

The value of AI for FC of the antrum or corpus mucosa showed no significant changes at two time points of observation either in persons without atrophy ($p=0.74$ for the antrum and $p=0.62$ for the corpus) or in persons who developed atrophy ($p=0.10$ for the antrum and $p=0.18$ for the corpus). AI for PZ and GP in the corpus mucosa specimens of 1997 decreased significantly, compared with the specimens of 1979, irrespective of the dynamics of atrophic alterations, i.e. persons who developed atrophy ($p=0.005$ for PZ and $p=0.00008$ for GP) or did not develop atrophy ($p=0.02$ and $p=0.00007$ for PZ and GP, respectively). In the antrum mucosa there occurred a significant decrease in AI for GP in persons without development of atrophy ($p=0.0000005$), and an obvious decrease in AI also in persons who developed atrophy ($p=0.05$). No significant difference was observed between the mean values of AI for FC, PZ and GP in the antrum or corpus in persons either with or without development of atrophy.

According to multifactorial dispersion analysis (Paper VIII, Table III), the strongest independent effector of AI in the antrum mucosa in 1979 were the grade of activity of gastritis (in FC; $p=0.01$) and degree of chronic inflammation (in GP; $p=0.03$), in 1997, grade of *H. pylori* colonization (in FC; $p=0.02$ and in GP; $p=0.03$). In the corpus mucosa, the strongest independent effect on AI in 1979 was exerted by activity of gastritis (in FC; $p=0.02$) and by chronic inflammation (in PZ; $p=0.04$).

Age had no effect on AI. Correlation analysis did not reveal any correlation between age and the values of AI for FC, PZ and GP. Multifactorial dispersion analysis showed that only grade of *H. pylori* colonization had a significant effect on the difference between AI values for FC in the antrum during follow-up.

Apoptotic index and CagA status in the samples of 1997

Seropositivity for CagA did not influence the mean value of AI in the studied compartments either in antrum or in corpus mucosa in samples from 1997 (Table 6; $p>0.05$). Nor did multiple regression analysis reveal any significant association between CagA status and the value of AI.

Table 6. Apoptotic index (AI) for different compartments of antrum and corpus mucosa specimens by different CagA status in the samples of 1997.

CagA status (OD>0.300 positive)	No. of persons studied	ANTRUM			CORPUS		
		Apoptotic index mean ±SD (No. of sections studied)			Apoptotic index mean±SD (No. of sections studied)		
		FC	PZ	GP	FC	PZ	GP
CagA+	47	8.7±6.6 (37)	2.1±2.7 (11)	1.3±1.5 (38)	7.8±7.2 (46)	2.3±3.0 (24)	1.2±1.8 (46)
CagA-	21	7.5±6.3 (20)^{p1}	1.7±1.6 (6)^{p2}	1.4±2.6 (20)^{p3}	7.4±7.1 (20)^{p4}	1.5±2.0 (14)^{p5}	1.4±2.4 (20)^{p6}

p values for the difference between the values of AI in CagA+ and CagA– groups according to Mann-Whithney test: p1=0.55; p2=1.0; p3=0.59; p4=0.86; p5=0.89; p6=0.66. FC – foveolar cells; PZ – proliferating zone; GP – glandular part.

Apoptotic index for the corpus mucosa and the status of anticanalicular autoantibodies (ACAB)

Presence of ACAB did not influence the value of AI in the studied compartments of corpus mucosa (Table 7; p>0.05):

ACAB status 1997	No. of persons studied for ACAB and apoptosis	Apoptotic index (1997) for corpus mucosa Mean±SD		
		Foveolar cells	Proliferating zone	Glandular part
ACAB +	6	7.1±6.5	2.3±3.1	1.1±1.9
ACAB –	14	8.1±7.6	1.8±2.5	1.7±3.5

8. Seropositivity for *H. pylori* HSP 60 and its correlation with gastritis parameters (Paper IX)

Forty-four out of 68 (65%) persons from Saaremaa were positive for *H. pylori* HSP 60 antibodies compared to 63 out of 68 (92%) positive sera in ELISA using *H. pylori* glycine extracted cell surface proteins as the antigen. Seventy per cent (44 out of 63) of sera, positive for the glycine extracted cell surface proteins of *H. pylori* possessed IgG antibodies to *H. pylori* HSP 60.

Grading of *H. pylori* colonization, activity of gastritis, chronic inflammation and atrophy, as well as odds ratios (OR) for the association between *H. pylori* HSP 60 and gastritis in the antrum and corpus mucosa are presented in Table 1 (Paper IX). A significant difference was found in the seroprevalence of *H. pylori* HSP 60 between persons with histological presence and absence of *H. pylori* in gastric biopsies (41/59 versus 3/9 in the antrum, p=0.03; 43/62 versus 1/6 in the corpus, p=0.01). Among six persons whose antrum as well as corpus was morphologically *H. pylori* negative (4/6 also without chronic inflammation both in the antrum and corpus), only one was seropositive for *H. pylori* HSP 60. The mean RAA value of anti-*H.pylori* HSP 60 IgG in persons without histologically observable *H. pylori* colonization in the antrum or corpus mucosa was significantly (p=0.01) lower than the respective value in persons with *H. pylori.* However, grade of histological *H. pylori* colonization did not correlate significantly with seropositivity for *H. pylori* HSP 60 (r=0.12; p=0.29 for the antrum and r=0.07; p=0.52 for the corpus).

The correlation between anti-*H. pylori* HSP 60 IgG antibodies and grade of chronic inflammation was significant (r=0.35, p=0.003 for the antrum mucosa and r=0.24; p=0.04 for the corpus mucosa; Paper IX, Figs. 1 and 2). Persons with a severe grade of chronic inflammation expressed simultaneously in the antrum and corpus displayed significantly higher values of RAA of anti-*H. pylori* HSP 60 (73.8±20.8) compared with those without of chronic inflammation (15.5±21.2; p=0.004) (Paper IX, Table 2).

There was found no correlation between seropositivity to *H. pylori* HSP 60 and grade of atrophy (r=0.013; p=0.91 for the antrum and r=0.04; p=0.70 for the

corpus), or activity of gastritis (r= 0.20; p=0.08 for the antrum; r=0.12; p=0.29 for the corpus).

In multiple regression analysis, *H. pylori* HSP 60 was significantly associated with grade of chronic inflammation (for the antrum: β=0.635; p=0.003; for the corpus: β=0.474; p=0.01). Grade of chronic inflammation in the antrum was the strongest independent predictor of seropositivity to *H. pylori* HSP 60 (β=0.626; p=0.01). Moreover, a strong correlation between grade of chronic inflammation and grade of *H. pylori* was found for the antrum mucosa(r=0.45; p=0.00008) while the correlation for the corpus mucosa was weaker (r=0.19 p=0.11, Spearman correlations analysis; r=0.29; p=0.01, Pearson correlations analysis).

Analogously, immune response to the acid glycine extracted cell surface proteins of *H. pylori* showed a significant correlation of RAA values with grade of chronic inflammation (r=0.36; p=0.002 for the antrum and r=0.50; p=0.000014 for the corpus) as well as with grade of activity of gastritis (r=0.30; p=0.01 for the antrum mucosa and r=0.29; p=0.01 for the corpus mucosa). According to multiple regression analysis the strongest independent effect on RAA values, reflecting immune response to the acid glycine extracted cell surface proteins of *H. pylori,* was exerted by grade of chronic inflammation in the corpus mucosa (β=0.673; p=0.0002).

Mean values of RAA to the acid glycine extracted cell surface proteins of *H. pylori* at different grades of *H. pylori* colonization separately and simultaneously for the antrum and corpus mucosa according to the samples of 1997 are presented in Table 8.

H. pylori colonization Grading	N	RAA value Mean±SD (median)
ANTRUM		
0	9	51.8±43.7 (32)*
1 mild	18	102.0±33.3 (112)**
2 moderate	9	114.8±19.1 (107)¤
3 severe	32	95.8±22.1 (96.5)¤¤
CORPUS		
0	6	29.1±17.7 (23)#
1 mild	21	100.9±31.5 (112)##
2 moderate	10	104.5±19.7 (105.5)
3 severe	31	99.2±26.6 (100)
Simultaneously in ANTRUM and CORPUS		
0	6	29.1±17.7 (23) ◊
1 mild	13	107.1±30.0 (115) ◊◊
2 moderate	4	117±20.7 (114)
3 severe	21	97.1±22.5 (94) ◊◊◊

Significant difference (Mann — Whithney method): ** > * p=0.008; ¤ > ¤¤ p=0.03; ## > # p=0.0005; ◊◊ > ◊ p=0.0009; ◊◊◊ > ◊ p=0.0004.

Immune response to the acid glycine extracted cell surface proteins of *H. pylori* was not significantly influenced by grade of *H. pylori* colonization.

Progression of chronic inflammation and seropositivity to H. pylori HSP 60
Increase in the grade of chronic inflammation in the antrum and corpus mucosa, as well as the positivity and mean values of RAA to *H. pylori* HSP 60 in serum samples from 1997 are presented in Table 3 (Paper IX). Persons who had no chronic inflammation during 18 years of observation, were negative also for *H. pylori* HSP60 in 1997 (17.4±18.8), while those whose degree of chronic inflammation in the antrum increased from grade 0 in 1979 to grade 3 in 1997 showed significantly higher values of RAA to *H. pylori* HSP 60 (65.7±21.2; p=0.008)

The corpus mucosa of persons whose chronic inflammation increased from mild to severe displayed significantly higher mean values of RAA to *H. pylori* HSP 60 (72.5±34.8) compared with persons in whom chronic inflammation remained mild during the follow-up period (24.6±25.8; p=0.04). Also, persons with severe chronic inflammation in the corpus at both time points showed more pronounced immune response to *H. pylori* HSP 60 (62.7±30.6) than those who had moderate chronic inflammation in 1979 and 1997 (29.0±16.5; p=0.02).

Dynamics of atrophy and seropositivity for H. pylori HSP 60
Seropositivity for *H. pylori* HSP 60 did not differ in persons who developed atrophy in the antrum or corpus during 18 years of observation (4/5 versus 24/37 cases for the antrum; OR=2.16; 95% CI 0.29–15.9 and 17/23 versus 16/27 for the corpus; OR=1.94; 95% CI 0.60–6.2).

Seropositivity for H. pylori HSP 60 in the samples of 1985 and 1997 and 12-year follow-up of antigastric autoantibodies
Among 52 sera positive for the cell surface proteins of *H. pylori,* studied for *H. pylori* HSP 60 in 1985 and in 1997, 40 (77%) were positive in 1985 and 36 (69%) were positive in 1997. Five out of 52 persons who were negative in 1985 developed IgG antibodies to *H. pylori* HSP60 in 1997, and 9 out of 52 persons who were positive in 1985 became negative for *H. pylori* HSP 60 in 1997. According to multifactorial dispersion analysis the highest association occurred between RAA to *H. pylori* HSP60 and grade of chronic inflammation in the antrum mucosa (F=3.58; p=0.03 for 1985 and F=4.06; p=0.01 in 1997).

In the group of 37 *H. pylori* infected persons who were studied for presence of ACAB in the 12-year follow-up, no significant association was found between seropositivity for *H. pylori* HSP 60 and ACAB (p=0.98; Table 9).

Table 9. Presence of ACAB and seropositivity for *H. pylori* HSP 60 in persons followed up in 1985 and 1997.

Person no.	ACAB in 1985	ACAB in 1997	*H. pylori* HSP 60 in 1985 (RAA value)	*H. pylori* HSP 60 in 1997 (RAA value)
1	–	–	46	45
2	–	–	**22**	29
3	–	–	41	80
4	–	–	**19**	35
5	–	–	59	35
6	–	–	66	102
7	–	–	60	81
8	–	–	30	**13**
9	–	–	59	46
10	–	–	**14**	**5**
11	–	–	59	34
12	–	–	71	**22**
13	–	–	24	**8**
14	–	–	66	72
15	–	–	106	72
16	–	–	51	**17**
17	–	–	66	44
18	–	–	34	**19**
19	–	–	**21**	**5**
20	–	–	67	91
Mean value			48.6±23.1*	42.7±30.2#
21	–	+	42	35
22	–	+	25	37
23	–	+	64	87
24	–	+	35	**16**
25	–	+	44	38
26	–	+	68	43
27	–	+	**13**	99
28	–	+	58	30
29	–	+	61	45
Mean value			45.5±18.8**	47.7±27.1##
30	+	+	43	30
31	+	+	**17**	**18**
32	+	+	26	**11**
33	+	+	70	65
34	+	+	27	42
35	+	+	51	62
36	+	+	**9**	**19**
37	+	+	61	70
Mean value			38.0±21.6***	39.6±23.5###

Between * and ** p= 0.83; between * and *** p= 0.35; between ** and *** p= 0.56;
Between # and ## p= 0.50; between # and ### p= 0.79; between ## and ### p= 0.77.

In 68 samples investigated in 1997 both for IgG to *H. pylori* HSP60 and ACAB, no association was found between these two parameters either: ACAB were found in *H. pylori* HSP60 positive persons in 22/44 (50%) cases and in HPS60 negative persons in 10/24 (42%) cases (OR=1.4 (95% CI 0.5–3.72; p=0.51).

9. Immune response to different antigens of *H. pylori* and age

The mean RAA values of IgG antibodies to cell surface proteins of *H. pylori*, *H. pylori* HSP 60 and the mean values of OD, reflecting IgG antibody response to the CagA protein for different age groups of 68 persons from Saaremaa, are presented in Table 10.

Table 10. Value of immune response to different *H. pylori* antigens in different age groups of an adult sample from Saaremaa in 1997.

Age groups	No	Acid glycine extracted cell surface proteins of *H. pylori* Mean RAA value±SD	CagA protein Mean OD±SD	*H. pylori* HSP 60 Mean RAA value±SD
30–40	6	106.8±31.3	0.703±0.493	52.1±40.2
41–50	11	96.6±30.5	0.738±0.634	35.4±28.0
51–60	22	88.2±36.1*	0.738±0.551	31.9±24.9#
61–70	20	87.5±34.2**	0.615±0.549	52.0±32.3##
71–80	9	112.3±21.7***	0.911±0.523	43.4±26.7
Total	68	94.2±33.1	0.720±0.547	41.7±29.9

*** > * p=0.07; *** > ** P=0.05;
> # p=0.02.

The only significant difference in immune response to *H. pylori* HSP 60 was observed between the age group 61–70 and the age group 51–60 years (p=0.02). According to correlation analysis there occurred no significant correlation between age and immune response to *H. pylori* cell surface proteins (r=0.01; p=0.9), *H. pylori* HSP 60 (r=0.1; p=0.37) or the CagA protein (r=0.01; p=0.89).

10. Glandular proliferation and density of specific cells in the antrum and corpus in *H. pylori* associated gastritis (Paper X)

The state of the antrum and corpus mucosa and the presence of *H. pylori* in the studied biopsy specimens are presented in Table 1 (Paper X). There was a significant positive correlation between density of *H. pylori* and severity of gastritis in the antrum (r=0.37, p=0.006) but not in the corpus mucosa (r=0.13, p=0.37).

Gastric epithelial cell proliferation in association with severity of gastritis and H. pylori colonization

Mean PCNA and Ki-67 LI in different parts of antral and corpus glands, and their relation to gastritis and *H. pylori* status are presented in Table 2 (Paper X). Overall mean PCNA LI was significantly higher in the antrum than in the corpus mucosa (p=0.02 for PZ; p=0.01 for GP). PCNA LI in FC and GP of corpus glands in presence of atrophic alterations was significantly higher compared with non-atrophic and normal mucosa (p=0.007 and p=0.03 respectively). Correlation analysis showed also a significant correlation of PCNA LI in corpus glands with severity of chronic inflammation and atrophy (r=0.34; p=0.03 for FC and r=0.46; p=0.001 for GP). However, there was no correlation of PCNA LI with severity of gastritis in the antrum (p=0.40, p=0.81; p=0.29 for FC, PZ and GP respectively). PCNA LI and Ki-67 LI in FC, PZ and GP of the corpus mucosa did not correlate with *H. pylori* colonization (p=0.47, p=0.53; p=0.09 for PCNA LI and p=0.99; p=0.51; p=0,14 for Ki-67). GP of antral glands, however, revealed a tendency for a negative correlation between mean PCNA LI and *H. pylori* density score (r=–0.23; p=0.09). The mean values of PCNA and Ki-67 LI in the antrum and corpus were significantly correlated (p=0.01 to 0.004 depending on the gland part). A significant positive correlation was found between Ki-67 LI and severity of chronic inflammation and atrophy in FC and GP of the corpus mucosa (r=0.32; p=0.04; r=0.46; p=0.01) but not with *H. pylori* density score (p=0.12; p=0.70; p=0.51 in FC, PZ and GP).

Multiple regression analysis showed that proliferation activity in the corpus mucosa depended significantly on severity of chronic inflammation and atrophy but not on *H. pylori* density (for PCNA LI in FC β=0.51; p=0.0007; in GP β= 0.46; p=0.002; for Ki-67 LI in FC β=0.33; p=0.03; for GP β=0.36; p=0.01). In the antrum mucosa, proliferation activity showed a tendency for a negative association with antrum gastritis only for Ki-67 in PZ (β= –0.23; p=0.09).

Immunoreactivity of gastric epithelial cells and interstitial lymphocytes for the bcl-2 protein

Bcl-2 positive staining was found in *epithelial cells,* i.e. in parietal cells of the corpus mucosa and in neck cells of the antral mucosa (16 cases: 8 in the antrum mucosa and 8 in the corpus mucosa; (Paper X Table 3). In the antrum, positive staining for bcl-2 positive epithelial cells was observed predominantly in the atrophic PZ of the mucosa (4/8 cases). In the corpus, bcl-2-positive staining of epithelial cells occurred in the normal and in the non-atrophic GP of the glands (3/8 and 2/8 cases, respectively), in 2 out of 8 cases in the non-atrophic PZ and only in one case in the atrophic PZ of corpus mucosa.

The density of bcl-2 positive *interstitial lymphocytes* in the corpus mucosa was positively correlated with severity of chronic inflammation and atrophy in PZ and GP (r=0.37, p=0.01; r=0.29, p=0.04). The mean value of the score for bcl-2 positive staining of interstitial lymphocytes (Paper X, Table 3) was

significantly higher in atrophic corpus gastritis than in non-atrophic gastritis (for FC, PZ and GP, p=0.01, p=0.02 and p=0.002 respectively). The score for overall mean bcl-2 positive staining of interstitial lymphocytes for PZ of the antrum mucosa was significantly higher compared with the respective score for PZ of the corpus mucosa (p=0.02).

Multiple regression analysis demonstrated that bcl-2 positive staining of interstitial lymphocytes in the corpus mucosa depended significantly on severity of gastritis but not on *H. pylori* density score (for FC β=0.45; p=0.002; for PZ β=0.47; p=0.001; for GP β=0.58; p=0.00003). In the antral mucosa, positive staining of interstitial lymphocytes for bcl-2 was associated more with *H. pylori* score (in PZ β=0.24; p=0.08).

Gastrin, somatostatin and β- H+, K+ ATP-ase positive cell densities and association with gastritis and H. pylori score

The densities of G- and D- cells in PZ of antral glands revealed a significant negative correlation with score for severity of atrophic alterations (r=–0.34, p=0.009, r=–0.52, p=0.0004, respectively). Mean G- and D-cell density (Paper X, Table 4) was significantly lower in non-atrophic and in atrophic gastritis compared with the normal mucosa (p=0.04; p=0.02 for G-cells and p=0.02 and p=0.0005 for D-cells). Mean G- cell number in the *H. pylori* positive atrophic mucosa tended to be lower (16.2±14.7) compared with the *H. pylori* negative atrophic mucosa (26.2±25.2, p=0.22), and D- cell density in the antrum tended to be a negatively correlated with *H. pylori* score in the antrum (r=–0.24, p=0.06, respectively).

The density of β- H+, K+ ATP-ase positive parietal cells in the atrophic corpus mucosa was significantly lower compared with the normal mucosa (p=0.0004) and non-atrophic gastritis (p=0.0001). There were no significant differences between the mean densities of β-H+, K+ ATP-ase positive parietal cells in the *H. pylori* positive and *H. pylori* negative mucosa (p=0.29). Also, multiple regression analysis showed that the density of β- H+, K+ ATP-ase positive cells was significantly dependent on grade of atrophy in the corpus (β=–0.65; p=0.000007) but not on *H. pylori* score (β=0.053; p=0.66).

A comparison of the antrum and corpus with regard to proliferation activity, expression of bcl-2 and density of specific cells in association with severity of gastritis and *H. pylori* colonization is presented in Table 5 (Paper X).

DISCUSSION

Seropositivity to *H. pylori* and CagA protein in adult populations from Southern Estonia and Saaremaa Island

The present study showed an extremely high frequency (87%–94%) of IgG antibodies to *H. pylori* in two Estonian adult populations aged 15 years and older, as determined by ELISA using a *H.pylori* acid glycine extracted cell surface proteins as the antigen. Serum IgG antibodies to *H.pylori* are used most frequently for locating persons with *H.pylori* infection (Perez-Perez *et al.* 1988, Mendall *et al.* 1992, Mégraud 1993, Rosenstock *et al.* 2000). Our results are in concordance with the data of previous studies performed in Estonia with the use of morphological method for assessment of *H. pylori* (Maaroos *et al.* 1990a,b, Villako *et al.* 1990). Thus, it can be concluded that Estonia is among the countries with high *H.pylori* prevalence, like some other East European countries where socio-economic conditions were not comparable with those of developed countries during long period. There is evidence that socio-economic factors play an important role in acquisition of this bacterium (Jones *et al.* 1987, Fiedorek *et al.* 1991, Mendall *et al.* 1992, Miyajj *et al.* 2000, Opekun *et al.* 2000). At the same time, it has been established that acquisition of *H. pylori* is a birth cohort-dependent phenomenon (Banatvala *et al.* 1993, Sipponen 1995; Maaroos 1995). According to the analysis, performed by Maaroos (1995), on Estonian populations from Kambja and Saaremaa, the cohort specific prevalence of *H. pylori* has remained high (60–80%) in cohorts born after 1945. However, on the basis of our results, the youngest birth cohort (born in 1975–1984) in the Karksi-Nuia population displays the lowest seroprevalence of *H. pylori* (69%) compared with 83% to 94% for the studied older cohorts.

This study and previous studies on occurrence of *H. pylori* in Estonian children (Maaroos *et al.* 1991b, 1993) showed that 41% to 59% of the children aged 5 to 15 years possessed *H.pylori* infection, i.e. its frequency was nearly the same as that found for youngest age group of our adult population, which indicates that most Estonian people are infected early in their life. According to a three-year follow-up study of Estonian children from Saaremaa Island from birth, 59% of children under the age of 3 years are *H. pylori* infected, while *H. pylori* seroprevalence among their mothers (mean age 24) was 83% (Lindkvist 1999). Taking into account the opinion of Mitchel (1999) that the relative risk of a child to acquire *H.pylori* is proportional to the prevalence of *H.pylori* in the adult population of childbearing age, as well as recent study of Miyaji *et al.* (2000), who showed that acquisition of *H. pylori* infection occurs in early childhood, especially via contact with infected mothers, it can be supposed that Estonian children acquire *H.pylori* often from their parents. In the present study the occurrence of *H.pylori* in the age groups 20–29 years and 30–39 years was 83% and 89%, respectively, which is in concordance with the data of Lindkvist (1999).

Among adult persons in the older age group a certain number of subjects were seronegative for *H.pylori.* There are several ways to explain this, but two

seem to be the most likely: either the gastric mucosa of these persons is not suitable for *H.pylori* colonization, or their immune system (possibly local IgA response) eliminates the infection in its early stages. Progression of gastritis to atrophic gastritis and the unsuitability of the atrophic gastric mucosa for maintaining *H.pylori* in the stomach, shown in several studies (Siurala *et al.* 1988, Tytgat 1992, Wyatt *et al.* 1992, Solnick and Tompkins 1993, Varis *et al.* 1993), may be the reason for *H.pylori* clearance and elimination of infection in some cases (Sipponen 2001).

The fact that already young Estonian people (aged 15 to 29) display high prevalence of *H.pylori* infection is of great importance, because it is related to possible early development of chronic gastritis and, subsequently, to high gastric cancer risk (Parsonnet *et al.* 1991, Forman *et al.* 1991, Graham *et al.* 1992, Sipponen and Marshall 2000). Gastric cancer is a common form of carcinoma in Estonia with a mean age-standardised incidence rate of 31.9 for males and 15.0 for females per 100.000 inhabitants in the period of 1993–1997 (Aarelaid and Mägi, 2000).

The prevalence of anti-CagA antibodies in a random adult population in Estonia was 63%, and anti-CagA positivity among *H. pylori* infected persons was 71%, which is significantly higher than average CagA seropositivity (49%) for 17 populations evaluated by the Eurogast Study Group (Crabtree *et al.* 1996b, Webb *et al.* 1999). Besides, anti-CagA and anti-VacA positivity in our gastric cancer group was significantly higher than in the non-cancer control. This result confirms the data of the higher percentage of serological recognition of cytotoxin associated *H. pylori* proteins in gastric cancer patients compared with control subjects (Crabtree *et al.* 1993, Blaser *et al.* 1995, de Figueiredo *et al.* 1998, Grimley *et al.* 1999). Our data on seropositivity for the acid glycine extracted cell surface proteins of *H. pylori* showed no significant difference between gastric cancer patients and the random population sample. This finding is in accordance with a study of Klaamas *et al.* 1996, which shows that overall *H. pylori* seropositivity for blood donors and for gastric cancer patients was not significantly different in Estonia.

Basing on the data of Crabtree *et al.* (1993), that recognition of 120 kD protein did not vary with age in gastric cancer patients, as well as on the data of the Eurogast Study Group concerning similar CagA positivity for the age groups 25–34 and 55–64 in 17 studied populations (Crabtree *et al.* 1996b), we supposed that the higher prevalence of CagA antibodies in gastric cancer patients was not influenced by the age difference between the gastric cancer group and population as a whole. This supposition is supported also by a significant association between CagA positivity and gastric cancer in *H. pylori* positive persons aged 50 and older.

It is notable that 20–29-year-old persons in the Estonian population appeared to have the highest prevalence of CagA antibodies. Taking into account the evidence that CagA positive strains nearly doubled the risk for developing gastric cancer over the ensuing 21 years (Blaser *et al.* 1995), we suggest that

young people (aged 20–29) with a high prevalence of CagA antibodies may be at high risk for developing gastric cancer.

A comparison of the evaluation of the antibodies to CagA protein in sera using both ELISA and immunoblot showed that the results of these tests agreed in 80% of cases. This is comparable with the results of the study by Xiang *et al.* 1993, where the agreement between the results in ELISA (using a recombinant fragment of CagA) and immunoblot (using total *H. pylori* extract as the antigen) was 85%. The cause of the 20% disagreement between the results in ELISA and immunoblot in our study may be due to the fact that in our CagA ELISA we used a recombinant CagA fragment (37.5 kD fused protein) as the antigen, whereas in immunoblot we employed acid glycine extracted cell surface protein. The overall good agreement between the results in the anti-CagA-ELISA and immunoblot in our study indicates that the use of a recombinant fragment of CagA protein in ELISA might prove a useful tool for evaluation of CagA status, especially in epidemiological studies.

Seropositivity for *H. pylori* and CagA protein in a non-selected group of schoolchildren

The seroprevalence of *H.pylori* in a nonselected group of schoolchildren was 56%, which did not differ significantly from the histological finding of *H.pylori* (58%) among Estonian children with abdominal complaints, obtained in a previous study (Maaroos *et al.* 1991b). According to literature data, the percentage of *H.pylori* infection in children, aged 9–15 years, without abdominal complaints varies from 3% in Sweden (Granstrom *et al.* 1997), 8.3–16.7% in Germany (Hornemann *et al.* 1997), 12% in children of Texas (Opekun *et al.* 2000), 11–23% in Holland (Roosendaal *et al.* 1997) and 16.7% in the UK (O'Donohoe *et al.* 1996) to 26% in Japan (Okuda *et al.* 1996), 36.8–68.4% in Turkey (Gurakan *et al.* 1996) and 34%–52% in China (Li *et al.* 1995). Thus the seroprevalence of *H.pylori* in schoolchildren in our study is higher than it is in most European countries.

The specific finding of the present study was that half of *H.pylori* positive schoolchildren, living in Southern Estonia, possess anti-CagA antibodies. These children should be given further attention with respect to development of gastritis, peptic ulcer or gastric cancer later in life, taking into account the association between infection with a cytotoxic strain and development of the above-mentioned diseases (Crabtree *et al.* 1991, Kuipers *et al.* 1995, Figura 1996, Parsonnet *et al.* 1997).

Literature data regarding CagA positivity in *H pylori* infected children (mostly children with recurrent abdominal pain or peptic ulcer) reveal the presence of infection with cytotoxic strains in 28.5 to 80.6% of cases when using different methods for the investigation of CagA positivity, such as ELISA, PCR and Western Blot (Oderda *et al.* 1993, Czkwianianc *et al.* 1997, Gzyl *et al.* 1997, Alarcon *et al.* 2000). CagA positivity among asymptomatic children was 54%, as evaluated by Elitsur *et al.* (1999), and 83%, as evaluated by (Karczewska *et al.*

1997), which is higher than the prevalence rate found in our study. This might be explained by the use of other methods for the establishment of CagA positivity in their studies, such as Western Blot (Helico blot 2.0, GeneLabs Diag. S. A.), as well as by the use of another CagA antigen in ELISA.

We compared *H.pylori* and CagA prevalence in healthy schoolchildren living in urban and rural areas of the same geographical region. It was found that children from rural areas (in villages and farms) were more often infected with *H.pylori* and with cytotoxic strains. The fact that children in rural areas had higher seroprevalence of *H.pylori* might hypothetically be explained by socio-economic differences as well as by the use of different water sources (here: external) in farms compared with urban households. Klein *et al.* (1991) showed that children living in homes with external water sources were three times more likely to be infected with *H.pylori* compared with households with internal water sources. However, the study of Lingkvist (1999), performed on a children's cohort from Saaremaa, found no correlation between seroprevalence to *H. pylori* and the kind of water used, while non-use of toothbrush was a strong independent predictor for *H. pylori* infection in Estonian children under 3 years of age.

Literature data point out that a major proportion of *H.pylori* infection is acquired in early childhood (Klein *et al.* 1991, Graham *et al.* 1992, Mendall *et al.* 1992, Mitchell *et al.* 1992, Neale *et al.* 1995, Ashorn *et al.* 1996). An extensive seroepidemiological study of *H.pylori* infection, conducted on various populations by Mégraud *et al.* (1989) showed that in France 3.5% of children were infected in the first decade of life. In the second decade, *H.pylori* prevalence increased to 16.3% and in the third decade, to 24.8%, which is still a markedly lower percentage compared with our results. The sharpest rise of antibodies to *H.pylori* in Sweden appears between 9 and 10 years of age (20%) (Lindkvist *et al.* 1996). In a study of Graham *et al.* (1992) it was shown that the prevalence of *H.pylori* infection was stable during a variable period in late childhood but increased in early adulthood. According to Sipponen *et al.* (1996b) *H.pylori* gastritis is acquired in childhood (age less than 20) in more than 50% of cases. On the basis of the present study it can be supposed that half Estonian schoolchildren are infected with *H.pylori*, while half of them possess cytotoxin-producing strains in the first decade of life.

H. pylori in the gastric mucosa of children with abdominal complaints

In the retrospective study of children with abdominal complaints, we compared the detection rate of *H. pylori* in the antrum and corpus mucosal biopsies from children using two methods, Giemsa staining and immunohistochemical investigation. It turned out that *H. pylori* was more frequently found in Giemsa preparations than at immunohistochemical investigation employing a polyclonal antibody directed against *H. pylori*. The concordance of the two methods in the detection of *H. pylori* in antrum biopsies was 70%, and in corpus biopsies 73%.

The fact that the use of polyclonal antibodies for assessment of *H. pylori* resulted in an *H. pylori* yield inferior to that obtained by Giemsa staining is not in

line with the data of other authors, who showed that immunohistochemistry is a more sensitive and specific staining method for evaluation of *H. pylori* in biopsies compared with conventional staining (Andersen *et al.* 1988, Barbarosa *et al.* 1988, Loffeld *et al.* 1991, Ashton-Key *et al.* 1996). One possible reason for this disagreement includes the hypothesis that *H. pylori* strains colonizing the gastric mucosa of the analysed children might have contained antigenic components different from the strains of *H. pylori* against which the polyclonal antibodies used in this study were produced. This possibility, based on certain disagreement between recognition of *H. pylori* in haematoxylin-eosin and peroxidase-stained sections, was pointed out by Andersen *et al.* (1988). Another reason why some Giemsa-positive cases were immunostain-negative could be the fact that in most cases of discrepancy a low grade of *H pylori* density was observed in the studied sections. Giemsa staining detects both intact and damaged *H. pylori,* while immunohistochemical reactivity may depend on presence of fully intact organisms harbouring a still complete set of respective antigens.

An additional aspect of the differential epitope reactivity of the antibody used in the present study was revealed on the staining of gastric epithelial cells subsets. Staining of the corpus gland neck cells by anti-*H. pylori* antibodies was found in 11% of biopsies, which suggests the cross reactivity of anti-*H. pylori* antibodies with gastric epithelial epitopes, as proposed previously by Negrini *et al.* (1989, 1993, 1996). The reaction may be related to the autoimmune mechanisms playing a pathogenic role of *H pylori* in progression of chronic gastritis (Negrini *et al.* 1993, 1996). This hypothesis is partly supported by our study of the Karksi-Nuia population, showed that long-term *H. pylori* infection may be a predictor for development of gastric autoimmunity (Paper II). It is, however, notable that in 8 out of 12 cases with intracytoplasmic staining of corpus mucosal cells, the corpus mucosa was otherwise normal, i.e. without acute or chronic inflammation which might indicate impairment of the immune response of these children to *H. pylori* (Torres *et al.* 2000). This observation does not preclude that such children with the sofar normal mucosa may be predisposed to develop autoimmune gastritis later in life. In this light, the finding of neck cell reactivity in the young age group is interesting, especially when considering that the use of anti- *H. pylori* antibodies on adult mucosal sections did not reveal immunostaining of epithelial cells in the neck part of corpus or antrum mucosa in any case. These facts might point out the peculiarities of immune response to *H. pylori* infection in childhood (Meining *et al.* 1996).

An alternative mechanism involved in staining of gastric gland neck cells by anti-*H. pylori* antibodies may be related to epithelial invasion by *H. pylori* (Andersen and Holck 1990, Ko *et al.* 1999, Su *et al.* 1999, Björkholm *et al.* 2000), or to endocytosis of *H. pylori* by epithelial cells followed by degradation of the microorganisms. In fact, internalisation of *H. pylori* by cells has been observed earlier (Bode *et al.* 1988, Andersen and Holck 1990, Evans and Graham 1992). We can suppose that the children's mucus layer is more predisposed to *H. pylori* penetration and invasion, which contributes to acquisition

of this bacterium predominantly in childhood. In a study of Blom *et al.* (2000) different patterns of *H. pylori* adherence to gastric mucosa cells were shown in children and adults. The authors found that a contact of *H. pylori* with the microvilli of gastric mucosa cells was significantly higher in children compared with adults, whereas adhesive pedestals dominated in adults, which can contribute to the lifelong infection of humans. The finding that *H. pylori* was not detected within cells by Giemsa staining in our study may indicate intracellular bacterial breakdown.

The association of CagA positivity with development of atrophy, activity of gastritis and chronic inflammation

The prospective 18-year follow-up study of an adult sample from Saaremaa Island shows that chronic *H. pylori* gastritis progresses gradually into atrophic gastritis and that this progression seems to be related particularly to presence of CagA-positive strains. The specific finding of this population study was that progression of atrophy as well as activity of gastritis in the antrum and corpus mucosa were associated with CagA positivity

Progression of gastritis to atrophic gastritis has been known for a long time (Siurala *et al.* 1980, Villako *et al.* 1982, Ihamäki *et al.* 1985, Sipponen *et al.* 1994a, Valle *et al.* 1996). The reason why atrophic gastritis develops in some but not in all affected individuals is unknown. Infection with *H. pylori*, particularly presence of CagA and cytotoxicity of the bacterium, are one way to affect the state of the gastric mucosa and dynamics of gastritis (Cover *et al.* 1990, Crabtree *et al.* 1991, Kuipers *et al.* 1995, Figura 1996, Sozzi *et al.* 1998). The association between systemic IgG response to CagA and gastric atrophy has also been shown (Beales *et al.* 1996, Oksanen *et al.* 2000). Our data are in accordance with studies indicating that the CagA-positive *H. pylori* status causes more aggressive gastritis than the CagA-negative status suggesting that CagA positive gastritis tends to progress to atrophic gastritis more frequently than CagA negative gastritis (Kuipers *et al.* 1995, Beales *et al.* 1996, Yamaoka *et al.* 1997, Warburton *et al.* 1998).

According to our study, development of atrophy in the corpus mucosa was significantly associated with activity of corpus gastritis. Furthermore, the association between activity of gastritis and CagA positivity was particularly significant for the corpus. There is evidence that *H. pylori* CagA protein stimulates well-pronounced active and chronic inflammatory response in the gastric mucosa (Crabtree *et al.* 1994, Sharma *et al.* 1995, Peek *et al.* 1995, Marchetti *et al.* 1995, Rautelin *et al.* 1996, Yamaoka *et al.* 1997, Ando *et al.* 2000). Since local immune response to *H. pylori* reflects antigenicity of the infecting organisms and taking into account that CagA induces strong humoral response, Figura (1995) proposed to determine indirectly the degree of polymorphonuclear cell infiltration in the stomach by the levels of systemic IgG to this protein. In our study the mean values of OD of anti-CagA IgG (which reflect the level of antibodies in sera) was associated with higher grade of activity of gastritis and atrophy, as well as with higher grade of chronic

inflammation, particularly in the corpus mucosa. This is in concordance with the results of Ando *et al.* (2000), which showed a good correlation of anti-CagA response with neutrophil infiltration, intestinal metaplasia, *H. pylori* density and IL-8 levels, suggesting that the absolute levels of these antibodies may be markers for gastric inflammation.

There is evidence that patients infected by cytotoxic strains and/or patients with anti-CagA antibodies are more likely to have active gastritis and to develop peptic ulcer or gastric cancer (Cover *et al.* 1990, Crabtree *et al.* 1991, Blaser *et al.* 1995, Figura *et al.* 1996, Orsini *et al.* 1998, Parsonnet *et al.* 1997). Both direct bacterial cytotoxicity with direct delivery of CagA into gastric epithelial cells and inflammatory cell aggression against the gastric epithelium may predispose the patient to peptic ulcer disease (Fiocca *et al.* 1994, Segal *et al.* 1999, Stein *et al.* 2000, Asahi *et al.* 2000, Odenbreit *et al.* 2000). The association between CagA, peptic ulcer and gastric cancer is most logical assuming that CagA is related to aggressiveness of gastritis. Occurrence of CagA infection results in more active gastritis which is more likely to ends up in atrophic gastritis. This sequence of events is supported by the present results.

The 18-year follow-up study of gastritis revealed a difference in the course of atrophy between the antrum and corpus mucosa, and established its relation to activity of gastritis. Thus 11% of the subjects developed atrophy in the antrum, while 35% developed it in the corpus. CagA positivity was also more closely associated with activity of gastritis and development of atrophy in the corpus than in the antrum mucosa.

The fact that the relationship between CagA and atrophic antral gastritis was not as strong as the relationship between CagA and atrophic corpus gastritis is somewhat unlogical but is obviously based on the natural course of gastritis in the present study population. There may occur healing of antral gastritis, while atrophic gastritis is progressing in the corpus (Kekki *et al.* 1984, Siurala *et al.* 1985, Maaroos *et al.* 1986, Valle *et al.* 1996). Such healing of antral gastritis may hence take place particularly in presence of a CagA positive strain. In other words, a significant worsening of atrophic gastritis in the corpus, as is the case in presence of CagA strains, is associated with opposite events in the antrum. If analysis were restricted to cases with atrophic antral gastritis only, presence of CagA strains would reveal the association with atrophic antral gastritis as well. Although the present material did not allow such analysis, there occurred a significant association between CagA and intestinal metaplasia in the antral mucosa even in the our series.

Prevalence of anticanalicular autoantibodies and their significance for development of gastric mucosa atrophy

An important finding of the present study was that the prevalence of ACAB increased significantly during the follow-up period of 12 years. One can suppose that ACAB are formed in the course of time, and their appearance may be related to the duration of *H. pylori* infection. However, as the exact time of

the onset of *H. pylori* infection in persons enrolled in this study is unknown, the duration of infection cannot be established either. It should be pointed out that the mean age of persons who were positive for ACAB at the beginning of the study, or turned positive for ACAB during 12 years, was not significantly different from the mean age of those who remained negative for ACAB during the whole period. This is in agreement with earlier data published by Faller *et al.* 1997, indicating that the age profile of *H. pylori* infected patients with antigastric autoantibodies was not significantly different from the age profile of patients without these autoantibodies. As most *H. pylori* infections occur in childhood (Sipponen 1995) and can persist throughout life, the age of an infected adult person and the duration of *H. pylori* infection can be regarded as roughly equivalent. The evidence of the role of the person's age or the duration of *H. pylori* gastritis in formation of ACAB is based on studies of infected children, reporting a lower prevalence of ACAB (Faller *et al.* 1999, Ierardi *et al.* 1998). Furthermore, a significant age-dependent increase in PCA positivity was shown in *H. pylori* infected persons but not in *H. pylori* negative persons in the studied Karksi-Nuia population, which suggests that long-lasting *H. pylori* gastritis might represent an important factor for formation of PCA (Paper II). Other studies in which formation of classical PCA was followed up over time have revealed similar results (Hawkins *et al.* 1979, Uibo *et al.* 1989).

