UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de São José do Rio Preto

Aline Cristina Targa Cadamuro

Expressão gênica e proteica e cinética celular no processo inflamatório induzido pela *Helicobacter pylori* antes e após terapia de erradicação

São José do Rio Preto, SP
2014

Aline Cristina Targa Cadamuro

Expressão gênica e proteica e cinética celular no processo inflamatório induzido pela *Helicobacter pylori* antes e após terapia de erradicação

Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Genética, junto ao Programa de Pós-Graduação em Genética, Área de Concentração – Genética Humana, do Instituto de Biociências, Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

Orientadora: Profª Drª Ana Elizabete Silva

São José do Rio Preto, SP
2014

Cadamuro, Aline Cristina Targa.
Expressão gênica e proteica e cinética celular no processo inflamatório induzido pela Helicobacter pylori antese após terapia de erradicação/ Aline Cristina Targa Cadamuro. -- São José do Rio Preto, 2014
177 f. : il., gráfs., tabs.

Orientador: Ana Elizabete Silva
Tese (doutorado) - Universidade Estadual Paulista “Júlio de Mesquita Filho”, Instituto de Biociências, Letras e Ciências Exatas

1. Genética humana. 2. Expressão gênica. 3. Helicobacter pylori. 4. Mucosa gástrica – Inflamação. 5. Células – Divisão. 6. Cinética. I. Silva, Ana Elizabete. II. Universidade Estadual Paulista "Júlio de Mesquita Filho". Instituto de Biociências, Letras e Ciências Exatas. III.Título.

CDU – 575.113

Ficha catalográfica elaborada pela Biblioteca do IBILCE
UNESP - Câmpus de São José do Rio Preto

Aline Cristina Targa Cadamuro

Expressão gênica e proteica e cinética celular no processo inflamatório induzido pela *Helicobacter pylori* antes e após terapia de erradicação

Tese apresentada como parte dos requisitos para obtenção do título de Doutor em Genética junto ao programa de Pós-Graduação em Genética, Área de Concentração - Genética Humana, do Instituto de Biociências Letras e Ciências Exatas da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de São José do Rio Preto.

Comissão Examinadora

Profª Drª Ana Elizabete Silva
Professor Adjunto Livre-Docente
Orientadora

Profª Drª Lúcia Regina Martelli
Professora Associada Livre-Docente
Universidade de São Paulo – Ribeirão Preto

Prof. Dr. Spencer Luiz Marques Payão
Professor Adjunto Livre-Docente
Faculdade de Medicina de Marília

Profª Drª Dorotéia Rossi S. Souza
Professor Adjunto Livre-Docente
Faculdade de Medicina de São José Rio Preto

Profª Drª Debora Ap. Pires de C. Zuccari
Professor Adjunto Doutor
Faculdade de Medicina de São José Rio Preto

São José do Rio Preto
27 de junho de 2014.

O presente trabalho foi realizado no Laboratório de Citogenética e Biologia Molecular Humana, do Departamento de Biologia, IBILCE/UNESP, na Unidade de Pesquisa Genética e Molecular (UPGEM), do Departamento de Biologia Molecular da Faculdade de Medicina de São José do Rio Preto-SP e no GENOA -Laboratório de Patologia Molecular e Celular Humana, SP. O projeto foi desenvolvido com bolsa e auxílio CNPq (processo nº 474776/2013-1) e auxílio FAPESP (processo n° 2012/15036-8).

Sumário

Dedicatória..... i

Agradecimentos..... ii

Epígrafe..... vii

Lista de Abreviaturas e Símbolos..... viii

Prefácio..... xiii

Resumo..... xvi

Abstract..... xviii

I. Introdução..... 1

II. Objetivos..... 20

III. Artigos..... 22

Artigo I..... 25

Artigo II..... 40

Artigo III..... 84

IV. Conclusões..... 131

VI. Referências Bibliográficas..... 134

Apêndices..... 145

Apêndice 1: Questionário de entrevista..... 146

Apêndice 2: Características demográficas e clinicopatológicas dos grupos..... 148

Anexos..... 149

Anexo 1: Parecer do Comité de Ética em Pesquisa Institucional..... 150

Anexo 2: Parecer consubstanciado do Comitê de Ética Ambiental..... 151

Anexo 3: Comprovante de submissão do artigo..... 152

Dedico este trabalho

Ao meu filho, Arthur, que me mostrou a existência do amor incondicional e das verdadeiras emoções, por me mostrar o que realmente importa;

Ao Rodrigo, companheiro inseparável, por me apoiar, entender minhas necessidades e prioridades, e permanecer ao meu lado;

Aos meus pais, exemplos a serem seguidos, por me estimular no crescimento profissional, pessoal, apoiando-me sempre.

AGRADECIMENTOS

A realização deste trabalho contou com a colaboração direta ou indireta de várias pessoas, reconheço a importância e agradeço:

À minha orientadora, Profª. Drª. Ana Elizabete Silva, meu respeito e reconhecimento pela competência, ética e profissionalismo. Meu agradecimento pelo carinho e acolhida após tantos anos, pela orientação, incentivo, amizade, permitindo meu crescimento profissional e enriquecendo minhas experiências pessoais.

À banca examinadora, Prof. Dr. Spencer Luiz Marques Payao, Profª. Drª. Lúcia Regina Martelli, Profª. Drª. Dorotéia Rossi Silva Souza e Profª. Drª. Débora A. P. de Campos Zuccari, por terem aceitado participar da análise e enriquecimento deste trabalho.

Às Profª. Drª. Lilian Madi-Ravazzi e Cláudia Márcia Aparecida Carareto, coordenadoras do Programa de Pós-graduação em Genética do Instituto de Biociências, Letras e Ciências Exatas (IBILCE/UNESP), durante a realização do meu doutorado, por possibilitar a realização desse trabalho.

À Profª Dra Patrícia Fucuta, à Dra Edla Bedin Polsinelli, e o Dr. Fausto Nasser, do Ambulatório de Dispepsia do Serviço de Gastrohepatologia - Hospital de Base de São José do Rio Preto pelo auxílio na seleção e inclusão dos pacientes, durante as consultas e entrevistas com os pacientes, pelo convívio gratificante e, principalmente pela amizade.

Ao Dr. Kenji Miyakazi e a toda equipe do Setor de Endoscopia do Hospital de Base de São José do Rio Preto, pela coleta das biópsias gástricas e pelos momentos de aprendizagem e descontração.

À Gizelda Warick Mazzale, enfermeira do Hospital Estadual João Paulo II, pelo auxílio na coleta das biópsias gástricas e levantamento de dados importantes para este trabalho.

Ao Dr. Ricardo Acayaba, diretor clínico, à Dra Jordana Melo, gastroenterologista e ao Dr André Volpato, endoscopista pelo auxílio na seleção e inclusão dos pacientes, durante as consultas e entrevistas com os pacientes e também, nas coletas no AME (Ambulatório Médico de Especialidades).

À Profª Dra. Kátia Ramos Moreira Leite e ao técnico Isaque Santana, pelo acolhimento e disponibilização do laboratório para a realização da técnica de imuno-histoquímica e pela valiosa contribuição na análise de expressão protéica.

Às Profª. Drª. Eny Maria Golloni Bertollo e Érika Cristina Pavarino por disponibilizar equipamentos, contribuindo para a realização deste trabalho e também, pela amizade.

À Drª. Patrícia Matos Biselli Chicote, profissional dedicada e competente, pelos ensinamentos e auxílio de grande importância na técnica de PCR em tempo real e, principalmente pela amizade e momentos agradáveis compartilhados.

Ao Prof. Dr. Sebastião Roberto Taboga e ao técnico Luiz Roberto Falleiros Júnior, pelos cortes histológicos necessários para posterior análise imunohistoquímica.

À Drª. e querida amiga, Joice Matos Biselli Périco, pelo carinho que sempre se dirigiu a mim, pela disponibilidade em me ensinar e auxiliar ao longo desse trabalho, pela assessoria nas análises estatísticas e, principalmente, pela agradável convivência, tornando tudo isso mais fácil.

À mestranda e amiga Ana Flávia Rossi, por quem tenho carinho, pessoa dedicada, organizada, competente e equilibrada, pelo auxílio fundamental nas análises de imuno-histoquímica, contribuindo muito com a realização deste trabalho.

Aos amigos do Laboratório de Citogenética e Biologia Molecular, onde este trabalho foi realizado: Ana Flávia, Ayla, Denis Bruno, Evelyn, Fernanda, Luiz Otávio, Marcela e Nathália. Agradeço a todos, de coração, pelo convívio gratificante, atenção, alegria constante e participação nas descobertas. Em especial, às minhas co-orientadas Ana Flávia, Fernanda e Evelyn que auxiliaram no desenvolvimento deste trabalho, tenham a certeza de que sem o esforço e a dedicação de vocês eu não conseguiria terminar este estudo.

Também agradeço as amigas do Laboratório de Genômica Estrutural e Comparativa: Nedenia, Mariana e Bruna, como também à Profa. Maria Elizabete Jorge Amaral, pela convivência diária e principalmente pelo conhecimento compartilhado e disponibilização de equipamentos sempre que preciso.

À MsC. e amiga do coração Marcela Alcântara Proença, pessoa equilibrada e coerente, pela amizade conquistada durante meu doutorado, pela ajuda em todos os momentos e pela agradável companhia em muitos congressos que contribuíram para nosso desenvolvimento científico.

A todos os professores e alunos da pós-graduação, os quais contribuíram para minha formação, em especial aos amigos Mariana Oliveira Gomes, Leiza Penariol, Elias Carnelossi pela convivência, ensinamentos e a amizade que se fortaleceu. Não poderia esquecer a Simone, secretária do Departamento de Biologia, que sempre me ajudou prontamente em tudo o que precisei.

Também agradeço aos funcionários da Seção de Pós-Graduação, pelo atendimento cordial e disponibilidade sempre dispensada.

Ao IBILCE/UNESP por fazer parte dos ensinamentos pessoais e acadêmicos, enriquecendo minha formação profissional.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e à Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) pela bolsa de doutorado e auxílios concedidos (CNPq, processo nº 474776/2013-1 e FAPESP, processo n° 2012/15036-8).

Ao meu sogro, Durvalino e à minha sogra, Idinir, pessoas boníssimas a quem tenho estima e consideração. Minha gratidão pelo carinho e apoio sempre presentes.

Aos amigos queridos, Ana e Ernesto, pela amizade, incentivo, pelos inúmeros momentos gratificantes, enfim só tenho a agradecer à minha "família do coração".

Ao Rodrigo, meu marido, minha segurança, por estar presente em mais esta etapa da minha vida, pelo amor, carinho, compreensão e incentivo. Trilhamos um lindo caminho juntos. Aprendo a cada dia com você, obrigada meu amor.

Aos meus queridos pais, Ademélio e Maria Célia, e ao meu irmão Flávio, por todas as razões deste mundo, não tenho palavras para descrever a importância de vocês em minha vida. Obrigada pelo carinho e apoio incondicional, pela educação de qualidade proporcionada, pelo incentivo, e por nunca me deixarem desistir. Vocês são minha vida.

Não poderia deixar de agradecer a Deus por abençoar a mim e à minha família, por me dar força nos momentos mais difíceis, e por iluminar sempre os meus caminhos.

"A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original."

Albert Einstein

LISTA DE ABREVIATURAS E SÍMBOLOS

°C	do inglês Celsius grade
µg	Micro-grama
µl	Micro-litro
µM	Micro-molar
ACTB	do inglês actin, beta
Akt/PI3K	do inglês serine-threonine kinase / Phosphatidylinositol-4,5-bisphosphate 3-kinase
ANOVA	Análise de variância
AP-1	do inglês Activator protein 1
BabA	do inglês *blood group antigen-binding adhesion*
bp	do inglês base pairs
cagA	do inglês *cytotoxin-associated gene A antigen*- CagA
cagPAI	do inglês cag Pathogenicity Island
CD25	do inglês interleukin 2 receptor, alpha
CD4+	do inglês CD4 molecule
CG	do inglês Chronic Gastritis
c-Jun/JNKs	do inglês N-terminal kinases
COX-2	do inglês Cyclooxygenase-2
CXCL1	do inglês chemokine (C-X-C motif) ligand 1
CXCL10	do inglês chemokine (C-X-C motif) ligand 10
CXCL14	do inglês chemokine (C-X-C motif) ligand 14
CXCL8	do inglês Chemokine (C-X-C motif) ligand 8
CYP2C19	do inglês *Cytochrome P450 2C19*
DAB	do inglês 3,3'diaminobenzidine tetrahydrochloride
DupA	do inglês *duodenal ulcer-promoting gene*
EMT	do inglês Epithelial to mesenchymal transitions
Foxa1	do inglês forkhead box A1
FoxM1	do inglês forkhead box M1

FOXP3	do inglês forkhead box P3
g	Grama
GAPDH	do inglês glyceraldehyde-3-phosphate dehydrogenase
GC	Gastrite Grônica
GC	do inglês Gastric Cancer
GSK3β	do inglês Glycogen synthase kinase 3 beta
hBD2	do inglês defensin, beta 2
hBD3	do inglês defensin, beta 3
IARC	do inglês International Agency for Research on Cancer
iceA	do inglês induced by contact with epithelium
IFITM1	do inglês Interferon Induced Transmembrane Protein 1
IFN-γ	do inglês Interferon
IGLC2	do inglês immunoglobulin lambda constant 2
IKKβ	do inglês I kappa B kinase
IL-10	do inglês Interleukin 10
IL-12	do inglês Interleukin 12
IL-12p40	do inglês Interleukin 12
IL-12p70	do inglês Interleukin 12
IL-1β	do inglês Interleukin beta 1
IL-23	do inglês Interleukin 23
IL-4	do inglês Interleukin 4
IL-6	do inglês Interleukin 6
IL-8	do inglês Interleukin 8
IP-10	do inglês Interferon gamma-induced protein 10
IRAK-1	do inglês Interleukin-1 receptor-associated kinase 1
IRAK-4	do inglês interleukin-1 receptor-associated kinase 4
IRF1	do inglês Interferon regulatory factor 1
ITGB2	do inglês *Integrin beta 2*
JAK/STAT	do inglês Janus kinase/signal transducers and activators of transcription
Ki-67	do inglês Antigen KI-67

LATS2	do inglês large tumor suppressor kinase 2
LOC400986	do inglês Ankyrin repeat domain-containing protein 36C-like
LPS	do inglês Lipopolysaccharide
LRRs	do inglês Leucine-rich repeat receptor
LY96	do inglês Lymphocyte antigen 96
MALT-linfoma	do inglês Musoca-associated lymphoid tissue
MAPK8	do inglês Mitogen-activated protein kinase 8
MDR1	do inglês *Multidrug resistance*
miRNA	do inglês small non-coding RNA molecule
mM	Mili-molar
MMP	do inglês Matrix Metalloproteinase
MyD88	do inglês Myeloid Differentiation primary response 88
NFAT	do inglês Nuclear factor of activated T-cells
NF-kB	do inglês Factor nuclear kappa B
NOD1	do inglês Nucleotide oligomerization domain 1
OAS1	do inglês 2'-5'-oligoadenylate synthetase 1
OipA	do inglês outer inflammatory protein
PAMPs	do inglês Pathogen-associated Molecular Pattern
PAP	do inglês Pancreatitis-associated Protein
PLAT	do inglês Plasminogen Activator, tissue
PPARγ	do inglês Peroxisome proliferator-activated receptor
PPI	do inglês Proton-pump inhibitor
qPCR	do inglês *quantitative polymerase chain reaction*
RAC2	do inglês ras-related C3 botulinum toxin substrate 2
Ras-ERK-MAPK	do inglês Mitogen-activated protein kinase
RECK	do inglês reversion-inducing-cysteine-rich protein with kazal motifs
REG3A	do inglês Regenerating islet-derived 3 alpha
RNS	do inglês reactive nitrogen species
ROS	do inglês reactive oxygen species

RT	do inglês Reverse transcriptase
SabA	do inglês sialic acid-binding adhesion
SD	Desvio padrão
siRNA	do inglês Small interfering RNA
SLA	do inglês Src-like-adaptor
STAT3	do inglês Signal transducer and activator of transcription 3
T4SS	do inglês *type-IVsecretion system*
TGF-β	do inglês Transforming Growth Factor beta
TGFβR1	do inglês transforming growth factor, beta receptor 1
TGFβR2	do inglês transforming growth factor, beta receptor 2
Th1	do inglês T helper type 1 cells
Th17	do inglês T helper type 17 cells
TLR1	do inglês *Toll like receptor 1*
TLR10	do inglês *Toll like receptor 10*
TLR2	do inglês *Toll like receptor 2*
TLR4	do inglês *Toll like receptor 4*
TLR5	do inglês *Toll like receptor 5*
TLR9	do inglês *Toll like receptor 9*
TNF	do inglês Tumor necrosis factor
TNFSF10	do inglês *Tumor necrosis factor (ligand) superfamily, member 10*
TOLLIP	do inglês Toll-interacting protein
TRAF6	do inglês TNF receptor-associated factor 6, E3 ubiquitin protein ligase
TsaA	do inglês Alkyl hydroperoxide reductase
TUNEL	do inglês Terminal Uridine Deoxynucleotidyl Nick end-Labeling
UreA	do inglês Urease subunit alpha
vacA	do inglês *vacuolating cytotoxin*
VCAM1	do inglês Vascular Cell Adhesion Molecule 1

Wnt/β-catenin	do inglês Wingless-type MMTV integration site family
ZEB1	do inglês zinc finger E-box binding homeobox

PREFÁCIO

Avaliamos, neste trabalho, as alterações nos níveis de expressão de genes e de proteínas envolvidas na resposta imune, bem como em processos celulares, como proliferação celular, apoptose, diferenciação, regeneração, metástase e angiogênese devido à infecção pela *Helicobacter pylori* em pacientes com lesões gástricas antes e cerca de três meses após a terapia de erradicação da bactéria.

As amostras foram obtidas a partir da coleta de biópsias gástricas durante exame endoscópico dos pacientes. A expressão dos genes *TLR2, TLR4, IL-8, TGF-β, PLAT, PAI-1*, *REG3A* e *IFITM1* foi avaliada pela técnica de PCR em tempo real, a expressão das respectivas proteínas, a cinética celular, como índice de proliferação e apoptose foram avaliadas por imuno-histoquímica.

Este estudo resultou em três manuscritos, um deles o artigo de revisão "*Helicobacter pylori infection: Host immune response, implications on gene expression and microRNAs*", publicado em volume especial em comemoração aos 20 anos do periódico *World Journal of Gastroenterology* (2014), em colaboração com as pós-graduandas Ana Flávia Rossi, Nathália Maciel Maniezzo e a Profª Drª Ana Elizabete Silva. São destacados alguns aspectos da infecção pela *H. pylori*, como os fatores de virulência bacterianos e, principalmente, sobre a modulação da resposta imune do hospedeiro e alterações no padrão de expressão de genes na mucosa gástrica infectada por esta bactéria. Também se destacou o papel da erradicação da bactéria para a normalização dos níveis de expressão gênica e o recente envolvimento de diferentes microRNAs na regulação da expressão gênica

em condições de mucosa gástrica normal, em lesões pré-cancerosas, no câncergástrico, e em processos inflamatórios induzidos pela bactéria.

Parte dos dados da tese, incluindo a expressão gênica e protéica dos receptores TLR2 e TLR4 e da cinética celular foi submetida à publicação à revista *Helicobacter,* em março de 2014. O artigo "*Helicobacter pylori eradication does not change TLR2 and TLR4 expression in chronic gastritis patients*" apresenta os dados referentes às alterações nos níveis de expressão gênica e protéica de TLR2 e TLR4 e também os índices de proliferação celular e apoptose em pacientes submetidos à terapia de erradicação da *H. pylori*. Este artigo mostra que a presença da bactéria aumenta os níveis de expressão desses receptores, os quais não são reduzidos após a terapia de erradicação. Além disto, evidencia redução do índice apoptótico e consequente aumento na taxa de proliferação celular em pacientes que erradicaram a bactéria.

Outro artigo "*Effect of H. pylori eradication on expression of the cytokines and cellular mediators in patients with gastric lesions*", ainda em sua primeira versão, será submetido a uma revista indexada internacional e reúne os resultados das alterações na expressão de mediadores celulares, como citocinas e fatores de crescimento (IL-8, TGF-β, PLAT, PAI-1, REG3A e IFITM1) em pacientes com lesões gástricas após a erradicação da bactéria.

O conjunto dos resultados obtidos das pesquisas sobre expressão gênica e protéica e cinética celular envolvida no processo inflamatório induzido pela *H. pylori* antes e após a terapia de erradicação, evidencia mudanças na expressão de mediadores celulares decorrentes da colonização persistente pela *H. pylori* no

epitélio gástrico, e que tendem a ser restaurados após a terapia de erradicação, evidenciando a importância do tratamento e contribuindo para o desenvolvimento de terapias mais efetivas relacionadas à erradicação desta bactéria.

RESUMO

Helicobacter pylori (*H. pylori*) é o principal patógeno associado ao câncer, devido mudanças inflamatórias e atróficas na mucosa gástrica, que por meio de progressão pode culminar no adenocarcinoma gástrico. A erradicação da *H. pylori* é considerada um tratamento de primeira linha para reverter as lesões pré-neoplásicas e prevenir a progressão maligna. Poucos estudos avaliaram a ocorrência de alterações genéticas antes e após a erradicação da bactéria, que possam evidenciar mudanças importantes relacionadas à resposta a terapia. Portanto, os objetivos deste estudo foram: avaliar a expressão do RNAm de genes que atuam na resposta inflamatória e imune e processos biológicos como proliferação celular, migração, invasão, apoptose, regeneração e angiogênse (*TLR2, TLR4, IL-8, TGF-β, REG3A, PLAT, PAI-1* e *IFITM1)* em pacientes com lesões gástricas antes e após a terapia de erradicação da *H. pylori*; investigar a expressão proteica dos respectivos genes; avaliar a influência dos genótipos bacterianos *CagA* e *VacA* na resposta à erradicação da bactéria e nos níveis de expressão gênica, e avaliar a cinética celular, como o índice de proliferação celular (PI) e o índice apoptótico (AI). Foram estudadas 38 biópsias gástricas de pacientes com gastrite crônica-Hp+ (GC-Hp+) antes do tratamento padrão de erradicação da bactéria e, 3 meses após a terapia. Como controle, foram obtidas quatro biópsias de mucosa gástrica normal-Hp- (MN-Hp). A quantificação relativa (RQ) do RNAm foi avaliada pelo ensaio TaqMan e a expressão proteica, proliferação celular e apoptose por imuno-histoquímica. Antes do tratamento foram observados níveis de expressão relativa aumentados do RNAm de *TLR2*, *TLR4*, *IL-8*, *TGF-β, REG3A* e *IFITM1* em pacientes GC-Hp+ em relação a MN-Hp- ($p< 0,0001$), exceto para *PLAT* e *PAI-1*, que apresentaram expressão reduzida ($p< 0,0001$). Os receptores *TLR2* e *TLR4*, assim como *TGF-β* e *IFITM1* apresentaram expressão aumentada do RNAm tanto antes como após o tratamento para erradicação da bactéria ($p< 0,05$). Os níveis elevados de expressão de *IL-8* e *REG3A* detectados antes da terapia (RQ= 20,42 e 7,68, respectivamente) foram

reduzidos nos pacientes que erradicaram a bactéria (RQ= 6,51, p= 0,013 e 0,61, p< 0,001), mas não nos não-erradicados. *PLAT* e *PAI-1* mostraram hipoexpressão antes do tratamento (RQ= 0,64 e 0,15, respectivamente), mas houve um aumento do RNAm de *PLAT* após a terapia de erradicação (RQ= 1,12, p= 0,003), porém os níveis de *PAI-1* não foram significantemente alterados (RQ= 0,22, p= 0,340). Também não se observou diferenças nos níveis de expressão dos genes avaliados quanto ao tipo histológico da lesão (gastrite crônica, gastrite atrófica e gastrite associada a metaplasia, p> 0,05). Quanto aos genótipos de virulência bacterianos *CagA* e *VacA* não houve diferenças significantes na taxa de erradicação da bactéria, assim como nos níveis de expressão gênica, exceto para *PAI-1*, em que níveis mais reduzidos do RNAm foram encontrados em pacientes erradicados portadores do genótipo *VacA* s1/m1 (p= 0,035). A imunoexpressão das proteínas TLR2 e TLR4 foram concordantes com os resultados de expressão gênica, enquanto que para as demais proteínas analisadas, verificou-se redução da expressão da proteína IFITM1 nas células do estroma em pacientes que erradicaram a bactéria (p= 0,0007), embora as demais proteínas analisadas não mostraram diferenças significantes após o tratamento (p> 0,05). O índice de proliferação celular não foi significantemente alterado após o tratamento da bactéria (p> 0,05), porém redução significante do índice apoptótico foi encontrada após a terapia de erradicação. Em conclusão, a infecção pela *H. pylori* desregula os níveis de expressão de genes envolvidos em processos biológicos como inflamação, proliferação e regeneração tecidual e aumenta a taxa de apoptose. Porém, a terapia de erradicação da bactéria não restabelece completamente os níveis de expressão gênica nos pacientes erradicados.

Palavras-chave: *Helicobacter pylori*, inflamação, lesões gástricas, terapia de erradicação, expressão gênica, cinética celular.

ABSTRACT

Helicobacter pylori is the main pathogen associated with cancer due inflammatory and atrophic changes in the gastric mucosa, which through of progression may result in gastric adenocarcinoma. The eradication of H. pylori is considered a first-line treatment to reverse precancerous lesions and prevent malignant progression, but a portion of patients has no effective response to therapy. Few studies have evaluated the occurrence of genetic changes before and after eradication of the bacteria, which can evidence important changes related to response to therapy. Therefore, the objectives of this study were to assess the mRNA expression of genes that participate in the inflammatory and immune response and biological processes such as cell proliferation, migration, invasion, apoptosis, regeneration and angiogenesis (TLR2, TLR4, IL-8, TGF-β, REG3A, PLAT, PAI-1 and IFITM1) in dyspeptic patients before and after eradication therapy of H. pylori; to investigate the protein expression of the respective genes; to evaluate the influence of bacterial CagA and VacA genotypes in response to H. pylori eradication and levels of gene expression, and assess cell kinetics, as cell proliferation index (PI) and apoptotic index (AI). 38 gastric biopsies from patients with chronic gastritis-Hp+ (CG-Hp+) before the standard treatment for eradication of the bacteria, and 3 months after therapy were studied. As control, four biopsies from normal gastric mucosa without infection (NM-Hp-) were obtained. Relative quantification (RQ) of mRNA was assessed by TaqMan assay and protein expression, cell proliferation and apoptosis by immunohistochemistry. Before treatment increased mRNA relative expression levels of TLR2, TLR4, IL-8, TGF-β, REG3A and IFITM1 were observed in CG-Hp+ compared to NM-Hp- (p <0.0001), except for PLAT and PAI-1, which showed reduced expression (p <0.0001). The TLR2 and TLR4 receptors, as well as TGF-β and IFITM1 showed increased expression of mRNA both before and after treatment for eradication (p< 0.05). High levels of IL-8 and REG3A expression detected before therapy (RQ= 20.42 and 7.68, respectively) were reduced in patients who has eradicated

bacteria (RQ= 6.51, p= 0.013 and 0.61, p <0.001), but not in non-eradicated. PLAT and PAI-1 showed down-regulation before treatment (RQ= 0.64 and 0.15, respectively), but there was an increase in PLAT mRNA after eradication therapy (RQ= 1.12, p= 0.003), but the PAI-1 levels were not significantly altered (RQ= 0.22, p= 0.340). Also, no differences were observed in the expression levels of genes evaluated according to histological type of the lesion (chronic gastritis, atrophic gastritis and metaplastic atrophic gastritis, p> 0.05). With regards to CagA and VacA strains there was no significant difference in the bacterial eradication rate as well as in the levels of gene expression, except for PAI-1, in which lower mRNA levels were found in eradicated patients carriers of VacA s1/m1 (p= 0.035). The immunoreactivity of TLR2 and TLR4 proteins were concordant with the results of gene expression, while for others proteins analyzed, there was reduced expression of IFITM1 protein in stromal cells in eradicated patients (p= 0.0007), although the others proteins showed no significant differences after treatment (p> 0.05). The cell proliferation rate was not significantly changed after treatment (p> 0.05), but a significant reduction in the apoptotic index was found after eradication therapy. In conclusion, the H. pylori infection deregulates the expression levels of genes involved in biological processes such as inflammation, proliferation, and tissue regeneration and increases the apoptosis. However, the eradication therapy tends to restore the levels of gene expression in eradicated patients, highlighting that the treatment plays an important role in the control of inflammation and homeostasis of the gastric mucosa, thus may prevent cancer progression.

Key-words: *Helicobacter pylori, inflammation, gastric lesions, eradication therapy, gene expression, cell kinetics.*

Introdução

I. INTRODUÇÃO

I.1. Inflamação e câncer gástrico

A inflamação, em decorrência de alguma lesão na mucosa ou presença de agentes infecciosos, tem como objetivo principal restabelecer a homeostase do tecido (VAGO et al., 2012). Contudo, quando a resposta inflamatória ocorre de maneira descontrolada ou não é resolvida corretamente, células inflamatórias permanecem no local injuriado originando a inflamação crônica. Esta resposta persistente resulta na produção de grande quantidade de espécies reativas de oxigênio (ERO) e nitrogênio (ERN) (BARCELLOS-HOFF; LYDEN; WANG, 2013), induzindo alterações genéticas e epigenéticas em vários proto-oncogenes e genes supressores tumorais, o que favorece o surgimento de diversos tipos de cânceres (CHIBA; MARUSAWA; USHIJIMA, 2012). No microambiente tumoral, a ocorrência de células e mediadores inflamatórios favorece a transformação celular e a propagação de doenças invasivas (FERRONE; DRANOFF, 2010; BALKWILL; MANTOVANI, 2012).

Agentes infecciosos causadores de inflamação crônica estão associados à cerca de 10-15% dos cânceres (CHIBA; MARUSAWA; USHIJIMA, 2012). Muitas neoplasias surgem a partir de sítios de infecção, irritação crônica e inflamação em órgãos como o fígado, pâncreas, estômago, esôfago, cólon e próstata, aumentando o risco de câncer, e pesquisas têm demonstrado o papel dos componentes importantes da inflamação na carcinogênese (COUSSENS; WERB, 2002; QUANTE; WANG, 2008). Neste modelo enquadra-se o câncer gástrico como consequência da infecção pela *Helicobacter pylori* (*H. pylori*). Esta bactéria

produz uma forte resposta inflamatória no seu hospedeiro (AMIEVA; EL-OMAR, 2008), estimulando a produção de muitos mediadores inflamatórios, além de ERO e ERN, o que influencia na carcinogênese de estômago pelo aumento no crescimento e na mobilidade celular, angiogênese e inibição da apoptose (CHIBA; MARUSAWA; USHIJIMA, 2012).

O câncer gástrico, cuja taxa de mortalidade permanece alta mundialmente, especialmente na Ásia (Japão, Coréia e China) e em vários países em desenvolvimento, é o quarto tipo de câncer mais frequente e a segunda causa de morte dentre as relacionadas ao câncer (JEMAL et al., 2011; SIEGEL; NAISHADHAM; JEMAL, 2012). No Brasil, em 2014 estima-se que ocorram 20.390 casos novos, representando o terceiro tipo de neoplasia com maior incidência no país (INCA, 2014). A maioria dos casos é diagnosticada entre os 50 ou 70 anos de idade, afetando duas vezes mais homens do que mulheres (HATAKEYAMA, 2009).

A incidência elevada é preocupante, pois o prognóstico da doença é pobre e a sobrevida de pacientes afetados após cirurgia e terapia é menor que 40% (JANG; KIM, 2011). Esta neoplasia é caracterizada como uma doença multifatorial (HERRERA; PARSONNET, 2009; JANG; KIM, 2011), com participação de fatores genéticos e ambientais. Dentre os fatores ambientais destaca-se o consumo elevado de sal e nitratos (HERRERA; PARSONNET, 2009; RESENDE et al., 2011), alimentos defumados, álcool (MILNE; CARNEIRO; O'MORAIN, 2009) e fumo (TRAMACERE; LA VECCHIA; NEGRI, 2011), sendo a infecção pela *H. pylori* o principal fator de risco exógeno (SMITH et al.,

2006). De modo contrário, a ingestão de frutas e vegetais está relacionada a uma diminuição do risco de câncer, pois contém compostos como ácido ascórbico, carotenoides, folatos e tocoferois que possuem efeito antioxidante (WU et al., 2011).

O adenocarcinoma gástrico correspondente a 95% das neoplasias malignas de estômago (INCA, 2014), é uma doença heterogênea (JANG; KIM, 2011), classificada conforme Lauren (1964) em dois tipos histológicos distintos: intestinal e difuso. Estes subtipos tumorais diferem quanto à epidemiologia, etiologia e alterações moleculares. O tipo difuso é etiologicamente atribuído com maior frequência a fatores genéticos do hospedeiro, sendo que uma pequena parcela de carcinomas do tipo difuso é de origem familial, causados por mutações no gene *E-caderina* (GUILFORD et al, 1998). O tipo intestinal apresenta-se bem diferenciado, é mais frequente e está associado mais comumente a perturbações ambientais e a progressão de lesões gástricas pré-neoplásicas. É composto por células malignas que se unem formando estruturas semelhantes a glândulas funcionais do trato gastrointestinal (SMITH et al., 2006). Acredita-se que a infecção pela *H. pylori* esteja vinculada ao surgimento dos dois tipos de adenocarcinoma (FUENTES-PANANÁ;CAMORLINGA-PONCE; MALDONADO-BERNAL, 2009).

A carcinogênese gástrica do tipo intestinal é decorrente de múltiplas etapas, sendo esta cascata iniciada pela gastrite superficial associada à infecção pela *H. pylori*. Esta lesão pode progredir para gastrite atrófica crônica, metaplasia intestinal, displasia e, por fim, carcinoma (CORREA, 1988), sendo

estes estágios sequenciais e separados por longos períodos de tempo. A gastrite crônica é uma lesão pré-maligna (GENTA, 1998) bastante frequente que engloba desde inflamação superficial até atrofia do epitélio, sendo caracterizada pela presença de linfócitos e plasmócitos (CHELI; GIACOSA, 1983). Cerca de 10% dos pacientes com atrofia gástrica desenvolvem adenocarcinoma em um período de 15 anos (CHELI; GIACOSA, 1983). Juntamente com a gastrite ou durante a cicatrização da mucosa é observada a metaplasia intestinal, caracterizada pela transformação das células do epitélio gástrico em células colunares absortivas que se intercalam com células caliciformes de morfologias intestinais, sendo também associada com infecção pela *H. pylori* (GUTIERREZ-GONZALEZ et al., 2011).

I.2. *Helicobacter pylori*

A *H. pylori* é uma bactéria Gram-negativa, de forma espiral, coloniza a mucosa gástrica e é considerada a infecção bacteriana mais comum no mundo todo. Geralmente, é adquirida na infância, podendo persistir no ambiente gástrico ao longo da vida se não tratada (EVERHART, 2000; RICCI; ROMANO; BOQUET, 2011). A presença persistente da *H. pylori* no estômago pode causar gastrite crônica, permanecendo silenciosa por décadas, devido ao balanço sincronizado entre o patógeno e seu hospedeiro, ou pode causar doenças mais severas, tais como gastrite atrófica, úlcera péptica, linfoma de tecido linfóide associado à mucosa gástrica (linfoma-MALT) e o adenocarcinoma gástrico (ZARRILLI; RICCI; ROMANO, 1999; RICCI; ROMANO; BOQUET, 2011). Portanto, a infecção por esta bactéria é considerada o fator mais fortemente

associado com esta neoplasia, em decorrência do processo inflamatório desencadeado na mucosa gástrica, aumentando o risco de câncer gástrico em mais de seis vezes comparando-se com indivíduos não infectados (KUIPERS et al., 1999; SUERBAUM; MICHETTI, 2002). Em consequência desta associação, a *H. pylori* foi classificada, em 1994, pela Agência Internacional de Pesquisa sobre o Câncer como um carcinógeno do tipo I (IARC, 1994).

Mundialmente, a *H. pylori* é responsável por infectar mais da metade da população, porém sua incidência é variável entre as diferentes regiões geográficas (VAN DOORN et al., 1999). Sabe-se que a infecção pode permanecer assintomática no decorrer da vida em 85% dos indivíduos, enquanto que, apenas 1% desenvolverá câncer gástrico (EL-OMAR et al., 2001) e 10% úlcera péptica (SACHS; SCOTT, 2012).

De fato, as consequências clínicas da infecção pela *H. pylori* são determinadas por múltiplos fatores, incluindo predisposição do hospedeiro, especialmente certos polimorfismos de citocinas e de genes receptores (LI et al., 2010; OLIVEIRA et al., 2012; de OLIVEIRA et al., 2012; de OLIVEIRA et al., 2013), regulação gênica e fatores ambientais, e heterogeneidade das linhagens de *H. pylori* (HERRERA; PARSONNET, 2009; RICCI; ROMANO; BOQUET et al., 2011).

Na maioria dos casos, a infecção pode persistir ao longo da vida do hospedeiro na ausência do uso de antibióticos para erradicação (EVERHART, 2000; DE FLORA; BONANNI, 2011). Tal persistência é atribuída à capacidade desta bactéria adaptar-se ao ambiente inóspito do estômago com variação de pH,

devido à atividade da enzima urease e à presença de flagelos que facilitam sua penetração na camada de muco, atingindo o epitélio gástrico (HATAKEYAMA, 2009). A urease hidroliza a ureia, produzindo amônia e dióxido de carbono, neutralizando o pH, permitindo assim, a sobrevivência e a proliferação bacteriana (HATAKEYAMA, 2009).

De modo geral, a colonização pela *H. pylori* causa forte resposta imune sistêmica, originando um ambiente cronicamente inflamado com acidez reduzida, o que favorece o crescimento de outras bactérias no nicho gástrico, mantendo a inflamação e reduzindo, portanto, o nível de vitamina C no suco gástrico. A inibição da secreção de ácido gástrico favorece a mudança de uma gastrite predominante do antro para uma gastrite na região do corpo do estômago, iniciando a atrofia gástrica e metaplasia intestinal, caracterizadas como lesões pré-cancerosas (KIM; KIM, 2009).

Além disso, os fatores de virulência bacterianos, como os codificados pelos genes do antígeno A associado à citotoxina (*cytotoxin-associated gene A antigen- CagA*) e da citotoxina de vacuolização (*vacuolating cytotoxin- VacA*) desempenham papel crucial na patogênese induzida pela *H. pylori* e outros, como o *IceA* (*induced by contact with epithelium*), o *BabA* (*blood group antigen-binding adhesion*), o *SabA* (*sialic acid-binding adhesion*), o *DupA* (*duodenal ulcer-promoting gene*) e o *OipA* (*outer inflammatory protein*), garantem também, o sucesso da colonização da mucosa (BACKERT; MEYER, 2006; AMIEVA; EL-OMAR, 2008). As populações bacterianas são altamente heterogêneas com relação aos fatores de virulência *CagA* e *VacA* (WEN , MOSS,

2009), e muitas evidências mostram que essas diferenças genéticas são importantes nas consequências clínicas da infecção (HERRERA; PARSONNET, 2009; BAUER; MEYER, 2011).

O gene *cagA* produz um dos fatores de virulência mais importantes da *H. pylori*, localiza-se no segmento de DNA conhecido como *ilha de patogenicidade cag* (cagPAI), contém também, genes que originam um sistema de secreção bacteriano do tipo IV (*T4SS-type-IVsecretion system*) (CENSINI et al., 1996). Este sistema funciona como uma seringa molecular, injetando CagA, peptideoglicanos e outros fatores dentro das células epiteliais do hospedeiro (BACKERT et al., 2000). Após a entrada na célula, CagA pode ser fosforilada por tirosinas quinases, interage com proteínas celulares, atuando nas vias de transdução de sinal para o núcleo. Assim, os fatores injetados e os componentes de T4SS influenciam a dinâmica da célula, ocasionando mudanças no citoesqueleto e o rompimento das junções célula-célula, bem como as respostas anti-apoptótica e inflamatória no hospedeiro (ATHERTON; BLASER, 2009; POLK; PEEK, 2010; BACKERT; TEGTMEYER; SELBACH, 2010). Por causa dos efeitos causados, cepas que portam cagPAI são associadas a altos graus de inflamação da mucosa gástrica (HATAKEYAMA, 2009), conferindo maior risco para o desenvolvimento de câncer de estômago (TRUONG et al., 2009; BAUER; MEYER, 2011).

O gene *vacA*, responsável pela expressão da citotoxina indutora de vacuolização em células epiteliais (POLK; PEEK, 2010) secreta a proteína VacA que estimula a apoptose de células gástricas e exerce efeitos na resposta imune do

hospedeiro pela perturbação da apresentação de antígenos, garantindo, assim, a evasão da *H. pylori* da resposta imune adaptativa (AMIEVA; EL-OMAR, 2008; WEN; MOSS, 2009; POLK; PEEK, 2010). Diferentemente de *cag-A*, todas as linhagens de *H. pylori* possuem o gene *vacA*, embora a proteína VacA seja expressa em apenas 50% delas. Esta diferença resulta de polimorfismos existentes no gene (WEN; MOSS, 2009), sendo a linhagem s1/m1 altamente toxigênica (BASSO et al., 2008). Cepas que expressam as formas ativas de VacA são associadas com maior risco à gastrite e câncer gástrico (PEEK, 2002; POLK; PEEK, 2010).

