

Vacuolating Cytotoxin Genotypes Are Strong Markers of Gastric Cancer and Duodenal Ulcer-Associated *Helicobacter pylori* Strains: a Matched Case-Control Study

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The *Helicobacter pylori* virulence gene, *cagA*, and active forms of the vacuolating cytotoxin gene, *vacA*, are major determinants of pathogenesis. However, previous studies linking these factors to disease risk have often included patients using aspirin/non-steroidal anti-inflammatory agents (NSAIDs) or acid-suppressing drugs, both of which may confound results. Also, particularly for gastric cancer (GC), controls have often been of quite different ages. Here, we performed a careful study in a “clean” Belgian population with gastric cancer cases age and sex matched to 4 controls and with a parallel duodenal ulcer (DU) group. As in other populations, there was a close association between the presence of *cagA* and the *vacA* s1 genotype. For GC, associations were found for *vacA* s1-positive ($P = 0.01$, odds ratio [OR], 9.37; 95% confidence interval [CI], 1.16 to 201.89), i1-positive ($P = 0.003$; OR, 12.08; 95% CI, 1.50 to 259.64), and *cagA*-positive status ($P < 0.05$; OR, infinity; 95% CI, 0.76 to infinity). For DU, associations were found with *vacA* s1 ($P = 0.002$; OR, 6.04; 95% CI, 1.52 to 27.87) and i1 ($P = 0.004$; OR, 4.35; 95% CI, 1.36 to 14.78) status but not with *cagA* status. Neither condition showed independent associations with the *vacA* m1 allele or with more biologically active forms of *cagA* with longer 3' variable regions. In this Belgian population, the best markers of gastric cancer- and duodenal ulcer-associated strains are the *vacA* s1 and i1 genotypes. This fits with experimental data showing that the s and i regions are the key determinants of vacuolating cytotoxin activity.

There are about 0.9 million new cases of gastric adenocarcinoma each year, and of these, at least 75% of cases are attributable to *Helicobacter pylori* (1). *H. pylori* is also the leading worldwide cause of peptic ulceration. However, only a small proportion of infected people develop disease, and the disease outcome is influenced by a combination of host, bacterial, and environmental factors (2, 3). Treating whole populations with complex antibiotic regimens against *H. pylori* is potentially problematic. The best approach, either at an individual level or to guide the public health strategy in populations, is to identify who is at risk of *H. pylori*-induced disease and treat these individuals selectively.

A large number of studies have assessed the excess risk of disease conferred by specific *H. pylori* virulence factors (4–7). However, several factors render these studies inaccurate. For example, many patients undergoing endoscopy are taking, or have taken, acid-suppressing drugs such as proton pump inhibitors (8). These people may carry pathogenic strains but will wrongly be assumed to have benign strains, because no ulcer is found. On the other hand, patients taking aspirin or other nonsteroidal anti-inflammatory agents (NSAIDs) may have an ulcer due to these drugs, although they may be carrying nonpathogenic strains. Also, careful studies of cancer patients are relatively uncommon as it is sometimes difficult to culture strains from these patients (9). Finally, most studies do not match the cases and controls for age and gender. Although the best studies correct for these factors in multivariate analysis (10), risk estimation is inaccurate when the ages of disease and control populations vary markedly. Therefore, to accurately estimate the excess disease risk from specific virulence factors, a careful case-control study was needed in “clean” populations not taking acid-suppressing medications or aspirin or NSAIDs. For the estimation of risk, a clean population with no

biases is much more useful than a large population with systematic biases.

The *Helicobacter pylori* vacuolating cytotoxin (VacA) is a difficult virulence factor to study as nearly all strains express a form of VacA, but only some forms are toxigenic and pathogenic. VacA and its gene *vacA* have been the subjects of extensive research, and it is known that several regions of *vacA* are important for VacA toxicity. The *vacA* signal region encodes the N terminus of the toxin and may be active (type s1) or encode an N-terminal extension which blocks activity (s2) (11). The *vacA* mid region may bind to a wide range of cells, causing toxicity (type m1), or to a smaller range (type m2) (12). Rhead and colleagues described a novel determinant of VacA toxicity, called the intermediate (i) region (13). They showed that two allelic variants of this region (i1 and i2) exist. Using exchange mutagenesis, they showed that this novel region determines toxic activity, and most importantly, they

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showed a significant correlation between the i1 region and gastric cancer (GC) in an Iranian population (13). A study from Iraq found that *vacA* i1 strains were significantly associated with gastric ulcers (14). Basso et al. confirmed disease associations in an Italian population and reported a significant association between the i1 type and duodenal ulcers (DU) (10).

