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Iron Deficiency and IL1β Polymorphisms in *Helicobacter pylori*-infected Children

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Abstract

Background: *Helicobacter pylori* infection has been associated with an imbalance of iron homeostasis. IL-1β has been related with iron absorption disturbances through a variety of mechanisms. The aim of this study was to evaluate the presence of polymorphic variants for IL-1β cluster and gastric IL1β mRNA expression in *H. pylori*-infected children and their relationship with hypochlorhydria and iron deficiency (ID).

Patients and Methods: Prospective study of 123 symptomatic children. At endoscopy, antral biopsies were taken for urease test, pathology and culture and blood for analysis of ferritin, transferrin, serum iron, and total iron-binding capacity. Polymorphisms in the IL-1β cluster (positions −511, −31, +3954, ILRN) were determined by PCR-RFLP. Gastric mucosal expression of IL-1β mRNA was determined by RT-PCR.

Results: After exclusions, of 105 patients, 33 (31.4%) were *H. pylori* positive. Nine (8.6%) children were classified as iron deficient (ID). *Helicobacter pylori* positivity was associated with ID (OR: 5.1; 95% CI: 1.2–21.9) (p = .04). No significant differences were found in allele frequency for IL1β gene cluster polymorphisms between infected and uninfected children. *Helicobacter pylori*-infected children with ID had significantly increased gastric IL1β mRNA in comparison with infected children without ID. In addition, a significant positive correlation was observed between mucosal IL-1β mRNA and fasting gastric juice pH. Gastric pH values were significantly increased in *H. pylori*-infected patients with ID compared to uninfected children.

Conclusions: The established association between *H. pylori* infection and ID in children may be mediated by increased gastric mucosal IL-1β.

Helicobacter pylori infection has been associated with an imbalance of iron homeostasis of the host. Recent meta-analyses indicate eradication of *H. pylori* infection improves serum iron parameters and resolves unexplained, or refractory to treatment iron, deficiency/iron deficiency anemia (ID/IDA) both in children and adults. This positive outcome depends on eradication of *H. pylori* infection, as iron supplementation on its own is not able to generate the same effect [1–3]. Most recent clinical guidelines recommend *H. pylori* eradication in the presence of unexplained or refractory ID/IDA [4,5]. A recent multicenter study identified that *H. pylori* infection is a significant predictor of low ferritin and hemoglobin concentrations in children from Latin America [6].

Infection with *H. pylori* induces several changes in the gastric mucosa that lead to altered gastric physiological responses, hence impacting on iron homeostasis. For intestinal absorption, 80% of dietary iron requires reduction to the ferrous state in the gastric lumen, a process dependent on gastric acidity and luminal ascorbic acid [7]. *Helicobacter pylori* infection is associated with the development of both transient and chronic hypochlorhydria [8,9]. *Helicobacter pylori* represses the expression of the α subunit of the H,K-ATPase in gastric parietal cells in a NF-κβ and *cag* pathogenicity island-dependent manner [10,11]. Low serum iron and transferrin in childhood *H. pylori* infection is associated with hypochlorhydria; however, in

uninfected children, hypochlorhydria does not alter serum iron parameters, indicating a combination of *H. pylori* infection and/or inflammation impacts on iron status [12].

Helicobacter pylori infection generates a strong inflammatory response in the gastric mucosa characterized by the secretion of several pro-inflammatory cytokines such as interleukin (IL)-8, IL-1β, and tumor necrosis factor (TNF)-α [13,14]. Both IL-1β and TNF-α are potent inhibitors of acid secretion by parietal cells induced by both histamine and pentagastrin in a mechanism dependent on phospholipids, Ca^{2+} and IP3 production [15–17]. In the gerbil model, Takashima et al. [18] showed that 12 weeks after *H. pylori* infection, gastric IL-1β mRNA abundance was elevated and gerbils were hypochlorhydric. Gastric juice acidity was restored after IL-1 receptor antagonist treatment. In adults, several polymorphisms in the IL-1β gene cluster have been described as risk factors for cancer gastric development and hypochlorhydria [19].

