THE DETECTION AND ANALYSIS OF PATHOGEN-REACTIVE IMMUNOGLOBULINS IN THE URINE OF MEN WITH NONGONOCOCCAL URETHRITIS

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John D. Ryan

THE DETECTION AND ANALYSIS OF PATHOGEN-REACTIVE IMMUNOGLOBULINS IN THE URINE OF MEN WITH NONGONOCOCCAL URETHRITIS

Inflammation of the urethra—*urethritis*—is commonly diagnosed in men and women who have sexually transmitted infections (STI). Characteristic signs and symptoms of urethritis include urethral discharge and burning pain during urination (dysuria). However, these findings are non-specific and can be elicited by STI for which optimal treatment approaches differ. We wanted to investigate if immunoglobulins (antibodies) in the urine of men with acute urethritis could determine the etiologies of these cases. Previously, we conducted an observational case-control study of biological males to compare the urethral microbiota of participants with unambiguous, laboratory-confirmed urethritis (cases) and participants without urethral inflammation (controls). This revealed that nearly 2 in 5 men with nongonococcal urethritis tested negative for all common STI. We identified atypical urethral pathogens in approximately 1/3 of these STI-negative individuals using shotgun metagenomic sequencing. However, we did not detect microorganisms suspected to be urethral pathogens in the remaining 2/3 of STI-negative participants. We hypothesized that these men with "pathogen-negative" urethritis had persisting inflammation from a recent STI that already cleared spontaneously by the time of testing. We observed that urine IgA antibodies against *Chlamydia trachomatis* (*Ctr*) infectious particles were significantly more prevalent among men with pathogen-negative urethritis compared to controls. In contrast, we found

that the prevalence of urine anti-*Ctr* IgA was similar between controls and urethritis cases with atypical infections. However, our efforts to detect antibodies against another common STI, *Mycoplasma genitalium* (*Mgen*), were complicated by low abundance in urine and the unexpected prevalence of *Mgen*-reactive antibodies among controls. Collectively, our results suggest that signs and symptoms of urethritis can continue after the causative STI(s) have been eliminated. Furthermore, male urine represents a practical, non-invasive source of pathogen-reactive antibodies that could be evaluated using point-of-care diagnostic tests to elucidate urethritis etiologies. Importantly, our results also suggest that sexual partners of men with pathogen-negative, nongonococcal urethritis are an unrecognized chlamydia reservoir.

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LIST OF ABBREVIATIONS

AZM..... azithromycin

BCR.....B cell receptor

BFC.....Bell Flower Clinic (Indianapolis, IN)

CASI ..... computer-assisted self-interviewing

CDC .....Centers for Disease Control and Prevention

CLSI..... Clinical and Laboratory Standards Institute

Ctr .....*Chlamydia trachomatis*

EB .....elementary body

ELISA .....enzyme-linked immunosorbent assay

FcRn.....neonatal Fc receptor

FCU.....first-catch urine

HIV .....human immunodeficiency virus

HRP.....horseradish peroxidase

HSV..... herpes simplex virus

HTM.....*Haemophilus* Test Medium

ID_{50}.....median (50^{th} percentile) infectious dose

IDRL ..... Infectious Diseases Research Laboratory (IUSM)

IFN-γ .....interferon-gamma

Ig ..... immunoglobulin

IHC ..... immunohistochemical

IU ..... idiopathic urethritis

IUMP ..... Idiopathic Urethritis Men's Project (R01 AI116706)

LGV ..... lymphogranuloma venereum

Mgen ..... *Mycoplasma genitalium*

MHC ..... major histocompatibility complex

MOMP ..... major outer membrane protein

MMP ..... matrix metalloproteinase

MRM ..... macrolide resistance-mediating mutation

MSM ..... men who have sex with men

MXF ..... moxifloxacin

NAAT ..... nucleic acid amplification test(ing)

NGU ..... nongonococcal urethritis

P^+ IU ..... pathogen-positive idiopathic urethritis

PAMP ..... pathogen-associated molecular pattern

PBS(T) ..... phosphate-buffered saline (plus Tween-20)

PID ..... pelvic inflammatory disease

pIgR ..... polymeric immunoglobulin receptor

PMBC ..... peripheral blood mononuclear cells

PMN ..... polymorphonuclear (leukocytes)

PN-IU ..... pathogen-negative idiopathic urethritis

PRR ..... pattern-recognition receptor

q-PCR ..... quantitative polymerase chain reaction

RB ..... reticulate body

STI ..... sexually transmitted infection

T3SS ..... type III secretion system

TCR ..... T cell receptor

TE ..... tetracycline

TFI ..... tubal factor infertility

Th ..... T helper cell

TLR ..... Toll-like receptor

TMB ..... tetramethylbenzidine

TV ..... *Trichomonas vaginalis*

UU ..... *Ureaplasma urealyticum*

WT ..... wild-type

CHAPTER I – Introduction

Section 1: Overview of the Male Urethra and Urethritis

The **urethra** is the terminal organ of the urinary tract and serves as a conduit for urine between the bladder and exterior of the body. Urine forms in the kidneys and is stored in the urinary bladder until it passes through the urethra. A healthy pair of adult human kidneys generates between 1–2 L of urine per day filtering nitrogenous wastes out of the bloodstream and maintaining the homeostatic balance of various compounds in the blood. The male urethra is approximately 20 cm in length. From its origin at the neck of the bladder, it passes through the prostate gland then exits the pelvic cavity. The **penile urethra** is the longest segment of the urethra and is encircled by the corpus spongiosum of the penis.

The urethra is a mucosal surface lined by a protective epithelium [1]. Despite frequent flushing by voided urine, the healthy urethra supports a microbiome of commensal microorganisms that can make use of the limited nutrients available in this niche [2]. During unprotected sexual contact, the urethra is exposed to sex partners' mucosal secretions. This can cause changes in the composition of the urethral microbiome. It can also result in transmission of sexually transmitted infections (STI) [3].

Inflammation of the urethra—**urethritis**—occurs in both sexes but is more common in men [4]. Urethritis is the leading diagnosis among men who present for medical care because of dysuria (*i.e.,* the sensation of pain or burning during urination) and/or purulent or mucopurulent urethral discharge. Other symptoms sometimes reported by male patients include stinging or itching of the urethra and irritation of the urinary meatus [5]. However, a recent study of patient-reported symptoms by Jordan and

colleagues found that symptoms other than dysuria and urethral discharge, including genital irritation or itching, genital lesions, urinary frequency, and urinary odor were poorly predictive of clinical urethritis [6].

Formal diagnosis of urethritis requires microscopic evidence of acute inflammation in the distal urethra. Urethritis is diagnosed by confirming an objective threshold of polymorphonuclear leukocytes (PMN) per high-power field (1000x) in a magnified, Gram-stained specimen of urethral swab material [5, 7, 8]. In many healthcare settings, however, the presumptive diagnosis of urethritis is established on the basis of urethral signs and symptoms (*e.g.,* dysuria or discharge), alone. According to the most recent version of the Centers for Disease Control and Prevention (CDC) STI treatment guidelines, patients with urethritis should undergo nucleic acid amplification testing (NAAT) for the bacterial pathogens, *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. Since the results of NAAT are not immediately known, patients with urethritis should also receive empiric therapy with antibiotics active against both of these bacteria [7].

In clinical and epidemiological practice, urethritis cases are broadly divided into two groups [9]. Traditionally, this distinction was made on the basis of physical examination and microscopy findings; NAAT is now the preferred method. **Gonococcal urethritis** is specifically caused by infections with the bacterium, *N. gonorrhoeae*, the microorganism known historically as the "gonococcus." *N. gonorrhoeae* has a distinct appearance and morphology in Gram-stained specimens of material collected from the distal urethra. Thus, urethral gonorrhea infections can be identified by the presence of intracellular Gram-negative diplococci inside PMN. However, this approach is only feasible in healthcare settings, such as specialized STI clinics, where microscopy is

accessible to examine urethral secretions. The emergence of highly antibiotic-resistant strains of *N. gonorrhoeae* over the past decade represents a serious ongoing threat in many parts of the world [10]. In the United States, nearly 700,000 gonorrhea infections were reported to the CDC in 2021, the highest-ever annual total [11]. Although not a focus of this dissertation, gonococcal urethritis is mentioned here for clarity as the counterpart to *nongonococcal* urethritis.

Nongonococcal urethritis (NGU) encompasses all other causes of urethral inflammation besides gonorrhea and is estimated to affect nearly three (3) million men each year in the US [12]. Infections with the sexually transmitted bacteria *Ctr* and *Mgen* are accepted as common causes of NGU. Other human pathogens including *Trichomonas vaginalis* and *Treponema pallidum*, which cause the diseases trichomoniasis and syphilis, respectively, also cause NGU occasionally [5, 13]. Some NGU cases are likely attributable to non-infectious etiologies such as urethral irritation or tissue damage [9].

Idiopathic urethritis (IU) is a non-specific description assigned to cases of NGU without a confirmatory NAAT diagnosis (Figure 2). In epidemiologic studies, as many as half of NGU cases do not have a clear infectious etiology [14]. Using culture-independent techniques to evaluate the urethral microbiome, some researchers have identified microorganisms that are more commonly found in men with inflamed urethrae compared to men without urethral disease symptoms [15-17]. Several bacterial and viral pathogens identified using these approaches have been speculated to cause NGU, but definitive evidence is lacking. The complete spectrum of microorganisms that are transmitted by sexual contact and cause NGU in men remains unknown.

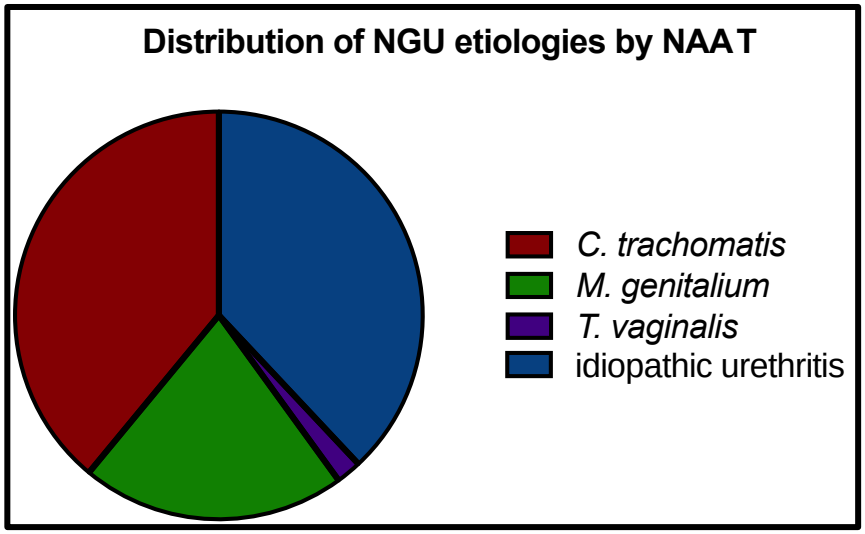

Figure 1. More than 1/3 of men with nongonococcal urethritis test negative for STI-causing pathogens.

Ctr and *Mgen* were the most-commonly identified etiologic agents of NGU in men with urethritis who enrolled in a clinical study. Approximately 38% of participants tested negative for known STI-causing pathogens; these cases were characterized as idiopathic urethritis (IU). (Adapted from Jordan *et al*. *Sex Transm Infect*. 2020. [18]).

Regardless of etiology, treatment for NGU is typically initiated at the point of care using empiric oral antibiotics. Indeed, antibiotic treatment is associated with the resolution of urethral symptoms. However, this practice may provide patients with a false sense of security about returning to sexual activity because many NGU cases do not have a clear bacterial cause [5, 18] and antibiotic treatment can sometimes alleviate infection-associated symptoms in men who remain infectious [19]. Further complicating efforts to develop effective, evidence-based treatment strategies, many men who acquire an STI at the urethral site remain asymptomatic and do not develop overt pathology. The same is likely true in adolescent males, but this population is difficult to study, especially in the context of STI research. In general, screening of asymptomatic males for bacterial sexually transmitted infections is usually not recommended under CDC guidelines [7].

Although serious complications of NGU in men are infrequently reported, efforts to better understand the pathogenesis and etiologies of this syndrome are areas of considerable research interest. This is because STIs that most commonly cause urethritis in men, including chlamydia as well as gonorrhea, are transmissible to female sexual partners. In some women, cervical infections spread to the upper reproductive tract and cause pelvic inflammatory disease (PID). Possible consequences include chronic pelvic pain and impaired fertility [20]. Concerningly, the highest rates of chlamydia incidence, by far, are in adolescent and young women who may not be conscious of the potential long-term complications of these infections [21].

In the remainder of this chapter, I will review the cell biology, epidemiology, and clinical features of the most frequently identified NGU-causing pathogens. I will then discuss mucosal immunity with a particular focus on the male and female reproductive tracts and how human immune responses against bacterial STIs can, paradoxically, be both protective and pathologic. Finally, I will review ongoing efforts to elucidate novel etiologies of NGU and how we will use evidence from a recently completed case-control study of men in Indianapolis—the Idiopathic Urethritis Men's Project—to further develop our understanding of this prevalent condition.

Section 2: *Chlamydia trachomatis* & Chlamydia

Chlamydia trachomatis (*Ctr*) causes chlamydia, the most prevalent bacterial STI worldwide [22]. *Ctr* is also the most frequently identified cause of nongonococcal urethritis [5, 14]. *Ctr* and the closely related species, *C. pneumoniae* and *C. psittaci*, are human pathogens that can cause serious disease in susceptible individuals. Consequently,

much research has been dedicated to understanding chlamydial pathogenesis and developing approaches to prevent future infections and their associated morbidity and mortality. In this section, I will discuss key elements of *Ctr* cell biology and epidemiology and important questions that remain unanswered about optimal strategies for chlamydia prevention and control.

Like the STIs gonorrhea and syphilis, chlamydia is a reportable disease. Positive identification of chlamydia infection requires reporting to local health departments and eventually to the CDC for contact tracing and case surveillance purposes. More than 1.6 million chlamydia cases were reported in 2021, down slightly from the all-time high in 2019 [11]. However, this decline was likely attributable to the de-prioritization of asymptomatic chlamydia screening services during the pandemic of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory coronavirus 2 (SARS-CoV-2) [11]. Although acute chlamydia does not cause mortality, it is responsible for preventable morbidity and significant healthcare expenditures. Direct medical costs associated with treating chlamydia and its resulting complications exceed $1 billion annually in the United States [23].

Like all Chlamydiales, *Ctr* is a Gram-negative, obligate intracellular pathogen of eukaryotic cells. Along with *C. pneumoniae* and *C. psittaci, Ctr* is one of three known human pathogens in this phylum. These Chlamydiae share an unusual bacterial developmental cycle in which DNA replication and cell division—essential functions of bacterial survival—must occur inside susceptible host cells [24]. However, any single host cell has finite resources (*e.g.*, lipids, amino acids, or ATP) that can be scavenged by chlamydial invaders. Thus, the continued proliferation of these parasites depends on their

ability to cycle continuously between new host cells and to survive periods of extracellular exposure.

The **elementary body** (EB) is the extracellular, non-replicative, and infectious form of *Ctr*. A new cycle of *Ctr* infection begins when an EB encounters a susceptible host cell (Figure 2) [25]. Human cells most vulnerable to *Ctr* are found in the mucosal epithelia of the male and female urogenital tracts, alimentary canal, and conjunctiva. Although the exact mechanism(s) by which EBs attach to host cells are unclear, research into the pathogenesis of *Ctr* and closely related *Chlamydia* spp. has uncovered several different receptors on human epithelial cells that might be exploited [26-28].

EBs exhibit minimal metabolic activity but can tolerate osmotic stress in the environment [29]. Although they cannot synthesize new proteins, EBs are preloaded with virulence factors including potent effectors that can manipulate specific biochemical pathways inside human epithelial cells. Some *Ctr* effector proteins are delivered directly from EBs through type III secretion systems (T3SS) into the cytosol of epithelial cells [30]. These effectors have roles such as the manipulation of host cells' actin machinery to mediate the process of *Ctr* invasion [31]. This allows infectious EBs bound to the outer membranes of host cells to force their own internalization via endocytosis. Through this specific mechanism of entry, an invading EB enters its host surrounded by a membrane-bound vesicle—the **inclusion**—that provides physical separation from the cytosol of the infected cell. Inside this nascent inclusion, the EB transforms into its metabolically active, replicative form: the **reticulate body** (RB). Newly differentiated RBs begin their intracellular phase by transcribing and translating a set of temporally regulated *Ctr* early genes [32]. This generates effector proteins that modify the inclusion membrane in order

to prevent fusion with lysosomes and degradation. Other effectors have roles in extracting nutrients from host cells.

