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ORIGINAL ARTICLE

Helicobacter pylori's virulence and infection persistence define pre-eclampsia complicated by fetal growth retardation

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Abstract

AIM: To better understand the pathogenic role of *Helicobacter pylori* (*H. pylori*) in pre-eclampsia (PE), and whether it is associated or not with fetal growth retardation (FGR).

METHODS: Maternal blood samples were collected from 62 consecutive pregnant women with a diagnosis of PE and/or FGR, and from 49 women with uneventful pregnancies (controls). Serum samples were evaluated by immunoblot assay for presence of specific antibodies against *H. pylori* antigens [virulence: cytotoxin-associated antigen A (CagA); ureases; heat shock protein B; flagellin A; persistence: vacuolating cytotoxin A (VacA)]. Maternal complete blood count and liver enzymes levels were assessed at delivery by an automated analyzer.

RESULTS: A significantly higher percentage of *H. pylori* seropositive women were found among PE cases (85.7%) compared to controls (42.9%, $P < 0.001$). There were no differences between pregnancies complicated by FGR without maternal hypertension (46.2%) and controls. Importantly, persistent and virulent infections (VacA/CagA seropositive patients, intermediate leukocyte blood count and aspartate aminotransferase levels) were exclusively associated with pre-eclampsia complicated by FGR, while virulent but acute infections (CagA positive/VacA negative patients, highest leukocyte blood count and aspartate aminotransferase levels) specifically correlated with PE without FGR.

CONCLUSION: Our data strongly indicate that persistent and virulent *H. pylori* infections cause or contribute to PE complicated by FGR, but not to PE without feto-placental compromise.

Key words: *Helicobacter pylori*; Virulence factors; Pre-eclampsia; Fetal growth retardation; Cytotoxin-associated antigen A; Vacuolating cytotoxin A

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INTRODUCTION

Pre-eclampsia (PE) is a severe hypertensive pregnancy-re-

lated disorder that affects 5%-8% of women worldwide, thus representing the main cause of feto-maternal mortality and morbidity[1,2]. PE is often associated with fetal growth retardation (FGR), defined as failure of the fetus to achieve its genetically determined growth potential[3,4]. FGR is commonly considered a severe complication of PE, but whether or not PE and FGR are manifestations of the same disorder, or two distinct pathologies, still remains unclear.

PE is characterized by excessive maternal inflammatory response, with high circulating levels of pro-inflammatory cytokines and endothelial injury[1,2]. Despite being an object of intense investigation, the etiopathogenetic mechanisms of PE are still poorly understood. Several lines of evidence suggest that subclinical infections could play a role in the onset of PE[5,6].

We previously reported a strong association between *Helicobacter pylori* (*H. pylori*) infection and PE[7]. *H. pylori* is a Gram-negative bacterium responsible for the large majority of peptic ulcers, gastric cancer, and gastric mucosa-associated lymphoid tissue lymphoma[8]. It has been demonstrated that this pathogen enhances platelets activation and thrombus formation[9,10], thus inducing endothelial inflammation and injury. Therefore, *H. pylori* could directly cause or intensify the generalized inflammation and endothelial dysfunction typical of PE[7]. Furthermore, it was recently observed that *H. pylori* seropositive PE subjects are characterized by a more severe inflammatory status[11] and lipid peroxidation[12].

The role of cytotoxin-associated antigen A (CagA) in inducing a severe immunogenic response in patients infected by *H. pylori* is now well established[13]. Nevertheless, other virulence factors could be involved in the severe inflammatory response mediated by this bacterium. The vacuolating cytotoxin A (VacA) is a protein produced by *H. pylori* with several effects on vulnerable cells, such as vacuolation with alteration of the endo-lysosomal function and mitochondrial damage accompanied by cytochrome C release and apoptosis[14].

Ureases allow colonization of the gastric mucosa by catalyzing the hydrolysis of urea and help to recruit neutrophils and monocytes in the mucosa, thus inducing pro-inflammatory cytokines production[15].

Heat shock protein B (HspB) has been shown to increase the risk of gastric carcinoma, by directly inducing hyper-proliferation of gastric cells[16]. Moreover, it strongly activates the immune system and stimulates a massive immune response in patients with gastritis and gastric cancer[17-19].

To better understand the pathogenic role of *H. pylori* in pre-eclampsia, we investigated maternal serum positivity for antibodies against CagA, VacA, HspB, ureases A, C, E and H (UreA, UreC, UreE, UreH), and for flagellin A (FlagA). FlagA is the major *H. pylori* flagellin isoform, mainly expressed during late exponential growth phase and represents a good *H. pylori* virulence index[20].

To correlate *H. pylori* virulence with PE severity, and to detect differences in *H. pylori* profiles between PE and FGR pregnancies, we determined seropositivity for the above mentioned antigens in three populations: PE without FGR, PE complicated by FGR, and FGR without PE.

Finally, we verified the reported association between *H. pylori* infection and elevated leukocyte blood count and serum amino-transferases levels[21].

MATERIALS AND METHODS

Population and samples

The study was approved by our Hospital Ethics Committee "Comitato Etico Interaziendale AA.OO O.I.R.M./S.Anna di Torino and Ordine Mauriziano di Torino" and written informed consent was obtained from each participating woman.

Maternal blood samples (5 mL) were collected before delivery from 62 consecutive pregnant women with diagnosis of PE and/or FGR, and from 49 women with normotensive pregnancies with normal fetal growth and normal uterine and umbilical Doppler flow velocimetry (FVW).

PE was diagnosed when hypertension (systolic blood pressure $\geqslant$ 140 mmHg or diastolic blood pressure $\geqslant$ 90 mmHg) and proteinuria ($\geqslant$ 300 mg/24 h) appeared after 20 wk of gestational age in previously normotensive women, according to the American College of Obstetricians and Gynecologists criteria[22]. PE was considered severe when one or more of the following criteria were present: systolic pressure $\geqslant$ 160 mmHg or diastolic pressure $\geqslant$ 110 mmHg on two occasions at least 6 h apart, or significant proteinuria ($\geqslant$ 3 + on urine dipstick or > 5 g in a 24-h urine)[22]. Patients with PE were further classified as either having early-onset ($\geqslant$ 34 wk), or late-onset (> 34 wk) disease according to the gestational age of PE diagnosis.

