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Serum antibodies to surface proteins of *Chlamydia trachomatis* as candidate biomarkers of disease: results from the Baltimore Chlamydia Adolescent/Young Adult Reproductive Management (CHARM) cohort

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One sentence summary: The serum antibody response of Chlamydia trachomatis-infected patients against selected chlamydial surface proteins is associated with gender and disease severity.
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Abstract

We previously observed that the nine-member family of autotransported polymorphic membrane proteins (Pmps) of *Chlamydia trachomatis* is variably expressed in cell culture. Additionally, *C. trachomatis*-infected patients display variable Pmp-specific serum antibody profiles indirectly suggesting expression of unique Pmp profiles is an adaptive response to host-specific stimuli during infection. Here, we propose that the host response to Pmps and other outer surface proteins may correlate with disease severity. This study tests this hypothesis using an ELISA that measures serum IgG antibodies specific for the nine *C. trachomatis* Pmp subtypes and four immunodominant antigens (MOMP, OmcB, Hsp60, ClpP) in 265 participants of the *Chlamydia* Adolescent/Young Adult Reproductive Management (CHARM) cohort. More *C. trachomatis*-infected females displayed high Pmp-specific antibody levels (cut-off Indexes) than males (35.9%–40.7% of females *vs.* 24.2%–30.0% of males), with statistical significance for PmpC, F and H ($P < 0.05$). Differences in Pmp-specific antibody profiles were not observed between *C. trachomatis*-infected females with a clinical diagnosis of pelvic inflammatory disease (PID) and those without. However, a statistically significant association between high levels of OmcB-specific antibody and a PID diagnosis ($P < 0.05$) was observed. Using antibody levels as an indirect measure of antigen expression, our results suggest that gender- and/or site-specific (cervix in females *vs.* urethra in males) stimuli may control *pmp* expression in infected patients. They also support the possible existence of immune biomarkers of chlamydial infection associated with disease and underline the need for high resolution screening in human serum.

Keywords: *Chlamydia trachomatis*, polymorphic membrane proteins, surface proteins, serology, ELISA, antibodies, pelvic inflammatory disease

Introduction

In 2018, more than 1.8 million cases of sexually transmitted *Chlamydia trachomatis* infection in the USA were reported to the Centers for Disease Control and Prevention (2018). Chlamydial infections, which are often asymptomatic, can lead to pelvic inflammatory disease (PID), a precursor of female infertility, ectopic pregnancy, and chronic pelvic pain in 10%–20% of infected women. Screening for *C. trachomatis* using nucleic acid amplification tests followed by treatment, can reduce the incidence of PID by as much as 60%, suggesting that detection of an active chlamydial infection is an effective means of reducing future sequelae. To fully control chlamydial disease, however, host or chlamydial

biomarkers that can discriminate between different clinical outcomes early in, or during, infection remain highly desirable.

A panoply of secreted and/or surface proteins of *C. trachomatis* are thought to act early in infection (Su *et al.* 1990, Ting *et al.* 1995, Crane *et al.* 2006, Moelleken and Hegemann 2008) as adhesins. These include the elementary body (EB)-specific cysteine-rich outer membrane complex protein OmcB (Moelleken and Hegemann 2008), the major outer membrane protein MOMP (OmpA) (Baehr *et al.* 1988), and members of the polymorphic membrane protein family (Pmps) (Becker and Hegemann 2014). The late-expressed cysteine-rich protein OmcB was previously suggested as a candidate adhesin for *C. trachomatis* (Fadel and Eley 2007),

Table 1. Demographics and sexual behavior compared between genders.

	Overall	Male N=120 (%)	Female N=145 (%)	P-value[a]
Age, median (IQR)	19.5 (17–21)	20.2 (18–22)	18.9 (17–21)	**0.0047[b]**
Age groups				**0.001**
≤20 years old	150 (56.60)	55 (45.83)	95 (65.52)	
>20 years old	115 (43.40)	65 (54.17)	50 (34.48)	
Sexual history				
Age of sexual debut				**0.007**
<13 years old	223 (84.15)	93 (77.50)	130 (89.66)	
13+ years old	42 (15.85)	27 (22.50)	15 (10.34)	
Sexual partners lifetime				**<0.001**
<6 partners	94 (35.47)	26 (21.67)	68 (46.90)	
6+ partners	171 (64.53)	94 (78.33)	77 (53.10)	
Oral sex	233 (87.92)	109 (90.83)	124 (85.52)	0.186
Anal sex	79 (29.81)	41 (34.17)	38 (26.21)	0.159
Condom use in past 3 months	32 (11.99)	21 (17.50)	11 (7.59)	**0.014**
Self-reported STI and reproductive health history				
Bacterial STI[c]	206 (77.74)	88 (73.33)	118 (81.38)	0.117
Viral STI[d]	27 (10.19)	9 (7.50)	18 (12.41)	0.188
Trichomonas vaginalis	N/A[e]	N/A	28 (19.31)	N/A
BV	N/A	N/A	15 (10.34)	N/A
PID	N/A	N/A	6 (4.14)	N/A
Physician assessment[f]				
PID	N/A	N/A	33 (22.76)	N/A
STI Diagnostic Laboratory tests (current)				
HIV	18 (6.79)	8 (6.67)	10 (6.90)	0.941
RPR/ syphilis	2 (0.75)	1 (0.83)	1 (0.69)	0.893
GC/ gonorrhoea	20 (7.55)	10 (8.33)	10 (6.90)	0.659

