THE ROLE OF DELAYED CARE SEEKING AND TOLL-LIKE RECEPTORS IN PELVIC INFLAMMATORY DISEASE AND ITS SEQUELAE

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THE ROLE OF DELAYED CARE SEEKING AND TOLL-LIKE RECEPTORS IN PELVIC INFLAMMATORY DISEASE AND ITS SEQUELAE

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Pelvic Inflammatory Disease (PID), the infection and inflammation of the female upper genital tract, can result in serious sequelae. Markers to predict sequelae following PID are greatly needed. The goal of this research is to explore the role of host genetic factors and delayed care seeking in the development of sequelae following clinically suspected PID. We studied the microbial correlates of delayed care and long-term outcomes among 298 women with histologically confirmed endometritis from the PID Evaluation and Clinical Health (PEACH) study. Mean days of pain prior to care were compared by microbial pathogen, with the longest times among women infected by *Chlamydia trachomatis* (CT) only (12.3±9.4 days) and *Mycoplasma genitalium* (MG) only (10.9±8.9 days), and the shortest among women infected by *Neisseria gonorrhoeae* (NG) only (4.6±5 days) or co-infection (5.6±5.1 days, $p<0.001$). Infertility, recurrent PID, and chronic pelvic pain were frequent (17%, 20%, and 36%), albeit non-significantly elevated after delayed care. PID patients infected with CT or MG were more likely to delay care, possibly increasing persistent inflammation which may permanently damage the reproductive tract before patients seek care.

Toll-like receptors (TLR) eliminate microbes through inflammatory responses. As genetic variations may increase TLR signaling, we determined if 18 tagging single nucleotide polymorphisms assayed in 4 TLR genes (TLR1, TLR2, TLR4, TLR6) and 2 adaptor molecules

(TIRAP, MyD88) were associated with CT, endometritis, or infertility among 205 African Americans with PID from the PEACH study. An empirical p-value <0.004 was significant. Logistic regression revealed that the TLR4 rs1927911 CC genotype was associated with CT (odds ratio (OR) 3.7, 95% confidence interval (CI) 1.6-8.8, p=0.0021). Further, the TLR1 rs4833095 TT genotype displayed trends towards increased CT (OR 2.8, 95% CI 1.3-6.2, p=0.0084). Predicted carriers of the TLR4 GTC haplotype (p=0.006) and the TLR1 TGT haplotype (p=0.04) were more likely to be CT positive. Genetic variations in TLR genes may play a role in CT pathogenesis.

This dissertation yields public health significance by demonstrating the need for increased efforts for early identification and treatment of genital tract infections and providing a novel exploration into the role of genetic variants in CT pathogenesis.

TABLE OF CONTENTS

1.0 INTRODUCTION 1

1.1 SPECIFIC AIMS 1

1.2 BACKGROUND 3

1.2.1 Pelvic Inflammatory Disease 3

1.2.2 Microbiological Etiology and Pathogenesis of PID 4

1.2.2.1 *Chlamydia trachomatis* and PID 4

1.2.2.2 Other Organisms and PID 8

1.2.3 Diagnosis of PID 17

1.2.4 Treatment of PID 18

1.2.4.1 Delayed Treatment of PID 24

1.2.5 Morbidity following PID 25

1.2.6 Toll-like Receptors, the Reproductive Tract, and PID 27

1.2.6.1 Toll-like Receptors and *Chlamydia trachomatis* 30

1.2.6.2 Toll-like Receptors and Other PID Organisms 34

1.2.6.3 Toll-like Receptor Polymorphisms 36

1.2.7 Summary 48

2.0 MANUSCRIPT 1: MICROBIAL CORRELATES OF DELAYED CARE FOR PELVIC INFLAMMATORY DISEASE 50

2.1 ABSTRACT 51

2.2 INTRODUCTION 52

2.3 METHODS 53

2.4 RESULTS 57

2.5 DISCUSSION 59

2.6 TABLES 63

3.0 MANUSCRIPT 2: ARE VARIATIONS IN INNATE IMMUNE RECEPTOR GENES ASSOCIATED WITH *CHLAMYDIA TRACHOMATIS* AMONG WOMEN WITH PELVIC INFLAMMATORY DISEASE? 67

3.1 ABSTRACT 68

3.2 INTRODUCTION 69

3.3 METHODS 71

3.4 RESULTS 76

3.5 DISCUSSION 78

3.6 TABLES 85

3.7 FIGURES 92

4.0 MANUSCRIPT 3: TOLL-LIKE RECEPTOR GENE POLYMORPHISMS IN PREGANANCY AND INFERTILITY AMONG WOMEN WITH PELVIC INFLAMMATORY DISEASE 93

4.1 ABSTRACT 94

4.2 INTRODUCTION 95

4.3 METHODS 97

4.4 RESULTS 102

4.5 DISCUSSION 104

4.6 TABLES 108

4.7 FIGURES 114

5.0 CONCLUSIONS 115

5.1 FUTURE RESEARCH 117

5.2 APPLICATION TO PUBLIC HEALTH 119

APPENDIX A : SUPPLEMENTARY FIGURES AND TABLE TO MANUSCRIPT 1 122

APPENDIX B : SUPPLEMENTARY TABLES TO MANUSCRIPT 2 126

APPENDIX C : SUPPLEMENTARY TABLES TO MANUSCRIPT 3 134

BIBLIOGRAPHY 136

LIST OF TABLES

Table 1. Baseline characteristics of participants presenting for care 63

Table 2. Mean time to treatment by organism 65

Table 3. Microbial correlates of participants presenting for care ≥14 days 66

Table 4. Baseline demographic and clinical characteristics by chlamydial status 85

Table 5. Association between genotypes and cervical and/or endometrial *C. trachomatis* infection among women with pelvic inflammatory disease 87

Table 6. Associations between genotypes and endometritis among women with pelvic inflammatory disease 89

Table 7. Associations between TLR haplotypes and *C. trachomatis* 91

Table 8. Baseline demographic and clinical characteristics by pregnancy status 108

Table 9. A ssociations between genotypes and time-to-pregnancy among women with pelvic inflammatory disease 110

Table 10. A ssociations between genotypes and infertility among women with pelvic inflammatory disease 112

Table 11. Effect of delayed care on time-to-pregnancy and time-to-recurrent PID 125

Table 12. Allele and genotype frequencies, of SNPs included in regression analyses, among the entire cohort, chlamydial cases and controls, and outside control groups 126

Table 13. Allele frequencies, of SNPs included in regression analyses, among patients genotyped with buffy coats vs. serum samples 131

Table 14. Associations between selected SNPs and upper genital tract infection..... 131

Table 15. Haplotype associations with endometritis 133

Table 16. Associations between selected SNPs and pregnancy among a subset of women with *C. trachomatis* infection 134

Table 17. TLR1 haplotypes and pregnancy 135

LIST OF FIGURES

Figure 1. Percentage of chlamydial positive women by predicted TLR4 diplotype 92

Figure 2. Percentage of women who achieved pregnancy by predicted TLR1 diplotype 114

Figure 3. Microbial correlates for delayed care among women with clinically suspected PID (n=774)..... 122

Figure 4. Microbial correlates for prompt care among women with clinically suspected PID (n=774)..... 123

Figure 5. Ratio of sequelae among women with histologically confirmed endometritis (n=298) 124

1.0 INTRODUCTION

1.1 SPECIFIC AIMS

Pelvic Inflammatory Disease (PID) is the infection and inflammation of the female upper genital tract and can cause serious reproductive morbidity including infertility, ectopic pregnancy, recurrent PID, and chronic pelvic pain (1-5). PID has a multimicrobial etiology and is associated with *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, and bacterial vaginosis (5-8). There is a significant need for biomarkers to predict morbidity following an episode of PID. However, little is known about differences in morbidity by pathogen (1). One strong predictor of sequelae following PID may be delayed care (1). Further, the delayed care association may vary by microbial pathogen (9). However, these associations have not been replicated in a contemporary cohort and the distribution of microbes among women with PID appears to have changed over time. In addition, new microorganisms including *M. genitalium* have recently been identified, and the care seeking pattern and long term outcomes of *M. genitalium* upper genital tract infection is largely unknown.

Chlamydia trachomatis is a frequent cause of PID and the most common bacterial sexually transmitted disease in the United States (10). There is great variability in the course and outcome of chlamydial infections. Further, the role of host genetic factors in chlamydial PID and post-PID sequelae development is not understood. The goal of the proposed research will be to

explore the relationships between host genetic factors and delayed care seeking in the development of reproductive sequelae following clinically suspected PID. Further, the role of host genetic factors in upper genital tract infection will be explored. As few studies have examined these associations, there is an opportunity to provide novel information regarding the pathogenesis of chlamydial PID and the development of post-PID sequelae.

The following research aims and hypotheses will be used to achieve this goal:

1. Determine the microbial correlates of time to treatment and the impact of delayed care for pelvic inflammatory disease (PID) on long term outcomes (infertility, chronic pelvic pain, and recurrent PID) among 298 women with histologically confirmed endometritis. *We hypothesize that self-reported timing of treatment for a current PID episode will vary by microbial pathogen among women with clinically suspected PID. We hypothesize that women who have Chlamydia trachomatis or Mycoplasma genitalium monoinfection will have waited longer to seek treatment for PID symptoms than women with Neisseria gonorrhoeae monoinfection or co-infection with two or more pathogens. Women who delay care, defined as 14 or more days of pain before seeking treatment, will have an increased risk for reproductive morbidity.*
2. Determine if specific Toll-like receptor (TLR) and TLR adaptor molecule single nucleotide polymorphisms (SNPs) are associated with histologically confirmed endometritis or *Chlamydia trachomatis* among 290 women (205 black, 51 white, 34 other) with clinically suspected PID. *As variations in toll-like receptor genes are suggested to be associated with disease progression, we believe that TLRs and TLR adaptor molecule polymorphisms will be associated with histologically*

confirmed endometritis or Chlamydia trachomatis among women with clinically suspected PID.

3. Determine if specific Toll-like receptor (TLR) and TLR adaptor molecule single nucleotide polymorphisms (SNPs) are associated with infertility or reduced pregnancy following clinically suspected pelvic inflammatory disease (N = 290; 205 black, 51 white, 34 other) *We hypothesize that genetic variations in TLRs and TLR adaptor molecules will be associated with infertility following pelvic inflammatory disease. Variations in innate immune receptors may be responsible for inadequate or overt immune responses, possibly increasing the risk for reproductive morbidity.*

1.2 BACKGROUND

1.2.1 Pelvic Inflammatory Disease

Pelvic inflammatory disease (PID) is the infection and inflammation of the female upper genital tract including the tubes and ovaries (salpingitis) and the uterine lining (endometritis) (1), which can cause serious reproductive sequelae including infertility, chronic pelvic pain, recurrent PID and ectopic pregnancy (2, 3). PID generally occurs when microorganisms ascend from the lower genital tract to the upper genital tract, infecting the uterus, fallopian tubes, and ovaries. PID is a fairly common disease with an estimated 8% of American women developing PID at some time in their reproductive lives (4). It is estimated that 1 million women will be treated annually for PID in the United States (US) (5). However, diagnosis and treatment can be difficult, as

symptoms of PID may be mild or absent. Thus, the condition may be unrecognized (6). Also challenging the diagnosis and management of PID is the multimicrobial etiology of the disease (5). Various organisms have been implicated in the etiology of PID including *Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma genitalium,* and anaerobic and aerobic bacteria commonly associated with bacterial vaginosis (BV) (5-8). Although *C. trachomatis* and *N. gonorrhoeae* have been studied more extensively, the etiology and pathogenesis of PID has not been fully delineated (5).

1.2.2 Microbiological Etiology and Pathogenesis of PID

1.2.2.1 *Chlamydia trachomatis* and PID

Chlamydia trachomatis is the most common bacterial sexually transmitted infection in the United States (10). In women, *C. trachomatis* can ascend from the endocervix to the upper genital tract and cause PID and serious reproductive morbidity including infertility, ectopic pregnancy, and chronic pelvic pain (5, 10). Although *C. trachomatis* is a frequent pathogen associated with PID, and is isolated in 10-27% of PID patients, rates of progression vary widely between patients (10-12). Among high risk populations the progression to PID in asymptomatic *C. trachomatis* positive women is generally low, at 2-4.5% (10, 13-15). Hook et al, in an eight month prospective cross-over trial, found that within 14 days after testing positive for chlamydia, 3.2% of those women developed PID (13). Similarly, Geisler et al conducted a prospective study of 129 adults who screened positive for *C. trachomatis* (15). Among the study population, two women (2%) developed PID within the interval between screening and treatment (15). Further, a retrospective study among 67 women who came in for follow-up care following a positive chlamydia test, three (4.5%) had clinically diagnosed PID (14). Among a lower risk Swedish

population of 109 asymptomatic chlamydial positive adolescents, four (3.7%) reported salpingitis within a three month period (16). Studies have also been conducted among women with treated *C. trachomatis* infection. A prospective study of 1,170 women at high risk for sexually transmitted infections from five U.S. sites found that 23(19%) of 122 women whom tested positive for *C. trachomatis* at baseline developed PID within three years (17).

Data on progression of *C. trachomatis* to reproductive sequelae is limited. *C. trachomatis* antibodies have been found to be more common among infertile women (10). However, there are few prospective studies linking chlamydia to infertility. Haggerty et al, found that among patients with clinically suspected PID, those with endometritis and/or upper genital tract chlamydial or gonococcal infection were no more likely to experience reduced pregnancy or elevated infertility, chronic pelvic pain, or recurrent PID compared to women without endometritis and/or upper genital tract chlamydial or gonococcal infection (1). Bias towards the null is possible as the cohort may have had prior chlamydial infections that resulted in tubal damage preceding the PID episode. In fact a study among this same cohort found that PID recurrence was higher (hazard ratio (HR) 2.48, 95% confidence interval (CI) 1.00-6.27) and pregnancy rates were significantly lower (HR 0.47, 95% CI 0.28-0.79) among women whose antibody titers to chlamydial elementary bodies (EB) collected at the end of the study were in the highest tertile (18). Other studies have found chlamydial infections to be associated with infertility among women diagnosed with PID (10, 19, 20). Brunham et al suggested that among women with PID, those with culture and/or serological evidence of chlamydial infection may have a poor fertility prognosis (19). The authors found that 7 of 13 women with non-gonococcal PID had an adverse reproductive outcome, compared with none out of 10 women with gonococcal PID (P=0.007) (19). Further, of the seven infertile women, four had tubal abscess, and three of the four had

evidence of chlamydial infection. A retrospective study of 51 women with PID found that those who were positive for *C. trachomatis* by culture were more likely to experience involuntary infertility compared to those who tested negative for chlamydia (relative risk (RR) 2.5, 95% CI 1.0-6.2) (20).

As not all women with chlamydial PID develop sequelae, host genetic factors or pathogen-specific virulence factors likely contribute to variability in outcomes. Immune responses to chlamydia specific proteins may be responsible for the progression of *C. trachomatis* infections. It has been hypothesized that immune reactions to chlamydia heat shock protein 60 (cHSP60) may cause chronic inflammation or persistent infection, leading to the progression of disease (21, 22). Several retrospective studies have found cHSP60 to be linked with chlamydia-associated tubal infertility and pelvic inflammatory disease (23-28). For example, Dutta et al, found that among 52 women with PID and infertility, there were significantly higher antibody titers for cHSP60 and cHSP10 when compared to 107 patients with uncomplicated cervicitis at dilutions of 1 in 50, 1 in 250, 1 in 1250 ($P<0.001$) and 1 in 6250 ($P<0.01$) (24). However, prospective data examining this relationship has been limited. One prospective study among 302 female sex workers in Nairobi, Kenya, found cHSP60 antibodies to be associated with PID (odds ratio (OR) 3.9, 95% CI 1.04-14.5; $P=0.04$) (29). However, this study did not examine reproductive sequelae following chlamydial PID. Ness et al, did examine cHSP60 and reproductive sequelae prospectively, but did not find cHSP60 antibody titers to be significantly associated with sequelae following clinically suspected PID (18). This suggests that the retrospective nature of the other studies may have caused them to measure surrogates for prior chlamydial exposure (18). Certain biases, such as diagnostic bias, were also suggested to overestimate the effect size (18). For example, diagnosis of clinically suspected PID based on

symptoms may have been influenced by the knowledge of a previous chlamydial exposure. If cHSP60 is a surrogate for that prior exposure then this would have biased the results away from the null.

Other proteins have been examined in the pathogenesis of *C. trachomatis*. C-reactive protein (CRP) is an acute phase protein and is an indicator of inflammation (26). In a study of 55 patients with PID, CRP >10 mg/1 had good sensitivity and specificity in the diagnosis of PID (30). den Hartog et al found the presence of elevated CRP (> 10 mg/1) to be significantly greater in women with distal tubal pathology than in controls without tubal pathology (50.8% vs. 15.0%; P<0.05) (26). Although CRP is a general marker of inflammation and is not pathogen or condition specific, this suggests that *C. trachomatis* is associated with an increased inflammatory resposnse which may lead to upper genital tract pathology.

Host genetic factors may also play a role in the development of sequelae following PID. It has been hypothesized that human leukocyte antigen (HLA) molecules may control the balance in Th1 (pro-inflammatory) and Th2 (anti-inflammatory) immune response to *C. trachomatis* (31). Studies have also shown a link between HLA class II alleles and tubal factor infertility (TFI) following chlamydial infection. A case control study of 52 women with TFI attending an in vitro fertilization lab found that HLA DQA*0102 and HLA DQB*0602 together with the interleukin (IL)-10-1082 AA genotype were significantly more frequent in the TFI patients than 61 Finnish controls (0.18 and 0.02; p=.005) (31). Similarly, results from the PID Evaluation and Clinical Health (PEACH) Study show that HLA DQA*0301 is associated with chlamydial/gonococcal cervicitis, endometritis and reduced fertility (32). In contrast, a prospective study which followed 113 female sex workers to determine incident *C. trachomatis* infection and PID found that HLA DQA1*0401 and DQB1*0402 alleles were significantly

associated with an increase in Chsp60 antibody (33). However, they did not find any significant associations with microimmunofluorescent antibody titers (MIF) to chlamydia elementrary bodies (33). Further, these alleles did not significantly alter the risk for chlamydial PID (OR 0.84, 95% CI 0.15-4.60; P=0.84). The authors contributed this finding to their small sample size, suggesting that further studies are needed to delineate this relationship (33). The authors note that in mice, cHSP60 antibody is influenced by genes (33). Therefore, HLA genes may indeed be correlated with response to cHSP60. However, the contrasting results over associations with PID may be due to genetic differences in study populations as well as differences in the comparison groups used in the studies. As little is known about the natural history and pathogenesis of untreated chlamydial infections, future studies should continue to examine the role of host genetic influences in the progression of *C. trachomatis*.

1.2.2.2 Other Organisms and PID

C. trachomatis and *N. gonorrhoeae* are the two most common reportable diseases in the United States (US), and chlamydial infection often accompanies 20% to 40% of gonococcal infections (34). In the US there was a steady decline in the number of reportable cases of *N. gonorrhoeae* between 1975 a nd 1997 (34). Since then, the rates have begun to plateau (34). Still, *N. gonorrhoeae* is considered a prevalent sexually transmitted infection and can lead to serious reproductive morbidity. *N. gonorrhoeae* has been isolated from the cervix, endometrium and fallopian tubes of women with PID (35-37). Women with *N. gonorrhoeae* and PID tend to have onset of pain in the first part of the menstrual cycle, suggesting that loss of the cervical mucus plug may promote ascension into the upper genital tract (36). Approximately 10-19% of women with *N. gonorrhoeae* isolated in the cervix have signs of clinical PID (36). Further, among PID

cases with positive lower genital tract *N. gonorrhoeae* culture, approximately 42% will have *N. gonorrhoeae* isolated from the Fallopian tubes (36).

While serological studies suggest a role for chlamydial PID in the development of sequelae, fewer studies have addressed the importance of other microorganisms such as *N. gonorrhoeae*. Although, serological testing exists for *N. gonorrhoeae* this method is not widely used. It is suggested that long term sequelae occurs more frequently among those with non-gonococcal PID, as those with *N. gonorrhoeae* present with more overt symptoms and acute inflammation and are possibly treated earlier (36, 38). In a study of 82 w omen with laparoscopically confirmed salpingitis *N. gonorrhoeae* in the upper genital tract appeared to be isolated more frequently in women with patent tubes (21/39; 54%) compared to women with tubal occlusion (4/14; 29%) (39). However, no statistically significant associations were found between *N. gonorrhoeae* and tubal disease severity. The authors report that low statistical power may have limited their ability to detect differences between groups. The study did find that women with gonorrhea were more likely to have free exudate compared to no exudate or exudate limited to the tubes (P=0.04) (39). Severity of tubal damage was negatively associated with the presence of free pelvic-abdominal exudate (39). Further, women with free exudate tended to present earlier after the onset of symptoms and presented with signs of peritonitis and elevated white blood cell (WBC) count, suggesting an earlier stage of acute infection (39). Still, *N. gonorrhoeae* has been isolated from women with infertility. Miettinen et al reported that among 76 women with tubal factor infertility, 14% tested positive for *N. gonorrhoeae* and 46% tested positive for *C. trachomatis* (40). Among these women, 26% of gonococcal positive women and 58% of chlamydial positive women reported a history of PID (40). In a Nigerian study, *N.*

gonorrhoeae was isolated in 17.4% of infertile women compared with 10.5% of pregnant women ($P<0.05$) (41).

Like *C. trachomatis*, host immune responses and bacterial virulence factors may play a role in the progression of *N. gonorrhoeae* infection. *N. gonorrhoeae* is considered to be versatile and can undergo a great degree of antigenic and phase variation (42). The mechanisms responsible for the progression of *N. gonorrhoeae* to PID and subsequent sequelae have not been completely delineated. It is known that in the Fallopian tubes ciliary activity is significantly decreased after exposure to gonococcal supernatant (36). Further, gonococcal infection can induce cell death in the fallopian tubes through necrosis, which then will trigger an inflammatory response and apoptosis (43). Aggressive inflammatory responses in the fallopian tubes are also produced when host antibodies bind to gonococcal lipooligosaccharide and petidoglycan (44). This may in part explain why women with gonococcal PID have more overt symptoms and acute inflammation, compared to those with *C. trachomatis* associated-PID who have less severe symptoms and longstanding low level inflammation (36).

Gonococcal proteins have been examined to determine their role in PID and subsequent sequelae. In a cohort of 243 sex workers from Nairobi, Kenya, antibodies to opacity proteins (Opa) were found to significantly decrease the risk of gonococcal salpingitis (OR 0.35, 95% CI 0.17-0.76) (45). However, the reduction in risk was only statistically significant for two Opa variants (1B-5 and 1A-6) (45). The authors also found the risk of salpingitis to be dramatically reduced in women with seropositivity to all nine Opa variants compared to women with antibody to less than one (RR 0.13, 95% CI 0.02-0.96, $p<0.01$) (45). In a separate study, Plummer et al reported that antibody response to outer membrane protein 3 (Rmp) was associated with an increased risk of gonococcal salpingitis (OR 3.4, 95% CI 1.1-10.4, $P < 0.05$), although this was

of borderline significance (46). Antibodies to gonococcal pili have also been examined in PID. One study of 35 women with PID and 115 normal controls found no difference in antibody response (IgM, IgG, and IgA) to gonococcal pili between groups (47). Despite the potential role for several *N. gonorrhoeae* proteins in virulence, management of the disease through vaccine development has been unsuccessful (48). Further investigation into both bacterial virulence factors and host immune response to *N. gonorrhoeae* infection is needed.

Chlamydia and gonorrhea are identified in approximately a third to a half of cases of PID. Thus, up to 70% of PID can be non-gonococcal and non-chlamydial in nature (5). Bacterial vaginosis (BV) is characterized by an imbalance in vaginal microflora, and occurs when hydrogen producing lactobacilli decrease in concentration and are replaced by anaerobic and facultative aerobic bacteria including *Gardnerella vaginalis* and *Mycoplasma* organisms (49). BV can be diagnosed in several ways. Amsel's criteria requires three of the following four criterion: 1) homogenous, thin, white discharge that smoothly coats vaginal walls; 2) presence of clue cells on microscopic examination (> 20 percent of epithelial cells with adherent bacteria); 3) pH >4.5; and 4) upon slide preparation a positive whiff test (production of a fishy odor when 10% potassium hydroxide is added) (6). Gram stain can also be interpreted using Nugent's criteria to determine the relative concentration of lactobacilli, Gram-negative and Gram-variable rods and cocci (*G. vaginalis, Prevotella, Porphyromonas*, and peptostreptococci) and curved Gram-negative rods (Mobiluncus) (6). However, this method cannot detect organisms such as ureaplasmas and mycoplasmas (50). Recently, cultivation independent studies using 16S rDNA sequences, polymerase chain reaction (PCR) amplified from vaginal DNA have found a range of novel bacteria associated with BV including *Atopobium vaginae*, *Leptotrichia sanguinegens*,

Leptotrichia amnionii, and three new species called Bacteria Vaginosis Associated Bacteria (BVAB) 1, 2, and 3 (50-52).

BV diagnosed by Nugent's criteria has been associated with clinically suspected PID (5, 53-55). Haggerty et al, found the association between BV and acute endometritis to be independent of *C. trachomatis* and *N. gonorrhoeae* (OR 2.4, 95% CI 1.3-4.3) (7). Further this study found significant associations between endometrial diptheriods, black-pigmented gram-negative rods, anaerobic gram-positive cocci, and acute endometritis independent of *N. gonorrhoeae* and *C. trachomatis* (7). BV is frequent among women with *N. gonorrhoeae* and *C. trachomatis* infections (56), but it is unclear whether anaerobes and facultative bacteria cause PID or if they ascend as a consequence of *N. gonorrhoeae* or *C. trachomatis* infection (57, 58). In a prospective study of 1,179 women from the Gynecologic Infection Follow-Through (GIFT) study, Ness et al reported no increase in the risk of developing incident PID over a three year period among women with BV diagnosed by Nugent criteria after adjustment for *C. trachomatis* and *N. gonorrhoeae* infection (HR 0.89, 95% CI 0.55-1.45) (57). However, acute carriage of pigmented, anaerobic gram negative rods was associated with PID (57). Further analysis in this cohort found that women with the highest growth of a cluster of BV-associated microorganisms (Lactobacillus, *Gardnerella vaginalis*, *Mycoplasma hominis*, pigmented and non-pigmented anaerobic gram negative rods, and *Ureaplasma urealyticum*) were significantly more likely to develop PID (RR 2.03, 95% CI 1.16-3.53) (58).

BV is a polymicrobial condition and it may be optimal to examine the relationships between individual BV-associated bacteria and PID. A pilot study among the PEACH population, found that women with clinically suspected PID infected with fastidious bacteria *L. sanguinegens/amnionii, A. vaginae*, and BVAV1 were more likely than women testing negative

by PCR to have BV (50). As these bacteria were not associated with vaginal discharge, cases of BV caused by these organisms may go undetected (50). This could leave women untreated possibly increasing their risk of PID. Further, not all PID treatment provides coverage against BV-associated bacteria. The Centers for Disease Control (CDC) does recommend an optional treatment of metronidazole for coverage against a broader spectrum of microorganisms (6). However, if BV goes undiagnosed among women with PID and metronidazole is not administered, treatment may be suboptimal for a number of women.