The hemolysis-elevated liver enzymes-low platelets (HELLP) syndrome was defined by the following criteria: hemolysis (characteristic peripheral blood smear and serum lactate dehydrogenase $\geqslant$ 600 U/L), elevated liver enzymes (serum aspartate aminotransferase $\geqslant$ 70 U/L), and low platelet count (< 100 000/μL)[23].

The diagnosis of FGR was made according to the following criteria: ultrasound measurement of fetal abdominal circumference below the 10th centile[24] or growth velocity below the 10th percentile[25] and/or birth weight below the 10th centile, according to Italian reference values[26] with abnormal umbilical arteries Doppler FVWs[27] and/or abnormal uterine artery Doppler FVWs (resistance index of > 0.58)[28]. Exclusion criteria were: multiple pregnancies, congenital malformations, and prenatal or postnatal diagnosis of chromosomal anomalies in number and/or structure.

For all cases and controls, the following data were collected: maternal age at delivery, gestational age at birth, week of PE onset, mode of delivery, neonatal sex, birth weight, placental weight, parity, blood pressure, urinary protein, complete blood count and differentials (count and percentage of neutrophils, lymphocytes, monocytes, eosinophils, and basophils), liver enzymes levels, risk factors for PE (previous pregnancy with PE, autoim-

Table 1 Clinical characteristics of study populations (continuous variables)

Variable	Controls (n = 49) Median (25th-75th)	PE-only (n = 17) Median (25th-75th)	PE FGR (n = 32) Median (25th-75th)	FGR-only (n = 13) Median (25th-75th)	P value[1]
Maternal age at delivery (yr)	30 (28-33)[4]	29 (24-32)	30 (26-34)[7]	25 (24-26)[4,7]	[4]< 0.001; [7]0.002
Gestational age at delivery (wk)	40 (39-41)[2,3,4]	30 (28-31)[2,5,6]	32 (31-34)[3,5]	34 (32-39)[4,6]	[2,3,4]< 0.001; [5]0.045; [6]0.009
Neonatal weight (g)	3380 (3170-3700)[2,3,4]	1140 (1045-1570)[2]	1278 (920-1668)[3]	1600 (1060-2730)[4]	[2,3,4]< 0.001
Placental weight (g)	600 (500-650)[2,3,4]	300 (240-410)[2]	280 (215-360)[3]	345 (300-470)[4]	[2,3,4]< 0.001
Systolic blood pressure (mmHg)	120 (110-120)[2,3]	160 (150-160)[2,6]	150 (148-160)[3,7]	120 (120-125)[6,7]	[2,3,6,7]< 0.001
Diastolic blood pressure (mmHg)	75 (70-80)[2,3]	100 (100-100)[2,6]	100 (95-105)[3,7]	77 (75-80) [6,7]	[2,3,6,7]< 0.001
Proteinuria (g/24 h)	0 (0-0)[2,3]	2.21 (1.52-3)[2,6]	1.34 (0.79-2.38)[3,7]	0 (0-0)[6,7]	[2,3,6,7]< 0.001

[1]P values were calculated by non-parametric Kruskal-Wallis H test, with post-hoc analysis by Mann-Whitney U test. [2]Comparison between controls and PE-only group; [3]Comparison between Controls and PE FGR group; [4]Comparison between controls and FGR-only group; [5]Comparison between PE-only and PE FGR groups; [6]Comparison between PE-only and FGR-only groups; [7]Comparison between PE-only and FGR-only groups. PE: Pre-eclampsia; FGR: Fetal growth retardation.

mune diseases, diabetes, cardiovascular diseases, or other common risk factors for PE), and family history of pre-eclampsia and/or cardiovascular diseases.

Venous blood samples were collected into Vacutainer tubes (Becton Dickinson, Plymouth, United Kingdom) without anticoagulant. Serum was separated by centrifugation immediately after clotting and stored at -30 ℃ until assayed.

Serology

Serum samples were evaluated for specific antibodies against *H. pylori* antigens by commercially available Heli-Blot assay (Nurex; Sassari, Italy). *H. pylori* seropositivity was determined according to manufacturer instructions. Briefly, diluted serum samples (1:100) were incubated with Heli-Blot strips for 30 min. The strips were then incubated consecutively with anti-IgG for 30 min, with substrate for range of minutes, and then dried. Results were read according to the standard control protein bands provided. The standard *H. pylori* antigens available in the strip included: 120 kDa (CagA), 89 kDa (VacA), 60 kDa (Urease C), 54 kDa (HSP), 35 kDa (Flagellin), 30 kDa (Urease H), 26 kDa (Urease A), and 19 kDa (Urease E). The presence of one of the three most specific antigens (CagA, VacA, or flagellin) or the presence of two of the three smallest antigens was considered a positive test for the diagnosis of *H. pylori* infection.

Statistical analysis

Data analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, Illinois, United States). Continuous variables were reported as medians and interquartile ranges (25th-75th percentiles). Medians among groups were analyzed by non-parametric Kruskal-Wallis H test, with post-hoc analysis by Mann-Whitney U test. Categorical variables are presented as frequencies (percentages) and the comparison between different groups was done with a χ^2 test by means of a 2 × 2 contingency table; Fisher's exact test was used for small sample sizes. All tests were 2-tailed and results were considered significant for a P value less than 0.05. The odds ratios (OR) and 95% confidence intervals (CI), adjusted for maternal age at delivery, pre-pregnancy body mass index, parity, presence of maternal and family risk factors, were calculated using logistic regression analysis to assess the risk of PE and/or FGR associated with *H. pylori* infection.

RESULTS

Population

A total of 111 serum samples from pregnant women were examined: 49 uneventful pregnancies (Ctrl) and 62 pathological pregnancies complicated by fetal growth retardation (FGR-only, n = 13), pre-eclampsia (PE-only, n = 17), or both (PE-FGR, n = 32). Characteristics of the study population are summarized in Tables 1 and 2.

We found that normotensive women with pregnancy complicated by FGR were significantly younger (median of 25 years with an interquartile range of 24-26 years) compared to controls and PE women (both with a median age of 30 years). As expected, pregnancies complicated by PE and/or FGR were delivered more often by caesarean section. Moreover, pathological cases led to lower neonatal and placental weight compared to controls, due to lower gestational age at delivery and reduced fetal growth.