A ligação da *H. pylori* as células gástricas epiteliais por meio de BabA e outras adesinas permite a introdução de CagA e outras moléculas efetoras, dentro das células do hospedeiro ativando a resposta imune com a produção de interleucinas, fator de necrose tumoral (TNF), citocinas pró-inflamatórias, ativação de NF-kB, AP-1, c-Jun, NH2-quinase terminal e de fatores de proliferação celular e sobrevivência. Além disso, as toxinas bacterianas, os níveis elevados de superóxidos, radicais, metabólitos de oxigênio reativo, compostos N-nitroso e óxido nítrico são potentes carcinógenos na mucosa gástrica, que podem causar danos no DNA. Também os fatores de virulência CagA e VacA induzem hiperproliferação e expressão de oncogenes (KIM; KIM, 2009; BEN-NERIAH; KARIN, 2011; KURAISHY; KARIN; GRIVENNIKOV, 2011). Outros estudos ainda têm mostrado que a presença da *H. pylori* na mucosa gástrica pode promover alterações epigenéticas induzindo metilação de regiões promotoras com ilhas CpG pela ativação da DNA metiltransferase (USHIJIMA; HATTORI, 2012).

O fato de a bactéria permanecer no estômago em alta densidade e a inflamação persistir por muitos anos suporta a ideia de que a resposta imune do hospedeiro seja desregulada e inefetiva (WILSON; CRABTREE, 2007).

I.3. Alterações genéticas e tratamento da *Helicobater pylori*

Embora a associação entre inflamação e carcinogênese esteja bem estabelecida, os mecanismos envolvidos ainda não estão elucidados. Porém são observadas alterações celulares consideradas como fatores carcinogênicos, como estresse oxidativo, danos no DNA, mudanças epigenéticas e proliferação celular induzida por citocinas. Além disso, a inflamação crônica gastrointestinal parece promover apoptose de células normais levando a proliferação compensatória para regeneração do tecido (QUANTE; WANG, 2008; CHEN et al., 2012).

Estudos também evidenciam mudanças na expressão gênica em resposta a infecção pela *H. pylori*, que podem estar associadas com a resposta do hospedeiro ao tratamento de erradicação da bactéria. A erradicação da *H. pylori* é considerada um tratamento de primeira linha para reverter as lesões pré-neoplásicas, prevenir a progressão maligna, assim reduzindo o risco de câncer gástrico (TSAI et al., 2006, KABIR, 2009, FUCCIO et al., 2009). Porém, o tratamento não é preconizado entre os portadores assintomáticos em países em desenvolvimento, devido seu custo elevado (WU et al, 2008).

Uma das opções de tratamento para esta infecção é a terapia tripla, considerada o tratamento padrão de primeira linha que consiste na administração de dois ou três antibióticos (claritromicina e amoxicilina) associados a um

inibidor de bomba de próton-PPI, durante uma ou duas semanas (RIMBARA et al, 2011). Contudo, a resistência da bactéria a alguns antibióticos, tem direcionado as pesquisas para terapias alternativas, incluindo a extensão da duração do tratamento para 14 dias, o uso de um esquema de quatro medicamentos contendo bismuto, terapia sequencial ou concomitante, assim como o uso de novos antibióticos, como a levofloxacina (VAIRA et al., 2007; JAFRI et al., 2008; GREENBERG et al., 2011; MALFERTHEINER et al., 2011), mas nem sempre a erradicação é bem sucedida. O sucesso terapêutico depende de vários fatores, incluindo a susceptibilidade aos antibióticos e o genótipo do hospedeiro, como polimorfismos de *CYP2C19*, *MDR1* e de citocinas pró-inflamatórias (GRAHAM et al, 2008; SUGIMOTO et al, 2009), sendo a quimiorresistência o fator mais importante (VAKIL; MÉGRAUD, 2007; WU et AL., 2010; SONG et al., 2011). Ainda outros fatores, como o tabagismo, submissão do paciente, duração da terapia, o esvaziamento gástrico, a inibição insuficiente de ácido gástrico durante o tratamento, assim como os fatores de virulência da bactéria podem interferir nas taxas de cura (HUNT, 1993; GRAHAM et al, 2008).

Poucos estudos têm avaliado a ocorrência de alterações genéticas e de proteínas no hospedeiro em decorrência da infecção pela *H. pylori*. A partir da construção de uma rede de interações de proteínas em resposta à infecção produzida pela *H. pylori* foi possível constatar a participação de proteínas principalmente relacionadas à resposta imune, ativação de vias como JAK/STAT acionada pelas interleucinas, ativação de NF-kB e TLR4, dentre outras. As proteínas associadas à resposta imune interagem com vias e proteínas relacionadas

com o ciclo celular, manutenção e proliferação celular, reguladores de transcrição e proteínas *zinc finger*. Portanto, tais proteínas são potencias alvos para drogas contra a inflamação gástrica e câncer (KIM; KIM. 2009). Em outra abordagem para caracterizar o perfil de expressão gênica na mucosa gástrica de pacientes infectados pela *H. pylori* foi evidenciado padrão transcricional distinto entre as biópsias da região antral e fúndica associado com a densidade bacteriana e aos fatores de virulência como C*agA, BabA2* e *VacA*. Expressão diferencial foi observada em classes de transcritos associados com os receptores do tipo *toll-like*, transdução de sinal, resposta imune, apoptose e ciclo celular, como por exemplo, *TLR2* e *TLR4*, *LY96, ITGB2* (*integrin 2*), *VCAM1*, *MAPK8, RAC2*, *SLA*, vários transcritos de quimiocinas e receptores, *IL-8*, transcritos das famílias *ADAM* e *MMP*, *PLAT*, *IFITM1* e *PAP*, todos com expressão aumentada na mucosa antral, exceto *PLAT*, que apresentou expressão reduzida (HOFFMAN et al., 2007).

Estes achados nos motivaram a investigar a ocorrência de mudanças nos níveis de expressão em genes que participam da resposta imune induzida pela *H. pylori* na mucosa gástrica infectada, assim como avaliar se após a erradicação da bactéria há uma normalização na expressão gênica. Portanto, foram selecionados os genes *TLR2, TLR4, IL-8, TGF-β, REG3A, PAI-1, PLAT* e *IFITM1* (Tabela 1) devido ao envolvimento nos processos inflamatório e carcinogênico para avaliação em pacientes *H. pylori*-positivos antes e após o tratamento de erradicação da bactéria.

Tabela 1. Denominação oficial dos genes avaliados na infecção pela *H. pylori*, localização cromossômica e função.

Gene	Nome oficial	Localização	Função
TLR2	*Toll-like receptor 2*	4q32	Reconhecimento de agentes patogênicos e ativação do sistema imune inato.
TLR4	*Toll-like receptor 4*	9q33.1	Reconhecimento de agentes patogênicos e ativação do sistema imune inato.
IL8	*Chemokine (C-X-C motif) ligand 8*	4q13-q21	Mediador da resposta inflamatória, atua como um quimiotáxico e como fator angiogênico.
TGFB1	*Transforming growth factor, beta 1*	19q13.1	Citocina, regulação da proliferação, diferenciação, adesão e migração.
PLAT	*Plasminogen activator, tissue*	8p12	Serina-protease que converte o plasminogênio em plasmina, papel na migração celular e remodelação do tecido.
PAI-1	*Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1*	7q22.1	Principal inibidor do ativador do plasminogênio tecidual (tPA) e uroquinase (uPA), regulação de processos biológicos como coagulação, invasão, metástase e angiogênese.
REG3A	*Regenerating islet-derived 3 alpha*	2p12	Proteína de secreção pancreática, atua na proliferação e diferenciação celular.
IFITM1	*Interferon induced transmembrane protein 1*	11p15.5	Transdução de sinais de adesão e antiproliferativos em linfócitos, inibição da replicação viral, promoção da invasão celular e resposta à droga.

http://www.ncbi.nlm.nih.gov/pubmed/

I.4. Genes envolvidos na inflamação e carcinogênese

Os genes *TLR2* e *TLR4* são membros da família dos receptores TLRs (*toll-like receptors*), compreendendo 10 tipos em humanos, TLR1 a TLR10, os quais são componentes essenciais da resposta imune inata e adaptativa (KANG; LEE, 2011). Eles reconhecem estruturas moleculares de padrões moleculares associados à patógenos (PAMPs), como lipopolissacarídeos (LPS), lipoproteínas, ácido lipoteicoico, peptideoglicanos, lipoarabinomanano e flagelina (TAKEDA; KAISHO; AKIRA, 2003). O LPS da *H. pylori* e outros componentes da parede celular são reconhecidos principalmente por TLR4 e também por TLR2 (YOKOTA et al., 2007). Os TLRs reconhecem seus ligantes através de LRRs (*leucine-rich repeats*) e os transmitem por meio de proteínas adaptadoras, como MyD88 desencadeando uma cascata de eventos de sinalização, como fosforilação de IRAK-1 (*interleukin-1 receptor-associated kinase 1*) e ativação de NF-kB ou de IRF3 (*interferon regulatory factor 3*), resultando na produção de mediadores imunes, como a indução de citocinas pró-inflamatórias e de genes induzíveis pelo IFN (AKIRA; UEMATSU; TAKEUCHI, 2006). Assim, a associação direta ou indireta de um ligante com o seu TLR correspondente atua como um sinalizador iniciando a resposta imune.

A interleucina 8 (IL-8; símbolo oficial *CXCL8*), uma quimiocina do grupo de citocinas pró-inflamatórias, compreende mediadores com habilidade de migração e ativação leucocitária, podendo estimular células que liberam enzimas proteolíticas, permitindo a digestão da matriz extracelular e providenciando uma via para a migração de células inflamatórias, crescimento tumoral e metástase

(YUAN et al., 2005). Esta quimiocina é produzida por diferentes tipos celulares, como monócitos, macrófagos, neutrófilos, fibroblastos, queratinócitos (DAN et al., 2010) e também, pelas células epiteliais gástricas, durante a infecção pela *H. pylori* (SUGIMOTO et al., 2010), onde os níveis de IL-8 na mucosa gástrica correlacionam-se com a severidade histológica em pacientes com gastrite induzida pela *H. pylori* (FAN et al., 1995). A associação entre os altos níveis na expressão do RNAm de *IL-8* com o risco de câncer gástrico tem sido relatada (YAMADA et al., 2013), uma vez que esta citocina apresenta papel crítico na angiogênese e progressão tumoral (SPARMANN; BAR-SAGI, 2004; SUGIMOTO et al., 2010) por meio da indução de proliferação celular e migração (TAGUCHI et al., 2005).

A citocina multifuncional TGF-β regula vários processos biológicos, incluindo crescimento celular, ciclo celular, desenvolvimento precoce, diferenciação, formação da matriz extracelular, hematopoese, angiogênese e função imune (MASSAGUE et al., 2005), modulando a resposta inflamatória pela inibição da proliferação de linfócitos T e B e suprimindo a atividade de macrófagos e células NK (KIM; KIM, 1996). TGF-β funciona tanto como um supressor quanto um promotor tumoral, inibindo o crescimento de células pré-malignas e promovendo a indução da apoptose, ao mesmo tempo que, promove metástase tumoral pela imunossupressão, indução da transição epitélio-mesenquimal e aumento da angiogênese (ACHYUT; YANG, 2011). Estudos clínicos mostraram correlação positiva entre a expressão de TGF-β com metástase de linfonodos e prognóstico ruim em carcinoma gástrico (MAEHARA et al., 1999; SAITO et al., 2000).

REG3A (*Regenerating islet-derived 3 alpha*, conhecido também como PAP), membro da família gênica de regeneração (Reg) foi identificado primeiramente no suco pancreático em pancreatite induzida experimentalmente em ratos (KEIM et al., 1984). Reg representa um grupo de proteínas secretórias essenciais para a regeneração e proliferação celular, além de atuar na formação do sistema imune inato (BROEKAERT et al., 2002). Estudos de expressão gênica têm mostrado a relação entre a expressão aumentada de transcritos de *REG3A* e atividade proliferativa, como ploidia do DNA (CAO et al., 2009), assim como aumento da síntese de DNA, atuando como um fator de crescimento *in vivo*, levando a regeneração do fígado (SIMON et al., 2003). Alguns estudos mostram também, a associação desse gene com vários mecanismos de efeito proliferativo, antiinflamatório, anti-apoptótico e antibacteriano (CLOSA; MOTOO; IOVANNA, 2007). As propriedades anti-apoptóticas e antimicrobianas de *REG3A* foram observadas em estudos *in vivo* (ZHANG et al., 2004), porém sua função biológica no câncer não está muito bem esclarecida.

O gene *PLAT* (*Plasminogen activator, tissue*) codifica o ativador do plasminogênio tecidual (t-PA), uma protease serina que converte o plasminogênio em plasmina, uma enzima fibrinolítica (SHIM et al., 2005). O sistema ativador do plasminogênio (PA) apresenta dois tipos principais de PA, o tipo tecido (tPA) e o tipo uroquinase (uPA), os inibidores do ativador do plasminogênio (PAI-1, 2 e 3) e o receptor (uPAR), que liga o PA nas células em migração (SMITH; MARSHALL, 2010). A resposta excessiva decorrente de um quadro inflamatório resultará na destruição do tecido e fibrose. Nesse sentido,

estudos relatam a função de tPA na regulação da matriz extracelular (FREDRIKSSON et al., 2004; HU et al., 2008) e na modulação da resposta inflamatória em resposta à lesão do tecido em vários modelos animais (ROELOFS et al., 2006; HIGAZI et al., 2008). Além disto, outros estudos demonstraram que tPA pode atuar como uma molécula que apresenta dupla função (HU et al., 2008; LIN et al., 2010; LIN; WU; HU, 2012). Como uma protease serina, desempenhando papel crucial na homeostase da coagulação do sangue/fibrinólise e regulação da matriz extracelular (HU et al., 2008) e também, como uma citocina, ligando-se aos seus receptores de membrana, e desencadeando eventos de sinalização intracelular (HU et al., 2008; SHI et al., 2009; LIN et al., 2010; LIN; WU; HU, 2012). Há indicações que o aumento na atividade ou nos níveis de expressão de *PLAT* seja benéfico, provavelmente devido à estimulação da produção de plasmina, uma vez que esta enzima induz a degradação da matriz de fibrina pró-angiogênica, resultando na inibição da angiogênese (GINGRAS et al., 2004).

O inibidor do ativador de plasminogênio-1 (*PAI-1*; símbolo oficial *SERPINE1*) é codificado pelo gene *SERPINE1* e pertence a uma classe de proteínas conhecidas como inibidores de serina protease (serpins). A principal função da proteína PAI-1 no tecido é inibir a ação do ativador de plasminogênio tipo uroquinase (u-PA) e tipo tecido (t-PA), inibindo assim o processo de fibrinólise. A proteína PAI-1 é sintetizada por uma variedade de células e induzida por fatores de crescimento, citocinas, hormônios e outros estímulos (KEATES et al., 2008). Além de sua função na coagulação, o aumento da

expressão de PAI-1 está associado com prognóstico ruim em muitos tipos de cânceres associados à inflamação (BINDER; MIHALY, 2008), uma vez que pode ter sua expressão aumentada por sinais presentes na inflamação crônica, incluindo NF-κB (CHEN et al., 2011), hipóxia (UENO et al., 2011) e estresse oxidativo (DU et al., 2000).

A proteína IFITM1, também conhecida como 9-27 ou Leu13, é um membro da família de proteínas transmembrana induzidas pelo interferon inicialmente conhecido como um antígeno leucocitário envolvido na transdução de sinais de adesão e antiproliferativos em linfócitos (MATSUMOTO et al., 1993; DEBLANDRE et al., 1995). A família *IFITM* em humanos consiste de, pelo menos, três outros genes funcionais (*IFITM1*, *IFITM2* e *IFITM3*). *IFITM1* tem sido estudado pelo seu envolvimento na inibição da replicação viral (BRASS et al., 2009), promoção da invasão celular (HATANO et al., 2008) e resposta à droga (FUMOTO et al., 2008). Além disto, estudos sugerem uma associação entre *IFITM1* e vários cânceres, como o câncer de cabeça e pescoço (HATANO et al., 2008), câncer de ovário (JOHNATTY et al., 2010), leucemia e linfoma (GOMES et al., 2010) e carcinoma cervical de células escamosas (PAN et al., 2010), além de modular a invasão celular no câncer gástrico (YANG et al., 2005).

Como exposto, várias alterações moleculares são desencadeadas após infecção pela *H. pylori* e associadas à carcinogênse do estômago, incluindo também a perda da homeostase entre proliferação e morte das células epiteliais gástricas. Contudo, poucos estudos têm avaliado tais aspectos antes e após a erradicação da bactéria. Por exemplo, a avaliação do perfil de expressão gênica

pela técnica de *microarray* em lesões gástricas pré-neoplásicas após erradicação da *H. pylori* identificou 30 genes cuja expressão foi alterada significantemente um ano após o tratamento. A maioria dos genes apresentava expressão reduzida e estavam associados com resposta imune e inflamatória, e outros associados com adesão celular, receptor de superfície celular, transdução de sinal, progressão e diferenciação celular, transcrição, citoesqueleto e metabolismo e transporte de lipídeos (TSAI et al., 2006).

O aumento dos níveis do RNAm de *IL-1β* e de *iNOS* e a ocorrência de gastrite mais grave foram observados em camundongos infectados pela *H. pylori*. Contudo, após a terapia de erradicação houve melhora na inflamação da mucosa gástrica e redução significante na expressão desses genes nos camundongos tratados comparados com os controles não tratados (HUANG et al. 2013).

Uma vez que o desenvolvimento do câncer ocorre como resultado de múltiplas alterações genéticas, a identificação de alterações na expressão de genes que codificam proteínas que atuam na resposta inflamatória e imune e controle do ciclo celular em lesões pré-neoplásicas, como a gastrite crônica em decorrência da infecção da *H. pylori,* poderá auxiliar na detecção inicial de tais mudanças, contribuindo para o diagnóstico precoce do câncer gástrico e desenvolvimento de terapias mais efetivas para o tratamento de erradicação da *H. pylori*.

Objetivos

II. OBJETIVOS

O presente projeto teve por objetivos:

1- Avaliar a expressão do RNAm dos genes *TLR2, TLR4, IL-8, TGF-β, PAP, PAI-1, PLAT* e *IFITM1* em pacientes com lesões gástricas antes e após a terapia de erradicação da *H. pylori*, pela técnica de PCR em tempo real;

2- Investigar a ocorrência de associação dos níveis de expressão desses genes conforme o tipo histológico da lesão gástrica;

3- Avaliar a associação dos genótipos de virulência *CagA e VacA* da *H. pylori* com a erradicação da bactéria, assim como com os níveis de expressão gênica;

4- Analisar a expressão protéica dos referidos genes por método imuno-histoquímico;

5- Avaliar a cinética celular, como o índice de proliferação celular (PI) e índice apoptótico (AI), antes e após a terapia de erradicação da *H. pylori*.

Artigos

ARTIGOS

Os resultados referentes aos objetivos desta tese serão apresentados a seguir na forma de três artigos científicos, conforme as normas de publicações específicas de cada periódico:

Artigo I

Título: *Helicobacter pylori* infection: Host immune response, implications on gene expression and microRNAs.

Autores: Aline Cristina Targa Cadamuro, Ana Flávia Teixeira Rossi, Nathália Maciel Maniezzo e Ana Elizabete Silva.

Periódico: *World Journal of Gastroenterology*, 20 (6): 1424-1437, 2014.

Artigo II

Título: *Helicobacter pylori* eradication does not change TLR2 and TLR4 expression in chronic gastritis patients.

Autores: Aline Cristina Targa Cadamuro, Ana Flávia Teixeira Rossi, Joice Matos Biselli-Périco, Patrícia Fucuta Pereira, Edla Bedin Polsinelli Mascarin, Ricardo Acayaba, Kátia Ramos Moreira Leite, Eny Maria Goloni-Bertollo e Ana Elizabete Silva

Periódico: *Helicobacter*, submetido em 18/03/2014.

Artigo III

Título: Effect of *H. pylori* eradication on expression of the cytokines and cellular mediators in patients with gastric lesions.

Autores: Aline Cristina Targa Cadamuro, Ana Flávia Teixeira Rossi, Joice Matos Biselli-Périco, Patrícia Fucuta Pereira, Edla Bedin Polsinelli Mascarin, Kátia Ramos Moreira Leite, Eny Maria Goloni-Bertollo, Ana Elizabete Silva.

Periódico: primeira versão do artigo a ser submetido à publicação.

Artigo I

World Journal of Gastroenterology

Online Submissions: http://www.wjgnet.com/esps/
bpgoffice@wjgnet.com
doi:10.3748/wjg.v20.i6.1424

World J Gastroenterol 2014 February 14; 20(6): 1424-1437
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (6): *Helicobacter pylori*

Helicobacter pylori infection: Host immune response, implications on gene expression and microRNAs

Aline Cristina Targa Cadamuro, Ana Flávia Teixeira Rossi, Nathália Maciel Maniezzo, Ana Elizabete Silva

Aline Cristina Targa Cadamuro, Ana Flávia Teixeira Rossi, Nathália Maciel Maniezzo, Ana Elizabete Silva, Department of Biology, UNESP-São Paulo State University, Campus São José do Rio Preto, 15054-000 São José do Rio Preto, SP, Brazil
Author contributions: All authors contributed equally to wrote this review.
Correspondence to: Ana Elizabete Silva, PhD, Department of Biology, UNESP-São Paulo State University, Campus São José do Rio Preto, Rua Cristóvão Colombo, 2265, 15054-000 São José do Rio Preto, SP, Brazil. anabete@ibilce.unesp.br
Telephone: +55-17-32212384 Fax: +55-17-32212390
Received: October 11, 2013 Revised: December 18, 2013
Accepted: January 3, 2014
Published online: February 14, 2014

Abstract

Helicobacter pylori (*H. pylori*) infection is the most common bacterial infection worldwide. Persistent infection of the gastric mucosa leads to inflammatory processes and may remain silent for decades or progress causing more severe diseases, such as gastric adenocarcinoma. The clinical consequences of *H. pylori* infection are determined by multiple factors, including host genetic predisposition, gene regulation, environmental factors and heterogeneity of *H. pylori* virulence factors. After decades of studies of this successful relationship between pathogen and human host, various mechanisms have been elucidated. In this review, we have made an introduction on *H. pylori* infection and its virulence factors, and focused mainly on modulation of host immune response triggered by bacteria, changes in the pattern of gene expression in *H. pylori*-infected gastric mucosa, with activation of gene transcription involved in defense mechanisms, inflammatory and immunological response, cell proliferation and apoptosis. We also highlighted the role of bacteria eradication on gene expression levels. In addition, we addressed the recent involvement of different microRNAs in precancerous lesions, gastric cancer, and inflammatory processes induced by bacteria. New discoveries in this field may allow a better understanding of the role of major factors involved in the pathogenic mechanisms of *H. pylori*.

Key words: *Helicobacter pylori*; Inflammation; Virulence factors; Immune response; Gastric lesions; Gastric cancer; Gene expression; MicroRNAs

Core tip: In this review, we focused some aspects of *Helicobacter pylori* (*H. pylori*) infection as bacterial virulence factor and mainly on modulation of host immune response and changes in the pattern of gene expression in *H. pylori*-infected gastric mucosa, with activation of gene transcription involved in inflammatory and immunological response, cell proliferation and apoptosis. We also highlighted the role of bacteria eradication for the normalization of gene expression levels. In addition, we addressed the recent involvement of different microRNAs in normal gastric mucosa, precancerous lesions, gastric cancer, and inflammatory processes induced by bacteria, showing deregulated expression.

Cadamuro ACT, Rossi AFT, Maniezzo NM, Silva AE. *Helicobacter pylori* infection: Host immune response, implications on gene expression and microRNAs. *World J Gastroenterol* 2014; 20(6): 1424-1437 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i6/1424.htm DOI: http://dx.doi.org/10.3748/wjg.v20.i6.1424

INTRODUCTION

Infection by *Helicobacter pylori* (*H. pylori*), a Gram-negative, microaerophilic, spiral-shaped bacteria that colonizes the gastric mucosa, is considered the most common bacterial infection worldwide. It is usually acquired during

childhood and may persist in the gastric environment throughout life if not treated[1,2]. The persistent presence of *H. pylori* in the stomach can result in chronic gastritis and may remain silent for decades after infection, due to the synchronized balance between the pathogen and its host, or cause more severe diseases such as atrophic gastritis, peptic ulcer, mucosa-associated lymphoid tissue lymphoma or gastric adenocarcinoma[2,3]. Therefore, *H. pylori* infection is considered the strongest factor associated with this neoplasm, mainly due to the inflammatory process triggered in the gastric mucosa, increasing the risk of gastric cancer over six-fold compared to individuals without this infection[4,5]. Gastric cancer is considered the first one among the several cancer types associated with infection in the world, with almost 75% of cases being attributable to *H. pylori* infection[6]. As a consequence of this association, *H. pylori* was classified in 1994 by the International Agency for Research on Cancer as a type I carcinogen[7].

H. pylori infects over half of the world population, but there is variation in incidence among different geographic regions[8]. Eighty-five percent of *H. pylori*-infected individuals remain lifelong asymptomatic, while only 1% of these individuals develop gastric cancer[9] and 10% develop peptic ulcer[10].

Indeed, the clinical consequences of infection by *H. pylori* are determined by multiple factors, including genetic predisposition of the host, especially regarding certain cytokine, and receptor gene polymorphisms[11-15], gene regulation, environmental factors such as high dietary salt intake, and heterogeneity of *H. pylori* strains[2,16,17].

In most cases, *H. pylori* infection can persist lifelong in its host in the absence of eradicating antibiotics[1,18], because it is capable of adaptations to colonize the adverse environment of the stomach. *H. pylori* can survive in the gastric environment at a wide range of pHs, due to urease enzyme activity and the presence of flagella which facilitate the penetration into the mucus layer and reaching the gastric epithelium[19]. Urease hydrolyzes urea to ammonia and carbon dioxide, neutralizing the pH, which allows the bacterial survival and proliferation[19], circumventing host defenses such as the immune response[20].

In general, it is noted that colonization by *H. pylori* causes a strong systemic immune response, creating a chronically inflamed environment with reduced stomach acidity that favors the growth of other bacteria in the gastric environment, maintaining the inflammation and thereby reducing the level of vitamin C in the gastric juice. The inhibition of gastric acid secretion favors a change from antrum-predominant to corpus-predominant gastritis, initiating gastric atrophy and intestinal metaplasia, which characterize precancerous lesions[21].

Furthermore, the bacterial virulence factors cytotoxin-associated gene A antigen (CagA) and vacuolating cytotoxin (VacA) play a pivotal role in *H. pylori*-induced pathogenesis, and others, such as IceA (induced by contact with epithelium), blood group antigen-binding adhesion (BabA), sialic acid-binding adhesion (SabA), duodenal ulcer-promoting gene (DupA) and outer inflammatory protein (OipA), also allow a successful colonization of the mucosa[22,23]. These bacteria populations are highly heterogeneous with respect to virulence factors VacA and CagA[24], and several substantial pieces of evidence show that these genetic differences play an important role in the clinical outcome of the infection[17,25].

The *cagA* gene produces one of the most important virulence factors of *H. pylori*, being located in a segment of DNA called the cag pathogenicity island (cagPAI) that contains, besides the *cagA* gene, genes which give rise to the bacterial type IV secretion system (T4SS-type-IV secretion system)[26]. This system functions like a molecular syringe, injecting *CagA*, peptidoglycans and other factors into host epithelial cells[27]. After its entry into the cell, CagA can be phosphorylated by tyrosine kinases and interact with cellular proteins, acting in the signal transduction pathways to the nucleus, changes in the cytoskeleton, disruption of cell-cell junctions[28,29], stimulating the growth factor signaling, leading to changes in cell morphology and increased cell proliferation[30], as well as anti-apoptotic responses[29,31]. CagA is not found in all strains of Western *H. pylori* population[32]. Its occurrence is associated with more severe inflammation of the gastric mucosa[33,34], conferring a greater risk of developing stomach cancer[32,35,36].

The second most studied virulence factor is the VacA, encoded by *vacA* gene that induces the formation of vacuoles in eukaryotic cells and stimulates apoptosis in epithelial cells[37]. Unlike *cag-A*, all *H. pylori* strains possess the *vacA* gene, although only about 50% of them express the VacA protein. The regions with the highest diversity are located at the 5' terminus signal (allele types s1a, s1b, s1c and s2), the mid-region (allele types m1 and m2) and the intermediate region (allele types i1 and i2)[38]. This combination of sequence diversity in *vacA*, considering that each gene contains a single allele (signal, mid-region and intermediate region allele), causes variations in cytotoxic activity[39], the s1m1 strain being highly toxigenic[40]. Humans infected with *H. pylori*-VacA$^+$ strains are more prone to gastritis than those infected with strains that do not express this protein[41]. VacA may interfere with phagocytosis and antigen presentation[42,43], reducing the activation of Jurkat cells, thereby inhibiting the activation of NFAT, an important transcription factor that is necessary for the expression of genes involved in the expansion of T cells activated by bacterial antigens[44], thereby ensuring the evasion of *H. pylori* from the adaptive immune response.

The BabA and SabA adhesins are encoded by the *babA* and *sabA* genes that encodes an outer membrane protein, BabA, which binds to the type B blood group antigen in gastric cells[1], while sabA binds to the sialyl-Lewis x/a antigens[45]. The adhesion of bacteria to the gastric epithelium allowing the release of the CagA and VacA factors into the host cells is mediated by BabA, which facilitates colonization, induces mucosal inflammation and can influence the severity of the disease[46,47]. *H. pylori* strains which carry *babA*, *vacAs1* and *cagA* together are associated with duodenal ulcer and present a higher risk of gastric cancer[48]. The inflammatory response may be increased due to sabA-mediated adhesion, by facilitating the utilization of nutrients exudated from damaged host

cells. Thus, as the inflammatory response increases, the *sabA* expression may be switched off, allowing the contact between the bacteria and the inflamed epithelium to be broken, thus maintaining prolonged infection[45]. However, there is no clinical or epidemiological evidence associating *sabA* to gastric cancer. Another gene that encodes an outer membrane protein is *oipA*, located near *cag*PAI[49]. It is regulated by a slipped-strand repair mechanism based on the number of Cysteine-Threonine dinucleotide repeats in the 5' regions of the gene[49]. The *oipA* gene has the ability to induce interleukin (IL)-8 from gastric epithelial cells, as *cagA* and its status have been linked to the discrimination of duodenal ulcer and gastritis[49,50].

The *dupA* gene, located in the plasticity region of the *H. pylori* genome, represents a marker of virulence with pathogenic potential[51]. This gene was reported to be associated with increased risk of duodenal ulcer[51], with lower gastric cancer incidence and lower acid output, including patients with peptic ulcer[52]. As opposed to these findings, there are studies of *dupA* status in which no association with any gastroduodenal disease was found[53].

The *iceA* gene, another virulence factor, has two variants, *iceA1* and *iceA2*[54]. However, the function of *iceA2* remains undefined[55], while the expression of *iceA1* is increased in some populations by the contact of *H. pylori* with human gastric epithelial cells and is associated with peptic ulcer[56]. Nevertheless, the development of erosive gastritis has been related to strains carrying genes *iceA1*, *cagA* and *vacAs1a/m1*, while enanthematous gastritis is associated with *vacAs2/m2* and *iceA2* genotypes[57]. Moreover, the severity of gastritis is related with the coexistence of the *iceA2* gene with *cagA*, *vacAs1/m1* and *babA2*[58].

In this review, we first approached about the *H. pylori* infection and its virulence factors, topics widely addressed in other recent reviews[2,18,19]. Thus, we will focus mainly on modulation of host immune response triggered by *H. pylori,* and the advances in the fast developing field of gene expression profiles in gastric mucosa, which can change as a consequence of *H. pylori* infection, leading to the activation of transcription of genes involved in defense mechanisms, inflammatory and immunological responses, cell proliferation and apoptosis. Moreover, we highlighted the importance of the eradication of *H. pylori*, which plays an important role in the restoration of gastric mucosa inflammation and on gene expression levels. In light of the increasing involvement of microRNAs (miRNAs) in the regulation of posttranscriptional gene silencing, we addressed the action of different miRNAs in precancerous lesions, gastric cancer, and inflammatory processes induced by *H. pylori,* evidencing its participation in several steps of gastric carcinogenesis.

MODULATION OF *H. PYLORI*-TRIGGERED HOST IMMUNE RESPONSE

As soon as *H. pylori* bacteria colonize the stomach, the epithelial cells and their innate immune receptors, mainly the toll-like receptors (TLRs)[19], recognize the bacteria (Figure 1). This attachment process can be facilitated by the action of adhesins (SabA and BabA) expressed by bacteria, which favor the action of other virulence factors (CagA and VacA). Soon after, the host's innate and adaptive immune systems are activated, leading to the recruitment of a wide variety of inflammatory cells and mediators, and the activation of transcription factor nuclear factor (NF)-κB and pro- and anti-inflammatory cytokines, cell proliferation and survival factors. The activation of the immune system in response to the presence of the bacteria increases the production of reactive oxygen and nitrogen species [reactive nitrogen species (RNS) and reactive oxygen species (ROS)] by increasing oxidative/genotoxic stress, which can cause cell and DNA damage, favoring the appearance of mutations that may facilitate the carcinogenic process. In addition, the expression of the immune response mediators can be regulated by miRNAs, and inflammatory mediators can change the miRNAs expression[59-61].

Members of the TLR family are essential components of the innate and adaptive immune response and comprise 10 types in humans, TLR1 to TLR10[62]. They recognize molecular structures of pathogenic microbe-associated molecular patterns (PAMPs), like lipopolysaccharides (LPS), lipoproteins, lipoteichoic acid, peptidoglycan, lipoarabinomannan and flagellin[63]. *H. pylori* LPS, as cell wall components, are recognized mainly by TLR4; however modifications of the LPS structure can alter this recognition and poorly stimulate the host immune response, enhancing the bacterial evasion and pathogenicity[64]. *H. pylori* is also recognized by TLR2 through other forms of LPS structurally different from those recognized by TLR4[65]. TLRs are dependent on the presence of MyD88 (myeloid differentiation primary-response gene 88) for efficient signal transduction. The MyD88 complex is associated with interleukin-1-receptor-associated kinase-1 (IRAK1) and IRAK4. IRAK1 is phosphorylated and then dissociated from MyD88. Subsequent dissociation of protein complexes occur by phosphorylation, and, as the last step, NF-κB is translocated into the nucleus, activating the expression of genes related to the inflammatory process[66] (Figure 1A).

CagA-positive strains contribute to the inflammatory response, since this virulence factor causes an increase in the production of certain cytokines such as IL-1β and IL-8[67,68] and activation of NF-κB, which can confer a proliferative phenotype to the bacteria, important in the process of carcinogenesis[69], promoting induction of growth factors and suppression of apoptosis[70]. Thus, CagA deregulates the cell signaling pathways and favors the arising of oncogenic cells, which is important in the pathogenesis of *H. pylori*[71]. The VacA factor induces a pro-inflammatory response[72] and multiple cellular activities that facilitate chronic colonization of the gastric mucosa by bacteria[68]. A recent study showed that overexpression of VacA led to the production of tumor necrosis factor (TNF)-α, IL-1β, nitric oxide, reactive oxygen species and the activation of NF-κB, which can be associated to pro-inflammatory cytokines and cell apoptosis[73]. VacA also can affect the immune system

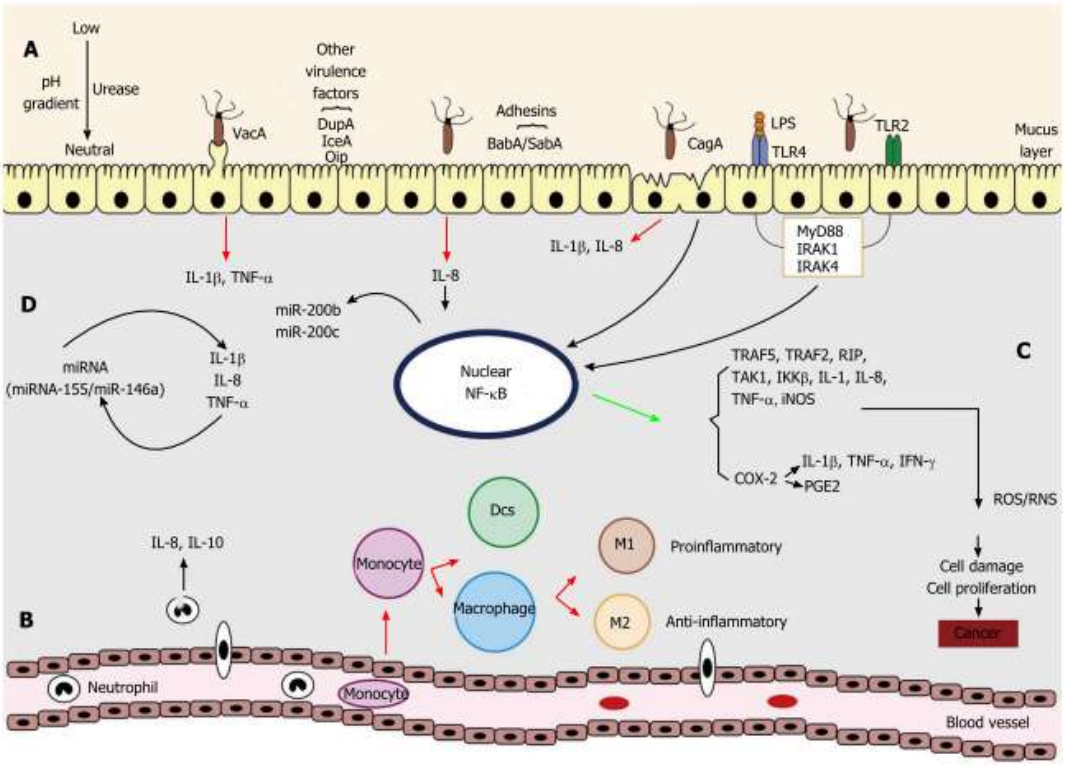


Figure 1 Pathogenesis of *Helicobacter pylori* infection and host immune response. A: Bacterial urease neutralizes the gastric pH, enabling the colonization of gastric epithelial cells by the bacteria and their motility in the mucus layer. Adhesion of the bacteria to the gastric epithelium is mediated by BabA and SabA adhesins, allowing the release of factors CagA and VacA into the host cells, which causes a strong systemic immune response and inflammation of the gastric mucosa. *Helicobacter pylori* LPS is recognized by toll-like receptors, mainly TLR4 and TLR2, in cooperation with the adapter molecule MyD88 associated with IRAK1 and IRAK4 that leads to activation of transcription factor NF-κB, activating inflammatory signaling pathways; B: The immune response is also activated, with the recruitment of inflammatory cells at the infection site, inducing the production of various pro- and anti-inflammatory mediators; C: After NF-κB activation, rapid expression of multiple pro-inflammatory cytokines, chemokines such as the tumor necrosis factor alpha (TNF-α) and interleukins, and consequently activation of oncogenic pathways may culminate in cancer; D: The expression of some miRNAs is changed by *H. pylori* infection and the host immune response is regulated accordingly. LPS: Lipopolysaccharides; IL: Interleukin; COX-2: Cyclooxygenase; RNS: Reactive nitrogen species; ROS: Reactive oxygen species; IFN: Interferon.

enabling *H. pylori* evade the adaptive immune response to establish persistent infection, since can interfere with phagocytosis and antigen presentation and also inhibit T cell proliferation[24].

H. pylori infection, after activation of NF-κB and cytokines production, causes chemotaxis of monocytes/macrophages and infiltration of polymorphonuclear leucocytes[74], recruitment of neutrophils and lymphocytes[75]) that also induces the production of IL-8 and IL-10 by neutrophils[76] (Figure 1B). In this pathway, monocytes secret interleukins such as IL-1β, IL-6, IL-10, and IL-12p40 (partially secreted as IL-23), dentritic cells (DCs) secret IL-1β, IL-6, IL-10, IL-12p40, IL-12p70, and IL-23, while M1 macrophages produce mainly IL-1β, IL-6, IL-10, IL-12p40 and IL-23. M2 macrophages synthesize IL-10 but produce less pro-inflammatory cytokines than M1 macrophages, which can control inflammation, leading to a chronic inflammatory response[77].

The activation of DCs and M1 macrophages is correlated with an increased capacity to induce T-cell proliferation like T helper cells and decreased phagocytosis[78,79], as well as Th17 that can promote chronic infection triggered by a chemotaxis system[80]. Even though *H. pylori* avoids phagocytosis and prevents the induction of an adaptive immune response, macrophages engulf the bacterium but cannot kill it, which facilitates the chronic infection[81]. This infection results in a predominantly T cell-mediated immunity rather than humoral immunity, with Th1 and Th17 responses, which increase the production of IL-1β, TNF-α and IL-8[82,83]. While Th17 cell differentiation is promoved by TNF-α and IL-6 from activated macrophages/dentritic cells, Th1 cell development is triggered by IL-12 and interferon (IFN)-γ[2]. In addition, the recruitment of antigen-specific regulatory T cells has also been reported, facilitating the permanent colonization of the stomach through direct cell-to-cell contact or by secreting cytokines [transforming growth factor (TGF)-β1 and IL-10] that modulate the immune response[84].

Moreover, NF-κB regulates the expression of several genes, for example *TRAF5*, *TRAF2*, *RIP*, *TAK1*

and *IKK*β, some of which are associated with inflammation and cancer[85]. Its activation by LPS leads to the synthesis of IL-1, IL-8, IL-10 and TNF-α, and iNOS (Figure 1C). NF-κB also upregulates the expression of the pro-inflammatory cyclooxygenase (COX-2) enzyme, whose function is to induce cytokines such as TNF-α, interferon-γ and IL-1, inhibiting apoptosis, maintaining cell proliferation and stimulating angiogenesis in favor of carcinogenesis[20]. In *H. pylori*-associated gastric cancer there are reports showing increased expression of COX-2 and prostaglandin E2 activated by TLR2/TLR9 and NF-κB, and this induction is mediated by the activation of the epidermal growth factor receptor in gastric epithelial cells[86,87]. Added to inflammatory stress effects, influences on the cell cycle and cell polarity, *H. pylori* also activates multiple oncogenic mechanisms, such as the PI3K/AKT/GSK3β pathway that regulates many functions like cell growth, proliferation, differentiation and motility, and its aberrant activation is associated with various types of cancer, including stomach cancer[88,89]. The presence of these bacteria also affects the STAT3 protein pathway that regulates cell growth, differentiation and apoptosis, in which a high expression of STAT3 is associated with advanced stage and poor prognosis of gastric cancer[90]. All the high immune stimulation produced by these molecules results in the production of ROS and RNS by neutrophils attracted to the infection site, which can cause cell damage, leading to gene mutations and cell proliferation, favoring the emergence of gastric cancer[91].

