Clinical Infectious Diseases Advance Access published April 13, 2015

MAJOR ARTICLE

Persistent and Transient *Helicobacter pylori* Infections in Early Childhood

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Background. *Helicobacter pylori*, the main cause of peptic ulcer disease and gastric cancer in adult populations, is generally acquired during the first years of life. Infection can be persistent or transient and bacterial and host factors determining persistence are largely unknown and may prove relevant for future disease.

Methods. Two cohorts of healthy Chilean infants (313 total) were evaluated every 3 months for 18–57 months to determine pathogen- and host-factors associated with persistent and transient infection.

Results. One-third had at least one positive stool ELISA by age 3, with 20% overall persistence. Persistent infections were acquired at an earlier age, associated with more household members, decreased duration of breastfeeding, and *nonsecretor* status compared to transient infections. The *cagA* positive strains were more common in persistent stools, and nearly 60% of fully characterized persistent stool samples amplified *cagA/vacAs1m1*. Persistent children were more likely to elicit a serologic immune response, and both infection groups had differential gene expression profiles, including genes associated with cancer suppression when compared to healthy controls.

Conclusions. These results indicate that persistent *H. pylori* infections acquired early in life are associated with specific host and/or strain profiles possibly associated with future disease occurrence.

Keywords. *Helicobacter pylori*; persistence; asymptomatic; virulence genes; children.

Nearly 50% of the adult population worldwide is infected with *Helicobacter pylori* (*H. pylori*) at some point during their lifetime [1, 2]. The vast majority of information on *H. pylori* infection has been derived from symptomatic populations, providing valuable information, but lacking the ability to determine the age of infection onset and to characterize pathogen characteristics and host responses to infection before the occurrence of symptoms. Only a few studies have included prospective cohorts with significant numbers of healthy children and/or with continuous monitoring for several years [3–11].

In a prospective pilot cohort study of Chilean children followed during their first 5 years with stool collection every three months, we reported that 41% had at least 1 positive *H. pylori* stool sample by enzyme-linked immunosorbent assay (ELISA) [7]. Two patterns of infection were identified in asymptomatic children: (1) *transient-* characterized by 1–3 nonconsecutive ELISA positive stool samples followed by persistently negative stool samples, and (2) *persistent-* with a minimum of 3 consecutive positive samples. Nearly 20% of asymptomatic children had a persistent infection, 90% of which were acquired during the first 2 years of life [7]. A full understanding of transient and persistent *H. pylori* infections is relevant foremost to clarify their subclinical and clinical impact throughout childhood and beyond. Genetic predisposition seems to play an important role

Received 27 November 2014; accepted 21 March 2015.

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Clinical Infectious Diseases®

DOI: 10.1093/cid/civ256

in *H. pylori* acquisition among symptomatic individuals, as fucosyltransferase 2 (FUT2) associated *secretor* status, has been reported to be more common among infected individuals [12, 13]. Whether this is the case in asymptomatic children and/or whether it plays a role in persistence is unknown. Strains containing virulence-associated genes, *cagA* and the more virulent version of *vacA* (*s1m1*), have been documented in isolated samples of asymptomatic Brazilian and Colombian children, but it is unclear if these strains are associated with persistence [14, 15].

The immune response to *H. pylori* in apparently healthy children has been shown to parallel infection [12], although whether it differs between persistent and transient infections is unknown. In addition, the differential expression of potentially relevant genes (associated with inflammation and/or cancer, among others) is also unknown. Whole blood gene expression has proven useful in assessing specific host transcriptional profiles induced by specific pathogens [13, 16].

This study is, to our knowledge, the first comprehensive evaluation of host- and strain-related factors associated with persistence as compared to transient *H. pylori* infections or lack of infection among asymptomatic children <5 years of age.