Pre-eclamptic mothers presented higher blood pressure values and urine protein concentrations. The presence of family risk factors was increased in PE cases without FGR (Table 2), while maternal risk factors for PE did not differ among groups (Table 2). The percentage of nulliparous women was significantly higher in the PE group than in controls. In 45 PE mothers, hypertension and proteinuria appeared early (before 34 wk) and in 32 of them these symptoms were severe; moreover five PE pregnancies were complicated by HELLP syndrome.

Leukocyte blood count, platelet count, and serum amino-transferases values in normal and pathologic pregnancies

Pre-eclamptic pregnancies were characterized by significantly higher values of total leukocyte count (P = 0.004) and serum amino-transferases [alanine aminotransferase

Table 2 Clinical characteristics of study populations (categorical variables) *n* (%)

Variable		Controls (*n* = 49)	PE-only (*n* = 17)	PE FGR (*n* = 32)	FGR-only (*n* = 13)	*P* value[5]
Cesarean section delivery		15 (30.6)[6,7,8]	16 (94.1)[6]	29 (90.6)[7]	9 (69.2)[8]	[6,7]< 0.001; [8]0.022
Neonatal sex	Male	19 (38.8)[7]	7 (41.2)	20 (62.5)[7]	6 (46.2)	[7]0.043
	Female	30 (61.2)	10 (58.8)	12 (37.5)	7 (53.8)	NS
Nulliparae		31 (63.3)[6,7]	16 (94.1)[6]	27 (84.4)[7]	10 (76.9)	[6]0.015; [7]0.047
Maternal risk factors		4 (8.2)	2 (11.8)[1]	8 (25.0)	1 (7.7)	NS
	Autoimmune diseases	1 (2.0)	2 (11.8)	4 (12.5)	0 (0)	NS
	Cardiovascular diseases	3 (6.1)	1 (5.9)	4 (12.5)	1 (7.7)	NS
Family risk factors		20[2] (40.8)[6]	12[3] (70.6)[6,10]	13[4] (40.6)	2 (15.4)[10]	[6]0.049; [10]0.004
	Hypertension	9 (18.4)	8 (47.1)	10 (31.3)	2 (15.4)	NS
	Diabetes	10 (20.4)	3 (17.6)	5 (15.6)	0 (0)	NS
	Cardiovascular diseases	5 (10.2)	2 (11.8)	3 (9.4)	0 (0)	NS
Other complications:	FGR	0 (0.0)[7,8]	0 (0.0)[9,10]	32 (100)[7,9]	13 (100)[8,10]	[7,8,9,10]< 0.001
	Early onset PE	-	16 (94.1)	29 (90.6)	-	NS
	Severe PE	-	13 (76.5)	19 (59.4)	-	NS
	HELLP syndrome	-	3 (17.6)	2 (6.3)	-	NS

[1]One patient presented both maternal risk factors (autoimmune and cardiovascular diseases); [2]Four patients presented two family risk factors (3 hypertension and diabetes; 1 diabetes and cardiovascular disease); [3]One patient presented two family risk factors (hypertension and diabetes); [4]Five patients presented two family risk factors (4 hypertension and diabetes; 1 hypertension and cardiovascular disease). [5]*P* values were calculated by chi-square test (χ^2). [6]Comparison between controls and PE-only group; [7]Comparison between controls and PE FGR group; [8]Comparison between controls and FGR-only group; [9]Comparison between PE-only and PE FGR groups; [10]Comparison between PE-only and FGR-only groups. NS: Non significant; PE: Pre-eclampsia; FGR: Fetal growth retardation; HELLP: Hemolysis-elevated liver enzymes-low platelets.

(ALT), aspartate aminotransferase (AST) $P = 0.006$ and $P = 0.029$, respectively], while eosinophil count and percentage were significantly lower ($P = 0.028$ and $P = 0.02$, respectively) compared to controls. However, if we exclude pathological cases complicated by HELLP syndrome, only ALT levels remained significantly higher in PE (Table 3). Normotensive pregnancies complicated by FGR showed significantly higher leukocyte levels compared to controls ($P = 0.045$, Table 3). Moreover, the FGR-only lymphocyte percentage was significantly higher relative to PE-only ($P = 0.047$, Table 3).

H. pylori seropositivity was increased in PE-FGR but not in FGR-only pregnancies

H. pylori seropositivity was significantly more frequent in PE women with or without FGR (85.7%) ($P < 0.001$; OR 9.22, 95% CI: 2.83-30.04), while it did not differ between FGR-only (46.2%) and controls (42.9%) (Table 4, Figure 1A). Further subdivision of PE group showed a higher prevalence of seropositive subjects among PE-FGR cases (93.8%) ($P < 0.001$; OR 35.56, 95% CI: 5.22-242.43) compared to controls; while in the PE-only group, the percentage of *H. pylori* seropositive women was higher, but not statistically significant (70.6%), relative to controls (Table 4, Figure 1A).

CagA and VacA seropositivity was increased in pre-eclamptic but not in FGR-only pregnancies

Similar to *H. pylori* seropositivity, the presence of antibodies against CagA antigen was prevalent only in PE pregnant women (81.6%) relative to controls (22.4%) ($P < 0.001$; OR 17.66, 95% CI: 5.25-59.49), while there were no differences between FGR-only cases (38.5%) and controls (Table 4, Figure 1B). CagA seropositivity was significantly more frequent in both PE-FGR (90.6%) ($P < 0.001$; OR 54.97, 95% CI: 9.24-326.88) and PE-only groups (64.7%) ($P = 0.038$; OR 5.20, 95% CI: 1.09-24.69), relative to controls. VacA seropositivity was significantly higher in PE-FGR cases (87.5%) ($P < 0.001$; OR 19.64, 95% CI: 3.75-102.98), while there were no differences between PE-only (55.6%) and FGR-only cases (53.8%), relative to controls (40%) (Table 4, Figure 1C).