Although decreases in gastric acid have been reported before in patients with ID, the majority of these studies have been in adults with a long-term chronic *H. pylori* infection. As *H. pylori* infection is acquired mainly in childhood, the study of the relationship between ID and infection in childhood is important. Recent studies in *H. pylori*-infected children have identified gastric mucosal IL-1β concentrations are inversely associated with blood ferritin and hemoglobin concentrations and linked to pro-inflammatory *IL-1β* gene cluster polymorphisms [20]. In this study, the presence of polymorphic variants of the IL-1β gene cluster and gastric mucosal IL-1β mRNA expression in *H. pylori*-infected children and their relationship with hypochlorhydria and ID in pediatric patients have been investigated.

Patients and Methods

Patients

One hundred and five consecutive children with abdominal symptoms referred for Upper GI endoscopy were enrolled in this IRB approved study at the Pontificia Universidad Catolica de Chile Endoscopic facilities after signing informed consent forms by the patients, or their parents/legal guardians. Inclusion and exclusion criteria for participation in this study are described elsewhere [12]. Briefly, inclusion criteria included symptoms suggestive of peptic disease such as nocturnal burning, abdominal pain, or chronic vomiting associated with food intake. Patients with a history of functional recurrent abdominal pain plus a first-degree relative with an endoscopically proven diagnosis of peptic ulcer disease were also considered as eligible for inclusion. Exclusion criteria included antibiotic use, H2 antagonist, or proton-pump inhibitors (PPI) in previous 28 days, antacids on day of endoscopy, peptic ulcers, known celiac disease, enteropathies with villous atrophy, confounding enteric infections, esophageal varices, coagulation disorders, acquired or congenital immunosuppression, renal failure, hematologic disorders, and neoplasias. Additional exclusion criteria after endoscopic procedures were undiagnosed celiac disease and any nonspecific duodenal inflammation.

Helicobacter pylori Infection and Gastric pH Status

At endoscopy, antral biopsies were taken for rapid urease test (Pronto Dry, Ecifarma, Chile), histology, RT-PCR, and culture. A subject was considered *H. pylori* positive if one of the three tests was positive. For gastric pH determination, fasting gastric juice was obtained as previously described [12] and pH was determined by pH-indicator strips (pH 0–14 Universal indicator, Merck, Darmstadt, Germany). Noninfected patients were defined as children with no infection and normal upper GI endoscopy.

Iron Status

Each subject underwent blood collection at the time of venous puncture for sedation during endoscopic procedures. Serum markers such as transferrin, serum iron, and total iron-binding capacity (TIBC) were measured by colorimetric spectrophotometer (MODULAR P, Roche Diagnostics GmbH, Mannheim, Germany). Ferritin was assayed by direct chemiluminescence (ADVIA Centauro, Siemens Healthcare Diagnostics Inc. Munich, Germany.). Each individual serum marker was considered altered when it was below the control group (children uninfected with *H. pylori,* parasites or rotavirus) mean −1 standard deviation (SD) for ferritin (<18.6 ng/mL), serum iron (<68 μg/dL), transferrin (<20.3% of saturation), or above mean +1SD for TIBC (>358 μg/dL). A child was considered ID when three or more of the serum markers were altered. Non-ID cases were defined as infected children with normal upper GI endoscopy who do not meet above criteria.

Cytokine Polymorphisms Analysis

Total genomic DNA was extracted from heparinized whole blood obtained at the time of venous puncture

for sedation during endoscopic procedures. Polymorphisms in the IL-1β cluster (positions −511, −31, +3954, ILRN) were determined by PCR-RFLP [19, 21].

