haematologica
Journal of the Ferrata Storti Foundation

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

by Dulciene M.M. Queiroz, Lucia L.C. Braga, Gifone A. Rocha, Sergio A. Batista, Andreia M.C. Rocha, Mayuko Saito, William Checkley, Robert H. Gilman, and Jean E. Crabtree

Received: April 19, 2024.
Accepted: August 5, 2024.

Citation: Dulciene M.M. Queiroz, Lucia L.C. Braga, Gifone A. Rocha, Sergio A. Batista, Andreia M.C. Rocha, Mayuko Saito, William Checkley, Robert H. Gilman, and Jean E. Crabtree. Infection by Helicobacter pylori cytotoxin-A-associated antigen-positive strains is associated with iron deficiency anemia in a longitudinal birth cohort in Brazil. Haematologica. 2024 Aug 22. doi: 10.3324/haematol.2024.285446 [Epub ahead of print]

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

Dulciene M. M. Queiroz[1*], Lucia L. C. Braga[2], Gifone A. Rocha[1], Sérgio A. Batista[1], Andreia M. C. Rocha[1], Mayuko Saito[3], William Checkley[3,4], Robert H. Gilman[3,5], Jean E. Crabtree[6*]

[1]Laboratory of Research in Bacteriology, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil; [2]Clinical Research Unity, Department of Internal Medicine, Universidade Federal do Ceará, Fortaleza, Brazil; [3] Laboratories of Investigation and Development, Universidad Peruana Cayetano Heredia, Lima, Peru; [4]Johns Hopkins University, School of Medicine, Baltimore, USA; [5]Johns Hopkins University, Bloomberg School of Public Health, Baltimore, USA; [6]Leeds Institute for Medical Research, Molecular Gastroenterology Section, St. James's University Hospital, University of Leeds, Leeds, United Kingdom

Corresponding author:

Professor Dulciene Maria de Magalhães Queiroz.

e-mail: dqueiroz@medicina.ufmg.br

Laboratory of Research in Bacteriology

Faculdade de Medicina, Universidade Federal de Minas Gerais

Av. Alfredo Balena, 190 s/216

30130-110 – Belo Horizonte, Brazil.

Phone/FAX: +5531-3409- 9784

Keywords: *Helicobacter pylori,* CagA, Iron deficiency (ID), Iron deficiency anemia (IDA) /children, longitudinal birth cohort, *cag* Pathogenicity Island (*cag* PAI), *cag* type IV secretion system (*cag* T4SS*)*.

Funding

This work was funded by the Sixth Framework Program of the European Union, Project CONTENT (INCO-CT-2006-032136), the European Union Project INCA (LSHC-CT-2OO5-018704) and the Special Trustees St. James's University Hospital. Funds were also provided by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil

Acknowledgments

We thank Dr G. del Guidice, Novartis Vaccines, Siena, Italy, who kindly provided recombinant CagA protein.

Contributors

RG, MS, WC, DMMQ, JEC: Design of birth cohort study. DMMQ, LLBCB: Direction of birth cohort study in Brazil. GAR, AMCR, SAB: Organised and undertook ^{13}C-UBT and stool antigen tests. JEC, DMMQ: Study conception and design, obtained funding, data analysis and interpretation. DMMQ, JEC, GAR wrote the manuscript. All authors read and approved the final version of manuscript.

Disclosure

The authors declare no conflict of interest

Data base sharing: The data that support the findings of this study are available from the corresponding author, D.M.M.Q, upon reasonable request

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 being 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 the 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 the 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.

Because, 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 comprised 123 children from randomly selected healthy mothers who had attended the antenatal follow-ups, living in a poor urban community in Fortaleza, Ceará, in the northeast, 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 six-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 for were performed for *H. pylori* diagnosis.[7] Children were considered *H. pylori*-positive when both tests were positive and -negative when both tests were negative. A questionnaire with demographic and clinical data (illness, antibiotic use) was administered at three-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 analysed 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). Two-tailed Student's 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. The level of significance was set at $p \leq 0.05$.