Many studies have also assessed the excess risk of disease conferred by the *cag* pathogenicity island (*cagPAI*) and its translocated effector protein CagA (10, 15, 16). *H. pylori* strains may possess or lack CagA and the *cagPAI* and may also have different forms of CagA: those with longer 3' variable regions due to possession of two or more EPIYA C segments have been shown to be more closely associated with disease (10, 17–19). However, to add an additional complication, CagA and the active forms of VacA often are found together in strains (16, 20). Not only do these bacterial factors coexist in certain *H. pylori* strains, but they also modulate each other's effects on target cells (21, 22). For instance, by suppressing the activity of the growth factor receptors epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2)/Neu, VacA inhibits CagA-mediated cell elongation (22). Thus, it is difficult to determine whether these virulence factors are independently important in conferring disease risk.

The aim of this project was to study the size of the excess gastric cancer risk conferred by *H. pylori* strains with specific *vacA* genotypes, with *cagA*, or with the more active "long" form of *cagA*. To achieve this, we planned a careful *H. pylori* genotyping study on strains from a clean Belgian population not taking disease-modifying drugs, and we collected GC cases and age- and gender-matched controls. We also studied whether there was an excess risk of duodenal ulceration using a parallel unmatched DU group.

MATERIALS AND METHODS

Population characteristics. A very well-defined and clinically and endoscopically characterized Belgian population was assessed in this study. In total, we studied *H. pylori* isolates from 129 Belgian patients, with positive cultures for *H. pylori*. Genomic DNA was extracted from all clinical *H. pylori* isolates. We had isolates from 19 patients with GC (male/female ratio, 0.82; mean age, 67.7 years; age range, 23 to 89 years) and a control group of 72 patients with nonulcer dyspepsia (NUD), who were matched for ethnicity, age, and gender. The control group did not have a present gastric or duodenal ulcer or a history of gastric or duodenal ulcer or a family history of gastric or duodenal ulcer or gastric cancer in their first-degree relatives and did not have atrophy or intestinal metaplasia in biopsy samples from the antrum and corpus (male/female ratio, 0.78; mean age, 67 years; age range, 23 to 89 years). Also included were 38 unmatched patients with DU (male/female ratio, 0.71; mean age, 53 years; age range, 25 to 81 years). All cases and controls included in this study were recruited from the period of 1990 through 2005, and none had received treatment with NSAIDs, proton pump inhibitors, or antibiotics within 4 months prior to diagnosis. The study protocol was approved by the hospital ethics and research committees of the Nottingham and Brugmann University hospitals, and all patients gave informed consent for the study.

Genotyping. PCR-based typing of the virulence genes was performed using genomic DNA extracted from multiple colony sweeps. The amplification of *vacA* signal (s), intermediate (i), and mid (m) region types was performed using primer sets as described previously (13, 23). To determine the *cagA* status and the size of the *cagA* 3' variable region, previously described primers *cag2* and *cag4* and primers *cagA28F* and *cagA-P3E*, respectively, were used (24, 25).

Histopathology. Additional biopsy specimens taken from the antrum and corpus of these patients were used to evaluate histopathological

TABLE 1 *vacA* allelic types of *H. pylori* strains isolated from the Belgian patients and their association with the *cagA* status and the size of the *cagA* 3' variable region^a

<i>vacA</i> type	No. of <i>H. pylori</i> strains	No. (%) of <i>cagA</i> ⁺ strains	No. (%) of <i>cagA</i> ⁺ strains with long 3' variable regions
s1/i1/m1	52	49 (94)	15 (31)
s1/i1/m2	26	24 (92)	7 (29)
s1/i2/m2	6	6 (100)	3 (50)
s2/i2/m2	29	15 (52)	2 (13)
Total	113	94 (83)	27 (29)

^a Of the eight possible combinations of signal (s), intermediate (i), and mid (m) regions, only these four were found. The presence of *cagA* was closely associated with the s1-type of *vacA*. *vacA* s2/i2/m2 types were less likely to be *cagA* positive than strains with any other *vacA* structure. Similarly, the *cagA*-positive strains with long *cagA* 3' variable region possessing two or more EPIYA C segments were more likely to have an s1-type *vacA* allele than an s2.

changes, including inflammation and activity. All slides were prepared and interpreted by the same pathologist according to the updated Sydney System scoring (26).