Other important members of the class of immune regulators are miRNA[92]. Recent reports have highlighted the regulatory role of miRNAs in *H. pylori* infection and associated diseases (Figure 1D). For example, a strong inflammatory response characterized by the early production of pro-inflammatory TNF-α and IL-6 cytokines, followed by IL-10, IL-1β and IL-23 secretion as a consequence of miR-146a up-regulation and strong miR-155 induction, which raised the TNF-α production[93]. In contrast, IL-8, TNF-α and IL-1β could contribute to the induction of miR-146a during *H. pylori* infection[94]. Therefore, miRNAs modulate the *H. pylori* infection and are also affected by these bacteria, as, for example, the synthesis of the transcription factor NF-κB that can act as a transactivator of miR-200b and miR-200c[95]. This issue will be discussed in more detail in the last section of this review. Thus, all the pathways reported above show the need for new approaches in order to reach a better understanding of the influence of *H. pylori* on the host immune system, allowing the working out of preventive measures and efficient new strategies of *H. pylori* eradication.

H. PYLORI INFECTION DEREGULATES THE EXPRESSION OF GENES INVOLVED IN INFLAMMATORY RESPONSE AND CELL KINETICS

In addition to a marked inflammatory response of the host, activation of signaling pathways and gastric mucosa injury, *H. pylori* infection can enhance cell proliferation and apoptosis of gastric epithelial cells[95]. Thus, to counteract *H. pylori* infection, the host activates gene transcription involved in his defense mechanisms, inflammatory and immunological response and control of cell kinetics[31,96,88]. Gene expression profiling analysis in gastric biopsies and cell lines in response to *H. pylori* infection might be one approach to better understand the role of important factors involved in the pathogenic mechanism of these bacteria.

In this respect, Hofman *et al*[97] (2007) evaluated the gene expression profile of the gastric mucosa of *H. pylori*-infected compared to noninfected patients and highlighted a distinct transcriptional pattern in biopsies of the antral and fundic regions, associated also with bacterial density and virulence factors such as *cagA*, *vacA* and *babA2*. The authors reported up-regulation in receptors and co-receptors involved in bacterial recognition such as *TLR2*, *TLR4*, *LY96*, *ITGB2*, *VCAM1*, *MAPK8*, *RAC2*, *SLA*, *ADAM*, *MMP*, *IFITM1* and *PAP*, signal transduction, inflammation and immune response, proteolysis, apoptosis and cell proliferation in antral biopsies from infected patients in comparison with biopsies from noninfected individuals. It was also observed that several transcripts encoding chemokines and their receptors were up-regulated in response to *H. pylori* infection. More recently, microarray data of gene expression profiling in gastric antral mucosa from chronic superficial gastritis patients infected by *H. pylori* and uninfected subjects revealed 38 differentially expressed genes, including 23 up-regulated and 15 down-regulated genes related to protein metabolism, inflammatory and immunological reaction, signal transduction, gene transcription and trace element metabolism[98]. These data indicate that *H. pylori* infection could induce carcinogenesis by altering cellular gene expression processes, evade the host defense mechanism, increase inflammatory and immune responses, activate NF-κB and Wnt/β-catenin signaling pathways, and disturb the metal ion homeostasis. However, the functional significance of these selected genes needs to be further evaluated in other studies.

TLRs expression has been evaluated in *H. pylori* infection due to its relevant role in the recognition of pathogenic components such as bacterial LPS. In *H. pylori*-negative normal gastric mucosa, *TLR5* mRNA is the most expressed, followed by *TLR2* and *TLR4*, whereas in *H. pylori*-infected normal gastric mucosa, intestinal metaplasia, independently of *H. pylori* infection, and in the dysplasia/cancer sequence *TLR2* and *TLR4* are the most overexpressed[99]. Therefore, these findings suggest that progressive activation of these receptors, initially by *H. pylori*, but also by other PAMPs or damage-associated molecular patterns at later stages, may have an important role in gastric carcinogenesis and tumor progression[99]. However there is also indication of no quantitative differences in the *TLR4* and *TLR5* mRNA levels, regardless of the presence or absence of *H. pylori*, in both gastric epithelial cell biopsies and AGS cells, suggesting that the

mRNA levels of these receptors may not be influenced by the infection process, or at least not at the time points selected for analysis[100].

H. pylori-CagA$^+$ strains often trigger more potent inflammatory and immune responses, leading to a more severe disease, which may be mediated by nucleotide oligomerization domain 1 (NOD1) by recognizing the intracellular pathogen and initiating pro-inflammatory signaling cascades[101,102]. Gastric epithelial cells co-cultured with *H. pylori*-CagA$^+$ strains show increased production of IFN-γ-inducible chemokines, IP-10 and MIG, in response to IFN-γ stimulation. In addition, gastric biopsies from infected and non-infected patients with gastritis or gastric cancer show increased mRNA expression levels of *NOD1, CXCL8, IRF1* and *CXCL10*, when compared with normal tissue[103]. Likewise, up-regulation of pro-inflammatory molecules expression also occurs in gastric tumor tissues compared to matched non-tumor samples such as *IRF1, NOD1* and *CXCL8*. Thus it is proposed that NOD1 and the IFN-γ signaling pathway regulate the expression levels of the tumor suppressor gene *IRF1*. That could, in some instances, potentiate oncogenic changes in the gastric mucosa as a consequence of infection with virulent *H. pylori*-CagA$^+$ strains and exacerbate disease severity and progression during chronic *H. pylori* infection.

In addition, *H. pylori*-CagA$^+$ strains also appear to be related with differential activation of two signaling proteins, STAT3 and ERK1/2 in gastritis patients[104]. The differential activation of these two signaling proteins may in part explain the increased predisposition to gastric cancer when infected with *H. pylori*-CagA$^+$ strains compared to their CagA$^-$ counterparts, due to the activation of epithelial cell turnover, thus increasing the likelihood of gaining somatic mutations and subsequent cellular transformation. Recently, in AGS cells incubated with *H. pylori*-CagA$^+$ strains 147A$^-$ and 147C was observed specific and significant alterations in gene expression profiles[105]. Up-regulated genes primarily encoded signal transduction (23.2%), transport (13.8%), transcription (12.6%), metabolic (11.3%), immune and inflammatory responses (6.9%), adhesion and migration (5.9%), and development proteins (5.0%), while down-regulated genes encoded metabolic (16.1%), transcription (14.6%), transport (14.6%), signal transduction (10.6%), translation (5.9%), cell cycle (5.1%), and apoptosis (3.0%). Among the differentially expressed genes compared to non-treated AGS cells, the *EMT* (epithelial-mesenchymal transition) gene was selected because it seems to facilitate the invasion of cancerous cells into both local and distant tissues. Thus, the *H. pylori*-CagA$^+$ strain plays a significant role in epithelial-mesenchymal transition, so the prevention of *H. pylori*-CagA$^+$ infection may be an effective approach in preventing the progression or metastasis of tumor cells that occurs *via* EMT-inducing genes.

Considering the role of *H. pylori* infection as a key event in triggering all these changes in gene expression of the infected gastric mucosa, and even the risk of malignant progression, the eradication of these bacteria has been recommended in various countries[106]. Once the gastric colonization by the pathogen is rarely eliminated spontaneously, *H. pylori* eradication is regarded as a first-line therapy to reverse the pre-neoplastic lesions and prevent malignant progression[107]. The standard triple treatment regimen of infection consists of two or three antibiotics (amoxicillin or clarithromycin) and a proton pump inhibitor, associated or not with bismuth salts, for 1 or 2 wk[108], reaching an eradication rate higher than 90%[109,110].

Although the eradication of *H. pylori* can result in partial regression of pre-neoplastic lesions, to this date few studies have evaluated the role of treatment for the restoration of gastric mucosa inflammation and normalization of gene expression levels. Tsai *et al*[107] (2006) employed microarray technology to investigate changes in gene expression profiles using samples from a double-blinded, placebo-controlled clinical trial, associated with *H. pylori* infection and eradication of the bacteria. One year after the bacteria eradication therapy, were identified 30 genes whose expression was significantly down-regulated, the majority of which were associated with immune response and inflammation (*CXCL1, CXCL14, IGLC2, LOC400986, TNFSF10* and *OAS1*), while in the placebo group the expression of 55 genes differed significantly in the same period (32 up-regulated and 23 down-regulated). Among them, genes involved in cell-cell adhesion and lining, cell cycle differentiation, and lipid metabolism and transport were down-regulated over time in the treatment group but up-regulated in the placebo group. Taken together, these findings showed that *H. pylori* infection and its subsequent eradication resulted in alterations of gene expression associated with cell damage, inflammation, proliferation, apoptosis and intestinal differentiation, suggesting that *H. pylori* eradication may stop or reverse ongoing malignancy-related molecular processes in the stomach. In this respect, further studies are needed to evaluate the use of these genes as possible markers for gastric cancer risk.

The eradication therapy also appears to influence the expression of the transcription factor FOXP3 by CD4$^+$CD25 regulatory T cells in the gastric and duodenal mucosa leading to reduced expression in response to treatment[111]. Moreover, was observed a decrease of IFN-γ and *IL-10* gene expression in the antral mucosa after eradication of *H. pylori*. Thus, it is possible that in the infected mucosa the overall immune response may be shifted towards an anti-inflammatory response. This could indicate that a moderate regulatory mechanism is induced in the presence of the bacteria, keeping an immunologic balance where the inflammation is maintained at a controlled level by the suppressive regulatory T cells. This effect may explain why *H. pylori* infections become chronic.

The effect of *H. pylori* eradication therapy was also observed on receptors expression levels such as genes human beta defensin 2 (*hBD2*) and *hBD3*, which codify antimicrobial peptides on the mucosal surface and act in

Table 1 MicroRNAs involved in *Helicobacter pylori*-induced inflammatory and carcinogenic processes

miRNAs	Regulation	Targets and action	Ref.
let-7b	Down	*TLR4, NF-κB, COX-2, Cyclin D1, IL1B* Initiation of immune response	[128,129]
has-miR-17	ND	ND	[130]
hsa-miR-21	Up	*RECK, TGFBR1, TGFBR2* Promotes proliferation, migration and invasion, inhibits apoptosis	[123,130]
hsa-miR-25	Up	ND	[130]
has-miR-93	Up	ND	[130]
has-miR-103	Down	TNFα ND	[128]
hsa-miR-146a	Up	*IRAK1, CARD10, COPS8, PTGS2* Inhibits tumor-promotes cytokines and growth factors	[131,132]
hsa-miR-155	Up	*SMAD2* Attenuation of the inflammatory response	[133]
hsa-miR-194	Up	ND ND	[130]
hsa-miR-196	Up	ND	[130]
hsa-miR-200b	Up	ZEB1 Promotes EMT	[60]
has-miR-200c	Up	*ZEB1, IL6* ND	[60,128]
has-miR-222	Up	*RECK* Promotes cell proliferation	[134]
has-miR-223	Up	*IL6, IL1B,* ND	[135,136]
hsa-miR-370	Down	*FoxM1* Promotes proliferation	[137]
has-miR-371-5p	Down	*LATS2* Inhibits cell cycle progression	[138]
has-miR-372	Down	LATS2 Inhibits cell cycle progression	[138]
has-miR-373	Down	*LATS2* Inhibits cell cycle progression	[138]
hsa-miR-375	Down	*IL8* ND	[128]
has-miR-449b	Down	*MET, GMNN, CCNE2, SIRT1,* HDAC1 Promotes proliferation Inhibits senescence and apoptosis	[139]
has-miR-584	Up	PPP2a, Foxa1 Promotes EMT and stem cells differentiation	[140]
hsa-miR-1290	Up	*NKRF, Foxa1* Promotes EMT and stem cells differentiation	[140]

EMT: Epithelial to mesenchymal transitions; ND: Not determined (in gastric mucosa); NF: Nuclear factor; COX-2: Cyclooxygenase; TNF: Tumor necrosis factor.

the innate immune responses to human pathogens[112]. Up-regulation of both *hBD2* and *hBD3* transcripts were observed in *H. pylori*-positive subjects that correlated with the degree of gastritis in corpus and antrum. However, after successful eradication therapy, while the mucosal hBD2 transcript levels returned to normal, the hBD3 protein expression level remained unchanged. In addition, while infiltrating granulocytes disappeared completely, higher lymphocytic infiltration still persisted compared to *H. pylori*-negative subjects[112]. Possibly *H. pylori*-positive patients were most likely infected in their early childhood and had carried the bacteria for decades, speculating whether the decreased expression of hBD3 after 3 mo of treatment should be attributed to long-lasting effects on the epithelial cells that had not been completely renewed or to the lymphocytic infiltration still present at the time of study.

In a broader perspective, despite the still limited studies on the role of *H. pylori* eradication in the normalization of gene expression levels in gastric mucosa, such studies showing genes with significant changes of expression over time may help reveal molecular markers involved in inflammatory processes and mechanisms of progression from precancerous lesions to malignancy.

MIRNAS REGULATING THE INFLAMMATORY AND CARCINOGENIC PROCESSES INDUCED BY *H. PYLORI*

miRNAs, non-coding ribonucleic acids with about 22 nucleotides[113], are involved in the process of post-transcriptional gene silencing through the pairing with mRNA target, promoting its degradation[114,115] or, mostly in animals, causing repression of mRNA translation[116,117]. Since the discovery of miRNAs, their key role in the regulation of gene expression[118,119] and their participation in various cellular and systemic functions, they have been associated with various pathologies, such as inflammation and cancer[120,121].

The expression of miRNAs is tissue-specific and they have different cellular functions, such as regulation of proliferation, apoptosis[122,123], differentiation[124,125] and carcinogenesis, and can be used as biomarkers for tumor origin[120,126]. With particular regard to the stomach, there are various studies reporting different miRNAs in normal mucosa[127], *H. pylori*-induced precancerous lesions and gastric cancer (Table 1)[60,125,128-140]. Studies on miRNA in precancerous gastric lesions are still scarce. For example, chronic gastritis experimentally induced by *H. pylori* showed the action of hsa-miR-155 in regulating the response of Th1 and Th17 cells to control infection and, in the meantime, induced precancerous pathologies associated with this bacterium by IFN-γ production[121,141]. In intestinal metaplasia was demonstrated that the CagA bacterial protein stimulates the expression of hsa-miR-584 and hsa-miR-1290, which results in down-regulation of the forkhead box A1 (*Foxa1*) gene, thus inducing transdifferentiation of gastric epithelial cells[140].

In *H. pylori*-associated gastric cancer, an increasing number of studies have described the occurrence of deregulation of miRNA expression and its involvement in the regulation of gene expression. *H. pylori* and CagA genotype inhibit has-miR-370 expression in both gastritis and gastric cancer, which led to overexpression of this target *FoxM1*. This increased expression was gradual from inflammation to cancer, resulting in cell proliferation for

gastric carcinogenesis[137]. In gastric cancer cell line, non-malignant gastric cell line, as well as in human gastric mucosal tissue, *H. pylori* is able to increase expression of has-miR-222 promoting cell proliferation by gradually decrease the expression of their target *RECK*[134], so *H. pylori* infection can induce carcinogenesis through altering expression of some miRNAs. Also *H. pylori*-infected AGS cell line results in the repression of hsa-miR-371-5p, hsa-miR-372 and hsa-miR-373, which leads to the inhibition of cell cycle progression by up-regulation of their target *LATS2* (serine-threonine kinase)[138]. hsa-miR-200b and hsa-miR-200c that have a common target, *ZEB1*, are transactivated by transcription factor NF-κB due to the presence of the *cagA* genotype, so that the gastric epithelial cells begin to undergo mesenchymal transition[66].

Considering the importance of the treatment and eradication of *H. pylori* to restore gastric tissue homeostasis, Matsushima *et al*[135] (2011) found 31 miRNAs differentially expressed in infected- noncancerous gastric mucosa compared to non-infected individuals. Of these miRNAs, only has-miR-223 showed increased expression in *H. pylori*-positive individuals. In a subgroup of four patients in which *H. pylori* was eradicated, was observed that 14 miRNAs that were down-regulated in the presence of the pathogen had their levels increased after four weeks of eradication therapy. However, in a patient in whom the therapy was not satisfactory, the levels of these miRNAS were unaltered[135]. However, eradication of the bacteria year after treatment did not change the expression of oncogenic miRNAs in metaplastic glands, but it was decreased in non-metaplastic glands, indicating that the treatment was effective in restoring the miRNAs expression only in the early stages of gastric transformation[130]. In addition, hsa-miR-21, hsa-miR-25, hsa-miR-93, hsa-miR-194 and hsa-miR-196 were overexpressed in gastric cancer in comparison to *H. pylori*-positive gastric ulcer or atrophic gastritis, and the eradication decreased the expression of these miRNAs only in atrophic gastritis[130]. These findings evidence that *H. pylori* is able to change the expression of miRNAs in noncancerous gastric mucosa, and this is one of the possible mechanisms for manipulating the host response.

H. pylori can remain in the stomach at high density levels and for a long time, indicating that the host immune response is not effective in eliminating the pathogen. This may be due to the deregulation caused by the bacteria in the expression pattern of miRNAs which target cytokines and other mediators of the immune response. The miRNA has-miR-21 is a possible regulator of *H. pylori*-induced inflammation, targeting the receptor of the TGFβ signaling pathway (TGFβR1 and TGFβR2)[142], and the mature form of this miRNA shows increased expression in both gastric cancer and *H. pylori*-infected gastric tissue[125]. hsa-miR-155 and hsa-miR-146a are also involved in the attenuation of the inflammatory response against *H. pylori*. In this process, the MyD88 complex and adaptor proteins (IRAK-1 and TRAF6) of the TLRs signaling cascade are targeted by these miRNAs, resulting in decreased NF-κB activation. In contrast, *H. pylori* also up-regulates hsa-miR-155 expression, which occurs in an NF-κB-dependent manner, resulting in decreased levels of pro-inflammatory mediators IL-8 and growth-related oncogene-α[131-133]. Moreover, *H. pylori* infection decreases the expression of let-7b, increasing the production of TLR4, NF-κB, COX-2 and Cyclin D1, thus contributing to the initiation of the immune response and the inflammation of the gastric mucosa[129]. Particularly, Isomoto *et al*[128] (2012) investigated the association of various miRNAs with cytokine expression in *H. pylori*-positive gastric mucosa and found a negative correlation among let-7b, hsa-miR-200c, hsa-miR-375 and hsa-miR-103 and interleukins IL-1β, IL-6, IL-8 and TNF-α, respectively. Other relationships between inflammatory mediators and miRNAs are described, as for example has-miR-370 and reduced expression of TGFβR2[143], has-miR-365 and negative regulation of IL-6[144], and has-miR-223 and the reduction of IL-6 and IL-1β[136].

Therefore, inflammatory process induced by *H. pylori* leading to precancerous gastric lesions and gastric cancer can alter the expression pattern of miRNAs in order to influence biological processes by changing the expression of mRNA targets. Eradication of the bacteria may be a strategy for restoring normal levels of these miRNAs in the gastric mucosa at early stages of malignant transformation, reducing the risk of gastric cancer.

CONCLUSION

After millennia of co-evolution of *H. pylori* bacteria with human hosts, complex mechanisms of interaction between pathogen and host developed, allowing its persistence and subversion of the immune system and successful colonization in the human stomach. Numerous studies about colonization and adhesion of bacteria in gastric epithelial cells, diversity of virulence factors, activation of signaling pathways, evasion and subversion of the immune system and, more recently, about changes in the gene expression profile of infected mucosa and participation of miRNAs have contributed to a better understanding of the host-pathogen relation. Taken together, these data may help to clarify pivotal biological and molecular mechanisms of infection pathogenesis and to identify clinically significant biomarkers, with the possibility of disclosing novel therapeutic targets for treatment strategies, especially in patients who developed resistance mechanisms. Taking into account that *H. pylori* infection is a relevant risk factor for the development of gastric cancer, strategies aiming for a better understanding of the mechanisms involved in its pathogenesis and effective eradication therapies are critical for the prevention of this type of malignancy.

ACKNOWLEDGMENTS

The authors thank PROPE/UNESP and FUNDUNESP (Process No. 0302/019/13-PROPe/CDC) by support of english revision.

REFERENCES

1 **Everhart JE**. Recent developments in the epidemiology of Helicobacter pylori. *Gastroenterol Clin North Am* 2000; **29**: 559-578 [PMID: 11030073 DOI: 10.1016/S0889-8553(05)70130-8]

2 **Ricci V**, Romano M, Boquet P. Molecular cross-talk between Helicobacter pylori and human gastric mucosa. *World J Gastroenterol* 2011; **17**: 1383-1399 [PMID: 21472096 DOI: 10.3748/wjg.v17.i11.1383]

3 **Zarrilli R**, Ricci V, Romano M. Molecular response of gastric epithelial cells to Helicobacter pylori-induced cell damage. *Cell Microbiol* 1999; **1**: 93-99 [PMID: 11207544 DOI: 10.1046/j.1462-5822.1999.00018.x]

4 **Kuipers EJ**. Review article: exploring the link between Helicobacter pylori and gastric cancer. *Aliment Pharmacol Ther* 1999; **13** Suppl 1: 3-11 [PMID: 10209681 DOI: 10.1046/j.1365-2036.1999.00002.x]

5 **Suerbaum S**, Michetti P. Helicobacter pylori infection. *N Engl J Med* 2002; **347**: 1175-1186 [PMID: 12374879 DOI: 10.1056/NEJMra020542]

6 **de Martel C**, Ferlay J, Franceschi S, Vignat J, Bray F, Forman D, Plummer M. Global burden of cancers attributable to infections in 2008: a review and synthetic analysis. *Lancet Oncol* 2012; **13**: 607-615 [PMID: 22575588 DOI: 10.1016/S1470-2045(12)70137-7]

7 Schistosomes, liver flukes and Helicobacter pylori. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7-14 June 1994. *IARC Monogr Eval Carcinog Risks Hum* 1994; **61**: 1-241 [PMID: 7715068]

8 **Van Doorn LJ**, Figueiredo C, Mégraud F, Pena S, Midolo P, Queiroz DM, Carneiro F, Vanderborght B, Pegado MD, Sanna R, De Boer W, Schneeberger PM, Correa P, Ng EK, Atherton J, Blaser MJ, Quint WG. Geographic distribution of vacA allelic types of Helicobacter pylori. *Gastroenterology* 1999; **116**: 823-830 [PMID: 10092304 DOI: 10.1016/S0016-5085(99)70065-X]

9 **El-Omar EM**, Carrington M, Chow WH, McColl KE, Bream JH, Young HA, Herrera J, Lissowska J, Yuan CC, Rothman N, Lanyon G, Martin M, Fraumeni JF, Rabkin CS. The role of interleukin-1 polymorphisms in the pathogenesis of gastric cancer. *Nature* 2001; **412**: 99 [PMID: 11808612 DOI: 10.1038/35083631]

10 **Sachs G**, Scott DR. Helicobacter pylori: Eradication or Preservation. *F1000 Med Rep* 2012; **4**: 7 [PMID: 22500191 DOI: 10.3410/M4-7]

11 **Li ZW**, Wu Y, Sun Y, Liu LY, Tian MM, Feng GS, You WC, Li JY. Inflammatory cytokine gene polymorphisms increase the risk of atrophic gastritis and intestinal metaplasia. *World J Gastroenterol* 2010; **16**: 1788-1794 [PMID: 20380014 DOI: 10.3748/wjg.v16.i14.1788]

12 **Crusius JB**, Canzian F, Capellá G, Peña AS, Pera G, Sala N, Agudo A, Rico F, Del Giudice G, Palli D, Plebani M, Boeing H, Bueno-de-Mesquita HB, Carneiro F, Pala V, Save VE, Vineis P, Tumino R, Panico S, Berglund G, Manjer J, Stenling R, Hallmans G, Martínez C, Dorronsoro M, Barricarte A, Navarro C, Quirós JR, Allen N, Key TJ, Binghan S, Caldas C, Linseisen J, Kaaks R, Overvad K, Tjønneland A, Büchner FC, Peeters PH, Numans ME, Clavel-Chapelon F, Trichopoulou A, Lund E, Jenab M, Rinaldi S, Ferrari P, Riboli E, González CA. Cytokine gene polymorphisms and the risk of adenocarcinoma of the stomach in the European prospective investigation into cancer and nutrition (EPIC-EURGAST). *Ann Oncol* 2008; **19**: 1894-1902 [PMID: 18628242 DOI: 10.1093/annonc/mdn400]

13 **Oliveira JG**, Duarte MC, Silva AE. IL-1ra anti-inflammatory cytokine polymorphism is associated with risk of gastric cancer and chronic gastritis in a Brazilian population, but the TNF-β pro-inflammatory cytokine is not. *Mol Biol Rep* 2012; **39**: 7617-7625 [PMID: 22327782 DOI: 10.1007/s11033-012-1596-x]

14 **de Oliveira JG**, Rossi AF, Nizato DM, Miyasaki K, Silva AE. Profiles of gene polymorphisms in cytokines and Toll-like receptors with higher risk for gastric cancer. *Dig Dis Sci* 2013; **58**: 978-988 [PMID: 23086128 DOI: 10.1007/s10620-012-2460-5]

15 **de Oliveira JG**, Silva AE. Polymorphisms of the TLR2 and TLR4 genes are associated with risk of gastric cancer in a Brazilian population. *World J Gastroenterol* 2012; **18**: 1235-1242 [PMID: 22468087 DOI: 10.3748/wjg.v18.i11.1235]

16 **Forman D**, Burley VJ. Gastric cancer: global pattern of the disease and an overview of environmental risk factors. *Best Pract Res Clin Gastroenterol* 2006; **20**: 633-649 [PMID: 16997150 DOI: 10.1016/j.bpg.2006.04.008]

17 **Herrera V**, Parsonnet J. Helicobacter pylori and gastric adenocarcinoma. *Clin Microbiol Infect* 2009; **15**: 971-976 [PMID: 19874380 DOI: 10.1111/j.1469-0691.2009.03031.x]

18 **De Flora S**, Bonanni P. The prevention of infection-associated cancers. *Carcinogenesis* 2011; **32**: 787-795 [PMID: 21436188 DOI: 10.1093/carcin/bgr054]

19 **Hatakeyama M**. Helicobacter pylori and gastric carcinogenesis. *J Gastroenterol* 2009; **44**: 239-248 [PMID: 19271114 DOI: 10.1007/s00535-009-0014-1]

20 **Smith MG**, Hold GL, Tahara E, El-Omar EM. Cellular and molecular aspects of gastric cancer. *World J Gastroenterol* 2006; **12**: 2979-2990 [PMID: 16718776 DOI: 10.3748/wjg.v12.i19.2979]

21 **Kim KK**, Kim HB. Protein interaction network related to Helicobacter pylori infection response. *World J Gastroenterol* 2009; **15**: 4518-4528 [PMID: 19777610 DOI: 10.3748/wjg.15.4518]

22 **Backert S**, Meyer TF. Type IV secretion systems and their effectors in bacterial pathogenesis. *Curr Opin Microbiol* 2006; **9**: 207-217 [PMID: 16529981 DOI: 10.1016/j.mib.2006.02.008]

23 **Amieva MR**, El-Omar EM. Host-bacterial interactions in Helicobacter pylori infection. *Gastroenterology* 2008; **134**: 306-323 [PMID: 18166359 DOI: 10.1053/j.gastro.2007.11.009]

24 **Wen S**, Moss SF. Helicobacter pylori virulence factors in gastric carcinogenesis. *Cancer Lett* 2009; **282**: 1-8 [PMID: 19111390 DOI: 10.1016/j.canlet.2008.11.016]

25 **Bauer B**, Meyer TF. The human gastric pathogen Helicobacter pylori and its association with gastric cancer and ulcer disease. *Ulcer* 2011; 2011: 1-23 [DOI: 10.1155/2011/340157]

26 **Censini S**, Lange C, Xiang Z, Crabtree JE, Ghiara P, Borodovsky M, Rappuoli R, Covacci A. cag, a pathogenicity island of Helicobacter pylori, encodes type I-specific and disease-associated virulence factors. *Proc Natl Acad Sci USA* 1996; **93**: 14648-14653 [PMID: 8962108 DOI: 10.1073/pnas.93.25.14648]

27 **Backert S**, Ziska E, Brinkmann V, Zimny-Arndt U, Fauconnier A, Jungblut PR, Naumann M, Meyer TF. Translocation of the Helicobacter pylori CagA protein in gastric epithelial cells by a type IV secretion apparatus. *Cell Microbiol* 2000; **2**: 155-164 [PMID: 11207572 DOI: 10.1046/j.1462-5822.2000.00043.x]

28 **Higashi H**, Nakaya A, Tsutsumi R, Yokoyama K, Fujii Y, Ishikawa S, Higuchi M, Takahashi A, Kurashima Y, Teishikata Y, Tanaka S, Azuma T, Hatakeyama M. Helicobacter pylori CagA induces Ras-independent morphogenetic response through SHP-2 recruitment and activation. *J Biol Chem* 2004; **279**: 17205-17216 [PMID: 14963045 DOI: 10.1074/jbc.M309964200]

29 **Backert S**, Tegtmeyer N, Selbach M. The versatility of Helicobacter pylori CagA effector protein functions: The master key hypothesis. *Helicobacter* 2010; **15**: 163-176 [PMID: 20557357 DOI: 10.1111/j.1523-5378.2010.00759.x]

30 **Li N**, Han L, Chen J, Lin X, Chen H, She F. Proliferative and apoptotic effects of gastric epithelial cells induced by coccoid Helicobacter pylori. *J Basic Microbiol* 2013; **53**: 147-155 [PMID: 22581720 DOI: 10.1002/jobm.201100370]

31 **Maeda S**, Yoshida H, Mitsuno Y, Hirata Y, Ogura K, Shiratori Y, Omata M. Analysis of apoptotic and antiapoptotic signalling pathways induced by Helicobacter pylori. *Mol Pathol* 2002; **55**: 286-293 [PMID: 12354930 DOI: 10.1136/gut.50.6.771]

32 **Truong BX**, Mai VT, Tanaka H, Ly le T, Thong TM, Hai HH, Van Long D, Furumatsu K, Yoshida M, Kutsumi H, Azuma T. Diverse characteristics of the CagA gene of Helicobacter pylori strains collected from patients from southern vietnam with gastric cancer and peptic ulcer. *J Clin Microbiol* 2009; **47**: 4021-4028 [PMID: 19846630 DOI: 10.1128/JCM.00504-09]

33 **Peek RM**, Miller GG, Tham KT, Perez-Perez GI, Zhao X, Atherton JC, Blaser MJ. Heightened inflammatory response and cytokine expression in vivo to cagA+ Helicobacter pylori strains. *Lab Invest* 1995; **73**: 760-770 [PMID: 8558837]

34 **Brandt S**, Kwok T, Hartig R, König W, Backert S. NF-kappaB activation and potentiation of proinflammatory responses by the Helicobacter pylori CagA protein. *Proc Natl Acad Sci USA* 2005; **102**: 9300-9305 [PMID: 15972330 DOI: 10.1073/pnas.0409873102]

35 **Yang JJ**, Cho LY, Ma SH, Ko KP, Shin A, Choi BY, Han DS, Song KS, Kim YS, Chang SH, Shin HR, Kang D, Yoo KY, Park SK. Oncogenic CagA promotes gastric cancer risk via activating ERK signaling pathways: a nested case-control study. *PLoS One* 2011; **6**: e21155 [PMID: 21698158 DOI: 10.1371/journal.pone.0021155]

36 **Karami N**, Talebkhan Y, Saberi S, Esmaeili M, Oghalaie A, Abdirad A, Mostafavi E, Hosseini ME, Mohagheghi MA, Mohammadi M. Seroreactivity to Helicobacter pylori antigens as a risk indicator of gastric cancer. *Asian Pac J Cancer Prev* 2013; **14**: 1813-1817 [PMID: 23679279]

37 **Yamaoka Y**. Mechanisms of disease: Helicobacter pylori virulence factors. *Nat Rev Gastroenterol Hepatol* 2010; **7**: 629-641 [PMID: 20938460 DOI: 10.1038/nrgastro.2010.154]

38 **Rhead JL**, Letley DP, Mohammadi M, Hussein N, Mohagheghi MA, Eshagh Hosseini M, Atherton JC. A new Helicobacter pylori vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. *Gastroenterology* 2007; **133**: 926-936 [PMID: 17854597 DOI: 10.1053/j.gastro.2007.06.056]

39 **Polk DB**, Peek RM. Helicobacter pylori: gastric cancer and beyond. *Nat Rev Cancer* 2010; **10**: 403-414 [PMID: 20495574 DOI: 10.1038/nrc2857]

40 **Basso D**, Zambon CF, Letley DP, Stranges A, Marchet A, Rhead JL, Schiavon S, Guariso G, Ceroti M, Nitti D, Rugge M, Plebani M, Atherton JC. Clinical relevance of Helicobacter pylori cagA and vacA gene polymorphisms. *Gastroenterology* 2008; **135**: 91-99 [PMID: 18474244 DOI: 10.1053/j.gastro.2008.03.041]

41 **Peek RM**. Helicobacter pylori strain-specific modulation of gastric mucosal cellular turnover: implications for carcinogenesis. *J Gastroenterol* 2002; **37** Suppl 13: 10-16 [PMID: 12109657 DOI: 10.1007/BF02990093]

42 **Allen LA**, Schlesinger LS, Kang B. Virulent strains of Helicobacter pylori demonstrate delayed phagocytosis and stimulate homotypic phagosome fusion in macrophages. *J Exp Med* 2000; **191**: 115-128 [PMID: 10620610 DOI: 10.1084/jem.191.1.115]

43 **Zheng PY**, Jones NL. Helicobacter pylori strains expressing the vacuolating cytotoxin interrupt phagosome maturation in macrophages by recruiting and retaining TACO (coronin 1) protein. *Cell Microbiol* 2003; **5**: 25-40 [PMID: 12542468 DOI: 10.1046/j.1462-5822.2003.00250.x]

44 **Gebert B**, Fischer W, Weiss E, Hoffmann R, Haas R. Helicobacter pylori vacuolating cytotoxin inhibits T lymphocyte activation. *Science* 2003; **301**: 1099-1102 [PMID: 12934009 DOI: 10.1126/science.1086871]

45 **Mahdavi J**, Sondén B, Hurtig M, Olfat FO, Forsberg L, Roche N, Angstrom J, Larsson T, Teneberg S, Karlsson KA, Altraja S, Wadström T, Kersulyte D, Berg DE, Dubois A, Petersson C, Magnusson KE, Norberg T, Lindh F, Lundskog BB, Arnqvist A, Hammarström L, Borén T. Helicobacter pylori SabA adhesin in persistent infection and chronic inflammation. *Science* 2002; **297**: 573-578 [PMID: 12142529 DOI: 10.1126/science.1069076]

46 **Prinz C**, Schöniger M, Rad R, Becker I, Keiditsch E, Wagenpfeil S, Classen M, Rösch T, Schepp W, Gerhard M. Key importance of the Helicobacter pylori adherence factor blood group antigen binding adhesin during chronic gastric inflammation. *Cancer Res* 2001; **61**: 1903-1909 [PMID: 11280745]

47 **Rad R**, Gerhard M, Lang R, Schöniger M, Rösch T, Schepp W, Becker I, Wagner H, Prinz C. The Helicobacter pylori blood group antigen-binding adhesin facilitates bacterial colonization and augments a nonspecific immune response. *J Immunol* 2002; **168**: 3033-3041 [PMID: 11884476]

48 **Gerhard M**, Lehn N, Neumayer N, Borén T, Rad R, Schepp W, Miehlke S, Classen M, Prinz C. Clinical relevance of the Helicobacter pylori gene for blood-group antigen-binding adhesin. *Proc Natl Acad Sci USA* 1999; **96**: 12778-12783 [PMID: 10535999 DOI: 10.1128/IAI.00334-07]

49 **Yamaoka Y**, Kwon DH, Graham DY. A M(r) 34,000 proinflammatory outer membrane protein (oipA) of Helicobacter pylori. *Proc Natl Acad Sci USA* 2000; **97**: 7533-7538 [PMID: 10852959 DOI: 10.1073ypnas.130079797]

50 **Yamaoka Y**, Kikuchi S, el-Zimaity HM, Gutierrez O, Osato MS, Graham DY. Importance of Helicobacter pylori oipA in clinical presentation, gastric inflammation, and mucosal interleukin 8 production. *Gastroenterology* 2002; **123**: 414-424 [PMID: 12145793 DOI: 10.1053/gast.2002.34781]

51 **Lu H**, Hsu PI, Graham DY, Yamaoka Y. Duodenal ulcer promoting gene of Helicobacter pylori. *Gastroenterology* 2005; **128**: 833-848 [PMID: 15825067 DOI: 10.1053/j.gastro.2005.01.009]

52 **Imagawa S**, Ito M, Yoshihara M, Eguchi H, Tanaka S, Chayama K. Helicobacter pylori dupA and gastric acid secretion are negatively associated with gastric cancer development. *J Med Microbiol* 2010; **59**: 1484-1489 [PMID: 20829397 DOI: 10.1099/jmm.0.021816-0]

53 **Nguyen LT**, Uchida T, Tsukamoto Y, Kuroda A, Okimoto T, Kodama M, Murakami K, Fujioka T, Moriyama M. Helicobacter pylori dupA gene is not associated with clinical outcomes in the Japanese population. *Clin Microbiol Infect* 2010; **16**: 1264-1269 [PMID: 19832706 DOI: 10.1111/j.1469-0691.2009.03081.x]

54 **Peek RM**, Thompson SA, Donahue JP, Tham KT, Atherton JC, Blaser MJ, Miller GG. Adherence to gastric epithelial cells induces expression of a Helicobacter pylori gene, iceA, that is associated with clinical outcome. *Proc Assoc Am Physicians* 1998; **110**: 531-544 [PMID: 9824536]

55 **Figueiredo C**, Machado JC, Pharoah P, Seruca R, Sousa S, Carvalho R, Capelinha AF, Quint W, Caldas C, van Doorn LJ, Carneiro F, Sobrinho-Simões M. Helicobacter pylori and interleukin 1 genotyping: an opportunity to identify high-risk individuals for gastric carcinoma. *J Natl Cancer Inst* 2002; **94**: 1680-1687 [PMID: 12441323 DOI: 10.1093/jnci/94.22.1680]

56 **Sgouros SN**, Bergele C. Clinical outcome of patients with Helicobacter pylori infection: the bug, the host, or the environment? *Postgrad Med J* 2006; **82**: 338-342 [PMID: 16679473 DOI: 10.1136/pgmj.2005.038273]

57 **Ramis IB**, Fonseca TL, de Moraes EP, Fernandes MS, Mendoza-Sassi R, Rodrigues O, Juliano CR, Scaini CJ, da Silva PE. Molecular Basis of pathogenicity in Helicobacter pylori clinical isolates. *J Clin Microbiol* 2010; **48**: 3776-3778 [PMID: 20686086 DOI: 10.1128/JCM.00472-10]

