The role of cytokines and pattern-recognition receptors in inflammatory gastrointestinal diseases: clinical and *in vitro* investigations

Ph.D. Thesis

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ABBREVIATIONS

ABC	ATP-binding cassette
AP	acute pancreatitis
ATP	adenosine triphosphate
BCG	Bacillus Calmette-Guérin
BSA	bovine serum albumin
CagA	cytotoxin-associated protein A (the product of the *cagA* gene)
CARD	caspase-recruitment domain
CARD4, -15	caspase-recruitment domain 4 and 15 genes
CATERPILLER	protein family, named after CARD, transcription enhancer, R (purine)-binding, pyrin, lots of leucine repeats
CD	Crohn's disease
CHO	chinese hamster ovary
CI	confidence interval
DAP	diaminopimelic acid
DC	dendritic cell
DU	duodenal ulcer
EAEC	enteroaggregative *Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular-signal-regulated kinase
HMG	high-mobility group
HMG-1	high-mobility group 1 protein
HMGB	high-mobility group box
HMGB-1, -2, -3	high-mobility group box -1,-2 and -3 proteins
HWE	Hardy-Weinberg equilibrium
IBD	inflammatory bowel disease
iE-DAP	γ-D-glutamyl-*meso*-diaminopimelic acid
IFN-γ	interferon-γ
IgG,-Y	immunoglobulin G and Y
IL-1α, -1β, -2, -4, -6, -8, -10, -12, -14	interleukin-1α, -1β, -2, -4, -6, -8, -10, -12, -14
LPS	lipopolysaccharide
LRR	leucine-rich repeat
MALT	mucosa-associated lymphoid tissue
MAPK	mitogen-activated protein kinase
MCP, -1	monocyte chemoattractant protein, -1
MDP	muramyl dipeptide
MIP-1α, -1β	macrophage inflammatory protein-1α and -1β
MOF	multiple organ failure
NACHT-LRR	protein family, named after neuronal apoptosis inhibitory protein (NAIP), CIITA, HET-E and TP1 – leucine-rich repeat
NF-κB	nuclear factor-κB
NLS, -1, -2	nuclear localization signal, -1 and -2
NLR	NOD-like receptor

NOD	nucleotide binding and oligomerization domain
NOD1, -2	nucleotide binding and oligomerization domain (containing) protein 1 and 2
OR	odds ratio
PAF	platelet activating factor
PAI	pathogenecity island
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline solution
PCR	polymerase chain reaction
PGN	peptidoglycan
PMA	phorbol-12-myristate-13-acetate
PRR	pattern-recognition receptor
PSD	post source decay
RA	rheumatoid arthritis
RAGE	receptor for advanced glycation end-products
RecAtn	recombinant rat amphoterin
SA	heat-killed *Staphylococcus aureus*
SAP	severe acute pancreatitis
SIRS	systemic inflammatory response syndrome
SNP	single nucleotide polymorphism
TFSS	type IV secretion system
Th1	T helper 1 cell
Th2	T helper 2 cell
TLR, -2, -4, -5, -9	toll-like receptor, -2, -4, -5 and -9
TNF-α	tumor necrosis factor-α
UBT	urea breath test
UC	ulcerative colitis

1. INTRODUCTION

In order to counteract events that disturb homeostasis such as infections or tissue injury, multicellular organisms have developed a mechanism called inflammatory response. Invading pathogens come into contact first with the mucosal epithelium and with cells of the innate immune system. These cells recognize endogenous danger signals, for example the high-mobility group box 1 protein (HMGB-1) [1, 2], and pathogen-associated molecular patterns (PAMPs) by their germ-line encoded pattern-recognition receptors (PRRs). PAMPs are evolutionary conserved, often structural motifs found in wide range of different microbes [3]. In mammals PRRs include Toll-like receptors (TLRs) and NOD (nucleotide binding and oligomerization domain)-like receptors (NLRs) [4, 5]. The receptor signaling leads to the production of inflammatory mediators such as tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8) and to the recruitment of inflammatory cells.

Individual differences in the receptor signaling and in the cytokine production can be genetically determined, which further influences the host response in inflammatory diseases. Therefore the aims of our study were to investigate the genetic polymorphisms of *IL-8*, *TLR4* and caspase-recruitment domain 4 gene (*CARD4/Nod1*) in such gastrointestinal diseases, where the intensity of host response definitely determines the consequences of infection or tissue necrosis, i.e. in *Helicobacter pylori*-induced gastritis and duodenal ulcer (DU), in acute pancreatitis (AP) and in Crohn's disease (CD). In addition, we investigated the production of the „newly" recognized danger signal and late cytokine HMGB-1 following *in vitro* bacterial infection and in pancreatitis.

1.1. Pattern-recognition receptors (PRRs)

1.1.1. Toll-like receptors (TLRs)

Toll was originally described as a receptor involved in the embryonic development [6] and in the defence mechanism against fungal infection in *Drosophila melanogaster* [7]. Since then, ten homologues of the Toll protein (TLRs) have been identified in humans. TLRs are transmembrane proteins that are located on the cell-surface or act as endosomal receptors,

recognizing a diverse group of microbial and endogenous ligands. TLR4 recognizes lipopolysaccharide (LPS), TLR5 detects flagellin, while TLR9 senses viral and bacterial DNA among others. Similarly to NOD containing proteins, TLRs possess LRR (leucine-rich repeat) domains for the recognition of their ligands. The downstream signaling pathways lead to nuclear factor-κB (NF-κB) activation and expression of genes that are involved in the pro-inflammatory responses [4, 8] .

Data on the role of different TLRs in the recognition of *H. pylori* are numerous but controversial. Bäckhed et al. demonstrated that primary gastric antral epithelial cells do not express TLR4, while the gastric epithelial cell lines AGS, MKN45 and NCI-N87 express the nonsignaling form of the receptor. Both primary gastric epithelial cells and cell lines were induced to secrete IL-8 following infection with *H. pylori* in a cytotoxin-associated gene pathogenecity island (*cag* PAI)-dependent manner, indicating that the recognition of *H. pylori* is independent of the TLR4-mediated LPS signaling [9]. In contrast, Su et al. found that AGS cells constitutively express TLR4 mRNA and *H. pylori* infection induces increase in transcription, translation and expression of TLR4 independently of *cagA* [10]. TLR4 was demonstrated to be expressed at the apical as well as at the basolateral pole of gastric surface epithelium *in vivo*, both in noninflamed gastric mucosa and in chronic active gastritis by Schmausser et al. The expression of TLR5 and TLR9 was identical to that of TLR4 in noninflamed gastric mucosa, but changed to an exclusive basolateral localization in *H. pylori* gastritis. *H. pylori* bacteria became directly attached to the apically expressed TLR4 receptors in patients with gastritis, supporting their role as binding sites for the bacteria *in vivo* [11]. This correlates with the finding that TLR4 expression promotes increased adherence of *H. pylori* to the surface of chinese hamster ovary (CHO) fibroblast cells [10].

TLR4 was shown to display an early, transient upregulation in experimental rat pancreatitis, suggesting its functional role in the development of the disease [12]. In addition, Johnson et al. described an endogenous pathway mediated by TLR4 which leads to systemic inflammatory response syndrome (SIRS)-like syndrome, the triggering factor of which is released into the blood and tissues during both pancreatitis and systemic inflammation [13]. This finding further supports that TLR4 is potentially associated with AP.

1.1.2. NOD-like receptors (NLRs)

The NOD (nucleotide binding and oligomerization domain)-like receptor (NLR) family, also described as NACHT-LRR receptors or CATERPILLER, is involved in bacterial sensing in the cytoplasm. The NLR family has more than 20 members, including NOD1 and NOD2 [14]. NOD1 is encoded by the caspase-recruitment domain 4 gene (*CARD4*/*Nod1*), which is located on chromosome 7p14-15, extends over 55 kb of genomic DNA and is composed of 14 exons. The protein is built of 965 amino acids [15]. NOD2, which is encoded by *CARD15*/*Nod2* [16], is composed of 1040 amino acids [17].

Members of the NLR family, including NOD1 have tripartite domain structure. The carboxy-terminal LRR domain participates in the recognition and detection of ligands, while there is a NOD (or NACHT) in central position of the molecule, which posesses ATP-ase activity and facilitates self-oligomerization. The amino-terminal effector domain is built from protein-protein interaction cassettes and can be represented either by CARD or pyrin (e.g. in case of cryopyrin) domain. NOD1 has one, while NOD2 has two CARDs. Following the interaction between the ligand and LRR domain, a complex conformational change of the protein is thought to be initiated: binding of ATP to an ATP-binding cassette (ABC) and self-oligomerization of the molecules are proposed to be the initial steps of binding and activating downstream effector molecules through the amino-terminal domain(s) [14, 18].

NOD1 and NOD2 are expressed in the cytosol of epithelial [19, 20] and antigen presenting cells [21], human gingival fibroblasts [22], myofibroblasts [23], astrocytes [24] and microglia [25]. The baseline expression of NOD1 is constitutive but variable, which is enhanced by IFN-γ, but not by TNF in epithelial cells [26]. NOD2 posesses low baseline expression, the upregulation of which can be exerted by TNF and further augmented by IFN-γ [27].

The minimal peptidoglycan (PGN) moieties recognized by NOD1 and NOD2 are γ-D-glutamyl-*meso*-diaminopimelic acid (iE-DAP) [28, 29] and muramyl dipeptide (MDP) [30, 31], respectively. There is species specifity in the recognition of ligands by NOD1 receptors: human NOD1 requires a tripeptide (L-Ala-D-Glu-*meso*-DAP), while the murine receptor needs a tetrapeptide (L-Ala-D-Glu-meso-DAP-D-Ala) for optimal sensing of PGN [32]. PGN in both Gram-negative and Gram-positive bacteria contains MDP, but the presence of iE-DAP is restricted only to the PGN of Gram-negatives (except for *Listeria* spp, *Bacillus* spp. and

Gram-positive bacteria in the soil). That is why NOD2 should be considered as a general sensor for most bacteria and NOD1 for mainly Gram-negatives [14]. The intracellular pathogen *Shigella flexneri* [33], the enteroinvasive *Escherichia coli* (EIEC) [19], *Chlamydia pneumoniae* [34] and *Pseudomonas aeruginosa* [35] have been shown to be detected by NOD1. In particular, NOD1 was shown to have a crucial role in the containment of *H. pylori*. After injected by the type IV secretion system (TFSS) of *H. pylori* into gastric epithelial cells, PGN fragments are specifically recognized by NOD1 [36]. Following activation of NOD1 and -2 by their ligands, they recruit a downstream effector molecule, the receptor-interacting serine/threonine kinase (RICK or RIP2), which triggers a cascade leading to the translocation of NF-κB to the nucleus [14], controlled by centaurin β1 [37], erbin [38] or by the transforming growth factor-β (TGF-β)-activated kinase 1 [39] among others. The activation of the mitogen-associated protein kinase (MAPK) pathways can be exerted through NOD1 and -2. Extracellular signal-regulated kinase (ERK) [40] and p38MAPK [41] pathways are involved in signaling processes by NOD2, while the activation of NOD1 may result in the activation of JUN amino-terminal kinase (JNK) [33]. NOD1 is thought to mediate a restricted pattern of immune responses, the primary role of which is to induce the recruitment and interaction of immune cells by enhancing the expression of IL-8, CXCL1/Groα, CXCL2/Groβ, monocyte chemoattractant protein 1 (MCP-1/ CCL2) and CD83 [42].