Statistical analysis. The association between *H. pylori* strain genotypes and disease was analyzed using Fisher's exact test. The association between genotypes and histopathological parameters was assessed with Minitab 14 software using the Mann-Whitney U test. In all cases, a significant difference was taken to exist when *P* was ≤ 0.05 .

RESULTS

Our first aim was to perform virulence typing on all the Belgian isolates and to assess whether the *H. pylori* strains were similar to those found in other populations in terms of *vacA* and *cagA* typing. We determined the *vacA* signal (s), intermediate (i), and mid (m) region types, the *cagA* status, and the size of the *cagA* 3' variable region by PCR of the genomic DNA extracted from multiple colony sweeps. A total of 16 isolates (3, 6, and 7 for GC, DU, and NUD, respectively) gave PCR results implying multiple *H. pylori* strain infections (that is, were positive for *vacA* of types s1 and s2, m1 and m2, or i1 and i2) and were excluded from further analysis. The remaining isolates contained one virulence genotype, and these are referred to henceforth as "strains."

First, we examined what combinations of *vacA* regions existed in our sampled Belgian population. In accordance with our previous work, we found that strains had 1 of 4 combinations of *vacA* regions: s1/i1/m1 (52 strains), s1/i1/m2 (26 strains), s1/i2/m2 (6 strains), and s2/i2/m2 (29 strains) (Table 1). The other 4 possible combinations (s1/i2/m1, s2/i1/m1, s2/i1/m2, and s2/i2/m1) were not found. Next, we examined whether our strains possessed the virulence gene *cagA*, and if so, whether this was associated with the s1 form of *vacA*, as previously described. Of the 113 strains studied, 94 (83%) were *cagA* positive. As expected, most (79/94, 84%) of these possessed the s1 form of *vacA*, whereas of the 19 *cagA* negative strains only 5 (26%) contained *vacA* s1 ($P < 0.0001$) (Table 1). The *vacA* i1 and m1 types were also associated with *cagA*-positive status, but numerically the association was less strong (Table 1). Finally, we examined how many *cagA*-positive strains possessed the more active form of *cagA* with a long 3' variable region, consistent with possessing two or more EPIYA C motifs. We found this in only 27 (29%) of the 94 *cagA*-positive strains. Of these, 25 (93%) had s1 *vacA*, a proportion similar to

TABLE 2 Association between *H. pylori vacA* s, i, and m regions and disease outcome^a

Disease status	No. of <i>H. pylori</i> strains	No. (%) of <i>H. pylori</i> strains possessing:		
		<i>vacA</i> s1	No. (%) of <i>vacA</i> i1	<i>vacA</i> m1
NUD	65	40 (62)	36 (55)	23 (35)
GC	16	15 (94) ^b	15 (94) ^c	9 (56)
DU	32	29 (91) ^c	27 (84) ^c	20 (63) ^b
Total	113	84 (74)	78 (69)	52 (46)

^a The number and percentage of *H. pylori* strains possessing *vacA* s1, i1, and m1 alleles in patients with each disease state are shown. The duodenal ulcer (DU) and gastric cancer (GC) groups were compared with a nonulcer dyspepsia (NUD) group.

^b $P < 0.05$.

^c $P < 0.005$.

that found in *cagA*-positive strains which did not have a long 3' variable region (54/67, 81%).

