ACTA ABP BIOCHIMICA POLONICA

Vol. 63, No 1/2016
145–152
http://dx.doi.org/10.18388/abp.2015_1122

Regular paper

Helicobacter pylori antigens, acetylsalicylic acid, LDL and 7-ketocholesterol — their potential role in destabilizing the gastric epithelial cell barrier. An *in vitro* model of Kato III cells*

Adrian Gajewski[1], Eliza Mnich[1], Karol Szymański[1], Krzysztof Hinc[2], Michał Obuchowski[2], Anthony P. Moran[3†] and Magdalena Chmiela[1]✉

[1]Division of Gastroimmunology, Department of Immunology and Infectious Biology, Institute of Microbiology, Biotechnology and Immunology, Faculty of Biology and Environmental Protection, University of Lodz, Łódź, Poland; [2]Intercollegiate Faculty of Biotechnology UG&MUG, Department of Molecular Bacteriology, Gdańsk, Poland; [3]Department of Microbiology, School of Natural Sciences, National University of Ireland Galway, Galway, Ireland

Colonization of gastric tissue in humans by *H. pylori* Gram-negative bacteria initiates gastric and duodenal ulcers and even gastric cancers. Infections promote inflammation and damage to gastric epithelium which might be followed by the impairment of its barrier function. The role of *H. pylori* components in these processes has not been specified. *H. pylori* cytotoxicity may potentially increase in the milieu of anti-inflammatory drugs including acetylsalicylic acid (ASA). The lipid transport-associated molecule such as low density lipoprotein (LDL), which is a classic risk factor of coronary heart disease (CHD) and 7-ketocholesterol (7-kCh) a product of cholesterol oxidation, which may occur during the oxidative stress in LDL could also be considered as pro-inflammatory. The aim of this study was to evaluate the cytotoxicity of *H. pylori* antigens, ASA, LDL and 7-kCh towards Kato III gastric epithelial cells, on the basis of the cell ability to reduce tetrazolium salt (MTT) and morphology of cell nuclei assessed by 4′,6-diamidino-2-phenylindole (DAPI) staining. Kato III cells were stimulated for 24 h, at 37°C and 5% CO_2, with *H. pylori* antigens: cytotoxin associated gene A (CagA) protein, the urease A subunit (UreA), lipopolysaccharide (LPS) and ASA, LDL or 7-kCh. *H. pylori* LPS, ASA, LDL and 7-kCh, but not *H. pylori* glycine acid extract (GE), demonstrated cytotoxicity against Kato III cells, which was related to a diminished percentage of MTT reducing cells and to an increased cell population with the signs of DNA damage. The results suggest that damage to gastric epithelial cells can be induced independently by *H. pylori* antigens, ASA and endogenous lipid transport-associated molecules. During *H. pylori* infection *in vivo,* especially in CHD patients, synergistic or antagonistic interactions between these factors might possibly influence the disease course. Further study is necessary to explain these potential effects.

Key words: *Helicobacter pylori*, acetylsalicylic acid, 7-ketocholesterol, gastric barrier

Received: 22 July, 2015; revised: 09 October, 2015; accepted: 27 October, 2015; available on-line: 30 November, 2015

INTRODUCTION

Helicobacter pylori Gram-negative bacteria have a high affinity to gastric epithelium. In humans, chronic colonization of the gastric tissue by *H. pylori* may be associated with both, hypochlorhydria and hyperchlorhydria, depending on the distribution of inflammation, and thus initiates gastric and duodenal ulcers and even gastric cancers (Neuman & Crabtree 2004; Derakhshan *et al.*, 2006). *H. pylori* infections promote an excessive inflammatory response which is deleterious to gastric epithelial cells (Israel & Peek, 2001; Chmiela & Michetti, 2006). Changes occurring in the gastric epithelium colonized by *H. pylori* may promote the penetration of *H. pylori* antigens into the basal membrane, where they interact with extracellular matrix proteins (ECM) (Valkonen *et al.*, 1994). This may be followed by an impairment of the gastric barrier function. *H. pylori* bacteria also interact with and activate the infiltrating immune cells *via* Pathogen Recognition Receptors (PRR) (Dubreuil *et al.*, 2002; Wessler & Backert, 2008). The role of individual *H. pylori* compounds in these processes has not been fully specified. Urease is a major *H. pylori* virulence factor, which increases gastric pH and may be involved in degradation of intracellular tight junctions (Posselt *et al.*, 2013). Cytotoxin associated gene A protein (CagA) is the most extensively studied virulence factor of *H. pylori.* The *cagA* is located at the cag pathogenicity island (PAI) encoding a type IV secretion system (T4SS), through which CagA is delivered into host cells. A higher risk of gastric ulcers and stomach cancer is related to infections caused by CagA producing, rather than CagA non-producing, *H. pylori* strains (Yamaoka, 2010). *H. pylori* CagA positive strains have functional *vacA*, which encodes a vacuolating cytotoxin A (VacA). In addition to vacuolation, VacA can induce multiple cellular activities, including membrane channel formation, cytochrome c release from mitochondria leading to apoptosis, and binding to

✉e-mail: chmiela@biol.uni.lodz.pl

*The results were presented at the 6th International Weigl Conference on Microbiology, Gdańsk, Poland (8–10 July, 2015).

Abbreviations: ASA, acetylsalicylic acid; CagA, cytotoxin associated gene A protein; CCUG, Culture Collection University of Gothenburg; CHD, coronary heart disease; DAPI, 4′,6-diamidino-2-phenylindole; ECM, extracellular matrix; EDTA, ethylenediaminetetraacetic acid; GE, glycine acid extract; 7-kCh, 7-ketocholesterol; LPS, lipopolysaccharide; LDL, low density lipoprotein; LRP1, low-density lipoprotein receptor-related protein-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide salt; NF-κB, nuclear factor kappa B; NK, natural killer lymphocytes; NSAID, nonsteroidal anti-inflammatory drugs; PAI, pathogenicity island, PCR, polymerase chain reaction; PRR, pathogen recognition receptors; rCagA, recombinant CagA protein; ROS, reactive oxygen species; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; T4SS, type IV secretion system; UreA, subunit A of *H. pylori* urease; VacA, vacuolating cytotoxin A

cell-membrane receptors, which is followed by the initiation of proinflammatory response (Yamaoka, 2012). In addition, VacA can modulate antigen presentation by B lymphocytes and inhibit T-cell activation as well as proliferation (Gebert *et al*., 2003). During natural infection *in vivo* this toxin may possibly modulate the influence of CagA on gastric epithelial barrier.

H. pylori lipopolysaccharide (LPS) is an important proinflammatory compound. However, its lipid A has probably evolved in the mode which promotes persistence of the infection by regulating the expression of adhesins and modulating the secretion of cytokines by host immune cells as well as their phagocytic, proliferative and cytotoxic activities (Taylor *et al*., 2006; Grebowska *et al*., 2008; Grebowska *et al*., 2010; Rudnicka *et al*., 2015).

H. pylori cytotoxicity may potentially increase in the milieu of anti-inflammatory drugs such as acetylsalicylic acid (ASA), which is widely used to reduce fever, pains and inflammation. In addition, ASA is preventively used as an anticoagulant in patients with coronary heart disease (CHD) (Baigent *et al*., 2009). The use of aspirin increases a risk of adverse reactions in the gastrointestinal tract such as erosions and ulcers due to irritation, impaired blood flow, accumulation of neutrophils and inhibition of prostaglandin synthesis (Perini *et al*., 2004; Vella, 2005). However, literature about interactions between *H. pylori* infections and a low dose of ASA is scarce and controversial (Sosters *et al*., 2014, Buzas 2014).