The pathogenesis of BV is not well understood and to date no single host factor has been found to increase susceptibility. The course and outcome of BV varies widely. It has been suggested that BV may increase the susceptibility of the upper genital tract to bacterial invasion through production of enzymes and cytokines (36, 59). In a clinical trial of 102 w omen randomized to either oral or vaginal metronidazole for BV, cervical levels of IL-1beta, IL-6, and IL-8 were significantly decreased after treatment among 72 women cured of BV ($P<0.001$, $P=0.001$, and $P=0.2$) (60). This study suggests a link between BV and elevated cytokine levels. In fact, Sturm-Ramirez et al found that cytokine levels, such as tumor necrosis factor-alpha (TNF-α) and IL-1β were significantly associated with BV (OR 4.17, 95% CI 1.69-10.30) among 196 women (61). Cauci et al, examining 51 w omen with BV and 60 healthy controls found similar results. Vaginal IL-1β concentrations were significantly higher in BV positive women ($P<0.001$) (62). However, there was no difference in IL-8 concentrations between BV positive women and healthy controls ($P=0.189$) (62). The authors suggested that this finding may explain the low inflammation seen in many BV positive women as low levels of IL-8 may be responsible for low counts of leukocytes (62). Further, the decrease in recruitment of neutrophils found in this study may impair the defense system of the vaginal mucosa (62). Collectively these studies

and others may suggest a role for host immune response in the development and progression of BV. However, the mechanisms leading to these observed responses need to be further investigated.

Mycoplasma genitalium is a sexually transmitted bacterial pathogen that is associated with non-gonococcal chlamydia-negative urethritis, mucopurulent cervicitis, endometritis, PID, and tubal factor infertility in humans (5, 63-69). *M. genitalium* was first isolated from the urethral discharge from men with non-gonococcal urethritis (66, 68). As *M. genitalium* is difficult to culture, it wasn't until recently when PCR became available that researchers began to examine this microorganism in women. A study of 170 w omen presenting with genital symptoms (abnormal vaginal discharge, dysuria, or pelvic pain) and attending a sexually transmitted disease clinic in France detected *M. genitalium* by PCR in 38% of women (68). *M. genitalium* has also been associated with cervicitis (66, 69), and PID (63-67). While *M. genitalium* has been detected frequently in women with gynecological conditions, it was found to be absent in healthy pregnant and non-pregnant women as well as women without endometritis (65, 68, 69). Work by Simms et al, suggests that the association between *M. genitalium* and PID is independent of chlamydial infection (64). Using PCR, the authors found that 13% (6/45) of women with PID had evidence of *M. genitalium* compared to none (0/37) of the controls (64). Further 27% (12/45) had *C. trachomatis* detected in the endocervix compared to none of the controls, and 16% of cases and 5% of controls had serological evidence of *C. trachomatis* infection (64). A study of 115 w omen in Nairobi, Kenya detected *M. genitalium, N. gonorrhoeae,* or *C. trachomatis* in the cervix, endometrium, or both in nine (16%), nine (16%), and four (7%) of 58 w omen with histologically confirmed endometritis and in one (2%), four (7%), and two (4%) of 57 women without endometritis (P=0.02 for *M. genitalium*) (65). Further

analysis from this group found among 126 women with acute salpingitis, *M. genitalium* was detected in 7% (9/126) of women, while *N. gonorrhoeae* was detected in 16% (21/126) and *C. trachomatis* in 6% (8/126) (63). A pilot study using data from the PEACH study detected *M. genitalium* in 14% (7/50) of women with clinically suspected PID (67). A more recent study from this group of 682 women found that *M. genitalium* was associated with baseline endometritis (OR 2.6, 95% CI 1.5-4.6) (8). After adjustments for age, race, *N. gonorrhoeae*, and *C. trachomatis,* results were similar (OR 2.0, 95% CI 1.0-4.2) (8). In addition, the authors reported that those whom tested positive for *M. genitalium* had an increased risk for short term treatment failure, defined as the presence of both endometritis and pelvic pain 30 days following treatment for PID, compared to those whom tested negative (RR 4.6, 95% CI 1.1-20.1) (8). Further, 44% of women with baseline endometrial positive specimens tested positive at 30 days post-treatment (8). Interestingly, among these women with mild to moderate PID, those with *M. genitalium* monoinfection had significantly less pelvic pain than women with *N. gonorrhoeae* (58.0±21.9 vs. 72.3±23.9; P=0.01), but not *C. trachomatis* (P=0.05).

It is unclear what the risk of sequelae is following *M. genitalium*-associated PID. Clausen et al examining 308 women with tubal factor infertility undergoing invitro fertilization (IVF), reported that 22% (29/132) displayed seropositivity to the major adhensin protein, MgPa, of *M. genitalium* compared to 6.3% of women with normal tubes (70). Among this same group, a prospective study examining 212 couples attending infertility clinics found that 17% of women with tubal factor infertility were seropositive to *M. genitalium* compared to 4% of women with normal tubes (71). Among these women 14%, who were seropositive for *M. genitalium,* reported a history of PID. However, detecting *M. genitalium* by serology can be difficult due to its cross-reactivity with *M. pneumoniae* (72). Using PCR, in the PEACH study, rates of sequelae were

higher among those testing positive for *M. genitalium*; 22% infertility, 31% recurrent PID, 42% chronic pelvic pain (8). Although there were increasing trends towards these sequelae they were non-significant; infertility (RR 1.4, 95% CI 0.6-2.9), recurrent PID (RR 1.6, 95% CI 0.8-3.1, and chronic pelvic pain (RR 1.6, 95% CI 0.3-1.3) (8). However, all women in this study had signs and symptoms of PID and all may have been at increased risk for reproductive sequelae biasing the results towards the null.

Few studies have examined the pathogenesis of *M. genitalium*. However, it is known that *M. genitalium* has the ability to produce pathological changes although it generally takes a more chronic course. *M. genitalium* has several adhesion proteins which are clustered in a tip structure (73). Among these proteins MgPa and P110 are considered to be immunogenic and are needed for adherence to host cells (72). These proteins are located on the cell surface and are potential targets for host antibodies. However, *M. genitalium* has been shown to be able to persist for long lengths of time in an infected individual (72, 74). Thus, *M. genitalium* is thought to be able to evade the host immune response through antigenic variation (72, 74). Less is known about the role of host response to *M. genitalium* in the course and outcome of infection. In the case of *M. pneumoniae,* it has been suggested that much of the tissue damage may actually be due to the host cell response (75). Further, *M. pneumoniae* lipids have been found to adhere to surface protein D (76), which is part of the innate immune system and may enable the organism to use the host defense mechanism for pathogenesis (77). As *M. genitalium* is genetically similar to *M. pneumoniae*, it ma y have similar functions (77). It is imperative that research continue to investigate the role of host immunity in *M. genitalium* upper genital tract infection, as these relationships have not been completely elucidated.

1.2.3 Diagnosis of PID

Diagnosis of PID is often difficult as signs and symptoms vary, may be mild or absent, and are generally based on clinical findings (5, 6). The CDC recommends that empiric treatment of PID be initiated in women at risk for sexually transmitted infections if they have pelvic or lower abdominal pain, and one or more of the following are present; cervical motion tenderness, uterine tenderness, or adnexal tenderness (6). To enhance the specificity the following criteria can be used; oral temperature > 101°F, abnormal cervical or vaginal mucopurulent discharge, presence of white blood cells, elevated erythrocyte sedimentation rate (ESR), elevated C-reactive protein and laboratory documentation of cervical infection with *N. gonorrhoeae* or *C. trachomatis* (6). These guidelines aim to diagnose with a high sensitivity at the expense of low specificity, in order to treat women early and prevent sequelae. These criteria have a positive predictive value to detect salpingitis of 65%-90% compared with laparoscopy (6). The PEACH study has shown that adnexal tenderness has a sensitivity of 96% for the detection of endometritis (77). In a recent study by Yudin et al, vaginal neutrophils had a sensitivity of 90.9% and a negative predictive value of 94.5% in the diagnosis of upper genital tract infection (78). However, this method had a low specificity of 26.3% and a low positive predictive value of 17.1% (78).

The most specific methods for the diagnosis of PID include the following; endometrial biopsy with histopathologic evidence of endometritis, transvaginal sonography, magnetic resonance imaging, or laparoscopy (6). Laparoscopy is considered to be the gold standard; however this method is invasive and not widely used in the United States (6). Further, laparoscopy is not standardized, is subjective, and will not detect endometritis or possibly even subtle inflammation in the Fallopian tubes (6). Laparoscopy was also found to have low

sensitivity for the diagnosis of PID (25-50%) when compared to fimbrial minibiopsy (79, 80). A good alternative for laparoscopy is histologically confirmed endometritis. Endometrial biopsy has been found to have a sensitivity of 70-89% and a s pecificity of 67-92% compared to laparoscopy (81-83). Due to the difficulties of diagnosis of PID, new methods have been developed. Transvaginal Doppler ultrasound which detects hyperaemia associated with fallopian tube inflammation is reported to have a high sensitivity and specificity (100% and 80%) compared to laparoscopy (84). Although it is not widely available, magnetic resonance imaging (MRI) can also be used as an alternative diagnosis of PID. Compared to laparoscopy, MRI is estimated to have a sensitivity of 95% and specificity of 89% for the diagnosis of PID (85). Endovaginal sonography is another minimally invasive procedure that may improve diagnosis of PID, although it has a lower sensitivity (32-81%) when compared to laparoscopy (86).

1.2.4 Treatment of PID

It is recommended that PID treatment provide a broad spectrum of coverage against *C. trachomatis, N. gonorrhoeae,* mycoplasmas and anaerobic and aerobic bacteria (6). Most PID patients are treated as outpatients. Findings from the PEACH study demonstrated that among women with mild to moderate PID, there is no difference in clinical and microbiological cure rates or rates of long term outcomes between inpatient and outpatient treatment of PID (87). However, the CDC recommends hospitalization for certain women, such as those with surgical emergencies (not excluding appendicitis), pregnant patients, those who do not respond clinically to oral antimicrobial therapy, those who cannot tolerate oral outpatient regimens, patients who have severe illness, nausea, or vomiting, and those with tubo-ovarian abscess (6). For inpatient treatment, the CDC recommends regimens of cefotetan (2g IV every 12 hours), cefoxitin (2g IV

every 6 hours) plus doxycycline (100mg orally or IV every 12 hours), or clindamycin (900mg IV every 8 hours) plus gentamicin (2mg/kg of body weight loading dose IV or IM followed by 1.5mg/kg maintenance does every 8 hours or 3-5mg/kg single daily dose). Gentamicin can also be given as a single daily dose, although there are no trials of once daily gentamicin dosing in PID (6). However, the regimen of clindamycin plus gentamicin has better coverage for anaerobic infections and facultative Gram-negative rods (88). Some studies have examined the efficacy of inpatient treatment trials, although most have focused on outpatient treatment. One study among 103 patients with PID randomized to ampicillin-subactam or cefoxitin (2g every 6 hours) plus doxycycline (100mg every 12 hours), found that the clinical cure/improvement rates were similar (85.5% and 89.6%) (89). In another study of 84 women with PID, IV meropenem had similar clinical cure rates (88% vs. 90%) and microbiological cure rates (88% vs. 86%) compared to clindamycin plus gentamicin (90).

For outpatient therapy, the CDC recommends several regimens including levofloxacin (500mg orally 1/day for 14 days), ofloxacin (400mg orally 2/day for 14 days), ceftriaxone (250mg IM single dose) plus doxycycline (100mg orally 2/day for 14 days), cefoxitin (2g IM single dose and probenecid 1g orally single dose) plus doxycycline (100mg oral 2/day for 14 days), or parenteral third generation cephalosporins plus doxycycline (100mg oral 2/day for 14 days) (6). The CDC provides the option that all recommended regimens can be given with metronidazole (500mg orally 2/day for 14 days) to provide coverage against anaerobes (6). In a meta-analysis, cefoxitin and doxycycline used in the PEACH study and the treatment recommended by CDC, was found to have a clinical cure of 93% and a microbiological cure of 98% for *C. trachomatis* and *N. gonorrhoeae* (91). In this same meta-analysis the clinical cure and microbiological cure rates for clindamycin and aminoglycoside, cefotetan and doxycycline,

and ciprofloxacin were 92% and 97%, 94% and 100%, and 94% and 96%. In general many trials have focused on ofloxacin due to its in vitro activity against *C. trachomatis*, *N. gonorrhoeae*, and anaerobic and aerobic bacteria (5). In one randomized clinical trial, oral ofloxacin was compared with cefoxitin followed by doxycycline among 295 women with clinically suspected PID and reported high rates of clinical cure (95% vs. 93%) and 100% *N. gonorrhoeae* eradication (92). Ofloxacin was associated with a higher microbiological cure rate for *C. trachomatis* (100% vs. 88%) and lower prevalence of side effects (7% vs. 14%). Ofloxacin has also been found to eradicate a range of anaerobic and aerobic pathogens (93). The CDC suggests that levofloxacin can be substituted for ofloxacin. In a pilot study, levofloxacin plus metronidazole, administered to 40 women with uncomplicated PID, has a 37% clinical cure rate and 100% microbiological cure (94). At follow-up 100% of patients were clinically cured and side effects were minimal. This study suggested that levofloxacin was well tolerated and effective for treatment of PID.

The multimicrobial nature of PID makes it difficult to successfully treat PID. Although about a third to a half of PID cases are caused by chlamydia or gonorrhea (5, 36), many cases are non-gonococcal and non-chlamydial in nature. For example BV, anaerobic bacteria, and mycoplasmas have been implicated in the etiology of PID (5, 7, 8, 36). Further, the microbiological etiology of many cases of PID is unknown. Metronidazole can optionally be added to all CDC recommended regimens to provide coverage against anaerobes (6). This is recommended as studies have found that BV-associated microorganisms are associated with PID and endometritis (5, 7, 54, 56, 58). However, in the same meta-analysis mentioned previously, a regimen consisting of metronidazole and doxycycline was found to have low efficacy, with a clinical cure rate of 75% and a microbiological cure rate of 71% (91). Studies conducted after the meta-analyses have also examined the efficacy of metronidazole. In one study, combined therapy

of doxycycline and metronidazole also exhibited a low clinical cure rate (35%) and clinical improvement rate (50%) among 40 women with laparoscopically diagnosed salpingitis (95). In a study of 135 women with PID from the United Kingdom (UK), the clinical cure rate of doxycycline and metronidazole was only 55%, although the addition of ceftriaxone increased the cure rate to 72% (96). The combination of doxycycline and metronidazole does not provide sufficient coverage for *N. gonorrhoeae*, as doxycycline itself is less effective without the addition of other drugs including cefoxitin. In a more recent analysis among women with uncomplicated PID, the cure rate for doxycycline (100mg 2/day) and metronidazole (400mg 3/day for 14 days) plus one single dose of 500mg ciprofloxacin was higher at 98% 2-14 days post therapy and 93.8% 21-35 days post therapy (97). However, the microbiological cure rate was only 88.2%, 2-14 days post therapy, with 95.5% *C. trachomatis* eradication and 91.7% *N. gonorrhoeae* eradication. In another randomized trial, women who received ofloxacin (14mg 2/day) plus metronidazole (400mg 1/day) for 14 days had a clinical resolution rate of 90.7% 5-24 days post therapy (98). During follow-up (28-42 days post therapy) the resolution rate was 87.9% and microbiological cure rate was 82.1% (98). Specifically the cure rates against *C. trachomatis* and *N. gonorrhoeae* was 85.7% and 81.8%.

Data from the National Hospital Ambulatory Medical Care Survey reported in 2004 that the compliance rate with the CDC PID treatment regimens is only 35% among women attending emergency departments (99). This is of great concern, especially for teenagers and young adults as they are at an increased risk for sexually transmitted diseases and PID. Therefore, short term monotherapies may be important for those with low compliance, such as teenagers and women of low socio-economic status (SES) (5). Some studies have examined single or short-term therapies for treatment of PID. In a randomized clinical trial of 165 Indian women with clinically

suspected PID, a regimen of fluconazole (1 tablet 150mg), azithromycin (1 tablet 1g) and secnidazole (2 tablets 2g) had a clinical cure rate of 94% (100). This was similar to a group who was treated with 500mg of ciprofloxacin and 600mg of tinidazole for 7 days (96%), and better than a group treated with 100mg of doxycycline (2/day) and 200mg of metronidazole (3/day) for 7 days (91%) (100). A study among 309 patients with clinically suspected PID (75% with laparoscopically confirmed PID) from the United Kingdom, compared azithromycin monotherapy (500mg IV single dose followed by 250mg oral dose for 6 days), and azithromycin in combination with metronidazole, with standard regimens (101). This study found similar rates of clinical success among the azithromycin monotherapy group (97%) compared to azithromycin and metronidazole (98%) and the comparator regime consisting of metronidazole, doxycycline, cefoxitin, and probenecid or doxycycline and amoxicillin and clavulanate (75%). Azithromycin is also suggested to be effective for treatment of anaerobes. In a study of 106 women with mild PID who received intramuscular injection of 250mg of ceftriaxone, azithromycin was found to have a better clinical cure rate (98.2% vs. 85.7%) compared to doxycycline (102).

There is numerous data on clinical and microbiologic cure following a variety of PID treatments with various regimens. However, data on the efficacy of PID treatment regimens in the prevention of sequelae is limited, as few studies have examined reproductive morbidity after PID treatment. Data from the PEACH study shows that among women treated with cefoxitin and doxycycline, endometritis and upper genital tract infection with chlamydia or gonorrhea was not associated with reproductive morbidity (1). This may suggest that current PID treatments are effective for the prevention of reproductive sequelae (1, 5). However, this study may have been biased as women could have had previous infections or PID that resulted in tubal damage prior to enrollment (1). Heinonen et al, found that among women treated for salpingitis with a regimen of

doxycycline plus metronidazole 28% had recurrent PID at follow-up (103). However, 89% of these women reported conception, which again may suggest the PID treatment is effective. It should be noted that the average time to pregnancy was 38 months, with greater times among women with severe salpingitis. Infertility is defined as lack of conception within 12 months; therefore many of these women would have been considered infertile (5). Further, high conception rates could be attributed to fertility treatments (5). Therefore, it is suggested that women with PID (chlamydial, gonococcal, or non-chlamydial/gonococcal), despite treatment may be at higher risk for reproductive morbidity than women without PID (5). There may also be subgroups of women who are more likely to have sequelae following PID. Those with non-gonococcal PID were more likely to experience sequelae in PEACH (infertility rates: *N. gonorrhoeae* 13%, *C. trachomatis* 19%, anaerobic bacteria 22%, *U. urealyticum* 27%, *M. hominis* 17%) (5). Similar results by Brunham et al., reported that 54% of women with non-gonococcal infections suffered adverse reproductive sequelae (19). Further, in the Heinonen study, among those with recurrent PID at follow-up, two had *C. trachomatis*, two had *N. gonorrhoeae*, and seven had unknown etiology (103). This suggests that among women with non-gonococcal PID, standard PID treatment may not be effective in the prevention of long term sequelae.

The current recommended therapies for PID may not provide sufficient coverage against all PID-associated pathogens. Haggerty et al, reported that among women treated with cefoxitin and doxycycline, women who tested positive for *M. genitalium* had an increased risk for short term treatment failure compared to those whom tested negative for *M. genitalium* (RR 4.6, 95% CI 1.1-20.1) (8). Studies among men with non-gonococcal urethritis found the *M. genitalium* can persist after treatment with levofloxacin (96, 102) and tetracyclines (104, 105). It has been

reported that in vitro *M. genitalium* is susceptible to azithromycin but not doxycycline or ciprofloxacin (106). However, in a study among 120 m ales and females infected with *M. genitalium* and treated with azithromycin, *M. genitalium* was reported to be eradicated in 84% and persisted in 16%, although 8% of those with persistent infection may have been at risk from untreated or doxycycline treated partners (107). Among those with persistent infection, moxifloxacin was effective in eradicating *M. genitalium* completely. Moxifloxacin has been studied in women with PID. Moxifloxacin monotherapy (400mg 1/day for 14 days) was found to have similar clinical resolution rates 5-24 days post treatment (90.2% vs. 90.7%) and similar clinical resolution rates 28-42 days post treatment (85.8% vs. 87.9%) compared to oral ofloxacin (400mg 2/day) plus metronidazole (500mg 2/day) (99). Microbiological cure rates were slightly higher for the moxifloxacin group (87.5% vs. 82.1%). In another study moxifloxacin also had similar clinical cure rates (96.6% vs. 98.0%) and microbiological cure rates (92.5% vs. 88.2%) compared to women treated with doxycycline and metronidazole (98).

1.2.4.1 Delayed Treatment of PID

Women who delay seeking treatment for PID may be at increased risk for adverse sequelae. For example, those who delay care could have chronic low level inflammation which may permanently damage the reproductive tract prior to receiving treatment. Among women with PID, pelvic pain may be mild or absent (55), and PID symptoms vary by microbial etiology (36, 108-110). Therefore, PID may go undetected in many women. Chlamydial infections are known to elude detection because they produce few or no symptoms or because the symptoms are non-specific (111). Further, in chlamydial salpingitis, clinical manifestations are often mild but associated with severe tubal damage (36, 111). This is in contrast to gonococcal salpingitis which produces more overt and severe acute inflammation and symptoms but leads to less damage to

the fallopian tubes (36). In fact, *C. trachomatis* has been found to increase the odds of subclinical "silent" PID six-fold (OR 6.1, 95% CI 1.8-2.1) compared to a four-fold increase among women with *N. gonorrhoeae* (OR 3.9, 95% CI 1.7-8.9) (108). Studies have reported similar symptoms between *C. trachomatis* and *M. genitalium* (109, 110). This may indicate that among women with PID, those infected with *N. gonorrhoeae* present with more overt and severe symptoms, leading to earlier treatment than women with *C. trachomatis* or *M. genitalium* (36). In fact, Hillis et al reported that delayed treatment for more than 3 days increases the risk of impaired fertility following an episode of PID (9). In this case-control study nested within a Scandinavian cohort conducted between the 1960s and early 1980s (3), delayed care was associated with a three-fold increase in infertility or ectopic pregnancy among 443 women with one known episode of clinical or laparoscopically confirmed PID (9). Further, the association was strongest among 114 women infected with *C. trachomatis*, with 17.8% of *C. trachomatis* positive women who delayed seeking care experiencing impaired fertility compared to 0% who sought care promptly (9). However, these associations have not been replicated in a contemporary cohort, and the distribution of microbes among women with PID appears to have changed over time. Therefore, the microbial correlates of time to treatment and long term outcomes among PID patients should continue to be examined.

1.2.5 Morbidity following PID

PID can cause serious reproductive sequelae such as infertility, chronic pelvic pain, and ectopic pregnancy (3, 5). These complications can result from damage to the cilia lining of the Fallopian tubes, Fallopian tube blockage, or adhesion formation among pelvic organs (5). A landmark Scandinavian cohort study among 2500 women with clinically suspected PID, found that 16% of

women with laparoscopically verified salpingitis versus 2.7% of controls became infertile (3). In addition, 20% and 25% developed recurrent PID and chronic pelvic pain respectively (2). Tubal factor infertility was also found to double with each PID episode and reached an estimated 40% after three or more episodes (3). Results from the PEACH study found that the mean physical and mental health composite scores progressively lowered as the grade of chronic pelvic pain increased (112). This highlights chronic pelvic pain as an important post-PID morbidity (5).

Among patients with clinically suspected PID from the PEACH study, those with endometritis were no more likely than those without endometritis to experience reduced pregnancy (OR 0.8, 95% CI 0.6-1.2), infertility (OR 1.0, 95% CI 0.6-1.6), or chronic pelvic pain (OR 0.6, 95% CI 0.6-1.6) (1). Although salpingitis has been found to be associated with morbidity, and endometritis is an accepted alternative to laparoscopically diagnosed salpingitis, this study suggests that endometritis may not be a good predictor of morbidity following PID. These findings may partially be a result of the study population. The PEACH population is likely a different patient population than the Scandinavian women recruited a generation ago. In addition, endometrial histology was used to confirm PID in PEACH, whereas 1,844 patients in the Westrom study had laparoscopically confirmed salpingitis and not all women with endometritis have salpingitis (1). Salpingitis may indicate more severe disease and lead to tissue damage, while endometritis may have lesser effects on tubal pathology (1). Further, the PEACH study treatment covered a broader range of microbes than did those used in the 1960-1980's. Differences in findings between the two studies may also be explained by the comparison group in the PEACH study. Women in PEACH had clinically suspected PID and studies have suggested that some women with clinically suspected PID may actually have ovarian cysts, pelvic adhesions, or endometriosis all of which may be associated with infertility (81). As these

women would be more likely to be in the comparison group and may have had infertility or chronic pelvic pain prior to enrollment, the results may have been biased towards the null. Attempts were made to reduce this bias by excluding women with greater than 30 days of pain, as these women could have had chronic pelvic pain caused by other conditions (1). Although endometritis was not associated with morbidity in the PEACH study, rates of sequelae were high (17% infertility, 20% recurrent PID, and 36% chronic pelvic pain). Therefore, despite treatment, women with PID may still be at high risk for reproductive morbidity.

Obstruction of the fallopian tubes following PID is a preventable cause of infertility and ectopic pregnancy. The role of individual microbes in the development of sequelae following PID is not well understood. Studies have shown that *C. trachomatis, N. gonorrhoeae, M. genitalium*, and *M. hominis* antibodies have been identified in women with tubal factor infertility or tubal occlusion (26, 39, 70, 113-116). Chlamydial antibodies have been found to be significantly more common among patients experiencing ectopic pregnancy as compared to controls with normal pregnancies (OR 3.1, 95% CI 2.2-4.3) (117). Low et al, in a study of 43,715 women from the general population, found that *C. trachomatis*, diagnosed by culture or PCR, was associated with infertility (HR 1.31, 95% CI 1.09-1.57) and ectopic pregnancy (HR 1.26, 95% CI 0.94-1.67) (118). Chlamydia is a leading cause of permanent tubal damage (36), and therefore antibody testing has been used in the evaluation of infertility (119, 120).

1.2.6 Toll-like Receptors, the Reproductive Tract, and PID

Interaction between the immune system and the reproductive tract is important for fertility and reproductive health. The innate immune system serves as the first line of defense after exposure to pathogens and depends on pattern recognition receptors (PRRs) for microbial recognition

(121-123). The understanding of the innate immune system has increased due to the discovery of a highly conserved family of PRRs call the Toll-like receptor (TLR) family (122). TLRs are characterized by an evolutionary conserved cytoplasmic Toll/interleukin-1 receptor homology (TIR) signaling domain, as well as an external antigen recognition domain which is comprised of 19-25 tandem leucine-rich repeat (LRR) motifs (123). TLRs are able to detect microorganisms by recognizing non-specific pathogen-associated molecular patterns (PAMPs). These ligands can include lipopolysaccharide (LPS), mannans, flagellins, carbohydrates, peptides, nucleic acid structures, and several others (122, 123). Thus, TLRs play a critical role in the recognition of pathogens and are responsible for direct microbial elimination through induction of pro-inflammatory cytokines, adaptive immunity, and resolution of inflammation through apoptosis. However, the exact mechanism of activity and function of TLRs in relation to fertility and reproductive health are unknown.