[a]P-values from chi-square, unless otherwise indicated
[b]P-values from Rank-sun Wilcoxon
[c]gonorrhoea, chlamydia, and syphilis
[d]HPV, Herpes, and HIV
[e]*Trichomonas vaginalis* was only diagnosed in women, hence is not applicable (N/A) to male participants
[f]PID assessment was performed by a physician as described in Materials and Methods: "PID diagnosis was based on the minimum clinical criteria (cervical motion tenderness, uterine tenderness, or adnexal tenderness) as set by the Centers for Disease Control and Prevention", according to CDC (2020)

Chlamydia caviae (Ting *et al.* 1995), and more recently for *Chlamydia pneumoniae* (Moelleken and Hegemann 2008). MOMP displays sequence-variable domains defining 17 serovars categorized according to tissue tropism (A-C, ocular; D-K, urogenital; L1-L3, inguinal lymph nodes), and was also proposed to function as an adhesin for *C. trachomatis* (Su *et al.* 1990). The 9-member *pmp* gene family of *C. trachomatis* (Pmp subtypes A-I; (Stephens *et al.* 1998)) is predicted to be associated with tissue tropism (Gomes *et al.* 2006). *Chlamydia trachomatis* PmpD-specific antibody has been shown to dramatically reduce infection *in vitro* (Crane *et al.* 2006). These properties and the observed on/off switching of expression of the Pmps in cell culture (Tan *et al.* 2010, Carrasco *et al.* 2011) suggest that production of a specific Pmp subtype(s) may be required and/or selected for at different sites or stages of infection. Consistent with variable expression in cell culture, we have previously shown that all *C. trachomatis*-infected patients mount a strong serologic response against different subsets of Pmps (Tan *et al.* 2009) with a suggested correlation between disease (PID) and PmpI antibody titer (Tan *et al.* 2009, Taylor *et al.* 2011).

The presence at the chlamydial surface of specific OmcB and MOMP types combined with the expression of diverse PmpA-I profiles may provide the outer coat diversity that is necessary for adherence to, internalization by and colonization of diverse mucosal sites. Since sites of infection may be associated with a spectrum of pathologies ranging from none to severe, we further propose that distinct outer surface protein profiles may also correlate with disease severity. Here, we indirectly test these two linked hypotheses using an antibody capture ELISA to evaluate the host response to OmcB and MOMP, and as a surrogate for the expression of specific Pmp subtypes. We use serum samples from participants in the *Chlamydia* Adolescent/Young Adult Reproductive Management (CHARM) cohort enrolled in the Baltimore, Maryland area. To address site/gender specific expression, sera from *C. trachomatis*-infected men and women are compared. To address the possible link with disease severity, sera from women with a lower genital tract *C. trachomatis* infection and a reported PID diagnosis are compared with those without a PID diagnosis. Because of their previously reported association with disease in other studies, the *C. trachomatis* Heat Shock Protein Hsp60 (also known as GroEL) (Wagar *et al.* 1990, Peeling *et al.* 1997, Rodgers *et al.* 2010) and the Caseinolytic protease P (ClpP) (Rodgers *et al.* 2010) are also included.