The role of lipid transport-associated molecules, including low density lipoprotein (LDL), which is a classic risk factor for CHD in the development of gastric disorders is a new approach. It has been shown that in the gastric epithelium, there might be present specific structures called lipid islands. The precise mechanism of their formation is not clear. It is hypothesized that the lipid islands might be formed by the accumulation of cholesterol, LDL and oxidized LDL (ox-LDL) in the gastric epithelium damaged due to inflammatory or degenerative processes (Kaiserling *et al*., 1996). It has been suggested that rather than being eliminated *via* reverse cholesterol transport machinery using LDL receptor, ox-LDL is recognized and captured by scavenger receptors abundant in peripheral tissues. In vascular cells, internalization of ox-LDL has been shown to trigger signaling events resulting in overproduction of reactive oxygen species, inflammation and proliferation (Khaidakov & Mehta, 2012). Various epithelial cells have been also shown to express ox-LDL specific scavenger receptors such as CD36 molecule (Wadsack *et al*., 2003; Susztak *et al*., 2005). CD36 is a transmembrane protein of class B scavenger receptor family and it is involved in multiple biological processes: angiogenesis, atherosclerosis, inflammation, lipid metabolism (Febbraio *et al*., 2001).

Gastric damage is observed in patients with chronic diseases (gastritis, peptic ulcer disease, and also after partial gastrectomy) (Chandan *et al*., 2004). It has been shown that infection and inflammation induce LDL oxidation and it is known that oxidative modification of lipoproteins plays a major role in atherosclerosis (Memon *et al*., 2000). The association between *H. pylori* infection and the serum lipid, lipoproteins, apolipoprotein A1 and apolipoprotein B in patients with gastritis has also been indicated (Memon *et al*., 2000; Kucukazman *et al*., 2008; Ansari *et al*., 2010; Kim HL *et al*., 2011). By the ability to create an atherogenic lipid profile and insulin resistance, *H. pylori* has been considered as an infectious agent promoting atherosclerosis, type 2 diabetes mellitus and a metabolic syndrome. However, the epidemiologic link between *H. pylori* infection and these syndromes is inconsistent and controversial (Buzas, 2014). While *H. pylori* does not enter blood circulation, these extragastric manifestations are probably mediated by the cytokines and acute phase proteins produced by the inflammatory mucosa (Lamb & Chen, 2013). However, it could not be ruled out that gastric epithelial damage due to *H. pylori* infection may be enhanced by the effect of drugs and some endogenous factors occurring in circulation. Their effects on the gastric epithelium may manifest in the situation of erosions and ulcers or as a consequence of activation of immune cells infiltrating the gastric mucosa.

Considering various effects of infectious agents that chronically colonize the gastric epithelium, as well as pharmacological agents and lipid transport-associated molecules on the gastric barrier function, in this study we evaluated the cytotoxic effects of selected and well defined *H. pylori* antigens, as well as ASA, LDL and 7-ketocholesterol (7-kCh) using an *in vitro* model of Kato III gastric epithelial cells. The ability of the cells to reduce tetrazolium salt (MTT) and morphology of cell nuclei assessed by 4',6-diamidino-2-fenyloindol (DAPI) staining were selected as cytotoxicity markers. We expect that this study will demonstrate the properties of individual compounds used as cell stimulators and will enable to determine the conditions to explore their putative synergistic or antagonistic effects on the integrity and function of gastric epithelial cells. Simultaneous evaluation of these effects is a new approach, which will help to better understand the relationship between *H. pylori* infection and CHD.

MATERIALS AND METHODS

***H. pylori* culture conditions.** A reference *Helicobacter pylori* strain CCUG 17874 (Culture Collection, University of Gothenburg, Gothenburg, Sweden), VacA and CagA positive, was used in this study. *H. pylori* bacteria were stored at –80°C in Tris-buffered saline (TBS) containing 10% glycerol. Before being used in the experiments, *H. pylori* bacteria were grown for 5 days on modified *Helicobacter* agar (Becton Dickinson, Heidelberg, Germany) under microaerophilic conditions (Gas Pak, Becton Dickinson, Heidelberg, Germany), at 37°C. Bacteria were harvested by scraping from agar plates, suspended in 0.85% sodium chloride (NaCl), pelleted by centrifugation ($4000 \times g$, for 15 min), and then washed twice under the same conditions. Bacterial pellet was used for the preparation of antigens.

Bacterial stimulators. Surface *H. pylori* antigens present in a glycine acid extract (GE) were extracted from the reference *H. pylori* strain CCUG 17874 using 0.2 M glycine buffer, pH 2.2, as previously described (Logan & Trust 1983, Rechciński *et al*., 1997), with evaluation of protein composition by SDS-PAGE and a Western blot — Immuno blot (Milenia®Blot *H. pylori*, DPC Biermann, GmbH, Bad Nanheim, Germany) performed with the reference serum samples from patients infected with *H. pylori* (Czkwianianc *et al*., 1997). Major proteins in GE recognized by sera from *H. pylori* positive patients were 120 kDa, 80 kDa, between 66–42 kDa and 29–26 kDa. The protein content in GE was 600 μg/ml (NanoDrop 2000c Spectrophotometer, ThermoScientific, Wlatman, WY, USA). The GE preparation contained < 0.001 EU ml of LPS as shown by the chromogenic *Limulus amebocyte* lysate test (Lonza, Braine-Alleud, Belgium). The *H. acynonychis* UreA subunit was amplified by a polymerase chain reaction (PCR) as previously described (Hinc

et al., 2010), and used as a homologue of *H. pylori* UreA protein. Recombinant CagA protein (rCagA) was from IRIS, Siena, Italy. LPS from the reference strain of *H. pylori* CCUG 17874 (courtesy of AP. Moran) was prepared by hot phenol-water extraction after pre-treatment of the bacterial biomass with a protease. Then, the crude LPS preparation was purified by RNAse, DNAse and proteinase K treatment and by ultracentrifugation, as previously described (Moran *et al.*, 1992). The *E. coli* LPS derived from the O55:B5 strain (Sigma, St. Louis, MI, USA) was used as control.

Culture and stimulation conditions of Kato III cells. Human gastric epithelial cells Kato III, ATCC®HTB™ (American Type Cell Culture Collection, Rockville, MD, USA) were used in this study. The cells were cultured in RPMI-1640 medium containing 10% fetal calf serum, L-glutamine and standard antibiotics, penicillin/streptomycin, at a concentration of 1×10^6 cells ml in tissue culture flasks up to 90% of confluence. The cells were detached from the tissue culture flasks by 0.25% trypsin solution in ethylenediaminetetraacetic acid (EDTA), 10 min, 37°C, and then washed twice with a culture medium by centrifugation (10 min, $300\times g$) and evaluated for cell viability by trypan blue staining. For experiments, a cell suspension containing 1×10^6 cells/ml was distributed into the wells of 96-well culture plates (100 µl/well) and attached for 24 h, at 37°C and 5% CO_2. The cells were stimulated for 24 h with *H. pylori* stimulators used at a concentration adjusted in the previous cellular experiments: GE (10 µg/ml), UreA (10 µg/ml), CagA (1 µg/ml), *H. pylori* LPS (1 ng/ml, 25 ng/ml) and *E. coli* LPS as control (at the same concentrations) as well as ASA (1, 2.5 and 5 mM), LDL (5 and 50 µg/ml) and 7-kCh (2.5 and 20 µg/ml) (Sigma, St Louis, Michigan, US). The primary solution of LDL in 0.85% NaCl/1%EDTA was diluted in RPMI-1640. This solvent was not cytotoxic as shown in a previous study. The stock solution of lyophilized 7-kCh (1mg/ml) was prepared in 96% ethanol and then diluted in a culture medium. The cells were incubated in the milieu of stimulators for 24 h, at 37°C and 5% CO_2. Then, the wells were emptied and filled with the culture medium without stimulators. The cytotoxic effects were assessed after 24, 48 and 72 h of a continued cell culture and assessed in comparison to control, i.e. untreated cells.