METHODS

Overall Study Design, Participants and Procedures

A fully prospective cohort of healthy 12-month-old children (±3 months) living in the city of Colina, a semirural, middle- to low-income area, were enrolled starting in April 2009, and followed every 3 months (referred to as the *1-yr-old cohort*). Where noted, we also include novel unpublished data from our previously published *birth cohort* [7], which includes 92 healthy newborns enrolled from 2006 to 2007 [7].

Well-baby visits were scheduled every 3 months. A stool sample was collected at the time of the visit or at home, in which case the parent/guardian obtained the stool sample within the 24-hour period prior to the scheduled visit and stored the sample in an ad hoc recipient in their home refrigerator. Children whose stool sample was ELISA positive for *H. pylori* submitted an additional stool sample within a 2-week window that was stored in RNA*later*. Saliva was obtained once during follow-up and a blood sample divided into a serum aliquot, and a whole blood sample collected in Tempus tubes (Applied Biosystems, California) was obtained following a first positive stool for *H. pylori*. In selected children a second and/or third blood sample was obtained. All samples were stored at −20°C until future testing. An ad hoc patient case report form was administered at every clinic visit. Upon identification of a new positive *H. pylori* child, an age-matched control, never positive for *H. pylori* was selected for a blood sample processed as described above. All children with persistent infection were given the opportunity to see a gastroenterologist.

H. pylori Detection

Stool samples were tested for *H. pylori* by HpSA ELISA (Premier Platinum HpSA, Meridian Diagnostics, Ohio), according to manufacturer's instructions. An optical density of ≥0.14 was considered positive. We tested a subset of samples by real-time polymerase chain reaction (PCR) to determine concordance with ELISA; further details are provided in the supplement.

Helicobacter pylori infection was defined as positive when a child had ≥1 ELISA positive stool, as *persistent* if ≥3 of 4 consecutive stool samples tested positive at any time point during follow-up, and as *transient* if 1–3 nonconsecutive samples tested positive followed by persistently negative samples. Age of onset of both transient and persistent infection was defined as the first positive detection. *Controls* were those who never tested positive. If a control became *H. pylori* positive he was recategorized and excluded from the control group.

H. pylori Virulence Genes

Stool samples collected in RNA*later* positive for *23S/16S rRNA* genes using real-time PCR were tested for presence of *cagA* and *vacA s/m* using PCR as described in the supplement.

Secretor Status Determination

Saliva samples were centrifuged and supernatants were processed by ELISA using monoclonal antibodies as previously described [7]. The *secretor* phenotype was defined by the presence of H, A, B, or Lewis b antigens and *nonsecretor* phenotype was defined as the absence of these antigens, or if only Lewis a antigen was detected.

Secretor phenotype of a subset of samples was confirmed by FUT2 genotyping as described in the supplement.

Determination of Serum Anti- *H. pylori* Antibodies

Serum samples were tested for anti-*H. pylori* immunoglobulin G (IgG) antibodies by Premier *H. pylori* enzyme immunosorbent assay (EIA; Meridian Diagnostics, Ohio). Values ≥0.07 were considered seropositive.

Gene Expression Profiles in Blood

Whole blood RNA was extracted and hybridized into Illumina Human HT12 V4 beadchips and scanned on the Illumina Beadstation 500. Microarray analyses were performed according to Mejias et al [13] in a subset of samples from children with persistent and transient *H. pylori* infection and healthy control children matched for age, gender, and ethnicity. Study personnel collecting clinical information and bioinformaticians analyzing the data were blinded to the transcriptional and clinical data.

Statistical Analysis

Subjects providing ≥6 stool samples (18 months of follow-up) were included for analysis. Statistical differences by *H. pylori*

detection status were tested using Pearson χ^2 test for categorical variables, or Fisher exact test, and independent sample t-test for continuous variables. Sample size calculations described in supplement were performed in Epi Info 7; statistical analysis was performed in R Version 3.0.0 [17].