Seropositivity for both CagA and VacA antibodies was associated with higher risk of PE-FGR (OR 45.44; 95% CI: 7.79-265.18). In fact, 87.5% of PE-FGR pregnancies were CagA and VacA seropositive, compared to 22.4% in Ctrl group (Table 5, Figure 1D). Patients seropositive for VacA, but not for CagA, were nine controls (18.4%), one FGR (7.7%), and no PE women, while seropositivity for CagA only was a specific feature of the PE-only group (Table 5). Seronegative women for both anti-CagA and VacA antibodies were only 9.4% in the PE-FGR group, while they were 59.2%, 35.3% and 53.8% in the Ctrl, PE-only and FGR-only groups, respectively (Table 5, Figure 1D). Importantly, CagA and VacA seronegativity was associated with a lower risk of developing pre-eclampsia complicated by fetal growth retardation (OR 0.04; 95% CI: 0.01-0.22).

UreC and UreE seropositivity was higher in PE-FGR pregnancies

We found significantly higher UreC and UreE seropositivity in PE-FGR patients (46.9%; $P = 0.018$ and 56.3%; $P = 0.003$, respectively) relative to controls (26.5% and 24.5%, respectively) (Figure 2B and C), while there were no differences among groups for HspB, FlagA, UreA, and UreH (Table 4, Figure 2A and D-F). Odds ratios calculation showed higher risk of developing PE-FGR in

Table 3 Leukocytes, platelets and liver enzymes in normal and pathological pregnancies

Variable	Normal values in Italian female population range	Controls (n = 49) Median (25th-75th)	All PE (n = 49) Median (25th-75th)	PE-only (n = 17) Median (25th-75th)	PE FGR (n = 32) Median (25th-75th)	FGR-only (n = 13) Median (25th-75th)	P value[2]
Total leukocyte count ($1 \times 10^3/\mu L$)	4.00-11.00	10.56 (9.21-11.65)[3,7,5,6]	12.03 (10.69-14.1)[3]	12.34 (10.71-13.83)[5]	11.83 (10.2-14.51)[6]	12.27 (11.21-13.47)[7]	[3]0.004; [7]0.045 [5]0.007; [6]0.024
Neutrophils ($1 \times 10^3/\mu L$)		8.27 (7.45-9.19)	10.09 (7.30-11.6)	10.15 (7.81-12.10)	9.92 (7.30-11.51)	9.34 (6.27-9.48)	NS
(%)	45.0-73.0	75.8 (68.1-78.8)	76.7 (68.37-87.1)	80 (72.5-90.1)	72.1 (68.2-83.6)	64.4 (57.3-77.3)	NS
Lymphocytes ($1 \times 10^3/\mu L$)		2.02 (1.68-2.26)	2.08 (1.3-3.06)	1.86 (1.22-2.34)	2.08 (1.31-3.07)	3.15 (2.13-3.79)	NS
(%)	19.0-47.0	18.7 (14-23.5)	17.4 (10.8-22.43)	14.55 (8.9-18.15)[8]	18.8 (11.1-23.2)	25.9 (17.4-34.3)[8]	[8]0.047
Monocytes ($1 \times 10^3/\mu L$)		0.54 (0.47-0.74)	0.59 (0.3-0.85)	0.61 (0.25-0.81)	0.58 (0.3-0.88)	0.89 (0.54-1.05)	NS
(%)	3.0-9.0	5.4 (4.5-6)	5.3 (3-7.3)	4.15 (2.15-7.75)	5.4 (3.5-7.3)	6.1 (4.9-7.9)	NS
Eosinophils ($1 \times 10^3/\mu L$)		0.15 (0.09-0.19)[3]	0.05 (0.02-0.1)[3]	0.04 (0.02-0.11)	0.05 (0.03-0.1)	0.17 (0.03-0.33)	[3]0.028
(%)	0.2-4.4	1.2 (0.9-1.8)[3]	0.5 (0.2-0.8)[3]	0.35 (0.1-0.8)	0.5 (0.2-0.8)	1.7 (0.2-2.4)	[3]0.020
Basophils ($1 \times 10^3/\mu L$)		0.02 (0.01-0.03)	0.02 (0.01-0.03)	0.03 (0.01-0.03)	0.02 (0.01-0.03)	0.01 (0-0.04)	NS
(%)	0.1-1.3	0.2 (0.1-0.3)	0.2 (0.1-0.3)	0.2 (0.15-0.25)	0.2 (0.1-0.3)	0.1 (0-0.3)	NS
Platelets ($1 \times 10^3/\mu L$)	150-400	219 (170-240)[5]	187 (126-228)	170 (111-214)[5,8]	191 (143-234)	235 (177-285)[8]	[5]0.024; [8]0.031
Platelets[1] ($1 \times 10^3/\mu L$)	150-400	219 (170-240)	191 (161-234)	180.5 (154-228)	191 (165-242)	235 (177-285)	NS
ALT (U/L)	< 34	15 (10-19)[3,5]	23 (14-46)[3]	26 (19-150)[5,8]	19.5 (13.5-35)	14 (10-21.5)[8]	[3]0.006; [5]0.002; [8]0.026
ALT[1] (U/L)	< 34	15 (10-19)[3]	20 (14-31)[3]	25 (15-46)	18 (13-27)	14 (10-21.5)	[3]0.023
AST (U/L)	< 31	17.5 (14-19)[3]	21 (16-39)[3,4]	25 (16-123)[8]	20 (16-35.5)[9]	14 (12-18)[4,8,9]	[3]0.029; [4]0.018; [8]0.031; [9]0.026
AST[1] (U/L)	< 31	17.5 (14-19)	19 (15.5-33)	19 (15-39)	18.5 (16-32)	14 (12-18)	NS

[1]Hemolysis-elevated liver enzymes-low platelets cases excluded; [2]P values were calculated by non-parametric Kruskal-Wallis H test, with post-hoc analysis by Mann-Whitney U test. [3]Comparison between controls and all PE group; [4]Comparison between all PE and FGR-only groups; [5]Comparison between controls and PE-only group; [6]Comparison between controls and PE FGR group; [7]Comparison between controls and FGR-only group; [8]Comparison between PE-only and FGR-only groups; [9]Comparison between PE-only and FGR-only groups. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NS: Non significant; PE: Pre-eclampsia; FGR: Fetal growth retardation.

