FABRÍCIO FREIRE DE MELO

ESTUDO DA RESPOSTA IMUNOLÓGICA À INFECÇÃO PELO *Helicobacter pylori*: COMPARAÇÃO ENTRE CRIANÇAS E ADULTOS

DEPARTAMENTO DE MICROBIOLOGIA
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ESTUDO DA RESPOSTA IMUNOLÓGICA À INFECÇÃO PELO *Helicobacter pylori*: COMPARAÇÃO ENTRE CRIANÇAS E ADULTOS

Tese apresentada ao Programa de Pós-Graduação em Microbiologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, como requisito parcial para a obtenção do grau de Doutor em Microbiologia

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À Dulciene;

À Andreia;

Ao Gifone;

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Aos pacientes, razão desse estudo.

“Querem que vos ensine o modo de chegar à ciência verdadeira? Aquilo que se sabe, saber que sabe; aquilo que não se sabe, saber que não se sabe; na verdade é este o saber”

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Resumo

A infecção pelo *H. pylori* é adquirida na infância, persiste pela vida do indivíduo e é responsável pelo aparecimento tardio de úlcera péptica gástrica e duodenal e carcinoma gástrico distal.

O tipo de resposta imunológica à infecção na infância deve contribuir de maneira decisiva para a susceptibilidade das crianças em se infectarem e o desfecho em doenças graves na idade adulta. Portanto, foram estudadas 245 crianças (142 *H. pylori*-negativas e 103 *H. pylori*-positivas) e 140 adultos (40 *H. pylori*-negativos e 100 *H. pylori*-positivos) quanto ao grau de gastrite, concentração gástrica de citocinas avaliada por ELISA e o número de células $Foxp3^+$ determinado por imuno-histoquímica. O grau de gastrite nas mucosas gástricas antral e oxíntica foi mais intenso nos adultos infectados que nas crianças *H. pylori*-positivas. As concentrações gástricas de todas as citocinas investigadas foi maior nos grupos de adultos e crianças infectados quando comparados com os respectivos grupos *H. pylori* negativos. Entretanto, foram observadas diferenças significativas entre as crianças e os adultos infectados. As concentrações gástricas das citocinas da resposta inata (IL-1α, IL-6, TNF-α) bem como as associadas às células T_{reg} (IL-10, TGF-β1) e Th2 (IL-4) foram significativamente maiores nas crianças que nos adultos. O número de células $T_{reg}Foxp3^+$ foi também maior na mucosa gástrica das crianças que dos adultos infectados. Por outro lado, a concentração gástrica das citocinas associadas à resposta Th1 (IL-2, IL-12p70 e IFN- γ) e Th17 (IL-1β, IL-17A e IL-23) foi maior nos adultos que nas crianças. Estratificando os grupos por idade, observou-se, nas crianças infectadas, aumento progressivo dos níveis gástricos de IL-2, IFN-γ e IL-17A e, nos adultos, diminuição das concentrações de IL-2, IL-12p70 e IFN-γ, mais intensamente a partir dos 55 anos de idade. Porque a IL-2 participa na diferenciação de células Th1 e T_{reg} e dados da literatura demonstram a presença de um polimorfismo no

gene que codifica a citocina (*IL2*-330T/G) que poderia ser funcional, investigou-se também em um grupo de 150 doadores de sangue associações entre a presença do polimorfismo e risco de infecção pelo *H.pylori*; tendo sido demonstrado que o polimorfismo foi inversamente associado à infecção. Para avaliar o efeito do polimorfismo na concentração sérica de IL-2 bem com das citocinas associadas à resposta Th1 e T_{reg}, 100 crianças também foram incluídas no estudo. Os níveis séricos de IL-2 foram maiores nos carreadores dos genótipos TG ou GG que naqueles com genótipo selvagem (TT). Os níveis séricos de IL-10 e TGF-β1 foram maiores nas crianças, mas não nos adultos *H. pylori*-positivos carreadores dos genótipos TG ou GG que nos indivíduos com o genótipo selvagem. Entretanto, nos adultos infectados, os genótipos TG ou GG se associaram a níveis séricos aumentados de INF-γ, o que não foi visto nas crianças. Concluindo, os resultados desse estudo demonstram que a resposta imunológica à infecção pelo *H. pylori* varia de acordo com a idade, o que pode explicar, ao menos em parte, as diferenças observadas entre crianças e adultos, como a maior susceptibilidade à infecção na infância, maior gravidade das lesões gástricas e prevalência das doenças graves associadas à infecção (úlcera péptica e carcinoma gástrico) nos adultos.

Abstract

Helicobacter pylori infection is acquired in childhood and may persist lifelong and causes severe diseases such as peptic ulcer and distal gastric carcinoma in adulthood. The immune events that occur in childhood may be crucial to determine the susceptibility to the infection and the outcome to the severe *H. pylori*-associated diseases in adults. Therefore, we evaluated the histology, the mean levels of cytokines, assayed by ELISA, and the number of Foxp3 cells by immunohistochemistry in the gastric mucosa of 245 children (142 *H. pylori*-negative and 103 *H. pylori*-positive) and 140 adults (40 *H. pylori*-negative and 100 *H. pylori*-positive). The degree of mononuclear and polymorphonuclear cells was significantly higher in the gastric mucosa of infected children and adults than in that of the *H. pylori*-negative groups, respectively. Also, the gastric mean levels of all cytokines evaluated were significantly higher in the *H. pylori*-positive children and adults than in the non-infected groups, respectively. Otherwise, significant differences in the gastric levels of the cytokines were observed between *H. pylori*-positive the children and the adults. The gastric levels of cytokine of the innate immune response (IL-1α, IL-6 and TNF-α) as well as of those linked to T_{reg} (IL-10 and TGF-β) and Th2 (IL-4) cell commitments were significantly higher in children than in adults. The proportion of gastric Foxp3 cells was also significantly higher in the children than in the adults. Otherwise, the gastric concentration of cytokines linked to Th1 (IL-2, IL-12p70 e IFN-γ) and Th17 (IL-1β, IL17A, and IL-23) cells was higher in adults than in children. When the *H. pylori*-positive group was stratified according to the age, the gastric levels of IL-2, IFN-γ and IL17A increased with increasing age in children, whereas in adults the levels of IL-2, IL-12-p70, and IFN-γ decreased with increasing age. The decrease of the IFN-γ levels was more pronounced in patients over 55 years of age. Because IL-2 participates in the differentiation of Th1 and T_{reg} cells and it has been demonstrated that *IL2*-330 T/G

polymorphism might be functional, we also evaluated the associations between the presence of the polymorphism and risk of *H. pylori* infection in a group of 150 blood donors, and showed an inverse association between the two variables. In order to investigate the effect of the polymorphism on the serum concentration of IL-2 as well as of cytokines related to Th1 and T_{reg} cells, 100 children were also evaluated. The serum levels of IL-2 were significantly higher in carriers of the TG or GG genotypes than in those harboring the wild genotype (TT). The serum levels of IL-10 and TGF-β1 were higher in *H. pylori*-positive children but not in adults carrying the polymorphic genotypes than in the carriers of the wild genotype. However, in the infected adults, the polymorphic genotypes associated with increased levels of IFN-γ, finding not observed in the children. In conclusion, the results of the present study demonstrated that the immune response to the *H. pylori* infection varies according to the age, which may explain, at least in part, differences such as an increased susceptibility to the infection in childhood, and a more pronounced gastric lesions and an augmented prevalence of the severe diseases associated to the infection, peptic ulcer and gastric carcinoma, in adulthood.

Introdução e Justificativa

O isolamento do *Helicobacter pylori* a partir de fragmentos de biópsia gástrica de pacientes com gastrite e úlcera duodenal, por dois pesquisadores australianos Barry Marshall e Robin Warren (Marshall & Warren, 1982), foi considerado um marco na história da Medicina. A confirmação de que úlcera péptica é causada pela bactéria melhorou consideravelmente as possibilidades de tratamento e cura da doença. Devido à relevância da descoberta, no ano de 2005, os dois pesquisadores foram agraciados com o Prêmio Nobel de Fisiologia ou Medicina.

1.1. A infecção pelo *H. pylori*

O *H. pylori* é, atualmente, aceito como o principal agente de gastrite em seres humanos e um fator essencial na patogênese de úlcera péptica, desempenhando, também papel fundamental na cadeia de eventos que culminam com o desenvolvimento de carcinoma gástrico e linfoma gástrico do tipo MALT (tecido linfóide associado à mucosa) (Forman *et al*., 1991; Parsonet *et al*., 1991; Marshall, 1994; Cover & Blaser, 1995; Wotherspoon & Path, 1998; Higashi *et al*., 2002a; Franco *et al*., 2008). Em 1994, a Agência Internacional para Pesquisa de Câncer da Organização Mundial de Saúde considerou a infecção pelo *H. pylori* carcinogênica do tipo I (IARC, 1994) com base em evidências epidemiológicas e plausibilidade biológica (Correa, 1991; Parsonnet *et al*., 1994; Blaser, 1995; Correa, 1995; Tsuji *et al*., 1997; Fujioka *et al.*, 2000; Suerbaum *et al*., 2002).

A infecção pelo *H. pylori* é uma das mais frequentes nos seres humanos, com uma prevalência global de aproximadamente 50%. Nos países em desenvolvimento a maioria da população encontra-se infectada (60% a 90%) (Blaser, 1993; Cunha *et al*., 2003; Rodrigues *et*

al., 2005a), ao contrário dos países desenvolvidos onde a infecção atinge 25% a 50% da população (Hamilton-Miller, 2003). No Brasil, a prevalência gira em torno de 60% nos estados do sul e sudeste atingindo quase 100% em algumas áreas, como no norte de Minas Gerais e regiões norte e nordeste do Brasil (Oliveira *et al*., 1999; Cunha *et al*., 2003; Rodrigues *et al*., 2005b).

A aquisição do *H. pylori* ocorre predominantemente na infância, na idade pré-escolar, por via oral-oral ou fecal-oral (Lee, 1994; Rocha *et al.*, 2003; Queiroz *et al*., 2006) e está associada a fatores como baixo nível socioeconômico, maior aglomeração familiar, condições de higiene precárias e ausência ou deficiência de saneamento básico (Malaty & Graham, 1994; Goodman *et al*., 1996; Sarker *et al*., 1997; Queiroz *et al*., 2006; Fialho *et al*., 2007; Braga e*t al*., 2007). Deve-se ressaltar, ainda, o papel da mãe ou irmãos infectados na transmissão da infecção pelo *H. pylori* (Rocha *et al*., 2003; Rodrigues *et al*., 2004; Konno *et al*., 2005; Weyermann *et al*., 2009).

O *H. pylori* é capaz de se estabelecer no estômago humano permanecendo para o resto da vida dos indivíduos, já que casos de cura espontânea são raros (Ilver *et al.*, 1998). A gastrite induzida pelo *H. pylori*, na maioria das vezes, não leva a consequências adversas (Dunn *et al.*, 1997). Entretanto, 15 a 20% dos indivíduos *H. pylori*-positivos irão desenvolver doenças graves, o que corresponde a aproximadamente 7 milhões de casos novos de úlcera péptica e carcinoma gástrico a cada ano (Parsonnet *et al*., 1991; Wotherspoon & Path, 1998; Ilver *et al.*, 1998; Parkin *et al*., 2005). No Brasil foram registrados em 2010 cerca de 13.820 casos novos de câncer de estômago entre os homens e 7.680 casos novos entre as mulheres. Estes valores correspondem a um risco estimado de 14 casos novos a cada 100 mil homens e 8 casos novos para cada 100 mil mulheres (INCA, 2010). Como a presença da infecção simplesmente não é suficiente para explicar porque apenas uma pequena porcentagem dos

indivíduos infectados irá desenvolver doenças mais graves, a influência de fatores do hospedeiro, ambientais e de virulência bacteriana tem sido investigada.

1.2. Características do *H. pylori*

O *H. pylori* é um micro-organismo Gram-negativo, espiralado, não esporulado (Marshall & Warren, 1983) que coloniza cronicamente a mucosa gástrica de seres humanos. Quando cultivada em meio sólido, a bactéria assume forma semelhante a bastonete, sendo menos frequentes as formas espiraladas (Goodwin & Armstrong, 1990). Formas cocóides podem se tornar predominantes em culturas velhas, tanto em meio sólido como líquido (Dunn *et al.*, 1997). É móvel, apresenta superfície lisa e mede aproximadamente 0,5 μm de largura e 2,0 a 3,0 μm de comprimento (Goodwin *et al*., 1985). Apresenta um número variável de 4 a 6 flagelos uni ou bipolares embainhados e com bulbos terminais nas extremidades distais (Taylor & Parsonnet, 1992). Os flagelos e a morfologia em espiral conferem motilidade à bactéria, permitindo que penetre na camada viscosa de muco gástrico e se localize junto às células epiteliais do estômago (Taylor & Parsonnet, 1992; Covacci *et al.*, 1999; Ferrero, 2005).

É uma bactéria microaerófila que cresce a 37ºC, necessitando de um período de incubação de três a sete dias. Por ser fastidiosa, é necessário para o seu crescimento e isolamento, o uso de meios de cultura enriquecidos, contendo agentes antimicrobianos para inibir o crescimento de fungos e bactérias contaminantes que podem competir por nutrientes ou produzir substâncias tóxicas (Marshall & Warren, 1983; Goodwin *et al.*, 1985a; Queiroz *et al*., 1987).

Embora o genoma do *H. pylori* seja pequeno (1,67 megabases), contendo um repertório mínimo de genes metabólicos, a diversidade genética do micro-organismo é maior

que a maioria das espécies bacterianas (Tomb *et al*., 1997). O micro-organismo apresenta numerosas enzimas pré-formadas como urease, oxidase, catalase, fosfatase alcalina, superóxido dismutase, aminopeptidase e desoxirribonuclease. Apesar de ser catalase e oxidase positivo, não reduz nitrato a nitrito, não fermenta a glicose e não hidrolisa o hipurato de sódio (McNulty & Dent, 1987; Goodwin & Armstrong, 1990).

O *H. pylori* pode ser isolado do antro, do corpo ou do fundo gástricos, embora a maior densidade bacteriana seja observada no antro (Taylor *et al*., 1987; Queiroz *et al*., 1988). O micro-organismo distribui-se de forma focal, segmentar ou difusa na superfície da mucosa gástrica (Marshall & Warren, 1983), localizando-se no interior ou abaixo da camada de muco que recobre o epitélio de superfície e das fovéolas gástricas (Hazell *et al.*, 1986), e parece proliferar quase que exclusivamente nas células superficiais do epitélio gástrico, não invadindo tecidos (Blaser & Berg, 2001).

1.3. Doenças associadas à infecção pelo *H. pylori*

H. pylori coloniza a superfície da mucosa gástrica sob a camada de muco, sem invadir o epitélio de revestimento. O micro-organismo interfere na integridade da mucosa pela produção de uma variedade de enzimas e toxinas, incluindo amônia, derivada da degradação de uréia pela urease bacteriana, desencadeando inflamação caracterizada por infiltrado de células mono e polimorfonucleadas, denominada gastrite crônica em atividade (Scheiman & Cutler, 1999).

A infecção por *H. pylori* inicialmente se estabelece no antro gástrico onde o pH é menos ácido. Nessa fase, a mucosa do corpo gástrico é parcialmente protegida devido à produção elevada de ácido pelas células parietais, ficando a reação inflamatória mais restrita ao antro gástrico (gastrite antral). Entretanto, a infecção pode se estender ao corpo gástrico,

levando a uma reação inflamatória mais generalizada, denominada pangastrite (Cover & Blaser, 1996; Howden, 1996; Dunn *et al.*, 1997). No primeiro caso a infecção pode se associar à úlcera péptica duodenal (NHI, 1994; Dunn *et al.*, 1997) e a erradicação da bactéria é acompanhada de cura da doença. Quando o corpo gástrico é acometido, a gastrite pode evoluir para atrofia, lesão que precede o carcinoma gástrico (El-Omar, 2001). Estima-se que 10% dos pacientes com gastrite atrófica irão desenvolver carcinoma gástrico em um período máximo de 15 anos (Sheiman & Cutler, 1999).

1.4.Fatores de virulência do *H. pylori*

Vários fatores de patogenicidade são comuns a todas as amostras de *H. pylori* como a motilidade e a morfologia em espiral, a produção da enzima urease e a habilidade para aderir ao tecido do hospedeiro (Hazel *et al*., 1986; Bode *et al*., 1989). Outros são identificados somente em algumas amostras de *H. pylori* e parecem relacionadas ao surgimento das doenças graves associadas à infecção. Dentre os fatores de virulência que não são expressos por todas as linhagens de *H. pylori* devem ser mencionadas a citotoxina vacuolizante (VacA), os genes *babA*¸ *sabA*, *oipA*, *dupA* e a ilha de patogenicidade (PAI) *cag*, que contém vários genes de virulência, entre eles o *cagA* (Tomb *et al*., 1997; Covacci *et al*., 1999, Plummer *et al*., 2007).

O gene *vac*A, presente em todas amostras de *H. pylori*, codifica uma citotoxina vacuolizante capaz de induzir diretamente a formação de vacúolos intracitoplasmáticos, a destruição de mitocôndrias, a liberação de citocromo c e a morte de células epiteliais por apoptose, eventos que lesam a mucosa gástrica. Além disso, a toxina aumenta a permeabilidade epitelial, o que pode facilitar tanto a passagem de substância tóxicas para dentro do epitélio como a difusão de nutrientes para a camada mucosa favorecendo a

sobrevivência do *H. pylori* (Covacci *et al*., 1997; Kuck et al., 2001; Gebert *et al*, 2003; Basso & Plebani, 2004; Nakayama *et al*., 2004). VacA ainda estimula a resposta inflamatória da mucosa gástrica por diferentes mecanismos, como por exemplo, pelo aumento da expressão da enzima ciclooxigenase 2 (COX-2) não somente em células T, mas também em neutrófilos e macrófagos (Montecucco & Bernard, 2003). Sun *et al*. (2006) demonstraram que a citotoxina é capaz de induzir a expressão de citocinas pró-inflamatórias como IL-1β, IL-8 e TNF-α em cultura de células epiteliais gástricas. Também, há fortes evidências de que VacA tenha atividade imunossupressora. Testes realizados *in vitro* demonstram que a toxina inibe a proliferação de linfócitos T induzidos por ativadores policlonais (Boncristiano *et al*., 2003; Gebert *et al*., 2003). No gene *vac*A há duas famílias sinalizadoras, denominadas s1 e s2, com variações s1a, s1b e s1c; bem como, duas regiões médias, m1 e m2. Recentemente, foi descrita uma outra região polimórfica, denominada região i, com dois genótipos distintos; i1 e i2 (Rhead *et al*., 2007). Padrões distintos estão associados com amostras produtoras ou não da toxina e diferenças quantitativas de produção (Atherton *et al*., 1995). As amostras de *H. pylori* tipo s1 são consideradas mais virulentas que as s2 e são mais frequentemente observadas em pacientes com úlcera péptica (Atherton *et al*., 1995; van Doorn *et al*., 1999; Gusmão *et al*., 2000; Erzin *et al*., 2006) e carcinoma gástrico (Evans *et al*., 1998; Kidd *et al*., 1999; van Doorn *et al*., 1999; Nogueira *et al*., 2001; Rhead *et al*., 2007; Basso *et al*., 2008) que naqueles com gastrite.