58 **Garcia GT**, Aranda KR, Gonçalves ME, Cardoso SR, Iriya K, Silva NP, Scaletsky IC. High prevalence of clarithromycin resistance and cagA, vacA, iceA2, and babA2 genotypes of Helicobacter pylori in Brazilian children. *J Clin Micro-*

biol 2010; **48**: 4266-4268 [PMID: 20826649 DOI: 10.1128/JCM.01034-10]
59 **Chen L**, Yang Q, Kong WQ, Liu T, Liu M, Li X, Tang H. MicroRNA-181b targets cAMP responsive element binding protein 1 in gastric adenocarcinomas. *IUBMB Life* 2012; **64**: 628-635 [PMID: 22539488 DOI: 10.1002/iub.1030]
60 **Baud J**, Varon C, Chabas S, Chambonnier L, Darfeuille F, Staedel C. Helicobacter pylori initiates a mesenchymal transition through ZEB1 in gastric epithelial cells. *PLoS One* 2013; **8**: e60315 [PMID: 23565224 DOI: 10.1371/journal.pone.0060315]
61 **Liu X**, Ru J, Zhang J, Zhu LH, Liu M, Li X, Tang H. miR-23a targets interferon regulatory factor 1 and modulates cellular proliferation and paclitaxel-induced apoptosis in gastric adenocarcinoma cells. *PLoS One* 2013; **8**: e64707 [PMID: 23785404 DOI: 10.1371/journal.pone.0064707]
62 **Kang JY**, Lee JO. Structural biology of the Toll-like receptor family. *Annu Rev Biochem* 2011; **80**: 917-941 [PMID: 21548780 DOI: 10.1146/annurev-biochem-052909-141507]
63 **Takeda K**, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* 2003; **21**: 335-376 [PMID: 12524386]
64 **Matsuura M**. Structural Modifications of Bacterial Lipopolysaccharide that Facilitate Gram-Negative Bacteria Evasion of Host Innate Immunity. *Front Immunol* 2013; **4**: 109 [PMID: 23745121 DOI: 10.3389/fimmu.2013.00109]
65 **Yokota S**, Ohnishi T, Muroi M, Tanamoto K, Fujii N, Amano K. Highly-purified Helicobacter pylori LPS preparations induce weak inflammatory reactions and utilize Toll-like receptor 2 complex but not Toll-like receptor 4 complex. *FEMS Immunol Med Microbiol* 2007; **51**: 140-148 [PMID: 17645528]
66 **Kumar Pachathundikandi S**, Brandt S, Madassery J, Backert S. Induction of TLR-2 and TLR-5 expression by Helicobacter pylori switches cagPAI-dependent signalling leading to the secretion of IL-8 and TNF-α. *PLoS One* 2011; **6**: e19614 [PMID: 21573018 DOI: 10.1371/journal.pone.0019614]
67 **Bartchewsky W**, Martini MR, Masiero M, Squassoni AC, Alvarez MC, Ladeira MS, Salvatore D, Trevisan M, Pedrazzoli J, Ribeiro ML. Effect of Helicobacter pylori infection on IL-8, IL-1beta and COX-2 expression in patients with chronic gastritis and gastric cancer. *Scand J Gastroenterol* 2009; **44**: 153-161 [PMID: 18985541 DOI: 10.1080/00365520802530853]
68 **Isomoto H**, Moss J, Hirayama T. Pleiotropic actions of Helicobacter pylori vacuolating cytotoxin, VacA. *Tohoku J Exp Med* 2010; **220**: 3-14 [PMID: 20046046]
69 **Argent RH**, Thomas RJ, Letley DP, Rittig MG, Hardie KR, Atherton JC. Functional association between the Helicobacter pylori virulence factors VacA and CagA. *J Med Microbiol* 2008; **57**: 145-150 [PMID: 18201978 DOI: 10.1099/jmm.0.47465-0]
70 **Greten FR**, Eckmann L, Greten TF, Park JM, Li ZW, Egan LJ, Kagnoff MF, Karin M. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell* 2004; **118**: 285-296 [PMID: 15294155 DOI: 10.1016/j.cell.2004.07.013]
71 **Ding SZ**, Goldberg JB, Hatakeyama M. Helicobacter pylori infection, oncogenic pathways and epigenetic mechanisms in gastric carcinogenesis. *Future Oncol* 2010; **6**: 851-862 [PMID: 20465395 DOI: 10.2217/fon.10.37]
72 **Sundrud MS**, Torres VJ, Unutmaz D, Cover TL. Inhibition of primary human T cell proliferation by Helicobacter pylori vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. *Proc Natl Acad Sci USA* 2004; **101**: 7727-7732 [PMID: 15128946]
73 **Luo JJ**, Li CY, Liu S, Yu W, Tang SY, Cai HL, Zhang Y. Overexpression of Helicobacter pylori VacA N-terminal fragment induces proinflammatory cytokine expression and apoptosis in human monocytic cell line through activation of NF-κB. *Can J Microbiol* 2013; **59**: 523-533 [PMID: 23898995 DOI: 10.1139/cjm-2013]
74 **Freedman DO**. Imported malaria--here to stay. *Am J Med* 1992; **93**: 239-242 [PMID: 1524073]
75 **Telford JL**, Covacci A, Rappuoli R, Chiara P. Immunobiology of Helicobacter pylori infection. *Curr Opin Immunol* 1997; **9**: 498-503 [PMID: 9287174]
76 **Alvarez-Arellano L**, Camorlinga-Ponce M, Maldonado-Bernal C, Torres J. Activation of human neutrophils with Helicobacter pylori and the role of Toll-like receptors 2 and 4 in the response. *FEMS Immunol Med Microbiol* 2007; **51**: 473-479 [PMID: 17892476]
77 **Fehlings M**, Drobbe L, Moos V, Renner Viveros P, Hagen J, Beigier-Bompadre M, Pang E, Belogolova E, Churin Y, Schneider T, Meyer TF, Aebischer T, Ignatius R. Comparative analysis of the interaction of Helicobacter pylori with human dendritic cells, macrophages, and monocytes. *Infect Immun* 2012; **80**: 2724-2734 [PMID: 22615251 DOI: 10.1128/IAI.00381-12]
78 **Soehnlein O**, Lindbom L. Phagocyte partnership during the onset and resolution of inflammation. *Nat Rev Immunol* 2010; **10**: 427-439 [PMID: 20498669 DOI: 10.1038/nri2779]
79 **Wilson KT**, Crabtree JE. Immunology of Helicobacter pylori: insights into the failure of the immune response and perspectives on vaccine studies. *Gastroenterology* 2007; **133**: 288-308 [PMID: 17631150]
80 **Rolig AS**, Cech C, Ahler E, Carter JE, Ottemann KM. The degree of Helicobacter pylori-triggered inflammation is manipulated by preinfection host microbiota. *Infect Immun* 2013; **81**: 1382-1389 [PMID: 23429529 DOI: 10.1128/IAI.00044-13]
81 **Weiss G**, Forster S, Irving A, Tate M, Ferrero RL, Hertzog P, Frøkiær H, Kaparakis-Liaskos M. Helicobacter pylori VacA suppresses Lactobacillus acidophilus-induced interferon beta signaling in macrophages via alterations in the endocytic pathway. *MBio* 2013; **4**: e00609-e00612 [PMID: 23760466 DOI: 10.1128/mBio.00609-12]
82 **Lindholm C**, Quiding-Järbrink M, Lönroth H, Hamlet A, Svennerholm AM. Local cytokine response in Helicobacter pylori-infected subjects. *Infect Immun* 1998; **66**: 5964-5971 [PMID: 9826379]
83 **Galgani M**, Busiello I, Censini S, Zappacosta S, Racioppi L, Zarrilli R. Helicobacter pylori induces apoptosis of human monocytes but not monocyte-derived dendritic cells: role of the cag pathogenicity island. *Infect Immun* 2004; **72**: 4480-4485 [PMID: 15271906]
84 **Bornschein J**, Kandulski A, Selgrad M, Malfertheiner P. From gastric inflammation to gastric cancer. *Dig Dis* 2010; **28**: 609-614 [PMID: 21088411 DOI: 10.1159/000320061]
85 **Au PY**, Yeh WC. Physiological roles and mechanisms of signaling by TRAF2 and TRAF5. *Adv Exp Med Biol* 2007; **597**: 32-47 [PMID: 17633015]
86 **Chang YJ**, Wu MS, Lin JT, Sheu BS, Muta T, Inoue H, Chen CC. Induction of cyclooxygenase-2 overexpression in human gastric epithelial cells by Helicobacter pylori involves TLR2/TLR9 and c-Src-dependent nuclear factor-kappaB activation. *Mol Pharmacol* 2004; **66**: 1465-1477 [PMID: 15456896]
87 **Sierra JC**, Hobbs S, Chaturvedi R, Yan F, Wilson KT, Peek RM, Polk DB. Induction of COX-2 expression by Helicobacter pylori is mediated by activation of epidermal growth factor receptor in gastric epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 2013; **305**: G196-G203 [PMID: 23681474 DOI: 10.1152/ajpgi.00495.2012]
88 **Nagy TA**, Frey MR, Yan F, Israel DA, Polk DB, Peek RM. Helicobacter pylori regulates cellular migration and apoptosis by activation of phosphatidylinositol 3-kinase signaling. *J Infect Dis* 2009; **199**: 641-651 [PMID: 19199544 DOI: 10.1086/596660]
89 **Tabassam FH**, Graham DY, Yamaoka Y. Helicobacter pylori activate epidermal growth factor receptor- and phosphatidylinositol 3-OH kinase-dependent Akt and glycogen synthase kinase 3beta phosphorylation. *Cell Microbiol* 2009; **11**:

70-82 [PMID: 18782353 DOI: 10.1111/j.1462-5822.2008.01237.x]
90 **Kim DY**, Cha ST, Ahn DH, Kang HY, Kwon CI, Ko KH, Hwang SG, Park PW, Rim KS, Hong SP. STAT3 expression in gastric cancer indicates a poor prognosis. *J Gastroenterol Hepatol* 2009; **24**: 646-651 [PMID: 19175826 DOI: 10.1111/j.1440-1746.2008.05671.x]
91 **Jang J**, Lee S, Jung Y, Song K, Fukumoto M, Gould VE, Lee I. Malgun (clear) cell change in Helicobacter pylori gastritis reflects epithelial genomic damage and repair. *Am J Pathol* 2003; **162**: 1203-1211 [PMID: 12651612 DOI: 10.1016/S0002-9440(10)63916-8]
92 **Baltimore D**, Boldin MP, O'Connell RM, Rao DS, Taganov KD. MicroRNAs: new regulators of immune cell development and function. *Nat Immunol* 2008; **9**: 839-845 [PMID: 18645592 DOI: 10.1038/ni.f.209]
93 **Hocès de la Guardia A**, Staedel C, Kaafarany I, Clément A, Roubaud Baudron C, Mégraud F, Lehours P. Inflammatory cytokine and microRNA responses of primary human dendritic cells cultured with Helicobacter pylori strains. *Front Microbiol* 2013; **4**: 236 [PMID: 23970881 DOI: 10.3389/fmicb.2013.00236]
94 **Li X**, Zhang Y, Zhang H, Liu X, Gong T, Li M, Sun L, Ji G, Shi Y, Han Z, Han S, Nie Y, Chen X, Zhao Q, Ding J, Wu K, Daiming F. miRNA-223 promotes gastric cancer invasion and metastasis by targeting tumor suppressor EPB41L3. *Mol Cancer Res* 2011; **9**: 824-833 [PMID: 21628394 DOI: 10.1158/1541-7786.MCR-10-0529]
95 **Yokota S**, Okabayashi T, Rehli M, Fujii N, Amano K. Helicobacter pylori lipopolysaccharides upregulate toll-like receptor 4 expression and proliferation of gastric epithelial cells via the MEK1/2-ERK1/2 mitogen-activated protein kinase pathway. *Infect Immun* 2010; **78**: 468-476 [PMID: 19858308 DOI: 10.1128/IAI.00903-09]
96 **Frey MR**, Golovin A, Polk DB. Epidermal growth factor-stimulated intestinal epithelial cell migration requires Src family kinase-dependent p38 MAPK signaling. *J Biol Chem* 2004; **279**: 44513-44521 [PMID: 15316018 DOI: 10.1074/jbc.M406253200]
97 **Hofman VJ**, Moreilhon C, Brest PD, Lassalle S, Le Brigand K, Sicard D, Raymond J, Lamarque D, Hébuterne XA, Mari B, Barbry PJ, Hofman PM. Gene expression profiling in human gastric mucosa infected with Helicobacter pylori. *Mod Pathol* 2007; **20**: 974-989 [PMID: 17643099 DOI: 10.1038/modpathol.3800930]
98 **Yang ZM**, Chen WW, Wang YF. Gene expression profiling in gastric mucosa from Helicobacter pylori-infected and uninfected patients undergoing chronic superficial gastritis. *PLoS One* 2012; **7**: e33030 [PMID: 22438889]
99 **Pimentel-Nunes P**, Gonçalves N, Boal-Carvalho I, Afonso L, Lopes P, Roncon-Albuquerque R, Henrique R, Moreira-Dias L, Leite-Moreira AF, Dinis-Ribeiro M. Helicobacter pylori induces increased expression of Toll-like receptors and decreased Toll-interacting protein in gastric mucosa that persists throughout gastric carcinogenesis. *Helicobacter* 2013; **18**: 22-32 [PMID: 23061653 DOI: 10.1111/hel.12008]
100 **Garza-González E**, Bocanegra-García V, Bosques-Padilla FJ, Flores-Gutiérrez JP, Moreno F, Perez-Perez GI. mRNA levels of TLR4 and TLR5 are independent of H pylori. *World J Gastroenterol* 2008; **14**: 5306-5310 [PMID: 18785283 DOI: 10.3748/wjg.14.5306]
101 **Masumoto J**, Yang K, Varambally S, Hasegawa M, Tomlins SA, Qiu S, Fujimoto Y, Kawasaki A, Foster SJ, Horie Y, Mak TW, Núñez G, Chinnaiyan AM, Fukase K, Inohara N. Nod1 acts as an intracellular receptor to stimulate chemokine production and neutrophil recruitment in vivo. *J Exp Med* 2006; **203**: 203-213 [PMID: 16418393 DOI: 10.1084/jem.20051229]
102 **Fritz JH**, Le Bourhis L, Sellge G, Magalhaes JG, Fsihi H, Kufer TA, Collins C, Viala J, Ferrero RL, Girardin SE, Philpott DJ. Nod1-mediated innate immune recognition of peptidoglycan contributes to the onset of adaptive immunity. *Immunity* 2007; **26**: 445-459 [PMID: 17433730 DOI: 10.1016/j.immuni.2007.03.009]
103 **Allison CC**, Ferrand J, McLeod L, Hassan M, Kaparakis-Liaskos M, Grubman A, Bhathal PS, Dev A, Sievert W, Jenkins BJ, Ferrero RL. Nucleotide oligomerization domain 1 enhances IFN-γ signaling in gastric epithelial cells during Helicobacter pylori infection and exacerbates disease severity. *J Immunol* 2013; **190**: 3706-3715 [PMID: 23460743 DOI: 10.4049/jimmunol.1200591]
104 **Jackson CB**, Judd LM, Menheniott TR, Kronborg I, Dow C, Yeomans ND, Boussioutas A, Robb L, Giraud AS. Augmented gp130-mediated cytokine signalling accompanies human gastric cancer progression. *J Pathol* 2007; **213**: 140-151 [PMID: 17724739 DOI: 10.1002/path.2218]
105 **Sohn SH**, Lee YC. The genome-wide expression profile of gastric epithelial cells infected by naturally occurring cagA isogenic strains of Helicobacter pylori. *Environ Toxicol Pharmacol* 2011; **32**: 382-389 [PMID: 22004957 DOI: 10.1016/j.etap.2011.08.006]
106 **Hunt RH**, Xiao SD, Megraud F, Leon-Barua R, Bazzoli F, van der Merwe S, Vaz Coelho LG, Fock M, Fedail S, Cohen H, Malfertheiner P, Vakil N, Hamid S, Goh KL, Wong BC, Krabshuis J, Le Mair A. Helicobacter pylori in developing countries. World Gastroenterology Organisation Global Guideline. *J Gastrointestin Liver Dis* 2011; **20**: 299-304 [PMID: 21961099 DOI: 10.1097/MCG.0b013e31820fb8f6]
107 **Tsai CJ**, Herrera-Goepfert R, Tibshirani RJ, Yang S, Mohar A, Guarner J, Parsonnet J. Changes of gene expression in gastric preneoplasia following Helicobacter pylori eradication therapy. *Cancer Epidemiol Biomarkers Prev* 2006; **15**: 272-280 [PMID: 16492915 DOI: 10.1158/1055-9965.EPI-05-0632]
108 **Rimbara E**, Fischbach LA, Graham DY. Optimal therapy for Helicobacter pylori infections. *Nat Rev Gastroenterol Hepatol* 2011; **8**: 79-88 [PMID: 21293508 DOI: 10.1038/nrgastro.2010.210]
109 **Yoon JH**, Baik GH, Sohn KM, Kim DY, Kim YS, Suk KT, Kim JB, Kim DJ, Kim JB, Shin WG, Kim HY, Baik IH, Jang HJ. Trends in the eradication rates of Helicobacter pylori infection for eleven years. *World J Gastroenterol* 2012; **18**: 6628-6634 [PMID: 23236238 DOI: 10.3748/wjg.v18.i45.6628]
110 **Kim SY**, Lee SW, Hyun JJ, Jung SW, Koo JS, Yim HJ, Park JJ, Chun HJ, Choi JH. Comparative study of Helicobacter pylori eradication rates with 5-day quadruple "concomitant" therapy and 7-day standard triple therapy. *J Clin Gastroenterol* 2013; **47**: 21-24 [PMID: 22647826 DOI: 10.1097/MCG.0b013e3182548ad4]
111 **Kindlund B**, Sjöling A, Hansson M, Edebo A, Hansson LE, Sjövall H, Svennerholm AM, Lundin BS. FOXP3-expressing CD4(+) T-cell numbers increase in areas of duodenal gastric metaplasia and are associated to CD4(+) T-cell aggregates in the duodenum of Helicobacter pylori-infected duodenal ulcer patients. *Helicobacter* 2009; **14**: 192-201 [PMID: 19702849 DOI: 10.1111/j.1523-5378.2009.00673.x]
112 **Bauer B**, Wex T, Kuester D, Meyer T, Malfertheiner P. Differential expression of human beta defensin 2 and 3 in gastric mucosa of Helicobacter pylori-infected individuals. *Helicobacter* 2013; **18**: 6-12 [PMID: 23067102 DOI: 10.1111/hel.12000]
113 **Ambros V**, Bartel B, Bartel DP, Burge CB, Carrington JC, Chen X, Dreyfuss G, Eddy SR, Griffiths-Jones S, Marshall M, Matzke M, Ruvkun G, Tuschl T. A uniform system for microRNA annotation. *RNA* 2003; **9**: 277-279 [PMID: 12592000 DOI: 10.1261/rna.2183803]
114 **Elbashir SM**, Lendeckel W, Tuschl T. RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* 2001; **15**: 188-200 [PMID: 11157775 DOI: 10.1101/gad.862301]
115 **Elbashir SM**, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T. Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. *EMBO J* 2001; **20**: 6877-6888 [PMID: 11726523 DOI: 10.1093/

emboj/20.23.6877]
116 **Selbach M**, Schwanhäusser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. Widespread changes in protein synthesis induced by microRNAs. *Nature* 2008; **455**: 58-63 [PMID: 18668040 DOI: 10.1038/nature07228]
117 **Beilharz TH**, Humphreys DT, Clancy JL, Thermann R, Martin DI, Hentze MW, Preiss T. microRNA-mediated messenger RNA deadenylation contributes to translational repression in mammalian cells. *PLoS One* 2009; **4**: e6783 [PMID: 19710908 DOI: 10.1371/journal.pone.0006783]
118 **Lee RC**, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 1993; **75**: 843-854 [PMID: 8252621 DOI: 10.1016/0092-8674(93)90529-Y]
119 **Zhou P**, Xu W, Peng X, Luo Z, Xing Q, Chen X, Hou C, Liang W, Zhou J, Wu X, Songyang Z, Jiang S. Large-scale screens of miRNA-mRNA interactions unveiled that the 3' UTR of a gene is targeted by multiple miRNAs. *PLoS One* 2013; **8**: e68204 [PMID: 23874542 DOI: 10.1371/journal.pone.0068204]
120 **Lu J**, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. *Nature* 2005; **435**: 834-838 [PMID: 15944708 DOI: 10.1038/nature03702]
121 **Oertli M**, Engler DB, Kohler E, Koch M, Meyer TF, Müller A. MicroRNA-155 is essential for the T cell-mediated control of Helicobacter pylori infection and for the induction of chronic Gastritis and Colitis. *J Immunol* 2011; **187**: 3578-3586 [PMID: 21880981 DOI: 10.4049/jimmunol.1101772]
122 **Brennecke J**, Hipfner DR, Stark A, Russell RB, Cohen SM. bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. *Cell* 2003; **113**: 25-36 [PMID: 12679032 DOI: 10.1016/S0092-8674(03)00231-9]
123 **Zhang Z**, Li Z, Gao C, Chen P, Chen J, Liu W, Xiao S, Lu H. miR-21 plays a pivotal role in gastric cancer pathogenesis and progression. *Lab Invest* 2008; **88**: 1358-1366 [PMID: 18794849 DOI: 10.1038/labinvest.2008.94]
124 **Kawasaki H**, Taira K. Hes1 is a target of microRNA-23 during retinoic-acid-induced neuronal differentiation of NT2 cells. *Nature* 2003; **423**: 838-842 [PMID: 12808467 DOI: 10.1038/nature01730]
125 **Wang YS**, Li SH, Guo J, Mihic A, Wu J, Sun L, Davis K, Weisel RD, Li RK. Role of miR-145 in cardiac myofibroblast differentiation. *J Mol Cell Cardiol* 2014; **66**: 94-105 [PMID: 24001939 DOI: 10.1016/j.yjmcc.2013.08.007]
126 **Schetter AJ**, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N, Yuen ST, Chan TL, Kwong DL, Au GK, Liu CG, Calin GA, Croce CM, Harris CC. MicroRNA expression profiles associated with prognosis and therapeutic outcome in colon adenocarcinoma. *JAMA* 2008; **299**: 425-436 [PMID: 18230780 DOI: 10.1001/jama.299.4]
127 **Ribeiro-dos-Santos Â**, Khayat AS, Silva A, Alencar DO, Lobato J, Luz L, Pinheiro DG, Varuzza L, Assumpção M, Assumpção P, Santos S, Zanette DL, Silva WA, Burbano R, Darnet S. Ultra-deep sequencing reveals the microRNA expression pattern of the human stomach. *PLoS One* 2010; **5**: e13205 [PMID: 20949028 DOI: 10.1371/journal.pone.0013205]
128 **Isomoto H**, Matsushima K, Inoue N, Hayashi T, Nakayama T, Kunizaki M, Hidaka S, Nakayama M, Hisatsune J, Nakashima M, Nagayasu T, Nakao K, Hirayama T. Interweaving microRNAs and proinflammatory cytokines in gastric mucosa with reference to H. pylori infection. *J Clin Immunol* 2012; **32**: 290-299 [PMID: 22161133 DOI: 10.1007/s10875-011-9626-3]
129 **Teng GG**, Wang WH, Dai Y, Wang SJ, Chu YX, Li J. Let-7b is involved in the inflammation and immune responses associated with Helicobacter pylori infection by targeting Toll-like receptor 4. *PLoS One* 2013; **8**: e56709 [PMID: 23437218 DOI: 10.1371/journal.pone.0056709]
130 **Shiotani A**, Uedo N, Iishi H, Murao T, Kanzaki T, Kimura Y, Kamada T, Kusunoki H, Inoue K, Haruma K. H. pylori eradication did not improve dysregulation of specific oncogenic miRNAs in intestinal metaplastic glands. *J Gastroenterol* 2012; **47**: 988-998 [PMID: 22382634 DOI: 10.1007/s00535-012-0562-7]
131 **Liu Z**, Xiao B, Tang B, Li B, Li N, Zhu E, Guo G, Gu J, Zhuang Y, Liu X, Ding H, Zhao X, Guo H, Mao X, Zou Q. Up-regulated microRNA-146a negatively modulate Helicobacter pylori-induced inflammatory response in human gastric epithelial cells. *Microbes Infect* 2010; **12**: 854-863 [PMID: 20542134 DOI: 10.1016/j.micinf.2010.06.002]
132 **Liu Z**, Wang D, Hu Y, Zhou G, Zhu C, Yu Q, Chi Y, Cao Y, Jia C, Zou Q. MicroRNA-146a negatively regulates PTGS2 expression induced by Helicobacter pylori in human gastric epithelial cells. *J Gastroenterol* 2013; **48**: 86-92 [PMID: 22699322 DOI: 10.1007/s00535-012-0609-9]
133 **Xiao B**, Liu Z, Li BS, Tang B, Li W, Guo G, Shi Y, Wang F, Wu Y, Tong WD, Guo H, Mao XH, Zou QM. Induction of microRNA-155 during Helicobacter pylori infection and its negative regulatory role in the inflammatory response. *J Infect Dis* 2009; **200**: 916-925 [PMID: 19650740 DOI: 10.1086/605443]
134 **Li N**, Tang B, Zhu ED, Li BS, Zhuang Y, Yu S, Lu DS, Zou QM, Xiao B, Mao XH. Increased miR-222 in H. pylori-associated gastric cancer correlated with tumor progression by promoting cancer cell proliferation and targeting RECK. *FEBS Lett* 2012; **586**: 722-728 [PMID: 22321642 DOI: 10.1016/j.febslet.2012.01.025]
135 **Matsushima K**, Isomoto H, Inoue N, Nakayama T, Hayashi T, Nakayama M, Nakao K, Hirayama T, Kohno S. MicroRNA signatures in Helicobacter pylori-infected gastric mucosa. *Int J Cancer* 2011; **128**: 361-370 [PMID: 20333682 DOI: 10.1002/ijc.25348]
136 **Chen Q**, Wang H, Liu Y, Song Y, Lai L, Han Q, Cao X, Wang Q. Inducible microRNA-223 down-regulation promotes TLR-triggered IL-6 and IL-1β production in macrophages by targeting STAT3. *PLoS One* 2012; **7**: e42971 [PMID: 22937006 DOI: 10.1371/journal.pone.0042971]
137 **Feng Y**, Wang L, Zeng J, Shen L, Liang X, Yu H, Liu S, Liu Z, Sun Y, Li W, Chen C, Jia J. FoxM1 is overexpressed in Helicobacter pylori-induced gastric carcinogenesis and is negatively regulated by miR-370. *Mol Cancer Res* 2013; **11**: 834-844 [PMID: 23576572 DOI: 10.1158/1541-7786.MCR-13-0007]
138 **Belair C**, Baud J, Chabas S, Sharma CM, Vogel J, Staedel C, Darfeuille F. Helicobacter pylori interferes with an embryonic stem cell micro RNA cluster to block cell cycle progression. *Silence* 2011; **2**: 7 [PMID: 22027184 DOI: 10.1186/1758-907X-2-7]
139 **Bou Kheir T**, Futoma-Kazmierczak E, Jacobsen A, Krogh A, Bardram L, Hother C, Grønbæk K, Federspiel B, Lund AH, Friis-Hansen L. miR-449 inhibits cell proliferation and is down-regulated in gastric cancer. *Mol Cancer* 2011; **10**: 29 [PMID: 21418558 DOI: 10.1186/1476-4598-10-29]
140 **Zhu Y**, Jiang Q, Lou X, Ji X, Wen Z, Wu J, Tao H, Jiang T, He W, Wang C, Du Q, Zheng S, Mao J, Huang J. MicroRNAs up-regulated by CagA of Helicobacter pylori induce intestinal metaplasia of gastric epithelial cells. *PLoS One* 2012; **7**: e35147 [PMID: 22536353 DOI: 10.1371/journal.pone.0035147]
141 **Sayi A**, Kohler E, Hitzler I, Arnold I, Schwendener R, Rehrauer H, Müller A. The CD4+ T cell-mediated IFN-gamma response to Helicobacter infection is essential for clearance and determines gastric cancer risk. *J Immunol* 2009; **182**: 7085-7101 [PMID: 19454706 DOI: 10.4049/jimmunol.0803293]
142 **Olivieri F**, Spazzafumo L, Santini G, Lazzarini R, Albertini

MC, Rippo MR, Galeazzi R, Abbatecola AM, Marcheselli F, Monti D, Ostan R, Cevenini E, Antonicelli R, Franceschi C, Procopio AD. Age-related differences in the expression of circulating microRNAs: miR-21 as a new circulating marker of inflammaging. *Mech Ageing Dev* 2012; **133**: 675-685 [PMID: 23041385 DOI: 10.1016/j.mad.2012.09.004]

143 **Lo SS**, Hung PS, Chen JH, Tu HF, Fang WL, Chen CY, Chen WT, Gong NR, Wu CW. Overexpression of miR-370 and downregulation of its novel target TGFβ-RII contribute to the progression of gastric carcinoma. *Oncogene* 2012; **31**: 226-237 [PMID: 21666718 DOI: 10.1038/onc.2011.226]

144 **Xu Z**, Xiao SB, Xu P, Xie Q, Cao L, Wang D, Luo R, Zhong Y, Chen HC, Fang LR. miR-365, a novel negative regulator of interleukin-6 gene expression, is cooperatively regulated by Sp1 and NF-kappaB. *J Biol Chem* 2011; **286**: 21401-21412 [PMID: 21518763 DOI: 10.1074/jbc.M110.198630]

P- Reviewers: Samulski RJ, Shimoyama S, Zhong L
S- Editor: Cui XM **L- Editor**: A **E- Editor**: Wu HL

Artigo II

Helicobacter pylori eradication does not change TLR2 and TLR4 expression in chronic gastritis patients

Running Head: TLR2 and TLR4 after *H. pylori* eradication

Aline Cristina Targa Cadamuro[a], Ana Flávia Teixeira Rossi[a], Joice Matos Biselli-Périco[a], Patrícia Fucuta Pereira[b], Edla Bedin Polsinelli Mascarin[b], Ricardo Acayaba[c], Kátia Ramos Moreira Leite[d], Eny Maria Goloni-Bertollo[e], Ana Elizabete Silva[a #].

[a]UNESP, São Paulo State University, Department of Biology, São José do Rio Preto Campus, SP, Brazil; [b]Base Hospital, São José do Rio Preto, SP, Brazil; [c]João Paulo II State Hospital, Medical Specialties Outpatient Clinic, São José do Rio Preto, SP, Brazil; [d]USP, São Paulo University, School of Medicine, São Paulo, SP, Brazil; [e]FAMERP, School of Medicine, São José do Rio Preto, SP, Brazil.

#Address correspondence to Ana Elizabete Silva

Rua Cristovão Colombo, 2265, São José do Rio Preto, SP, CEP: 15054-000, Brazil

E-mail: anabete@ibilce.unesp.br

Abstract

Background. *Helicobacter pylori* is the main cause of gastric cancer and precancerous lesions in consequence of persistent chronic inflammation. The recognition of the bacteria in gastric epithelial cells is performed by toll-like receptors, such as TLR4 and TLR2, which trigger the activation of genes involved in the host immune response. Therefore, *H. pylori* eradication has been one of the strategies for the prevention of gastric cancer. The goal of this study was evaluate for the first time the mRNA and protein expression of TLR2 and TLR4 in *H. pylori*-infected chronic gastritis patients (CG-Hp+) before and 3 months after eradication therapy, as well as the cell kinetics, using cell proliferation (PI) and apoptosis (AI) indices. **Materials and Methods.** 59 gastric biopsies from patients with chronic gastritis-Hp+ (CG-Hp+) before the standard treatment for eradication of the bacteria, and 3 months after therapy were studied. As control, four biopsies from normal gastric mucosa without infection (NM-Hp-) were obtained. Relative quantification (RQ) of mRNA was assessed by TaqMan assay and protein expression, cell proliferation and apoptosis by immunohistochemistry. **Results.** The relative quantification (RQ) of *TLR2* and *TLR4* mRNA in CG-Hp+ patients (median RQ: *TLR2*= 1.32 and *TLR4*=1.26) was not significantly changed after successful eradication therapy (median RQ: *TLR2*=1.47 and *TLR4*=1.53). Moreover, after treatment, both *TLR2* and *TLR4* were upregulated in comparison to an Hp-negative normal gastric mucosa group. In addition, we observed a positive correlation between the RQ values of *TLR2* and *TLR4*, both before and after treatment. Immunoexpression of the TLR2 and TLR4 proteins confirmed the

gene expression results, and regarding cell kinetics, there was no change in PI, while AI showed a significant reduction after treatment. **Conclusions.** In conclusion, *H. pylori* eradication therapy changed the cell kinetics, but did not reduced the *TLR2* and *TLR4* expression levels within a short period of time.

Key words: *H. pylori*, eradication therapy, gastric lesions, TLR2, TLR4, gene expression, cell kinetics.

Introduction

The *Helicobacter pylori* (*H. pylori*) bacterium is responsible for 5.5% of all infection-associated cancers (1) and is the major cause of gastric cancer in consequence of chronic inflammation. Persistent gastric mucosa inflammation results in chronic gastritis and progresses through a multistep process to gastric atrophy, intestinal metaplasia, dysplasia, and finally to carcinoma (2). The clinical consequences of *H. pylori* infection are determined by bacteria virulence genes as well as by host genetic factors such as immune response genes, besides environmental factors (3-5). Among the bacterial products, the CagA (cytotoxin-associated gene A) and VacA (vacuolating cytotoxin) proteins are the major virulence factors related to the severity of gastric lesions and cell responses (6,7).

The gastric epithelium cells provide the first point of contact for *H. pylori* adhesion through interaction with Toll-like receptors (TLRs), responding to the infection by activating various signaling pathways (8). TLRs are key regulators of both innate and adaptive immune responses, recognizing several microbial products, such as lipoproteins, peptidoglycans and lipopolysaccharides (LPS) (9). The LPS of *H. pylori*, as a cell wall component, is recognized mainly by TLR4 (10), but also by TLR2, which recognizes other forms that are structurally different from those recognized by TLR4 (11). Both TLR2 and the TLR4 are activated, after the bacteria recognition, in cooperation with the adapter molecule MyD88, triggering the mitogen-activating protein kinase (MAPK) signaling pathway. At this point, there is a subsequent activation of the transcription factor NF-kB, which leads to the rapid expression of inducible nitric oxide synthase

(iNOS) and proinflammatory cytokines, chemokines and their receptors, and interleukins (12,13). When these factors are stimulated, they initiate a marked inflammatory response of the mucosa, characterized as chronically active gastritis, and may acquire oncogenic potential (14,15). Moreover, TLR2 and TLR4 also contribute to the maintenance of the tissue homeostasis, regulating apoptosis and tissue regeneration and repair (16,17).

In addition, *H. pylori* infection is responsible for inflammatory and atrophic changes in the gastric mucosa, together with increased expression of pro-tumorigenic agents such as cyclooxygenase-2 (COX-2) and the anti-apoptotic protein (Bcl-2), resulting in uncontrolled cell proliferation with atrophic changes, suppression of apoptosis, and excessive angiogenesis. Consequently, these changes may lead to the development of cancer (18,19).

So far, the strategy for prevention of *H. pylori*-associated gastric cancer has been the eradication of these bacteria, regarded as a first-line therapy to reverse the pre-neoplastic lesions and prevent malignant progression (16). However, treatment is not adopted for asymptomatic carriers in developing countries, due to its high cost (20). *H. pylori* is susceptible to most antibiotics, although resistance has been common, and triple or quadruple therapy consisting of two antibiotics, a proton pump inhibitor and bismuth have lately been used to eradicate the bacteria (21). Unfortunately, the eradication is not always successful, mainly due to chemoresistance (22).

Studies to evaluate changes in expression levels of genes involved in the recognition of the bacteria and the immune response of the host in patients

infected by *H. pylori* are scarce, both before and after eradication treatment. Moreover, there are no reports about the expression of TLR2 and TLR4 in gastric lesions before and after bacterial clearance. Therefore, the main goal of the present study was to evaluate, for the first time, the mRNA and protein expression levels of TLR2 and TLR4 in *H. pylori*-positive chronic gastritis patients. In addition, we also evaluated the occurrence of changes in the expression levels of these receptors after successful *H. pylori* eradication therapy and the cell kinetics assessed by cell proliferation and apoptosis indices.

Materials and Methods

Patients

At first, 59 patients scheduled for upper endoscopy with positive histological and molecular diagnosis for *H. pylori* and not yet submitted to eradication therapy were enrolled prospectively between May 2010 and December 2012 from the Gastro-Hepatology Outpatient Clinic at the Base Hospital and the João Paulo Segundo Hospital, both at São José do Rio Preto, SP, Brazil.

From each patient, gastric biopsies of the antrum region were collected for histological analyses, molecular and immunohistochemichal study. None of the individuals had taken any antibiotics, nonsteroidal anti-inflammatory drugs, or corticosteroids during the two months prior to endoscopy, nor proton-pump inhibitors, H_2 antagonists in the 10-15 days preceding the procedure. Patients with gastric cancer and infectious diseases were excluded from this study. Gastric biopsy specimens were examined histologically by a specialized pathologist for

the presence of the bacteria and histopathologically classified as: superficial chronic gastritis (*n*=45; mean age 44 years; 19 females and 17 males), atrophic gastritis (*n*=8; mean age 50 years; 3 females and 5 males), and atrophic gastritis with intestinal metaplasia (*n*=6; mean age 50 years; 4 females and 2 males), according to the Sydney system (23), constituting the so-called CG-Hp+ group. Of the 59 CG-Hp+ patients, only 37 (63%) concluded the treatment, and called completed treatment group, and 23/37 (62%) of them had the bacteria eradicated, as evidenced by concordant histological and molecular *H. pylori*-negative diagnosis, so-called successful treatment group (Table 1). Four gastric biopsy specimens presented histologically normal, *H. pylori*-negative gastric mucosa (normal-Hp- group) and were used as control (mean age 35.6 years; 3 females and 1 male). Epidemiological data of patients and controls were collected using a standard interviewer-administered questionnaire, containing questions about smoking habits, alcohol intake, previous or ongoing treatment, use of medications, previous surgeries and family history of cancer.

The CG-Hp+ group was submitted to standard triple therapy consisting of amoxicillin (1g), clarithromycin (500 mg) and omeprazole (20 mg), all given twice daily for seven days. 3 months after treatment, the individuals underwent another endoscopy for collection of gastric biopsies of the antrum region. Immediately after collection, the biopsy specimens were placed into RNA Later solution (*Applied Biosystems*) and stored at -20ºC until nucleic acid extraction.

The study protocol was approved by the local Research Ethics Committee (CEP/IBILCE/UNESP number 030/10), and written informed consent was obtained from all participating individuals.

Molecular diagnosis of *H. pylori*

DNA/RNA extraction from the gastric biopsies was performed according to the protocol accompanying the reagent Trizol (*Invitrogen*) and the concentrations were determined in a NanoDrop® ND1000 spectrophotometer (*Thermo Scientific*). First, multiplex PCR was performed, using 100 ng of DNA in a final volume of 25μL containing specific primers for *H. pylori* genes such as *UreA* and *tsaA*, besides the constitutive human *CYP1A1* gene (Table 2). In brief, the PCR solution contained 1X buffer, 0.15 μM of each deoxyribonucleotide, 2 mM $MgCl_2$, 0.6 μM of each genomic DNA primer, and 1.8 U *Taq* Platinum DNA Polymerase (*Invitrogen*), in a final volume of 25 μL. The reaction consisted of an initial denaturation at 94ºC for 3 minutes, followed by 35 cycles at 94ºC for 45 seconds, 60ºC for 30 seconds and 72ºC for 1 minute, and a final extension step for 10 minutes at 72ºC. The reaction products were subjected to electrophoresis on 2% agarose gel stained with ethidium bromide. Molecular diagnosis was considered positive when at least one gene (*UreA* or *tsA*) had been amplified.

TaqMan quantitative real-time PCR (qPCR) for *TLR2* and *TLR4* mRNA

Reverse transcription (RT) of total RNA was performed using a High Capacity cDNA Archive Kit (*Applied Biosystems*), in a total volume of 25 μl,

according to the manufacturer's protocol. Then, qPCR was carried out in a *StepOnePlus Real Time PCR System 2.2.2* (*Applied Biosystems*), using specific TaqMan probes for target genes *TLR2* (assay ID Hs00610101_m1, *Applied Biosystems*) and *TLR4* (assay ID Hs01060206_m1, *Applied Biosystems*), and two reference genes, *ACTB* (part number: 4352935E, *Applied Biosystems*) and *GAPDH* (*Glyceraldehyde 3-phosphate dehydrogenase*) (part number: 4352934E, *Applied Biosystems*), used as endogenous controls according to the manufacturer's instructions. All reactions were performed in triplicate in a final volume of 20 µl, using 100 ng/µl cDNA and a blank to ensure the absence of contamination. Relative quantification (RQ) of *TLR2* and *TLR4* mRNA was obtained according to the model proposed by Livak & Schmittgen (24) and normalized to the *ACTB* and *GAPDH* reference genes and a pool of normal Hp- samples. The RQ was expressed as median (range) and considered upregulated for values of RQ > 2.

Immunohistochemical assay for TLR2, TLR4, Ki-67 antigen and TUNEL apoptosis

Immunohistochemical analysis was performed in 14 samples from the CG-Hp+ group before and after bacteria eradication and four samples from the normal Hp- group. Consecutive 4 µm-thick sections were cut from each trimmed paraffin block. Deparaffinized tissue slides were then submitted to antigen retrieval, using a high-temperature antigen-unmasking technique. The sections were incubated with specific primary antibodies overnight: rabbit polyclonal antibody anti-TLR2 (06-1119, 1:50 dilution; *Millipore*), mouse monoclonal anti-TLR4 (76B357.1,

1:200 dilution; *Abcam*), and polyclonal antibody for antigen Ki-67 (SP6, 1:100; *Cell Marque*). Then the slides were incubated with biotinylated secondary antibody (Picture Max Polymer Detection Kit, *Invitrogen*) for 30 minutes, following the manufacturer's protocol. Immunostaining was done with 3,3'diaminobenzidine tetrahydrochloride (DAB) containing 0.005% H_2O_2, counterstained with hematoxylin. Placenta mucosa and appendix tissue were used, respectively, as positive controls for the TLR2 and TLR4 proteins. The immunostaining was evaluated in the cytoplasm of the epithelial cells by densitometric analysis with an arbitrary scale going from 0 to 255, performed with Axio Vision software under a Zeiss-Axioskop II light microscope. Sixty equally distributed points were scored in each one of the regions, and the results were expressed as mean±SE.

For Ki-67, only nuclear, strong staining was considered positive. Five hundred epithelial cells were counted for each sample, and the percentage of positive cells defined the proliferating index (PI) (25). Apoptosis was detected by Terminal Uridine Deoxynucleotidyl Nick end-Labeling (TUNEL) using a FragEL™ DNA Fragmentation Detection Kit, Colorimetric –TdT Enzyme (*Calbiochem*), according to the manufacturer's protocol. Sections of human tonsils were used as positive controls. The apoptotic index (AI) was calculated as the percentage of TUNEL-positive cells (nuclear staining) in about 500 epithelial cells counted for each sample. All analyses were performed under a light microscope (x400 magnification). The proliferation rate was determined by dividing the proliferation index (PI) by the apoptotic index (AI).

Statistical analysis

Data analysis was performed using the computer software GraphPad Prism 5 version 5.01. The distribution of continuous data was evaluated using the D'Agostino & Pearson omnibus normality test or Shapiro-Wilk normality test. Data are presented as median and range, as mean ± standard deviation (SD), or as frequencies, according to the data distribution. Student's *t*-test for paired and unpaired data or correspondent nonparametric tests, such as the Mann-Whitney test and the Wilcoxon signed rank test, were used for comparisons between groups. To evaluate the association between relative gene expression and risk factors such as age, gender, smoking and drinking, the Mann-Whitney test was performed. The correlation between TLRs gene expression before and after eradication therapy was analyzed using Pearson's or Spearman's Correlation, as appropriate. For protein expression, the means obtained from the densitometry analysis were compared before and after treatment and with the normal Hp- group using ANOVA followed by the Bonferroni test. The level of significance was set at $p \leq 0.05$.