Table 4 Seropositivity against *Helicobacter pylori*, cytotoxin-associated antigen A, vacuolating cytotoxin A, ureases A, C, E and H, heat shock protein B and flagellin A n (%)

	Controls (n = 49)	All PE (n = 49)	PE-only (n = 17)	PE FGR (n = 32)	FGR-only (n = 13)	P value[1,2]	Odds ratio[1] (95% CI)
Helicobacter pylori	21 (42.9)[3,5]	42 (85.7)[3]	12 (70.6)	30 (93.8)[5]	6 (46.2)	[3,5]< 0.001	[3]9.22 (2.83-30.04) [5]35.56 (5.22-242.43)
CagA	11 (22.4)[3,4,5]	40 (81.6)[3]	11 (64.7)[4]	29 (90.6)[5]	5 (38.5)	[3,5]< 0.001 [4]0.038	[3]17.66 (5.25-59.49) [4]5.20 (1.09-24.69) [5]54.97 (9.24-326.88)
VacA	20 (40.8)[3,5]	37 (75.5)[3]	9 (52.9)	28 (87.5)[5]	6 (46.2)	[3]0.005 [5]< 0.001	[3]4.89 (1.62-14.73) [5]19.64 (3.75-102.98)
HspB	15 (30.6)	21 (42.9)	5 (29.4)	16 (50.0)	6 (46.2)	NS	
FlagA	13 (26.5)	22 (44.9)	6 (35.3)	16 (50.0)	5 (38.5)	NS	
UreA	10 (20.4)	13 (26.5)	4 (23.5)	9 (28.1)	3 (23.1)	NS	
UreC	13 (26.5)[3,5]	19 (38.8)[3]	4 (23.5)	15 (46.9)[5]	4 (30.8)	[3]0.042 [5]0.018	[3]2.84 (1.04-7.75) [5]4.02 (1.27-12.80)
UreE	12 (24.5)[3,5]	26 (53.1)[3]	8 (47.1)	18 (56.3)[5]	3 (23.1)	[3]0.004 [5]0.003	[3]4.41 (1.59-12.26) [5]6.29 (1.88-21.04)
UreH	8 (16.3)	13 (26.5)	5 (29.4)	8 (25.0)	4 (30.8)	NS	

[1]Adjusted for maternal age at delivery, pre-pregnancy body mass index, parity, and presence of maternal and family risk factors; [2]P values were calculated by χ^2 test; [3]Comparison between controls and all PE group; [4]Comparison between controls and PE-only group; [5]Comparison between controls and PE FGR group. CI: Confidence intervals; NS: Non significant; PE: Pre-eclampsia; FGR: Fetal growth retardation; CagA: Cytotoxin-associated antigen A; VacA: Vacuolating cytotoxin A; HspB: Heat shock protein B; FlagA: Flagellin A; Ure: Ureases.

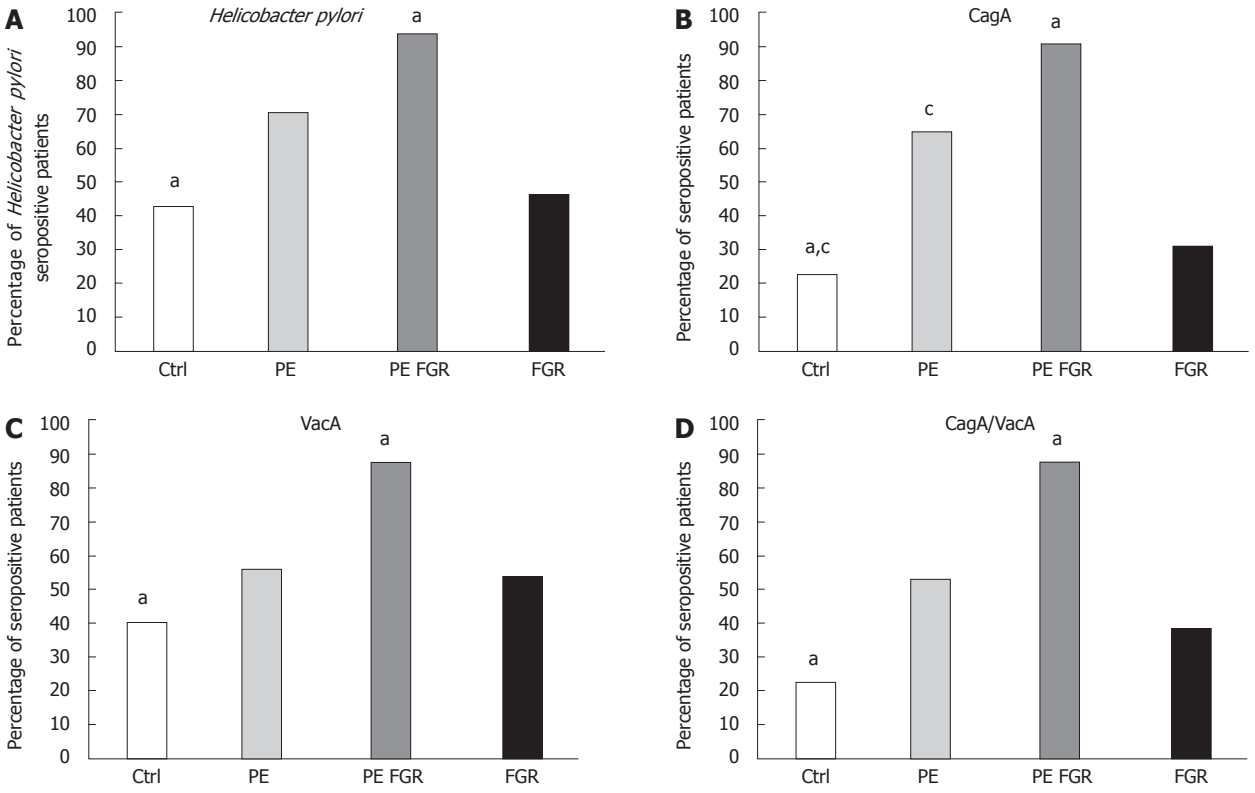

Figure 1 Percentage of *Helicobacter pylori* (A), CagA (B), VacA (C), and CagA/VacA (D) seropositive women in control, PE, PE FGR, and FGR groups. [a]*P* < 0.05 between controls and PE-FGR; [c]*P* < 0.05 between controls and PE without FGR. CagA: Cytotoxin-associated antigen A; VacA: Vacuolating cytotoxin A; PE: Pre-eclampsia; FGR: Fetal growth retardation.