MTT reduction assay. The cytotoxic activity of stimulators was estimated on the basis of the live target cells' ability to reduce MTT, using the TACS™ MTT Cell Proliferation Assay (R&D Systems, USA), as recommended by the manufacturer. The intensity of MTT reduction was estimated spectrophotometrically at 570 nm. The correlation between the number of viable target cells and the absorbance intensity was used for the standard curve construction. The level of cytotoxicity was expressed as downregulation of absorbance values and a percentage of dead target cells.

Cell nuclei staining by DAPI. Cell nuclei damage was considered as a sign of apoptosis, which was determined by staining the cells 24 h after the challenge with 4',6-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MI, USA), a fluorescent dye, which has a strong affinity to the AT base pairs in DNA. The cells were fixed with 4% formaldehyde, and stained with a DAPI solution (2.5 µg/ml) for 15 minutes, at room temperature. The samples were viewed under a fluorescent microscope at 358 nm (excitation) and 461 nm (emission). A percentage of cells with damaged nucleus was assessed.

Statistical analysis. The Statistica 10 PL program with non-parametric tests was used: the Mann-Whitney U test (for impaired data) to verify the hypothesis that two samples being compared came from two statistically different populations.

RESULTS

Cytotoxic effects of *H. pylori* antigens, ASA, LDL and 7-kCh towards Kato III cells assessed by MTT reduction

Our study demonstrated the cytotoxic effect of *H. pylori* CagA, UreA and LPS on Kato III cells. These antigens diminished the number of cells reducing MTT in the cell cultures 24 h, 48 h and 72 h after antigen challenge (Fig. 1). CagA significantly reduced the percentage of viable cells by 21.6%, 27.6% and 16.37%, respectively, and UreA by 23.3%, 10.4% and 11.2%, respectively. The differences for UreA 48 h and 72 h after challenge were significant. Among *H. pylori* antigens used in this study, the LPS of *H. pylori* at a concentration of 25 ng ml caused the greatest loss of MTT reducing cells. The percentages of cells which did not reduce MTT after the challenge with *H. pylori* LPS at this concentration, were 88.5%, 74.3% and 78.5% in 24 h, 48 h and 72 h cultures, respectively. By comparison, the percentages of MTT non reducing cells in the cultures treated with *E. coli* LPS at a concentration of 25 ng/ml at 24 h, 48 h and 72 h before the evaluation were 14.9%, 64.2%, 62.5%, respectively. *E. coli* LPS at a lower concentration (1 ng/ml) showed cytotoxic effect only 24 h after the challenge. In contrast, the cytotoxic effect of a lower dose of *H. pylori* LPS was shown after 24 h and persisted after 48 and 72 h. The mixture of *H. pylori* antigens

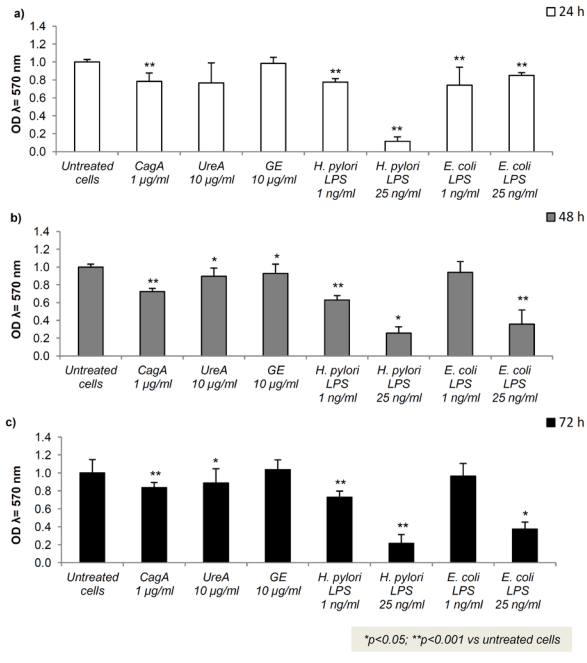

Figure 1. Cytotoxic effects of *H. pylori* antigens towards Kato III cells assessed by the MTT reduction assay.
Human gastric epithelial cells Kato III were stimulated for 24 h with *H. pylori* antigens: glycine acid extract (GE), subunit A of urease (UreA), cytotoxin associated gene A antigen (CagA), *H. pylori* lipopolysaccharide (LPS) or control *E. coli* LPS. Then, stimulators were removed and the cytotoxic effects were assessed after 24 h (**a**), 48 h (**b**) and 72 h (**c**) of continued culture of the cells on the basis of cells' ability to reduce tetrazolium salt (MTT). Statistical significance: *$p<0.05$; **$p<0.001$.

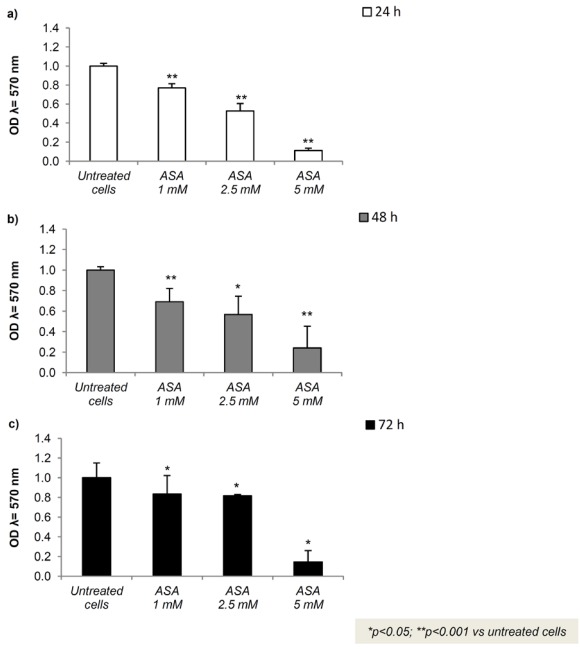

Figure 2. Cytotoxic effects of acetylsalicylic acid (ASA) towards Kato III cells assessed by MTT reduction assay.
Human gastric epithelial cells Kato III were incubated in the milieu of various ASA concentrations for 24 h. Then, stimulators were removed and the cytotoxic effects were assessed after 24 h (**a**), 48 h (**b**) and 72 h (**c**) of continued cell culture on the basis of cells' ability to reduce tetrazolium salt (MTT). Statistical significance: **p*<0.05; ***p*<0.001.