Although *Ctr* has limited ability to synthesize essential metabolites *de novo*, RBs express several genes to scavenge host resources such as lipids, amino acids, and nucleic acids. These anabolites are subsequently converted into the macromolecules required for chlamydial proliferation. As metabolically active cells, RBs undergo genome replication and cell division, which produces daughter cells through binary fission or budding mechanisms [33].

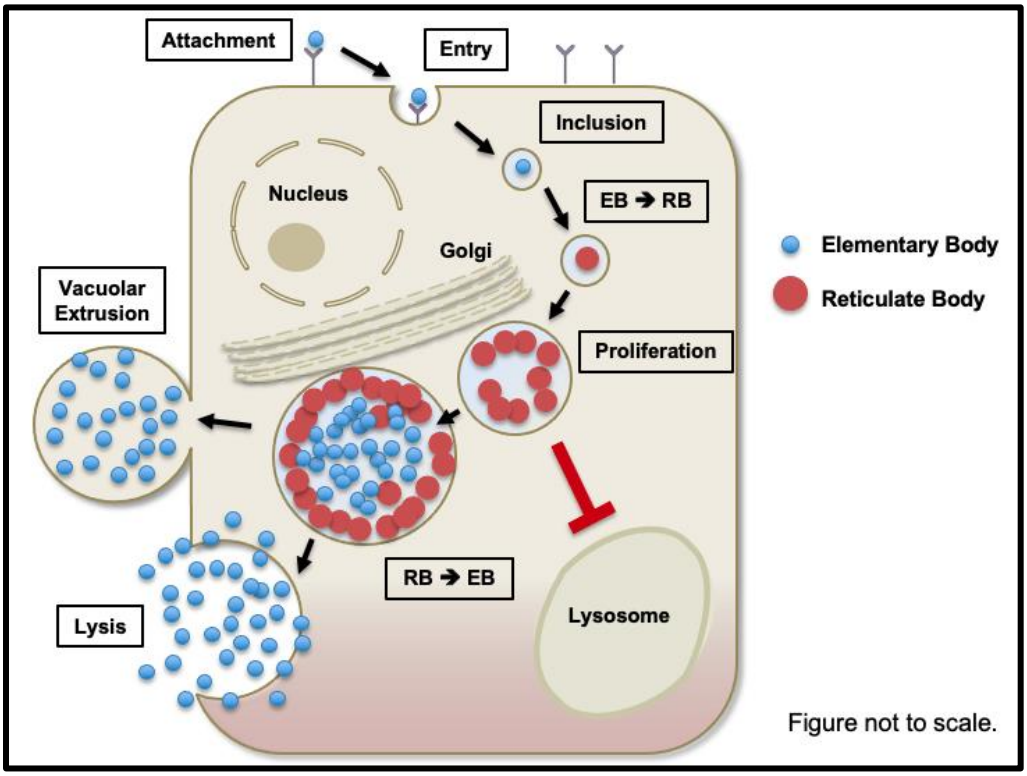

Figure 2. *Chlamydia trachomatis* is an obligate intracellular pathogen that infects human reproductive tract epithelia and causes chlamydia.

(Adapted from A. Banerjee, ProQuest Dissertation No. 30248435. 2023. [34])

As development continues inside the inclusion, RBs shift towards transcribing mid-cycle genes. These include genes for Incs, which maintain inclusion stability, and for major outer membrane protein (MOMP), which provides structural integrity for the RBs. In the late stages of RB development, gene expression shifts toward pre-forming effector proteins and components of the T3SS machinery that EBs will need to establish subsequent infections. After several rounds of replication, late-stage RBs asynchronously disconnect from the inclusion membrane and transition back into compact, spore-like EBs. Finally, the progeny EBs are released from their host cells by lysis or by vacuolar extrusion [35].

Not only does MOMP help stabilize dividing RBs, but it is also the primary constituent of the EB cell envelope. MOMP accounts for approximately 60% of the mass of an EB [36]. In pioneering work on human chlamydial infections, Wang and Grayston used monoclonal antibodies to characterize *Ctr* strains with 15 different variants of MOMP [37]. Variation between these MOMP antigens derives from sequence heterogeneity in four variable regions of the *Ctr* gene, *ompA*. Recombination of *ompA* sequences between different serovars can also occur, and hybrid *ompA* alleles have been documented [38]. Many sources now recognize 18 or 19 distinct *Ctr* serovars (or "genovars"). Due to differences in their respective tissue tropism, *Ctr* serovars are generally grouped according to the human diseases they cause.

Ctr serovars A–C infect human epithelial cells in the conjunctiva and cause the ocular disease, trachoma. Although rarely diagnosed in the United States, trachoma remains endemic in many parts of the developing world including sub-Saharan Africa, Asia, and Central and South America [39]. Since trachoma is spread not only by direct

human contact but also by flies, improved facial hygiene is considered a critical step towards preventing these infections. A single, 1-gram dose of azithromycin typically cures trachoma, but even this relatively inexpensive antibiotic is not available in many resource-limited settings. Furthermore, since the human immune response against *Ctr* does not generate sterilizing immunity, even for specific serovars, re-infections with trachoma are highly common. After many infections, fibrosis and subsequent scarring of the conjunctiva can cause inversion of eyelashes and repeated mechanical abrasion of the cornea. In some individuals, continued abrasion ultimately progresses to corneal opacification and blindness. Trachoma remains the most common cause of blindness worldwide due to a preventable infection.

Infections with *Ctr* serovars L1, L2/L2a, and L3 are transmitted by sexual activity and cause the invasive disease, lymphogranuloma venereum (LGV). Like trachoma, LGV is also rare in developed countries. However, its incidence is increasing among men who have sex with men (MSM), especially those who are coinfected with human immunodeficiency virus (HIV) [40, 41]. Classical symptoms of LGV include genital ulcers and lymphadenopathy of inguinal or femoral lymph nodes. Untreated, LGV-infected lymph nodes can become necrotic and rupture, resulting in draining fistulae. However, recent evidence suggests that proctitis and proctocolitis are now the predominant manifestations of LGV in North America and Europe [42]. Symptoms of rectal LGV include mucosal ulcerations, bleeding, and tenesmus with possible progression to perirectal abscesses or fissures. However, LGV remains treatable with an extended course of doxycycline antibiotic therapy.

Chlamydia

Ctr serovars D–K primarily infect epithelial cells in the male (urethra) and female (cervix) reproductive tracts. These serovars cause the STI, chlamydia, and are most frequently observed in adolescents and young adults. Although complications including epididymitis and reactive arthritis are occasionally recognized, symptomatic chlamydia infections in men usually present as uncomplicated urethritis, [43, 44]. However, an estimated 50% of urethral *Ctr* infections in males are asymptomatic [45]. In women, chlamydia clinically presents most often as cervicitis or urethritis, but an even larger proportion of women with *Ctr* infections—estimated in one study at nearly 70%— do not report any symptoms [46]. For this reason, the CDC estimates that the true incidence of chlamydia in the United States may be as high as four (4) million cases annually, compared to just 1.6 million reported cases [11].

Urogenital chlamydia is common in both men and women and can be long-lasting in the absence of effective antimicrobial therapy. In most cases, however, chlamydia is self-limited and elicits no symptoms. Thus, the main reason that chlamydia is regarded as a significant public health concern is the possibility of irreversible reproductive tract damage in women. By mechanisms that are not entirely understood, endocervical chlamydia occasionally ascends to the uterus and fallopian tubes. Inflammation of these organs causes PID, which is not only acutely painful but also can induce permanent damage of the fallopian tubes through the effects of inflammatory mediators and tissue remodeling. Even a single episode of PID can have dramatic consequences for future fertility [47]. Scarring of the oviducts can cause tubal factor infertility (TFI) if it impairs their physiologic function of transporting ova (eggs) from the ovaries to the uterus. This

scarring can also cause ectopic pregnancy if a fertilized ovum (zygote) implants in the fallopian tube. Rupture of an ectopic pregnancy can cause severe, life-threatening hemorrhaging and is therefore a medical emergency.

As clearer evidence emerged of the association between *Ctr*, PID, and reproductive sequelae, many countries established chlamydia screening programs to preemptively test asymptomatic women in certain high-risk groups [7, 48]. Surprisingly, although some of these screening programs have successfully reduced chlamydia-related reproductive complications [49], they have coincided with increases in incident case rates that cannot be explained by an increased uptake of STI screening services. Instead, Brunham and colleagues observed that the continuously increasing chlamydia case rate in British Columbia, Canada, was driven by a dramatic increase in the frequency of re-infections [50]. Thus, these authors formulated the **arrested immunity hypothesis** of chlamydia infection [51]. Briefly, this hypothesis postulates that aggressively seeking out and treating asymptomatic *Ctr* infections in women may have resulted in reduced levels of population-level acquired immunity. However, the development of acquired immunity against a primary chlamydia infection and correlates of protection against subsequent *Ctr* re-infection remain poorly understood.

Infection of men with *Ctr* might also increase the likelihood that they will acquire another STI. Multiple studies suggest that *Ctr* infections are associated with increased risk of acquiring [52] or transmitting [53, 54] HIV.

Sexual transmission of chlamydia infection

Ctr is highly transmissible between hosts, and sexual intercourse is the primary mechanism by which uninfected individuals acquire chlamydia. This suggests that *Ctr* EBs enter host cells very efficiently and/or that the number of these infectious particles required to establish a new infection is low. Although the median infectious dose (ID_{50}) of *Ctr* is unknown in humans, it has been calculated to be as low as 79 infectious EBs in the guinea pig model of urethral infection using the guinea pig pathogen, *Chlamydia caviae* [55]. This corresponds well with a previous finding that sexual transmission from male guinea pigs required as few as 10^2 infectious EBs to establish an active infection in female animals [56].

Human sexual networks are complex, and it is difficult to know with certainty how frequently chlamydia is transmitted to exposed individuals [57]. Most chlamydia infections, although likely still transmissible, are asymptomatic. However, the human experiments required to conclusively address this question are impermissible. Mathematical models of chlamydia infection vary widely, but recent work suggests that the *per-act* probability of *Ctr* transmission is approximately 10% [57, 58]. However, various host factors that are not well understood might affect the risk of acquiring chlamydia from an infected partner. Indeed, in observational studies involving sexual partnerships, many men and women with chlamydia-infected partners are not infected, themselves [59-61]. This implies that some individuals are innately more resistant to chlamydia infection or that some, but not all, mount an immune response that successfully clears *Ctr*.

Spontaneous clearance of chlamydia

Given the ethical constraints associated with infectious diseases and STI research, in particular, experiments to definitively establish the entire natural course of human chlamydia infection from transmission to resolution are rarely permissible. As a result, it is unclear how chlamydia develops over time or what factors influence why some cases remain uncomplicated and others progress to severe pathology. Evidence from human observational studies and experimental animal infection models suggests that *Ctr* can persist for extended periods (years) in the male and female reproductive tracts but will eventually resolve without antibiotic therapy in many individuals [62, 63].

More recently, mathematical modeling studies have suggested that the natural course of chlamydia in men and in women follows a bimodal distribution with distinctly "fast-clearing" and "slow-clearing" infections [64, 65]. Moreover, Price *et al*. calculated that these fast-clearing and slow-clearing infections in men corresponded to strikingly different average durations: 7.45 days and 2.84 years, respectively, in the absence of treatment [64]. One intriguing possibility is that fast-clearing chlamydia infections are eliminated by a rapid and potent immune response, possibly developed through previous exposure(s) to *Ctr*. However, the development of immunity and mechanisms of protection against bacterial pathogens in the male urethra are not well understood.

Section 3: Other Etiologies of NGU

Most symptoms of nongonococcal urethritis are not specific to any particular microorganism. Although *Ctr* is the best known and most extensively studied etiologic

agent of NGU, other microorganisms have also been identified as causes of this syndrome. As I will discuss in the sub-section on *Ureaplasma urealyticum*, however, new evidence continues to emerge which challenges current understanding about the causative roles of microorganisms in NGU.

Mycoplasma genitalium

The sexually transmitted bacterium, *Mycoplasma genitalium* (*Mgen*), is a species in the phylum, *Mollicutes*. It was first identified as a putative etiologic agent of urethritis in 1981 after it was cultured from the urethral secretions of a small number of men with NGU [66]. *Mgen* is now the second-most commonly identified cause of this syndrome and is a frequent cause of persistent urethritis that does not resolve with empiric antibiotic therapy [67]. In women, *Mgen* has been reported to be a common cause of cervicitis. Although asymptomatic *Mgen* infections are observed in both sexes, screening of asymptomatic individuals is not recommended. Thus, the true incidence and prevalence of *Mgen* infections are unknown [7]. The CDC now recommends the antibiotic, doxycycline, as first-line empiric therapy for NGU, in part because of increasing rates of azithromycin treatment failures observed among men with *Mgen* urethritis.

Data are mixed concerning whether infections with *Mgen* cause long-term complications in men. It is also unclear whether these infections cause female reproductive tract complications similar to those associated with urogenital chlamydia [68, 69]. Thus, *Mgen* infections, even in asymptomatic women, could cause PID and subsequent damage to upper reproductive tract tissues. Furthermore, *Mgen* infection, like chlamydia, is associated with increased risk of HIV acquisition and transmission [70].

Despite these potential health threats, however, *M. genitalium* remains relatively unknown as an STI [71].

Trichomonas vaginalis

Trichomonas vaginalis (TV) is a protozoan parasite that causes the sexually transmitted infection, trichomoniasis ("trich"). Despite its large case burden, TV is considered to be a neglected disease. Approximately 250 million new cases of TV occur annually worldwide [72], while the estimated prevalence of this parasite in the United States is 3.7 million cases [73]. TV remains poorly studied despite evidence for potential long-term reproductive complications in women similar to those associated with *Ctr*. In fact, the genome of TV was not published until 2007 [74].

T. vaginalis infections in men are primarily asymptomatic but, like other STIs, can be long-lasting. In some men, TV causes NGU and occasionally epididymitis or prostatitis [75]. However, testing for TV by NAAT is not widely available. Instead, diagnosis requires microscopic identification of the parasite which has low sensitivity in men [75]. Treatment with the anti-parasitic medication, metronidazole, is typically effective at resolving NGU symptoms caused by TV [72]. However, the identification of TV isolates that exhibit resistance to nitroimidazole-class drugs has raised concerns about the lack of other approved therapies [76].

Ureaplasma urealyticum

Ureaplasma urealyticum (UU) is another species of *Mollicutes* that is transmitted by sexual activity. UU and the related bacterium, *U. parvum*, commonly colonize the male urethra [77]. Early studies that indicated this bacterium causes urethritis were met with enthusiasm but have been called into question by more recent evidence [16, 18]. In some case-control studies, UU was more prevalent among men with NGU compared to controls. Other studies—including one from our laboratory—have suggested that UU is not an independent risk factor for urethritis. In order to reconcile this discrepancy, it has been hypothesized that UU may cause urethritis only under particular conditions, such as when the urethral UU organism load is high [78, 79].

Section 4: Mucosal Immunity & STI Immunopathology

Mucous membranes in the urogenital tract, alimentary canal, and elsewhere are important barriers against environmental insults and against invasion by bacteria, viruses, fungi, and parasites. In the previous sections, I discussed microbial pathogens, particularly *Ctr*, that are most closely associated with the syndrome of NGU in men. These microorganisms share a common route of transmission by sexual activity and can cause pelvic inflammatory disease and severe complications in infected women. They also share the abilities to resist immune-mediated clearance and to overcome, sometimes for extended periods, the various defensive mechanisms that provide protection at human mucosal surfaces.

Among the known etiologies of NGU, *Ctr* is the best-studied pathogen and will be the main focus of this section. *Ctr* poses a particular challenge for the human immune system because it is an intracellular bacterium that replicates inside specially modified inclusions and uses secreted virulence factors to manipulate host cells. Although little is known about the pathophysiology of *Ctr* infection in the male urethra or how immunity develops at this site, much work has been performed to elucidate the mechanisms by which urogenital chlamydia causes lasting reproductive sequelae in women. In general, it is now believed that tissue damage caused when *Ctr* ascends to the female upper reproductive tract is primarily mediated by the host's own immune response. Importantly, this type of pathologic immune response may or may not be associated with patient-reported symptoms of PID. Although *Ctr* infections in most women remain asymptomatic and resolve inconsequentially, even asymptomatic infections can induce inflammatory mucosal immune responses that damage tissue. However, the factors that might predispose certain women to PID and other sequelae are not well understood.

In the remainder of this section, I will review current understanding about how the innate and adaptive branches of the immune system can have both protective and pathologic effects upon exposure to *Ctr*. Much of this understanding has been derived from animal and tissue-culture models of female *Ctr* infection but appears to correlate relatively well with available human data. In contrast, very limited evidence is available about the immune response to chlamydia in men, and only a few groups have investigated chlamydia infection using male models of urogenital disease. Furthermore, little is known about protective immune responses against *M. genitalium* in either sex or how it develops during the course of infection. However, men are a critical vector of *Ctr*

and *Mgen* transmission to new hosts. Thus, further investigation is warranted to understand how the immune response develops against these pathogens in the urethra and why the overt signs and symptoms of NGU are observed only in some individuals.