The association of ACAB with *H pylori* gastritis, particularly with atrophic gastritis, in the corpus mucosa has been established in several previous studies (Faller *et al.* 1996, 1997, Negrini *et al.* 1996, Clayeas *et al.* 1998, Steininger *et al.* 1998) and was proved again on a group of 62 persons in this investigation.

Progression of superficial gastritis to atrophic gastritis has been shown in several previous follow-up studies (Villako *et al.* 1982, 1991, 1995, Ihamäki *et al.* 1985, Kuipers *et al.* 1995, Valle *et al.* 1996). In the present study we attempted to find an answer to the question why gastritis remains non-atrophic and confined to superficial mucosa layers in one patient, while gastric atrophy develops in another. Since ACAB are correlated with atrophic corpus gastritis, and since autoimmune reaction can lead to loss of parietal cells and evolution of gastric atrophy, then ACAB can serve as relevant markers for defining patients who are at higher risk for development of gastric atrophy (Faller *et al.* 1997). However, in this follow-up study we could not establish a significant association between presence of ACAB and development of atrophy over twelve study years. The lack of such a correlation is obviously due to the low number of enrolled persons, since such correlation was observed when the number of patients with the same epidemiological background increased from 37 to 62 at the end of the study. Also, the study period might have been too short. Uibo *et al* (1989) reported that in a six-year follow-up period, changes in the gastric mucosa in autoimmune gastritis and in autoantibody negative gastritis revealed no significant differences. The antrum and corpus mucosa of two persons who developed PCA did not reveal any alterations over six years. In contrast, in a much longer, 32-year follow-up study, the appearance of PCA

occurred in parallel with progression of corpus atrophy, disappearance of *H. pylori* and improvement of the antral mucosa (Valle *et al.* 1996).

Like in previous investigations, all PCA positive persons in our study were also positive for ACAB (Faller *et al.* 1997) On the other hand, only three out of all 29 ACAB positive persons were positive for PCA. This can be explained by the different binding sites of ACAB and classical PCA within parietal cells (Claeys *et al.* 1998). Interestingly, three persons who had classical PCA in 1997 showed very intensive staining for ACAB throughout the cytoplasma (Fig. 4). Two of them had moderate or severe corpus atrophy. In the follow-up group only two persons turned out to be positive for PCA in 1997. However, although both had displayed intensive staining for ACAB already in 1985 and 1997, one developed corpus atrophy, but the other did not. It could be hypothesized that ACAB precede classical PCA, and that epitope switching takes place in the process of *H. pylori* associated antigastric autoimmunity. This hypothesis could also explain why not all ACAB+ persons were PCA+ in our study and in previous research (Faller *et al.* 1997, Claeyes *et al.* 1998).

In our study we were able to confirm previous reports on a significant correlation between ACAB and gastric mucosa atrophy (Faller *et al.* 1997, 1998, Steininger *et al.* 1998). However, the data of this study do not allow to clearly define whether ACAB are the cause or the consequence of gastric atrophy. There are some patients who are ACAB positive but do not yet have atrophy. In these patients antigastric autoimmunity precedes atrophy. On the other hand, there are also patients who have atrophy but are still negative for ACAB. We can surmise that in this subgroup of persons ACAB can develop during further progression of atrophy and appear at the time when gastric cells are sufficiently degraded and autoantigens are presented to immunocompetent cells.

The association of apoptosis in different compartments of the antrum and corpus mucosa with development of atrophy, grade of activity of gastritis, chronic inflammation and *H. pylori* colonization

This study represents the first prospective long-term investigation of apoptosis in three different compartments of both antrum and corpus mucosa specimens, taken from a random adult cohort at the initial and end points of an 18-year follow-up of chronic *H. pylori* gastritis. Although the results of a 31-year follow-up study of gastric cellular turnover and development of atrophy were published by Moss *et al.* 1999, these authors examined apoptosis at one (initial) time point and only in corpus biopsies.

The specific finding of the present study was that AI for PZ and GP of the corpus mucosa and for GP of the antrum mucosa decreased significantly during 18 years of follow-up, irrespective of the dynamics of atrophic alterations (in persons without development of atrophy versus in persons with development of atrophy).

There is evidence that cell turnover in the gastric mucosa is related to a delicate balance between cell proliferation and apoptosis. More intensive cell

proliferation may induce increase in apoptosis, i.e. programmed cell death, in order to maintain tissue homeostasis (Kerr *et al.* 1972, Thompson 1995, Moss *et al.* 1996). It can be also supposed that cell turnover is more intensive in younger persons. Consequently, to keep cell turnover in balance, apoptosis must be more intensive. There exists evidence that in some cell types the ability to undergo apoptosis declines and upregulation of the bcl-2 gene due to chromosome translocation becomes more frequent with age (Warner 1997). It could be hypothesized that there may occur also reduction of the intensity of cell turnover in PZ and GP of the mucosa, because gradual cell turnover through the lifespan of a mammal eventually exhausts the finite capacity of somatic cells to replicate (Ostler *et al.* 2000). The subjects of our study were 18 years younger in 1979 than in 1997. Therefore one explanation of the significantly higher AI for PZ and GP of antrum and corpus mucosa specimens, sampled in 1979, compared with those sampled in 1997, could be the age difference. One can suppose that cell turnover in the FC compartment, which is continuously more exposed to several damaging factors, has to maintain its intensity also in old age. Also, it can be hypothesized that sustainment of intensive cell turnover in the FC compartment might be important for a better survival of bacteria on the non-damaged epithelium of the host (Parente *et al.* 2001). From methodological point of view, comparison of paraffin-embedded biopsy specimens, obtained with a 18-year interval, should not affect the results, because according to the data of Bardales *et al.* (1997), the use of formalin-fixed and paraffin-embedded archival material as old as 25 years yielded a staining pattern by the TUNEL method similar to that of freshly prepared tissues.

AI was not significantly different in the group without atrophy and in the group who developed atrophy 18 years later, which refutes our hypothesis that increased apoptosis might induce progression of atrophic gastritis. An analogous result was demonstrated in a follow-up study of Moss *et al.* (1999), in which the ratio of apoptosis to proliferation was not a determinant of risk for development of atrophy 31 years later.

It seems that development of atrophy cannot be explained only by violation of the balance between apoptosis and proliferation. As far as the association of apoptosis with gastritis is concerned, the results of several studies are quite different. For example, the findings of a study of Steininger *et al.* (1998) suggests that gastric atrophy might be the result of apoptosis in the gastric epithelium. Also, according to the opinion of Genta (1997), increased apoptosis in the gastric mucosa can lead to development of atrophy. However, Moss *et al.* (1996) found that apoptotic cell number does not correlate with degree of histological gastritis and that compensatory decrease in apoptotic activity takes place during progression of atrophic gastritis and intestinal metaplasia. A recent study of Scotiniotis *et al.* (2000) showed that in *H. pylori* antral gastritis, apoptosis was significantly reduced within the foci of intestinal metaplasia due to increased cell proliferation, which could contribute to *H. pylori* associated gastric carcinogenesis. Neither a study of Moss *et al.* (1996) nor a study of Anti

et al. (1998) established the correlation of AI with acute or chronic inflammation, whereas Jang *et al.* (2000) found an association between increased apoptosis in antral epithelial cells with degree of acute inflammation and density of *H. pylori* colonization.

Most authors have found a significant association between apoptosis and presence of *H. pylori* in the gastric mucosa (Moss *et al.* 1996, Chen *et al.* 1997, Konturek *et al.* 1999a, Scotiniotis *et al.* 2000, Von Herbay and Rudi 2000), suggesting a direct effect (Mannick *et al.* 1996, Moss *et al.* 1996, Smoot *et al.* 1997, Kohda *et al.* 1999) of *H. pylori* on mucosal cell apoptosis, or on further modulation of gastric epithelial cell apoptosis by immune mediators produced by neutrophils in response to *H. pylori* (Kim *et al.* 2000). The finding of Konturek *et al.* (1999a) suggests that *H. pylori* induces apoptosis in the gastric epithelium due to upregulation of proapoptotic Bax protein, while Bax overexpression is stronger in the antrum than in the corpus. The authors also found that activity of gastritis was higher in the antrum than in the corpus. These results are in accordance with our results concerning the association of AI in the FC compartment with grade of *H. pylori* colonization and activity of gastritis, which was more pronounced in the antrum than in the corpus mucosa.

A significant finding of our study was that the association of apoptosis with *H. pylori* colonization was expressed differently in the antrum and corpus mucosa, and that the effect of grade of activity of gastritis and chronic inflammation on AI was more expressed in the antrum than in the corpus, which may support the evidence of a different environment in the antrum and in the corpus (Stolte *et al.* 1990, Bayerdörffer *et al.* 1992).

Literature offers data about the role of CagA status in inducing or changing apoptosis in gastric epithelial or infiltrating T-cells (Peek *et al.* 1997, Anti *et al.* 1998, Figura *et al.* 1998, Rokkas *et al.* 1999; Ernst *et al.* 2000b, Kim *et al.* 2000). A recent study of Moss *et al.* (2001) found increased apoptosis in the antrum and corpus only in patients with *cagA*-positive *H. pylori* strains. The results of our study did not reveal a significant effect of CagA status on the value of AI either in the antrum, or in the corpus. According to the data of Peek *et al.* (1997) and Rokkas *et al.* (1999), CagA positive strains induce gastric cell proliferation which, however, is not accompanied by a simultaneous increase in apoptosis. On the basis of the data of Peek *et al.* (1999), the association of apoptosis with *cag*A positive strains is dependent upon the expression of *vac*A and genes within *cag*A PAI. Therefore these authors found heterogeneity of the levels of gastric epithelial cell proliferation and apoptosis within the *H. pylori* colonized mucosa, as well as a different effect of *H. pylori* on apoptosis at different time points since co-culturing of epithelial cells with *H. pylori* reference strains. One can surmise that the relationship between the CagA status and apoptosis of epithelial cells cannot be unambiquously characterized by a simple comparison of both of these statuses. Our results about the association of apoptosis with activity of gastritis are in better agreement with a study of Kim *et al.* (2000), who found that extent of apoptosis was similar in case of CagA

positive and CagA negative *H. pylori* infected cells, while apoptosis of gastric epithelial cells and caspase-3 activation can be modulated by the immune response of neutrophils.

Literature data suggest that autoimmune corpus gastritis develops in the course of time, as a result of antigastric autoimmune reaction against parietal cells (Strickland and Mackay 1973). Anticanalicular autoantibodies too are associated with atrophic corpus gastritis, as was shown in the present study. In a study of Steininger *et al.* (1998) apoptotic cell loss in the glandular zone of the corpus mucosa correlated with anticanalicular autoantibodies. However, in our study AI in the corpus mucosa revealed no association with presence or absence of ACAB in the studied persons; nor was there found any correlation between AI and atrophic alterations in the corpus.

Seropositivity for *H. pylori* HSP 60 and its correlation with gastritis parameters

The specific finding of our study was that immunological response to *H. pylori* HSP 60 was associated with *H. pylori,* which is strongly correlated with grade of chronic inflammation, particularly in the antrum mucosa.

Our results concerning seropositivity for *H. pylori* HSP 60 among *H. pylori* infected persons are in accordance with the data of Macchia *et al.* (1993), who found that 50% of *H. pylori* infected patients with gastroduodenal diseases displayed immune response to *H. pylori* HSP 60. The authors noted that presence of *H. pylori* HSP 60 positivity varied greatly among different individuals. Suerbaum *et al.* (1994) demonstrated that 76.3% of *H. pylori* infected patients were positive for MBP-HspB recombinant proteins. Why only some infected persons develop immunological response to HSP 60 is not known. Kansau and Labigne (1996) explained this by host specific factors.

We established a significant difference in seropositivity for *H. pylori* HSP 60 between persons in whom *H. pylori* was morphologically present in the gastric mucosa and those in whom it was absent, but it was not affected by grade of *H. pylori* colonization. Our finding that immunological response to *H. pylori* HSP 60 is related to chronic inflammation, particularly in the antrum mucosa, confirms the view that *H. pylori* HSP 60 is able to evoke inflammatory response in the gastric mucosa (Engstrand *et al.* 1991, 1993, Dunn *et al.* 1992, Perez-Perez *et al.* 1994, Leri *et al.* 1996, Hayashi *et al.* 1998, Kamiya *et al.* 1998, Kawahara *et al.* 1999, Yamaguchi *et al.* 1999), as well as supports previous studies where immunologic response of the gastric mucosa to luminal antigens and inflammatory reactions involved was more pronounced in the antrum than in the corpus (Valnes *et al.* 1984, Bayerdörffer *et al.* 1992). Analogously, immune response to the cell surface proteins of *H. pylori* was strongly associated with chronic inflammation in the antrum and corpus, but was not significantly influenced by grade of *H. pylori* colonization, particularly in the corpus. This fact may reflect the circumstance that immune response to *H. p*ylori is influenced rather by host response than by grade of *H. pylori* colonization.

A good correlation between serological response to *H. pylori* Hp54K (analogue to HSP 60) and mucosal inflammation (both acute and chronic) was found in a study of Perez-Perez *et al.* (1994). In our study, however, we could not establish a correlation between serological response to *H. pylori* HSP 60 and grade of activity of gastritis in the antrum or corpus mucosa. Nor did we detect any association with atrophy or with dynamics of atrophic alterations during 18 years of follow-up, whereas in a study of Barton et al. (1998), IgA antibodies to mycobacterial and human HSP 60 (sharing 75% of sequence homology with *H. pylori* HSP 60) were associated with the presence of gastric atrophy.

The particular association of serological response to *H. pylori* HSP 60 with chronic gastric inflammation can be explained by the fact that *H. pylori* HSP 60 induces IL-8 secretion by human gastric cells (Yamaguchi *et al.* 1999). The IL-8 is known as an pro-inflammatory cytokine (Crabtree *et al.* 1994). The important finding of our study was that presence of antibodies to *H. pylori* HSP 60 was associated also with increase in chronic inflammation during 18 years of follow-up. Persons who developed higher grade inflammation during 18 years displayed also a higher level of IgG antibodies to *H. pylori* HSP 60 at the end point of observation. These follow-up data support the view that *H. pylori* HSP 60 plays an important role in triggering of gastric mucosal inflammation (Perez-Perez *et al.* 1994, Kamiya *et al.* 1998).

An additional point for discussion is the possible involvement of *H. pylori* HSP60 in induction of autoimmunity-mediated gastritis (Engstrand *et al.* 1991, Macchia *et al.* 1993, Kansau and Labigne, 1996). Engstrand *et al.* (1991) showed that intraepithelial gamma/delta T-cells play a role in host defence against *H. pylori,* and that *H. pylori* itself may trigger an autoimmune response to heat shock proteins as stress proteins expressed by gastric epithelial cells. However, the studies of Taylor *et al.* (1999) as well as of Dunn *et al.* (1997) showed that the antibody, raised to *H. pylori* HSP60, reacted only with *H. pylori* cells but not with gastric mucosal tissue. A study of Kamoshida *et al.* (1999) did not find any significant difference in HSP60-like immunoreactivity in the gastric epithelium between *H. pylori* positive and *H. pylori* negative biopsies either. We tested the hypothesis whether there exists an association between presence of anticanalicular autoantibodies and seroprevalence of antibodies to *H. pylori* HSP60. The results of our study did not support this hypothesis. Since we did not examine local response to the *H. pylori* HSP60 derived antigen on the level of the gastric mucosa, our results point only indirectly to absence of this association. Studies of Faller *et al.* (1998) and Claeys *et al.* (1998) did not confirm the cross-reaction of ACAB with *H. pylori* antigens either.

Differences in glandular proliferation and homeostasis of specific cells between the gastric antrum and corpus in *H. pylori* associated gastritis

Based on the study of gastric epithelial cell proliferation and expression of bcl-2 protein in the antrum and corpus mucosa in patients undergoing gastroscopy, we found that severity of gastritis and presence of *H. pylori* influenced gastric

epithelial cell proliferation and expression of the bcl-2 protein in epithelial cells as well as in interstitial lymphocytes differently in the antrum and corpus mucosa. In the corpus, a significant positive correlation was found between proliferation activity and grade of chronic inflammation and atrophy (atrophic mucosa exhibited higher PCNA and Ki-67 LI's), whereas in the antrum mucosa, proliferation indices tended to correlate negatively with grade of gastritis (atrophic mucosa displayed lower Ki-67 LI). These findings are in accordance with the data of Bechi *et al.* (1996), which suggest differences in the proliferative activity of epithelial cells between the gastric antrum mucosa and corpus mucosa, but show discordance with the data of other authors, which show enhancement of LI with increasing degrees of gastritis both in the antrum and corpus mucosa (Hart-Hansen *et al.* 1979, Lipkin *et al.* 1985).

Proliferation rates for the corpus glandular epithelium were not related to *H. pylori* status in our study. In the antrum glands, Ki-67 LI tended to be inversely related to higher *H. pylori* score as well as to gastritis score. A study of Chow *et al.* (1995) showed too that *H. pylori* does not increase antrum cell proliferation. Reduced proliferative activity of antrum gland cells with increasing grade of gastritis is not an unexpected finding because it represent gastritis harbouring a significant proportion of involutes glands in probable proliferative quiescence, since severe cell loss does not permit normal cell maturation and differentiation (Bechi *et al.* 1996). The contrastive findings for the gastric corpus, namely increased proliferative activity, especially in FC and GP, suggest that corpus gland cell populations maintain active cell turnover despite reduction in cellular mass as established through grading criteria. Extension of the proliferative compartment with consequent displacement of proliferating cells to the epithelial surface has indeed been reported in advanced gastritis (Hart-Hansen *et al.* 1979, Deschner *et al.* 1972). Moreover, corpus gastritis may be in an earlier phase of inflammatory evolution compared with antrum gastritis, retaining still its proliferative repertoire: there is evidence that lesions in the antrum can be considered older than lesions in the corpus (Lipkin *et al.* 1985). Indeed, in our group of patients, cases with atrophic antrum alterations prevailed over atrophic corpus alterations.

It was our aim to test whether cell protection from apoptosis is related to gastritis and *H. pylori* colonization. Immunoreactivity for the apoptosis protector, bcl-2 protein, was detected in a subset of gastric epithelial cells, mainly in the glandular neck region. The noteworthy finding of our study was that both glandular proliferation and bcl-2 protein reactivity were differently expressed already in the normal (i.e. without chronic inflammation or atrophy of the glands) corpus and antrum mucosa. In normal corpus glands, the balance between cell proliferation and programmed cell death was manifested as lower PCNA and Ki-67 LI's and higher bcl-2 protein staining of epithelial cells in GP compared with the atrophic mucosa. However, compared with atrophic glands, the balance between cell proliferation and apoptosis in the normal antrum mucosa was attained in a different way: there was a tendency for increased cell

proliferation and reduced bcl-2 protein staining of epithelial cells. In our study, overall mean PCNA LI too was significantly higher in the antrum than in the corpus. This finding may support the evidence of presence of a different environment in the antrum mucosa and in the corpus mucosa (Stolte *et al.* 1990), as well as of dissimilarities in the antrum and corpus regarding the interaction between the bacterium and the host (Sipponen *et al.* 1996a).

For the atrophic corpus mucosa, we established higher proliferation index compared with respective index for normal corpus glands, and simultaneous increase in bcl-2 positivity of interstitial lymphocytes. While upregulation of the bcl-2 as the apoptosis protector factor suggests reduced apoptosis, its association with prolonged higher proliferation activity can be regarded as a factor contributing to development of MALT lymphoma and carcinogenesis (Lauwers *et al.* 1994, Banerjee *et al.* 2000). A positive correlation of positive staining for bcl-2 in interstitial lymphocytes of the corpus with grade of gastritis may also indicate that these lymphocytes are protected from programmed cell death and may hence be responsible for increase in chronic inflammation.

Lower Ki-67 proliferation index for the atrophic antral mucosa was associated with more frequent bcl-2 protein staining of epithelial cells in the neck part of glands (PZ) than in the normal mucosa, which indicates possible protection from cell loss and may thus reflect an important mechanism for retaining the renewal potential of antrum glands (Lauwers *et al.* 1994).

Our investigation showed that antral G- and D-cell densities decrease significantly already at the stage of non-atrophic gastritis and reach a minimum in atrophic gastritis. This finding confirms previous data demonstrating decrease in G-cells, particularly in atrophic gastritis (Crivelli *et al.* 1977, Stave *et al.* 1978, Marotta *et al.* 1990, Graham *et al.* 1993). We detected only a few G- and D-cells in the corpus, predominantly in atrophic areas, probably in the transition area between the antrum and the corpus. In our study the presence of *H. pylori* tended to be associated with decrease in G- and D- cell densities in the antrum, however, this decrease was significantly affected by advanced grade of atrophic gastritis. Our results are consistent with the observation of Wyatt *et al.* (1989), who found also significantly fewer gastrin cells in *H. pylori* positive patients, while this decrease was positively correlated with degree of antral atrophic gastritis. The lower density of D-cells in the *H. pylori* positive antrum mucosa, found in our study, is in agreement with the evidence that *H. pylori* induces reduction of somatostatin cells (Moss *et al.* 1992, Ødum *et al.* 1994, Park *et al.*1999, Tham *et al.* 1998).

Concerning the third type of specific gastric cells, i.e. β- H+, K+ ATP-ase-reactive parietal cells, we found that their density was dependent rather on grade of atrophic gastritis than on *H. pylori* density. Atrophy in the corpus mucosa may represent a more powerful factor for inducing decrease in specific cells, and advanced stages of mucosal atrophy exhibit depletion of specific cells irrespective of *H. pylori* status.

SUMMARY

This investigation was undertaken to determine the seroprevalence of *H. pylori* infection among Estonian population, both in adults and in children. The aim was to establish, whether present *H. pylori* infection is aggressive or not by detecting the antibodies to cytotoxin associated protein, to heat shock protein 60 and to the acid glycine extracted cell surface proteins of *H. pylori*. Also, we attempted to establish possible factors which play a role in progression of gastritis into atrophic gastritis (presence of autoantibodies, apoptosis), as well as to assess the association of host immune response to different *H. pylori* antigens with histological parameters of gastritis, and to find out whether there exist some immunological factors differing for various topographic types of gastritis. In addition, the influence of *H. pylori* on cell turnover in the antrum and corpus mucosa was studied.

Altogether, we studied 1461 persons from Karksi-Nuia and 497 persons from Abja-Paluoja (from an unselected population of two small towns in Southern Estonia), 70 adult Estonians from Saaremaa Island (from a primary sample of 227 subjects endoscoped in 1979 and reinvestigated by endoscopy and biopsy in 1997), 421 schoolchildren selected randomly from a population of urban or rural areas in Southern Estonia, 112 children with abdominal complaints, endoscoped in Tartu Children's Hospital, and 45 consecutive gastric cancer patients from the Tartu University Clinic of Oncology, as well as 45 age and sex matched non-gastric controls (from the serum bank of the Department of Immunology, University of Tartu). Also, 59 patients investigated by routine endoscopy in the Department of Gastroenterology of the University Hospital, Bern, were included in the study.

The state of the gastric mucosa and presence of *H. pylori* in histological sections were assessed in accordance with the updated Sydney system. Seroprevalence of IgG antibodies to *H. pylori* acid glycine extracted cell surface proteins and IgG antibodies to the recombinant fragment of 128 kD cytotoxin-associated (CagA) protein were detected by ELISA and immunoblot. Immunological response to *H. pylori* HSP 60 was evaluated using purifed *H. pylori* HSP 60 in ELISA.

H. pylori on histological sections was evaluated using peroxidase-anti-peroxidase method and polyclonal anti- *H. pylori* antibodies. Anticanalicular autoantibodies (ACAB) in the serum were evaluated immunohistochemically on the gastric corpus mucosa paraffin sections from patient cases without pathological alterations. Classical IgG-type parietal cell antibodies (PCA) were examined by indirect immunofluorescence method. Apoptosis was evaluated using TUNEL method. Gastric epithelial cell proliferation (with PCNA and Ki-67 labelling), bcl-2 protein staining and immunostaining for G, D cells and β-H+, K+ ATP-ase in parietal cell canaliculi were evaluated immunohistochemically.

The results showed that the seroprevalence of *H. pylori* infection in Estonian adult population is extremely high. IgG antibodies to the acid glycine extracted cell surface proteins of *H. pylori* were detected in 87% of inhabitants from Karksi-Nuia, in 89% of inhabitants from Abja-Paluoja and in 94% of an adult sample from Saaremaa. A significant increase was established in the seroprevalence of *H. pylori* from 69% in the youngest age group of 15–19 years to 83% in 20–29 year-olds, while persons aged 20–29 years are characterised by the highest prevalence (76%) of anti-CagA IgG in the population, which may indicate high risk for developing gastric cancer among them. Seroprevalence to *H. pylori* in gastric cancer patients was 91%, which was similar to seroprevalence in a random adult population, while the prevalence of anti-CagA antibodies was significantly higher in gastric cancer patients (87%) compared with adult population from Karksi-Nuia (63%) and from Saaremaa (70%). Additionally, seropositivity for cytotoxin associated CagA and VacA proteins was significantly more frequent among gastric cancer patients than among age and sex matched non-gastric controls. This support the view that cytotoxic strains of *H. pylori* constitutes factors particularly associated with increased risk of the development of gastric cancer.

Of schoolchildren, aged 9–15 years, living in Southern Estonia, 56% were seropositive for *H. pylori*, while 46% of them were anti-CagA positive. Neither *H. pylori* nor CagA positivity was significantly different in girls and boys, or in children aged 9, 12 or 15 years. Schoolchildren from rural areas were infected significantly more often with CagA seropositive strains compared with those living in towns.

Gastritis of any degree and localization was found in 75% of children with abdominal complaints (median age 12), while the most frequent morphological and topographical type of gastritis was nonatrophic gastritis localized simultaneously in the antrum and corpus mucosa (46% of the cases studied). The prevalence of *H. pylori* among Estonian children with abdominal complaints, determined by Giemsa staining, was higher (74%) than the prevalence obtained by the use of immunohistochemical method (49%). In 11% of cases the staining of epithelial cells in the neck part of corpus glands was established by anti-*H. pylori* IgG, which may indicate the possible immunological cross-reactivity of anti-*H. pylori* IgG with gastric epithelial cell antigen epitopes or internalization of *H. pylori* by epithelial cells in the children's gastric mucosa. These facts might point to peculiarities of interactions between *H. pylori* and host in childhood, considering that the use of anti-*H. pylori* antibodies on adult mucosa sections did not reveal immunostaining of epithelial cells in any one case.

Immune response to the CagA protein of *H. pylori* was strongly associated with activity of gastritis, presence of atrophy and damage of surface epithelial cells in the antrum and corpus mucosa, as well as with presence of intestinal metaplasia in the antrum. In the antrum mucosa, the strongest association was established between CagA positivity and presence of atrophy, whereas in the

corpus the strongest association occurred between CagA positivity and grade of activity of gastritis. Moreover, progression of atrophy in the antrum mucosa and particularly in the corpus mucosa was associated with CagA positivity. This supports the view about the role of the cytotoxin-associated CagA protein in enhanced gastric inflammatory response and atrophic alterations of the stomach mucosa.

The prevalence of anticanalicular autoantibodies (ACAB) increased significantly with duration of *H. pylori* gastritis from 22% in 1985 to 46% in 1997. A significant association was found between ACAB and corpus mucosa atrophy as well as presence of intestinal metaplasia in the corpus. A significant age-dependent increase in PCA positivity was also shown in *H. pylori*-infected persons but not in *H. pylori* negative persons in the study of the Karksi-Nuia population. This fact implies that long-lasting *H. pylori* gastritis may represent an important factor for formation of PCA and possibly also ACAB. Comparison of presence of ACAB and classical IgG type PCA antibodies in the follow-up group allowed to hypothesize that ACAB preceded classical PCA and that epitope switching takes place in the process of *H. pylori*-associated antigastric autoimmunity.

The analysis of apoptotic index (AI) for the foveolar cells, the proliferating zone and the glandular part of the antral and corpus mucosa in 18-year follow-up did not reveal any difference between persons with and without development of atrophy. The AI in the antrum was higher in persons with high activity of gastritis, chronic inflammation and grade of *H. pylori* colonization. The highest independent effect on apoptosis in the corpus was exerted also by activity of gastritis and chronic inflammation. Neither CagA status nor the presence of ACAB influenced the value of AI for all studied compartments in the antrum and in corpus mucosa.

Seropositivity for *H. pylori* HSP 60 in the studied persons was 65%. Serological response to *H. pylori* HSP 60 was seen only in subjects with *H. pylori* in the antrum or corpus and it was strongly correlated with grade of chronic inflammation, but not with activity of gastritis or development of atrophy. Grade of chronic inflammation in the antrum was the highest independent predictor of seropositivity for *H. pylori* HSP 60. No significant association was found between seropositivity to HSP 60 and presence of ACAB in the 12-year follow-up study. According to our study, *H. pylori* HSP 60 does not appear to be involved in molecular mimicry with antigens in the canalicular structure of the parietal cells in the corpus mucosa.

The intensity of serological immune response to *H. pylori* HSP 60 and to the acid glycine extracted cell surface protein antigens of *H. pylori* was associated not so much with grade of *H. pylori* colonization as with grade of chronic inflammation. This allowed to suppose that in serological response to both of these *H. pylori* antigens the intensity of host response plays a more important role than grade of *H. pylori* colonization.

Severity of gastritis and density of *H. pylori* colonization influence gastric epithelial cell proliferation and expression of bcl-2 differently in the antrum and corpus mucosa. In the corpus mucosa, proliferation activity and expression of bcl-2 in interstitial lymphocytes was significantly higher in atrophic than in non-atrophic gastritis but did not depend on *H. pylori* density, while bcl-2 positive epithelial cells predominated in the non-atrophic corpus glands. In contrast, the proliferative activity of the antral mucosa showed a tendency toward a negative association with severity of gastritis, and expression of bcl-2 positive interstitial lymphocytes was more associated with *H. pylori* density. Homeostasis of antral G- and D- cells as well as loss of β- H+, K+ ATP-ase — reactive cells in the corpus was shown to be a function of the degree of atrophic alterations rather than a function of *H. pylori.*

Summarizing the present results about the association between immune response to different *H. pylori* antigens and its histological appearance in the antrum and corpus mucosa the following can be concluded:

The state of corpus mucosa was particularly strongly associated with immune response to CagA protein, while the state of the antrum mucosa was associated with immune response to *H. pylori* HSP60;

Atrophy in the antrum as well as in the corpus mucosa was strongly associated with CagA positivity, while the atrophy in the corpus was associated also with presence of ACAB. The other factors, influencing development of atrophy in addition to CagA positivity were activity of gastritis for the corpus mucosa, and age for the antrum mucosa. The association of ACAB with progression of corpus atrophy was not significant. Nor did apoptotic index appear to be associated with atrophy, or to predict atrophy. Immunological response to *H pylori* HSP 60 did not correlate with development of atrophy.

The results of our research showed that different histological signs (as evaluated by the Sydney classification system of gastritis) are associated with immune response to *H. pylori* antigens in different ways. Activity of gastritis, (particularly in the corpus), atrophy (in the antrum and corpus) and intestinal metaplasia (in the antrum mucosa) are associated with immune response to CagA protein. Grade of chronic inflammation, particularly in the antrum mucosa, is associated with serological response to HSP 60 of *H. pylori*, while chronic inflammation in the corpus mucosa is associated predominantly with immune response to the acid glycine extracted cell surface proteins of *H. pylori* (Fig. 14).

Thus, *H. pylori* harbouring highly immunogenic proteins, like CagA and HSP60, induce local immune response in the stomach mucosa, appearing as acute and chronic inflammation, as well as systemic serological response. Seropositivity for CagA protein may serve as a sign of activity of gastritis and atrophy and might even predict development of atrophy, particularly in the corpus. Positivity of patients' sera for anticanalicular antibodies may suggest also presence of corpus atrophy, while positivity for *H. pylori* HSP60 indicates chronic inflammation in the antrum mucosa. Seropositivity for different

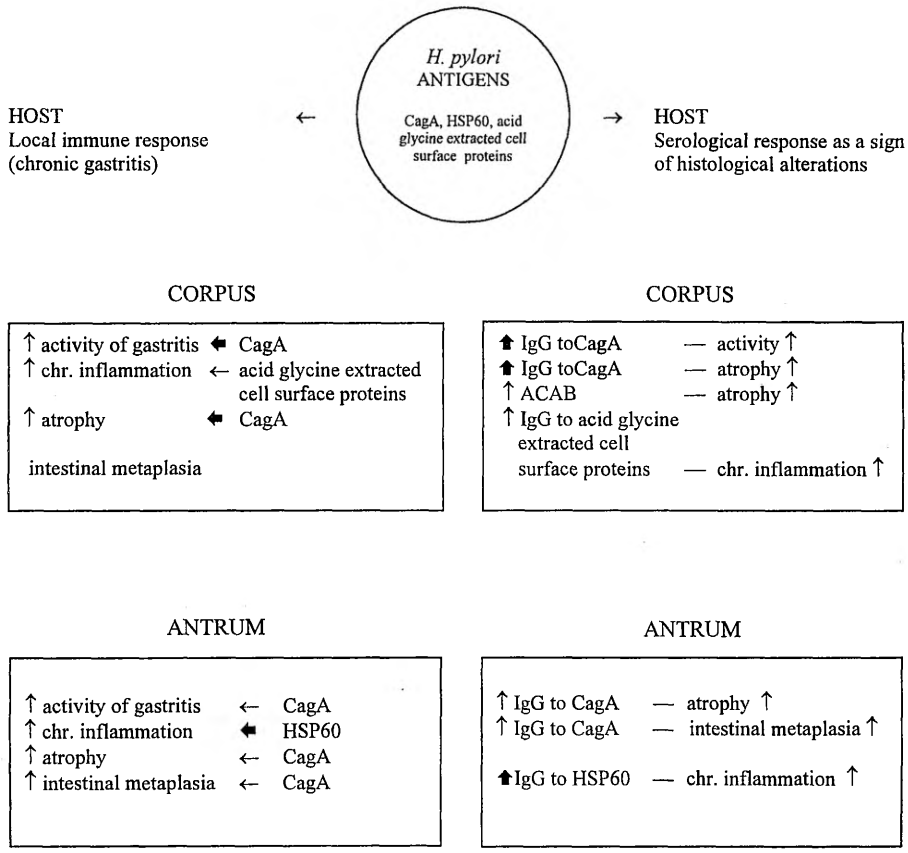


Figure 14. Scheme of associations between the histological parameters of gastritis in the antrum and corpus and serological response to different *H. pylori* antigens.

H. pylori antigens serves as a marker of histological appearance in the antrum and corpus mucosa in *H. pylori* infection. However, apoptotic index is not associated with development of atrophy but is largely dependent on grade of activity of gastritis and, particularly in the antrum, on grade of *H. pylori* colonization.

The antrum and the corpus mucosa displayed some differences in progression of atrophy. According to the 18-year follow-up of an adult population from Saaremaa, atrophy developed in the antrum mucosa in 11% of cases and in the corpus mucosa in 35% of cases. Persons who developed atrophy in antrum mucosa during follow-up were older at the beginning of observation, than those whose did not develop atrophy. In contrast, for the corpus mucosa there was revealed no age difference between persons who developed atrophy or did not develop it, but the subjects who had corpus atrophy already in 1979 were older than those without atrophy at the beginning of observation. It was noteworthy

that immune response to different *H. pylori* antigens revealed no association with age. Activity of gastritis exerted an effect on development of atrophy only in the corpus mucosa.

The association of apoptosis in gastric epithelial cells with grade of *H. pylori* colonization was also expressed differently in the antrum and corpus mucosa. The peculiarity of the antrum was a strong association of apoptotic index with *H. pylori* colonization. Also, the effect of activity of gastritis and chronic inflammation on apoptotic index was more expressed in the antrum than in the corpus. Moreover, epithelial cell proliferation and expression of the apoptosis protector oncoprotein bcl-2 in interstitial lymphocytes in the antrum was correlated predominantly with *H. pylori,* while in the corpus they were correlated with gastritis score.

The findings of our investigation point to several peculiarities of the antrum versus corpus in immune response to different *H. pylori* antigens and the association of this response with mucosal alterations, especially with cellular turnover and development of atrophy in the two parts of the stomach mucosa, which may support the evidence of presence of a different environment in the antrum and corpus.

CONCLUSIONS

1. The seroprevalence of *H. pylori* infection in Estonian adult population is extremely high, 87% to 94%, from which 63% to 71% account for CagA positive cases. The lowest seroprevalence of *H. pylori* (69%) was established for the youngest studied birth cohort (15–19 years). Most Estonian people are infected early in their life. Of schoolchildren aged 9–15 years and living in Southern Estonia 56% are seropositive for *H. pylori* and 46% of them have antibodies to CagA protein. Schoolchildren from rural areas possess significantly more often CagA seropositive strains compared with those from towns.

2. The prevalence of *H. pylori* among Estonian children with abdominal complaints (median age 12 years) was higher (74%) when it was assessed from gastric mucosa samples using Giemsa staining, than when it was assessed with immunohistochemical method employing anti-*H. pylori* polyclonal antibodies (49%). The staining of epithelial cells in the neck part of corpus glands with the use of anti-*H. pylori* IgG indicates the possible immunological cross-reactivity of anti-*H. pylori* IgG with epithelial cell epitopes, or internalization of *H. pylori* by these cells and may point to the peculiarities of interactions between *H. pylori* and host in childhood.

3. A significant association occurs between seropositivity for *H. pylori* CagA protein and activity of gastritis, presence of atrophy and damage of surface epithelial cells in the antrum and corpus. Unlike CagA-negative strains, CagA-positive strains of *H. pylori* enhance development of atrophic gastritis of the antrum and corpus. This association is more pronounced for the corpus mucosa. Development of atrophy in the corpus mucosa is significantly associated also with activity of gastritis, and in the antrum mucosa also with age.

4. The occurrence of autoantibodies reacting against canalicular structures within the human parietal cells increased significantly with duration of *H. pylori* gastritis. The correlation between these antibodies and presence of gastric corpus atrophy was confirmed. A significant age-dependent increase in parietal cell antibodies positivity was shown in *H. pylori*-infected persons.

5. Antibody response to *H. pylori* HSP 60 was associated with histological presence of *H. pylori* in the antrum and corpus mucosa and was strongly correlated with grade of chronic inflammation, particularly in the antrum mucosa, as well as with progression of chronic inflammation, but not with the development of atrophy during 18 years of follow-up, or with activity of gastritis.

6. The apoptotic index of epithelial cells for the proliferating zone as well as for the glandular part of the corpus mucosa and for the glandular part of the antrum mucosa decreased significantly during 18 years of follow-up irrespective of the dynamics of atrophic alterations. *H. pylori* colonization in the antrum during 18

years had a significant effect on the difference in apoptotic index between the two time points of observation only for the foveolar cells of the antrum mucosa. Apoptotic index is related to activity of gastritis in general but does not appear to be associated with atrophy or to predict atrophy. The association of apoptosis with *H. pylori* colonization was expressed differently in the antrum and corpus mucosa, being more pronounced in the antrum.

7. In the antrum, proliferation activity of epithelial cells revealed a tendency to be negatively correlated with gastritis, and the expression of bcl-2 positive interstitial lymphocytes was more strongly associated with *H. pylori* score, while in the corpus, proliferation activity of epithelial cells as well as expression of bcl-2 in interstitial lymphocytes was strongly dependent on severity of gastritis but not on *H. pylori* density. This indicates that severity of gastritis and *H. pylori* colonization influence cellular turnover differently in the antrum and corpus.

8. Seropositivity for different *H. pylori* antigens may indicate presence of certain histological manifestations in the antrum and corpus mucosa.

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SUMMARY IN ESTONIAN

IMMUUNVASTUS *HELICOBACTER PYLORI'* le, SELLE SEOS KROONILISE GASTRIIDI DÜNAAMIKAGA JA EPITEELRAKKUDE APOPTOOSI NING PROLIFERATSIOONIGA ANTRUMIS JA KORPUSES

Käesolev töö on järjeks gastriidi uurimise pikaajalisele traditsioonile Tartu Ülikoolis, millele pani 1930 aastatel aluse Lauri Walk ja mida jätkas professor Kaljo Villako eestvedamisel ülikooli töögrupp koostöös professor Max Siurala juhitud Soome gastroenteroloogide rühmaga. Juhusliku valikuga moodustatud eestlaste rühmade uurimisel selgus, et maal (Kambja arstijaoskonnas) elavatest isikutest esineb antrumi gastriiti 68%-l ja korpuse gastriiti 45%-l, Saaremaa linnaelanikel vastavalt 64% ja 62% (Villako *et al.* 1976, 1982).

Tänaseks on teada, et Marshalli ja Warreni poolt 1982. a. avastatud *Helicobacter pylori (H. pylori)* osaleb gastriidi, peptilise haavandi ja maovähi patogeneesis. Eestis alustati *H. pylori* uurimist juba 1986. a. ja histoloogilist meetodit kasutades tehti kindlaks, et *H. pylori*ga on täiskasvanud eestlaste juhusliku valikuga moodustatud rühmas nakatunud 73%, maohaavandi haigetest 91% ja kaksteistsõrmikhaavandi all kannatajatest 96%. Samuti leiti, et eestlaste nakatumine lastel on kõrge, mis on üheks gastriidi tekkimise riskifaktoriks juba lapseeas (Maaroos *et al.* 1990a, 1991a, 1991b).

H. pylori infektsioon on kohordifenomen, s.t. nakatumine toimub lapseeas ja täiskasvanutel lisandub uusi esmajuhte vähe. Eestis ja Soomes tehtud gastriidi ja *H. pylori* esinemise histoloogiliste uurimistega tehti kindlaks, et gastriit ja *H. pylori* esines neil, kes olid sündinud eelmise sajandi algusest kuni Teise maailmasõjale järgnenud perioodini, mõlemal maal ühesuguse sagedusega. Pärast seda vähenes gastriidi esinemine Soomes, mitte aga Eestis (Maaroos 1995).