Gene Expression of IL-1β in Gastric Mucosa

Gastric mucosal expression of IL-1β mRNA was determined by real-time RT-PCR. Gastric antral biopsies taken at endoscopy were snap frozen in liquid nitrogen and stored at −70 °C until processed. RNA extractions were performed using a commercial kit (RNAeasy kit, Qiagen Venlo, Netherlands.) according to manufacturer instructions. RNA samples were quantified by measuring the absorbance at 260 and 280 nm and its integrity assessed by agarose gel electrophoresis. RNA was treated with DNAse I (Invitrogen) for 15 minutes and inactivated at 65 °C to avoid genomic DNA amplification. Reverse transcription was undertaken using the affinityScript QPCR cDNA synthesis kit (Agilent Technologies, Stratagene Santa Clara, CA, USA) with oligo DT as primers. Real-time PCR was performed using SYBR green detection method with standard curve quantification (Brilliant II SYBR green QPCR master mix; Agilent technologies, Stratagene). Primer pairs for specific cytokines were designed using the Perl primer software [22] spanning the exon–exon junction. GAPDH amplicons were used for normalization. IL-1β and GAPDH forward and reverse primer sequence were as follows: IL-1β F:5′-ACAGATGAAGTGCTCCTTCC-3′; IL-1β R:5′-CACATAAG CCTCGTTATCCC-3′ and GAPDH F:5′-AACCTGCCAAA-TATGATGAC-3′; R:5′-GTTGTCATACCAGGAAATGAG-3′. PCRs were performed in an Mx3000p Real-Time PCR System (Agilent technologies, Stratagene). For the standard curve construction, PCR fragments of each cytokine and the reference gene obtained by RT-PCR were quantified densitometrically to determine the necessary concentration for cloning into the pGEM cloning vector. Plasmids extracted from selected clones containing expected inserts were quantified spectrophotometrically. Serial dilutions of the vector-insert were used as standard curves to quantify the levels of gene expression.

Statistical Analysis

Comparisons between groups were undertaken using Student's t-test for continuous parametric data and with Mann–Whitney test for continuous nonparametric data. Categorical data were analyzed using a chi-square test and Fisher's exact test. A p value less than .05 was considered significant. Odds ratios and their 95% confidence interval were calculated to determine the association between *H. pylori* infection and ID.

Results

Iron Deficiency in Children

One hundred and five consecutive patients were included in this study. Thirty-three patients (31.4%) were infected by *H. pylori*. As previously described [12], there was no significant difference in gender distribution (43 and 45% males in noninfected and in *H. pylori*-infected patients respectively), mean age (10.5 + 3.0 years old and 10.8 + 3.4 years old in noninfected and in *H. pylori*-infected patients, respectively), symptoms, and clinical referral for endoscopy between infected and noninfected children. According to the definition of ID (see Patients and Methods), 9 (8.6%) children had ID. Of the ID-positive patients, 6 (66%) were *H. pylori* positive. *Helicobacter pylori* infection was significantly associated with ID (OR: 5.1; 95% CI: 1.2–21.9) (p = .04).

ID and Gastric pH Levels

As alterations in gastric pH levels in *H. pylori*-infected children have previously been correlated with changes in iron blood parameters [12], gastric juice pH of *H. pylori*-infected children with, and without ID, and uninfected children were compared. Gastric juice pH in *H. pylori*-infected children with ID was significantly higher (p = .04) than in *H. pylori*-negative children (Fig. 1A). Furthermore, in *H. pylori*-infected children with ID, the percentage of children with pH over four was significantly higher (p = 0.03) than in *H. pylori*-infected patients without ID (Fig. 1B).

Pro-inflammatory Cytokines Gene Polymorphisms and Iron Deficiency in *Helicobacter pylori*-infected Children

To analyze further the relation between *H. pylori* and ID, gastric cancer- and hypochlorhydria-related IL-1β gene cluster (positions −511, −31, +3954, ILRN) polymorphisms were examined in children (n = 91) with available DNA. Carriage of the polymorphic alleles (allele C for IL-1β -31, T for IL-1β -511, T for IL-1β +3954, 2 for ILRN) did not increase the risk of ID in *H. pylori*-infected children (Table 1).

