Infection by *Helicobacter pylori* cytotoxin-A-associated antigen-positive strains is associated with iron deficiency anemia in a longitudinal birth cohort in Brazil

Globally, anemia affects 40.0% of the child population.[1] In developing countries, anemia is a major public health problem in infants and young children. Iron is the most common cause of single-nutrient deficiency[1] due to persistent negative iron balance in association with rapid child growth that leads to high iron requirements. In parallel, in developing countries, *Helicobacter pylori* infection prevalence rates are as high as 70.0-90.0%, and the infection is mainly acquired early in childhood.[1,2] This lends considerable support to the hypothesis that *H. pylori* infection plays a role in anemia in infants / young children in low resource settings. However, the mechanism by which the infection contributes to anemia remains to be elucidated.

Gastric acidity is essential for iron absorption by the reduction and solubilisation of the non-heme iron, the main form of iron in the dietary intake, and consequently for the iron duodenal absorption.

*H. pylori cag*A that encodes the major *H. pylori* virulence factor, cytotoxin-associated gene A (CagA), is a component of the cag pathogenicity island (*cag* PAI). *cag* PAI genes are mechanistically involved in the repression of the transcription of H^+K^+a (H^+K^+ alpha-subunit) of the parietal cell H^+K^+-ATPAse leading to the hypochlorydria that impairs iron absorption.[3] Additionally, CagA is able to alter the polarity of the transferrin / transferrin receptor-iron uptake system, which allows the bacterium to shuttle iron across the epithelium.[4-6]

Notably, the *H. pylori* virulent proteins, CagA and vacuolating cytotoxin (Vac) A, may act in concert to alter the host transferrin trafficking allowing the bacterium to use the cell surface as a replicative niche,[5] which may explain why $CagA^+$ strains colonise the stomach at higher density.

Since there are no studies evaluating CagA-positive *H. pylori* infection and anemia in infants and young children, we investigated whether infection with CagA-positive *H. pylori* strains is associated with anemia in a longitudinal birth cohort of children living in a poor community in Brazil.

The prospective birth cohort study was made up of 123 children from randomly selected healthy mothers who had attended the antenatal follow-ups, living in a poor urban community in Fortaleza, Ceará, in northeastern Brazil. We evaluated the influence of *H. pylori* infection upon the risk of iron deficiency (ID) and iron deficiency anemia (IDA) in young children. Exclusions included preterm infants, low birth weight (<2500 g), severe disease that required hospitalization or chronic severe illness, among them congenital hemolytic anemia. The study was approved by the National Ethics Committee on Research of the Health Ministry of Brazil / European Union Ethics Committee.

Blood samples were obtained at 6-month intervals from six until 36/40 months of age for iron and CagA status assessment. ^{13}C-urea breath test (^{13}C-UBT) and stool antigen test were performed for *H. pylori* diagnosis.[7] Children were considered *H. pylori*-positive when both tests were positive and *H. pylori*-negative when both tests were negative. A questionnaire with demographic and clinical data (illness, antibiotic use) was administered at 3-month intervals.

CagA IgG serological status was determined by a validated immunoassay (cut-off value=7.5 units),[8] using recombinant CagA protein (kindly provided by Dr G. del Guidice, Novartis Vaccines, Siena, Italy).

Hemoglobin and hematocrit were determined in an automated electronic counter (Sysmex XT 1800i; Sysmex Corporation, Kobe, Japan). Serum concentrations of ferritin, iron and total iron binding capacity (TIBC) were assayed as previously described,[9] as well as transferrin saturation (TSat).

Data were analyzed by the public domain statistical software R version 4.0.2 (www.r-project.org)[10] and the SPSS statistical software package version 20.0 (SPSS Inc., Chicago, IL, USA). Two-tailed Student *t* test or Mann-Whitney U test, as well as χ^2-test with Yates' correction or Fisher's exact test was employed as indicated. Log-rank test was assessed to estimate the Kaplan-Meier survival. $P \leq 0.05$ was considered statistically significant.

Notably, to avoid maternal antibody interference, only children ≥11 months of age were followed at different intervals at 18, 24, 30 and 36-40 months - mean of evaluations of 4.68 times along the time, which gave us the opportunity to identify whether ID/IDA followed the acquisition of CagA-positive *H. pylori* infection. The cut-offs for diagnosing anemia was hemoglobin <105.0 g/L (children aged 6-26 months) and <115.0 g/L (children aged 24-36 months). Anemia was defined as IDA when TSat <15% or serum ferritin levels ≤7 ng/mL or serum iron levels and <40 μg/dL.