H. pylori on tugevate immunogeensete omadustega, põhjustades antikehade produktsiooni ja paikse rakulise immuunvastuse maolimaskestas. Samuti on teada, et just *H. pylori* tsütotoksilistel tüvedel on oluline osa gastriidi, peptilise haavandi ja maovähi patogeneesis (Cover *et al.* 1990, Crabtree *et al.* 1991, Sipponen and Hyvärinen 1993, Vaucher *et al.* 2000). Seetõttu oli vaja määrata *H. pylori* tsütotoksiiniga assotsieeritud proteiini (CagA) antikehade esinemist populatsioonis nii täiskasvanutel kui ka lastel. Vereseerumis antikehade määramise kaudu saab suure täpsusega diagnoosida *H. pylori* infektsiooni (Perez-Perez *et al.* 1988, Mégraud 1993). Seni aga puudusid seroepidemioloogilised uurimised *H. pylori* leviku kohta eestlastest täiskasvanute ja laste hulgas.

H. pylori gastriit on kohalik immuunvastus selle bakteri infektsioonile (Sipponen *et al.* 1998). *H. pylori* tüvede antigeensed komponendid on mitmesugused. Kroonilise gastriidi ja eriti maolimaskesta atroofia patogeneesi seisukohalt on oluline teada, millised *H. pylori* antigeensed komponendid (nagu CagA,

kuumašoki-proteiin) on seotud selliste gastriiti iseloomustavate näitajatega nagu gastriidi aktiivsus, krooniline infiltratsioon limaskestas, atroofia, intestinaalne metaplaasia, ja seda nii antrumis kui ka korpuses. Mainitud seoste teadmine võimaldaks prognoosida erisuguste *H. pylori* tüvede mõju maolimaskesta muutustele.

Artoofia tekke võimalikuks põhjuseks on peetud parietaalrakkude antigeenidevastaseid immuunreaktsioone (Salupere 1968, Uibo *et al.* 1991), sh. antikanalikulaarseid antikehi (Faller *et al.* 1996, Steininger *et al.* 1998). Oluliste tegurite hulka on arvatud ka *H. pylori* võimet mõjutada maolimaskesta rakkude apoptoosi ja proliferatsiooni (Moss *et al.* 1996, Konturek *et al.* 1999, Lauwers *et al.* 1994). Seni ei ole uuritud *H. pylori* seost apoptoosi ja gastriidi dünaamikaga longitudinaaluurimise alg- ja lõpp-punktis ning ühel ajal nii antrumis kui ka korpuses.

Uurimuse eesmärk

1. Kindlaks teha *H. pylori* antigeenidele vastavate antikehade levik täiskasvanud eestlastel, mitteselekteeritud koolilaste rühmas ja maovähihaigetel.
2. Määrata *H. pylori* esinemissagedus ülakõhuvaevustega lastel antrumis ja korpuses, kasutades *H. pylori* immunohistokeemilist uuringut ning Giemsa värvimismeetodit, ja võrrelda nende meetodite tundlikkust ning eripära.
3. Kindlaks teha *H. pylori* antigeenidega (rakupinnaproteiin, CagA, kuumašokivalk) seotud immuunvastuse ja antikanalikulaarsete antikehade seos gastriidi dünaamikaga.
4. Hinnata apoptoosi antrumi- ja korpuselimaskesta epiteelrakkudes, selle seost *H. pylori* kolonisatsiooni, gastriidi aktiivsuse, kroonilise põletiku ning atroofiaga täiskasvanute 18-aastasel longitudinaalsel uurimisel.
5. Kindlaks teha *H. pylori* ja gastriidi mõju rakkude proliferatsioonile ja apoptoosi pärssiva proteiini bcl-2 ekspressioonile antrumis ja korpuses ning antrumi ja korpuse spetsiifiliste rakkude esinemissagedusele.

Uuritavad ja meetodid

Uuriti 1461 (vanuse mediaan 42 a) Karksi-Nuia ja 497 (vanuse mediaan 61 a) Abja-Paluoja elanikku (selekteerimata populatsiooni uurimine), 70 täiskasvanut (vanuse mediaan 1979. a 57,5 a) Saaremaalt (longitudinaalne uurimine aastatel 1979–1997), 421 linnas ja maal elavat selekteerimata koolilast (vanuses 9, 12 ja 15 a), 112 ülakõhuvaevuste tõttu TÜ Lastekliinikus gastroskopeeritud last (vanuse mediaan 12 a), 45 järjestikku TÜ Onkoloogia Kliinikus hospitaliseeritud maovähihaiget (vanuse mediaan 64,5 a) ja viimastele vanuse ja soo poolest vastavat kontrollisikut (TÜ ÜMPI immunoloogia uurimisgrupi seerumipangast)

ning 59 haiget (vanuse mediaan 47 a), kes olid endoskopeeritud ülakõhuvaevuste tõttu Berni Ülikooli Kliiniku gastroenteroloogia osakonnas.

H. pylori IgG tüüpi antikehi (pinnaproteiini, CagA ja kuumašokivalgu vastaseid) määrati ELISA meetodil. Osal uuritavatest määrati täiendavalt ka *H. pylori* pinnaproteiini, CagA, vakuoliseeriva tsütotoksiini (VacA) vastaseid antikehi immunobloti-meetodil. Antrumi- ja korpuselimaskesta seisundit ning *H. pylori* hinnati hematoksüliin-eosiiniga ja Giemsa järgi värvitud histoloogilistel preparaatidel, tuginedes gastriidi Sidney klassifikatsiooni kriteeriumidele. Samuti määrati *H. pylori* esinemine koelõikudes immunohistokeemiliselt, kasutades *H. pylori* vastaseid antikehi ja peroksüdaas-antiperoksüdaas-meetodit. Antikanalikulaarsed antikehad määrati uuritavate seerumis immunohistokeemiliselt, kasutades maoresektsioonil saadud normaalsest korpuselimaskestast valmistatud parafiinlõike. Parietaalrakkudevastased (nn klassikalised) antikehad määrati kaudsel immuunfluorestsentsmeetodil. Apoptoos tehti kindlaks TUNEL-meetodil, epiteelrakkude proliferatsioon, bcl-2 ekspressioon epiteelrakkudes ja interstitsiaalsetes lümfotsüütides määrati immunohistokeemiliselt (APAAP- ja avidiin-biotiin-meetodid), kasutades proliferatsiooni markerit PCNA ja Ki-67 ning bcl-2 proteiini vastaseid antikehi. Immunohistokeemiliselt (avidiin-biotiin-meetod) määrati gastriini- ning somatostatiinirakkude ja H+, K+ ATPaasi sisaldavate parietaalrakkude hulk, kasutades vastavaid antikehi.

Uurimuse peamised tulemused

H. pylori pinnaproteiinivastaste IgG tüüpi antikehade esinemissagedus täiskasvanud eestlastel on väga suur (84–94%) ja enamik nendest on CagA seropositiivsed (63–70%). Madalaim *H. pylori* seropositiivsus (69%) on uuritavast populatsioonist kõige nooremate (15–19-aastastel). Koolilaste hulgas (9–15 a) on *H. pylori* suhtes seropositiivseid 56%, 46% nendest on ka CagA-positiivsed, mis tähendab, et enamus eestlastest on saanud *H. pylori* nakkuse juba lapsena. Koolilastel, kes elavad maal, on võrreldes linnalastega suurem CagA antikehade levimus.

Maovähihaigetest leiti seropositiivsus *H. pylori* pinnaproteiini vastu 91%-l, mis ei erinenud täiskasvanute kontrollgrupis leitud tulemusest, kuigi neil haigeil oli tunduvalt sagedasem CagA seropositiivsus (87%), võrreldes Karksi-Nuia (63%) ja Saaremaa elanikega (70%). Samuti oli maovähihaigetel, võrreldes kontrollrühma isikutega, sagedamini VacA-vastaseid antikehi. See tulem kinnitab tsütotoksiliste tüvede rolli maovähi riskifaktorina.

H. pylori määramine histoloogilistes lõikudes ülakõhuvaevustega lastel Giemsa meetodil võimaldas bakterit sagedamini kindlaks teha (74%) kui immunohistokeemilise meetodiga (49%). See võib olla tingitud sellest, et antigeensed komponendid *H. pylori* nendel tüvedel, mis koloniseerivad uuritud laste maolimaskesta, erinevad nendest, mille vastu meie poolt kasutatud *H. pylori* vastased antikehad olid produtseeritud. 11%-l uuritud juhtudest reageerisid *H. pylori*

antikehad korpuselimaskesta proliferatiivse tsooni epiteelrakkudega, mis võib olla tingitud immunoloogilisest ristreaktsioonist või peegeldada *H. pylori* tungimist epiteelrakkudesse. See viitab *H. pylori* ja peremeesorganismi suhete eripärasusele lapseeas.

Gastriidi aktiivsus, maolimaskesta atroofia ja selle areng aastate jooksul ning nii antrumi kui ka korpuse epiteelrakkude kahjustus on rohkem väljendunud CagA-positiivsetel isikutel. Korpuselimaskesta atroofia arenemine on seotud ka gastriidi aktiivsusega, antrumilimaskesta atroofia lisaks veel isikute vanusega. Nimetatud tulemus toetab arvamust *H. pylori* tsütotoksilise proteiiniga seotud antigeenide mõju kohta maolimaskestas toimuvatele muudatustele ja atroofia tekkele.

Parietaalrakkude kanaliikulitega reageerivate antikehade levimus suureneb tunduvalt *H. pylori* tekitatud gastriidi pikajalisel kestmisel. Meie uuringud näitasid, et nende antikehade esinemine on sagedasem korpuselimaskesta atroofia korral. Seega on kanaliikulitega reageerivate antikehade esinemine maokorpuse limaskesta atroofia markeriks. Varasemate uuringutega on tõestatud parietaalraku autoantikehade ja atroofia seos. Käesoleva uuringu järgi suureneb *H. pylori* positiivsetel isikutel seoses vanusega parietaalraku autoantikehade esinemissagedus, mis omakorda võiks olla nendel oleva korpuselimaskesta atroofia kaudseks tunnuseks.

Antikehi *H. pylori* kuumašokivalgu vastu oli täiskasvanutest 65%-l, kusjuures antikehade leid oli spetsiifiline *H. pylori* olemasolule limaskestas (antikehi ei leitud *H. pylori* negatiivsetel isikutel). *H. pylori* kuumašokivalguvastaste antikehade esinemine sõltus *H. pylori*ga seotud põletiku astmest. Eriti tugevasti oli see seis väljendunud antrumilimaskesta põletiku korral. See toetab arvamust, et *H. pylori* antikehade tekkes on oluline peremehe organismi immuunvastuse tugevus, mitte *H. pylori* kolonisatsiooni aste. *H. pylori* kuumašokivalguvastaste antikehade esinemine ei korreleerunud gastriidi aktiivsusega, ega atroofia arenguga.

Apoptoosiindeks (korpuselimaskesta rakkude proliferatsiooni piirkonnas ning korpuse ja antrumi näärmete piirkonnas) vähenes tunduvalt uuritavate 18-aastase jälgimise jooksul ning see võib olla seotud rakkude asendumisprotsessi aeglustumisega patsientide vananemisel. Samas aga apoptoosiindeksi vähenemine ei olnud seotud mao antrumi- või korpuselimaskesta atroofia arenguga. Aktiivse gastriidiga isikutel ning maolimaskesta *H. pylori* intensiivsema kolonisatsiooni korral oli apoptoos rohkem väljendunud, iseäranis antrumis.

Gastriidi väljendusaste ja *H. pylori* kolonisatsiooni aste on seotud epiteelrakkude proliferatsiooniga ning bcl-2 ekspressiooniga antrumi- ja korpuselimaskestas erinevalt. Rakuproliferatsiooni aktiivsus antrumis on negatiivses korrelatsioonis gastriidi raskusastmega, bcl-2 ekspressioon interstitsiaalkoe lümfotsüütides ei ole seotud gastriidi raskusega. Seejuures on korpuses proliferatsiooni aktiivsus ja bcl-2 ekspressioon seotud tugevasti gastriidi, mitte aga *H. pylori* kolonisatsiooni astmega. Gastriini- ning somatostatiinirakkude ja H+,

K+ ATPaasi reaktiivsete parietaalrakkude hulk vastavas maoosas sõltub pigem atroofia astmest kui *H. pylori* kolonisatsiooni tihedusest.

Uuringute tulemuste põhjal võib väita, et antrumis- ja korpuselimaskesta atroofia tekib eri sagedusega (18-aastase longitudinaaluurimise põhjal antrumis 11%, korpuses 35%). Kuigi nii antrumis kui ka korpuses oli atroofiline gastriit enam väljendunud CagA-positiivsetel isikutel, mõjutas CagA positiivsus atroofia arengut korpuses rohkem kui antrumis. Gastriidi aktiivsus korpuses korreleerub rohkem kui antrumis CagA olemasoluga. Immuunvastus *H. pylori* kuumašokiproteiinile ja sellega seotud muutused korreleeruvad rohkem kroonilise infiltratsiooniga antrumis kui ka korpuses. Samuti mõjutavad gastriidi raskus ja *H. pylori* kolonisatsiooni tihedus rakuproliferatsiooni ja bcl-2 ekspressiooni antrumis ja korpuses erinevalt. Niisugune leid võib tähendada seda, et *H. pylori* infektsiooni tulemusena tekkiv immuunvastus on antrumi- ja korpuselimaskestas erinev.

H. pylori, mis omab tugevalt immunogeenseid proteiine, nagu CagA, kuumašokivalk, kutsub esile maolimaskesta paikse immunvastuse, mis ilmneb nii akuutse kui ka kroonilise põletikureaktsioonina (gastriidi näol), samuti tekitab süsteemse seroloogilise immunvastuse. Seropositiivsus CagA proteiini suhtes võib olla gastriidi aktiivsuse ja atroofia märgiks, võimaldades isegi prognoosida atroofia arengut, iseäranis korpuselimaskestas. Parietaalrakkude kanaliikulitega reageerivate antikehade korral võib oletada maokorpuse atroofiat, seropositiivsus *H. pylori* kuumašokivalgu suhtes aga võib olla kroonilise infiltratsiooni indikaatoriks, iseäranis antrumis. Seega, seropositiivsus erinevate *H. pylori* antigeenide suhtes võib peegeldada erisugused histoloogilisi muutusi antrumi ja korpuse limaskestas. Seevastu apoptootiline indeks ei ole seotud atroofia arenguga, olles aga rohkem väljendunud gastriidi suurema aktiivsuse korral. Apoptootiline indeks antrumilimaskestas sõltub *H. pylori* kolonisatsiooni astmest.

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PUBLICATIONS

I

Vorobjova T, Kisand K, Haukanõmm A, Maaroos H-I, Wadström T, Uibo R.
The prevalence of *Helicobacter pylori* antibodies
in a population from southern Estonia.
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The prevalence of *Helicobacter pylori* antibodies in a population from southern Estonia

Tamara Vorobjova, Kalle Kisand, Alar Haukanõmm, Heidi-Ingrid Maaroos, Torkel Wadström* and Raivo Uibo

Objectives: Previous retrospective histological studies have revealed a *Helicobacter pylori* infection rate of 73–79%. Cross-sectional studies on *H. pylori* prevalence are still lacking in Estonia.

Design: A total of 1461 inhabitants between the ages of 15 and 95 years from the village of Karksi-Nuia and 497 between the ages of 50 and 91 years from Abja-Paluoja were examined for IgG antibodies to *H. pylori*. This study was performed on a quasi-global sample of the general adult rural population in two villages in southern Estonia.

Method: A cell-surface glycine extract of *H. pylori* strain NCTC 11637 was used as antigen in an enzyme-linked immunosorbent assay.

Results: IgG antibodies to *H. pylori* strain NCTC 11637 were detected in 87.0±2.3% of the inhabitants of Karksi-Nuia and in 89.3±3.6% of those from Abja-Paluoja. IgG prevalence rates increased from 69.0±9.5% in the 15- to 19-year-old age group to 83.0±7.1% in the 20- to 29-year-olds ($P<0.05$).

Conclusion: We found an extremely high prevalence rate of *H. pylori* infection in this Estonian adult rural population. *H. pylori* infection was very prevalent among young people (aged 15–29 years).

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Keywords: *Helicobacter pylori*, epidemiology, serology, enzyme-linked immunosorbent assay

Introduction

The strong association between *Helicobacter pylori* infection and chronic gastritis and peptic ulcer has been extensively documented [1,2]. Recently, several studies [3–9] have observed a close relationship between *H. pylori* infection, chronic gastritis and gastric cancer, thus, indicating a significant new area for investigations related to this microorganism.

In Estonia, the frequency of *H. pylori* has been studied retrospectively from gastric biopsy specimens in random samples, collected from the rural population of southern Estonia in 1972 (154 participants, 79% *H. pylori*-positive) and from the adult urban population of Kuressaare, the island of Saaremaa (227 participants, 73% *H. pylori*-positive) in 1979 [10,11].

The present study is a cross-sectional investigation of *H. pylori* seropositivity in an Estonian adult population. The frequency of histologically verified *H. pylori* infection is high in Estonia and serology is the most suitable indicator of the rate of infection in epidemiological studies [12–14].

For this purpose, representative populations from two southern Estonian villages were studied. This study was part of a large epidemiological investigation of several immunologically mediated diseases begun in 1990 in these two villages.

Methods

All inhabitants aged 15–95 years from Karksi-Nuia and those aged 50–91 years from Abja-Paluoja were invited to participate in this study. The response rate was 84% from Karksi-Nuia [1461 participants; 637

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men (median age 41 years), 824 women (median age 42 years)] and 77% from Abja-Paluoja [497 participants; 189 men (median age 60 years), 308 women (median age 62 years)]. Serum samples were taken from all participants. Sixty-four subjects (28 men and 36 women) from Karksi-Nuia were invited for further study and underwent gastroscopy because of elevated serum IgA or IgG anti-gliadin antibody levels for diagnosis of coeliac disease. Although no cases of coeliac disease were diagnosed, four people were found to have peptic ulcer disease [15]. Two biopsy specimens were taken from the gastric corpus and the antral mucosa from all subjects. The results obtained from the two villages were analysed separately since the subjects participating in the study in Abja-Paluoja were older.

IgG antibodies to *H. pylori* were determined using an enzyme-linked immunosorbent assay (ELISA) with a cell-surface glycine extract of *H. pylori* strain NCTC 11637 as antigen [16,17]. The protein concentration of both antigens was 5 μg/ml. A commercial human IgG (Kabi Pharmacia AB, Stockholm, Sweden) was used at a concentration of 100 units as a positive control for both antigens. The absorbance values were expressed as relative antibody activities (RAA) [16]. RAA values of 25 and over were regarded as positive [18]. The interassay variation in the assay using the antigen preparation from strain NCTC 11637 was 13.5%; the intra-assay variation was 5.0±1.73%.

The gastric biopsy specimens were stained using standard haematoxylin and eosin and Giemsa methods. The severity of *H. pylori* infection was evaluated as follows: 0, absence of bacteria; 1, <20 per field; 2, 20–60 per field; 3, >60 per field [19]. Four to six fields were examined. An objective of 40× and an ocular with 10× magnification were used. If *H. pylori* colonization was patchy, the grade was classified according to the most pronounced grade of colonization. Changes in the mucosa were graded as follows: 0, normal mucosa; 0.5, slightly superficial gastritis; 1, moderate; 1.5, severe round cell infiltration; 2, slight atrophic gastritis; 3, moderate; 4, severe atrophy of glands. This system for grading gastritis is comparable with the Sydney system, however, in the estimation of the grade of atrophic gastritis, the level of infiltration was not taken into account. The morphologic criteria used for discriminating between different grades of superficial and atrophic gastritis have been described in more detail elsewhere [19].

Each participant was questioned about the past and present history of gastric diseases, including peptic ulcer disease, as well as other illnesses, surgery and medications.

Statistical analyses were performed using the statistical package Statgraphics. Tests included the Chi-squared test, the Student's t-test and Spearman's rank correlation test. This study was approved by the Committee of Ethics at Tartu University.

Results

IgG antibodies to *H. pylori* strain NCTC 11637 were detected in 1271 (87.0±2.3%; median age 43 years) out of the 1461 subjects studied in Karksi-Nuia. The prevalence rates of antibodies to NCTC 11637 significantly increased from 69.0±9.5% in those aged 15–19 years to 83.0±7.1% in those aged 20–29 years ($\chi^2 = 9.26$, $P = 0.002$), and to 89.0±7.7% in those aged 30–39 years ($\chi^2 = 4.1$, $P = 0.04$). There was no significant difference between any of the older age groups ($P > 0.05$; Table 1).

In Abja-Paluoja, 444 (89.3±3.6%) out of 497 (269 women, 175 men; median age 61 years) subjects had IgG antibodies to *H. pylori* strain NCTC 11637. There was no significant difference in the prevalence of *H. pylori* antibodies in the different age groups studied (Table 2).

Table 1. Age-specific prevalence rates of *Helicobacter pylori* antibodies to the NCTC 11637 strain in the Karksi-Nuia population.

Age group (years)	No. of subjects	Age distribution (%)		No. *H. pylori*-positive (%)			Mean values of RAA±SD
		Men	Women	Total	Men	Women	
15–19	159	12	10	109 (69)*	54/74 (73)	55/85 (65)	51.7±35.8
20–29	184	13	12	152 (83)*	72/85 (85)	80/99 (81)	63.5±31.0
30–39	327	24	21	291 (89)*	138/153 (91)	153/174 (87)	71.4±58.5
40–49	275	17	20	247 (90)	99/108 (92)	148/167 (89)	71.7±29.5
50–59	289	20	20	264 (91)	116/126 (92)	148/163 (91)	78.2±40.3
60–69	146	10	10	135 (92)	59/63 (94)	76/83 (91)	76.9±31.2
70–79	63	3	5	59 (94)	21/22 (95)	38/41 (93)	73.9±31.2
80–89	16	1	1	13 (81)	4/6 (67)	9/10 (90)	56.0±27.0
90–99	2	0	1	1 (50)	0	1/2 (50)	69.5±48.5
Total	1461	100	100	1271	563/637 (88)	708/824 (86)	

RAA, relative antibody activities. *Significantly different (between those aged 15–19 and 20–29 years: $\chi^2 = 9.26$, $P = 0.002$; between those aged 20–29 and 30–39 years: $\chi^2 = 4.1$, $P = 0.04$).

Table 2. Age-specific prevalence rates of *Helicobacter pylori* antibodies to the NCTC 11637 strain in the Abja-Paluoja population.

Age group (years)	No. of subjects	Age distribution (%)		No. *H. pylori*-positive (%)			Mean values of RAA±SD
		Men	Women	Total	Men	Women	
50–59	200	48	35	182 (91)	82/90 (91)	100/110 (91)	72.5±29.7
60–69	191	33	41	170 (89)	59/63 (94)	111/128 (87)	72.5±31.7
70–79	86	15	19	75 (87)	27/29 (93)	48/57 (84)	75.1±33.3
80–89	19	4	4	16 (84)	7/7 (100)	9/12 (75)	69.9±29.8
90–99	1	0	1	1 (100)	0	1/1 (100)	40.0±0
Total	497	100	100	444 (89)	175/189 (92)	269/308 (87)	

There was no statistically significant difference in *H. pylori* positivity between any of the age groups studied. RAA, relative antibody activities.

Table 3. A comparison of histological and serological results of *Helicobacter pylori* detection and the state of the gastric mucosa in the 64 subjects who underwent gastroscopy.

Evalutation of *H. pylori*		State of the gastric mucosa (grade)			
Serology IgG to NCTC 11637	Histology†	Normal (0)	Superficial gastritis (0.5–1.5)	Atrophic gastritis (2–4)	Total
Antral mucosa					
–	–	8	0	1	9
–	+	0	1	0	1
+	–	1	0	1	2
+	+	5	45	1	51*
Total		14	46	3	63*
Corpus mucosa					
–		6	1	2	9
–	–	1	0	0	1
+	+	0	2	0	2
+	–	9	35	6	50*
Total	+	16	38	8	62*

*Determination of the state of the gastric mucosa was impossible due to inadequate size of biopsy specimens from the antrum in one subject and from the corpus mucosa in two others. †Histology for both the antrum and corpus.

There was a tendency towards a higher prevalence of *H. pylori*-positive subjects among men when compared with women in each age group in both villages (Tables 1 and 2). The median age of men did not differ from that of women in either village (41 and 42 years, respectively, for Karksi-Nuia, 60 and 62 years, respectively, for Abja-Paluoja). The age distribution between men and women was not significantly different (Tables 1 and 2).

A comparison of the results of serological and histological *H. pylori* determinations, and the state of the gastric mucosa in the 64 participants from Karksi-Nuia subjected to endoscopy is presented in Table 3. As can be seen, there was a good agreement (95%) between the histological and serological findings for *H. pylori*. Disagreement was revealed in three subjects only, two of whom were found to be *H. pylori*-positive on serology and negative on histology, and one histologically proven case had no IgG antibodies to *H. pylori*. The histological absence or presence of *H. pylori* was the same in the biopsy specimens taken from the antrum and from the corpus mucosa.

The sensitivity of the ELISA test with the NCTC 11637 strain antigen when compared with the results of morphology was 98% (52 out of 53); the specificity was 82% (nine out of 11); the positive predictive value was 96%; the false-positive rate with NCTC 11637 antigen was 18%, whereas the false-negative rate was 2%.

We found statistically significant correlations between the RAA values for the ELISA and the grade of *H. pylori* colonization when evaluated histologically as the sum of the grades in the antrum and corpus ($r = 0.389$; $P = 0.002$). The grade of *H. pylori* colonization in the antrum significantly correlated with scores of mucosal changes in the antrum ($r = 0.482$; $P = 0.0001$). *H. pylori* grades in the corpus correlated with scores of mucosal changes in the corpus ($r = 0.310$; $P = 0.015$). No significant correlation was observed between the RAA values on ELISA and the severity of gastritis (a total score of antral and corpus gastritis; $r = 0.220$; $P = 0.08$).

The follow-up studies, where *H. pylori* antibodies to NCTC 11637 strain were investigated twice within 6 months, showed a concordance of results in 61 out of 64 subjects studied. There were also no significant differences between the RAA values in both serum samples ($t = 1.89$, $P = 0.06$). In three subjects, the im-

mune response to *H. pylori* strain NCTC 11637 was changed during the follow-up period. In two subjects with antibodies to NCTC 11637 at the beginning of the study, the second blood sample was free of antibodies and there was no histological evidence of *H. pylori* colonization. One subject, originally antibody-negative, became seropositive for NCTC 11637. The presence of *H. pylori* was also confirmed histologically.

Discussion

The present study revealed an extremely high frequency (87.0±2.3%) of IgG antibodies to *H. pylori* in an Estonian population aged 15 years and over, as determined by an ELISA using a *H. pylori* standard strain NCTC 11637 antigen. Serum IgG antibodies to *H. pylori* are most frequently used for the diagnosis of *H. pylori* infection [12–14]. These antibodies correlate well with the persistence of *H. pylori* in the gastric mucosa [14,20]. This was confirmed by the present study, where only in three out of 64 subjects with a histological and serological evaluation of *H. pylori* infection, there was no concordance between the two methods.

Thus, 87.0±2.3% of the Estonian population is infected with *H. pylori*. This is a higher *H. pylori* prevalence rate than that observed in previous studies performed in Estonia [10,11] and is due to the use of the more sensitive serological method for the detection of *H. pylori* in the present study. Hence, it can be concluded that Estonia is among those countries with a high *H. pylori* prevalence rate, such as Poland, and developing countries with poor socioeconomic conditions [12,21–25]. In similar studies performed in areas with high *H. pylori* prevalence rates, there are always some individuals without *H. pylori* infection, especially in the younger age groups [20,25].

In studies performed in several developed countries, it has been shown that until the third decade of life, *H. pylori* infection is uncommon and increases at approximately 1% per year [2,12,13,20,26–29]. So far, the prevalence of *H. pylori* infection in children in Estonia has been studied in 5- to 15-year-olds with abdominal complaints [30,31]. In 41–56% of the children studied, *H. pylori* infection was detected histologically or serologically, i.e. at almost the same frequency as detected in the youngest age group in our study. Taking into account the opinion of Mitchel *et al.* [32] that the relative risk of a child acquiring *H. pylori* infection is proportional to the prevalence of *H. pylori* in the adult population of childbearing age, we can assume that Estonian children easily acquire *H. pylori* from their parents; in the present study in those aged 20–29 years and 30–39 years, the prevalence of *H. pylori* is 83.0±7.1 and 89.0±7.7%, respectively.

However, there are also a number of subjects without *H. pylori* infection in the higher age groups. These subjects deserve special attention because presumably they have been in continuous contact with *H. pylori* without developing an infection. There are several ways to explain this, but two possibilities are most likely: either the gastric mucosa of these persons is not suitable for *H. pylori* colonization or their immune system cleans out the infection in its early stages. The unsuitability of atrophic gastric mucosa for the persistent colonization of *H. pylori* in the stomach has been shown in several studies [7,33–35] and this may be a reason for *H. pylori* clearance in some of the older individuals. In others, genetically determined incompatibility between gastric mucous glycoproteins and *H. pylori* might be of importance, but this is a field which has not been extensively studied to date. The lack of colonization in some individuals may also be explained by differences in cell-surface adhesins of various *H. pylori* strains [36].

The fact that young Estonian people (aged 15–29 years) already have a high prevalence of *H. pylori* infection is of great importance because this is related to the possible early development of chronic gastritis with a high risk of gastric cancer [1–4,7,37,38]. Indeed, chronic gastritis is very common in Estonia [39] and gastric cancer is a common form of carcinoma here, with an incidence rate of 37.0 for men and 18.6 for women per 100 000 inhabitants in 1987 [40].

In conclusion, the serological evaluation of IgG antibodies to *H. pylori* standard strain NCTC 11637 among the rural population of southern Estonia showed an extremely high (87.0±2.3%) prevalence rate of *H. pylori* infection in the population studied.

That young Estonian people (aged 15–29 years) already have a high prevalence of *H. pylori* infection (69–83%) is important for the possible development of chronic gastritis and risk of gastric cancer. However, in spite of a very high infection rate of *H. pylori* in this population, some of the subjects are still free of infection. To elucidate the reasons for this resistance, further studies should pay more attention to the immune mechanisms and genetically determined differences of *H. pylori* receptor structures in the gastric mucosa.

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II

Uibo R, **Vorobjova T**, Metsküla K, Kisand K, Wadström T, Kivik T.
Association of *Helicobacter pylori* and
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Association of *Helicobacter pylori* and gastric autoimmunity: A population-based study

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Abstract

Based on clinical studies, a negative association between *Helicobacter pylori* and autoimmune corpus gastritis is described. In the present investigation of an unselected population of 1461 adults we can state, however, that there exists a relationship between *H. pylori* infection and the development of gastric corpus autoimmunity. As confirmation for the gastric autoantibody development through molecular mimicry, a high homology (72% in 25 amino acid overlap) between the beta subunit of *H. pylori* urease and that of H + K + ATPase, the gastric parietal cell autoantigen, was revealed.

Keywords: *Helicobacter pylori*; IgG antibody; Parietal cell antibody; Gastric autoimmunity; Chronic gastritis; Amino acid sequence

1. Introduction

There is a large amount of experimental and clinical data confirming that *Helicobacter pylori* is the major etiological factor in the development of both chronic non-immune (type B) gastritis and its important associates, duodenal ulcer and gastric cancer. On the contrary, few studies have been performed so far to elucidate the role of *H. pylori* in autoimmune (type A) gastritis and, as a rule, only patients with pernicious anaemia have been studied [1,2]. Pernicious anaemia, however, is characterised by deep atrophic gastritis which is not convenient for the existence of *H. pylori* [3]. Therefore it is evident that, in order to get a more realistic picture about the role of *H. pylori* in gastric autoimmune processes, autoimmune gastritis should be investigated for *H. pylori* at much earlier stages. In clinical material this is not easy to do because uncomplicated autoimmune gastritis is an incidental finding [3]. Thus, the way to address the issue is to perform a study of a large unselected population group. For these reasons, the population of a small town, Karksi-Nuia, in Estonia was chosen for the present investigation (with the permission of the Committee of Ethics for Medical Investigations at the University of Tartu, Estonia).

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2. Materials and methods

All inhabitants of age 15 years or older of Karksi-Nuia were asked, by personal invitation letters, to participate in the study. A total of 1461 (84%) individuals, median age 43 years, 637 males and 824 females, responded. All the participants were informed about the content of the investigations, their subjective health status was interviewed and analysed in relation to their personal out-patient records, samples of a venous blood were taken for serological studies (stored at $-20°$ C), and, if necessary, routine out-patient investigations were prescribed.

As a marker of autoimmune (type A) gastritis [3,4], parietal cell antibodies (PCA) were determined by the indirect immunofluorescence method using unfixed cryostate sections from mouse stomach fundal region as an antigen. Rat kidney and liver sections were explored as controls. The dilution of the tested sera was 1:10 and fluorescein isothiocyanate-conjugated sheep anti-human immunoglobulin was used as the secondary antibody. Every set of tests contained a known PCA positive as well as a PCA negative serum. Other tissue antibodies (mitochondrial, smooth muscle antibodies) were excluded since they gave no reaction with control antigens [5].

The *H. pylori* infection was serologically verified by the enzyme-linked immunosorbent assay with a cell surface glycine extract of *H. pylori* strain NCTC 11637 as an antigen [6]. This assay detects antibodies of IgG type and the results are in a high correlation (sensitivity 98%) with the presence of *H. pylori* in the gastric mucosa (unpublished). The test results were expressed in relative antibody activities (RAA), i.e. the ratio of the tested serum result in optical density (OD) units to the positive control serum result in OD units considered equal to 100 units. If the RAA value for the tested serum was 25 or over, the test result was regarded as indicative of the presence of *H. pylori*.

The statistical analysis of the significance of the results was performed by χ^2 and Fisher's exact tests as appropriate.

3. Results and discussion

Our studies revealed PCA in 36 (2.5%) from the 1461 individuals: in 22 females (2.7% from the 824 females studied) and in 14 males (2.2% from the 637 males studied), including a pair of close relatives (a mother of 42 years and her son of 17 years of age). He was the only person with PCA in the youngest group of the inhabitants studied (PCA frequency in this group was 0.3%). A continuous increase of the frequency of PCA was observed in the older age groups: at age 30–49 years it was 2.0%, at age 50–69 years 4.1%, and among persons of 70 years and older it was 6.2%. None of the persons with PCA had any clinical signs of anaemia which could have indicated the possibility of pernicious anaemia. According to their case histories, no diagnoses of pernicious anaemia had been made before the study, but in one female, iron deficiency anaemia had been diagnosed and she had been receiving treatment for it during the last two years.

IgG antibodies to *H. pylori* were detected in a total of 1271, i.e. in 87% of the persons studied. The lowest percentage of *H. pylori* antibodies (76%) was revealed in the age group of 15–29 years, which differed significantly ($\chi^2 = 28.47$, $P < 0.001$) from the occurrence of these antibodies in the next age

Table 1
The distribution of parietal cell antibody (PCA) positivity among persons with and without *Helicobacter pylori* antibodies

	Age groups (years)							
	15–29		30–49		50–69		> 70	
	Studied	PCA +	Studied	PCA +	Studied	PCA +	Studied	PCA +
H. pylori positive	261	1 (0.4%)	538	6 (1.1%) [a]	399	15 (3.8%) [a]	73	4 (5.5%) [a]
H. pylori negative	82	0 (0%)	64	6 (9.4%) [b]	36	3 (8.3%) [b]	8	1 (12.5%) [b]
All	343	1 (0.3%)	602	12 (2.0%)	435	18 (4.1%)	81	5 (6.2%)

[a] $\chi^2 = 9.577$; $P = 0.008$. [b] $\chi^2 = 0.138$; $P = 0.933$.

group of 30–49 years (89%). No statistically significant differences of *H. pylori* antibody incidence were seen between the older age groups where *H. pylori* was detected in at least 90% of the persons studied (Table 1). Among the 36 PCA positive persons, antibodies to *H. pylori* were detected in 26 (10 males and 16 females, median age 60 years), whereas 10 (5 males and 5 females, median age 44 years) were seronegative for *H. pylori*. In 7 of the 10 seronegative persons, antibodies to *H. pylori* were revealed in their family members (the family members of the remaining three were not studied). A comparative analysis of the incidence of PCA among the *H. pylori* positive and *H. pylori* negative persons showed an age dependency in the *H. pylori* positives above 30 years (Table 1). Thus, in the age group of 30–49 years, 1.1% of *H. pylori* positives have PCA in comparison to 3.8% and 5.5% in the next age groups (significant difference, $\chi^2 = 9.577$, $P = 0.008$). In *H. pylori* negatives, the PCA incidence was practically similar in three subsequent age groups, respectively 9.4%, 8.3% and 12.5% (non-significant difference, $\chi^2 = 0.138$, $P = 0.933$).

As far as we are aware, this is the first study where the *H. pylori* and gastric autoimmunity incidence has been analysed in a large population sample without participant selection. Thanks to the high participation rate in all main age groups (mean 84%), we were able to obtain material which should well describe the natural relationship between these two pathogenetically important parameters in chronic gastritis. In selected (clinical) material these relationships could not be revealed because such selected material includes only a part of the patients with autoimmune gastritis due to absence of complications in its earlier stages.

Consequently, based on our results, we can state that there exists a relationship between the *H. pylori* infection and the development of gastric autoimmunity. First, we could demonstrate a high coincidence of PCA and *H. pylori* antibodies which is not seen in patients with pernicious anaemia [1,2]. If not detected in persons with PCA, *H. pylori* was revealed in those of their family members who were available for serological studies. It shows that the *H. pylori* seronegative persons, too, have also been exposed to *H. pylori* and are likely to have been infected with *H. pylori* at some point of their life. The fact that they were free from *H. pylori* during the study can be accounted for the existence of deep gastric corpus atrophy most characteristic for autoimmune gastritis [3,5].

Secondly, starting from the age of 30 years where the *H. pylori* infection was revealed in 89–91% of the inhabitants, there was a significant age-dependent increase of PCA positivity among the *H. pylori* infected persons but not among the *H. pylori* negative ones suggesting that the long-lasting *H. pylori* infection might be an important factor for the development of PCA. Accordingly, the longer the duration of the infection, the greater the possibility to develop PCA. Once developed, PCA will promote the atrophy of the gastric corpus mucosa through the immune reaction toward its autoantigenic target [4]. During the due course of corpus atrophy, the gastric milieu becomes more and more unfavourable for the *H. pylori* colonisation until its total disappearance. Thus, we have reached the situation which is operative in the group of *H. pylori* negative and PCA positive persons. This is probably the group where pernicious anaemia appears in the part of persons who are under the influence of yet unknown genetic and immunological factors.

What could be the mechanism of induction of autoimmune reactions against the gastric mucosa by *H. pylori* infection? As in many other similar situations [7], molecular mimicry between some of the *H. pylori* antigens and the PCA autoantigen, gastric H + K + ATPase [4], could be involved. To evaluate this possibility, we compared, by PROSIS microcomputer software, all the known sequences of *H. pylori* proteins (EMBL release of March 3, 1994) with that of gastric H + K + ATPase. The highest homology (72% in 25 amino acid overlap) was revealed between the beta subunit of *H. pylori* urease and the beta subunit of H + K + ATPase including 8 identical amino acids and 10 conservative replacements (|, identical amino acids; ·, conserved substitutions):

H. pylori urease beta subunit

```
    30                  40                  50
L I A E V E H D Y T I Y G E E L K F G G G K T L R
| . . . . . .   .  |  . . | |  .  |  | | |
L M Q T I D P Y T P D Y Q D Q L K - S P G V T L R
            70                  80
```

H + K + ATPase beta subunit

Lower homologies (6–7 identical amino acids with some conservative replacements within 25 comparable amino acids) were revealed on many occasions, including the already published homology with *H. pylori* vacuolating toxin [8]. Since urease is a common surface component of different strains of *H. pylori* as well as a putative virulence factor and immune reactions against *H. pylori* urease are universally seen in nearly all persons with *H. pylori* infection [9], we propose that immune reactions to urease trigger the autoimmune reactivity against H + K + ATPase in susceptible persons. To prove this hypothesis, concomitant antibody studies with both purified enzyme preparations as well as with the overlapping region of these enzymes need to be further performed in groups of persons with different *H. pylori* and PCA states. Moreover, similar experiments with activated T cells should also be performed since cell-mediated immunity might be of primary importance in the development of gastric autoimmunity [4].

Taken together, our results suggest that PCA and autoimmune corpus gastritis development could be related to the gastric mucosal pathogen *H. pylori*. Although there have recently been a few other studies that have pointed to this possibility [10], much more molecule-oriented confirmative investigations are needed. We hope that the present report might provide some useful suggestions for analogous studies. As one can see from the review by Oldstone [7], such suggestions have led to a number of studies of different autoimmune diseases, some of which have produced valuable results for the understanding of disease pathogenesis, the relationship between *Klebsiella pneumonia* and ankylosing spondylitis being an example.