Gastric Mucosal IL-1β mRNA in *Helicobacter pylori*-infected Children With, and Without, ID

As IL-1β is considered a potent inhibitor of gastric acid [15–18], IL-1β mRNA expression was examined in gastric biopsies of *H. pylori*-infected children (n = 28). *Helicobacter pylori*-infected children with ID (n = 4) had

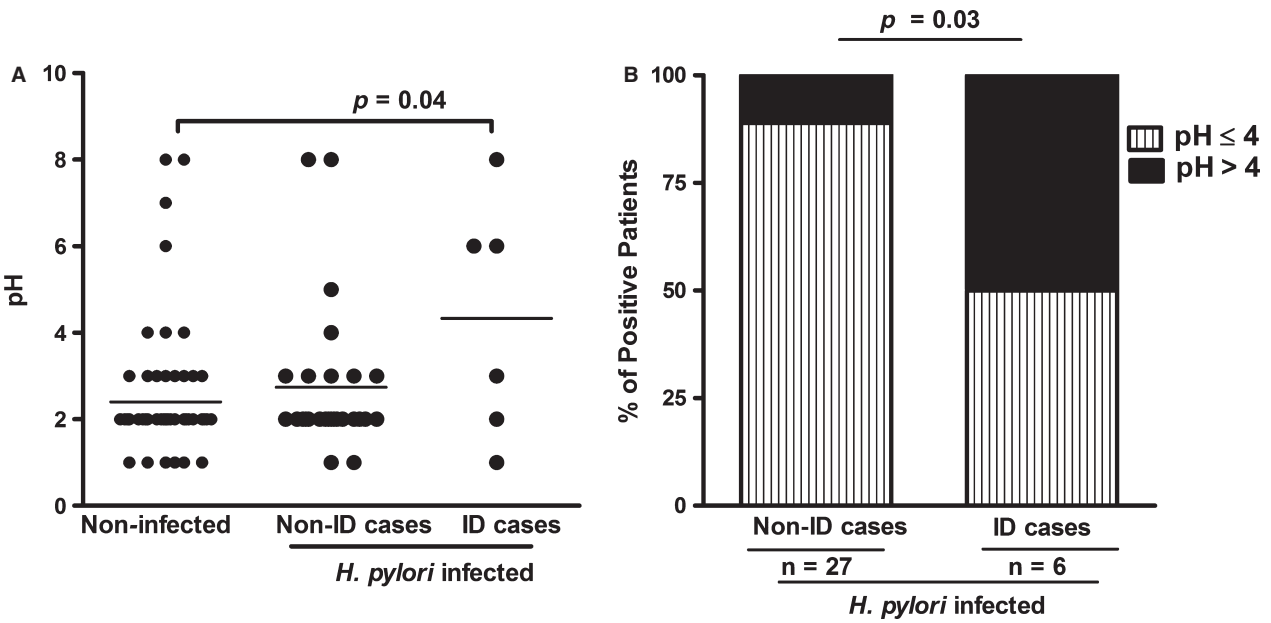

Figure 1 (*A*) Iron deficiency (ID) and gastric juice pH. Fasting gastric juice pH of *Helicobacter pylori*-infected children with, and without, ID and *H. pylori*-negative children (noninfected). (*B*) Percentage of *H. pylori*-positive children with fasting gastric juice pH >4 or <4 with, or without, ID.