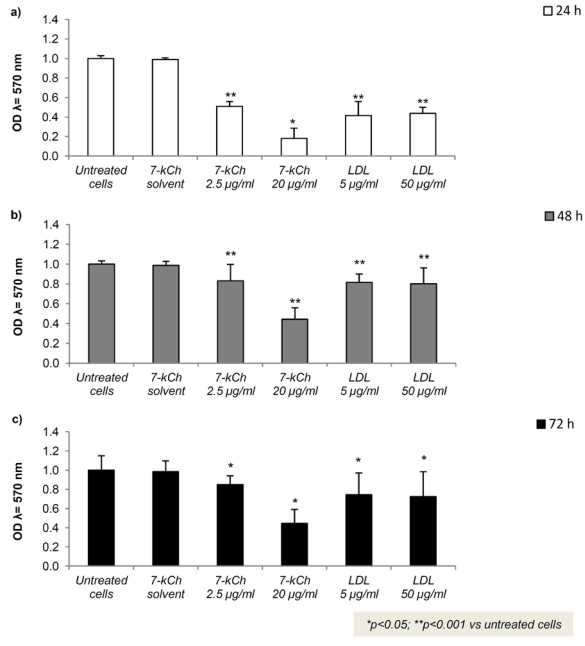

Figure 3. Cytotoxic effects of low density lipoprotein (LDL) and 7-ketocholesterol (7-kCh) towards Kato III cells assessed by MTT reduction assay.
Human gastric epithelial cells Kato III were stimulated for 24 h with different 7-kCh and LDL concentrations. Then, stimulators were removed and the cytotoxic effects were assessed after 24 h (**a**), 48 h (**b**) and 72 h (**c**) of continued culture of the cells on the basis of cells' ability to reduce tetrazolium salt (MTT). Statistical significance: **p*<0.05; ***p*<0.001.

in GE did not decrease the viability of Kato III cells as assessed by MTT reduction.

The cytotoxic effect of ASA used at concentrations of 1, 2.5 and 5 mM towards Kato III cells was dose dependent. The strongest impact was observed in the cell cultures treated with 5 mM ASA 24 h, 48 h and 72 h before the assessment (Fig. 2). In these cell cultures, 88.9%, 75.9% and 85.6% of the primary cell population, respectively, did not reduce MTT. The cytotoxic effect of ASA at a concentration of 1 mM or 2.5 mM was weaker as compared to 5 mM ASA, but the percentage of dead cells was significantly higher than in the control culture in RPMI-1640 culture medium alone.

Also, 7-kCh and LDL downregulated the ability of Kato III cells to reduce MTT (Fig. 3). The effect of 7-kCh was time and dose dependent. In the cell cultures 24 h after the challenge with 2.5 µg/ml of 7-KCh, the number of inactive cells was equal to 49.2% and was significantly lower than in the cultures treated with the 7-kCh solvent alone. By comparison, in 48 h and 72 h cell cultures treated with 2.5 µg/ml of 7-kCh, the percentages of dead cells were 16.9% and 15.0%, respectively.

Both, lower (5 µg/ml) and higher (50 µg/ml) doses of LDL affected the metabolic activity of Kato III cells to a similar degree: 58.4% and 56.3%, 24 h after challenge; 18.5% and 19.9%, 48 h after challenge; 25.6% and 27.6%, 72 h after challenge, respectively. With regard to the control cultures, the differences were statistically significant.

Genetic material damage in Kato III cells treated with *H. pylori* antigens, ASA, LDL or 7-kCh visualized by DAPI staining

Figure 4 shows the percentage of Kato III cells with damaged genetic material in cultures treated 24 h before assessment with *H. pylori* antigens, ASA, LDL and 7-kCh. Figure 5 presents the representative images of the cells exposed for 24 h to *H. pylori* antigens, LDL, 7-kCh or ASA, stained by DAPI. In the cultures treated with CagA, UreA, and GE, the percentage of cells with damaged nuclei was above 20%, whereas in the control cell cultures in RPMI-1640 culture medium alone it was 8.67%. The diminished number of viable cells after treatment with 1 ng/ml and 25 ng/ml *H. pylori* LPS was correlated with the increased number of cells with the signs of DNA destruction. The percentage of the cells with damaged DNA was equal to 30.67% and 89.33%, respectively. *E. coli* LPS also showed genotoxic activity, but it was lower than that of the LPS of *H. pylori*. Genotoxic properties of ASA detected by DAPI staining were in the range of 46.67% and 59.33% of the cells with disrupted DNA. The strongest genotoxic effect (over 90%) in the cells with damaged DNA was demonstrated in the cultures treated with 20 µg/ml 7-kCh. It was reduced up to 40% in the cultures treated with 2.5 µg/ml 7-kCh. In the cell cultures exposed to 5 µg/ml or 50 µg/ml LDL, the cell nuclei were affected in 25% and 41% of the cells.

DISCUSSION

H. pylori infection and nonsteroidal anti-inflammatory drugs (NSAID)/low-dose aspirin use are associated with a peptic ulcer disease (Sosters *et al.*, 2015). The results of many studies indicate that long-term infections with *H. pylori* may contribute to the development of CHD (Mendall *et al.* 1994; Longo-Mbenza *et al.*, 2012; Ayada *et al.*, 2009). CHD pa-

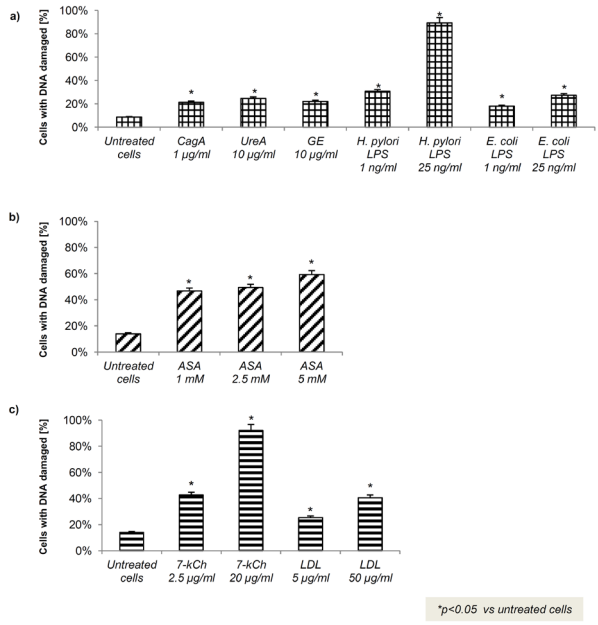

Figure 4. The percentages of Kato III cells with damaged genetic material.
The cells were stimulated for 24 h with *H. pylori* antigens: glycine acid extract (GE), subunit A of urease (UreA), cytotoxin associated gene A antigen (CagA), *H. pylori* lipopolysaccharide (LPS) or control *E. coli* LPS or by acetylsalicylic acid (ASA), 7-ketocholesterol (7-kCh) ,as well as low density lipoprotein (LDL), and then stained by 4′,6-diamidino-2-phenylindole (DAPI). Statistical significance: **p*<0.05.

tients are treated with ASA, which represents the most frequently identified cause of gastric and duodenal ulcers not associated with *H. pylori* infections (Papatheodoritis *et al.*, 2006). Given that *H. pylori* infections remain the most common chronic bacterial infections worldwide, a synergistic or additive effect of *H. pylori* and ASA in peptic ulcer development has been suggested (Sosters *et al.*, 2014; 2015). This correlation has been considered to be of great clinical importance since eradication of the bacterium would reduce the risk of upper gastrointestinal complications in infected ASA-treated patients (Cekin *et al.*, 2012). It can be assumed that changes in the gastric epithelium induced by a *H. pylori* infection may be intensified by ASA. On the other hand, its toxicity may predispose to *H. pylori* infections and accumulation of infection related deleterious effects. Persistent epithelial cell defects can promote deeper penetration of *H. pylori* antigens, where they can interact with the immune cells and even enter the circulation, provoking the development of a systemic inflammatory response.