Ten different TLRs (TLRs 1-10) have been identified in humans and the overlap between them allows recognition of a diverse range of pathogens (121-123). TLRs 1, 2, 4-6, and 10 are expressed on the cell surface of a number of immune effector cells, while TLRs 3, and 7-9 are expressed inside of the cell in endosomes or endoplasmic reticulum (123-126). Lipids and lipoproteins can act as ligands for TLRs 1, 2, 4, and 6; proteins can act as ligands for TLR5 and nucleic acid for TLRs 3 and 7-9 (123, 124, 127, 128). There is no known ligand for TLR10. In addition to be able to identify lipids and lipoproteins, TLR2 can form noncovalent hetrodimers with TLR1 or TLR6, enabling TLR2 to recognize a diverse range of pathogens (127).

After ligand binding, TLRs can recruit other TIR domain containing molecules to the site. These are called adaptor molecules and they include myeloid differentiation primary response protein 88 (MyD88), TIR domain-containing adaptor protein (TIRAP or MAL), TIR

domain-containing adaptor protein interferon-beta (IFN)-β (TRIF), and TRIF-related adaptor molecule (TRAM) (123, 127). TIRAP and TRAM are needed to recruit either MyD88 or TRIF to the site and therefore are called bridging adaptor molecules. Almost all TLRs signal via MyD88, with the exception of TLR3. Therefore, variations in MyD88 genes and other adaptor molecules may also play a role in disease pathogenesis.

After MyD88 is recruited to the complex it will initiate a cascade of signaling events, which will eventually dissociate nuclear factor κ-B (NF-κB) from its inhibitor, translocating it into the nucleus, releasing proinflammatory cytokines and chemokines. Specifically, upon ligand binding MyD88 is joined by IL-1 associated protein kinase 1 (IRAK-1), IRAK-4, and TNF-associated factor 6 (TRAF-6) (129). IRAK-1 and TRAF-6 will then dissociate from the complex and activate transforming growth factor-β-activated kinase (TAK-1), which in turn activates IκB kinase kinase (IKK) complex (129). This complex is modulated by the transcription factor NF-κB essential modulator (NEMO) (129). IKK-mediated phosphorylation of IκB eventually leads to the translocation of NF-κB into the nucleus, activating cytokine genes (129).

As TLRs are expressed throughout the female reproductive tract (121,125,126), there is a possible role for TLRs in gynecological disease and fertility. TLRs 1-3, 5 and 6 are expressed in vaginal and cervical epithelial cells, further TLRs 1-3, and 6 are expressed by primary endocervical epithelial cells (121, 130-133). TLR4 has been reported to be present in the endocervix, endometrium, and Fallopian tubes (121, 130-133). TLRs 7-10 have been identified in the endometrial epithelia and stroma (134).

TLR2 and TLR4 are suggested to have differential expression, with low expression in the lower genital tract and higher expression in the upper genital tract (131). Studies have also shown variability in expression of TLRs during the menstrual cycle (133, 134). For example,

during the menstrual phase there is an increase in TLR expression possibly as a defense mechanism (133). In contrast, during the periovulatory phase there is a decrease in expression that may prevent unfavorable inflammation (133). However, Aflatoonian et al reported that TLRs 2-6, 9, and 10 have higher expression during the secretory phase compared to other phases of the menstrual cycle (134). These contrasting results may have been due to differences in study methodology. Overall, the variability in expression may suggest that TLRs hold different functions in different parts of the genital tract.

1.2.6.1 Toll-like Receptors and *Chlamydia trachomatis*

To our knowledge, no studies have examined the role of TLRs in PID. However, limited studies have examined the role of TLRs in infection with PID-associated pathogens (*C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*), BV, and tubal factor infertility. It is known that *Chlamydia sp*. can infect epithelial cells causing them to secret proinflammatory cytokines (135-137). Researchers have suggested that this inflammatory response may be responsible for disease progression (137), especially among those with chronic or persistent infections. As TLRs are expressed in the genital tract and can stimulate proinflammatory genes after binding to a bacterial ligand, it is possible that they may play a role in chlamydial pathogenesis.

TLRs have been examined in chlamydial infections, although most have focused on TLR2 and TLR4. Studies have shown that TLRs can bind to several possible chlamydial ligands. TLR4 have been suggested to recognize LPS and HSP60 (138). TLR2 can respond to a variety of ligands such as lipoproteins and lipopeptides, lipoarabinomannan, lipoteichoic acid, and bacterial prion (139-143). Further, TLR2 can dimerize with TLRs 1 and 6, possibly to recognize a more diverse range of ligands (144). Interestingly, one study found that LPS from the lymphogranuloma venereum (LGV)-1 strain of *C. trachomatis* activated the NF-kB pathway via

TLR2 (145). Therefore, *C. trachomatis* may be able to express a variety of ligands for TLR recognition.

It is known that inflammatory responses are necessary for the elimination of primary *C. trachomatis* infections, although inflammation leads to long-term damage in chlamydial genital tract infections (146, 147). Several studies have examined TLR pathways in chlamydial infections and have suggested a role for TLR2 in cytokine production. For example, Derbigny et al found that cloned murine epithelial cells infected with *C. muridarum* secreted cytokines IL-6 and GM-CSF in a MyD88-dependent fashion (148). Similarly, O'Connell et al found that TLR2 was required for chlamydial induced IL-8 expression, while TLR4/MD-2 had minimal effects on cytokine production (149). Further, TLR2 and MyD88 both localized to chlamydial inclusions during active infection (149). This study suggested that both TLR2 and its downstream adaptor, MyD88 are required for cellular activation. However, this response did not occur with irradiated bacteria or bacteria treated with antibiotics.

While TLRs are necessary for a healthy immune response, TLRs may also be involved in overt immune responses that could lead to tissue damage. Animal models have been able to provide us with some information regarding the role of TLRs in the progression of *C. trachomatis* infections. For example, Darville et al found that TLR2 knockout (KO) mice had significantly lower levels of inflammatory mediators in genital tract secretions during the first week of chlamydial infection, as well as a significant reduction in oviduct and mesosalpinx pathology at late time points compared with mice with the TLR2 gene (150). This essentially caused a reduction in reproductive sequelae caused by chlamydial infection. Further studies from the same group found that mutant plasmid deficient C. *muridarum* strains (CM972 and CM3.1) infected the murine genital tract but did not cause disease because of a failure to stimulate TLR2-

dependent cytokine production in mice (151). Darville et al, concluded that TLR2 signaling is essential for the development of oviduct pathology. In chlamydial genital tract infection, plasmid deficient strains fail to stimulate TLR2 and do not induce oviduct pathology, and mice primarily infected with plasmid-deficient strains are protected against oviduct pathology upon challenge with virulent *C. muridarum* (150, 151). In contrast, TLR4 KO mice had similar pathology compared to infected controls with TLR4 genes (150). Further, a similar in vivo cytokine response was observed in TLR4 KO mice and controls and reflected similar outcomes of infection. Therefore, these data do not suggest a direct role for chlamydial HSP60-induced TLR4 mediated tissue damage in genital tract infection (150). Interestingly, data from the PEACH study shows that chlamydial HSP60 is not associated with sequelae among women with clinically suspected PID (18). Therefore, if TLR2 stimulation is associated with chlamydial-induced tissue damage and TLR4 is not, this could suggest that HSP60 is not a major player in chlamydial-induced disease pathogenesis.

Although animal studies may not completely translate into human in vivo situations, they can certainly give insight into the biology and pathogenesis of chlamydial infection. Unfortunately, there are only a limited number of epidemiological studies examining the role of TLRs in *C. trachomatis* infection. There is a role for genetic variations in immunologically important host genes in the pathogenesis of disease (152). Single nucleotide polymorphisms (SNPs) are a type of variation, in which one nucleotide has been substituted, inserted or deleted and variations exist in the number of repetitive DNA sequences. Carrying a specific SNP may have direct or indirect biological consequences for the host. It has been suggested that TLR4 homozygote (A>G) and Thr399I1e SNPs affect LPS receptor function, whereas heterozygous carriage had no effect (153). Morre et al examined 35 Dutch women with tubal pathology and 49

Dutch women without tubal pathology and found that the TLR4 Asp299Gyl polymorphism was not associated with tubal infertility (P>0.5) (Allele frequency: 7.1% vs. 10.2%) (154). In a cohort of 227 subfertile women, among *C. trachomatis* positive women there was an increasing risk for tubal pathology (64% risk for normal genotype vs. 83% risk for heterozygous SNP carrier), although this did not reach statistical significance (154). Read et al examined this polymorphism in meningococcal disease (gram-negative bacteria) and did not find an association with the TLR4 polymorphism (155). The study results were not what the authors expected. However, the lack of associations in these studies may be explained by Erridge et al, who found that TLR4 heterozygote mutations do not affect recognition of chlamydial LPS (153). As the rare homozygotes may be less responsive to LPS, they may be more likely to experience adverse effects. The lack of association may have been due to the fact that almost all carriers of the common TLR4+896 A>G SNP were heterozygous. Thus, the authors simply did not have enough genotypic diversity to detect any significant results. The protein CD14 is a co-receptor for TLR4 and confers responsiveness to LPS leading to the activation of NF-κB associated immune response (156). Polymorphisms in this co-receptor may lead to sequelae following infection. Outburg et al, compared 253 Dutch women with subfertility to 170 fertile women to examine the CD14 functional polymorphism -260C>T (156). However, this study found no association between this polymorphism and subfertility following chlamydial infection (156).

Fewer studies have examined polymorphisms in *C. trachomatis* infections. In one study examining TLR4 polymorphisms, no a ssociations were found between TLR4 SNPs and *C. trachomatis* infection among a Dutch population (157). However, women with tubal pathology who were *C. trachomatis* Ig positive were twice as likely to be carriers of the TLR4 +896 G allele compared to women without tubal pathology (157). Karimi et al examined the role of

several TLR2 SNPs in 468 Dutch women (158). This study found no associations with any SNP genotypes and susceptibility to *C. trachomatis* infections (158). However, a haplotype formed by -16934 T>A and +2477 G>A SNPs was significantly associated with protection against tubal disease following *C. trachomatis* infection (P=0.015) (158). Further, this haplotype was associated with a decrease in severity of *C. trachomatis* infection (P=0.021), suggesting that this haplotype has a possible protective function (158). Results from theres studies need to be replicated. These limited studies among Dutch Caucasian populations have been unable to show any significant associations between innate immune receptor functional polymorphisms and tubal factor infertility. These studies have been limited by small sample sizes and low power. Further, the homogeneous population used in these studies is likely not generalizable to women with sexually transmitted diseases and PID in the US. Further studies should examine a full panel of candidate TLR genes in *C. trachomatis* infection and disease progression in different racial groups.

1.2.6.2 Toll-like Receptors and Other PID Organisms

Fewer studies have been conducted to examine the association between TLRs and other PID associated pathogens. Using real time PCR, Muenzner et al found that *N. gonorrhoeae* activated NF-κB possibly via TLR4 or TLR2 (159). However, the authors did not allow for determination of levels of expression (159). A study that did examine the expression found that response to *N. gonorrhoeae* at the mucosal surface is independent of TLR4 (160). Pridmore et al found that lipooligosaccharide (LOS) from different strains of *Neisseria*, including gonococcal strains, induced TLR4/MD2 signaling (161). However, the level of signaling differed significantly depending on t he strain of *Neisseria* (P<0.01). Further, the level of TNF was significantly associated with the level of TLR signaling (161).

TLR2 may be important in producing an inflammatory response during gonococcal infection. Fisette et al suggested that TLR2 cooperates with TLR1 and/or TLR6 to respond to lipoproteins (162). However, they found that the Lip lipoprotein signals through TLR2 with TLR1 but not TLR6, suggesting that this lipoprotein induced inflammation in a TLR2 dependent manner (162). Continued investigation of the interactions between TLRs and *N. gonorrhoeae* are needed.

Stimulation of cells and release of certain proinflammatory cytokines or alterations in the response by TLRs may mediate the adverse effects caused by BV. Genc et al reported that women with the TLR4 4795A>G polymorphisms who were homozygotes had increasing levels of IL-1β and IL-1ra (163). In addition, those who were carriers for the TLR4 4795 G allele had higher concentrations of *G. vaginalis*, although no significant associations were found with BV (163). This may suggest that the G allele increases susceptibility to BV. The small numbers in this study did not allow them to find significant differences in allele frequencies between races, although there appeared to be a greater percentage of blacks who had the G allele (14%) compared to whites (8.5%) (163). Allelic variation by race may explain the higher incidence of BV among black women, but it has not been studied widely. In a study of 885 predominantly African American women, the TLR4 4795A>G polymorphism was not associated with BV (164). The authors did find a slight protective effect with the TLR4 5095C>T polymorphism, however after controlling for ethnicity the association was no longer significant (164). In a study of 144 Caucasian women, no significant associations were found with TLR4A>G and *Gardnerella vaginalis* or *Atopobium vaginae* (165). Further, this study found no significant associations between several other SNPs in TLR2 (-15607A>G, 1350T>C, 2258G>A, 2029C>T), TLR4 (-2026A>G, 5095C>T), or TLR6 (-502T>C, 745C>T, 1083C>G, 1280T>C)

(165). The authors did find a significant association between *A. vaginae*, during the first half of pregnancy, and the TLR1 743A>G (P=0.038) polymorphism, as well as the TLR1 -720A>G (p=0.062) polymorphism. However, the authors did not find any significant associations between BV and any of the TLR SNPs. BV is not a condition of a single pathogen and the microorganisms which cause BV may differ between women. This may make it difficult to find associations between TLRs and BV.

M. genitalium has a large amount of lipoproteins on the cell membrane, some of which can interact with TLRs leading to inflammatory cytokine production (166). Although the possibility exists that TLRs may be associated with the pathogenesis of *M. genitalium,* few studies have examined these associations. McGowin et al found that the C-terminal portion of the antigenic protein encoded by MG309 activates NF-κB via TLR2 and 6, resulting in cytokine secretion from genital epithelial cells (166). However, the authors report that although NF-κB activation was observed via TLR2 alone, results were not significant and were only observed at the highest concentrations of *M. genitalium*, suggesting TLR2 homodimers are not sufficient to recognize the microbe (166). Further, another experimental study found that the activation of NF-κB by *M. genitalium* lipoprotein MG149 was inhibited by a dominant negative construct of TLR1 and TLR2 but not TLR6, thus suggesting that NF-κB is activated through TLRs 1 and 2 (167). To date no studies have examined TLRs, *M. genitalium* and PID or subsequent development of sequelae

1.2.6.3 Toll-like Receptor Polymorphisms

Variations in TLR genes or their adaptor molecules may lead to overt or inadequate immune responses, possibly influencing disease progression. For the proposed research study, several

SNPs from four TLR genes and two adaptor molecule genes will be examined. These SNPs include three from TLR1 (rs5743618, rs5743817, rs4833095), three from TLR2 (rs3804099, rs11938228, rs1898830), three from TLR6 (rs1039559, rs5743810, rs3775073), four from TLR4 (rs5030728, rs4986790, rs11536889, rs1927911), two from MyD88 (rs7744, rs4988457) and three from TIRAP or MAL (rs3802813, rs7932976, rs8177374). The exact function of these SNPs in reproductive health is unknown and none of these SNPs have been studied in women with PID. Further, very few studies have examined these SNPs to determine their role in sexually transmitted diseases or infertility. Although the exact function of TLR SNPs in reproductive health has yet to be completely elucidated, it is important to take into consideration recent research exploring these SNPs in other disease states in order to gain insight into the development and progression of gynecological diseases.

TLR2 can recognize a variety of ligands and is considered an inducer of proinflammatory signaling (144). Further, TLR2 was found to increase murine oviduct pathology following chlamydial infection (150 151). Human studies examining TLR2 variants in PID or PID-associated pathogens are limited. However, several TLR2 SNPs including SNP rs3804099 (Asn199Asn) have been studied in a variety of other diseases. Asn199Asn so far has not been found to be associated with atopic asthma (168), rheumatoid arthritis (169), pulmonary tuberculosis (TB) (170), normal tension glaucoma (171), or allergic rhinitis (172). On the other hand Asn199Asn has been found to be associated with the occurrence of leprosy reversal reaction among Ethiopian patients (173). In particular, the T allele displayed a protective effect under the dominant model (OR 0.34, 95% CI 0.17-0.68; P=0.002) (173). Reversal reaction occurs when there is a shift in the immune system leading to increased cell mediated response that can eventually lead to severe tissue damage (173). In a study of 410 Chinese patients with

major trauma, the Asn199Asn C allele was associated with increased production of IL-10 (P=0.002), IL-8 (P=0.004), and TNF-α (P=0.005) compared to the wild T allele (174). In addition, the C allele was also associated with higher sepsis morbidity rates compared to the T allele (P=0.004; dominant model) (174). Not surprisingly, the wild-type haplotype ATT (1898830A 3804099T 7656411T), which includes the T allele for Asn199Asn, was significantly associated with lower production of cytokines (174). This SNP was also found to be associated with sepsis in preterm infants using transmission disequilibrium test (175). However, when individual alleles were examined no significant associations with sepsis were found. It was unclear in this study if race was considered in the allelic analyses. Further, power may have limited in this study. The CC genotype of this SNP has been found to be associated with tuberculosis meningitis among a Vietnamese population (176). This association was increased when the C allele interacted with TIRAP SNP 558T (OR 5.4, 95% CI 1.34-21.77; P=0.008). A subsequent study from this same group which examined interactions between host genotypes and *Mycobacterium tuberculosis* genotypes, reported that those who carried the C allele were more likely to have tuberculosis meningitis caused by the East-Asia/Beijing genotype (OR 1.57, 95% CI 1.15-2.15) (177). No associations were found between the development of bronchiolitis obliterans following lung transplantation and Asn199Asn (178). Overall the function of this SNP is not well understood. As this SNP is located in the exon and induces synonymous variation (174), it is possible that it is in linkage disequilibrium with another functional SNP.

Fewer studies have examined the other two TLR2 polymorphisms. Rs1898830 is located in intron 1 and has no known function. No associations have been found with this SNP and atopic asthma (168) or normal tension glaucoma (171). Chen et al, did find an association between the rs1898830 G allele and increased cytokine production (IL-10; P=0.013, IL-8;

P=0.014, and TNF-α; P=0.018) among a Chinese population (174). However, there were no associations found with sepsis morbidity rates or multiple organ dysfunction scores following major trauma (174). The authors report that low statistical power may have limited their analysis. Ryckman et al examined 97 Caucasian women in their first trimester of pregnancy and reported the rs1898830 G allele was associated with increased IL1-α, IL-10, and IP10 (179). However, theses associations did not remain significant after correction for multiple comparisons. Again, power may have contributed to these findings. In another study among 144 pregnant Caucasian women, the AA genotype was not associated with *Atopobium vaginae* (165). In contrast, a study among 110 Dutch lung transplant patients found that homozygotes for the major A allele for rs1898830 had an increased risk of bronchiolitis obliterans compared to carriers of the minor G allele (178). Less is known about SNP rs11938228, which is located in the intron. The function is unknown and only two studies have examined this SNP. In these studies no significant associations were found with either normal tension glaucoma (171) or Behcet's disease (180).

TLR1 and TLR6 can both form a heterodimer with TLR2, to recognize a range of pathogens and activate inflammatory responses. TLR1, TLR2, TLR6, and TLR10 are all located on chromosome 4, while TLR1/TLR6/TL10 form a gene cluster that is located on the P arm (181). TLR1 and TLR6 are suggested to have evolved from the same orthologous gene, as they share 69% of their amino acid sequence (181). TLR1 SNPs have been associated with several diseases, while the role of TLR6 and TLR10 is less clear.

TLR1 SNP rs5743618 (Ser602Ile), is non-synonymous and results in an amino acid change. The G allele has been reported to be associated with deficient TLR signaling in comparison to the T allele (182-185). Johnson et al, reported that the G allele displayed significantly lower levels of TNF-a ($P<0.05$) in response to a synthetic triacylated lipopeptide,

TLR1/TLR2 agonist Pam_3CSK_4 (182). Further, these authors report that among 57 Caucasians with leprosy and 90 asymptomatic controls, homozygous carriers of the G allele had a decreased incidence of leprosy (P=0.017). Similarly, the G allele was also found to be protective against leprosy in an Indian population consisting of 258 leprosy cases and 300 controls (OR 0.31, 95% CI 0.20-0.48) (186). The G allele was also protective against leprosy reversal reaction in 1171 patients from Nepal (184). Hawn et al, reported that the T allele expressed significantly greater NF-κB signaling in transfected HEK293 cells compared to the G allele (183). In a subsequent study from this group among 638 Caucasian women, the TT genotype was found to be protective against pyelonephritis (OR 0.53, 95% CI 0.29-0.96) (187). In another study among patients with sepsis, the T allele was found to be associated with higher mortality (OR 1.79, 95% CI 1.02-3.13; P=0.042) (185). However, the authors suggest the T allele does not affect phenotype independently, as Ser602Ile is in linkage disequilibrium with TLR1 SNP rs5743551 (r^2=0.76), which showed a much stronger association with organ dysfunction and death. This SNP was not found to be associated with rheumatoid arthritis (169) or invasive aspergillosis (188).

TLR1 SNP rs4833095 (Asn248Ser), is non-synonymous and results in an amino acid change. Like the Ser602Ile variant, Asn248Ser may alter TLR signaling increasing the risk of infection. Compared to the wild-type, the A allele was found to impair TLR response to Pam_3CSK_4 (P<0.05) (189). Several diseases have been found to be associated with this variant (181, 182, 188, 190 -193). A study among 302 primiparous women from Ghana, reported that heterozygotes had increased risk of placental *P. falciparum* infection (OR 2.01, 95% CI 1.01-4.0) compared to carries of the wild-type, although no associations were found with low birth weight or preterm delivery (191). Homozygotes in this population were too few for analysis. In a population based case control study among 1312 tuberculosis patients and controls, the G allele

significantly increased risk of tuberculosis in African Americans (P=0.009) (181). Further, the authors found that the G allele was more frequently transmitted in diseased children compared to the A allele during a transmission disequilibrium test (61 vs. 38 times; P=0.021). In a study of 842 leprosy patients and 543 controls from Bangladesh, the GG genotype significantly increase leprosy (OR 0.78, 95% CI 1.06-1.70), while the heterozygotes had a decreased leprosy risk (OR 0.78, 95% CI 0.63-0.96) (190). However, the AA genotype was not significantly associated with leprosy (OR 1.01, 95% CI 0.79-1.29). Neither the A nor G allele alone significantly altered risk. It should be mentioned that Asn248Ser and Ser602Ile are reported to be in strong linkage disequilibrium with each other as well as with TLR1 SNP rs5743551, which was found to be associated with poor sepsis outcomes (185). These SNPs may be measuring the same association. Haplotypes containing these SNPs may also be responsible for the significant associations (185). In fact, Pino-Yanes et al, reported that the 248Ser-602Ile haplotype was associated with circulatory dysfunction among 218 sepsis patients (P<0.022), decreased IL-10 (P<0.047), and increased CRP (P<0.036) (194). TLR1 SNP rs5743817 is another SNP that results in an amino acid change. However, the function of this SNP is unknown and no studies have examined it for disease associations.

Little is known about the function of TLR6 SNP rs1039559, and so far three studies have failed to find any significant associations with non-Hodgkin's lymphoma (195), *Atopobium vaginae* (165) or bronchiolitis obliterans (178). TLR6 SNP rs5743810, also known as Ser249Pro, is non-synonymous and results in an amino acid change. There is no association between this SNP and rheumatoid arthritis (169) or *Atopobium vaginae* (165). However, Sales et al examined 443 hypertensive Brazilian subjects and found that the TT genotype of Ser249Pro was associated with reduced posterior wall thickness (P=0.02), reduced intreventricular septum thickness

(P=0.03), and reduced left ventricle wall thickness (P=0.02) (196). In addition the authors found that monocytes from hypertensive women with the TT genotype had reduced IL-6 and TNF-α release compared to sex matched cells carrying the C allele (P<0.05). These results were not observed in hypertensive men. Similarly in a study among 100 subjects from South Africa, the T allele of Ser249Pro was associated with decreased NF-κB signaling activity (197). The authors also suggested this SNP may be associated with reduced IL-6 levels in response to lipopeptide. However, they were unable to reproduce their results to confirm their findings (197). Tantisira et al, reported that the T allele decreased the risk of asthma in African Americans (OR 0.38, 95% CI 0.16-0.84; P=0.01), however this was not observed for European Americans (OR 0.72, 95% CI 0.48-1.09) (198). A study among 1,872 Germans reported that the minor allele was associated with an increase in atopic childhood asthma (OR 1.79, 95% CI 1.24-2.58) under the dominant model (168). Lastly, an American study found that at least one copy of the T allele in combination with at least one copy of the minor allele for TLR1 SNP Asn248Ser increased susceptibility to invasive aspergillosis (OR 1.30, 95% CI 1.13-1.50) (188). This study did not indicate if race was considered in their analysis.

There is no evidence that TLR6 SNP rs3775073 is associated with any disease. However, two studies have examined the possible functional role of this SNP. One study found that the rs3775073 G allele was associated with differential responses to tri-acylated lipopeptide Pam_3CSK_4, mediating a decreased effect on IL-6 (185). In contrast, Shey et al did not find this variant to be associated with differential response to Pam_3CSK_4 (197). The contradicting results were suggested to be a result of different methodologies and patient populations (197). One population was recruited from Seattle, WA (185) and the other from South Africa (197).

TLR4 has been one of the most studied TLR genes, recognizing a wide range of pathogens and playing a role in the activation of inflammatory genes. However, not all TLR4 polymorphisms have been studied extensively. The function of TLR4 SNP rs5030728 is not well known and very few studies have examined this SNP to determine associations with disease. In one study Cheng et al examined 506 men with prostate cancer and 506 controls from hospitals in Cleveland, OH and found that those who carried the AA genotype for rs5030728 had a significantly decreased risk of prostate cancer (OR 0.60, 95% CI 0.37-0.97) compared to those who carried the GG genotype (199). Similarly, little is known about the function or clinical relevance of SNP rs11536889, which is located in the 3'UTR of the TLR4 gene. The C allele for this variant has been found to increase the risk of severe gastric atrophy in Japanese patients (200). Further, the G/C genotype was found to be associated with moderate or severe periodontitis (201). However, no associations have been found with this variant and prostate cancer (199), autoimmune pancreatitis (202), or liver fibrosis (203). The minor T allele of SNP rs1927911, which is located in the intron, has been found to decrease the risk of myocardial infarction (OR 0.88, 95% CI 0.77-0.99) among 1,216 cases and 2,682 controls from Washington state (204). In contrast, those who carried the TT genotype were found to have increased odds of bronchiolitis obliterans (BOS) (OR 4.20, 95% CI 1.43-12.35; p=0.005) among 110 lung transplantation patients from the Netherlands (178). In this study the authors noted that acute rejection is a risk factor for BOS and reduced frequency of rejection has been shown to be linked to an increase in pro-inflammatory cytokines and chemokines (178). In this study, heterozygous carriers of the G allele for TLR4 SNP Asp299Gly had reduced risk of rejection and others have noted that the G allele is associated with reduced BOS (178). The G allele of Asp299Gly was

reported to be completely linked to the C allele of rs1927911 (D'=1). Further, carriers of the C allele for rs1927911 had reduced risk of BOS.