Materials and methods
CHARM cohort
The Chlamydia Adolescent & Young Adult Reproductive Management (CHARM) cohort consists of 265 *Chlamydia trachomatis*-infected men and women recruited between September 2010 and December 2013 in the Baltimore area (Table 1). CHARM inclusion and exclusion criteria, informed consent, treatment, data, and specimens collected at entry and at intervals, follow-up activities, and statistical analyses were published independently (Mark *et al.* 2019). CHARM (HP-00042320) was reviewed and
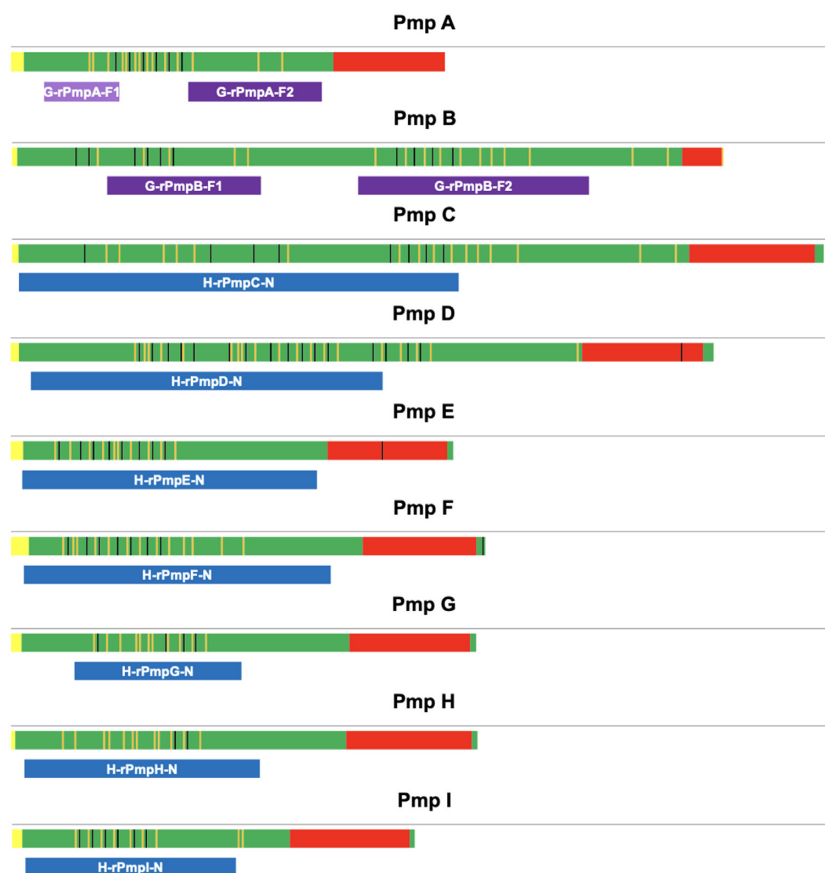
Figure 1. Schematic representation of the Pmp proteins and recombinant polypeptides used in this study. Structural features of Pmps are shown; Predicted signal peptide using Signal 4.0 software (yellow), passenger domain (green), GGA[I/L/V] (black bars), and FXXN (orange bars) tetrapeptide repeats and autotransporter domain (red). Recombinant Pmp derivatives based on predicted hydrophilic and antigenic domains located within the Pmp passenger domain, expressed in GST• tag pGEX-2t vector (GE Healthcare Biosciences) are shown in light purple (G-rPmpA-F1) and purple (G-rPmpA-F2, G-rPmpB-F1 and G-rPmp-F2). Passenger domain recombinant proteins expressed as inclusion bodies in His• tag pET30 (a or b) are in blue (H-rPmp(C to I)-N).

approved by the Institutional Review Board of the University of Maryland Baltimore. For the purpose of this study, blood was collected without additives at enrollment and prior to antibiotic treatment. After centrifugation, serum samples were stored at –20°C until analyzed. PID diagnosis was based on the minimum clinical criteria (cervical motion tenderness, uterine tenderness, or adnexal tenderness) as set by the Centers for Disease Control and Prevention (2020).

Recombinant polypeptides

The predicted passenger domains of PmpC, D, E, F, H, and I, smaller fragments of PmpA (*pmpA-F2),* PmpB (*pmpB-F1* and *pmpB–F2*), and PmpG (Fig. 1) (Tan *et al.* 2009), rMOMP, rHsp60, rOmcB, and rClpP (from *C. trachomatis* serovar D; kindly provided by Dr Guangming Zhong) were expressed in *E. coli* BL21(DE3) and purified as indicated in (Tan *et al.* 2009). The genome sequence of *C. trachomatis* D/UW-3/CX was used as the reference genome to predict the protein sequences (Table S1).

Characteristics and purification conditions of the recombinant polypeptides.

Insoluble polypeptides (all except rHsp60) were solubilized overnight at 42°C in 8 M urea/10 mg/ml Octyl beta-D-glucopyranoside (OGP) in buffer (50 mM Tris-HCl, pH 8/1 mM EDTA/1 mM DTT), dialyzed against 0.01% OGP and affinity purified (Table S1). Purification was monitored by SDS-PAGE

and immunoblot (Figure S1, Expression and purification of the recombinant proteins and peptides used in this study).

ELISA

Microplates PS (F-form; Greiner Bio-One North America, Inc.) coated with EBs purified from 48h *C. trachomatis* serovar E cultures and 72h *C. pneumoniae* AR39 cultures, respectively (Mahony *et al.* 1983) (Table S2, ELISA optimized conditions) or purified recombinant polypeptides (Table S2) were incubated overnight at 4°C, washed with PBS-T and blocked with 2.5% milk. ELISA was performed as described (Frikha-Gargouri *et al.* 2008) using diluted CHARM sera (Table S2) and goat anti-human IgG peroxidase labeled antibody (1:2000 dilution; 100 μl/well; KPL, Inc.). Results were read in a Beckman DTX 880 plate reader (Beckman Coulter, Inc.) at 450 and 620 nm (background).

Results obtained by ELISA were comparable to those previously obtained by immunoblot analysis and densitometry (Tan *et al.* 2009) (not shown), thus supporting the validity of results obtained by either method. ELISA results generated with GST• tagged and His• tagged OmcB were positively correlated (*P* < 0.001; Figure S2, Correlation between G-OmcB and H-OmcB ELISA results), further validating the ELISA.

Statistical analysis

Chi-squared test was used to compare the demographic, sexual behavior and biological factors of the study groups. Median and

Table 2. Association of demographic, biological, and sexual behaviors in 33 women with a PID diagnosis among the 145 women in the study.