1.1.3. Cooperation between NOD containing proteins and TLRs

Due to the synergistic effects of the TLR- and NOD containing protein-mediated signaling pathways, the cellular response to whole microbes is not only recognizing their individual PAMPs.

NOD1 and NOD2 agonists act cooperatively with the TLR4 agonist LPS to induce the release of proinflammatory and anti-inflammatory cytokines from CD14+ monocytes and CD1a+ immature DCs, and synergize with sub-active doses of LPS to induce DC maturation [43]. Yang et al. reported that MDP alone is hardly able to elicit cytokine production in human monocytic cell lines, but exerted priming effects on IL-8 secretion by THP-1 cells upon stimulation with LPS, which may be partially explained by the up-regulation of MyD88 by MDP [44]. In addition, Uehara et al. demonstrated that synthetic MDP and DAP-containing

desmuramylpeptides, signaling through NOD2 and NOD1 respectively, exhibited synergism with TLR2 (synthetic *E. coli*-type triacyl lipopeptide), TLR4 (synthetic *E. coli*-type lipid A) and TLR9 agonits (bacterial CpG DNA) to induce the production of IL-8 in THP-1 cells [45]. *Bordatella pertussis* tracheal cytotoxin (*N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-(L)-alanyl-γ-(D)-glutamyl-*meso*-diaminopimelyl-(D)-alanine) and endotoxin were synergistic in the induction of IL-1α mRNA and protein, production of nitric oxide and inhibition of DNA synthesis in hamster trachea epithelial cells [46], in accordance with the results of Dokter et al., where the *N*-acetylglucosaminyl-1,6-anhydro-*N*-acetylmuramyl-(L)-alanyl-γ-(D)-glutamyl-*meso*-diaminopimelyl-(D)-alanine induced IL-1β and IL-6 mRNA expression was further enhanced by costimulation with LPS or MDP in human monocytes [47]. MDP had also strong synergistic effects with the mycobacterial TLR2 ligand 19-kDa lipoprotein on the production of TNF, IL-1β and IL-6 in mononuclear cells [48].

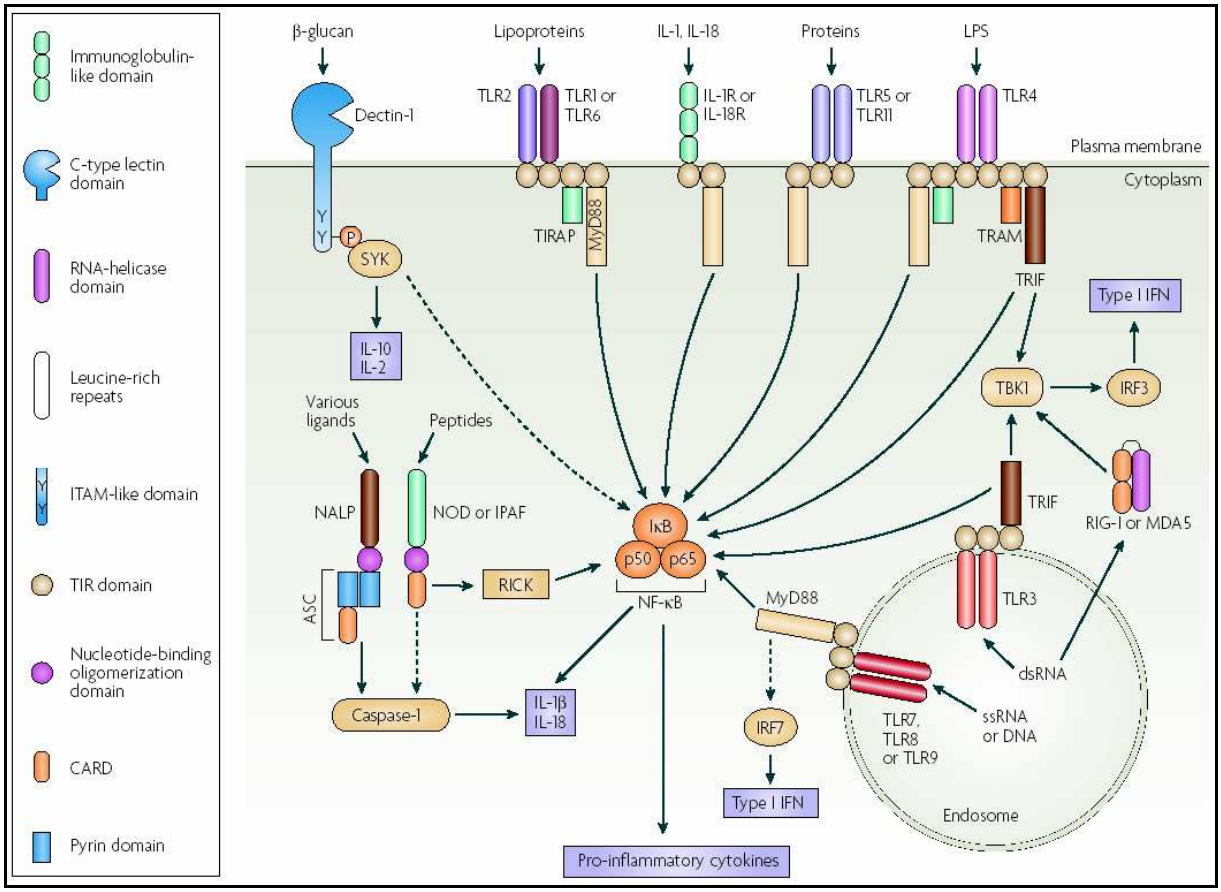

Figure 1 The structure and main signaling pathways of PRR families [49]

1.2. *Helicobacter pylori* infection

1.2.1. General features of *H. pylori* infection

Helicobacter pylori is a Gram-negative, microaerophilic, neutrophilic, spiral-shaped, flagellated [50, 51] bacterium that colonizes the human gastric mucosa and induces chronic inflammation. Since its isolation from human stomach biopsies by Marshall and Warren in 1983, *H. pylori* was shown to be an etiological agent in the development of gastritis, peptic ulcer [52], mucosa-associated lymphoid tissue (MALT) lymphoma [53], gastric atrophy with/without intestinal metaplasia [54] and gastric cancer [55, 56, 57]. *H. pylori* was declared to be the first bacterial class1 carcinogen by the World Health Organisation/ International Agency for Research on Cancer (WHO/IARC) in 1994 [58]. *H. pylori* infection is one of the most common bacterial infections worldwide affecting over half of the whole human population [59]. The infection is mostly acquired during childhood and is connected with low socioeconomic status. The transmission routes are fecal-oral, oral-oral and gastric-oral. If the infection is acquired, it can persist for the entire life of the host unless the eradication therapy is successful [60 61].

1.2.2. The *cag* pathogenecity island (*cag* PAI)

The cytotoxin-associated gene pathogenecity island (*cag* PAI) of *H. pylori* is a 40 kb cluster of 31 genes that encodes components of the TFSS [62, 63] among others, and was suggested to modulate the TLR2-agonist activity of *H. pylori* [64]. The presence of *cag* PAI is associated with increased mucosal inflammation and confers elevated risk for the development of peptic ulcer disease [65] and gastric cancer [66]. The TFSS can be depicted as a syringe that enables bacteria to deliver the CagA protein and bacterial breakdown products into the host cells. Knockout mutants for the *virD4* or *cagG* lose the ability to translocate CagA and cause temporally retarded or abolished gastric inflammation, respectively. Once CagA is injected into the gastric epithelial cells, it causes tight junction dysfunction and becomes phosphorylated by Src kinases on the tyrosine residues of the carboxy-terminal Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs, which enables it to participate in processes leading to host cell elongation (hummingbird phenotype) and changes in cell motility [63].

In gastric epithelial cells, the activation of the transcription factors NF-κB and activating protein-1 (AP-1), which leads to the expression of proinflammatory cytokines and exerts apoptotic and antiapoptotic effects, is *cag* PAI-dependent. These comprize the upregulation of the antiapoptotic gene that encodes the cellular inhibitor of apoptosis protein 2 (*c-IAP2*) in gastric cancer cells [67], apoptotic processes exerted mainly through the mitochondrial pathway [68] and the transcriptional regulation of *IL-8* [69], among others. In addition, the *cag* PAI-positive *H. pylori* causes inflammation by a synergistic mechanism that activates IL-8 and its receptors IL-8RA (CXCR1) and IL-8RB (CXCR2) by independent pathways [70]. The levels of TNF-α, IL-1β and IL-6, the genes of which are also NF-κB-responsive, are also elevated in the gastric mucosa of *H. pylori* infected patients [71].

1.2.3. Host responses to *H. pylori*

H. pylori infection alternates and evades the host responses, which enables the bacteria to perform a long-term colonization up to several decades even though local immune responses are present [72]. These responses are not only ineffective in eliminating the bacterium from its preferred niche, but actually contribute to the development of the gastric mucosal lesions observed in *H. pylori*-infected individuals.

The function of NOD1 in the host defence against *H. pylori* seems to be important, because *Nod1*-deficient mice have higher bacterial loads after *H. pylori* infection than wild-type mice [36]. NOD1 stimulation results in the production of other chemokines, and not merely IL-8. NOD1 induction stimulates human intestinal epithelial cells to produce CXCL1 (Groα) and CD83 as well [42]. These molecules are important in the recruitment and interaction of immune cells and this very probably holds true in the case of *H. pylori*-induced diseases. The role of TLR2, TLR4 and TLR5 is controversial in the recognition of *H. pylori* [73].

The local inflammation in *H. pylori* infection is characterized by the infiltration of neutrophils and lymphocytes into the gastric mucosa and by increased production of the proinflammatory cytokines TNF-α [74], IL-1β [75], IL-6 [76], IL-8 [77] and interferon-γ (IFN-γ) [78]. Lymphocytic derived IL-2 has also been detected [79]. IL-8, as a potent neutrophil chemotactic and activating factor secreted by epithelial cells, has been suggested to play a central role in *H. pylori*-associated diseases [80]. *H. pylori* also elicits the production of the

proinflammatory IL-12 by dendritic cells (DCs) in the gastric mucosa, which further induces the production of IFN-γ [81]. The effects of proinflammatory cytokines might be counteracted by the locally produced IL-10, a Th2 cytokine with anti-inflammatory effects [82].