Much more is known about TLR4 SNP rs4986790 (Asp299Gly). Asp299Gly is a missense mutation and it has been reported that the AG genotype decreases responsiveness to inhaled LPS in humans, while the wild type (AA) is capable of secreting cytokines in response to LPS (205). This may lower cytokine production for heterozygotes. In fact, Genc et al reported that women who carried the AA genotype and were colonized with *G. vaginalis* or gram negative rods had significantly higher levels of IL-1β, compared with women who carried the AG or GG genotypes (163). Further, a study among 115 Sweedish children reported a decrease in LPS-induced IL-12 and IL-10 among heterozygous carriers (206). An Austrian study reported no significant differences in early cytokine release for wild type or those with the mutant G allele (207). However, carriers of the G allele had significantly lower IL-6 concentrations at 6 hours, and lower CRP and IL-1β at 24 hours, suggesting a possible role in chronic but not acute disease (207). Studies have challenged the effects of Asp299Gly on LPS signaling. In a study among 80 Scottish subjects, Erridge et al reports that heterozygotes do not exhibit reduced recognition of LPS after challenge from *E. coli, N. meningitides, B. fragilis, Y. pestis, C. trachomatis, P. gingivalis*, or *P. aeruginosa* (153). The contrasting results could be due to a possible difference in response by human airways vs. human monocytes (153). Further, the LPS used in the first study was commercial bought and may have been contaminated (153). Thus, Erridge et al suggested that the blunting effect was caused by another TLR4 ligand and not LPS. However in study among 22 Dutch subjects, van der Graaf et al reports that Asp299Gly does not influence the production of TNF or IL-10 when human blood cells are stimulated with a variety of exogenous and endogenous TLR4 ligands (208). This study had a very small number of

volunteers, including only one who was homozygous. In another study among 160 German subjects, von Aulock et al also reported that heterozygotes did not differ significantly from the wild-type for any cytokines (TNF-α, IL-1β, IL-6, INF-γ, G-CSF) (209). However, heterozygous subjects produced less anti-inflammatory IL-10 (P=0.002), although this was only at the highest concentrations.

Similar to the controversy over the function of Asp299Gly, reports of associations with various diseases have also been conflicting. The Asp299Gly G allele has been found to be associated with increased vaginal Ph (p=0.05) and *G. vaginalis* (p<0.0001) among 238 pregnant women (163). Similar trends were observed for anaerobic Gram-negative rods, *Prevotella, Bacteroides*, and *Porphyromonas,* although these did not reach statistical significance (p=0.08) (163). In addition, an American study reported that the G allele was present in 23.8% (67/282) premature infants and 15.9% (55/345) term infants (p=0.024) (210). A higher frequency either the GG or AG genotype was also observed in singleton preterm infants (p=0.028) compared to term infants. The G allele was also identified in 24.2% (15/62) of mothers with a preterm infant and 15.0% (3/20) of mothers with a term infant, although results were not significantly different. In a South American study also examining fetal genotype, Asp299Gly was not associated with preterm birth (OR 0.99, 95% CI 0.6-1.7) (211). However, significant associations were found between heterozygous carriers and premature rupture of membranes (PROM) among severely premature infants (< 33 weeks gestation) (OR 4.6, 95% CI 1.2-16.6; p=0.032). In contrast, there was no association between the G allele and PROM among 131 African American premature infants (< 37 weeks gestation) and 246 controls (212). Severe premature birth was not examined in that study. Further, none of the 3 studies examined associations with maternal genotype. Among 115 Swedish children, this variant was associated with asthma (OR 4.5, 95% CI 1.1-

17.4) (206). Associations have also been found with septic shock and gram negative infections (213), as well as an increased risk of gram negative infections in critically ill patients (p=0.004) (214). In a Dutch study, Asp299Gly was not associated with susceptibility to *C. trachomatis* (157). However, subfertile women with tubal infertility who were positive for *C. trachomatis* immunoglobulin (Ig) G were twice as likely to be carriers for the mutant Asp299Gly allele. These results were not statistically significant. No significant associations have been found between this variant and rheumatoid arthritis (169, 215, 216), systemic lupus erythematosus (216), meningococcal disease (155), aspergillosis (188), or atopic dermatitis (217).

TLR adaptor molecules such as TIRAP and MyD88 are critical for innate immune response. Variations in these receptors may also affect TLR signaling. Unfortunately, little is known about the function and clinical relevance of most of these SNPs. In 187 rheumatoid arthritis patients from the United Kingdom, the MyD88 SNP rs7744 G allele was associated with response to anti-TNF treatment (218). Specifically, the GG genotype was associated with greater improvement (OR 1.5, 95% CI 1.1-2.3; p=0.020) (218). However, as this was an exploratory analysis no correction for multiple testing was made. The rs7744 SNP is located in the 3'UTR and was suggested to influence mRNA stability (218). On the other hand, this SNP is also in LD with rs156265, which may influence gene expression (218). MyD88 SNP rs4988457 was not found to be associated with Hodgkin's lymphoma (219). No other studies to our knowledge have examined either rs7744 or rs4988457. Two SNPs in the TIRAP gene, rs3802813 and rs7932976 also have no supporting studies to determine possible function or clinical relevance. However, rs7932976 is a missense mutation that results in an amino acid change (Val197Ile). Therefore, Val197Ile may be a functional variant. TIRAP SNP rs8177374, also known as Ser180Leu, has been studied more extensively. This variant is thought to attenuate TLR2 signal transduction and

suggested that homozygotes have an over-active response, resulting in more severe outcomes (220). Heterozygous carriers of this variant were found to have a reduced risk of invasive pneumococcal disease (OR 0.59, 95% CI 0.42-0.83, p=0.003) bacteraemia (OR 0.40, 95% CI 0.21-0.77, p=0.003), malaria (OR 0.47, 95% CI 0.28-0.76, p=0.002), and tuberculosis (OR 0.23, 95% CI 0.07-0.73, p=0.008) among 6,106 subjects from Gambia, Kenya, the United Kingdom, and Vietnam (220). The T allele was also found to be protective against systemic lupus erythematosus (SLE) (OR 0.29, 95% CI 0.14-0.61; p=0.0002) and TB (OR 0.53, 95% CI 0.29-0.97; p=0.04) in a case-control study among 1325 Columbians (221). Genotype analysis show that the CT genotype was protective against SLE (OR 0.27, 95% CI 0.13-0.57; p=0.0002) and TB (OR 0.50, 95% CI 0.27-0.94; p=0.03) compared to the CC genotype. The T allele was shown to have significantly increased cytokine production (TNF, IFN-y, and IL-6), indicating a protective effect against infection (222).

As the function and clinical relevance of many of these polymorphisms remains unknown it is imperative the research continue to examine TLR and adaptor molecule SNPs in disease progression and inflammatory response. Contrasting findings in some of these association studies is likely a result of different patient populations, differences in gender or ethnicity, differences in doses, differences in heterogeneity, population stratification, population admixture, and/or chance findings. SNPs which result in an amino acid change may alter the function of TLR signaling and downstream cytokine responses. Those that do not result in an amino acid change may still be responsible for disease pathogenesis. However, it is possible that some of these SNPs are in linkage disequilibrium with a disease variant. Research should continue to delineate whether these SNPs are in proximity of a disease variant or have their own functional consequences.

1.2.7 Summary

Organisms such as *Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma genitalium* and anaerobic bacteria associated with bacterial vaginosis are prevalent and can lead to PID, and in turn major reproductive morbidity such as infertility, ectopic pregnancy, and chronic pelvic pain. The role of individual microbes in the development of sequelae following PID is not well understood. *C. trachomatis* is often asymptomatic and chlamydial salpingitis clinical manifestations are reported to be mild but associated with severe tubal damage. In contrast, gonococcal salpingitis may produce more overt and severe symptoms but leads to milder tubal damage. Studies have consistently reported similar symptoms between *C. trachomatis* and *M. genitalium.* Therefore, many women with *C. trachomatis* or *M. genitalium* may have chronic low level inflammation which could permanently damage the reproductive tract before treatment is initiated. In fact, delayed care has been implicated as a predictor of impaired fertility following PID, with the strongest association among women with *C. trachomatis*. However, these associations have not been replicated in a contemporary cohort and the distribution of microbes appears to have changed over time.

Variability exists in the course and outcome of *C. trachomatis*. For example, 80% or more of women with chlamydia do not develop PID and not all women with chlamydial PID go on to develop reproductive morbidity. It is suggested that differences in *C. trachomatis* progression may be explained by variations in host genetic genes. Further, chlamydial pathogenesis has been suggested to be immunologically driven. However, the role of host immunity in the pathogenesis of *C. trachomatis* has not been extensively studied. Gaps in our knowledge of the natural history of *C. trachomatis* have made management and control a challenge. Examining the role of innate immune genes is important to further understand the

pathogenesis of *C. trachomatis* and may bring researcher closer to developing a safe and effective vaccine. The proposed specific aims were designed to examine the role of innate immune receptor genes in endometritis, *C. trachomatis*, and infertility among women with PID. Further, the role of delayed care seeking in a contemporary cohort of women with clinically suspected PID will be examined.

2.0 MANUSCRIPT 1: MICROBIAL CORRELATES OF DELAYED CARE FOR PELVIC INFLAMMATORY DISEASE

Sexually Transmitted Diseases 2011, In Press

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2.1 ABSTRACT

Objective: We studied the microbial correlates of time to care and long term outcomes among pelvic inflammatory disease (PID) patients, as delayed care may increase the risk for reproductive sequelae.

Methods: Mean days of pain prior to care were compared by microbial pathogen (*Chlamydia trachomatis* only, *Neisseria gonorrhoeae* only, *Mycoplasma genitalium* only, co-infection with two or more pathogens, or no pathogens) among 298 w omen with histologically confirmed endometritis from the PID Evaluation and Clinical Health (PEACH) study. Times to pregnancy and recurrent PID were assessed over a mean of 84 months and compared between women who delayed care (≥14 days) and women who sought early care, in the entire cohort and in subsets defined by microbial infection. Analyses were adjusted for age and race, additionally time to pregnancy was adjusted for self-reported baseline infertility.

Results: Patients waited a mean of 7 days before seeking care for symptoms. Time to care was longest among women infected by *C. trachomatis* only (12.3±9.4 days) and *M. genitalium* only (10.9±8.9 days) and the shortest among women infected by *N. gonorrhoeae* only (4.6±5 days) or co-infection (5.6±5.1 days, $p<0.001$). Rates of infertility, recurrent PID, and chronic pelvic pain were frequent overall (17%, 20%, and 36%) and tended to be higher, albeit non-significantly, after delayed care.

Conclusions: Among women with clinically suspected PID, time to care was generally high. *C. trachomatis* and *M. genitalium* positive women had the longest times to care. Although reproductive morbidity was high in this cohort, associations with delayed care were non-significant.

2.2 INTRODUCTION

Pelvic Inflammatory Disease (PID), infection and inflammation of the female upper genital tract (1), is a common condition affecting an estimated 8% to 11% of American women at some time in their reproductive lives (4). Approximately 1 million women will be treated annually for PID in the United States (5). Various organisms have been implicated in the etiology of PID including *Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma genitalium* and anaerobic bacteria associated with bacterial vaginosis (5).

PID is a disease that can cause serious reproductive morbidity. In a landmark Scandinavian cohort study conducted between 1960 and 1984 (2, 3), of 2500 women with clinically suspected PID, 16% with laparoscopically verified salpingitis versus 2.7% of controls became infertile (2, 3). In addition, 20% and 25% with salpingitis developed recurrent PID and chronic pelvic pain respectively (2, 3).

Hillis et al reported that one strong predictor of sequelae following PID is delayed care (9). In this case-control study nested within the Scandinavian cohort (2), delayed care was associated with a three-fold increase in infertility or ectopic pregnancy among 714 women with one known episode of PID (9). Further, the association was strongest among 114 women infected with *C. trachomatis*, with 17.8% of *C. trachomatis* positive women who delayed seeking care experiencing impaired fertility compared to 0% who sought care promptly (9). As these associations have not been replicated in a contemporary cohort, and since the distribution of microbes among women with PID appears to have changed over time, our objective was to examine the microbial correlates of time to treatment and long term outcomes among PID patients.

2.3 METHODS

This study utilized data from the PID Evaluation and Clinical Health (PEACH) study. This was the first randomized clinical trial to compare inpatient and outpatient treatment in preventing long-term complications among 831 women with mild to moderate PID. The methods of subject recruitment, data collection, and follow-up have been reported elsewhere (87). Briefly, between March 1996 and February 1999 women aged 14-37 years were recruited from emergency departments, obstetrics and gynecology clinics, sexually transmitted disease clinics, and private practices at 7 primary and 6 secondary sites throughout the eastern, southern, and central regions of the United States. Women who had suspected PID and gave informed consent were eligible for the PEACH study. Eligible women had a history of pelvic discomfort for less than 30 days, findings of pelvic organ tenderness (uterine or adnexal) on bimanual examination, and leukorrhea and/or mucopurulent cervicitis and/or untreated but documented gonococcal or chlamydial cervicitis. The University of Pittsburgh Institutional Review Board approved the study.

A total of 2941 women were screened for study entry, of those 346 (11.8%) did not meet the clinical inclusion criteria for randomization. Women were additionally excluded if they were pregnant (n = 141, 4.8%); had taken antimicrobials within the past 7 days (n = 248, 8.4%); had a history of hysterectomy or bilateral salpingectomy (n = 248, 8.4%); had an abortion, delivery, or gynecologic surgery within the past 14 days (n = 51, 1.7%); had a suspected tubo-ovarian abscess or other condition requiring surgery (n = 191, 6.5%); had an allergy to the study medications (n = 163, 5.5%); were homeless (n = 29, 1%); or had vomiting after a trial of antiemetic treatment (n = 11, 0.4%). A total of 831 women were enrolled in the PEACH study. Our analyses are among 298 women with histologically confirmed endometritis and complete

time to treatment data. These women did not differ on important demographic, clinical, and behavioral characteristics compared to women without complete data. Among our cohort, 251 had *N. gonorrhoeae* culture, 271 had *C. trachomatis* polymerase chain reaction (PCR) and 240 had *M. genitalium* PCR results available for analysis.

Women were randomized to either inpatient treatment of intravenous cefoxitin every 6 hours and doxycycline orally twice a day for 14 days; or outpatient treatment consisting of a single intramuscular injection of cefoxitin and oral doxycycline twice a day for 14 days. Because the treatment modality was not associated with reproductive morbidities in the PEACH study (87), we do not include them as a covariate in these analyses. Participants were followed-up with in-person visits at 5 and 30 days after treatment. At the 30-day follow-up the gynecological exam was repeated. Telephone follow-ups were conducted by the study nurses every 3 months during the first year after enrollment and then every 4 months until June 2004. At that point information was obtained by self-report for 69.1% of the cohort, with a mean follow-up of 84 months.

A pelvic examination and interview were conducted at the baseline visit. The interview collected information on reason for visit, brief pain history, demographics, history of PID/sexually transmitted diseases (STDs), sexual and contraceptive history, reproductive decisions, douching history, pregnancy history, medical and gynecological history and lifestyle habits. Baseline demographic and clinical characteristics used in this analysis included age, race, marriage, education, insurance utilization, microbiological etiology, clinical findings (abnormal vaginal discharge, elevated temperature (>100.4^{o}F), elevated white blood cell count (WBC) (> 10,000mm^{3}), and bilateral adnexal tenderness), smoking, drug use, history of PID, history of bacterial vaginosis, history of *C. trachomatis*, history of *N. gonorrhoeae*, and history of pelvic surgery. Data on prior *M. genitalium* infection was not available. Follow-up interviews collected

self-reported information on pelvic pain, pregnancy and births, signs and symptoms of PID, STDs, contraceptive use, pattern of sexual intercourse, and health care utilization.

Gynecological examinations were performed at baseline and 5 and 30 days post treatment. Vaginal smears were gram stained for bacterial vaginosis in a central laboratory by standardized methods described by Nugent et al (223). Endometrial biopsy specimens were obtained for histological examination including, chlamydial PCR and gonococcal culture. PCR and cultures were performed at a central reference laboratory. For the patients with endometrial biopsies, two reference pathologists separately evaluated at least one section stained with hematoxylin and eosin and at least one stained with methyl green pyronine. Disagreements about the presence or absences of neutrophils and plasma cells were settled by both pathologists reading the slides together and coming to an agreement. Histological endometritis was based on a modification of the criteria proposed by Kiviat et al (224). Endometritis was defined as the presence of at least five neutrophils in the endometrial surface epithelium in the absence of menstrual endometrium and/or at least two plasma cells in the endometrial stroma. Cervical and endometrial specimens were stored and later used to test for *M. genitalium* (8), using a microwell-plate-based PCR assay (MgPa-IMW) targeting the MgPa gene (225).

In the current paper, the main outcome variable is delayed care. Sensitivity analyses were conducted to define delayed care in our cohort. Participants were considered to have delayed care if they reported that treatment was sought after having pain between14 and 30 days (n = 70), as women with over 30 days of pain were not included in PEACH. Participants who sought treatment before experiencing 14 days of pain were considered to have sought early care (n = 228). The variable was categorized by using the 75th percentile of the distribution of the days until treatment reported during the baseline questionnaire.

Reproductive outcomes included pregnancy, infertility, live birth, recurrent PID, and chronic pelvic pain. Self-reported pregnancy was determined by a positive urine/blood test or physician's diagnosis. Pregnancy was determined among women reporting no birth control or methods considered being unreliable, including withdrawal, rhythm method, vasectomy, or using the following methods rarely or occasionally – diaphragm, condoms, spermicidal foam/cream/jelly/suppositories, or cervical cap. Infertility was defined by lack of conception (positive urine test or blood test or doctor's diagnosis of pregnancy) despite sexual activity with rare or never use of methods of contraception considered reliable during 12 or more months of follow-up. Frequency of sexual activity did not differ significantly between groups (P = 0.6180). Live birth was determined by self-report, and recurrent PID was self-reported and verified whenever medical records were available (45% of cohort). Recurrent PID was confirmed in 76% of medical records. Chronic pelvic pain was defined by two or more consecutive reports of pelvic pain during follow-up. Data from at least 2 follow-up visits were required to determine chronic pelvic pain.

Frequencies of baseline characteristics including age, race, marriage, education, insurance, microbiological etiology, vaginal discharge, temperature, elevated WBC, bilateral adnexal tenderness, smoking, drug use, and gynecological history were compared between patients seeking delayed care or early care, using the X^2 test of proportions.

Delayed care was compared among the following groups; women with *C. trachomatis* identified in the cervix and/or endometrium who had test results that were negative for both *N. gonorrhoeae* and *M. genitalium*, women with *N. gonorrhoeae* identified in the cervix and/or endometrium who had test results that were negative for both *C. trachomatis* and *M. genitalium,* women with *M. genitalium* identified in the cervix and/or endometrium who had test results that

were negative for both *C. trachomatis* and *N. gonorrhoeae,* and women with co-infection with two or more of these organisms. A total of 188 women had complete data on all three organisms. Important demographic, behavioral, and clinical characteristics of these women did not differ from women without complete data on all three organisms. Kruskal Wallis test was used to compare the mean time to treatment between microbial pathogens. Cox regression models were used to compare times to pregnancy and times to recurrent PID between groups among the entire cohort and by groups defined by microbial pathogen. All models were adjusted for race and age, and models predicting infertility, pregnancy, and live birth were additionally adjusted for self-reported infertility at baseline. All analyses were conducted using SAS Version 9.2 (Cary, NC).

2.4 RESULTS

Treatment group, age, education, marriage, insurance, vaginal discharge, elevated temperature, history of PID, history of bacterial vaginosis, history of *C. trachomatis*, history of *N. gonorrhoeae*, history of pelvic surgery, and behavioral characteristics did not differ significantly between groups (Table 1). Women with delayed care (n = 70) compared to women with early care (n = 228) were significantly less likely to be black (68.6% vs. 82%; p = 0.016), less likely to have an elevated WBC count (26.3% vs. 43.9%; p = 0.017), and less likely to have bilateral adnexal tenderness (74.3% vs. 85.1%; p = 0.037).

Time to care varied within our cohort, ranging from 1 to 30 days. Overall, women waited a mean of 7 days before seeking care for symptoms. The mean time to treatment differed significantly between microbial pathogen (p<0.0001), with those who had *C. trachomatis* monoinfection exhibiting the longest time to treatment (12.3 days) followed by patients with *M.*

genitalium monoinfection(10.86 days), co-infection patients (5.56 days), and patients with *N. gonorrhoeae* monoinfection (4.56 days; Table 2). Women who tested negative for all three pathogens delayed care for 8.83 da ys. While these women tested negative for all three of our pathogens, we cannot rule out that they aren't infected with other pathogens which were not tested for or tested negative due to a small pathogen load. Pairwise comparisons revealed that time to care among patients with *C. trachomatis* monoinfection significantly differed from *N. gonorrhoeae* monoinfection and co-infection ($p<0.05$), but not *M. genitalium* monoinfection. Similarly, *M. genitalium* monoinfection significantly differed from *N. gonorrhoeae* monoinfection and co-infection ($p<0.05$), but not *C. trachomatis* monoinfection.

Among women with co-infection, those infected with both *C. trachomatis* and *M. genitalium*, but not *N. gonorrhoeae*, had the longest times to treatment (6.8±5.7 days), followed by infection with both *C. trachomatis* and *N. gonorrhoeae*, but not *M. genitalium*, (5.8±5.6 days), infection with both *N. gonorrhoeae* and *M. genitalium*, but not *C. trachomatis*, (4.7±3.7 days), and co-infection with all three pathogens (2.7±1.7 days). Results were non-significant ($p=0.6638$). Pairwise comparisons revealed similar but non-significant results.

Microbial correlates of participants presenting for care differed significantly between groups ($p = 0.006$; Table 3). Compared to women with prompt care those who delayed care were more likely to have *C. trachomatis* (23.8% vs. 8.9%) or *M. genitalium* (7.1% vs. 2.7%) monoinfection. In contrast, women with delayed care were less likely to be infected with *N. gonorrhoeae* alone (9.5% vs. 28.8%) or to be co-infected with two or more pathogens (19.1% vs. 28.8%).

Although infertility, recurrent PID, and chronic pelvic pain were frequent overall (17%, 20%, & 36%), rates did not significantly differ between women with delayed care and early care.

In the entire cohort, there was a non-significant decrease in pregnancy rates (adjusted hazards ratio (AHR) 0.79, 95% CI 0.55-1.13) and increase in recurrent PID (AHR 1.46, 95% CI 0.83-2.58) among women who delayed care. Among *C. trachomatis* patients there was a 6-fold increase in recurrent PID among those who delayed care (AHR 6.11, 95% CI 0.21-187.41), although this was non-significance. A non-significant increase in recurrent PID was also observed among *M. genitalium* (AHR 1.59, 95% CI 0.02-125.52) and *N. gonorrhoeae* (AHR 1.23, 95% CI 0.15-10.05) patients who delayed care. However, the sample size was small for monoinfections, limiting our power and resulting in large confidence intervals.

2.5 DISCUSSION

In this cohort of women with mild to moderate PID, times to care varied by pathogen, with the longest times among women with *C. trachomatis* monoinfection, followed by *M. genitalium* monoinfection, and the shortest times among women with *N. gonorrhoeae* monoinfection and co-infection with two or more pathogens. Overall, rates of infertility, recurrent PID, and chronic pelvic pain were high in our cohort.

Our findings demonstrate that women infected with *C. trachomatis* monoinfection had the longest times to care following the onset of pelvic pain. This is similar to Hillis *et al*. who reported that *C. trachomatis* was associated with delayed care (OR 2.1, 95% CI 1.0-4.1) (9). Our results indicate trends towards decreased pregnancy rates and increased recurrent PID. This is similar to Hillis *et al*, who found that delayed care was associated with impaired fertility (OR 2.8, 95% CI 1.3-6.1) (9). In contrast to Hillis *et al*. our results were non-significant.

There are several possible explanations for these not entirely concordant results. Our sample size limited the power of our analysis. Further, our population of women is likely a different patient population than the Scandinavian women recruited a generation ago. In addition, endometrial histology was used to confirm PID in our study, whereas 1,844 patients in the Westrom study had laparoscopically confirmed salpingitis (2). Although, the use of histologically confirmed endometritis to diagnose PID is an appropriate alternative to laparoscopy (5), not all women with endometritis have salpingitis (1). Salpingitis may indicate more severe disease and lead to tissue damage, while endometritis may have lesser effects on tubal pathology (1). In fact, endometritis was previously reported to not be associated with reproductive morbidity in the PEACH study (1).

Lastly, our studies differed in our definition of delayed care. Hillis *et al.* defined delayed care as 3 or more days of pain before seeking treatment. This cut-point was not appropriate for our population as 76% of patients delayed care for 3 days or more and waited a mean of 7 days before seeking treatment. We ran several sensitivity analyses and using a definition of 3 or more days of pain before seeking care did not change our results.

Our finding that patients with chlamydial or *M. genitalium*-associated PID were more likely to delay care was not surprising. Among women with PID, pelvic pain may be mild or absent (55), and PID symptoms vary by microbial etiology (108, 36, 109, 110). Chlamydial infections are known to elude detection because they produce few or no symptoms or because the symptoms are non-specific (111). Further, in chlamydial salpingitis, clinical manifestations are often mild but associated with severe tubal damage (36). This is in contrast to gonococcal salpingitis which produces more overt and severe inflammation and symptoms but leads to milder disease (36).

We found that the time to treatment for *M. genitalium* was similar to that of *C. trachomatis*. This finding is consistent with other studies reporting similar symptoms between *C. trachomatis* and *M. genitalium* (109, 110). This may indicate that among women with PID, those infected with *N. gonorrhoeae* or co-infection present with more overt and severe symptoms, leading to earlier treatment than women with *C. trachomatis* or *M. genitalium*. Indeed we found that the clinical characteristics such as elevated temperature, elevated WBC count, and bilateral adnexal tenderness were lower among women who delayed care compared to those who presented with prompt care.

The role of individual microbes in the development of sequelae following PID is not well understood. In the PEACH study, approximately 6% of participants had *N. gonorrhoeae* or *C. trachomatis* and 45.9% had persistent endometritis at the 30-day follow-up following baseline treatment with cefoxitin and doxycycline (87). Despite demonstrated efficacy of this Centers for Disease Control recommended regimen (6), there may be several reasons for long term sequelae in some patients. First, as patients with chlamydial or *M. genitalium* infection were more likely to delay care, persistent, low level inflammation may have caused permanent damage to the reproductive tract prior to enrollment. Further *M. genitalium* is resistant to tetracycline and lacks the cell wall target of cefoxitin (8). Not surprisingly, PEACH study patients with endometrial *M. genitalium* were previously reported to suffer treatment failure (8). Although the effect of delayed care on sequelae among microbial subgroups was non-significant, rates of sequelae were high.