Innate Immunity

Mucosal immunity encompasses multiple lines of defense to provide comprehensive host protection against microorganisms. Broadly, these defenses can be divided into two systems—innate and adaptive—that have distinct, but interconnected, roles in immune defense. Innate immune cells, such as macrophages, respond quickly and non-specifically against invading pathogens. Foreign microbes can be marked for destruction by opsonization with complement proteins or with immunoglobulins (antibodies) in the blood or mucosal secretions. The barrier functions provided by the mucosal epithelia (and also by the skin) are another key component of the innate system. Goblet cells in the epithelium produce mucus to help immobilize foreign bodies [80], and epithelial cells secrete various antimicrobial peptides including defensins and lysozyme [81]. Although they are not professional immune cells, mucosal epithelial cells also express cell-autonomous immune defenses to defend against viruses and other intracellular pathogens [82].

Many types of innate immune cells express pattern-recognition receptors (PRRs) to detect conserved microbial structures called pathogen-associated molecular patterns (PAMPs). PRRs, including Toll-like receptors (TLRs) are also expressed on the surface of mucosal epithelial cells and in the cytoplasm. Although the corresponding chlamydial ligand(s) have not been clearly identified, activation of TLR2 appears to be a key

mechanism by which infected epithelial cells sense *Ctr* [83]. Through the adaptor protein, MyD88, activation of TLR2 induces a signaling cascade that results in transcription of many defense-related genes. These genes include the sequences encoding several pro-inflammatory cytokines (*e.g.*, TNF-α) and chemokines. These compounds alert nearby epithelial cells and professional immune cells, such as neutrophils, to the ongoing infection. Some of these cytokines also induce changes in endothelial (blood vessel) cells to help neutrophils and other immune cells extravasate in response to an infection.

Activated neutrophils secrete chemicals including matrix metalloproteases and neutrophil elastase that are toxic to many bacterial pathogens. However, neutrophil elastase, in particular, can damage connective tissues in the mucosa. The **cellular paradigm of chlamydia pathogenesis** introduced by Stephens posits that chlamydia-infected epithelial cells, themselves, are primarily responsible for the tissue damage that eventually leads to oviduct scarring. This may result from continuous expression of pro-inflammatory cytokines and prolonged recruitment of immune cells [84]. Data regarding innate immunity against chlamydia in males are limited, although the observed influx of neutrophils into the urethral lumen in men with chlamydia suggests that some similar mechanisms might be involved.

In the only study to date of cytokine responses in men with chlamydia urethritis, only IL-8, among nine different cytokines tested, was measured at higher concentrations in urethral swab specimens from men with chlamydia versus controls who did not have urethritis [85]. However, this study was observational and was conducted among a population of STI clinic patients. As a result, the time elapsed since acquisition of *Ctr*

infection was generally unknown. Furthermore, prior clinical histories of participants enrolled in this study were also unavailable.

In another investigation, immortalized epithelial cells (THUEC) derived from the prostatic segment of the human urethra expressed IL-6 and TNF-α, in addition to IL-8, following *in vitro* infection with *Ctr* [86]. Similar results were demonstrated by Wang and colleagues using the guinea pig model of urethral chlamydia infection [55]. In these animals, an influx of neutrophils into the urethral lumen coincided with secretion of IL-8, consistent with what has been observed in female guinea pigs and generally similar to observations made in studies of human *Ctr* infection.

Infection of cultured epithelial cells with *Mgen* also induces pro-inflammatory and chemotactic cytokines that attract and activate neutrophils [87]. Unlike Ctr, however, *Mgen* is not an obligate intracellular pathogen. Thus, the inflammatory response generated against *Mgen* appears to result not from epithelial cell invasion, but instead from adherence to epithelial surfaces. Here, sustained activation of surface-expressed PRRs including TLR2 and TLR6 could result in continuous cytokine secretion and PMN recruitment [88]. As mentioned previously, however, little is known about human immune responses against *Mgen*.

Adaptive Immunity

The innate immune mechanisms discussed above provide rapid, but non-specific, defense by recognizing conserved microbial structures (*e.g.*, PAMPs). Generally, the goal of these systems was to identify and then promptly limit the spread of acute infectious

threats. The adaptive immune system, in contrast, is slower to activate but eventually generates a response that is highly pathogen-specific. The adaptive immune response comprises cell-mediated and humoral elements, which are primarily mediated by T cells and B cells, respectively. The goals of the adaptive immunity are to eliminate infectious threats and to generate immunologic memory that prevents future infections.

T cells are activated to perform their effector functions and proliferate when their T cell receptors (TCR) recognize cognate antigens displayed by major histocompatibility complex (MHC) molecules. CD8+ T cells—also known as killer T cells or cytotoxic T lymphocytes—recognize cognate antigens expressed on target cells' class I MHC molecules. When activated, CD8+ T cells release cytotoxins including perforin and granzymes that ultimately induce target cell elimination by apoptosis. CD4+ "helper" T (Th) cells have TCRs that recognize antigens displayed on class II MHC molecules. MHC class II are primarily found on antigen-presenting dendritic cells but are also expressed by some B cells and epithelial cells. Under the influence of cytokines and co-stimulatory receptors present during activation, naive Th cells acquire different roles to direct the immune response. Th1 CD4+ T cells are particularly important for protective immunity against *Ctr*. This class of CD4+ T cells secretes large quantities of interferon-gamma (IFN- γ), a cytokine that promotes the activation of macrophages to upregulate phagocytosis. In contrast, Th2 CD4+ T cells primarily activate B cells. Mice that are T cell-deficient or lack the ability to activate their CD4+ T cells via class II MHC cannot resolve a primary infection with *Ctr* [89, 90]. In observational studies, women whose peripheral blood mononuclear cells (PMBCs) produced IFN-γ after stimulation with *Ctr*

antigens were less likely to be infected with *Ctr* [91] or to be re-infected when tested at 3- or 6-month follow-up [92].

B cells that recognize their cognate antigen in a lymph node can be activated with co-stimulation from Th cells to secrete immunoglobulins (antibodies). Each B cell produces a unique antibody with an antigen-binding region that recognizes a specific microorganism-associated epitope. Antibodies can have several roles in the protection of mucosal surfaces. These include the neutralization of intracellular pathogens by blocking entry to host cells, opsonization of extracellular pathogens to promote their phagocytosis, and activation of the complement system proteins.

Although protective immunity against chlamydia is primarily mediated by CD4+ T cell responses, humoral immune mechanisms may have a complementary role in preventing *Ctr* re-infection. In the female mouse model of chlamydia, B cell-deficient mice resolved primary *Ctr* infections similarly to wild-type (WT) animals [93, 94]. As demonstrated by Su and colleagues, however, these B cell-deficient mice were more susceptible than WT to re-infection upon *Ctr* re-challenge 70 days after resolving the primary infection [93]. Furthermore, although CD4+ T cells are required to resolve a primary *Ctr* infection, Morrison *et al*. demonstrated that WT mice depleted of CD4+ T cells following the resolution of a primary infection were still able to clear a secondary challenge infection, while B cell-deficient mice depleted of CD4+ T cells could not [95]. Taken together, these results suggest that B cells and/or the antibodies they produce could be important for host protection against subsequent *Ctr* exposures. More recently, it has been proposed that populations of B cells play a supportive, albeit indirect, role in resolving a primary *Ctr* infections by improving CD4+ T cell activation [96].

The roles of different antibody isotypes in protection against *Ctr* infection remain incompletely understood. In a classic study of human anti-chlamydia antibody responses, Brunham *et al*. showed that *Ctr* infectious loads were positively correlated with serum titers of immunoglobulin (Ig)G and IgM in women. Meanwhile, *Ctr* loads were inversely correlated with cervical levels of secretory IgA [97]. Another study showed that women who cleared chlamydia within 48 hours of antibiotic therapy had significantly higher cervicovaginal IgA titers at presentation (and for at least 6 months following diagnosis) compared to women who remained infected at 48 hours post-treatment [98]. However, in the mouse model of chlamydia using the murine pathogen, *Chlamydia muridarum*, mice deficient in IgA cleared both primary chlamydial infections and secondary challenge infections as well as WT mice [99].

In a study of men with and without chlamydia reported by Pate and colleagues [85], men with chlamydia had elevated levels of chlamydia-specific IgA and IgG antibodies in their urethral swab, but not serum, specimens compared to *Ctr*-negative controls. This suggests that the localized immune response against *Ctr* infection in the male urethra might differ from the systemic response to this pathogen.

Section 5: Idiopathic Urethritis

Idiopathic urethritis (IU) is a non-specific finding in patients who present with objective urethritis or evidence of urethral disease but test negative for known pathogens by NAAT. Across populations of men in North America, Europe, and Australia, IU accounts for a significant proportion—as much as 50%—of all NGU cases. This has

raised concerns that many cases of NGU are caused by overlooked urethral pathogens and/or unfamiliar STIs.

Case-control studies are epidemiologic tools that help investigators determine whether a particular observable feature is significantly more or less common among individuals affected with some disease compared to "healthy" individuals (controls) who are not affected. In this way, it is possible to infer associations between a given disease and factors that could potentially cause or influence its course. However, case-control studies cannot determine causation or the directionality of observed associations.

Recently, many groups have conducted case-control studies to evaluate the types of microorganisms that are present in men with documented, objective NGU and/or NGU-related symptoms compared to men who do not have NGU and/or symptoms. In the past, this type of work relied on culture-based methods with various growth media to identify as many distinct microorganisms as possible. However, many human pathogens are difficult to culture or cannot be cultivated at all. This may be due to nutritional requirements or environmental conditions that are not adequately replicated *in vitro*.

The development of "next-generation" sequencing technologies has allowed for intensive bioinformatic analyses of the metagenomes of urethral microbial communities. Importantly, these methods can identify microorganisms regardless of their ability to be isolated or cultured from human specimens. These studies have revealed that some bacteria (*e.g., Haemophilus influenzae* and *Mycoplasma penetrans*) [16, 17] and viruses (*e.g.*, herpes simplex virus and adenovirus) are more prevalent in men with NGU compared to control men without NGU. Many of these findings corroborate previous case reports and case series that associated these microorganisms with urethritis.

However, future studies will be required to determine whether these microorganisms are "true" etiologic agents of NGU that are sufficient to elicit inflammation in the penile urethral mucosa.

Management of idiopathic urethritis

Since IU is defined by the absence of known pathogens, evidence-based strategies for the clinical management of this condition are incomplete. However, treatment with various antibiotics has been shown to correlate with the resolution of NGU symptoms. In one study in Japan, 32/38 men with "nonmycoplasmal, nonureaplasmal, nonchlamydial NGU" treated with 1g azithromycin experienced resolution of their urethral inflammation [100]. The same research group also demonstrated that treatment with clarithromycin, levofloxacin, gatifloxiacin, and minocycline were all effective in resolving urethral inflammation and urethritis symptoms in most men with IU [101]. However, since no untreated controls were included in these studies, it is difficult to determine conclusively whether resolution of the participants' symptoms reflected the antibiotic actions of these drugs or was spontaneous. Although technically challenging to perform, carefully designed naturally history studies will likely be required to understand the development and resolution of NGU symptoms relative to the acquisition of urethral pathogens.

Section 6: The Idiopathic Urethritis Men's Project (IUMP)

The Idiopathic Urethritis Men's Project (IUMP) is an NIH-funded, clinical investigation designed to evaluate how the urethral microbiome might influence IU

pathogenesis (R01 AI116706; PI: D. Nelson). From 2016 – 2020, adult men presenting for care at the Marion County Public Health Department (MCPHD) Bell Flower Clinic (BFC) in Indianapolis, Indiana, were invited to participate in this prospective case-control study. By definition, idiopathic NGU cases cannot be identified at the point of care prior to diagnostic NAAT evaluation. Thus, men with objective evidence of NGU were enrolled irrespective of etiology. All participants provided written consent, underwent a genital examination, and provided first-catch urine and urethral swab specimens. Participants also completed a thorough questionnaire of demographic, behavioral, and clinical factors using computer-assisted self-interviewing (CASI).

One question posed by the IUMP study was whether the signs and symptoms of idiopathic urethritis might differentiate this condition from known etiologies of NGU. A clear difference in the clinical signs and symptoms of IU might suggest a unique etiology or pathologic mechanism. However, when Jordan *et al.* investigated this question using the IUMP cohort, no clear difference was observed among men with defined mono-microbial urethritis compared to IU [102]. Another question investigated by this study was whether IU might result from an overgrowth of commensal bacteria in the urethra or a pathogenic imbalance (dysbiosis) in the urethral microbiota. However, bioinformatic analysis of men in the idiopathic urethritis cohort did not support these commensal-load or dysbiosis hypotheses (D. Nelson, personal communication). Thus, the causes of nearly 2/3 of the IU cases enrolled in this study remain unexplained.

In light of these observations, I speculated that some cases of idiopathic urethritis may not be caused by active infection. Instead, I hypothesized that symptomatic IU could reflect residual immune responses against an infection that has already resolved.

Evidence from trachoma studies and cell culture models of *Ctr* suggests that inflammation may persist due to the continued presence of antigenic chlamydial components even after the active infectious process has been terminated. Interestingly, a Swedish investigation indicated that male partners of women who were hospitalized with symptomatic, chlamydia-associated PID often had non-specific (*Ctr*-negative) urethritis [103]. This could suggest sexual transmission of an unknown pathogen that causes both NGU in men and PID in women. An alternative explanation is that the men with non-specific urethritis previously had chlamydia, like their partners, but had already cleared their infection prior to contact tracing and STI testing. Thus, we wanted to determine if men in our study with IU had evidence of a recent chlamydia infection.

Section 7: Research Goals and Significance

Following the design of the IUMP study and initiation of enrollment, Albritton *et al*. developed a novel method for detecting and quantifying *Ctr*-specific antibodies using enzyme-linked immunosorbent assays (ELISA) [104]. ELISA is an analytical technique that takes advantage of the specificity of the interactions between antibodies and their cognate antigens. Antibody-based strategies for detecting *Ctr* were previously explored as an alternative diagnostic modality to inoculating tissue cultures with specimens of cervicovaginal secretions. However, the sensitivity achieved by these techniques was unacceptably low, and NAAT is now the diagnostic method of choice. Nonetheless, ELISA remains a popular tool in research settings.

Albritton and colleagues demonstrated that *Ctr*-specific IgG and IgA antibodies could be reliably detected in serum, genital tract secretions (endocervical and vaginal),

and cytobrush samples collected from women with chlamydia. Using the same approach, Darville *et al*. showed that mucosal titers of *Ctr*-specific IgG and IgA were inversely correlated with cervical *Ctr* loads [105]. Thus, I hypothesized that this method could be applied to detect antibodies in the inflamed male urethra that are indicative of an ongoing or recently resolved chlamydia infection.

Since the IUMP study was designed primarily to investigate urethral microbiome compositions in men with NGU versus controls, limited urethral swab material was available for further evaluation by ELISA. However, first-catch urine (FCU) samples were obtained from IUMP participants at enrollment and at any subsequent follow-up visits and continuously stored frozen at –80˚ C. Previous studies have demonstrated that urine specimens contain sufficient quantities of antibodies to detect some infectious agents by ELISA. For example, antibodies against the bacterial pathogen, *Helicobacter pylori*, have been detected in urine samples using ELISA [106, 107], and this method has been applied to create diagnostic assays for several protozoan and helminthic parasites [108]. Thus, the sample repository generated by the IUMP presented a unique opportunity to study the urethral immune response against chlamydia among a well-characterized cohort of men with *Ctr* mono-infection. Furthermore, a separate cohort of men with *M. genitalium* mono-infection offered a natural control group of participants with NAAT-confirmed, non-chlamydia NGU.

In summary, the goals of this research were two-fold. The first goal was to determine whether the approach of Albritton *et al*. [104] could be modified to detect anti-*Chlamydia* antibodies in the urine specimens of men with chlamydia. Assuming that these antibodies were specific for *Ctr* (*i.e.*, more commonly observed in men with

chlamydia infection compared to healthy controls), the second goal of this project was to evaluate the hypothesis that some men with idiopathic urethritis were recently infected with *Ctr* but had mounted an effective immune response that successfully cleared the pathogen. In other words, despite testing negative for *Ctr* by NAAT, we reasoned that men with idiopathic urethritis could have persisting urethral inflammation that was originally instigated by chlamydia infection. In this scenario, the signs and symptoms of NGU result from continued activation of the innate immune system after the active infection was resolved.