1.3. The role of cytokines and PRRs in acute pancreatitis

AP remains a significant clinical challenge, because it has a complex and poorly understood pathophysiology, an unpredictable outcome and no specific treatment [83]. It has an annual incidence of 10-20 cases per 100000 people in the Western world [84]. The etiology is mostly alcoholic [85] or related to obstruction by gallstones [86]. Although the clinical course of AP is often mild with only minimal organ dysfunction, a significant proportion of these patients develop severe disease often associated with multiple organ failure (MOF) and infections [87]. The initial prediction about the mild or severe outcome of an attack has important implications for management, prognostication and use of health care resources. Different methods of prognostic classification have been developed, but so far, none has shown optimal overall predictive accuracy and usefulness for prediction in individual patients [88].

MOF and septic complications in AP do not differ from the systemic complications of other diseases such as sepsis itself, trauma or burn, which are included in SIRS. The systemic manifestations are responsible for the majority of pancreatitis-associated morbidity and mortality, and are due to the actions of the proinflammatory cytokines TNF-α, IL-1 and IL-8 [89, 90]. The levels of cytokines depend on a number of factors, including genetic background, particularly polymorphisms of cytokine genes. Genetic polymorphisms within the promoter region of inflammation-related cytokine genes are considered to influence the expression of the encoded cytokines [91]. IL-8 is an important neutrophil-activating cytokine and a potent chemoattractant, the increased levels of which have been demonstrated in AP [92, 93]. Other factors, including TLR4, that regulate the innate immune responses [94], may also be involved in the complications of AP. In view of the inflammatory nature of AP, we postulated that single nucleotide polymorphisms (SNPs) in the promoter region of *IL-8* and in the third exon of *TLR4* might have some association with the development of AP.

1.4. The role of cytokines and NOD1 in Crohn's disease

Inflammatory bowel disease (IBD) is a collective term for at least three heterogenous gastrointestinal disorders including Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis, which result in several distinct clinical phenotypes and lead to persistent and sometimes irreversible impairment of the gastrointestinal structure and function. IBD is thought to be a multifactorial disease, triggered by the inappropriate and exaggerated mucosal immune response to the normal microflora, which is partly determined by genetic and environmental factors. The importance of the commensal flora and its communication strategies with the host is illustrated by several transgenic mouse models and innate immunodeficiency syndromes. Innate immunity is much more complex than a mere mechanism which is fully capable to distinguish self from non-self. *CARD4/Nod1* is a perfect candidate as a susceptibility gene for CD. The latest microbiological studies failed to find a potential pathogen microorganism which leads to development of IBD, perhaps because there is no such a common microbe. As each bacterial sensor triggers the activation signal of different bacteria, theoretically the altered function of any of them may result in defective recognition of at least one part of the commensal flora. This theory might explain how IBD can develop in those patients who do not have NOD2 polimorphism: other receptors of PAMPs are responsible for the causative defect, and this receptor can be the NOD1 in individual cases [95].

In CD, manifestations can occur in any part of the gastrointestinal tract, but most commonly the terminal ileum, the cecum, the peri-anal area and the colon are affected, where the alteration of linear ulcers with islands of normal or edematous mucosa produces cobblestone-like appearance. By the progression of the disease, the bowel narrows, which leads to bowel obstruction, abscess formation and fistulization. Extraintestinal manifestations of CD affect the joints, the skin, the eyes, the mouth and the liver [96, 97].

The inflammation observed is dominated by an excessive Th1-mediated effector cell response. Increased amounts of the Th1 polarizing cytokine IL-12 were shown to be produced by DCs and macrophages in CD [98]. The mucosal expression of IL-18 - which is considered to be an activator of Th1 responses - becomes also upregulated in patients with CD [99]. The TNF superfamily member TL1A protein, the expression of which correlated positively with

the intensity of inflammation in CD, was suggested to maintain and perpetuate the IFN-γ mediated Th1 immune responses in the intestinal mucosa [100]. The production of IFN-γ provokes downstream effector cells such as macrophages to induce further production of the proinflammatory cytokines TNF-α, IL-1β and IL-6 [101]. Supporting the significant role of TNF-α in the pathogenesis of CD, infliximab, a chimeric monoclonal immunoglobulin G1 (IgG1) antibody proved to be effective in inducing and maintaining the remission of CD in 60% of the patients, by binding and neutralizing soluble and membrane-bound TNF-α [102, 103]. Although IL-10 was shown to downregulate the elevated secretion and mRNA levels of proinflammatory cytokines by CD mononuclear cells *in vitro* [104, 105], clinical trials with recombinant human IL-10 yielded only modest results [106].

1.5. Single nucleotide polymorphisms (SNPs)

DNA sequence polymorphisms are variations present at greater than 1% frequency in the population, while the frequency of mutations is less than 1%. By comparing two DNA sequences, wherever found different nucleotides at the same position, that is a SNP. SNPs are responsible for over 80% of the 0.1 % difference that is present between the DNA sequences of two individuals. They can occur anywhere in the genom: both in coding and noncoding regions of genes or in intergenic spacers [107]. Non-conservative mutations within the coding region of genes can result in loss, abrogation or change of function in the expressed protein as a result of change in protein structure. Although conservative mutations do not affect the amino acid sequence, they may influence protein expression in a variety of other ways: they can alter mRNA splicing, mRNA stability and levels of gene expression. Polymorphisms within the 5'- and 3'-regulatory sequences or introns of genes may have a significant effect on the transcription, since they may alter the structure of transcription factor binding sites within gene promoters or the structure of enhancers and silencers within introns [108]. SNPs are estimated to occur as frequently as every 100-300 bases [109]. More than 4 million SNPs have been identified to date with the leader participation of The SNP Consortium (TSC).

The clinical outcome of many infectious, autoimmune or malignant diseases appears to be influenced by the overall balance of production of proinflammatory and anti-inflammatory cytokines. A significant number of studies have addressed whether genetic polymorphisms

within the genes encoding these cytokines might influence the levels of expression, and therefore the overall immune response [108].

One potential application of SNPs is to develop individualised medicine by pharmacogenomics, in order to understand adverse drug responses, with respect to SNPs in the genes of drug-metabolizing enzymes [110]. SNPs represent useful tool in DNA fingerprinting for criminal investigations [111] and paternity testing [112]. With the help of SNPs, we can also gain insight into the steps of evolution: DNA sequence variants that provided benefit for the individual for succesful reproduction can even be selected by natural selection for retention. SNPs profiles characteristish for a disease trait can help to identify the relevant genes associated with polygenic diseases [113, 114].

1.5.1. SNPs of *CARD4*/*Nod1* and *CARD15*/*Nod2*

The deletion allele of the complex insertion-deletion polymorphism (*ND_1+32656*) of *CARD4* was significantly associated with susceptibility to inflammatory bowel disease [115], while the insertion allele was found to be associated with the presence of asthma and elevated IgE levels [15]. Focusing on five non-synonymous mutations of *CARD4*, Zouali et al. could not reveal any contribution to the genetic predisposition to IBD [116]. Polymorphisms with database accession numbers rs2736726 and rs2075817 of *CARD4* showed weak associations with atopic eczema [117]. Rosenstiel et al. reported that no mutations of *CARD4* or *CARD15* contribute to the development of gastritis or gastric ulcer in *H. pylori*-infected patients. There was no association found between SNPs of *CARD4* and the development of MALT lymphoma, while the R702W polymorphism of *CARD15* was significantly associated with the development of gastric lymphoma: carriers of the rare T allele had more than doubled risk to develop the disease than controls [118].

Mutations of *CARD15/Nod2* confer genetic predisposition for CD [31, 119], Blau syndrome [120], early-onset sarcoidosis [121], graft-versus-host disease [122], allergic rhinitis and atopic dermatitis [123].

1.5.2. SNPs of *TLR4*

The human gene encoding TLR4 is located on chromosome 9q32-q33 and contains four exons [124]. The A→G substitution at nucleotide 896 from the start codon of the human *TLR4* gene (in the fourth exon of *TLR4*) leads to the replacement of a conserved aspartic acid residue with glycine at position 299 (Asp299Gly; NCBI SNP cluster ID: rs4986790; also referred to as A12874G according to GenBank acc. no. AF177765 [125]). The C→T conversion at 1196 position results in the replacement of a nonconserved threonine with an isoleucine at position 399 (Thr399Ile; NCBI SNP cluster ID: rs4986791; also referred to as C1196T according to GenBank acc. no. AF177765 [125]). Both SNPs affect the extracellular domain of the receptor [126], presumably by altering its structure, which may cause altered ligand binding or protein interactions, resulting in impaired LPS signaling.

Peripheral monocytes from donors homozygous for the *TLR4* 896 G allele were LPS hyporesponsive, exhibiting fourfold higher median effective concentrations for TNF-α, IL-1β and IL-6 production compared to monocytes from patients with heterozygous or wild-type genotypes [127]. Upon *in vitro* stimulation with LPS, Kroner et al. detected significantly lower proliferation indices of peripherial blood mononuclear cells (PBMCs) from individuals heterozygous for A896G, in comparison with cells from individuals with the wild-type genotype [128]. Surgical intensive care unit patients who were heterozygous for both the A896G and C1196T polymorphisms (no homozygous mutant was found) had higher risk for subsequent Gram negative infections [129]. These two *TLR4* polymorphisms were shown to be associated with decreased airway responsiveness to inhaled LPS in humans by Arbour et al. [130] These generally accepted results and even studies relied on their citation were questioned by Erridge et al., who found that monocytes from individuals heterozygous for both polymorphisms of *TLR4* do not exhibit deficit in the recognition of LPS [131].

The frequency of the *TLR4* 896 G allele was significantly higher in patients with CD and UC than in their respective control populations [132]. CD patients with the A896G SNP had more often ileal envolvement or fistulizing disease, compared to wild-type patients [133].

The presence of the *TLR4* C1196T SNP was shown to be associated with UC and its increased frequency was observed in patients with CD, without reaching significance [134].

In contrast to the defective TLR4-mediated signal transduction in the murine experimental model of disseminated candidiasis [135], the *TLR4* Asp299Gly polymorphism was shown not to play a role in susceptibility to and severity of human urogenital *Candida albicans* infection [136].