	Overall	Unadjusted analysis Odd ratio (95% CI)	P-value	Adjusted analysis Odd ratio (95% CI)	P-value[a]
Age groups			0.796		0.523
≤20 years old	95 (65.52)	1		1	
>20 years old	50 (34.48)	1.11 (0.50, 2.50)		0.75 (0.35, 1.83)	
Sexual history					
Age of sexual debut			0.135		0.326
<13 years old	107 (73.79)	1.89 (0.82, 4.35)		1.60 (0.63, 4.05)	
13+ years old	38 (26.21)	1		1	
Sexual partners lifetime			0.079		0.274
<6 partners	68 (46.90)	1		1	
6+ partners	77 (53.10)	2.07 (0.92, 4.67)		1.64 (0.67 4.00)	
Receptive oral sex ever (vs. never)	119 (82.07)	2.58 (0.72, 9.22)	0.144	2.00 (1.00, 1.00)	0.596
Anal sex ever (vs. never)	38 (26.21)	1.07 (0.45, 2.57)	0.874	0.89 (0.36, 2.24)	0.808
No condom use in past 3 months	134 (92.41)	N/A[b]	N/A	N/A[b]	N/A
Self-reported prior STI					
Bacterial STI[c]	118 (81.38)	2.73 (0.77, 9.71)	0.121	2.19 (0.59, 8.22)	0.244
Viral STI[d]	18 (12.41)	0.97 (0.29, 3.16)	0.954	0.54 (0.14, 2.09)	0.373
Trichomonas vaginalis	28 (19.31)	2.79 (1.15, 6.80)	**0.023**	2.36 (0.89, 6.25)	**0.083**
BV	15 (10.34)	2.54 (0.83, 7.77)	0.101	2.19 (0.68, 7.04)	0.191
PID	6 (4.14)	19.82 (2.23, 176.52)	**0.007**	-	
STI Diagnostic Laboratory tests (current)[c]					
HIV	10 (6.90)	0.36 (0.04, 2.93)	0.338	0.25 (0.03, 2.32)	0.223
GC/ gonorrhea	10 (6.90)	0.84 (0.17, 4.16)	0.829	1.00 (0.19, 5.20)	0.998

[a]P-values from logistic regression
[b]None of the female participants positive for PID used condoms
[c]gonorrhea and chlamydia (syphilis was not analyzed because it was only detected in one female)
[d]HPV, Herpes, and HIV

inter-quartile ranges were used to describe continuous variables. A non-parametric test was used to compare the median by gender.

Antibody levels defined as the cut-off Indexes for rPmpA to I, rClpP, rHsp60, rMOMP, rOmcB, and *C. trachomatis* purified EBs were used as categorical variables and split into tertiles. The antibody response was separated into three levels with similar number of samples, 48 to 49 samples per tertile for females only (n = 145), and 87 to 90 samples per tertile for both females and males (n = 265). The first tertile cut-point represented the lower, while the third tertile represented the higher cut-off indexes. Logistic regression models were then used to determine the association between cut-off indexes in the first and third tertiles (highest/lowest) and PID diagnosis (outcome). Unadjusted and adjusted Odds Ratios (ORs) and their 95% confidence intervals (CIs) were calculated. In adjusted analysis, results were presented after accounting for potential confounding effects of age, *Trichomonas vaginalis* infection and a diagnosis of bacterial vaginosis (BV) based on Amsel's criteria (Amsel *et al.* 1983).

All analyses were performed using Stata 12.0 (College Station).

Results

Demographics and sexual behavior in the CHARM cohort

A total of 265 participants (120 males, 45.3% and 145 females, 54.7%) of the CHARM cohort were included in the study. All participants were positive for *Chlamydia trachomatis* using PCR analysis (Mark *et al.* 2019). The overall median age of the study population was 19.5 years old (IQR: 17-21). Males were more likely to be older (median: 20.2 years, IQR: 18-22) compared to women

(18.9 years, IQR: 17–21, respectively) ($P = 0.0047$). Table 1 compares demographic data, sexual behavior, sexual history, and past and current diagnosis of sexually transmitted infections (STIs) in men and women of the CHARM cohort (Mark *et al.* 2019). Most of the study population reported having first vaginal intercourse before age 13 years (84.2%). Females were more likely to report a sexual debut before age 13 years (86.7 females vs. 77.5%, males, $P = 0.007$). The majority of the participants (64.5%) reported having six or more lifetime sexual partners. Male participants were more likely than females to report six plus sexual partners (53.1% females vs. 78.3% males, $P < 0.001$). There were no significant differences reported for receptive oral or anal sex based on gender. Of the 145 CHARM female participants whose samples were analyzed by ELISA, 126 (86.9%) were heterosexual, 1 (0.7%) was homosexual and 18 (12.4%) were bisexual. Thirty seven (25.5%) reported receptive anal sex. Of the CHARM 120 male participants, 105 (87.5%) were heterosexual, 9 (7.5%) were homosexual, and 6 (5%) bisexual. Thirteen (10.8%) reported receptive anal sex. Overall, 12.0% of the participants reported condom use in the last 3 months and females were significantly less likely to report having sex with partners who used condoms compared to males (7.6% females *vs.* 17.5% males, $P = 0.014$). Most of the CHARM participants were African Americans or of Hispanic/Latino ethnicity.