For microarray data the Illumina GenomeStudio software was used to subtract background and scale average samples' signal intensity and, GeneSpring GX 7.3 software was used to perform further normalization and analyses (for details see supplement). The expression of each individual gene is normalized to the median expression of that individual gene in all of the healthy controls.

RESULTS

Study Cohorts' Characteristics

A total of 302 children were enrolled in our *1-year-old cohort*, of which 228 children completed 6 follow-up visits (reasons for drop-out in supplement). Seven children had an indeterminate *H. pylori* detection status and were excluded, leaving a final total of 221 evaluable children. The median number of visits completed was 7 (interquartile range [IQR]: 6–8), and the median age of last follow-up was 33 months (IQR: 30–39). Within this cohort 74 (33%) children had at least 1 stool sample test positive for *H. pylori*. Among the 74 children who tested positive for *H. pylori*, 49 (22% overall) had persistent and 25 (11%) transient infections.

For the *birth cohort* the median number of visits completed was 17 (IQR: 7.8–19.3), and the median age of last follow-up was 60 months (IQR: 29.3–72). Nineteen (21%) had persistent and 18 (20%) transient infections.

Of 579 samples positive by ELISA, a subset of 138 samples was analyzed by real-time PCR for the presence of *23S* and/or *16S rRNA*. Compared to real-time PCR, ELISA was highly concordant; 88% of persistent and 86% of the transient samples positive by ELISA also tested positive for *23S* or *16S* (P = NS). We also tested 113 ELISA negative samples, of which 27 (33%) were real-time PCR positive. For further details on confirmation of ELISA results by real-time PCR, as well as an analysis of differences in optical density between persistent and transitory samples, please see the supplement.

Persistent infections began significantly earlier, median age: 20.5 months (IQR: 15–24), than transient episodes, median age: 27 months (IQR: 17.5–38) (P = .001) (Figure 1). Only one persistently positive subject reverted to negative (column 19 in Figure 1).

Active reporting of gastrointestinal symptoms (vomiting, stomach pain, and abdominal distension) at any time point occurred in 24% of controls, 35% of persistent, and 28% of transient infections (P = NS; symptomatology details are summarized in Supplementary Note 1). Clinical and pathological findings were detected in 1/49 (2%) persistently infected children and 0/25 transient children by the gastroenterologist (for subject descriptions see Supplementary Note 2).

Host Factors Associated with *H. pylori* Infection

The only significant difference between controls and infected children (transient and persistent) was co-sleeping with a parent or sibling (Table 1). Persistent children were more likely than transient children to not attend daycare at 18 months of age, to live in a household with a greater number of inhabitants,