1.5.3. SNPs of *IL-8*

The human gene encoding IL-8 is localized in the CXC chemokine locus on chromosome 4q12-q21 and consists of 4 exons and 3 introns [137]. The SNP at -251 nucleotide relative to the transcription start site (T-251A), in the promoter region of *IL-8* was identified by Hull et al. The presence of the mutant A allele was associated with increased IL-8 production by LPS stimulated whole blood, with the rising severity of respiratory syncytial virus acute bronchiolitis [138] and with increased risk for human tuberculosis [139]. Matheson et al. found no association between the T-251A SNP and respiratory symptoms or lung function in middle-aged and older adults [140].

The chance of having enteroaggregative *E. coli* (EAEC)-associated diarrhea significantly increased among those harbouring the -251 A allele, compared to the individuals with TT genotype [141]. Jiang et al. found significantly higher occurence of the homozygous genotype for the -251 A allele among patients with *Clostridium difficile* toxin-induced diarrhea, compared to the control groups of *C. difficile*-negative diarrhea and nondiarrhea [142]. Fecal IL-8 levels were significantly higher in subjects with *C. difficile* toxin-induced or EAEC diarrhea who were homozygous for the -251 A allele, compared to patients with heterozygous or wild-type genotype [141].

Suggesting the involvement of IL-8 in the development and progression of human malignancies, the -251 A allele was found to be associated with higher risk for nasopharyngeal carcinoma [143], prostate cancer [144], oral squamous cell carcinoma [145] and breast carcinoma [146]. Individuals homozygous for the A allele at -251 or for the G allele at +396 on *IL-8* were at twofold increased risk of cardia adenocarcinoma compared to those harbouring the wild-types, furthermore the risk for the AGT/AGC haplotype of *IL-8* -251/+396/+781 was fourfold in a chinese population (T-251A, T+396G and C+781T SNPs on *IL-8*) [147].

Renzoni et al. identified three polymorphisms in the noncoding areas of *IL-8* (A-353T in the promoter region, G+293T and T+678C in the first intron) that were shown not to be associated with cryptogenic fibrosing alveolitis and fibrosing alveolitis associated with systemic sclerosis [148]. Rovin et al. identified the *IL-8* T-738A and T-845C biallelic polymorphisms and demonstrated that the frequency of the -845 C allele was significantly higher in African American patients with severe, inflammatory systemic lupus erythematosus nephritis compared to healthy individuals, indicating that the T-845C polymorphism might influence the expression of IL-8 [149].

1.6. High-mobility group box 1 protein (HMGB-1)

High-mobility group (HMG) chromosomal proteins were described as abundant components of the chromatin by Goodwin et al. [150] . Their name indicates the rapid electrophoretic mobility on polyacrilamide gel. The HMG proteins are grouped into 3 families. The HMGB family has 3 members: HMGB-1, -2 and -3 [151].

HMGB-1 (named also HMG-1 or amphoterin) is a highly conserved protein featuring >98 % identity among mammals. It has two DNA-binding domains (named A- and B-box) and a negatively charged, acidic, repetitive carboxy-terminal tail. The nuclear translocation of the protein is under the control of two nuclear localization signals (NLS) on the molecule: NLS1 is localized to the A-box, while NLS2 can be found between the B-box and the acidic tail [152].

HMGB-1 has different properties depending on its localization. The intranuclear form binds to the minor groove of DNA without sequence specificity, and acts as an architectural protein stabilizing the nucleosome formation, facilitating DNA bending and taking part in the interaction between transcription factors and DNA [153, 154, 155, 156, 157].

HMGB-1 can get out of the cells by two mechanisms: it can be secreted actively by monocytes [158], macrophages [159], pituicytes [160], human umbilical vein endothelial cells (HUVEC) [161] and murine erythroleukemia cells [162] following appropriate stimulation, or released passively by damaged or necrotic cells [163]. Rouhiainen et al. demonstrated that HMGB-1 present in human platelets is exported to the cell surface upon activation [164].

Proinflammatory mediators can co-operate in order to promote HMGB-1 secretion: IFN-γ induces HMGB-1 release partly through a TNF-dependent mechanism in murine macrophages [165]. HMGB-1 released in response to proinflammatory stimuli also induces the release of proinflammatory mediators by macrophages and neutrophils such as TNF-α, IL-1α, IL-1β, IL-6**,** IL-8, macrophage inflammatory proteins MIP-1α and MIP-1β and HMGB-1 [166, 167, 168], thus prolonging and sustaining inflammatory processes. When released passively, HMGB-1 acts as an endogenous danger signal which triggers inflammation and reparative processes [163] .

HMGB-1 lacks a secretory signal peptide (similarly to IL-1β [169]), so it cannot be secreted via the Golgi-endoplasmic reticulum (ER) pathway [162]. In resting monocytes HMGB-1 shuttles through the nuclear membrane to the cytosol or into the nucleus. Triggered by an activation signal, lysine residues within the two NLS sites of the HMGB-1 molecule become acethylated, which is thought to inhibit the relocalization of the protein into the nucleus. This hyper-acethylated form will accumulate in the cytosol and will be packed into secretory lysosomes that fuse with the cell membrane and release their content into the extracellular space [152, 170].

HMGB-1 enhances its own degradation by accelerating the tissue plasminogen activator (t-PA)-catalysed plasminogen activation: the protein is suggested to be a target of the lysine-specific serine protease plasmin [171].

HMGB-1 was reported to play an important role in the pathomechanism of atherosclerosis [167, 172] and rheumatoid arthritis (RA) [173], and its elevated serum levels were measured in patients with hemorrhagic shock [174], community-acquired pneumonia [175] and Churg-Strauss syndrome [176]. HMGB-1 was demonstrated to be a late mediator of endotoxin lethality in mice and of sepsis in humans by Wang et. al [159]. Sundén-Cullberg et al. found that serum levels of HMGB-1 remained high in the majority of patients with severe sepsis and septic shock up to one week after admission [177]. In the pathomechanism of SAP, the release of HMGB-1 by injured pancreas or other damaged organs as well as its secretion by activated monocytes/ macrophages can be taken into consideration. [178].

The receptor for advanced glycation end-products (RAGE) was shown to be the major functional receptor responsible for the proinflammatory effects of HMGB-1 in rhodent

macrophages [179]. HMGB-1 enhances the maturation of DCs [180], induces the extensive spreading and mediates the transendothelial migration of monocytes also with the involvement of RAGE [158]. In addition, HMGB-1 enhances RAGE expression in human microvascular endothelial cells [181] and in synovial fluid macrophages [166]. TLR4 mediates the early inflammatory effects of HMGB-1 during hepatic ischemia/reperfusion injury [182], and together with TLR2 might serve as an alternative HMGB-1 receptor [183, 184].

AIMS

The present study was designed to address the following aims:

1. To determine the association between *CARD4/Nod1*, *TLR4* and *IL-8* polymorphisms and the development of gastritis and DU in *H. pylori*-infected patients.

2. To determine the association between *IL-8* and *TLR4* polymorphisms and the development of AP.

3. To determine the association between *CARD4/Nod1* polymorphism and the development of CD.

4. To evaluate the role of HMGB-1 protein in AP, which required the elaboration of reliable *in vitro* methods for the detection of HMGB-1.

2. PATIENTS AND METHODS

2.1. Patients and controls

2.1.1. Patient groups with duodenal ulcer or chronic active gastritis

85 *H. pylori*-positive patients with DU and 136 with chronic active gastritis were enrolled in the study. Biopsies were taken during upper gastrointestinal endoscopy from adjacent sites of the gastric antrum and corpus for histology. In addition, ^{13}C-Urea Breath Test (UBT) was carried out. Patients with *H. pylori* infection confirmed by histology and ^{13}C-UBT were considered to be eligible for the study. The presence of *H. pylori* and the severity of gastritis were graded with the updated Syney Classification System [185].

The 75 members of the control population were serologically *H. pylori*-positive, without any gastrointestinal symptoms. Their status of *H. pylori* infection was determined by serology, with an enzyme-linked immunosorbent assay (ELISA) kit [HP IgG ELISA (Dia.Pro, Milan, Italy)]. They were recruited from the Blood Transfusion Center at the University of Szeged.

Sera either from patients and control people were tested for CagA by serology, with a commercial ELISA kit (Dia.Pro, Milan, Italy). Only *H. pylori*-positive and CagA-positive patients and controls were considered to be eligible for the study.

All cases and controls were of Hungarian ethnic origin and resident in Hungary. Informed consent was obtained from all patients. The project was approved by the Clinical Ethical Committee of the Medical Faculty of the University of Szeged and by the Clinical Ethical Committee of Semmelweis University.

2.1.2. Patient group with acute pancreatitis

92 patients with AP were enrolled. Diagnostic criteria of AP included clinical history consistent with the disease, radiological evidence and serum amylase level greater than 660 U/L. According to the original criteria of Ranson [186], patients were classified into groups with mild or severe pancreatitis: patients with fewer than three positive prognostic signs (n=42) were considered to have mild pancreatitis; while those with three or more positive prognostic signs (n=50) were classified into the severe pancreatitis group. Patients with severe

acute necrotizing pancreatitis were divided into aseptic (n=28) or infected (n=22) groups on the basis of the results of bacterial cultures of the necrotic pancreatic tissue sampled during surgery or ultrasound- or computed tomography (CT)-guided biopsies.

The patients entered this prospective study at the Department of Surgery and at the First Department of Internal Medicine of Albert Szent-Györgyi Medical Center, University of Szeged between March 2003 and January 2005. The control cohort consisted of a random, unrelated population sample of 200 healthy blood donors. All cases and controls were of Hungarian ethnic origin and resident in Hungary.

2.1.3. Patient group with Crohn's disease

434 CD patients were investigated. The diagnosis was set up on the basis of Lennard-Jones criteria [187]. The age at onset, duration of the disease, presence of extraintestinal manifestations, frequency of flare-ups, effectiveness of therapy, need for surgery, occurence of familial IBD, smoking habits and peri-anal involvement were investigated by the review of the medical charts and questionnaries. The disease phenotype was determined according to the Vienna classification [188].

The control group consisted of 200 age- and gender-matched healthy blood donors without gastrointestinal or liver diseases. They were selected from consecutive blood donors in Szeged. The second, non-IBD control group included 136 patients with chronic active gastritis.

2.2. Genotyping procedures

2.2.1. DNA extraction

For the examination of *CARD4*/*Nod1, TLR-4* and *IL-8* polymorphisms, leukocyte DNA from peripheral blood was isolated using the High Pure PCR Template Preparation Kit according to the manufacturer's instructions (Roche Diagnostics GmbH). The genomic DNA was stored at –20 °C until further use.

2.2.2. Determination of *CARD4*/*Nod1* G796A polymorphism

The G→A SNP on *CARD4* at position 796 (NCBI SNP cluster ID: rs2075820) was analysed by PCR-restriction fragment length polymorphism (PCR-RFLP). The 379 bp amplified product was digested with *AvaI* restriction endonuclease enzyme and analysed on 2% agarose gel under UV illumination. The restriction site was present only together with the G allele and was indicated by the cleavage of the 379 bp product, giving fragments of 209 bp and 170 bp. The *CARD4* A allele gives a single 379 bp fragment due to the absence of restriction [116].