Table 1 Percentage of pro-inflammatory IL-1β gene cluster polymorphisms in children

Polymorphisms	Non infected patients (n = 65)	ID Cases[a] (n = 5)	Non-ID Cases[a] (n = 24)	*p* value[b]	Odds Ratio[b]
IL-1β position -31					
T/T (Native)	17 (26)	1 (20)	3 (12.5)	.6	0.6 (0.05–6.9)
C carrier	48 (74)	4 (80)	21 (87.5)		
IL-1β position -511					
C/C (Native)	18 (28)	1 (20)	3 (12.5)	.6	0.6 (0.05–6.9)
T carrier	47 (72)	4 (80)	21 (87.5)		
IL-1β position +3954					
C/C (Native)	51 (77)	3 (60)	20 (83)	.2	3.3 (0.41–27)
T carrier	15 (23)	2 (40)	4 (17)		
IL-1RN					
Alleles (1, 3) (Native)	19 (30)	3 (60)	13 (54)	.8	0.8 (0.11–5.6)
Allele 2 carrier	44 (70)	2 (40)	11 (45)		

[a]*Helicobacter* pylori-infected children.
[b]Comparison between infected patients with and without ID.

significantly increased ($p = .01$) gastric mucosal IL-1β mRNA abundance compared to infected children without ID (Fig. 2A). In addition, there was a significant positive correlation ($p = .03$; $r = .4$, n = 28) between gastric mucosal IL-1βmRNA and gastric juice pH (Fig. 2B).

Discussion

In this study, a significant correlation between the presence of *H. pylori* infection and the presence of ID in symptomatic children has been observed. ID was not defined utilizing the traditional threshold of low level ferritin. Although this may limit the comparability with other studies, ferritin is considered an acute-phase reactant that might change in the presence of concomitant infection such as *H. pylori* [23]. Recent studies have shown in a murine *Helicobacter* infection model that initial gastric *Helicobacter* infection is associated with an increase in serum ferritin which is followed by a marked decrease in serum ferritin relative to uninfected controls [24]. In *H. pylori*-positive children included in this study, the duration of infection will be variable. To get a more comprehensive view of iron stores in the

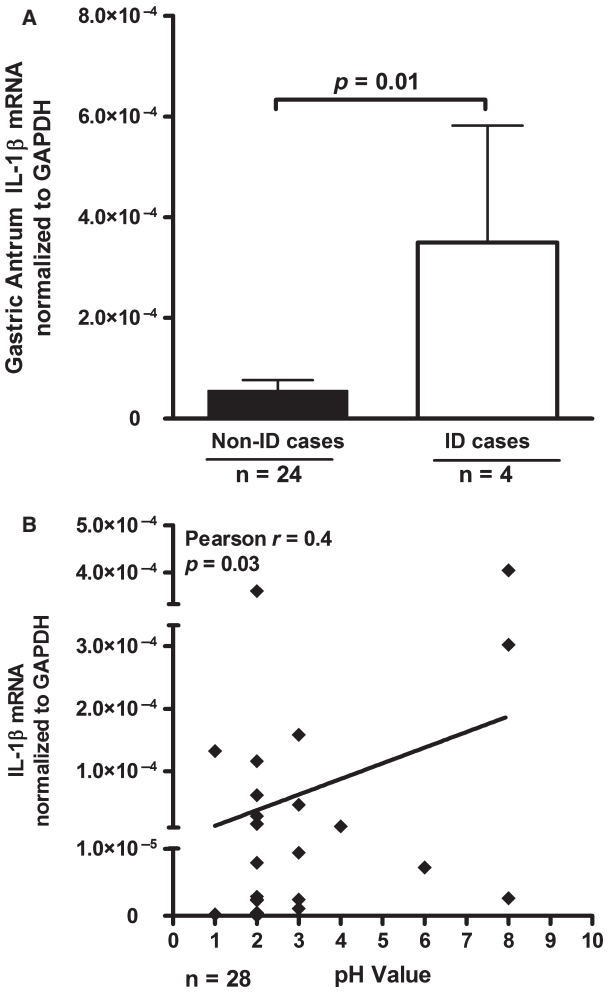

Figure 2 Gastric mucosal IL-1β mRNA is increased in iron deficiency (ID). (*A*) IL-1β mRNA abundance was analyzed by real-time RT-PCR in gastric antral biopsies of *Helicobacter pylori*-infected children with (n = 4), and without (n = 24) ID. (In two patients with ID, no gastric mRNA was available for analysis). (*B*) Relation between gastric mucosal IL1β mRNA and fasting gastric juice pH in *H. pylori*-infected children (n = 28).

body of investigated children, ID was defined as a summary of several altered serum iron parameters in relation to noninfected patients (children with no infection and normal upper GI endoscopy).