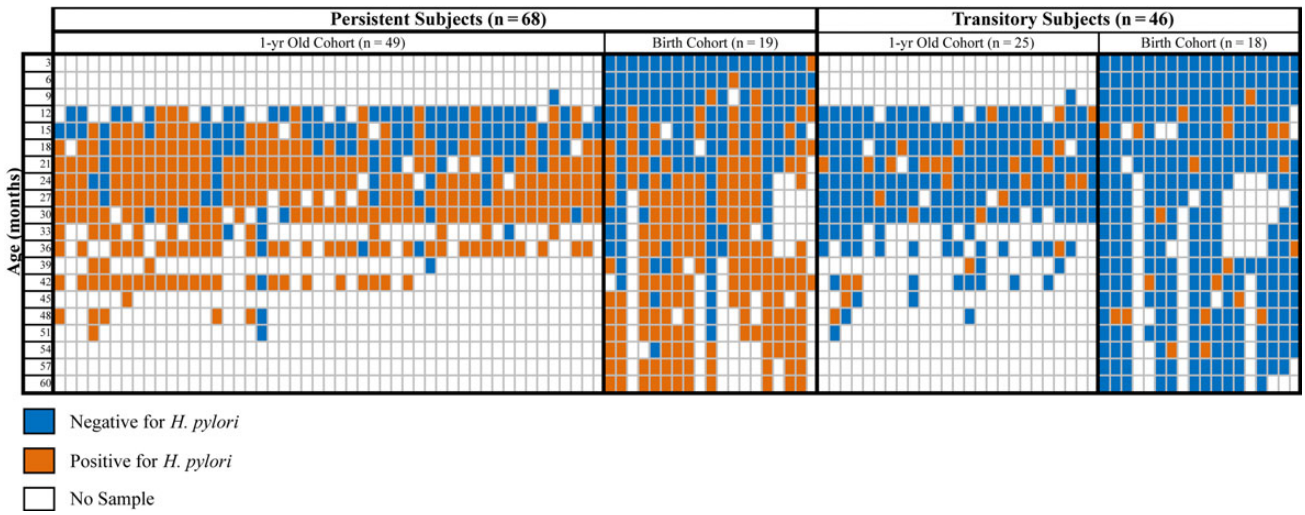

Figure 1. Dynamics of *Helicobacter pylori* infection for 68 children with persistent and 46 with transient infection. Each column represents 1 child, age in months increases down the vertical axis. Blue indicates a negative sample, orange positive, and white either no sample or the subject has not yet reached that month of age.

Table 1. Patient Characteristics by *Helicobacter pylori* Detection Status for the 1-yr-old Cohort, n = 221

Characteristic	Presence of Characteristic by *H. pylori* Detection Status (n/obtained (%))[a]			*P* Value[b]		
	Control 147 (67)	Persistent 49 (22)	Transient 25 (11)	Infected vs Control[c]	Persistent vs Transient	Persistent vs All Others
Male	82/147 (56)	26/49 (53)	16/26 (64)	NS	NS	NS
Solid housing construction	113/145 (78)	39/49 (80)	16/26 (64)	NS	NS	NS
Number of people living in household at 18 mo, median (IQR)	4.5 (4–6) n = 136	5 (4–6) n = 45	4 (4–5) n = 23	NS	.036	NS
Co-sleeping at 18 mo	72/138 (52)	30/45 (67)	16/23 (70)	.035	NS	NS
Mother completed high school	58/86 (67)	27/34 (79)	10/15 (67)	NS	NS	NS
Receiving breastmilk at 18 mo	43/141 (31)	12/46 (26)	13/23 (57)	NS	.013	NS
Attending daycare at 18 mo	110/139 (79)	32/46 (70)	21/21 (100)	NS	.004	.070
Nonsecretor[d]	32/132 (24)	19/49 (39)	4/24 (17)	NS	.056	.031
Age of first positive sample, median (IQR)[e]	. . .	20 (17–24) n = 45	26 (20–30) n = 25	. . .	.036	. . .

Statistical differences by *H. pylori* detection status were tested using Pearson's Chi-squared test for categorical variables and an independent t-test for the continuous variable *age of first positive sample*.

Abbreviations: IQR, interquartile range; NS, not significant.

[a] Except where otherwise noted.

[b] Only values <0.1 are listed.

[c] Infected includes both transient and persistent subjects.

[d] Secretor phenotype was confirmed by *FUT2* genotyping in 87 children. All *nonsecretor* individuals were homozygotes AA at position 428. There were no homozygotes TT at position 385.

[e] 4 persistent subject were positive at the time of 1st sample, therefore their month of 1st positive sample is unknown.

to not be breastfeeding at 18 months of age, and to have been infected at a younger age. Persistent children were more likely than transient and noninfected controls combined to be *nonsecretors*.

These results from this new cohort confirm and expand our previous findings [7], as the trends for the variables "number of people living with the child" and "whether the child received breastmilk at 18 months of age" observed in the birth cohort became significant in the *1-yr-old cohort*.

Combining both cohorts, the number of times antibiotics were administered per year tended to be higher, but was not significant, in persistent (median: 0.5, range 0–3.5, $P = .074$) compared to transient children (median: 0.09, range 0–3.5).