2.2.3. Determination of *TLR4* A12874G and C13174T polymorphisms

The *TLR4* 12874 A→G (according to GenBank acc. no. AF177765, NCBI SNP cluster ID: rs4986790; also referred to as A896G) and 13174 C→T (according to GenBank acc. no. AF177765, NCBI SNP cluster ID: 4986791; also referred to as C1196T) polymorphisms were genotyped simultaneously by a real-time PCR (RT PCR) assay using specific fluorescence-labelled hybridization probes, followed by melting point analysis with the help of a LightCycler instrument. By displaying the negative first derivative of the melting curve data versus temperature [-(dF/dT) vs T], wild-type samples showed a peak at 64 °C (A12874G) or at 67 °C (C13174T), while heterozygous samples were characterized by an additional peak at 61 °C (A12874G and C13174T) [125].

2.2.4. Determination of *IL-8* T-251A polymorphism

The T→A SNP on *IL-8* at position -251 (relative to the transcription start site) was genotyped by amplification refractory mutation system (ARMS). The allele-specific primers were: 5'-CCACAATTTGGTGAATTATCAAT-3' (-251A) and 5'-CCACAATTTGGTGAATTATCAAA-3' (-251T). The consensus primer was: 5'-TGCCCCTTCACTCTGTTAAC-3'. The amplified PCR product consisted 336 bp. In each reaction, a second set of primers for exon 3 of the HLA-DRB1 gene was applied (forward: 5'-TGCCAAGTGGAGCACCCAA-3', reverse: 5'-GCATCTTGCTCTGTGCAGAT-3', product size: 796 bp) as control for the PCR efficiency [138].

2.3. Determination of HMGB-1 secretion by monocytic cells and plasma HMGB-1 levels in patients with acute pancreatitis

2.3.1. Cell line

Human monocytic U-937 cells were propagated in RPMI 1640 medium (GIBCO) supplemented with 100 μg/ml ampicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum at 37 °C in a humidified CO_2 incubator.

2.3.2. Cell stimulation

Before analysing HMGB-1 secretion by U-937 cells, the culture media were replaced by OptiMEM (GIBCO) medium, and the number of the cells was adjusted to 10^6 cells/ml. The cells were stimulated for 24 hours with 5 ng/ml phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich), 10 μg/ml LPS *E. coli* O111:B4 (Sigma-Aldrich), heat-killed *Staphylococcus aureus* (SA, 10^8/ml) or with *Mycobacterium bovis* BCG (10^7/ml, Pasteur strain of *M. bovis* BCG, provided by David G. Russel, Department of Microbiology and Immunology, Cornell University, Ithaca, NY, USA).

2.3.3. HMGB-1 Western blot analysis

The supernatants of stimulated U-937 cells were concentrated 10-fold on Centricon 10 filters (Millipore Corporation), with subsequent processing in Laemmli buffer. Recombinant human HMG-1 (Sigma-Aldrich) and recombinant rat amphoterin (RecAtn, provided by Ari Rouhiainen, Neuroscience Center, University of Helsinki, Helsinki, Finland) served as standard antigens. The standards and the concentrated supernatants were resolved in 12.5 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The proteins were transferred to nitrocellulose blotting membrane that were then blocked with 5% nonfat dry milk. The filters were stained firstly with chicken polyclonal anti-RecAtn antibodies (provided by Ari Rouhiainen, Neuroscience Center, University of Helsinki, Helsinki, Finland), secondly by horseradish peroxidase-conjugated goat anti-chicken antibodies (Zymed Laboratories), developed with ECL Plus reagents (Amersham

Biosciences) and exposed to X-ray film. The densitometric analysis of the immunoreactive bands was performed by ImageQuant 5.2 software (Molecular Dynamics).

2.3.4. HMGB-1 ELISA / a.

The wells of the 96-well MaxiSorp plate (Nunc) were coated with mouse monoclonal anti-human HMG-1 antibodies (R&D Systems) at 10 μg/ml, then blocked with 2% nonfat milk. The recombinant human HMG-1 (Sigma-Aldrich) was used as a standard substance, serially diluted from 200 to 1.6 ng/ml. After keeping the standard dilutions and the samples in the wells, the steps separated by washing procedures were as follows: the addition of rabbit anti-HMG-1 polyclonal antibodies (BD Biosciences), horseradish peroxidase-labelled goat anti-rabbit IgG antibodies (Bio-Rad Laboratories) and then TMB substrate (BD Biosciences) to the microtitration plates. After the reaction was stopped by adding 2N sulphuric acid, the absorbance was measured at 450 nm (650 nm reference). HMGB-1 concentrations of the samples were calculated from the standard curve.

2.3.5. HMGB-1 ELISA / b.

Plasma HMGB-1 concentrations were determined by the HMGB-1 ELISA kit (Shino-Test Corporation), according to the instructions of the manufacturer [189, 190].

2.3.6. TNF-α ELISA

TNF-α concentrations in the cell culture supernatants were quantified by the use of a TNF-α ELISA kit (BioSource), according to the instructions of the manufacturer.

2.3.7. Immunofluorescence

Either stimulated and control cells were fixed in 4% paraformaldehide and permeabilized with 0.3% Triton X-100 on glass coverslips. The coverslips were saturated with 10% bovine serum albumin (BSA) and the cells were stained with chicken polyclonal anti-RecAtn antibodies (provided by Ari Rouhiainen, Neuroscience Center, University of Helsinki, Helsinki, Finland). Fluorescein isothiocyanate (FITC)-conjugated donkey anti-chicken IgY (Jackson Immunoresearch Laboratories) was applied for the detection of the bound antibodies.

Between all incubation step, the cells were washed three times with PBS containing 0.2% BSA. Fluorescence signals were detected by confocal microscopy.

2.3.8. Confocal microscopy and semiquantitative assessment of fluorescence intensities

Serial images of the immunostained samples were captured by Olympus FV1000 confocal laser scanning microscope with standard parameter settings. The fluorescent signal of control, *M. bovis* BCG and PMA treated cells were quantitatively analysed by ImageQuant software (Molecular Dynamics). The mean and standard deviation of these data were calculated by the Microsoft Excel program. The level of statistical significance was determined by two-tailed T-probe.

2.4. Statistical analysis

In order to analyse the level of significance for genotype and allele frequencies between groups, chi-square test or Fisher exact test was applied. The genotype frequencies for each polymorphisms were tested for deviation from the HWE by the χ^2 test, with 1 degree of freedom. An α level of $p<0.05$ was taken as an indication of statistical significance. The relationship between genotypes and disease severity is presented as the odds ratio (OR), with a 95% confidence interval (CI).

The Mann-Whitney test was used to evaluate the differences in HMGB-1 levels between the group of patients with acute pancreatitis and the control groups. An α level of $p<0.05$ was taken as an indication of statistical significance.

All statistical analyses were performed using GraphPad Prism 4 statistical program (GraphPad Software Inc.).

3. RESULTS

3.1. Association of *CARD4*/*Nod1*, *TLR4* and *IL-8* polymorphisms with *Helicobacter pylori*-induced duodenal ulcer and gastritis

3.1.1. *CARD4/Nod1* G796A polymorphism

The distribution of *CARD4* 796 genotypes was in accordance with the HWE both in the control population and in the patient group with gastritis, but not in the group of patients with DU. Significant difference was found in the genotype distribution between the patients overall and the controls. There was a higher frequency of the homozygote mutant AA genotype among patients with DU, compared to the controls or to the gastritis group. Conversely, the prevalence of the wild-type GG genotype proved to be significantly lower in the group of DU patients, either in comparison with the control group or patients with gastritis. However, no significant difference was observed between the genotypes of controls and gastritis patients.

	GG	GA	AA
DU	38/85 (45%) p=0.017 versus control[a] p=0.012 versus gastritis[a]	30/85 (35%) p=0.020 versus control[a] p=0.010 versus gastritis[a]	17/85 (20%)
Gastritis	85/136 (63%)	41/136 (30%)	10/136 (7%)
Control	48/75 (64%)	22/75 (29%)	5/75 (7%)
χ^2=13.46, p=0.009: comparison between the patients overall (DU+gastritis) and the controls [a]Fisher test			

Table 1 *CARD4* G796A genotypes in patients with DU or gastritis and in the control population

3.1.2. *TLR4* A12874G polymorphism

The distribution of the *TLR4* 12874 genotypes was in accordance with the HWE in the two groups of patients and in the controls. There were no significant differences in the genotype

frequencies of *TLR4* between the patients overall and the healthy controls, nor between the patients with DU and gastritis (Manuscript II. / Table 4).

3.1.3. *TLR4* C13174T polymorphism

The distribution of the *TLR4* 13174 genotypes was in accordance with the HWE either in the patient groups and in the control group. No significant difference was found in the genotype distribution between the patients and the controls, nor between the DU and gastritis group (Manuscript II. / Table 5).

3.1.4. *IL-8* T-251A polymorphism

The genotype frequencies for the *IL-8* -251 polymorphism did not deviate significantly from those expected for the HWE in the two groups of patients and in the controls.

The genotypic frequencies were significantly different between the patients taken as a whole and the healthy controls. There was a higher frequency of the heterozygote AT genotype among the patients with DU, compared to the controls. Similarly, significant difference was found between the gastritis patients and healthy blood donors, concerning the frequency of the AT genotype. Conversely, the wild-type TT genotype was present with significantly lower frequency among patients with DU or gastritis than in the control group.

	TT	TA	AA
DU	15/85 (17%) p=0.002 versus control[a]	49/85 (58%) p=0.027 versus control[a]	21/85 (25%)
Gastritis	31/136 (23%) p=0.011 versus control[a]	76/136 (56%) p=0.0314 versus control[a]	29/136 (21%)
Control	30/75 (40%)	30/75 (40%)	15/75 (20%)
χ^2=11.98, p=0.017: comparison between the patients overall (DU+gastritis) and the controls [a]Fisher test			

Table 2 *IL-8* T-251A genotypes in patients with DU or gastritis and in the control population

These data suggest that NOD1 rather than TLR4 has functional significance in the recognition of *H. pylori*. On the other side, they indicate the importance of IL-8 in *H. pylori*-associated gastrointestinal diseases.

3.2. Association of *IL-8* and *TLR4* polymorphisms with the severity of acute pancreatitis

3.2.1. *IL-8* T-251A polymorphism

The genotype frequencies for the *IL-8* -251 polymorphism did not deviate significantly from those expected for the HWE in the control population and in the patient group.