Our main aim was to use our gastric cancer-associated strains and our age- and sex-matched controls to assess accurately the association between different virulence factors and gastric cancer (GC). We were interested specifically in the *vacA* allelic types, *cagA*, and the size of the *cagA* 3' variable region. In a univariate analysis, GC was significantly associated with the presence of the *vacA* s1 allele ($P = 0.01$; odds ratio [OR], 9.37; 95% confidence interval [CI], 1.16 to 201.89) and the i1 allele ($P = 0.003$; OR, 12.08; 95% CI, 1.50 to 259.64) but not the m1 allele ($P = 0.10$; OR, 2.34; 95% CI, 0.68 to 8.21) (Table 2). There was also a significant association with *cagA*-positive status ($P < 0.05$; OR, infinity; 95% CI, 0.76 to infinity) but not with the long *cagA* 3' variable region, consistent with two or more EPIYA C motifs ($P = 0.18$; OR, 0.42; 95% CI, 0.08 to 1.19) (Table 3).

Next we wanted to assess which *vacA* allelic types were the most important in terms of their association with GC. Since all 16 GC strains were concordant for *vacA* s and i regions and only 4 of the 65 NUD strains were discordant (Table 4), it was not possible to assess the independent importance of these two regions in this Belgian population. However, we were able to examine whether the s1/i1 strains were associated with GC independent of m region status, by comparing the disease associations of s1/i1/m2 and s2/i2/m2 strains. Of the GC patients, 6/7 (86%) had an s1/i1 strain compared to only 13/38 (34%) NUD patients ($P < 0.02$). We did a similar analysis to assess whether the m region type was impor-

TABLE 3 Association between *H. pylori cagA*, the size of the *cagA* 3' variable region, and disease outcome^a

Disease status	No. of <i>H. pylori</i> strains	No. (%) of <i>cagA</i> ⁺ strains	No. (%) of <i>cagA</i> ⁺ strains with long 3' variable region
NUD	65	51 (78)	18 (35)
GC	16	16 (100) ^b	3 (19)
DU	32	27 (84)	6 (22)
Total	113	94 (83)	27 (29)

^a The number and percentage of *H. pylori cagA*-positive strains and the size of *cagA* 3' variable region in patients with each disease state are shown. The duodenal ulcer (DU) and gastric cancer (GC) groups were compared with a nonulcer dyspepsia (NUD) group.

^b $P < 0.05$.

TABLE 4 Full *vacA* allelic types of *H. pylori* strains in patients with each disease state^a

Disease status	No. of <i>H. pylori</i> strains	No. of allelic types:			
		s1/i1/m1	s1/i1/m2	s1/i2/m2	s2/i2/m2
NUD	65	23	13	4	25
GC	16	9	6	0	1
DU	32	20	7	2	3

^a Patients were categorized according to their disease states including duodenal ulcer (DU), gastric cancer (GC), and nonulcer dyspepsia (NUD).

tant for otherwise toxic (s1/i1) strains. Among *vacA* s1/i1 strains, 9/15 (60%) GC strains were also m1 compared with 23/36 (64%) NUD strains ($P =$ not significant [NS]). In summary, as suggested to be likely by experimental data on *in vitro* toxicity, *vacA* s1 and i1 regions appear to be important markers for GC-associated strains: we did not have enough discordant cases here to show which (if either) is more important, although the experimental data show that both are needed for vacuolating activity *in vitro*. Our analyses showed no independent GC association for the m1 mid region.

Next, we assessed the association between the various *vacA* allelic types, the *cagA* status, the long *cagA* 3' variable region, and DU. DU patients were not matched with controls for age and gender in our study design, making this analysis less robust. Nevertheless, disease associations were similar to those found for gastric cancer, giving good biological plausibility to both sets of results. In a univariate analysis, DU was significantly associated with the presence of the *vacA* s1 allele ($P = 0.002$; OR, 6.04; 95% CI, 1.52 to 27.87), i1 allele ($P = 0.004$; OR, 4.35; 95% CI, 1.36 to 14.78), and m1 allele ($P = 0.01$; OR, 3.04; 95% CI, 1.16 to 8.07) (Table 2) but not with the *cagA* status ($P = 0.34$; OR, 1.48; 95% CI, 0.43 to 5.32) or with the long *cagA* 3' variable region ($P = 0.18$; OR, 0.52; 95% CI, 0.15 to 1.7) (Table 3).