H. pylori Virulence Genes Associated with Persistence

Persistent children were more likely to be infected with a *cagA* positive strain of *H. pylori* than transient children (84% vs 48%, $P = .002$) with a trend toward higher detection of *vacA s1* (66% vs 20%, $P = .067$) (Table 2). No difference was observed for carriage of *vacA m1/m2*. In 34 children we fully characterized all three genes, all but one of which had persistent infection. Overall the most prevalent genotype for persistent infection episodes was *cagA* positive *vacA s1m1* (19/33, 58% of fully characterized samples; details in Supplementary Table 3).

Of 14 persistent children with either two or three fully characterized samples over a 4–36 month period: 5 children changed from *cagA* positive to *cagA* negative, 3 changed in *vacA s*, one in *vacA m*, and 7/14 children (50%) maintained the same genotypes (Supplementary Table 4).

Host Response to *H. pylori* Infection

Persistent children were more commonly positive for *H. pylori* IgG than controls prior to first detection ($P = .014$; Table 3);

Table 2. *H. pylori* Virulence Genes Amplified in Stools of Persistent and Transient Children

Virulence Genes	Detection of Indicated Gene by *H. pylori* Detection Status (n/tested (%))		*P* Value[a]
	Persistent 49 (22)	Transient 25 (11)	
cagA positive	37/44 (84)	10/21 (48)	.002
vacA s1[b]	29/44 (66)	1/5 (20)	.067
vacA s2[b]	23/44 (52)	4/5 (80)	NS
vacA m1[c]	35/40 (88)	5/5 (100)	NS
vacA m2[c]	8/40 (20)	0/5 (0)	NS

Statistical differences by *H. pylori* detection status were tested using Pearson χ^2 test.

Abbreviation: NS, not significant.

[a] Only values <0.1 are listed.

[b] 7 persistent subjects were positive for both *s1* and *s2* in distinct samples.

[c] 3 persistent subjects were positive for both *m1* and *m2* in distinct samples.

Table 3. Seropositivity to *H. pylori* Before and After a First Stool Sample Positive by ELISA in Persistent and Transient Subject and Age-matched, Non-infected Controls

	Seropositivity at Indicated Time			*P* Value[a]		
Timing and Age of Serum Sample	Control	Persistent	Transient	Persistent vs Control[c]	Transient vs Control	Persistent vs Transient
Before first positive stool sample, n/tested (%)	1/26 (4)	5/16 (31)	1/12 (8)	.014	NS	NS
Child age at time of serum sample, median (IQR)	16 (13–22)	20 (16–23)	23 (18–36)	NS	NS	NS
1 to 6 mo after first positive stool sample, n/tested (%)	3/16 (19)	10/17 (59)	3/12 (25)	.019	NS	.071
Child age at time of serum sample, median (IQR)	20 (17–26)	24 (22–29)	31 (26–36)	NS	.078	NS
>6 mo after first positive stool sample, n/tested (%)	2/19 (11)	17/22 (77)	6/17 (35)	<.001	.074	.008
Child age at time of serum sample, median (IQR)	40 (27–47)	40 (28–45)	40 (30–58)	NS	NS	NS

Abbreviations: ELISA, enzyme-linked immunosorbent assay; IQR, interquartile range; NS, not significant.

[a] Only values <0.1 are listed.

however, 2/5 samples were obtained during the previous 3-month period possibly after infection onset. Seropositivity was more common in persistent vs control children 1–6 months after ($P = .019$) and >6 months after ($P < .001$) first *H. pylori* detection. No significant differences were observed in transient vs control children. Seropositivity was more common in persistent compared to transient children >6 months after their first stool positive sample ($P = .008$). These seropositivity differences were not due to age differences.