Of the 102 children (54 girls) older than 11 months who neither had other infections nor had been previously treated with iron, 30 (29.4%) had IDA and 17 (16.7%) had ID, in at least 2 evaluations; 55 (53.9%) did not have ID/IDA in all evaluations. In the group of IDA, 23 (76.7%) and 7 (23.3%) children were CagA positive and CagA-negative, respectively. A similar result was observed in the ID group (13 [76.5%] and 4 [23.5%], respectively, with no difference between groups [two-tailed Fisher test, P=1.0]). In the ID/IDA negative children, 11 (30.9%) were CagA positive and 44 (69.1%) were CagA-negative; these values became significantly different when compared with

either ID (P<0.001, two-tailed Fisher's exact test) or IDA (Odds Ratio [OR] = 13.14, 95%Confidence Interval [CI] = 4.49-38.45, P<0.001, two tailed χ^2-test).

Because the data were longitudinal, they were analyzed by regression models of generalized estimating equation (GEE).[11] To assess the fit of the models, quality indications of adjustment of the models in the adjustment phase were evaluated by the area under the ROC curve. The quality measure was ≥0.72 in all GEE models.

A logistic marginal regression was adjusted to evaluate the categorical dependent variable CagA status. CagA-positive *H. pylori* infection was significantly more frequent in children with ID/IDA than in those without anemia (Table 1).

Next, to explain the categories in function of the time and of each individual variable, other GEE models were used to identify the predictors that were independently affected by CagA. In the univariate analysis, male gender, hemoglobin, iron and ferritin values were selected for the multivariate analysis. In the initial multivariate model, predictors with P values ≤0.20 were included in the final model. Hemoglobin and iron values remained independent and negatively associated with CagA IgG seropositive status (Table 2).

To demonstrate that the association between anemia and CagA seropositive status was not due to an overlapping between CagA seropositive status, we used GEE models to evaluate *H. pylori*-positive and *H. pylori*-negative children in the group of CagA-negative children. *H. pylori* infection was not associated with anemia in this group confirming absence of overlapping between CagA and *H. pylori*-positive status. However, *H. pylori* infection was associated with age, which was expected because in childhood the prevalence of infection increases as children grow older (*Online Supplementary Table S1*).

When the hemoglobin concentration was below the reference values and the results were confirmed in the subsequent evaluation, the child was treated with oral ferrous sulphate (3 mg/kg/dose, maximum, 60 mg/dose, twice daily). Among 30 IDA children, 2 recovered from IDA after they had spontaneously cleared the infection (negative ^{13}C-UBT and stool antigen test, and CagA IgG serum reversion) and remained without *H. pylori* infection and IDA in further evaluations. Among 28 children who were treated, 23 returned to be re-evaluated. The levels of hemoglobin, iron and ferritin increased to normal values in 19 (82.61%) children. Therapeutic failure was observed in 4 (17.39%) children; 3 of them remained CagA IgG-positive until 40 months of age despite receiving iron replacement therapy. Figure 1 shows the Kaplan-Meier curves demonstrating that the time required to achieve increased ferritin and iron levels as well as IDA cure in the children infected with CagA-positive *H. pylori* strains (50% of probability of IDA cure after around 16 months) is longer (P<0.001) than that observed in the CagA-negative children (i.e., around six months).

The role of CagA in iron acquisition should be considered in the light of the strong association between anemia and CagA-positive status we observed. Because gastric acidity is essential for the reduction and solubilisation of non-heme dietary iron, reduced non-heme iron absorption may be attributable to the gastric hypochlorhydria that occurs in the early phase of *H. pylori* infection.[12-14]

We speculate that CagA may impair the activity of the endogenous alpha-subunit (H^+,$K^+\alpha$) of the gastric H^+,K^+-ATPase, the parietal cell enzyme that mediates acid secretion[3] and it increases iron uptake via transferrin endocytosis, as well as lysosomal iron thorough augmented expression of H-ferritin. Although in this study the CagA status was evaluated by serology, which would be considered one limitation, the rigorous experimental protocol and the data analysis strengthen the

Table 1. Association between iron deficiency and iron deficiency anemia and HP^+/$CagA^+$ status and HP^+/$CagA^-$ * status in young children.