In view of the chronic nature of *H. pylori* infection and its putative association with the development or maintenance of the CHD symptom, in this study we focused on the influence of *H. pylori* antigens and ASA on the gastric epithelial cells using an *in vitro* cell culture model of Kato III cells.

Our *in vitro* study demonstrated that *H. pylori* CagA, UreA and LPS were cytotoxic to Kato III cells. The cytotoxicity of these stimulators was related to a diminished percentage of MTT reducing cells and to an increased number of cells with DNA damage, as visualized by DAPI staining. Various mechanisms can be involved in the cell cytotoxicity. DNA damage often reflects cell apoptosis. Apoptosis induced by *H. pylori in vivo* may

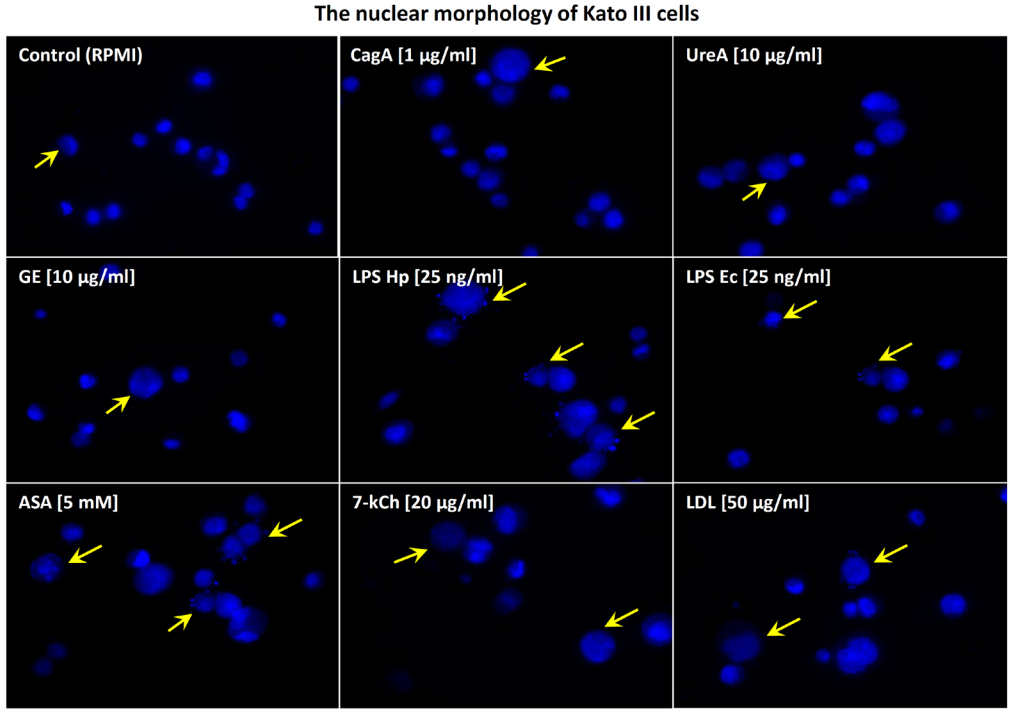

Figure 5. Microscopic images of Kato III cells with a sign of cell nuclei damage.
Human gastric epithelial cells unstimulated (control) or stimulated with *H. pylori* antigens: glycine acid extract (GE), subunit A of urease (UreA), cytotoxin associated gene A antigen (CagA), *H. pylori* lipopolysaccharide (LPS); or control *E. coli* LPS; or with acetylsalicylic acid (ASA), and 7-ketocholesterol (7-kCh); or low density lipoprotein (LDL). Morphology of cell nuclei was assessed by 4′,6-diamidino-2-phenylindole (DAPI) staining. Samples were viewed under a fluorescent microscope (Axio Scope A1, Zeiss). The arrows indicate cells with damaged DNA (x1000 magnification).

stimulate an excessive hyperproliferative response. Increased cell apoptosis has been observed in gastric tissues isolated from patients infected with *H. pylori* as well as in guinea pigs infected experimentally with these bacteria (O'Connor *et al*., 1997; Miszczyk *et al*., 2014). Alternatively, apoptosis may be viewed as the response to hyperproliferation in an attempt to reduce tissue growth (Neuman and Crabtree, 2004). Whether apoptosis is the primary or secondary event is not clear. The induction of excessive apoptosis by *H. pylori* could be related to secondary hyperproliferative response in order to maintain cellular mass. Once hyperproliferation is established, the increased rate of cell cycling might predispose gastric epithelial cells to genotoxic damage. It is possible that gastric epithelial cells exposed to carcinogenic *H. pylori* bacteria are more sensitive to apoptosis. In our previous study we demonstrated using the *in vitro* model of epithelial HeLa cells that exposition of these cells to *H. pylori* antigens was related to cell cycle arrest in the S (GE) or G2/M (CagA and LPS) phase (Miszczyk *et al*., 2014a). Some experimental data indicate that individual *H. pylori* components may promote their effects *in vivo*. Previously Wessler and Backert (2008) showed that soluble factors of *H. pylori,* such as urease and VacA, can open the tight and adherens junctions allowing direct contact between CagA ligand and beta1 – integrin in the basolateral membrane of epithelial cells (Wessler and Backert, 2008). At the apical side of polarized cells, CagA might translocate in a soluble form *via* phosphatidylserine and cholesterol. Phosphorylated CagA dysregulates signal transduction pathways, leading to alterations in gene expression, and interferes with the cytoskeletal rearrangement, which is important for the mitogenic response to *H. pylori* antigens. Another *H. pylori* compound, i.e. peptidoglycan, binds Nod1 and activates NF-$\varkappa$B signaling (Viala *et al*., 2004; Backert *et al*., 2011). Local *H. pylori* induced inflammation in gastric epithelium might be reflected on the periphery by systemic inflammatory markers, which may enhance the development of inflammatory lesions in vascular endothelium. Also, it cannot be excluded that certain *H. pylori* components crossing the epithelial barrier in the stomach or intestine could have a direct influence on the vascular endothelial cells, as well as circulating immune cells, maintaining their constant activation.