Table 5 Cytotoxin-associated antigen A/vacuolating cytotoxin A dual seropositivity *n* (%)

	Controls (*n* = 49)	All PE (*n* = 49)	PE-only (*n* = 17)	PE FGR (*n* = 32)	FGR-only (*n* = 13)	*P* value[1,2]	Odds ratio[1] (95% CI)
CagA+VacA+	11 (22.4)[3,4]	37 (75.5)[3]	9 (52.9)	28 (87.5)[4]	5 (38.5)	[3]< 0.001	12.10 (3.76-38.91)
						[4]0.001	45.44 (7.79-265.18)
CagA-VacA+	9 (18.4)	0 (0)	0 (0)	0 (0)	1 (7.7)	NS	
CagA+VacA-	0 (0.0)	3 (6.1)	2 (11.8)	1 (3.1)	0 (0.0)	NS	
CagA-VacA-	29 (59.2)[3,4]	9 (18.4)[3]	6 (35.3)	3 (9.4)[4]	7 (53.8)	[3]0.001	0.13 (0.04-0.42)
						[4]< 0.001	0.04 (0.01-0.22)

[1]Adjusted for maternal age at delivery, pre-pregnancy body mass index, parity, and presence of maternal and family risk factors; [2]*P* values were calculated by chi-square test (χ^2); [3]Comparison between controls and all PE group; [4]Comparison between controls and PE FGR group. CI: Confidence intervals; NS: Non significant; CagA: Cytotoxin-associated antigen A; VacA: Vacuolating cytotoxin A; PE: Pre-eclampsia; FGR: Fetal growth retardation.

patients seropositive for UreC (OR 4.02, 95% CI: 1.27-12.8) and UreE (OR 6.29, 95% CI: 1.88-21.04) (Table 4).

Association among CagA/VacA seropositivity and leukocyte blood count, platelet count, and serum amino-transferases values

Considering seropositivities for CagA and/or VacA antigens, we found that the total leukocyte count was significantly decreased in VacA only seropositive patients relative to seronegative, CagA+/VacA- and CagA+/VacA+ patients ($P = 0.003$; $P = 0.014$ and $P = 0.012$, respectively, Table 6). Moreover, the basophiles percentage, but not total count, was significantly increased in CagA/VacA double seropositive compared to seronegative patients ($P = 0.002$, Table 6). No differences among groups were found for the other investigated parameters (Table 6). Analyzing amino-transferases levels after HELLP cases exclusion, ALT was significantly increased in CagA only seropositive patients relative to the other groups ($P = 0.02$; $P = 0.025$; $P = 0.023$, respectively, Table 6), while no differences were found for AST levels (Table 6).

DISCUSSION

In the present study, we reported a direct association between *H. pylori* virulence and the onset of pre-eclampsia complicated by FGR. Moreover, by investigating seropositivity for *H. pylori* virulence factors, we were able to

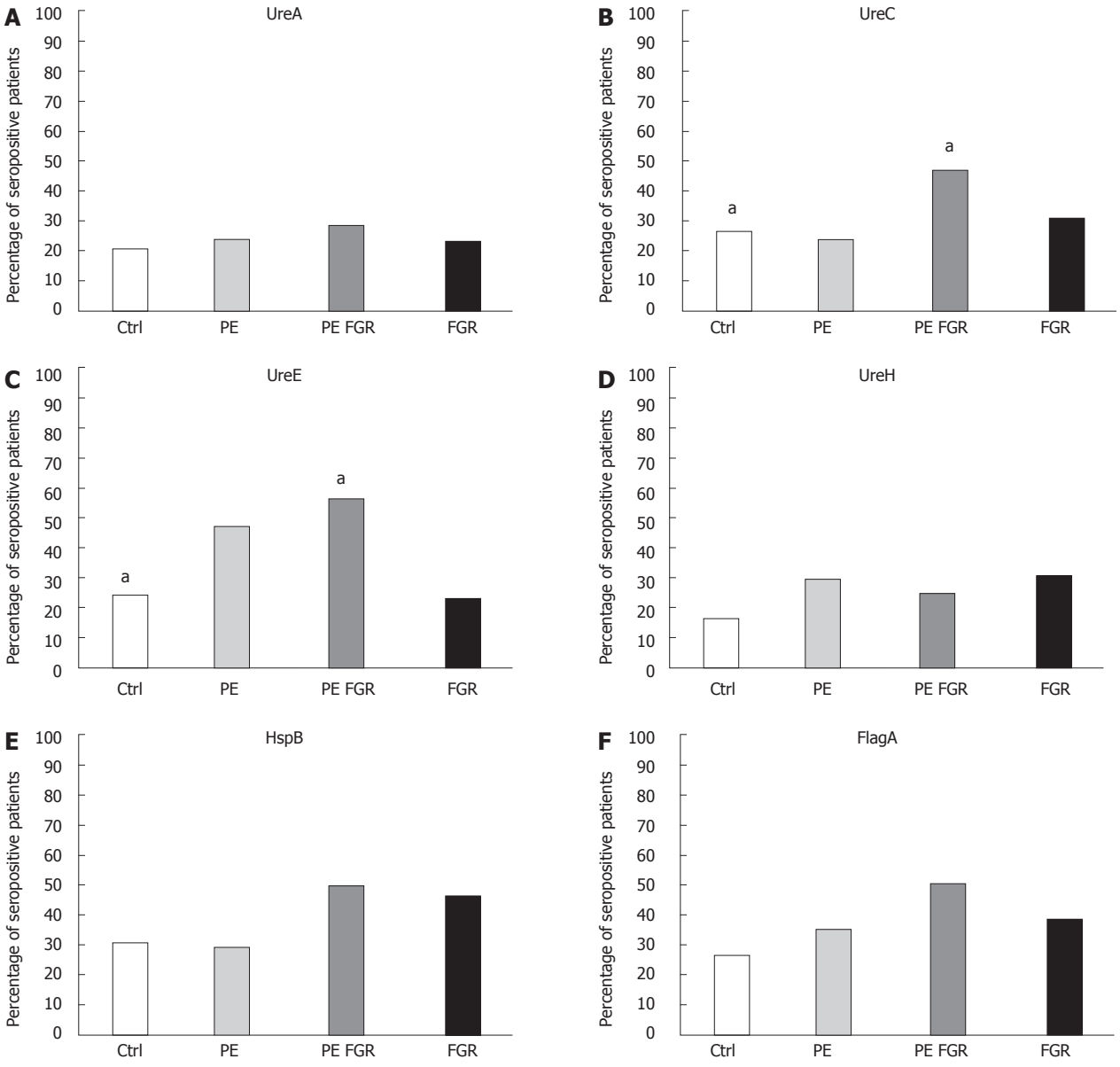

Figure 2 Percentage of Ureases (A-D), HspB (E), and FlagA (F) seropositive women in control, PE, PE FGR, and FGR groups. $^{a}P < 0.05$ *vs* controls. HspB: Heat shock protein B; FlagA: Flagellin A; Ure: Urease; PE: Pre-eclampsia; FGR: Fetal growth retardation.

distinguish pre-eclampsia and FGR without hypertension as different pathologies.