Results

The relative expression of *TLR2* and *TLR4* mRNA is not altered after successful eradication therapy

Table 3 shows the data regarding the relative expression levels of *TLR2* and *TLR4* mRNA of 37 CG-Hp+ patients who concluded the treatment

(completed treatment group) and 23 CG-Hp+ patients in which the bacteria were eradicated (successful treatment group), allowing paired analysis before and after eradication therapy. The relative expression levels of *TLR2* and *TLR4* mRNA after normalization with the *ACTB* and *GAPDH* reference genes and comparison with normal mucosa *H. pylori*-negative in both completed and successful treatment, either before or after treatment were increased significantly (data not shown). In the completed treatment group, no significant reduction was found after treatment in the relative expression levels of either *TLR2* or *TLR4* mRNA (*TLR2* RQ median= 1.55; and *TLR4* RQ median= 1.64) with, respectively, 38% and 46% of the cases being upregulated in comparison to the cases before the treatment (*TLR2* RQ median= 1.31, p= 0.291 and *TLR4* RQ median= 1.45, p= 0.084) and with, respectively, 32% and 40% of the cases showing upregulation. Therefore, for both genes, no significant differences were found before and after the treatment (p= 0.291 and p= 0.084, respectively).

In the successful treatment group, heterogeneity of relative expression levels for both *TLR2* and *TLR4* mRNAs can be observed before and after the treatment (Fig. 1A and B). The *TLR2* and *TLR4* relative expression levels in this group were slightly lower after treatment (*TLR2* RQ median= 1.47; and *TLR4* RQ median= 1.53) than in the completed treatment group, but the differences were not statistically significant. When we compared the relative expression levels for both the *TLR2* and *TLR4* mRNAs before and after the treatment in the group in which *H. pylori* was eradicated (successful treatment group), no significant differences were either observed for both genes (p= 0.533 and p= 0.094 for *TLR2* and *TLR4*,

respectively) (Fig. 1C and D). Furthermore, a positive correlation between the RQ values of *TLR2* and *TLR4* mRNA before and after in the successful treatment group (before: r^2= 0.85, p<0.0001; after: r^2= 0.554, p= 0.006) were found.

In another analysis, we evaluated the association between relative expression levels of *TLR2* and *TLR4* mRNA and the risk factors as age, gender, smoking and drinking (Table 4). A significant, about 3-fold increase in the median RQ of the *TLR4* mRNA was observed in males compared to females (p= 0.043) after the *H. pylori* eradication therapy. None of the other factors investigated showed significant differences, as well as no significant difference was observed in the levels of gene expression according to histological type of lesion (CG, AG and MAG (data not shown).

TLR2 and TLR4 protein and mRNA relative expressions are concordant

In normal mucosa, the TLR2 and TLR4 protein expression was weak or absent, mainly in the foveolar epithelium (Fig. 2A and B). Nevertheless, the CG-Hp+ samples collected before the treatment showed a cytoplasmatic, perinuclear and focal immunostaining pattern, mostly in the basal area of the foveolar epithelium. A strong expression in the inflammatory cells was also observed (Fig. 2C and D). After the eradication of *H. pylori*, an immunostaining pattern similar to the one observed before the treatment was found for both the TLR2 and TLR4 proteins (Fig. 2E and F).

The mean optical densitometry values observed in the normal Hp- group for TLR2 and TLR4 were 105.6 ± 2.7 and 101.4 ± 6.5, respectively. While the

CG-Hp+ group before treatment presented significantly increased mean values for both TLR2 (151.7 ± 6.1) and TLR4 (132.2 ± 4.7) in comparison with the normal Hp- group (p= 0.020 and p= 0.007, respectively). After eradication of the bacteria, both the TLR2 and TLR4 proteins showed a slight reduction in their mean optical densitometry values (136.1 ± 6.1 and 122.8 ± 5.8, respectively), but without significant differences (p= 0.064 and p= 0.198, respectively) (Fig. 2G and H), confirming the findings regarding the mRNA relative expression.

Eradication therapy leads to an increase in cell proliferation rate, measured by Ki-67 antigen and TUNEL assay.

The cell proliferation index measured before and after treatment by antigen Ki-67 in the normal Hp- group and in the CG-Hp+ group showed positive brown immunostaining in the nuclei of the foveolar epithelial cells and in the crypt glands (Fig. 3A, C and E).

In the normal Hp- group, the PI mean was 17.40 ± 2.86, in which 50% of the samples showed PI>20% (Table 5). In the CG-Hp+ successful treatment group the PI mean of 18.23 ± 2.85 was observed before treatment, however, after treatment (PI= 19.92 ± 2.0) there was no significant difference (p=0.379). The apoptotic nuclei detected by the TUNEL assay were evaluated in the same samples for Ki-67 and were mainly observed in the foveolar epithelium (Fig. 3B, D and F). The AI mean in the normal Hp- group was 1.00 ± 0.24 , while in the CG-Hp+ group before the treatment it was increased to 2.02 ± 0.34, decreasing significantly after the eradication therapy (0.88 ± 0.15; p=0.013) (Table 5). As a

consequence, the cell proliferation rate calculated by dividing PI by AI showed a significant increase after the treatment (p=0.027).

Discussion

In this study we investigated for the first time the occurrence of alterations in the *TLR2* and *TLR4* mRNA and protein expression in *H. pylori*-infected patients with chronic gastritis, before and after successful bacteria eradication treatment, besides evaluating the cell kinetics. Our results did not reveal significant changes in the relative expression levels of either *TLR2* or *TLR4* mRNA after treatment, which was confirmed by immunohistochemistry. Moreover, the mRNA expression of both receptors remained increased after eradication therapy compared to the normal Hp- group, showing that the eradication of the bacteria did not normalize the expression of these receptors, at least under the conditions evaluated. Additionally, we also observed a positive correlation between the mRNA expression values of *TLR2* and *TLR4*, both before and after treatment, and found after *H. pylori* eradication an increase in proliferation rate in consequence of reduction of apoptosis.

TLRs are transmembrane proteins that play a critical role in the recognition of pathogen components (26). LPS of Gram-negative bacteria are recognized mainly by TLR4 and also TLR2 activating signaling pathways that culminate in an inflammatory response (27). It is believed that the interaction between bacterial virulence and a genetically susceptible host is associated with more severe chronic inflammation, which may, in the long run, lead to cancer

(28). Under normal physiological conditions, the expression of these receptors in the mucosa of the gastrointestinal tract is low, due to the action of their antagonists, such as TOLLIP (Toll-interacting protein) and PPARγ (Peroxisome proliferator-activated receptor) that show higher levels, in order to prevent inappropriate activation of nonpathogenic antigens (29-31).

In our study, we observed a significantly increased expression for both *TLR2* and *TLR4* in CG-Hp+ patients even after successful *H. pylori* eradication compared to the noninfected normal mucosa samples. In children infected by *H. pylori*, Lagunes-Servin et al. (32) found an increase in the expression of the TLR2, TLR4, TLR5, and TLR9 in the gastric epithelium comparing with noninfected children, and also an association with pro- and anti-inflammatory cytokines (IL-8, TNF-α and IL-10). These findings confirm that *H. pylori* has the ability of to increase the *in vivo* expression of TLRs by gastric epithelial cells early during infection in children, starting a chronic and balanced inflammatory process that will continue for decades, and so may contribute to the development of *H. pylori*-associated diseases later in adulthood. Pimentel-Nunes et al. (33) observed that, considering the different TLRs of normal *H. pylori*-negative mucosa, the mRNA of *TLR5* was the most expressed, followed by those of *TLR2* and *TLR4*. Furthermore, these authors found *TLR2* and *TLR4* overexpression in intestinal metaplasia, independently of the *H. pylori* status, and in the dysplasia/cancer sequence. Moreover, upregulation of *TLR2* and *TLR4* mRNA was also observed in *H. pylori*-associated normal mucosa. These results were confirmed by immunohistochemical analyses, which found an increase in protein

expression in *H. pylori*-infected normal mucosa, further increasing in intestinal metaplasia and dysplasia/carcinoma. These findings suggest that progressive activation of these receptors, initially by *H. pylori*, but also by other PAMPs (pathogen-associated molecular patterns) or DAMPs (damage-associated molecular patterns), at later stages, may play an important role in gastric carcinogenesis and tumor progression (33).

Several groups of researchers have demonstrated TLRs, mainly TLR4 and TLR2, as pattern recognition receptors for *H. pylori*. Upregulation of *TLR4* expression responsiveness to LPS and *H. pylori* in gastric cell lines has been reported (34,35). *H. pylori* infection induced both *TLR4* mRNA and protein expression in AGS cells that were dependent on bacterial load and infection duration. However, the transfection of AGS cells with *TLR4* siRNA followed by the bacterial infection suppressed the expression of this receptor (34). Moreover, LPS of *H. pylori* upregulate TLR4 expression via TLR2 signaling in MKN28 gastric cell lines by the MEK1/2-ERK1/2 MAP kinase pathway (36), leading also to an increase in cell proliferation. Conversely, previous studies (37-39) did not observe any relevant role of TLR4 in the cellular recognition of *H. pylori* in AGC cells. These controversial results may be due to differences in the lipid A structures produced by distinct *H. pylori* strains (40-42). Therefore, the interaction of the bacteria with TLR2 should also be considered, mainly after the first contact with the gastric mucosa, triggering immunologic responses (43) such as induction of IL-8 and subsequent activation of NF-kB (11).

Our study revealed no reduction of the transcript levels of *TLR2* and *TLR4* or their proteins 2 to 3 months after treatment, showing that the successful eradication of *H. pylori* does not change the expression of these receptors within a short period after the treatment. Similarly, Garza-Gonzales et al. (44) found no quantitative differences in the *TLR4* and *TLR5* mRNA levels either, regardless of the presence or absence of *H. pylori* in gastric epithelial cells biopsies and AGS cells, suggesting that the mRNA levels of both receptors may not be influenced by the infection process, or at least not at the time points selected for analysis. However, in our study, we observed higher levels of both *TLR2* and *TLR4* mRNA and proteins in *H. pylori*-infected mucosa compared to non-infected normal mucosa. It should however be taken into consideration that the post-treatment time elapsed until biopsy collection may not have been sufficient for mucosal renovation and transcription level normalization.

Generally, the imbalance between proliferation and apoptosis is associated with the development of cancer (45). One of the pathways by which *H. pylori* is associated to gastric carcinogenesis may involve the disruption of cell kinetics, with activation of cell proliferation and induction of apoptosis as a response to the *H. pylori*-induced mucosal damage (46). However, the effect of the eradication therapy on these processes remains unclear.

Our results demonstrated that, after eradication of *H. pylori*, the proliferation index of the gastric epithelial cells no showed significant changes in the chronic gastritis patients, while the apoptotic index was reduced. Similar results were reported by Sougioultzis et al. (46) who observed a significant

reduction of apoptosis, but not of the proliferation index, in gastric epithelial cells after eradication of *H. pylori,* as well as in multifocal atrophic gastritis patients one year after treatment (47), suggesting that the continuous cell proliferation can provide genetic transformations and could eventually lead to histopathologic events, such as atrophy, metaplasia, or dysplasia. The authors also observed a decrease in the apoptosis of inflammatory cells that may reduce inflammatory triggers, although, one year after treatment, no significant reduction of B lymphocytes and macrophages had occurred.

In contrast, in patients with *H. pylori*-infected gastric cancer (49) and in patients with gastritis-associated intestinal metaplasia (50), a reduction in the proliferation index was reported after treatment, but the apoptotic index remained unchanged. While a significant reduction of both Ki-67 labeling and the apoptotic index in the gastric mucosa after *H. pylori* eradication in patients with gastritis was shown by Ohkura et al. (45).

In gastric cancer patients, Watari et al. (50) reported higher cellular proliferation rates compared to chronic gastritis patients before *H. pylori* eradication, whereas the apoptotic index presented no significant changes. However, after *H. pylori* eradication, there were no significant changes in the proliferation index between groups, but the apoptotic index increased in both groups after treatment. These divergent results may be related to a variety of factors, such as the histological type of the gastric lesion, bacterial virulence factors, and the use of different markers to evaluate cell proliferation and apoptosis, as well as the time elapsed after the eradication of the bacteria. In

general, it is expected that, after *H pylori* eradication, patients with precancerous gastric lesions show improvements in their lesions, accompanied by suppression of cell proliferation and induction of cell apoptosis, as reported by Zhang et al. (19).

When we compared the expression levels of *TLR2* and *TLR4* mRNA with risk factors, we did not find any association, except for a positive association between an about 3-fold upregulated expression of *TLR4* mRNA in males after successful treatment of *H pylori*. We did not find any explanation for the influence of gender on the TLR receptor expression or response to treatment for *H pylori*. However, this association may be due to hormonal differences, such as the interference of sex hormones with the secretion of IL-8 and mRNA levels (52), as well as other inflammatory mediators, such as annexin-A1 (53).

Despite the importance of this study, some limitations, also observed in other studies of this kind (54,55) should be considered, such as the number of patients who completed the treatment and had the bacteria successfully eradicated; the effect of inflammatory cells on gene expression levels in gastric mucosa, although this can be differentiated by immunohistochemical analysis; and also the follow-up time to obtaining biopsies after treatment. Moreover, alterations in mRNA expression levels after *H. pylori* infection eradication therapy have been demonstrated, involving genes associated with cell damage, inflammation, proliferation, apoptosis and intestinal differentiation (55,56).

In conclusion, we report here an increase in *TLR2* and *TLR4* mRNA and protein expression in CG-Hp+ patients before eradication therapy and the

maintaining of this expression pattern even after treatment, suggesting that these receptors remain expressed in the gastric mucosa even after eradication of the bacteria, at least for the period evaluated. In addition, the alteration in cellular kinetics resulting from the infection by the pathogen may be a response to damage caused by the bacteria to the gastric mucosa. So, the next step is to evaluate other genes related with the inflammatory cascade induced by *H. pylori*, such as those encoding cytokines, malignant transformation processes, as well as the involvement of microRNAs. Therefore, considering the higher risk of malignant progression in patients infected by *H. pylori* for a long time, further investigations are needed to clarify the changes in gene expression occurred in response to the eradication treatment.

Acknowledgments and Disclosure

The authors are grateful to Dr. Sebastião Roberto Taboga and Luiz Roberto Faleiros Junior for their help with the histological sections, Isaque Santana for technical assistance during the immunohistochemical assay, and nurse Gizelda Warick Mazzale for her help in contacting patients. This study was supported by grants from the Brazilian agencies FAPESP (2012/15036-8) and CNPq (474776/2013-1).

The authors declare no competing interests.

REFERENCES

1. **Parkin DM.** The global health burden of infection-associated cancers in the year 2002. International Journal of Cancer 2006; **118**:3030-3044.

2. **Correa P.** Human model of gastric carcinogenesis. Cancer Res 1988; **48**:3554-3560.
3. **de Oliveira JG, Silva AE.** Polymorphisms of the TLR2 and TLR4 genes are associated with risk of gastric cancer in a Brazilian population. World J Gastroenterol 2012; **18**:1235–1242.
4. **Xue H, Liu J, Lin B, Wang Z, Sun J, Huang G.** A meta-analysis of interleukin-8-251 promoter polymorphism associated with gastric cancer risk. PLoS ONE 2012; 7:e28083. doi: 10.1371/journal.pone.0028083.
5. **Lamb A, Chen LF.** Role of the *Helicobacter pylori*-induced inflammatory response in the development of gastric cancer. J Cell Biochem 2013; **114**:491-497.
6. **Moss SF, Blaser MJ.** Mechanisms of disease: inflammation and the origins of cancer. Nat Clin Pract Oncol 2005; **2**:90-97.
7. **Peek RM Jr, Crabtree JE.** *Helicobacter* infection and gastric neoplasia. J Pathol 2006; **208**:233-248.
8. **Wilson KT, Crabtree JE.** Immunology of *Helicobacter pylori*: insights into the failure of the immune response and perspectives on vaccine studies. Gastroenterology 2007. **133**:288-308.
9. **Akira S, Uematsu S, Takeuchi O.** Pathogen recognition and innate immunity. Cell 2006; **124**:783-801.
10. **Matsuura M.** Structural modifications of bacterial lipopolysaccharide that facilitate Gram-Negative bacteria evasion of host innate immunity. Front Immunol 2013; **4:**109.

11. **Yokota S, Ohnishi T, Muroi M, Tanamoto K, Fujii N, Amano K.** Highly-purified *Helicobacter pylori* LPS preparations induce weak inflammatory reactions and utilize Toll-like receptor 2 complex but not Toll-like receptor 4 complex. FEMS Immunol Med Microbiol 2007; 51:140-148.
12. **Re F, Strominger JL.** Heterogeneity of TLR-induced responses in dendritic cells: from innate to adaptive immunity. Immunobiology 2004; 209:191-8.
13. **Ding SZ, Torok AM, Smith MF Jr, Goldberg JB.** Toll-like receptor 2-mediated gene expression in epithelial cells during *Helicobacter pylori* infection. Helicobacter 2005; 10:193-204.
14. **Akira S, Takeda K.** Toll-like receptor signalling. Nat Rev Immunol 2004; 4: 499–511.
15. **Fukata M, Abreu MT.** Pathogen recognition receptors, cancer and inflammation in the gut. Curr Opin Pharmacol 2009; 9:680-687.
16. **Tsan MF.** Toll-like receptors, inflammation and cancer. Seminars in Cancer Biology 2006; 16:32-7.
17. **Ioannou S, Voulgarelis M.** Toll-like receptors, tissue injury and tumourigenesis. Mediators of Inflammation 2010; pii: 581837. doi: 10.1155/2010/581837.
18. **Konturek PC, Konturek SJ, Brzozowski T.** Gastric cancer and Helicobacter pylori infection. J Physiol Pharmacol 2006; 57:51-65.
19. **Zhang LJ, Wang SY, Huo XH, Zhu ZL, Chu JK, Ma JC, Cui DS, Gu P, Zhao ZR, Wang MW, Yu J.** Anti-*Helicobacter pylori* therapy followed by

celecoxib on progression of gastric precancerous lesions. World J Gastroenterol 2009; 15:2731-38.

20. **Wu MS, Chow LP, Lin JT, Chiou SH.** Proteomic identification of biomarkers related to *Helicobacter pylori*-associated gastroduodenal disease: Challenges and opportunities. J Gastroenterol Hepatol 2008; 23:1657-61.
21. **Chuah SK, Tsay FW, Hsu PI, Wu DC.** A new look at anti-*Helicobacter pylori* therapy. World J Gastroenterol 2011; 17:3971-5.
22. **Vakil N, Mégraud F.** Eradication treatment for *Helicobacter pylori*. Gastroenterol 2007; 133:985-1001.
23. **Dixon MF, Genta RM, Yardley JH, Correa P.** Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. Am J Surg Pathol 1996; 20:1161-1181.

24. Livak K, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCt method. Methods 2001;25:402–408.

24. **Bellini MF, Cury PM, Silva AE.** Expression of Ki-67 antigen and caspase-3 protein in benign lesions and esophageal carcinoma. Anticancer Res 2010; 30:2845-2850.
25. **Medzhitov R, Janeway C Jr.** Innate immunity. N Engl J Med 2000; 343**:**338-344.
26. **Amieva MR, El-Omar EM.** Host-bacterial interactions in *Helicobacter pylori* infection. Gastroenterology 2008; 134:306–323.

27. **Machado JC, Figueiredo C, Canedo P, Pharoah P, Carvalho R, Nabais S, Castro Alves C, Campos ML, Van Doorn LJ, Caldas C, Seruca R, Carneiro F, Sobrinho-Simões M.** A proinflammatory genetic profile increases the risk for chronic atrophic gastritis and gastric carcinoma. Gastroenterology 2003; 125:364–371.

28. **Pimentel-Nunes P, Soares JB, Roncon-Albuquerque R Jr, Dinis-Ribeiro M, Leite-Moreira AF.** Toll-like receptors as therapeutic targets in gastrointestinal diseases. Expert Opin Ther Targets 2010; 14:347–368.

29. **Melmed G, Thomas LS, Lee N, Tesfay SY, Lukasek K, Michelsen KS, Zhou Y, Hu B, Arditi M, Abreu M.** 2003. Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor2-dependent bacterial ligands: implications for host-microbial interactions in the gut. J Immunol 2003; 170:1406–15.

30. **Liew FY, Xu D, Brint EK, O'Neill LA.** Negative regulation of toll-like receptor mediated immune responses. Nat Rev Immunol 2005; 5:446–458.

31. **Lagunes-Servin H, Torres J, Maldonado-Bernal C, Perez-Rodriguez M, Huerta-Yepez S, la Garza AM, Munoz-Perez L, Flores-Luna L, Ramon-Garcia G, Camorlinga-Ponce M.** Toll-Like Receptors and Cytokines are Upregulated during *Helicobacter pylori* Infection in Children. Helicobacter 2014; 18: 423–432. doi:10.1111/hel.12067

32. **Pimentel-Nunes P, Gonçalves N, Boal-Carvalho I, Afonso L, Lopes P, Roncon-Albuquerque R Jr, Henrique R, Moreira-Dias L, Leite-Moreira AF, Dinis-Ribeiro M.** *Helicobacter pylori* induces increased expression of

Toll-like receptors and decreased Toll-interacting protein in gastric mucosa that persists throughout gastric carcinogenesis. Helicobacter 2012; 18:22-32. doi: 10.1111/hel.12008.

33. **Su B, Ceponis PJ, Lebel S, Huynh H, Sherman PM.** *Helicobacter pylori* activates Toll-like receptor 4 expression in gastrointestinal epithelial cells. Infect Immun 2003; 71:3496 –3502.
34. **Lu DY, Chen HC, Yang MS, Hsu YM, Lin HJ, Tang CH, Lee CH, Lai CK, Lin CJ, Shyu WC, Lin FY, Lai CH.** Ceramide and Toll-like receptor 4 are mobilized into membrane rafts in response to *Helicobacter pylori* infection in gastric epithelial cells. Infect Immun 2012; 80:1823–1833.
35. **Yokota S, Okabayashi T, Rehli M, Fujii N, Amano K.** *Helicobacter pylori* lipopolysaccharides upregulate Toll-like receptor 4 expression and proliferation of gastric epithelial cells via the MEK1/2-ERK1/2 Mitogen-Activated Protein Kinase pathway. Infect Immun 2010; 78:468-76.
36. **Maeda S, Akanuma M, Mitsuno Y, Hirata Y, Ogura K, Yoshida H, Shiratori Y, Omata M.** Distinct mechanism of *Helicobacter pylori*-mediated NF-kappa B activation between gastric cancer cells and monocytic cells. J Biol Chem 2001; 276:44856–44864.
37. **Bäckhed F, Rokbi B, Torstensson E, Zhao Y, Nilsson C, Seguin D, Normark S, Buchan AM, Richter-Dahlfors A.** Gastric mucosal recognition of *Helicobacter pylori* is independent of Toll-like receptor 4. J Infect Dis 2003; 187:829–836.

38. **Otte JM, Neumann HM, Brand S, Schrader H, Schmidt WE, Schmitz F.** Expression of beta-defensin 4 is increased in human gastritis. European Journal of Clinical Investigation 2009; 39:126-138.

39. **Kawahara T, Teshima S, Oka A, Sugiyama T, Kishi K, Rokutan K.** Type I *Helicobacter pylori* lipopolysaccharide stimulates Toll-like receptor 4 and activates mitogen oxidase 1 in gastric pit cells. Infect Immun 2001; 69:4382-4389.

40. **Ishihara S, Rumi MA, Kadowaki Y, Ortega-Cava CF, Yuki T, Yoshino N, Miyaoka Y, Kazumori H, Ishimura N, Amano Y, Kinoshita Y.** Essential role of MD-2 in TLR4-dependent signalling during *Helicobacter pylori*-associated gastritis. J Immunol 2004; 173:1406–1416.

41. **Hirata Y, Ohmae T, Shibata W, Maeda S, Ogura K, Yoshida H, Kawabe T, Omata M.** MyD88 and TNF receptor-associated factor 6 are critical signal transducers in *Helicobacter pylori*-infected human epithelial cells. J Immunol 2006; 176:3796–3803.

42. **Smith MF Jr, Mitchell A, Li G, Ding S, Fitzmaurice AM, Ryan K, Crowe S, Goldberg JB.** Toll-like receptor (TLR) 2 and TLR5, but not TLR4, are required for *Helicobacter pylori*-induced NF-kappa B activation and chemokine expression by epithelial cells. J Biol Chem 2003; 278:32552–32560.

43. **Garza-González E, Bocanegra-García V, Bosques-Padilla FJ, Flores-Gutiérrez JP, Moreno F, Perez-Perez GI.** mRNA levels of TLR4 and TLR5 are independent of H pylori. World J Gastroenterol 2008; 14:5306-10.

44. Wyllie AH. The biology of cell death in tumors. Anticancer Res 1996; 5:131–136.

45. Ohkura Y, Furihata T, Kawamata H, Tabuchi M, Kubota K, Terano A, Sakai T, Fujimori T. Evaluation of cell proliferation and apoptosis in *Helicobacter pylori* gastritis using an image analysis processor. Gastric Cancer 2003; 6:49–54.

46. Sougioultzis S, Foukas PG, Tzivras M, Kourtessas D, Gorgoulis VG, Davaris P, Archimandritis AJ. Alterations in the proliferating compartment of gastric mucosa during *Helicobacter pylori* infection: the putative role of epithelial cells expressing p27(kip1). Mod Pathol 2003; 16:1076-1085.

47. Guarner J, Bartlett J, Seitz R, Whistler T, Herrera-Goepfert R, Mohar A, Sanchez L, Halperin D, Parsonnet J. Cell proliferation and inflammation on biopsy samples with multifocal atrophic gastritis before and 1 year after *Helicobacter pylori* eradication. Arch Pathol Lab Med 2005; 129:1451-6.

48. Mitani T, Shirasaka D, Aoyama N, Miki I, Morita Y, Ikehara N, Matsumoto Y, Okuno T, Toyoda M, Miyachi H, Yoshida S, Chayahara N, Hori J, Tamura T, Azuma T, Kasuga M. Role of metallothionein in *Helicobacter pylori*-positive gastric mucosa with or without early gastric cancer and the effect on its expression after eradication therapy. J Gastroenterol Hepatol 2008; 23:e334-9. DOI: 10.1111/j.1440-1746.2007.05124.x

49. **Leung WK, Yu J, To KF, Go MY, Ma PK, Chan FK, Sung JJ.** Apoptosis and proliferation in *Helicobacter pylori*-associated gastric intestinal metaplasia. Aliment. Pharmacol Ther 2001; 15:1467-1472.

50. **Watari J, Tanaka A, Tanabe H, Sato R, Moriichi K, Zaky A, Okamoto K, Maemoto A, Fujiya M, Ashida T, Das KM, Kohgo Y.** K-ras mutations and cell kinetics in *Helicobacter pylori* associated gastric intestinal metaplasia: a comparison before and after eradication in patients with chronic gastritis and gastric cancer. J Clin Pathol 2007; 60:921-26.

51. **Campa D, Zienolddiny S,Maggini V, Skaug V, Haugen A, Canzian F.** Association of a common polymorphism in the cyclooxygenase 2 gene with risk of non-small cell lung cancer. Carcinogenesis 2004; 25:229-235.

52. **Jorge YC, Mataruco MM, Araújo LP, Rossi AFT, de Oliveira JG, Valsechi MC, Caetano A, Miyazaki K, Fazzio CSJ, Thomé JA, Rahal P, Oliani SM, Silva AE.** Expression of Annexin-A1 and Galectin-1 anti-inflammatory proteins and mRNA in Chronic Gastritis and Gastric Cancer. Mediators of Inflammation 2013; doi: 10.1155/2013/152860.

53. **Mannick EE, Schurr JR, Zapata A, Lentz JJ, Gastanaduy M, Cote RL, Delgado A, Correa P, Correa H.** Gene expression in gastric biopsies from patients infected with *Helicobacter pylori*. Scand J Gastroenterol 2004; 39:1192–200.

54. **Tsai CJ, Herrera-Goepfert R, Tibshirani RJ, Yang S, Mohar A, Guarner J, Parsonnet J.** Changes of gene expression in gastric preneoplasia following

Helicobacter pylori eradication therapy. Cancer Epidemiology, Biomarkers & Prevention 2006; 15**:**272-80.

55. **Kim SS, Meitner P, Konkin TA, Cho YS, Resnick MB, Moss SF.** Altered expression of Skp2, c-Myc and p27 proteins but not mRNA after *H. pylori* eradication in chronic gastritis. Mod Pathol 2006; 19:49-58.

Legend of Figures

Figure 1 Relative expression levels of *TLR2* and *TLR4* RNAm in patients with chronic gastritis before and after *H. pylori* eradication therapy of the successful treatment group. Gene expression levels (Log2RQ) of (A) *TLR2* and (B) *TLR4* per individual evaluated; RQ median of (C) *TLR2* and (D) *TLR4* mRNA before and after *H. pylori* eradication. Data are presented as median and range for experiments performed in triplicate. Statistical significance was determined using Wilcoxon's signed rank test.

Figure 2 Immunohistochemistry images of Toll-like receptors (TLRs) in normal gastric mucosa (NM) and chronic gastritis (CG). Normal mucosa (A – TLR2; B – TLR4) – normal glands with no staining or low expression intensity; *H. pylori*-positive chronic gastritis (C – TLR2; D – TLR4) - foveolar epithelial cells and glands (arrows) and inflammatory cells (arrowhead) before treatment, presenting moderate to strong TLR expression compared to normal mucosa; (E – TLR2; F – TLR4) - foveolar epithelial cells and glands and inflammatory cells after treatment (no significant reduction of protein expression was detected). Counterstain: Hematoxylin. Bars: 50 µm. (G-H) Densitometry analyses (Mean±SE). *: $p<0.05$. a.u.= arbitrary unit.

Figure 3 Evaluation of cell proliferation and apoptosis. (A, C and E) Ki-67 immunohistochemistry images of normal gastric mucosa and *H. pylori*-positive chronic gastritis. (A) Normal mucosa (NM) - immunostaining of nuclei (arrowhead) was detected in crypt glands; (C) *H. pylori*-positive chronic gastritis before treatment, and (E) after treatment showing immunostaining of nuclei (arrowhead) in the crypt glands. Note the presence of labeled nuclei in the basal area glands of the crypt. Counterstain: Hematoxylin. Bars: 50 µm. G) Statistical analysis (paired *t*-test) no showed significant difference in the mean of proliferative activity in the successful treatment group ($p=0.379$). (B, D and F)

Detection of apoptotic cells by TUNEL assay. Normal mucosa (B); *H. pylori*-positive chronic gastritis [D: detail of the apoptotic nucleus (arrowhead)] – epithelial cells and crypt glands showed significantly increased apoptotic index before compared to after eradication therapy (F). Counterstain: Methyl Green. Bars: 50 µm. (H) Reduction of apoptotic nuclei in gastric lesions after successful *H. pylori* eradication therapy. (I) Increased cell proliferation rate observed in chronic gastritis after successful *H. pylori* eradication therapy. *:$p < 0.05$.

Table 1. Demographic and clinicopathological data of *H. pylori*-positive patients with chronic gastritis.

Patients	**Total N= 59**
***Age**, mean (SD), years*	48.0 ± 15.9
Range	21 – 82
Gender	
Male	26 (44%)
Female	33 (56%)
Drinking	
Yes	19 (32%)
No	36 (61%)
Not available	4 (7%)
Smoking	
Yes	21 (36%)
No	36 (61%)
Not available	2 (3%)
Histological Diagnosis	
Chronic gastritis	45 (76%)
Atrophic gastritis	8 (14%)
Atrophic gastritis-associated intestinal metaplasia	6 (10%)
Eradication therapy	
Completed treatment	37 (63%)
Successful treatment	23 (62%)

N: number of individuals.

Table 2: Primer sequences and size of the fragments generated to determine *H. pylori* infection.

Gene	Position	GenBank* Access	Sequence 5'- 3'	Fragment size (bp)
UreA	754333 754648	CP006610.1	F: TTCCTGATGGGACCAAACTC R: TTACCGCCAATGTCAATCAA	316
tsaA	37 449	AY762757.1	F: CCTGCCGTTTTAGGAAACAA R: TCCGCATTCCTACCTAATGG	413

*http://www.ncbi.nlm.nih.gov/genbank/

Table 3. Comparison of *TLR2* and *TLR4* mRNA relative expression levels before and after *H. pylori* eradication therapy.

	Before treatment		**After treatment**
TLR2	N		N
Completed Treatment	37		37
Upregulated	12 (32%)		14 (38%)
Median RQ	1.31		1.55
Range	0.37 – 23.05		0.34 – 43.12
P value		0.291	
Successful Treatment	23		23
Upregulated	8 (35%)		9 (39%)
Median RQ	1.32		1.47
Range	0.37 – 12.63		0.34 – 43.12
P value		0.533	
TLR4			
Completed Treatment	37		37
Upregulated	15 (40%)		17 (46%)
Median RQ	1.45		1.64
Range	0.50 – 11.09		0.56 – 28.07
P value		0.084	
Successful Treatment	23		23
Upregulated	8 (35%)		10 (43%)
RQ Median	1.26		1.53
Range	0.50 – 7.17		0.64 – 28.07
P value		0.094	

N: number of individuals; p: probability; RQ: relative quantification; Statistical analysis by Wilcoxon signed rank test.

Table 4. Comparison of *TLR2* and *TLR4* mRNA relative expression levels according to variables age, gender, drinking and smoking, before and after *H. pylori* treatment.

Variables	*TLR2*		*TLR4*	
	Before	**After**	**Before**	**After**
Age (years)				
< 46	9 (37%)	9 (39%)	9 (37%)	9 (37%)
(median; range)	1.33 (0.44 - 3.02)	0.83 (0.50- 3.97)	1.53 (0.65 – 3.26)	1.42 (090 – 4.11)
≥ 46	15 (63%)	14 (61%)	15 (63%)	15 (63%)
(median; range)	1.31 (0.37 - 12.63)	2.17 (0.34- 43.12)	1.19 (0.50 – 7.17)	1.62 (0.64 – 28.07)
P	0.952	0.361	0.676	0.720
Gender				
Female	13 (54%)	12 (52%)	13 (54%)	13 (54%)
(median; range)	1.18 (0.44 – 3.02)	1.24 (0.34– 4.75)	1.08 (0.50 – 3.23)	0.98 (0.72 – 4.11)
Male	11 (46%)	11 (48%)	11 (46%)	11 (46%)
(median; range)	1.32 (0.37 – 12.63)	2.46 (0.50– 43.12)	1.49 (0.85 – 7.17)	3.10 (0.64 – 28.07)
P	0.385	0.735	0.247	0.043
Drinking				
Yes	8 (35%)	8 (36%)	8 (35%)	8 (35%)
(median; range)	1.89 (0.37 – 12.63)	0.74 (0.50 – 43.12)	1.84 (0.86 – 7.17)	1.52 (0.64 – 28.07)
No	15 (65%)	14 (64%)	15 (65%)	15 (65%)
(median; range)	1.07 (0.44 – 3.02)	1.51 (0.34 – 7.95)	1.19 (0.50 – 3.22)	1.46 (0.72 - 4.49)
P	0.208	0.609	0.186	0.641
Smoking				
Yes	9 (39%)	9 (41%)	9 (39%)	9 (39%)
(median; range)	2.30 (0.37 – 12.63)	2.46 (0.34 – 43.12)	2.19 (0.50 – 7.17)	1.62 (0.64 – 28.07)
No	14 (61%)	13 (59%)	14 (61%)	14 (61%)
(median; range)	1.19 (0.54 – 2.70)	1.01 (0.50 – 7.95)	1.18 (0.52 – 3.26)	1.44 (0.72 – 4.49)
P	0.508	0.423	0.361	0.359

p: probability (statistical analysis by Mann-Whitney test and Wilcoxon's signed rank test)

Table 5. Distributions of proliferation index determined by antigen Ki-67 and apoptotic index by TUNEL assay in normal *H. pylori*-negative (normal Hp-) gastric mucosa and in *H. pylori*-positive (CG-Hp+) chronic gastritis, before and after *H. pylori* eradication therapy.

Groups	**N**	**Ki-67 (PI)**		**Mean**	**TUNEL (AI)**	**Proliferation Rate**
		10-20%	**> 20%**		**Mean**	**(PI/AI) Mean**
Normal Hp-	4	2 (50%)	2 (50%)	17.40 ± 2.86	1.00 ± 0.24	22.33 ± 7.01
CG-Hp+						
Before	14	3 (21%)	11 (79%)	18.23 ± 2.85	2.02 ± 0.34	14.28 ± 4.41
After	14	1 (7%)	13 (93%)	19.92 ± 2.07	0.88 ± 0.15	34.35 ± 11.16
P *value*				0.379	0.013	0.027

N: number of individuals; PI: proliferation index; AI: apoptotic index; p: probability; statistical analysis using *t*-test and Wilcoxon signed rank test.

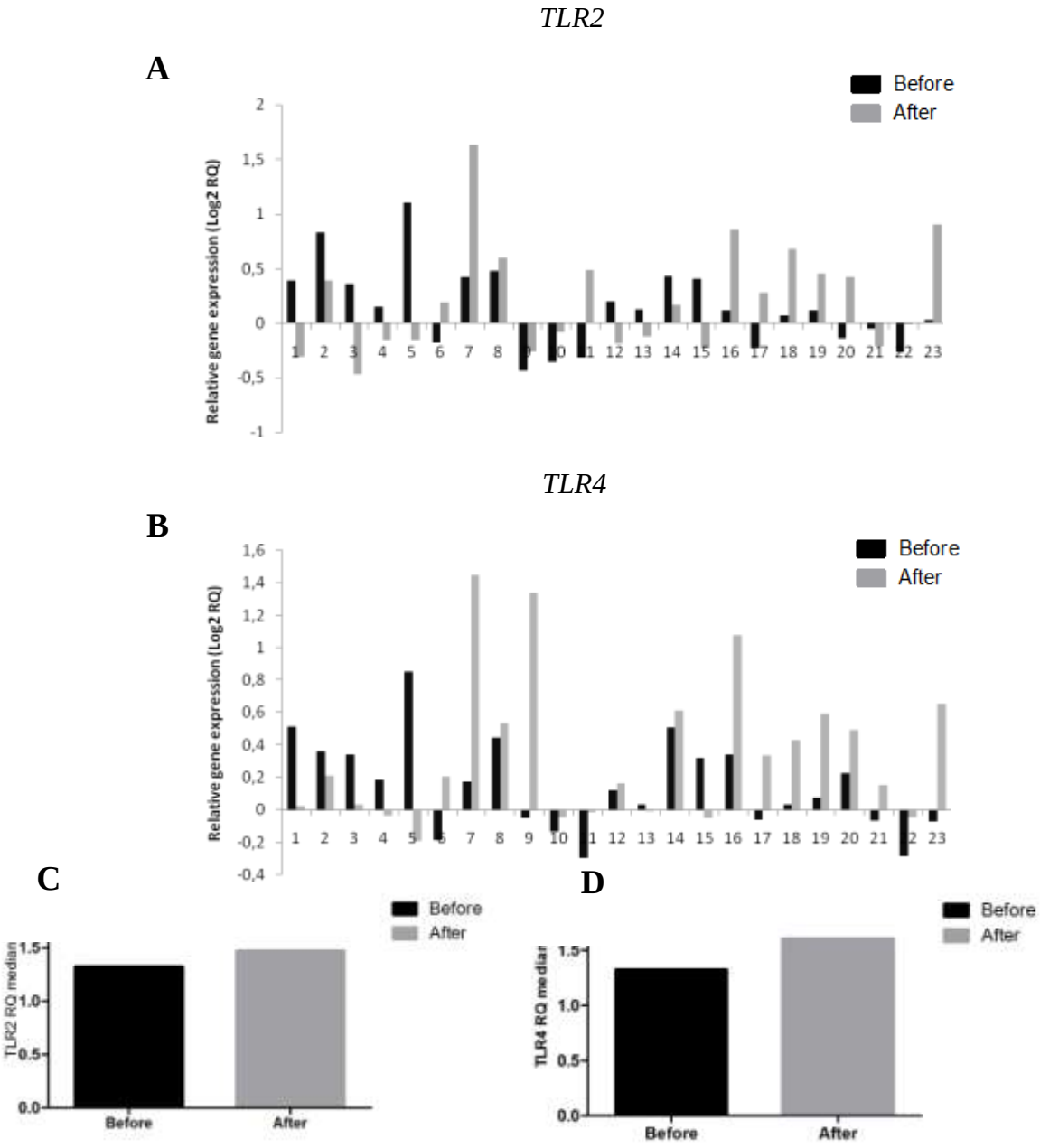
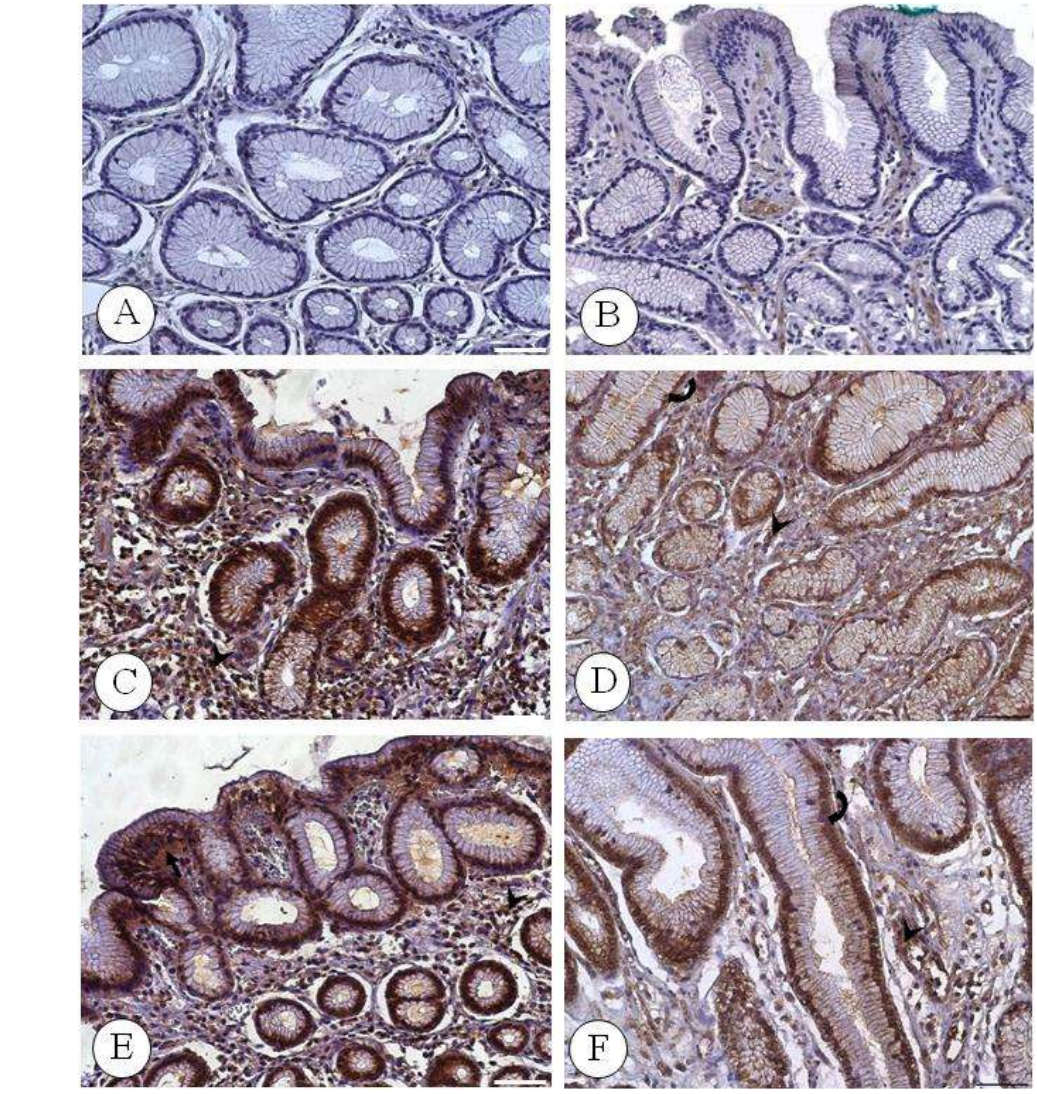


Figure 1. Relative expression levels of *TLR2* and *TLR4* RNAm in patients with chronic gastritis before and after *H. pylori* eradication therapy of the successful treatment group. Gene expression levels (Log2RQ) of (A) *TLR2* and (B) *TLR4* per individual evaluated; RQ median of (C) *TLR2* and (D) *TLR4* mRNA before and after *H. pylori* eradication. Data are presented as median and range for

experiments performed in triplicate. Statistical significance was determined using Wilcoxon's signed rank test.