Significant difference was observed in the genotype distribution between the pancreatitis patients overall and the healthy controls. After dividing the patients according to the severity of the disease, the significant difference in genotype frequencies was present only between the controls and the patients with severe pancreatitis, but it could not be detected between the control group and the patient group with mild pancreatitis.

	TT	AT	AA	p
Mild	15/42 (36%)	15/42 (36%)	12/42 (28%)	0.220[a]
Severe	8/50 (16%) p=0.0009 vs.control[b] p=0.051 vs. mild[b]	30/50 (60%) p=0.0264 vs. control[b] p=0.0232 vs. mild[b]	12/50 (24%)	0.004
Total	23/92 (25%)	45/92 (49%)	24/92 (26%)	0.020
Control	82/200 (41%)	84/200 (42%)	34/200 (17%)	
[a] Chi-square test versus controls [b]Fisher test				

Table 3 *IL-8* T-251A genotypes in patients with AP and in the control population

The heterozygote AT genotype was present with a significantly higher frequency among patients with severe disease, compared to the controls. There was also a significant difference between the patients having severe and those with mild form of the disease. In accordance with this, the rate of carriage of the high IL-8 secreting A allele proved to be significantly

higher in patients with severe pancreatitis than in the control population. Conversely, the prevalence of the wild-type TT genotype was significantly lower among patients with the severe disease form than in the control population or in the patient group with mild pancreatitis.

3.2.2. *TLR4* A12874G polymorphism

The distribution of the *TLR4* 12874 genotypes was in accordance with the HWE neither in the control nor in the patient group. No significant difference was found in the genotype distribution between the pancreatitis patients overall and the healthy controls, nor between the patient group with severe pancreatitis and the control group. It is worth mentioning that six of the seven AG heterozygote patients were found in the severe pancreatitis group (six of fifty), in contrast to the only one heterozygote among the 42 patients with mild pancreatitis (Manuscript I. / Table 2).

3.2.3. *TLR4* C13174T polymorphism

The distribution of the *TLR4* 13174 genotypes both in the control population and in the patient group was in accordance with the HWE. There were no significant differences in the genotype frequencies of the *TLR4* 13174 polymorphism either between the pancreatitis patients overall and the healthy control population, or between the patients with severe pancreatitis and the controls. In spite of the relatively low number of CT heterozygotes, it is striking that six of the seven heterozygotes were found in the severe pancreatitis group, while there was only one among the 42 patients with mild pancreatitis (Manuscript I. / Table 3). So we can assume that the activation of TLR4 might not have importance, while the IL-8 producing capacity has a definite role in the pathomechanism of AP.

3.3. Association of a *CARD4/Nod1* polymorphism with the susceptibility and phenotype of Crohn's disease

The distribution of *CARD4* 796 genotypes was in accordance with the HWE in the patient group with CD and in the control groups of healthy blood donors. An additional group of

patients without IBD (n=136) was included in the study, whose genotypes were determined previously, during the course of *H. pylori*-related diseases.

Significant difference in the genotype distribution was found between the CD patients and the healthy controls, as well as between CD patients and non-IBD controls. When frequencies of the homo- and heterozygous mutations were examined together, the carriage of *CARD4* G796A polymorphism proved to be a highly significant risk factor for CD. In addition, the A allele was significantly more frequent among patients with CD, compared to both control populations.

No significant associations were found between the different genotypes and the demographic data of the patients or the clinical characteristics of CD (Manuscript III. / Table 4).

	Genotype[*]			Allele frequency[#]	
	GG	AG	AA	G	A
CD	215/434 (49.5%)	180/434 (41.5%)	39/434 (9%)	610 (70.3%)	258 (29.7%)
Controls	134/200 (67%)	56/200 (28%)	10/200 (5%)	324 (81%)	76 (19%)
Non-IBD GI	85/136 (62.5%)	41/136 (30%)	10/136 (7.5%)	211 (77.6%)	61 (22.4%)

[*]CD vs. controls: χ^2<0.0001, p<0.0001, $OR_{hetero/homozygous\ vs.\ wild\text{-}type}$: 2.07, 95% CI: 1.46-2.93.
CD vs. Non-IBD GI controls: χ^2=6.977, p=0.008, $OR_{hetero/homozygous\ vs.\ wild\text{-}type}$:1.70, 95% CI: 1.14-2.52.
[#]CD vs. controls: χ^2=16.229, p<0.0001, $OR_{A\ vs.\ G}$: 1.80, 95% CI: 1.35-2.41.
CD vs. Non-IBD GI controls: χ^2=5.472, p<0.019, $OR_{A\ vs.\ G}$: 1.46, 95% CI: 1.06-2.01.

Table 4 *CARD4* G796A genotypes in patients with CD and controls

3.4. The secretion of HMGB-1 by monocytic cells

In order to investigate the role of HMGB-1 in pancreatitis, it was necessary to elaborate a reliable *in vitro* model to detect HMGB-1. Therefore at first we set up experiments to measure HMGB-1 levels in the supernatants of U-937 cells, following stimulation of the cells with PMA, LPS, SA or with *M. bovis* BCG.

3.4.1. HMGB-1 Western blot analysis

Western blotting of the 10-fold concentrated cell culture supernatants revealed 25 kDa bands that migrated together with the recombinant rat amphoterin (RecAtn, kindly provided by Ari Rouhiainen, Neuroscience Center, University of Helsinki, Helsinki, Finland). The *E. coli*-derived recombinant human HMG-1 gave also a considerable signal, featuring different molecular weight at 30 kDa. Chicken polyclonal anti-RecAtn antibodies were applied, because of their exclusive potential to recognize HMGB-1 secreted by the monocytic cells in Western blot experiments. These chicken antibodies were raised against recombinant rat amphoterin, and the rat protein differs only in two amino acids (Asp189Glu and Glu201Asp) from the human one. The commercial affinity purified rabbit anti-HMG1 polyclonal (BD Biosciences), mouse anti-human HMG-1 monoclonal (R&D Systems) and mouse monoclonal (clone 4C3) anti-HMGB-1 antibodies failed to form considerable bands. The densitometric analysis of the immunoreactive bands revealed higher concentrations of HMGB-1 in the supernatants of cell cultures stimulated either with PMA or *Mycobacterium bovis* BCG bacteria than in those of non-stimulated, control cells (Manuscript IV. / Figure 1).

3.4.2. Mass spectrometry

Because of the two standard antigens featured different molecular weights in the Western blotting experiments, mass spectrometry analysis was performed in order to verify the identity of these proteins and to explain the differences in their molecular weights.

Matrix assisted laser desorption/ionization – time of flight (MALDI-TOF) analysis of the unfractionated tryptic digest of the appropriate gel band identified 79% of the masses detected as predicted tryptic cleavage products of the recombinant human HMGB-1. These peptides represented approximately 56% of the protein sequence. The identity of four peptides was further confirmed by collision-induced dissociation (CID) analysis. Masses corresponding to predicted His-tag tryptic peptides were also detected, and their identity was confirmed by post source decay (PSD) analysis (Manuscript IV. / Figure 2).

82% of the masses detected in the RecAtn matched predicted tryptic peptides of the rat HMGB-1 protein. The identity of m/z 1520.76 as predicted Ile^{113}-Lys^{127} was further

confirmed by PSD analysis. These peptides represented approximately 47.6% of the protein sequence (Manuscript IV. / Figure 3).

These experiments were performed by the Proteomics Research Group, Biological Research Center of the Hungarian Academy of Sciences.

3.4.3. The levels of HMGB-1 and TNF-α following bacterial induction

In order to obtain more sensitive and quantitative detection of HMGB-1, an in home ELISA was developed. We wanted to reveal how the incubation with LPS, SA, *M. bovis* BCG or PMA affects the secretion of HMGB-1 by U-937 cells. It was also checked whether the induction of HMGB-1 secretion proceeded in parallel with that of TNF-α.

Induction by LPS resulted in moderate TNF-α (5.5 ± 6.6 pg/ml) and weak HMGB-1 secretion (4.5 ± 0.5 ng/ml). There was a higher concentration of TNF-α in the supernatants of the cells incubated with SA (650 ± 40.5 pg/ml), and considerable amounts of HMGB-1 (6.2 ± 0.9 ng/ml) were detected in the supernatants of the same cell cultures. The induction by *M. bovis* BCG rather than by SA resulted in pronounced HMGB-1 (56 ± 11 ng/ml) and decreased TNF-α secretion (120 ± 11 pg/ml). Much greater amounts of TNF-α and HMGB-1 were measured in the PMA-treated cell supernatants (350 ± 50 pg/ml and 65 ± 19 ng/ml respectively). Mycobacteria induced almost the same magnitude of HMGB-1 secretion as PMA did. PMA and *M. bovis* BCG added at the same time resulted in more elevated HMGB-1 secretion.

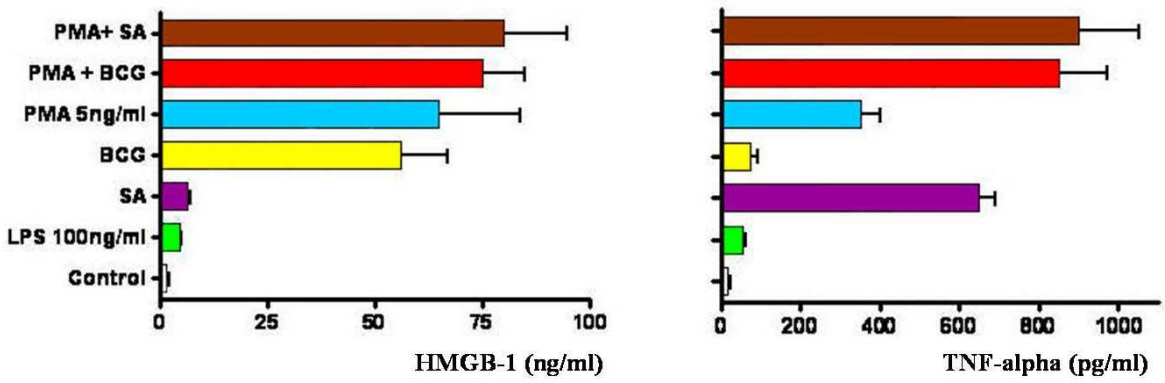

Figure 2 HMGB-1 and TNF-α ELISAs

3.4.4. Immunofluorescence

U-937 cells were incubated with *M. bovis* BCG, the impact of which on the HMGB-1 secretion was examined in the preliminary experiments. Non-stimulated cells displayed strong staining for HMGB-1 mostly restricted to the nucleus. Eighteen hours after stimulation by *M. bovis* BCG, the HMGB-1 protein appeared to be translocated from the nucleus (it was still partly positive) to the periphery of the cells, displaying patchy staining in the cytoplasm. Due to the dispersity of the fluorescence, its intensity was significantly lower in the cells stimulated with *M. bovis* BCG (Manuscript IV. / Figure 4).