Significance

Nongonococcal urethritis is a common syndrome in men. It often reflects infection with pathogens that are sexually transmissible to female partners. While many of these infections never become symptomatic and eventually resolve inconsequentially, they can also cause severe complications, including irreversible infertility and ectopic pregnancy. Thus, improved understanding about the causes, appropriate management, and prevention of NGU is essential for reducing preventable morbidity and mortality in women, limiting the overuse of antibiotics in men, and improving overall antibiotic stewardship. The work presented in this dissertation will help to address important gaps in knowledge about NGU in the following ways:

1. Reporting and contact tracing. Identified cases of the STIs chlamydia, gonorrhea, and syphilis must be reported to local health authorities in order to perform

contact tracing of sexual partners who might also be infected. Cases of *M. genitalium* infection and idiopathic urethritis do not require contact tracing. However, if it is determined that IU in men is associated with recent spontaneous clearance of chlamydia, then it may be essential to evaluate these individuals' sexual partners for asymptomatic *Ctr* infection. Not only could these asymptomatic infections later cause sequelae, but they might also represent key reservoirs from which this pathogen can be transmitted to other susceptible individuals.

2. Improved management of NGU cases. Men with symptomatic NGU are typically treated empirically with antibiotics at the point of care. Although usually well-tolerated, the use of these drugs is not without risk. Furthermore, the long-term use of azithromycin as first-line therapy for NGU was a likely driver of the rRNA mutations in *Mgen* that make many isolates resistant to macrolide antibiotics. Although many men with IU experience symptomatic relief after taking antibiotics, it is unclear whether this is due to direct antimicrobial effects against IU-associated pathogens or indirect anti-inflammatory side effects of these mediations. If idiopathic urethritis is determined to result from spontaneous immune clearance of a previous infection, then antibiotic therapy may not be warranted at all in these individuals.

3. Non-invasive study of NGU immune responses. In the past, studies of men with urethritis have often relied on swabbing the distal urethra to collect secreted material for analysis. However, this practice is often painful and can rarely lead to iatrogenic damage of the urethra. Collection of urine that passes through the urethra could represent an

alternative approach to sampling the urethral site for biological analysis. Collection of first-catch urine is non-invasive, generally tolerable, and could expand the scope of NGU research to broader populations. In particular, although adolescent males are at especially high risk for NGU, no information about the immunological parameters of the adolescent urethra is available. Furthermore, there are significant ethical concerns surrounding adolescent research that further limit investigation of this population. Thus, a non-invasive method for analyzing immune responses against NGU-causing urethral pathogens could be paradigm-shifting.

CHAPTER II – Materials and Methods

Preparation of *C. trachomatis* EB stocks

HeLa 229 (HeLa) cells were obtained from the American Type Culture Collection (ATCC) and grown to confluency in high-glucose DMEM (HyClone) supplemented with 10% fetal bovine serum, HEPES, and non-essential amino acids. Confluent monolayers were pre-treated with DEAE-dextran for 5 minutes, then infected with purified *Ctr* serovar D (D/UW3) EBs at a multiplicity of infection (MOI) of ~2. Infected monolayers were incubated at 37˚C in a 5% CO_2 humidified incubator and harvested at approximately 48 hours post-infection. *Ctr* EBs were released from harvested HeLa monolayers by mechanical agitation and sonication, then purified by density gradient centrifugation in MD76-R (Mallinckrodt) as described previously [36]. Purified EB stocks were maintained at –80˚C.

Preparation of *M. genitalium* G37 stocks

Mycoplasma genitalium strain G37 (kind gift from Dr. Mitchell Balish) was cultured in SP4 growth medium [109] at 37˚C with rocking. SP4 contains the pH indicator phenol red, and *Mgen* growth was assessed by the change in media color. Following incubation, *Mgen* cells were manually dislodged from the flask surface by scraping, and 1 mL aliquots of the resulting suspension were centrifuged at 13,000 rpm at 4˚C for 20 minutes. Stocks were concentrated by removing 800 µL of SP4 supernatant, followed by resuspension of cell pellets in the remaining volume. Concentrated *Mgen*

suspensions were passed through a 20 gauge needle several times to homogenize. Homogenized stocks were maintained at –80˚C.

Protein concentration of *Ctr* EB and *Mgen* G37 stocks

Aliquots of purified *Ctr* serovar D EBs (75 μL) or homogenized *Mgen* G37 cells (50 μL) were centrifuged at 12,000 rpm at 4˚C for 30 minutes. The resulting cell pellets were resuspended in 10 μL of sucrose-phosphate-glutamine buffer (SPG) and treated overnight at 4˚C with 190 μL of RIPA lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). Total protein concentrations in the overnight lysates were determined by comparison to bovine serum albumin (BSA) standards using bicinchoninic acid (BCA) assay.

Preparation of plates and specimens for total urine immunoglobulin ELISA

Concentrations of total IgA, IgG, and IgM in neat FCU specimens were measured as described previously in [104], with modifications. Briefly, the wells of tissue culture-treated 96-well plates were coated overnight at 4˚C with unlabeled, affinity-purified goat antibodies (Southern Biotech). These antibodies were specific for the heavy chains of human IgA (0.5 μg/well) (#2050-01), human IgG (0.25 μg/well) (#2044-01), or human IgM (1.25 μg/well) (#2020-01) diluted in phosphate-buffered saline (PBS). Coated plates were washed three times with PBS plus 0.05% Tween-20 (PBST), then blocked for 1 hour with PBST containing 2% goat serum (PBST-S). Freshly thawed specimens of first-catch urine (FCU) were initially evaluated at dilutions of 1/10,000 (IgA), 1/50,000 (IgG),

or 1/250 (IgM) in PBST-S. Diluted samples were loaded into blocked 96-well plates in duplicate. Additionally, PBST-S (negative control) blank and isotype-specific human antibody reference standards were loaded in each plate. All plates were incubated overnight at 4˚C, then developed as described below.

Preparation of plates and specimens for anti-*Ctr* immunoglobulin ELISA

Anti-*Ctr* IgA, IgG, and IgM in neat FCU specimens were measured using a modified version of the whole-EB ELISA method described in [104]. Briefly, the wells of tissue-culture treated 96-well plates were coated overnight at 4˚C with 75 μL/well of poly-L-lysine (EMD Millipore) diluted to 1 μg/mL in 0.05 M carbonate-bicarbonate buffer, pH 9.6. Coated plates were washed three times with PBST, then freshly thawed *Ctr* serovar D EBs (50 μL/well diluted in PBS to 4.5 μg/mL) were added to each well. EB-treated plates were centrifuged at 900 x *g* at room temperature for 5 minutes. Without removing the PBS supernatant from the wells, EBs were fixed by adding 50 μL/well of glutaraldehyde (0.1% *v/v* in PBS) and incubating the plates at room temperature for 20 minutes. EB-loaded plates were washed three times with PBST, then blocked for 1 hour with PBST-S. Freshly thawed FCU specimens were diluted 1/50 (for anti-*Ctr* IgA and IgG) or 1/2 (anti-*Ctr* IgM) in PBST-S. The diluted urine specimens were loaded into prepared 96-well plates in duplicate. Additionally, PBST-S (negative control) blank and isotype-specific human antibody reference standards were loaded in each plate. All plates were incubated overnight at 4˚C, then developed as described below.

Preparation of plates and specimens for anti-*Mgen* immunoglobulin ELISA

Anti-*Mgen* IgA, IgG, and IgM in neat FCU specimens were captured using a whole-*Mycoplasma* ELISA approach based on the whole-EB ELISA method described above. The wells of tissue-culture treated 96-well plates were coated overnight at 4˚C with 75 μL/well of poly-L-lysine (EMD Millipore) diluted to 1 μg/mL in 0.05 M carbonate-bicarbonate buffer, pH 9.6. Coated plates were washed three times with PBST, then freshly thawed *Mgen* G37 cell suspension (50 μL/well diluted in PBS to 20 μg/mL) was added to each well. *Mgen*-treated plates were centrifuged at 900 x *g* at room temperature for 5 minutes. Without removing the PBS supernatant from the wells, the *Mgen* cells were fixed by adding 50 μL/well of glutaraldehyde (0.1% *v/v* in PBS) and incubating the plates at room temperature for 20 minutes. *Mgen*-loaded plates were washed three times with PBST, then blocked for 1 hour with PBST-S. Freshly thawed FCU specimens were diluted 1/3 (for anti-*Mgen* IgA), 1/10 (anti-*Mgen* IgG) or 1/2 (anti-*Mgen* IgM) in PBST-S. The diluted urine specimens were loaded into prepared 96-well plates in duplicate. Additionally, PBST-S (negative control) blank and isotype-specific human antibody reference standards were loaded in each plate. All plates were incubated overnight at 4˚C, then developed as described below.

Development of colorimetric ELISA to detect bound immunoglobulin

To detect bound antibody following overnight incubation at 4˚C, ELISA plates for evaluating total urine immunoglobulins, anti-*Ctr* immunoglobulins, and anti-*Mgen* immunoglobulins were developed using the same procedure. Briefly, plates were washed three times with PBST, then treated with 10 ng/well (diluted in PBST) of biotinylated,

affinity-purified goat antibodies (Southern Biotech) specific for the heavy chains of human IgA (#2050-08), IgG (#2040-08), or IgM (#2020-08) and incubated for 1 hour at 37˚C with rocking. The plates were then washed three times with PBST, treated with 25 ng/well (diluted in PBST) of Neutralite avidin-horseradish peroxidase (Southern Biotech #7200-05) and incubated for 30 minutes at room temperature with rocking. After three final washes with PBST, plates were developed by adding 100 μL/well of tetramethylbenzidine (Invitrogen 00-4201-56) and incubating for 20 minutes at room temperature. The developing reactions were quenched by adding 100 μL/well of 1 M hydrochloric acid (HCl), and absorbance in each well at 450 nm was measured using a Synergy HTX (Biotek) automated plate reader. Total urine concentrations of each antibody isotype were determined using the standard curves of antibody reference standards generated for each plate. Positive anti-*Ctr* or anti-*Mgen* antibody responses were defined as sample $absorbance_{450}$ values greater than the average $absorbance_{450}$ of blank wells plus three (3) standard deviations after quenching.

<u>*ompA* genotyping of IUMP *Ctr* isolates</u>

FCU specimens were collected from IUMP participants with NAAT-confirmed chlamydia. *Ctr* DNA in FCU (3 μL urine/reaction) was amplified for *ompA* by PCR using the primers ompA_P1 and ompA_P2 (Table 1). PCR cycling reactions were performed using Phusion DNA polymerase (New England BioLabs) according to manufacturer recommendations. Amplicons were confirmed by agarose gel electrophoresis and purified using the Bio Basic PCR Purification Kit (9K-006-0003) according to manufacturer instructions. Cycling reactions that did not yield detectable amplicons by

gel electrophoresis were repeated using the primers MOMP87 and MOMP1059 to amplify 3 μL of the raw PCR product generated previously. Column-purified PCR products were Sanger sequenced by a commercial vendor (Eurofins Genomics, Louisville, KY); sequencing primers used in this analysis are listed in Table 2. *ompA* genotypes for each IUMP *Ctr* isolate were assigned by alignment of the nucleotide sequences obtained here with published *Ctr* strains using the online "nucleotide Basic Local Alignment Search Tool" (nBLAST) (NCBI).

Primer	**Direction**	**Sequence (5' → 3')**
191S	Forward	GCTYTSTGGGARTGTGGRTGTGC
MOMP1059	Reverse	GCAATACCGCAAGATTTTCTAGATTTCATC
MOMP87	Forward	TGAACCAAGCCTTATGATCGACGGA
C214	Reverse	TCTTCGAYTTTAGGTTTAGATTGA

Table 1: Primers used for amplifying *C. trachomatis ompA* in IUMP specimens

Primer	**Direction**	**Sequence (5' → 3')**
ompA_P1	Forward	ATGAAAAAATCTCTTGAAATCGG
ompA_P2	Reverse	ACTGTAACTGCGTATTTGTCTG
MOMP87	Forward	TGAACCAAGCCTTATGATCGACGGA
MOMP1059	Reverse	GCAATACCGCAAGATTTTCTAGATTTCATC

Table 2: Primers used for sequencing *C. trachomatis ompA* amplicons

Statistical analysis

Statistical analyses were performed using the GraphPad Prism (v 9.0) software package. Data were analyzed according to the tests indicated in their respective figure legends, and *P values* for each statistical test performed are indicated.

CHAPTER III – Pathogen-Reactive Immunoglobulins in Male First-Catch Urine

Section 1: Study Population

From 2016 – 2020, 451 men (164 non-NGU controls and 287 NGU cases) who presented to the Bell Flower Clinic (BFC) were enrolled in the IUMP study [3, 18, 19, 102]. For this analysis, we evaluated 289 men from the IUMP cohort: 102 non-NGU controls, 67 cases with chlamydia, 38 with *Mgen* infections, and 82 with idiopathic urethritis. Sixty-two controls were excluded for various reasons. These include: 1) microscopic evidence of urethral inflammation, 2) signs of urethritis such as urethral discharge on physical exam, 3) positive/incomplete urine NAAT testing, and 4) protocol deviations. Some cases were excluded due to mixed infections, incomplete NAAT testing, and/or because participants did not return for requested follow-up.

Section 2: IgA & IgG Concentrations in the Urine of Men with Urethritis

Understanding about the immune response elicited by *Ctr* infection of the male urethra remains limited. In one previous report, urethral swab specimens collected from men with chlamydia contained higher concentrations of total immunoglobulin (Ig) A and IgG than swabs from uninfected controls [85]. Higher concentrations of total IgA and IgG relative to uninfected controls were also observed among men with NGU who did not have chlamydia. This suggests that urethral inflammation is associated with increased local antibody secretion. Voided urine passes through the urethra, including the distal portion (urethral fossa) from which secretions are collected using urethral swabs. Thus, I

wanted to determine if the concentrations of IgA and/or IgG in first-catch urine (FCU) would also differ between men with and without NGU.

Colorimetric ELISA methods have been described previously for quantifying the total Ig concentrations in biological samples including cell culture supernatants [110] and female genital tract secretions [104]. This approach uses microtiter plates pre-treated with unlabeled polyclonal antibodies to capture the constant (Fc) region of human Ig molecules in an isotype-specific manner. Captured human antibodies are subsequently bound using isotype-matched, biotinylated antibodies. The biotin (*i.e.*, vitamin B_7) molecules conjugated to these antibodies serve as molecular scaffolds for the chromogenic reaction. Avidin, a protein produced by many non-mammalian vertebrates, binds biotin strongly and can be conjugated with enzymes such as horseradish peroxidase (HRP). HRP, in turn, is used to oxidize chemicals like 3,3',5,5'-Tetramethylbenzidine (TMB) that change color in different oxidation states. Thus, by this technique, the concentration of human immunoglobulin in a particular sample is ultimately proportional to the intensity of the colorimetric reaction that is generated.

Diagnostic testing of all BFC specimens by NAAT is performed at the Indiana University Infectious Diseases Research Laboratory (IDRL). We obtained residual urine specimens from the IDRL that tested NAAT-positive or NAAT-negative for chlamydia and used these to calibrate the ELISA approach described by Albritton *et al.* [71] for detection of urine antibodies. Initially, I tested the intensity of colorimetric responses across a range of urine dilutions according to the procedure and reagent concentrations as described. This resulted in weak responses at all tested dilutions (data not shown). However, I noted that the units in this report describing concentrations of unlabeled

capture antibody differed somewhat from contemporary practice and reasoned that a typographical error in the manuscript may have been made. Thus, I decided to proceed under the assumption that μg (micrograms), rather than ng (nanograms), were the intended unit of measurement.

Using this modified approach, I empirically determined that urine dilutions of 1/10,000 and 1/50,000 were optimal for measuring total concentrations of IgA and IgG, respectively. These dilutions resulted in most specimens giving a colorimetric response within the range detectable by our spectrophotometer (data not shown). At a wavelength of 450 nm, the upper limit of detection for our instrument is 4.0 absorbance units.

Urine specimens were collected from men who enrolled in the Idiopathic Urethritis Men's Project (IUMP) as either NGU cases or non-NGU controls. After determining the appropriate urine dilutions for measuring total IgA and IgG by ELISA, I performed these assays on the stored FCU specimens provided by IUMP participants. I analyzed cases according to the NAAT result and excluded participants who enrolled with NGU and were subsequently lost to follow-up. Men who were co-infected with *Ctr* and *Mgen* were excluded, as well as men with either *Ctr* or *Mgen* who were co-infected with *Ureaplasma urealyticum* (UU). However, men who tested positive only for UU by NAAT were included in the idiopathic urethritis group. This was because two recent studies, including one from our group about IUMP participants, concluded that the prevalence of UU did not differ among men with NGU compared to similar men without urethritis [16, 18].

First, I found that the FCU collected from men with chlamydia or with idiopathic urethritis at study enrollment contained higher concentrations of total IgA ($p < 0.0001$ &

p = 0.0011, respectively) than the urine of non-NGU controls (Figure 3). In contrast, total IgA antibody concentrations in the urine of men with *Mgen* infections did not differ from controls (p = 0.2185).