Gene expression profiles obtained from 53 children (9 controls, 22 transient, and 22 persistent) identified 108 differentially expressed genes between children who had persistent and/or transient infections and *H. pylori* negative controls (Figure 2). Cluster analysis identified 61 genes by function and/or disease, of which 10 potentially relevant genes, composed of 2 clusters, presented the highest scores by *Davis Tools* and *Ingenuity Systems*. The first cluster with an enrichment score of 1.44 included the following genes related to regulation and cell cycle:

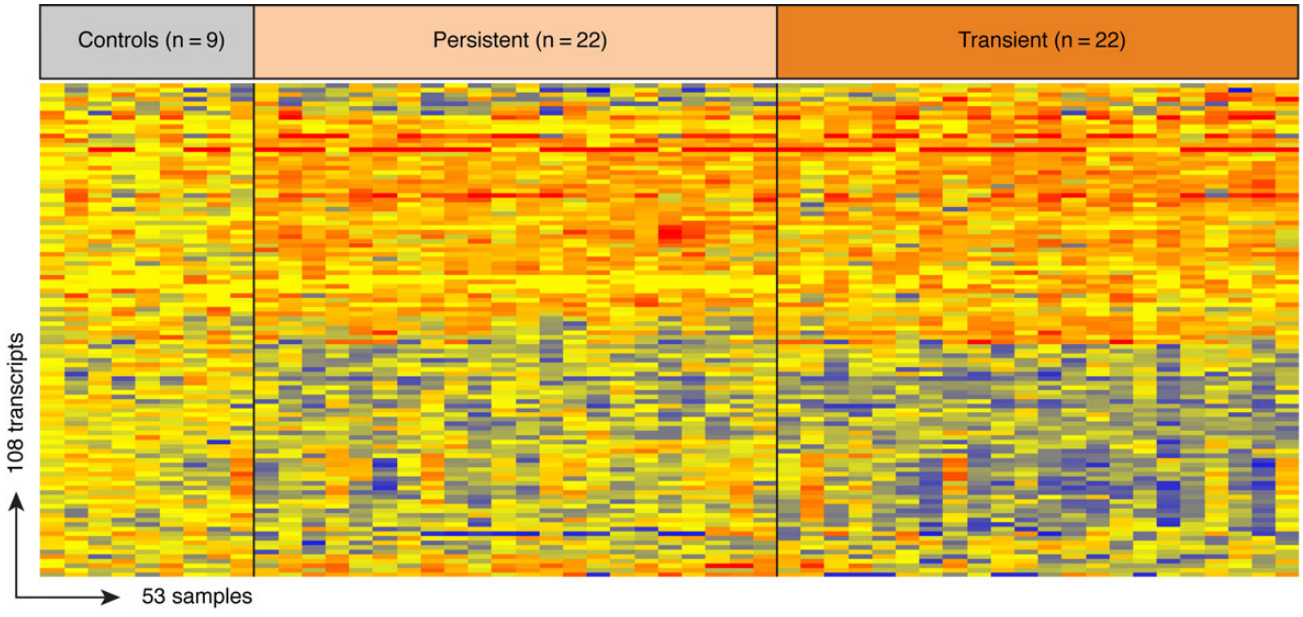

Figure 2. Gene expression profiles in children with persistent and transient *H. pylori* infection compared to healthy controls. The expression of each individual gene is normalized to the median expression of that individual gene in all of the healthy controls. Statistical group comparisons between children (Kruskal–Wallis $P < .05$) yielded 108 significantly differentially expressed transcripts. Transcripts were organized by hierarchical clustering, where each row represents a single transcript and each column an individual participant. Normalized expression levels are indicated as overexpressed (red) or underexpressed (blue) compared to the median expression.

(1) chromatin licensing and DNA replication factor 1 (CDT1); (2) nucleolar and spindle associated protein 1 (NUSAP 1); (3) transforming growth factor, alpha; (4) BTG family, member 3. The second cluster with an enrichment score of 1.28 included the following genes related to apoptosis and programmed cell death: (5) T-cell lymphoma invasion and metastasis 1 (TIAM 1); (6) chromosome 3 open reading frame 38 (C3ORF38); (7) granzyme H (cathepsin G-like 2, protein h-CCPX); (8) homeodomain interacting protein kinase 2 (HIPK2); (9) interferon, gamma; and (10) solute carrier family 5 (iodide transporter), member 8 (SLC5A8).