Factors associated with PID in the females of the CHARM cohort

Of the 145 CT+ female participants from the CHARM cohort, 33 (22.8%) were clinically diagnosed as having PID at the study visit (Table 2). We assessed associations between a PID diagnosis with

Table 3. *Chlamydia* antigenic biomarkers compared between genders (categorized by tertiles).

	Overall	Male N=120 (%)	Female N=145 (%)	P-value[a]
G-rPmpA-F2				0.068
<0.41	89 (33.58)	45 (37.50)	44 (30.34)	
0.41–1.04	88 (33.21)	44 (36.67)	44 (30.34)	
>1.04	88 (33.21)	31 (25.83)	57 (39.31)	
G-rPmpB-F1				0.586
<0.28	89 (33.58)	43 (35.83)	46 (31.72)	
0.28–0.69	88 (33.21)	41 (34.17)	47 (32.41)	
>0.69	88 (33.21)	36 (30.00)	52 (35.86)	
G-rPmpB-F2				0.417
<0.55	90 (33.96)	42 (35.00)	48 (33.10)	
0.55–2.40	87 (32.83)	43 (35.83)	44 (30.34)	
>2.40	88 (33.21)	35 (29.17)	53 (36.55)	
H-rPmpC-N				0.001
<8.75	89 (33.58)	54 (45.00)	35 (24.14)	
8.75–20.47	88 (33.21)	35 (29.17)	53 (36.55)	
>20.47	88 (33.21)	31 (25.83)	57 (39.31)	
H-rPmpD-N				0.195
<1.88	89 (33.58)	47 (39.17)	42 (28.97)	
1.88–4.10	88 (33.21)	38 (31.67)	50 (34.48)	
>4.10	88 (33.21)	35 (29.17)	53 (36.55)	
H-rPmpE-N				0.063
<6.13	89 (33.58)	46 (38.33)	43 (29.66)	
6.13–14.94	88 (33.21)	43 (35.83)	45 (31.03)	
>14.94	88 (33.21)	31 (25.83)	57 (39.31)	
H-rPmpF-N				0.020
<5.52	89 (33.58)	51 (42.50)	38 (26.21)	
5.52–19.85	88 (33.21)	34 (28.33)	54 (37.24)	
>19.85	88 (33.21)	35 (29.17)	53 (36.55)	
H-rPmpG-N				0.199
<0.79	89 (33.58)	44 (36.67)	45 (31.03)	
0.79–3.69	88 (33.21)	43 (35.83)	45 (31.03)	
>3.69	88 (33.21)	33 (27.50)	55 (37.93)	
H-rPmpH-N				0.018
<1.93	89 (33.58)	46 (38.33)	43 (29.66)	
1.93–5.17	88 (33.21)	45 (37.50)	43 (29.66)	
>5.17	88 (33.21)	29 (24.17)	59 (40.69)	
H-rPmpI-N				0.105
<4.19	89 (33.58)	46 (38.33)	43 (29.66)	
4.19–10.68	88 (33.21)	42 (35.00)	46 (31.72)	
>10.68	88 (33.21)	32 (26.67)	56 (38.62)	
G-rClpP				0.709
<0.68	89 (33.58)	39 (32.50)	50 (34.48)	
0.68–2.08	88 (33.21)	38 (31.67)	50 (34.48)	
>2.08	88 (33.21)	43 (35.83)	45 (31.03)	
G-rHsp60				0.952
<4.11	89 (33.58)	40 (33.33)	49 (33.79)	
4.11–13.58	88 (33.21)	39 (32.50)	49 (33.79)	
>13.58	88 (33.21)	41 (34.17)	47 (32.41)	
G-rMOMP				0.011
<1.64	89 (33.58)	51 (42.50)	38 (26.21)	
1.64–4.61	88 (33.21)	32 (25.83)	57 (39.31)	
>4.61	88 (33.21)	38 (31.67)	50 (34.48)	
G-rOmcB				0.830
<1.94	89 (33.58)	40 (33.33)	49 (33.79)	
1.94–4.44	88 (33.21)	42 (35.00)	46 (31.72)	
>4.44	88 (33.21)	38 (31.67)	50 (34.48)	
H-rOmcB				0.835
<7.00	89 (33.58)	38 (31.67)	51 (35.17)	
7.00–24.83	88 (33.21)	41 (34.17)	47 (32.41)	
>24.83	88 (33.21)	41 (34.17)	47 (32.41)	
Ct purified EBs[b]				0.308
<1.96	89 (33.58)	43 (35.83)	46 (31.72)	
1.96–5.22	88 (33.21)	43 (35.83)	45 (31.03)	
>5.22	88 (33.21)	34 (28.33)	54 (37.24)	

[a]P-values from chi-square
[b]*Chlamydia trachomatis* serovar E purified EBs

Table 4. Assessing associations between the levels of the factors (in tertiles) associated with PID diagnosis (tertiles calculated only for females, described on Table S4).