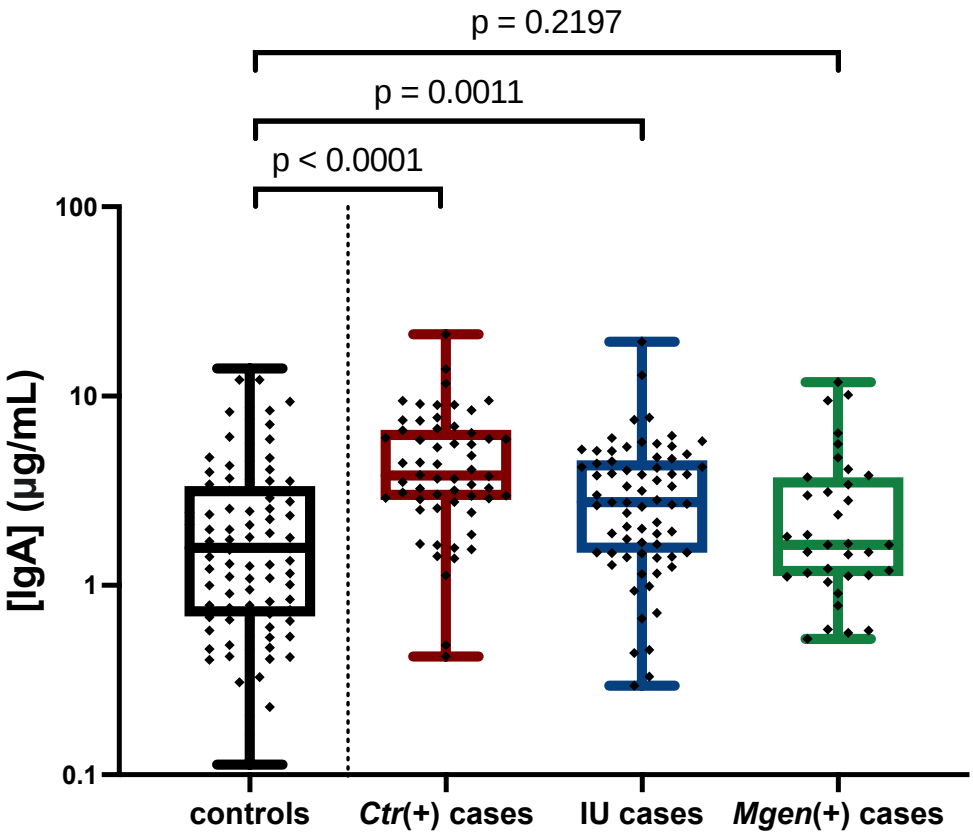

Figure 3. Total IgA concentrations were elevated in the urines of men with chlamydia or with idiopathic urethritis.

FCU specimens were obtained from men who enrolled in the IUMP study. Concentrations of total IgA in the urines of non-NGU controls (N = 92) or NGU cases with chlamydia (N = 58), idiopathic urethritis (N = 69), or *Mgen* (N = 35) were determined using quantitative ELISA. Total IgA concentrations were normalized by log transformation, and statistical significance was assessed by unpaired T-test of each NGU case group versus non-NGU controls. *P* values for each comparison are indicated.

Men with chlamydia or with idiopathic urethritis also had higher concentrations of total urine IgG at enrollment ($p < 0.0001$) compared to non-NGU controls (Figure 4). Meanwhile, total urine IgG concentrations did not differ between non-NGU controls and men with *Mgen* ($p = 0.6971$). In line with previous observations that IgG, rather than IgA, is the predominant immunoglobulin isotype in human genital-tract mucosae [111], I observed that overall concentrations of IgG were greater than concentrations of IgA in each group of cases and the non-NGU controls.

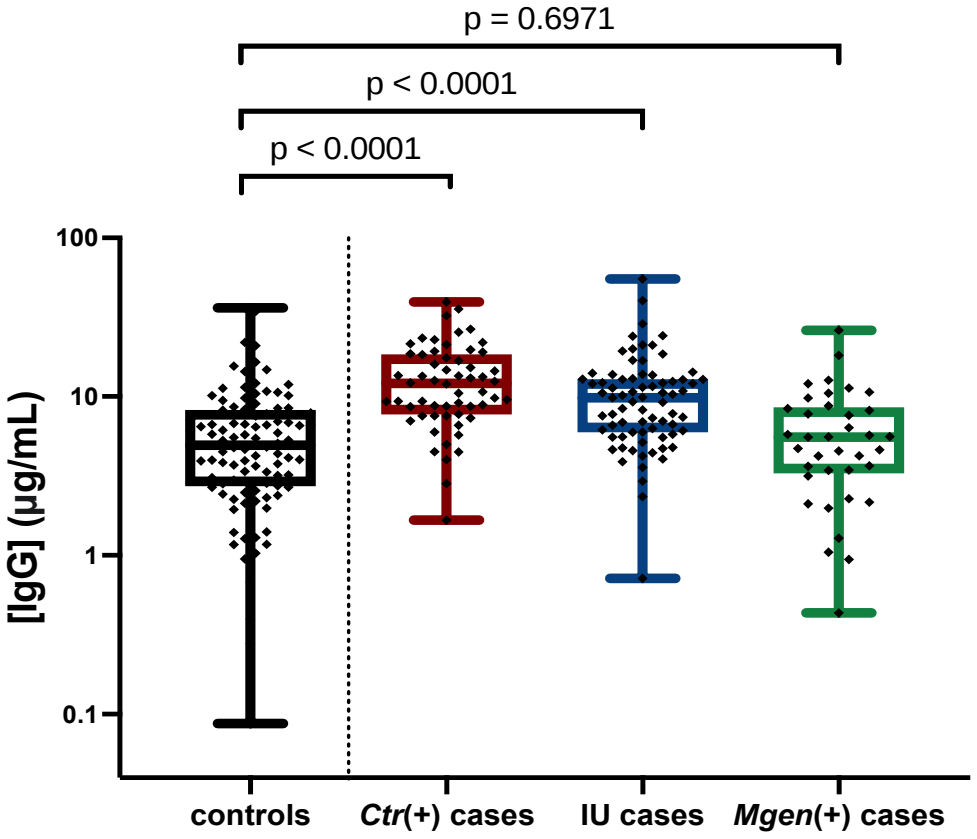

Figure 4. Total IgG concentrations were elevated in the urines of men with chlamydia or with idiopathic urethritis.

FCU specimens were obtained from men who enrolled in the IUMP study. Concentrations of total IgG in the urines of non-NGU controls (N = 98) or NGU cases with chlamydia (N = 58), idiopathic urethritis (N = 75), or *Mgen* (N = 37) were determined using quantitative ELISA. Total IgG concentrations were normalized by log transformation, and statistical significance was assessed by unpaired T-test of each NGU case group versus non-NGU controls. *P* values for each comparison are indicated.

Men who enrolled in the IUMP study as NGU cases were treated empirically with azithromycin according to the standard-of-care and asked to return for a follow-up test-of-cure evaluation at approximately one month (range: 21–35 days). Men who are included in this analysis returned to BFC and provided additional specimens at follow-up.

No previous studies have investigated changes in urine Ig concentrations over time in men diagnosed with NGU. Thus, I wanted to determine if urine Ig concentrations among men with NGU differed between enrollment and follow-up. To test this, I used the same ELISA approach to quantify total IgA and IgG in follow-up specimens from the participants in each NGU group. Since non-NGU controls who enrolled in the IUMP study were not requested to return for follow-up, we could not evaluate the variation in urine Ig concentrations among non-inflamed men.

Among the NGU cases with chlamydia, I did not observe a difference in urine IgA concentrations between enrollment and follow-up ($p = 0.3295$) (Figure 5). However, I found that FCU collected at one-month follow-up from men with idiopathic urethritis or with *Mgen* infection contained higher concentrations of total IgA than matched enrollment specimens ($p = 0.0008$ & $p = 0.0005$, respectively).

Azithromycin is a macrolide antibiotic that inhibits bacterial protein synthesis by binding to the large (50S) subunit of the prokaryotic ribosome. Importantly, the use of this antibiotic in men with *Mgen* infection has caused selection for macrolide resistance-mediating mutations (MRM) [112]. These MRM are single-nucleotide polymorphisms (SNP) that modify the structure of the *Mgen* ribosome and diminish the effectiveness of azithromycin therapy. In a recent study from our laboratory, 17 of 24 (70.8%) *Mgen* isolates obtained from IUMP participants were found to have MRM [19]. In accordance

with this finding, I performed a manual review clinical records for the 38 *Mgen*-infected men included in this analysis and discovered that approximately ¾ continued to have signs of urethritis and/or positive *M. genitalium* NAAT results when they were evaluated for test-of-cure (data not shown). This factor may have confounded the finding of increased urine IgA at follow-up among NGU case men with *Mgen* infections.

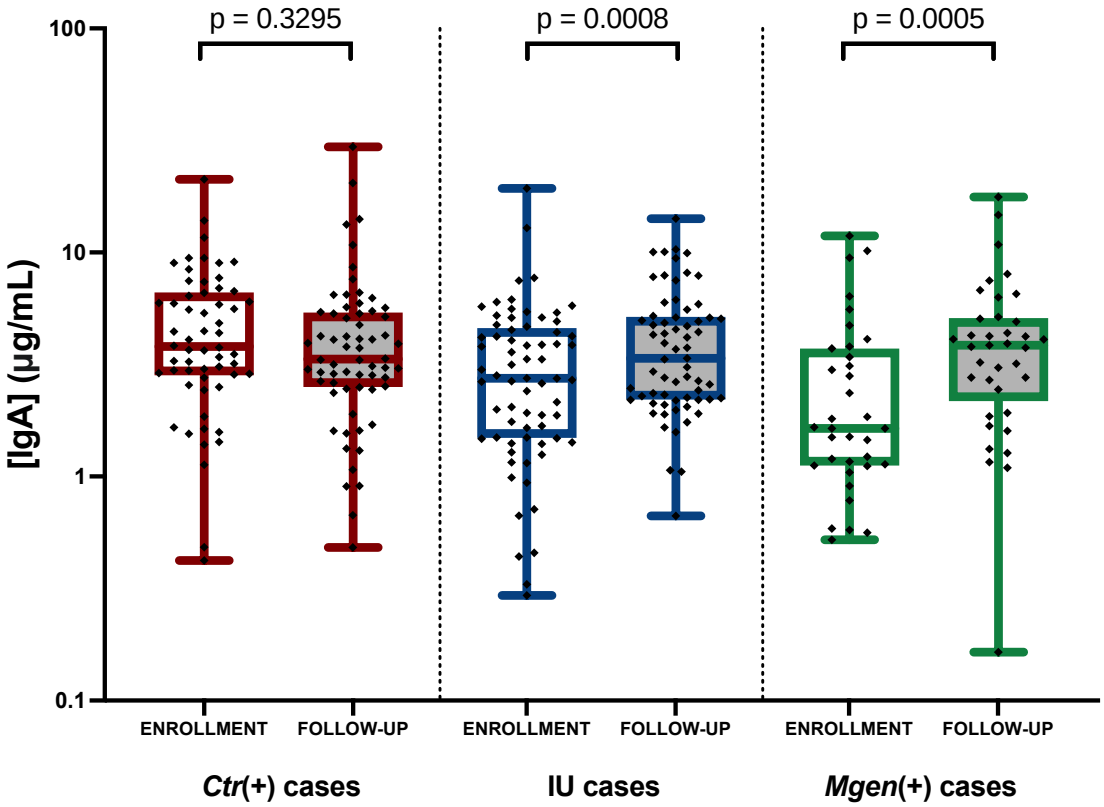

Figure 5. Men with idiopathic urethritis or *Mgen*, but not chlamydia, had higher concentrations of total urine IgA at follow-up versus enrollment.

Follow-up FCU specimens were obtained from participants in the IUMP study. Concentrations of total urine IgA at follow-up among NGU cases with chlamydia (N = 64), idiopathic urethritis (N = 65), or *Mgen* (N = 37) were determined using quantitative ELISA. Total IgA concentrations were normalized by log transformation. Statistical significance was assessed by paired T-test of enrollment versus follow-up IgA concentrations; *Ctr*(+) cases, N = 55; IU cases, N = 53; *Mgen*(+) cases, N = 34. *P* values for each comparison are indicated.

Strikingly, I found that men with chlamydia had much lower concentrations of urine total IgG at one-month follow-up compared to matched specimens from enrollment ($p < 0.0001$) (Figure 6). In contrast, urine concentrations of total IgG did not differ between enrollment and follow-up among men with idiopathic urethritis ($p = 0.5939$). Additionally, total IgG concentrations in the urine specimens of men infected with *Mgen* were trending towards an increase at follow-up compared to enrollment ($p = 0.0963$).

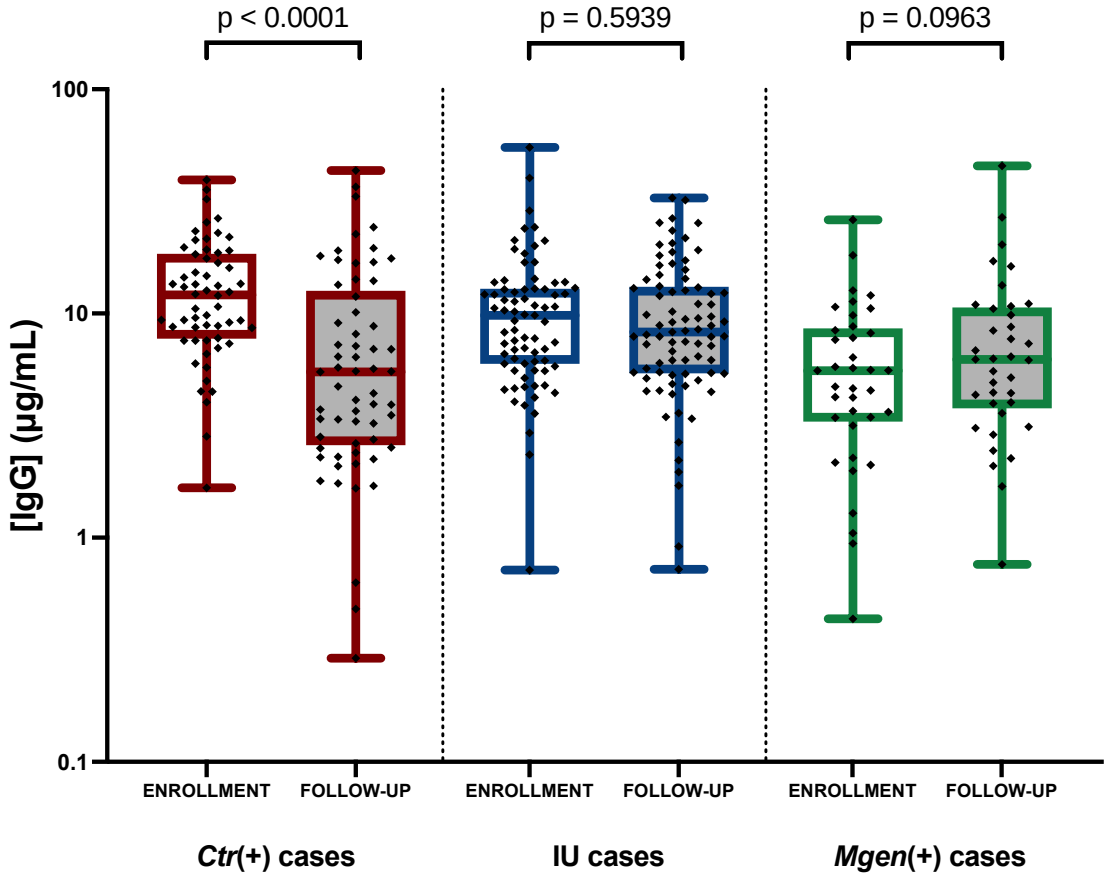

Figure 6. Men with chlamydia had lower concentrations of total IgG in their urines at follow-up versus enrollment.

Follow-up FCU specimens were obtained from participants in the IUMP study. Concentrations of total urine IgG at follow-up among NGU cases with chlamydia (N = 61), idiopathic urethritis (N = 81), or *Mgen* (N = 37) were determined using quantitative ELISA. Total IgG concentrations were normalized by log transformation. Statistical significance was assessed by paired T-test of enrollment versus follow-up IgG concentrations; *Ctr*(+) cases, N = 52; IU cases, N = 74; *Mgen*(+) cases, N = 36. *P* values for each comparison are indicated.