Among *H. pylori* antigens, LPS showed the strongest cytotoxic and genotoxic properties towards Kato III cells. In a previous study we have shown that *H. pylori* LPS affected the cell cycle of HeLa cells and guinea pig fibroblasts (Miszczyk *et al*., 2014a), and downregulated the cytotoxic activity of NK cells and proliferation of human and guinea pig T lymphocytes (Grebowska *et al*., 2008; Grebowska *et al*., 2010; Rudnicka *et al*., 2015; Miszczyk *et al*., 2014; Chmiela *et al*., 2014). It is possible that *in vivo* domination of antigens negatively modulating the growth of epithelial cells and the activity of immune cells, comprising the first line of an immune response, promotes the development of a chronic infection. In this study, GE showed no cytotoxic and genotoxic effects against Kato III cells. This might be due to the low concentration of individual antigens in GE preparation, which is composed of surface antigens that may probably modulate the effects of each other. Several surface antigens have been reported for *H. pylori*: 120 kDa, 110 kDa, 94 kDa, 83 kDa, 63 kDa, 61 kDa, 28 kDa, and multiple determinant antigenic cell fragments have been found throughout the *H. pylori* outer membrane proteins and flagellar structures (Lelwala-Guruge *et al*., 1992). Major proteins in GE, recognized by sera from *H. pylori* positive patients, were 120 kDa, 80 kDa, between 66–42 kDa and 29–26 kDa. It is worth mentioning that many *H. pylori* surface components are lectin-like molecules, which play a role of adhesins and modulate both, proliferative and cytokine activities of the immune cells, without any cytotoxic effects (Chmiela *et al*., 1996; Hynes *et al*., 2003; Walz *et al*., 2009).

The intriguing question is how *H. pylori* and low dose ASA exert the gastric damage. For instance, different phenotypes of *H. pylori* may induce various types of gastritis and gastric damage in low dose ASA users. Furthermore, there are various possibilities how low dose ASA damages the gastric mucosa, such as enhanced exposure of gastric epithelial cells to luminal acid, reduction of mucosal blood flow, increase of apoptosis of epithelial cells and recruitment of polymorphonuclear cells, generating the conditions of oxidative stress. Moreover, *H. pylori* may also further increase local injury by impairing the gastric adaptation to ASA (Sostres *et al*., 2014). Several clinical studies proved the benefit of *H. pylori* eradication in ASA users (Lim & Hong, 2014). However, the exact pathophysiological relationship between a *H. pylori* infection and ASA use has not yet been fully elucidated. Probably, through different mechanisms both, *H. pylori* components and ASA, may interact in a synergistic or antagonistic manner.

In this study, cytotoxic and genotoxic effect of LDL and 7-kCh, which is a product of cholesterol oxidation, was demonstrated on Kato III cells. During *H. pylori* infection, the conditions of oxidative stress are exacerbated because the gastric mucosa is extensively infiltrated by polymorphonuclear cells, mainly neutrophils and eosinophils. These cells provide soluble inflammatory mediators that aggravate the influx of other immune cells, resulting in gastric epithelial cell damage. Granulocytes, which are excessively activated and undergo degranulation, provide conditions of oxidative stress due to a release of reactive oxygen species (ROS). They can also secrete excessively proteolytic enzymes. ROS affect the expression of pro- and anti-apoptotic proteins and represent a major cause of cellular damage and death due to lipid peroxidation. ROS are also responsible for oxidative DNA damage (Yoshikawa & Naito, 2002). Previously, a significant association between *H. pylori* infections and high LDL-cholesterolemia, low HDL-cholesterolemia and elevated levels of C-reactive protein was shown, indicating a potential impact of a chronic infection on lipid metabolism. By modulation of lipid metabolism, *H. pylori* can create an atherogenic lipid profile promoting atherosclerosis (Laurilla *et al*., 1999; Ansari *et al*., 2010; Buzas, 2014). It was also revealed that *H. pylori* VacA could specifically utilize the low-density lipoprotein receptor-related protein-1 (LRP1), acting as a receptor which promotes cell autophagy and apoptosis (Cover and Blanke, 2005; Yahiro *et al.,* 2003, 2012). Laurilla *et al* (2001), using LDL receptor negative (LDLR-/-) mice as model for *H. pylori* infection, provided for the first time evidence that a standard high-fat, high-cholesterol diet used for atherosclerosis induction is associated with a high incidence of gastritis, which is independent on infection. In these animals, NF-$\varkappa$B, a common signaling factor, was upregulated in both, coronary vascular endothelium and gastric mucosa, in response to high level of cholesterol (Laurilla *et al*. 2001). These studies indicate that the role of endogenous factors, such as LDL or 7-kCh, in the development of local inflammation in the stomach is possible. On the other hand, our results allow to suggest that epithelial cell damage due to these factors can be involved in the initiation or maintenance of a pre-existing inflammatory response. Whether the cells are damaged directly or indirectly by inflammato-

ry mediators secreted by stimulated cells should be addressed in a further study.

In conclusion, the results obtained in this *in vitro* cellular study, using the Kato III cell line, suggest that *in vivo* damage to the gastric epithelial cells due to *H. pylori* infection can be modulated by LDL and 7-kCh, as well as pharmacological agents, such as ASA. The limitation of this study is that the findings do not explain the molecular mechanism of cell damage. However, diminished metabolic activity of the cells and DNA damage indicate that these factors may contribute *in vivo* to impairment of gastric epithelial barrier function, diminishing of wound healing and upregulation of inflammatory response, with a potentially stronger pathological effects both local and systemic. Further studies are necessary to explore the nature of these processes more deeply.

Acknowledgements

This project was financed from the funds of the National Science Centre, Poland, granted on the basis of the decision number DEC-2013/09/N/NZ6/00805 and DEC-2015/17/N/NZ6/03490 as well as the statutory funds of University of Lodz. The authors have no conflicting financial interests.

REFERENCES

Ansari MHK, Omrani M, Sayyah B, Ansari SK (2010) Effect of *Helicobacter pylori* infection on the lipid, lipoproteins, apolipoprotein-A1, lipoprotein (a) and apolipoprotein-B in patients with gastritis. *African J Microbiol Res* **4**: 084–087.

Ayada K, Yokota K, Kobayashi K, Shoenfeld Y, Matsuura E, Oguma K (2009) Chronic infections and atherosclerosis. *Clin Rev Allergy Immunol* **37**: 44. http://dx.doi.org/10.1196/annals.1422.062.

Baigent C, Blackwell L, Collins R, Emberson J, Godwin J, Peto R, Bunng J, Hennekens C, Kearney P, Meade T, Patrono C, Roncaglioni MC, Zanchetti A (2009) Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomized trials. *Lancet* **373**: 1849–1860. http://dx.doi.org/10.1016/S0140-6736(09)60503-1.

Backert S, Clyne M, Tegtmeyer N (2011) Molecular mechanisms of gastric epithelial cell adhesion and injection of CagA by *H. pylori*. *Cell Commun Signal* **9**: 28. http://dx.doi.org/10.1186/1478-811X-9-28.

Buzas GM (2014) Metabolic consequences of *Helicobacter pylori* infection and eradication. *W J Gastroenterol* **20**: 5226–5234. http://dx.doi.org/10.3748/wjg.v20.i18.5226.

Cekin AH, Taskoparan M, Duman A, Sezer C, Cekin Y, Yolcular BO, Can H, Pehlivan FS, Cayirci M (2012) The role of *Helicobacter pylori* and NSAID in the pathogenesis of uncomplicated duodenal ulcer. *Gastroentero Res and Practice* **2012**: http://dx.doi.org/10.1155/2012/189373.

Chandan VS, Wang W, Landas SK (2004) Gastric lipids islands. *Arch Pathol Lab Med* **128**: 937–938.

Chmiela M, Lelwala-Guruge JA, Wadstrom T, Rudnicka W (1996) The stimulation and inhibition of T cell proliferation by *Helicobacter pylori* components. *J Physiol Pharmacol* **47**: 195–202.

Chmiela M, Michetti P (2006) Inflammation, immunity, vaccines for *Helicobacter* infection. *Helicobacter* **11** (Suppl. 1): 21–26. http://dx.doi.org/10.1111/j.1478-405X.2006.00422.x.