O gene *babA* codifica uma adesina que permite a ligação específica da bactéria a antígenos de Lewis b e H–1, expressos na superfície das células da mucosa gástrica (Ilver *et al.*,1998). A aderência do *H. pylori* ao epitélio gástrico, mediada pela proteína BabA (*blood-group antigen-binding adhesin A*), parece desempenhar um papel crítico na transferência de fatores de virulência bacterianos, que produzem lesões no tecido do hospedeiro, seja diretamente ou por meio de reação inflamatória incluindo auto-imunidade. Além disso,

bactérias que se mantêm mais aderidas conseguem se proteger melhor da acidez gástrica e da eliminação decorrente dos movimentos peristálticos (Ilver *et al.*,1998; Rad *et al*., 2002; Erzin *et al*., 2006).

O gene *sabA* codifica a proteína SabA (*sialic acid-binding adhesin A*), uma adesina que se liga aos resíduos glicoconjugados de ácido siálico expressos na superfície das células epiteliais gástricas na vigência de processo inflamatório ou neoplásico. A expressão de ácido siálico, rara na mucosa gástrica normal, é induzida pela infecção pelo *H. pylori*, o que contribui para a cronicidade da infecção. A adesina SabA participa da ativação de neutrófilos por mecanismos outros que não envolvem a opsonização da bactéria (Mahdavi *et al*., 2002; Unemo *et al*., 2005).

O gene *oipA* (*outer inflammatory protein A*) que codifica proteínas da membrana externa do micro-organismo, bem como o gene *ice*A (*inducible by contact with epithelium*), têm sido associados à maior virulência das amostras, embora não se conheçam as funções dos seus produtos (Figueiredo *et al*., 2005).

Em 2005 foi descrito um novo possível fator de virulência de *H. pylori* denominado *dupA* (*duodenal ulcer promoting gene*). O *dupA*, localizado na região de plasticidade do genoma bacteriano é constituído por dois genes homólogos ao *vir*B4, *jhp0*917 e *jhp*0918 que formam um gene contínuo pela inserção de uma base C ou T na posição 1385 da região 3’ do *jhp*0917. A função do gene é desconhecida, mas de acordo com Lu e colaboradores (2005), produtos do *dupA* são homólogos a uma ATPase denominada VirB4 com função de produção de energia para a formação de um aparato secretor envolvido na transferência de DNA. Os autores mostraram associação entre infecção por amostras *dupA*-positivas e risco aumentado de úlcera duodenal, bem como proteção contra câncer gástrico (Lu *et al*., 2005). Recentemente, em um trabalho feito pelo grupo do Laboratório de Pesquisa em Bacteriologia da Faculdade de Medicina (LPB-UFMG), os autores demonstraram que uma frequência muito

alta de linhagens *dupA*-positivas foram isoladas da mucosa gástrica de 100% e 92% das crianças e adultos avaliados e não observaram associação entre linagens *dup*A-positivas e úlcera duodenal (Gomes *et al*., 2008). À semelhança do que se verifica com outros marcadores de virulência do micro-organismo, existem grandes diferenças regionais na distribuição de *dupA*. Entretanto, quando um fragmento do gene foi sequenciado para confirmar a presença da inserção T ou C na posição 1385, observou-se, em algumas amostras, uma deleção de uma adenina na posição 1311 e/ou uma inserção de uma adenina na posição 1426 que criam "stop codon" precoce o que pode comprometer a função ou a expressão da proteína DupA (Queiroz *et al*., 2008). Assim, outro trabalho realizado no LPB investigou a frequência dessas mutações do *dup*A e sua associação com doenças graves decorrentes da infecção pelo *H. pylori* (Queiroz *et al*., 2011). A deleção de uma adenina na posição 1311 não se associou com úlcera duodenal, nem com câncer gástrico. Entretanto, a frequencia da inserção de uma adenina na posição 1426 foi significativamente maior nas amostras isoladas de pacientes com câncer gástrico quando comparados com gastrite e com úlcera duodenal. Além disso, não foi observada diferença na frequência do polimorfismo nas amostras isoladas de pacientes com úlcera duodenal quando comparados com gastrite. Com base no fato de que esses polimorfismos criam um stop códon prematuro, foram consideradas *dup*A-positivas, somente as amostras que não apresentavam os polimorfismos (Queiroz *et al*., 2011). Estudos subsequentes do grupo mostraram a presença de outras mutações com "stop códons" e as análises mostraram que amostras com o dupA intacto estão associados com úlcera duodenal. A presença de *dup*A intacto também se associou positivamente com infiltrado de células mononucleadas no antro gástrico e negativamente com atrofia da mucosa oxìntica, indicando que o *dup*A intacto é um fator de risco para úlcera duodenal (Avelar, 2011).

O gene *cag*A é um marcador da *cag* PAI, um fragmento de DNA de 40Kb e que contém cerca de 31 genes. O conteúdo de G + C (35%) da *cag* PAI difere do conteúdo de G + C do

resto do genoma da bactéria (39%), sugerindo que *cag* PAI tenha sido adquirida horizontalmente e integrada ao cromossomo de *H. pylori* (Tomb *et al*., 1997; Covacci *et al*., 1999). Alguns genes da ilha codificam proteínas que formam um sistema de secreção do tipo IV (Censini *et al*., 1996; Backert *et al*., 2000; Odenbreit *et al*., 2000) responsável pela translocação da proteína CagA para dentro do citoplasma das células epiteliais gástricas.

CagA possui sítios de fosforilação denominados de sequências EPIYA, região constituída de cinco aminoácidos Glu-Pro-Ile-Tyr-Ala localizada na porção carboxi-terminal da proteína. Depois de CagA ser translocada para o interior das células epiteliais gástricas pelo SST4, os sítios EPIYA são fosforilados no aminoácido Tirosina por quinases da família Src como s-Src, Fyn, Lyn e Yes ou por quinase Abl das células do hospedeiro (Selbach *et al*., 2002; Stein *et al*., 2002; Tammer *et al*., 2007; Poppe *et al*., 2007). Uma vez fosforilada, a proteína CagA é recrutada na membrana celular onde interage com proteínas da família tirosina fosfatase (SHP-2) que apresentam dois domínios SH2. Essa interação de CagA com os domínios SH2 induz mudanças na conformação da fosfatase SHP-2, estimulando sua atividade (Higashi *et al*., 2002b) e desencadeando mudanças no citoesqueleto celular que levam à formação de pedestais que permitem maior aderência bacteriana, bem como ao alongamento das células epiteliais que adquirem o fenótipo denominado “hummingbird” (beija-flor) (Segal *et al*., 1996; 1999; Backert *et al*., 2001; Saadat *et al*., 2007). As alterações do citoesqueleto celular são acompanhadas de fenômeno que desregulam o crescimento celular, o contato célula/célula e a migração celular, e que se associam com risco aumentado de mutações genéticas pré-cancerosas (Feng *et al*., 1993; Yu *et al*., 1998).

Vários genes da ilha estão, ainda, envolvidos na estimulação da produção da quimiocina IL-8 pelas células epiteliais gástricas. A IL-8 é um potente fator quimiotático e ativador de leucócitos polimorfonucleares e macrófagos contribuindo para uma resposta inflamatória mais acentuada nos pacientes colonizados por amostras *cag* PAI positivas.

Outras atividades associadas à PAI *cag* incluem a ativação da transcrição do fator AP-1 e ativação da expressão dos proto-oncogenes *c-fos* e *c-jun*, que desempenham papel crucial na proliferação e transformação celular predispondo também à oncogênese (Crabtree *et al*., 1994; Rautelin *et al*., 1994; Fan *et al*., 1995; Husson *et al*., 1995; Torres *et al*., 2000; Gerhard *et al*., 2002; Suerbaum & Michetti, 2002; Basso & Plebani, 2004; Bagnoli *et al*., 2005). A infecção por amostras *cagA*-positivas está associada à gastrite atrófica (Kupiers *et al*., 1995) e carcinoma gástrico (Blaser *et al*., 1995; Queiroz *et al*., 1995; Queiroz *et al*., 1998; Hatakeyama, 2004; Rocha *et al*., 2005).

A presença de marcadores de virulência; entretanto, não é suficiente para predizer se um paciente irá desenvolver doença grave ou não. Outros fatores, como o ambiente e a genética do hospedeiro influenciam na evolução da infecção.

1.5. Fatores do hospedeiro: resposta imunológica inata e adaptativa na infecção pelo *H. pylori*

1.5.1. Características gerais das respostas inata e adaptativa

O sistema imunológico inato representa a primeira linha de defesa contra os micro-organismos patogênicos. Na vigência de infecção, a resposta imunológica inata é estimulada por uma grande variedade de componentes bacterianos denominados padrões moleculares associados a patógenos (PAMPs), que incluem lipopolissacarídeos (LPS) de bactérias Gram-negativas, peptideoglicano (PGN) de bactérias Gram-positivas, ácido lipoteitóico e lipoarabinomananas que compõem a parede celular de micobactérias, lipopeptídeos e DNA bacteriano, dentre outros. O reconhecimento e a resposta a essas moléculas ocorrem principalmente via receptores do tipo "*Toll like*" (TLR) e NOD (*nucleotide-binding oligomerization domain*). Ao reconhecerem produtos microbianos, esses receptores

desencadeiam uma cascata de eventos intracelulares compreendendo a ativação de NF-κB (*nuclear factor-kappa* B) e consequentemente ativação de genes relacionados à produção de citocinas pró-inflamatórias (Smith *et al.*, 2003; Sánches *et al.*, 2004; Eckmann, 2006). Enquanto os TLRs estão associados com a membrana plasmática ou lisossomos e vesículas endossomais, os receptores NOD são expressos somente no citosol (Strober *et al*., 2006). Esses receptores estão presentes nos macrófagos, neutrófilos, células dendríticas, células endoteliais e células epiteliais de mucosa. Recentemente receptores TLR-2 foram observados em linfócitos T naïve e de memória. Embora a imunidade inata seja a primeira linha de defesa contra os micro-organismos, nem sempre o agente causador da infecção é eliminado, sendo então requerida uma resposta imunológica denominada adaptativa, que reconhece estímulos específicos de um determinado patógeno.

Há estreita relação entre as respostas inata e adaptativa. Células da resposta inata, como as células apresentadoras de antígenos - células dendríticas - estimuladas pelos PAMPs migram para os linfonodos onde sofrem processo de maturação. Uma vez maduras, recrutam e ativam linfócitos denominados T-helper (Th) responsáveis pela resposta específica. Classicamente, a diferenciação das células Th depende, além das células apresentadoras de antígenos, de moléculas co-estimuladoras e/ou citocinas (Murphy, 2002). As citocinas produzidas na resposta inata ou no início da resposta adaptativa influenciam a diferenciação das células Th em linhagens responsáveis por diferentes respostas efetoras denominadas Th1, Th2, Th17 e T reguladoras (T_{reg}) (Chen & O'Shea, 2008), como mostra a Figura 1.

A IL-12p70 (a forma ativa da IL-12) produzida por macrófagos ativados e células dendríticas induz a diferenciação de células T em células Th1 que produzem principalmente IFN-γ (*interferon-gamma*), bem como IL-2, IL-12p70 e TNF-α (*Tumor Necrosis Factor-α*) por uma via dependente de STAT4 (*Signal Transducers and Activators of Transcription factor* 4) e do fator de transcrição T-bet (*T-box Transcription Factor*) (Chen & O'Shea, 2008).

As citocinas produzidas pelas células Th1 são pró-inflamatórias e fundamentais na imunidade celular, atuando principalmente contra patógenos intracelulares (Zhu *et al*., 2010).

A IL-4 participa no processo de diferenciação em células Th2 que secretam citocinas anti-inflamatórias como IL-4, IL-5, e IL-13 associadas à imunidade humoral, desempenhando, também, papel importante nas infecções causadas por parasitas como os helmintos. Essa diferenciação é dependente de STAT6 e do fator de transcrição GATA3 (*Trans-acting T-cell-specific transcription factor 3*) (Chen & O'Shea, 2008; O'Keeffe & Moran, 2008; Zhu *et al*., 2010; Littman *et al*., 2010).

Recentemente, foi descrita uma nova linhagem de células Th, distinta das células Th1 e Th2, denominada Th17. A diferenciação de células Th17 depende dos fatores de transcrição STAT3 e RORγt (*retinoid orphan nuclear receptor*) e das citocinas IL-1β, IL-6, IL-23 e TGF-β (Volpe *et al*., 2008; Korn *et al*., 2009; Littman *et al*., 2010). Embora haja divergência entre os trabalhos quanto à participação da IL-23 na diferenciação das células Th17 em seres humanos, há consenso de que é importante para a manutenção/estabilização da síntese de citocinas pró-inflamatórias pelas células Th17 (McGeachy *et al*., 2007; Awasthi & Kuchroo, 2009). As células Th17 produzem quimiocinas e citocinas pró-inflamatórias como IL-17A, IL-17F, IL-6 e TNF-α que atuam na resposta imunológica contra bactérias extracelulares e fungos. Ainda, há evidências de que são importantes mediadoras inflamatórias nas doenças da auto-imunidade (Wilson *et al*., 2007; Hashimoto *et al*., 2010).

A ação de citocinas imunomoduladoras como IL-10 e TGF-β sobre células T $CD4^+$ naïve pode induzir diferenciação para outro grupo de células, denominadas células T reguladoras (T_{reg}). As células T_{reg} podem ser distinguidas de outras células T $CD4^+$ por apresentarem expressão de receptores CD25 e do fator de transcrição Foxp3 (*transcription factor forkhead box P3*). Células T $CD4^+CD25^-$ na presença de TGF-β e IL-2 se diferenciam em células reguladoras $CD4^+CD25^+Foxp3^+$ (Chen *et al*., 2003; Wahl, 2007), capazes de inibir

várias células, tanto relacionadas à imunidade inata quanto à adaptativa (Itoh *et al*., 1999). As células T_{reg} têm papel essencial na manutenção da resposta imunológica, funcionando como reguladoras de células T efetoras por meio de uma variedade de processos, que incluem: a produção de citocinas moduladoras, como a IL-10 e TGF-β, a indução de citólise pela produção de granzimas, bem como a interrupção do metabolismo das células efetoras CD4/CD8 (Yamaguchi & Sakaguchi, 2006; O'Keeffe & Moran, 2008; Feuerer e*t al*., 2009). Células T_{reg} participam do controle da resposta imunológica em doenças da auto-imunidade, alergia, rejeição de transplantes de órgãos, bem como na supressão da resposta imunológica ao câncer (Yamaguchi & Sakaguchi, 2006; Sakaguchi *et al*., 2008).

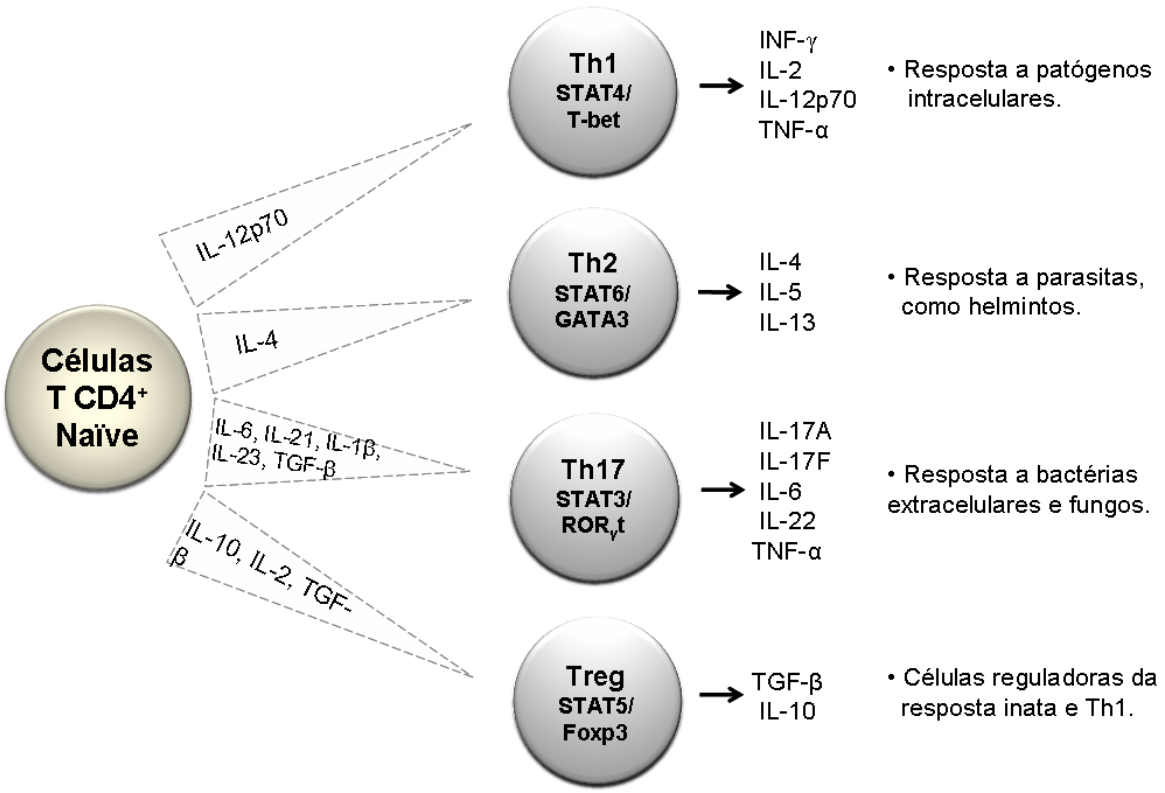

Figura 1: Diferenciação das células T $CD4^+$: Células T $CD4^+$ naïve depois de ativadas, na presença de IL-12p70, são diferenciadas em células Th1 que têm como principal produto o IFN-γ. Essa diferenciação é regulada principalmente por STAT4 e T-bet. IFN-γ tem grande importância na defesa

contra patógenos intracelulares. Em resposta a IL-4, células T $CD4^+$ naïve se diferenciam em células do tipo Th2 através da ativação dos fatores de transcrição STAT6 e GATA3. IL-4 é a principal citocina produzida pelas células Th2 e tem papel fundamental na defesa contra parasitas como os helmintos. A diferenciação das células T $CD4^+$ naïve em células Th17 ocorre pela ativação dos fatores de transcrição STAT3 e RORγt por diferentes citocinas. IL-17 é uma das principais citocinas produzidas pelas células Th17 e tem grande importância na resposta contra bactérias extracelulares. Em resposta à ativação dos fatores de transcrição STAT5 e Foxp3 pelas citocinas IL-10, TGF-β e IL-2, as células T $CD4^+$ naïve se diferenciam em células T_{reg}. As citocinas TGF-β e IL-10 são produtos das células T_{reg} que tem como principal função a modulação da resposta inflamatória regulando a resposta inata e adaptativa.