Next, we wanted to assess which *vacA* allelic types were most important in terms of their association with DU. Unfortunately, as for GC, the close concordance of the *vacA* s1 and i1 types made it impossible to assess the relative merits of the s and i regions as markers of DU-associated strains in this population. However, it was possible to test whether the s1/i1 strains were associated with DU independent of the m region status by comparing the disease associations of s1/i1/m2 and s2/i2/m2 strains. When analysis was confined to these strains, 7/10 (70%) ulcer patients had s1/i1 strains compared to only 13/38 (34%) NUD patients ($P < 0.05$) (Table 4). We did a similar analysis to assess whether the m region type was important for otherwise toxic (s1/i1) strains. Among the *vacA* s1/i1 strains, 20/27 (74%) DU strains were also m1 compared with 23/36 (64%) NUD strains ($P =$ NS), implying that the *vacA* s1 and i1 regions (but not the m1 region) are important markers of DU-associated strains in this population.

To investigate the association between *H. pylori* virulence genes and histopathological parameters, all patients with GC, DU, and NUD (excluding those infected with multiple *H. pylori* strains) were considered according to the presence of their *vacA* allelic types, their *cagA* statuses, and the sizes of their 3' variable region. The analysis was performed for 99 of 113 patients since full histopathology data were not available for all cases. *H. pylori* mononuclear cell infiltration (inflammation) and neutrophil infiltration (activity) in the antrum and corpus were assessed and graded according to the updated Sydney System scoring. Al-

TABLE 5 *H. pylori* virulence genes and histopathological parameters^a

<i>H. pylori</i> virulence genotype	No. of <i>H. pylori</i> strains	Histological parameters (mean [median]) in:			
		Antrum		Corpus	
		Inflammation	Activity	Inflammation	Activity
<i>vacAs1</i>	77	2.33 (2)	1.84 (2)	1.41 (1)	1.00 (1)
<i>vacAs2</i>	22	2.13 (2)	1.72 (2)	1.22 (1)	0.72 (1)
<i>vacAi1</i>	71	2.34 (2)	1.81 (2)	1.14 (1)	1.01 (1)
<i>vacAi2</i>	28	2.17 (2)	1.82 (2)	1.32 (1)	0.82 (1)
<i>vacAm1</i>	47	2.27 (2)	1.83 (2)	1.53 (1)	1.11 (1)
<i>vacAm2</i>	52	2.30 (2)	1.79 (2)	1.26 (1)	0.79 (1)
<i>cagA</i> positive	84	2.28 (2)	1.81 (2)	1.43 (1)	0.98 (1)
<i>cagA</i> negative	15	2.33 (2)	2.00 (2)	1.20 (1)	0.73 (1)
Short <i>cagA</i>	60	2.25 (2)	1.77 (2)	1.33 (1)	0.87 (1)
Long <i>cagA</i>	24	2.29 (2)	1.87 (2)	1.58 (1)	1.17 (1)

^a *H. pylori* mononuclear cell infiltration (inflammation) and neutrophil infiltration (activity) in antrum and corpus are shown.

though the mean and median values of inflammation and activity in both the antrum and the corpus were found to be numerically higher in patients infected with *H. pylori vacA* s1-type strains, these associations were not statistically significant (Table 5). Similarly, no significant correlation was found between the other *vacA* allelic types, the *cagA* status, or the 3' variable region size and histology (Table 5).

DISCUSSION

Studies to determine any association between the presence of several *H. pylori* virulence genes and clinical outcome have been contradictory, and several authors have reported that these virulence markers were not useful predictors of disease-associated *H. pylori* strains (27, 28). While the relationship of the *vacA* genotype, the *cagA* status, and the 3' variable region size with clinical outcomes and histopathological changes has been studied in the past, it has proven difficult to obtain completely clean populations, not recently taking acid-suppressing drugs or aspirin/NSAIDs. Also, most populations vary markedly between patients (particularly gastric cancer patients) and controls in gender and particularly age. Although these factors can be corrected for in multivariate analysis, large differences make the numerical estimation of risk unreliable. Careful matching removes this problem. The features of our study should give an unbiased and accurate estimation of the contribution of strain virulence to gastric cancer risk.