It is accepted that pre-eclamptic pregnancies, complicated or not by FGR, are characterized by severe maternal inflammation[1]. Less is known about "pure" FGR pregnancies, probably because of a biased classification system that considers FGR a secondary disease or a complication of pre-eclampsia. We found elevated maternal leukocytes count, typical sign of inflammation, in all pathological pregnancies relative to controls. However, while leukocytosis in PE patients, as previously reported[29,30], was mainly due to elevated neutrophils levels, a typical marker of bacterial infection[31], in FGR-only mothers, leukocytosis was due to increase in monocytes, eosinophils, and lymphocytes. Moreover, we found significantly higher transaminases levels in the PE group, even after the exclusion of HELLP cases, known to be characterized by elevated hepatic enzymes. The trigger of this exacerbated inflammatory response still remains unknown.

Graham *et al*[21] previously demonstrated a direct association between abnormal total leukocyte count and *H. pylori*-infection in patients with duodenal ulcer disease. They reported a significant fall in total white cell and neutrophils counts in patients successfully treated by *H. pylori* antibiotic therapy[21]. Moreover, they observed higher AST levels in CagA-positive patients, even after antibiotic treatment, thus assuming that AST levels are not directly associated with *H. pylori* infection[21]. Furthermore, we previously reported a correlation between *H. pylori* infec-

Table 6 Hematological values and cytotoxin-associated antigen A/vacuolating cytotoxin A antigens

Variables	Normal values in Italian female population range	CagA-VacA- (n = 45) Median (25th-75th)	CagA-VacA+ (n = 10) Median (25th-75th)	CagA+VacA- (n = 3) Median (25th-75th)	CagA+VacA+ (n = 53) Median (25th-75th)	P value[2]
Total leukocyte count ($1 \times 10^3/\mu L$)	4.00-11.00	12.02 (10.6-13.13)[3]	8.95 (7.75-10.5)[3,5,6]	14.1 (12.4-16.34)[5]	11.27 (9.62-13.64)[6]	[3]0.003; [6]0.012; [5]0.014
Neutrophils ($1 \times 10^3/\mu L$)		10.02 (8.88-11.40)	7.45 (4.87-9.48)	12.75 (11.03-14.48)	8.43 (6.9-10.5)	NS
(%)	45.0-73.0	78.8 (67.05-88.85)	71.9 (68.1-77.3)	83.4 (78.2-88.6)	72.05 (67.4-80)	NS
Lymphocytes ($1 \times 10^3/\mu L$)		1.72 (1.11-2.85)	2.13 (1.68-2.13)	1.61 (0.93-2.28)	2.34 (1.63-3.07)	NS
(%)	19.0-47.0	15.3 (8.9-23.2)	20.6 (17.4-23.5)	10.95 (5.7-16.2)	18.75 (14-23.3)	NS
Monocytes ($1 \times 10^3/\mu L$)		0.57 (0.26-0.89)	0.54 (0.43-0.6)	0.78 (0.68-0.88)	0.6 (0.44-0.84)	NS
(%)	3.0-9.0	4.75 (2.15-6.85)	5.2 (4.9-6)	5.1 (4.8-5.4)	5.81 (4.3-7.4)	NS
Eosinophils ($1 \times 10^3/\mu L$)		0.05 (0.02-0.18)	0.09 (0.03-0.21)	0.06 (0.03-0.08)	0.06 (0.03-0.15)	NS
(%)	0.2-4.4	0.55 (0.1-1.45)	1.2 (0.2-2)	0.4 (0.2-0.6)	0.58 (0.3-1.17)	NS
Basophils ($1 \times 10^3/\mu L$)		0.01 (0-0.02)	0.03 (0.01-0.03)	0.02 (0.02-0.03)	0.03 (0.01-0.04)	NS
(%)	0.1-1.3	0.1 (0-0.2)	0.2 (0.2-0.3)	0.15 (0.1-0.2)	0.2 (0.2-0.3)	0.002
Platelets[1] ($1 \times 10^3/\mu L$)	150-400	222 (169-249)	175 (154-209.5)	214 (210-228)	191 (166-242)	NS
ALT[1] (U/L)	< 34	16 (12.5-26)[4]	11 (9-12)[5]	32 (30-178)[4,5,7]	17 (11.5-24)[7]	[4]0.020; [5]0.025; [7]0.023
AST[1] (U/L)	< 31	18 (15.5-23.5)	13 (12-19)	58 (15-143)	18 (14.5-26)	NS

[1]Hemolysis-elevated liver enzymes-low platelets cases excluded; [2]P values were calculated by non-parametric Kruskal-Wallis H test, with post-hoc analysis by Mann-Whitney U test; [3]Comparison between CagA-VacA- and CagA-VacA+ groups; [4]Comparison between CagA-VacA- and CagA+VacA- groups; [5]Comparison between CagA-VacA+ and CagA+VacA- groups; [6]Comparison between CagA-VacA+ and CagA+VacA+ groups; [7]Comparison between CagA+VacA- and CagA+VacA+ groups. CagA: Cytotoxin-associated antigen A; VacA: Vacuolating cytotoxin A; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; NS: Non significant.

tion and the onset of pre-eclampsia during pregnancy, suggesting that this Gram-negative bacterium could cause or contribute to the etiopathogenesis of pre-eclampsia[7], by inducing the pro-inflammatory state.

In the present study, we further investigated *H. pylori* and pre-eclampsia association by considering the main markers of *H. pylori* virulence and infection persistence, which are useful for understanding the severity and characteristics of the infection.