1.5.2. Resposta imunológica inata e adaptativa na infecção pelo *H. pylori*

Estudos têm demonstrado que a infecção pelo *H. pylori* induz inflamação por vários mecanismos, entre eles o contato direto do micro-organismo com células epiteliais gástricas, bem como com células específicas da resposta imunológica. Há evidências de que antígenos da bactéria são reconhecidos por receptores TLR 2 e 4 (extra-celulares) e TLR 7, 8 e 9 (intracelulares) (Rad *et al*., 2009). Embora haja discordância entre os estudos, há, também, evidências da participação tanto de receptores NOD1 (Viala *et al*., 2004, Rosenstiel *et al*., 2006) como de NOD2 (Rosenstiel *et al*., 2006).

A maioria das bactérias intracelulares induz respostas do tipo Th1. Baseado no fato de que o *H. pylori* é um micro-organismo não-invasivo e que a infecção é acompanhada por uma exuberante resposta humoral, esperava-se que não houvesse predomínio de resposta do tipo Th1. No entanto, a maioria dos clones de células T específicos presentes na mucosa gástrica infectada pela bactéria produz níveis elevados de IFN-γ, principal produto das células Th1 (D'Elios et al., 1997). *H. pylori* também estimula a produção *in vitro* de IL-12p70, considerada a principal citocina que promove a diferenciação de células Th1. Entretanto, essa

resposta aparentemente não é efetiva na eliminação do micro-organismo resultando em uma infecção crônica acompanhada de gastrite (Bamford *et al*., 1997).

1.5.2.1. Infecção pelo *H. pylori* e níveis gástricos de citocinas em adultos

O perfil de citocinas na infecção pelo *H. pylori* tem sido estudado, sobretudo em adultos. Crabtree *et al*., (1991), usando ensaio imunoenzimático, demonstraram níveis gástricos aumentados de IL-6 e TNF-α em pacientes *H. pylori*-positivos na Inglaterra. Noach *et al*. (1994) observaram níveis de IL-1β e TNF-α significativamente maiores na mucosa gástrica de pacientes holandeses com a infecção pelo *H. pylori* quando comparados com aqueles sem a infecção. No estudo de Fan *et al*. (1995), além do aumento na concentração de TNF-α, os autores observaram aumento de IL-8 na vigência da infecção em pacientes irlandeses.

Resultados semelhantes foram relatados por Peek *et al*. (1995b) quando estudaram uma população americana, avaliando, por ensaio imunoenzimático, os níveis de IL-8 na mucosa gástrica de pacientes *H. pylori*-positivos e negativos. Ainda, os autores observaram que os níveis de IL-8 eram significativamente maiores nos pacientes colonizados por amostras *cag*A-positivas. No mesmo estudo, embora tenha sido demonstrado aumento de expressão de mRNA de IL-1α e IL-1β na mucosa gástrica dos indivíduos infectados pelo *H. pylori*, não houve diferença significativa na concentração de IL-1α entre o grupo infectado e não infectado. Também foi observado aumento da expressão de mRNA de IL-2 e IL-10 na mucosa gástrica de pacientes *H. pylori*-positivos; entretanto, os níveis dessas citocinas não foram avaliados. Resta ressaltar que a determinação foi feita em um número muito pequeno de indivíduos (7 *H. pylori*-negativos e 14 *H. pylori*-positivos). Entretanto, em outro estudo

realizado nos Estados Unidos, Karttunen *et al*. (1997) não observaram expressão aumentada de mRNA de IL-10 em pacientes infectados quando comparados com os não infectados. Os autores, também, não observaram expressão aumentada de mRNA de INF-γ nos pacientes infectados pelo *H. pylori*.

Em um trabalho realizado na Inglaterra, Hida *et al*. (1999) demonstraram expressão mais elevada de mRNA de IL-10 e IL-12p40 na mucosa gástrica de pacientes infectados pelo *H. pylori* que naqueles *H. pylori*-negativos. Além disso, os autores observaram expressão mais elevada das duas citocinas na mucosa gástrica de pacientes infectados por amostras de *H. pylori* cagA-positivas. Ao estudarem pacientes italianos, além do aumento dos níveis gástricos de IL-12, Pellicano e colaboradores (2007) também observaram níveis gástricos aumentados de IL-4 e INF-γ. Por outro lado, Serrano *et al*. (2007) não observaram diferença significativa nos níveis gástricos de IL-10 e IL-12 entre pacientes mexicanos infectados ou não pela bactéria. Os autores observaram níveis gástricos de IL-4 e INF-γ mais elevados nos pacientes *H. pylori*-positivos que nos pacientes *H. pylori*-negativos; entretanto, a diferença não foi significativa. Vale ressaltar que o estudo conduzido por Serrano *et al*. (2007) avaliou um número pequeno de pacientes (4 *H. pylori*-negativos e 41 *H. pylori*-positivos).

Na população asiática, Yamaoka *et al*. (1997) observaram que as concentrações de IL-1β, IL-6, IL-8 e TNF-α, mas não de IL-10, eram significativamente maiores na mucosa gástrica dos pacientes japoneses infectados que daqueles sem a infecção. À semelhança do estudo de Peek *et al*. (1995b), os níveis de IL-1β e IL-8 eram maiores nos pacientes colonizados por amostras de *H. pylori cag*A-positivas. Também estudando pacientes japoneses, Katagiri *et al*. (1997) observaram que os níveis de TNF-α eram significativamente maiores nos pacientes infectados quando comparados com aqueles sem infecção.

Quanto à principal citocina que caracteriza o perfil Th17, aumento significativo nos níveis de IL-17A e expressão elevada de mRNA de IL-17A foram observados na mucosa

gástrica de pacientes japoneses e italianos *H. pylori*-positivos, respectivamente (Mizuno *et al*., 2005; Caruso *et al*., 2008). No estudo de Caruso *et al*. (2008), além do aumento da expressão de mRNA de IL-17A, os autores observaram aumento de IL-23 na vigência da infecção pelo *H. pylori*. Entretanto, pouco se conhece sobre a participação das células Th17 na resposta à infecção pela bactéria.

1.5.2.2. Infecção pelo *H. pylori* e níveis gástricos de citocinas em crianças

Há poucos trabalhos avaliando a participação de citocinas na infecção por *H. pylori* em crianças, poucos pacientes foram avaliados, e os resultados dos estudos são discordantes. Deve ainda ser ressaltado que há grandes variações nos métodos adotados nos diferentes estudos e populações geneticamente distintas foram incluídas.

Kutukçuler *et al*. (1997) observaram níveis elevados de TNF-α em sobrenadante de cultura de mucosa gástrica de crianças polonesas *H. pylori*-positivas. Guiraldes *et al*. (2001), em um estudo realizado no Chile, demonstraram que níveis de IL-1β, IL-8 e TNF-α elevados na mucosa gástrica de crianças associaram-se com a infecção pela bactéria. Luzza *et al.* (2001), em um estudo realizado na Itália, demonstraram que a expressão de mRNA de IL-2, IL-8 e IL-17 e INF-γ era mais elevada em crianças *H. pylori*-positivas que nas negativas; entretanto, não foi observada diferença significativa na expresão de mRNA de IL-4 e IL-10 entre os grupos. Shimizu *et al.* (2004) também encontraram um aumento significativo do nível de IFN- γ na mucosa gástrica de crianças japonesas positivas em comparação com as negativas. Oderda *et al*. (2007), em um estudo desenvolvido na Itália, demonstraram expressão gástrica aumentada de mRNA INF-γ, bem como de IL-10 em crianças *H. pylori*-

positivas. A expressão de IL-10 foi vista ser foi maior em crianças com idade maior ou igual a 4 anos.

1.5.2.3. Comparação entre os níveis gástricos de citocinas em crianças e adultos

Há, até o momento, apenas um estudo compararando a expressão de citocinas entre crianças e adultos com infecção pelo *H. pylori*. Além de os autores confirmarem que o grau de gastrite é mais moderado nas crianças infectadas pela bactéria que nos adultos, observaram um número maior de células T_{reg}, bem como concentração mais aumentada dos seus dois principais produtos, IL-10 e TGF-β1, na mucosa gástrica de crianças quando comparadas com os adultos *H. pylori*-positivos (Harris *et al*. 2008).

1.5.2.4. Polimorfismo no gene que codifica IL-2: comparação entre adultos e crianças

A IL-2 tem funções distintas na resposta adaptativa. Por um lado, tem uma função redundante envolvida na reposta pró-inflamatória Th1 participando no aumento da produção de INF-γ. Participa também de maneira não redundante na diferenciação de células T $CD4^+$ naïve em $T_{reg}Foxp3^+$.

É importante salientar que estudos realizados "*in vitro*" demonstraram que o polimorfimo do gene *IL2* na posição -330 (substituição de T→ G) aumenta a expressão de IL-2 (Hoffmann *et al*., 2001).

Portanto, vários pontos merecem ser investigados, visto que a infecção é adquirida na infância, o polimorfismo de IL2 aumenta a produção da citocina e IL-2 pode atuar positivamente na diferenciação tanto de uma resposta pró-inflamatória Th1 quanto de uma

resposta moduladora, T_{reg}, o que pode repercutir na aquisição ou resistência à infecção na dependência da polarização.

2. Objetivos

1- Avaliar as concentrações de citocinas associadas à resposta imunológica inata e adaptativa (Th1, Th2, Th17 e T_{reg}) na mucosa gástrica de crianças e adultos *H. pylori*-negativos e -positivos;

2- Comparar os resultados observados nas crianças com aqueles obtidos nos adultos;

3- Avaliar, por imunohistoquímica, a expressão de Foxp3 nos linfócitos da mucosa gástrica de crianças e adultos infectados ou não pela bactéria, comparando os grupos;

4- Investigar se a presença do polimorfismo no gene que codifica IL-2 na posição -330 T/G está associada à susceptibilidade à infecção pelo *H. pylori*.

5- Avaliar se o polimorfismo *IL2*-330T/G é funcional, pelo efeito na concentração sérica de IL-2, IL-10, INF-γ e TFG-β1 em crianças e adultos *H. pylori*-positivos e -negativos.

3. Trabalhos científicos

3.1. Trabalho 1

A regulatory instead of an IL-17 T response predominates in *H. pylori*-associated gastritis in children

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Abstract

Th17 cells seem to have an important role in the efficacy of vaccines against *Helicobacter pylori*. Because children are a target group for human vaccination and Th17/T_{reg} cells have intrinsically linked and antagonic commitments, we compared the gastric levels of Th17- and T_{reg}-associated cytokines of children and adults. IL-6, IL-10 and TGF-β1 levels and $Foxp3^+$ cell numbers were higher, but IL-1β, IL-17A and IL-23 were lower in infected-children than in infected-adults. In conclusion T_{reg} instead of Th17 cell response to *H. pylori*-infection predominates in children.

Keywords: *Helicobacter pylori*; Th17; T_{reg}; Children; Adults.

1. Introduction

Helicobacter pylori is a well recognized gastric pathogen that infects more than 50% of the world's population. The infection is acquired predominantly in childhood, persists throughout life and predisposes to severe diseases such as peptic ulcer or gastric carcinoma in adulthood [1]. Most infected children do not develop complication; but, the immunological events that take place in the child gastric mucosa might be decisive in the immune response and might determine the final infection's outcome in adulthood.

The exposure to bacterial antigens induces in the host the innate immune response that strongly participates in the development of the adaptive immunity by activating T lymphocytes to differentiate into T helper (Th) effector cells categorized mainly by the cytokines they produce. The Th1 cell subset protects the host against intracellular bacteria and the recently discovered proinflammatory Th17 cells are involved in the protection against extracellular bacteria [2]. The Th1 cell response to *H. pylori* infection has been largely studied [3-6]; but, although *H. pylori* is an extracellular pathogen, there are few studies evaluating the Th17 cell response to the infection [6-8]. It has also to be emphasized that in mouse models Th17 cells have been considered to have a more important role in the efficacy of vaccines [9-11], which indicates that Th17 cell subset needs to be better investigated, especially in children, who are the target group for vaccination. The development of Th17 cells in human beings depends on a cytokine milieu rich in IL-1β, IL-6 and TGF-β that initiates the differentiation process and IL-23 that participates in the expansion and maintenance of the Th17 cells [12-13]. Although apparently paradoxical, TGF-β participates in both Th17 and T_{reg} cell differentiation. In the former by activating the transcription factor RORγt (retinoid-related orphan receptor γ) or RORc, the human

homologue of RORγt, and in the later, by activating the Foxp3 (forkhead box 3/ winged helix) transcription factor [14-16]. Because T_{reg} limits bacterium elimination and T_{reg}/Th17 cell commitments are intrinsically linked, we aimed to determine the gastric levels of the proinflammatory Th17 cell signature cytokine, IL-17A, and the cytokines associated with Th17 and T_{reg} cell differentiation in children comparing the results with those obtained in adults.

2. Materials and methods

This study was approved by the Ethics Committee of the Universidade Federal de Minas Gerais, Belo Horizonte, Brazil. Signed informed consent to participate was obtained from the adults as well as from the children (whenever possible) and their parents.

2.1. Study population

We studied prospectively 245 children [142 *H. pylori*-negative (62 female, 9.5 ± 3.4 years, range 1 - 18 years)] and 103 *H. pylori*-positive (43 female, 10.6 ± 3.4 years, range 2 - 18 years)] and 140 adults [40 *H. pylori*-negative (20 female, 42.2 ± 15.1 years, range 19 - 69 years) and 100 *H. pylori*-positive (63 female, 52.3 ± 16.2 years, range 19 - 87 years)] who underwent endoscopy to clarify the origin of symptoms referable to the upper gastrointestinal tract. Patients with peptic ulcer, gastric cancer and other complications and those who received antimicrobial drugs, anti-cholinergic and anti-inflammatory agents or proton pump inhibitors for at least 30 days before endoscopy were not included. All patients were natives of the Minas Gerais state with the same genetic background, approximately 33% of Portuguese,

33% of Amerindian and 33% of African ancestry, homogenously present in each subject [17]. Biopsy specimens were obtained from the antral and oxyntic gastric mucosa of all patients for evaluation of the *H. pylori* status and histological parameters and from the antral mucosa for the cytokine concentration determination and $T_{reg}Foxp3^{+}$ immunohistochemistry.

2.2. H. pylori status

H. pylori status was evaluated by culture, preformed urease test, carbolfuchsin-stained smear, polymerase chain reaction (PCR) for *ure*A, and ^{13}C-urea breath test as previously described [18]. Patients were considered *H. pylori*-positive when culture was positive or at least two of the other tests were positive and *H. pylori*-negative when the results of all tests were negative.

2.3. DNA extraction

Tissue and bacterial culture DNA was extracted with QIAamp® DNA mini kit (Quiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations. The presence of the *ure*A *H. pylori* specific gene was evaluated, according to Clayton *et al.* [19].

2.4. Histology

Fragments from the antral and oxyntic mucosa were fixed in 10% formalin and embedded in paraffin wax, and 4-μm-thick histological sections were stained with hematoxylin and eosin. The sections were analyzed according to the revised Sydney System [20]. Mononuclear and polymorphonuclear cell infiltrations as well as

intestinal metaplasia and atrophy were graded as none (0), mild (1), moderate (2), or marked (3).

2.5. Determination of the gastric cytokine levels

Two antral biopsy fragments were immediately placed into cryotubes, frozen in liquid nitrogen and stored at -80ºC until used. Aliquots of homogenate supernatant in 1.5 mL PBS, pH 7.4 containing 2 μg/mL aprotinin were obtained by centrifugation (10,000 g for 10 minutes). The total protein concentration was measured by Bradford's method. The gastric levels of the cytokines involved in the Th17 and T_{reg} cell commitment, IL-1β, IL-6, IL-10, IL-17A, IL-23, and TGF-β1 (after activation), were assayed in duplicate by ELISA (Biosource, Camarillo, CA). The cytokine mucosal levels were expressed as picogram of cytokine per milligram of protein (pg/mg protein). IL-1β and IL-6 levels were evaluated by ultra sensitive kits. The minimum detectable levels of the cytokines are 0.06 pg/mL (IL-1β), 104 fentogram per millilitre -fg/mL (IL-6), 0.2 pg/mL (IL-10), 2.0 pg/mL (IL-17A), 4.0 pg/mL (IL-23) and 15.6 pg/mL (TGF-β1). All values below the detection levels were regard as undetectable and were ascribed the value zero.

2.6. T_{reg}Foxp3+ cell number

The T_{reg}Foxp3 cell number was assessed in formalin-fixed paraffin-embedded sections of antral mucosa of 50 children and 50 adults, who were randomly selected, by conventional immunohistochemistry using as first antibody 1:35 diluted mouse anti-human Foxp3 IgG (mAbcam 22509, Abcam, Cambridge, UK) with modifications including incubation with Novocastra Post Primary Block for 30 minutes and with

NovoLink polymer for 30 minutes (Novocastra Laboratories Ltd, São Paulo, Brazil). Sections were counterstained with Meyer's hematoxycillin and then mounted. Fragments of inflamed ileum from patients with active Crohn's disease were included as positive control and slides containing tissue sections without addition of primary antibody, as negative control. The mononuclear cells with the nucleus stained in brown were considered positive for Foxp3. The number of Foxp3-positive cells (proportional to the number of all lymphocytes) was evaluated in 20 representative visual fields at a magnification of 1,000x in an Olympus CX41RF microscope.