(G) (H)

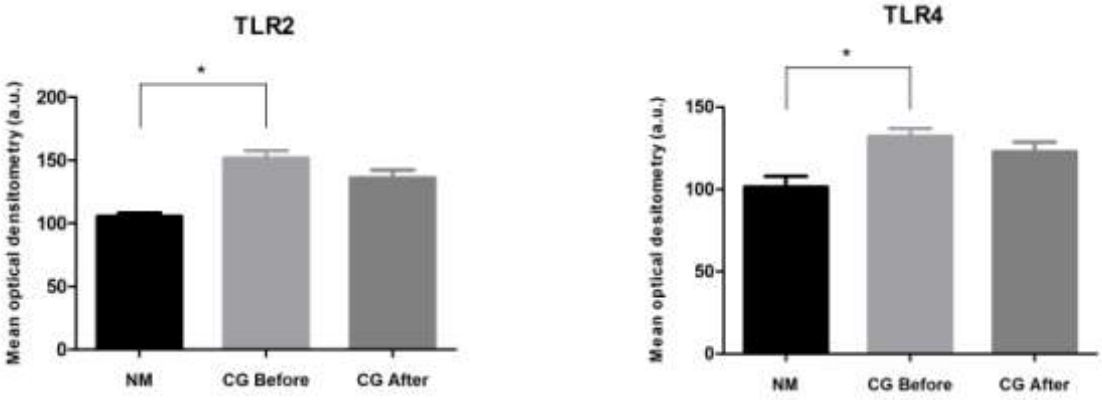


Figure 2. Immunohistochemistry images of Toll-like receptors (TLRs) in normal gastric mucosa (NM) and chronic gastritis (CG). Normal mucosa (A – TLR2; B – TLR4) – normal glands with no staining or low expression intensity; *H. pylori*-

positive chronic gastritis (C – TLR2; D – TLR4) - foveolar epithelial cells and glands (arrows) and inflammatory cells (arrowhead) before treatment, presenting moderate to strong TLR expression compared to normal mucosa; (E – TLR2; F – TLR4) - foveolar epithelial cells and glands and inflammatory cells after treatment (no significant reduction of protein expression was detected). Counterstain: Hematoxylin. Bars: 50 µm. (G-H) Densitometry analyses (Mean±SE). *: $p<0.05$. a.u.= arbitrary unit.

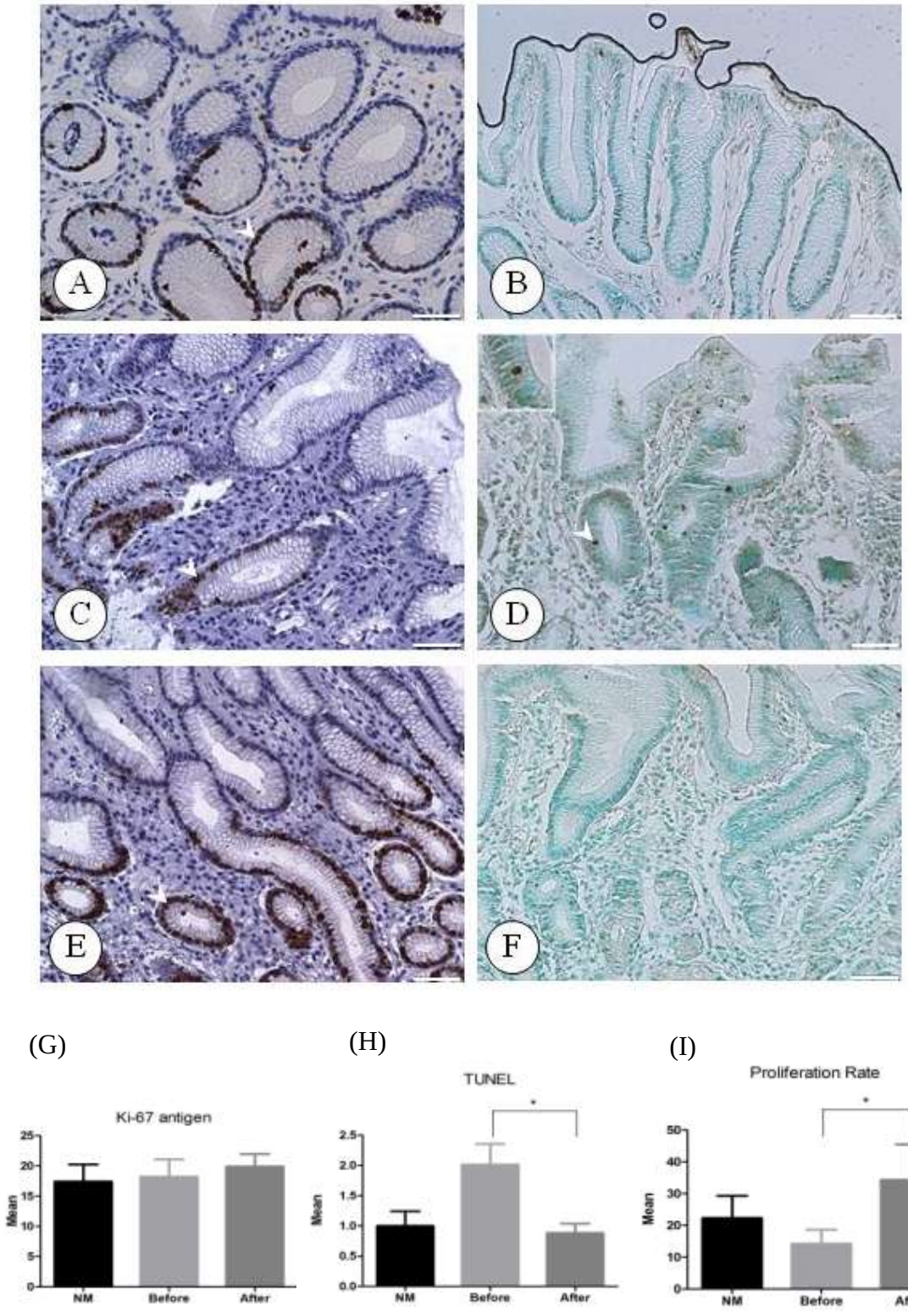


Figure 3. Evaluation of cell proliferation and apoptosis. (A, C and E) Ki-67 immunohistochemistry images of normal gastric mucosa and *H. pylori*-positive chronic gastritis. (A) Normal mucosa (NM) - immunostaining of nuclei

(arrowhead) was detected in crypt glands; (C) *H. pylori*-positive chronic gastritis before treatment, and (E) after treatment showing immunostaining of nuclei (arrowhead) in the crypt glands. Note the presence of labeled nuclei in the basal area glands of the crypt. Counterstain: Hematoxylin. Bars: 50 µm. G) Statistical analysis (paired *t*-test) no showed significant difference in the mean of proliferative activity in the successful treatment group (p=0.379). (B, D and F) Detection of apoptotic cells by TUNEL assay. Normal mucosa (B); *H. pylori*-positive chronic gastritis [D: detail of the apoptotic nucleus (arrowhead)] – epithelial cells and crypt glands showed significantly increased apoptotic index before compared to after eradication therapy (F). Counterstain: Methyl Green. Bars: 50 µm. (H) Reduction of mean of apoptotic nuclei in gastric lesions after successful *H. pylori* eradication therapy. (I) Increased cell proliferation rate observed in chronic gastritis after successful *H. pylori* eradication therapy. *:p< 0.05.

Artigo III

Effect of *H. pylori* eradication on expression of the cytokines and cellular mediators in patients with gastric lesions.

Aline Cristina Targa Cadamuro[a], Ana Flávia Teixeira Rossi[a], Joice Matos Biselli-Périco[a], Fausto Nasser[b], Patrícia Fucuta Pereira[b], Edla Bedin Polsinelli Mascarin[c#], Kátia Ramos Moreira Leite[d], Eny Maria Goloni-Bertollo[e], Ana Elizabete Silva[a #].

[a]UNESP, São Paulo State University, Department of Biology, São José do Rio Preto Campus, SP, Brazil; [b]Base Hospital, São José do Rio Preto, SP, Brazil; [c#]João Paulo II State Hospital, Medical Specialties Outpatient Clinic, São José do Rio Preto, SP, Brazil; [d]USP, São Paulo University, School of Medicine, São Paulo, SP, Brazil; [e]FAMERP, School of Medicine, São José do Rio Preto, SP, Brazil.

#Address correspondence to Ana Elizabete Silva

Rua Cristovão Colombo, 2265, São José do Rio Preto, SP, CEP: 15054-000, Brazil

E-mail: anabete@ibilce.unesp.br

Abstract

Helicobacter pylori (*H. pylori*) infection is the main causative agent of gastric malignancies through the chronic inflammation induced by the host immune response. *H. pylori* and its *CagA* and *VacA* virulence factors trigger the induction of cytokines, may deregulate gene expression, and activate multiple intracellular pathways, so starting the inflammatory and carcinogenic processes. Once that *H. pylori* eradication can prevent the progression of premalignant lesions, the goal of this study was investigate whether *H. pylori* eradication interferes on the mRNA and protein expression of genes involved with inflammatory and immune response, cell proliferation and regeneration, and apoptosis. Thus, we evaluated the mRNA and protein expression of *IL-8*, *TGF-β*, *REG3A*, *PLAT*, *PAI-1* and *IFITM1* genes in patients with *H. pylori*-infected chronic gastric (CG-Hp+), both before and 3 months after bacterial eradication therapy. Moreover, the influence of *CagA* and *VacA* strains on the bacteria eradication and gene expression levels was also investigated. Relative quantification (RQ) of mRNA was performed by qPCR-real time (TaqMan assay), while proteins expression was assessed by immunohistochemical. Before treatment, higher mRNA levels of these genes were observed in CG-Hp+ patients than normal gastric mucosa Hp- ($p < 0.0001$), while *PLAT* and *PAI-1* were down-regulated ($p < 0.0001$). After treatment, only *IL-8* and *REG3A* mRNA were significantly reduced in eradicated patients ($p < 0.05$), but not in non-eradicated patients ($p > 0.05$). Unlike, *PLAT* and *PAI-1* mRNA levels were increased in eradicated patients ($p < 0.05$) compared to non-eradicated, while *TGF-β* and *IFITM1* did not show differences before and after

treatment. The *CagA* and *VacA* genotypes not influenced the bacteria eradication rate nor the gene expression levels, but lower levels of *PAI-1* mRNA was observed after treatment in eradicated carriers of *VacA* s1/m1 strain. Immunoexpression of proteins occurred mainly in the cytoplasm of epithelial cells of the gastric glands of the CG-Hp+ patients, however not found an absolute agreement between the results of gene and protein expression before and after eradication therapy. In conclusion, our results show deregulation in the gene expression due *H. pylori* infection, highlighting that there is a trend towards normalization of *IL-8*, *REG3A* and *PLAT* genes after bacteria eradication, which play important roles in biological processes for gastric mucosa homeostasis.

Key-words: *H. pylori* infection, inflammatory response, cell regeneration, cell proliferation, eradication therapy, gastric lesions.

Introduction

Helicobacter pylori (*H. pylori*) causes a wide range of gastric malignancies including chronic gastritis, gastric and peptic ulcer disease and gastric cancer (PEEK; CRABTREE, 2006; BRONTE-TINKEW; TEREBIZNIK; FRANCO, 2009) through the chronic inflammation induced by the host immune response (ADAMU et al., 2010). During early stages of infection, bacterial virulence factors triggers the induction of cytokines by inflammatory infiltrate and gastric epithelial cells, such as IL-8, IL-6, IFN-γ, TNF-α, NF-kB, IL-1β, and IL-4, reactive oxygen species (ROS) and reactive nitrogen species (RNS) (BASSO et al., 2010). These inflammatory mediators exert important effects on cancer development through various mechanisms as cell growth and mobility, induction of angiogenesis, and inhibition of apoptosis.

Among *H. pylori* virulence factors, the product of the cytotoxin-associated gene A (*CagA*) and the vacuolating cytotoxin A (*VacA*) are the major pathogenic factors may induce a more potent inflammatory response (GEBERT et al., 2003; YAMAOKA, 2012). In addition, the *H. pylori* and its virulence factors may activate multiple intracellular pathways in epithelial cells such as Ras-ERK-MAPK, NF-kB, Wnt/β-catenin, Akt/PI3K, signal transducers and transcription activators, such as STAT3 (TABASSAM; GRAHAM; YAMAOKA, 2009; BRONTE-TINKEW; TEREBIZNIK; FRANCO, 2009; LIU et al., 2012), genetic alterations as oncogenes activation and tumor suppressor inactivation (HAYASHI et al., 2013), epigenetic changes as aberrant DNA methylation profiles (QU; DANG; HOU, 2013) and deregulation of the microRNA expression (THORNS et

al., 2012), so may alter cellular function and triggers inflammatory and carcinogenic processes. Consequently, *H. pylori* strains expressing *CagA* and *VacA* s1/m1 are associated with higher risk for gastric cancer development (Rhead et al., 2007).

The eradication therapy of *H. pylori* is recommended in symptomatic patients and in those at risk for gastric cancer, may reverse precancerous lesions and prevent malignant progression (KABIR, 2009; FUCCIO et al., 2009; FORD et al., 2014). The standard triple therapy includes the use of antibiotics amoxicillin and clarithromycin associated with a proton-pump PPI inhibitor for 7-14 days (RIMBARA et al, 2011). While the quadruple therapy containing a PPI, bismuth salt, tetracycline and metronidazole is recommended as a first-line therapy in regions where clarithromycin resistance is high (MALFERTHEINER et al., 2012). However, therapeutic success depends on several factors as the poor compliance due to complicated treatment regimens and antimicrobial resistance (GRAHAM, LEE, WU, 2014; SMITH et al., 2014), besides polymorphisms of *CYP2C19*, *MDR1* and pro-inflammatory cytokines (Graham et al, 2008; SUGIMOTO et al, 2009), chemoresistance (WU et aL, 2010; SONG et al, 2011), as well as bacterial virulence factors (GRAHAM et al, 2008).

Studies have shown deregulation on gene expression profiling in response to *H. pylori* infection. The gastric mucosa of *H. pylori*-infected compared to non-infected patients presents up-regulation in receptors and co-receptors involved in bacterial recognition such as *TLR2, TLR4, LY96, ITGB2, VCAM1, MAPK8, RAC2, SLA, ADAM, MMP, IFITM1* and *PAP*, signal transduction, inflammation and

immune response, proteolysis, apoptosis and cell proliferation (HOFFMAN et al., 2007). In addition, genes related to protein metabolism, gene transcription and trace element metabolism has been reported to be changed (YANG et al., 2012).

Although is well known that infection by *H. pylori* is responsible for many molecular alterations associated with gastric carcinogenesis few studies have evaluated changes in the gene and protein expression pattern in patients after eradication therapy. Considering that *H. pylori* eradication can prevent the progression of premalignant lesions and reduce the risk of gastric cancer development, the goal of this study was investigate whether *H. pylori* eradication interfere on the mRNA and protein expression of genes involved with inflammatory and immune response, cell proliferation, apoptosis and cell regeneration. Thus, we evaluated the gene and protein expression of *IL-8*, *TGFB*, *REG3A*, *PLAT*, *PAI-1* and *IFITM1* genes in patients with precancerous gastric lesions, both before and after bacterial eradication therapy. Moreover, the influence of *CagA* and *VacA* bacterial virulence genotypes on the bacteria eradication and gene expression levels was also investigated.

Materials and Methods

Patients

A total of 38 patients undergoing routine upper gastrointestinal endoscopy with positive histological and molecular diagnosis for *H. pylori* and not yet submitted to eradication therapy were enrolled prospectively between May 2010 and December 2012 from the Gastro-Hepatology Outpatient Clinic at the Base

Hospital and the State Hospital João Paulo II, both at São José do Rio Preto, SP, Brazil. None of the individuals had taken any antibiotics, nonsteroidal anti-inflammatory drugs, or corticosteroids during the two months prior to endoscopy, nor proton-pump inhibitors, H_2 antagonists in the 10-15 days preceding the procedure. Patients with gastric cancer and infectious diseases were excluded from this study. The study protocol was approved by the local Research Ethics Committee (CEP/IBILCE/UNESP number 030/10), and written informed consent was obtained from all participating individuals.

From each patient, gastric biopsies of the antrum region were collected for histological, molecular and immunohistochemichal analyses. Gastric biopsy specimens were examined histologically by a specialized pathologist for the presence of the bacteria and histopathologically classified as: superficial chronic gastritis (*n*= 24), atrophic gastritis (*n*= 8), and metaplastic atrophic gastritis (*n*= 6), according to the Sydney system (DIXON et al., 1994), constituting the so-called CG-Hp+ group (mean age 48±15.9 years; 24 females and 14 males). Four gastric biopsy specimens presented histologically normal, *H. pylori*-negative normal mucosa (NM-Hp- group) and were used as control (mean age 29.5±12.5 years; 3 females and 1 male).

The CG-Hp+ group was submitted to standard triple therapy consisting of amoxicillin (1g), clarithromycin (500 mg) and omeprazole (20 mg), all given twice daily for seven days. 3 months after treatment, the individuals underwent another endoscopy for collection of gastric biopsies of the antrum region.

Immediately after collection, the biopsy specimens were placed into RNA Later solution (*Applied Biosystems*) and stored at -20ºC until nucleic acid extraction.

Molecular diagnosis of *H. pylori* and *CagA* and *VacA* virulence factors

DNA/RNA extraction from the gastric biopsies was performed according to the protocol accompanying the Trizol reagent (*Invitrogen*) and the concentrations were determined in a NanoDrop® ND1000 spectrophotometer (*Thermo Scientific*). First, multiplex PCR was performed, using 100 ng of DNA in a final volume of 25μL containing specific primers for *H. pylori* genes such as *UreA* and *tsaA*, besides the constitutive human *CYP1A1* gene, according our protocol described in previous study (Rossi et al., 2014) Molecular diagnosis was considered positive when at least one gene (*UreA* or *tsaA*) had been amplified. The *H. pylori*-positive samples were then subjected to a second PCR run, to investigate the virulence genotype *cagA* (Rossi et al, 2014), and after also for *vacA* s/m regions as previously described (Gatti et al., 2005). Primers amplify s1 fragment of 176 bp or s2 fragment of 203 bp, while primers for "m" alleles amplify m1 fragment of 400 bp or m2 fragment of 475 bp. Positive and negative controls were used in all experiments.

Quantitative real-time PCR (qPCR) for quantification of mRNA expression

Reverse transcription (RT) of total RNA was performed using a High Capacity cDNA Archive Kit (*Applied Biosystems*), in a total volume of 25 μl, according to the manufacturer's protocol. Then, qPCR was carried out in a

StepOnePlus Real Time PCR System 2.2.2 (*Applied Biosystems*), using specific TaqMan probes for target genes *IL-8* (assay ID Hs00174103_m1, *Applied Biosystems*), *TGF-β* (assay ID Hs00998133_m1, *Applied Biosystems*), *PLAT* (assay ID Hs00263492_m1, *Applied Biosystems*), *PAI-1* (assay ID Hs01126607_g1, *Applied Biosystems*), *REG3A* (assay ID Hs01055563_gH, *Applied Biosystems*), *IFITM1* (assay ID Hs00705137_s1, *Applied Biosystems*) and two reference genes, *ACTB* (part number: 4352935E, *Applied Biosystems*) and *GAPDH* (part number: 4352934E, *Applied Biosystems*), used as reference genes according to the manufacturer's instructions. All reactions were performed in triplicate in a final volume of 20 µl, using 100 ng/µl cDNA and a negative control without cDNA to ensure the absence of contamination. Relative quantification (RQ) of *IL-8, TGF-β, PLAT, PAI-1, REG3A* and *IFITM1* mRNA was obtained according to the model proposed by Livak & Schmittgen (2001) and normalized to the *ACTB* and *GAPDH* reference genes and a pool of normal mucosa Hp- samples. The RQ was expressed as median (range).

Immunohistochemical evaluation of IL-8, TGF beta 1, PLAT, PAI-1, Reg3A and IFITM1

Immunohistochemical analysis was performed in 24 samples from the CG-Hp+ group before and after bacteria eradication and in the four samples from NM-Hp-. Consecutive 4 µm-thick sections were cut from each trimmed paraffin block. Deparaffinized tissue slides were then submitted to antigen retrieval, using a high-temperature antigen-unmasking technique. The sections were incubated with

specific primary antibodies overnight: rabbit polyclonal IL-8 antibody (NBP1-19757, 1:200 dilution; Novus Biologicals), mouse monoclonal TGF beta 1 antibody (NB110-59988, 1:50 dilution; *Novus Biologicals*), rabbit polyclonal Tissue Plasminogen Activator antibody (PLAT) (NBP2-20648, 1:100 dilution; Novus Biologicals), goat polyclonal PAI-1/Serpine 1 antibody (NB100-1498, 1:200 dilution, Novus Biologicals), rabbit polyclonal Reg3A (ab134309, 1:100 dilution; Abcam) and rabbit polyclonal IFITM1 antibody (NBP2-16922, 1:500; Novus Biologicals). Then the slides were incubated with biotinylated secondary antibody (Picture Max Polymer Detection Kit, *Invitrogen*) for 30 minutes, following the manufacturer's protocol. Immunostaining was done with 3,3'diaminobenzidine tetrahydrochloride (DAB) containing 0.005% H_2O_2, counterstained with hematoxylin. Human lung was used as positive control for IL-8, colon was used for TGF-B1, PLAT and REG3A, placenta was used for PAI-1 and tonsil was used for IFITM1 proteins. The immunostaining was evaluated in the epithelial cytoplasm and also in the stroma for PLAT and IFITM1 proteins, by densitometric analysis with an arbitrary scale going from 0 to 255, performed with Axio Vision software under a Zeiss-Axioskop II light microscope. Sixty equally distributed points were scored in each one of the regions, and the results were expressed as median and range.

Statistical Analysis

The data were analyzed using the box-plot graphic method for outliers detection (Moroco, 2003) and the outliers were removed from the subsequent

analyses. Data analysis was performed using the computer software GraphPad Prism 5 version 5.01. The distribution of continuous data was evaluated using the D'Agostino & Pearson omnibus normality test. Data are presented as median and range, mean±standard deviation (SD), or frequencies, according to the data distribution. Student's *t*-test for paired and unpaired data or correspondent nonparametric tests, such as the Mann-Whitney test and the Wilcoxon signed rank test, were used for comparisons between groups. The comparisons of gene expression levels CG-Hp+ before treatment, eradicated and non-eradicated patients with histological types of lesion were evaluated by Kruskal-Wallis test. To evaluate the association between *CagA* and *VacA* genotypes with eradication rate it was employed Fisher Exact test, and the comparisons of RQ median values and influence of these genotypes was performed using Mann Whitney test. For protein expression, the medians obtained from the densitometry analysis were compared before *vs* normal Hp- group, before *vs* after eradicated, and after eradicated *vs* after non- eradicated using Mann-Whitney test. The level of significance was set at $p \leq 0.05$.

Results

mRNA expression of *IL-8, TGF-β, PLAT, PAI-1, REG3A* and *IFITM1* before and after eradication therapy

At first, the relative gene expression levels of *IL-8, TGF-β, PLAT, PAI-1*, *REG3A* and *IFITM1* mRNA of the CG-Hp+ patients before treatment were compared with pool of samples of the NM-Hp-. We found that the expression

levels of these genes were significantly higher in CG-Hp+ group than in non-infected mucosa pool ($p<0.0001$), except for *PLAT* and *PAI-1*, whose expression levels were lower than NM-Hp- ($p< 0.0001$).

The Table 1 presents the data regarding the relative gene expression levels of *IL-8, TGF-β, PLAT, PAI-1, REG3A* and *IFITM1* mRNA in CG-Hp+ group before and after treatment, which was stratified in those that bacteria was eradicated (Hp-) and non-eradicated (Hp+).

The mRNA expression levels of *IL-8* and *REG3A* were significantly increased before therapy (RQ median= 20.42 and 7.68, respectively), and were significantly reduced after treatment in the patients that eradicated the bacteria (RQ median= 6.51, $p= 0.013$, and 0.61, $p< 0.001$). However, in the non-eradicated patients the mRNA levels of these genes remained high (Figure 1A and E).

TGF-β and *IFITM1* mRNA that had slightly high expression before treatment (RQ median= 1.58 and 1.38) maintained high expression after treatment in both eradicated (RQ median= 2.74 and 1.36), and non-eradicated patients (RQ median= 3.01 and 1.24), thus no significant differences were observed (Figure 1B and F).

On contrary, *PLAT* and *PAI-1* mRNA were down-regulated before treatment (RQ median= 0.64, and 0.15, respectively). After therapy, while *PLAT* mRNA was significantly increased in the eradicated patients (RQ median= 1.12, $p= 0.003$), the *PAI-1* expression levels were not significantly increased (RQ median= 0.22, $p= 0.340$) (Figure 1C and D).

The paired analysis of patients before and after therapy considering only the patients in which the bacteria was eradicated confirmed the results of the gene expression presented in Table 1, except for *TGF-β* that for this analysis showed significant increased expression after bacteria eradication (before treatment: RQ median= 1.47; after eradication: RQ median= 2.37, p= 0.010) (data not shown).

When we compared the mRNA relative expression levels after treatment between eradicated and non-eradicated patients, while *REG3A* mRNA was significantly up-regulated in non-eradicated patients (RQ median= 4.59) compared to eradicated (RQ median= 0.61, p= 0.009), *PAI-1* mRNA showed down-regulation in non-eradicated patients (RQ median= 0.12) relative to eradicated (RQ median= 0.22, p= 0.017) with RQ median similar to before treatment. However, no significant differences were observed for other genes analyzed (p> 0.05).

mRNA expression levels according histological classification and *CagA* and *VacA* genotypes of *H. pylori*

In the Table 2 are presented the comparisons of relative gene expression levels stratified by histological type of lesion, as normal mucosa (NM), chronic gastritis (CG), atrophic gastritis (AG), and metaplastic atrophic gastritis (MAG). No significant difference was observed for the analyzed genes, although higher expression levels of *IL-8* and *REG3A* mRNA were observed in MAG before treatment. Moreover, after treatment, eradicated patients with AG showed higher expression for *IL-8* and *PLAT* mRNA.

Considering that gastric lesions may regress after bacteria eradication, it was observed regression of the lesion in 10/38 (26.3%) of samples analyzed according histopathological report, which 37.5% (3/8) of patients with previous histological diagnosis of AG were diagnosed after therapy as CG, 83.3% (5/6) with previous histological diagnosis of MAG, were posteriorly diagnosed as AG or CG, and 8.3% (2/24) with previous histological diagnosis of CG showed NM after *H. pylori* eradication. Although, there was no significant reduction of the gene expression levels.

Table 3 presents the frequencies for the *CagA* and *VacA* virulence genotypes of *H. pylori* in the CG-Hp+ patients and its influence on bacteria eradication after treatment. The polymorphic genotypes for *VacA* were grouped as s1/m1 and others (s1/m2, s2/m2, and s2/m1) due the low frequency of others polymorphisms. The *CagA*-positive genotype was detected in about 50% of both patients that eradicated and non-eradicated the bacteria, so there was no significant difference (p= 1.000). Although *VacA* s1/m1 genotype was detected in 50% (7/14) of eradicated patients and in only 25% (3/12) those that non-eradicated the bacteria after treatment, no significant difference was observed between the groups (p= 0.247).

In another investigative approach, the influence of *CagA* and *VacA* genotypes on the gene expression levels, both before and after treatment was evaluated (Tables 4 and 5). In general, *CagA* did not influence the gene expression in patients analyzed both before and after treatment, thus no significant difference was observed between *cagA*+ and *cagA*- genotypes (p>0.05) for all

analyzed genes. Regarding *VacA* s/m genotype, it was verified lower levels of *PAI-1* mRNA after treatment in patients that eradicated the bacteria carriers of *vacA* s1/m1 genotype than carriers *vacA* others genotypes (RQ median= 0.13 *versus* 0.29, p= 0.035), while for others genes no change was observed (p> 0.05).

Protein expression and densitometry analysis

Qualitative and quantitative analyses of the expression of IL-8, TGF-β, PLAT, PAI-1, Reg3A and IFITM1 proteins were assessed by immunohistochemistry, both before and after *H. pylori* eradication therapy. Various dilutions for PAI-1 antibody were tested (1:100, 1:200 and 1:500), however, it was not possible to analyze the results due to problems in detecting the antibody, which show no adequate standard of staining.

In normal mucosa, the IL-8 protein expression was weak or absent, mainly in the epithelial cells in gastric glands, in foveolar epithelium and inflammatory cells (Figure 2A). The CG-Hp+ patients before treatment showed a cytoplasmatic immunostaining pattern, in the epithelial cells in gastric glands, in foveolar epithelium and, a strong expression in the inflammatory cells was also observed (Figure 2B). After eradication of bacterium a similar immunostaining pattern those before treatment was observed (Figure 2C).

For TGF-β, in normal mucosa the protein expression was absent in epithelial cells of gastric glands, foveolar epithelium and inflammatory cells (Figure 2E). However, it was expressed in cytoplasm of gastric epithelial cells of the CG-Hp+ patients before treatment (Figure 2F). Eradicated patients showed no

reduction in the TGF-β expression with similar immunostaining pattern those observed before treatment (Figure 2G).

In the normal mucosa, IFITM1 protein showed weak or absent staining (Figure 2I), while those CG-Hp+ patients presented positive cytoplasm immunostainig in epithelial cells of glands and inflammatory cells of lamina propria (Figure 2J) in majority of samples analyzed. After treatment, immunopositive pattern was reduced in the eradicated patients (Figure 2K).

Immunohistochemical for PLAT in normal mucosa showed expression only in epithelial cells in the glands (Figure 3A). In CG-Hp+ patients the immunoreactivity occurred predominantly in epithelial cells in the glands, including parietal cells besides inflammatory infiltrated of the stroma (Figure 3B). The majority of *H. pylori* infected samples showed positive PLAT expression, which remained immunopositive after eradication therapy (Figure 3 C).

Reg3A protein displayed low or no immunoreactivity in the normal mucosa (Fig. 3E), while in CG-Hp+ patients before eradication therapy was predominantly immunostained in the cytoplasm of the epithelial cells in the glands and foveolar portion. In addition, it was verified perinuclear positivity in the bottom of the crypt cells, suggesting a labeling of replacement cells (Fig 3F). However, the majority of patients presenting negative immunostaining both before and after treatment (Fig 3G).

The densitometry analysis of the cytoplasm of gastric epithelial cells showed no significant difference of median optical densitometry values of the proteins evaluated in patients CG-Hp+ before treatment compared to normal gastric mucosa

Hp-, as well as between eradicated and non-eradicated patients (p> 0.05) (Fig 2D, H, L; and 3D,H). The PLAT and IFITM1 proteins showed a positive immunostaining also of the stromal cells, but only IFITM1 protein presented significant difference between CG-Hp+ patients before treatment compared to eradicated patients (p= 0.0007), due a reduction of median optical densitometry values in the eradicated patients (data not shown).

I. DISCUSSION

Previous studies have shown that *H. pylori* and its virulence factors can promote changes in the expression of genes associated with inflammatory and immune responses and other cellular processes. Nevertheless there is little information about the effect of bacteria eradication on the restoration of gene expression in the gastric mucosa. In this context, we analyzed the mRNA and protein expression of *IL-8*, *TGF-β, PLAT, PAI-1*, *REG3A* and *IFITM1* in patients with precancerous gastric lesions infected by *H. pylori* before and after eradication therapy, and also the influence of *CagA* and *VacA* virulence genotypes on the gene expression and *H. pylori* eradication rates. Before treatment, we observed significantly higher mRNA levels of these genes in CG-Hp+ patients relative to the normal gastric mucosa Hp-, except for *PLAT* and *PAI-1*, which were down-regulated. Two to three months after treatment, only *IL-8* and *REG3A* mRNA were significantly reduced in those patients who eradicated the bacteria, but not in non-eradicated patients, while for *PLAT*, the mRNA levels were statistically increased only in eradicated patients. Despite *PAI-1* mRNA was down-regulated before treatment, in the eradicated patients there was a significant

increase in gene expression compared to non-eradicated patients. With respect to *CagA* and *VacA* bacterial virulence factors we found that they did not influence the bacteria eradication rate nor the gene expression levels, except for *PAI-1*, which lower levels of mRNA was observed after treatment in eradicated carriers of *VacA* s1/m1. Regarding the protein expression we have not found an absolute agreement with the results of gene expression, since the densitometry analysis revealed no significant differences for evaluated proteins before and after eradication therapy of *H. pylori*.

Some studies have demonstrated an association of gastrointestinal cancers with chronic inflammation, accompanied by abnormal cellular changes such as oxidative stresses, mutations, epigenetic alterations and activation of inflammatory cytokines (Lippitz, 2013). The gastric carcinogenesis as a result of *H. pylori* infection can occur in two distinct pathways. One way is by activation a chronic inflammatory response, causing several molecular and morphological changes in the inflamed epithelium, leading to mucosa atrophy, metaplasia, dysplasia and eventually gastric cancer. The other one, the bacteria may modify the epithelial cell function by interfering in genes that regulate cellular processes such as apoptosis, cell cycle control, tumor suppression and cell-to-cell contact, thereby promoting carcinogenesis (Mueller; Falkow; Amieva, 2005).

In this respect, IL-8 is an important pro-inflammatory cytokine, acting as major inflammatory mediator involved in the induction of *H. pylori*-associated gastritis. Our results showed higher levels of *IL-8* mRNA in CG-Hp+ patients than normal gastric mucosa non-infected, while that after treatment, it was

observed decreased in the eradicated patients, but remained high in non-eradicated patients. Therefore, our results indicate that up-regulation of *IL-8* expression levels reflect increased inflammatory responses of gastric mucosa in infected patients. These results are supported by strong immunoexpression of this protein by inflammatory cells and epithelial cells in gastric glands before treatment, which showed also a similar immunostaining pattern after eradication of bacteria, indicating that IL-8 cytokine keeps expressed for a period after eradication of the bacteria due the infiltrated of inflammatory cells.

Up-regulation of IL-8 due *H. pylori* infection has been reported in some studies (Goll et al., 2007a; Lu et al., 2012; Eftang et al., 2012; Cook et al., 2013). Goll et al. (2007a) found increased *IL-8* levels and other cytokines in peptic ulcer associated with *H. pylori*. In Hp+ AGS cells or co-incubated to *H. pylori* high levels of IL-8 have been reported (Lu et al., 2012; Eftang et al., 2012), as well as deregulation of apoptosis, tumor suppressor genes and oncogenes in the first 24h of *H. pylori* infection, which may represent early signs of gastric tumorigenesis (Eftang et al., 2012). High IL-8 levels in Treg cells were detected in the mucosal response to *H. pylori* (Cook et al., 2013). Given that Tregs reduce inflammation to avoid excess damage to the host, the authors believe that looking at Treg migrant might help to identify patients at risk of developing more extensive disease. In contrast, reduction in the *IL-8* levels after bacteria eradication has also been reported (Resnick et al., 2006; Goll et al., 2007b), evidencing a chronic low-grade inflammation with reduced Th1, prolonged Th2 and disappearance of the T-regulatory response (Goll et al., 2007b).

TGF-β is a multifunctional cytokine with critical roles in many cellular pathways including cellular growth, apoptosis, differentiation, and immune reactions (Guasch et al., 2007; Yang et al., 2010; Lee et al., 2013). Under normal conditions, it acts as an anti-inflammatory cytokine with suppressive effect of carcinogenesis inhibiting abnormal cell growth. However, TGF-β production by tumor cells results in suppression of anti-tumor immune response creating a local environment of immune tolerance (Lippitz, 2013).

In the current study, increased levels of *TGF-β* mRNA was found in CG-Hp+ patients compared to Hp- normal mucosa, showing a slight increase after treatment in both eradicated and non-eradicated patients, although no significant difference has been observed. TGF-β protein was immunoexpressed in cytoplasm of gastric epithelial cells and infiltrated inflammatory cells of the CG-Hp+ patients both before and after treatment, whose the eradicated patients showed similar immunostaining pattern those observed before treatment.

In contrast, Nakachi et al. (2000) showed that infection by the bacteria does not increased levels of *TGF-β* mRNA in human gastric epithelial cells. Similarly to our results higher *TGF-β* mRNA levels in *H. pylori*-infected patients were demonstrated by Rahimian et al. (2014) that correlated positively with *VacA* bacterial genotype, types of disease and grade of inflammation, indicating that vacA secretion by *H. pylori* may induce the mucosa *TGF-β* mRNA and contribute to persistent infection by inhibiting the proliferation and immune response of T cells. In *H. pylori*-associated duodenal ulcer patients TGF-β immunostaining was detected mainly in infiltrated inflammatory cells and lymphoid follicles. After

pathogen eradication immunoexpression of TGF-β was markedly increased in gastric surface epithelial cells and glandular mucosa and could be still seen in the remaining inflammatory cells, whereas the degree of inflammation was significantly decreased (Jo et al., 2010). In addition, the authors observed TGF-β levels decreased in gastric antral tissues of patients with *H. pylori*-associated gastritis compared to both normal mucosa and *H.pylori*-negative gastritis. Thus, these results suggest that the attenuated levels of gastric antral mucosal TGF-β associated with *H. pylori* infection could be an important determinant for the degree of inflammation provoked by *H. pylori* and the associated clinical outcomes.

TGF-β seems do play a paradoxical role, during *H. pylori* infection, its induction may be a mechanism by which the bacteria manipulates host response to enhance its own survival, enhancing bacterial colonization and attachment to host cells, while attenuation of TGF-β expression could be the host defense reaction to avoid the attachment of *H. pylori* and to prevent subsequent injuries including apoptosis or inflammation. Even though the suppression of this cytokine promotes increased gastric inflammation (Jo et al., 2010).

Studies evaluating the expression of members of plasminogen activator system in gastric lesions associated with *H. pylori* infection are scarce. Therefore, to address this issue, we analyzed the expression of *PLAT* and *PAI-1*. mRNA expression of both genes was lower in gastric tissue infected by the bacteria than in the Hp- normal gastric mucosa. However, after treatment, up-regulation of both *PLAT* and *PAI-1* mRNA levels were observed in eradicated patients.

Immunohistochemical assay evidenced positive PLAT expression in both cytoplasm of epithelial cells and stroma for the majority of *H. pylori* infected samples, remaining immunopositive after eradication therapy.

In agreement with previous studies, PLAT protein levels were significantly decreased in the extracts of gastric antrum from *H. pylori*+ patients (COLUCCI et al., 2005). Analysis of the expression of genes responsible for invasive phenotype of primary cultured hepatocellular carcinoma cells showed differential expression of several groups of genes, among them *PLAT* (Lin; Chuang, 2012). Indeed, some studies confirmed that up-regulation of *PLAT* gene promote proliferation, migration, invasion and angiogenesis in cancer cells (Roda et al., 2009; Sharma et al., 2010).