Section 3: The Detection of Anti-*Chlamydia* IgA & IgG in Male Urine

Pate and colleagues observed higher levels of *Ctr*-specific IgA and IgG antibodies in urethral swab specimens, but not in the sera, of men with chlamydia compared to uninfected controls [85]. I was curious if we could detect *Ctr*-specific antibodies in the urine specimens that were collected from IUMP participants at enrollment or at follow-up. I hypothesized that detection of *Ctr*-specific antibodies in the urine of men with NGU could identify individuals who have or recently had chlamydia.

To conduct this analysis, I slightly modified the indirect ELISA approach developed by Albritton *et al*. This strategy uses whole, glutaraldehyde-fixed EBs as the target antigen for reactive anti-*Ctr* antibodies [104]. By using whole, conformationally intact EBs, this assay specifically detects anti-*Ctr* antibodies which have corresponding epitopes exposed on the EB surface. Albritton and colleagues showed that the ability to detect anti-*Ctr* antibodies was similar when using serovar D EBs or serovar E EBs (or a combination of these EBs) as the indirect ELISA target antigen. Thus, because serovar D is the most widely used chlamydia-biovar (serovars D–K) strain in STI studies, I selected serovar D EBs to be the representative chlamydial antigen in this study.

In order to maximize the efficiency of this screening approach, I decided to test FCU specimens, in duplicate, at a consistent level of dilution across samples. Based on some preliminary optimization studies using residual, *Ctr*-NAAT-positive urine specimens from the IDRL (data not shown), I decided to assess *Ctr*-specific IgA and IgG antibodies in the IUMP urine specimens at dilutions of 1/50. Positive anti-*Ctr* antibody responses were defined as specimens yielding colorimetric ELISA reactions with

absorbance$_{450}$ values for both duplicates greater than or equal to the mean absorbance$_{450}$ of negative control wells (loaded with PBST-S diluent only) plus 3 standard deviations.

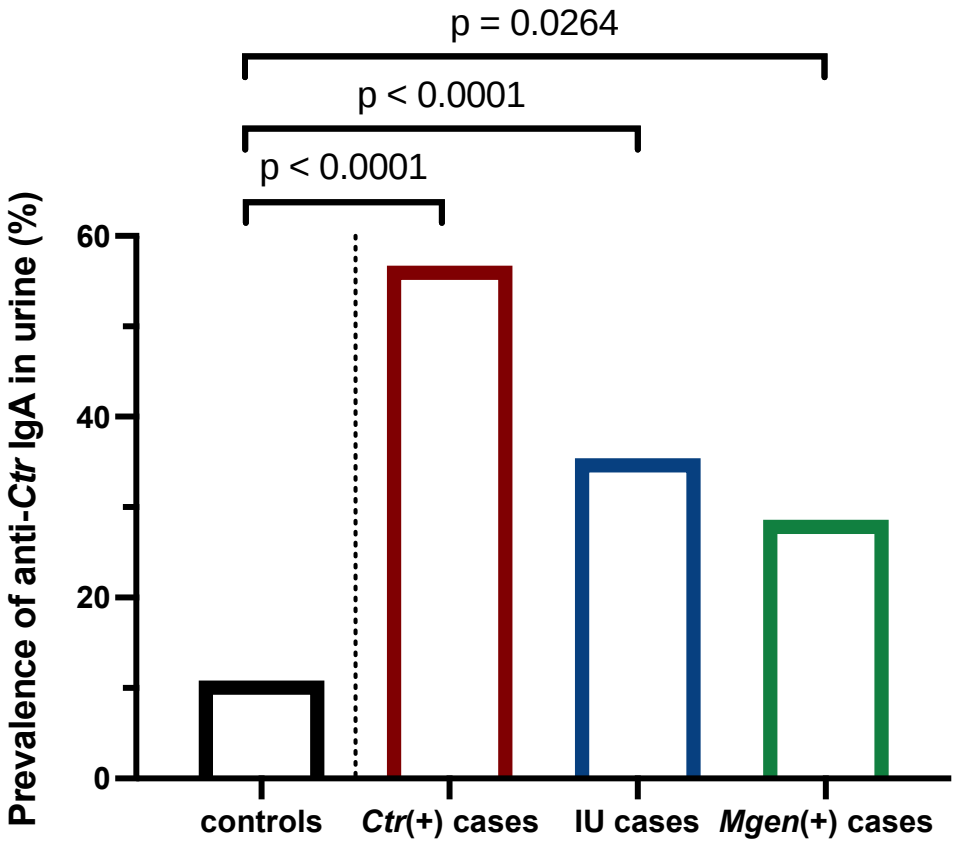

Figure 7. The prevalence of urine anti-*Chlamydia* IgA at enrollment was higher among NGU cases than non-NGU controls.

FCU specimens were obtained from men who enrolled in the IUMP study. Prevalence of anti-*Ctr* IgA in the urines of non-NGU controls (N = 102) and NGU cases with chlamydia (N = 60), idiopathic urethritis (N = 82), or *Mgen* (N = 35) was determined at a dilution of 1/50 using indirect ELISA for antibodies to serovar D EBs. Positive detection of anti-*Ctr* IgA was delimited by sample absorbance values ≥ the mean absorbance of blank wells (background) plus 3 standard deviations. Statistical significance was assessed by Fisher's exact test for the proportions of positive responses in each NGU case group versus non-NGU controls. *P* values for each comparison are indicated.

Using this approach and defined positivity cut-off for *Ctr*-specific Igs, I detected anti-*Ctr* IgA antibodies in the diluted, first-catch urines of 56.7% (34/60) of men with chlamydia who enrolled as cases in the IUMP study (Figure 7). In comparison, anti-*Ctr* IgA antibodies were detected in only 10.8% (11/102) of FCU specimens from non-NGU controls, a lower proportion than I observed among men with chlamydia ($p < 0.0001$). Interestingly, anti-*Ctr* IgA antibodies were also detected in higher proportions of the FCU specimens from participants with non-chlamydia NGU compared to non-NGU controls. I detected these *Ctr*-specific antibodies in 35.4% (29/82) of men with idiopathic urethritis ($p < 0.0001$ vs. controls) and 28.6% (10/35) of men infected with *Mgen* ($p = 0.0264$).

At the same dilution factor of 1/50, we detected anti-*Ctr* IgG antibodies in less than 1% (1/102) of FCU specimens from non-NGU controls (Figure 8). In contrast, anti-*Ctr* IgG was found in 58.9% (33/56) of the enrollment FCU specimens from men with chlamydia ($p < 0.0001$). Anti-*Ctr* IgG antibodies were also detected in 9.8% (8/82) of the enrollment urine specimens from men with idiopathic urethritis and 10.5% (4/38) of specimens from men with *Mgen* infection. Both of these proportions differed significantly from non-NGU controls ($p = 0.0114$ & $p = 0.0193$, respectively), but were lower than the detection rate observed among men with chlamydia.

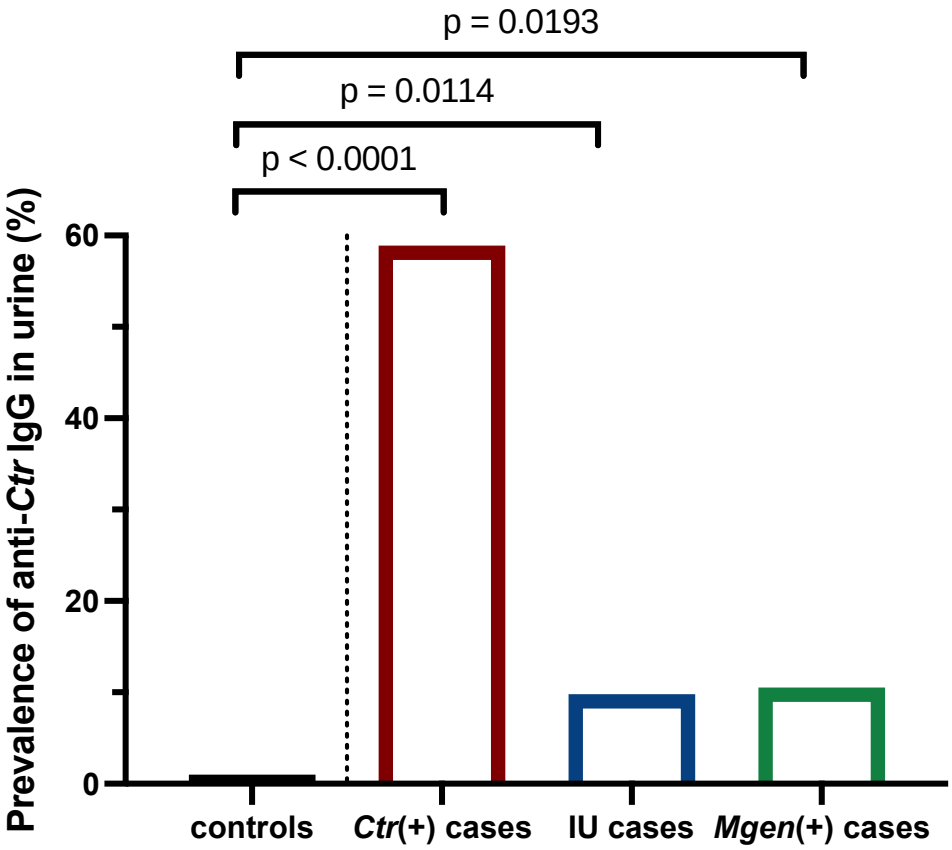

Figure 8. The prevalence of urine anti-*Chlamydia* IgG at enrollment was higher among NGU cases than non-NGU controls.

FCU specimens were obtained from men who enrolled in the IUMP study. Prevalence of anti-*Ctr* IgG in the urines of non-NGU controls (N = 102) and NGU cases with chlamydia (N = 56), idiopathic urethritis (N = 82), or *Mgen* (N = 38) was determined at a dilution of 1/50 using indirect ELISA for antibodies to serovar D EBs. Positive detection of anti-*Ctr* IgG was delimited by sample absorbance values ≥ the mean absorbance of blank wells (background) plus 3 standard deviations. Statistical significance was assessed by Fisher's exact test of the proportions of positive responses in each NGU case group versus non-NGU controls. *P* values for each comparison are indicated.

By definition, the eighty-two participants with idiopathic urethritis included in this analysis had objective NGU but tested negative for *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, and *T. vaginalis* by NAAT. A key objective of the IUMP study was to evaluate how the urethral microbiome influences the development of idiopathic urethritis.

Thus, in a separate arm of the study, urethral swab specimens collected at enrollment were analyzed by shotgun metagenomic sequencing. Intriguingly, this revealed that 34.1% (28/82) of the participants in our idiopathic urethritis cohort were colonized by pathogens that have previously been associated with atypical cases of NGU. These included *Haemophilus influenzae* [16, 17], *Mycoplasma penetrans* [16], herpes simplex virus (HSV) [113, 114], and adenovirus [115, 116]. We classified these men as having "pathogen-positive IU." Men with idiopathic urethritis that did not have a potential explanation from the results of urethral microbiome sequencing were categorized as having "pathogen-negative IU."

We wondered if the presence of a urethral pathogen in men with pathogen-positive IU (P^+ IU) would affect the likelihood of detecting anti-*Ctr* IgA antibodies in their enrollment specimens. Thus, I decided to perform a sub-analysis to compare men with P^+ IU against men with pathogen-negative IU (PN-IU).

First, I compared concentrations of total IgA (see Figure 1) between P^+ IU and PN-IU participants. I found that urine concentrations of total IgA did not differ between these groups at enrollment ($p = 0.2585$) (Figure 9A). Next, I compared the proportions of men in each group whose urine contained detectable anti-*Ctr* IgA at the 1/50 test dilution. This revealed a striking difference between men with P^+ IU versus PN-IU. Whereas only 21.4% (6/28) of the men with P^+ IU had detectable anti-*Ctr* IgA antibodies in their urine at enrollment ($p = 0.2012$ vs. non-NGU controls), I detected these antibodies in over 42.6% (23/54) of participants with PN-IU ($p < 0.0001$) (Figure 9B).

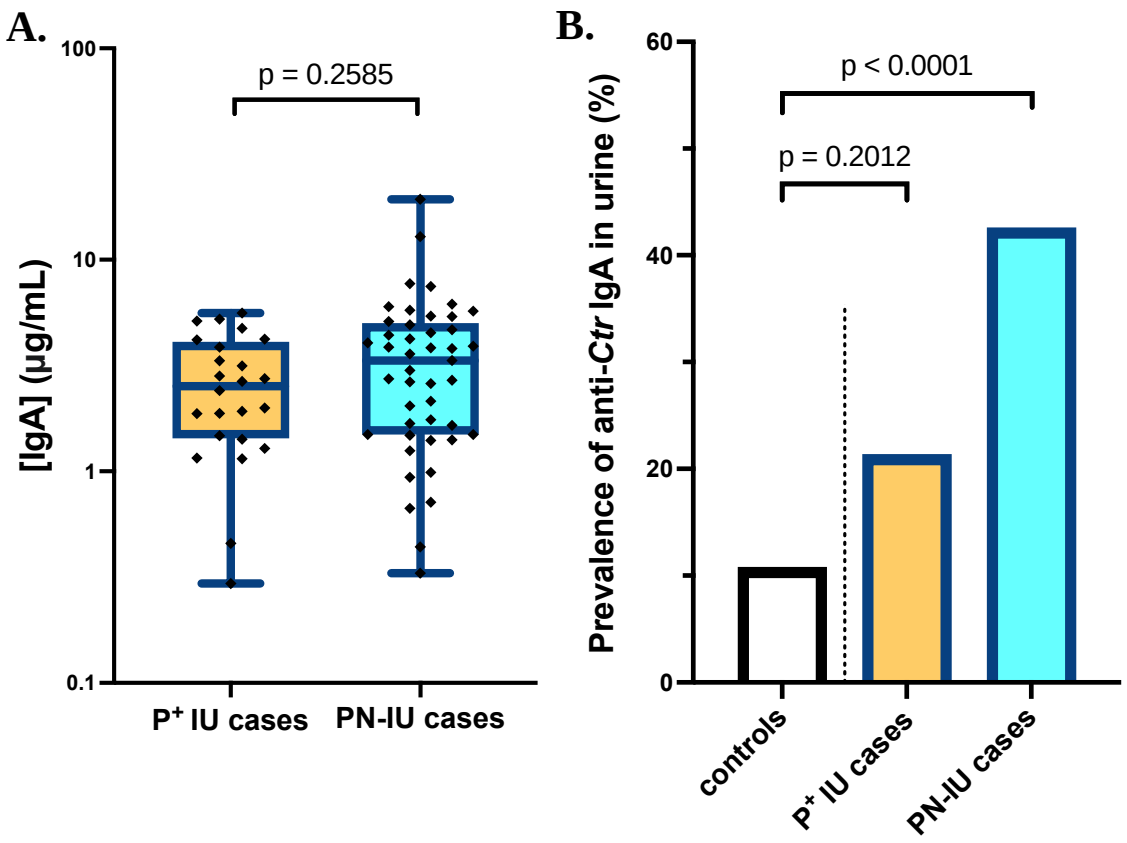

Figure 9. The prevalence of urine anti-*Chlamydia* IgA at enrollment was higher among men with pathogen-negative, but not pathogen-positive, idiopathic urethritis compared to non-NGU controls.

Idiopathic urethritis cases were classified as P^+ IU or PN-IU according to the urethral microorganisms, if any, identified by shotgun metagenomic sequencing. Concentrations of total urine IgA (see Figure 3) were similar between P^+ IU (N = 24) and PN-IU (N = 45) cases (A). The prevalence of anti-*Ctr* IgA (see Figure 7) in the urines of PN-IU cases (N = 28), but not P^+ IU cases (N = 54), differed from controls (B). Statistical significance was assessed by unpaired T-test of log-transformed IgA concentrations (A) or by Fisher's exact test for the proportions of positive responses in each IU case group versus non-NGU controls (B). *P* values for each comparison are indicated.

I observed an analogous pattern when comparing total urine IgG concentrations and positive anti-*Ctr* IgG responses among IU sub-groups. In enrollment FCU specimens, total IgG did not differ between men with P^+ IU versus PN-IU (p = 0.6403) (Figure 10A). At the test dilution of 1/50, I detected anti-*Ctr* IgG antibodies in only 1 of 28 enrollment

FCU specimens (3.6%) from men with P^+ IU (Figure 10B). This proportion of positive responses did not differ from non-NGU controls (p = 0.3857). In contrast, anti-*Ctr* IgG antibodies were detected in 13.0% (7/54) of PN-IU urine specimens and were more prevalent among these men than non-NGU controls (p = 0.0026).