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III

Vorobjova T, Nilsson I, Kull K, Maaroos H-I, Covacci A, Wadström T, Uibo R.
CagA protein seropositivity in a random sample of adult population and gastric cancer patients in Estonia.
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CagA protein seropositivity in a random sample of adult population and gastric cancer patients in Estonia

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Objective **The prevalence of antibodies to CagA protein, associated with the risk of developing gastric cancer (GC), was studied in an Estonian adult population with a high prevalence of *Helicobacter pylori* (HP) infection and in a group of GC patients.**

Design **In a representative sample of a random adult population from the South Estonian town of Karksi-Nuia, containing 199 subjects (86 M, 113 F, mean age 42.4) and in 45 (22 M, 23 F, mean age 64.5) consecutive patients with gastric adenocarcinoma, recruited during the periods 1986–87 and 1995–96 in the Hospital of Oncology, University of Tartu, anti-CagA IgG antibodies were determined by enzyme-linked immunosorbent assay (ELISA) using a recombinant fragment of CagA protein. The occurrence of anti-CagA IgG in ELISA was compared with immunoblot results for 141 subjects.**

Results **Seropositivity to acid glycine extracted cell surface proteins of HP was 85% in the population and 91% in GC patients ($p = 0.39$). Anti-CagA IgG antibodies were present in 63% of the population and in 87% of GC patients ($p = 0.004$). The highest prevalence of anti-CagA IgG in the population sample occurred in the age group 20–29 (76%). A comparison of anti-CagA positivity evaluated by using ELISA and immunoblot showed an agreement of results in 80% of cases.**

Conclusion **HP seropositivity was similarly high in the Estonian random adult population sample and in GC patients, however, the prevalence of anti-CagA IgG was significantly higher in GC patients. Moreover, persons aged 20–29 years in the population possess the highest prevalence of anti-CagA IgG and should be given further attention with respect to the development of GC later in life.**

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Keywords: CagA antibodies, enzyme-linked immunosorbent assay, gastric cancer, *Helicobacter pylori*, immunoblot

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Introduction

There is much evidence that *Helicobacter pylori* (HP) is involved in the development of gastric cancer (GC) [1–6]. Infection with HP strains expressing the 128 kDa cytotoxin-associated (CagA) protein constitutes a factor associated with increased risk of the development of peptic ulcer disease and GC [7–17]. In a previous study we found a very high seroprevalence (87%) of HP in an Estonian adult population [18]. It is remarkable that population studies in both Kuressaare (1979) [19] and Karksi-Nuia (1990) [18] showed that 58–69% of young Estonians aged 15–19 years are infected with HP and that there is a first significant peak (83%) of HP infection in the 20–29 year age group. This fact is of great importance as it is related to the possible risk of developing GC later in life [20, 21]. Indeed, the incidence rate of stomach cancer for males and females in Estonia is higher than the average of the other European countries [22].

Taking into account the above facts, the aim of this study was to evaluate the prevalence of antibodies to a recombinant fragment of the CagA protein in a random sample of the Estonian adult population and to compare it with the presence of antibodies to CagA in GC patients. As an additional task, the presence of antibodies to the CagA evaluated by using enzyme-linked immunosorbent assay (ELISA) was compared with the results of immunoblot.

Subjects and material

The study included 199 subjects (86 males, 113 females, mean age 42.4 years) forming a representative sample of adult population from the South Estonian town of Karksi-Nuia. We selected these subjects from 1461 persons (637 males, 824 females, mean age 42.3 years) who had participated in and donated blood for our large seroepidemiolog-

ical study the results of which were published elsewhere [18]. In the latter study, 84% of all inhabitants of Karksi-Nuia were involved [18]. The relative proportion of males/females of different age was calculated on the original material of 1461 samples and this proportion was extrapolated to a set of 200 samples. According to these proportions, serum samples from each age/sex group of the whole material were randomly selected by the computer. In the present study, 199 sera out of the 200 samples were available for the determination of HP and CagA status. Additionally, 45 consecutive patients (22 males, 23 females, mean age 64.5 years) with gastric adenocarcinoma, operated on and diagnosed pathohistologically in the Hospital of Oncology, University of Tartu, and recruited during two periods, in 1986–87 and 1995–96, were studied. GC patients came from the town of Tartu and from South Estonian counties, including Karksi-Nuia. Serum samples from GC patients were collected before gastric surgery and were stored at –20 °C until assayed.

Adenocarcinoma was localized in the antrum in 14 cancer patients, in the corpus in 17 cases and in the antrum and corpus in 14 cases. The gastric carcinomas were classified histologically according to the Laurén system [23]. The intestinal type of adenocarcinoma was diagnosed in 18 cases, the diffuse type in nine cases and the indeterminate type in 14 cases; four cases were inoperable.

In order to compare the presence of IgG antibodies to CagA, using ELISA and immunoblot, 141 sera which included all 45 sera from GC patients, 52 sera from a representative population sample with borderline absorbance values (OD 0.245–0.345) in CagA ELISA, nine sera from gastric and duodenal ulcer patients (all HP-positive in ELISA with glycine-extracted cell surface antigen) and 35 sera from children with abdominal complaints, treated in Tartu Children's Hospital (10 HP-positives and 25 HP-negatives both in ELISA and morphologically) were consequently analysed by immunoblot.

This study was approved by the Committee of Ethics at the University of Tartu.

Methods

ELISA

HP status was determined using a serological evaluation of IgG antibodies to HP (strain NCTC 11637) by ELISA as reported previously [18, 24]. Briefly, 0.5 μg of acid glycine (pH 2.2)-extracted cell surface proteins per well were used for coating microtitre plates (NUNC, Roskilde, Denmark). The sera were diluted to 1:800; the second antibody was alkaline phosphatase-labelled anti-human IgG (DAKO, Glostrup, Denmark) which was diluted to 1:500. The results were expressed by corrected mean absorbance values as a percentage of a reference standard (human γ-globulin, KABI/Pharmacia, Stockholm, Sweden). The cut-off value for seropositivity was set to a relative antibody activity (RAA) of 25. Interassay variation was 13%; intraassay variation was 5% [18].

Anti-CagA IgG were detected by ELISA using a recombinant fragment of the CagA antigen His-17/12 of the HP CCUG 17874 strain following the methodology, described by Xiang *et al.* [25], with modifications. In our study the recombinant CagA antigen was diluted in 0.1 mol/l carbonate buffer of pH 9.6 to a final concentration of 1.25 μg/ml. Maxisorp Immunoplates (NUNC, Roskilde, Denmark) were coated with 100 μl/well in duplicate and incubated for 16 h at 4 °C, washed three times with PBS (pH 7.4) containing 0.5% Tween 20. The wells were saturated with 200 μl/well of 1% bovine serum albumin (BSA) in PBS–Tween at 37 °C for 2 h. After washing the plates three times, 100 μl of each serum sample diluted to 1:300 in 1% BSA–PBS was added to the wells in triplicate (two wells with an antigen and one without an antigen) and incubated at 37 °C for 2 h. The plates were then washed three times and 100 μl/well of alkaline phosphatase-conjugated anti-human IgG (DAKO), diluted to 1:1000 in 1% BSA–PBS, was added and the plates were incubated at 37 °C for 1.5 h. After three washes, 100 μl/well of a substrate solution containing *p*-nitrophenyl phosphate (SigmaChemical, St. Louis, MO, USA) in diethanolamine–$MgCl_2$ buffer was added to each well and incubated at 37 °C for 30 min. Each plate contained a positive and a negative control serum. Absorption was read at 405 nm. The absorption value derived from the mean value of absorption in two wells with antigen minus the absorption value in a well without antigen was taken as the absorbance value of the studied sera. Instead of the cut-off 0.250 OD, used by Xiang *et al.* [25], the absorbance value 0.300 (*A* 405 nm) was taken as the cut-off point in our study, based on the calculation of ELISA results, derived from the mean absorbance value of 25 HP-negative persons (histologically and serologically) plus two standard deviations, by using square root transformation [26]. Interassay variation was 11.5%.

Immunoblot

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a Protean II Cell Vertical Electrophoresis equipment (Bio-Rad, Richmond, CA, USA). Glycine-extracted proteins from seven pooled antigen batches of HP strain NCTC 11637 were separated in a gradient gel (5–20%) with a 5% stacking gel (acrylamide–bis 29:1, Bio-Rad) and were used for protein separation. The HP strain NCTC 11637 is known as a cytotoxic strain expressing CagA protein [27]. The antigen (140 μg/gel) was diluted four times in a sample buffer (0.5 mol/l Tris–HCl pH 6.8, 0.5% bromphenol blue, 8% glycerol, 4% SDS, 4% 2-mercaptoethanol) and heated at 95 °C for 3 min. After cooling, the proteins were loaded on the gel and separated for 16 h at 80 V. Molecular weight standards (Promega, Scandinavian

Diagnostic Service, Falkenberg, Sweden), including proteins ranging from 14.3 to 97.4 kDa in size, were treated similarly.

The proteins were transferred electrophoretically to the Immobilon PVDF membrane (0.45 μm, Millipore Intertech, Bedford, MA, USA) using a semi-dry electro-blotter equipment (Ancos, Vig, Denmark) for 1.5 h at a constant current of 0.8 mA/cm^2.

The membrane was saturated by incubation for 2 × 15 min in blocking buffers I and II (from M. Rucheton, Orstom Laboratories, Montpellier, France) [28]. Saturated membranes were rinsed once for 10 min in a washing buffer and cut into strips. The strips were overlaid with sera diluted to 1:100 in a washing buffer under gentle agitation for 16 h at 4 °C. The strips were then rinsed for 3 × 5 min. and incubated for 2 h at 4 °C with horseradish peroxidase-labelled anti-human IgG antibodies (DAKO) diluted to 1:600. After repeated rinsing, bound antibodies were detected by adding 50 mmol/l sodium acetate buffer (pH 5.0) containing 0.04% 3-amino-9-ethylcarbazole (Sigma Chemical) and 0.015% H_2O_2. The intensity of the stained bands obtained with patient sera was compared with that of the strip incubated with human γ-globulin (KABI/Pharmacia, Stockholm, Sweden) included as a calibrator in each analysis.

At analysing, special attention was paid to the presence of antibodies to the cytotoxin-associated high molecular protein band of 128 kDa.

Statistical analysis

Differences in HP status and in the prevalence of antibodies to CagA between groups were tested by Yates's corrected χ^2 test. Odds ratios (OR) with 95% confidence intervals (CI) were estimated using Cochran–Mantel–Haenszel statistics with the SAS computer program to examine the magnitude of the associations. Effects were considered to be statistically significant if the 95% CI did not include the value 1.0.

Results

The prevalence of IgG antibodies to acid glycine-extracted cell-surface HP proteins was 85% (169/199) in a random adult population sample and 91% (41/45) in GC patients (Table 1), which was not significantly different ($p = 0.39$).

Anti-CagA IgG were seen in 63% (126/199) of the population sample. In GC patients the prevalence of anti-CagA IgG was significantly higher, 87% (39/45; $p = 0.004$). HP-positive GC patients showed anti-CagA IgG reactivity in 90% (37/41) of cases, whereas in the population sample 71% of HP-positive persons (120/169) were CagA positive ($p = 0.019$) (Table 1).

The OR for the association between HP infection and GC was 1.82 (95% CI 0.61–5.39), which was not statistically significant, but we found significant association between CagA positivity and GC, where OR was 3.76 (95% CI 1.59–8.91).

In the population sample the highest prevalence of CagA antibodies was found in the 20–29 year age group: 19/25 (76%) in all persons studied, 18/22 (82%) in persons defined as HP positive, which was statistically not different from the corresponding proportions in the other age groups ($p = 0.65$, $p = 0.57$, $p = 0.52$, $p = 0.52$, $p = 0.99$, $p = 0.08$ comparing with age groups 15–19, 30–39, 40–49, 50–59, 60–69 and ≥70 years, respectively).

The difference in the prevalence of anti-CagA IgG between the corresponding age groups in the population sample and in GC patients (Table 1) was not statistically significant ($p > 0.05$ for groups 30–39 to ≥70 years).

Table 1 The prevalence of IgG antibodies to glycine-extracted cell-surface proteins of HP and to a recombinant fragment of CagA protein in a representative sample of adult Estonian population and in gastric cancer patients

Subjects, age groups	*n*	HP+	CagA+	CagA+/HP+
Population				
15–19	22	13/22 (59%)	11/22 (50%)	9/13 (69%)
20–29	25	22/25 (88%)	19/25 (76%)	18/22 (82%)
30–39	44	39/44 (89%)	28/44 (64%)	28/39 (72%)
40–49	38	34/38 (89%)	26/38 (68%)	24/34 (71%)
50–59	39	34/39 (87%)	24/39 (62%)	23/34 (68%)
60–69	20	17/20 (85%)	13/20 (65%)	13/17 (68%)
≥70	11	10/11 (91%)	5/11 (45%)	5/10 (50%)
Total	199	169/199 (85%)*	126/199 (63%)†	120/169 (71%)‡
Cancer patients				
30–39	2	2/2 (100%)	2/2 (100%)	2/2 (100%)
40–49	4	4/4 (100%)	4/4 (100%)	4/4 (100%)
50–59	6	5/6 (83%)	6/6 (100%)	5/5 (100%)
60–69	13	11/13 (85%)	10/13 (77%)	10/11 (91%)
70–79	20	19/20 (95%)	17/20 (85%)	16/19 (84%)
Total	45	41/45 (91%)**	39/45 (87%)††	37/41 (90%)‡‡

Statistical comparison: * and ** $\chi^2 = 0.71$; $p = 0.39$; † and †† $\chi^2 = 8.1$; $p = 0.004$; ‡ and ‡‡ $\chi^2 = 5.49$; $p = 0.019$.

However, if only subjects aged 50 years and older from the population and GC patients were taken into account, a significant difference in the prevalence of anti-CagA IgG was revealed between the cancer group and the population sample defined as HP positive (31/35 and 41/62, respectively; $p = 0.02$; OR = 3.96; 95% CI 1.24–12.5).

Anti-CagA positivity in GC patients' sera with adenocarcinoma in the antrum region occurred in 11/14 cases (OR = 0.39; 95% CI 0.08–1.87), in the corpus in 14/17 (OR = 0.56; 95% CI 0.11–2.67) and in the antrum and corpus in 14/14 (OR = 3.36; 95% CI 0.39–28.5) cases (no significant difference; $p > 0.05$). We did not observe a significant difference between the histological type of cancer and anti-CagA positivity. Anti-CagA IgG were found in sera from patients with intestinal adenocarcinoma in 16/18 (OR = 1.39; 95% CI 0.27–6.96) cases, in the diffuse type in 9/9 (OR = 1.80; 95% CI 0.19–16.9) cases and in the indeterminate type in 10/14 (OR = 0.17; 95% CI 0.03–0.86) cases ($p > 0.05$). All four inoperable cases with indeterminate type of carcinoma were anti-Cag A positive.

A comparison of the occurrence of IgG antibodies to a recombinant fragment of CagA antigen, evaluated by ELISA, and to CagA protein in acid glycine extracted cell-surface proteins of HP, evaluated by using immunoblot, both of which were performed on 141 sera, is presented in Table 2. Anti-CagA positivity in ELISA was found in 79/141 (56%) cases and the CagA protein band was recognized in immunoblot in 90/141 (64%) cases. Disagreement of results occurred in 28/141 cases (20%). Nineteen ELISA-negative patients were positive in immunoblot and nine CagA ELISA-positive patients were negative in immunoblot. ELISA and immunoblot showed an agreement of results in 113/141 (80%) cases.

Discussion

In the present study we compared the prevalence of anti-CagA antibodies in an adult random population sample with a high prevalence of HP antibodies and in GC patients using a recombinant fragment of the CagA antigen by ELISA.

The prevalence of anti-CagA antibodies in a random adult population in Estonia was 63% and anti-CagA positivity among HP-infected persons was 71%, which is significantly higher ($\chi^2 = 61.3$, $p = 0.000$) than the average CagA seropositivity (49%) of 17 populations evaluated by the Eurogast Study Group [29]. Anti-CagA positivity in our GC group was significantly higher than in the population. This result confirms earlier data regarding the higher percentage of serological recognition of the cytotoxin associated 128 kDa HP protein in GC patients when compared with control subjects [6, 12, 13, 30]. Our data on seropositivity to glycine-extracted cell surface proteins of HP in GC patients and in the random population sample showed no significant difference. This finding is in accordance with a recent study by Klaamas *et al.* [31] which shows that overall HP seropositivity for blood donors and for GC patients did not differ significantly in Estonia.

Using reported data on the increasing prevalence of HP antibodies with age [20], we discussed the possible influence of age on anti-CagA prevalence in the group of elderly GC patients. A significant difference in the prevalence of anti-CagA IgG between the cancer group and controls was found when only subjects aged 50 and older in the population sample were taken into account. Based on the data of Crabtree *et al.* [12] that 120 kDa protein recognition did not vary with age in GC patients and on the recent data of the Eurogast Study Group concerning similar CagA positivity for age groups 25–34 and 55–64 years in 17 studied populations [29], we considered that the higher prevalence of CagA antibodies in GC patients was not influenced by the age difference between the GC group and the population as a whole. This is supported also by a significant association (OR = 3.96; 95% CI 1.24–12.5) between CagA positivity and GC in HP-positive persons aged 50 and older.

It is notable that 20–29-year-old persons in the Estonian population seem to have the highest prevalence of CagA antibodies and that CagA positivity among HP-positive persons is the highest also in this age group. In our previous random population study [18], the first statistically significant increase in antibodies to glycine-extracted

Table 2 Comparison of IgG antibody response to a recombinant fragment of CagA protein (ELISA) and to CagA protein of acid glycine-extracted cell surface protein of HP (immunoblot)

Sera studied	*n*	CagA positivity in ELISA	CagA protein band recognition in immunoblot	Agreement of results (both positive, or both negative)	Disagreement of results
Karksi-Nuia Population sample	52	17/52 (35%)	25/52 (48%)	35/52	17/52
Gastric cancer	45	39/45 (87%)	44/45 (98%)	40/45	5/45
Peptic ulcer	9	9/9 (100%)	8/9 (89%)	8/9	1/9
Children HP–	25	5/25 (20%)	4/25 (16%)	22/25	3/25
Children HP+	10	9/10 (90%)	9/10 (90%)	8/10	2/10
Total	141	79/141 (56%)	90/141 (64%)	113/141 (80%)	28/141 (20%)

cell surface proteins of HP (83%) was demonstrated in the 20–29 year age group. Also, according to another Estonian population study carried out in Kuressaare [19], the morphological evaluation of HP infection showed the first significant increase in HP positivity (83%) in the age group 20–29 years. Taking into account the evidence that CagA-positive strains nearly doubled the risk of developing GC over the ensuing 21 years [13], we suggest that young people (aged 20–29 years) with a high prevalence of CagA antibodies may be infected with a CagA-positive strain and therefore may have a higher risk of developing GC later in life.

A comparison of the evaluation of antibodies to the CagA protein in sera using both ELISA and immunoblot showed that the results of these tests agreed in 80% of cases. This is comparable to the results of the study by Xiang *et al.* [25], where the agreement between the results in ELISA (using a recombinant fragment of CagA) and immunoblot (using total HP extract as an antigen) was 85% for positive cases. The cause of the 20% disagreement between the results in ELISA and immunoblot in our study may be due to the fact that in our CagA ELISA we used a recombinant CagA fragment (37.5 kDa fused protein) as an antigen [25], whereas in immunoblot glycine-extracted cell surface protein was employed. Therefore, it is not precluded that some sera containing antibodies to the fragment of CagA protein may not have reacted with the entire 128 kDa protein owing to configuration differences between these two antigenic targets and, vice versa, epitopes in the recombinant fragment of the CagA protein were not recognized by some sera reacting with the entire 128 kDa protein. It is possible that in immunoblot sera react with CagA proteins through a different spectrum of epitopes compared with that in ELISA, which renders immunoblot more sensitive [32].

The overall good agreement between the results in the anti-CagA-ELISA and immunoblot in our study indicates that the use of a recombinant fragment of CagA protein in ELISA might prove a useful tool for the evaluation of CagA status, especially in epidemiological studies.

Conclusion

This study has shown that HP seropositivity was similarly high in an Estonian adult random population sample and in GC patients, whereas the prevalence of anti-CagA IgG was significantly higher in GC patients than in the random population sample studied (87% versus 63%; OR = 3.76; 95% CI 1.59–8.91). Persons aged 20–29 years in the population with the highest prevalence of anti-CagA IgG should be given further attention with respect to the development of cancer later in life. A comparison of the evaluation of IgG antibodies to CagA ELISA and immunoblot showed good agreement of results.

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IV

Vorobjova T, Grünberg H, Oona M,
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Seropositivity to *Helicobacter pylori* and CagA protein in schoolchildren
of different age living in urban and rural areas in southern Estonia.
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Seropositivity to *Helicobacter pylori* and CagA protein in schoolchildren of different ages living in urban and rural areas in southern Estonia

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Objective To evaluate *Helicobacter pylori* and CagA seropositivity in a non-selected group of schoolchildren in southern Estonia, with reference to previous studies where high seroprevalence to *H. pylori* (87%) and anti-CagA positivity (63%) in an adult population from the same region were found.

Study population A total of 421 schoolchildren selected haphazardly from a random population ($n = 1018$, ages 9, 12 or 15 years) and living in urban or rural areas.

Methods *H. pylori* status was determined by evaluation of IgG antibodies against cell surface proteins of *H. pylori*, strain CCUG 17874, using standard ELISA. Anti-CagA IgGs were determined by ELISA using a recombinant fragment of CagA (CCUG 17874) as solid-phase antigen. Absorbance values > 0.3 (405 nm) were taken as a CagA-positive result based on a study of 25 sera from *H. pylori*-negative children.

Results Of the 421 subjects, 235 (56%) were *H. pylori*-ELISA positive, and 109 out of the 235 (46%) were anti-CagA positive. Neither *H. pylori* nor CagA positivity were significantly different in girls and boys, or in children aged 9, 12 or 15 years. The *H. pylori* prevalence rate (118/181, 65%) as well as CagA positivity (64/181, 35%) in rural areas were higher compared with those in towns (117/240, 49% and 54/240, 22%, respectively; $P = 0.001$ and $P = 0.005$).

Conclusion Of schoolchildren living in southern Estonia, 56% were seropositive to *H. pylori*. Half of them had anti-CagA antibodies. Schoolchildren living in rural areas were infected significantly more often with CagA-seropositive strains compared with those living in towns. *Eur J Gastroenterol Hepatol* 12:97–101 © 2000 Lippincott Williams & Wilkins

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Introduction

There is ample evidence that *Helicobacter pylori* is the main cause of chronic gastritis and is involved in the development of gastric cancer [1–4]. Infection with *H. pylori* strains expressing cytotoxin-associated CagA protein complex is more virulent and is associated with increased risk of development of atrophic gastritis, peptic ulcer disease and gastric cancer [5–8]. Acquisition of the infection in childhood appears to be a risk factor for gastric cancer [9–12].

Considering the high prevalence of *H. pylori* (87%) and of anti-CagA seropositivity (63%) in the adult population from a region of southern Estonia in our previous studies [13,14], the aim of this study was to evaluate *H. pylori* and CagA protein seropositivity in a non-selected group of schoolchildren of different ages and places of residence in southern Estonia.

The study was presented in part at the XIth International Workshop on Gastroduodenal Pathology and *Helicobacter pylori*, Budapest, 2–5 September 1998 (Abstract A76) and at the World Congress of Gastroenterology, Vienna, Austria, 6–11 September 1998 (Abstract ExhB3302).

Methods

Subjects

A cross-sectional study of schoolchildren was carried out from Autumn 1993 to Spring 1996 in five southern Estonian counties. Twenty secondary schools were selected assuming that they were of similar size in each county. Within each school, children were selected randomly from the school roll, and every third 9-, 12- and 15-year-old child was afforded an opportunity to participate. The overall response rate of 78% yielded a total of 1074 children who agreed to participate in the study. However, 56 of these children were excluded for the following reasons: absence from school on the day of the study (16 children), chronic disease (diabetes, asthma, etc.; seven children), reluctance to undergo

blood sampling despite prior agreement (13 children), and inadequately completed questionnaires (20 children). The final group of 1018 children made up almost 10% of the children living in this region: 319 aged 9 (154 boys and 165 girls, age range 8.5–9.6 years), 343 aged 12 (160 boys and 183 girls, age range 11.5–12.6 years) and 356 aged 15 (155 boys and 201 girls, age range 14.5–15.6 years). The investigated children were all ethnic Estonians.

A further random selection was performed among the 1018 children to reduce the study size, resulting in a total of 421 children (231 girls, 190 boys) from whom serum samples were tested and analysed. The distribution of the studied 421 schoolchildren according to age, gender and place of residence is presented in Table 1.

The Committee of Ethics at the University of Tartu approved the study.

Procedures

The serum samples collected from the children were stored at −20°C until assayed.

H. pylori status was determined using a serological evaluation by ELISA of IgG antibodies to *H. pylori* (strain CCUG 17874) as reported previously [13,14]. Briefly, 0.5 μg of acid (pH 2.2) glycine-extracted cell-surface proteins per well was used for coating microtitre plates (NUNC, Roskilde, Denmark). The studied sera were diluted 1:800; the second antibody was alkaline phosphatase-labelled anti-human IgG (DAKO, Glostrup, Denmark) diluted 1:500. ELISA results were expressed as the corrected mean absorbance values as a percentage of the reference standard (human γ-globulin, Pharmacia & UpJohn, Stockholm, Sweden). The absorbance values were expressed as relative antibody activity (RAA) [15]. RAA = 25 was taken as the cut-off value for seropositivity, derived from the mean value of RAA units plus two standard deviations in 39 histologically *H. pylori*-negative children (median age 11) endoscoped because of abdominal complaints in Tartu Children's Hospital. Inter-assay variation was 13% and intra-assay variation was 5%.

Anti-CagA IgG were detected by ELISA using a recombinant fragment of the CagA antigen His-17/12 of *H. pylori* strain CCUG 17874 following the modified methodology of Xiang *et al.* [16]. The recombinant CagA antigen was diluted in 0.1 mol/l carbonate buffer with pH 9.6 to a final concentration of 1.25 μg/ml. Maxisorp Immunoplates (NUNC, Roskilde, Denmark) were coated with 100 μl/well in duplicate and incubated for 16 h at 4°C, then washed three times with phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Tween-20. The wells were saturated with 200 μl/well of 1% bovine serum albumin (BSA) in PBS–Tween at 37°C for 2 h. After washing the plates three times, 100 μl of each serum sample diluted to 1:300 in 1% BSA–PBS was added to the wells in triplicate (two wells with the antigen and one without the antigen) and incubated at 37°C for 2 h. The plates were then washed three times, and 100 μl/well of alkaline phosphatase-conjugated anti-human IgG (DAKO), diluted to 1:1000 in 1% BSA–PBS, was added, and the plates incubated at 37°C for 1.5 h. After three washes, 100 μl/well of a substrate solution containing *p*-nitrophenyl phosphate (Sigma Chemical Co., St Louis, Missouri, USA) in a diethanolamine–$MgCl_2$ buffer was added to each well and incubated at 37°C for 30 min. Each plate contained a positive and a negative control serum. Absorption was measured at 405 nm. The absorption value derived from the mean value of absorption for two wells with the antigen minus the absorption value for the well without the antigen was taken as the absorbance value of the sample. Rather than the cut-off of 0.250 OD used by Xiang *et al.* [16], the absorbance value 0.300 (405 nm), based on the calculation derived from the mean absorbance value of the serum samples of 25 histologically and serologically *H. pylori*-negative children (median age 12) plus two standard deviations was taken as the cut-off point in this study, employing square root transformation method [17]. Inter-assay variation was 8%.

Statistical analysis

Differences in *H. pylori* status and in the prevalence of antibodies to CagA between various groups were tested by the χ^2 test. Odds ratios (OR) with 95% confidence intervals (CI) were estimated using Mantel–Haenszel statistics with the Exact software (Exact v.2.0 © David D. Martin, 1988–1994) to examine the magnitude of

Table 1 Distribution of the studied schoolchildren ($n = 421$) according to age, gender and place of residence

	Gender		Place of residence			
Age	Girls	Boys	Town	Village	Farm	Total
9 years	47 (50%)	47 (50%)	39 (42%)	35 (37%)	20 (21%)	94 (22%)
12 years	78 (53%)	69 (47%)	78 (53%)	51 (35%)	18 (12%)	147 (35%)
15 years	106 (59%)	74 (41%)	123 (69%)	33 (18%)	24 (13%)	180 (43%)
Total	231 (55%)	190 (45%)	240 (57%)	119 (28%)	62 (15%)	421 (100%)

the association. Effects were considered to be statistically significant if the 95% CI did not include the value 1.0.

Results

The prevalence of IgG antibodies to acid glycine-extracted cell-surface *H. pylori* proteins in the studied schoolchildren was 56% (235/421). Anti-CagA IgG were found in 28% (118/421). Of the 235 *H. pylori*-positive schoolchildren, 109 were CagA-positive (46%). Nine children negative to the glycine-extracted cell-surface protein in the *H. pylori* ELISA were positive in the CagA ELISA. The *H. pylori* and CagA seropositivity in ELISA in schoolchildren of different ages and places of residence in southern Estonia is presented in Table 2.

There was no significant difference in *H. pylori* and CagA positivity between girls and boys. Schoolchildren aged 9, 12 and 15 years revealed no significant difference in *H. pylori* status and CagA positivity either. A slight tendency to an increase in *H. pylori* and CagA positivity was observed in 12- and 15-year-old children compared with 9-year-old children ($P > 0.05$).

Schoolchildren living in rural areas (including both villages and farms) had a significantly higher *H. pylori* prevalence rate (118/181; 65%) as well as CagA positivity (64/181; 35%) compared with those living in towns (117/240 (49%) and 54/240 (22%), respectively) ($P = 0.001$ for *H. pylori* prevalence rate and $P = 0.005$ for CagA prevalence rate). The odds ratio (OR) for the association between *H. pylori* infection and the place of residence in rural areas was 1.96 (95% CI 1.32–2.92). We also found a significant association between CagA positivity and the place of residence in rural areas: OR = 1.88 (95% CI 1.22–2.88).

Discussion

In the present study, we determined *H. pylori* and CagA seropositivity in a non-selected group of schoolchildren aged 9, 12 and 15 years, who are living in neighbouring urban and rural areas in southern Estonia, using cell-surface proteins of *H. pylori* and the recombinant fragment of the CagA antigen in ELISAs.

The seroprevalence of *H. pylori* was 56%, which did not differ significantly from the histological finding of *H. pylori* (58%) among Estonian children with abdominal complaints, obtained in a previous study [18]. According to literature data, the percentage of *H. pylori* infection in children, aged 9–15 years, without abdominal complaints varies from 3% in Sweden [19], 8.3–16.7% in Germany [20], 11–23% in the Netherlands [21] and 16.7% in the UK [22] to 26% in Japan [23], 36.8–68.4% in Turkey [24] and 34–52% in China [11]. Thus the seroprevalence of *H. pylori* in schoolchildren according to our study is higher than it is in most European countries.

The specific finding of the present study was that half of the *H. pylori*-positive schoolchildren living in southern Estonia have anti-CagA antibodies. These children should be given further attention with respect to the development of gastritis, peptic ulcer or gastric cancer later in life, taking into account the association between infection with a cytotoxic strain of *H. pylori* and development of the above-mentioned diseases [5–8,25].

Literature data regarding CagA positivity in *H. pylori*-infected children (most studies being concerned with children with recurrent abdominal pain) reveal the presence of infection with cytotoxic strains in 28.5–80.6% of cases when using different methods for the investigation of CagA positivity such as ELISA, PCR and Western blot [26–34]. CagA positivity among asymptomatic children was 64% as evaluated by Elitsur and Neace [32] and 82.6% as evaluated by Karczewska *et al.* [33], which is higher than the prevalence rate found in our study. This might be explained by the use of other methods for the detection of CagA positivity in their studies, such as Western blot or another CagA antigen in ELISA.

We compared *H. pylori* as well as CagA prevalence in

Table 2 ***H. pylori*** **and CagA seropositivity in schoolchildren of different ages and in different places of residence in southern Estonia evaluated by ELISA**

	Gender		Age (years)			Place of residence			
	Girls	Boys	9	12	15	Town	Village	Farm	Total
Number of persons	231	190	94	147	180	240	119	62	421
H. pylori-positive	127 (55%)	108 (57%)	46 (49%)	81 (55%)	108 (60%)	117* (49%)	79 (66%)	39 (63%)	235 (56%; 95%CI 51.3–60.7)
CagA-positive	71 (31%)	47 (25%)	20 (21%)	40 (27%)	58 (32%)	54[†] (23%)	42 (35%)	22 (35%)	118[‡] (28%; 95% CI 23.8–32.3)
CagA-positive in *H. pylori*-positive sera	65 (51%)	44 (41%)	19 (41%)	39 (48%)	51 (47%)	47 (40%)	40 (51%)	22 (56%)	109 (46%; 95% CI 39.8–52.2)

* $P = 0.01$ compared with village residents, $P = 0.04$ compared with farm residents. [†] $P = 0.009$ compared with village residents, $P = 0.03$ compared with farm residents.
[‡]Nine sera were negative to cell-surface proteins in ELISA but positive in CagA ELISA.

healthy schoolchildren living in the urban and rural areas of the same geographical region. It was found that children living in rural areas (in villages and farms) were more often infected with *H. pylori* and with cytotoxic strains. In a study by Mitchell *et al.* [35], a significantly higher prevalence of *H. pylori* was found in a city in southern China compared with that in rural areas. However, this difference was found in children aged 0–5 years. In the study by Elitsur and Neace [32], the prevalence of CagA-positive *H. pylori* strains in asymptomatic children of West Virginia was 64%, and the location of the community (urban/rural) did not influence significantly the distribution of CagA-positive strains. Thus, literature data do not substantiate the tendency observed in the present study.

The fact that children living in rural areas had a higher seroprevalence of *H. pylori* and anti-CagA antibodies might hypothetically be explained by the use of different water sources (i.e. external ones) in farms compared with urban households. Klein *et al.* [36] showed that children living in homes with external water sources were three times more likely to be infected with *H. pylori* compared to households with internal water sources.

Comparison of *H. pylori* and CagA prevalence in the age groups of 9, 12 and 15 years showed only a tendency to increase in positivity with age in our study. The literature indicates that a major proportion of *H. pylori* infection is acquired in early childhood [9,35–40]. Based on a three-year follow-up of Estonian children from birth, Lindkvist *et al.* [41] found very high seroconversion rates during the children's first years of life: first year 27%, second year 25%, third year 12%. The sharpest rise of antibodies to *H. pylori* in Sweden appears between 9 and 10 years of age (20%) [42]. In a study by Graham *et al.* [9], it was shown that the prevalence of *H. pylori* infection was stable during a variable period in late childhood but increased in early adulthood. According to Sipponen *et al.* [43,44], *H. pylori* gastritis is acquired in childhood and adolescence (age less than 20) in more than 50% of cases. In the present study, half of the schoolchildren in the first decade of life were infected with *H. pylori* and half of these possessed cytotoxin-producing strains.

Taking into account the results of our previous study [14] on *H. pylori* and CagA prevalence in a random sample of adults from a region of southern Estonia, it is evident that children are less infected with cytotoxic strains compared with total prevalence in the random sample of adults (46% versus 71%; $P = 0.000008$). However, our previous study [14] showed that 59% of young people aged 15–19 years were infected with *H. pylori*, 69% of them being CagA-positive, which is not significantly higher compared with the values for 15-year-old schoolchildren in the present study ($P = 0.9$ for *H. pylori* status; $P = 0.22$ for CagA status).

According to the data of our previous studies, the first significant increase in *H. pylori* positivity in adults was shown to occur at the age of 20–29 years (88% in a Karksi-Nuia population and 83% in a Kuressaare population study) [13,45]. CagA positivity among *H. pylori* positive persons was also the highest (82%) in this age group [14]. It is possible that young people in Estonia achieve a high seroprevalence of *H. pylori*, including infection with cytotoxic strains, during the third decade of life. However, this fact needs to be confirmed during a prospective study of *H. pylori* incidence in the group of the studied schoolchildren. The cohort effect also should be taken into account [44]. In an extensive seroepidemiological study of *H. pylori* infection in various populations, Mégraud *et al.* [46] showed that in France only 3.5% of children were infected in the first decade of life. In the second decade, *H. pylori* prevalence increased to 16.3%, and to 24.8% in the third decade, which is however a markedly lower percentage compared with our results.

Conclusion

This study showed that 56% of schoolchildren in southern Estonia had *H. pylori* infection and 46% of these were anti-CagA-positive. Neither *H. pylori* nor CagA positivity was significantly different in girls and boys, or in children aged 9, 12 or 15 years. The *H. pylori* prevalence rate and CagA positivity were significantly higher in children living in villages and farms compared with those living in towns. Children with anti-CagA antibodies should be given further attention with respect to the development of atrophic gastritis, peptic ulcer or gastric cancer later in life.

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Helicobacter pylori (*H. pylori*) in Gastric Mucosa of Children with Abdominal Complaints: Immunohistochemistry Detects Antigen-Reactive Corpus Mucosa Cells

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ABSTRACT

Background. Previous morphological and serological studies of gastric *Helicobacter pylori* (*H. pylori*) colonization among Estonian children with abdominal complaints, as well as among populations of schoolchildren, have shown a high prevalence of *H. pylori* (49–60%). Based on published data concerning the high specificity and sensitivity of immunohistochemical detection of *H. pylori*, we examined the prevalence of *H. pylori* in gastric biopsy specimens of Estonian children by different localization and morphological type of gastritis comparing Giemsa staining with immunohistochemistry.

Material and Methods. Formalin-fixed biopsies (107 antral, 108 corpus mucosa) of 112 children (41 boys, 71 girls, age range 1–16 years, median age 12 years) with abdominal complaints were stained with hematoxylin & eosin and Giemsa stains, as well as examined using the peroxidase antiperoxidase (PAP) method with polyclonal antibodies to *H. pylori*.

Results. Gastritis of any degree and localization was found in 84/112 (75%) children. Using Giemsa staining *H. pylori* were detected in 83/112 (74%) of all children, and by use of the PAP method in 55/112 (49%) ($p = .001$). Concordance of the results of immunohistochemical and Giemsa methods in antrum biopsies was 70%, in corpus biopsies 73%. In 12 out of 108 (11%) corpus mucosa specimens a positive staining with anti-*H. pylori* IgG was localized in the cytoplasma of corpus mucosal cells in the neck part of the glands.

Conclusions. The prevalence of *H. pylori* was higher when employing the Giemsa stain in comparison with immunohistochemistry. Antibody reactivity of cells in the neck part of the corpus glands may either be due to cross-reactivity of anti-*H. pylori* IgG with epithelial cell epitopes, or to internalization of *H. pylori* by these cells, suggesting a pathogenic role of neck cells in an anti-*H. pylori* immune response.

The prevalence of *Helicobacter pylori* (*H. pylori*) infection in Estonian children is high. Over 50% of children are affected based on either histological [1] or serological [2] methods.

Several studies have reported on immunohistochemical methods using polyclonal or monoclonal antibodies to *H. pylori* for identification of this agent in biopsy specimens and documented their high specificity and sensitivity [3–9]. Immunohistochemistry allows the detection of even single bacteria and to identify the coccoid form of *H. pylori* [5,9]. However, Negrini et al. [10–12] demonstrated that some of the monoclonal antibodies against *H. pylori* cross-react with epithelial cells of the human and murine gastric mucosa; this and other groups therefore suggested a possible role of *H. pylori* to induce autoantibodies involved in the progression of chronic gastritis [12–16].

The aim of the present study was to assess the prevalence of *H. pylori* in gastric biopsies of children as a function of gastritis type and of gastric anatomical site. In addition, we compared the efficacy of *H. pylori* detection based on Giemsa staining with that of immunohistochemistry. Thirdly, we examined the reactivity of polyclonal antibodies to *H. pylori* in the cytoplasm of antral and corpus epithelial cells.

Subjects, Materials and Methods

One hundred twelve children (41 boys and 71 girls; age range 1–16 years, median age 12 years) with abdominal complaints defined according to Apley's criteria [17] and endoscoped in Tartu Children's Hospital, were studied.

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Two biopsy specimens each from antral and corpus mucosa were taken. In 4 children only biopsy specimens from antrum and in 5 children only specimens from corpus mucosa were examined due to inadequate size of specimens. Formalin-fixed, paraffin-embedded biopsy specimens (107 from the antral and 108 from the corpus mucosa) were stained with hematoxylin and eosin for assessing type and grade of gastritis. Parallel sections were either stained with Giemsa or processed for immunohistochemistry (see below).

Gastric mucosa was graded as normal or as showing superficial or atrophic gastritis [18]. The severity of *H. pylori* colonization in Giemsa-stained sections was graded as follows: 0 (absence of bacteria); 1 (<20 per field); 2 (20–60 per field); 3 (>60 per field) [18]. Four to six fields were examined per specimen (×40 objective, ×10 eyepiece) by one investigator (H.-I. M.). The section was regarded as *H. pylori* positive by detection of *H. pylori* colonization of grade 1 or more.

Immunohistochemical Detection of H. pylori

Formalin-fixed, paraffin-embedded biopsy specimens were studied using the peroxidase-antiperoxidase (PAP) method. After deparaffinization, tissue sections 5 μm thick were pretreated with 0.1% trypsin (Spofa, Praha, Czech Republic) during 15 min at 37°C and with hydrogen peroxide in methanol (30 min at 20°C) to prevent nonspecific reactions. As primary antibodies rabbit anti-*H. pylori* polyclonal antibodies (anti-*H. pylori* IgG; DAKO, Glostrup, Denmark) were used (dilution: 1:50; incubation overnight at 4°C). Sections from the same biopsy specimens incubated without primary antibody served as negative controls. As secondary antibody swine-anti rabbit IgG (DAKO) was used (dilution: 1:100; incubation 1 hour at 20°C), followed by incubation with peroxidase antiperoxidase rabbit complex (DAKO; dilution: 1:500; incubation 1 hour at 20°C). Binding of specific antibodies was visualized using 3,3- diaminobenzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO, USA). Sections were counterstained with hematoxylin and examined by one investigator (T.V.) without knowing the results of gastritis grading and the results of *H. pylori* positivity, based on Giemsa preparations. The entire surface of all sections present on the slide was examined. The staining was considered positive if brown-stained organisms of typical shape were seen on the luminal surface of corpus or antrum glands. Additionally, we assessed immunostaining of antral or corpus epithelial cells with anti-*H. pylori* IgG.