DISCUSSION

The comprehensive approach undertaken in this study provides new findings related to *H. pylori*-host interactions occurring in early childhood. We demonstrated that nearly one-third of children were infected, as determined by at least 1 positive stool ELISA, that nearly 20% had a persistent *H. pylori* infection, that transient infections are not due to increased antibiotic use, and that persistent but not transient infections are mostly associated with significant seroresponses. We conclusively show that these persistent infections are acquired during the first 5 years of life, mostly within the first 2 years.

Persistent infections were associated with indirect evidence of early familial exposure and lower exposure to breastfeeding, as previously reported [18]. However, a novel finding was the increased risk for persistence in *nonsecretors*, replicated in both of our cohorts. In vitro, animal models and human studies have demonstrated the binding of *H. pylori* to Le^b antigens through the BabA adhesin [19–23], which has been associated with more severe lesions, duodenal ulcer, and gastric adenocarcinoma, in *H. pylori* infected patients [24]. However, fucosylated antigens can also be secreted in the mucous layer and expressed by the death of epithelial cells [25], possibly acting as decoy receptors and protecting against *H. pylori* infection. *Nonsecretor* individuals may be more susceptible to *H. pylori* attachment through other "Le^b-independent" adhesins, such as SabA, AlpA/B, OipA, and HopZ among others [26, 27]. Cederberg et al reported a positive association between *nonsecretor* status, *H. pylori* infection, and active peptic ulcer disease in young men [28]. Our results strongly suggest that in apparently healthy children, persistence of *H. pylori* may not be strongly related to BabA- Le^b interactions, and that other adhesins attaching to non- Le^b receptors may be playing a more relevant role, opening additional avenues of research.

Helicobacter pylori strains harboring recognized virulence genes, as detected by PCR amplification in stool in our asymptomatic population, is also consistent with other reports [13, 15]. This study further adds that stool amplification of these genes, specifically *cagA* and *vacA s1*, was associated with persistence, opening several avenues of future research aimed at pinpointing the subgroup that will develop disease later in life, and that could thus potentially benefit from preemptive treatment and/or preventive strategies such as vaccines [28]. Potential vaccine strategies aimed at preventing infection with "aggressive" *H. pylori* strains will need to consider that most persistent infections begin within the first 2 years of life. Importantly, although virulence traits tended to vary over time, in nearly half of a subset of longitudinally followed children, the other half maintained *cagA* and *vacA* associated traits from 4 to 34 months of follow-up, possibly representing a subgroup at higher risk for disease [28]. Future studies should include other relevant genes possibly associated with persistence, including adhesins [26–28].

A previous study concluded that most *H. pylori* infections in children were associated with an immune response [12]. We demonstrate that most transient infections are not paralleled by an immune response while most persistent infections are. When comparing gene expression profiles this study showed that *H. pylori* infection (persistent and transient) displayed differential expression of several genes compared to noninfected controls. Four genes are related to the cell cycle (TGFA, CDT1, NUSAP1, BTG3) and 6 related to apoptosis and programed cell death (C3ORF38, GZMH, TIAM 1, IFNG, HIPK2, SLC5A8), which will need confirmation in future studies. Future targeting of these genes, in both symptomatic and asymptomatic *H. pylori* infected individuals, will allow us to confirm or discard whether they are over- or underexpressed; such results would provide a molecular basis for disease development at older ages; such studies are currently underway in our laboratory.