In agreement with previous studies, our study confirmed that all strains with s1/m1 *vacA* alleles were type i1 and all s2/m2 alleles were type i2, whereas s1/m2 alleles were either i1 or i2. The occurrence of s1/i1/m1, s1/i1/m2, s1/i2/m2, and s2/i2/m2 *vacA* alleles but not alleles with other combinations of s, i, and m regions would superficially appear likely because of recombination between s1/i1/m1 and s2/i2/m2 alleles with a single breakpoint. However, this does not explain why all observed s2 strains are also i2 and m2 nor why we and others have occasionally found strains with other allelic structures such as s1/i2/m1, s2/i1/m2, or s2/i2/m1 (29). We therefore suggest an alternative theory, whereby the "common" *vacA* alleles encode a protein which, whether strongly toxic or not *in vitro*, benefits the strain. In contrast, we suggest that other *vacA* structures are selected against if they arise through recombination.

In the Belgian population, the *vacA* s1 and i1 alleles, but not the

m1 alleles, were significantly associated with GC as previously reported in Italy and Iran (10, 13). The *vacA* s1, i1, and m1 regions were significantly associated with DU, confirming the previously published data from Italy, but the within-group analysis showed that the m1 association was not independent. Using exchange mutagenesis, previous studies have shown that the s1 region is permissive for toxin activity and that the s2 region blocks activity, rendering VacA nontoxic (30). Among the *vacA* s1 strains, Rhead et al. showed that the i1 strains are vacuolating, whereas the naturally occurring i2 strains or natural i1 strains in which the i1 region is exchanged for an i2 region are nonvacuolating (13). Unfortunately, the Belgian strains studied here were mostly concordant for the s and i regions so we were not able to assess whether they had independent associations with disease (although, of course, the tight association also meant that this question was not relevant in this Belgian population). As previously published experimental data on *in vitro* toxicity show that both an s1 and an i1 region are important for toxicity, we think it likely that both will be important markers for disease-associated strains. In this Belgian population, the *vacA* i region was found numerically to be a slightly better disease discriminator than the s region, although this difference was not significant. The *vacA* m1 genotype, which enables the toxin to bind to a broader range of cells *in vitro*, was not found to be independently associated with disease. Larger studies in other populations are required to determine whether the m region typing of strains in any population provides additive information as a marker of *H. pylori* disease-associated risk.

In addition to the *vacA* s and i regions, *cagA*⁺ strains were also significantly associated with GC, but not with DU, although since 83% of strains in this population were *cagA*⁺, showing an association was always likely to be difficult. Since a close concordance was observed between the presence of *cagA* and *vacA* s1/i1, it was not possible to assess which is more important in determining disease outcome. However, *vacA* genotypes are clearly an important marker of DU- and GC-associated *H. pylori* strains, and in this Belgian population, they appear to be a better marker than the *cagA* status. In this population, possession of a long *cagA* with multiple tyrosine phosphorylation (activation) sites did not predict disease risk.

Our study also allowed us to assess the association between gastric pathology and the more virulent *H. pylori* strains. Both the

s1 type *vacA* allele and the *cagA*⁺ strains with more than one EPIYA C motif were found to have numerically increased lymphocyte infiltration (inflammation) and neutrophil infiltration (activity) in the antrum and corpus. However, this association failed to reach statistical significance, perhaps due to the insufficient sample size. Numerically slightly increased inflammation in the antrum and activity in the corpus were also observed for the *vacA* i1-type strains, but as for the s1 and *cagA* strains with a long *cagA*, a significant association was not found. This again may be a type II error, although even if this is the case, our study implies that any “real” differences would be likely to be small. Another possibility is that our results are correct and that mechanisms other than an inflammatory process underlie the disease risk. This would appear feasible for VacA, which is thought to have a directly toxic rather than a proinflammatory effect.

In conclusion, our data have several important implications. First, the *vacA* genotypes are clearly an important marker of GC- and DU-associated *H. pylori* strains. In this Belgian population they are a better marker than the *cagA* status or the size of the *cagA* 3' variable region. Second, the s1 and i1 genotypes, which confer vacuolating toxicity to *vacA*, appear most relevant. In this population at least, the m1 genotype is of no demonstrated pathogenic importance. In other populations, for example, populations containing more *cagA*-negative strains, the balance in importance between *vacA* and *cagA* may be different, and it is likely that both are important virulence determinants. Indeed, it is likely that there are multiple interactions between the virulence factors, host immune responses, and environmental factors that influence clinical outcomes.

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We declare no conflicts of interest.

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