PAI-1, a serine protease belonging to the family of serine protease inhibitors, is synthesized by many cells and can be induced by growth factors, cytokines, hormones and other stimuli. High levels of PAI-1 were detected in patients with acute or chronic inflammatory conditions. The PAI-1 has inhibitory action on urokinase-type plasminogen activator (u-PA), a serine protease involved in tissue remodeling and cell migration in tissue, interacting with integrins and extracellular matrix components. Therefore, PAI-1 is considered one of the key regulators of tumor invasion and metastasis, as well as cancer-related angiogenesis (Chorostowska-Wynimko; Skrzypczak-Jankun; Jankun, 2004).

PAI-1 overexpression in *H. pylori*-infected gastric epithelial cells has been shown in some studies (Keates et al., 2008; Kenny et al., 2008). Keates et al. (2008) observed an increase in both *PAI-1* mRNA and protein expression in

patients with *H. pylori*-associated gastritis. Furthermore, the authors confirmed increased *PAI-1* mRNA expression and the secretion of protein after infection of AGS cells with *H. pylori*, suggesting that the up-regulation of PAI-1 may contribute to the carcinogenic process (Keates et al., 2008). In contrast, the PAI-1 potential effect on wound healing process and gastric mucosal protection has been studied. Kenny et al. (2013) found elevated *PAI-1 mRNA* levels in patients taking aspirin or NSAIDs (Nonsteroidal anti-inflammatory drugs) and a protective effect of this inhibitor was confirmed in two experimental models of gastric mucosa damage (Kenny et al., 2008; Norsett et al., 2011). This findings show that PAI-1 prevents fibrinolysis by mucosa injury and its increase in the gastric epithelial might therefore be therapeutically useful in preventing ulcerogenesis. However, our results demonstrated down-regulation of *PAI-1* mRNA in patients with *H. pylori*-infected gastritis regarding to non-infected normal gastric mucosa, and lower *PAI-1* mRNA levels were detected in non-eradicated patients than eradicated patients. It is noteworthy that were excluded from our study patients under treatment with anti-inflammatory drugs.

We also found that *H. pylori* infection induces *REG3A* mRNA expression in chronic gastritis, and was significantly reduced after bacteria eradication. Moreover, we verified that levels of *REG3A* mRNA remained increased in non-eradicated patients. In contrast, the most of patients presenting negative immunostaining for Reg3A protein both before and after treatment, and densitometry analysis also showed no significant difference in relation to treatment. As *H. pylori* colonizes mainly on gastric surface epithelium (Ishihara et

al., 2001) and the Reg3A -producing cells are located in the deeper part of gastric fundic glands, it is suggested that infection by *H. pylori* may indirectly stimulate the expression of this protein in the stomach. The majority of studies have been mainly conducted in animal models, and little is known regarding the link between Reg3A protein expression and *H. pylori* infection. Reg3A is a potent growth factor up-regulated in *H. pylori*-infected gastric mucosa and it may play an important role in promoting epithelial proliferation in the stomach and lead to the development of *H. pylori*-associated gastric cancer (Kinoshita et al., 2004). Besides the proliferative activity and cell regeneration, it also exerts anti-inflammatory, antibacterial and anti-apoptotic function (Lee et al., 2012; van Beelen et al., 2013; Murano et al., 2014).

Similarly to our findings Yoshino et al. (2005) showed that Reg3A protein production was significantly higher in gastric biopsies *H. pylori*+ than *H. pylori*- and was up-regulated by the pro-inflammatory cytokine IL-8 in ECC10 cells. In our study, although no correlation between expression levels of *IL-8* and *REG3A* has been evaluated, both showed increased expression levels in infected patients before treatment and in those non-eradicated patients. These results should be consequence of gastric mucosa injury and inflammation, which was reduced in patients who eradicate the bacteria indicating the restoration of the mucosa. Studies in experimental models evaluated the effect of acute inflammation on gastric mucosa and reported increase in Reg3A protein expression during the healing process of damaged rat gastric mucosa induced by water-immersion stress

(WRS) or administration of indomethacin (Fukui et al., 1998; Kazumori et al., 2000) evidencing the anti-inflammatory effect of this protein.

Our results demonstrated moderate expression of *IFITM1* transcripts in Hp+ chronic gastritis patients and no significant change was observed after eradication therapy. However, IFITM1 protein showed positive immunostaining in inflammatory cells in majority of infected patients, and the densitometry analysis showed reduction of its expression after treatment in both eradicated and non-eradicated patients.

IFITM1, (interferon-induced transmembrane protein 1) has important functions in regulating cell proliferation and differentiation, and the interferons (IFNs) induced IFITM1 expression is considered important in cell adhesion, proliferation, tumor immunity and antiviral activity (Yang et al., 2005; Brass et al., 2009), and in the antiproliferative activity (Yang et al., 2007). It has been considered as a novel candidate gene for invasion in some studies. Hatano et al. (2008) demonstrated that IFITM1 overexpression promoted the invasion and migration of HNSCC (squamous cell carcinoma of the head and neck) cells *in vitro*. The authors suggested that *IFITM1* may be involved in the initial step of invasion, such as the degradation of the basement membrane and the interstitial extracellular matrix, which results in cellular infiltration into the adjacent tissue. In contrast, Yang et al. (2007) had observed that overexpression of *IFITM1* inhibited cell proliferation in hepatoma cells, while suppression of *IFITM1* accelerated cell growth, and conferred tumorigenicity to normal liver cells in nude mice. Despite these contradictory results on the role of IFITM1 in the

carcinogenic process, our findings show that the treatment of *H. pylori* reduces the production of this protein by inflammatory infiltrated cells.

Even though *H. pylori* is completely eradicated, the gastric inflammation may remain, thus it seems that improvement of gastric inflammation itself seems to be far more essential in achieving cancer prevention than the eradication of the pathogen (Hong et al., 2010). Although we did not evaluate the degree of inflammation of gastritis before and after eradication therapy, this is an important factor that may have contributed to the changes of gene expression evaluated.

We found no complete agreement between mRNA expression evaluated by real time qPCR and protein expression assessed by immunohistochemistry. While for *IL-8*, *PLAT*, *PAI-1* and *REG3A* transcripts showed statistically significant differences before and after eradication therapy of *H. pylori*, only the IFITM1 protein showed a significant reduction in the immunostaining of the stromal cells in patients after treatment. This discrepancy has been reported in various studies (Guo et al., 2008; Kordek et al. 2010), which can be due the mRNA expression originate from different cell types, heterogeneity of the tissue, since immunohistochemistry is not exactly the same sample which is examined by real time q-PCR. Others factors such as transcriptional and post-transcriptional splicing, translational modifications and regulation, protein complex formation, and also the degradation rates of mRNA and protein might affect the relative quantities of these molecules. In addition, the post-transcriptional gene silencing performed by miRNAs, causing degradation or repression of mRNA translation

might affect its correlations with protein levels (Guo et al., 2008), thus the relative expression level of mRNA not always directly reflects protein expression.

The studies that assess the effects of *CagA* and *VacA* virulence factors on the gene and protein expression are controversial. Ours results evidenced that *CagA* and *VacA* genotypes no influenced on the eradication rate of bacteria nor in mRNA expression levels of these genes evaluated, except for *PAI-1* that showed down-regulation in eradicated patients harbor *VacA* s1/m1. In general, *VacA* s1/m1/*Cag*A^{+} strains have been associated with deregulation of gene expression, as *MAPK8* (JNK) (HOFFMAN et al., 2007) and *TGF-β* (RAHIMIAN et al., 2014). Similarly, the bacteria eradication rate can be affected by many factors, as infection by *Cag*A strain and absence of organisms with coccoid morphology (HUANG et al, 2005; ZHAO et al, 2007; SUGIMOTO; YAMAOKA, 2009; FIGURA et al., 2012). The strains carrying the *CagA* genotype increase cytokine production in gastric mucosa, resulting in suppression of acid secretion (FURUTA et al, 2002), so increasing the effectiveness of treatment (SUGIMOTO et al, 2007). In this context, we must consider some limitations of our study, due to the reduction of the number of patients in the subgroup stratification after treatment, which may have contributed during the statistical analysis of *CagA* and *VacA* genotypes.

In conclusion, ours results show changes in the mRNA expression of *IL-8*, *TGF-β*, *REG3A*, *PLAT*, *PAI-1* and *IFITM1* genes due *H. pylori* infection in patients with chronic gastritis independent of *CagA* and *VacA* status. However, *H. pylori* eradication not completely restores the expression of these gene that play

important roles in biological processes such as inflammatory and immune response, cell proliferation, regeneration, differentiation, migration and invasion, suggesting that inflammation of the mucosa, beyond the presence of the pathogen contribute to deregulation of gene expression. More importantly, should be highlighted that a trend towards normalization of *IL-8, REG3A* and *PLAT* genes after treatment was observed. Thus emphasizing that *H. pylori* eradication therapy is a relevant strategy for homeostasis of the gastric mucosa and possibly prevents malignant progression.

Acknowledgments

The authors are grateful to Dr. Sebastião Roberto Taboga and Luiz Roberto Faleiros Junior for their help with the histological sections, Isaque Santana for technical assistance during the immunohistochemical assay, and nurse Gizelda Warick Mazzale for her help in contacting patients. This study was supported by grants from the Brazilian agencies FAPESP (2012/15036-8) and CNPq (304870/2012-9).

The authors declare no competing interests.

V. REFERENCES

ADAMU, M.A. et al. Incidence of chronic atrophic gastritis: Systematic review and meta-analysis of follow-up studies. **Eur J Epidemiol,** v.25, p.439–448, 2010.

BASSO, D.; PLEBANI, M.; KUSTERS, J.G. Pathogenesis of *Helicobacter pylori* infection. **Helicobacter, v.**15(Suppl. 1), p.14–20, 2010.

BRASS, A.L., et al. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. **Cell.** v.139p., p.1243-1254, 2009.

BRONTE-TINKEW, D.M. et al. *Helicobacter pylori* cytotoxin-associated gene A activates the signal transducer and activator of transcription 3 pathway in vitro and in vivo. **Cancer Res**., v.69, p.632–9, 2009.

CHOROSTOWSKA-WYNIMKO, J.; SKRZYPCZAK-JANKUN; JANKUN, J. Plasminogen activator inhibitor type-1: its structure, biological activity and role in tumorigenesis. **Int J Mol Med**, 13:759-766, 2004.

COLUCCI, M., et al. Changes in coagulation-fibrinolysis balance in blood mononuclear cells and in gastric mucosa from patients with *Helicobacter pylori* infection. **Thromb Res**, v.116(6), p.471-7, 2005.

COOK, K.W. et al. CCL20/CCR6-mediated migration of regulatory T cells to the *Helicobacter pylori*-infected human gastric mucosa. **Gut**, v.0p.1–10, 2014.

DIXON, M.F. et al. Classification and grading of gastritis; the updated Sydney system. **Am J Surg Pathol**, v.20, p.1161-1181, 1996.

EFTANG, L.L. Interleukin-8 is the single most up-regulated gene in whole genome profiling of *H. pylori* exposed gastric epithelial cells. **BMC Microbiology**, v.12, p.9, 2012.

FIGURA, N. et al. Factors modulating the outcome of treatment for the eradication of *Helicobacter pylori* infection. **New Microbiol**, v.35, p.335-40, 2012.

FORD, A.C. et al. *Helicobacter pylori* eradication therapy to prevent gastric cancer in healthy asymptomatic infected individuals: systematic review and meta-analysis of randomised controlled trials. **BMJ**, v.348, p.g3174, 2014.

FUCCIO, L. et al. Meta-analysis: can *Helicobacter pylori* eradication treatment reduce the risk for gastric cancer? **Ann. Intern. Med**, v.151, p.121e8, 2009.

FUKUI, H. et al. Regenerating gene protein may mediate gastric mucosal proliferation induced by hypergastrinemia in rats. **Gastroenterology**, v.115, p.1483–93, 1998.

FURUTA, T. et al. Interleukin 1beta polymorphisms increase risk of hypochlorhydria and atrophic gastritis and reduce risk of duodenal ulcer recurrence in Japan. **Gastroenterol**, v.123, p.92-105, 2002.

GATTI, L.L. et al. cagA vacA alelles and babA2 genotypes of *Helicobacter pylori* associated with gastric disease in Brazilian adult patients. **Diagn Microbiol Infect Dis**., v.51(4), p.231-5, 2005.

GEBERT, B. et al. *Helicobacter pylori* vacuolating cytotoxin inhibits T lymphocyte activation. **Science**, v.301, p.1099–102, 2003.

GOLL, R et al. Alterations in antral cytokine gene expression in peptic ulcer patients during ulcer healing and after *Helicobacter pylori* eradication. **Scand J immunol**, v.67, p.57-62, 2007b.

GOLL, R. et al. *Helicobacter pylori* stimulates a mixed adaptative immune response with a strong T-regulatory component in human gastric mucosa. **Helicobacter**, v.12, p.185-92, 2007a.

GRAHAM, D.Y.; LEE, Y.C.; WU, M.S. Rational *Helicobacter pylori* therapy: evidence-based medicine rather than medicine-based evidence. **Clin Gastroenterol Hepatol**, v.12, p.177–186, 2014.

GRAHAM, D.Y.; LU, H.; YAMAOKA, Y. Therapy for *Helicobacter pylori* infection can be improved: sequential therapy and beyond. **Drugs**, v.68, p.725-36, 2008.

GUASH, G., et al. Loss of TGFβ signaling destabilizes homeostasis and promotes squamous cell carcinomas in stratified epithelia. **Cancer Cell**. v.12, p. 313–327, 2007.

GUO, Y. et al. How is mRNA expression predictive for protein expression? A correlation study on human circulating monocytes. **Acta Biochim Biophys Sin**, v.40, p.426-36, 2008.

HAYASHI, Y. et al. CagA mediates epigenetic regulation to attenuate let-7 expression in *Helicobacter pylori*-related carcinogenesis. **Gut**, v.62(11), p.1536-46, 2013.

HOFFMAN, V.J. et al. Gene expression profiling in human gastric mucosa infected with *Helicobacter pylori*. **Mod. Pathol**, v.20, n.9, p.974-989, 2007.

HONG, S.J. The overmethylated genes in *Helicobacter pylori* infected gastric mucosa are demethylated in gastric cancers. **BMC Gastroenterol**, v.10, p.137, 2010.

HU, K. et al. tPA protects renal interstitial fibroblasts and myofibroblasts from apoptosis. **J. Am. Soc. Nephrol**, v.19, n.3, p.503-514, 2008.

HUANG, J.Q. et al. Do patients with non-ulcer dyspepsia respond differently to *Helicobacter pylori* eradication treatments from those with peptic ulcer disease? A systematic review. **World J Gastroenterol**, v.11, p.2726-32, 2005.

ISHIHARA, S. et al. Intragastric distribution of *Helicobacter pylori* during short-term omeprazole therapy: Study using Carnoy's fixation and immunohistochemistry for detection of bacteria. **Aliment Pharmacol Ther**, v.15, p.1485–91, 2001.

JO, Y., et al. Suppressed gastric mucosal TGF-beta1 increases susceptibility to *H. pylori*-induced gastric inflammation and ulceration: a stupid host defense response. **Gut Liver**, v.4, p.43–53, 2010.

KABIR, S. Effect of *Helicobacter pylori* eradication on incidence of gastric cancer in human and animal models: underlying biochemical and molecular events. **Helicobacter**, v.14(3), p.159-71, 2009.

KAZUMORI, H. et al. Localization of Reg receptor in rat fundic mucosa. **J Lab Clin Med**, v.139, p.101–8, 2002.

KEATES, A.C. et al. *Helicobacter pylori* infection stimulates plasminogen activator inhibitor 1 production by gastric epithelial cells. **Inf Immun**, v.76, p.3992-3999, 2008.

KENNY, S. et al. Increased expression of the urokinase plasminogen activator system by *Helicobacter pylori* in gastric epithelial cells. **Am J Physiol Gastrointest Liver Physiol., v.**295, p.G431–G441, 2008.
KENNY, S. et al. The role of plasminogen activator inhibitor-1 in gastric mucosal protection. **Am J Physiol Gastrointest Liver Physiol**, v.304, p.G814-22, 2013.

KINOSHITA, Y. et al. Reg protein is a unique growth factor of gastric mucosal cells. **J Gastroenterol**, v.39, p.507–13, 2004.

KORDEK, R. et al. Basal keratin expression in breast cancer by quantification of mRNA and by immunohistochemistry. **J Exp Clin Cancer Res**, v.28, p.29:39, 2010.

LEE, J.; CHOI, J.H.; JOO, CK. TGF-β1 regulates cell fate during epithelial-mesenchymal transition by upregulating survivin. **Cell Death Dis**, v.4, p.714. 2013.

LEE, K.S., et al. *Helicobacter pylori* CagA triggers expression of the bactericidal lectin REG3γ via gastric STAT3 activation. PLoS One.,v.7(2), 2012.

LIN Z-Y, CHUANG W-L. Genes responsible for the characteristics of primary cultured invasive phenotype hepatocellular carcinoma cells. **Biomedicine & Pharmacotherapy**, v.66, p.454–458, 2012.

LIPPITZ, B.E. Cytokine patterns in patients with cancer: a systematic review. **Lancet Oncol**, v.14, p.e218-28, 2013.

LIU, Z. et al. *Helicobacter pylori* CagA inhibits the expression of Runx3 via Src/MEK/ERK and p38 MAPK pathways in gastric epithelial cell. **J Cell Biochem**, v.113(3), p.1080-6, 2012.

MALFERTHEINER, P. et al. Management of *Helicobacter pylori* infection – the Maastricht IV/Florence Consensus Report. **Gut,** v.61p.646–664, 2012.

MUELLER, A.; FALKOW, S.; AMIEVA, M.R. *Helicobacter pylori* and gastric cancer: what can be learned by studying the response of gastric epithelial cells to the infection? **Cancer Epidemiol Biomarkers Prev**, v.14(8), p.1859-64, 2005.

MURANO, T. et al. Hes1 promotes the IL-22-mediated antimicrobial response by enhancing STAT3-dependent transcription in human intestinal epithelial cells. **Biochem Biophys Res Commun**. v.17;443, p.840-846, 2014.

NAKACHI, N. et al. *Helicobacter pylori* infection of human gastric epithelial cells induces IL-8 and TNF-a but not TGF-b 1 mRNA. **FEMS Immunol Med Microbiol**, v.29, p.23–6, 2000.

NORSSET, K.G. et al. Gastrin stimulates expression of plasminogen activator inhibitor-1 in gastric epithelial cells. **Am J Physiol Gastrointest Liver Physiol., v.**301, p.446–G453, 2011.
PEEK, R.M.JR; CRABTREE, J.E. *Helicobacter infection* and gastric neoplasia**. J Pathol**., v.208, p.233–48, 2006.

RAHIMIAN, G. et al. Virulence factors of *Helicobacter pylori* vacA increase markedly gastric mucosal TGF-β1 mRNA expression in gastritis patients. **Microb Pathog.**, v.67-68, p.1-7, 2014.

RESNICK, M.B. et al. Global analysis of the human gastric epithelial transcriptome altered by *Helicobacter pylori* eradication in vivo. **Gut**, v.55, p.1717-24, 2006.

RHEAD, J.L. et al. A new *Helicobacter pylori* vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. **Gastroenterology**, 133:926-36, 2007.

RHEAD, J.L., et al. A new *Helicobacter pylori* vacuolating cytotoxin determinant, the intermediate region, is associated with gastric cancer. **Gastroenterology**, v.133, p.926-36, 2007.

RIMABRA, E.; FISCHBACH, L.A.; GRAHAM, D.Y. Optimal therapy for *Helicobacter pylori* infections. **Nat Rev Gastroenterol Hepatol**, v.8, p.79-88, 2011.

RODA, O. et al. Galectin-1 is a novel functional receptor for tissue plasminogen activator in pancreatic cancer. **Gastroenterology**, v.136, p.1379–90, 2009.

ROSSI, A.F., et al. Deregulation of annexin-A1 and galectin-1 expression in precancerous gastric lesions: intestinal metaplasia and gastric ulcer. **Mediators Inflamm**, v.478138, 2014.

SHARMA, M.; OWNBEY, R.T.; SHARMA, M.C. Breast cancer cell surface annexin II induces cell migration and neoangiogenesis via tPA dependent plasmin generation. **Exp Mol Pathol**, v.88, p.278–86, 2010.

SMITH, S.M., et al. Practical treatment of *Helicobacter pylori*: a balanced view in changing times. **Eur J Gastroenterol Hepatol**, 2014.

SONG, Z. et al. A study to explore Hp antibiotic resistance and efficacy of eradication therapy in China (multicenter, nation-wide, randomized, control study). **Helicobacter**, v.16, (Suppl. 1), p.117, 2011.

SUGIMOTO, M. et al. Treatment strategy to eradicate *Helicobacter pylori* infection: impact of pharmacogenomics-based acid inhibition regimen and alternative antibiotics. **Expert Opin. Pharmacother**, v.8, p.2701-2717, 2007.
SUGIMOTO, M.; YAMAOKA, Y. Virulence factor genotypes of *Helicobacter pylori* affect cure rates of eradication therapy. **Arch Immunol Ther Exp**, v.57, p.45-56, 2009.

TABASSAM, F.H. et al. *Helicobacter pylori* activate epidermal growth factor receptor- and phosphatidylinositol 3-OH kinase-dependent Akt and glycogen synthase kinase 3beta phosphorylation. **Cell Microbiol** 11(1):70–82, 2009.

THORNS, C. et al. Deregulation of a distinct set of microRNAs is associated with transformation of gastritis into MALT lymphoma. **Virchows Arch**, v.460(4), p.371- 372, 2012.

VAN, B.G.A. et al. REG gene expression in inflamed and healthy colon mucosa explored by in situ hybridisation. **Cell Tissue Res**. V.352(3), p.639-46, 2013.

WU, D.C. et al. Sequential and concomitant therapy with four drugs is equally effective for eradication of *H. pylori* infection. **Clin. Gastroenterol. Hepatol**., v.8, n.1, p.36–41, 2010.

YANG, G. et al. IFITM1 plays an essential role in the antiproliferative action of interferon-γ. **Oncogene**, v.26, p.594–603, 2007.

YANG, L.; PANG, Y.; MOSES, H.L. TGF-β and immune cells: an important regulatory axis in the tumor microenvironment and progression. **Trends Immunol,** v.31, p.220–227, 2010.

YANG, ZM; CHEN, W.W.; WANG, Y.F. Gene expression profiling in gastric mucosa from *Helicobacter pylori*-infected and uninfected patients undergoing chronic superficial gastritis. **PLoS One**, v.7, p.e33030, 2012.

YOSHINO, N. et al. Interleukin-8 Regulates Expression of Reg Protein in *Helicobacter pylori*-Infected Gastric Mucosa. Am J Gastroenterol 2005;100:2157–2166.

ZHAO, J.J. et al. Influence of *Helicobacter pylori* genotype on triple eradication therapy. **J Gastroenterol Hepatol**, v. 22, p. 2251-55, 2007.

Table 1. Comparisons of *IL-8, TGF-β, PLAT, PAI-1*, *REG3A* and *IFITM1* mRNA relative expression levels in patients with gastric lesions before and after *H. pylori* eradication therapy.

Genes	Before treatment (Hp+)	After treatment	
		Eradicated (Hp-)	Non-eradicated (Hp+)
IL-8			
Samples (n)	29	17	12
Median RQ	20.42	6.51	15.02
Range	0.65 - 236.60	0.91 - 18.44	0.61 - 49.03
P-value	0.013	0.076	
TGF-β			
Samples (n)	32	19	13
Median RQ	1.58	2.74	3.01
Range	0.71 - 7.04	0.10 - 8.56	0.56 - 20.88
P-value	0.106	0.497	
PLAT			
Samples (n)	38	24	14
Median RQ	0.64	1.12	0.60
Range	0.27 - 1.75	0.45 - 6.28	0.05 - 2.18
P-value	0.003	0.119	
PAI-1			
Samples (n)	38	24	14
Median RQ	0.15	0.22	0.12
Range	0.05 - 0.50	0.04 - 1.23	0.00 - 0.21
P-value	0.340	0.017	
REG3-A			
Samples (n)	38	24	14
Median RQ	7.68	0.61	4.59
Range	0.13 - 110.80	0.09 - 7.33	0.01 - 45.89
P-value	0.0001	0.009	
IFTM1			
Samples (n)	37	23	14
Median RQ	1.38	1.361	1.24
Range	0.47 - 2.44	0.39 - 2.29	0.00 - 2.89
P-value	1.000	0.991	

RQ: relative quantification; P-*value*= Mann Whitney test; $p < 0.05$.

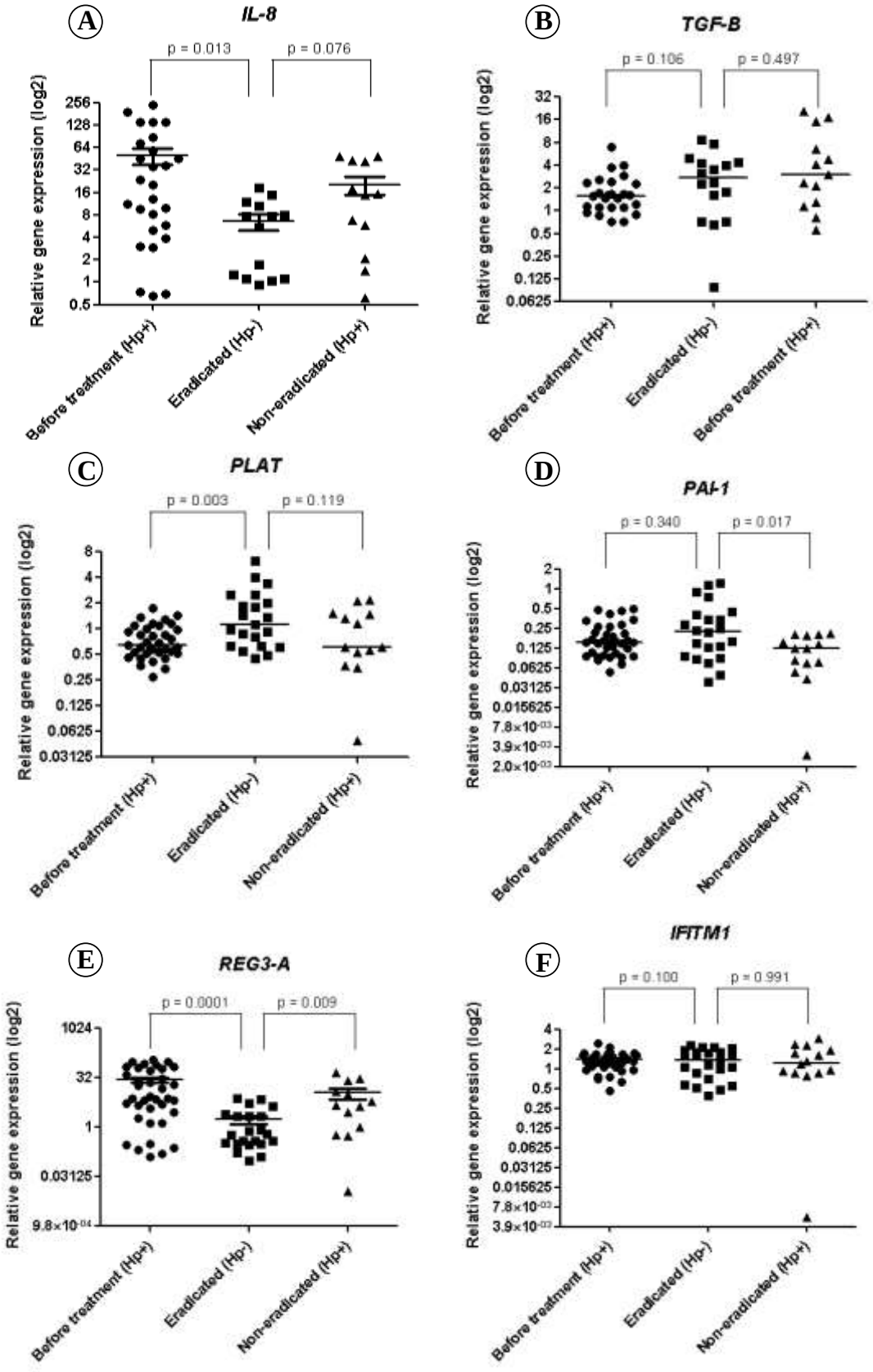


Figure 1. Relative expression levels of *IL-8, TGF-β, PLAT, PAI-1*, *REG3A* and *IFITM1* RNAm in patients infected with *H. pylori* before treatment (Hp+)

compared to eradicated patients (Hp-) and non-eradicated patients (Hp+) after eradication therapy. (A) *IL-8,* (C) *PLAT-1* and (E) *REG3A* mRNA levels are statistically different between CG-Hp+ patients before treatment and eradicated patients after treatment. For *REG3A* mRNA expression was detected reduction in eradicated patients relative to non-eradicated. (D) *PAI-1* mRNA levels was higher in eradicated patients than non-eradicated. (B) *TGF-β* and (F) *IFITM1* mRNA showed no change in gene expression levels before and after treatment. Data are presented as median and range for experiments performed in triplicate. Levels are normalized to *GAPDH* and *β-actin* reference genes. P values <0.05 was considered statistically significant using the Mann Whitney test.

Table 2. Comparisons of relative gene expression levels stratified by histological type of lesion, as normal mucosa (NM), chronic gastritis (CG), atrophic gastritis (AG), and metaplastic atrophic gastritis (MAG).

	Before treatment (Hp+)			**After treatment**					
				Eradicated (Hp-)			**Non-eradicated (Hp+)**		
	CG	**AG**	**MAG**	**NM**	**CG**	**AG**	**CG**	**AG**	**MAG**
IL-8									
Samples (%)	19/28	4/28	5/28	2/16	13/16	1/16	7/12	4/12	1/12
Median RQ	16.67	3.84	45.21	1.10	6.51	14.87	15.31	21.78	1.43
Range	0.65-236.6	2.88-140.7	8.17-138.3	1.09-1.10	0.91-12.05	-	5.76-49.03	0.61-48.59	-
P value		0.613			0.188			0.350	
TGF-β									
Samples (%)	19/32	7/32	6/32	2/19	13/19	4/19	8/13	4/13	1/13
Median RQ	1.51	1.35	1.99	3.04	2.74	2.36	4.83	2.68	1.16
Range	0.71-7.04	0.85-1.71	0.88-2.88	1.75-4.34	0.10-7.63	0.66-8.56	0.82-20.88	0.56-4.82	-
P value		0.618			0.950			0.291	
PLAT									
Samples (%)	24/38	8/38	6/38	2/24	18/24	4/24	9/14	4/14	1/14
Median RQ	0.83	0.59	0.67	1.41	0.97	5.21	1.24	0.49	0.53
Range	0.27-1.75	0.44-0.77	0.34-1.09	1.37-1.45	0.45-3.99	0.60-6.28	0.05-2.18	0.35-1.52	-
P value		0.537			0.911			0.396	
PAI-1									
Samples (%)	24/38	8/38	6/38	2/24	18/24	4/24	9/14	4/14	1/14
Median RQ	0.16	0.15	0.15	0.15	0.27	0.17	0.15	0.10	0.14
Range	0.07-0.50	0.09-0.48	0.05-0.21	0.07-0.23	0.04-1.23	0.09-0.75	0.00-0.21	0.07-0.12	-

P value		0.829			0.628			0.680	
REG3-A									
Samples (%)	24/38	8/38	6/38	4/24	18/24	2/24	9/14	4/14	1/14
Median RQ	6.78	7.15	27.14	0.68	0.71	0.39	2.79	5.29	11.98
Range	0.13-110.8	0.24-100.2	6.78-65.14	0.30-2.10	0.10-7.33	0.38-0.39	0.01-45.89	2.85-30.61	-
P value		0.501			0.875			0.595	
IFITM1									
Samples (%)	23/37	8/37	6/37	2/23	17/23	4/23	9/14	4/14	1/14
Median RQ	1.30	1.38	1.52	1.81	1.28	0.93	1.61	1.14	0.93
Range	0.47-2.44	0.74-1.82	0.70-2.11	1.74-1.88	0.47-2.29	0.39-1.76	0.01-2.89	0.86-2.28	-
P value		0.63			0.278			0.799	

Hp+: *H. pylori* infection; Hp-: uninfected patients; RQ: relative quantification; P-*value*= Kruskal-Wallis test.

Table 3. Frequencies of the *CagA* and *VacA* virulence genotypes of *H. pylori* and evaluation of its influence on bacteria eradication after treatment.

Genotype	Eradicated N (%)	Non-Eradicated N (%)
CagA		
Positive	6 (46,1%)	6 (54,5%)
Negative	7 (53,9%)	5 (45,5%)
Total	13	11
P-value	1.000	
VacA		
s1/m1	7 (50%)	3 (25%)
others	7 (50%)	9 (75%)
Total	14	12
P-value	0.247	

P-value = Fisher's exact test; $p<0.05$

Table 4. Relative gene expression levels according *CagA* genotype of *H. pylori* in infected patients before and after bacteria eradication treatment.

Genes	Before treatment (Hp+)		After treatment			
			Eradicated (Hp-)		Non-Eradicated (Hp+)	
	cagA+	***cagA-***	***cagA+***	***cagA-***	***cagA+***	***cagA-***
IL-8						
Samples (%)	10/19 (52.6)	9/19 (47.4)	4/9 (44.4)	5/9 (55.6)	6/10 (60)	4/10 (40)
RQ Median	21.98	45.09	1.05	7.58	28.10	3.59
Range	0.70-141.5	3.84-236.6	0.92-5.44	1.27-7.87	2.10-49.03	0.61-17.69
P-value	0.762		0.143		0.114	
TGF-β						
Samples (%)	10/21 (47.6)	11/21 (52.4)	4/10 (40)	6/10 (60)	6/11 (54.5)	5/11 (45.5)
RQ Median	1.55	1.71	1.16	4.00	5.73	2.11
Range	0.72-4.00	0.95-7.04	0.66-2.30	0.10-4.97	0.56-20.88	1.16-17.12
P-value	0.424		0.191		0.329	
PLAT						
Samples (%)	12/24 (50)	12/24 (50)	6/13 (46.2)	7/13 (53.8)	6/11 (54.5)	5/11 (45.5)
RQ Median	0.54	0.69	0.96	1.90	0.94	0.60
Range	0.34-1.70	0.27-1.45	0.60-3.99	0.45-3.40	0.05-2.18	0.53-1.16
P-value	0.325		0.937		1.000	
PAI-1						
Samples (%)	12/24 (50)	12/24 (50)	6/13 (46.2)	7/13 (53.8)	6/11 (54.5)	5/11 (45.5)
RQ Median	0.15	0.12	0.13	0.23	0.10	0.07
Range	0.08-0.32	0.05-0.50	0.04-1.15	0.15-1.23	0.00-0.15	0.04-0.19
P-value	0.793		0.180		0.931	
REG3A						
Samples (%)	12/24 (50)	12/24 (50)	7/13 (46.2)	6/13 (53.8)	6/11 (54.5)	5/11 (45.5)
RQ Median	30.84	14.90	0.34	3.01	5.29	4.60
Range	1.37-83.77	3.65-100.2	0.09-4.35	0.13-7.33	0.01-45.89	0.59-11.98
P-value	0.325		0.937		1.000	
IFITM1						
Samples (%)	12/24 (50)	12/24 (50)	7/13 (46.2)	6/13 (53.8)	6/11 (54.5)	5/11 (45.5)
RQ Median	1.35	1.54	1.59	1.66	1.24	0.96
Range	0.70-1.82	0.94-6.91	0.51-2.07	0.47-2.13	0.00-2.28	0.78-1.89
P-value	0.312		0.836		0.931	

P-value = Mann Whitney test; $p < 0.05$

Table 5. Relative gene expression levels according *VacA* genotype of *H. pylori* in infected patients before and after bacteria eradication treatment.

Genes	Before treatment (Hp+)		After treatment			
			Eradicated (Hp-)		Non-Eradicated (Hp+)	
	***vacA* s1/m1**	***vacA* others**	***vacA* s1/m1**	***vacA* others**	***vacA* s1/m1**	***vacA* others**
IL-8						
Samples (%)	8/20 (60)	12/20 (40)	5/10 (50)	5/10 (50)	3/10 (30)	7/10 (70)
RQ Median	27.52	34.26	6.652	1.729	48.59	6.728
Range	0.70-141.5	3.84-236.6	1.05-10.44	0.91-7.82	1.43-49.03	0.61-41.47
P-valeu	0.482		0.413		0.383	
TGF-β						
Samples (%)	8/22 (36.4)	14/22 (63.6)	5/11 (45.5)	6/11 (54.5)	3/11 (27.3)	8/11 (72.7)
RQ Median	3.22	1.66	1.60	4.00	4.82	3.21
Range	0.72-258.8	0.95-48.41	0.10-3.11	0.72-4.97	1.16-20.88	0.56-17.12
P-value	0.433		0.095		0.776	
PLAT						
Samples (%)	10/26 (38.5)	15/26 (61.5)	7 /14 (50)	7/14 (50)	3/12 (25)	9/12 (75)
RQ Median	0.69	0.64	0.62	1.90	0.53	0.88
Range	0.34-1.75	0.27-1.45	0.49-3.99	0.45-3.40	0.37-2.11	0.05-2.18
P-value	0.905		0.628		0.921	
PAI-1						
Samples (%)	10/26 (38.5)	16/26 (61.5)	7/14 (50)	7/14 (50)	3/12 (25)	9/12 (75)
RQ Median	0.21	0.13	0.13	0.29	0.12	0.08
Range	0.05-0.48	0.09-0.50	0.04-1.15	0.15-1.23	0.08-0.14	0.00-0.20
P-value	0.340		0.035*		0.600	
REG3A						
Samples (%)	10/26 (38.5)	16/26 (61.5)	7/14 (50)	7/14 (50)	3/12 (25)	9/12 (75)
RQ Median	14.81	27.38	0.34	0.80	11.98	4.40
Range	1.36-110.8	3.65-100.2	0.09-4.35	0.13-7.33	6.19-45.89	0.01-30.61
P-value	0.515		0.181		0.145	
IFITM1						
Samples (%)	10/26 (38.5)	16/26 (61.5)	7/14 (50)	7/14 (50)	3/12 (25)	9/12 (75)
RQ Median	1.39	1.51	1.00	1.66	1.16	1.31
Range	0.70-2.44	0.94-1.82	0.51-2.07	0.47-2.13	0.93-2.28	0.00-2.89
P-value	0.978		0.383		0.864	

P-value = Mann Whitney test; $p < 0.05$

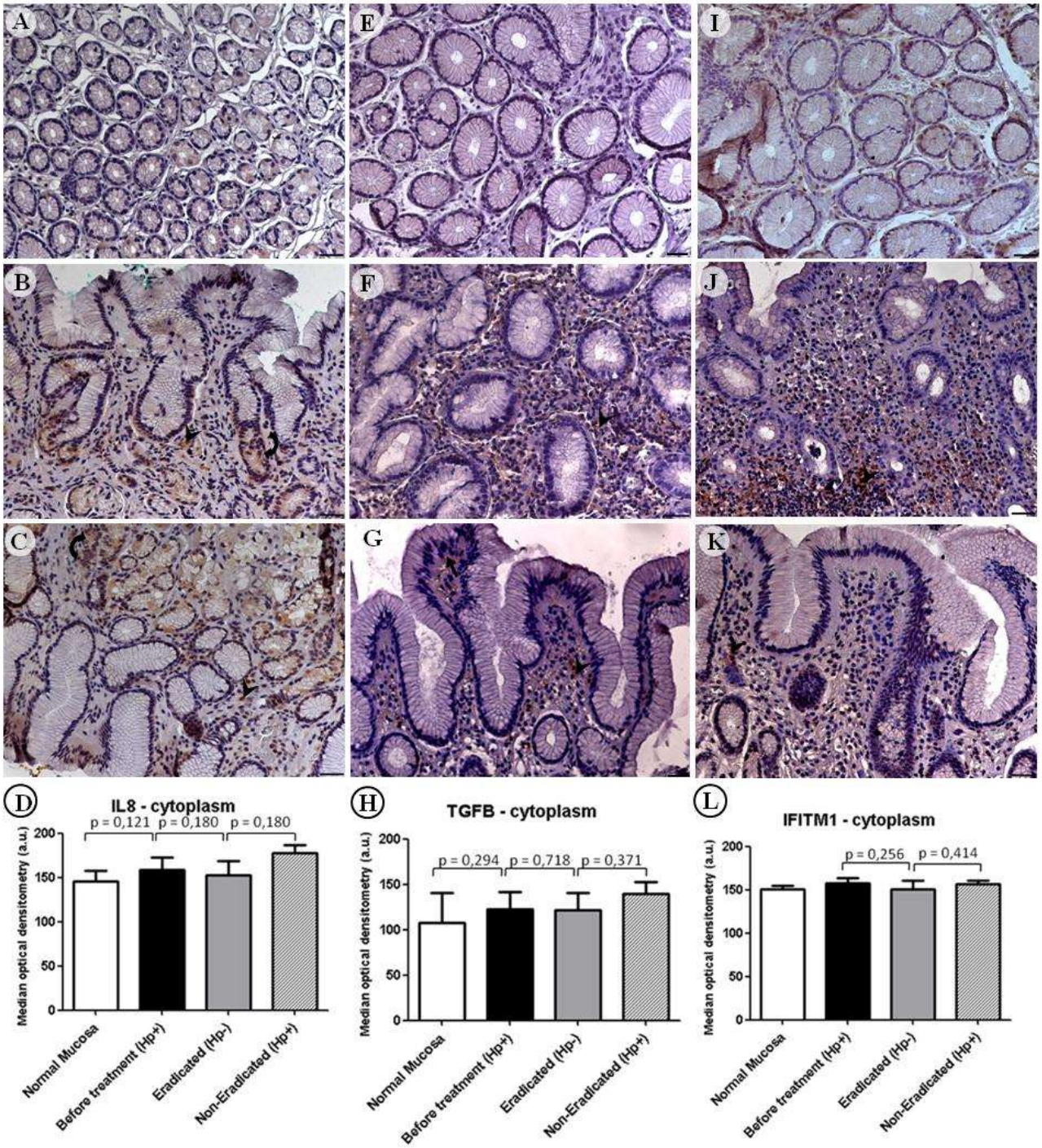
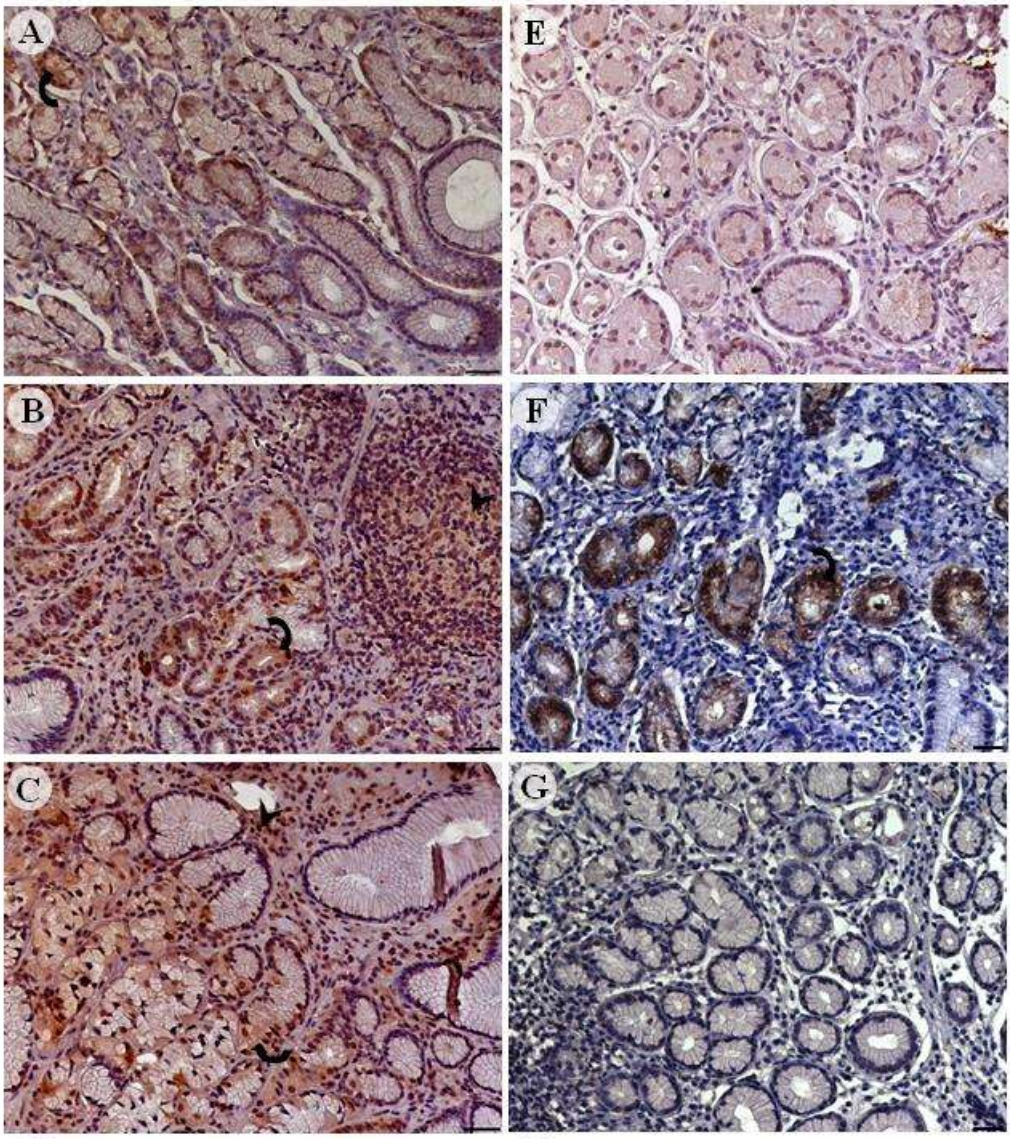
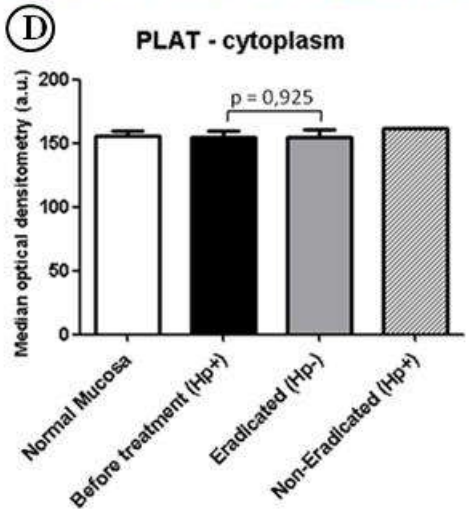


Figure 2. Immunohistochemical staining of IL-8, TGF-β1 and IFITM1 proteins in normal gastric mucosa (**A, E, I**) and *H. pylori*-associated chronic gastritis (CG-Hp+) before treatment (**B, F, J**) in comparison to eradicated patients after treatment (**C, G, K**). In the normal mucosa the proteins IL-8, TGF-β and IFITM1 (**A, E, I**, respectively) showed no staining or low expression intensity in the

epithelial cells of gastric glands and foveolar epithelium. Before treatment, these proteins (**B:** IL-8; **F:** TGF-β; **J:** IFITM1) in the CG-Hp+ patients presented cytoplasmic immunostaining pattern in the epithelial cells in gastric glands (arrows), in foveolar epithelium (arrows), and also in the inflammatory cells (arrowhead). After treatment (**C:** IL-8; **G:** TGF-β) a similar immunostaining pattern those before treatment was observed for most samples, while for IFITM1 (**K**) was reduced mainly in the inflammatory infiltrate. Densitometry analysis of cytoplasmic immunostaining (**D, H, L**) showed no significant change of protein expression between the patients before treatment and eradicated and non-eradicated patients. Counterstain: Hematoxylin. Bars: 50 μm. Densitometry analysis (Median; range); a.u.= arbitrary unit.