3.5. Plasma HMGB-1 levels in patients with acute pancreatitis

Though we elaborated an in home HMGB-1 ELISA, meanwhile a commercial ELISA kit became available in the market developed by Shino-Test Corporation. The sensitivity proved to be higher, therefore when measuring plasma samples, we applied this commercial one in spite of its expense. The mean value of plasma HMGB-1 levels was significantly higher (P=0.0038) in patients with AP (2.889±1.976 ng/ml) than in healthy subjects (0,039±0.014 ng/ml).

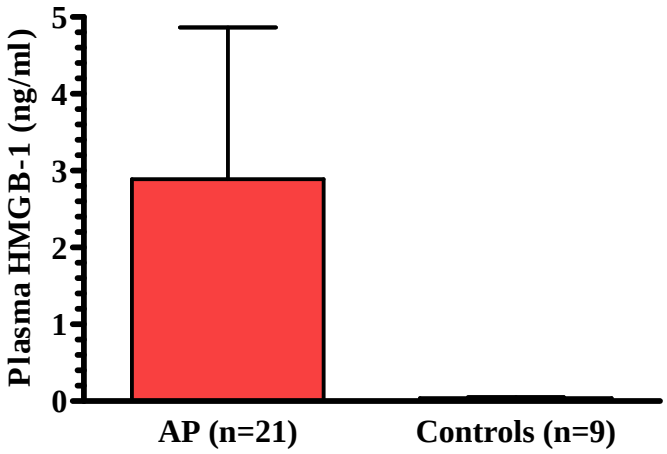

Figure 3 Plasma HMGB-1 levels in patients with AP and in the control population

It was also higher than that measured in a second control group of patients with sepsis (2.583±0.874 ng/ml) without reaching statistical significance (P=0.4724). The mean value of plasma HMGB-1 levels was also significantly higher (P=0.0007) in patients with sepsis than

in the control group of healthy subjects. The mean value of plasma HMGB-1 concentrations was higher in the group of patients with SAP (6.014±5.177 ng/ml) than among patients with the mild form of the disease (0.966±0,295 ng/ml), but the difference was not statistically significant (P=0.7170). In the SAP group, the mean value of HMGB-1 concentrations was higher than that measured among healthy control people (P=0.0055) or in the control group with sepsis (P=0.9058). In the patient group with mild AP, the mean value of HMGB-1 concentrations was higher than the value obtained in the control group of healthy people (P=0.0161), but was lower than that measured in the group of septic patients (P=0.3738).

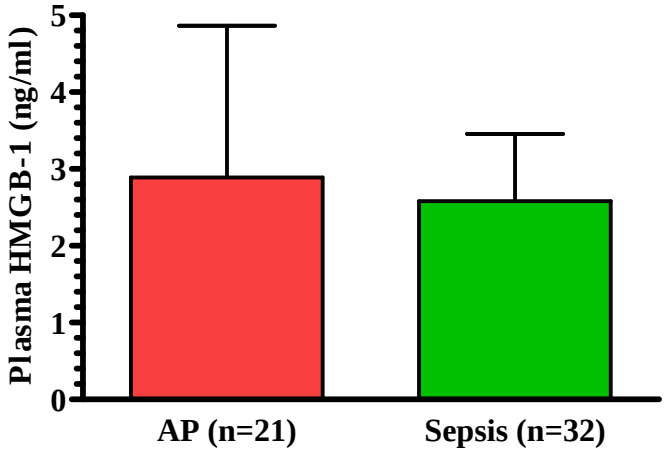

Figure 4 Plasma HMGB-1 levels in patients with AP and with sepsis

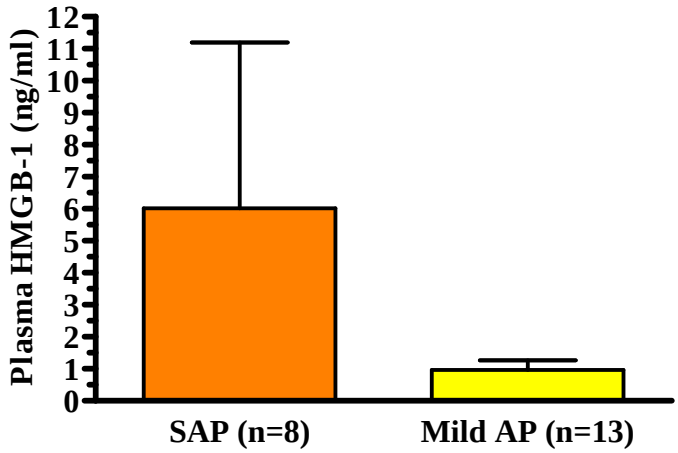

Figure 5 Plasma HMGB-1 levels in patients with SAP and mild AP

To our knowledge, this is the first study in Europe investigating HMGB-1 levels in AP.

4. DISCUSSION

4.1. *CARD4/Nod1, TLR4* and *IL-8* polymorphisms in *H. pylori*-induced duodenal ulcer and gastritis

The outcome of gastrointestinal diseases in *H. pylori*-infected persons is determined by the variations in the immunological responses of the host, environmental influences and genetic composition of the infecting strains. That is why many infected individuals have few if any gastrointestinal symptoms, and among *H. pylori*-infected patients, the incidence of ulcer formation is 15-20% [191].

4.1.1. *CARD4/Nod1* G796A polymorphism

The homozygote mutant AA genotype was present with significantly higher frequency among patients with DU, either in comparison with the group of patients with gastritis or with the control population.

There are not any exact data in the literature demonstrating how this polymorphism alters the function of the receptor. The Chou-Fasman algorithm indicated a slight decrease in the helix-forming potential by the replacement of the glutamic acid residue to lysine, which may influence the ability of the NOD to facilitate the self-oligomerization of the molecule. The fact that 15 of 17 biopsy samples obtained from DU patients with the AA genotype were scored between 10 and 19 on the updated Sydney system scale, suggest that the SNP studied may contribute to enhanced inflammatory response.

The lack of significant difference between the genotypes of controls and patients with gastritis indicates that the presence of this polymorphism is not associated with the onset of gastritis. This is in good accordance with the findings of Rosenstiel et al. that no mutations of *CARD4* or *CARD15* contribute to the development of gastritis or gastric ulcer in *H. pylori*-infected patients. [118].

4.1.2. *TLR4* A12874G and C13174T polymorphisms

There was no significant difference in the *TLR4* A12874G and C13174T genotypes between the patients overall and the controls.

In accordance with our results, Kato et al. did not reveal significant association between the Asp299Gly polymorphism and various stages of gastric premalignant lesions [192]. Similarly, there was not found any association between the Asp299Gly and Thr399Ile polymorphisms and distal gastric cancer [193].

Since these two SNPs of *TLR4* bring about diminished LPS-responsiveness [130], our results support that TLR4 does not play an important role in the recognition of *H. pylori*, neither in the outcome of gastrointestinal diseases in *H. pylori*-infected individuals. Besides, it is worth mentioning that the LPS of *H. pylori* posesses lower immunological activity than that of other intestinal bacteria such as *E. coli* or *Campylobacter jejuni* [194, 195]. This can be explained by the absence of ester-bound 4'-phosphate and by the presence of tetraacyl lipid A with fatty acids of 16 to 18 carbons in lenght, which differentiate the lipid A of *H. pylori* from those of other bacterial species [196].

These data support the observations that NOD1 rather than TLR4 is of functional significance in sensing *H. pylori*. Our results emphasize the importance of the *CARD4* G796A polymorphism in the genetic susceptibility to a more severe disease manifestation that is DU following *H. pylori* infection.

4.1.3. *IL-8* T-251A polymorphism

There was a significantly higher frequency of the heterozygote AT genotype and a significantly lower frequency of the wild-type TT genotype in the patient group with DU or with gastritis than in the control population. The genotype frequencies in the patient group with gastritis differ significantly ($P=0.0213$, $\chi^2=7.696$) from those reported by Kamali-Sarvestani et al. in an iranian population [197].

The presence of the -251 mutant A allele was associated with increased IL-8 production by LPS stimulated whole blood [138], with higher mucosal IL-8 levels in gastric biopsy specimens from *H. pylori*-infected Japanese patients with gastric cancer [198], and with

higher *IL-8* promoter activity in transfected human gastric AGS cells upon stimulation by IL-1β or TNF-α [199]. These are consistent with the findings of Hamajima et al, who concluded that the wild type TT genotype might display milder inflammatory reaction [200]. These findings and our observations are connected by the work of Klausz et al., who detected higher inducible IL-8 levels in patients with DU than in *H. pylori*-positive healthy subjects [201].

Consistently with the higher IL-8 secretion associated with the A allele, the AA genotype was shown to be associated with significantly elevated risk of atrophic gastritis and gastric cancer [197, 202]. There was a significant decrease in the neutrophil infiltration score among the AA, AT and TT genotypes [198]**.** Similarly, there were associations between the IL-8 -251 A allele and the development of gastric cancer in Mexican [193], Iranian [197] and in Japanese populations [198, 199], and increased risk for having gastric ulcer among Japanese patients [199]. Leung et. al did not find any association between gastric intestinal metaplasia and *IL-8* -251 polymorphism among subjects with the mean age of 51 from a Chinese region with high gastric cancer incidence [203].

These data together with our results suggest that the -251 TT genotype is associated with a relative protection from a spectra of gastrointestinal diseases associated with *H. pylori* infection. The fact that the *IL-8* –251 SNP was observed in higher frequency in both the gastritis and the DU groups draws attention to the possibility that the higher IL-8 producing ability might result not only in ulcerative processes, but also in chronic gastritis. In our previous work, a close connection was also obsereved between DU and the *IL-8* –251 SNP [204].

4.2. *IL-8* and *TLR4* polymorphisms in acute pancreatitis

4.2.1. *IL-8* T-251A polymorphism

Although the importance of IL-8 production and neutrophil activation in acute pancreatitis have been well demonstrated, it is indicated at the first time that a polymorphism in the promoter region of the *IL-8* contributes to the development of the disease. Kim et al. did not

find any difference in the genotype and allele frequencies of *IL-8* polymorphisms between patients with alcoholic pancreatitis and the group of healthy blood donors [205].

Because of the -251 T →A conversion was shown to be accompanied with increased IL-8 production, the significantly higher frequencies of the heterozygote AT genotype and A allele among patients with SAP suggest that the exaggerated IL-8 response might serve as a predisposing factor to the severe complications of pancreatitis. In accordance with this, the -251 TT genotype occured significantly more frequently among the healthy control population and even in the group of patients with mild pancreatitis. It is noteworthy that only one of the patients with TT genotype in the severe pancreatitis group showed the symptoms of SIRS.