2.7. Statistical analysis

Data were analysed with SPSS (SPSS Inc., Chicago, IL) statistical software package version 17.0. In addition to the visual examination of the histograms and box plots, the Kolmogorov-Smirnov goodness-of-fit was used to assess the normality of the data. When significant departures from normality were detected the data were log transformed. The two-tailed Student's t test was used to compare the sub groups of patients. The score of mononuclear and polymorphonuclear cells in the antral and corpus mucosa, as well as the number of Foxp3 positive cells was compared by the Mann Whitney *U* test. The level of significance was set at $p < 0.05$.

3. Results

3.1. Population

No difference in the sex frequency was observed between infected and uninfected children (p = 0.65) and adults (p = 0.28); but, the mean age was higher in *H. pylori*-positive children (p = 0.002) and adults (p = 0.005) than in the -negative groups.

The score of the antral and corpus mononuclear and polymorphonuclear cells was higher in *H. pylori*-positive than in *H. pylori*-negative children and adults. The degree of mononuclear cells in the antrum and the corpus as well as of polymorphonuclear cells in the antrum and the corpus were significantly higher in infected-adults than in infected-children (Table 1). Also, atrophy and intestinal metaplasia were observed only in the gastric mucosa of adults

3.2. Cytokine levels in the gastric mucosa of children and adults

3.2.1. H. pylori-*negative group*

In the *H. pylori*-negative group, IL-1β, IL-6, IL-10, IL-17A, IL-23 and TGF-β1 were naturally expressed in the gastric mucosa of 44.4%, 3.5%, 52.8%, 86.6%, 12.7% and 59.2% of the children and in the gastric mucosa of 97.5%, 82.5%, 42.5%, 100%, 22.5% and 100% of the adults, respectively. When children and adults were compared, the mean gastric levels (pg/mg of protein) of IL-1β (19.2 ± 56.9 vs. 227.5 ± 112.4, $p < 0.001$), IL-6 (2.8 ± 15.3 vs. 21.0 ± 14.5, $p < 0.001$), IL-17A (152.8 ± 98.2 vs. 192.9 ± 124.5, p = 0.03), IL-23 (26.2 ± 73.6 vs. 34.7 ± 67.0, p = 0.05) and TGF-β1 (1101.7 ± 1194.3 vs. 3742.1 ± 1601.3, $p < 0.001$) were significantly lower, but IL-10 levels (34.5 ± 43.9 vs. 18.0 ± 24.3, $p < 0.001$) were higher in the children than in the adults.

3.2.2. H. pylori-*positive group*

All cytokines, but IL-23 (detected in 86.4% of children and 82.0% of adults), were detected in the gastric mucosa of all *H. pylori*-positive children and adults.

The gastric levels of IL-6 ($p < 0.001$), IL-10 ($p < 0.001$) and TGF-β1 ($p = 0.04$) were significantly higher in the gastric mucosa of children than in adults. Otherwise, IL-1β ($p < 0.001$), IL-17A ($p < 0.001$) and IL-23 ($p = 0.001$) gastric levels were significantly higher in adults than in children (Fig. 1).

3.3. Comparison of the gastric cytokine levels between H. pylori-*positive and -negative patients*

The gastric concentrations of all cytokines were significantly higher in infected than in non-infected children ($p < 0.001$ for all) (Fig. 2A) and adults ($p = 0.004$ for IL-1β and $p < 0.001$ for the other cytokines) (Fig. 2B).

An 11.5-, 307.1-, 20.6-, 3.2-, 15.8- and 7.1-fold increased gastric levels of IL-1β, IL-6, IL-10, IL-17A, IL-23 and TGF-β1, respectively, was observed in infected children when compared with non-infected ones.

The concentration of IL-1β, IL-6, IL-10, IL-17A, IL-23 and TGF-β1 were 1.3-, 18.6-, 6.3-, 4.2-, 17.5- and 1.8-fold increased, respectively, in the gastric mucosa of *H. pylori*-positive adults when compared with the bacterium-negative ones.

3.4. Foxp3 cells

The proportion of $Foxp3^+$ stained/mononuclear cells was significantly higher in the *H. pylori*-positive children (median: 0.30; range: 0.15-0.75; $p = 0.001$) and adults (0.14; 0.05-0.23; $p = 0.02$) than in the *H. pylori*-negative groups (median: 0.02, range 0.01-0.03; 0.015, 0.00-0.05; respectively).

The proportion of Foxp3 stained/mononuclear cells was significantly higher ($p = 0.009$) in the antral mucosa of *H. pylori*-positive children (median: 0.30; range: 0.15-0.75) than in that of adults (0.14; 0.05-0.23) (Fig.3).

4. Discussion

Because *H. pylori* infection is usually acquired in early childhood, events that take place in this age might influence or even determine susceptibility to the infection and might also contribute to the clinical outcomes in adulthood. Therefore, a better understanding of the child's immune response to *H. pylori* infection is the first step in the development of an effective vaccine to target children. It has been recently demonstrated that the efficacy of a vaccine against *H. pylori* in mouse models relies more on Th17 than on Th1 cell response [9-11]. However, to date, we are aware of only one study evaluating IL-17 cell response to *H. pylori* in children [6]. Luzza *et al.* demonstrated an increased IL-17A mRNA gastric expression in *H. pylori*-positive children; but, other cytokines linked to the Th17 cell commitment were not investigated by the authors. In adults, higher levels of IL-17A and expression of IL-17A mRNA were observed in the gastric mucosa of *H. pylori*-positive than in that of *H. pylori*-negative Japanese [7] and Italian [8] patients. In the later, increased levels of IL-23 was also observed in the gastric mucosa of *H. pylori*-positive adults and the authors also demonstrated the role of IL-23 in increasing the production of IL-17 by gastric mononuclear cells "*in vitro*".

In agreement with the above cited studies, higher IL-17A gastric levels were observed in infected than in uninfected children and adults.

Th17 cell commitment pathway is well known in mice. The cell originates from naïve CD4 T cells stimulated by IL-6 and TGF-β and IL-23 seems to be essential for

cell stabilization/amplification [21]. There are evidences that IL-1β, IL-6, TGF-β and IL-23 are all also essential for human Th17 differentiation and maintenance [2,12], as our results in adults suggest. However, in children, the picture is a quite little different. Of note, IL-6 was not naturally detected in the gastric mucosa of most non-infected children, but the infection induced a huge cytokine expression. IL-6 is produced by different cells, including those of the innate immune system, which might explain the results we observed in children because *H. pylori* infection is mainly acquired in childhood. However, other cells of the adaptive immunity are also important source of IL-6 such as Th17 cells. In turn, there are consistent evidences that IL-6 participates in the human Th17 cell commitment. IL-6 is extremely potent in suppressing TGF-β-driven induction of Foxp3 resulting in strong induction of Th17 [2]. Thus, the current consensus is that IL-6 induces Th17 differentiation together with TGF-β. TGF-β is required not only for Th17, but also for T_{reg} differentiation, by inducing both key transcription factors, RORγt/RORc and Foxp3, respectively [18-20]. However, in the absence of IL-6, an exclusive T_{reg} differentiation occurs as Foxp3 is able to associate with and to inhibit RORγt. Otherwise, in the presence of IL-6, this inhibition is abrogated allowing Th17 differentiation [22].

Unexpectedly, the IL-6 rich gastric milieu of *H. pylori*-infected children did not substantially inhibit the T_{reg} commitment as it is confirmed by the increased number of $Foxp3^+$ cells and high gastric concentration of IL-10 and TGF-β induced by the infection as also observed by Harris *et al.* in Chilean children [23]. One might hypothesize; however, that the disturbance is due to the lower gastric levels of IL-23 in children when compared with adults, which prevents the amplification/stabilization of the shifted Th-17 cells. Another possibility is the high concentration of TGF-β in the gastric milieu of infected children. At low concentrations, TGF-β synergizes with IL-6

to promote IL-23 receptor (*IL-23r*) expression, favouring Th17 cell commitment. High concentrations of TGF-β; however, repress *IL-23r* expression and favor $Foxp3^+$ T_{reg} cell differentiation [24]. Finally, a recent study has demonstrated that IL-6 overproduction *in vivo* by an IL-6 transgenic mouse does not affect the development and function of natural T_{reg} [25].

The predominant T_{reg} instead of Th17 cell differentiation in *H. pylori*-infected children might account to the susceptibility of children to the infection as well to the bacterium persistence. It may also explain the lower degree of gastritis observed in infected children than in infected adults, especially in respect to the number of polymorphonuclear cells that depends on the recruitment and activation induced by IL-17A. It has to be highlighted that polymorphonuclears cells are considered relevant to the clearance of the infection. In mice, the IL-17 production and the associated neutrophil infiltration seem to be essential in the *H. pylori* clearance induced by the vaccine [10-11].

Finally, all the results we observed could be attributed to infection by a more virulent *H. pylori* strain because neither difference in the prevalence of the infection by a CagA-positive strains between adults and children nor to association between CagA status and cytokine concentration (data not shown) was observed.

In conclusion T_{reg} instead of Th17 cell response to *H. pylori*-infection predominates in children.

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Legend for figures

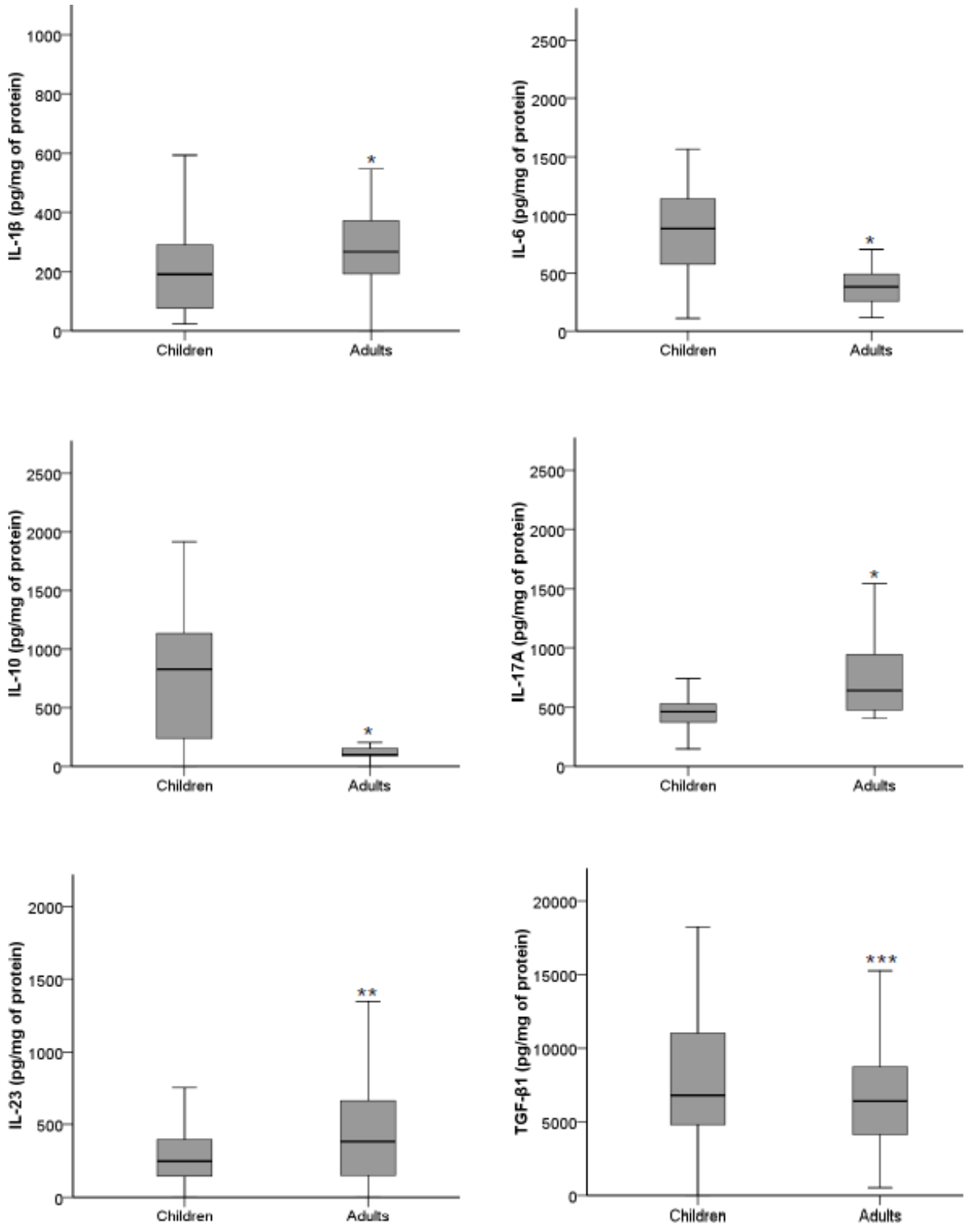
Figure 1 - Box plots representing the comparison of the antral gastric cytokine concentrations (pg/mg of protein) between *H. pylori*-positive children (n = 103) and adults (n = 100). The upper and lower limits of the boxes represent the 75^{th} and 25^{th} percentiles, respectively. The horizontal bar across the box indicates the median and the capped bars indicate the minimum and maximum data values. The data were analyzed by the two-tailed Student's t. * $P < 0.001$, ** $P = 0.001$ and *** $P = 0.04$.

Figure 2A - Box plots representing the comparison of the antral gastric cytokine concentrations (pg/mg of protein) between *H. pylori*-positive children (n = 103) with children without *H. pylori* infection (n = 142). The upper and lower limits of the boxes represent the 75^{th} and 25^{th} percentiles, respectively. The horizontal bar across the box indicates the median and the capped bars indicate the minimum and maximum data values. The data were analyzed by the two-tailed Student's t test. * $P < 0.001$. 2B - Box plots representing the comparison of the antral gastric cytokine concentrations (pg/mg of protein) between *H. pylori*-positive (n = 100) and –negative (n = 40) adults. The upper and lower limits of the boxes represent the 75^{th} and 25^{th} percentiles, respectively. The horizontal bar across the box indicates the median and the capped bars indicate the minimum and maximum data values. The data were analyzed by the two-tailed Student's t. * $P < 0.001$, **$P = 0.004$.

Figure 3 - Immunohistochemistry for T_{reg} Foxp3+ cell in the gastric mucosa of a *H. pylori*-negative child (A); *H. pylori*-positive child (B); *H. pylori*-positive adult (C); brown

staining indicates positive cells whereas negative cells are counterstained with hematoxylin. Magnification: 1,000x.

Figure 1:


IL-1β (pg/mg of protein)
1000
800
600
400
200
0
*
Children
Adults
IL-6 (pg/mg of protein)
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500
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Children
Adults
IL-10 (pg/mg of protein)
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Children
Adults
IL-17A (pg/mg of protein)
2500
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Children
Adults
IL-23 (pg/mg of protein)
2000
1500
1000
500
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Children
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TGF-β1 (pg/mg of protein)
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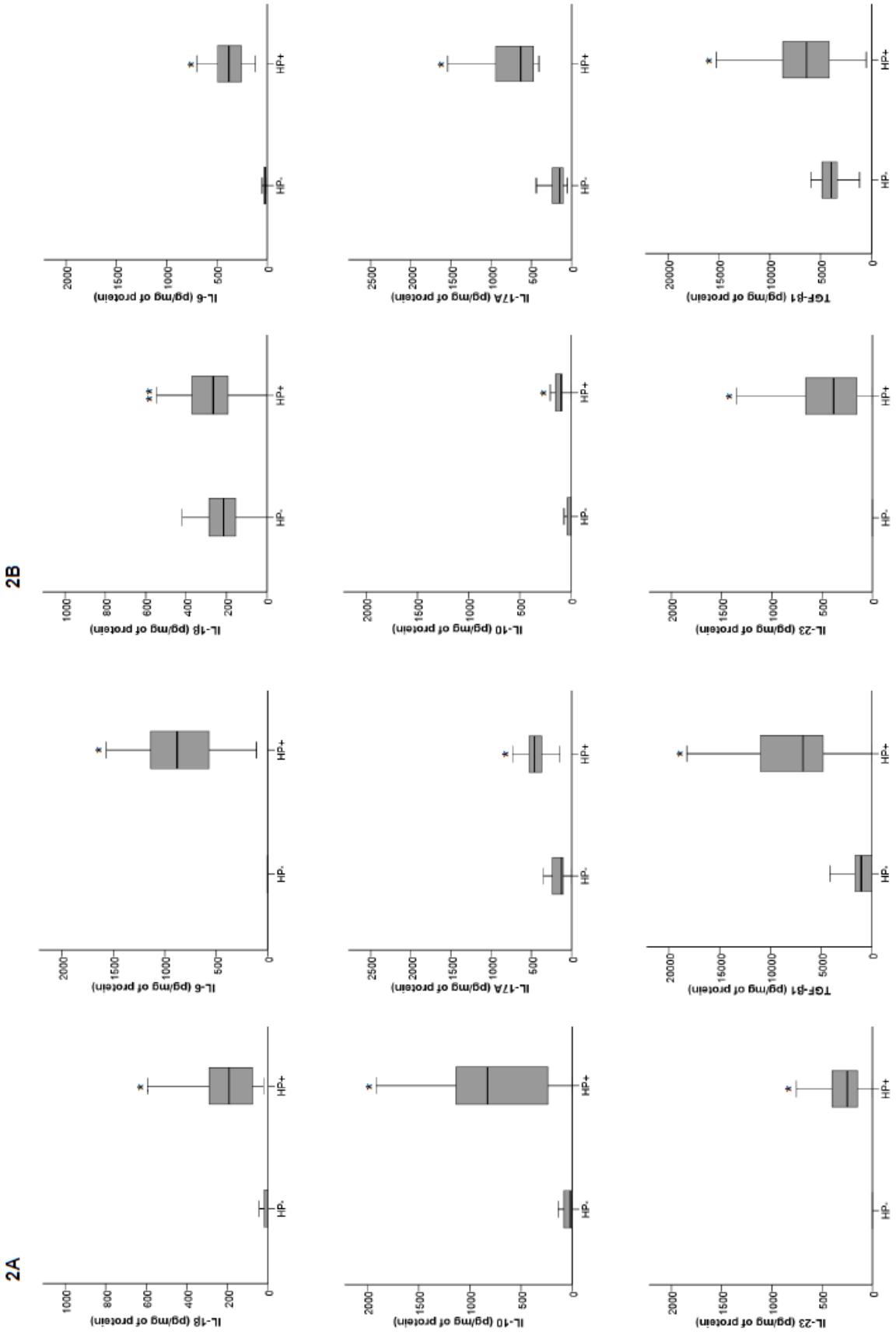
Children
Adults