A
E
B
F
C
G


D
PLAT - cytoplasm
Median optical densitometry (a.u.)
200
150
100
50
0
p = 0,925
Normal Mucosa
Before treatment (Hp+)
Eradicated (Hp-)
Non-Eradicated (Hp+)

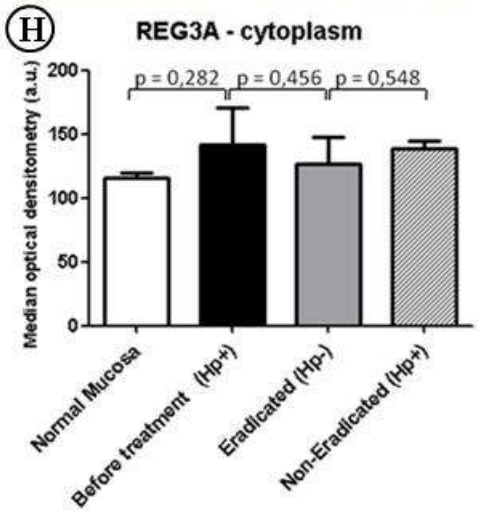

H
REG3A - cytoplasm
Median optical densitometry (a.u.)
200
150
100
50
0
p = 0,282
p = 0,456
p = 0,548
Normal Mucosa
Before treatment (Hp+)
Eradicated (Hp-)
Non-Eradicated (Hp+)

Figure 3. Immunohistochemical staining of PLAT and Reg3A in normal gastric mucosa (**A, E**) and *H. pylori*-associated chronic gastritis (CG-Hp+) before treatment (**B, F**) in comparison to eradicated patients after treatment (**C, G**). In the normal mucosa PLAT protein (**A**) showed positive immunoexpression in epithelial cells of gastric glands (arrow), while Reg3A (**E**) presented no staining or low expression intensity. CG-Hp+ patients before treatment (**B:** PLAT; **F:** Reg3A) cytoplasmic immunostaining pattern in the epithelial cells in gastric glands (arrows), in foveolar epithelium (arrows) and also in the inflammatory cells for PLAT protein was observed. After treatment, PLAT protein (**C**) remained immunopositive, while for Reg3A protein (**G**) negative immunostaining was detected for most of patients. Densitometry analysis of cytoplasmic staining (**D, H**) showed no significant difference of protein expression between the patients before treatment and eradicated and non-eradicated patients. Counterstain: Hematoxylin. Bars: 50 µm. Densitometry analysis (Median; range); a.u.= arbitrary unit.

Conclusões

IV. CONCLUSÕES

No presente estudo, considerando-se o grupo de indivíduos com lesões gástricas pré-cancerosas com infecção pela *H. pylori* avaliados antes e após o tratamento para erradicação da bactéria e as técnicas empregadas, é possível obter-se as seguintes conclusões:

1. Pacientes com gastrite crônica infectados pela *H. pylori* apresentam níveis aumentados da expressão relativa do RNAm de *TLR2, TLR4, IL-8, TGF-β, REG3A* e *IFITM1* e reduzidos de *PLAT* e *PAI-1* em comparação com a mucosa gástrica normal não infectada, assim evidenciando desregulação gênica decorrente da colonização pela bactéria. Após a erradicação da bactéria, apenas *IL-8, REG3A* e *PLAT* mostram mudanças para normalização nos níveis dos transcritos na mucosa gástrica;

2. Os níveis de expressão do RNAm dos genes avaliados não diferem conforme os tipos das lesões gástricas dos pacientes estudados (gastrite crônica, gastrite atrófica e gastrite atrófica associada à metaplasia intestinal);

3. Não há evidências de associação entre os genótipos de virulência bacteriano *CagA* e *VacA* com os níveis de expressão dos genes analisados, nem com a taxa de erradicação da bactéria, exceto *PAI-1* em que

indivíduos portadores do genótipo *VacA* s1/m1 apresentam menores níveis de expressão desse gene.

4. Em geral, observa-se imunoexpressão das proteínas TLR2, TLR4, IL-8, TGF-β, PLAT e IFITM1 nas células epiteliais das glândulas gástricas e também em células do infiltrado inflamatório do estroma para as proteínas PLAT e IFTM1, mas em geral, não é observada concordância absoluta da expressão dessas proteínas com os níveis de expressão do RNAm, exceto para os receptores TLR2 e TLR4.

5. A infecção pela *H. pylori* aumenta o índice apoptótico das células epiteliais gástricas, os quais são reduzidos após a terapia de erradicação da bactéria, mas não altera significantemente o índice de proliferação celular após o tratamento, sugerindo que a bactéria induz danos na mucosa gástrica, estimulando a morte celular.

Referências Bibliográficas

V. REFERÊNCIAS BIBLIOGRÁFICAS

ACHYUT, B.R. et al. Transforming growth factor-B1 and matrix metalloproteinase-7 promoter variants induce risk for *Helicobacter pylori*-associated gastric precancerous lesions. **DNA Cell Biol**, v.28, p.295-301, 2009.

AMIEVA, M.R.; EL-OMAR, E.M. Host-bacterial interactions in *Helicobacter pylori* infection. **Gastroenterology**, v.134, p.306-323, 2008.

ATHERTON, J.C.; BLASER, M.J. Coadaptation of *Helicobacter pylori* and humans: ancient history, modern implications. **The Journal of Clinical Investigation**, v.119, p.2475-2487, 2009.

BACKERT, S. et al. Translocation of the *Helicobacter pylori* CagA protein in gastric epithelial cells by a type IV secretion apparatus. **Cell Microbiol**, v.2, p.155-164, 2002.

BACKERT, S.; MEYER, T.F. Type IV secretion systems and their effectors in bacterial pathogenesis. **Curr Opin Microbiol**, v.9, p. 207-217, 2006.

BACKERT, S.; TEGTMEYER, N.; SELBACH, M. The versatility of *Helicobacter pylori* CagA effector protein functions: The master key hypothesis. **Helicobacter**, v.15(3),p.163-76, 2010.

BALKWILL, F.R.; MANTOVANI, A. Cancer-related inflammation: Common themes and therapeutic opportunities. **Semin Cancer Biol**, v.22, p.33-40, 2012.

BARCELLOS-HOFF, M.H.; LYDEN, D.; WANG, T.C. The evolution of the cancer niche during multistage carcinogenesis. **Nat. Rev. Cancer**, v.13, n.7, p.511–518, 2013.

BASSO, D. et al. Clinical relevance of *Helicobacter pylori cagA* and *vacA* gene polymorphisms. **Gastroenterology**, v.135, n.1, p.91-99, 2008.

BAUER, B.; MEYER, T.F. The human gastric pathogen Helicobacter pylori and its association with gastric cancer and ulcer disease. **Ulcer**, v. 2011, p. 1-23, 2011.

BEN-NERIAH, Y.; KARIN, M. Inflammation meets cancer, with NF-κB as the matchmaker. **Nat. Immunol**, v.12, n.8, p.715–723, 2011.

BINDER, B.R.; MIHALY, J. The plasminogen activator inhibitor "paradox" in cancer. **Immunol. Lett**, v.118, n.2, p.116–124, 2008.

BRASS, A.L. et al. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. **Cell**, v.139(7), p.1243-54, 2009.

BROEKAERT, D. et al. Comparison of leptin- and interleukin-6-regulated expression of the rPAP gene family: evidence for differential co-regulatory signals. **Eur Cytokine Netw**, v.13, p.78-85, 2002.

CAO, G. et al. Pancreatitis-associated protein is related closely to neoplastic proliferative activity in patients with colorectal carcinoma. **Anat Rec (Hoboken)**, v.292, n.2, p.249-253, 2009.

CENSINI, S.et al. cag, a pathogenicity island of *Helicobacter pylori*, encodes type I-specific and disease-associated virulence factors. **Proc Natl Acad Sci USA,** 1996.

CHELI, R.; GIACOSA, A. Chronic atrophic gastritis and gastric mucosal atrophy-one and the same. **Gastrointest Endosc**, v.29, p.23-25, 1983.

CHEN, F. JNK-induced apoptosis, compensatory growth, and cancer stem cells. **Cancer Res.**, v.72, n.2, p.379–386, 2012.

CHEN, Y. et al. 1,25-Dihydroxyvitamin D_3 suppresses inflammation-induced expression of plasminogen activator inhibitor-1 by blocking nuclear factor-κB activation. **Arch Biochem Biophys**. 2011.

CHIBA, T.; MARUSAWA, H.; USHIJIMA, T. Inflammation-Associated Cancer Development in Digestive Organs: Mechanisms and Roles for Genetic and Epigenetic Modulation. **Gastroenterology,** v. 143, p. 550-563**,** 2012.

CLOSA, D.; Motoo, Y.; Iovanna, J.L. Pancreatitis-associated protein: from a lectin to an anti-inflammatory cytokine. **World J. Gastroenterol**, v13, p.170–174, 2007.

CORREA, P. Human model of gastric carcinogenesis. **Cancer Res,** v.48, p.3554-3560, 1988.

COUSSENS, L.M.; WERB, Z. Inflammation and cancer. **Nature**, v.420, n.6917, p.860-867, 2002.

DAN, H. et al. Association of Interleukin-8 Gene Polymorphisms and Haplotypes with Oral Lichen Planus in a Chinese Population. **Inflammation**, v.33, p.76-81, 2010.

de OLIVEIRA, J.G. et al. Profiles of gene polymorphisms in cytokines and Toll-like receptors with higher risk for gastric cancer. **Dig Dis Sci**. v.58; p.978-988, 2013.

de OLIVEIRA, J.G.; Duarte, M.C.; Silva, A.E. IL-1ra anti-inflammatory cytokine polymorphism is associated with risk of gastric cancer and chronic gastritis in a Brazilian population, but the TNF-β pro-inflammatory cytokine is not. **Mol Biol Rep**, v. 39, p.7617-7625, 2012.

de OLIVEIRA, J.G.; Silva, A.E. Polymorphisms of the TLR2 and TLR4 genes are associated with risk of gastric cancer in a Brazilian population. **World J Gastroenterol**. v.18, p.1235-1242, 2012.

DEBLANDRE, G.A. et al. Expression cloning of an interferon-inducible 17-KDa membrane protein implicated in the control of cell growth. **J. Biol. Chem.**, v.270, n.40, p.23860-23866, 1995.

DU, X.L. et al. Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. **Proc. Natl. Acad. Sci. U.S.A.**, v.97, n.22, p.12222–12226, 2000.

EL-OMAR, E.M. et al. The role of interleukin-1 polymorphisms in the pathogenesis of gastric cancer. **Nature,** v.412, p.99, 2001.

EVERHART, J.E. Recent developments in the epidemiology of *Helicobacter pylori*. **Gastroenterol. Clin. North Am**, v.29, n.3, p.559-578, 2000.

FAN, X.G. et al. Increased gastric production of interleukin-8 of tumor necrosis factor in patients with *Helicobacter pylori* infection. **J. Clin. Pathol.**, v.48, n.2, p.133-136, 1995.

FERRONE, C.; DRANOFF, G. Dual roles for immunity in gastrointestinal cancers. **J Clin Oncol,** v.28, p.4045-4051, 2010.

FREDRIKSSON, L. et al. Tissue plasminogen activator is a potent activator of PDGF-CC. **Embo J**. v.23, n.19, p.3793-3802, 2004.

FUCCIO, L. et al. (2009) Meta-analysis: can *Helicobacter pylori* eradication treatment reduce the risk for gastric cancer? **Ann. Intern. Med. 151, 121e8**; erratum in (2009) Ann. Intern. Med. 151, 516.

FUMOTO S. et al. Selection of a novel drug-response predictor in esophageal cancer: a novel screening method using microarray and identification of IFITM1 as a potent marker gene of CDDP response. **Int J Oncol**, v.32, p.413-423, 2008.

GENTA, R.M. Atrophy, metaplasia and dysplasia: are they reversible? **Ital J Gastroenterol Hepatol**, v.3, p.324-325, 1998.

GINGRAS D. et al .Activation of tissue plasminogen activator gene transcription by Neovastat, a multifunctional antiangiogenic agent. **Biochem Biophys Res Commun**, v.16, p.205-212, 2004.

GOMES, A.Q. et al. Identification of a panel of ten cell surface protein antigens associated with immunotargeting of leukemias and lymphomas by peripheral blood γδ T cells. **Haematologica,** v.95, p.1397–1404, 2010.

GRAHAM, D.Y.; LU, H.; YAMAOKA, Y. Therapy for *Helicobacter pylori* infection can be improved: sequential therapy and beyond. **Drugs**, v.68, N.6, p.725-736, 2008.

GREENBERG, E.R. et al. 14-day triple, 5-day concomitant, and 10-day sequential therapies for Helicobacter pylori infection in seven Latin American sites: a randomised trial. **Lancet**, v.378, n. 9790, p.507-514, 2011.

GUILFORD, P. et al. E-cadherin germline mutations in familial gastric cancer. **Nature**, v.392, n. 6674, p.402-405, 1998.

GUTIÉRREZ-GONZÁLEZ, L.; WRIGHT, N.A. Biology of metaplasia in 2008: more than a simple phenotypic alteration. **Digest Liver Dis,** v.40, p.510-522, 2008.

HATAKEYAMA, M. *Helicobacter pylori* and gastric carcinogenesis. **J. Gastroenterol.**, v.44, n.4, p.239-248, 2009.

HATANO, H. et al. IFN-induced transmembrane Protein 1 Promotes Invasion at early stage of head and neck cancer progression. **Clin. Cancer Res.**, v.14, n.19, p.6097-6105, 2008.

HERRERA, V.; PARSONNET, J. *Helicobacter pylori* and gastric adenocarcinoma. **Clin Microbiol Infect**. v.15, p. 971-976, 2009.

HIGAZI, A.A. et al. Immunomodulatory effects of plasminogen activators on hepatic fibrogenesis. **Clin. Exp. Immunol.**, v.152, n.1, p.163-173, 2008.

HOFFMAN, V.J. et al. Gene expression profiling in human gastric mucosa infected with *Helicobacter pylori*. **Mod. Pathol.**, v.20, n.9, p.974-989, 2007.

HU, K. et al. tPA protects renal interstitial fibroblasts and myofibroblasts from apoptosis. **J. Am. Soc. Nephrol.**, v.19, n.3, p.503-514, 2008.

HUANG, F., et al. Characterization of interleukin-1β in *Helicobacter pylori*-induced gastric inflammation and DNA methylation in interleukin-1 receptor type 1 knockout (IL-1R1(-/-)) mice. **Eur J Cancer**, v.49, p.2760-2670, 2013.

INTERNATIONAL AGENCY FOR RESEARCH ON CANCER (IARC). Schistossomes, liver flukes and *Helicobacter pylori*. Lyon: **IARC**, 1994.

JAFRI, N.S; HORNUNG, C.A.; HOWDEN, C.W. Meta-analysis: sequential therapy appears superior to standard therapy for *Helicobacter pylori* infection in patients naive to treatment. **Ann. Intern. Med.**, v.148, n.8, p.923-931, 2008.

JANG, B-G; KIM, W.H. Molecular Pathology of Gastric Carcinoma. **Pathobiology,** v.78, p.302-310, 2011.

JEMAL, A. et al. Global Cancer Statistics. **CA Cancer J Clin.**, v.61, n.2, p.69–90, 2011.

JOHNATTY SE. et al. Evaluation of candidate stromal epithelial cross-talk genes identifies association between risk of serous ovarian cancer and TERT, a cancer susceptibility "hot-spot." **PLoS Genet**, v.6, p.e1001016, 2010.

KABIR, S. Effect of *Helicobacter pylori* eradication on incidence of gastric cancer in human and animal models: underlying biochemical and molecular events. **Helicobacter**. v.14(3), p.159-71, 2009.

KANG, J.Y.; LEE, J.O. Structural biology of the Toll-like receptor family. **Ann Rev Biochem**. v.80, p.917-941, 2011.

KEATES, A.C. et al. *Helicobacter pylori* infection stimulates plasminogen activator inhibitor 1 production by gastric epithelial cells. **Inf Immun**, v.76, p.3992-3999, 2008.

KEIM, V. et al. An additional secretory protein in the rat pancreas. **Digestion**. v.29, p.242–249, 1984.

KIM, D.H; KIM, S.J. Transforming growth factor-beta receptors: role in physiology and disease. J Biomed Sci, v.3, p.143-158, 1996.

KIM, K.K.; KIM, H.B. Protein interaction network related to *Helicobacter pylori* infection response. **World J Gastroenterol**, v.15, p.4518-4528, 2009.

KUIPERS, E.J. Exploring the link between *Helicobacter pylori* and gastric cancer. **Aliment Pharmacol Ther**, v.13, p.3-11, 1999.

KURAISHY, A.; KARIN, M.; GRIVENNIKOV, S.I. Tumor promotion via injury and death-induced inflammation. **Immunity.**, v.35, n.4, p.467–477, 2011.

LAUREN, P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma: an attempt at a histo-clinical classification. **Acta Pathol Microbiol Scand**, v.64, p.31-49, 1965.

LI, Z.W. et al. Inflammatory cytokine gene polymorphisms increase the risk of atrophic gastritis and intestinal metaplasia. **World J Gastroenterol,** v.16, p.1788-1794, 2010.

LIN, L.; WU, C.; HU, K. Tissue Plasminogen Activator Activates NF-kappaB through a Pathway Involving Annexin A2/CD11b and Integrin-Linked Kinase. **J. Am. Soc. Nephrol.**, v.23, n.8, p.1329-1338, 2012.

MAEHARA, Y. et al. Role of transforming growth factor-beta 1 in invasion and metastasis in gastric carcinoma. **J Clin Oncol**, v.17, p.607-614, 1999.

MALFERTHEINER, P. et al. Helicobacter pylori eradication with a capsule containing bismuth subcitrate potassium, metronidazole, and tetracycline given with omeprazole versus clarithromycin-based triple therapy: a randomised, open label, non-inferiority, phase 3 trial. **Lancet**, v.377, n. 9769, p.905-913, 2011.

MASSAGUE, J.; SEOANE, J.; WOTTON, D. Smad transcription factors. **Genes Dev**, v.19, p.2783-2810, 2005.

MATSUMOTO, A. K. et al. Functional dissection of the CD21/CD19/TAPA-1/Leu-13 complex of B lymphocyte. J Exp Med, v.178, p.1407-1417, 1993.

MILNE, A.N.; CARNEIRO, F.; O'MORAIN, C. Nature meets nurture: molecular genetics of gastric cancer. **Hum genet**, v.126, p.615-628, 2009.

PAN, Z. et al. Differential gene expression identified in Uigur women cervical squamous cell carcinoma by suppression subtractive hybridization. **Neoplasma**, v.57, p.123–128, 2010.

PEEK, R.M. *Helicobacter pylori* strain-specific modulation of gastric mucosal cellular turnover: implications for carcinogenesis. **J Gastroenterol**. V. 37, Suppl 13.p. 10-16, 2002.

POLK, D.B.; PEEK, R.M. *Helicobacter pylori:* gastric cancer and beyond. **Nature Reviews**, v.10, p.403-414, 2010.

QUANTE, M.; Wang, T.C. Inflammation and stem cells in gastrointestinal carcinogenesis. **Physiol**, v.23, p.350-359, 2008.

RESENDE, C. et al. Gastric Cancer: Basic Aspects. **Helicobacter,** v.16, p.38-44, 2011.

RICCI, V.; ROMANO, M.; BOQUET, P. Molecular cross-talk between *Helicobacter pylori* and human gastric mucosa. **World J Gastroenterol,** v.17, p.1383-1399, 2011.

RIMABRA, E.; FISCHBACH, L.A.; GRAHAM, D.Y. Optimal therapy for *Helicobacter pylori* infections. **Nat Rev Gastroenterol Hepatol**, v.8, p.79-88, 2011.

ROELOFS, J.J. et al. Tissue-type plasminogen activator modulates inflammatory responses and renal function in ischemia reperfusion injury. **J. Am. Soc. Nephrol.**, v.17, n.1, p.131-140, 2006.

SACHS, G.; SCOTT, DR. *Helicobacter pylori*: Eradication or Preservation. **F1000 Med Rep, v.**4, p.7, 2012.

SAITO, H. et al. An elevated serum level of transforming growth factor-beta 1 (TGF-beta1) significantly correlated with lymphonode metastasis and poor prognosis in patients with gastric carcinoma. **Anticancer Res**, v.20, p.4489-4493, 2000.

SHI, Y. et al. Ligand binding to LRP1 transactivates Trk receptors by a Src family kinase-dependent pathway. **Sci Signal**. 2009; 2: ra18.

SHIM, B.C. et al. The kringle domain of tissue-type plasminogen activator inhibits in vivo tumor growth. **Biochem Biophys Res Commum**, v.327, p.1155-1162, 2005.

SIEGEL, R.; NAISHADHAM, D.; JEMAL, A. Cancer statistics, 2012. **CA Cancer J Clin.**, v.62, n.1, p.10–29, 2012.

SIMON, M.T. et al. HIP/PAP stimulates liver regeneration after partial hepatectomy and combines mitogenic and anti-apoptotic functions through the PKA signaling pathway. **Faseb J**, v.17, p.1441-1450, 2003.

SMITH, H.W.; MARSHALL, C.J. Regulation of cell signaling by uPAR. **Nat Rev Mol Cell Biol**, v.11, p.23-36, 2010.

SMITH, M.G. et al. Cellular and molecular aspects of gastric cancer. **World J Gastroenterol**, v.12, p.2979-2990, 2006.

SONG, Z. et al. A study to explore Hp antibiotic resistance and efficacy of eradication therapy in China (multicenter, nation-wide, randomized, control study). **Helicobacter.**, v.16, p.117, 2011.

SPARMANN, A.; BAR-SAGI. D. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. **Cancer Cell**, v.5, p.447-458, 2004.

SUERBAUM, S.; MICHETTI, P. Helicobacter pylori infection. **N Engl J Med,** v**.**347, p.1175-1186, 2002.

SUGIMOTO, M.; YAMAOKA, Y. Virulence factor genotypes of *Helicobacter pylori* affect cure rates of eradication therapy. **Arch Immunol Ther Exp**, v.57, p.45-56, 2009.

SUGIMOTO, M.; YAMAOKA, Y.; FURUTA, T. Influence of interleukin polymorphisms on development of gastric câncer and peptic ulcer. **Gastroenterology**, v.16, p.1188-1200, 2010.

TAGUCHI, A. et al. Interleukin-8 Promoter Polymorphism Increases the Risk of Atrophic Gastritis and Gastric Cancer in Japan. **Cancer Epidemiol Biomarkers Prev**, v.14, p.2487-2493, 2005.

TAKEDA, K.; KAISHO, T.; AKIRA, S. Toll-like receptors. **Annu Rev Immunol**. v. 21, p.335-376, 2003.

TRAMACERE, I.; La VECCHIA, C.; NEGRI, E. Tobacco smoking and esophageal and gastric cardia adenocarcinoma: a meta-analysis. **Epidemiology**, v.22, p. 344–349, 2011.

TRUONG, B.X. et al. Diverse Characteristics of the *cagA* Gene of *Helicobacter pylori* Strains Collected from Patients from Southern Vietnam with Gastric Cancer and Peptic Ulcer. **J Clin Microbiol,** v.47, p.4021-4028, 2009.

TSAI, M.Y. et al. A mitotic lamin B matrix induced by ranGTP required for spindle assembly. **Science**, v.311, p.1887-1893, 2006.

UENO, M. et al. Hypoxia-inducible factor-1α mediates TGF-β-induced PAI-1 production in alveolar macrophages in pulmonary fibrosis. **Am. J. Physiol. Lung Cell Mol. Physiol.**, v.300, n.5, p.L740–752, 2001.

USHIJIMA, T.; HATTORI, N. Molecular pathways: involvement of *Helicobacter pylori*-triggered inflammation in the formation of an epigenetic field defect, and its usefulness as cancer risk and exposure markers. **Clin. Cancer Res.**, v.18, n.4, p.923–929, 2002.

VAGO, J.P. et al. Annexin A1 modulates natural and glucocorticoid-induced resolution of inflammation by enhancing neutrophil apoptosis. **J Leukocyte Biol,** v.92, p.1-10, 2012.

VAIRA, D. et al. Sequential therapy versus standard triple-drug therapy for *Helicobacter pylori* eradication: a randomized trial. **Ann. Intern. Med.**, v.146, n.8, p.556-563, 2007.

VAKIL, N.; MÉGRAUD, F. Eradication treatment for *Helicobacter pylori*. **Gastroenterol**, v. 133, p. 985-1001, 2007.

VAN DOORN, L.J. et al. Geographic distribution of vacA allelic types of *Helicobacter pylori*. **Gastroenterology,** v.116, p.823-830, 1999.

WEN, S.; MOSS, S.F. Helicobacter pylori virulence factors in gastriccarcinogenesis. **Cancer Lett**. V. 282, p. 1-8, 2009.

WILSON, K.Y.; CRABTREE, J.E. Immunology of *Helicobacter pylori:* Insights Into the Failure of the Immune Response and Perspectives on Vaccine Studies. **Gastroenterol,** v.133, p.288-308, 2007.

WU, D.C. et al. Sequential and concomitant therapy with four drugs is equally effective for eradication of *H. pylori* infection. **Clin. Gastroenterol. Hepatol.**, v.8, n.1, p.36–41, 2010.

WU, M.S. et al. Proteomic identification of biomarkers related to *Helicobacter pylori*-associated gastroduodenal disease: Challenges and opportunities. **J Gastroenterol Hepatol**, v.23, p.1657-1661, 2008.

WU, S. et al. Fish consumption and the risk of gastric cancer: systematic review and meta-analysis. **BMC Cancer**. v.11, p.1657-61, 2011.

YAMADA S, et al. Predominant mucosal IL-8 mRNA expression in non-cagA Thais is risk for gastric cancer.**World J Gastroenterol**., v.19(19), p. 2941-9, 2013.

YANG, Y. et al. The interferon-inducible 9–27 gene modulates the susceptibility to natural killer cells and the invasiveness of gastric cancer cells. Cancer Lett, v.21, p.191–200, 2005.

YOKOTA, S. et al. Highly-purified *Helicobacter pylori* LPS preparations induce weak inflammatory reactions and utilize Toll-like receptor 2 complex but not Toll-like receptor 4 complex. **FEMS Immunol Med Microbiol**, v.51p.140-148, 2007.

YUAN, A. et al. The role of interleukin-8 in cancer cells and microenvironment interaction. **Front Biosci**, v.10, p.853-865, 2005.

ZARRILI, R.; RICCI, V.; ROMANO, M. Molecular response of gastric epithelial cells to *Helicobacter pylori*-induced cell damage. **Cell Microbiol**. v.1, p.93-99, 1999.

ZHANG, H. et al. Targeted inhibition of gene expression of pancreatitis-associated proteins exacerbates the severity of acute pancreatitis in rats. **Scand J Gastroenterol**, v.39, p.870–81, 2004.

ZORIO, E. et al. Fibrinolysis: the key to new pathogenetic mechanisms. **Curr Med Chem**, v.15, p.923-929, 2008.

Apêndices

Apêndice 1. Questionário do projeto: Expressão gênica e cinética celular no processo inflamatório induzido pela *Helicobacter pylori* antes e após terapia de erradicação.

Responsáveis: Doutoranda Aline Cristina Targa Cadamuro, Profa. Dra. Ana Elizabete Silva (Departamento de Biologia IBILCE-UNESP, São José do Rio Preto-SP)

Colaboradores: Profa. Dra. Eny Maria Goloni Bertollo (FAMERP), Dr. Fausto Nasser (Hospital de Base, São José do Rio Preto, SP)

I. IDENTIFICAÇÃO

Nome:.....Prontuário:.....

Data de nascimento:...../...../..... Sexo: ( ) F ( ) M

Grupo étnico: ( ) caucasóide ( ) negróide ( ) asiático

Endereço:.....Fone:.....

Cidade:.....Estado:.....

Profissão atual:.....tempo de tuação:.....

Profissão anterior:.....tempo de atuação:.....

II. DADOS PESSOAIS E FAMILIAIS

- Consumo de bebida alcoólica: ( ) sim ( ) não ( ) ex-etilista

Há quantos anos:.....Tipo de bebida.....dose/dia.....

- Consumo de cigarro: ( ) sim ( ) não ( ) ex-fumante

Há quanto anos:.....Quantidade (un/dia):.....

- Doenças anteriores ou presentes:

( ) úlcera ( ) gastrite ( ) câncer (tipo:.....)

( ) outras (tipo:.....)

- Tratamentos anteriores ou em andamento: ( ) sim, período:..... ( ) não

Tipo:.....

- Uso de medicamentos: ( ) sim, período..... ( ) não

Tipo:.....

- Cirurgias anteriores: ( ) sim ( ) não

Tipo:.....

História de câncer ou outras doenças na família (grau de parentesco)

( ) câncer (tipo:.....) ( ) úlcera ( ) gastrite

( ) outras (tipo:.....

Apêndice 2. Características demográficas dos pacientes com infecção pela *H. pylori* incluídos neste estudo e submetidos à terapia de erradicação.

Pacientes	**Total n= 38**
Idade (anos), média ± SD	48 ± 15,9
Variação	21 - 82
Gênero	
Masculino	14 (37%)
Feminino	24 (63%)
Etilistas	
Sim	10 (26%)
Não	27 (71%)
Não avaliados	1 (3%)
Fumantes	
Sim	13 (34%)
Não	25 (66%)
Diagnóstico Histológico	
Gastrite Crônica (GC)	6 (16%)
Gastrite Atrófica (GA)	8 (21%)
Gastrite Atrófica Metaplásica (GAM)	24 (63%)
Terapia de Erradicação	
Erradicados	24 (63%)
Não-Erradicados	14 (37%)
Genótipos de virulência (*H. pylori*)	
CagA-positivo	12 (50%)
CagA-negativo	12 (50%)
VacA-s1/m1	10 (39%)
VacA-(s1/m2, s2/m2, s2/m1)	16 (61%)

Anexos

Anexo 1. Parecer consubstanciado do Comitê de Ética em Pesquisa Institucional.

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Câmpus de São José do Rio Preto

PARECER CONSUBSTANCIADO
PROJETO DE PESQUISA

IDENTIFICAÇÃO

Nome do pesquisador: Aline Cristina Targa Cadamuro
Departamento: Biologia
Instituição: IBILCE / UNESP – São José do Rio Preto
Projeto: "Avaliação da cinética celular e expressão de genes envolvidos na resposta inflamatória induzida pela *Helicobacter pylori* antes e após terapia de erradicação da bactéria e associação com o genótipo de virulência *CagA*"
Protocolo: 0007.0.229.000-10 de 18/março/2010

PARECER nº 030/10

O presente parecer refere-se ao projeto de Doutorado da pesquisadora Aline Cristina Targa Cadamuro desenvolvido junto ao programa de Pós Graduação em Genética do IBILCE/UNESP, com orientação da Profa. Dra. Ana Elizabete Silva.
O estudo tem por objetivo avaliar a expressão protéica (imunohistoquímica) e do RNAm de genes (PCR em tempo real) que atuam na resposta inflamatória que ocorre na mucosa gástrica de indivíduos com infecção pela bactéria *H. pylori*. Serão avaliados os mediadores inflamatórios (NOS-2, IL-1, IL-8, TGF-beta, TNF-alfa, TLR-2, TLR-4 e PAI-1), o genótipo *CagA* da *H. pylori* e a cinética celular (índice de proliferação e índice apoptótico), antes a após a terapia de erradicação do H. pylori.
Serão avaliadas biópsias da mucosa gástrica obtidas de pacientes submetidos à Endoscopia Digestiva Alta realizada no Hospital de Base.
A análise do material será realizada no laboratório de citogenética e biologia molecular com a colaboração da Profa. Dra. Eny M. G. Bertollo e Profa. Dra. Patrícia M. Cury (FAMERP) e Profa. Dra. Kátia R. M. Leite (Hospital Sírio Libanês).
O projeto apresenta-se de maneira bem estruturada com Introdução, Justificativa, Material e Métodos, Casuística e Bibliografia.
Foram apresentados: cronograma de execução, orçamento financeiro, declaração de infra-estrutura e termo de compromisso conforme os termos da resolução 196/96 do CNS.
O TCLE está redigido de forma clara e compreensível com o nome do responsável pela obtenção do mesmo.
Desta maneira o CEP aprova o projeto.

(X) APROVADO
( ) COM PENDÊNCIA, máximo de 60 dias para atendimento
( ) RETIRADO
( ) NÃO APROVADO
( ) APROVADO, aguardar apreciação final da CONEP

Datas previstas para apresentação dos relatórios
1° relatório: abril/2011 – 2° relatório: abril/2012 - 3° relatório: abril/2013
Obs.: Para facilitar a apresentação do relatório poderá seguir como orientação o roteiro no site http://www.ibilce.unesp.br/instituicao/comissoes/etica/arquivos/Roteiro_02.doc

São José do Rio Preto, 14 de abril de 2010.

Prof. Dr. Raul Aragão Martins
Coordenador do CEP

Rua Cristóvão Colombo, 2265 – Jardim Nazareth - CEP 15054-000
São José do Rio Preto – SP – Brasil
Tel (17) 3221-2456

Anexo 2. Parecer consubstanciado do Comitê de Ética Ambiental

unesp UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" Campus de São José do Rio Preto

Formulário 2

OFÍCIO: 07/2010
INTERESSADOS: Orientadora: Profa. Dra. Ana Elizabete Silva
Aluna: Aline Cristina Targa Cadamuro
ASSUNTO: Parecer

São José do Rio Preto, 29 de junho de 2010.

PARECER DA COMISSÃO DE ÉTICA AMBIENTAL (CEA)

Trata o presente da análise do potencial de risco dos resíduos químicos a serem gerados do projeto de pesquisa intitulado, "Expressão gênica e cinética celular no processo inflamatório induzido pela *Helicobacter pylori* antes e após terapia de erradicação". Este projeto de pesquisa será enviado à FAPESP para solicitação de bolsa de doutorado a ser desenvolvido pela aluna Aline Cristina Targa Cadamuro, pós-graduanda do curso de Genética. O projeto será executado no Laboratório de Citogenética e Biologia Molecular Humana, do Departamento de Biologia do Instituto de Biociências, Letras e Ciências Exatas da UNESP – São José do Rio Preto, sob coordenação da Profa. Dra. Ana Elizabete Silva, também deste Departamento, no período de 24/02/2010 a 01/03/2014. Os resíduos químicos gerados em função de procedimentos de laboratório serão: RNA later, Trizol, Gel de agarose corado com brometo de etídio, Tampão TBE (ácido bórico, EDTA e Tris-Base), Etanol, Citrato de sódio 0,1M em etanol 10%, Tampão PBS 0,1M, Peróxido de Hidrogênio a 3%, SYBR Green e Clorofórmio. A metodologia para descarte dos resíduos deverá ser realizada de acordo com pesquisa realizada pelo solicitante e encaminhada a Comissão de Ética Ambiental, que avaliou e aprovou o tratamento proposto.

Sendo assim, esta Comissão julga que os procedimentos estão de acordo com as normas estabelecidas pela CEA/IBILCE.

Atenciosamente,

Profª. Drª. Márcia Cristina Bisinoti
Comissão de Ética Ambiental
Presidente
IBILCE/UNESP

Instituto de Biociências, Letras e Ciências Exatas – Departamento de Química e Ciências Ambientais
Rua Cristovão Colombo, 2265 – Jardim Nazareth. CEP 15054-000 – São José do Rio Preto SP Brasil
Tel 17 221 2350 fax 17 221 2356

Anexo 3. Comprovante de submissão do artigo "*Helicobacter pylori* eradication

Submission Confirmation

Thank you for submitting your manuscript to *Helicobacter*.

Manuscript ID:	HEL-OA-14-0059
Title:	Helicobacter pylori eradication does not change TLR2 and TLR4 expression in chronic gastritis patients
Authors:	Cadamuro, Aline Rossi, Ana Biselli-Périco, Joice Pereira, Patrícia Mascarin, Edla Acayaba, Ricardo Leite, Kátia Goloni-Bertollo, Eny Silva, Ana
Date Submitted:	18-Mar-2014