Chmiela M, Miszczyk E, Rudnicka K (2014) Structural modifications of *H. pylori* lipopolysaccharide: An idea for how to live in peace. *W J Gastroenterol* **2014**: http://dx.doi.org/10.3748/wjg.v20.i29.0000.

Cover TL, Blanke SR (2005) *Helicobacter pylori* VacA, a paradigm for toxin multifunctionality. *Nat Rev Microbiol* **3**: 320–332. http://dx.doi.org/10.1038/nrmicro1095.

Czkwianianc E, Chmiela M, Lawnik M, Planeta-Malecka I, Rudnicka W (1997) Serodiagnosis of *Helicobacter pylori* in children with gastroduodenitis. *Centr Eur J Immunol* **22**: 240–247.

Derakhshan MH, , El-Omar E, Oien K, Gillen D, Fyfe V, Crabtree JE, McColl KE (2006) Gastric histology, serological markers and age as predictors of gastric acid secretion in patients infected with *Helicobacter pylori*. *J Clin Pathol* **59**: 1293–1299. http://dx.doi.org/10.1136/jcp.2005.036111.

Dubreuil JD, Giudice GD, Rappuoli R (2002) *Helicobacter pylori* interactions with host serum and extracellular matrix proteins: potential role in the infectious process. *Microbiol Mol Biol Rev* **66**: 617–629. http://dx.doi.org/10.1128/MMBR.66.4.617-629.2002

Febbraio M, Hajjar DP, Silverstein RL (2001) CD36: A class B scavenger receptor involved in angiogenesis, atherosclerosis, inflammation, and lipid metabolism. *J Clin Invest* **108**: 785–791. http://dx.doi.org/10.1172/JCI14006.

Gebert B, Fischer W, Weiss E, Hoffmann R, Haas R (2003) *Helicobacter pylori* vacuolating cytotoxin inhibits T lymphocyte activation. *Science* **301**: 1099–1102. http://dx.doi.org/10.1126/science.1086871.

Grebowska A, Moran AP, Matusiak A, Bak-Romaniszyn L, Czkwianianc E, Rechcicnski T, Walencka M, Planeta-Malecka I, Rudnicka W, Chmiela M (2008) Anti-phagocytic activity of *Helicobacter pylori* lipopolysaccharide (LPS) — possible modulation of the innate immune response to these bacteria. *Polish J Microbiol* **57**: 185–192.

Grebowska A, Moran AP, Bielanski W, Matusiak A, Rechcinski T, Rudnicka K, Baranowska A, Rudnicka W, Chmiela M (2010) *Helicobacter pylori* lipopolysaccharide activity in human peripheral blood mononuclear leukocyte cultures. *J Physiol Pharmacol* **61**: 437–442.

Hinc K, Isticato R, Dembek M, Karczewska J, Iwanicki A, Peszyńska-Sularz G, De Felice M, Obuchowski M, Ricca E (2010) Expression and display of UreA of *Helicobacter acinonychis* on the surface of *Bacillus subtilis* spores. *Microbial Cell Factories* **9**: 2. http://dx.doi.org/10.1186/1475-2859-9-2.

Hynes SO, Teneberg S, Roche N, Wadstrom T (2003) Glycoconjugate binding of gastric and enteropathic *Helicobacter* spp. *Infect Immun* **71**: 2976–2980. http://dx.doi.org/10.1128/IAI.71.5.2976-2980.2003.

Israel DA, Peek RM (2001) Pathogenesis of *H. pylori*-induced gastric inflammation. *Aliment Pharmacol Ther* **15**: 1271–1290. http://dx.doi.org/10.1046/j.1365-2036.2001.01052.x.

Kaiserling E, Heinle H, Itabe H, Takano T, Remmele W (1996) Lipid islands in human gastric mucosa: morphological and immunohistochemical findings. *Gastroenterology* **2**: 369–374. http://dx.doi.org/10.1053/gast.1996.v110.pm8566582.

Khaidakov M, Mehta JL (2012) Oxidized LDL triggers pro-oncogenic signaling in human breast mammary epithelial cells partly via stimulation of MiR-21. *PLOS ONE* **7**: e46973. http://dx.doi.org/10.1371/journal.pone.0046973.

Kim HL, Jeon HH, Park IY, Choi JM, Kang JS, Min KW (2011) *Helicobacter pylori* infection is associated with elevated low density lipoprotein cholesterol levels in elderly Koreans. *J Korean Med Sci* **26**: 654–658. http://dx.doi.org/10.3346/jkms.2011.26.5.654.

Kucukazman M, Yavuz B, Sacikara M, Asilturk Z, Ata N, Ertugrul DT, Yalcin AA, Yenigun EC, Kizilca G, Okten H, Akin KO, Nazligul Y (2008) The relationship between updated Sydney System score and LDL cholesterol levels in patients infected with *Helicobacter pylori*. *Dig Dis Sci* **2008**: http://dx.doi.org/10.1007/s10620-008-0391-y.

Lamb A, Chen LF (2013) Role of the *Helicobacter pylori* induced inflammatory response in the development of gastric cancer. *J Cell Biochem* **3**: 491–497. http://dx.doi.org/10.1002/jcb.24389.

Laurilla A, BloiguA, Nayha S, Hassi J, Leinonen M, Saikku P (1999) Association of *H. pylori* infection with elevated serum lipids. *Atherosclerosis* **142**: 207. http://dx.doi.org/10.1016/S0021-9150(98)00194-4.

Laurilla A, Cole SP, Merat S, Obonyo M, Palinski W, Fierer J, Witzum JL (2001) High-fat, high-cholesterol diet increases the incidence of gastritis in LDL receptor-negative mice. *Arterioscler Thromb Vasc Biol* **21**: 991–996. http://dx.doi.org/10.1161/01.ATV.21.6.991.

Lelwala-Guruge J, Nilsson I, Ljungh A, Wadstrom T (1992) Cell surface proteins and *Helicobacter pylori* as antigens in the ELISA and a comparison with three commercial ELISA. *Scan J Infect Dis* **24**: 457–465. http://dx.doi.org/10.3109/00365549209052632.

Lim YJ, Hong SJ (2014) *Helicobacter pylori* infection in nonsteroidal anti-inflammatory drug users. *Korean J Gastroenterol* **64**: 70–75. http://dx.doi.org/10.4166/kjg.2014.64.2.70.

Logan SM & Trust TJ (1983) Molecular identification of surface protein antigens of *Campylobacter jejuni*. *Infect Immun* **42**: 675–682.

Longo-Mbenza B, Nsenga JN, Makondijmobe E, Gombet T, Assori IN, Ibara JR, Ellenga-Molla B, Vangu DN, Fuelle SM (2012) *H. pylori* infection is identified as a cardiovascular risk factor in Central Africans. *Vasc Health Risk Mangement* **8**: 455. http://dx.doi.org/10.2147/VHRM.S28680.

Memon RA, Staprans I, Noor M, Holleran WM, Uchida Y, Moser AH, Feingold KR, Grunfeld C (2000) Infection and inflammation induce LDL oxidation *in vivo*. *Arterioscler Thromb Vasc Biol* **20**: 1536–1542. http://dx.doi.org/10.1161/01.ATV.20.6.1536.

Mendall MA, Goggin PM, Molineaux N, Levy J, Toosy T, Strachan D, Camm AJ, Northfield TC (1994) Relation of *H. pylori* infection and coronary heart disease. *Heart J* **71**: 437.