Notably, to avoid maternal antibody interference, only children ≥11 months of age were followed at different intervals as 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 that 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 to 26 months) and <115.0 g/L (children aged 24 to 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, at least in two evaluations; 55 (53.9%) had not ID/IDA in all evaluations. In the group of IDA, 23 (76.7%) and seven (23.3%) children were CagA positive and -negative, respectively. A similar result was observed in the ID group [13 (76.5%) and four (23.5%), respectively, without difference between the 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 -negative, being significantly

different when compared with either ID ($p < 0.001$, two-tailed Fisher's exaxt test) or IDA (OR = 13.14, 95%CI = 4.49 - 38.45, $p < 0.001$, two tailed χ^2-test).

Because the data were longitudinal, they were analysed by regression models of GEE (generalized estimating equation).[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 evaluated by GEE models*, H. pylori* positive and –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 the

age, which was expected because the infection increases with increasing age in childhood (Supplemental Table 1).

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, two 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 the further evaluations. Among 28 children who were treated, 23 returned to be re-evaluated. The levels of hemoglobin, iron and ferritin increased to the normal values in 19 (82.61%) children. Therapeutic failure was observed in four (17.39%) children; three of them remained CagA IgG-positive until 40 months of age despite they were under 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 (around six months).

The role of CagA in iron acquisition should be considered in 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 the *H. pylori* infection.[12-14]

We speculate that CagA may impair the activity of the endogenous alpha-subunit (H^+,K^+α) 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 strength the 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.

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 pylor*i 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 Immuno.l 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 on 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.

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

	HP+, CagA+			HP+, $CagA^{-}$		
Diagnosis	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; N, children without ID/IDA; ID, iron deficiency; IDA, iron deficiency anemia; OR, odds ratio; CI, confidence interval, * none of 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 anaemia