Biomarkers	Second vs. first Unadjusted Odds Ratio (95% CI)	p-value	Third vs. first Unadjusted Odds Ratio (95% CI)	P-value[a]
G-rPmpA-F2	0.66 (0.26–1.67)	0.379	0.58 (0.22–1.50)	0.258
G-rPmpB-F1	0.64 (0.24–1.67)	0.362	0.82 (0.33–2.08)	0.680
G-rPmpB-F2	1.83 (0.70–4.75)	0.214	1.17 (0.43–3.19)	0.760
H-rPmpC-N	0.92 (0.36–2.34)	0.855	0.81 (0.31–2.11)	0.667
H-rPmpD-N	1.03 (0.41–2.59)	0.954	0.71 (0.27–1.89)	0.494
H-rPmpE-N	1.77 (0.70–4.47)	0.225	0.78 (0.28–2.18)	0.636
H-rPmpF-N	0.71 (0.27–1.89)	0.494	1.03 (0.41–2.59)	0.954
H-rPmpG-N	1.57 (0.63–3.89)	0.330	0.59 (0.21–1.68)	0.322
H-rPmpH-N	0.66 (0.26–1.67)	0.379	0.58 (0.22–1.50)	0.258
H-rPmpI-N	2.33 (0.88–6.16)	0.088	1.35 (0.48–3.77)	0.569
G-ClpP	0.92 (0.37–2.29)	0.863	0.55 (0.21–1.49)	0.242
G-rHsp60	1.48 (0.56 –3.93)	0.429	1.48 (0.56–3.93)	0.429
G-rMOMP	1.48 (0.56–3.93)	0.429	1.48 (0.56–3.93)	0.429
G-rOmcB	2.95 (1.03–8.49)	0.045	2.66 (0.92–7.72)	0.072
H-rOmcB	2.62 (0.83–8.21)	0.099	4.83 (1.61–4.47)	0.005
Ct purified EBs[b]	1.52 (0.55–4.20)	0.415	2.11 (0.79–5.63)	0.135
	Second vs. first Adjusted[c] Odds Ratio (95% CI)	P-value	Third vs. first Adjusted[c] Odds Ratio (95% CI)	P-value
G-rPmpA-F2	0.68 (0.26–1.79)	0.432	0.76 (0.27–2.14)	0.606
G-rPmpB-F1	0.80 (0.28–2.25)	0.668	0.98 (0.35–2.69)	0.962
G-rPmpB-F2	2.00 (0.72–5.52)	0.181	1.01 (0.35–2.92)	0.983
H-rPmpC-N	0.98 (0.36–2.66)	0.974	0.85 (0.31–2.33)	0.752
H-rPmpD-N	1.07 (0.41–2.81)	0.892	0.86 (0.31–2.40)	0.774
H-rPmpE-N	2.21 (0.80–6.10)	0.128	1.06 (0.35–3.19)	0.922
H-rPmpF-N	0.77 (0.28–2.16)	0.622	1.38 (0.51–3.75)	0.529
H-rPmpG-N	1.96 (0.74–5.17)	0.175	0.68 (0.22–2.03)	0.486
H-rPmpH-N	0.74 (0.28–1.98)	0.551	0.71 (0.25–1.99)	0.510
H-rPmpI-N	2.52 (0.89–7.11)	0.081	1.77 (0.59–5.36)	0.310
G-ClpP	0.80 (0.31–2.11)	0.657	0.51 (0.18–1.45)	0.208
G-rHsp60	1.28 (0.47–3.49)	0.634	1.40 (0.51–3.87)	0.517
G-rMOMP	1.32 (0.48–3.63)	0.592	1.39 (0.50–3.85)	0.529
G-rOmcB	2.58 (0.87–7.63)	0.087	2.56 (0.86–7.65)	0.091
H-rOmcB	2.31 (0.72–7.46)	0.160	4.22 (1.37–13.01)	0.012
Ct purified EBs[c]	1.59 (0.56–4.57)	0.385	2.23 (0.80–6.24)	0.127

[a]P-values from logistic regression
[b]*Chlamydia trachomatis* serovar E purified EBs
[c]Adjusted for age, sexual partners ever, *Trichomonas vaginalis*, and Bacterial Vaginosis (BV)

various demographic and sexual risk factors. Participants self-reporting previous *T. vaginalis* infection (OR: 2.79, 95% CI: 1.15–6.80, P-value = 0.0230), and previous PID (OR: 19.82, 95% CI: 2.23–176.52, P-value = 0.007) were more likely to be diagnosed with PID at study entry.

The serum Pmp-specific antibody profile of *C. trachomatis*-infected CHARM participants is associated with gender

Chlamydia trachomatis and *C. pneumoniae* purified EBs and recombinant antigen ELISA were used to analyze serum antibodies (IgG) of 265 patients (145 females and 120 males) from the CHARM cohort. Most participants had an antibody response against H-rOmcB, H-rPmpC, G-rHsp60, and H-rPmpI with sensitivity above 85% (Table S3). The antibody response against *C. pneumoniae* purified EBs was very low with only three samples (1.1%) with slightly elevated antibody levels.