Figure 2:


2A
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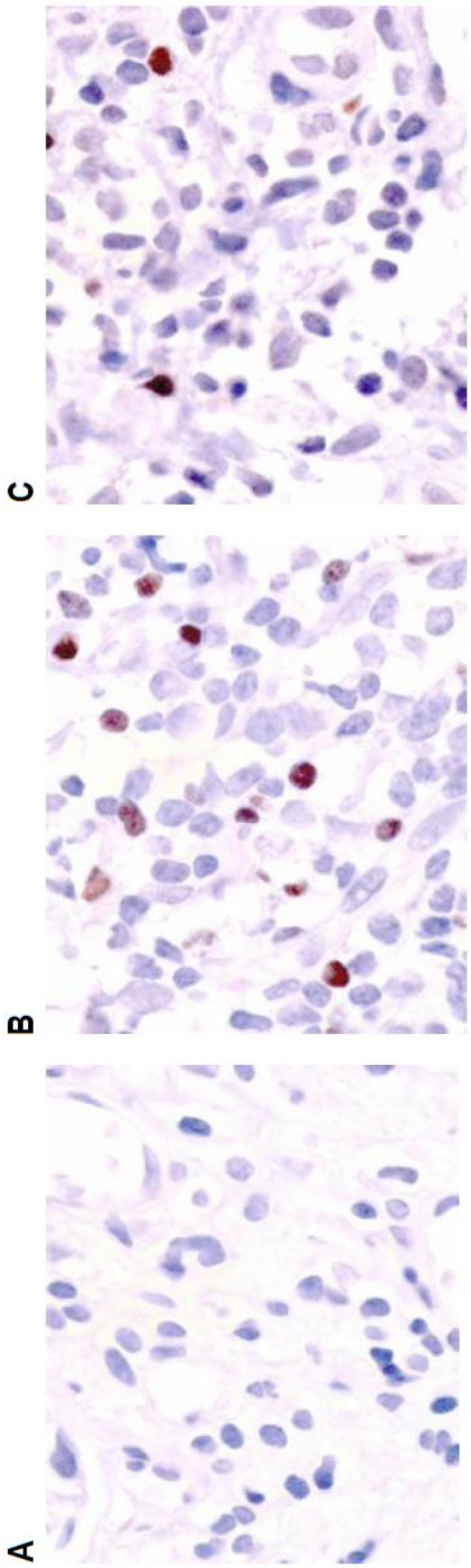
IL-1β (pg/mg of protein)
IL-10 (pg/mg of protein)
IL-23 (pg/mg of protein)
IL-6 (pg/mg of protein)
IL-17A (pg/mg of protein)
TGF-β1 (pg/mg of protein)
HP-
HP+

Figure 3:



3.2. Trabalho 2

Cytokine expression in *Helicobacter pylori* infected gastric mucosa: comparison between children and adults

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Abstract

We aimed to investigate to compare children and adults in respect to the effect of *H. pylori* infection on the gastric levels of cytokines linked to the innate immune response and to the adaptive Th1/Th2 response. We studied 245 children (142 *H. pylori*-negative and 103 *H. pylori*-positive) and 140 adults (40 *H. pylori*-negative and 100 *H. pylori*-positive). The cytokines representative of the innate compartment (IL-1α, IL-1β, IL-6, IL-12p70 and TNF-α), Th1 (IL-2, IFN-γ) and Th2 (IL-4) responses were higher in the *H. pylori*-positive than in the -negative children and adults. By comparing children and adults the mean levels of the Th2 cell (IL-4, $p < 0.001$) and the immune innate compartment (IL-1α, $p < 0.001$; IL-6, $p < 0.001$ and TNF-α, $p < 0.001$) cytokines were significantly higher in the *H. pylori*-positive children than in the infected adults. However, the gastric levels of the cytokines linked to the Th1 cell response (IL-2, $p = 0.005$; IL-12p70, $p < 0.001$ and IFN-γ, $p < 0.001$), and also IL-1β ($p < 0.001$), were significantly higher in adults than in children. In the children, the gastric concentration of IL-1β and IL-2 increased with the increasing age ($p = 0.003$ for both). The IFN-γ gastric concentration also increased in children older than 14 years of age ($p = 0.05$). Otherwise, in adults, the mean levels of Th1 linked cylokines, IFN-γ, IL-2 ($p = 0.01$ for both) and IL-12p70 ($p = 0.005$), decreased in elderly age.

Key words: *Helicobacter pylori*, cytokines, children, adults, innate immune response, adaptive immune response.

1. Introduction

Helicobacter pylori causes one of the most common chronic infections in human beings, being present in the gastric mucosa of more than 50% of the world's population. The infection is predominantly acquired in childhood and persists for life unless treated [1]. In most persons, the natural history of the infection is without complications, but peptic ulcer disease, distal gastric carcinoma, or mucosa-associated lymphoid gastric lymphoma may occur in a percentage of the infected individuals [2-3].

Although the infection is acquired early in life, the associated diseases will develop mainly in adulthood. Probably, the nature of the immune response and the amount of the diverse inflammatory mediators present in the gastric mucosa in childhood are determinant factors for the final infection's outcome in adulthood.

Immune response against *H. pylori* includes acute/innate [4-6] and chronic/adaptive components [7-8] The infection leads to gastric inflammation with increased number of inflammatory cells, such as neutrophils, monocytes/macrophages and plasma cells and increased production of mucosal inflammatory cytokines [9-14].

Several studies provided evidences that in adults, Th1, and more recently, Th17 cell responses contribute to the gastric inflammation during the infection [15-19]. However, although the early phases of the infection occur in childhood there are few studies evaluating the child immune response to the infection that may have a crucial role in modulating the adaptive response and ultimately in the development of the associated diseases in adulthood. Recently, we have demonstrated increased

levels of IL-17 cell-associated cytokines in infected children. However, the Th17 cell response was significantly higher in infected adults than in infected children [19].

Therefore, in this study, we aimed to investigate differences between children and adults in respect to the effect of *H. pylori* infection on the gastric levels of the representative cytokines of the innate response compartment as well as those associated with the adaptive Th1/Th2 response.

2. Patients and Methods

This study was approved by the Ethics Committee of the Universidade Federal de Minas Gerais, Belo Horizonte, Brazil. Signed informed consent to participate was obtained from the adults as well as from the children (whenever possible) and their parents.

2.1. Study population

We studied prospectively 245 children (142 *H. pylori*-negative and 103 *H. pylori*-positive) and 140 adults (40 *H. pylori*-negative and 100 *H. pylori*-positive) who underwent endoscopy to clarify the origin of symptoms referable to the upper gastrointestinal tract. Patients with peptic ulcer, gastric cancer, coagulation disorders and complications such as gastric perforation or haemorrhage, anatomical obstacle to endoscopy, or esophageal varices and patients who had already undergone gastric surgery were not included in the study. None of the patients had received antimicrobial drugs, anti-cholinergic and anti-inflammatory agents, proton pump inhibitors, or H_2-receptor antagonists for at least 30 days before endoscopy. All included patients were natives of the Minas Gerais state with the same genetic

background, approximately 33% of Portuguese, 33% of Amerindian and 33% of African ancestry, homogenously present in each subject [20].

Biopsy specimens were obtained from the antral and oxyntic gastric mucosa of all patients for evaluation of the *H. pylori* status and histological parameters and from the antral mucosa for the cytokine concentration determination.

2.2. *H. pylori* status

H. pylori status was evaluated by culture, preformed urease test, carbolfuchsin-stained smear, polymerase chain reaction (PCR) for *ure*A, and ^{13}C-urea breath test as previously described [21]. Patients were considered *H. pylori*-positive when culture was positive or at least two of the other tests were positive and *H. pylori*-negative when the results of all tests were negative.

2.3. DNA extraction

Tissue and bacterial culture DNA was extracted with QIAamp® DNA mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's recommendations with minor modifications (ref). The presence of the *ure*A *H. pylori* specific gene was evaluated, according to Clayton *et al.* [22] in all isolated strains to confirm *H. pylori* identification, as well as in gastric fragments of the patients in order to exclude false-negative results.

2.4.Determination of the gastric cytokine levels

Cytokines of the innate immune response (IL-1α, IL-1β, IL-6, IL-12p70 and TNF-α,) and those representative of the Th1 (IL-2, IFN-γ) and Th2 (IL-4) cell response were investigated. To determine the cytokine concentrations in the antral biopsy, the specimens were immediately placed into cryotubes, frozen in liquid nitrogen and stored at – 80ºC until used. Samples were homogenized using homogenizer tissue. Aliquots of homogenate supernatant in 1.5 mL PBS, pH 7.4 containing 2 μg/mL aprotinin were obtained by centrifugation (10,000 g for 10 minutes). The total protein concentration was measured by Bradford's method. Cytokine levels in the supernatant fluid were assayed in duplicate by enzyme-linked immunosorbent assay (Biosource, Camarillo, CA). For quantification of IL-4 and TNF-α levels, ultra sensitive kits were used. The mucosal levels of cytokines were expressed as picogram of cytokine per milligram of biopsy protein (pg/mg protein). The minimum detectable levels are 1.0 pg/mL (IL-1α), 4.0 pg/mL (IL-2), 0.27 pg/mL (IL-4), 4.0 pg/mL (IL-8), 0.2 pg/mL (IL12p70), 4 pg/mL (IFN-γ), and 0.09 pg/mL (TNF-α). All values below the detection levels were regard as undetectable and were ascribed the value zero.

2.5. Statistical analysis

Data were analysed with SPSS (SPSS Inc., Chicago, IL) statistical software package version 17.0. In addition to the visual examination of the histograms and box plots, the Kolmogorov-Smirnov goodness-of-fit was used to assess the normality of the data. When significant departures from normality were detected the data were log

transformed. The two-tailed Student's t test was used to compare the sub groups of patients. The level of significance was set at $p \leq 0.05$.

3. Results

3.1. Population

The mean age of *H. pylori*-positive children ($p = 0.002$) and adults ($p = 0.005$) was higher than that of the -negative children and adults, but no difference in respect to the gender was observed among the bacterium positive and negative patients ($p \geq 0.28$).

3.2 Cytokine levels in the gastric mucosa of children and adults

In the *H. pylori*-negative group, IL-1α, IL-6, IL-12p70 and TNF-α were naturally expressed in the gastric mucosa of few numbers of children and IL-1β in 44% of them. When children were compared with adults, the gastric levels of all cytokines, but not IL-12p70, were higher in the latter (figure 1). Similar results for IL-2 and IL-4 were observed in children and adults; but IFN-γ was neither detected in the gastric mucosa of children nor in that of adults.

In the *H. pylori*-positive group, almost all children and adults expressed all cytokines in the gastric mucosa; but IL-4 was detected only in 28.5% and 63.1% of the gastric mucosa of children and adults, respectively. When compared with the negative groups, significantly higher concentrations of all cytokines were observed in both *H. pylori*-positive children and adults (figure 2A and 2B).

By comparing the *H. pylori*-positive and -negative patients, the fold increase in the mean levels of all cytokines, but not IFN-γ, were higher in the children than in the adults (table 1).

The cytokine gastric mean levels of the Th2 cell response (IL-4, $p < 0.001$) and also the immune innate compartment (IL-1α, $p < 0.001$; IL-6, $p < 0.001$ and TNF-α, $p < 0.001$) were significantly higher, while IL-1β ($p < 0.001$) was lower in the *H. pylori*-positive children than in the infected adults. However, the gastric levels of the cytokines linked to the Th1 cell response (IL-2, $p = 0.005$; IL-12p70, $p < 0.001$ and IFN-γ, $p < 0.001$) were significantly higher in adults than in children (figure 2).

Therefore, next, the *H. pylori*-positive children and adults were stratified by age in order to identify more precisely when the changes occur. In the children, the gastric concentration of IL-1β increased with the increasing age from 186.88 pg/mg at ages 1 - 8 (n = 26), to 208.34 pg/mg, 9 - 13 (n = 59) and to 344.56 pg/mg, 14 - 18 (n = 18) ($p = 0.003$). The concentration of IL-2 also increased with increasing age from 591.85 pg/mg at ages 1-8, to 741.49 pg/mg, 9 - 13 and to 1159.95 pg/mg, 14 - 18 ($p = 0.003$). The IFN-γ gastric levels was similar ($p = 0.44$) between the two sub groups of young children (mean levels of 772.82 pg/mg in children at ages 1 – 8 and 883.80 pg/mg, at 9 – 13 years). However, the IFN-γ levels were significantly higher ($p = 0.05$) in children at ages equal or older than 14 years (1.191.37 pg/mg). No other significant difference among the groups was observed.

In adults, the mean levels of IFN-γ and IL-12p70 decreased with increasing age: IFN-γ from 2681.11 pg/mg; to 2248.93 pg/mg; to 1976.40 pg/mg and to 893.78 pg/mg ($p = 0.01$) and IL-12p70 from 90.86 pg/mg; to 88.85 pg/mg; to 54.87 pg/mg

and to 53.50 pg/mg ($p = 0.05$) at ages 19 - 30, 40 - 54, 55 – 67 and > 68, respectively. No other difference was observed.

4. Discussion

H. pylori infection is acquired usually in childhood and its prevalence varies in different regions of the world, showing predominance in the populations of developing countries.

The infection is characterized by mucosal infiltration of inflammatory cells, such as mononuclear and polymorphonuclear cells. The migration and activation of these cells into the mucosa depend on the expression of different cytokines [23]. The degree of gastric inflammation is lower in infected children than in infected adults [19, 24], suggesting that the immune/inflammatory response of the host to the *H. pylori* infection is linked to the age.

The comparative analyses of the effects of the infection on the gastric mucosa of children and adults can provide subsidies for the compression of the natural history of the infection as well as to elucidate the progression into severe diseases such as peptic ulcer or gastric carcinoma. Thus, we evaluated the cytokine gastric environment of children and adults infected by *H. pylori* in order to better understand the differences in the degree of gastritis according to age that we and others have previously demonstrated [19, 24].

Here, confirming previous studies [15-17], we observed increased levels of Th1 signature cytokine, IFN-γ, and of lL-12p70, a key inducer of Th1-associated response, in the gastric mucosa of infected adults and children. However, the levels of both cytokines were lower in infected children than in infected adults. Of note, the

IFN-γ levels increased with increasing age in childhood, but decreased in the elderly. Otherwise, the IL-4 gastric mean levels were higher in infected children than in adults. Furthermore, in a previous study, we also have demonstrated that the number of T_{reg}Foxp3+ cells and the levels of IL-10 and TGF-β were higher in the gastric mucosa of infected children than those of adults. Considering all data together, we might speculate that the differences according to the age observed in this study might explain the differences between children and adults in respect to the gastric inflammation and to the development of the severe infection outcomes. Thus, it seems that the young child low IFN-γ gastric concentration might facilitate the bacterium colonization and the infection establishment in childhood; the young/meddle age adult increased IFN-γ gastric production increases the gastric inflammation, that is the background for the development of gastric cancer; and the decreased IFN-γ gastric levels in the elderly adults might also contribute to gastric carcinogenesis, because IFN-γ is considered one of the most important molecules with antitumor activity.

Finally, we also evaluated the gastric content of the innate pro-inflammatory cytokines that were increased in the infected patients, but more significantly in children, which might contribute to the induction of the gastric inflammation induced by the infection in childhood even in a T_{reg} immune regulated gastric milieu. These cytokines participate in the antimicrobial activity by inducing the release of the acute phase reactants, by promoting inflammation and enhancing antimicrobial functions of macrophages and other effectors cells [25].

Acknowledgements

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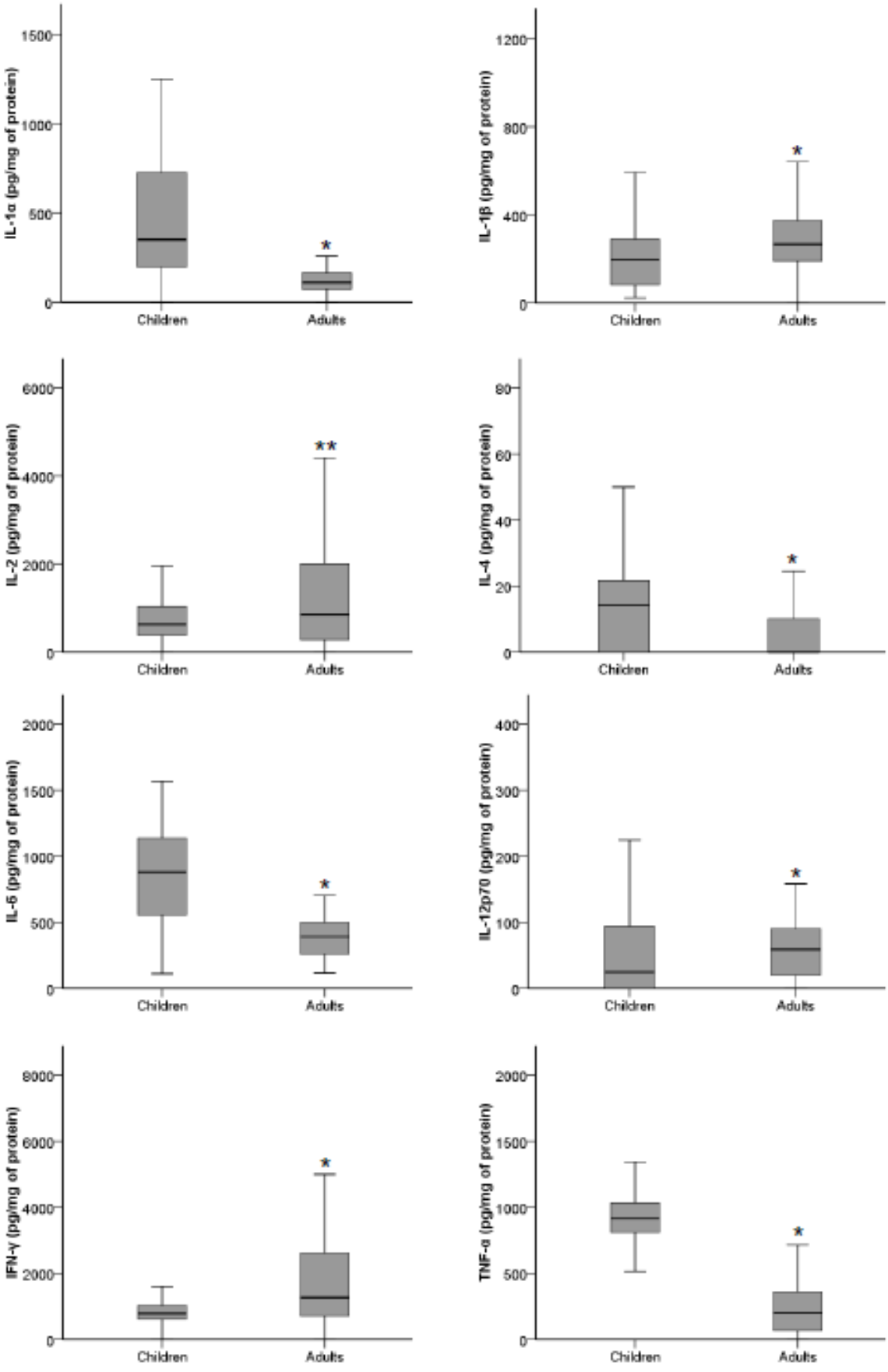
Legend for figures

Figure 1 - Box plots representing the comparison of the antral gastric cytokine concentrations (pg/mg of protein) between *H. pylori*-positive children (n = 103) and adults (n = 100). The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively. The horizontal bar across the box indicates the median and the capped bars indicate the minimum and maximum data values. The data were analyzed by the two-tailed Student's t. * $P < 0.001$, ** $P = 0.001$.