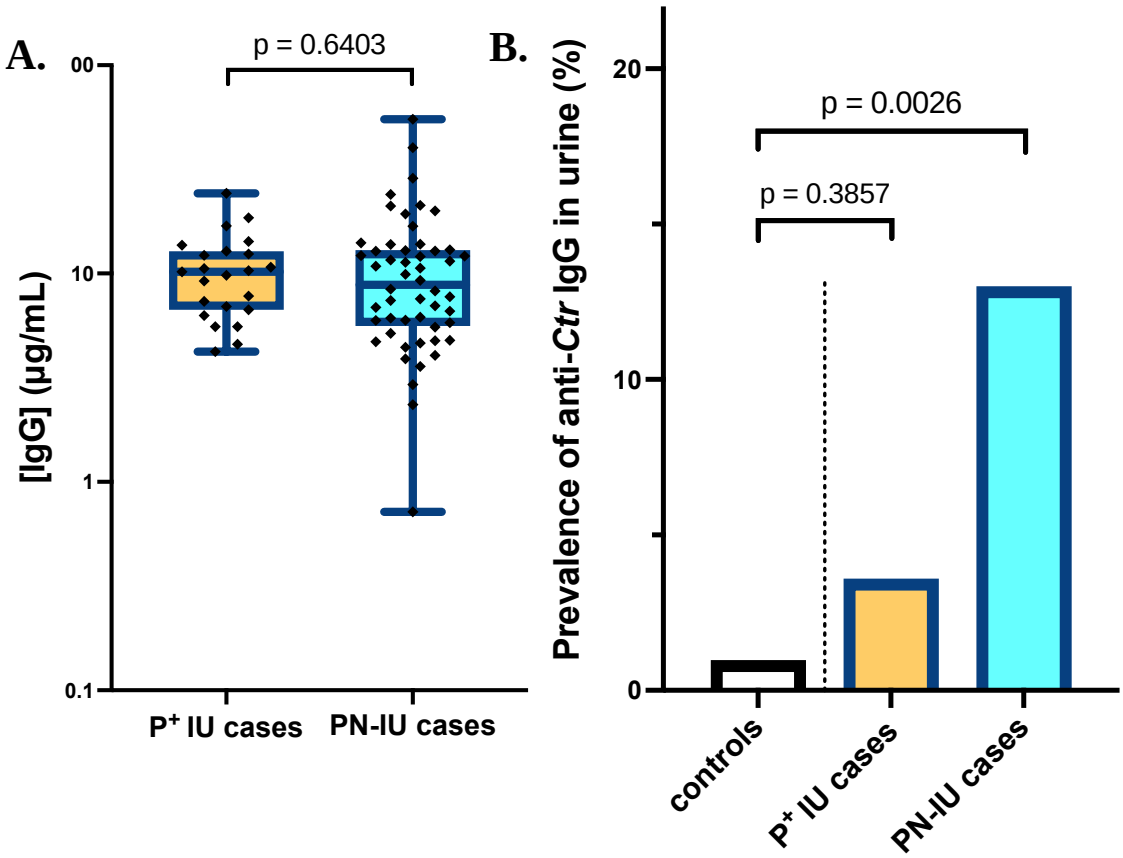

Figure 10. The prevalence of urine anti-*Chlamydia* IgG at enrollment was higher among men with pathogen-negative, but not pathogen-positive, idiopathic urethritis compared to non-NGU controls.

Idiopathic urethritis cases were classified as P^+ IU or PN-IU according to the urethral microorganisms, if any, identified by shotgun metagenomic sequencing. Concentrations of total urine IgG (see Figure 4) were similar between P^+ IU (N = 23) and PN-IU (N = 52) cases (A). The prevalence of anti-*Ctr* IgG (see Figure 8) in the urines of PN-IU cases (N = 28), but not P^+ IU cases (N = 54), differed from controls (B). Statistical significance was assessed by unpaired T-test of log-transformed IgG concentrations (A) or by Fisher's exact test for the proportions of positive responses in each IU case group versus non-NGU controls (B). *P* values for each comparison are indicated.

No data are available concerning the duration of anti-*Ctr* antibody responses in the urethral site in humans or how production of these antibodies is affected by antibiotic treatment. Thus, I decided to test urine specimens collected from men with NGU at follow-up for anti-*Ctr* IgA and IgG antibodies using the same 1/50 test dilution and method described above. Because of the differences in the prevalence of anti-*Ctr* antibodies that I observed previously, I decided to evaluate the P^+ IU and PN-IU participants separately in this analysis.

Interestingly, across each group of men with NGU, I found anti-*Ctr* IgA antibodies in a higher proportion of FCU specimens collected at follow-up compared to enrollment (Figure 11). However, comparison of discordant pairs by McNemar's test revealed that the change in anti-*Ctr* IgA prevalence was significant ($p = 0.0077$) only among men with P^+ IU. In men with PN-IU, the increased proportion of specimens containing detectable anti-*Ctr* IgA trended towards significance ($p = 0.0961$).

My analysis of *Ctr*-specific IgG antibodies in follow-up FCU specimens revealed that many men with chlamydia who previously had detectable anti-*Ctr* IgG in their urine at enrollment no longer exhibited these antibodies at follow-up (Figure 12). I detected anti-*Ctr* IgG in enrollment FCU from twenty-four men with chlamydia who were negative for these antibodies at one-month follow-up. Only three men exhibited the opposite trend ($p < 0.0001$). Interestingly, among the three participants with detectable anti-*Ctr* IgG at follow-up but not enrollment, all three still had signs or objective evidence of urethritis, and two were *Ctr*-positive by NAAT. However, it is unclear whether these participants experienced azithromycin treatment failures or were re-infected with chlamydia. Taken together, these results suggest that urethral *Chlamydia*-

specific IgG is indicative of acute infection. The proportions of FCU specimens with detectable anti-*Ctr* IgG antibodies did not differ between enrollment and follow-up in the other groups of NGU cases ($p > 0.2$).

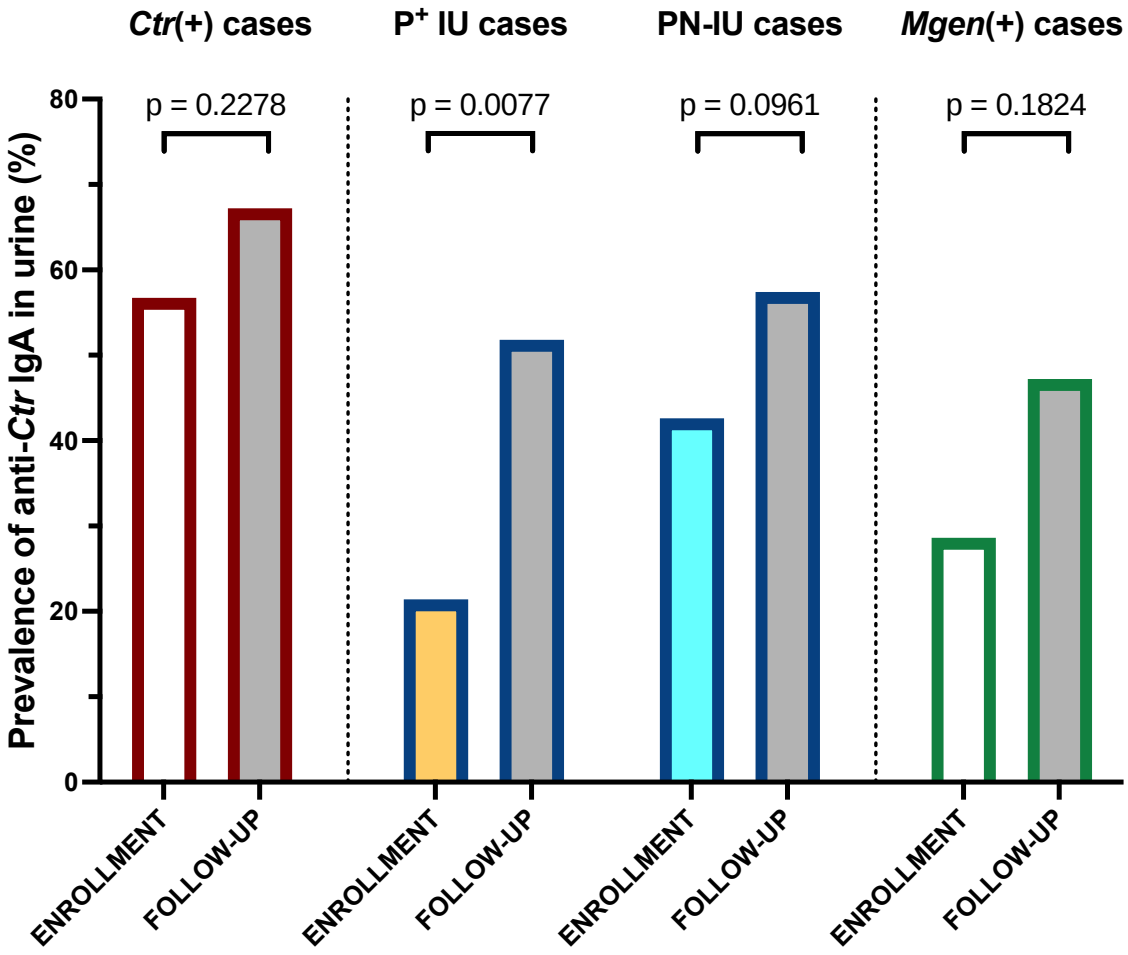

Figure 11. The prevalence of urine anti-*Chlamydia* IgA among men with NGU was similar or increased at follow-up compared to enrollment.

Follow-up FCU specimens were obtained from participants in the IUMP study. Prevalence of anti-*Ctr* IgA in urines of NGU cases with chlamydia (N = 64), P+ IU (N = 27), PN-IU (N = 47), or *Mgen* (N = 36) was determined at a dilution of 1/50 using indirect ELISA for antibodies to serovar D EBs. Positive detection of anti-*Ctr* IgA was delimited by sample absorbance values ≥ the mean absorbance of blank wells (background) plus 3 standard deviations. Statistical significance of the change in proportion with detectable anti-*Ctr* IgA at follow-up versus enrollment was assessed by McNemar's test: *Ctr*(+) cases, N = 57; P^+ IU cases, N = 27; PN-IU cases, N= 47; *Mgen* cases, N = 33). *P* values for each comparison of anti-*Ctr* IgA prevalence at follow-up versus enrollment are indicated.

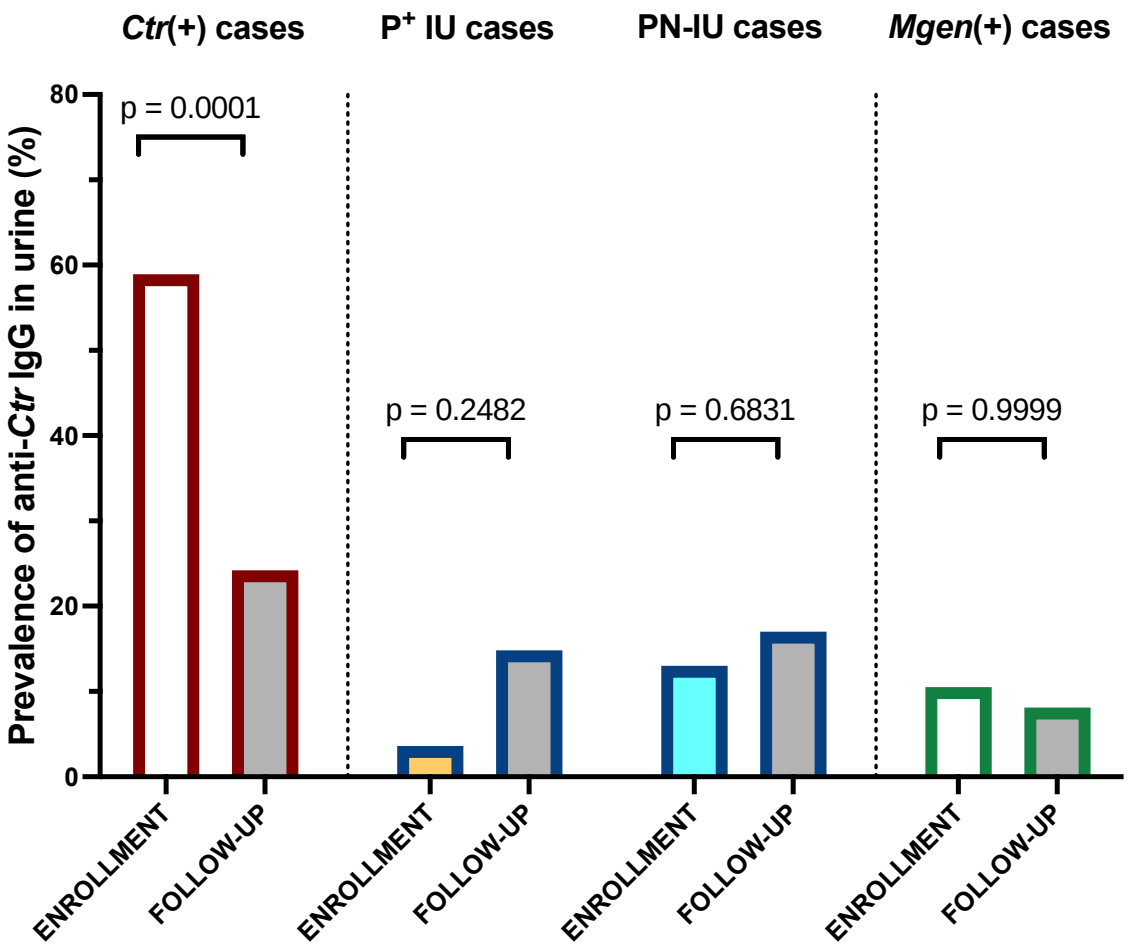

Figure 12. The prevalence of urine anti-*Chlamydia* IgG among men with chlamydia was significantly lower at follow-up compared to enrollment.

Follow-up FCU specimens were obtained from participants in the IUMP study. Prevalence of anti-*Ctr* IgG in urines of NGU cases with chlamydia (N = 66), P+ IU (N = 27), PN-IU (N = 53), or *Mgen* (N = 37) was determined at a dilution of 1/50 using an indirect ELISA for antibodies to serovar D EBs. Positive detection of anti-*Ctr* IgG was delimited by sample absorbance values ≥ the mean absorbance of blank wells (background) plus 3 standard deviations. Statistical significance of the change in proportion with detectable anti-*Ctr* IgG at follow-up versus enrollment was assessed by McNemar's test; *Ctr*(+) cases, N = 56; P+ IU cases, N = 27; PN-IU cases, N= 53; *Mgen* cases, N = 37). *P* values for each comparison of anti-*Ctr* IgG prevalence at follow-up versus enrollment are indicated.

Sixty-seven (67) men with NGU who enrolled as cases in the IUMP study and subsequently tested positive for chlamydia met the inclusion criteria for this investigation. FCU specimens were available from both the enrollment and one-month follow-up visits for 59 of these participants. One participant had only an enrollment, but not a follow-up, specimen available; six participants had specimens only from follow-up. As shown above, anti-*Ctr* IgA was detected in 34/60 FCU specimens from enrollment (see Figure 7) and in 43/64 specimens from follow-up (see Figure 11).

Because the prevalence of urine anti-*Ctr* IgA remained elevated at follow-up among IUMP participants with chlamydia, I wanted to know if the concentration of these antibodies was substantially different between visits. Concentrations of anti-*Ctr* IgA in enrollment (N= 34) and follow-up (N = 39) FCU specimens were estimated using standard curves of human total immunoglobulin reference standards. I found that the concentrations of detectable anti-*Ctr* IgA in the urines of men with chlamydia were similar between their enrollment (mean = 34.576 ng/mL) and follow-up (mean = 31.736 ng/mL) visits (Figure 13). Median concentrations of anti-*Ctr* IgA at these visits were 27.028 ng/mL and 18.882 ng/mL, respectively. Four IUMP participants with chlamydia mono-infection were requested to return for a second follow-up visit approximately one month after follow-up #1; three of these men exhibited detectable anti-*Ctr* IgA in their urine specimens at all three timepoints. Taken together, these results suggest that production of anti-*Ctr* IgA antibodies in the urethra persists following antibiotic treatment and infection resolution.