Statistical analyses were performed using the χ^2 test. The study was approved by the Committee of Ethics at the University of Tartu, Estonia.

Results

Gastritis of any degree and localization was found in 84/112 (75%) children with abdominal complaints. Both antrum and corpus mucosa were available in 103 children. The most frequent topographical and morphological type of gastritis was superficial gastritis localized simultaneously in antrum and corpus mucosa (47/103 or 45.6% cases studied). Superficial gastritis restricted to antrum was seen in 30/103 (29%) cases, and superficial gastritis restricted to corpus in 3/103 (3%) cases. The prevalence of different states of gastric mucosa and of *H. pylori* positivity as detected using the two different methods are presented in Table 1.

H. pylori was detected by Giemsa staining in 83/112 (74%) of children. Immunohistochemical assessment of *H. pylori* using anti-*H. pylori* IgG resulted in a significantly lower *H. pylori* yield (55/112 or 49%; $p = .001$). Children with superficial gastritis, localized simultaneously in antrum and corpus, were *H. pylori*-positive in 41/47 (87%) cases according Giemsa staining and in 31/47 (66%)

Table 1 *H. pylori* positivity found by Giemsa and immunohistochemical staining in children with topographically and morphologically different types of gastritis in antrum or/and in corpus mucosa

Topography of mucosa	Morphological type of mucosal change	No. of persons studied	*H. pylori* positivity using Giemsa staining	*H. pylori* positivity using anti-*H. pylori* IgG
Antrum	normal			
Corpus	normal	21	5/21	2/21
Antrum	superficial gastritis			
Corpus	normal	30	30/30*	19/30**
Antrum	normal			
Corpus	superficial gastritis	3	2/3	1/3
Antrum	superficial gastritis			
Corpus	superficial gastritis	47	41/47†	31/47††
Antrum	atrophic gastritis			
Corpus	normal	2	1/2	0/2
Antrum	normal			
Corpus	—	3	1/3	1/3
Antrum	superficial gastritis			
Corpus	—	1	1/1	0/1
Antrum	—			
Corpus	normal	4	1/4	0/4
Antrum	—			
Corpus	superficial gastritis	1	1/1	1/1
Total		112	83/112 (74%)‡	55/112 (49%)‡‡

*> **$p = .008$; † > ††$p = .01$; ‡ > ‡‡$p = .001$

Table 2 Comparison of detection of *H. pylori* using Giemsa staining and anti-*H. pylori* polyclonal antibodies in antrum and corpus biopsy specimens in different morphological types of gastric mucosa

Topography of mucosa	No. of biopsy specimens studied	Staining	Morphological type of gastric mucosa: Normal mucosa *H. pylori*+	Morphological type of gastric mucosa: Superficial gastritis *H. pylori*+	Morphological type of gastric mucosa: Atrophic gastritis *H. pylori*+	Total *H. pylori*+ in biopsy specimens
Antrum	107	Giemsa	8/27 (30%)	68/78 (87%)*	1/2	77/107 (72%)‡
	107	anti-*H. pylori* IgG	4/27 (15%)	46/78 (59%)**	0/2	50/107 (47%)‡‡
Corpus	108	Giemsa	16/57 (28%)	37/51 (73%)†	0/0	53/108 (49%)§
	108	anti-*H. pylori* IgG	13/57 (23%)	24/51 (47%)††	0/0	37/108 (34%)§§

* > **p = .0007; † > ††p = .008; ‡ > ‡‡p = .001; § > §§p = .02

cases using anti-*H. pylori* IgG (p = .01). Children with superficial gastritis restricted to the antrum were all *H. pylori*-positive, based on Giemsa staining, whereas immunohistochemically only 19/30 (63%) were *H. pylori*-positive (p = .008).

Comparison of positive staining for *H. pylori* using the Giemsa method and anti-*H. pylori* IgG in antrum and corpus biopsy specimens in different morphological types of gastric mucosal change is presented in Table 2. In antrum and in corpus biopsies detection of *H. pylori* by use of Giemsa staining sections was superior to immunohistochemistry (77/107 or 72% vs. 50/107 or 47% in antrum; 53/108 or 49% vs. 37/108 or 34% in corpus specimens; p = .001 and p = .02, respectively). *H. pylori* were found more often in antrum (77/107; 72%) than in corpus (53/108; 49%) biopsies (p = .005) using Giemsa staining. Immunohistochemical evaluation resulted in a tendency for a higher prevalence of *H. pylori* in antrum than in corpus (50/107; 47% vs. 37/108; 34%; p = .06).

When comparing the results of *H. pylori* evaluation using the two staining methods separately on antrum and on corpus biopsies of each patient, the concordance of *H. pylori* positivity in antrum mucosa was 45%, of *H. pylori* negativity 25%, whereas in corpus the concordance of *H. pylori* positivity was 34%, of *H. pylori* negativity 39%. (Table 3).

Among the 30 Giemsa-positive, immunostain-negative antrum samples, the bacteria grade as based on Giemsa-stained sections was as follows: grade 1 in 16 cases, grade 2 in 7 cases, and grade 3 in 7 cases. Among corpus biopsies, 19 cases were Giemsa-positive and immunostain-negative, and the respective bacteria grades as based on Giemsa-stained sections were grade 1 in 15 cases, grade 2 in 1 case, grade 3 in 3 cases. Thus, in antrum 16/30 (53%) and in corpus 15/19 (79%) of discrepant cases belong to the low Giemsa grade. Among antrum biopsies, 2 were Giemsa-negative, immunostain-positive.

In 12 out of 108 (11%) corpus mucosa specimens a positive staining with anti-*H. pylori* IgG was localized in the cytoplasma of corpus mucosal cells in the neck part of the glands. This staining was diffuse and affected mainly apical part of the cells. No *H. pylori* bacilli have been identified in cytoplasma of mucus neck cells. All negative controls sections (incubated without primary antibodies) were negative for such a staining (Figs. 1 and 2). In 8 out of these 12 cases corpus mucosa was normal, whereas in 4 out of 12 cases superficial gastritis was found, and in 2 out of these 12 cases *H. pylori* were detected in Giemsa-stained sections.

Discussion

In the present retrospective study we compared the detection yield of *H. pylori* in antrum and corpus mucosal biopsies of 112 children using two methods, Giemsa staining and immunohistochemistry. It turned out that *H. pylori* were more frequently found in Giemsa preparations than by immunohistochemistry employing a polyclonal antibody directed against *H. pylori*. The concordance of these two methods in detection of *H. pylori* in antrum biopsies was 70%, and in corpus biopsy sections concordance was 73%.

Table 3 Concordance in detection of *H. pylori* (HP) using Giemsa staining and anti-*H. pylori* IgG as compared separately in antrum and corpus biopsy specimens in the same person

Biopsy specimens	Concordance	No. of cases in concordance
Antrum biopsy specimen	in *H. pylori* positivity	48/107 (45%)
	in *H. pylori* negativity	27/107 (25%)
Total	concordance	75/107 (70%)
Corpus biopsy specimen	in *H. pylori* positivity	37/108 (34%)
	in *H. pylori* negativity	42/108 (39%)
Total	concordance	79/108 (73%)

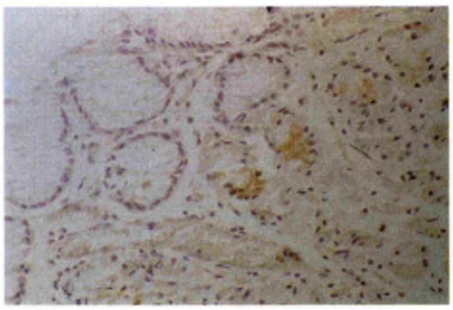

Figure 1 A positive staining with anti-*H. pylori* polyclonal DAKO antibodies localized in the cytoplasma of corpus mucosa cells in the neck part of the glands, affected mainly apical part of the cells. (PAP method, ×20 objective, ×10 eyepiece).

The observation that the most frequent morphological and topographical type of gastritis in Estonian children with abdominal complaints is superficial gastritis simultaneously affecting antrum and corpus, supports the findings of a previous investigation [1]. This type of gastritis was associated with *H. pylori*-positivity in our and the study of Maaroos [1] in 87% and 84%, respectively, however, based on Giemsa-stained sections.

Overall *H. pylori*-positivity in children with abdominal complaints detected morphologically using the Giemsa method was 74% and using anti-*H. pylori* IgG 49%, which is higher than in most developed countries [19–24]. Our study of seroprevalence of *H. pylori* in an Estonian population of schoolchildren [2] showed that children aged 9, 12 and 15 years were also frequently infected with *H. pylori* (in 49–60%).

The fact that in our study *H. pylori* was identified in 30% of cases with normal antrum and in 28% of cases with normal corpus using Giemsa staining hypothetically may be explained by the nonstable adherence capability of bacteria in these children, knowing that the adherence to mucosa is an important virulence factor in development of gastritis [25,26]. Recent study of children and adults with chronic gastritis by Blom et al. [27] showed that there exist different patterns of *H. pylori* adherence to gastric mucosa cells. Additionally, this situation could be explained by the fact that these children have been recently infected and their mucosa is still normal, but they can be predisposed to develop gastritis later in life. The grade of colonization in these cases in our study was predominantly low. The grade of colonization of normal antrum mucosa was as follows: grade 1 in 5 cases, grade 2 in 2 cases, and grade 3 in 1 case of 8 normal biopsies with identified organisms. In normal corpus mucosa grade 1 was found in 9, grade 2 in 5 (Fig. 3) and grade 3 in 2 cases of 16. It is known that the degree of gastric mucosal inflammation and severity of epithelial damage is positively correlated to the degree of colonization of the mucosa by *H. pylori* [28,29].

The evidence of normal-looking mucosa with identified organisms may be also due to the presence of *H. pylori* strains that cause less inflammatory response, e.g., strains not expressing the CagA and VacA proteins. There are literature data that the mucosal responses to different strains is variable and that strains expressing the CagA and VacA proteins are more pathogenic [30,31]. How-

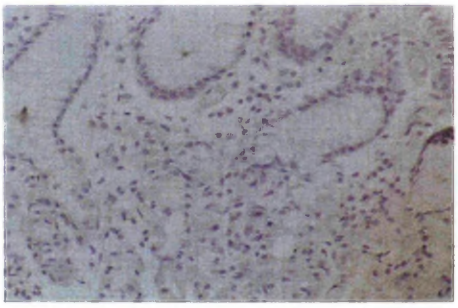

Figure 2 Negative staining of the corpus mucosa section incubated without anti-*H. pylori* polyclonal antibodies (PAP method, ×20 objective, ×10 eyepiece).

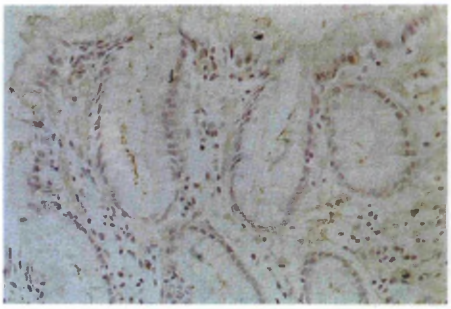

Figure 3 Normal corpus mucosa with presence of *H. pylori*, stained with anti-*H. pylori*.

ever, the data concerning CagA status in studied children were not available.

The fact that using polyclonal antibodies and the PAP method for the assessment of *H. pylori* resulted in an *H. pylori* yield inferior to that of Giemsa staining is not in line with data of other authors, who showed that immunohistochemistry is the most sensitive and specific staining method for the evaluation of *H. pylori* in biopsies as compared with conventional staining [5,9,32,33]. One possible reason for this disagreement includes the hypothesis that *H. pylori* strains colonizing the gastric mucosa of the children analyzed might contain different antigenic components than strains of *H. pylori* against which the polyclonal antibodies of this study have been raised. Andersen et al. [5] pointed out this possibility based on some disagreement between recognition of *H. pylori* in hematoxylin-eosin—and peroxidase-stained sections. A second reason why some of Giemsa-positive cases were immunostain-negative could be the fact that most of the Giemsa-positive cases belong to the low grade of *H. pylori* density. In some of these cases we cannot exclude the possibility that the immunohistochemical stain may be negative due to the different level of the section which was stained [33]. Giemsa staining detects both, intact and damaged or non-vital *H. pylori*, whereas immunohistochemical reactivity may depend on the presence of fully intact organisms harboring a still complete set of respective antigens.

An additional aspect of differential epitope reactivity of the antibody used in the present study is evident by the staining of subsets of gastric epithelial cells. Staining of corpus gland neck cells by anti-*H. pylori* antibodies were found in 11% of biopsies, suggesting cross-reactivity of anti-*H. pylori* antibodies with gastric epithelial epitopes, as previously proposed by Negrini et al. [11–13]. The reaction may be related to autoimmune mechanisms playing a pathogenic role of *H. pylori* in the progression of chronic gastritis [12,13]. This hypothesis is in part supported by our previous study that long term *H. pylori* infection may be a predictor for the development of gastric autoimmunity [14]. It is, however, notable that in 8 out of 12 cases with intracytoplasmic staining of corpus mucosal cells, corpus mucosa was otherwise normal, i.e., without gastritis. This observation does not preclude that these children with a still normal mucosa may be predisposed to develop autoimmune gastritis later in life. In this light, the finding of neck cell reactivity in a young age group is of particular significance. An alternative mechanism involved in staining of gastric gland neck cells by anti-*H. pylori* antibodies may be related to an epithelial invasion by *H. pylori* [7]. In fact, internalization of *H. pylori* by cells has been observed and discussed with respect to an inflammatory response [7,33–36]. The finding that *H. pylori* cannot be detected within cells by Giemsa staining may indicate intracellular bacterial breakdown. We regard it as promising to test whether gastric neck cells capable of internalizing *H. pylori* may express features of antigen-presenting cells operational in an immune response, as has been proposed for other epithelial cells. In case that immunoreactivity detected in mucus neck cells should in fact be due to the presence of *H. pylori*-related antigenic material instead of a so-far not further specified cross-reactivity, accumulation of *H. pylori* product in these cells would be a possible explanation. We are not aware that these cells are capable for the intake of bacterial components, but endocytosis of antigen, but not of particulate matter, may be one pathogenic mechanism, even though neck cell reactivity was also observed in *H. pylori*-negative cases and normal samples.

In conclusion, superficial gastritis, simultaneously affecting antrum and corpus, was the most frequent alteration (42%) in a subset of children with abdominal complaints. They were *H. pylori* positive in antrum and/or corpus in 87% cases according Giemsa-staining and in 66% using anti-*H. pylori* IgG.

Prevalence of *H. pylori* among Estonian children with abdominal complaints using Giemsa staining was higher (74%) in comparison with immunohistochemistry employing anti-*H. pylori* polyclonal antibodies (49%).

The concordance of *H. pylori* yields using these two staining methods by comparing histological sections from the same persons was 70% for antrum mucosa, and 73% for corpus mucosa sections.

The staining of epithelial cells in the neck part of corpus glands by use of anti-*H. pylori* IgG indicates a possible cross-reactivity of anti-*H. pylori* IgG with epithelial cell epitopes or the internalization of *H. pylori* by these cells.

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VI

Maaroos H-I, **Vorobjova T**, Sipponen P,
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An 18-year follow-up study of chronic gastritis and *Helicobacter pylori*:
association of CagA positivity with development of atrophy and activity of gastritis.
Scand J Gastroenterol 1999; 34: 864–869.

ORIGINAL ARTICLE

An 18-Year Follow-up Study of Chronic Gastritis and *Helicobacter pylori*: Association of CagA Positivity with Development of Atrophy and Activity of Gastritis

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Maaroos H-I, Vorobjova T, Sipponen P, Tammur R, Uibo R, Wadström T, Keevalik R, Villako K. An 18-year follow-up study of chronic gastritis and *Helicobacter pylori*: association of CagA positivity with development of atrophy and activity of gastritis. Scand J Gastroenterol 1999;34:864–869.

Background: We wanted to evaluate the course of chronic gastritis and its association with *Helicobacter pylori* and CagA seropositivity in an adult sample from Saaremaa (Estonia) during an 18-year follow-up. **Methods:** Seventy persons (31 men, 39 women; median age, 57.5 years) from a primary sample of 304 subjects endoscoped in 1979 were reinvestigated by endoscopy and biopsy in 1997. The state of the gastric mucosa and the presence of *H. pylori* in histologic sections from the antrum and corpus were assessed both in 1979 and 1997 in 66 subjects in accordance with the Sydney system, and *H. pylori* status in all 70 subjects was determined with the enzyme-linked immunosorbent assay (ELISA). Anti-CagA IgGs were determined with the ELISA, using the recombinant fragment of CagA. **Results.** During an 18-year follow-up 11% of the subjects developed atrophy in the antrum, whereas 35% developed it in the corpus. Development of atrophy in the corpus and the appearance of intestinal metaplasia in the antrum were associated with increased activity of gastritis both in the initial and last follow-up biopsies. Anti-CagA positivity was found in 71% of *H. pylori*-seropositive persons (94% of subjects). There was a significant association between CagA positivity and the activity of gastritis, the presence of atrophy or damage to surface epithelial cells in the antrum and in corpus mucosal biopsy specimens at the last follow-up endoscopy. **Conclusion:** The CagA-positive strains of *H. pylori* enhance the development of atrophic gastritis compared with CagA-negative strains.

Key words: Activity of gastritis; atrophy; CagA; follow-up of gastritis; *Helicobacter pylori*

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Long-term follow-up studies have shown that gastritis is a dynamic process. The main tendency is progression of gastritis with time from a non-atrophic to an atrophic form, although there are data indicating some regression of gastritis (1–6). There is ample evidence that infection with *Helicobacter pylori*, and especially with the strains expressing the 128-kDa cytotoxin-associated (CagA) protein, constitutes a factor associated with enhanced gastric inflammatory response and increased risk of development of atrophic gastritis (7–10), peptic ulcer (11–15), and even gastric cancer (16–22).

The aim of our study was to evaluate the course of gastritis during 18 years and its association with *H. pylori* and CagA seropositivity in an adult sample from Saaremaa, Estonia. This study is an extension of our earlier studies (1, 2, 4, 5) and is based on the hypothesis that the local immune response to the CagA protein of *H. pylori* is associated with increased recruitment of inflammatory cells in the gastric mucosa and with epithelial damage (7, 8, 13, 23–28) and on the possibility that CagA is highly immunogenic and stimulates a strong humoral response (11, 12, 29–31). We have tested the hypothesis that the CagA strain is associated with atrophic gastritis and progression of atrophic gastritis as a direct result.

Subjects and Methods

Subjects

Seventy persons (31 men, 39 women; median age, 57.5 years) of an adult sample of 304 from Saaremaa Island, most of whom had previously been investigated with endoscopy and biopsy in 1979, 1985, and 1991, were reinvestigated by means of endoscopy and biopsy in 1997. Of the initial 304

Table I. Grading of activity of gastritis, inflammation, atrophy, intestinal metaplasia, epithelial damage, and *Helicobacter pylori* in the antrum and corpus mucosa in samples from 1997

Grade	Activity of gastritis, *n*	Inflammation, *n*	Atrophy, *n*	Intestinal metaplasia, *n*	Epithelial damage, *n*	*H. pylori*, *n*
In antrum						
0	16/66	7/66	51/66	53/66	20/66	9/66
1, mild	45	10	13	10	44	17
2, moderate	5	38	2	3	2	9
3, severe	0	11	0	0	0	31
Total (1–3)	50/66	59/66	15/66*	13/66	46/66†	57/66
In corpus						
0	24/66	5/66	34/66	56/66	32/66	6/66
1, mild	29	10	17	9	29	19
2, moderate	13	28	10	1	5	10
3, severe	0	23	5	0	0	31
Total (1–3)	42/66	61/66	32/66**	10/66	34/66††	60/66

Statistically significant difference (on the basis of the chi-square test): ** > * $P = 0.003$, † > †† $P = 0.03$.

subjects, 227 were randomly selected and 77 wished to be investigated (2).

During the 18-year follow-up 39 of the original 304 subjects had died by 1997 (gastric carcinoma, 2; coronary heart disease and heart failure, 17; malignancies other than gastric causes or leukaemia, 11; cerebral insult, 6; and other causes, 3). The other subjects ($n = 195$) were lost to follow-up because of high age, severe illness, change of residence, and failure of communication or refusal. Gastroduodenal endoscopy was performed at the beginning of the study and subsequently at 6, 12, and 18 years. The results of a 6- and 12-year follow-up study of the course of gastritis are described elsewhere (4, 5). The results of the last follow-up examinations are analysed in the present study, and the course of gastritis is evaluated as the difference between the first and last follow-up examinations over 18 years.

The seventy persons who were investigated in 1997 were endoscoped, and technically acceptable data (biopsies) were obtained for 66 subjects.

At the last examination five subjects had duodenal ulcer or duodenal ulcer scar, four had antral polyps, and five had antral/prepyloric erosions. Three mucosal specimens were taken from the antrum and six from the corpus. Formalin-fixed, paraffin-embedded biopsy specimens were stained with haematoxylin and eosin and modified Giemsa. The state of the gastric mucosa and the presence of *H. pylori* in histologic sections were assessed in accordance with the Sydney system (32) and scored from 0 (no changes) through 1 (mild) and 2 (moderate) to 3 (severe changes).

Serum samples were collected from all 70 subjects and were stored at −20°C until assayed.

Methods

H. pylori status was determined on the basis of a serologic evaluation of IgG antibodies to *H. pylori* (strain CCUG 17874), using the enzyme-linked immunosorbent assay (ELISA), as reported previously (33). In brief, 0.5 µg of acid glycine (pH 2.2)-extracted cell surface proteins per well were used for the coating of microtitre plates (Nunc, Roskilde, Denmark). The studied sera were diluted to 1:800; the second antibody was alkaline phosphatase-labelled anti-human IgG (Dako, Glostrup, Denmark), which was diluted to 1:500. The results were expressed as corrected mean absorbance values as a percentage of the reference standard (human gammaglobulin, Pharmacia & Upjohn, Stockholm, Sweden). The cut-off value for seropositivity was set at a relative antibody activity (RAA) of 25. Interassay variation was 13%, and intra-assay variation 5%.

Anti-CagA IgG were detected with the ELISA using a recombinant fragment of the CagA antigen His-17/12 of the *H. pylori* strain CCUG 17874, kindly donated to us by Dr. A. Covacci (Chiron, Biocine, Siena, Italy), using the method described by Xiang et al. (34), with modifications. In our study the recombinant CagA antigen was diluted in 0.1 M carbonate buffer, pH 9.6, to a final concentration of 1.25 µg/ml. Maxisorp Immunoplates (Nunc) were coated with 100 µl/well in duplicate, incubated for 16 h at 4°C, and washed three times with phosphate-buffered saline (PBS) (pH 7.4) containing 0.05% Tween 20. The wells were saturated with 200 µl/well of 1% bovine serum albumin (BSA) in PBS–Tween at 37°C for 2h. After the plates had been washed three times, 100 µl of each serum sample, diluted to 1:300 in 1% BSA–PBS, was added in triplicate (two wells with the antigen and one without the antigen) and incubated at 37°C for 2 h. The plates were then washed three times, and 100 µl/well of alkaline phosphatase-conjugated anti-human IgG (Dako), diluted to 1:1000 in 1% BSA–PBS, was added, and the plates were incubated at 37°C for 1.5 h. After three washes, 100 µl/well of a substrate solution containing *p*-nitrophenyl phosphate (Sigma Chemical Co, St. Louis, Mo., USA) in diethanolamine–$MgCl_2$ buffer was added to each well and incubated at 37°C for 30 min. Each plate contained a positive and a negative control serum. Absorption was measured at 405 nm. The absorption value derived from the mean value of absorption in two wells with the antigen minus the absorption value for the well without antigen was taken as the absorbance

Table II. 18-year follow-up of gastritis and CagA status

Gastritis 1979→1997	Antrum			Corpus		
	n	%	No. of CagA+ in 1997	*n*	%	No. of CagA+ in 1997
No development	42*	66	24	25**	39	13‡
Development of AG	7†	11	6	22††	35	20‡‡
No change	5	8	5	4	6	3
Progression of AG	2	3	2	4	6	3
Decrease of AG	1	1	1	2	3	2
Disappearance of AG	7	11	6	7	11	3
Total	64	100	44	64	100	44

AG = atrophic gastritis of any degree.
Statistically significant difference (on the basis of the chi-square test): * > ** $P = 0.002$; †† > † $P = 0.001$; ‡‡ > ‡ $P = 0.009$.

value of the studied sera. Instead of the cut-off at 0.250 optical density (OD), used by Xiang et al. (34), the cut-off point in this study was set at an absorbance value of 0.300 (A 405 nm), on the basis of the calculation of the ELISA results, derived from the mean absorbance value of 25 *H. pylori* histologically and serologically negative subjects plus two standard deviations, using square-root transformation (35). Interassay variation was 8% and intra-assay variation was 3%.

Statistical analysis

Differences in *H. pylori* status and in prevalence of antibodies to CagA between the groups were checked by means of the chi-square test. Differences in the mean values of OD as the expression of anti-CagA IgG level were calculated with the Student *t* test. Odds ratios (OR) with 95% confidence intervals (CI) were estimated by using Mantel–Haenzel statistics with Exact software. Differences were considered statistically significant when 95% CI did not include the value 1.0. Association of the grade of activity of gastritis, inflammation, atrophy, intestinal metaplasia, and surface epithelial cell damage with CagA positivity was also evaluated by using multiple logistic linear regression analysis with the SAS package; a P value <0.05 was considered statistically significant.

The Committee of Ethics at the University of Tartu approved this study.

Results

Histologic observation

Histologic data and *H. pylori* status for the 1977 samples are presented in Table I.

The course of atrophy in the antrum and corpus mucosa during 18 years is presented in Table II. Between two observations, in 1979 and 1997, 7 of 64 (11%) subjects developed atrophy in the antrum, whereas 22 of 64 (35%) developed atrophy in the corpus ($P = 0.001$).

Analysis of the association between the development of atrophy in the antrum and corpus mucosa and the grade of activity of the antrum and corpus gastritis at the first and the last follow-up investigations is presented in Table III. The results indicated that the development of atrophy in the corpus but not in the antrum was significantly associated with a high activity of gastritis both in the initial and in the last periods of observation ($P = 0.0003$).

Serologic data

The prevalence of IgG antibodies in 1997 was 94% (66 of 70). Of *H. pylori*-positive persons 71% (47 of 66) were CagA-positive.

The OR for the association of the grade of activity of gastritis, inflammation, atrophy, intestinal metaplasia, or epithelial cell damage with CagA positivity for 1997 are presented in Table IV. Logistic linear regression showed that anti-CagA positivity was significantly associated with the activity of gastritis in the antrum and corpus ($P = 0.04$, $P = 0.0001$, respectively). In the corpus mucosa the gastritis activity was also associated with the level of CagA antibodies ($P = 0.002$). No association was found between CagA positivity and severity of chronic inflammation in the antrum or corpus ($P = 0.14$, $P = 0.18$, respectively). On the other hand, CagA positivity was significantly associated with the presence of atrophy in the antrum and corpus ($P = 0.04$, $P = 0.002$, respectively). In the corpus mucosa the association between the presence of atrophy and the level of anti-CagA antibodies was also significant ($P = 0.0007$). CagA positivity was associated with the presence of intestinal metaplasia (IM) in the antrum but not in the corpus. Multiple regression analysis showed the strongest association between CagA positivity and the presence of atrophy of the antrum mucosa ($P = 0.04$) and between CagA positivity and activity of gastritis in the corpus mucosa ($P = 0.01$).

The mean OD values of anti-CagA IgG for different grades of activity of corpus gastritis showed a significantly higher mean OD value of antibodies to CagA in persons with mild ($OD = 0.893 \pm 0.495$) or moderate ($OD = 1.028 \pm 0.563$) grade of activity than in those without polymorphonuclear cell infiltration ($OD = 0.331 \pm 0.366$; $t = 4.59$, $P = 0.0002$ and $t = 4.55$, $P = 0.0006$, respectively). However, there was no significant difference between the mean OD values of anti-

Table III. Association between development of atrophic gastritis in the antrum and corpus mucosa and grade of activity of gastritis in 1979 and 1997

Dynamics of grade of atrophy 1979→1997	Activity of gastritis in 1979					Activity of gastritis in 1997				
	Cases with grade 1–3	Cases with grade 0	Total	OR (95% CI)	*P* value for trend*	Cases with grade 1–3	Cases with grade 0	Total	OR (95% CI)	*P* value for trend*
Antrum										
No development of AG	19	22	41	1		29	13	42	1	
Development or progression of AG	5	4	9	1.4 (0.36–5.69)	0.89	7	2	9	1.6 (0.32–7.52)	0.90
Corpus										
No development of AG	4	21	25	1		9	16	25	1	
Development or progression of AG	17	7	24	12.7 (3.35–48.5)	0.0003	23	3	26	13.6 (3.4–54.7)	0.0003

AG = atrophic gastritis; OR = odds ratio; CI = confidence interval.
* *P* value for trends evaluated with the chi-square test.

CagA IgG corresponding to different grades of activity of gastritis in the antrum mucosa: for grade 0, 0.569 ± 0.622; for grade 1, 0.729 ± 0.519; by grade 2, 1.061 ± 0.468; $P = 0.32$, $P = 0.12$, $P = 0.17$, respectively).

Course of gastritis during follow-up and CagA status

There was an association between CagA positivity in 1997 and progression of atrophy in the antrum and corpus mucosa during follow-up from 1979 to 1997. Among persons who had no atrophy in the antrum mucosa in 1979 and in 1997, 24 of 42 (57%) were CagA-positive, whereas among those in whom atrophy appeared during follow-up 8 of 9 (89%) were CagA-positive. Among persons who did not develop atrophy in corpus mucosa, during follow-up 13 of 25 (52%) were CagA-positive. On the other hand, among persons who developed atrophy or in whom atrophy progressed 23 of 26 (88%) were CagA-positive ($P = 0.01$; OR = 7.07 (1.80–27.7)).

There were significantly more CagA-positive subjects among those in whom activity of corpus gastritis increased during follow-up (13 of 17) than among those in whom no activity was detected either at the beginning or at the end of the study period (7 of 21; $P = 0.02$; OR = 6.50 (1.63–25.91).

Discussion

The present study is a prospective 18-year endoscopic follow-up showing that chronic *H. pylori* gastritis progresses gradually but slowly into atrophic gastritis and that this progression particularly appears to be related to the presence of CagA-positive strains. The specific finding of the present population study is that the CagA strain was associated with progression of both atrophic gastritis in the corpus and atrophic gastritis in the antrum.

Progression of gastritis to atrophic gastritis has been known for a long time (1–6). Why atrophic gastritis develops in some but not in all affected individuals is unknown. Infection with *H. pylori*, especially in the presence of CagA, and the cytotoxicity of the bacterium, constitute one possible means

Table IV. Odds ratios (OR) for association between CagA positivity and grade of activity of gastritis, inflammation, atrophy, intestinal metaplasia, and epithelial damage in the antrum and corpus in follow-up biopsy specimens (1997)

Grading	Antrum				Corpus			
	n	CagA+	CagA−	OR (95% CI)	*n*	CagA+	CagA−	OR (95% CI)
No activity of gastritis (0)	16	7	9	1	24	8	16	1
Activity (1–3)	50	38	12	4.1 (1.3–12.7)	42	37	5	14.8 (4.5–50.3)
No inflammation (0)	7	3	4	1	5	2	3	1
Inflammation (1–3)	59	42	17	3.3 (0.7–14.6)	61	43	18	3.6 (0.7–19.5)
No atrophy (0)	51	31	20	1	34	17	17	1
Atrophy (1–3)	15	14	1	9.0 (1.4–57.9)	32	28	4	7.0 (2.1–23.1)
No intest. metapl. (0)	53	33	20	1	56	37	19	1
Intest. metapl. (1–3)	13	12	1	7.3 (1.1–46.9)	10	8	2	2.1 (0.4–9.4)
No epith. damage (0)	20	9	11	1	32	17	15	1
Epith. damage (1–3)	46	36	10	4.4 (1.4–13.1)	34	28	6	4.1 (1.4–12.2)

CI = confidence interval.

of affecting the state of gastric mucosa and the dynamics of gastritis (8–11). The present study supports this possibility and showed an association between CagA positivity and the activity of gastritis, the presence of atrophy, and epithelial cell damage in both the antrum and the corpus mucosa. Our data are in accordance with the studies indicating that CagA-positive *H. pylori* status causes more aggressive gastritis than the CagA-negative status. This finding suggests that CagA-positive gastritis tends to progress to atrophic gastritis more frequently than CagA-negative gastritis (7–10, 13, 25–28, 36–38).

According to our study the development of atrophy in the corpus mucosa was significantly associated with the activity of corpus gastritis. Furthermore, association between the activity of gastritis and CagA positivity was particularly marked in the corpus. CagA protein is highly immunogenic (12, 29–31) and may stimulate a strong humoral response. Indirect determination of the degree of polymorphonuclear cell infiltration in the stomach corresponding to the levels of systemic IgG antibodies to this cytotoxic protein has been proposed by Figura (25). There is evidence that CagA-positive strains have a higher pathogenic potential than CagA-negative strains. They increase mucosal cytokine expression and polymorphonuclear cell infiltration and induce an immune response leading to mucosal damage (7, 13, 39, 40, 41). In our study the mean OD values of anti-CagA IgG (which reflect the level of antibodies in sera) was associated with a higher grade of activity of gastritis and atrophy, especially in the corpus mucosa.

There is evidence that patients infected by cytotoxic strains and/or patients with anti-CagA antibodies are more likely to have active gastritis and to develop peptic ulcer or gastric cancer (11–22, 26). Both direct bacterial cytotoxicity and inflammatory cell aggression against gastric epithelium may predispose the patient to peptic ulcer disease (39). The association between CagA, peptic ulcer, and gastric cancer is most logical if it is assumed that CagA is related to aggressiveness of gastritis. Occurrence of CagA infection results in more active gastritis, which is more likely to end up as atrophic gastritis. Such a sequence of events is supported by the results of this study.

Our 18-year follow-up study of gastritis showed a difference in the course of atrophy between the antrum and corpus mucosa and established its relation to the activity of gastritis. In the study population 11% of the subjects developed atrophy in the antrum, whereas 35% developed it in the corpus. Analysis of the course of gastritis and CagA status in these patients showed that CagA positivity was more closely associated with the development of atrophy in the corpus than in the antrum.

A somewhat surprising finding was that the relationship between CagA and atrophic antral gastritis was not so strong as the relationship between CagA and atrophic corpus gastritis. This fact seems illogical but is obviously based on the natural course of gastritis in the present study population: in some patients healing of antral gastritis is accompanied by a rapid exacerbation of atrophic gastritis in the corpus (4, 6). Such healing of antral gastritis may take place in the presence of a CagA-positive strain and confound the analysis of events in the antrum. In other words, a significant progress of atrophic gastritis in the corpus, as is the case in the presence of CagA strains, is associated with opposite events in the antrum. If analysis were to be restricted to patients with atrophic antral gastritis only, the presence of CagA strains would show an association with atrophic antral gastritis too. However, the present material did not enable such an analysis. Still, even in our series, a significant association was observed between CagA and IM in the antral mucosa.

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ORIGINAL ARTICLE

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Significant increase in antigastric autoantibodies in a long-term follow-up study of *H. pylori* gastritis

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Abstract In 30% of *H. pylori*-infected patients a certain type of antigastric autoantibodies, reacting against canalicular structures within human parietal cells, is detectable. Furthermore, it has been shown that these autoantibodies are correlated with atrophy of the mucosa in the corpus. The aim of this study was to analyse the prevalence of these anticanalicular autoantibodies (ACAB) and their significance for development of gastric mucosa atrophy in a 12-year follow-up period. Gastric biopsy specimens from 62 persons in Saaremaa Island, Estonia, were collected in 1997 and assessed independently by two pathologists in accordance with the updated Sydney system. The sera of these persons were immunohistochemically screened for ACAB and for classic parietal cell antibodies (PCA). In addition, for 37 of the 62 persons, gastric biopsies and sera collected 12 years earlier (1985) were investigated in an analogous manner. ACAB increased significantly, from 8 out of 37 in 1985 to 17 out of 37 in 1997 (P=0.004; McNemar test). In 1997 a significant correlation existed between the presence of ACAB and corpus mucosa atrophy (19 out of 30 versus 10 out of 32 without atrophy; P=0.01; odds ratio (OR)=3.8, 95% CI 1.4–10.6). However, no correlation was found between ACAB and development of atrophy in the period from 1985 to 1997. All 37 persons were PCA negative in 1985, whereas in 1997, 2 turned out to be PCA positive. ACAB increased significantly with duration of *H. pylori* gastritis. The correlation between ACAB and presence of gastric corpus atrophy was confirmed. However, it is possible that ACAB are the consequence of and not a causative factor in gastric mucosa atrophy, insofar as the association of ACAB with progression of corpus atrophy was not significant.

Key words Anticanalicular autoantibodies · Corpus atrophy · Follow-up of gastritis · *Helicobacter pylori* · Parietal cell antibodies

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Introduction

H. pylori gastritis leads to the development of gastric mucosa atrophy [11, 17, 18, 24, 26]. Furthermore, recent studies have shown that a considerable proportion of *H. pylori*-infected patients develop autoimmune reactions against gastric epithelial cells [4, 14, 15, 23]. In particular, anticanalicular autoantibodies (ACAB) reacting against canalicular structures within human parietal cells can be demonstrated in 30% of infected patients. Gastric H^+,K^+-adenosine triphosphatase (H^+,K^+-ATPase) represents a major autoantigen of these autoantibodies. This particular type of antigastric autoimmunity is associated with atrophic corpus gastritis [2, 4, 5, 15].

It has been speculated that molecular mimicry between *H. pylori* and the host plays some part in the formation of ACAB in *H. pylori* gastritis [1, 14, 15, 23]. However, more recent data have provided evidence that pathogenic pathways other than molecular mimicry may also be responsible for this type of antigastric autoimmunity [2, 6].

The aim of the present study was to analyse the prevalence of ACAB and their significance for development of gastric corpus mucosa atrophy in a 12-year follow-up in an adult population from Saaremaa Island in Estonia. The study is an extension of earlier long-term follow-up research into chronic gastritis [12, 25–27] and parietal cell antibodies (PCA) in different Estonian populations

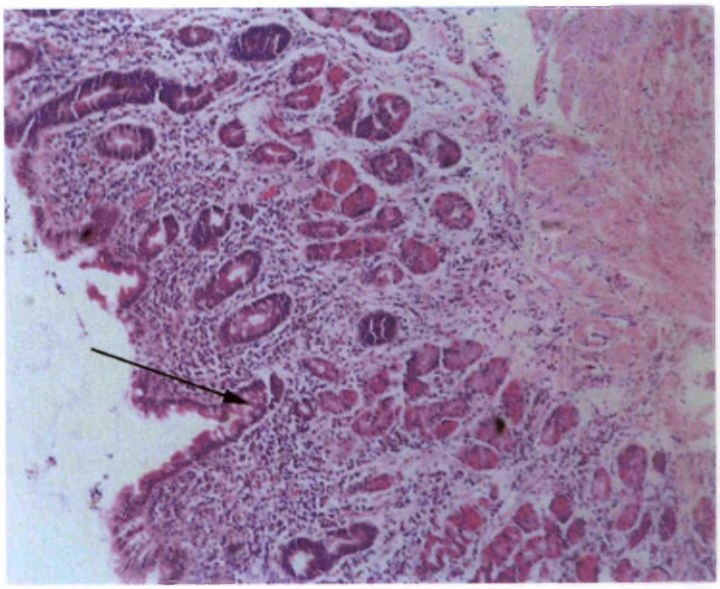

Fig. 1 Atrophic gastritis of moderate degree in the corpus. There is a remarkable loss of oxyntic glands and intestinal metaplasia (*arrow*) is present. Chronic inflammation is mild or moderate, and inactive. HE, original magnification ×100

[21–23] with a high prevalence of *H. pylori* infection [28].

Materials and methods

This study was approved by the Committee of Ethics of the University of Tartu. All persons gave their informed consent prior to their inclusion in the study.

Subjects

Seventy persons (mean age 57.8±11.4; 31 men, 39 women) who had been part of an adult random sample of 304 persons from Saaremaa Island, most of whom had previously been investigated by endoscopy and gastric biopsy in 1979, 1985 and 1991 [25–27], were re-investigated in 1997. In 1997, gastric biopsy specimens from the antrum and corpus mucosa and also serum samples were obtained from 66 of these 70 persons (mean age 57.7±11.3; 30 men, 36 women). Inclusion criteria for 62 (mean age 57.6±11.4, 30 men, 32 women) out of 66 persons for assessment of ACAB were: seropositivity for *H. pylori* in ELISA, presence of *H. pylori* in antrum and/or corpus biopsy specimens assessed by two pathologists.

Additionally, antrum and corpus biopsies and serum samples for 37 out of the 62 persons (mean age 43.7±10.6, 21 men, 16 women) were available from 1985, i.e. from a time 12 years earlier. Serum samples from both points of time were stored at –20°C and were then screened for ACAB and classic PCA.

Formalin-fixed, paraffin-embedded gastric biopsy specimens from the antrum (3 specimens) and corpus mucosa (6 specimens) were stained with haematoxylin and eosin and with a modified Giemsa stain. *H. pylori* colonisation, grade and activity of gastritis, and presence of gastric mucosa atrophy were assessed independently by two pathologists (P.S. and G.F.) in blinded fashion in accordance with the updated Sydney system [3] and scored from 0 (no changes) through 1 (mild) and 2 (moderate) to 3 (severe changes) (Fig. 1).