Multiple studies support the relationship between unexplained or iron refractory ID/IDA and *H. pylori* infection. Preliminary reports were based on clinical series of patients and descriptive epidemiological field studies [25–32]. Recently, meta-analysis studies of interventional *H. pylori* eradication trials have resulted in international acceptance of the evidence, and *H. pylori* eradication in children with unexplained or iron refractory ID/IDA is now recommended as a clinical guideline for most medical associations [1–5].

Bacterial as well as host factors have been implicated in the pathogenesis of *H. pylori*-associated ID/IDA, including disturbances in the gastric mucosal physiology related to iron metabolism as well as competition between the pathogen and host for limited iron stores [33]. The present study identifies that *H. pylori*-infected children that develop ID have higher gastric pH than uninfected children without ID. In addition, in *H. pylori*-infected children, those with ID have significantly greater percentage of children with fasting gastric juice ≥4 than those without ID. *H. pylori* infection-related hypochlorhydria may predispose children to ID/IDA due to the inability to form bioavailable ascorbate-complexed ferrous iron under nonacidic conditions limiting the absorption of dietary iron in the gastrointestinal tract [34]. Adults with chronic, long-standing infection develop hypochlorhydria associated with gastric atrophy after decades of infection [34], a well-known initial step in the cancer cascade promoting the development of metaplasia and dysplasia. However, the duration of hypochlorhydria in children with acute *H. pylori* infection is a transient event that can continue for several months [33]. The extent of the hypochlorhydria and its relation with age of acquisition as well as severity of the bacterial load has not been explored. However, the transient period of hypochlorhydria in acute *H. pylori* infection may explain at least partially the iron abnormalities in infected children.

Polymorphisms in pro-inflammatory cytokines have been linked to gastric cancer and corpus atrophy in *H. pylori*-infected adults [20,35]. Recent data from Latin American populations reported polymorphisms of IL-1β (−511 C/T, 14 studies; −31 T/C, 10 studies) and IL-1RN (variable number of tandem repeats, 17 studies) as the most represented ones [35]. In addition to their association with gastric cancer in adults [20], pro-inflammatory IL-1β gene cluster polymorphisms have been related to reduced hemoglobin and hematocrit in *H. pylori*-infected children [6]. In the present study, there was no relation between the IL-1β gene cluster pro-inflammatory alleles with ID. The lack of association among ID/IDA and IL-1β polymorphisms in *H. pylori*-infected children in the current study may relate to the low frequency of ID. This may reflect the accelerated improvement in health parameters in the country as the result of public health policies in the last decades, or the study's strict exclusion criteria. Hu et al. [36] described a lack of association between IL-1β polymorphisms and induced gastric acid secretion in young healthy adults regardless of *H. pylori* status. The null correlation between IL-1 cluster polymorphisms and ID in the present study may be a reflection of that characteristic. Moreover, no correlation between IL-1β

polymorphisms and mucosal IL1β mRNA in *H. pylori*-positive children was observed (data not shown).

In this study, *H. pylori*-infected children with ID have increased levels of antral mucosa IL-1β mRNA in comparison with their *H. pylori*-infected counterparts without ID. Concordantly, Queiroz et al. [20] showed an inverse correlation between gastric corpus IL-1β concentrations and blood ferritin and hemoglobin concentrations in *H. pylori*-infected children in Brazil. In addition, we showed a positive correlation between gastric mucosal IL-1β mRNA and fasting gastric juice pH. Although a relatively low number of patients were recruited for this study, the evidence presented suggests that *H. pylori* infection in children predisposes to hypochlorhydria associated with increased gastric IL-1β.

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