Our study has several strengths. First, data were obtained from a large, multicentre, prospective randomized clinical trial, with comprehensive demographic, clinical, and obstetric measurements. Further, our findings are generalizable to patients treated for clinically suspected

PID. We recognize that timing of treatment was based on self-reported data and that misclassification bias is possible.

Our study demonstrates that among patients with clinically suspected PID, those infected with *C. trachomatis* and *M. genitalium* were more likely to delay care compared to those with gonococcal infection, possibly increasing the likelihood that persistent, low level inflammation may permanently damage the reproductive tract before patients seek care. Our findings could be translated to a population with uncomplicated sexually transmitted infections, where those infected with *C. trachomatis* and *M. genitalium* may be more likely to delay or not seek treatment, putting them at higher risk for PID and its sequelae. Thus continued efforts aimed at early identifications and treatment of asymptomatic, mildly symptomatic, and symptomatic lower genital tract infections are needed for the prevention and progression to PID and its associated long term morbidities.

2.6 TABLES

Table 1. Baseline characteristics of participants presenting for care

Characteristics	**Delayed Care (≥ 14 days)** **N = 70** **n (%)**	**Early Care (< 14 days** **N = 228** **n (%)**	***p-value**
Treatment Groups			
Inpatient	31 (44.3)	121 (53.1)	0.198
Outpatient	39 (55.7)	107 (46.9)	
Demographics			
Age			
<25 years	52 (74.3)	158 (69.3)	0.423
25+ years	18 (25.7)	70 (30.7)	
Race/Ethnicity			
African American	48 (68.6)	187 (82)	0.016
White/Hispanic/Other	22 (31.4)	141 (18)	
Married	7 (11.3)	14 (6.7)	0.231
Education			
Less than high school	30 (72.7)	103 (45.2)	0.732
High school or greater	40 (57.1)	125 (54.8)	
Uninsured	36 (58.5)	114 (54.3)	0.599
Clinical Findings			
Abnormal vaginal discharge	47 (24.4)	23 (21.9)	0.634
Temperature (>100.4 F)	3 (4.7)	26 (11.9)	0.095

Table 1. continued

WBC (> 10,000 mm^3)	15 (26.3)	86 (43.9)	0.017
Bilateral adnexal tenderness	52 (74.3)	194 (85.1)	0.037
Gynecological History			
History of PID	16 (23.5)	53 (23.8)	0.968
[**]History of BV	15 (21.7)	43 (19.5)	0.679
History of *C. trachomatis*	22 (31.9)	100 (45.1)	0.053
History of *N. gonorrhoeae*	24 (34.3)	60 (26.8)	0.225
[§]History of pelvic surgery	14 (20.0)	51 (22.4)	0.650
Behavioral			
Current smoker	33 (47.1)	113 (49.8)	0.699
Drug use	21 (30)	67 (29.5)	0.938

[*]Chi-square was used to derive the p-value

[**]History of bacterial vaginosis (BV)

[§]Self-reported prior pelvic surgeries including but not limited to: Appendix, Bartho cyst, tubal, ovarian, uterine, fiscular, hernia, and colopscopy.

Table 2. Mean time to treatment by organism

Organism	**Days of pain** **Mean±SD**	***p-value**
C. trachomatis Only (N=23)	12.3±9.35	<.0001
M. genitalium Only (N=7)	10.86±8.90	
N. gonorrhoeae Only (N=45)	4.56±4.97	
**Co-Infection (N=50)	5.56±5.11	
None (N=63)	8.83±8.95	

*Kruskal Wallis test was used to derive the p-value.

* *Co-infection – participants with any combination of two or more of the following pathogens: *C. trachomatis*, *N. gonorrhoeae*, or *M. genitalium*

Table 3. Microbial correlates of participants presenting for care ≥14 days

Organism	**Delayed Care (≥14 days)** **[*]N=42** **n (%)**	**Early Care (< 14 days)** **[*]N=146** **n (%)**	****p-value**
C. trachomatis Only (N=23)	10 (23.8)	13 (8.9)	0.0186
M. genitalium Only (N=7)	3 (7.1)	4 (2.7)	0.1551
N. gonorrhoeae Only (N=45)	4 (9.5)	41 (28.8)	0.0122
[§]Co-Infection (N=50)	8 (19.1)	42 (28.8)	0.2090
None (N=63)	17 (40.5)	46 (31.5)	0.4613

[*]A total of 188 women had data on *C. trachomatis*, *M. genitalium*, and *N. gonorrhoeae* combined.

[**]Chi-square was used to derive the p-value

[§]Co-infection – participants with any combination of two or more of the following pathogens: *C. trachomatis*, *N. gonorrhoeae*, or *M. genitalium*

3.0 MANUSCRIPT 2: ARE VARIATIONS IN INNATE IMMUNE RECEPTOR GENES ASSOCIATED WITH *CHLAMYDIA TRACHOMATIS* AMONG WOMEN WITH PELVIC INFLAMMATORY DISEASE?

Manuscript in Preparation

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3.1 ABSTRACT

Background: Toll-like receptors (TLR) initiate microbial elimination through induction of inflammatory responses. As genetic variations may alter TLR signaling, we explored the role of TLR variants in *Chlamydia trachomatis* (CT) and endometritis among women with pelvic inflammatory disease (PID).

Methods: We determined if 18 tagging single nucleotide polymorphisms (tagSNP) assayed in 4 TLR genes (TLR1, TLR2, TLR4, TLR6) and 2 adaptor molecules (TIRAP, MyD88) were associated with CT or endometritis, among 205 African Americans with clinically suspected PID from the PID Evaluation and Clinical Health Study. Logistic regression was used to calculate odds ratios (OR) and 95% confidence intervals (CI). An empirical p-value of <0.004 was considered significant. Haplotype analyses were performed in SAS/Genetics v9.1.3.

Results: Women with PID who carried the TLR4 rs1927911 CC genotype had increased odds of CT (case:control frequency = 36:14; OR 3.7, 95% CI 1.6-8.8; p=0.002). The TLR1 rs5743618 TT genotype was also associated with CT (84:65; OR 2.8, 95% CI 1.3-6.2), but not after permutations (p=0.008). Haplotype analysis revealed that the TLR4 GAC haplotype was more frequent in CT negative women (0.59:0.43; p=0.012). In contrast, the TLR4 GAT haplotype and the TLR1 TGT haplotype (0.15:0.07; p=0.04) were more frequent in CT positive women (0.19:0.10; p=0.04). No significant associations were found between any TLR or adaptor molecule SNP and endometritis.

Conclusions: Among African American women with PID, variants in the TLR1 and TLR4 genes were associated with increased CT. Genetic variations in these TLR genes may increase signaling, possibly leading to upper genital tract inflammation.

3.2 INTRODUCTION

Chlamydia trachomatis is the most common bacterial sexually transmitted infection in the United States (10), and can lead to serious complications such as pelvic inflammatory disease (PID) and its subsequent sequelae including ectopic pregnancy, infertility, and chronic pelvic pain (2, 3). *C. trachomatis* can be asymptomatic in up to 80% of women, making diagnosis and management a challenge (226). Further, rates of progression from *C. trachomatis* infection to PID vary widely, and can range from 2-4.5% in asymptomatic populations (10, 13-15). As the mechanisms underlying the pathogenesis of *C. trachomatis* have yet to be completely elucidated, the reasons for this variability are unknown. It is widely thought that chlamydial pathogenesis is driven by inflammatory responses (36, 137), especially among those with chronic or persistent infections. Thus, variations in host innate immune receptor genes may not only play a role in chlamydia pathogenesis but may explain the wide variability in the course and outcome of infection.

The innate immune system serves as the first line of defense after exposure to pathogens and depends on pa ttern recognition receptors (PRRs) for microbial recognition (121-123). A conserved family of PRRs called the Toll-like receptor (TLR) family (122) is responsible for microbial elimination through induction of inflammatory cytokine and chemokine genes, as well as the priming of the adaptive immune system. Ten different TLRs (TLRs 1-10) have been identified in humans and the overlap between them allows identification of a diverse range of pathogens through ligand binding (121-123). Adaptor molecules including myeloid differentiation primary response protein 88 (MyD88) and TIR domain-containing adaptor protein (TIRAP or MAL) help to mediate TLR signaling (123, 127). However, as important as TLRs and

their adaptor molecules are for a healthy immune response, variations in these genes may lead to an overt or inadequate inflammatory response, possibly playing a role in disease progression.

Studies have shown that TLRs 2 and 4 can bind to several possible chlamydial ligands (138-143), thus making them likely candidates for *C. trachomatis* recognition. TLR4 may be able to bind to Lipopolysaccharide (LPS) and chlamydia heat shock protein 60 (cHSP60) (138). TLR2 can dimerize with TLRs 1 and 6, to recognize a more diverse range of ligands (144) including lipoproteins, lipopeptides, lipoteichoic acid, and bacterial prion (139-143). Still, the exact function of TLRs in *C. trachomatis* infection is unclear. O'Connell et al, using an experimental model to examine the role of TLRs in early infection, found that TLR2 was required for IL-8 expression, while TLR4/MD-2 had minimal effects on cytokine production (149). Further, TLR2 and MyD88 both localized within chlamydia inclusions during active infection (149). Darville et al, using a murine model, found that TLR2 signaling is necessary for the development of oviduct pathology and excessive cytokine production following chlamydial genital infection, while TLR4 has similar outcomes in TLR4 knockout mice (150). These studies may suggest that TLR2 is primarily responsible for signaling following *C. trachomatis* infection.

Several TLR SNPs have been found to be associated with inflammatory and infectious diseases (163, 173, 176, 186, 199). However, very few epidemiologic studies have examined TLRs in *C. trachomatis* pathogenesis. One group of researchers that conducted several limited studies among Dutch Caucasian populations has been unable to show any significant associations between innate immune receptor functional single nucleotide polymorphisms (SNPs) and *C. trachomatis* (154, 157, 158). One study among 48 women who were positive for *C. trachomatis* IG antibodies, found no significant associations between TLR4 SNP Asp299Gly (rs4986790) and tubal factor infertility (154). Similarly, in another study from this group Asp299Gly was not

associated with *C. trachomatis* susceptibility or subsequent pathology among 614 sexually transmitted disease (STD) patients (157). Karimi et al, examined the role of two TLR2 SNPs (rs5743708 and rs4696480) in 468 Dutch women (158), and found no associations with *C. trachomatis* infection (158). These homogenous Dutch populations may not be generalizable to more diverse populations of women with STDs in the United States.

Our objective was to explore the role of TLR1, TLR2, TLR4, TLR6, MyD88, and TIRAP gene polymorphisms in *C. trachomatis* among African American women with clinically suspected PID. Further, to explore SNP associations with upper genital tract inflammation, we also used histologically confirmed endometritis as an outcome. As few studies have examined these associations, there is an opportunity to provide novel information regarding the pathogenesis of *C. trachomatis*.

3.3 METHODS

This study utilized data from the PID Evaluation and Clinical Health (PEACH) study. This was the first randomized clinical trial to compare inpatient and outpatient treatment in preventing long-term complications among 831 women with mild to moderate PID. The methods of subject recruitment, data collection, and follow-up have been reported elsewhere (87). Briefly, between March 1996 and February 1999 women aged 14-37 years were recruited from emergency departments, obstetrics and gynecology clinics, sexually transmitted disease clinics, and private practices at 7 primary and 6 secondary sites throughout the eastern, southern, and central regions of the United States. Women who had suspected PID and gave informed consent were eligible for the PEACH study. Eligible women had a history of pelvic discomfort for less than 30 days,

findings of pelvic organ tenderness (uterine or adnexal) on bimanual examination, and leukorrhea and/or mucopurulent cervicitis and/or untreated but documented gonococcal or chlamydial cervicitis. The University of Pittsburgh Institutional Review Board approved the study.

A total of 2941 women were screened for study entry, of those 346 (11.8%) did not meet the clinical inclusion criteria for randomization. Women were additionally excluded if they were pregnant (n = 141, 4.8%); had taken antimicrobials within the past 7 days (n = 248, 8.4%); had a history of hysterectomy or bilateral salpingectomy (n = 248, 8.4%); had an abortion, delivery, or gynecologic surgery within the past 14 days (n = 51, 1.7%); had a suspected tubo-ovarian abscess or other condition requiring surgery (n = 191, 6.5%); had an allergy to the study medications (n = 163, 5.5%); were homeless (n = 29, 1%); or had vomiting after a trial of antiemetic treatment (n = 11, 0.4%). A total of 290 women (205 non-Hispanic black race, 51 non-Hispanic white race, 34 other race) with previously stored buffy coats (n=237) or serum samples (n=50) were genotyped for TLR and adaptor molecule SNPs in this study. There were no significant differences in the genotype frequencies between women genotyped with buffy coats or serum samples. Because the sample size for white race and other races was small they were excluded from this analysis. A total of 205 African American women were included in this analysis.

Women were randomized to either inpatient treatment of intravenous cefoxitin every 6 hours and doxycycline orally twice a day for 14 days; or outpatient treatment consisting of a single intramuscular injection of cefoxitin and oral doxycycline twice a day for 14 days. Because the treatment modality was not associated with reproductive morbidities in the PEACH study (87), we do not include them as a covariate in this analysis. Participants were followed-up with

in-person visits at 5 and 30 days after treatment. At the 30-day follow-up the gynecological exam was repeated. Telephone follow-ups were conducted by the study nurses every 3 months during the first year after enrollment and then every 4 months until June 2004. At that point information was obtained by self-report for 69.1% of the cohort, with a mean follow-up of 84 months.

A pelvic examination and interview were conducted at the baseline visit. The interview collected information on reason for visit, brief pain history, demographics, history of PID/sexually transmitted diseases, sexual and contraceptive history, reproductive decisions, douching history, pregnancy history, medical and gynecological history and lifestyle habits. Follow-up interviews collected self-reported information on pelvic pain, pregnancy and births, signs and symptoms of PID, STDs, contraceptive use, pattern of sexual intercourse, and health care utilization.

Gynecological examinations were performed at baseline and 5 and 30 days post treatment. Vaginal smears were gram stained for bacterial vaginosis in a central laboratory by standardized methods described by Nugent et al (223). Endometrial biopsy specimens were obtained for histological examination including, chlamydial polymerase chain reaction (PCR) and gonococcal culture. PCR and cultures were performed at a central reference laboratory. For the patients with endometrial biopsies, two reference pathologists separately evaluated at least one section stained with hematoxylin and eosin and at least one stained with methyl green pyronine. Disagreements about the presence or absences of neutrophils and plasma cells were settled by both pathologists reading the slides together and coming to an agreement. Histological endometritis was based on a modification of the criteria proposed by Kiviat et al (224). Endometritis was defined as the presence of at least five neutrophils in the endometrial surface epithelium in the absence of menstrual endometrium and/or at least two plasma cells in the

endometrial stroma. Cervical and endometrial specimens were stored and later used to test for *M. genitalium* (8), using a microwell-plate-based PCR assay (MgPa-IMW) targeting the MgPa gene (225).

A total of 205 African American women were genotyped for TLR and TLR adaptor molecule SNPs. For the proposed study 18 tagging SNPs (TagSNPs) including three from TLR1 (rs5743618, rs5743817, rs4833095), three from TLR2 (rs3804099, rs11938228, rs1898830), three from TLR6 (rs1039559, rs5743810, rs3775073), four from TLR4 (rs4986790, rs4986791, rs11536889, rs5030728), two from MyD88 (rs4988457, rs7744), and three from TIRAP (rs3802813, rs8171374, rs7932976), were chosen from Hapmap.org. TagSNPs were chosen based on their reported minor allele frequencies and relative distance to one another within each gene.

All SNPs were genotyped by fluorescence polarization (227). PCR conditions included 1 X PCR 2.5µl of buffer (Invitrogen) with 1.0µl of $MgCl_2$, 4µl of dNTPs, 1.5µl of each primer, 0.1µl of Taq polymerase (Invitrogen), and 13.4µl of dH_2O; for a total volume of 25.0µl. Amplification was performed using a Peltier Thermal Cycler (MJ Research). Thermal cycling conditions were 95°C for 3 minutes, then 35 cycles of 95°C/30 seconds for denaturing, 55°C/30 seconds for annealing, and 72°C/30 seconds for extension, then the final extension step of 72°C/1 minute. PCR products were visualized on a 3% agar gel by electrophoresis. To check for genotyping errors, allele frequencies in our cohort were compared to those reported on Hapmap.org from a population of African ancestry in Southwest USA and a healthy African American population from Pittsburgh, PA which was genotyped in the same lab as our samples. There were no significant differences in allele frequencies between the groups.

All analyses were conducted among 205 African American women. Demographic and clinical baseline characteristics including, age, education, insurance, marriage, white blood cell count (WBC), temperature, C-reactive protein (CRP), bilateral adnexal tenderness, cervicitis, erythrocyte sedimentation rate (ESR), *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, smoking status, and drug use were compared between chlamydial positive cases and chlamydial negative controls, using X^2 test of proportions. Our main analyses compared genotype frequencies between 94 *C. trachomatis* positive cases and 111 *C. trachomatis* negative controls. In addition, we also compared genotype frequencies between 91 cases with confirmed endometritis and 74 controls without histological evidence of endometritis. All SNPs were tested for Hardy-Weinberg equilibrium (HWE), using 10,000 permutations to approximate an exact p-value. Additive or dominant models were analyzed for all SNPs. Dominant models were defined by the minor allele, where if the minor allele was A the dominant model would compare AA+AG to GG. Logistic regression was used to calculate odds ratios and 95% confidence intervals. Any model with less than 5 in any cell was excluded from logistic regression analysis (TLR1 rs5743817, TLR4 rs11536889, TIRAP rs8171374, and TIRAP rs7932976). All models were adjusted for age. Permutations were used to correct for multiple comparisons. A p-value <0.004 based on 1000 permutations, was considered significant for SNP genotype associations. Haplotype analyses were also conducted. The expectation-maximization algorithm was used to generate maximum likelihood estimates of haplotype frequencies using SAS/Genetic v9.1.3 (228). Haplotypes were then tested for associations with each trait using chi-square test and 1000 permutations to approximate an exact p-value based on the Monte Carlo method. For genes with significant and frequent haplotypes, regression analysis was used to examine associations

between haplotype pairs and traits. All analyses were performed using SAS/Genetics v9.1.3 (Cary, NC).

3.4 RESULTS

Compared to chlamydial negative women, chlamydial positive women were more likely to be under the age of 25 ($p<0.001$), have elevated C-reactive protein (CRP; >5mg/dL) ($p=0.0003$), and endometritis ($P=0.006$), but less likely to have bilateral adnexal tenderness ($p=0.02$) or an elevated temperature ($p=0.02$) (Table 4). All other demographic variables were similar between cases and controls. All SNPs were in HWE in the control group.

Logistic regression revealed that among women with PID, the TLR4 rs1927911 CC genotype increased the odds of chlamydial infection (odds ratio (OR) 3.7, 95% confidence interval (CI) 1.6-8.8; $p=0.0021$) (Table 5). Results were similar for the TLR1 rs5743618 TT genotype (OR 2.8, 95% CI 1.3-6.2), although this association was not statistically significant after adjusting for multiple comparisons ($p=0.0084$). No significant associations were found between any other TLR or adaptor molecule SNP and chlamydia. We also failed to find any significant associations between TLR or adaptor molecule SNPs and endometritis (Table 6). However, a sub-analysis among 164 women who had data on upp er genital tract infection, revealed that after adjustments for age and *N. gonorrhoeae* the TLR1 rs5743618 TT genotype (case:control frequency = 87:69; OR 7.2, 95% CI 1.8-27.7; $p=0.004$) and the TLR4 rs1927911 CC genotype (case:control frequency = 56:27; OR 4.9, 95% CI 1.7-13.8; $p=0.003$) were both associated with upper genital tract infection.

Haplotype analyses revealed that women who carried the TLR1 (rs5743618/rs5743817/rs4833095) TGA haplotype were more likely to be chlamydial positive (case:control frequency = 0.15:0.07; exact p=0.04) (Table 7). However, the frequency of this haplotype was low in the population (10%), and regression analyses could not be conducted because of small cell size. Women who carried the TLR4 (rs5030728/rs4986790/rs1927911) GAC haplotype were more likely to be chlamydial positive (0.33:0.19, exact p=0.002), while those who carried the GAT haplotype were less likely to be chlamydial positive (0.43:0.59, exact p=0.01). Regression analyses revealed similar results for the GAC/GAC haplotype pair (OR 4.3, 95% CI 1.6-12.2) and the GAT/GAT haplotype pair (OR 0.2, 95% CI 0.1-0.6). Among women who carried the GAC/GAC haplotype pair, 86% (12/14) were chlamydial positive, while 39% (15/38) who carried the GAT/GAC haplotype pair and 35% (20/57) who carried the GAT/GAT haplotype pair were chlamydial positive (Figure 1). No other significant haplotype associations were found between TLR2, TLR6, TIRAP, or MyD88 and chlamydia.

Women who carried the TIRAP (rs3802813/rs7932976/rs8177374) GGC haplotype were more likely to have histologically confirmed endometritis (0.92:0.83, exact p=0.04). Regression analyses revealed similar results for the GGC/GGC haplotype pair (OR 5.1, 95% CI 1.3-19.4). However, this haplotype was very frequent in the population (89%) and almost all women with data on endometritis were predicted to carry 1 or more of the GGC haplotype. Women who carried the TLR6 (rs1039559/rs5743810/3775073) CTA haplotype were less likely to have endometritis (0.04:0.11, exact p=0.04). However, the frequency of the CTA haplotype in the population was low (7%) and regression analyses could not be performed. Haplotype analyses revealed no significant associations between TLR1, TLR2, TLR4, or MyD88 haplotypes and endometritis. However, we did find that women who were predicted to carry the TLR4

(rs5030728/rs4986790/rs1927911) GAC haplotype were more likely to have upper genital tract infection (0.35:0.21, exact p=0.02), while those with the GAT haplotype were less likely to have upper genital tract infection (0.59:0.42, exact p=0.01).

3.5 DISCUSSION

In this cohort of African American women with mild to moderate PID, TLR1 and TLR4 variants were associated with *C. trachomatis* infection. In addition, women predicted to carry haplotypes in the TLR1 and TLR4 genes were more likely to be *C. trachomatis* positive. Although these variants were not significantly associated with endometritis, they displayed trends towards increased odds of upper genital tract infection.

TLRs initiate microbial elimination through the production of inflammatory cytokines and chemokines via activation of nuclear factor κ-B (NF-κB) (121-123). Although this initially results in a healthy immune response, variations in these genes may alter TLR signaling possibly playing a role in disease progression. Variations in these genes may also explain the variability seen in the course and outcome of *C. trachomatis* infection (10, 13-15). Our results show that TLR1 and TLR4 variants may play a role in the development of chlamydial PID. The involvement of innate immune receptors in chlamydial pathogenesis makes sense. Chlamydia ssp. can infect epithelial cells leading to the secretion of proinflammatory cytokines (135-137). Further, this inflammatory response may be responsible for long-term damage of the reproductive tract following *C. trachomatis* infection (146, 147). Therefore, the initial inflammatory response following the recognition of *C. trachomatis* by TLRs is likely protective. However, as chlamydia has a self-limited acute course that often resolves into a chronic low-

grade infection (229), it is likely that TLR signaling eventually leads to chronic inflammation of the upper genital tract causing long-term sequelae. We did find that compared to chlamydial negative women, chlamydial positive women were more likely to have elevated CRP. CRP is an acute phase protein and is an indicator of inflammation, suggesting that these women may have had persistent chlamydial infections resulting in increased inflammation.

We found that among women with PID, the CC genotype of TLR4 SNP rs1927911 was associated with increased odds of *C. trachomatis* infection. Further, women who were predicted to carry the TLR4 (rs5030728/rs4986790/rs1927911) GAC haplotype were more likely to be chlamydial positive. As those with the TLR4 GAT haplotype were significantly less likely to be infected with *C. trachomatis*, this suggests that rs1927911 is driving the haplotype associations. It is possible that TLR4 plays a role in *C. trachomatis* pathogenesis. TLR4 is expressed in the female reproductive tract and has been reported to be present in the endocervix, endometrium, and Fallopian tubes (121, 130-133). In addition, TLR4 can recognize both LPS and cHSP60 (138). Several retrospective studies have found cHSP60 to be linked with chlamydia-associated tubal infertility and PID (23-28). However, prospective data examining the relationship has been limited. One prospective study among 302 female sex workers in Nairobi, Kenya, found cHSP60 antibodies to be associated with PID (OR 3.9, 95% CI 1.04-14.5; P=0.04) (29). In contrast, Ness et al did not find cHSP60 antibody titers to be significantly associated with sequelae following clinically suspected PID (18). Certain biases, such as diagnostic bias, were suggested to overestimate the effect size in some studies (18). For example, diagnosis of clinically suspected PID may have been influenced by the knowledge of a previous chlamydial exposure. If cHSP60 is a surrogate for that prior exposure then this would have biased the results away from the null.

Still, it may be possible that cHSP60 is recognized by TLR4, resulting in persistent inflammation that damages the reproductive tract following *C. trachomatis* infection. However, this does not correlate with mice models which suggest that TLR4 is not involved in genital tract pathology following genital tract chlamydial infection (149, 150). Darville et al found that TLR2 knockout (KO) mice had significantly lower levels of inflammatory mediators in genital tract secretions during the first week of chlamydial infection, as well as a significant reduction in oviduct and mesosalpinx pathology at late time points following challenge with *C. muridarum* (150). In contrast, after challenge with *C. muridarum*, TLR4 KO mice had similar pathology and cytokine production compared to infected controls with TLR4 genes (150). This suggests that there is no direct role for cHSP60-induced TLR4 mediated tissue damage in genital tract infection (150). Disease mechanism in mice may not always translate to human in vivo situations. Therefore, in humans TLR4 may indeed be involved in pathogenesis following *C. trachomatis* infection.

However, the relationship may not be that straight forward as interactions between several TLRs may be involve in the balance of cytokine production. TLR2 and TLR4 have been reported to interact with one another altering TLR signaling (144, 230, 231). Mu et al found that agonist interactions between TLR2 and TLR4, in mice infected with *Mycoplasma arthritidis,* leads to differential release of IL-17 and its associated cytokines (230). Specifically the authors found high levels of IL-6 in TLR2+/TLR4+ mice compared to high levels of IL1β and TNF-α in TLR2+/TLR4- mice. When TLR4 was blocked in the TLR2+/TLR4+ mice, IL-17 and IL-6 but not IL-23 were decreased, while IL-17 and IL-6 were increased in TLR2 KO mice. Reiling et al, found that interaction between TLR2 and TLR4 resulted in increased proinflammatory cytokine production following infection with *M. tuberculosis* (231). It is clear that associations between

cHSP60 and TLR4, as well as interactions between TLR2 and TLR4 following *C. trachomatis* infection need to be further explored. Due to our sample size we did not examine any interactions, but acknowledge that our results may be masked by gene-gene or gene-environment interactions.