Active questioning identified sporadic gastrointestinal symptoms in 24%–35% of children, with no difference between infected and noninfected children, suggesting that most symptoms were not related to *H. pylori*. A more in-depth evaluation by a gastroenterologist of persistently infected children identified one child with *H. pylori* associated disease. Thus, although uncommon, consequences of early infection can be detected before 5 years of age. Our current data do not support referral of asymptomatic children with persistent infection to a gastroenterologist during the first 5 years of life. This may change in the future if a significant proportion of these persistently infected children develop disease at older ages.

This study has limitations. Nearly 25% of enrolled children were not eligible for analysis for several reasons, mostly due to drop-out. We do not foresee higher or lower infection rates in this nonanalyzed population that could affect our conclusions. The significance of the 32% real-time PCR positive/ELISA negative samples is unclear. Premier ELISA has shown to be the most accurate test in adults and children with *H. pylori* disease compared to the "gold standard" detection in gastric biopsy tests [29, 30]. A real-time PCR positive/ELISA negative sample may be a false positive, a transitory infection with low bacterial load, or some other unknown condition. Few studies

have compared real-time PCR amplification with the "gold standard" (histopathology and/or rapid urea test); one study detected PCR positivity in 2/8 (25%) "gold standard" negative samples [31], whereas, to our knowledge, studies comparing real-time PCR with ELISA are nonexistent. The role of PCR detection vs ELISA has been an issue for other enteric pathogens as well, for example, rotavirus, where ELISA has become the gold standard as it has been associated more clearly with a true infection when compared to real-time PCR [32]. Thus, these results are interesting and suggest that possibly some additional children within our cohort may be *H. pylori* infected despite being ELISA negative, but these infections would most probably be of low viral load. Amplification of *H. pylori* "virulence genes" in stools proved to be difficult and the number of isolates fully characterized is lower than initially estimated, reducing the power for comparisons. Higher amplification rates in persistent children may be due to higher bacterial loads compared to transient children, which could be a bias favoring detection of virulence genes. In addition, *H. pylori* detection by ELISA and characterization by gene amplification in stools is most probably a partial reflection of what may be occurring at the gastric level and thus should be interpreted with caution. Several events, including degradation of nucleic acids, can occur during the passage of bacteria from the stomach to stool. Strain diversity, most probably an important factor in *H. pylori* pathogenesis, can be hinted at by amplification of several genes in one stool sample but cannot be characterized in detail as in biopsy specimens [33, 34]. We also recognize that an important number of factors previously associated with *H. pylori* persistence, including proteins associated with reactive oxygen species detoxification, DNA repair proteins and over 60 predicted adhesins, among others, were not studied here [34]. Nevertheless, the strength and uniqueness of this study comes from the inclusion of well-characterized, prospectively followed children, who provided biological samples for pathogen and host characterization. A more prolonged follow-up focused on older children is required in order to fully understand the clinical significance of *H. pylori* persistence acquired in early childhood.

In conclusion, persistent *H. pylori* infections are acquired early during childhood and are associated with pathogen and host-related factors and responses that may prove relevant for future development of ulcer disease or cancer, opening several research avenues for prevention, and/or selective preemptive treatment strategies.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We are indebted to the children of Colina and their parents for participating in this study. We thank the Director of the Centro de Salud Familiar (CESFAM) Colina, Carolla Ponce Madariaga, the Director of the CESFAM Esmeralda, Claudia Barra, and the medical director of the area of Colina, Jessica Pradenias Toro, for their permanent support of this study. We especially thank our research nurse Elizabeth Ceron for her extraordinary work with the families allowing adhesion to the study protocol throughout the years and Hector Cortes for administrative support. Lastly we thank Dr Francisca Blanco, Department of Plant Biotechnology, Universidad Andrés Bello for lending us equipment for real-time polymerase chain reaction analysis.

Financial support. This work was supported by the Fondo Nacional de Desarrollo Científico y Tecnológico [1061079, 1100514 and 1130561 to M. L. O. R.].

Potential conflicts of interest. All authors: No potential conflicts of interest.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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