Anticanalicular autoantibodies

ACAB reacting against canalicular structures within human parietal cells were detected using an immunohistochemical method described earlier [4]. Briefly, heterologous formalin-fixed and paraffin-embedded gastric corpus mucosa with no pathological alterations and not expressing blood groups A and B were incubated overnight at 20°C with sera diluted 1:100 in RPMI 1640 medium (Biochrom, Berlin, Germany). As the secondary antibody, alkaline phosphatase-conjugated rabbit anti-human IgG (DAKO, Hamburg, Germany), diluted 1:10 in RPMI 1640 medium was used (incubation at 20°C for 90 min). After washing, positive reaction was induced with fast red. Sections were counterstained with haematoxylin, mounted with Aquatex and examined by light microscopy, without knowledge of the respective patient data or histological alterations. One positive control serum was included in each immunohistochemical staining session, and negative controls were performed by omitting the human serum. Intensive red immunohistochemical staining, visible at the canaliculi of parietal cells in the corpus mucosa, was taken as a positive reaction (Fig. 2).

Parietal cell antibodies

Classic IgG-type PCA were examined in all sera by an indirect immunofluorescence method, as described previously [21].

H. pylori status

H. pylori status was determined on the basis of a serological evaluation of IgG antibodies to *H. pylori* (strain CCUG 17874), using the enzyme-linked immunosorbent assay (ELISA) method, as re-

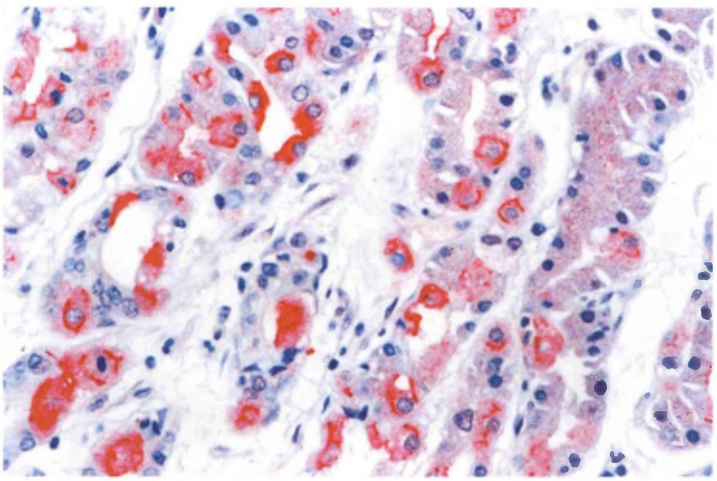

Fig. 2 In situ binding pattern of anticanalicular autoantibodies reacting against canalicular membranes within human parietal cells of the gastric corpus mucosa. Original magnification ×400

ported previously [28]. Briefly, 0.5 µg of acid glycine (pH 2.2)-extracted cell surface proteins per well were used for coating microtitre plates (NUNC, Roskilde, Denmark). The studied sera were diluted to 1:800; the secondary antibody was alkaline phosphatase-labelled anti-human IgG (DAKO, Glostrup, Denmark), which was diluted 1:500. The results were expressed by corrected mean absorbance values as percentages of the reference standard (human gamma globulin, Pharmacia and UpJohn, Stockholm, Sweden). The cut-off value for seropositivity was set at a relative antibody activity (RAA) of 25.

Statistics

Statistical differences between the ACAB-positive and ACAB-negative groups were evaluated by χ^2-and the McNemar tests using Statgraphics and SPSS statistical software. Odds ratios (OR) with 95% confidence intervals (CI) were estimated using the Mantel-Haenzel statistics with the Exact software. Differences were considered statistically significant if 95% CI did not include the value 1.0. Differences in the mean values of age were calculated by the Mann-Whitney test (Statgraphics software). The κ-value for measuring agreement between histological assessments was calculated using SPSS statistical software. *P*-values <0.05 were considered significant.

Results

H. pylori status

In the sample of 1997, positive *H. pylori* serology in ELISA was evaluated in 63 out of the 66 (95%) sera studied. Morphologically, *H. pylori* was confirmed in 62 of them. Three persons were serologically and morphologically negative for *H. pylori*, while 1 was serologically positive but morphologically negative.

The sensitivity of ELISA was 100%, specificity 75%, positive predictive value (PPV) 98%, and negative predictive value (NPV) 100%. Interassay variation was 13% and intra-assay variation was 5%.

In all 37 subjects who passed the follow-up study, *H. pylori* infection was confirmed histologically from the presence of bacteria in biopsy specimens from the antrum and/or corpus as well as from positive *H. pylori* serology in ELISA at both time points.

Gastric mucosa alterations

The grading of chronic inflammation, activity of gastritis, atrophy, intestinal metaplasia and *H. pylori* colonisation in the antrum and corpus mucosa samples in the follow-up group are presented in Table 1.

The grading of antrum and corpus mucosa atrophy, as evaluated independently by two pathologists, is displayed in Table 2. Interobserver agreement regarding atrophy in follow-up samples was as follows: κappa values for samples of 1985: 0.770 (agreement 94.5%) for antrum biopsies and 0.548 (agreement 89.2%) for corpus biopsies; kappa values for samples of 1997: 0.247 (agreement 67%) for antrum biopsies and 0.455 (agreement 73%) for corpus biopsies. Kappa values for 62 samples of 1997 were 0.376 (agreement 72.5%) for antrum biopsies and 0.416 (agreement 71%) for corpus biopsies.

Prevalence of ACAB

Prevalence of ACAB in the follow-up group (samples of 1985 and 1997) and in 62 persons investigated in 1997 are presented in Tables 1 and 3.

ACAB were immunohistochemically detectable in 29 of the 62 (47%) subjects investigated in 1997. In the follow-up group, made up of 37 infected persons, ACAB were detected in 8 of the 37 (22%) in 1985 and in 17 of

Table 1 Grading of chronic inflammation, activity of gastritis, atrophy, intestinal metaplasia, *Helicobacter pylori* and anticanicular autoantibody (*ACAB*) positivity in antrum and corpus mucosa samples in the follow-up group (n=37) in 1985 and 1997

Grade	Chronic inflammation		Activity of gastritis		Atrophy		Intestinal metaplasia		*Helicobacter pylori*	
	1985 ACAB+/n	1997 ACAB+/n	1985 ACAB+/n	1997 ACAB+/n	1985 ACAB+/n	1997 ACAB+/n	1985 ACAB+/n	1997 ACAB+/n	1985 ACAB+/n	1997 ACAB+/n
In antrum										
0	0/0	0/0	0/1	0/1	5/33	9/23	6/32	12/30	1/4	0/2
1, mild	0/1	0/1	1/2	6/11	2/2	5/11	2/5	5/7	3/14	6/12
2, moderate	6/27	13/29	6/26	11/25	1/2	3/3	0/0	0/0	3/12	1/3
3, severe	2/9	4/7	1/8	0/0	0/0	0/0	0/0	0/0	1/7	10/20
Total	8/37	17/37	8/37	17/37	8/37	17/37	8/37	17/37	8/37	17/37
In corpus										
0	0/0	0/0	1/4	0/0	7/34	10/23	6/33	14/33	1/4	0/2
1, mild	3/11	3/7	3/12	8/19	0/0	5/11	2/4	3/4	4/13	8/12
2, moderate	4/24	11/25	4/20	9/18	1/3	2/3	0/0	0/0	2/15	2/6
3, severe	1/2	3/5	0/1	0/0	0/0	0/0	0/0	0/0	1/5	7/17
Total	8/37	17/37	8/37	17/37	8/37*	17/37**	8/37	17/37	8/37	17/37

Statistically significant difference: **>*P=0.004 (on the basis of McNemar test)

Table 2 Grading of antrum and corpus mucosa atrophy in the samples of 1985 and 1997 as evaluated independently by two pathologists

Grade	Follow-up group				Samples of 1997 (n=62)	
	1985 (n=37)		1997 (n=37)		Pathologist I	Pathologist II
	Pathologist I	Pathologist II	Pathologist I	Pathologist II		
In antrum						
1, mild	3	2	7	11	14	14
2, moderate	3	2	1	3	2	6
3, severe	0	0	0	0	0	0
Total (1–3)	6	4	8	14	16	20
In corpus						
1, mild	4	0	10	11	17	19
2, moderate	2	3	5	3	9	6
3, severe	1	0	3	0	4	1
Total	7	3	18	14	30	26

them (46%) in 1997. All 8 patients who were positive for ACAB in 1985 remained positive for these autoantibodies, whereas 9 of the 29 patients who were negative in 1985 developed ACAB during the follow-up period. The increase in ACAB during this period was statistically significant (P=0.004; McNemar test). These results are presented in Fig. 3.

At the study point in 1985, the mean age (41.7±10.3) of the ACAB-negative persons who were also negative in 1997 did not differ significantly from the mean age (46.1±10.6) of those who were ACAB positive in 1985 or had become ACAB positive in 1997 (P=0.18).

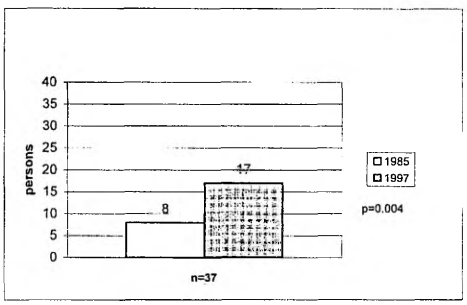

Fig. 3 Significant increase in anticanalicular autoantibodies in a 12-year follow-up of untreated *H. pylori* gastritis

Prevalence of PCA

Among the 62 serum samples examined in 1997, 3 were positive for classic PCA. All these 3 persons were also positive for ACAB in 1997. In the follow-up sample, all 37 patients were negative for classic PCA in 1985, while

Table 3 Grading of chronic inflammation, activity of gastritis, atrophy, intestinal metaplasia, *Helicobacter pylori*, as evaluated by two pathologists, and ACAB positivity in antrum and corpus mucosa samples in a group of 62 persons in 1997

Grade	Chronic inflammation		Activity of gastritis		Atrophy			Intestinal metaplasia		*Helicobacter pylori*	
	Pathologist		Pathologist		Pathologist			Pathologist		Pathologist	
	I ACAB+/*n*	II ACAB+/*n*	I ACAB+/*n*	II ACAB+/*n*	I ACAB+/*n*		II ACAB+/*n*	I ACAB+/*n*	II ACAB+/*n*	I ACAB+/*n*	II ACAB+/*n*
In antrum											
0	1/2	0/0	4/10	0/1	20/46		18/42	21/50	22/49	2/3	2/3
1, mild	3/9	0/0	22/47	10/19	8/14		7/14	6/9	7/13	11/18	11/18
2, moderate	19/40	20/48	3/5	19/41	1/2		4/6	2/3	0/0	3/9	3/9
3, severe	6/11	9/14	0/0	0/1	0/0		0/0	0/0	0/0	13/32	13/32
Total	29/62	29/62	29/62	29/62	29/62		29/62	29/62	29/62	29/62	29/62
In corpus											
0	0/1	0/0	7/20	0/1	10/32*		13/36†	21/54	20/49	0/0	0/0
1, mild	3/10	3/10	13/29	14/30	7/17		10/19	7/7	9/13	14/21	14/21
2, moderate	13/29	18/41	9/13	15/30	8/9	**	5/6	1/1	0/0	3/10	3/10
3, severe	13/22	8/11	0/0	0/1	4/4		1/1	0/0	0/0	12/31	12/31
Total	29/62	29/62	29/62	29/62	29/62		29/62	29/62	29/62	29/62	29/62

Statistically significant differences: **>*P=0.01; ††>†P=0.04; > P=0.004 (on the basis of the Chi-square test)

2 of the 37 had become PCA positive in 1997. The latter were also ACAB positive in 1985 and 1997. All PCA-positive persons revealed highly intensive staining for ACAB throughout the cytoplasm.

Among the persons who possessed PCA in 1997, 1 had grade 2, and another had grade 3 corpus atrophy, while the third showed grade 2 chronic inflammation in the corpus but no atrophy. In the follow-up group, of the 2 persons who developed PCA in 1997, 1 developed grade 2 corpus atrophy, but the other did not. The latter person had grade 2 chronic inflammation in the corpus at both time points.

Course of gastritis and ACAB

In samples for 62 persons examined in 1997 a significant association was found between ACAB and corpus mucosa atrophy (grades 1–3) by both pathologists. According to pathologist I, of 30 persons with corpus mucosa atrophy (grades 1–3), 19 (63%) had ACAB, while autoantibodies were found in only 10 of the 32 (31%) subjects without atrophy (P=0.01; OR=3.8; 95% CI 1.4–10.6). According to the diagnosis of atrophy established by pathologist II, 16 of 26 (62%) subjects with corpus atrophy grade 1–3 had ACAB, whereas only 13 of 36 (36%) persons without corpus atrophy had these autoantibodies (P=0.04; OR=2.8; 95% CI 1.02–7.8).

In Table 4 the relationship between ACAB and development of corpus atrophy of different grades is shown separately for 37 persons who were followed up for 12 years. In summary, according to pathologist I (the values given by pathologist II are presented in brackets) 2 (1) of 8 patients with ACAB had some grade of atrophy in 1985, while 5 (2) of 29 infected patients without ACAB also had corpus atrophy already in 1985 (P=1.0). In 1997, 10 (7) out of 17 ACAB-positive subjects had atrophy (grade 1, 4; grade 2, 4; grade 3, 2; according to pathologist I; grade 1, 5; grade 2, 2; according to pathologist II), while 8 (7) out of 20 ACAB-negative patients showed similar mucosal alterations (atrophy grade 1, 6; grade 2, 2 according to pathologist I; atrophy grade 1, 6 grade 2, 1; according to pathologist II; P=0.25). Thus, although the prevalence of atrophy determined by either pathologist was higher in the subset of infected persons with ACAB than in the subset without these autoantibodies, no statistically significant association between ACAB and corpus mucosa atrophy could be established either at the beginning of the study or at the end of the follow-up period.

Furthermore, out of 9 persons who developed ACAB during the follow-up period, 4 (3) subjects also developed corpus mucosa atrophy (grade 1, 2; grade 2, 1; grade 3, 1 according to pathologist I; grade 1, 2; grade 2, 1 according to pathologist II). On the other hand, out of the 14 (11) persons who developed atrophy (grade 1 , 9; grade 2, 1; grade 3, 1 according to pathologist I; grade 1, 9; grade 2, 2 according to pathologist II) in the follow-up period, 6 (6) had no autoantibodies either at the begin-

Table 4 Grading of corpus atrophy in persons with and without ACAB in 1985 and 1997 (n=37). Atrophy was graded from 0 (no atrophy) through 1 (mild) and 2 (moderate) to 3 (severe)

Pat. no.	ACAB in 1985	ACAB in 1997	Pathologist I		Pathologist II	
			Corpus atrophy 1985	Corpus atrophy 1997	Corpus atrophy 1985	Corpus atrophy 1997
1	–	–	0	1	0	0
2	–	–	0	1	0	1
3	–	–	2	0	2	1
4	–	–	0	0	0	0
5	–	–	0	2	0	2
6	–	–	0	0	0	0
7	–	–	0	0	0	0
8	–	–	0	0	0	0
9	–	–	0	0	0	1
10	–	–	0	0	0	0
11	–	–	0	0	0	0
12	–	–	1	2	0	1
13	–	–	0	0	0	0
14	–	–	0	1	0	0
15	–	–	0	1	0	0
16	–	–	1	0	0	1
17	–	–	0	1	0	0
18	–	–	0	0	0	0
19	–	–	1	1	0	1
20	–	–	0	0	0	0
21	–	+	0	1	0	1
22	–	+	0	3	0	2
23	–	+	0	0	0	0
24	–	+	0	1	0	0
25	–	+	0	2	0	1
26	–	+	0	0	0	0
27	–	+	3	2	2	2
28	–	+	0	0	0	0
29	–	+	0	0	0	0
30	+	+	0	2	0	1
31	+	+	0	0	0	0
32	+	+	0	1	0	0
33	+	+	2	3	2	1
34	+	+	0	1	0	0
35	+	+	0	2	0	1
36	+	+	1	0	0	0
37	+	+	0	0	0	0

ning or at the end of the study (P=1.0). Again, at follow-up no statistically significant correlation was found between evolution of ACAB and development of gastric atrophy, or vice versa.

Intestinal metaplasia and ACAB

The grading of intestinal metaplasia and its association with ACAB are presented in Tables 1 and 3. In the follow-up group, persons with corpus intestinal metaplasia grade 1 were ACAB positive in 2 out of 4 cases in 1985 and in 3 out of 4 in 1997 (P>0.05); persons with antrum intestinal metaplasia grade 1 were ACAB+ in 2 out of 5 cases in 1985 and in 5 out of 7 cases in 1997 (P>0.05). In the larger sample of 1997 (n=62) a significant association was noted between presence of intestinal metaplasia in the corpus (grades 1–2) and ACAB (8 ACAB positive out of 8 cases with intestinal metaplasia grade 1–2 versus 21 ACAB positive out of 54 cases without intestinal metaplasia; P=0.004). This association was not significant in the antrum mucosa: 8 ACAB positive out of 12 cases with intestinal metaplasia versus 21 out of 50 cases without intestinal metaplasia (P=0.22).

Isolated antrum atrophy and ACAB

With reference to the association between atrophic antrum gastritis and ACAB, cases with isolated atrophic antrum gastritis are of special interest. In the samples of 1997 (n=62), according to the data of pathologist I there were 4 cases of isolated antrum atrophy, while 2 of them were ACAB positive. According to the data of pathologist II, 4 out of 9 cases of isolated antrum atrophy were ACAB positive. In the follow-up group, according to the data of pathologist I, 2 persons developed isolated antrum atrophy (1 ACAB positive); according to the data of pathologist II, 4 persons developed isolated antrum atrophy (2 of them ACAB positive).

Chronic inflammation, activity of gastritis, *H. pylori* colonisation and ACAB

ACAB positivity at different grades of chronic inflammation and activity of gastritis and at different grades of *H. pylori* colonisation are presented in Tables 1 and 3. The tendency to an increase in the prevalence of ACAB in 1997 compared with 1985 was seen in a group of persons with grade 2 chronic inflammation in corpus mucosa (11/25 versus 4/24; P=0.14, McNemar test). During the follow-up period ACAB formed in 9 of the 37 persons studied. Among these 9 persons the grade of chronic inflammation in the corpus mucosa progressed in 5 cases, retained the same in 3 cases and decreased in 1 case. The grade of chronic inflammation in the antrum mucosa progressed in 2 cases, remained the same in 6 cases and decreased in 1 case.

In the corpus mucosa, periglandular lympocytic infiltrates were found in the samples of 1985 in 5 of the 37 cases, while all these 5 cases were ACAB negative both in 1985 and in 1997. In the 1997 sample periglandular lymphocytic infiltrates were found in 9 cases (7 of them newly diagnosed) and ACAB were present in 4 out of 9 cases. All persons with the finding of periglandular lymphocytic infiltrates were PCA negative.

Concerning changes in the activity of corpus gastritis in persons who developed ACAB, the grade of activity progressed in only 1 case, remained the same in 6 cases and decreased in 2 cases. In the antrum mucosa no progression of activity was observed, activity remaining the same grade in 4 cases and decreasing in 5 cases.

Thus, there was no significant association between grade of activity of gastritis and the presence or absence of ACAB at any time of observation. A tendency to an increase in the prevalence of ACAB during 12 years' follow-up was observed among persons with moderate grades of chronic inflammation.

In the sample of 1997 the number of persons with *H. pylori* colonisation grade 3 had increased significantly: in antrum specimens from 7 of 37 in 1985 to 20 of the 37 in 1997 (P=0.003); in corpus specimens from 5 of the 37 in 1985 to 17 in 1997 (P=0.005). However, development of ACAB in these persons was not statistically significant (P=0.11 for antrum specimens with *H. pylori* colonisation grade 3; P=0.42 for corpus mucosa specimens with *H. pylori* colonisation grade 3) and only 1 person in whom the grade of *H. pylori* colonisation progressed to severe developed a severe grade of atrophy in the corpus.

Discussion

The present study represents the first prospective investigation of anticanalicular autoantibodies (ACAB) and their significance for development of atrophic gastritis in a long-term follow-up of an infected random adult cohort with noneradicated *H. pylori*. In a previous study by Uibo et al. [22] the correlation between classic anti-PCA and development and progression of chronic gastritis was studied in a similar group followed up for 6 years. Furthermore, a 32-year follow-up study of chronic *H. pylori* gastritis has provided evidence that progression of corpus gastritis might be associated with the occurrence of PCA [24].

The most important finding of the present study was that the prevalence of ACAB increased significantly during the follow-up period of 12 years. One might suppose that ACAB are formed in the course of time and their appearance may be related to the duration of *H. pylori* infection. However, as the exact time of onset of *H. pylori* infection is unknown in the persons enrolled in this study, the duration of infection cannot be established either. It should be pointed out that the mean age of persons who were positive for ACAB at the beginning of the study, or became positive for ACAB during the next 12 years, did not differ significantly from the mean age of those who remained negative for ACAB during the whole period. This is in agreement with earlier data published by Faller et al. [5], indicating that the age profile of *H. pylori*-infected patients with antigastric autoantibodies was not significantly different from the age profile of patients without these autoantibodies. As most *H. pylori* infections occur in childhood [19] and can persist throughout life, the age of an infected adult person and duration of *H. pylori* infection can be regarded as roughly equivalent. The evidence of the role of the person's age or duration of *H. pylori* gastritis in formation of ACAB is based on studies of infected children, in which a lower prevalence of ACAB was reported [7, 9]. Furthermore, a significant age-dependent increase in PCA positivity was shown in *H. pylori*-infected persons but not in *H. pylori*-negative persons, which suggests that long-lasting *H. pylori* gastritis might represent an important factor for formation of PCA [23]. Other studies in which the formation of classic PCA was followed up over time have given similar results [8, 22].

The association of ACAB with *H. pylori* gastritis, particularly with atrophic gastritis, in the corpus mucosa has been established in several previous studies [2, 4, 5, 15, 20] and was confirmed again in a group of 62 persons in this investigation. The histological diagnosis of atrophy is difficult, and significant interobserver variation in the determination and grading of atrophy has been reported only recently [16]. The two pathologists who participated in this study attained interobserver agreement comparable to that seen in the last study. The fact that both investigators found a significant correlation between corpus atrophy and ACAB confirms the existence of a link between antigastric autoimmunity and gastric atrophy in *H. pylori* gastritis.

Progression of superficial gastritis to atrophic gastritis has been shown in several previous follow-up studies [10, 11, 24–27]. In the present study we attempted to find an answer to the question of why gastritis remains confined to superficial mucosal layers in some patients while gastric atrophy develops in others. Since ACAB are correlated with atrophic corpus gastritis, and an autoimmunological reaction can lead to loss of parietal cells and to evolution of gastric atrophy, then ACAB can

serve as relevant markers for defining patients who are at higher risk for development of gastric atrophy [5]. However, in this follow-up study we could not establish a significant association between presence of ACAB and development of atrophy over the 12 study years. The lack of any such correlation is obviously due to the low number of enrolled persons, since such a correlation was observed when the number of patients with the same epidemiological background was increased from 37 to 62 at the end of the study. Also, the study period might have been too short. Uibo et al. (1989) reported that in a 6-year follow-up period, changes in the gastric mucosa in autoimmune gastritis and in autoantibody-negative gastritis revealed no significant differences [22]. The antrum and corpus mucosa of 2 persons who developed PCA did not reveal any alterations over 6 years. In contrast, in a much longer, 32-year, follow-up study, the appearance of PCA occurred in parallel with progression of corpus atrophy, disappearance of *H. pylori* and improvement of the antral mucosa [24].

Although our study could not establish a significant correlation between ACAB and development of atrophic alterations in the corpus mucosa, we found a tendency to an increase in the prevalence of ACAB over the 12 study years in persons with moderate chronic lympocytic infiltration in the corpus mucosa and in the case of intestinal metaplasia in the corpus. The importance of lymphocytic infiltration in the corpus mucosa, especially in association with intestinal metaplasia, as an increased risk for development of gastric carcinoma, was stressed in a study conducted by Meining et al. [13].

As in previous investigations, all PCA-positive persons in our study were also positive for ACAB [5]. On the other hand, only 3 of all 29 ACAB-positive persons were positive for PCA. This can be explained by different binding sites of ACAB and classic PCA within parietal cells [2]. Interestingly, 3 persons who had classic PCA in 1997 showed very intensive staining for ACAB throughout the cytoplasm. Two of them had moderate or severe corpus atrophy. In the follow-up group only 2 persons turned out to be positive for PCA in 1997. However, although both had already revealed intensive staining for ACAB in 1985 and 1997, 1 developed corpus atrophy but the other did not. It could be hypothesised that ACAB precede classic PCA and that epitope switching takes place in the process of *H. pylori*-associated antigastric autoimmunity. This hypothesis could also explain why not all ACAB-positive persons were PCA positive in our study and in previous research [2, 5].

Our study confirmed previous reports of a significant correlation between ACAB and gastric mucosa atrophy [5, 6, 20]. However, from the data presented in this study we cannot clearly define whether ACAB are the cause or the consequence of gastric atrophy. There are some patients who are ACAB positive but (still) do not have atrophy. In these patients antigastric autoimmunity precedes atrophy. On the other hand, there are also patients who have atrophy but who are negative for ACAB. We can surmise that in this subgroup of persons ACAB might be developed during further progression of atrophy and appear when gastric cells are sufficiently degraded and autoantigens are presented to immunocompetent cells. It is evident that further research into antigastric autoimmunity is needed to get a better insight in the pathogenesis of chronic atrophic gastritis.

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VIII

Vorobjova T, Maaroos H-I, Sipponen P, Villako K, Uibo R.
Apoptosis in different compartments of antrum and
corpus mucosa in chronic *Helicobacter pylori* gastritis.
An 18-year follow-up study. Scand J Gastroenterol 2001, 36: 136–143.

ORIGINAL ARTICLE

Apoptosis in Different Compartments of Antrum and Corpus Mucosa in Chronic *Helicobacter pylori* Gastritis. An 18-Year Follow-up Study

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Vorobjova T, Maaroos H-I, Sipponen P, Villako K, Uibo R. Apoptosis in different compartments of antrum and corpus mucosa in chronic *Helicobacter pylori* gastritis. An 18-year follow-up study. Scand J Gastroenterol 2001;36:136–143.

Background: The association of apoptosis was analysed in three different compartments (foveolar cells—FC, proliferating zone—PZ and glandular part—GP) of antrum and corpus mucosa specimens with development of atrophy and the extent of apoptosis as depending on grade of chronic inflammation, activity of gastritis and *Helicobacter pylori* colonization at two time points of an 18-year follow-up in an adult population from Saaremaa, Estonia, with a high prevalence of *H. pylori* infection were compared. **Methods:** A total of 68 persons (31 men, 37 women; median age, 39 years in 1979) from a primary sample of 304 subjects, endoscoped in 1979 and reinvestigated by endoscopy and biopsy in 1997, were included in the study. The state of the gastric mucosa and the presence of *H. pylori* in the antrum and corpus mucosa were assessed in accordance with the Sydney system. The dynamics of apoptotic index (AI) between two time points in 1979 and 1997 was evaluated in antrum biopsies of 49 persons and in corpus biopsies of 64 persons. Apoptosis was measured using terminal deoxyuridine nucleotide nick end labelling (TUNEL) histochemistry. **Results:** The antrum as well as the corpus of 2/68 persons were *H. pylori* negative at both time points. Atrophy developed in 9/68 persons in the antrum and in 23/68 in the corpus. In PZ and GP of the corpus mucosa as well as in GP of the antrum mucosa, AI decreased significantly during 18 years compared with initial values ($P < 0.05$), which was not associated with development of atrophy. In all compartments of the antrum and corpus mucosa, studied at the initial and end points of observation, AI did not reveal a difference in persons with and without development of atrophy ($P > 0.05$). In the samples of 1979 the highest independent effect on the value of AI in the FC compartment for the antrum was exerted by grade of activity of gastritis ($P = 0.01$) and in GP by degree of chronic inflammation ($P = 0.03$), while in the samples of 1997 the highest effect was exerted by grade of *H. pylori* colonization ($P = 0.02$ and 0.03 in FC and GP, respectively). For the corpus mucosa AI was most strongly affected also by grade of activity of gastritis in FC compartment ($P = 0.02$) and by degree of chronic inflammation in PZ ($P = 0.04$), but not by grade of *H. pylori* colonization. **Conclusion:** AI was not associated with development of atrophy, but was largely dependent on grade of activity of gastritis and degree of chronic inflammation; in the antrum mucosa AI depended also on grade of *H. pylori* colonization.

Key words: Activity of gastritis; antrum and corpus mucosa; apoptosis; atrophy; follow-up of gastritis; *Helicobacter pylori*

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Helicobacter pylori gastritis leads to development of gastric mucosa atrophy (1–5). There is also evidence that *H. pylori* is associated with changes of epithelial cell turnover (6–9) and increased apoptosis (programmed cell death) in the gastric epithelium (10–16), which can lead to development of atrophy of the gastric mucosa (17). A recent study by Moss et al. (18) on gastric cellular turnover and development of atrophy after 31 years of follow-up showed that patients who developed atrophy later had had initially mildly increased gastric epithelial cell proliferation and apoptosis, however, the ratio of apoptosis to proliferation was not a determinant of risk for development of atrophy several decades later.

The aim of the present study was to analyse the association of apoptosis in the foveolar cells (FC), proliferating zone (PZ) and glandular part (GP) of antrum and corpus mucosa specimens with development of atrophy, as well as to compare apoptosis at different grades of chronic inflammation, activity of gastritis and *H. pylori* colonization at two time points of an 18-year follow-up in an adult population from Saaremaa, Estonia, with a high prevalence of *H. pylori* infection.

This study is an extension of our earlier long-term follow-up research into chronic gastritis in an Estonian adult sample from Saaremaa (1, 19–23). In the present study we tested the hypothesis that increased apoptosis may be responsible for

progression of atrophy and is associated with higher degree of chronic inflammation, activity of gastritis and *H. pylori* colonization in the antrum and corpus mucosa.

Subjects and Methods

Subjects

Of the initial 304 subjects from an adult sample from Saaremaa Island in Estonia, who had been investigated by endoscopy and gastric biopsy in 1979, 1985 and 1991, 70 persons were reinvestigated in 1997. Sixty-eight persons (31 men, 37 women; median age, 39 in 1979), whose antrum and corpus biopsy specimens were available for both time points, 1979 and 1997, were included in the study.

During the 18-year follow-up of the initial 304 subjects, 39 had died by 1997, the remaining 195 subjects were lost to follow-up because of high age, severe illness, change of residence, failure of communication or refusal. The studied persons had not received any *H. pylori* eradication therapy.

The course of gastritis was evaluated as the difference between the first (1979) and the last (1997) follow-up examination over 18 years.

Apoptotic index (AI) was evaluated in the samples of 1979 for 52 antrum and 64 corpus biopsy specimens and in the samples of 1997 for 58 antrum and 66 corpus biopsy specimens. The dynamics of AI between the two time points, 1979 and 1997, was evaluated in the antrum biopsies of 49 persons and in the corpus biopsies of 64 persons. The histological examination and evaluation of apoptosis were performed simultaneously in the tissue samples of 1979 and 1997.

Methods

Formalin-fixed, paraffin-embedded gastric biopsy specimens from the antrum (three specimens) and corpus mucosa (six specimens) were stained with haematoxylin and eosin, and with a modified Giemsa stain. The state of the gastric mucosa and the presence of *H. pylori* in histological sections were assessed in accordance with the updated Sydney system (24) and scored from 0 (no changes) through 1 (mild) and 2 (moderate) to 3 (severe changes).

TUNEL histochemistry

Apoptosis was evaluated with the use of the TUNEL (terminal desoxynucleotidyl-transferase mediated dUTP–biotin nick end labelling) histochemistry method (25). After deparaffinization and rehydration, the sections were digested with proteinase K (Sigma, St Louis, MO) for 30 min at 20 °C (20 μg/ml in TRIS–HCl). After washing with PBS (pH 7.4) a TUNEL reaction was performed using the In Situ Death Detection Fluorescein Kit (Boehringer Mannheim, Germany). The principle of the test: DNA strand breaks generated during apoptosis were identified by labelling with fluorescein dUTP (digoxigenin-11-deoxyuridine triphosphate) using terminal deoxynucleotidyl transferase (TdT). By an enzymatic reaction (incubation for 40 min at 37 °C) with TdT, fluorescein labelled dUTP was specifically transferred to the 3′-OH ends of DNA. After washing with PBS (pH 7.4) and treatment with BSA (bovine serum albumin, 1 mg/ml in PBS) for 10 min, the sections were subsequently incubated with monoclonal mouse antifluorescein antibodies (DAKO, Glostrup, Denmark, diluted 1:10, incubation for 30 min at 20 °C). After washing the sections were incubated with rabbit anti mouse biotinylated monoclonal antibodies (DAKO, Glostrup, Denmark, diluted 1:50, for 30 min at 20 °C), followed by incubation with the Strept AB Complex, labelled with alkaline phosphatase (DAKO, Glostrup, Denmark) and fast red as a substrate. Counterstaining was done with haematoxylin. Apoptotic cells with incorporated dUTP showed a distinct bright red signal in the nucleus (Fig. 1). For negative controls TdT was omitted. The corpus gastric mucosa with lymphofollicules, containing apoptotic lymphocytes, was used as positive control.

Morphometry. The rate of apoptotic cells in the antrum and corpus mucosa was quantified by counting three times 200 cells of the FC compartment, 200 cells in the PZ (neck part) (only for well-oriented glands) and 200 cells of the GP of the section, using a light microscope with magnification ×400. The mean value of positively labelled nuclei per 200 cells, calculated in three different section areas separately for FC, PZ and GP, were expressed as AI. Intra-assay variation for AI was 11%. Counting was done by one observer (T.V.) who was not aware of morphological data or year of sampling (done in mixed manner).

Statistical analysis

Data were presented as means with standard deviation. The Wilcoxon signed rank test served for evaluation of the differences between AI of the same persons in samples of 1979 and 1997, and the Mann–Whitney unpaired, nonparametric test served for analysis of AI for two groups according to different grades of atrophy, chronic infiltration, activity of gastritis and *H. pylori* colonization. Differences in the number of persons with a different grade of *H. pylori* colonization at two time points of observation were checked by means of the chi-square test. The effect of different variables on the expected values of AI and on the difference between the mean values of AI for different compartments of antrum and corpus mucosa sections in the samples of 1979 and 1997 was evaluated by multifactorial dispersion analysis by the SAS/LAB with the SAS package. Values of $P < 0.05$ were considered significant.

The Ethics Committee of the University of Tartu approved this study. All persons gave their informed consent prior to inclusion in the study.

Results

Gastric mucosa alterations and H. pylori *status*

Grading of chronic inflammation, activity of gastritis, atrophy, intestinal metaplasia and *H. pylori* colonization in

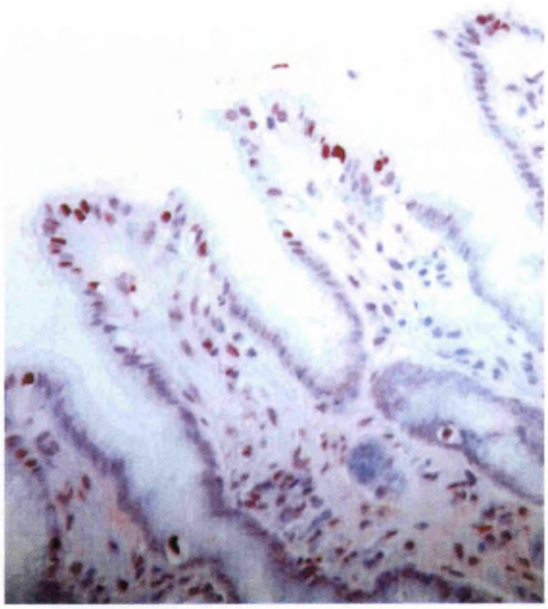

Fig. 1. Apoptotic cells in the foveolar cells compartment of the antrum mucosa (TUNEL histochemistry; original magnification ×400).

the antrum and corpus mucosa samples from 68 persons studied in 1979 and 1997 are presented in Table I. Both the antrum and corpus mucosa specimens of 9/68 persons in 1979 and 6/68 persons in 1997 were *H. pylori* negative. Both parts of the gastric mucosa remained *H. pylori* negative in only two persons at the initial and end points of observation. In 1997 the number of cases with a severe grade of *H. pylori* colonization was significantly higher than in 1979 (32/68 versus 16/68 for antrum mucosa specimens, $P = 0.004$; 31/68 versus 13/68 for corpus mucosa specimens, $P = 0.009$).

Table I. Grading of activity of gastritis, chronic inflammation, atrophy, intestinal metaplasia and *H. pylori* in the antrum and corpus mucosa in the samples of 1979 and 1997

	1979					1997				
Grade	Activity of gastritis	Chronic inflamm.	Atrophy	Intestinal metaplasia	*H. pylori*	Activity of gastritis	Chronic inflamm.	Atrophy	Intestinal metaplasia	*H. pylori*
Antrum										
0	31	24	53	58	15	16	7	51	55	9
1 (mild)	20	14	13	9	15	47	10	15	10	18
2 (moderate)	14	27	2	1	22	5	40	2	3	9
3 (severe)	3	3	0	0	16	0	11	0	0	32
Total	68	68	68	68	68	68	68	68	68	68
Corpus										
0	33	28	51	63	16	25	5	36	58	6
1 (mild)	20	17	10	5	16	29	11	17	9	21
2 (moderate)	12	15	5	0	23	14	29	10	1	10
3 (severe)	3	8	2	0	13	0	23	5	0	31
Total	68	68	68	68	68	68	68	68	68	68

Table II. Course of mucosal alterations in antrum and corpus during 18-year follow-up

Mucosal alterations 1979–1997	Antrum	Corpus
No development of atrophy	44	28
Development of atrophy	9	23
Progression of atrophy	2	4
Disappearance of atrophy	8	7
No changes	5	6
Total	68	68

Course of gastritis and dynamics of AI

The course of atrophy in the antrum and corpus mucosa during 18 years of observation is presented in Table II. During the follow-up period, development of any grade of atrophy in the antrum mucosa was established in 9/68 persons and in the corpus in 23/68 persons.

The dynamics of AI in three compartments of the studied sections in samples from persons without and with development of atrophy in the antrum mucosa are presented in Fig. 2; the dynamics of AI in the corpus is shown in Fig. 3.

Dynamics of AI in FC

The value of AI for FC of the antrum as well as of the corpus mucosa at two time points of observation showed no significant changes either in the persons without atrophy ($P = 0.74$ for the antrum and $P = 0.62$ for the corpus) or in the persons who developed atrophy ($P = 0.10$ for the antrum and $P = 0.18$ for the corpus).

Dynamics of AI in PZ and GP

AI for PZ and GP in the corpus mucosa specimens of 1997 decreased significantly compared with the specimens of 1979 irrespective of the dynamics of atrophic alterations, i.e. in persons who developed atrophy ($P = 0.005$ for PZ and $P =$

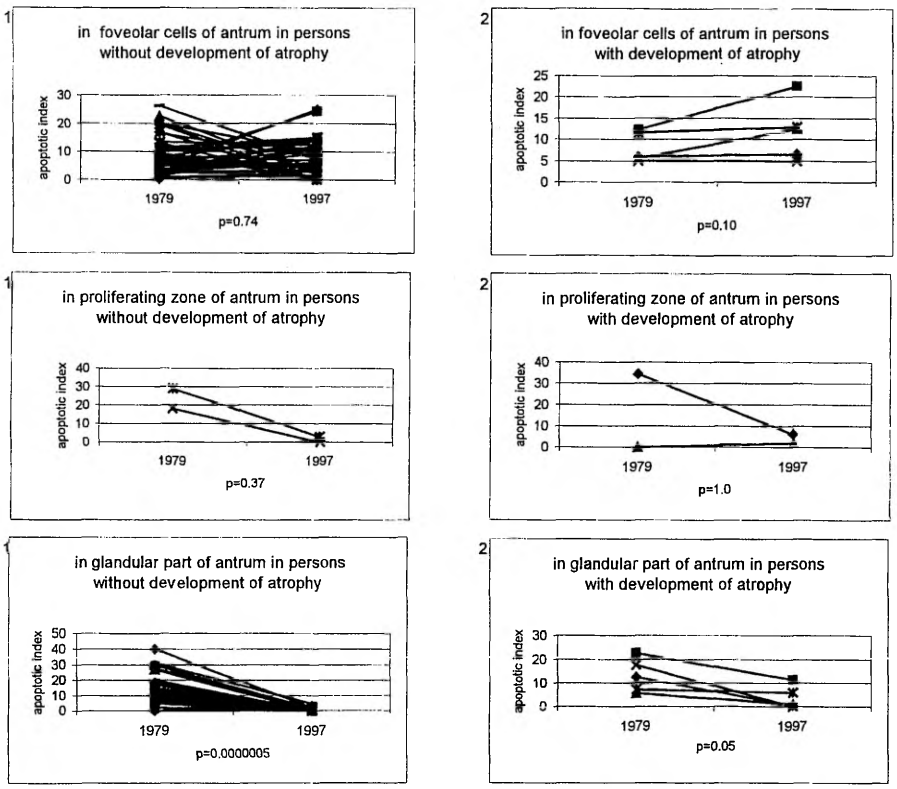

Fig. 2. Dynamics of apoptotic index for the antrum mucosa in persons without 1) and with development 2) of atrophy during 18 years of observation.