Figure 2A - Box plots representing the comparison of the antral gastric cytokine concentrations (pg/mg of protein) between *H. pylori*-positive children (n = 103) with children without *H. pylori* infection (n = 142). The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively. The horizontal bar across the box indicates the median and the capped bars indicate the minimum and maximum data values. The data were analyzed by the two-tailed Student's t test. * $P < 0.001$. 2B - Box plots representing the comparison of the antral gastric cytokine concentrations (pg/mg of protein) between *H. pylori*-positive (n = 100) and –negative (n = 40) adults. The upper and lower limits of the boxes represent the 75th and 25th percentiles, respectively. The horizontal bar across the box indicates the median and the capped bars indicate the minimum and maximum data values. The data were analyzed by the two-tailed Student's t. * $P < 0.001$.

Figure 1:


IL-1α (pg/mg of protein)
1500
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Children
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IL-1β (pg/mg of protein)
1200
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4000
2000
0
IL-4 (pg/mg of protein)
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IL-6 (pg/mg of protein)
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IL-12p70 (pg/mg of protein)
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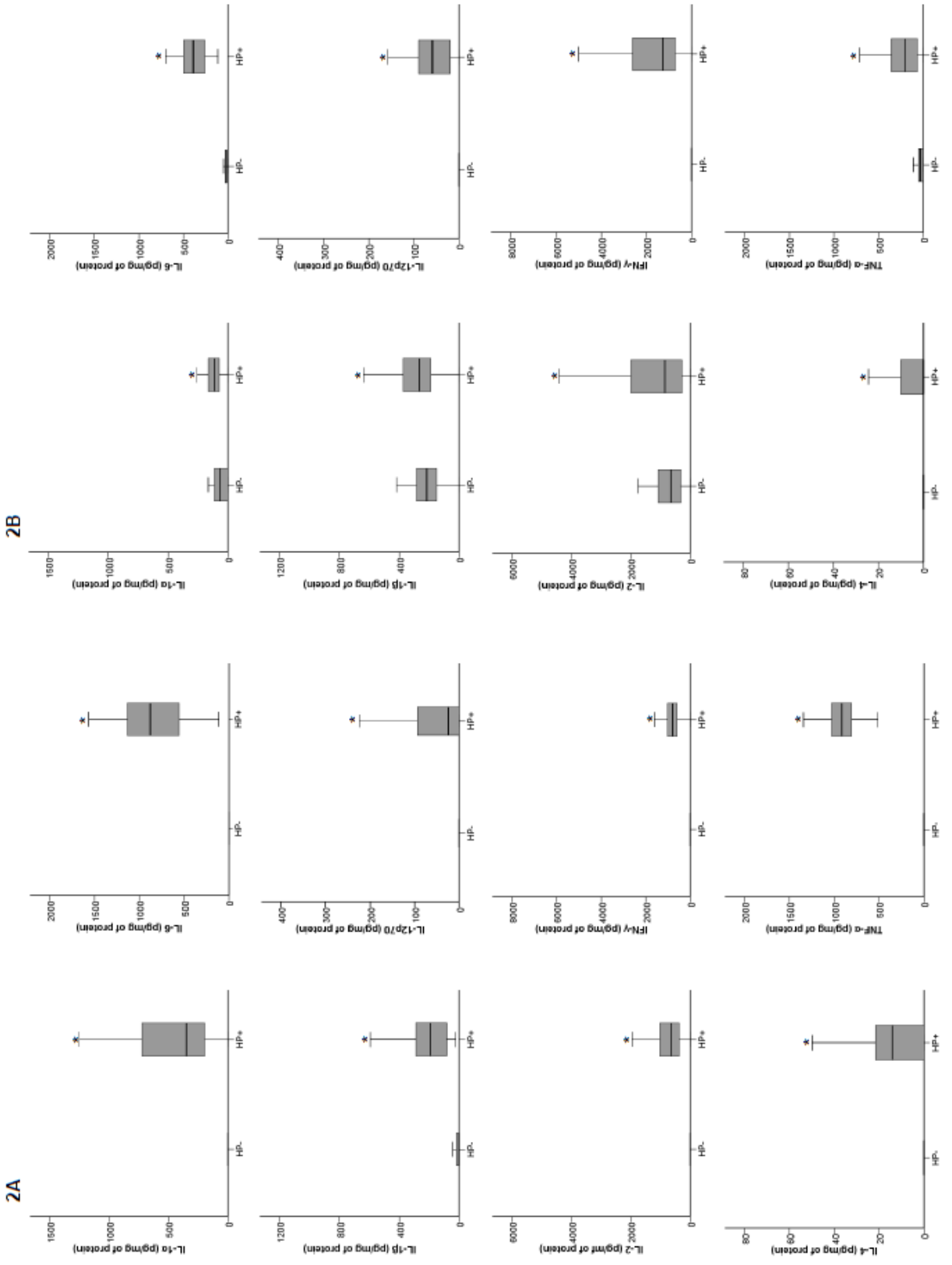
Figure 2:


2A
2B
IL-4 (pg/mg of protein)
IL-2 (pg/mg of protein)
IL-12p70 (pg/mg of protein)
IL-1β (pg/mg of protein)
IL-1α (pg/mg of protein)
TNF-α (pg/mg of protein)
IFN-γ (pg/mg of protein)
IL-6 (pg/mg of protein)
HP-
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3.3. Trabalho 3

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Original article

IL2-330G polymorphic allele is associated with decreased risk of *Helicobacter pylori* infection in adulthood

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Abstract

We evaluated whether polymorphisms in genes coding molecules linked to the innate and adaptive immune response are associated with susceptibility to *Helicobacter pylori* infection. *IL1B*-511C → T, *IL1B*-31 T → C, *IL1RN* allele 2, *IL2*-330 T → G, *TNFA*-307 G → A, *TLR2*Arg677Trp, *TLR2*Arg753Gln, *TLR4*Asp299Gly, and *TLR5*^{392STOP} polymorphisms were determined in 541 blood donors. *IL2*-330 T → G allele carriers had a decreased *H. pylori* infection risk (OR = 0.63, 95% CI = 0.43–0.93) after adjustment for demographic and environmental factors. Hence, we investigated whether the polymorphism is functional by evaluating IL-2 serum concentration in 150 blood donors and 100 children. IL-2 pro-inflammatory and anti-inflammatory properties were indirectly investigated by determining serum IFN-γ and IL-10/TGF-β levels. The polymorphism was associated with increased mean IL-2 levels in *H. pylori*-positive adults (2.65 pg/mL vs. 7.78 pg/mL) and children (4.19 pg/mL vs. 8.03 pg/mL). Increased IL-2 was associated with pro-inflammatory activity in adults (IFN-γ = 18.61 pg/mL vs. 25.71 pg/mL), and with anti-inflammatory activity in children (IL-10 = 6.99 vs. 14.17 pg/mL, TGF-β = 45.88 vs. 93.44 pg/mL) ($p < 10^{-3}$ for all). In conclusion, in the context of *H. pylori* infection, *IL2*-330 T → G polymorphism is functional and is associated with decreased risk of infection in adults.

Keywords: *Helicobacter pylori*; IL2-330T/G; IFN-γ; IL-10; TGF-β

1. Introduction

Helicobacter pylori infection is mainly acquired in childhood [1], and is followed by a lifelong persistent colonization of the gastric mucosa. In approximately 10–15% of the infected adults, it causes severe diseases such as peptic ulcer [2], distal gastric carcinoma [3] and mucosa-associated lymphoid tissue (MALT) lymphoma [4].

There are strong evidences of genetic influence on the susceptibility to *H. pylori* infection, as demonstrated in the Malaty's twin study [5]; however, only one genetic risk factor has been identified [6]. The presence of a polymorphic locus in the gene that codes the IFN-γ receptor 1 was seen to be associated with increased susceptibility to the infection in an African population. Although the authors did not evaluate functional implications of the polymorphisms, they argued in favor of loss of function.

Therefore, functional polymorphisms in genes linked to the innate and adaptive immune response may contribute to individual differences in the susceptibility to and persistence of *H. pylori* infection. Among the polymorphisms in genes encoding cytokines, in addition to those of *IL1B* [7], *IL1RN* [7,8], and *TNFA* [9] that are recognized as risk factors for *H. pylori*-associated gastric carcinoma, one that can be implicated in *H. pylori* infection as well is the *IL2*-330 T → G polymorphism, considered to increase the production of IL-2 [10].

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IL-2 is considered to be an important cytokine that plays a central role in orchestrating the T lymphocyte response. It has a broad proliferating effect on T cells including the Th1 subset. IFN-γ is a major product of Th1 cells and further skews the immune response toward a Th1 phenotype that dominates in the immune response to *H. pylori* infection [11]. Furthermore, a more specific, nonredundant IL-2 function was recently demonstrated, that is a regulatory immune effect mediated by an enhancement of Treg lymphocytes [12]. Which phenotype is more relevant in vivo and influences the clinical consequences of the infection deserves to be demonstrated.

In addition to cytokines, the toll-like receptors (TLRs) may be relevant in the context of *H. pylori* infection due to their properties in modulating the immune response by increasing nonspecific inflammation and triggering the adaptive immunity. TLRs lead to a translocation of NF-kB to the cell nucleus, with subsequent expression of TNF-α. Linking innate to adaptive immunity, activated TLRs up-regulate MHC and co-stimulatory molecules in dendritic cells, that subsequently migrate to draining lymph nodes and stimulate T cells against specific antigens. The described polymorphisms in the genes encoding TLRs have been considered to lead to truncated proteins and loss of function. It has been demonstrated that the *H. pylori* neutrophil-activating protein (HP-NAP) is a TLR-2 agonist able to induce a strong Th1 response [13]. However, the effect of the two known polymorphisms *TLR2* Arg677Trp and *TLR2* Arg753Gln on the *H. pylori* infection has not been evaluated yet. Also, the polymorphism 392STOP in *TLR5* that recognizes flagellin [14], the subunit of flagellum, deserves to be evaluated as a factor linked to susceptibility to the flagellated bacterium *H. pylori*. Although there are controversies with regard to the relevance of TLR-4 in the immune response to *H. pylori*, the *TLR4* Asp299Gly mutation has been recently linked to an increased susceptibility to gastric cancer in Caucasian populations [15].

Therefore, we investigated whether the *IL1B*-511C → T, *IL1B*-31 T → C, *IL1RN* allele 2, *IL2*-330 T → G, *TNFA*-307 G → A, *TLR2* Arg677Trp, *TLR2* Arg753Gln, *TLR4* Asp299Gly, and *TLR5*^{392STOP} polymorphisms influence the susceptibility to or the persistence of *H. pylori* infection by evaluating asymptomatic adult blood donors adjusting for environmental and demographic factors. Subsequently, we aimed to test whether significant associations observed in the first analyses would show functional relevance in terms of serum levels of target cytokines. Since it is well known that *H. pylori* infection is mainly acquired in childhood and persists throughout life unless treated, serum cytokine levels were also measured in a group of *H. pylori*-positive children to address the effect of the polymorphism on two different phases of the infection.

2. Patients and methods

2.1. Selection of subjects and gathering of specimens

The Ethics Committees of Hospital das Clínicas, Universidade Federal de Minas Gerais, and of Fundação Hemominas, Belo Horizonte, Minas Gerais, Brazil, approved this study.

Five hundred and forty-one consecutive voluntary asymptomatic adult blood donors from the Fundação Hemominas and 100 *H. pylori*-positive children (50 girls and 50 boys) randomly selected among those included in a previous study of our group evaluating cytokine and *TLR* gene polymorphisms [16] were included. Written informed consent was obtained from all adult blood donors, all parents of children and of children whenever possible. In the group of adult blood donors, demographic data, crowding index, socioeconomic level, educational level, dyspeptic symptoms and personal habits such as alcohol and tobacco use were obtained by a questionnaire that was validated according to Stolley and Schlesselman [17]. Exclusion criteria included history or previous diagnosis of peptic ulcer disease, previous treatment for *H. pylori* and use of proton-pump inhibitors or antibiotics in the last month. Parasitic infections, severe diseases or any immunological and infectious disorders were also exclusion criteria. Sera for *H. pylori* diagnosis and cytokine determination, and buff coat for DNA extraction were stored at −80 °C before processing. DNA was extracted with a QIAamp DNA Mini Kit (QIAGEN Inc. Valencia, CA), according to the manufacturer's instructions.

H. pylori status in the group of blood donors was evaluated by ELISA [15]. The sensitivity and specificity of the test were previously determined in endoscoped patients by comparing the ELISA results with those obtained by culture, urease test, histology and carbolfuchsin-stained smears. The sensitivity and specificity of the test were 95.4% and 100%, respectively, for the Brazilian adult population [18]. These results demonstrate that the accuracy of the test for *H. pylori* diagnosis in adults is very high. The same tests used for the validation of the ELISA in adults plus ^{13}C-urea breath test were used for the diagnosis of *H. pylori* infection in children. A patient was considered to be *H. pylori* positive when the culture was positive or two of the other tests were positive.

2.2. Polymorphism genotyping

The *IL1B*-31 T → C [19] and the *IL2*-330 T → G [20] polymorphisms were genotyped by PCR-CTPP (polymerase chain reaction with confronting two-pair primers). The PCR-RFLP (restriction fragment length polymorphism) method was used to genotype the *IL1B*-511C → T [21], *TNFA*-307 G → A [21], *TLR2*Arg677Trp, *TLR2*Arg753Gln [22], and *TLR4*Asp299Gly polymorphic alleles. The *IL1RN* penta-allelic variable number tandem repeats (VNTR) was genotyped according to Mansfield et al. [21]. For statistical analysis and due to the rarity of alleles 3, 4 and 5, this polymorphism was treated as bi-allelic by classifying the alleles in short (allele 2) and long (alleles 1, 3, 4 and 5) categories. The *TLR5*^{392STOP} polymorphism was genotyped by allele-specific-PCR. Primers binding either to the wild-type sequence beginning with C at position 1174 (5'-TTACA GACCTTGGATCTCC-3') or to the mutant sequence beginning with T at the position 1174 (5'-TTACAGACCTTGGATCTCT-3') were designed. The polymorphism-specific primers were

used together with a conserved reverse primer (5'-CAGAATCTGGAGATGAGGTACCCG-3') and internal control primers. Standard PCR cycling condition was used, with an annealing temperature of 65 °C. Allele-specific PCR products were separated on a 2% agarose gel.

All the results of PCR-RFLP were confirmed by sequencing. To confirm the presence of specific polymorphisms when the other methods were used, representative samples were sequenced. The samples were sequenced by cycle sequencing (ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kits) and analyzed on an ABI Prism 310 Automated DNA Sequencer (Applied Biosystems, Foster City, CA).

2.3. Determination of cytokine concentration in the serum

Since *IL2*-330 T → G polymorphism was observed to be associated with *H. pylori* infection, we decided to evaluate the serum concentrations of IL-2, IFN-γ (the signature cytokine produced by Th1 cells induced by IL-2), and IL-10 and TGF-β (regulatory cytokines produced by Treg cells stimulated by IL-2). The serum cytokine concentrations (pg/mL) were assayed in duplicate using sandwich-type ELISA kits (BioSource International, Camarillo, CA), according to the manufacturer's instructions. The minimum detectable levels for IL-2, IL-10, IFN-γ and TGF-β are <4.0 pg/mL, = 0.2 pg/mL, <4.0 pg/mL and 15.6 pg/mL, respectively, according to the BioSource's protocol booklet. All values below the detection levels were regarded as undetectable and were ascribed the value zero. The cytokine levels were evaluated in the serum from 100 *H. pylori*-positive (50 females and 50 males) and 50 *H. pylori*-negative (25 females and 25 males) subjects randomly selected among the adult blood donors and from the *H. pylori*-positive children (50 girls and 50 boys).

2.4. Statistical analysis

Increasing age was categorized according to 10 year groups. The cytokine variables were categorized according to the presence of none or at least one polymorphic allele, in each locus. Firstly, we evaluated the associations between the covariates and *H. pylori* seropositivity using univariate statistics. In this step, age, gender, crowding index, income, education, alcohol and tobacco use, ABO and Rh blood groups, *IL1B*-511C → T, *IL1B*-31 T → C, *IL1RN* allele 2, *IL2*-330 T → G, *TNFA*-307 G → A, *TLR2*Arg753Gln, *TLR4*Asp299Gly, and *TLR5*^{392STOP} polymorphisms were included. Every covariate that yielded a *p*-value of 0.25 or less was selected for inclusion in the full model of logistic regression. OR and 95% confidence interval (95% CI) were used as an estimate of the risk. The Hosmer–Lemeshow goodness-of-fit test was used to evaluate the fit of the model [23].

In addition to visual examination of histograms and box plots, the Kolmogorov–Smirnov goodness-of-fit test was used to assess the normality of the data. No significant departures from normality were detected for the serum cytokine concentrations, with the exception of IFN-γ that did not distribute normally in children, even after log-transformation. Therefore, two-tailed Student's *t* test and Mann–Whitney *U* test (only for IFN-γ serum levels in children) were used to detect differences between the sub-groups. Nonparametric statistics (χ^2 or Fisher's exact test) were used to compare groups formed by individuals in whom the cytokines were not detected with those in whom the cytokine levels were above the detection limit with regard to *H. pylori* status and presence/absence of *IL2*-330 T → G polymorphism. Correlations were evaluated by Pearson's correlation test. The level of significance was set at $p < 0.05$.