These are consistent with the results of Pooran et al., who found significantly higher serum IL-8 concentrations in the patient group with SAP than among patients with mild pancreatitis or in the control population. They did not reveal statistical difference between patients with mild pancreatitis and control persons [206]. Similarly, Stimac et al. reported that patients with severe pancreatitis had statistically higher median values of IL-8 than those with mild pancreatitis on clinical admisson [207]. This difference was demonstrated to remain statistically significant up to one day after admission by Berney et al., but peak IL-8 levels measured in the mild group did not reach the values of the severe group [208].

There are relatively few subjects with the AA genotype, which may be explained by the comparatively small number of patients examined.

It is very likely that merely one polymorphism cannot determine the final outcome of the disease. Due to other predisposing factors, patients carrying the -251 A allele may have higher risk for the development of the severe form of acute pancreatitis, and once it has developed, the elevated IL-8 production exacerbates the inflammatory processes.

4.2.2. *TLR4* A12874G and C13174T polymorphisms

Genetic predisposition of LPS immunity is a potent candidate to explain why certain patients with AP develop infection while others do not. These infections are likely to originate from the blood or from the gastrointestinal tract, due to the incresed intestinal permeability during AP [209]. The findings of Li et al. and Johnson et al. further support that TLR4 is potentially associated with AP [12, 13].

Although there was not found statistical difference in the *TLR4* A12874G and C13174T genotypes between the patients and the healthy control population in any form of AP investigated, the 12874 AG and 13174 CT heterozygote genotypes were present with higher frequency among patients with severe pancreatitis, compared to the patients with mild pancreatitis or to the control group. In spite of the low frequency of the heterozygote mutants, six out of seven were detected among severely ill patients who suffered from the severe, infected form of acute necrotizing pancreatitis with MOF. These results suggest that the carrier state of either *TLR4* 12874 or 13174 heterozygotes might be a risk factor for the development of severe septic necrotizing pancreatitis. This is supported by Gao et al., who reported that the incidence of Gram-negative infection was significantly higher in the 12874 AG heterozygote patients with severe AP than in the wild-type population [210].

4.3. Polymorphism of *CARD4/Nod1* in Crohn's disease

Although the 7p14 region, which encompasses the *CARD4* gene, has been linked to IBD by genome-wide scans involving patients from the United Kingdom [211] and North-America [212, 213], the prevalence of the *CARD4* G796A polymorphism is reported for the first time in patients with IBD from an Eastern European country. According to our study, the heterozygous/ homozygous carriage of the variant SNP as well as the *Nod1* 796 A allele is considered to be possible determinant of the susceptibility for CD in Hungarian patients compared to both the healthy and non-IBD controls.

However, due to the multifactorial nature of the disease, the literature is conflicting. Therefore it seems to be hard to reach an unanimous decision upon the possible role of any mutation of *CARD4/Nod1* in the development of CD at all. A multicenter Western European study involving 381 IBD families did not reveal association between the susceptibility of IBD and the E266K polymorphism, but when 63 unrelated index patients were subjected to mutation screening, the E266K variant was the only one out of other polymorphisms detected that encodes a changed protein, suggesting a potential functional effect of the mutation [116]. By examining seventy Turkish CD patients, Ozen et al. found that the genotype distribution of *CARD4* G796A and of three *CARD15* polymorphisms were similar in the groups of CD patients and healthy control subjects [214]. The relatively small number of patients and the

highly heterogenous population might explain why their results differ from ours. In the study of McGovern et al., the frequency of the G796A polymorphism proved to be less than 1% in a British population, and was not tested further. On the other hand, it was demonstrated that the deletion allele of the complex insertion-deletion polymorphism (*ND_1 + 32656*) of *CARD4* was significantly associated with the susceptibility to IBD [115]. A similar positive result was ruled out in a British population by Tremelling et al. [215] and in a Scottish and Swedish IBD population by Van Limbergen et al. [216].

There was not found any significant association between the different genotypes and the demographic data of the patients or the clinical characteristics of CD. Similarly, when 235 CD patients were subdivided into three groups on the grounds of the number of K variants by Zouali et al., no difference could be observed between these groups as regards sex, age at onset, family history, disease location at onset and at maximal severity, behaviour, granuloma formation, extraintestinal symptoms and therapeutic management [116].

4.4. HMGB-1: its detection, secretion by monocytic cells and role in AP

Three years ago far less details were revealed about HMGB-1 protein than today, and there was a lack of commercial kits for the routine screening of HMGB-1 levels in biological samples. Since then, the second ELISA kit of the Shino-Test Corporation with improved sensitivity has been released. HMGB-1 is now in the focus of various research groups, but many reports on the role, secretion and structure of the protein have not been so far confirmed. In consequence, they are either not taken into consideration or are excessively generalized. However the primary structure of HMGB-1 proteins of different origin are (on the whole) known, hardly any information do we have about their post-translational modifications. These structural changes – which may also be cell- and species-specific – might influence the translocation of HMGB-1 from the nucleus into the cytoplasm and from the cytoplasm to the extracellular space.

Even in the dipterous insects *Chironomus* and *Drosophila*, HMG1 proteins are constitutively phosphorylated within the carboxy-terminal tail of the molecules by casein kinase II, which alters their conformation, thermal stability and DNA-binding properties and stabilizes them

against digestion by some proteinases [217]. In macrophages, the hyperacethylation of the NLS regions causes the relocalization of the molecule to the cytosol due to the inhibition of the nuclear import [152]. Secretory lysosomes which are responsible for the HMGB-1 secretion by activated monocytes have not been described yet for all cell types secreting this protein, so there must exist yet uncharacterized mechanisms concerning the externalisation of HMGB-1 by living cells [218]. The results of Rouhiainen et al. suggest that the secretion of amphoterin and IL-1β requires the multidrug resistance protein ABC-1 [158]. It is worth mentioning that besides active secretion, passive release of the protein from necrotic cells has to be taken into account. On the basis of all these, it might be explained why certain anti-HMGB-1 antibodies tested by us – in spite of the manufacturer's recommendation – did not work in ELISA and Western blot. In accordance with this, Ito et al. reported that the same monoclonal antibody recognizes the epitope region of certain HMGB-1 residues in lymphocytes, while it does not in neutrophils, suggesting that the epitope or its peripherial structure is conformationally changed in the different cell-types [219]. Besides, HMGB-1 molecules of different origin can exert diverse proinflammatory activities. As demonstrated by Rouhiainen et al., the rat brain-derived and the recombinant rat HMGB-1 showed different effects on the secretion of TNF-α, IL-6 and MCP and on the release of nitric oxide from macrophages [220]. Zimmermann et al. reported that the native HMGB-1 proteins, purified from calf thymus and secreted by a CHO cell line, exert less pronounced biological activity than the recombinant ones [221].

Our pilot experiments draw attention to the HMGB-1-inducing ability of *M. bovis*, demonstrated in both Western blot and ELISA experiments. To date, this is supported only by Grover et al. who reported that mycobacterial infection resulted in HMGB-1 secretion in both macrophage and monocytic cell cultures, and the secretion of TNF-α and IL-1β increased significantly when murine macrophage cell cultures were incubated with HMGB-1 during infection with *M. bovis* BCG. Thus, HMGB-1 may enhance and perpetuate the immune response in tuberculosis [222]. Assessment of the pathophysiological role of this late cytokine in mycobacterial infections demands further *in vitro* and *in vivo* experiments.

Our findings suggest that HMGB-1 might be important in the pathogenesis of AP. Produced by activated monocytes/macrophages, HMGB-1 can amplify the inflammation and may contribute to the tissue injury and organ failure during AP. In the severe, necrotizing form of

pancreatitis, HMGB-1 may also be produced by the injured pancreas and other damaged organs. This is consistent with the results of Yasuda et al.: the mean value of serum HMGB-1 levels was significantly higher in patients with SAP than in healthy volunteers, and serum HMGB-1 concentrations were in significant positive correlation with the Japanese severity score and Glasgow score [223]. Further supporting the importance of HMGB-1 in SAP, Sawa et al. reported that anti-HMGB-1 neutralizing antibodies significantly reduced the elevation of serum amylase level, ameliorated significantly the elevations of serum alanine aminotransferase and creatinine and improved the histological alterations of pancreas and lung in experimental murine SAP [224]. Similarly, HMGB-1 was found to be a key mediator of inflammatory response and organ injury in the rat model of SAP [178].

In our pilot study, a significant elevation of HMGB-1 concentration was observed in plasma of patients with AP compared to healthy subjects. The HMGB-1 concentration was the highest in the plasma of patients with SAP. To the best of our knowledge, this is the first report in Europe in which the role of HMGB-1 is investigated in AP. However, it is necessary to bear in mind that it is a preliminary result and increase in the number of cases in the study group is therefore mandatory in the future. Additionally, studying SNPs of the gene of HMGB-1 would be intriguing, but until now no such mutations have been found in the databases.

5. SUMMARY: CONCLUSIONS AND POTENTIAL SIGNIFICANCE

1. Association between *CARD4/NOD1*, *TLR4* and *IL-8* polymorphisms and the development of gastritis and duodenal ulcer in *H. pylori*-infected patients

- Patients carrying the *CARD4/Nod1* 796 AA genotype are at an increased risk of the development of *H. pylori*-induced duodenal ulcer.
- Polymorphisms in *TLR4* do not play an important role in the outcome of diseases in *H. pylori*-infected individuals.
- In conclusion, NOD1 rather than TLR4 is important in the recognition of *H. pylori* by gastric epithelial cells.
- The *IL-8* –251 AT genotype and possibly the presence of the A allele contribute to the genetic predisposition to *H. pylori*-induced gastritis and duodenal ulcer.

2. Association between *CARD4/Nod1* polymorphism and Crohn's disease

- The *CARD4/Nod1* 796 A allele is a significant risk factor for Crohn's disease in the Hungarian population.

3. Association between *IL-8* and *TLR4* polymorphisms and acute pancreatitis

- Patients carrying the *IL-8* –251 A allele are at an increased risk of severe complications of acute pancreatitis.

4. HMGB-1 and acute pancreatitis

- A reliable *in vitro* method was elaborated to detect HMGB-1 in cell supernatants.
- Induction of HMGB-1 secretion was also observed *in vitro* by *Mycobacterium bovis* BCG.

- Elevated levels of HMGB-1 were observed in plasma of patients with AP, which suggests that HMGB-1 as a danger signal and as a “late cytokine” is an important inflammatory mediator in the pathogenesis of acute pancreatitis

General conclusion: Single nucleotide polymorphisms of genes of IL-8 and pattern-recognition receptors might be responsible for different host responses, which further determine the severity of gastrointestinal inflammation.

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