Potential association between serum antibody levels (grouped in tertiles) and gender was investigated. Overall, when compared to males, a higher proportion of females were in the top tertile across all Pmps (35.9%–40.7% for females vs. 24.2%–30.0% for males; Table 3). Significant gender differences were observed for rPmps subtypes H-rPmpC-N (39.3% vs. 25.8%, P = 0.001 for females and males, respectively), H-rPmpF-N (36.6% vs. 29.2%, P = 0.020) and rPmpH-N (40.7% vs. 24.2%, P = 0.018). Trending statistical significance was observed for G-rPmpA-F2 (39.3% vs. 25.8%, P = 0.068), and H-rPmpE-N (39.3% vs. 25.8%, P = 0.063). For G-MOMP, a significant proportion of males were in the lower tertile when compared to females (26.2% vs. 42.5%, P = 0.011). Gender-based differences were not detected for other recombinant peptides.

The serum OmcB-specific antibody response is associated with a clinical diagnosis of PID in *C. trachomatis*-infected female CHARM participants

High antibody levels against H-rOmcB (Fig. 2 and Table 4; Figure S3 and Table S4, showing associations between factors (in tertiles) associated with PID diagnosis with tertiles calculated only for females; OR: 2.31, 95% CI: 0.72–7.46, P = 0.160 and OR: 4.22, 95% CI: 1.37–13.01, P = 0.012, respectively) were significantly associated with a clinical diagnosis of PID. Similar results were obtained for
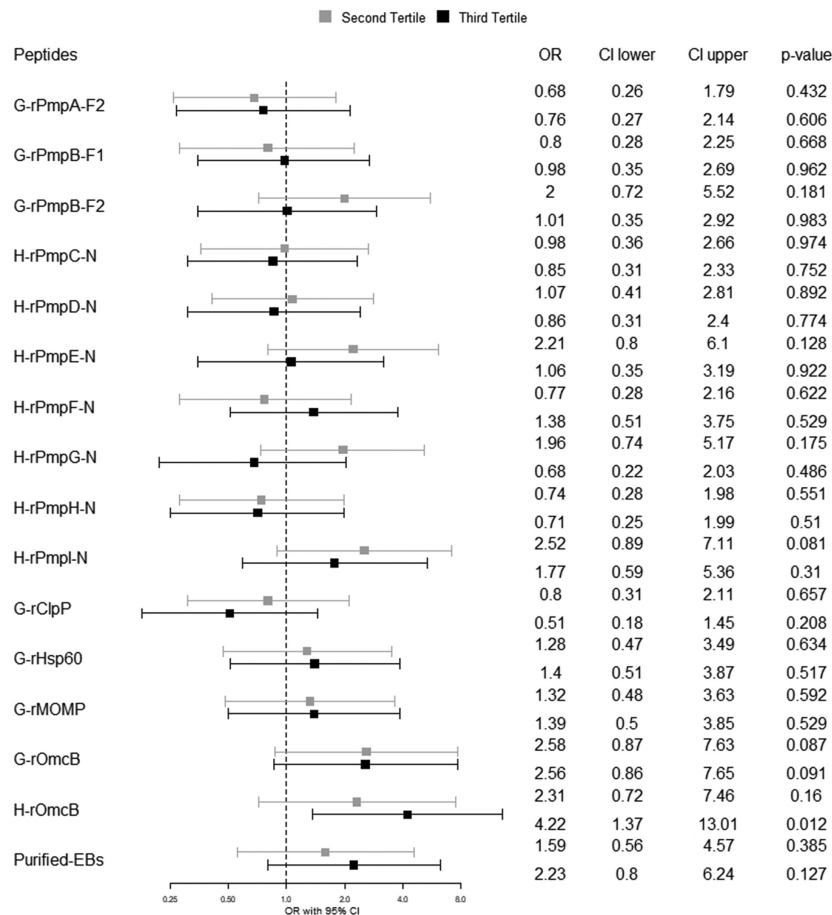
Figure 2. High cut-off index antibodies associated with a PID diagnosis. Sera from 145 female CHARM participants were analyzed by ELISA. Odds ratios (ORs) for PID positivity when a patient is in the second (black) or third (grey) tertile, compared to the first tertile are shown. The OR (95% confidence interval) was greater than o for G-rOmcB in both tertiles and H-rOmcB in the second tertile (*$P < 0.05$) suggesting that high antibody cut-off indexes for this antigen were significantly associated with PID. Results for H-rPmpI-N second tertile were borderline significant (‡ $P = 0.075$).

G-rOmcB (Fig. 2 and Table 4; Figures S3 and S4) associated with PID diagnosis with tertiles calculated only for females; OR: 2.58, 95% CI: 0.87–7.63, $P = 0.087$/second vs. first tertile, OR: 2.56, 95% CI: 0.86–7.65, $P = 0.091$/third vs. first tertile).

In contrast, antibodies to the other tested antigens were not individually associated with PID with any statistical significance. Antibody levels for the second and third tertiles were almost identical for Hsp60 and MOMP (Fig. 2). Similarly, antibody levels against each of the Pmp subtypes were not significantly associated with PID, although the response to H-rPmpI was borderline significant (Fig. 2; OR: 2.52, 95% CI: 0.89–7.11, $P = 0.075$/first vs. second tertile). Interestingly, however, antibody levels against 8 of the 9 rPmp subtypes (all but H-rPmpF) were slightly higher in women without a clinical diagnosis of PID, although none reached statistical significance individually. This suggests a global negative association of antibodies against the Pmp family and upper genital tract disease.