Diagnosis	HP^+, $CagA^+$			HP^+, $CagA^-$		
	OR	95%CI	*P*	OR	95%CI	*P*
N	1.00	-	-	1.00	-	-
ID	6.40	2.45-16.76	<0.001	-	-	0.02*
IDA	17.42	6.99-43.38	<0.001	0.54	0.13-2.25	0.40

HP: *Helicobacter pylori;* CagA: cytotoxin-associated protein; +: positive; -: negative; ID: iron deficiency; IDA: iron deficiency anemia; N: children without ID/IDA; OR: Odds Ratio; CI: Confidence Interval. *None of the children in the ID group was *H. pylori* positive/CagA negative, incalculable OR, negative association in comparison with the control group.

Table 2. Variables associated with serum CagA IgG positive status in infants and young children with iron deficiency and iron deficiency anemia.

Univariate		Multivariate					
		Initial model			Final model		
Variable	*P*	OR	95%CI	*P*	OR	95%CI	*P*
Girl	-	1.00	-	-	-	-	-
Boy	0.08	0.57	0.29-1.12	0.10	-	-	-
Hb	<0.001	0.73	0.55-0.98	0.04	0.72	0.54-0.96	0.03
Iron	<0.001	0.68	0.53-0.88	0.003	0.65	0.50-0.86	0.002
Ferritin	0.14	0.95	0.79-1.14	0.65	-	-	-

Hb: hemoglobin; OR: Odds Ratio; CI: Confidence Interval. Data were analyzed by generalized estimating equation.

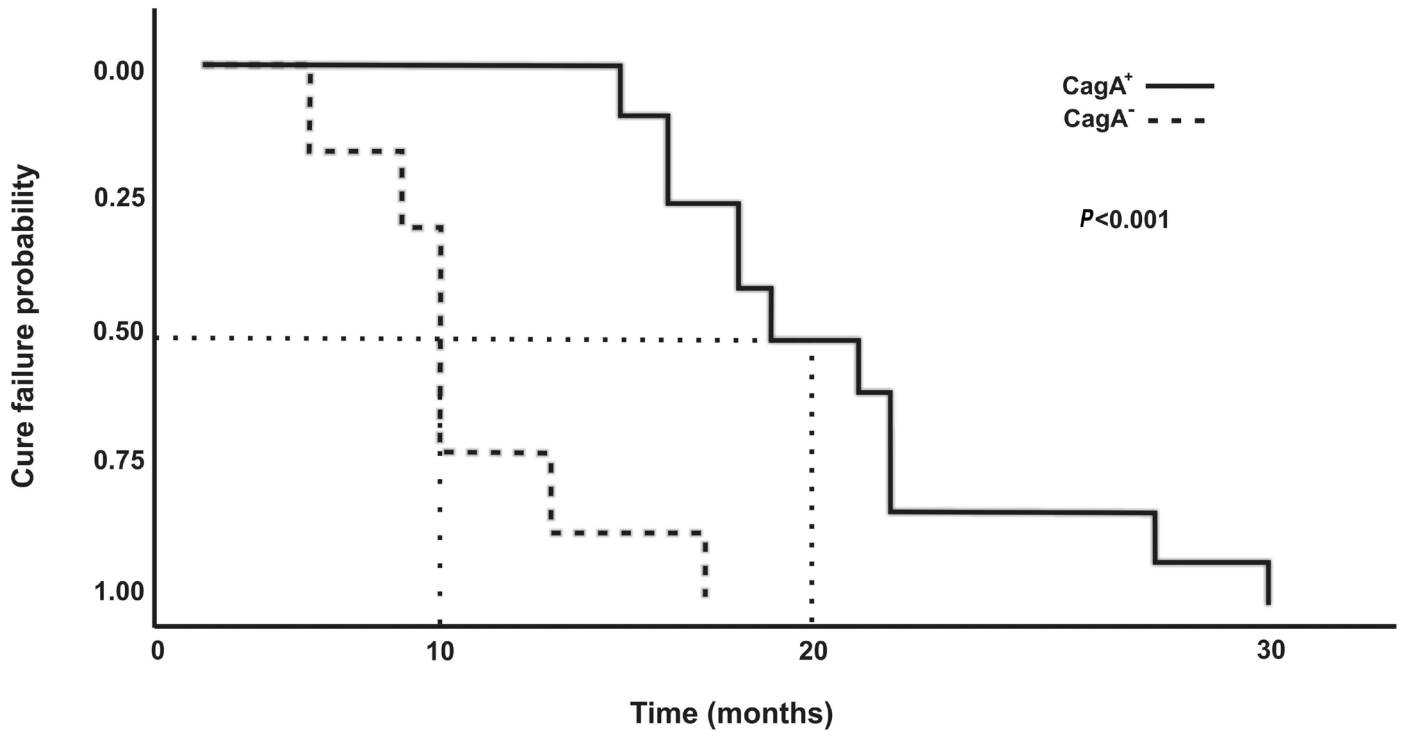

Figure 1. Estimation of iron deficiency anemia cure over time in the *H. pylori* CagA-positive children compared with CagA-negative children by Kaplan-Meier.