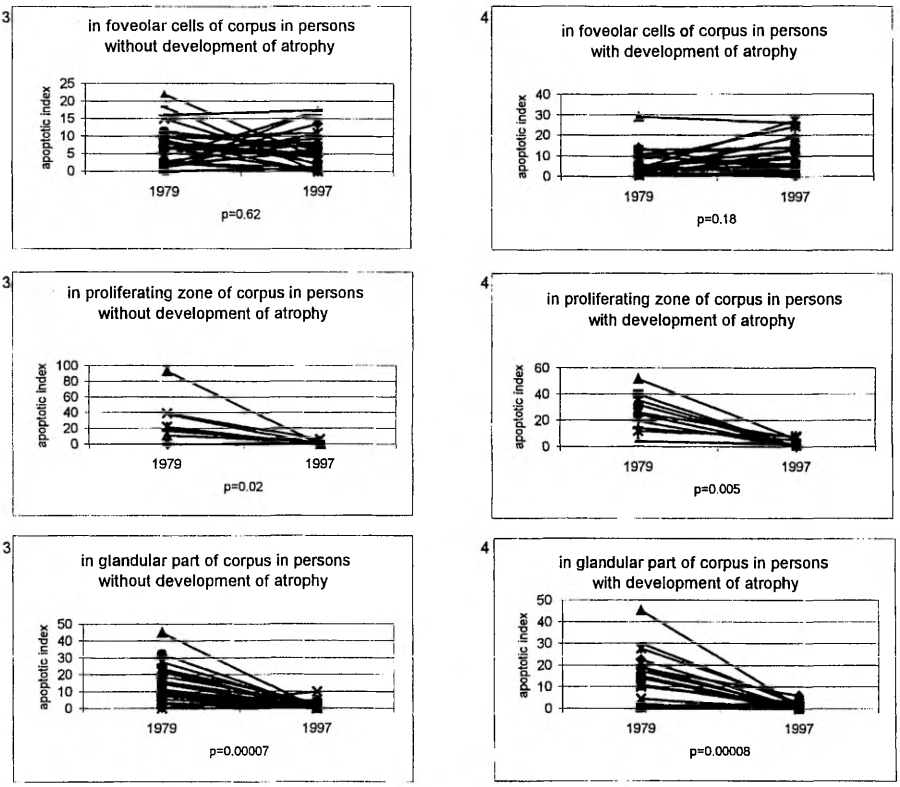

Fig. 3. Dynamics of apoptotic index for the corpus mucosa in persons without 3) and with development 4) of atrophy during 18 years of observation.

0.00008 for GP) as well as in persons without development of atrophy ($P = 0.02$ and $P = 0.00007$ for PZ and GP, respectively). In the antrum mucosa there occurred a significant decrease in AI for GP from the initial to the end point of observation in persons without development of atrophy ($P = 0.0000005$) and an obvious decrease of AI also in persons with development of atrophy ($P = 0.05$).

No significant difference was observed between the mean values of AI for FC, PZ and GP of the antrum in groups with and without development of atrophy either in the initial year of observation ($P = 0.98$, 1.0 and 0.63 for FC, PZ and GP, respectively) or at the end point ($P = 0.21$, 0.69 and 0.36 in FC, PZ and GP, respectively). Analogously, in FC, PZ and GP of the corpus, the mean values of AI did not differ significantly in groups with and without development of atrophy ($P = 0.33$, 0.82 and 0.71 for 1979 and $P = 0.30$, 0.52 and 0.94 for 1997).

According to multifactorial dispersion analysis (Table III), in 1979 the strongest independent effect on AI in the antrum mucosa was exerted by grade of activity of gastritis (in FC; $P = 0.01$) and degree of chronic inflammation (in GP; $P = 0.03$) and in 1997, by grade of *H. pylori* colonization (in FC and GP; $P = 0.02$ and 0.03); in the corpus mucosa, the strongest independent effect on AI in 1979 was exerted by activity of gastritis (in FC; $P = 0.02$) and chronic inflammation (in PZ; $P = 0.04$). In the samples of 1997 for corpus mucosa no analysed factor exerted a statistically significant effect on AI, only chronic inflammation tended to affect AI for PZ ($P = 0.09$).

In 1979, a significantly higher mean AI value for FC in the antrum was found in the case of higher activity of gastritis (sum of grades 1–3) compared with grade 0 activity (10.5 ± 6.9 versus 6.3 ± 5.8; $P = 0.01$). In the corpus mucosa higher AI for FC was observed in the case of

Table III. Results of multifactorial dispersion analysis of the effect of different variables on the expected values of AI in different compartments of antrum and corpus mucosa sections in the samples of 1979 and 1997

	1979						1997					
	Foveolar cells		Prolifer. zone		Glandular part		Foveolar cells		Prolifer. zone		Glandular part	
Factors affecting AI	*F* statistic	*P* value	*F* statistic	*P* value	*F* statistic	*P* value	*F* statistic	*P* value	*F* statistic	*P* value	*F* statistic	*P* value
					Antrum							
Age	2.80	0.09	0.69	0.42	0.006	0.93	1.22	0.27	4.83	0.06	0.24	0.61
Activity of gastritis	4.70	0.01	0.91	0.43	1.06	0.37	0.73	0.48	3.3	0.11	0.52	0.59
Chronic inflamm.	1.50	0.23	0.90	0.43	3.15	0.03	0.05	0.98	1.5	0.28	1.50	0.22
Atrophy	0.18	0.67	0.01	0.89	3.60	0.06	0.27	0.60	0.33	0.58	0.79	0.37
Intest. metaplasia	1.10	0.28	0.20	0.69	3.20	0.07	0.12	0.73	0.25	0.60	0.11	0.74
H. pylori	1.80	0.15	0.69	0.57	1.16	0.33	3.37	0.02	2.4	0.15	3.05	0.03
					Corpus							
Age	0.01	0.90	0.03	0.84	0.16	0.68	1.40	0.24	0.35	0.55	0.04	0.83
Activity of gastritis	1.54	0.22*	0.009	0.99	0.16	0.85	1.33	0.27	0.48	0.62	0.17	0.83
Chronic inflamm.	1.06	0.37	2.16	0.11†	1.27	0.29	0.96	0.41	2.35	0.09	0.94	0.42
Atrophy	0.15	0.85	0.79	0.46	0.47	0.62	0.25	0.85	0.28	0.83	0.30	0.82
Intest. metaplasia	0.10	0.74	1.38	0.24	0.004	0.94	0.23	0.63	1.11	0.30	1.20	0.27
H. pylori	0.49	0.68	0.80	0.50	0.62	0.60	0.75	0.52	0.15	0.92	0.30	0.82

AI = apoptotic index.
* After gradual exclusion of the other factors, the effect of activity of gastritis appeared to be significant ($P = 0.02$).
† The effect of chronic inflammation appeared to be significant ($P = 0.04$).

moderate activity of gastritis (9.2 ± 4.7 versus 6.4 ± 6.4 by grade 0; $P = 0.02$).

Concerning the grade of chronic inflammation, in 1979 mild grade of chronic inflammation had a significant positive effect on AI for GP of the antrum mucosa and for PZ of the corpus mucosa, then in 1997 a significantly lower AI was note in the case of severe grade of chronic inflammation in the corpus compared with grade 0 (1.1 ± 2.0 versus 2.9 ± 0.8; $P = 0.01$).

Significant association of AI with grade of *H. pylori* colonization was found only in antrum mucosa: in samples of 1979 in FC in the case of moderate *H. pylori* colonization compared with mild colonization (10.2 ± 6.9 versus 4.4 ± 3.7; $P = 0.01$) and in FC in the samples of 1997 in the case of severe (10.4 ± 6.7) versus mild colonization (3.6 ± 3.9; $P = 0.001$), as well as for GP in the case of severe (1.9 ± 2.4) versus mild colonization (0.3 ± 0.4; $P = 0.0006$).

Analysis of AI at different grades of atrophy and intestinal metaplasia in the samples of 1979 and 1997 revealed no significant association between the mean values of AI and grade of atrophy or presence of intestinal metaplasia either in the antrum or in the corpus ($P > 0.05$).

Multifactorial dispersion analysis of the effect of the 18-year dynamics of grade of the Sydney parameters (activity of gastritis, chronic inflammation, atrophy, intestinal metaplasia and *H. pylori* colonization) on the difference between the mean values of AI in 1979 and 1997 showed that only grade of *H. pylori* colonization had a significant effect on the difference between AI values for FC in the antrum. At higher scores of *H. pylori* colonization in 1997 the difference between mean AI values for FC in the antrum was significantly higher (F statistic = 3.21; $P = 0.03$).

Mild and moderate grades of activity of antrum gastritis in 1979 tended to affect the difference in mean AI values for FC in the antrum between the two time points ($F = 2.16$; $P = 0.12$) and severe grade of chronic inflammation in 1997 appeared to affect the difference in mean AI values for GP in the corpus mucosa between 1979 and 1997 ($F = 1.99$; $P = 0.05$).

Discussion

The present work represents the first prospective investigation of apoptosis in three different compartments of both antrum and corpus mucosa specimens, taken from a random adult cohort in the initial and end years of an 18-year follow-up of chronic *H. pylori* gastritis according to different grades of atrophy, chronic inflammation, activity of gastritis and *H. pylori* colonization. Although the results of a 31-year follow-up study of gastric cellular turnover and development of atrophy were recently published by Moss et al. (18), these authors examined apoptosis at one (initial) time point and only in corpus biopsies.

The specific finding of the present study was that compared with initial values AI for PZ and GP of the corpus mucosa as well as for GP of the antrum mucosa decreased significantly during 18 years of follow-up, irrespective of the dynamics of atrophic alterations (in persons without development of atrophy and in persons with development of atrophy), but not for the FC compartment.

There is evidence that cell turnover in the gastric mucosa is related to a delicate balance between cell proliferation and apoptosis. More intensive cell proliferation may induce increase in apoptosis, i.e. programmed cell death, in order to maintain tissue homeostasis (11, 26, 27). It can be supposed that cell turnover is more intensive in younger persons. Evidence also exists that some cell types may lose their ability to undergo apoptosis with increasing age (28). The subjects of our study were 18 years younger in 1979 than in 1997.

Therefore, one explanation of the significantly higher AI for PZ and GP of antrum and corpus mucosa specimens sampled in 1979, compared with those sampled in 1997, could have been the higher capacity of cells in PZ and GP for proliferation in the initial period of observation when persons were younger. Consequently, to keep cell turnover in balance, apoptosis must be more intensive. One can suppose that cell turnover in the FC compartment, which is continuously more exposed to several damaging factors, has to maintain intensive cell turnover also in old age. From a methodological point of view, comparison of paraffin-embedded biopsy specimens, obtained with an 18-year interval, should not affect the results, because according to the data of Bardales et al. (29) the use of formalin-fixed and paraffin-embedded archival material as old as 25 years yielded a staining pattern by the TUNEL method similar to that of freshly prepared tissues.

The fact that for all studied compartments of the antrum as well as of the corpus mucosa in the initial year of observation, AI was not significantly different in the group without atrophy and in the group who developed atrophy 18 years later, refutes our hypothesis that increased apoptosis might induce progression of atrophic gastritis. An analogous result was demonstrated in a follow-up study by Moss et al. (18), in which the ratio of apoptosis to proliferation was not a determinant of risk for development of atrophy 31 years later.

It seems that development of atrophy cannot be explained merely by violation of the balance between apoptosis and proliferation. An important scenario of development of autoimmune gastritis has been proposed by Judd et al. (30), namely that normal developmental pathways of the gastric mucosa are disrupted in autoimmune gastritis, resulting in an amplification of immature cell types. Differentiation of these immature cells appears to be blocked, which contributes to depletion of end-stage cells.

Concerning the association of apoptosis with gastritis the results of several studies are quite different. Thus the findings of a study of Steininger et al. (17) suggest that gastric atrophy might be the result of apoptosis in the gastric epithelium. Also, according to the opinion of Genta (31), increased apoptosis in the gastric mucosa can lead to development of atrophy. However, Moss et al. (11) found that apoptotic cell number does not correlate with the degree of histological gastritis and that a compensatory decrease in apoptotic activity takes place during progression of atrophic gastritis and intestinal metaplasia. A recent study of Scotiniotis et al. (14) showed that in *H. pylori* antral gastritis apoptosis was significantly reduced within foci of intestinal metaplasia due to increased cell proliferation, which could contribute to *H. pylori* associated gastric carcinogenesis. Neither the study by Moss et al. (11) nor the study by Anti et al. (7) established the correlation of AI with acute or chronic inflammation, whereas Jang & Kim (32) found an association between increased apoptosis in antral epithelial cells with the degree of acute inflammation and density of *H. pylori* colonization.

An important finding of our study was that the grade of activity of gastritis had the strongest effect on AI for FC, especially in the antrum mucosa, and the degree of chronic inflammation had the strongest effect on AI for GP in the antrum and for PZ in the corpus mucosa. According to our results, the grade of *H. pylori* colonization affects AI for FC and GP only in the antrum mucosa. The fact that increase in *H. pylori* colonization in the antrum during 18 years of observation had a significant effect on the difference in AI between two time points of observation only for FC of the antrum mucosa was also noteworthy.

Most authors have found a significant association between apoptosis and the presence of *H. pylori* in the gastric mucosa (11–15, 33–35), suggesting a direct effect (10, 11, 33, 35) of *H. pylori* on mucosal cell apoptosis, or on further modulation of gastric epithelial cell apoptosis by immune mediators produced by neutrophils in response to *H. pylori* (36).

The finding of Konturek et al. (13) suggests that *H. pylori* induces apoptosis in the gastric epithelium due to upregulation of proapoptotic Bax protein, while Bax overexpression is stronger in the antrum than in the corpus. The authors also found that activity of gastritis was higher in the antrum than in the corpus. These results are in accordance with our results concerning the association of AI in the FC compartment with grade of *H. pylori* colonization and activity of gastritis, which was more pronounced in the antrum than in the corpus mucosa.

A significant finding of our study was that the association of apoptosis with grade of *H. pylori* colonization was expressed differently in the antrum and corpus mucosa, and that the effect of grade of activity of gastritis and chronic inflammation on AI was more expressed in the antrum than in the corpus, which may support the evidence of a different environment in the antrum than in the corpus (37).

In conclusion, AI is related to intensity of gastritis in general but does not appear to be associated with atrophy or to predict atrophy. In the antrum, AI was also highly dependent on grade of *H. pylori* colonization.

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Seropositivity to *Helicobacter pylori* heat shock protein 60 is strongly associated with intensity of chronic inflammation, particularly in antrum mucosa: an extension of an 18-year follow-up study of chronic gastritis in Saaremaa, Estonia

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Abstract

Helicobacter pylori is a cause of chronic gastritis and leads to development of atrophy in some cases. There is evidence that the heat shock protein 60 (HSP60) of *H. pylori* is involved in induction of chronic inflammation. Seroprevalence of IgG antibodies to *H. pylori* HSP60 in an adult cohort from Saaremaa, Estonia (68 persons, median age 57 years), with a high prevalence of antibodies to cell surface proteins of *H. pylori* (92%) and a well characterized dynamics of chronic gastritis in an 18-year follow-up study, was tested using purified *H. pylori* HSP60 at a concentration of 1 μg ml^{-1} with ELISA. The state of the gastric mucosa and the presence of *H. pylori* in histological sections in the samples of 1979 and 1997 were assessed in accordance with the Sydney system. Seropositivity for *H. pylori* HSP60 was 65%. Immunological response to *H. pylori* HSP60 is associated with the morphological presence of *H. pylori* in the antrum and corpus ($P = 0.01$) and is strongly correlated with the grade of chronic inflammation, particularly in the antrum mucosa ($r = 0.34$; $P = 0.003$; OR = 5.97 (95% CI 1.21–29.3)), but is not associated with development of atrophy during 18 years of follow-up, or with the activity of gastritis. This finding supports the evidence that immunological response to *H. pylori* HSP60 may play a role in triggering of the inflammatory process in the gastric mucosa.

Keywords: *Helicobacter pylori* heat shock protein 60; IgG antibody titer; Chronic inflammation; Antrum; Corpus mucosa; Long-term follow-up of chronic gastritis

1. Introduction

Helicobacter pylori is a cause of chronic gastritis leading to development of gastric mucosa atrophy [1–4]. In 1992, Dunn et al. [5] identified a homologue of the chaperonin 60 family of heat shock proteins (HSP) in *H. pylori* and showed that this protein is immunogenic in individuals infected with *H. pylori*. Among other HSPs, *H. pylori* produces mainly two HSPs: a GroES analogue, class HSP A (HSP13), and a GroEL analogue, class HSP B (HSP60) [5–7]. Several reports have shown that *H. pylori* HSP60 plays a role in induction of chronic mucosal inflammation, suggesting also the triggering role of HSP60 in mucosal inflammation of an autoimmune type, which results in gastric atrophy [8–16]. Furthermore, considering the fact that HSP represents a dominant antigen in microbial infections, a role of pathogen-derived HSP, including HSPs of *H. pylori*, has been suggested in protective immunity and vaccination [17–19].

The aim of the present study was to evaluate the seroprevalence of IgG antibodies to *H. pylori* HSP60 in an adult cohort from Saaremaa, Estonia, with a high prevalence of antibodies to glycine-extracted cell surface proteins of *H. pylori* and a well characterized dynamics of chronic gastritis in an 18-year follow-up study [20–25]. Another aim was to investigate the association between

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the serological response to *H. pylori* HSP60 and the grade of *H. pylori* colonization, chronic inflammation, atrophy and activity of gastritis. Also, we analyzed whether the immunological response to *H. pylori* HSP60 was associated with the progression of chronic inflammation and atrophy during 18 years of follow-up.

2. Subjects and methods

2.1. Subjects

Of the initial 304 subjects of an adult sample from Saaremaa Island in Estonia, who had been investigated by endoscopy and gastric biopsy in 1979, 1985, and 1991, 70 persons were reinvestigated in 1997. The results of these investigations have been published previously [20–24]. Sixty-eight persons (31 men, 37 women, median age 57 in 1997) in whom the state of the gastric antrum and corpus mucosa was evaluated at the initial (1979) and end (1997) points of observation and from whom serum samples were collected in 1997, were included in the study.

During the 18-year follow-up of the 304 subjects, 39 had died by 1997, and of the remaining subjects 195 were lost to follow-up because of severe illness, change of residence, failure of communication or refusal. The studied persons had not received any *H. pylori* eradication therapy.

2.2. Histopathological examination

In 68 persons formalin-fixed, paraffin-embedded gastric biopsy specimens from the antrum (three specimens) and corpus mucosa (six specimens), taken in 1979 and 1997, were stained with hematoxylin and eosin, and with a modified Giemsa stain. The state of the gastric mucosa and the presence of *H. pylori* in histological sections were assessed in accordance with the updated Sydney system [26] and scored from 0 (no changes) through 1 (mild) and 2 (moderate) to 3 (severe changes). The course of gastritis was evaluated as the difference between the first (1979) and the last (1997) follow-up examination over 18 years [24].

2.3. Serological evaluation of IgG antibodies to glycine-extracted cell surface proteins of H. pylori

Seropositivity to *H. pylori* cell surface proteins (strain CCUG 17874) was evaluated using an enzyme-linked immunosorbent assay (ELISA) method as reported previously [24,25,27]. In brief, 0.5 μg of acid glycine-extracted cell surface proteins per well were used for coating microtiter plates (Nunc, Roskilde, Denmark). The studied sera were diluted to 1:800 and as the second antibody an alkaline phosphatase-labelled anti-human IgG (Dako, Glostrup, Denmark) diluted 1:500 was used. The results are expressed as corrected mean absorbance value as a percentage of the value for a reference standard (human γ-globulin, Pharmacia and Upjohn, Stockholm, Sweden) as relative antibody activity (RAA) [28]; the cut-off value for seropositivity was set at 25 RAA units [27]. The sensitivity of the test was 98% and the specificity 75%.

2.4. Serological evaluation of IgG antibodies to H. pylori HSP60

For determination of antibodies for *H. pylori* HSP60, LabSyst (Labsystems, Finland) 96-well plates were coated with HSP60 at a concentration of 1 μg ml^{-1} (0.1 μg per well) for 16 h at 4°C. The used HSP60 was purified from supernatant of sonicated *H. pylori* cells by gel filtration on Superose 12® and by ion exchange chromatography on MonoQ® at pH 6.7. The purity of antigen was checked by immunoblot using serum of *H. pylori*-infected patients and found to be approximately 97% homogeneous. After 1.5 h of blocking with 2% bovine serum albumin in phosphate-buffered saline (PBS), plates were washed four times with PBS-0.05% Tween 20 (Sigma, St. Louis, MO, USA). Serum samples were diluted to 1:800 in PBS-0.02% Tween and incubated for 1.5 h at 37°C. Alkaline phosphatase-conjugated rabbit anti-human IgG (Dako, Glostrup, Denmark) was used as the secondary antibody (dilution 1:500, incubation for 1 h at 37°C). Bound antibodies were visualized by addition of a substrate solution containing 1 mg ml^{-1} *p*-nitrophenylphosphatase (Sigma, St. Louis, MO, USA) in diethanolamine buffer, pH 9.8. The optical density was read at 405 and 492 nm after 50 min of incubation.

The results are expressed as corrected mean absorbance values as a percentage of the value for a reference standard (human γ-globulin, Pharmacia and Upjohn, Stockholm, Sweden) as relative antibody activity (RAA). The cut-off value for seropositivity to *H. pylori* HSP60 (RAA = 24) was based on results obtained by testing 28 sera, previously known to be negative for antibodies against the *H. pylori* glycine-extracted cell surface protein (mean absorbance value of 28 *H. pylori*-negative persons plus 2 S.D.). The sensitivity of the test was 69% and the specificity 83%.

2.5. Statistical analysis

Data are presented as positive or negative test results for antibodies against *H. pylori* glycine-extracted cell surface proteins and *H. pylori* HSP60 and as the mean values of RAA to *H. pylori* HSP60. Differences in the number of positive cases between the groups were tested using the χ^2 test. Differences in the mean values of RAA as the expression of the anti-*H. pylori* HSP60 IgG level were calculated with Student's *t*-test. Odds ratios (OR) with 95% confidence intervals (CI) were estimated using Mantel–Haenzel statistics with Exact software. Differences were considered statistically significant when the 95% CI did not include

the value 1.0. Correlation between the values of RAA of *H. pylori* HSP60 antibodies and the grade of *H. pylori* colonization, chronic inflammation, atrophy, and activity of gastritis, as well as their interrelationships were evaluated using Spearman's rank correlation test and multiple regression analysis (Statistika, Statsoft 99). P values < 0.05 were considered significant.

The Committee of Ethics of the University of Tartu approved this study.

3. Results

3.1. Seropositivity to H. pylori HSP60 in the serum samples of 1997 and its correlation with gastritis parameters

Forty-four out of 68 (65%) tested sera were positive for *H. pylori* HSP60 antibodies compared to 63 out of 68 (92%) positive sera in ELISA using *H. pylori* glycine-extracted cell surface proteins as the antigen. Seventy percent (44 out of 63) of sera positive for glycine-extracted cell surface proteins of *H. pylori* possessed IgG antibodies to *H. pylori* HSP60.

Grading of *H. pylori* colonization, activity of gastritis, chronic inflammation, and atrophy, as well as ORs for the association between positivity to *H. pylori* HSP60 and gastritis parameters and the mean values of RAA to *H. pylori* HSP60 in the antrum and corpus mucosa are presented in Table 1. A significant difference was found in seroprevalence with respect to *H. pylori* HSP60 between persons with histological presence and persons with histological absence of *H. pylori* in the gastric mucosa (41/59 versus 3/9 in antrum, $P = 0.03$; 43/62 versus 1/6 in corpus, $P = 0.01$). Among six persons whose antrum as well as corpus were morphologically *H. pylori*-negative, only one was seropositive for *H. pylori* HSP60, while two had antibodies to the *H. pylori* glycine-extracted cell surface antigen. The RAA value of anti-*H. pylori* HSP60 IgG in persons without histologically observable *H. pylori* colonization in antrum or in corpus mucosa was significantly lower than in persons with morphologically diagnosed *H. pylori* ($P = 0.01$ for the antrum and $P = 0.01$ for the corpus mucosa). However, the grade of histological *H. pylori* colonization did not correlate significantly with seropositivity for *H. pylori* HSP60 ($r = 0.12$, $P = 0.29$ for the antrum and $r = 0.07$, $P = 0.52$ for the corpus).

The correlation between anti-*H. pylori* HSP60 IgG antibodies and grade of chronic inflammation was significant ($r = 0.34$, $P = 0.003$ for the antrum and $r = 0.26$, $P = 0.04$

Table 1
Grade of activity of gastritis, chronic inflammation, atrophy, *H. pylori* colonization, and ORs for association between antibodies for *H. pylori* HSP60 and all these parameters for the antrum and corpus mucosa

Grading	Antrum				Corpus			
	n	IgG to HSP60 present	OR (95% CI)	RAA (mean ± S.D.)	*n*	IgG to HSP60 present	OR (95% CI)	RAA (mean ± S.D.)
Activity of gastritis								
0	16	8	1	35.3 ± 34.6	25	15	1	35.8 ± 28.5
1	47	31	1.93 (0.63–5.89)	42.7 ± 29.8	29	20	1.48 (0.49–4.40)	46.9 ± 31.1
2	5	4	4.0 (0.48–32.90)	52.2 ± 24.7	14	9	1.20 (0.32–4.30)	41.4 ± 29.7
3	0	0	–	–	0	0	–	–
1–3	52	35	2.05 (0.68–6.18)	43.6 ± 29.2	43	29	1.38 (0.5–3.740)	45.1 ± 30.4
Chronic inflammation								
0	7	2	1	13.4 ± 16.8*	5	1	1	12.8 ± 19.3†
1	10	6	3.75 (0.55–25.30)	44.6 ± 34.7**	11	6	4.8 (0.53–43.20)	38.4 ± 29.9
2	40	28	5.83 (1.13–29.90)	44.7 ± 30.0	29	20	8.8 (1.1–69.0)	42.5 ± 28.5
3	11	9	11.2 (1.41–89.50)	61.3 ± 31.4***	23	17	11.3 (1.37–93.40)	48.5 ± 31.0††
1–3	61	43	5.97 (1.21–29.30)	47.7 ± 31.2****	63	43	8.6 (1.18–61.50)	43.9 ± 29.5†††
Atrophy								
0	51	33	1	42.5 ± 31.2	36	22	1	41.2 ± 30.6
1	15	10	1.09 (0.33–3.50)	45.0 ± 30.2	17	11	0.33 (0.08–1.33)	38.0 ± 26.0
2	2	1	0.54 (0.05–5.59)	24.0 ± 8.4	10	8	2.54 (0.53–12.10)	48.0 ± 32.4
3	0	0	–	–	5	3	0.95 (0.16–5.40)	45.6 ± 38.4
1–3	16	11	1 (0.32–3.39)	42.5 ± 29.2	32	22	1.4 (0.51–3.720)	42.3 ± 29.4
H. pylori								
0	9	3	1	20.8 ± 24.2#	6	1	1	14.3 ± 17.7•
1	18	13	5.2 (1.02–26.40)	48.7 ± 33.7##	21	16	16 (1.92–133.0)	50.6 ± 33.0••
2	9	7	7.0 (1.0–48.60)	47.5 ± 26.3	10	8	20 (1.82–218.0)	38.9 ± 21.9
3	32	21	3.82 (0.87–16.60)	42.0 ± 28.4###	31	19	7.9 (1.07–58.20)	41.9 ± 29.3•••
1–3	59	41	4.55 (1.1–18.50)	44.8 ± 29.5####	62	43	11.3 (1.62–78.90)	44.3 ± 29.5••••

Significant difference: * < ** $P = 0.02$; * < *** $P = 0.003$; * < **** $P = 0.003$; # < ## $P = 0.03$; # < ### $P = 0.01$; # < #### $P = 0.01$; † < †† $P = 0.02$; † < ††† $P = 0.01$; • < •• $P = 0.01$; • < ••• $P = 0.02$; • < •••• $P = 0.01$.

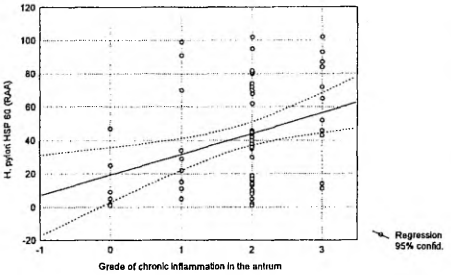

Fig. 1. Correlation between anti-*H. pylori* HSP60 RAA and grade of chronic inflammation in the antrum mucosa according to the samples of 1997. $r = 0.34$, $P = 0.003$.

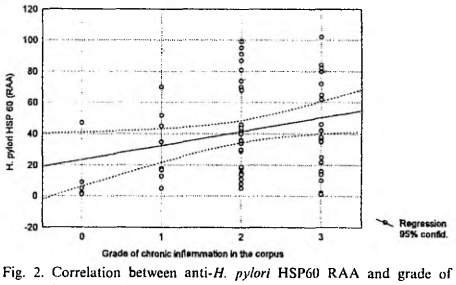

Fig. 2. Correlation between anti-*H. pylori* HSP60 RAA and grade of chronic inflammation in the corpus mucosa according to the samples of 1997. $r = 0.26$, $P = 0.04$.

for the corpus mucosa) (Figs. 1 and 2). The OR for the association of seropositivity for *H. pylori* HSP60 and chronic inflammation was particularly high in the case of a severe grade of chronic inflammation both for the antrum (OR = 11.2 (95% CI 1.41–89.5)) and for the corpus mucosa (OR = 11.3 (95% CI 1.37–93.4)). Persons with a severe grade of chronic inflammation expressed simultaneously in both parts of the stomach displayed significantly higher values of RAA of anti-*H. pylori* HSP60 (73.8 ± 20.8) compared with those with absence of chronic inflammation (15.5 ± 21.2, $P = 0.004$) (Table 2).

No significant correlation was found of seropositivity to *H. pylori* HSP60 with grade of atrophy ($r = 0.013$, $P = 0.91$ for the antrum and $r = 0.04$; $P = 0.70$ for the corpus), or with activity of gastritis ($r = 0.20$, $P = 0.08$ for the antrum; $r = 0.12$, $P = 0.29$ for the corpus).

In multiple regression analysis, *H. pylori* HSP60 was significantly associated with the grade of chronic inflammation (for the antrum: $\beta = 0.635$, $P = 0.003$; for the corpus: $\beta = 0.474$, $P = 0.01$). The grade of chronic inflammation in the antrum was the highest independent predictor of seropositivity to *H. pylori* HSP60 ($\beta = 0.628$, $P = 0.01$). Moreover, a correlation between grade of chronic inflammation and grade of *H. pylori* was found for the antrum ($r = 0.45$, $P = 0.00008$) but not for the corpus mucosa ($r = 0.19$, $P = 0.11$).

3.2. Progression of chronic inflammation and seropositivity to H. pylori HSP60

The progression of chronic inflammation in grade in the antrum and corpus mucosa, positivity, and the mean values of RAA to *H. pylori* HSP60 in the serum samples of 1997 are presented in Table 3. Persons whose grade of chronic inflammation in the antrum increased from grade 0 in 1979 to grade 3 in 1997 showed significantly higher values of RAA to *H. pylori* HSP60 (65.7 ± 21.2) compared with those who had no inflammation (17.4 ± 18.8, $P = 0.008$) or with those in whom inflammation regressed.

The corpus mucosa of persons whose chronic inflammation increased from mild to severe displayed significantly higher mean values of RAA to *H. pylori* HSP60 (72.5 ± 34.8) compared with persons in whom chronic inflammation remained mild during the follow-up period ($P = 0.04$). Also, persons with severe chronic inflammation in the corpus at both time points showed a more pronounced response to *H. pylori* HSP60 (62.7 ± 30.6) than those who had moderate chronic inflammation in 1979 and 1997 (29.0 ± 16.5, $P = 0.02$).

3.3. Dynamics of atrophy and seropositivity to H. pylori HSP60

Additionally, we compared seropositivity for *H. pylori* HSP60 in persons who developed atrophy in the antrum during 18 years of observation (four out of five cases;

Table 2
Seropositivity for *H. pylori* HSP60 at different grades of chronic inflammation expressed simultaneously in the antrum and corpus mucosa accordingly to the samples of 1997

Grade of chronic inflammation		*n*	IgG to HSP60 present	OR (95% CI)	RAA (mean ± S.D.)
Antrum	Corpus				
0	0	4	1	1	15.5 ± 21.2*
1	1	3	2	6.0 (0.34–103.80)	48.3 ± 37.5
2	2	19	14	8.4 (0.94–74.80)	42.1 ± 25.2
3	3	5	5	15 (0.95–236.0)	73.8 ± 20.9**

** > * $P = 0.004$.

Table 3
Dynamics of grade of chronic inflammation in the antrum and corpus mucosa and seropositivity to *H. pylori* HSP60

Dynamics of chronic inflammation (grades 0–3)		Antrum				Corpus			
1979	1997	*n*	IgG to HSP60 present 1997	OR (95% CI)	RAA (mean ± S.D.)	*n*	IgG to HSP60 present 1997	OR (95% CI)	RAA (mean ± S.D.)
0	0	5	2	1	17.4 ± 18.8*	4	1	1	15.5 ± 21.2
0	1	2	1	3.2 (0.50–20.60)	40.5 ± 41.7	5	4	12.0 (0.74–192.30)	51.0 ± 33.5
0	2	13	8	2.4(0.35–16.40)	42.7 ± 35.0	9	6	6.0 (0.57–62.60)	49.3 ± 34.7
0	3	4	4	6.0 (0.49–72.90)	65.7 ± 21.2**	10	5	3.0 (0.31–28.70)	33.1 ± 30.0
1	1	4	4	6.0 (0.49–72.90)	56.0 ± 29.6	5	1	0.75 (0.05–10.50)	24.6 ± 25.8†
1	2	7	5	3.7 (0.41–33.70)	44.0 ± 26.8	7	6	18.0 (1.15–280.80)	46.7 ± 27.9
1	3	2	1	1.5 (0.09–24.00)	47.5 ± 51.6	4	3	9.0 (0.54–148.10)	72.5 ± 34.8††
2	2	17	12	3.6 (0.54–23.80)	38.1 ± 20.2	9	5	3.8 (0.37–37.30)	29.0 ± 16.5#
2	3	5	4	6.0 (0.49–72.90)	62.2 ± 34.9	5	5	15.0 (0.95–236.50)	48.8 ± 17.0
3	3	0	0	–	–	4	4	12.0 (0.74–192.30)	62.7 ± 30.6##
1	0	1	0	0	2.0	1	0	0	2.0
2	0	1	0	0	5.0	0	0	–	–
3	0	0	0	–	–	0	0	–	–
2	1	4	1	0.5 (0.04–6.00)	35.2 ± 43.0	1	1	0	45.0
3	2	3	2	3.0 (0.22–40.30)	38.6 ± 34.5	4	3	9.0 (0.54–148.10)	50.5 ± 35.8
Total		68	44			68	44		

** > * $P = 0.008$; †† > † $P = 0.04$; ## > # $P = 0.02$.

RAA = 49 ± 21.4) and in persons who did not develop it (24 out of 37 cases; RAA = 41.2 ± 29.5, $P = 0.57$, OR = 2.16 (95% CI 0.29–15.9)). Among persons who developed atrophy in the corpus mucosa, 17 out of 23 cases displayed seropositivity for *H. pylori* HSP60 (RAA = 44.7 ± 27.5), while among persons who did not develop atrophy, seropositivity was detected in 16 out of 27 cases (RAA = 40.0 ± 32.2, $P = 0.58$).

4. Discussion

In the present study the seroprevalence of IgG antibodies to *H. pylori* HSP60 was evaluated in a random adult cohort with knowledge of the dynamics of chronic gastritis during an 18-year follow-up and with a high prevalence of antibodies to the glycine-extracted cell surface proteins of *H. pylori* [20–26].

The specific finding of the study was that immunological response to *H. pylori* HSP60 was restricted to morphological presence of *H. pylori* and was strongly correlated with grade of chronic inflammation, particularly in the antrum mucosa.

Our results concerning seropositivity to *H. pylori* HSP60 among *H. pylori*-infected persons are in accordance with the data of Macchia et al. [6], who found that 50% of *H. pylori*-infected patients with gastroduodenal diseases displayed an immune response for *H. pylori* HSP60. These authors noted that the presence of *H. pylori* HSP60 positivity varied greatly among different individuals. Suerbaum et al. [7] demonstrated too that 76.3% of *H. pylori*-infected patients were positive for MBP-HspB recombinant proteins. Why only some of the infected persons develop an immunological response to HSP60 is not known. Kansau and Labigne [29] explained this by host-specific factors.

In our study we established a significant difference in seroprevalence for *H. pylori* HSP60 between persons in whom *H. pylori* was morphologically present in the gastric mucosa and those in whom it was absent. A noteworthy finding of our study is that the immunological response to *H. pylori* HSP60 is strongly correlated with grade of chronic inflammation, particularly in the antrum mucosa, supporting the view that *H. pylori* HSP60 is able to evoke an inflammation response in the gastric mucosa [5,8,9–11,13–16]. A good correlation between serological response to *H. pylori* Hp54K (an analogue of HSP60) and mucosal inflammation (both acute and chronic) was found in a study of Perez-Perez et al. [10]. In our study, however, we could not establish a correlation between serological response to *H. pylori* HSP60 and grade of activity of gastritis in the antrum or in the corpus mucosa. Nor did we detect any association with atrophy or with dynamics of atrophic alterations during 18 years of follow-up, whereas in a study of Barton et al. [12], IgA antibodies to mycobacterial and human HSP60 (sharing 75% sequence homology with *H. pylori* HSP60) were associated with the presence of gastric atrophy.

The particular association of a serological response to *H. pylori* HSP60 with chronic gastric inflammation can be explained by the fact that *H. pylori* HSP60 induces interleukin-8 (IL-8) secretion from human gastric cells [16]. IL-8 is known to be an inflammatory cytokine [30].

The important finding of our study was that the pres-

ence of antibodies to *H. pylori* HSP60 was also associated with an increase in chronic inflammation during 18 years of follow-up. Persons who developed a higher grade of inflammation during 18 years also had a higher level of IgG antibodies to *H. pylori* HSP60 at the end point of observation. Regrettably, since serum samples from 1979 were not available any more, it was not possible to follow the presence of antibodies to *H. pylori* HSP60 during this period. Nevertheless, the follow-up data support the view that *H. pylori* HSP60 plays an important role in triggering gastric mucosal inflammation [10,14].

In conclusion, the immunological response to *H. pylori* HSP60 was associated with the histological presence of *H. pylori* in the antrum and corpus mucosa and was strongly correlated with grade of chronic inflammation, particularly for the antrum mucosa, as well as with progression of chronic inflammation, but not with development of atrophy during 18 years of follow-up or with activity of gastritis.

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GLANDULAR PROLIFERATION AND HOMEOSTASIS OF SPECIFIC CELLS ARE DIFFERENTLY AFFECTED IN GASTRIC ANTRUM AND CORPUS IN *HELICOBACTER PYLORI* ASSOCIATED GASTRITIS

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Abstract

The aim of the study was to assess the effect of *H. pylori* on epithelial cell proliferation and on the expression of the apoptosis protector oncoprotein bcl-2 at different grades of chronic inflammation and atrophy in the antrum and corpus mucosa. Furthermore, the effect of *H. pylori* and of the severity of chronic gastritis on the density of gastrin (G) and somatostatin (S) cells as well as on the number of β- H+, K+ ATP-ase immunoreactive cells was tested. The antrum and corpus biopsies of 59 patients (34 men, median age 47) endoscoped at the Gastrointestinal Unit of University Hospital, Bern, were analysed for *H. pylori* density, presence of chronic inflammation and atrophy. Immunostaining for PCNA (APAAP method), Ki-67, bcl-2, G-, and D-cells, as well as for β- H+, K+ ATP-ase (Avidin-Biotin method) was performed on formalin fixed, paraffin embedded biopsy specimens. Gastric mucosal glands in the antrum and corpus were divided into the foveolar cell (FC) compartment, the proliferating zone (PZ) and the glandular part (GP). There was a significant correlation between the labelling index of PCNA and Ki-67, and severity of chronic inflammation and atrophy in the corpus ($p=0.03$; $p=0.01$) but not in the antrum mucosa. Bcl-2 positive epithelial cells were found in PZ of atrophic antrum and PZ and GP of non-atrophic and normal corpus glands. In the corpus mucosa, the density of bcl-2 positive interstitial lymphocytes in PZ and GP was significantly higher in atrophic than in non-atrophic mucosa ($p=0.01$; $p=0.002$), while in the antrum bcl-2 positivity of interstitial lymphocytes was more associated with *H. pylori* score ($p=0.08$). The densities of G and D cells in the PZ of antral glands and of β- H+, K+ ATP-ase positive parietal cells in the

corpus mucosa showed significant negative correlations with severity of atrophic alterations (p=0.009; p=0.0004; p=0.0009), but not with *H. pylori* density (p=0.87). We conclude, that severity of gastritis and presence of *H. pylori* affect gastric epithelial cell proliferation and the expression of bcl-2 protein in gastric epithelial cells and interstitial lymphocytes differently in the antrum and corpus mucosa.

Key words: *Helicobacter pylori*, chronic gastritis, antrum and corpus mucosa, PCNA, Ki-67, bcl-2, gastrin and somatostatin cells, β - H+, K+ ATP-ase.

Introduction

Helicobacter pylori (*H. pylori*) is associated with enhanced gastric inflammatory response, atrophy and increased risk for development of peptic ulcer and gastric cancer [10, 16, 36, 41, 43]. There is also evidence that *H. pylori* is associated with changes in epithelial cell turnover, including the influence on programmed cell death or apoptosis [1, 5, 9, 19, 20, 25, 28, 33, 50]. Up-regulation of apoptosis protectors may supply cells with a survival advantage and hence play a role in carcinogenic pathway [26]. The expression of the apoptosis protector, bcl-2 protein, is enhanced in the case of gastric carcinoma as well as in the proliferative zone of gastric glands in presence of chronic atrophic gastritis with intestinal metaplasia [26, 38, 40, 47].