Discussion

An ELISA was developed to measure antibody levels against recombinant polypeptides derived from the nine *C. trachomatis* Pmps, the EB-specific OmcB, MOMP, ClpP, and the stress response protein Hsp60, in 265 chlamydia-positive male and female participants of the CHARM cohort from the Baltimore area. All partic-

ipants exhibited an antibody response to *C. trachomatis* purified EBs and at least one of the Pmps. While female CHARM participants had proportionally higher antibody levels to all Pmps compared to males, statistically significant gender differences were observed for three Pmps (rPmpC, F and H). The higher antibody levels in females are consistent with previous reports of sex differences in antibody responses (reviewed in (Klein and Flanagan 2016)). However, since *C. trachomatis* infection in men and women is diagnosed at different sites (the cervix in women and urethra in men), these findings are also consistent with the hypothesis that different Pmps may be expressed differentially in different anatomical sites and/or physiological environments.

Statistically significant differences between antibody response levels were analysed using categorical variables split in tertiles. Significant differences in anti-Pmp antibody levels were not detected when comparing female CHARM participants with or without a positive clinical diagnosis for PID, indicating that the Pmp-specific antibody response is not significantly altered when the infection ascends to the upper genital tract compared to when it is limited to the lower genital tract. In turn, this suggests that the expressed Pmp profile of *C. trachomatis*-infected women is independent of the site of infection along the reproductive tract. Although Pmp-based differences were not observed, CHARM participants with a clinical PID diagnosis were more likely to have high antibody levels against OmcB than those without. This is

consistent with previous reports of OmcB being associated with CD8+ T cell-mediated upper genital tract immunopathology (Vlcek *et al.* 2016). In contrast, antibody levels were almost identical for Hsp60 and MOMP, suggesting that these antigens are not associated with upper genital tract infection and PID, consistent with previous findings for Hsp60 (Ness *et al.* 2008) and MOMP (Stothard *et al.* 1998). Our study failed to confirm the reported association of ClpP-specific antibodies with upper genital tract disease (Rodgers *et al.* 2010). However, this earlier study involved a different end point (tubal factor infertility) and a smaller cohort.

Overall, the observation of different antibody responses to specific Pmp subtypes in *C. trachomatis*-infected patients relative to gender and/or site of infection, while compatible with the hypothesized site-specific expression of Pmps in the context of infection, falls short of providing indisputable evidence for it, and does not lend confidence that these antigens are exploitable for serodiagnosis. Multiple behavioral, immunological and structural factors have historically confounded or limited serologic analyses of chlamydial antigens for diagnostic purposes. For instance, potential cross-reactivity with orthologous antigens owing to *Chlamydia pneumoniae* (Baud *et al.* 2010) or *Chlamydia psittaci* (Dumke *et al.* 2015) previous or concurrent infections, gender-associated differential immune responses (Klein and Flanagan 2016), co-infection with other STIs, repeat infection with *C. trachomatis* or concurrent infection at multiple sites, as well as non-native structural features of recombinant antigens, are potential limitations of any approach toward reliable sero-diagnosis. Conversely, any statistically significant serologic difference that is detectable above the background 'noise' generated by these confounding factors and limitations should be exploitable as a diagnostic or prognostic tool. This is particularly critical for the development of diagnostic tools for persistent chlamydial infections that are the cause of the most severe sequelae of *C. trachomatis* infection in women (Puolakkainen 2013). In this context, our findings that OmcB-specific serum antibody levels were elevated in women with a PID diagnosis suggests that this antigen is worthy of further study as a biomarker of upper genital tract disease. For instance, the possibility of determining whether an asymptomatic *C. trachomatis*-infected woman is likely to develop or already has subclinical upper genital tract pathology (silent PID; (Wiesenfeld *et al.* 2005)) via a simple measurement of OmcB-specific antibody would be of great benefit to the physician and ultimately to his/her patient. Although cross-reactive antibody responses against *C. pneumoniae* OmcB have been reported (Bas *et al.* 2001), Gijsen et al. (Gijsen *et al.* 2001) also showed that tubal pathology was more common in patients with both *C. pneumoniae* and *C. trachomatis* antibodies compared to patients with antibodies against OmcB from only one *Chlamydia* species, further strengthening a role for this well-conserved chlamydial protein in eliciting pathology.

In conclusion, our expanded analysis of the observed Pmp-specific antibody profile variation in *C. trachomatis*-infected men and women of the CHARM cohort may reflect the hypothesized differential expression of different Pmp profiles as a prerequisite or in response to infection of distinct urogenital sites. However other possibilities exist that suggest that these antigens may be impractical to use as serologic biomarkers. Our study also revealed an association between a high OmcB-specific serum antibody level and a PID diagnosis. This finding corroborates previous studies (Wagar *et al.* 1990) and indicates that this protein may provide an exploitable discriminatory biomarker of disease severity during *C. trachomatis* infection.

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