Little is known about the functional or clinical relevance of TLR4 rs1927911. The T allele has been found to decrease the risk of myocardial infarction (OR 0.88, 95% CI 0.77-0.99) among 1,216 cases and 2,682 controls from Washington state (204). Over 90% of this population was Caucasian. In another Caucasian population consisting of 110 lung transplant recipients and 422 healthy controls, the TT genotype was found to increase the odds of bronchiolitis obliterans (BOS) (OR 4.20, 95% CI 1.43-12.35; p=0.005) (178). Differences in the mechanisms underlying these diseases may explain the contrasting results. In latter study the authors noted that acute rejection is a risk factor for BOS and an increase in pro-inflammatory cytokines and chemokines may decrease acute rejection thus decreasing BOS (178). The C allele was found to decrease BOS in this study. In contrast, increases in inflammation may be associated with MI (204). Functional analyses are needed to determine the relevance of rs1927911. As this SNP is located in the intron, it may be in linkage disequilibrium (LD) with another functional SNP. However, using Haploview, we were unable to find any SNPs in strong LD with this variant in African Americans.

TLR2 has been recognized to play a role in chlamydial infections (144, 149, 150, 151) However, we did not find any significant associations between TLR2 variants and *C. trachomatis*. We do acknowledge that we only had 35% gene coverage for TLR2, and other TLR2 variants that were not tagged by our SNPs should be further examined. TLR2 can form a dimer with TLR1 to recognize a range of pathogens and activate inflammatory responses. We

did find that the TLR1 rs5743618 TT genotype was associated with increased *C. trachomatis* infection. Further, the TLR1 (rs5743618/rs5743817/ rs4833095) TGA haplotype was more frequent among chlamydial positive women. The association between the TT genotype and chlamydia did not reach statistical significance after permutations. However, our sample size limited our power. TLR1 rs5743618 is a non-synonymous mutation and results in an amino acid change. The G allele has been reported to be associated with deficient TLR signaling in comparison to the T allele (182-185), and has been reported to reduce leprosy (182, 186, 184). Hawn et al, reported that the T allele expressed significantly greater NF-κB signaling in transfected HEK293 cells compared to the G allele (183). Among sepsis patients, the T allele was found to be associated with higher mortality (OR 1.79, 95% CI 1.02-3.13; P=0.042) (185). As rs574618 has a possible functional relevance it should be further explored in *C. trachomatis* pathogenesis.

We found that TLR variants were associated with *C. trachomatis* among women with mild to moderate PID. However, as some women with clinically suspected PID may actually have ovarian cysts, pelvic adhesions, or endometriosis (81), some women in our cohort may not have had true upper genital tract infection and inflammation. Endometritis is an accepted measure of true PID. However, we found no significant associations found between any of our genotyped SNPs and endometritis. Endometritis is a spotty disease and may not indicate all cases of upper genital tract inflammation (1). Further, PID is polymicrobial and therefore women with endometritis could have had a variety of microorganisms. This could possibly bias our results, as different TLRs recognize different pathogens and have been shown to induce different sets of chemokines and cytokines (230-234), suggesting that immune response may be different depending on the pathogen present (150). Further, PID-associated pathogens may have different

mechanisms of disease (36). Therefore, it would have been optimal to examine upper genital tract inflammation specifically among women with *C. trachomatis* infection. We did not have the power to examine endometritis in a subset of women with chlamydial infection. However, we did find that rs1927911 and rs5743618 were associated with upper genital tract infection after adjusting for age and *N. gonorrhoeae*. This may give some suggestion that that these variants play a role in the progression of *C. trachomatis* to the upper genital tract.

Our study has several strengths. First, data were obtained from a large, multicenter, prospective randomized clinical trial, with comprehensive demographic, clinical, and obstetric measurements. These findings are generalizable to patients treated for clinically suspected PID. Not all women in the PEACH study had blood samples available for analyses. However, important demographic and clinical characteristics between women with and without blood samples did not differ. This is the first study to examine the role of several TLR and adaptor molecule SNPs in chlamydial-PID. However, our sample size limited our power. We also had low SNP coverage for our genes. Therefore, other TLR variants especially in the TLR2 gene should continue to be explored in chlamydial pathogenesis. We also relied on an internal control group for comparison. Therefore women in the controls groups all had clinically suspected PID.

Among African American women with PID, TLR1 and TLR4 variants were associated with *C. trachomatis*. These variants may increase TLR signaling leading to persistent inflammation which may permanently damage the reproductive tract. This study provides novel insight into the pathogenesis of *C. trachomatis* infection. However, our results need to be replicated. In addition, comparisons should be made between women with chlamydial PID and women with uncomplicated lower genital *C. trachomatis* infection. Further exploration into the role of innate immune receptors in the course and outcome of *C. trachomatis* is needed to

delineate its pathogenesis. This type of research may lead to better management and control of *C. trachomatis*, possibly through vaccine development.

3.6 TABLES

Table 4. Baseline demographic and clinical characteristics by chlamydial status

Characteristics	**Chlamydia Negative** **N = 111** **n (%)**	**Chlamydia Positive** **N = 94** **n (%)**	***p-value**
Demographics			
Age			
<25 years	53 (47.8)	81 (86.2)	
25+ years	58 (52.3)	13 (13.8)	<0.0001
Married	8 (7.8)	5 (5.6)	0.5704
Education			
Less than high school	44 (39.6)	45 (47.9)	
High school or greater	67(60.4)	49 (52.1)	0.2360
Uninsured	51 (50.0)	38 (43.7)	0.3855
Clinical Findings			
Temperature (>100.4 F)	15 (14.7)	4 (4.4)	0.0175
WBC (> 10,000 mm^3)	32 (38.1)	28 (33.3)	0.5195
C-reactive protein (>5mg/dL)	1 (8.3)	16 (72.7)	0.0003
Bilateral adnexal tenderness	92 (82.9)	65 (69.2)	0.0207
Neisseria gonorrhoeae	34 (35.1)	27 (34.6)	0.9520
Mycoplasma genitalium	12 (12.2)	8 (13.3)	0.8417
Bacterial Vaginosis	69 (65.7)	58 (66.7)	0.8896
Cervicitis	60 (59.4)	56 (65.1)	0.4226
Endometritis	42 (45.7)	49 (67.1)	0.0059

Table 4. continued

Behavioral			
Current smoker	50 (45.1)	39 (41.5)	0.6088
Drug use	34 (30.6)	31 (33.0)	0.7189

*Chi-square was used to derive the p-value. Fisher's Exact was used when cell size was less than 3

Table 5. Association between genotypes and cervical and/or endometrial *C. trachomatis* infection among women with pelvic inflammatory disease

SNPs and Genotypes	Chlamydial Negative (N=111)	Chlamydial Positive (N=94)	*Adjusted Odds Ratio (95% CI)	P-value
TLR 1				
rs5743618				
GG+GT	37(34.9)	12(16.0)	Referent	
TT	69(65.1)	63(84.0)	2.8 (1.3-6.2)	0.0084
rs4833095				
AA+AG	46(62.1)	63(56.3)	Referent	
GG	28(37.8)	49(43.8)	1.2 (0.6-2.3)	0.5287
TLR 2				
rs3804099				
CC	46 (41.5)	38(39.4)	Referent	
CT	49 (44.1)	25(32.5)	0.6 (0.3-1.3)	0.1855
TT	16(14.4)	14(18.2)	0.9 (0.4-2.2)	0.8818
rs11938228				
CC	82(73.9)	54(73.0)	Referent	
AA + AC	29(26.1)	20(27.0)	1.1 (0.6-2.4)	0.6994
rs1898830				
AA	84(75.7)	47(77.1)	Referent	
GG+AG	27(34.3)	14(22.9)	1.1 (0.5-2.6)	0.7267
TLR 6				
rs1039559				
TT	68(61.8)	43(64.2)	Referent	
CC+TC	42(38.2)	24(35.8)	0.8 (0.4-1.7)	0.6263
rs5743810				
CC	92(83.6)	59(81.9)	Referent	
TT + CT	18(16.4)	13(18.1)	1.2 (0.5-2.7)	0.7407
rs3775073				
GG	48(43.2)	25(34.3)	Referent	
AG	51(46.0)	39(53.4)	1.1 (0.6-2.2)	0.7504
AA	12(10.8)	9(12.3)	1.0 (0.4-3.0)	0.9331
TLR 4				
rs5030728				
GG	75(68.8)	58(75.3)	Referent	
AA + AG	34(31.2)	19(24.7)	0.7 (0.4-1.5)	0.4131
rs4986790				
AG	17(15.3)	14(16.7)	Referent	
AA	94(84.7)	70(83.3)	0.7 (0.3-1.7)	0.4547
rs1927911				
TT	47(42.7)	30(33.7)	Referent	

Table 5. continued

CT	47(42.7)	27(30.3)	1.0 (0.5-2.0)	0.9675
CC	16(14.6)	32(36.0)	3.7 (1.6-8.8)	0.0021
Myd88				
rs7744				
AA	102(91.9)	86(91.5)	Referent	
AG + GG	9(8.1)	8(8.5)	1.3 (0.4-3.9)	0.6288
rs4988457				
CG	25(22.5)	10(14.1)	Referent	
CC	86(77.5)	61(85.9)	1.9 (0.8-4.5)	0.1429
TIRAP				
rs3802813				
AG	15(13.5)	9(10.2)	Referent	
GG	96(86.5)	79(89.8)	1.7 (0.6-4.3)	0.2801

* Adjusted for age

** Significant based on an empirical P-value < 0.004

Logistic regression was used to calculate odds ratios and 95% CI. Any model with less than 5 observations in any cell was excluded (TLR1 rs5743817, TLR4 rs11536889, TIRAP rs8171374, and TIRAP rs7932976).

Table 6. Associations between genotypes and endometritis among women with pelvic inflammatory disease

SNPs and Genotypes	Endometritis Negative (N=74)	Endometritis Positive (N=91)	*Adjusted Odds Ratio (95% CI)	**P-value
TLR 1				
rs5743618				
GG+GT	21(31.8)	19(23.8)	Referent	
TT	45(68.2)	61(76.3)	1.5 (0.7-3.0)	0.3227
rs4833095				
AA+AG	31(51.7)	35(39.3)	Referent	
GG	29(48.3)	54(60.7)	1.7 (0.8-3.2)	0.1391
TLR 2				
rs3804099				
CC	29(41.4)	33(40.2)	Referent	
CT	31(44.3)	36(43.9)	1.1 (0.5-2.1)	0.8622
TT	10(14.3)	13(15.9)	1.2 (0.4-3.0)	0.8068
rs11938228				
CC	52(76.5)	58(71.6)	Referent	
AA + AC	16(23.5)	23(28.4)	1.4 (0.6-2.9)	0.4140
rs1898830				
AA	48(75.0)	54(72.0)	Referent	
GG+AG	16(25.0)	21(28.0)	1.2 (0.6-2.7)	0.5890
TLR 6				
rs1039559				
TT	36(57.1)	52(65.8)	Referent	
CC+TC	27(42.9)	27(34.2)	0.7 (0.3-1.3)	0.2467
rs5743810				
CC	51(77.3)	71(88.8)	Referent	
TT + CT	15(22.7)	9(11.3)	0.4 (0.2-1.1)	0.0739
rs3775073				
GG	27(40.9)	33(41.3)	Referent	
AG	30(45.5)	39(48.8)	0.9 (0.4-1.9)	0.8226
AA	9(13.6)	8(10.0)	0.5 (0.2-1.6)	0.2341
TLR 4				
rs5030728				
GG	51(73.9)	58(71.6)	Referent	
AA + AG	18(26.1)	23(28.4)	1.1 (0.5-2.4)	0.7203
rs4986790				
AG	10(14.3)	15(17.2)	Referent	
AA	60(85.7)	72(82.8)	0.8 (0.3-1.8)	05334
rs1927911				
TT	29(40.9)	33(37.1)	Referent	
CT	27(38.0)	35(39.3)	1.2 (0.6-2.4)	0.6865
CC	15(21.1)	21(23.6)	1.2 (0.5-2.8)	0.6367

Table 6. continued

Myd88				
rs7744				
AA	69(93.2)	81(89.0)	Referent	
AG + GG	5(6.8)	10(11.0)	1.7 (0.6-5.4)	0.3375
rs4988457				
CG	12(18.2)	16(20.0)	Referent	
CC	54(81.8)	64(80.0)	0.9 (0.4-2.1)	0.8042
TIRAP				
rs3802813				
AG	11(15.7)	10(11.2)	Referent	
GG	59(84.3)	79(88.8)	1.6(0.6-4.1)	0.3243

* Adjusted for age

**Significance is based on an empirical P-value of 0.004

Logistic regression was used to calculate odds ratios and 95% CI. Any model with less than 5 observations in any cell was excluded (TLR1 rs5743817, TLR4 rs11536889, TIRAP rs8171374, and TIRAP rs7932976).

Table 7. Associations between TLR haplotypes and *C. trachomatis*

Haplotype	Chlamydia negative frequencies	Chlamydial positive frequencies	*Exact P-value
**TLR1 haplotype*			
TGA	0.07	0.15	0.0440
§*TLR 4 haplotypes*			
GAC	0.19	0.33	0.006
GAT	0.59	0.43	0.0120

*Based on 1000 permutations

** TLR1 rs5743618/rs5743817/rs4833095 haplotype

§TLR 4 rs5030728/rs4896790/rs1927911 haplotypes

3.7 FIGURES

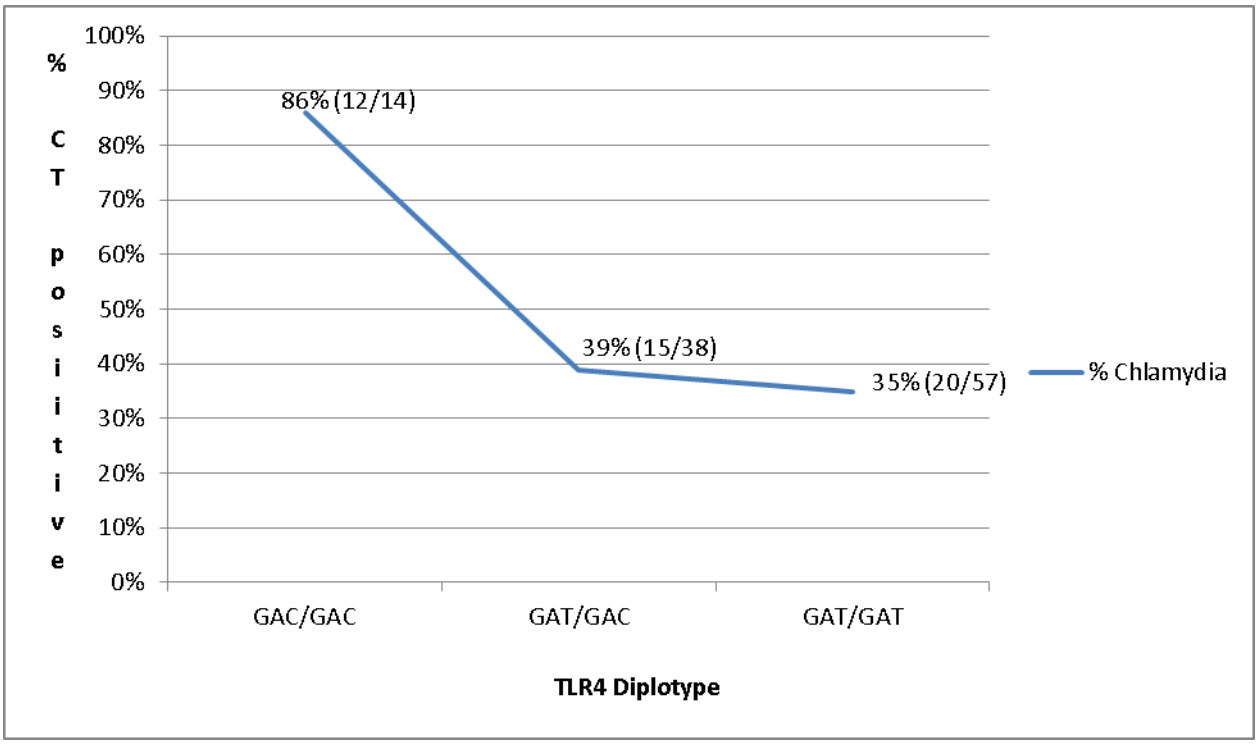

Figure 1. Percentage of chlamydial positive women by predicted TLR4 diplotype

4.0 MANUSCRIPT 3: TOLL-LIKE RECEPTOR GENE POLYMORPHISMS IN PREGANANCY AND INFERTILITY AMONG WOMEN WITH PELVIC INFLAMMATORY DISEASE

Manuscript in Preparation

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4.1 ABSTRACT

Background: Toll-like receptors (TLR) are expressed throughout the female reproductive tract and are responsible for microbial recognition. Genetic variations in TLR genes may alter inflammatory responses possibly influencing the development of sequelae following pelvic inflammatory disease (PID).

Methods: We explored the role of TLR single nucleotide polymorphisms (SNPs) in pregnancy and infertility among 205 African American women with clinically suspected PID from the PID Evaluation and Clinical Health Study. Outcomes were assessed over a median of 84 months. To determine associations with pregnancy, Cox regression methods were used to calculate hazard ratios (HR) and 95% confidence intervals (CI) for a total of 18 tagSNPs in 6 TLR genes (TLR1, TLR2, TLR4, TLR6, TIRAP, and MyD88). For infertility odds ratios (OR) and 95% CI were calculated using logistic regression. Significance was based on an empirical P-value of <0.003. All models were adjusted for age and history of infertility.

Results: Women with PID who carried the GG genotype for TLR1 rs4833095 had a non-significant trend towards decreased pregnancy rates (HR 0.7, 95% CI 0.5-1.1). Although power was limited, we found similar results among a subset of women with *C. trachomatis* (HR 0.5, 95% CI 0.3-0.9, p=0.04). Further, women predicted to carry the TLR1 (rs5743618/ rs5743817/rs4833095) TGG haplotype were less likely to achieve pregnancy (0.80:0.71, p=0.04), although this was non-significant (empirical p=0.2860).

Conclusions: Among African American women with PID, TLR and adaptor molecule variants did not appear to significantly alter the risk of infertility or pregnancy. As PID is polymicrobial these associations should be examined in cohorts defined by PID-associated pathogen.

4.2 INTRODUCTION

Pelvic inflammatory disease (PID) is the infection and inflammation of the female upper genital tract. PID is a multimicrobial condition and is associated with several pathogens including *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Mycoplasma genitalium*, and anaerobic and aerobic bacteria commonly associated with bacterial vaginosis (BV) (5-8). Complications following PID can result from damage to the cilia lining of the Fallopian tubes, Fallopian tube blockage, or adhesion formation among pelvic organs (5). A landmark Scandinavian cohort study among 2500 w omen with clinically suspected PID, found that 16% of women with laparoscopically verified salpingitis versus 2.7% of controls became infertile (3). In addition, 20% and 25% developed recurrent PID and chronic pelvic pain respectively (2). Tubal factor infertility was also found to double with each PID episode and reached an estimated 40% after three or more episodes (3).

Obstruction of the Fallopian tubes following PID is a preventable cause of infertility and ectopic pregnancy. There is a need for biomarkers to predict morbidity risk following an episode of PID. However, the role of individual microbes in the development of sequelae following PID is not well understood and although *C. trachomatis* and *N. gonorrhoeae* have been studied more extensively, the etiology and pathogenesis of PID has not been fully delineated (5). It is suggested that the presence of *C. trachomatis* or *N. gonorrhoeae* on the genital mucosal surface, invokes inflammatory responses which may clear infection and/or elicit tissue damage (36). As innate immune receptors results in expression of proinflammatory cytokine and chemokines, host genetic variations in innate immune receptor genes may play a role in the pathogenesis of PID, possibly through overt inflammatory responses.

Interaction between the immune system and the reproductive tract is important for fertility and reproductive health. The innate immune system serves as the first line of defense after exposure to pathogens and depends on pattern recognition receptors (PRRs) for microbial recognition (121-123). A conserved family of PRRs called the Toll-like receptor (TLR) family (122) is responsible for microbial elimination through induction of inflammatory cytokine and chemokine genes. Ten different TLRs (TLRs 1-10) have been identified in humans and the overlap between them allows identification of a diverse range of pathogens through ligand binding (121-123). Adaptor molecules including myeloid differentiation primary response protein 88 (MyD88) and TIR domain-containing adaptor protein (TIRAP) help to mediate TLR signaling (123, 127). Polymorphisms in TLR and adaptor molecule SNPs may alter TLR signaling and have been found to be associated with several inflammatory and infectious diseases (163, 173, 176, 186, 199).

TLRs are expressed throughout the female reproductive tract (121,125,126) and could play a role in gynecologic disease. To our knowledge no studies have examined the role of TLRs in PID. One group of researchers conducted several limited studies among Dutch Caucasian populations and has been unable to show any significant associations between innate immune receptor functional single nucleotide polymorphisms (SNPs) and tubal factor infertility (154, 156). For example, Morre et al examined 35 Dutch women with tubal pathology and 49 Dutch women without tubal pathology and found that the TLR4 Asp299Gyl polymorphism was not associated with tubal infertility ($P>0.5$) (Allele frequency: 7.1% vs. 10.2%) (154). In a cohort of 227 subfertile women, among those who were *C. trachomatis* positive women, there was an increasing risk for tubal pathology (64% risk for normal genotype vs. 83% risk for heterozygous SNP carrier), although this did not reach statistical significance (154). Outburg et al, compared

253 Dutch women with subfertility to 170 fertile women to examine the CD14 functional polymorphism -260C>T, and found no association between this polymorphism and subfertility (156). den Hartog et al reported that women with tubal pathology who were *C. trachomatis* Ig positive were twice as likely to be carriers of the TLR4 +896 G allele compared to women without tubal pathology, although this was not statistically significant (157).

As no studies have examined TLRs in PID, our objective was to explore the role of TLR1, TLR2, TLR4, TLR6, MyD88, and TIRAP gene polymorphisms in infertility and pregnancy among African American women with clinically suspected PID. This may provide novel information regarding the pathogenesis of PID.

4.3 METHODS

This study utilized data from the PID Evaluation and Clinical Health (PEACH) study. This was the first randomized clinical trial to compare inpatient and outpatient treatment in preventing long-term complications among 831 women with mild to moderate PID. The methods of subject recruitment, data collection, and follow-up have been reported elsewhere (87). Briefly, between March 1996 and February 1999 women aged 14-37 years were recruited from emergency departments, obstetrics and gynecology clinics, sexually transmitted disease clinics, and private practices at 7 primary and 6 secondary sites throughout the eastern, southern, and central regions of the United States. Women who had suspected PID and gave informed consent were eligible for the PEACH study. Eligible women had a history of pelvic discomfort for less than 30 days, findings of pelvic organ tenderness (uterine or adnexal) on bimanual examination, and leukorrhea and/or mucopurulent cervicitis and/or untreated but documented gonococcal or

chlamydial cervicitis. The University of Pittsburgh Institutional Review Board approved the study.

A total of 2941 women were screened for study entry, of those 346 (11.8%) did not meet the clinical inclusion criteria for randomization. Women were additionally excluded if they were pregnant (n = 141, 4.8%); had taken antimicrobials within the past 7 days (n = 248, 8.4%); had a history of hysterectomy or bilateral salpingectomy (n = 248, 8.4%); had an abortion, delivery, or gynecologic surgery within the past 14 days (n = 51, 1.7%); had a suspected tubo-ovarian abscess or other condition requiring surgery (n = 191, 6.5%); had an allergy to the study medications (n = 163, 5.5%); were homeless (n = 29, 1%); or had vomiting after a trial of antiemetic treatment (n = 11, 0.4%). A total of 290 women (205 non-Hispanic black race, 51 non-Hispanic white race, 34 other race) with previously stored buffy coats (n=237) or serum samples (n=50) were genotyped for TLR and adaptor molecule SNPs in this study. There were no differences in the genotype frequencies between women genotyped with buffy coats or serum samples. Because of small sample sizes, women of white race and other races were excluded. A total of 205 African American women were included in this analysis.

Women were randomized to either inpatient treatment of intravenous cefoxitin every 6 hours and doxycycline orally twice a day for 14 days; or outpatient treatment consisting of a single intramuscular injection of cefoxitin and oral doxycycline twice a day for 14 days. Because the treatment modality was not associated with reproductive morbidities in the PEACH study (87), we do not include them as a covariate in this analysis. Participants were followed-up with in-person visits at 5 and 30 days after treatment. At the 30-day follow-up the gynecological exam was repeated. Telephone follow-ups were conducted by the study nurses every 3 months during

the first year after enrollment and then every 4 months until June 2004. At that point information was obtained by self-report for 69.1% of the cohort, with a mean follow-up of 84 months.

A pelvic examination and interview were conducted at the baseline visit. The interview collected information on reason for visit, brief pain history, demographics, history of PID/sexually transmitted diseases (STDs), sexual and contraceptive history, reproductive decisions, douching history, pregnancy history, medical and gynecological history and lifestyle habits. Follow-up interviews collected self-reported information on pelvic pain, pregnancy and births, signs and symptoms of PID, STDs, contraceptive use, pattern of sexual intercourse, and health care utilization.

Gynecological examinations were performed at baseline and 5 and 30 days post treatment. Vaginal smears were gram stained for bacterial vaginosis in a central laboratory by standardized methods described by Nugent et al (223). Endometrial biopsy specimens were obtained for histological examination including, chlamydial polymerase chain reaction (PCR) and gonococcal culture. PCR and cultures were performed at a central reference laboratory. For the patients with endometrial biopsies, two reference pathologists separately evaluated at least one section stained with hematoxylin and eosin and at least one stained with methyl green pyronine. Disagreements about the presence or absences of neutrophils and plasma cells were settled by both pathologists reading the slides together and coming to an agreement. Histological endometritis was based on a modification of the criteria proposed by Kiviat et al (224). Endometritis was defined as the presence of at least five neutrophils in the endometrial surface epithelium in the absence of menstrual endometrium and/or at least two plasma cells in the endometrial stroma. Cervical and endometrial specimens were stored and later used to test for *M.*

genitalium (8), using a microwell-plate-based PCR assay (MgPa-IMW) targeting the MgPa gene (225).

Reproductive outcomes were assessed over a mean of 84 months and included infertility, pregnancy, live birth, recurrent PID, and chronic pelvic pain. Infertility was defined by lack of conception despite sexual activity with rare or never use of methods of contraception considered reliable during 12 or more months of follow-up. Self-reported pregnancy was determined by a positive urine/blood test or physician's diagnosis. Live birth was determined by self-report, and recurrent PID was self-reported and verified whenever medical records were available (45% of cohort). Recurrent PID was confirmed in 76% of medical records. Chronic pelvic pain was defined by two or more consecutive reports of pelvic pain during follow-up. Data from at least 2 follow-up visits were required to determine chronic pelvic pain.