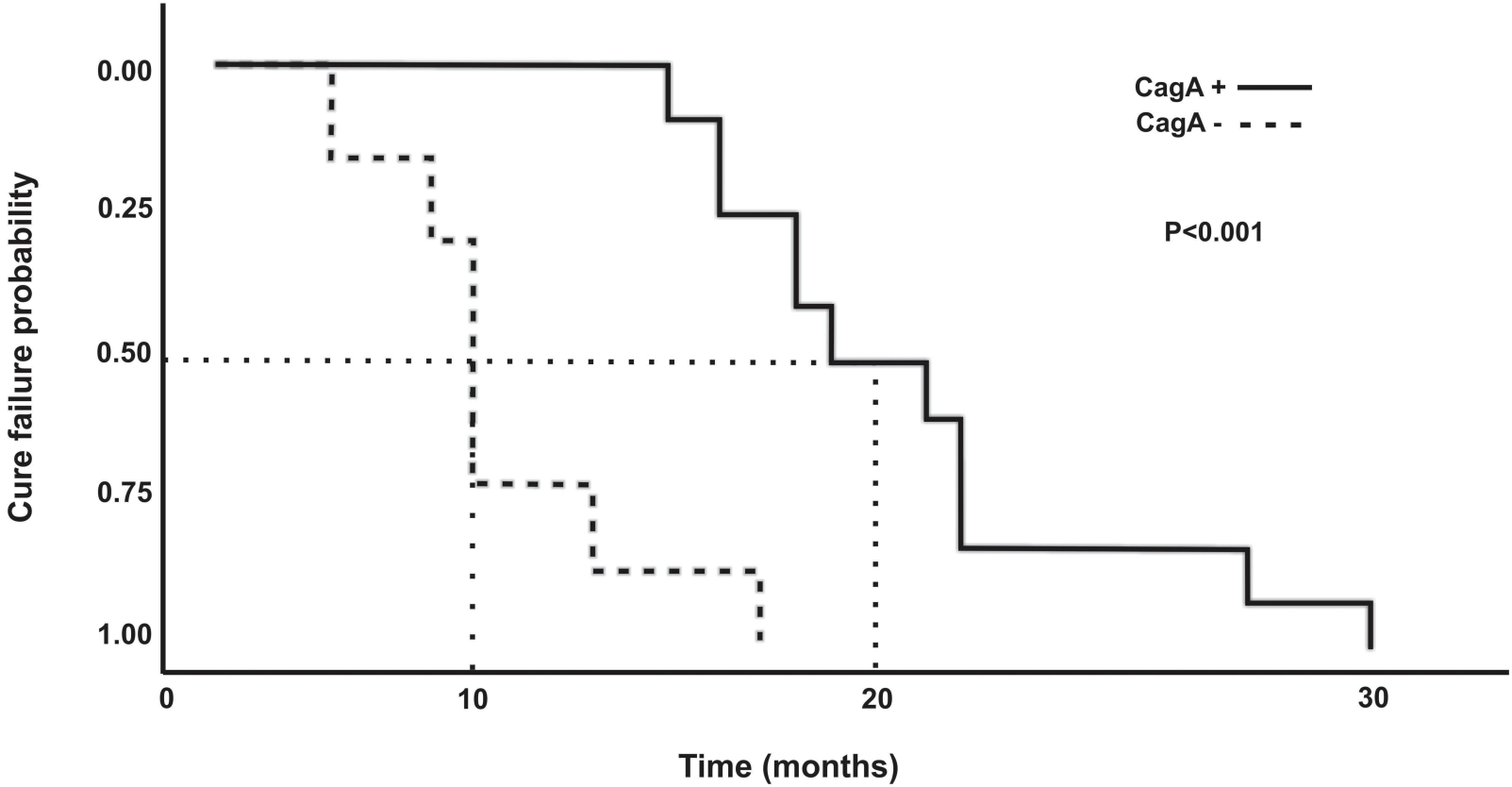
		Multivariate					
Univariate		Initial model			Final model		
Variable	p value	OR	95%CI	p value	OR	95%CI	p value
Girl		1.00	-	-	-	-	-
Boy	0.08	0.57	0.29-1.12	0.10			
Hb	<0.001	0.73	0.55-0.98	0.04	0.72	0.54-0.96	0.03
Iron	<0.001	0.68	0.53-0.88	0.003	0.65	0.50-0.86	0.002
Ferritin	0.14	0.95	0.79-1.14	0.65			

Hb, hemoglobin; OR, odds ratio; CI, confidence interval. The data were analysed by generalized estimating equation

Legend for Figure

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 method


0.00
0.25
0.50
0.75
1.00
Cure failure probability
0
10
20
30
Time (months)
CagA +
CagA -
P<0.001

Supplemental Table 1 - Variables associated with serum CagA IgG-negative/*Helicobacter pylori*-positive status in infants and young children with iron deficiency and iron deficiency anemia

		Multivariate					
Univariate		Initial model			Final model		
	p value	OR	95%CI	p value	OR	95%CI	p value
Girl		1.00	-	-	-	-	-
Boy	0.63						
Hb	0.001	1.26	0.94-1.68	0.13			
Iron	0.03	1.06	0.87-1.29	0.59			
Ferritin	0.70						
Age range I		1.00	-	-	-	-	-
II	0.008	2.17	1.20-3,90	<0.001	2.36	1.27-4.40	<0.001
III	<0.001	5.49	2.64-11.41	<0.001	6.66	3.14-14.11	<0.001
IV	<0.001	9.72	4.28-22.10	<0.001	11.94	5.05-28.24	<0.001

Hb, hemoglobin; OR; odds ratio; CI, confidence interval; group, age group; I, 12-18 month (mo.); II, 18-24 mo.; III, 24-30 mo.; IV, 30-36/40 mo. The data were analysed by generalized estimating equation.