Several earlier studies have shown that *H. pylori* can affect gastrin (G) cells as well as somatostatin (D) cell density, in particular with selective reduction and suppression of D-cells, which results in reduced inhibition of G-cell secretion by somatostatin [17, 21, 22, 32, 34, 35, 37, 39, 48]. In contrast, the influence of *H. pylori* on the density of parietal cells, expressing the β subunit of H+, K+ ATP-ase, is not so widely described. However, recent studies have demonstrated association between *H. pylori* and development of autoimmune reactions against canalicular structures within human parietal cells [14, 51]. Gastric H+, K+ ATP-ase represents a major autoantigen in the canaliculi of parietal cells and the association of anticanalicular autoantibodies with gastric corpus atrophy was confirmed [8, 14, 23, 51]. The significance of homology between the β subunit of H+, K+ ATP-ase of parietal cells and *H. pylori* urease was also shown [49]. These relationships suggest a role of *H. pylori* in development of gastric corpus autoimmunity [14, 49].

The aim of the present study was to assess the effect of *H. pylori* on epithelial cell proliferation and on the expression of the apoptosis protector oncoprotein bcl-2 as a function of gastric site (antrum versus corpus), grade of chronic inflammation and atrophy. Furthermore, using the same biopsy specimens, we aimed to assess the effect of *H. pylori* and the severity of gastritis on the density of G and D cells and β H+, K+ ATP-ase immunoreactive cells.

In this study we tested the hypothesis that *H. pylori* enhances epithelial cell proliferation and affects the expression of the bcl-2 protein as well as the density of specific glandular cells in the antrum and corpus at advanced grades of chronic inflammation and in case of antrum and corpus mucosal atrophy.

Subjects and Material

A total of 59 patients (34 men, median age 47), endoscoped at the Gastro-intestinal Unit of University Hospital, Bern, during 1993 (retrospective study – 39 persons) and in January-February 1994 (prospective study -20 persons) were studied. The group of patients included 20 persons with peptic ulcer (gastric ulcer – 4 cases; duodenal ulcer – 16 cases); 18 with erosion in the antrum or/and corpus mucosa and duodenitis; 5 with hiatus hernia; one with reflux oesophagitis; one with gastric corpus adenocarcinoma; one with a tumor of the liver and pancreas; one with colon carcinoma; and one with liver cirrhosis. In 11 patients no pathological gastroduodenoscopic findings were established.
In 40 patients, minimum two biopsies were sampled both from the antrum and corpus, in 16 patients only from the antrum, and in 3 patients only from the corpus. The 56 antrum and 43 corpus biopsies were fixed in 4% formalin and embedded in paraffin.

Methods

Histopathologic evaluation
The state of the gastric mucosa and the presence of *H. pylori* was examined in haematoxylin and eosin-stained sections and graded as normal — no loss of glands and no chronic inflammation (score 0), non-atrophic gastritis: chronic inflammation present without atrophy and scored as 0.5 (mild), 1.0 (moderate) and 1.5 (severe) and atrophic gastritis in three stages: mild, moderate, severe loss of mucosal glands, varying grade and extent of inflammation (score numbers correspondingly 2, 3, 4). The severity of *H. pylori* colonization was evaluated as follows: absence of bacteria (grade 0), mild (grade 1), moderate (grade 2) or severe (grade 3) colonization, according to evaluation of gastritis, described by Kekki *et al.* [24] and used in the study of Maaroos *et al.* [32]. These grades correspond to the Sydney classification system [13].

Immunohistochemistry
Gastric epithelial cell proliferation was assessed by employing both the Proliferating Cell Nuclear Antigen (PCNA) and Ki-67 (MIB 1) labelling. Since PCNA is increasingly expressed through the G_1 phase, reaching its maximum in the early S phase and persisting in the G_2 phase [31], while the Ki-67 antigen is expressed in all phases except G_0 and early G_1 [15], we used both antibodies.

PCNA immunostaining was performed using the alkaline phosphatase anti-alkaline phosphatase (APAAP) procedure. After deparaffinisation and pre-treatment with 20% acetic acid (15 sec, 4°C) and 3% BSA (Merck) in TRIS-NaCl buffer (20 min., 20°C), tissue sections were incubated overnight (4°C) with a primary mouse monoclonal antibody against PCNA (PCNA PC10, DAKO, Glostrup, Denmark, dilution 1:50). Rabbit anti-mouse immunoglobulin (DAKO, Glostrup, Denmark, dilution 1:30) was used as the secondary antibody (incubation 30 min, at 20°C). The APAAP mouse monoclonal antibody complex (DAKO, Glostrup, Denmark, dilution 1:50) was then applied for 60 min. To enhance the intensity of final staining, these two steps were repeated. New fuchsine (Merck)- naphthol AS-Bi phosphate (Sigma, St. Louis, USA), in TRIS-HCl buffer, pH 8.7, containing 2.1 mg/ml levamisole (Sigma, St. Louis, USA) was used as the alkaline phosphatase substrate for 20 min. at room temperature. The reaction was stopped by rinsing the sections in cold water. The sections were counterstained with haematoxylin (Merck) and mounted with Aquatex (Merck).

Immunostaining for Ki-67, bcl-2 oncoprotein, as well as for gastrin, somatostatin, and β-H+, K+ ATPase immunoreactive cells was performed using avidin-biotin procedure. After deparaffinisation and rehydration, the sections were treated in the microwave oven in 10 mM citrate buffer, pH 6.0 at 190 W for 5 min. and twice at 140 W for 5 min. before staining for Ki-67, bcl-2 and gastrin cells. Further, the plastic jar was allowed to cool for 15 min. at room temperature and was then washed for 10 min. in TRIS-NaCl buffer, pH 7.5.

As primary antibodies, monoclonal anti Ki-67 (MIB-1, Dianova, Hamburg, dilution 1:50), the anti-human bcl-2 oncoprotein (clone 124, DAKO, Glostrup, Denmark, dilution 1:50), the anti- β subunit of H+, K+ ATP-ase (AB 2611 kindly donated by Dr. J.G. Forte, Berkeley, USA; dilution 1:200), and polyclonal anti-gastrin (DAKO, Glostrup, Denmark; dilution 1:300) and anti-somatostatin (DAKO, Clostrup, Denmark; dilution 1:1000) were used. Anti- β-H+, K+ ATPase and anti-somatostatin antibodies were applied on tissue sections without microwave pre-treatment of the last. All primary antibodies were diluted in the TRIS-NaCl buffer with 0.1% casein and 0.1% NaN_3 (pH 7.5). The preparations were incubated with primary antibodies for 60 min. at 20°C. Subsequently, the sections were incubated with rabbit anti-mouse biotinylated immunoglobulins (DAKO, Glostrup, Denmark) for monoclonal primary antibodies, or with swine anti-rabbit immunoglobulins biotinylated immunoglobulins (DAKO, Glostrup, Denmark) for polyclonal primary antibodies at dilution 1:200 for 45 min. The Avidin-Biotin Complex (DAKO, Glostrup, Denmark) was applied for 45 min. at room temperature. Alkaline phosphatase reaction was developed for 30 min. in the New Fuchsine substrate solution. The specimens were then washed in tap water, counterstained with haematoxylin and mounted with Aquatex (Merck). Negative controls were performed by replacing the primary antibodies by the TRIS-NaCl buffer.

Evaluation of immunostained sections

The sections were examined using an objective x40 and an eyepiece x10. Antral and corpus mucosa glands were divided into the foveolar cells compartment (FC), the proliferating zone (PZ; neck-isthmus) and the glandular part (GP). In each of them five fields were examined.

Only bright red stained nuclei were considered positive either for PCNA or for Ki-67. PCNA and Ki-67 labelling index (LI) were defined as the proportion of bright red stained nuclei in relation to 100 nuclei counted per field. Bcl-2 positive staining for interstitial lymphocytes was scored as 0-5. Bcl-2 staining of epithelial cells in the antrum and corpus mucosa was considered positive (red staining of cytoplasm) or negative.

The densities of G, D and β- H+, K+ ATP-ase positive cells were expressed as the mean values of cells counted in five fields of each gland part. The slides were analysed by one investigator (T.V.) who was not aware of the data of histological alterations. The intraobserver variability was 12.6±7.0 %.

Statistics

Values of LI were indicated as mean ± SD. Statistical analysis was done with Student's t-test, χ^2, Spearman rank correlation analysis (Statgraphics software) as well as with the use of multiple regression analysis (Statistika, Statsoft 99). P-values <0.05 were considered significant.

The Ethics Committee of University Bern, Switzerland has approved the study protocol.

Results

The state of the antrum and corpus mucosa and the presence of *H. pylori* in the studied biopsy specimens are presented in Table 1. There was a significant positive correlation between density of *H. pylori* and severity of gastritis in the antrum (r=0.37, p=0.006) but not in the corpus mucosa (r=0.13, p=0.37).

Gastric epithelial cell proliferation in association with severity of gastritis and H. pylori colonization

Mean PCNA and Ki-67 LI in different parts of antral and corpus glands, and their relation to gastritis and *H. pylori* status are presented in Table 2. Overall mean PCNA LI was significantly higher in the antrum than in the corpus mucosa (p=0.02 for PZ; p=0.01 for GP). PCNA LI in FC and GP of corpus glands by presence of atrophic alterations was significantly higher as compared to non-atrophic and normal mucosa (p=0.007 and p=0.03 respectively). Also the correlation analysis showed a significant correlation of PCNA LI in corpus glands with severity of chronic inflammation and atrophy (r=0.34; p=0.03 for FC and r=0.46; p=0.001 for GP). However, there was no correlation of PCNA

LI and severity of gastritis in antrum (p=0.40, p=0.81; p=0.29 for FC, PZ and GP respectively). PCNA LI and Ki-67 LI in FC, PZ and GP of the corpus mucosa did not correlate with the *H. pylori* colonization (p=0.47, p=0.53; p=0.09 for PCNA LI and p=0.99; p=0.51; p=0,14 for Ki-67). GP of antral glands, however, revealed a tendency for a negative correlation between mean PCNA LI and *H. pylori* density score (r=-0.23; p=0.09). Mean values of PCNA and Ki-67 LI in the antrum and corpus were significantly correlated (p=0.01 to 0.004 depending on the gland part). A significant positive correlation was found between Ki-67 LI and severity of chronic inflammation and atrophy in FC and GP of corpus mucosa (r=0.32; p=0.04; r=0.46; p=0.01), but not with *H. pylori* density score (p=0.12; p=0.70; p=0.51 in FC, PZ and GP).

Multiple regression analysis showed that proliferation activity in the corpus mucosa depended significantly on the severity of chronic inflammation and atrophy but not on the *H. pylori* density (for PCNA LI in FC β=0.51; p=0.0007; in GP β= 0.46; p=0.002; for Ki-67 LI in FC β=0.33; p=0.03; for GP β=0.36; p=0.01). In the antrum mucosa proliferation activity showed a tendency for negative association with antrum gastritis only for Ki-67 in PZ (β= –0.23; p=0.09).

Immunoreactivity of gastric epithelial cells and interstitial lymphocytes for bcl-2 protein

Bcl-2 positive staining was found in *epithelial cells,* i.e. parietal cells in the corpus and neck cells in the antral mucosa (in 16 cases: 8 in the antrum and 8 in corpus mucosa; (Table 3). In the antrum positive staining for bcl-2 positive epithelial cells was observed predominantly in the atrophic PZ of the mucosa (4/8 cases), whereas in the corpus bcl-2-positive staining of epithelial cells occurred in the normal and non-atrophic GP of the glands (3/8 and 2/8 cases respectively), in 2 out 8 cases in non-atrophic PZ and only in one case in atrophic PZ of corpus mucosa.

The density of bcl-2 positive *interstitial lymphocytes* in the corpus mucosa was positively correlated with the severity of chronic inflammation and atrophy in PZ and GP (r=0.37, p=0.01; r=0.29, p=0.04). The mean value of bcl-2 positive staining score of interstitial lymphocytes (Table 3) was significantly higher in atrophic corpus gastritis than in non-atrophic gastritis (in FC, PZ and GP, p=0.01, p=0.02 and p=0.002 respectively). The overall mean bcl-2 positive staining score of interstitial lymphocytes for PZ of the antrum mucosa was significantly higher compared with the respective score for PZ of the corpus mucosa (p=0.02).

Multiple regression analysis showed that the bcl-2 positive staining of interstitial lymphocytes in the corpus mucosa depended significantly on the severity of gastritis but not on the *H. pylori* density score (for FC β=0.45; p=0.002; for PZ β=0.47; p=0.001; for GP β=0.58; p=0.00003). In the antral

mucosa, positive staining of interstitial lymphocytes for bcl-2 was more associated with *H. pylori* score (in PZ β=0.24; p=0.08).

G, D and β- H+, K+ ATP-ase –positive cell densities and association with gastritis and H. pylori scores.

The densities of G and D cells in PZ of antral glands revealed a significant negative correlation with the severity of atrophic alterations score (r=–0.34, p=0.009, r=–0.52, p=0.0004, respectively). Mean G and D cell density (Table 4) was significantly lower in non-atrophic and in atrophic gastritis compared with the normal mucosa (p=0.04; p=0.02 for G cells and p=0.02 and p=0.0005 for D cells). Mean G cell number in the *H. pylori* positive atrophic mucosa tended to be lower (16.2±14.7) compared with the *H. pylori* negative atrophic mucosa (26.2±25.2, p=0.22), and D cell density in the antrum tended to have a negative correlation with *H. pylori* score in the antrum (r=–0.24, p=0.06, respectively).

The density of β-H+, K+ ATP-ase positive parietal cells in the atrophic corpus mucosa was significantly lower compared with the normal mucosa (p=0.0004) and non-atrophic gastritis (p=0.0001). There were no significant differences between the mean densities of β-H+, K+ ATP-ase positive parietal cells in the *H. pylori* positive and *H. pylori* negative mucosa (p=0.29). Also, multiple regression analysis showed that the density of β- H+, K+ ATP-ase positive cells was significantly dependent on grade of atrophy in corpus (β=–0.65; p=0.000007) but not on *H. pylori* score (β=0.053; p=0.66).

The comparison of the antrum and corpus with regard to proliferation activity, expression of bcl-2 and density of specific cells in association with severity of gastritis and *H. pylori* colonization is summarized in Table 5.

Discussion

In the present study, gastric epithelial cell proliferation, the expression of the apoptosis protector, bcl-2 protein, as well as the density of specific cells in the antrum and corpus mucosa and their dependence on severity of gastritis and *H. pylori* colonization were studied with conventional morphologic and immuno-histochemic techniques in gastric biopsies from 59 patients who had an upper gastrointestinal endoscopy. The most important finding of our study was that the severity of gastritis and the presence of *H. pylori* have an influence on gastric epithelial cell proliferation and expression of the bcl-2 protein in gastric epithelial cells and in interstitial lymphocytes differently in the antrum and corpus mucosa. A significant positive correlation between proliferation activity and grade of chronic infiltration and atrophy was found in the corpus (atrophic mucosa exhibiting higher PCNA and Ki-67 LI's), while in the antrum mucosa proliferation indices tended to correlate negatively with grade of gastritis (atrophic mucosa in the antrum showed lower Ki-67 LI). These findings are in

accordance with the data of Bechi *et al.* [3], which suggest differences in the proliferative activity of epithelial cells between the mucosa of gastric antrum and corpus, but in contrast to the data of other authors, which show enhancement of LI with increasing degrees of gastritis both in the antrum and in corpus mucosa [18, 27].

Proliferation rates of corpus glandular epithelia were not related to *H. pylori* status in our study. In the antrum glands, Ki-67 LI tended to be inversely related to higher *H. pylori* score as well as to gastritis score. The study of Chow *et al.* [7] showed too that *H. pylori* do not increase antrum cell proliferation. Reduced proliferative activity of antrum gland cells with increasing grade of atrophic gastritis is not an unexpected finding, because it represent gastritis harbouring a significant proportion of involutes glands in probable proliferative quiescence, since severe cell loss does not permit normal cell maturation and differentiation [3]. The contrasting findings in the gastric corpus, namely an increased proliferative activity, especially in FC and GP, suggest that the corpus gland cell populations maintain active cell turnover despite reduction in cellular mass as established through grading criteria. Extension of the proliferative compartment with consequent displacement of proliferating cells to the epithelial surface has indeed been reported in advanced gastritis [12, 18]. Moreover, corpus gastritis may be in an earlier phase of inflammatory evolution compared with antrum gastritis, retaining still its proliferative repertoire: there is evidence that lesions in the antrum can be considered older than lesions in the corpus [27]. Indeed, in our group of patients, cases with atrophic antrum alterations prevailed over atrophic corpus alterations.

In the present study it was our aim to test whether cell protection from apoptosis is related to gastritis and *H. pylori* colonization. Immunoreactivity for the apoptosis protector, bcl-2 protein, was detected in a subset of gastric epithelial cells, mainly in the glandular neck region. The noteworthy finding of our study was that both glandular proliferation and bcl-2 protein reactivity were differentially expressed already in the normal (i.e. without chronic inflammation or atrophy of the glands) corpus and antrum mucosa. In normal corpus glands, the balance between cell proliferation and programmed cell death was manifested as lower PCNA and Ki-67 LI's and higher bcl-2 protein staining of epithelial cells in GP compared with the atrophic mucosa. However, compared with atrophic glands, in the normal antrum mucosa the balance between cell proliferation and apoptosis was attained in a different way: there was a tendency for elevated cell proliferation and reduced bcl-2 protein staining of epithelial cells. In our study overall mean PCNA LI too was significantly higher in the antrum than in the corpus. This finding may support the evidence for the presence of a different environment in the antrum and corpus mucosa [45], as well as dissimilarities in antrum and corpus regarding the interaction of the bacterium and host [42].

For the atrophic corpus mucosa, we established higher proliferation index, compared with the respective index for normal corpus glands, and simultaneous

increase in bcl-2 positivity of interstitial lymphocytes. While upregulation of the bcl-2 as apoptosis protector factor suggests reduced apoptosis, its association with prolonged higher proliferation activity can be regarded as a factor contributing to development of MALT lymphoma and carcinogenesis [2, 26]. A positive correlation of positive staining for bcl-2 in interstitial lymphocytes of the corpus with grade of gastritis may also indicated that these lymphocytes are protected from programmed cell death and may hence be responsible for increase in chronic inflammation.

The lower Ki-67 proliferation index for the atrophic antral mucosa was associated with more frequent bcl-2 protein staining of epithelial cells in the neck part of glands (PZ) than in the normal mucosa, which indicates possible protection from cell loss and thus may reflect an important mechanism for retaining the renewal potential of antrum glands [26].

Concerning specific gastric cells, the present investigation shows that antral G and D cell densities decrease significantly already in the stage of non-atrophic gastritis and reach a minimum in atrophic gastritis. This finding confirms previous data demonstrating a decrease in G cells, particularly in atrophic gastritis [11, 30, 44, 52]. We detected only a few G and D cells in the corpus, predominantly in atrophic areas, probably in the transition area between the antrum and the corpus. In our study the presence of *H. pylori* tended to be associated with decrease in G and D cell density in the antrum, however, this decrease was significantly affected by advanced grade of atrophic gastritis. Our results are consistent with observation of Wyatt *et al.* [52] who also found significantly fewer gastrin cells in *H. pylori* positive patients whereby this decrease was positively correlated to the degree of antral atrophic gastritis. The lower density of D-cells in *H. pylori* positive antrum mucosa found in our study is in agreement with the evidence that *H. pylori* induces reduction of somatostatin cells [32, 34, 35, 48].

Concerning the third type of specific gastric cells, β- H+, K+ ATP-ase — reactive parietal cells, we found that their density was rather dependent on the grade of atrophic gastritis. Atrophy in the corpus mucosa may represent a more powerful factor for inducing decrease in specific cells, and advanced stages of mucosal atrophy exhibit depletion of specific cells irrespective of *H. pylori* status.

In conclusion, the severity of gastritis and *H. pylori* colonization influence gastric epithelial cell proliferation and the expression of bcl-2 differently in the antrum and corpus mucosa. In the corpus mucosa, proliferation activity and the expression of bcl-2 in interstitial lymphocytes was significantly higher in atrophic than in non-atrophic gastritis, but did not dependent on *H. pylori* density. In contrast the antral mucosa proliferative activity showed a tendency toward a negative association with severity of gastritis, and the expression of bcl-2 positive interstitial lymphocytes was more associated with *H. pylori* density. Homeostasis of antral G and D cells as well as loss of β- H+, K+ ATP-

ase — reactive cells in the corpus was shown to be a rather a function of degree of atrophic alterations than of *H. pylori.*

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Table 1. State of antral and corpus mucosa and grade of *H. pylori* colonization in the studied patients.

State of gastric mucosa	ANTRUM					CORPUS				
		H. pylori grade					*H. pylori* grade			
	n	Absence	Mild	Moderate	Severe	**n**	Absence	Mild	Moderate	Severe
Normal (grade 0) (no chronic inflammation, no atrophy)	**4**	4	0	0	0	**11**	10	1	0	0
Non-atrophic gastritis (grade of chronic inflammation):										
mild (0.5)	**9**	9	0	0	0	**15**	8	2	4	1
moderate (1.0)	**11**	6	3	1	1	**6**	3	0	0	3
severe (1.5)	**8**	1	3	3	1	**5**	2	0	3	0
Atrophic gastritis (grade of atrophy):										
mild (2.0)	**15**	7	4	3	1	**0**	0	0	0	0
moderate (3.0)	**2**	0	0	0	2	**2**	2	0	0	0
severe (4.0)	**7**	4	1	2	0	**4**	4	0	0	0
Total	**56**	31	11	9	5	**43**	29	3	7	4

Table 2. PCNA and Ki-67 LI in different parts of antral and corpus glands and their relation to gastritis and *H. pylori* status.

State of gastric mucosa	ANTRUM						CORPUS					
	PCNA LI			Ki-67 LI			PCNA LI			Ki-67 LI		
	FC	PZ	GP	FC	PZ	GP	FC	PZ	GP	FC	PZ	GP
Normal (grade 0)	0.8±0.4	8.5±7.3	1.5±1.2	0.3±0.3	7.3±7.8	0.9±0.7	2.2±2.0*	9.2±8.8	0.9±0.8¤	0.6±0.7	4.4±4.8	0.1±0.3⌖
Non-atrophic gastritis (chr. inflammation grades 0.5–1.5)	2.6±2.3	12.8±8.7##	2.6±3.9	1.3±1.4	8.3±7.4	0.6±1.3	3.2±2.3**	7.5±5.9#	0.9±1.3¤	1.2±1.0	5.3±4.1	0.7±1.0
Atrophic gastritis (atrophy grades 2–4)	3.0±3.1✦	11.5±5.5	3.1±3.3	1.5±2.0	5.1±4.3	0.5±0.7	10.0±10.9 •	12.6±6.6	3.2±1.7¤¤	1.80±1.7	7.0±6.2	0.9±1.2⌖⌖
H. pylori +	2.2±1.8	11.6±7.5	2.6±4.2	1.5±2.0	5.7±4.6	0.4±0.7	2.9±2.3	9.7±8.6	1.2±1.7	1.5±1.2	4.9±3.3	0.6±1.1
H. pylori –	3.0±3.1	12.3±7.5	2.9±2.8	1.1±1.3	7.8±7.4	0.7±1.2	4.5±6.0	8.1±6.0	1.4±1.4	0.9±1.0	5.5±5.1	0.5±0.8
Totally	2.7±2.6	12.0±7.4★★	2.7±3.5	1.3±1.6	6.9±6.3	0.6±1.0	3.9±5.1	8.7±6.9★	1.3±1.5	1.1±1.1	5.7±4.9	0.5±0.8

Significant difference: in corpus: • > * p= 0.03 (as compared to normal); • > ** p= 0.007 (as compared to non-atrophic gastritis); ¤¤ > ¤ p= 0.001 (as compared to normal and non-atrophic gastritis); ⌖⌖ > ⌖ p=0.04 (as compared to normal).
Significant difference: between antrum and corpus: ## > # p= 0.01; • > ✦ p= 0.009; ★★ > ★ p= 0.02;

Table 3. Number of cases with bcl-2 positive staining of epithelial cells (EC) and mean score of bcl-2 stained interstitial lymphocytes (IL) in different parts of antral and corpus glands and their relation to gastritis and *H. pylori* status.

State of gastric mucosa	ANTRUM						CORPUS					
	Foveolar cells		Proliferating zone		Glandular part		Foveolar cells		Proliferating zone		Glandular part	
	EC	IL	EC	IL	EC	IL	EC	IL	EC	IL	EC	IL
Normal	0/4	0.0±0.0	0/4	1.0±0.7	1/4	0.2±0.5	0/11	0.09±0.3	0/11	0.2±0.6	3/11	0.0±0.0
Non-atropic gastritis (chr. inflammation grades 0.5–1.5)	0/27	1.3±1.3	1/28	2.3±1.2	1/28	2.3±1.3	0/24	0.9±0.9¤	2/25	1.6±1.1•	2/25	0.9±1.0#
Atrophic gastritis (atrophy grades 2–4)	0/23	1.3±1.4	4/23	2.3±1.2	1/23	1.7±1.3	0/6	2.1±1.6¤¤	1/6	2.8±1.5••	0/6	3.2±1.7##
H. pylori +	0/23	1.4±1.4	2/24	2.7±0.8* .	0/24	2.1±1.3	0/12	0.9±1.0	0/13	1.8±1.2	1/13	0.6±1.0
H. pylori –	0/31	1.4±1.1	3/31	1.9±1.3**	3/31	1.8±1.3	0/29	1.3±1.1	3/29	1.6±1.3	4/29	1.5±1.4
Total	0/54	1.3±1.2	5/55	2.3±1.2♦	3/55	1.9±1.3	0/41	1.1±1.1	3/42	1.7±1.2♦♦	5/42	1.2±1.3

Significant difference: in antrum: * > ** p=0.02 (as compared to *H. pylori* -); in corpus: ¤¤ > ¤ p= 0.01; •• > • p=0.01 (as compared to non-atrophic gastritis); ## > # p=0.002 (as compared to non-atrophic gastritis);
Significant difference between antrum and corpus: ♦ > ♦♦ p=0.02.

Table 4. Number of specific cells and grade of gastritis in *H. pylori* positive and *H. pylori* negative antrum and corpus mucosa (mean±SD).

Specific cells and state of gastric mucosa	ANTRUM			CORPUS		
Gastrin cells	Foveolar cells	Proliferating zone	Glandular part	Foveolar cells	Proliferating zone	Glandular part
Normal	0	54.2±46.5*	5.5±6.5	0	0.2±0.6	0
Non-atrophic gastritis	0.3±0.3	26.5±20.4**	2.9±3.3	0	0.7±2.1	0.9±2.4
Atrophic gastritis	0.4±0.6	21.6±20.8***	2.8±4.2	0	2.1±3.2	2.6±5.1
H. pylori +	0.2±0.3	20.6±20.0	2.4±2.4	0	0.1±0.1	0.1±0.2
H. pylori –	0.4±0.5	30.9±25.9	3.6±4.7	0	1.5±3.0	1.2±3.1
Somatostatin cells						
Normal	0.1±0.1	14.1±10.5¤	0.7±0.4	0	1.3±1.8	0.9±2.2
Non-atrophic gastritis	0.1±0.2	7.2±4.4¤¤	1.1±1.5	0.1±0.1	0.6±1.2	0.6±0.9
Atrophic gastric	0.2±0.7	4.1±3.1¤¤¤	0.8±0.9	0.1±0.1	1.9±2.5	0.9±1.6
H. pylori +	0.1±0.2	5.1±4.3	1.0±0.9	0	0.5±1.1	0.7±1.1
H. pylori –	0.2±0.6	7.4±5.7	0.8±1.4	0.1±0.1	1.2±1.7	0.8±1.5
β-H+, K+ ATP positive cells						
Normal	nd	nd	nd	0.2±0.4	100.6±26.7•	35.1±23.0
Non-atrophic gastritis	nd	nd	nd	0	84.7±27.8••	39.6±18.3
Atrophic gastritis	nd	nd	nd	0	22.8±23.7•••	2.0±4.0
H. pylori +	nd	nd	nd	0	89.7±30.5	40.2±13.2
H. pylori –	nd	nd	nd	0	76.8±37.1	31.8±24.5

Significant difference in antrum: * > ** p=0.04 (as compared to non-atrophic gastritis); * > *** p=0.02 (as compared to atrophic gastritis); ¤ > ¤¤ p=0.02 (as compared to non-atrophic gastritis); ¤ > ¤¤¤ p= 0.0005 (as compared to atrophic gastritis); in corpus: • > •• p= 0.0001 (as compared to non-atrophic gastritis); • > ••• p= 0.0004 (as compared to atrophic gastritis).

Table 5. Comparison of the antrum and corpus in relation to proliferation activity, expression of bcl-2 and density of specific cells in association with gastritis and *H. pylori* colonization.

	ANTRUM	CORPUS
Gastritis and *H. pylori*	Correlation with *H. pylori* score (p=0.006)	No correlation with *H. pylori* score (p=0.37)
Atrophic gastritis	More cases of atrophic gastritis in comparison with corpus (11/31; 35%); p=0.02	Less cases of atrophic gastritis in comparison with antrum (6/43; 14%)
Proliferation activity:		
PCNA LI	Overall mean PCNA LI higher than in corpus (p=0.02)	Overall mean PCNA LI lower than in antrum
	No correlation with gastritis score	Correlation with gastritis score (r= 0.46; p=0.001)
	Tendency to negative correlation with *H. pylori* score (r= –0.23; p=0.09)	No correlation with *H. pylori* score
Ki-67 LI	No correlation with gastritis score	Correlation with gastritis score (r= 0.46; p=0.01)
	No correlation with *H. pylori* score	No correlation with *H. pylori* score
Bcl-2 positive interstitial lymphocytes	No correlation with gastritis score,	Correlation with gastritis score (r=0.37; p=0.01)
	Tendency to positive correlation with *H. pylori* score (r=0.29; p=0.08)	No correlation with *H. pylori* score
	Overall mean bcl-2 positive staining score higher than in corpus (p=0.02)	Overall mean bcl-2 positive staining score lower as in antrum
Bcl-2 positive epithelial cells	4/8 cases of positive staining localized in atrophic proliferating zone	3/8 cases of positive staining localized in normal foveolar cell compartment (no chronic inflammation, no atrophy)
G and D cell density	Negative correlation with gastritis score (r= –0.36; p= 0.009)	
	Tendency to negative correlation with *H. pylori* score (r= –0.24; p= 0. 06)	
β- H+, K+, ATP-ase positive cells		Negative correlation with gastritis score (r= –0.53; p= 0.0009)
		No correlation with *H. pylori* score

CURRICULUM VITAE

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Education

1962–1972	Narva 7th Secondary School (*cum laude*)
1972–1978	University of Tartu, Faculty of Medicine (*cum laude*)
1978–1979	Tartu University Hospital, internship in internal diseases
1985	Academic dissertation and degree: M.D. Cand med (PhD) (internal diseases), University of Tartu ("Humoral and cellular immunity in gastric and duodenal ulcer patients with a different state of gastric mucosa").

Special courses

Training in immunohistochemistry and immunology and a visiting scientist at the Institute of Pathology, University of Leipzig; at the Institute of Clinical Immunology, University of Jena; at the Institute of Pathology, Medical Academy in Dresden; at the Institute of Medical Immunology in Berlin; at the Institute of Pathology, University of Rostock, Germany (1987–1988).

Course "Immunocytochemical methods and monoclonal antibodies in oncology, haematology and clinical immunology", Kiev, Ukraine (March 1990).

Institute of Pathology and Institute of Immunology, University of Göttingen, Germany (August, September 1992).

Summer School Course "Immunology-93", Palanga, Lithuania (June 1993).

Post-graduate course "Autoimmunity: Basic and Clinical", University of Tartu, Estonia (June 5–12, 1994).

1st International Technical Course on *Helicobacter pylori*, University of Bordeaux, France (September 4–16, 1994).

International Course on Gastric Cancer and *Helicobacter pylori*, San Marino (October 2–5, 1995).

Attending the "*Helicobacter pylori*: Basic mechanisms to clinical cure 1996" symposium in Ottawa, Ontario as continuing medical education activity for 20.5 hours as category 1 of the Physician's recognition Award of the Americal Medical Association (June 10–12, 1996).

Salzburg-Cornell Seminar in Oncology, Salzburg, Austria (July 27 – August 2, 1997).

SAS System computer course, University of Tartu (October 1997).

European *Helicobacter pylori* Study Group's Gastric Pathology Course, Northick Park & St Mark' Hospitals London, UK (February 7/8th 1998).

Nordic Summer School in Methods of Infectious Disease Epidemiology. Söderfors, Sweden (8–17 June 1998).

International Summer School of Immunology, Jurmala, Latvia (24–28 May 1999).

Language course "How to write scientific papers", organized in Tartu by Helsinki University (May 2000).

Seminar "2-D Electrophoresis in Proteome Analysis", organized by Amersham Pharmacia Biotech, Tartu (May 3th, 2001).

International Summer School "Autoimmunity: Molecular and Clinical Aspects", EFIS John Humphrey Course, Tartu (5–9 June, 2001).

Research activity

Medical University of Lübeck, Germany (July, August 1990).

Department of Medical Microbiology and Infectious Diseases, University of Lund, Sweden (March 1993, June–July 1994, July 1996).

Department of Gastroenterology, University Hospital, Bern and the Institute of Pathology, University of Bern, Switzerland (January–March 1994, May 1997).

Institute of Pathology, University Erlangen-Nürnberg, Germany (October–November, 1998).

Knowledge of languages: Estonian, Russian, English, German, French, and Italian.

Professional employment

1979–1981	Research Assistant, Laboratory of Immunology, University of Tartu
1981–1993	Junior Researcher, Department of Immunology, University of Tartu
1993– up to now	Senior Researcher, Immunology Research Group and Lecturer of Immunology, University of Tartu (since 1992; lectures in Estonian, Russian and English languages for students of Faculty of Medicine)

Major directions of research

Major areas of research: humoral and cellular immunity in gastric and duodenal ulcer patients with a different state of gastric mucosa (Academic dissertation 1985), seroepidemiology of *H. pylori* infection in Estonian adults and children, immune response to different *H. pylori* antigens and its association with gastritis, development of atrophy; cellular turnover in gastric mucosa in *H. pylori* gastritis.

Publications: 72 publications (24 papers, incl. 16 in peer reviewed international journals), 35 presentations at international scientific workshops and congresses.

Member of the Estonian Society of Gastroenterology (since 1980), Member and Secretary of the Estonian Society of Immunology (since 1984) and the Estonian Society of Immunology and Allergology (since 2001), Member of the Board of the Baltic Immunological Society (since 1991) and Member of the German-Baltic Medical Society (since 1991).

Member of the Organizing Committee of the 1st Congress of the Estonian Society of Immunology (1989), of the International Summer Schools "Autoimmunity: Basic and Clinical" (Tartu, 1994) and "Autoimmunity: Molecular and Clinical aspects" (Tartu, 2001).

Awards and scholarships

1994 Science Award of the Government of the Republic of Estonia to the group of authors: H.-I. Maaroos, T. Vorobjova, R.Uibo, K.Kisand, R. Tammur, T. Kivik, K. Villako for the study "Occurrence of *Helicobacter pylori* infection among the Estonian population".
1994 Scholarship of the Swiss National Foundation
1998 Scholarship of DAAD (German Academic Exchange Service)
1999 Award of Tartu University Faculty of Medicine.

CURRICULUM VITAE

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Haridus

1962–1972	Narva 7. Keskkool (kuldmedal)
1972–1978	Tartu Ülikooli Arstiteaduskond (*cum laude*)
1978–1979	Internatuur Tartu Ülikooli Kliinikutes.
1985	Meditsiiniteaduste kandidaat sisehaiguste alal, Tartu Ülikool ("Humoraalne ja rakuline immuunsus mao ja kaksteistsõrmiksoole haavandi haigetel, nende seos maolimaskesta seisundiga").

Erialane enesetäiendus

Täiendused:

Leipzigi Ülikooli Patoloogia Instituut, Jena Ülikooli Kliinilise Immunoloogia Instituut, Dresdeni Meditsiini Akadeemia Patoloogia Instituut, Berliini Meditsiini Immunoloogia Instituut, Rostocki Ülikooli Patoloogia Instituut — immunoloogia ja immunohistokeemia (1987–1988).

Kiiev, kursus "Imunotsütokeemilised meetodid ja monoklonaalsed antikehad onkoloogias, hematoloogias ja kliinilises immunoloogias" (1990).

Göttingeni Ülikooli Patoloogia Instituut ja Immunoloogia Instituut (1992).

Tartu Ülikool, rahvusvahelises suvekoolis "Autoimmunity: Basic and Clinical" (1994).

Bordeaux, esimene rahvusvaheline *Helicobacter pylori* tehniline kursus (1994).

San Marino, rahvusvaheline kursus "Maovähi ja *Helicobacter pylori* epidemioloogia" (1995).

Ottawa, "*Helicobacter pylori*: Basic mechanisms to clinical cure" (20.5 hours as category 1 of the Physician's recognition Award of the Americal Medical Association) (1996).
Salzburg-Cornell, seminar onkoloogia alal (1997).
London, "European *Helicobacter pylori* Study Group's Gastric Pathology Course" (1998).
Söderfors, Rootsi, "Nordic Summer School in Methods of Infectious Disease Epidemiology" (1998).
Jurmala, immunoloogia rahvusvaheline suvekool (1999).
Tartu Ülikool, rahvusvaheline suvekool "Autoimmunity: Molecular and Clinical Aspects" (2001).

Teadustöö

Lübecki Meditsiiniülikool, (kaks kuud 1990).
Lundi Ülikooli Meditsiinilise Mikrobioloogia Instituut (üks kuu 1993, kaks nädalat 1994, üks kuu 1996).
Berni Ülikooli gastroenteroloogia osakond ja patoloogia instituut, (kolm kuud 1994, üks nädal 1997).
Erlangeni-Nürnbergi Ülikoolis, patoloogia instituut (kaks kuud 1998).

Keelteoskus: eesti, vene, inglise, saksa, prantsuse, itaalia.

Teenistuskäik

1979–1981	TÜ ÜMPI immunoloogia labori vanemlaborant
1981–1993	TÜ ÜMPI immunoloogia labori nooremteadur
1993–tänaseni	TÜ ÜMPI immunoloogia uurimisgruppi vanemteadur

Teadustegevus

Põhiliseks uurimisvaldkonnaks on olnud humoraalne ja rakuline immuunsus mao- ja kaksteistsõrmikuhaavandi haigetel, nende seos maolimaskesta seisundiga, samuti *H. pylori* seroepidemioloogiline uuring Eestis, selle seos nii täiskasvanute kui ka laste kroonilise gastriidiga, *H. pylori* osatähtsus maolimaskesta rakkude proliferatsioonis ja apoptoosis ning atroofia arengus.

72 teaduspublikatsiooni, nendest 24 artiklit (16 rahvusvahelistes, kaks Eesti ja kuus Tartu Ülikooli väljaannetes).

35 ettekannet rahvusvahelistel konverentsidel ja kongressidel.

Eesti Immunoloogide (1984. aastast) ja Eesti Immunoloogide ja Allergoloogide Seltsi (2001. aastast), Eesti Gastroenteroloogide Seltsi (1980. aastast)

liige, Balti Immunoloogide Seltsi juhatuse liige (1991 aastast) ja Saksa-Balti Arstide Seltsi liige (1991. aastast). Esimese Eesti immunoloogide kongressi (1989), rahvusvaheliste suvekoolide "Autoimmunity: Basic and Clinical" (1994) ja "Autoimmunity: Molecular and Clinical Aspects" (2001) organiseerimiskomiteede liige.

Tunnustused

1994 Eesti Vabariigi Kultuuri- ja Haridusministeeriumi aastapreemia autorite kollektiivile H-I. Maaroos, T. Vorobjova, R. Uibo, K. Kisand, R. Tammur, T. Kivik, K. Villako, "*Helicobacter pylori* infektsiooni sagedus Eestis — epidemioloogiline populatsiooni juhuslikustatud valikul põhinev uurimus".
1994 Šveitsi Natsionaalfondi stipendium.
1998 DAADi (Deutscher Akademischer Austauschdienst) stipendium
1999 Tartu Ülikooli arstiteaduskonna preemia.

Õppetöö

Immunoloogia loengukursus arstiteaduskonna 2. kursuse üliõpilastele.

DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

1. **Heidi-Ingrid Maaroos.** The natural course of gastric ulcer in connection with chronic gastritis and Helicobacter pylori. Tartu, 1991.
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8. **Ruth Mikelsaar.** Etiological factors of diseases in genetically consulted children and newborn screening: dissertation for the commencement of the degree of doctor of medical sciences. Tartu, 1993.
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22. **Triin Remmel.** Primary biliary cirrhosis in Estonia: epidemiology, clinical characterization and prognostication of the course of the disease. Tartu 1996.
23. **Toomas Kivastik.** Mechanisms of drug addiction: focus on positive reinforcing properties of morphine. Tartu, 1996.
24. **Paavo Pokk.** Stress due to sleep deprivation: focus on $GABA_A$ receptor-chloride ionophore complex. Tartu, 1996.
25. **Kristina Allikmets.** Renin system activity in essential hypertension. Associations with atherothrombogenic cardiovascular risk factors and with the efficacy of calcium antagonist treatment. Tartu, 1996.
26. **Triin Parik.** Oxidative stress in essential hypertension: Associations with metabolic disturbances and the effects of calcium antagonist treatment. Tartu, 1996.
27. **Svetlana Päi.** Factors promoting heterogeneity of the course of rheumatoid arthritis. Tartu, 1997.
28. **Maarike Sallo.** Studies on habitual physical activity and aerobic fitness in 4 to 10 years old children. Tartu, 1997.
29. **Paul Naaber.** *Clostridium difficile* infection and intestinal microbial ecology. Tartu, 1997.
30. **Rein Pähkla.** Studies in pinoline pharmacology. Tartu, 1997.
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34. **Ülla Linnamägi.** Changes in local cerebral blood flow and lipid peroxidation following lead exposure in experiment. Tartu, 1998.
35. **Ave Minajeva.** Sarcoplasmic reticulum function: comparison of atrial and ventricular myocardium. Tartu, 1998.
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