A total of 205 African American women were genotyped for TLR and TLR adaptor molecule SNPs. For the proposed study 18 tagging SNPs (TagSNPs) including three from TLR1 (rs5743618, rs5743817, rs4833095), three from TLR2 (rs3804099, rs11938228, rs1898830), three from TLR6 (rs1039559, rs5743810, rs3775073), four from TLR4 (rs4986790, rs4986791, rs11536889, rs5030728), two from MyD88 (rs4988457, rs7744), and three from TIRAP (rs3802813, rs8171374, rs7932976), were chosen from Hapmap.org. TagSNPs were chosen based on their reported minor allele frequencies and relative distance to one another within each gene.

All SNPs were genotyped by fluorescence polarization (227). PCR conditions included 1 X PCR 2.5µl of buffer (Invitrogen) with 1.0µl of $MgCl_2$, 4µl of dNTPs, 1.5µl of each primer, 0.1µl of Taq polymerase (Invitrogen), and 13.4µl of dH_2O; for a total volume of 25.0µl. Amplification was performed using a Peltier Thermal Cycler (MJ Research). Thermal cycling

conditions were 95°C for 3 minutes, then 35 cycles of 95°C/30 seconds for denaturing, 55°C/30 seconds for annealing, and 72°C/30 seconds for extension, then the final extension step of 72°C/1 minute. PCR products were visualized on a 3% agar gel by electrophoresis. To check for genotyping errors, allele frequencies in our cohort were compared to those reported on Hapmap.org from a population of African ancestry in Southwest USA and a healthy African American population from Pittsburgh, PA which was genotyped in the same lab as our samples. No significant differences in the frequencies were found. Therefore, we do not believe that there were any genotyping errors.

All analyses were conducted among 205 African American women. Demographic and clinical baseline characteristics including, age, education, insurance, marriage, white blood cell count (WBC), temperature, C-reactive protein (CRP), bilateral adnexal tenderness, cervicitis, endometritis, erythrocyte sedimentation rate (ESR), *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, smoking status, and drug use were compared between pregnant women and women who did not achieve pregnancy, using X^2 test of proportions. Our main analyses compared genotype frequencies between 77 cases who did not achieve pregnancy and 128 pregnant controls. In addition, we also compared genotype frequencies between 39 infertile cases and 166 fertile controls. All SNPs were tested for Hardy-Weinberg equilibrium (HWE), using 10,000 permutations to approximate an exact p-value. Additive or dominant models were analyzed for all SNPs. Dominant models were defined by the minor allele, where if the minor allele was A the dominant model would compare AA+AG to GG. For the pregnancy models, Cox regression was used to calculate hazard ratios and 95% confidence intervals. For infertility, logistic regression was used to calculate odds ratios and 95% confidence intervals. Any model with less than 5 in any cell was excluded from regression analysis, this included

TLR1 rs5743817, TLR4 rs11536889, TIRAP rs8171374, and TIRAP rs7932976. In addition, TIRAP rs3802813 was also excluded for infertility. All models were adjusted for age and history of infertility. Permutations were used to correct for multiple comparisons. An empirical p-value <0.003 was considered significant for SNP genotype associations. Haplotype analyses were also conducted. The expectation-maximization algorithm was used to generate maximum likelihood estimates of haplotype frequencies using SAS/Genetic v9.1.3 (228). Haplotypes were then tested for associations with each trait using chi-square test and 1000 permutations to calculate exact p-values. For genes with significant haplotypes, regression analysis was used to examine associations between haplotype pairs and traits. All analyses were performed using SAS/Genetics v9.1.3 (Cary, NC).

4.4 RESULTS

Baseline demographic, clinical, and behavioral characteristics between cases and controls were similar (Table 8). However, pregnant women were more likely to be less than 25 years of age (78% vs 44%, p<0.001) compared to women who did not achieve pregnancy. No SNPs were out of HWE among controls.

Among women with PID, those who carried the TIRAP rs3802813 GG genotype had decreased odds of achieving pregnancy (hazard ratio (HR) 0.6, 95% confidence interval (CI) 0.3-0.9). However, this was not significant after correction for multiple comparisions (p=0.02) (Table 9). No other TLR or adaptor molecule SNP was significantly associated with pregnancy, although several non-significant trends were observed. Women who carried the TLR6 rs5743810 TT or CT genotype had increased pregnancy rates (HR 1.6, 95% CI 1.0-2.5, p=0.0599). Among

women who carried the TLR1 rs5743618 TT genotype, a non-significant trend towards decreased pregnancy rates (HR 0.7, 95% CI 0.4-1.1) was also observed. Although our power was limited, in a subset of 94 women with *C. trachomatis*, we found that the TT genotype was more frequent in women who did not achieve pregnancy (93% vs. 78%) compared to pregnant women. However, because of small cell size we were unable to perform logistic regression. Women who carried the GG genotype for TLR1 rs4833095 had a non-significant trend towards decreased pregnancy rates (HR 0.7, 95% CI 0.5-1.1). Results were similar among a subset of *C. trachomatis* positive women (HR 0.5, 95% CI 0.3-0.9, p=0.04). We did not find any significant associations between TLR and adaptor molecule SNPs and infertility (Table 10).

Women who carried the TLR1 (rs5743618/rs5743817/rs4833095) TGG haplotype were less likely to achieve pregnancy (0.80:0.71, p=0.04). However, this was no longer significant after correction for multiple comparisons (p=0.2860). Still, among women who carried the TGG/TGG haplotype pair 53% achieved pregnancy, compared to a 60% pregnancy rate among women who were predicted to carry only 1 TGG haplotype, and a 77% pregnancy rate among carriers of all other haplotypes (Figure 2). Cox regression analyses revealed decreased pregnancy rates among carriers of the TGG/TGG haplotype pair (HR 0.5, 95% CI 0.3-0.8, p=0.007) compared to all other haplotype pairs. However, this was not significant after correction for multiple comparisons. No other haplotype was significantly associated with pregnancy. Similarly, TLR or adaptor molecule haplotypes were not significantly associated with infertility.

4.5 DISCUSSION

In this cohort of African American women with clinically suspected PID, TLR and adaptor molecule variants did not appear to significantly alter the risk of pregnancy or infertility. However, women predicted to carry the TLR1 TGG/TGG haplotype pair had decreased pregnancy rates. Although our power was limited, we found that TLR1 variants were significantly associated with decreased pregnancy among a subset of women with chlamydial-PID. As PID is polymicrobial, TLR variants could play a role in PID pathogenesis in specific subsets of pathogens.

The exact mechanism of activity and function of TLRs in relation to fertility and reproductive health are unknown. However, as TLRs are expressed throughout the female reproductive tract (121,125,126) and are responsible for microbial elimination through inflammatory responses, there is a possible role for TLRs in reproductive health. TLRs 1-3, 5 and 6 are expressed in the vaginal and cervical epithelial cells, further TLRs 1-3, and 6 are expressed by primary endocervical epithelial cells (121, 130-133). TLR4 has been reported to be present in the endocervix, endometrium, and uterine tubes (121, 130-133). TLRs 7-10 have been identified in the endometrial epithelia and stroma (134). Studies have also shown that TLRs can bind to several PID-associated pathogens. TLR4 may recognize LPS and HSP60 (138). TLR2 can respond to a variety of ligands such as lipoproteins and lipopeptides, lipoarabinomannan, lipoteichoic acid, and bacterial prion (139-143). Further, TLR2 can dimerize with TLRs 1 and 6, possibly to recognize a more diverse range of pathogens (144). TLRs 2 and 4 have also been found to bind to *N. gonorrhoeae* ligands (161, 162), while TLR2 may bind to *M. genitalium* (167). Thus, during an upper genital tract infection TLR or adaptor molecule SNPs could alter TLR signaling, possibly leading to an overt inflammatory response and long-term sequelae.

We were unable to find any significant associations between TLR or adaptor molecule SNPs and pregnancy or infertility. There several explanations for these findings. First, due to our sample size our power to detect significant associations was limited. We did find two associations with borderline significance. Carrying T allele for TLR6 SNP rs5743810 appeared to increase pregnancy rates (HR 1.6, 95% CI 1.0-2.5). TLR6 SNP rs5743810, also known as Ser249Pro, is a non-synonymous polymorphism and results in an amino acid change. Sales et al found that monocytes from hypertensive women with the TT genotype had reduced IL-6 and TNF-α release compared to sex matched cells carrying the C allele ($P<0.05$). Similarly in a study among 100 subjects from South Africa, the T allele of Ser249Pro was associated with decreased NF-κB signaling activity (197). We also found that the TIRAP rs3802813 GG genotype was associated with decreased pregnancy rates (HR 0.6, 95% CI 0.3-0.9), but not after correction for multiple comparisons ($p=0.02$). However, the functional and clinical relevance of this SNP is unknown. Second, our gene coverage for the TLRs and adaptor molecules was low (ranged 15-35%). Variants in TLR genes may be responsible for infertility following an episode of PID, and our SNPs simply may not have tagged those disease variants if they exist. Therefore, larger studies with better gene coverage should examine the role of TLRs and their adaptor molecules in PID.

Lastly, the polymicrobial nature of PID complicates analyses. Although, PID-associated pathogens are recognized by several TLRs, their mechanisms of disease may differ. For example, *N. gonorrhoeae* causes a severe acute neutrophilic inflammatory response and tissue damage possibly through an endotoxic effect, while *C. trachomatis* causes a less severe, cell-mediated lymphocytic response that often resolves into a chronic infection (36). Although our power was limited, among a subset of 94 women with *C. trachomatis* infection, TLR1 variants rs5743618

and rs4833095 appear to alter the risk of pregnancy. Further, among the entire PID cohort we found that the TLR1 (rs5743618/rs5743817/rs4833095) TGG/TGG haplotype pair decreased pregnancy rates. TLR1 SNPs rs5743618 (Ser602Ile) and rs4833095 (Asn248Ser) are non-synonymous mutations and result in amino acid changes. The G allele of Ser602Ile has been reported to be associated with deficient TLR signaling in comparison to the T allele (182-185). Hawn et al, reported that the T allele expressed significantly greater NF-κB signaling in transfected HEK293 cells compared to the G allele (183). Like the Ser602Ile variant, Asn248Ser may alter TLR signaling increasing the risk of infection. Compared to the wild-type, the A allele was found to impair TLR response to Pam3CSK4 ($P<0.05$) (189). In a population-based case-control study among 1312 tuberculosis patients and controls, the G allele significantly increased risk of tuberculosis in African Americans ($P=0.009$) (181). Similar to our findings, Pino-Yanes et al reported that the 248Ser-602Ile haplotype (T and G alleles) was associated with circulatory dysfunction among 218 sepsis patients ($P<0.022$), decreased IL-10 ($P<0.047$), and increased CRP ($P<0.036$) (194).

Our study has several strengths. First, data were obtained from a large, multicenter, prospective randomized clinical trial, with comprehensive demographic, clinical, and obstetric measurements. These findings are generalizable to patients treated for clinically suspected PID. Not all women in the PEACH study had blood samples available for analyses, but demographics between women with and without blood samples did not differ (185). This is the first study to examine the role of several TLR and adaptor molecule SNPs in PID and its subsequent sequelae. However, our sample size limited our power. We also had low SNP coverage for our genes. Further, we relied on internal control groups. Therefore, all controls had clinically suspected PID.

Among African American women, TLR and adaptor molecule variants did not appear to significantly alter the risk of pregnancy or infertility following an episode of PID. However, our power was limited and we did observe trends towards decreased pregnancy rates for TLR1 variants. These TLR1 variants also appeared to decrease pregnancy among a subset of women with *C. trachomatis* infection. Further, the TLR1 TGG/TGG haplotype pair appeared to reduce pregnancy rates. TLR1 variants may increase TLR signaling leading to persistent inflammation which may permanently damage the reproductive tract following an episode of PID. As PID is polymicrobial future studies with larger samples sizes should examine the role of innate immune receptor variants in infertility among specific subsets of PID-associated pathogens. In particular, as chlamydia appears to be immunologically driven, the role of innate immune receptors should be explored in infertility and pregnancy following chlamydial-PID.

4.6 TABLES

Table 8. Baseline demographic and clinical characteristics by pregnancy status

Characteristics	**Pregnant** **N = 128** **n (%)**	**Not Pregnant** **N = 77** **n (%)**	***p-value**
Demographics			
Age <25 years 25+ years	 100 (78.1) 28 (21.9)	 34 (44.2) 43 (55.8)	 <0.0001
Married	8 (6.8)	5 (7.0)	0.9449
Education Less than high school High school or greater	 60 (46.9) 68 (53.1)	 29 (37.7) 48 (62.3)	 0.1975
Uninsured	49 (41.5)	40 (56.3)	0.0482
Clinical Findings			
Temperature (>100.4 F)	8 (6.7)	11 (15.1)	0.0601
WBC (> 10,000 mm^3)	37 (35.2)	23 (36.5)	0.8679
C-reactive protein (>5mg/dL)	10 (50.0)	7 (50.0)	1.000
Bilateral adnexal tenderness	98 (76.6)	59 (76.6)	0.9920
Chlamydia trachomatis	62 (48.4)	32 (41.6)	0.3384
Neisseria gonorrhoeae	39 (35.8)	22 (33.3)	0.7420
Mycoplasma genitalium	11 (11.1)	9 (15.3)	0.4487
Bacterial Vaginosis	86 (69.9)	41 (59.4)	0.1402
Cervicitis	73 (63.5)	43 (59.7)	0.6066

Table 8. continued

Endometritis	32 (53.3)	59 (56.2)	0.7226
Behavioral			
Current smoker	50 (39.1)	39 (50.7)	0.1050
Drug use	38 (29.7)	27 (35.1)	0.4230

*Chi-square was used to derive the p-value. Fisher's Exact was used when cell size was less than 3

Table 9. Associations between genotypes and time-to-pregnancy among women with pelvic inflammatory disease

SNPs and Genotypes	Not Pregnant (N=77)	Pregnant (N=128)	*Adjusted Hazard Ratio (95% CI)	**P-value
TLR1				
rs5743618				
GG+GT	15(20.8)	34(31.2)	Referent	
TT	57(79.2)	75(68.8)	0.7 (0.4-1.1)	0.1206
rs4833095				
AA+AG	26(35.1)	50(44.6)	Referent	
GG	48(64.9)	62(55.4)	0.7 (0.5-1.1)	0.1168
TLR 2				
rs3804099				
CC	32(43.2)	52(45.6)	Referent	
CT	31(41.9)	43(37.7)	0.9 (0.6-1.4)	0.7842
TT	11(14.9)	19(16.7)	1.0 (0.6-1.7)	0.9034
rs11938228				
CC	55(75.3)	81(72.3)	Referent	
AA + AC	18(24.7)	31(27.7)	1.2 (0.8-1.8)	0.4833
rs1898830				
AA	53(74.7)	78(77.2)	Referent	
GG+AG	18(25.4)	23(22.8)	1.1 (0.7-1.7)	0.7200
TLR 6				
rs1039559				
TT	50(69.4)	61(58.1)	Referent	
CC+TC	22(30.6)	44(41.9)	1.2 (0.8-1.9)	0.2543
rs5743810				
CC	67(90.5)	84(77.8)	Referent	
TT + CT	7(9.5)	24(22.2)	1.6 (1.0-2.5)	0.0599
rs3775073				
GG	35(46.7)	38(34.9)	Referent	
AG	33(44.0)	57(52.3)	1.0 (0.7-1.7)	0.9159
AA	7(9.3)	14(12.8)	1.0 (0.5-2.0)	0.9108
TLR 4				
rs5030728				
GG	52(70.3)	81(72.3)	Referent	
AA + AG	22(29.7)	31(27.7)	1.0 (0.7-1.5)	0.9773
rs4986790				
AG	15(19.7)	16(13.5)	Referent	
AA	61(80.3)	103(86.5)	1.5 (0.9-2.5)	0.1622
rs1927911				
TT	29(37.7)	48(39.3)	Referent	
CT	29(37.7)	45(36.9)	1.0 (0.7-1.5)	0.9354
CC	19(24.7)	29(23.8)	0.8 (0.5-1.4)	0.5766

Table 9. continued

Myd88				
rs7744				
AA	71(92.2)	117(91.4)	Referent	
AG + GG	6(7.8)	11(8.6)	1.4 (0.7-2.6)	0.3463
rs4988457				
CG	15(20.3)	20(18.5)	Referent	
CC	59(79.7)	88(81.5)	1.1 (0.7-1.9)	0.6291
TIRAP				
rs3802813				
AG	6(7.8)	18(14.8)	Referent	
GG	71(92.2)	104(85.3)	0.6 (0.3-0.9)	0.0235

* Adjusted for age and history of infertility

****Significance based on a permutated P-value of 0.004**

Cox regression was used to calculate hazard ratios and 95% CI. Any model with less than 5 observations in any cell was excluded (TLR1 rs5743817, TLR4 rs11536889, TIRAP rs8171374, and TIRAP rs7932976).

Table 10. Associations between genotypes and infertility among women with pelvic inflammatory disease

SNPs and Genotypes	Fertile (N=166)	Infertile (N=39)	*Adjusted Odds Ratio (95% CI)	**P-value
TLR 1				
rs5743618				
GG+GT	39(26.9)	10(27.8)	Referent	
TT	106(73.1)	26(72.2)	1.0 (0.4-2.3)	0.9938
rs4833095				
AA+AG	60(81.1)	87(77.7)	Referent	
GG	14(18.9)	25(22.3)	1.3 (0.6-2.7)	0.4916
TLR 2				
rs5743618				
CC	70(46.4)	14(37.8)	Referent	
TT+TC	81(53.6)	23(62.2)	1.3 (0.6-2.8)	0.4353
rs5743817				
CC	107(72.3)	29(78.4)	Referent	
AA+AC	41(27.7)	8(21.6)	0.7 (0.3-1.6)	0.3512
rs4833095				
AA	103(75.7)	28(77.8)	Referent	
GG+AG	33(24.3)	8(22.2)	0.8 (0.3-2.0)	0.6792
TLR 6				
rs1039559				
TT	87(62.1)	24(64.9)	Referent	
CC+TC	53(37.9)	13(35.1)	0.9 (0.4-1.9)	0.7505
rs3775073				
GG	58(39.7)	15(39.5)	Referent	
AA+AG	88(60.3)	23(60.5)	1.1 (0.5-2.4)	0.7920
TLR 4				
rs5030728				
GG	108(72.5)	25(67.6)	Referent	
AA + AG	41(27.5)	12(32.4)	1.2 (0.6-2.7)	0.6294
rs4986790				
AG	23(14.7)	8(21.1)	Referent	
AA	134(85.4)	30(78.9)	0.7 (0.3-1.7)	0.4359
rs1927911				
TT	61(38.1)	16(41.0)	Referent	
CT	59(36.9)	15(38.5)	1.0 (0.4-2.2)	0.9615
CC	40(25.0)	8(20.5)	0.7 (0.3-1.9)	0.4871
Myd88				
rs4988457				
CG	29(20.0)	6(16.2)	Referent	
CC	116(80.0)	31(83.8)	1.2 (0.5-3.2)	0.7089

* Adjusted for age **Significance based on a permutated P-value of 0.004

Logistic regression was used to calculate odds ratios and 95% CI. Any model with less than 5 observations in any cell was excluded TLR1 rs5743817, TLR4 rs11536889, TIRAP rs8171374, TIRAP rs7932976, and TIRAP rs3802813).

4.7 FIGURES

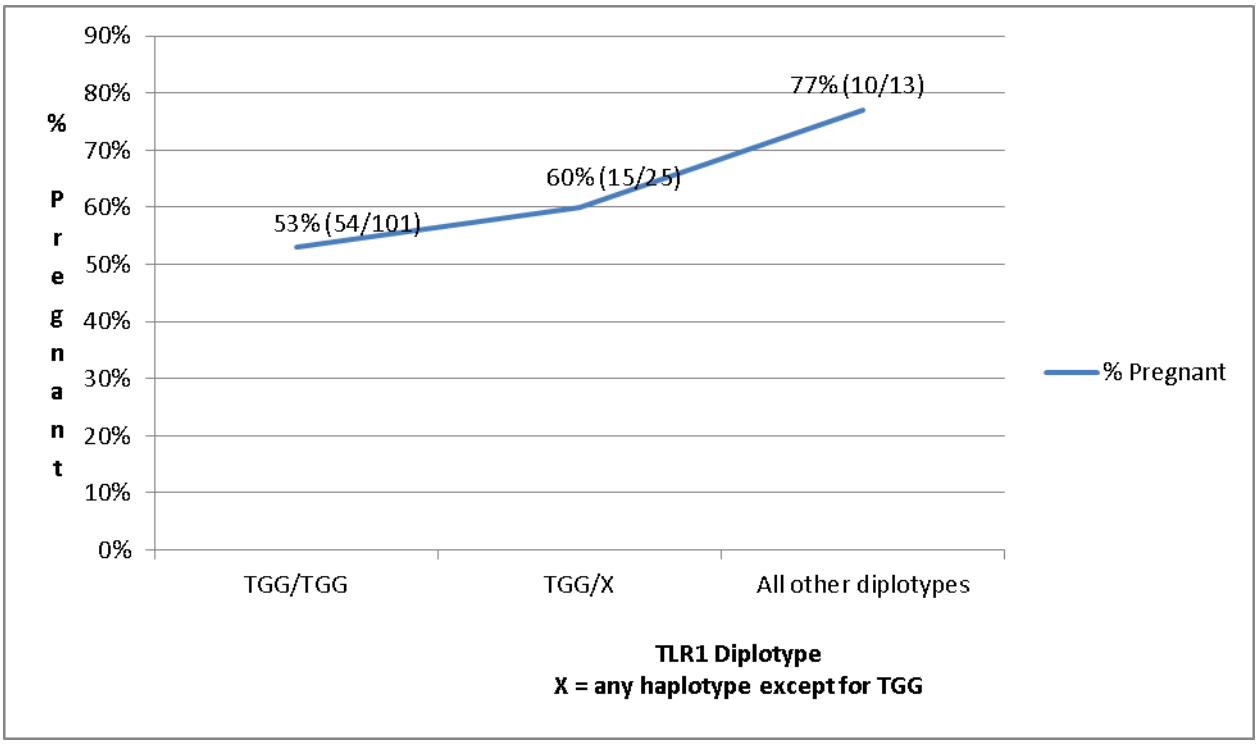

Figure 2. Percentage of women who achieved pregnancy by predicted TLR1 diplotype

5.0 CONCLUSIONS

For this study, data was obtained from the PID Evaluation and Clinical Health (PEACH) study. This cohort is generalizable to women with clinically suspected PID and obtained comprehensive data on demographic, clinical, and obstetric measurements. We used this data to examine important aspects of PID epidemiology including the microbial correlates of delayed care and the role of innate immune receptors in chlamydia, endometritis, and infertility. Results from this study suggest that women with *C. trachomatis* or *M. genitalium* monoinfection had longer times to care than women with *N. gonorrhoeae* monoinfection or co-infection with two or more pathogens. Furthermore, study results suggest that among women with PID variations in TLR1 and TLR4 genes may contribute to chronic inflammation following chlamydial infection, possibly leading to upper genital tract pathology.

Among women with mild to moderate PID, women waited a mean of 7 days before seeking treatment for symptoms. Women with *C. trachomatis* monoinfection, and *M. genitalium* monoinfection displayed the longest times to care, while the shortest times were among women with *N. gonorrhoeae* monoinfection and co-infection with two or more pathogens. We found that time to care differed significantly between *C. trachomatis* monoinfection or *M. genitalium* monoinfection and *N. gonorrhoeae* monoinfection. However, time to care did not significantly differ between *C. trachomatis* and *M. genitalium*. These results suggest that *M. genitalium* and *C. trachomatis* may have a similar course of infection. In general, the long time to care in this

cohort is of concern as treatment after the first 3 days of the onset of symptoms has been shown to increase the risk for impaired fertility (9). Although, we did not find any significant associations between delayed care of more than 14 days and reproductive sequelae, rates of infertility, recurrent PID, and chronic pelvic pain were high in our cohort. As PID is difficult to diagnose and treatment is often delayed, this data supports a continued need for early identification and treatment of lower genital tract infections.

Among African American women with mild to moderate clinically suspected PID, the TLR4 rs1927911 CC genotype significantly increased the odds of *C. trachomatis* infection. In addition, women who were predicted to carry the TLR4 GTC haplotype were significantly more likely to have *C. trachomatis* infection, while those predicted to carry the GTT haplotype were significantly less likely to have *C. trachomatis* infection. The exact function of TLR4 rs1927911 is unknown. The TLR1 rs5743618 TT genotype was significantly associated with *C. trachomatis*, but not after correction for multiple comparisons. In addition, the TLR1 TGA haplotype was significantly more frequent in chlamydial positive women. Although our power was limited, among a subset of 94 women with *C. trachomatis* infection, TLR1 variants rs5743618 and rs4833095 appear to alter the rates of pregnancy. Further, among the entire PID cohort we found that the TLR1 (rs5743618/rs5743817/rs4833095) TGG/TGG diplotype decreased pregnancy rates. Both of these variants result in an amino acid change and may increase TLR signaling (181-185, 189). No other variants in TLR or adaptor molecule genes were significantly associated with chlamydia, endometritis, infertility, or pregnancy. TLR and their adaptor molecules initiate microbial elimination through induction of inflammatory responses. As genetic variations may alter TLR signaling, they may play a role in *C. trachomatis* pathogenesis.

5.1 FUTURE RESEARCH

In the PEACH study, women with *C. trachomatis* or *M. genitalium* monoinfection had longer times to care compared to women with *N. gonorrhoeae* monoinfection. This data is consistent with previous research which suggests that *C. trachomatis* and *M. genitalium* symptoms are often mild, while *N. gonorrhoeae* symptoms are more overt. As this data can be translated to a population of women with uncomplicated sexually transmitted diseases, future studies with larger sample sizes, should prospectively follow women with uncomplicated lower genital tract infections to examine the role of delayed care in the development of PID and reproductive sequelae. This study may also suggest that women with *C. trachomatis* or *M. genitalium* may have low level chronic inflammation which may damage the reproductive tract prior to treatment. This reiterates the need for early identification and treatment of lower genital tract infections. Specifically, research should continue to delineate and compare the pathogenesis of asymptomatic, mildly symptomatic and symptomatic lower genital tract infections. This will enable public health professionals to determine the best approach for the prevention of sexually transmitted diseases. Currently, in the United States young women under the age of 26 are recommended to be screened for *C. trachomatis* (6). However, the evidence examining the effectiveness of chlamydia screening is limited, as few high quality randomized clinical trials have been conducted (234). Therefore, future research is needed to determine the best approach for chlamydia management. This can be achieved through research focused on host immunology, bacterial antigens, duration, and the role of pathogen load in the course and outcome of chlamydial infections. These factors should also be examined in *M. genitalium* as its symptoms consistently appear to be similar to C. *trachomatis* and little is known about its pathogenesis. Furthermore, as *M. genitalium* has also been found to be associated with PID, studies should also

examine the cost effectiveness of screening high risk women for *M. genitalium* in order to prevent fertility reducing sequelae.