H. pylori strains carrying the CagA antigen are known to be among the most virulent and are associated with increased inflammation[13]. VacA is a *H. pylori* toxin crucial to promote and maintain bacterial colonization[14]. Importantly, combined seropositivity for both CagA and VacA directly correlates with elevated morbidity[32-34]. We previously reported a strong association between CagA positive *H. pylori* infection and the onset of PE in Italian women[7]. In the present study, we also found that CagA/VacA dual seropositivity is specifically associated with pre-eclampsia and, in particular, with PE complicated by FGR. In contrast, the absence of both anti-CagA and anti-VacA antibodies is associated with a lower risk of PE. Interestingly, the association with CagA-/VacA+ was found only in controls and normotensive women with FGR pregnancies, while CagA+/VacA- patients belong to the PE groups. Our data suggest that the CagA antigen is associated with a more severe pattern, while VacA alone is not sufficient to cause the severe systemic inflammation typical of PE. The highest leukocyte count and ALT level observed in CagA+/VacA- patients further corroborated this hypothesis, while subjects seropositive only for VacA were characterized by the lowest median leukocyte values (Table 6). CagA/VacA dual seropositivity was the most frequent condition in PE complicated by FGR patients (Table 5), and was associated with intermediate leukocyte and ALT values (Table 6). Therefore, we speculate that CagA, with or without VacA, may contribute to the onset of pre-eclampsia, while VacA seropositivity could attenuate CagA virulence. Our results indicate that severe (CagA positive) and persistent (VacA positive) maternal *H. pylori* infections are strongly associated to pre-eclampsia complicated by feto-placental compromise, as indicated by FGR. Therefore, chronic and severe *H. pylori* infections could contribute not only to the exacerbated maternal inflammatory response leading to pre-eclampsia, but also to the abnormal placentation typical of FGR.

Importantly, FGR without PE does not present significant differences relative to physiological controls for either *H. pylori* (Table 3, Figure 1A) and CagA/VacA dual seropositivity (Figure 1D), suggesting that different etiopathogenetic mechanisms lead to "pure" FGR.

Another key *H. pylori* virulence factor is Urease, an enzyme that modifies environmental pH to allow *H. pylori* colonization[35]. Moreover, it helps to activate pro-inflammatory cytokines production[15]. We determined seropositivity for A, E and H urease subunits and for UreC in pre-eclamptic and/or FGR pregnant women relative to controls. In PE women relative to controls, we found significantly higher seropositivity only for the UreE subunit; the carrier of nickel ions and pivotal for proper enzyme activity[36-38]. The rate of seropositivity for UreC, the enzyme necessary for bacterial cell wall formation[39], was significantly higher in PE pregnancies complicated by FGR, as we previously showed for CagA/VacA dual-seropositivity. These data suggest that UreE and UreC contribute to the onset of both PE and PE-FGR.

Even though pre-eclampsia has been extensively investigated, the only effective therapeutic option remains a timed, programmed delivery. Our data clearly demonstrate a direct correlation between severe and persistent *H. pylori* infection and the onset of PE complicated by FGR, opening up attractive perspectives for the design of new preventive and therapeutic interventions for pre-eclampsia.

Although specific combinations of different antibiotics are effective in eradicating *H. pylori*, antibiotics-resistant strains are already emerging, thus decreasing the efficacy of existing therapies[40]. Pharmacogenomics-based treatments seem to increase the cure rates and new therapeutic approaches targeting *H. pylori* virulence factors are required[40]. In the case of pregnancy-related diseases, it would be preferable to prevent the exacerbated inflammation typical of PE, thus avoiding pharmacologic therapies during pregnancy. Recently, several clinical trials and animal studies have focused on generating *H. pylori* recombinant vaccines[41,42]. They demonstrated the possibility of eliciting an immunological response against *H. pylori* in humans, and to eradicate and protect against the infection in mice[43]. Experimental *H. pylori* vaccines have been created using bacterial urease and designed as oral preparations.

In conclusion, our results define pre-eclampsia complicated by FGR and "pure FGR" as different pathologies. Moreover, we demonstrated a direct role for *H. pylori* CagA/VacA positive strains in the etiopathogenesis of PE-FGR. Our data further emphasize the importance of an accurate classification of the multifactorial and multiform pre-eclamptic disease. It is generally accepted that PE is a syndrome that includes several pathologies with different etiopathogenesis but with similar clinical manifestations. For this reason, PE is usually classified on the basis of symptoms severity (moderate or severe) or of symptoms onset (early- or late-onset PE). We strongly believe that, as demonstrated by the present study, pre-eclampsia should also be classified as placental (with feto-placental involvement) or maternal (without feto-placental compromise)[44], both of which may have early or late onset. This classification will lead to a better management of this devastating pregnancy-related disorder. Further studies are required to identify specific *H. pylori*-related therapeutic targets.

COMMENTS

Background

Pre-eclampsia (PE), a severe hypertensive pregnancy-related syndrome that affects 5%-8% of women worldwide, represents the main cause of feto-maternal mortality and morbidity. Despite being the object of intense investigation, the etiopathogenesis of PE is still poorly understood, and no effective therapeutic interventions are available in clinical practice.

Research frontiers

Several lines of evidence suggest that maternal sub-clinical infections could play a pivotal role in the onset of PE. *Helicobacter pylori* (*H. pylori*) could directly cause or intensify the generalized inflammation and endothelial dysfunction typical of this syndrome.

Innovations and breakthroughs

The data represent a major advance in the understanding of PE etiopathogenesis and add pivotal information for an accurate classification of this multifactorial and multiform syndrome. In fact, the authors clearly demonstrated a direct correlation between severe and persistent *H. pylori* infection and the onset of PE complicated by fetal growth retardation (FGR).

Applications

The findings open up new, attractive perspectives regarding the design of effective preventive and therapeutic interventions for pre-eclampsia associated with *H. pylori* infection.

Terminology

FGR is defined as failure of the fetus to achieve its genetically determined growth potential and is commonly considered a severe complication of PE.

Peer review

The work is a contribution to the understanding of *H. pylori*'s pathogenic role in PE, associated or not with FGR. The association between maternal infection and PE has been evaluated by several researchers and is a good field to study the etiopathogenesis of this critical clinical condition. Authors confirmed that persistent and virulent *H. pylori* infections cause or contribute to PE complicated by FGR.

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