Data were analyzed with SPSS (SPSS Inc., Chicago, IL) statistical software package version 10.0. Hardy–Weinberg equilibrium of alleles at individual loci was assessed by χ^2 statistics.

3. Results

3.1. Population characteristics

The demographic data and distribution of the alleles are shown in Table 1. All polymorphisms were in Hardy–Weinberg equilibrium in the group of adult blood donors. *IL1B*-511C → T and *IL1B*-31 T → C were in almost complete linkage disequilibrium ($p < 10^{-6}$), thus we restricted further analyses to *IL1B*-31. The other loci segregated independently.

Table 1
Characteristics of the blood donors and children.

		HP- blood donors[a] ($n = 171$)	HP + blood donors[b] ($n = 370$)	HP+ children ($n = 100$)
Mean age (SD)		31.7 (10.1)	34.7 (9.8)	9.9 (3.4)
Male gender (%)		118 (69.0)	291 (78.6)	50 (50%)
IL1B-31	T/T	54	130	–
	T/C	77	188	–
	C/C	40	72	–
IL1RN VNTR	1/1	123	255	–
	1/2	37	104	–
	2/2	10	11	–
TNFA-307[c]	G/G	121	282	–
	G/A	42	81	–
	A/A	7	6	–
IL2-330	T/T	81	212	50
	T/G	82	139	45
	G/G	8	19	5
*TLR2*Arg753Gln	W/W[d]	168	363	–
	W/M[e]	3	7	–
	M/M	0	0	–
*TLR4*Asp299Gly	W/W	156	334	–
	W/M	15	36	–
	M/M	0	0	–
TLR5^{392STOP}	W/W	160	344	–
	W/M	10	24	–
	M/M	1	2	–

[a] HP-, *H. pylori* negative.
[b] HP+, *H. pylori* positive.
[c] The *n* for the blood donors for the *TNFA* locus was 539 due to the impossibility to genotype 2 subjects.
[d] W, wild-type allele.
[e] M, polymorphic (mutated) allele.

No individual harbored the *TLR2*Arg677Trp polymorphic genotype in our sample.

3.2. Risk factors associated with H. pylori infection

Univariate and regression analyses taking seropositivity to *H. pylori* as the dependent variable are shown in Table 2. Since the level of income and education were highly correlated (Pearson correlation = 0.53, $p < 10^{-3}$), we limited the analysis to the former. The *IL2*-330 T → G polymorphism was inversely associated with seropositivity to *H. pylori* ($p = 0.02$, OR = 0.63, 95% CI = 0.43–0.93), after controlling for the other factors in the full model of logistic regression.

3.3. The concentration of IL-2 and associated cytokines according to the H. pylori status

In the *H. pylori*-negative group, the cytokine concentrations were very low, being detected only in 14.0% (IL-2), 46.0% (IFN-γ) and 24.0% (TGF-β) and 36.0% (IL-10) of the individuals. Otherwise IFN-γ, TGF-β and IL-10 were detected in the serum of all *H. pylori*-positive subjects. With respect to IL-2, the serum levels were above the test detection limit in 52.0% of *H. pylori*-positive blood donors. By using a non-parametrical statistics, the *H. pylori* positive and negative groups differed significantly with regard to the number of subjects with detectable concentrations of IL-10, IFN-γ and TGF-β ($p < 10^{-6}$) and IL-2 ($p < 10^{-3}$).

When the mean concentrations of IL-2, IL-10, IFN-γ and TGF-β between *H. pylori*-positive (mean: 4.29 pg/mL, SD: 2.85 pg/mL; mean: 3.26 pg/mL, SD: 0.66 pg/mL; mean: 20.88 pg/mL, SD: 8.82 pg/mL and mean: 29.84 pg/mL, SD: 10.70 pg/mL, respectively) and *H. pylori*-negative (mean: 1.18 pg/mL, SD: 1.42 pg/mL; mean: 0.29 pg/mL, SD: 0.58 pg/mL; mean: 0.59 pg/mL, SD: 0.49 pg/mL and mean: 5.57 pg/mL, SD: 13.21 pg/mL, respectively) subjects were compared, the values were significantly higher ($p < 10^{-3}$ for all) in the *H. pylori*-positive adult blood donors.

Table 2
Logistic regression analysis of factors associated with *H. pylori*-positive status in the group of blood donors[a].

	Univariate	Multivariate		
	p	*p*	OR	95% CI
Increasing age	0.01	0.02	1.28	1.05–1.57
Female gender	0.01	0.02	0.60	0.39–0.92
Increasing income	0.00	0.00	0.60	0.49–0.73
Crowding index	0.19	0.83	1.04	0.72–1.50
ABO group	0.78	–	–	–
Rh group	0.34	–	–	–
Alcohol use	0.31	–	–	–
Tobacco use	0.17	0.41	1.26	0.73–2.16
IL1B-31C carrier	0.66	–	–	–
IL1RN VNTR 2 carrier	0.38	–	–	–
TNFA-307A carrier	0.15	0.11	0.70	0.43–1.08
IL2-330 G carrier	0.01	0.02	0.63	0.43–0.93
*TLR2*Arg753Gln M carrier	0.92	–	–	–
*TLR4*Asp299Gly M carrier	0.66	–	–	–
TLR5^{R392STOP} M carrier	0.91	–	–	–

[a] Hosmer–Lemeshow goodness-of-fit test, $p = 0.96$, 8 *df*, 10 steps.

3.4. IL-2 levels according to the IL2-330 T → G polymorphism

Among the 50 *H. pylori*-negative blood donors, 28 were IL2-330 T/T carriers and 22 were carriers of the IL2-330 T → G polymorphic allele. In the *H. pylori*-positive adult blood donors, 68 were carriers of the homozygous wild genotype and 32 of at least one polymorphic allele. Fifty children had the *IL2*-330 T/T genotype and 50 carried the *IL2*-330 T → G polymorphic allele.

In the *H. pylori*-negative adult blood donors, there was no difference in the mean serum IL-2 levels between *IL2*-330 T/T and G carrier subjects ($p = 0.86$, mean value = 0.43 pg/mL, SD = 1.20 pg/mL for T/T vs. mean value = 0.37 pg/mL, SD = 1.16 pg/mL for G carriers).

In the *H. pylori*-positive adult blood donors, a marked increase in IL-2 mean levels was associated with the *IL2*-330 T → G allele ($p < 10^{-3}$, mean value = 2.65 pg/mL, SD = 1.19 pg/mL for T/T vs. 7.78 pg/mL, SD = 2.10 pg/mL for G carriers). The IL-2 levels were above the detection limit of the test in only 14 (29.6%) of 68 wild *IL2*-330 T/T carriers in contrast to the *IL2*-330 T → G polymorphic carriers (100%) (Fisher's exact test, $p < 10^{-7}$).

The serum levels of IL-2 were significantly increased among *H. pylori*-positive children with at least one *IL2*-330 T → G allele compared with those harboring the wild *IL2*-330 T/T genotype ($p < 10^{-3}$, mean value = 4.19 pg/mL, SD = 2.55 pg/mL for T/T vs. mean value = 8.03 pg/mL, SD = 3.55 pg/mL for G carriers). Again, the number of wild *IL2*-330 T/T genotype carriers with detectable serum IL-2 concentration (26 of 50, 52.1%) was significantly lower than that observed in the group of *IL2*-330 T → G allele carriers (100%) (Fisher's exact test, $p < 10^{-6}$).

3.5. Concentration of IL-10, IFN-γ and TGF-β in the H. pylori-positive groups

Since we found that individuals carrying the *IL2*-330 T → G polymorphic allele exhibited increased serum levels of IL-2 in the presence of *H. pylori* infection, and considering that IL-2 has broad and complex immune effects such as to stimulate expansion of different cell populations that produce either pro-inflammatory (IFN-γ) or regulatory (IL-10 and TGF-β) cytokines, we measured the levels of these cytokines in the serum of children and adults representing the initial and the chronic phase of the infection, respectively.

All the determinations were above the detection limit of the tests. The mean levels of IL-10 ($p < 10^{-3}$, 6.99 ± 2.22 pg/mL for T/T vs. 14.17 ± 3.70 pg/mL for G carriers) and TGF-β ($p < 10^{-3}$, 45.88 ± 17.22 pg/mL for T/T vs. 93.44 ± 55.85 pg/mL for G carriers), but not of IFN-γ ($p = 0.45$, 9.43 ± 4.35 pg/mL for T/T vs. 10.46 ± 8.57 pg/mL for G carriers) were significantly increased in the children harboring the polymorphic *IL2*-330 T → G allele than in those harboring the IL2-330 T/T wild

allele. The opposite was observed in the group of adult blood donors. IFN-γ mean levels ($p < 10^{-3}$, 18.61 ± 8.57 pg/mL for T/T vs. 25.71 ± 7.36 pg/mL for G carriers) were significantly increased in the carriers of *IL2*-330 T → G allele than in those carriers of the *IL2*-330 T/T wild genotype. However, neither IL-10 ($p = 0.27$, 3.28 ± 0.67 pg/mL for T/T vs. 3.22 ± 0.65 pg/mL for G carriers) nor TGF-β ($p = 0.89$, 29.82 ± 10.67 pg/mL for T/T vs. 29.88 ± 10.94 pg/mL for G carriers) mean levels were increased in the carriers of the *IL2*-330 T → G polymorphic allele (Fig. 1).

3.6. Correlations between IL-2 and IL-10, TGF-β and IFN-γ in the H. pylori-positive groups

In order to confirm our results, we correlated the levels of IL-2 with those of IL-10, TGF-β and IFN-γ. The IL-2

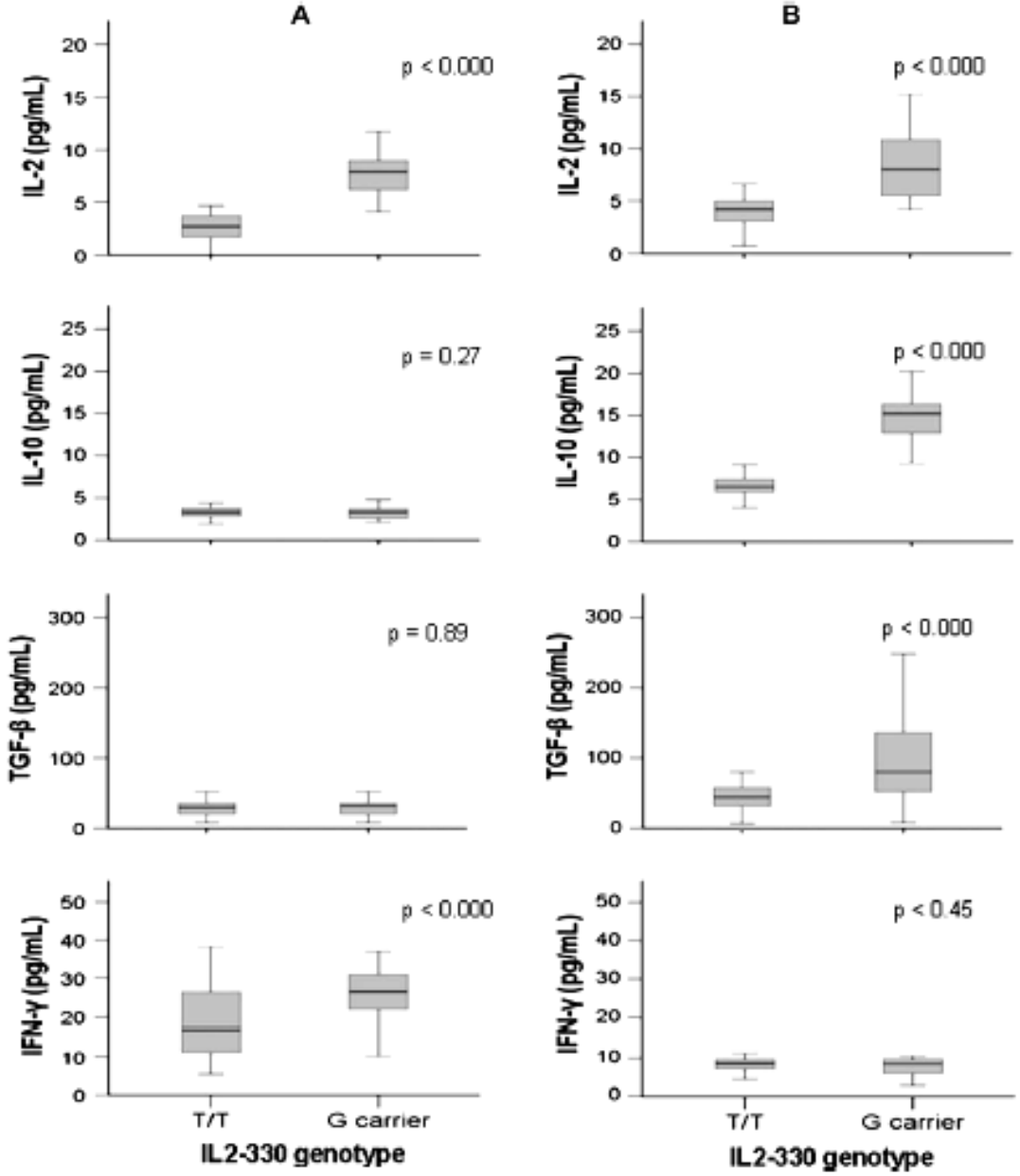

Fig. 1. Box plots representing the serum concentrations of cytokines according to the *IL2*-330 genotype. The upper and lower limits of the boxes represent the 25th and 75th percentiles, respectively, the horizontal bars represent the mean values, and the capped bars represent the 10th and 90th percentiles. (A) *H. pylori*-positive adults, subset ($n = 100$, 68 with genotype T/T, 32 carrying the polymorphic G allele) from the original group of blood donors. (B) *H. pylori*-positive children ($n = 100$, 50 T/T and 50 G carriers).

concentrations strongly correlated with the levels of IL-10 ($r = 0.59$, $p < 10^{-3}$) and TGF-β ($r = 0.63$, $p < 10^{-3}$) in children and with IFN-γ levels ($r = 0.57$, $p < 10^{-3}$) in adult blood donors. Otherwise, the IL-2 levels did not correlate with the levels of IFN-γ ($r = -0.058$, $p = 0.58$) in children as well as with IL-10 ($r = 0.031$, $p = 0.76$) and TGF-β ($r = 0.26$, $p = 0.62$) levels in adults (Fig. 2).

4. Discussion

H. pylori infects chronically the human stomach and the infection is associated with severe clinical outcomes, such as peptic ulcer disease and gastric carcinoma. Genetic host factors that contribute to the acquisition and maintenance of the infection are poorly known and difficult to be evaluated in populations under low exposure to the bacterium.

An advantage of the present study is the fact that our population lives in an area where the prevalence of *H. pylori* infection is high and the exposure of individuals to the bacterium occurs along their lives, which increases the chance of identifying host factors that protect against the infection.

We identified a factor, *IL2*-330 T → G polymorphism, that was negatively associated with the infection. We also demonstrated that this polymorphism is functional by increasing the serum levels of IL-2 in *H. pylori*-positive adults and children. The infection elicits an increased level of IL-2 that was higher in the carriers of the *IL2*-330 T → G polymorphic allele. IL-2 is a dual cytokine, showing both a redundant pro-inflammatory activity and a nonredundant anti-inflammatory property. Around 30 years ago, a series of in vitro experiments led to the discovery of IL-2 as a potent lymphoproliferative agent. In the early 2000s, the concept that

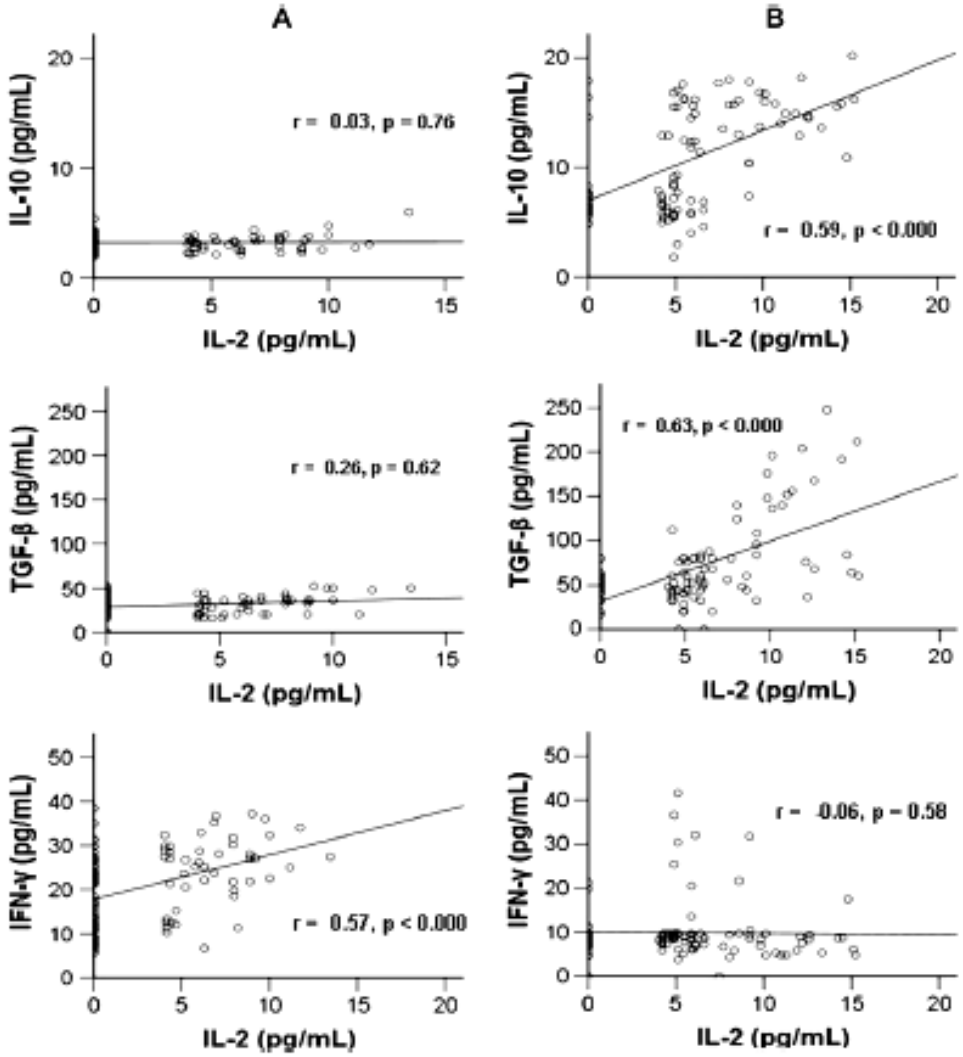

Fig. 2. Correlations between serum concentrations of IL-2 and the related cytokines in *H. pylori*-positive subjects, irrespective of *IL2*-330 genotype. (A) *H. pylori*-positive blood donors ($n = 100$). (B) *H. pylori*-positive children ($n = 100$).

lymphoproliferation depended exclusively on IL-2 was challenged with the results of studies showing massive in vivo lymphoproliferation in *IL2* knockout or *IL2* receptor knockout mice. Further characterization finally reconciled both observations in a model in which IL-2 shows both anti-inflammatory and pro-inflammatory actions. IL-2 has potent lymphoproliferative effects and may act as an inflammatory cytokine. Also, it is an important cytokine in the development of Treg cells, thus playing an anti-inflammatory, down-regulatory role in the immune response [12]. We provided evidences that carriers of *IL2*-330 T → G polymorphism might have amplified specific lymphocyte responses induced by *H. pylori* infection that varies according to age. In children, the increased IL-2 levels observed in the IL-2 polymorphism carriers seem to activate the expansion of Treg cells that produce increasing amounts of regulatory cytokines, IL-10 and TGF-β, in agreement with a study showing increased number of Foxp3 Treg cells and increased levels of IL-10 and TGF-β in the gastric mucosa of *H. pylori*-infected children compared with adults [24]. The more downregulated immune profile observed in children may account for the fact that the infection is predominantly acquired in childhood [1] and the gastric inflammation in response to the infection is much less marked in children than in adults [25]. Otherwise, the increased IL-2 levels observed in the *H. pylori*-positive adult carriers of *IL2*-330 T → G polymorphism probably induced a strong expansion of Th1 cells that led to an increased release of IFN-γ, the signature cytokine of Th1 cells, considered a relevant molecule in the effective immune response to several pathogens, including *H. pylori*. This result is in agreement with and complements the finding observed by Thye et al. [6], who identified a polymorphic locus in the gene coding the sequence of chain 1 of the IFN-γ receptor associated with increased susceptibility to *H. pylori* infection in an African population. Although the authors did not evaluate functional polymorphism implications, they argued that it leads to loss of function with consequent impairment of IFN-γ signalling pathway.