Miszczyk E, Walencka M, Rudnicka K, Matusiak A, Rudnicka W, Chmiela M (2014) Antigen-specific lymphocyte proliferation as a marker of immune response in guinea pigs with sustained *H. pylori* infection. *Acta Bioch Pol* **61**: 295–303.

Miszczyk E, Kowalewicz-Kulbat M, Walencka M, Sicińska P, Rudnicka K, Matusiak A, Mikołajczyk-Chmiela M (2014) Disorders of cell growth in response to *H. pylori* antigens on the guinea pig model in vivo and in the cell cultures *in vitro*. *Centr Eur J Immunol* **39**: 43(A96).

Moran AP, Helander IM, Kosunen TU (1992) Compositional analysis of *Helicobacter pylori* rough-form lipopolysaccharides. *J Bacteriol* **174**: 1370–1377.

Neuman M, Crabtree JE (2004) *Helicobacter pylori*-induced epithelial cell signaling in gastric carcinogenesis. *Trends Microbiol* **12**: 29–36. http://dx.doi.org/10.1016/j.tim.2003.11.005.

O'Connor F, Hyde D, Lee J, O'Morain C (1997) Increased gastric epithelial cell proliferation is directly associated with *Helicobacter pylori* but independent of the presence of the CagA gene. *Iris J Med Sci* **166**: 16.

Papatheodoridis GV, Sougioultzis S, Archimandritis AJ (2006) Effects of *Helicobacter pylori* and nonsteroidal anti-inflammatory drugs on peptic ulcer disease: a systemic review. *Clin Gastroenterol Hepatol* **4**: 130–142. http://dx.doi.org/10.1016/j.cgh.2005.10.006.

Perini R, Fiorucci S, Wallace JL (2004) Mechanisms of nonsteroidal anti-inflammatory drug-induced gastrointestinal injury and repair: A window of opportunity for cyclooxygenase-inhibiting nitric oxide donors. *J Malta College Pharmacy Practice* **10**: 15–19.

Posselt G, Backert S, Wessler S (2013) The functional interplay of *Helicobacter pylori* factors with gastric epithelial cells induces a multi-step process in pathogenesis. *Cell Commun and Signaling* **11**: 1–14. http://dx.doi.org/10.1186/1478-811X-11-77.

Rechcinski T, Chmiela M, Malecka-Panas E, Planeta-Malecka I, Rudnicka W (1997) Serological indicators of *Helicobacter pylori* infection in adult dyspeptic patients and healthy blood donors. *Microbiol Immunol* **41**: 387–393. http://dx.doi.org/10.1111/j.1348-0421.1997.tb01869.x.

Rudnicka K, Miszczyk E, Matusiak A, Walencka M, Moran AP, Rudnicka W, Chmiela M (2015) *Helicobacter pylori*-driven modulation of NK cell expansion, intracellular cytokine expression and cytotoxic activity. *Innate Immun* **21**: 127–139. http://dx.doi.org/10.1177/1753425913518225.

Sostres C, Gargallo CJ, Lanas A (2014) Interaction between *Helicobacter pylori* infection, nonsteroidal anti-inflammatory drugs and/or low dose aspirin use: Old questions new insights. *W J Gastroenterol* **20**: 9439–9450. http://dx.doi.org/10.3748/wjg.v20.i28.9439.

Sosters C, Carrera-Lasfuentes, Benito R, Roncales P, Arruebo M, Arroyo MT, Bujanda L, Garcia-Rodriguez LA, Lanas A (2015) Peptic ulcer bleeding risk. The role of *Helicobacter pylori* infection in NSAID/Low-dose aspirin users. *Am J Gastroenterol* **110**: 684–689. http://dx.doi.org/10.1038/ajg.2015.98.

Szustak K, Ciccone E, McCue P, Sharma K, Bottinger EP (2005) Multiple metabolic hits converge on CD36 as novel mediator of tubular epithelial apoptosis in diabetic nephropathy. *PLos Med* **2**: e45. http://dx.doi.org/10.1371/journal.pmed.0020045.

Taylor JM, Ziman ME, Hyff JL, Moroski NM, Vajdy M, Solnick JV (2006) *Helicobacter pylori* lipopolysaccharide promotes a Th1 type immune response in immunized mice. *Vaccine* **24**: 4987–4994. http://dx.doi.org/10.1016/j.vaccine.2006.03.043.

Valkonen KH, Wadstrom T, Moran AP (1994) Interaction of lipopolysaccharides of *Helicobacter pylori* with basement membrane protein laminin. *Infect Immun* **62**: 3640–3648.

Vella V (2005) Drug-induced peptic ulcer disease. *Journal of the Malta College of Pharmacy Practise* **10**: 15–19.

Viala J, Chaput C, Boneca IG, Cardona A, Girardin SE, Moran AP, Athman R, Memet S, Huerre MR, Coyle AJ (2004) Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island. *Nat Immunol*, **5**: 1166–1174. http://dx.doi.org/10.1038/ni1131.

Wadsack C, Hirschmugl B, Hammer A, Levak-Frank S, Kozarsky KF, Sattler W (2003) Scavenger receptor class B, type I on non-malignant and malignant lipoproteins. *Int J Biochem Cell Biol* **35**: 441–454. http://dx.doi.org/10.1016/S1357-2725(02)00272-8.

Walz A, Odenbreit S, Stuhler K, Wattenberg A, Mayer H, Mahdavi J, Boren T, Ruhl S (2009) Identification of glycoprotein receptors within human salivary proteome for the lectin-like BabA and SabA adhesisns of *Helicobacter pylori* by fluorescence-based 2-D bacterial overlay. *Proteomics* **9**: 1582–1592. http://dx.doi.org/10.1002/pmic.200700808.

Wessler S, Backert S (2008) Molecular mechanisms of epithelial-barrier disruption by *H. pylori*. *Trends Microbiol* **16**: 397-405. http://dx.doi.org/10.1016/j.tim.2008.05.005.

Yahiro K, Wada A, Nakayama M, Kimura T, Ogushi K, Niidome T, Aoyagi H, Yoshino K, Yonezawa K, Moss J, Hirayama T (2003) Protein-tyrosine phosphatise alpha, RPTP alpha, is a *H. pylori* VacA receptor. *J Biol Chem* **278**: 19183–19189. http://dx.doi.org/10.1074/jbc.M300117200.

Yahiro K, Satoh M, Nakano M, Hisatsune J, Isomoto H, Sap J, Suzuki H, Nomura F, Noda M, Moss J, Hirayama T (2012) Low-density lipoprotein receptor-related protein-1 (LRP-1) mediates autophagy and apoptosis caused by *H. pylori* VacA. *J Biol Chem* **287**: 31104–31115. http://dx.doi.org/10.1074/jbc.M112.387498.

Yamaoka Y (2010) Mechanisms of disease: *Helicobacter pylori* virulence factors. *Nat Rev Gastroenterol Hepatol* **7**: 629–641. http://dx.doi.org/10.1038/nrgastro.2010.154.

Yamaoka Y (2012) Pathogenesis of *Helicobacter*-related gastroduodenal diseases from molecular epidemiological studies. *Gastroenterol Res and Practice* **2012**: http://dx.doi.org/10.1155/2012/371503.

Yoshikawa T, Naito Y (2002) What is oxidative stress? *JMAJ* **45**: 271.