Chlamydia has been suggested to be a disease of immunopathology (229). Our study suggests that polymorphisms in innate immune receptor genes may play a role in *C. trachomatis* pathogenesis. Further studies with larger sample sizes and appropriate control groups are needed to reproduce these findings. It is very important that future studies compare innate immune receptor SNPs between women with uncomplicated *C. trachomatis* infection and women with chlamydial-PID. Adverse reproductive outcomes should also be explored in a larger subset of women with *C. trachomatis* infection. In addition, larger studies should examine a broader range of innate immune receptor SNPs in the course and outcome of *C. trachomatis*. Larger cohorts would also enable researchers to examine gene-gene and gene-environment interactions, as well as examine haplotypes with greater power. As the function and clinical relevance of many TLR and adaptor molecule SNPs is unknown, it is imperative that research continue to delineate their function. In future studies it would be optimal to examine cytokine expression in women with *C. trachomatis* and PID. As disparities exist in gynecological disease, these innate immune receptor variants should also be examined in other racial groups. Lastly, although it is important to further examine innate immune receptors in PID, it is a polymicrobial condition. Therefore, the role of innate immune receptors in the course and outcome of other lower genital tract infections should be explored. In particular, the role of innate immune receptors in *M. genitalium* should be examined as it appears to be similar to *C. trachomatis*.

5.2 APPLICATION TO PUBLIC HEALTH

Sexually transmitted diseases are a serious public health concern and can cause major reproductive morbidity. The anatomy and physiology of the female reproductive tract puts women at greater risk of complications following many lower genital tract infections (235). Women who have lower genital infections can develop PID and its sequelae including infertility, ectopic pregnancy, and recurrent PID (5-8). An estimated 8% of American women will develop PID at some time in their reproductive lives (4). Further, the estimated average per person life time cost of PID is $1060-$3180, suggesting that PID prevention would have substantial savings to the health care system (36). Various organisms have been implicated in the etiology of PID including *Neisseria gonorrhoeae, Chlamydia trachomatis, Mycoplasma genitalium,* and anaerobic and aerobic bacteria commonly associated with bacterial vaginosis (BV) (5-8). However, the pathogenesis of PID has not been completely elucidated (5) and the role of individual microbes in the etiology of PID is not well understood. Further, treatment of PID may not effectively reduce reproductive sequelae (36).

Prevention of PID and its morbidities is of great public health importance (36). It has been suggested that prompt treatment of PID is an important strategy for infertility prevention (36, 9). Compared to women with *C. trachomatis*, women with *N. gonorrhoeae* have a quicker onset of severe symptoms, possibly through an endotoxic effect, which may lead to earlier care and less tubal damage (36). However, only one study has actually examined the role of prompt care seeking in fertility preservation among women with PID (9). Hillis et al, in a cohort of women recruited in the 1960-1980's, found that delaying care for 3 or more days significantly increased the risk of impaired fertility, with the strongest associations being found among women infected with *C. trachomatis* (9). Our study was the first study to examine these

associations in a contemporary cohort of women with clinically suspected PID. Our study shows that women with clinically suspected PID waited a mean of 7 days before seeking treatment. Similar to Hillis et al, we were also able to show that women with *C. trachomatis* monoinfection waited longer to receive treatment compared to women with *N. gonorrhoeae* monoinfection. In addition, this is the first study to show that women with *M. genitalium* had significantly longer time to care compared to women with *N. gonorrhoeae,* while *M. genitalium* and *C. trachomatis* has similar times to care. This result may suggest women infected with *M. genitalium* or *C. trachomatis* have low levels of chronic inflammation that can lead to serious reproductive damage before treatment is sought. Therefore, continued efforts should be made at early identification and treatment of lower genital tract infections to prevent the progression to PID and its long term morbidities. Inflammatory processes are suggested to play a role in *C. trachomatis* pathogenesis (36, 229). If inflammation tends to peak within one to two weeks after infection, it makes sense that women with *C. trachomatis* would delay care (9). We are adding the observation that *M. genitalium* pathogenesis may also be driven by inflammatory processes, as these women were also likely to delay treatment.

Although screening for chlamydia is considered a cost effective way to prevent PID, cost-effectiveness is influenced by the rate of complications (236-238), which may be lower than previously estimated (237, 238). More recent studies using dynamic simulation models reported that chlamydia screening may not be as cost-effective as previously thought. (239, 240) Further, of the efficacy of chlamydia screening to reduce PID is limited (234). Aggressive screening and treatment has also been suggested to blunt the natural immunity to *C. trachomatis*, essentially increasing reinfection in the population (241). Reinfection rates are high in both men and women (242, 243) and may further increase the risks of reproductive sequelae (18, 29). The

asymptomatic nature of *C. trachomatis* and our incomplete understanding of its natural history further complicate control efforts. Therefore, the management and control of *C. trachomatis* may ideally be through a safe and effective vaccine (144), which can generate an immune response, better than what occurs naturally (229). However, epidemiologic research examining the role of host immunity and bacterial virulence factors in the pathogenesis of *C. trachomatis* is limited. Our study provided a novel exploration into the role of innate immune receptor variants in PID. We found that TLR1 and TLR4 SNPs increased the odds of *C. trachomatis* infection among women with clinically suspected PID. Further, TLR1 variants may decrease pregnancy rates among women with PID and among a subset of women with chlamydial-PID. Although these findings need to be reproduced, this study provides insight into the pathogenesis of *C. trachomatis*. This is significant as this type of research could aid in vaccine development. If researchers can delineate the role of TLRs in chlamydial pathogenesis, they may be able to be selectively target or manipulate TLR genes to induce an optimal immune response, thus enhancing host resistance to chlamydial infection (144). In addition, our study may help to better understand the etiology of PID and post-PID sequelae. This is particularly important as there is a great need for markers to predict sequelae following an episode of PID. Lastly, this research is of importance as the future may hold a place for SNPs in personalized medicine. Physicians one day may be able to use innate immune receptor SNPs in individual level risk estimation and prediction of sexually transmitted diseases, PID, and post-PID sequelae. This would enable doctors to identify patients who are the greatest risk for developing upper genital tract infection and infertility, thus ensuring that they are frequently tested for lower genital tract infections so that prompt and proper treatment can be initiated.

APPENDIX A: SUPPLEMENTARY FIGURES AND TABLE TO MANUSCRIPT 1

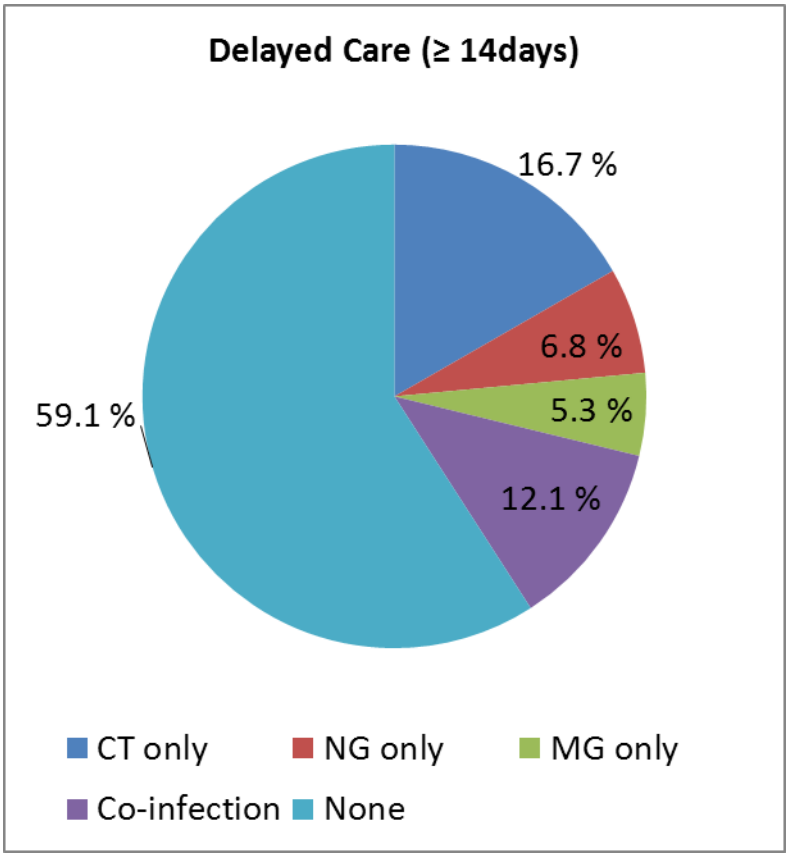

Figure 3. Microbial correlates for delayed care among women with clinically suspected PID (n=774)

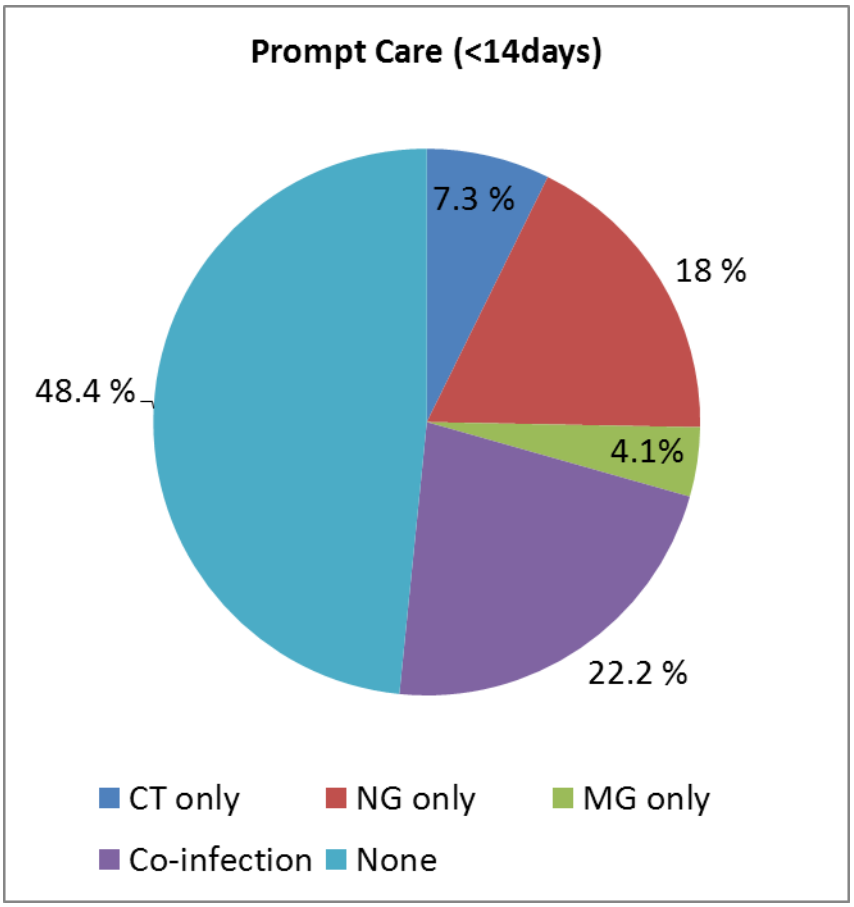

Figure 4. Microbial correlates for prompt care among women with clinically suspected PID (n=774)

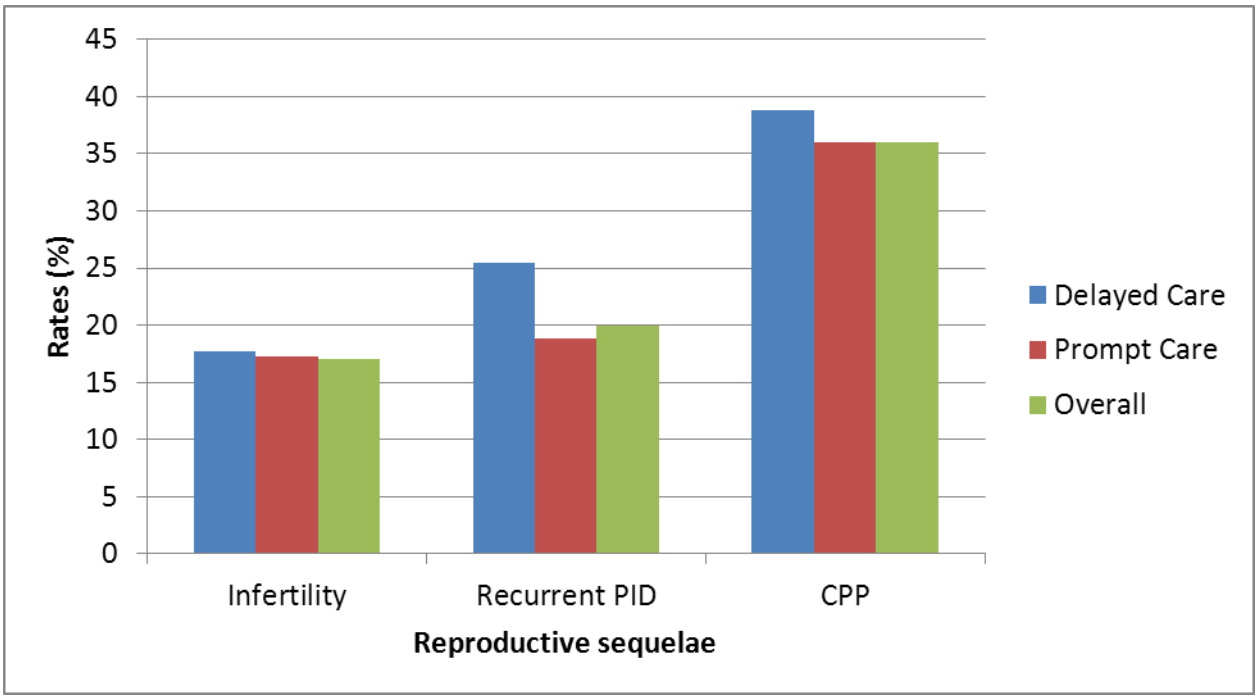

Figure 5. Ratio of sequelae among women with histologically confirmed endometritis (n=298)

Table 11. Effect of delayed care on time-to-pregnancy and time-to-recurrent PID

Subgroup	Pregnancy		Recurrent PID	
	Crude HR (95% CI)	*Adjusted HR (95% CI)	Crude HR (95% CI)	*Adjusted HR (95% CI)
Entire cohort (n=298)	0.79 (0.55 – 1.26)	0.79 (0.55 – 1.13)	1.49 (0.84 – 2.63)	1.46 (0.83 – 2.58)
C. trachomatis only (n = 23)	1.08 (0.39 – 2.99)	1.07 (0.32 – 3.61)	3.06 (0.28 – 33.86)	6.11 (0.21 – 184.41)
M. genitalium only (n = 7)	0.91 (0.12 – 6.64)	1.05 (0.06 – 19.93)	3.94 (0.04 – 358.5)	1.59 (0.02 – 125.52)
N. gonorrhoeae only (n = 45)	1.13 (0.34 – 3.77)	1.17 (0.34 – 4.03)	1.44 (0.18 – 11.71)	1.23 (0.15 – 10.05)
Co-infection (n=50)	0.78 (0.27 - 2.22)	0.79 (0.26 – 2.39)	1.56 (0.34 – 7.22)	2.38 (0.47 – 12.05)

Risk estimate is presented in Hazard Ratios (HR) with 95% confidence intervals (CI)

* Pregnancy and recurrent PID were both adjusted for age and race. Additionally pregnancy was adjusted for self-reported history of infertility at baseline.

APPENDIX B: SUPPLEMENTARY TABLES TO MANUSCRIPT 2

Table 12. Allele and genotype frequencies, only of SNPs included in regression analyses, among the entire cohort, chlamydial cases and controls, and outside control groups

SNPs and Genotypes	Entire PID cohort	Chlamydial positive cases	Chlamydial negative controls	*Healthy African American control group	Hapmap.org
rs5743618					
Alleles					
G	0.141	0.093	0.174	0.120	0.250
T	0.859	0.907	0.825	0.880	0.750
Genotypes	n=181	n=75	n=106	n=187	n=48
GG	2 (0.01)	2 (0.03)	0 (0.00)	5 (0.03)	4 (0.08)
GT	47 (0.26)	10 (0.13)	37 (0.35)	35 (0.19)	16 (0.33)
TT	132 (0.73)	63 (0.84)	69 (0.65)	147 (0.79)	28 (0.58)
rs4833095					
Alleles					
G	0.763	0.753	0.771	0.755	0.792
A	0.237	0.247	0.229	0.244	0.208
Genotypes	n=186	n=77	n=109	n=192	n=53
GG	112 (0.60)	49 (0.64)	63 (0.58)	112 (0.58)	32 (0.60)
AG	60 (0.32)	18 (0.23)	42 (0.39)	66 (0.34)	20 (0.38)
AA	14 (0.08)	10 (0.13)	4 (0.04)	14 (0.07)	1 (0.02)
rs3804099					
Alleles					
C	0.644	0.656	0.635	0.630	0.615
T	0.356	0.344	0.364	0.370	0.385
Genotypes	n=188	n=77	n=111	n=177	n=52
CC	84 (0.45)	38 (0.49)	46 (0.41)	71 (0.40)	20 (0.39)
CT	74 (0.39)	25 (0.32)	49 (0.44)	81 (0.46)	24 (0.46)
TT	30 (0.16)	14 (0.18)	16 (0.14)	25 (0.14)	8 (0.15)

Table 12. continued

SNPs and Genotypes	Entire PID cohort	Chlamydial positive cases	Chlamydial negative controls	*Healthy African American control group	Hapmap.org
rs11938228					
Alleles					**
A	0.148	0.169	0.135	0.171	
C	0.851	0.831	0.865	0.830	
Genotypes	n=185	n=74	n=111	n=173	**
AA	6 (0.03)	5 (0.07)	1 (0.01)	8 (0.05)	
AC	43 (0.23)	15 (0.20)	28 (0.25)	43 (0.25)	
CC	136 (0.74)	54 (0.73)	82 (0.74)	122 (0.71)	
rs1898830					
Alleles					
A	0.845	0.869	0.878	0.859	0.774
G	0.125	0.131	0.122	0.141	0.226
Genotypes	n=172	n=61	n=111	n=174	n=53
AA	131 (0.76)	47 (0.77)	84 (0.76)	131 (0.75)	34 (0.64)
AG	39 (0.23)	12 (0.20)	27 (0.24)	37 (0.21)	14 (0.26)
GG	2 (0.01)	2 (0.03)	0 (0.00)	6 (0.03)	5 (0.09)
rs1039559					
Alleles				**	
C	0.201	0.202	0.200		0.173
T	0.799	0.799	0.800		0.827
Genotypes	n=177	n=67	n=110	**	n=98
CC	5 (0.03)	3 (0.04)	2 (0.02)		2 (0.02)
CT	61 (0.34)	21 (0.31)	40 (0.36)		30 (0.31)
TT	111 (0.63)	43 (0.64)	68 (0.62)		66 (0.67)
rs5743810					
Alleles					
C	0.912	0.903	0.918	0.848	0.915
T	0.088	0.097	0.081	0.152	0.085
Genotypes	n=182	n=72	n=110	n=178	n=53
CC	151 (0.83)	59 (0.82)	92 (0.84)	129 (0.72)	44 (0.83)
CT	30 (0.16)	12 (0.17)	18 (0.16)	44 (0.25)	9 (0.17)
TT	1 (0.01)	1 (0.01)	0 (0.00)	5 (0.03)	0 (0.00)

Table 12. continued

SNPs and Genotypes	Entire PID cohort	Chlamydial positive cases	Chlamydial negative controls	*Healthy African American control group	Hapmap.org
rs3775073					
Alleles					
A	0.359	0.390	0.337	0.088	0.321
G	0.641	0.609	0.662	0.911	0.679
Genotypes	n=184	n=73	n=111	n=180	n=53
AA	21 (0.11)	9 (0.12)	12 (0.11)	1 (0.005)	4 (0.08)
AG	90 (0.49)	39 (0.53)	51 (0.46)	30 (0.17)	26 (0.49)
GG	73 (0.40)	25 (0.34)	48 (0.43)	149 (0.83)	23 (0.43)
rs5030728					
Alleles				**	
A	0.156	0.149	0.161		0.113
G	0.844	0.851	0.839		0.887
Genotypes	n=186	n=77	n=109	**	n=53
AA	5 (0.03)	4 (0.05)	1 (0.01)		0 (0.00)
AG	48 (0.26)	15 (0.19)	33 (0.30)		12 (0.23)
GG	133 (0.72)	58 (0.75)	75 (0.69)		41 (0.77)
rs4986790					
Alleles				**	
T	0.921	0.916	0.923		0.943
C	0.080	0.083	0.077		0.057
Genotypes	n=195	n=84	n=111	**	n=53
TT	164 (0.84)	70 (0.83)	94 (0.85)		48 (0.91)
TC	31 (0.16)	14 (0.17)	17 (0.15)		4 (0.08)
CC	0 (0.00)	0 (0.00)	0 (0.00)		1 (0.01)

Table 12. continued

SNPs and Genotypes	Entire PID cohort	Chlamydial positive cases	Chlamydial negative controls	*Healthy African American control group	Hapmap.org
rs1927911					
Alleles				**	
C	0.427	0.511	0.359		0.425
T	0.573	0.489	0.641		0.575
Genotypes	n=199	n=89	n=110	**	n=53
CC	48 (0.24)	32 (0.36)	16 (0.15)		9 (0.17)
CT	74 (0.37)	27 (0.30)	47(0.43)		27 (0.51)
TT	77 (0.39)	30 (0.34)	47 (0.43)		17 (0.32)
rs7744					
Alleles				**	
A	0.956	0.952	0.959		0.925
G	0.043	0.048	0.041		0.075
Genotypes	n=205	n=94	n=111	**	n=53
AA	188 (0.92)	86 (0.91)	102 (0.92)		45 (0.85)
AG	16 (0.08)	7 (0.07)	9 (0.08)		8 (0.15)
GG	1 (0.004)	1 (0.01)	0 (0.00)		0 (0.00)
rs4988457					
Alleles				**	
C	0.903	0.930	0.887		0.925
G	0.096	0.070	0.113		0.075
Genotypes	n=182	n=71	n=111	**	n=53
CC	147 (0.81)	61 (0.86)	86 (0.77)		45 (0.85)
CG	35 (0.19)	10 (0.14)	25 (0.23)		8 (0.15)
GG	0 (0.00)	0 (0.00)	0 (0.00)		0 (0.00)
rs3802813					
Alleles				**	
A	0.060	0.051	0.068		0.028
G	0.940	0.948	0.934		0.972
Genotypes	n=199	n=88	n=111	**	n=53
AA	0 (0.00)	0 (0.00)	0 (0.00)		0 (0.00)
AG	24 (0.12)	9 (0.10)	15 (0.14)		3 (0.06)
GG	175 (0.88)	79 (0.90)	96 (0.86)		50 (0.94)

* Group of healthy African American controls from Pittsburgh, PA

** Allele or genotype frequencies were not available

All frequencies taken from Hapmap.org were for African American ancestry, from the Southwest, USA

This table only displays frequencies of SNPs that were used in the regression analyses (SNPs not used in regression analyses included: TLR1 rs5743817, TLR4 rs11536889, TIRAP rs8171374, and TIRAP rs7932976).

Table 13. Allele frequencies, only of SNPs included in regression analyses, among patients genotyped with buffy coats vs. serum samples

SNPs & Alleles	Buffy Coats (n=157)	Serum Samples (n=48)
rs5743618		
G	0.1565	0.0735
T	0.8435	0.9265
rs4833095		
G	0.7810	0.6818
A	0.2190	0.3182
rs3804099		
C	0.6401	0.6613
T	0.3599	0.3387
rs11938228		
A	0.1401	0.1964
C	0.8599	0.8036
rs1898830		
A	0.8710	0.9118
G	0.1290	0.0882
rs1039559		
C	0.2000	0.2045
T	0.8000	0.7955
rs5743810		
C	0.9231	0.8462
T	0.0789	0.1538
rs3775073		
A	0.3408	0.4630
G	0.6592	0.5370
rs5030728		
A	0.1516	0.1774
G	0.8484	0.8226
rs4986790		
C	0.0828	0.0658
T	0.9172	0.9342
rs1927911		
C	0.3942	0.5465
T	0.6058	0.4335
rs7744		
A	0.9554	0.9583
G	0.0446	0.0417
rs4988457		
C	0.8942	0.9615
G	0.1058	0.0385
rs3802813		
A	0.0732	0.0119
G	0.9268	0.9881

This table only displays frequencies of SNPs that were used in the regression analyses (SNPs not used in regression analyses included: TLR1 rs5743817, TLR4 rs11536889, TIRAP rs8171374, and TIRAP rs7932976).

Table 14. Associations between selected SNPs and upper genital tract infection

SNPs and Genotypes	*UGTI Negative (N=101)	*UGTI Positive (N=64)	**Adjusted Odds Ratio (95% CI)	P-value
TLR 1				
rs5743618				
GG+GT	29(30.9)	6(12.2)	Referent	
TT	65(69.2)	43(87.8)	7.2 (1.8-27.7)	0.0043
rs4833095				
AA+AG	41(44.1)	17(31.5)	Referent	
GG	52(55.9)	37(68.5)	2.0 (0.8-5.0)	0.1368
TLR 4				
rs1927911				
TT	43(43.4)	18(30.0)	Referent	
CT	40(40.4)	19(31.7)	1.5 (0.5-4.2)	0.4568
CC	16(16.2)	23(38.3)	4.9 (1.7-13.8)	0.0026

*Upper genital tract infection (UGTI)

** Adjusted for age and *N. gonorrhoeae*

Logistic regression was used to calculate odds ratios and 95% CI.

Table 15. Haplotype associations with endometritis

Haplotype	Endometritis negative frequencies	Endometritis positive frequencies	*Exact P-value
**TIRAP haplotype*			
GGC	0.83	0.92	0.0420
§*TLR 6 haplotypes*			
CTA	0.11	0.03	0.0410

*Exact p-value based on 1000 permutations

**TIRAP haplotype (rs3802813/rs7932976/rs8177374)

§ TLR6 haplotype (rs109559/rs5743810/rs3775073)

APPENDIX C: SUPPLEMENTARY TABLES TO MANUSCRIPT 3

Table 16. Associations between selected SNPs and pregnancy among a subset of women with *C. trachomatis* infection

SNPs and Genotypes	Pregnant (N=62)	Not Pregnant (N=32)	*Adjusted Hazard Ratios (95% CI)	P-value
TLR 1				
rs5743618				
GG+GT	10(21.2)	2(7.1)	**	**
TT	37(78.7)	26(92.9)		
rs4833095				
AA+AG	19(41.3)	9(29.0)	Referent	
GG	27(58.7)	22(71.0)	0.5 (0.3-0.9)	0.0412
TLR 4				
rs1927911				
TT	18(31.6)	12(37.5)	Referent	
CT	17(29.8)	10(31.3)	1.2 (0.4-3.9)	0.7823
CC	22(38.6)	10(31.3)	1.0 (0.6-2.1)	0.7796

*Adjusted for age and history of infertility

**Cell size too small for logistic regression

Table 17. TLR1 haplotypes and pregnancy

Haplotype	Pregnancy frequencies	No Pregnancy frequencies	P-value	*Exact P-value
**_TLR1 haplotypes_*				
TGC	0.72	0.80	0.0494	0.2860
TGT	0.12	0.08	0.2626	0.3490

*Based on 1000 permutations

** TLR1 rs5743618/rs5743817/rs4833095 haplotype

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