results. The test has been previously validated for children using a culture as a gold standard, and the agreement between ELISA and Western blotting in detecting IgG anti-CagA was 100%.[8]

To the best of our knowledge, this is the first study to demonstrate that infection with CagA positive *H. pylori* strains is an independent risk factor for IDA in infants and young children. In conclusion, young children infected with CagA-positive *H. pylori* strains are at increased risk of developing iron deficiency anemia. Further exploration of our findings towards a mechanistic understanding of the underlying pathogenesis of CagA-associated IDA is warranted.

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Disclosures

No conflicts of interest to disclose.

Contributions

RG, MS, WC, DMMQ and JEC designed the birth cohort study. DMMQ and LLBCB directed the birth cohort study in Brazil. GAR, AMCR and SAB organized and undertook ^{13}C-UBT and stool antigen tests. JEC and DMMQ are responsible for study concept and design, obtained funding, analyzed and interpreted data. DMMQ, JEC and GAR wrote the manuscript. All authors read and approved the final version of manuscript.

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Data-sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. Stevens GA, Paciorek CJ, Flores MC, et al. National, regional, and global estimates of anaemia by severity in women and children for 2000-19: a pooled analysis of population-representative data. Lancet Glob Health. 2022;10(5):e627-e639.
2. Zimmermann MB, Hurrell RF. Nutritional iron deficiency. Lancet. 2007;370(9586):511-520.
3. Saha A, Hammond CE, Beeson C, Peek RM Jr, Adam J Smolka. Helicobacter pylori represses proton pump expression and inhibits acid secretion in human gastric mucosa. Gut. 2010;59(7):874-881.
4. Tan S, Tompkins LS, Amieva MR. Helicobacter pylori usurps cell polarity to turn the cell surface into a replicative niche. PLos Pathog. 2009;5(5):e1000407.
5. Tan S, Noto JM, Romero-Gallo J, Peek RM Jr, Amieva MR. Helicobacter pylori perturbs iron trafficking in the epithelium to grow on the cell surface. PLoS Pathog. 2011;7(5):e1002050.
6. Flores SE, Aitchison A, Day AS, Keenan JI. Helicobacter pylori infection perturbs iron homeostasis in gastric epithelial cells. Plos One. 2017;12(9):e0184026.
7. Queiroz DMM, Saito M, Rocha GA, et al. Helicobacter pylori infection in infants and toddlers in South America: concordance between [13C]urea breath test and monoclonal H. pylori stool antigen test. J Clin Microbiol. 2013;51(11):3735-3740.
8. Dzierzanowska-Fangrat K, Raeiszadeh M, Dzierzanowska DM, Gladkowska-Dura M, Celinska-Cedro D, Crabtree JE. IgG subclass response to Helicobacter pylori and CagA antigens in children. Clin Exp Immunol. 2003;134(3):442-446.
9. Queiroz DM, Harris PR, Sanderson IR, et al. Iron status and Helicobacter pylori infection in symptomatic children: an international multi-centered study. PLoS One. 2013;8(7):e68833.
10. Statistical software R version 4.0.2 (www.r-project.org). Accessed March 15, 2024.
11. Liang K-Y, Zenger SL. Longitudinal data analysis using generalized linear models. Biometrika. 1986;73(1):13-22.
12. Annibale B, Capurso G, Delle Fave G. The stomach and iron deficiency anaemia: a forgotten link. Dig Liver Dis. 2003;35(4):288-295.
13. Sobala GM, Crabtree JE, Dixon MF, et al. Acute Helicobacter pylori infection: clinical features, local and systemic immune response, gastric mucosal histology, and gastric juice ascorbic acid concentrations. Gut. 1991;32(11):1415-1418.
14. Windle HJ, Kelleher D, Crabtree JE. Childhood Helicobacter pylori infection and growth impairment in developing countries: a vicious cycle? Pediatrics. 2007;119(3):e754-759.