In conclusion, we demonstrated that the *IL2*-330 T → G polymorphism is associated with increased IL-2 and IFN-γ levels that might protect against *H. pylori* infection in adulthood.

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4. Discussão

A aquisição da infecção pelo *H. pylori* ocorre predominantemente na infância; entretanto, o aparecimento das doenças graves associadas à infecção se dá principalmente na vida adulta dos indivíduos infectados pela bactéria. Provavelmente, a natureza da resposta imunológica à infecção na infância é determinante para o surgimento das doenças associadas à infecção na idade adulta.

O *H. pylori* induz na mucosa gástrica uma resposta imunológica/inflamatória complexa, caracterizada pela presença de componentes da imunidade inata e adaptativa. Entretanto, essa resposta não é capaz de levar à erradicação da bactéria, podendo, paradoxalmente, contribuir para a patogênese das doenças associadas à infecção como ulcera péptica e carcinoma gástrico.

Não existem, até o momento, vacinas eficazes para prevenir a infecção pelo microrganismo, disponível para o uso em seres humanos. Resultados de estudos em camundongos avaliando a participação das células Th1 e Th2 na efetividade de vacinas anti-*H. pylori* são inconclusivos e controversos (Del Giudice *et al*., 2001; Lucas *et al*., 2001; Garhart *et al*., 2003a; Garhart *et al* 2003b; Panthel *et al*., 2003; Akhiani *et al*., 2004). Estudos mais recentes, em modelo animal, demonstram que as células Th17 têm papel central na eficácia da vacinação contra o *H. pylori* (Delyria *et al*., 2009; Velin *et al*., 2009; Flach *et al*., 2011). Foi observado que nos camundongos imunizados quando comparados com animais não imunizados havia diminuição da carga bacteriana e expressão gástrica aumentada de IL-17. Vale ressaltar que os níveis da citocina e de quimiocinas induzidas por células Th17 permaneceram elevados durante toda a fase de erradicação da bactéria, indicando que os mecanismos efetores das células Th17 podem desempenhar papel crítico na obtenção de proteção efetiva.

Apenas um estudo avaliou a expressão gástrica de IL-17 em crianças infectadas pelo *H. pylori*. Luzza *et al.* (2001), estudando crianças italianas, demonstraram a expressão de mRNA IL-17 mais elevada no grupo *H. pylori*-positivo que no grupo -negativo. Vale ressaltar que outras citocinas relacionadas à diferenciação das células Th17 não foram avaliadas no estudo de Luzza *et al*. (2001).

Em adultos, aumento nos níveis de IL-17A e expressão elevada de mRNA de IL-17A foram observados na mucosa gástrica de pacientes *H. pylori*-positivos japoneses e italianos, respectivamente (Mizuno *et al*., 2005; Caruso *et al*., 2008). No estudo de Caruso *et al*. (2008), além do aumento da expressão de mRNA de IL-17A, os autores observaram aumento das concentrações gástricas de IL-23 na vigência da infecção pelo *H. pylori*.

Os resultados do nosso primeiro estudo confirmam os escassos dados da literatura quanto ao aumento de IL-17 na infecção humana pelo *H. pylori*. Acrescentam dados relativos às citocinas envolvidades na diferenciação das células Th17. Demonstramos *in vivo* o aumento da concentração gástrica de IL-1β, IL-6, IL-23 e TGF-β, citocinas consideradas essenciais na diferenciação e atividade das células Th17, em estudos *in vitro* (Volpe *et al*., 2008; Korn *et al*., 2009). Tem sido proposto que para que ocorra a diferenciação de células Th17 é necessário um ambiente rico em IL-1β, IL-6 e TGF-β, que iniciam o processo, e em IL-23, que participa na expansão e estabilidade das células Th17 já diferenciadas (Volpe *et al*., 2008; Awasthi *et al*., 2009; Korn *et al*., 2009). Além de estar envolvido na diferenciação das células Th17 em seres humanos, TGF-β também participa do processo de diferenciação das células T_{reg}. Para a diferenciação das células Th17, TGF-β ativa o fator de transcrição RORc (retinoid-related orphan receptor c), e para a diferenciação das células T_{reg} ativa o fator de transcrição Foxp3 (forkhead Box 3/winged helix) (Chen *et al*., 2003; Ivanov *et al*., 2006; Veldhoen *et al*., 2006).

Vale ressaltar, que foram observadas diferenças no perfil de expressão de citocinas entre crianças e adultos infectados pelo *H. pylori.* Os níveis de IL-6 foram significativamente maiores na mucosa gástrica de crianças que de adultos. A IL-6 é uma citocina produzida principalmente por células da resposta imunológica inata, o que poderia explicar, pelo menos em partes, esses resultados, uma vez que a infecção pelo *H. pylori* é adquirida predominantemente na infância. Entretanto, outras células, como as Th17, também são fonte de IL-6; e, como já mencionado, a citocina tem importância crucial na diferenciação das células Th17, por mecanismos que incluem a inibição da ativação do fator de transcrição Foxp3 por TGF-β (Korn *et al*., 2009), o que, consequentemente, resulta na indução e diferenciação de células Th17. Na ausência de IL-6, TGF-β é capaz de ativar o fator de transcrição Foxp3, induzindo a diferenciação de células T_{reg}. Por outro lado, na presença de IL-6, essa ativação não ocorre, permitindo, assim, uma diferenciação de células Th17 (Weaver *et al*., 2007). Por mecanismos ainda não compreendidos, o ambiente gástrico rico em IL-6, como aqui observado nas crianças infectadas, não foi capaz de inibir a diferenciação de células T_{reg}, nem da expressão gástrica de IL-10 e TGβ. Um número aumentado de células Foxp3+, bem como aumento da concentração gástrica dos principais produtos das células Treg, IL-10 e TGF-β, foram observados tanto nos adultos como nas crianças infectadas. Entretanto, como previamente demonstrado por Harris *et al*. (2008) em pacientes Chilenos, esses valores foram significativamente maiores na mucosa gástrica de crianças que dos adultos. Uma provável explicação para esse fenômeno seria a menor habilidade das crianças produzirem IL-23, o que limitaria a expansão e atividade das células Th17 que parecem depender da IL-23. Também, o aumento de TGF-β observado na mucosa gástrica das crianças infectadas poderia suprimir a expressão do receptor da IL-23, como sugerido no estudo de Zhou *et al*. (2008), o que favoreceria a diferenciação de células T_{reg}. Ainda, há um estudo

recente demonstrando que produção aumentada de IL-6 *in vivo* não afeta a atividade de células T_{reg} naturais em camundongos transgênicos (Fujimoto *et al*., 2011).

Na infância uma resposta à infecção caracterizada por predomínio de células T_{reg} em detrimento de células Th17 poderia explicar, pelo menos em parte, a susceptibilidade das crianças à infecção, bem como o grau da inflamação gástrica decorrente da infecção que é menos intenso nas crianças que nos adultos. De fato, nesse estudo, observou-se que o infiltrado de células mono e polimorfonucleares foi mais intenso na mucosa gástrica de adultos infectados que nas crianças *H. pylori*-positivas. Lesões mais graves como atrofia e metaplasia intestinal foram observadas somente na mucosa gástrica de adultos.

Estudos comparativos entre crianças e adultos, como já mencionado, podem ajudar na compreensão da história natural da infecção pelo *H. pylori* nos seres humanos, como a progressão para doenças graves, como úlcera péptica e carcinoma gástrico. Assim, no segundo trabalho, avaliou-se a expressão de citocinas da resposta imunológica inata, bem como de citocinas associadas aos perfis Th1 e Th2 na mucosa gástrica de crianças e adultos infectados ou não pela bactéria. Demonstramos que os níveis gástricos de todas as citocinas avaliadas estavam aumentados nos pacientes infectados quando comparados com os *H. pylori*-negativos. Entretanto, à semelhança do observado no primeiro estudo, foram vistas diferenças significativas entre crianças e adultos.

Confirmando estudos prévios (Luzza *et al*., 2001; Shimizu *et al*., 2004; Oderda *et al*., 2007; Pellicano *et al*., 2007), observamos níveis aumentados de IFN-γ e IL-12p70 na mucosa gástrica de crianças e adultos infectados pelo *H. pylori*. Entretanto, os níveis gástricos dessas citocinas foram maiores nos adultos do que nas crianças. Por outro lado, as concentrações de IL-4 foram maiores na mucosa gástrica de crianças que na de adultos infectados. Demonstramos também no primeiro estudo um aumento de células T_{reg}, bem como de IL-10 e TGF-β, na mucosa gástrica das crianças que na de adultos infectados pela bactéria. As

diferenças observadas em relação à idade podem explicar as diferenças entre adultos e crianças relacionadas à inflamação gástrica e ao desenvolvimento das doenças graves associadas à infecção. Assim, a baixa concentração de IFN-γ na mucosa gástrica na primeira infância poderia facilitar a colonização pela bactéria, bem como o estabelecimento da infecção; o aumento dos níveis IFN-γ em adolescentes e adultos de meia idade poderia contribuir para uma inflamação gástrica acentuada, condição predisponente para o desenvolvimento do câncer gástrico; e os níveis reduzidos de IFN-γ em adultos mais velhos poderiam contribuir também para a carcinogênese gástrica, uma vez que o IFN-γ é considerado uma das mais importantes moléculas com atividade antitumoral.

Demonstramos, também, que a infecção é acompanhada por aumento nas concentrações de citocinas pró-inflamatórias da resposta imunológica inata em crianças e adultos. Entretanto, o aumento foi mais acentuado nas crianças. É provável que essas citocinas desempenhem papel importante na inflamação gástrica induzida pela infecção na infância, visto que tanto as respostas pró-inflamatórias adaptativas Th1 quanto Th17 são menos acentuadas nas crianças.

O fato de as crianças apresentarem uma resposta imunológica mais regulada com aumento de IL-4, IL-10, TGF-β e do número de células T_{reg} pode estar na dependência da dicotomia funcional da IL-2. Os níveis de IL-2 aumentam na infecção e a citocina tem funções distintas na resposta adaptativa. Por um lado, tem uma função redundante envolvida na reposta pró-inflamatória Th1, participando no aumento da produção de IFN-γ. Participa, também, de maneira não redundante, na diferenciação de células T $CD4^+$ naïve em $T_{reg}Foxp3^+$. Compatível com o perfil de citocinas observado nas crianças e adultos infectados, no terceiro trabalho dessa tese demonstramos que os níveis séricos de IL-2 se correlacionaram positivamente com os níveis séricos de IL-10 e TGF-β nas crianças, mas não nos adultos. Por outro lado, nos adultos infectados, os níveis de IL-2 se correlacionaram positivamente com os

níveis de IFN-γ. No mesmo estudo, foi também demonstrado que a presença do alelo polimófico *IL2*-330G do gene que codifica a IL-2 associou-se negativamente à infecção pelo *H. pylori* e positivamente com concentração sérica de IL-2. Quando um grupo de adultos foi estudado, a presença do polimorfismo foi vista estar também associada a níveis aumentados de IFN-γ. Nas crianças; entretanto, o polimorfismo se associou a aumento dos níveis de IL-10 e TGF-β.

O fato de a resposta imunológica das crianças à infecção ser mais "regulada" que a dos adultos pode explicar a susceptibilidade das crianças à infecção (Rocha *et al*., 2003), pela incapacidade de montarem uma resposta imunológica mais eficiente para impedir a colonização persistente da bactéria. Explicaria também o grau de gastrite menos intenso e consequentemente a raridade de complicações associadas à infecção nessa faixa etária (Queiroz *et al*., 1991; Harris *et al*., 2008). Por outro lado, a concentração aumentada de IFN-γ observada na mucosa gástrica dos adultos indica a maior habilidade de os adultos montarem uma resposta Th1, que é considerada essencial contra a infecção pelo *H. pylori*, o que poderia explicar porque a infecção é raramente adquirida na idade adulta. Como os adultos infectados, embora em menor intensidade que as crianças, também respondem com produção de IL-10 e TGF-β, a infecção estabelecida na infância se mantém para o resto da vida do hospedeiro.

Concluindo, os nossos resultados em conjunto fornecem dados que apontam que a resposta imunológica à infecção pelo *H. pylori* varia de acordo com a idade, o que pode explicar, ao menos em parte, as diferenças observadas entre crianças e adultos infectados, como a susceptibilidade à infecção, o grau de lesões gástricas e a evolução para doenças graves como úlcera péptica e carcinoma gástrico.

5. Conclusões

- A infecção pelo *H. pylori* é acompanhada de aumento da concentração gástrica de citocinas associadas às respostas Th1, Th2, Th17 e T_{reg} tanto em adultos quanto em crianças;

- Nas crianças infectadas pelo microrganismo a resposta imunológica é caracterizada principalmente por um aumento das citocinas associadas à imunidade inata, bem como às células T_{reg} e Th2, em detrimento das respostas adaptativas pró-inflamatórias Th1 e Th17, o que as protege dos efeitos deletérios da inflamação excessiva, mas contribui para a aquisição e persistência da infecção;

- Por outro lado, nos adultos a infecção cursa com concentrações elevadas das citocinas representativas das respostas pró-inflamatórias Th1 e Th17, criando condições favoráveis para o desenvolvimento de doenças graves como o carcinoma gástrico;

- A concentração gástrica de citocinas associadas à resposta Th1 aumenta com a idade na infância, mas diminuem depois dos 55 anos, o que também pode contribuir na carcinogênese gástrica dada à ação antitumoral do IFN-γ;

- O polimorfismo *IL2*-330G protege contra a infecção pelo *H. pylori* e é funcional, visto que os carreadores dos alelos polimórficos produzem concentrações aumentadas de IL-2 e de citocinas associadas como IL-10 e TGF-β1 nos casos das crianças e IFN-γ no caso dos adultos.

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7. Referência Eletrônica

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8. Anexos

Date: Jul 19, 2011 To: "Dulciene M Queiroz" dqueiroz@medicina.ufmg.br cc: microbes@pasteur.fr;Martin.Blaser@nyumc.org;Martin.Blaser@med.nyu.edu;stacy.bodziak @nyumc.org From: "Microbes and Infection" microbes@pasteur.fr Subject: Your Submission - MICINF-D-11-00170

Ms. Ref. No.: MICINF-D-11-00170
Title: A regulatory instead of a IL-17 T response predominates in H. pylori-associated gastritis in children
Microbes and Infection

Dear Dr. Queiroz,

Thank you for submitting your manuscript. Both reviewers believed that your manuscript had promise, but they had a number of areas of concern. On this basis and after my review, we would be willing to consider a revised version of this manuscript. We ask that you submit a revision within three months that addresses each of the reviewers' comments. At that time, we will determine whether the revised version will require re-review.

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Sincerely yours,

Martin J. Blaser, M.D.
Editor

Trabalho 1

Table 1: Histological comparison of the gastric mucosa of *Helicobacter pylori*-positive childrem (n=103) and adults (n=100)

Inflamation	Absent	Mild	Moderate	Marked	*p*
	n (%)	n (%)	n (%)	n (%)	
Antrum MN cells					
Children	5 (4.9)	30 (29.1)	63 (61.1)	5 (4.9)	
Adults	1 (1.0)	16 (16.0)	67 (67.0)	16 (16.0)	< 0.001
Antrum PMN cells					
Children	14 (13.6)	53 (51.5)	34 (33.0)	2 (1.9)	
Adults	5 (5.0)	53 (53.0)	36 (33.0)	6 (6.0)	< 0.01
Corpus MN cells					
Children	5 (4.8)	72 (69.9)	22 (21.4)	4 (3.9)	
Adults	3 (3.0)	47 (47.0)	41 (41.0)	9 (9.0)	< 0.001
Corpus PMN cells					
Children	28 (27.2)	62 (60.2)	9 (8.7)	4 (3.9)	
Adults	18 (18.0)	57 (57.0)	19 (19.0)	6 (6.0)	< 0.001

n, number; MN, mononuclear; PMN, polymorphonuclear

Trabalho 2

Table 01- Fold increase in the mean levels of the cytokines

	Innate immune response					Th1		Th2
	IL-1α	IL-1β	IL-6	IL-12p70	TNF-α	IL-2	IFN-γ	IL-4
Children	15.1	11.5	307.1	213.1	197.9	5.4	927.1	130.7
Adults	1.4	1.3	18.6	161.8	5.0	1.5	1,595.7	1.8