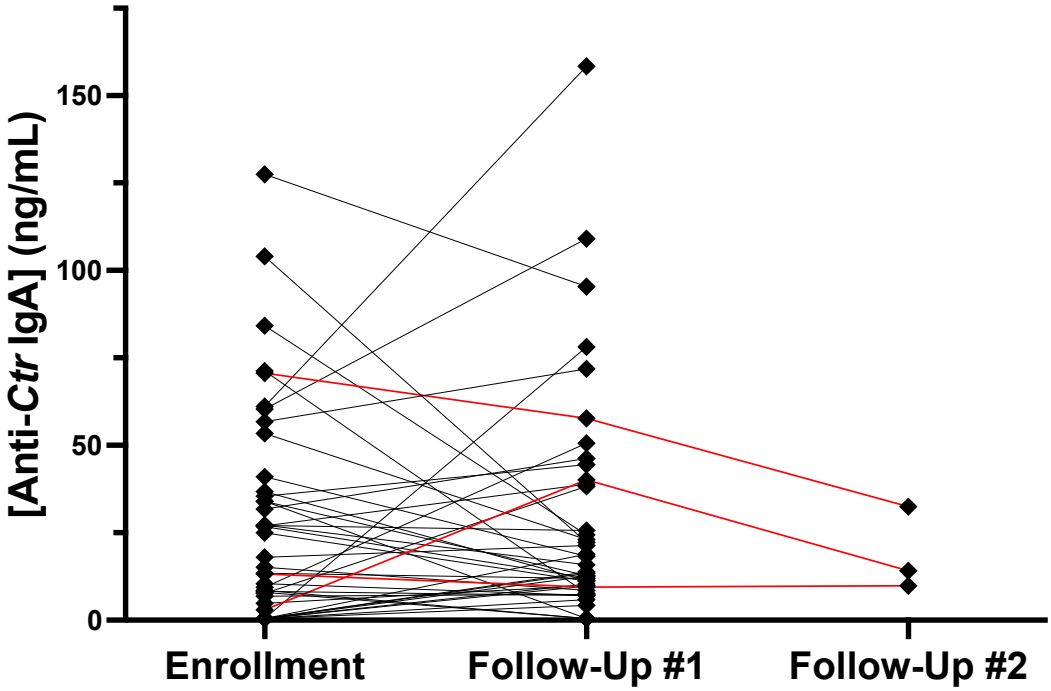

Figure 13. Estimated concentrations of anti-*Chlamydia* IgA at enrollment and follow-up in the urine of men with chlamydia.

FCU specimens were collected at enrollment and follow-up from participants in the IUMP study. In specimens with detectable anti-*Ctr* IgA (1/50 dilution), antibody concentrations were estimated using indirect ELISA. Concentrations of anti-*Ctr* IgA at enrollment (N = 34) and at follow-up (N = 39) in the urines of men with chlamydia are shown; a concentration of 0.5 ng/mL was arbitrarily assigned for matched specimens (enrollment or follow-up) that did not contain detectable antibodies. Three men with chlamydia who returned for a second follow-up visit (N = 4) still had detectable anti-*Ctr* IgA in their urines at ~2 months post-enrollment.

Section 4: IgM Concentrations in the Urine of Men with Urethritis

Somewhat unexpectedly, my analyses of *Chlamydia*-specific IgA and IgG antibodies revealed that one or both of these immunoglobulin isotypes were detected in only 66.7% (40/60) of men with NAAT-confirmed chlamydia at enrollment. I hypothesized that chlamydia-infected men without detectable anti-*Ctr* IgA or IgG antibodies were recently infected and had not yet class-switched from producing IgM to these other isotypes. Mature naive B cells secrete IgM antibodies with relatively low affinity until they receive activation signals to undergo class-switch recombination and somatic hypermutation [117]. Thus, I decided to evaluate the total IgM concentrations in the urine of men with and without NGU and to test these specimens for the presence of anti-*Ctr* IgM antibodies.

Preliminary optimization studies using residual IDRL specimens revealed that concentrations of total urine IgM are much lower than IgA or IgG (data not shown). As a result, I empirically determined that a urine dilution of just 1/250 was appropriate for the evaluation of total IgM concentrations by colorimetric ELISA.

Compared to non-NGU controls, I found that enrollment urine specimens from men with chlamydia contained higher concentrations of total IgM ($p < 0.0001$) (Figure 14). Total urine IgM at enrollment were also higher than controls among men with idiopathic urethritis or with *Mgen* infection ($p < 0.0001$). Thus, IgM was the only antibody isotype of the three I tested that differed in urine concentration between participants with *Mgen* infection and non-NGU controls.

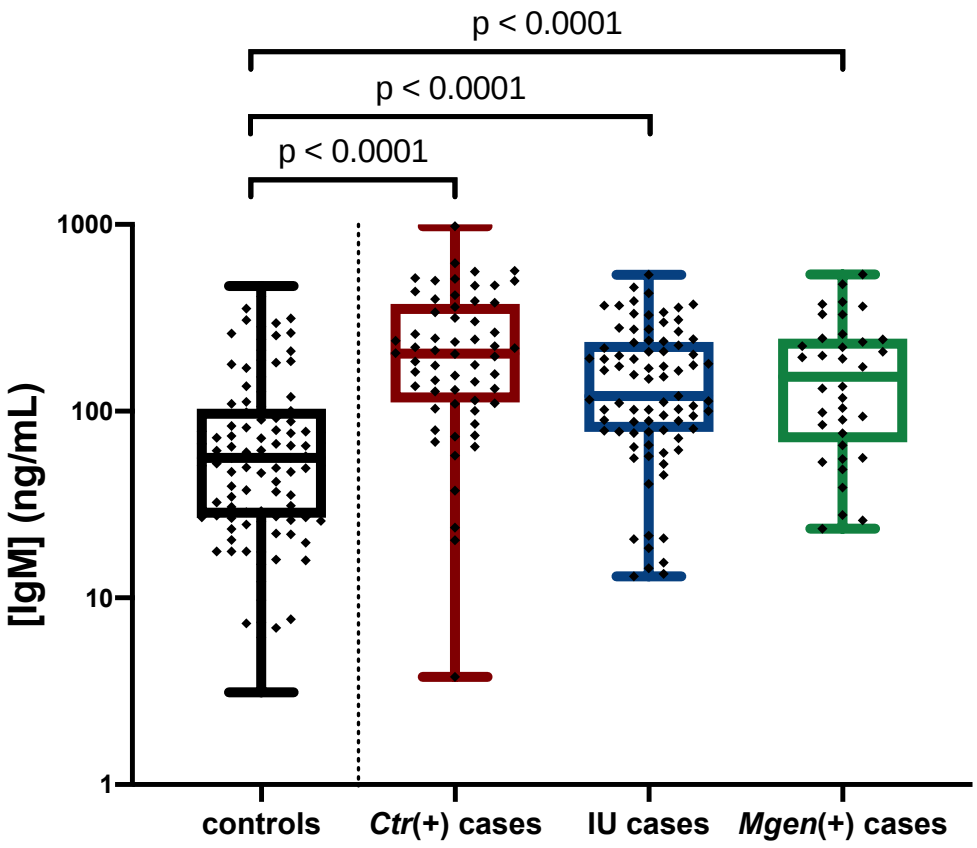

Figure 14. Total IgM concentrations were elevated in the urines of men with NGU compared to non-NGU controls.

FCU specimens were obtained from men who enrolled in the IUMP study. Concentrations of total IgM in the urines of non-NGU controls (N = 101) or NGU cases with chlamydia (N = 60), idiopathic urethritis (N = 81), or *Mgen* (N = 36) were determined using quantitative ELISA. Total IgM concentrations were normalized by log transformation, and statistical significance was assessed by unpaired T-test of each NGU case group versus non-NGU controls. *P* values for each comparison are indicated.

I previously observed that men with chlamydia had lower total IgG concentrations in their urine at follow-up compared to enrollment (see Figure 5). Mirroring this decline in total IgG, I found that men with chlamydia also had lower concentrations of IgM in their follow-up FCU compared to matched presentation specimens ($p < 0.0001$) (Figure 15). Meanwhile, I observed that urine concentrations of total IgM were trending higher

follow-up among men with idiopathic urethritis (p = 0.0515) but did not differ between enrollment and follow-up in men with *Mgen* infections (p = 0.9039).

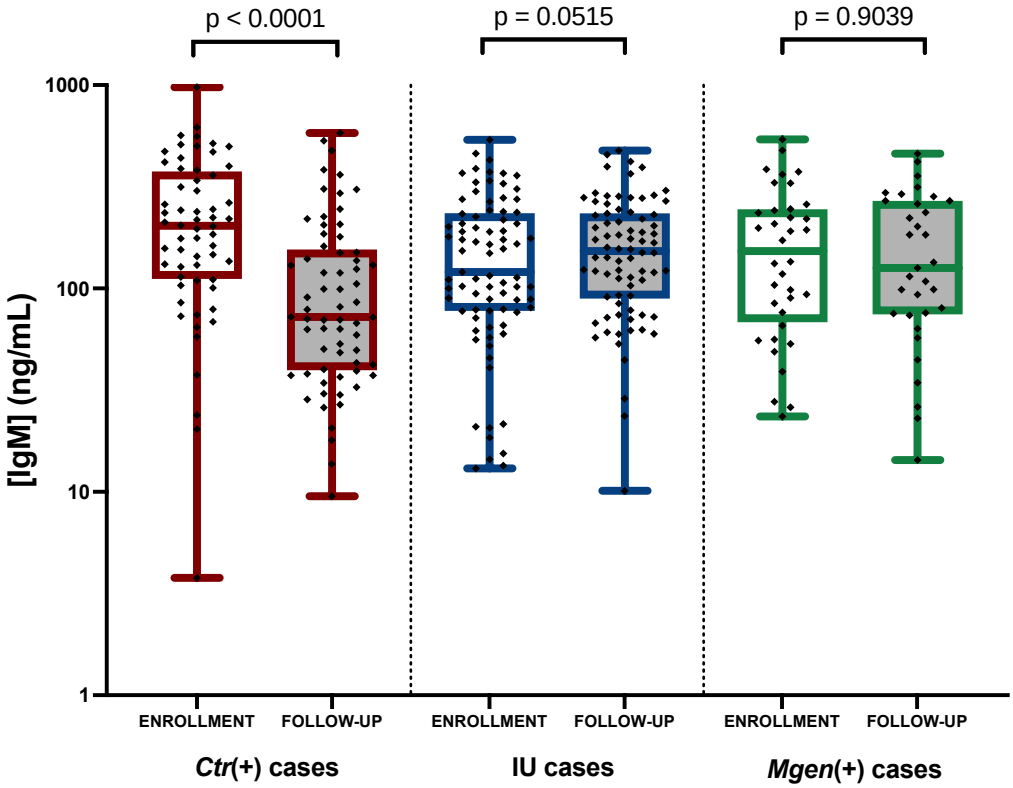

Figure 15. Men with chlamydia had lower concentrations of total IgM in their urines at follow-up versus enrollment.

Follow-up FCU specimens were obtained from participants in the IUMP study. Concentrations of total urine IgM at follow-up among NGU cases with chlamydia (N = 65), idiopathic urethritis (N = 78), or *Mgen* (N = 33) were determined using quantitative ELISA. Total IgM concentrations were normalized by log transformation. Statistical significance was assessed by paired T-test of enrollment versus follow-up IgM concentrations; *Ctr*(+) cases, N = 58; IU cases, N = 77; *Mgen*(+) cases, N = 31. *P* values for each comparison are indicated.

Section 5: The Detection of Anti-*Chlamydia* IgM in Male Urine

Although the study of men with chlamydia by Pate *et al.* [85] did not include an analysis of *Chlamydia*-specific IgM antibodies, multiple studies have documented the presence of anti-*Ctr* IgM in the sera of individuals with chlamydia [118]. Additionally,

serum-based diagnostic assays are now commercially available to assay anti-*Ctr* IgM. Furthermore, De Clerq and colleagues detected *Chlamydia*-specific IgM antibodies in the vaginal secretions from pigs experimentally infected with *Ctr* serovar L2 [119]. Thus, I wondered if anti-*Ctr* IgM could be detected in the IUMP participants' FCU using a similar ELISA approach as anti-*Ctr* IgA and IgG.

In accordance with my finding that total IgM is present in lower concentrations in the urine compared to total IgA and IgG, my optimization of this assay revealed that few IDRL residual urine specimens contained detectable anti-*Ctr* IgM antibodies when diluted beyond 1/2 (data not shown). Despite the increased potential for non-specific interactions at this dilution factor, I wanted to test the hypothesis that anti-*Ctr* IgM antibodies can identify men who are or were recently infected with chlamydia.

At the urine test dilution of 1/2, I detected anti-*Ctr* IgM antibodies in 33.3% (20/60) of enrollment FCU specimens from men with chlamydia (Figure 16). In contrast, I detected anti-*Ctr* IgM in a lower proportion, 5.0% (5/101), of urine specimens from non-NGU controls ($p < 0.0001$). Anti-*Ctr* IgM antibodies were detected in the specimens of 14.6% (12/82) of men with idiopathic urethritis ($p = 0.0383$ vs. non-NGU controls).

Confirming the potential for non-specific reactivity against serovar D EBs by low-affinity IgM produced innately or against other pathogens, anti-*Ctr* IgM antibodies were detected in 42.4% (14/33) of men with *Mgen* infection at enrollment.

Among the 20 chlamydia cases who had detectable anti-*Ctr* IgM in their FCU at enrollment, only four did not also have detectable anti-*Ctr* IgA or anti-*Ctr* IgG in these specimens. Intriguingly, while anti-*Ctr* IgA antibodies were detected in follow-up FCU

specimens from two of these four men (see Figure 9), none had detectable anti-*Ctr* IgG in their follow-up FCU (see Figure 10).

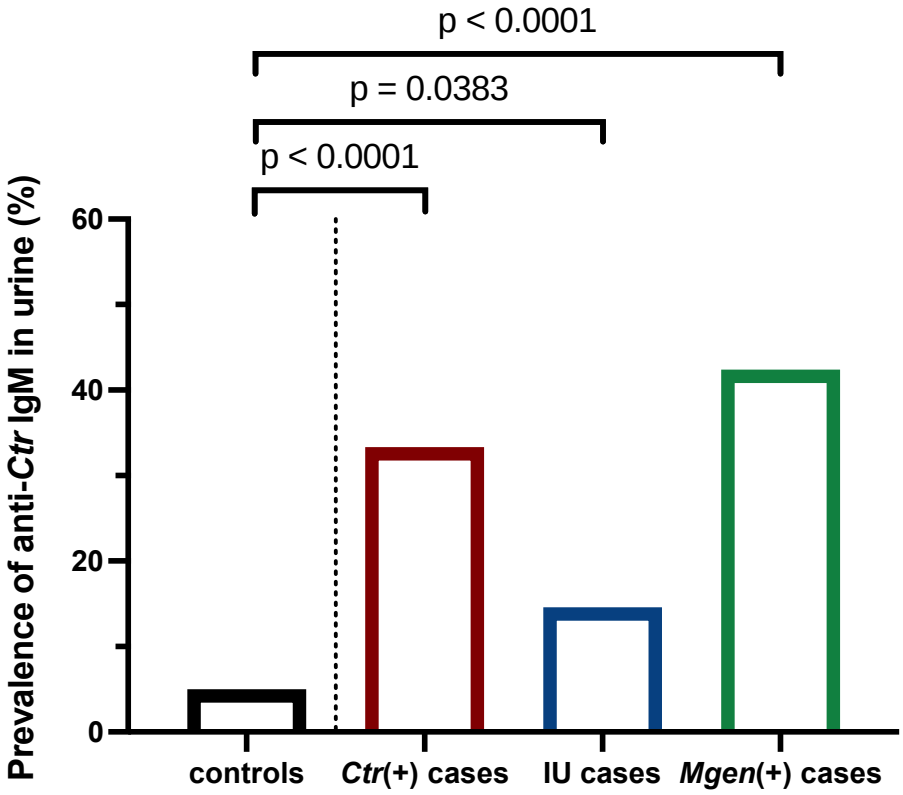

Figure 16. The prevalence of urine anti-*Chlamydia* IgM at enrollment was higher among NGU cases than controls and highest among men with *Mgen*.

FCU specimens were obtained from men with and without NGU who enrolled in the IUMP study. FCU specimens from non-NGU controls (N =101) and men with chlamydia infection (N = 60), idiopathic urethritis (N = 82), or *Mgen* infection (N = 33) were diluted 1/2 and tested for anti-*Ctr* IgM antibodies by indirect ELISA against serovar D EBs. Positive detection was defined by absorbance values greater than 3 standard deviations above the mean absorbance of blank wells. Statistical significance was assessed by Fisher's exact test of each NGU group versus non-NGU controls. *P* values for each comparison are indicated.

Concentrations of total urine IgM did not differ between men with P^+ IU versus PN-IU (p = 0.9783) (Figure 17A). However, as with the detection of anti-*Ctr* IgA and IgG, I observed an intriguing difference between the proportions P^+ IU or PN-IU men

with detectable anti-*Ctr* IgM (Figure 17B). Specifically, anti-*Ctr* IgM antibodies were detected in 10.7% (3/28) of P^+ IU cases, a rate that did not differ from non-NGU controls ($p = 0.3698$). However, compared to controls, these antibodies were detected in a higher proportion, 16.7% (9/54), of the men with PN-IU ($p = 0.0203$).

Finally, I evaluated whether the prevalence of anti-*Ctr* IgM antibodies differed between the FCU specimens collected at presentation versus follow-up among men with NGU (Figure 18). The proportion of men with chlamydia who had detectable anti-*Ctr* IgM at follow-up trended higher ($p = 0.0523$) compared to enrollment. The rates of detection for these antibodies did not differ between enrollment and follow-up among men with P^+ IU, PN-IU, or *Mgen*.

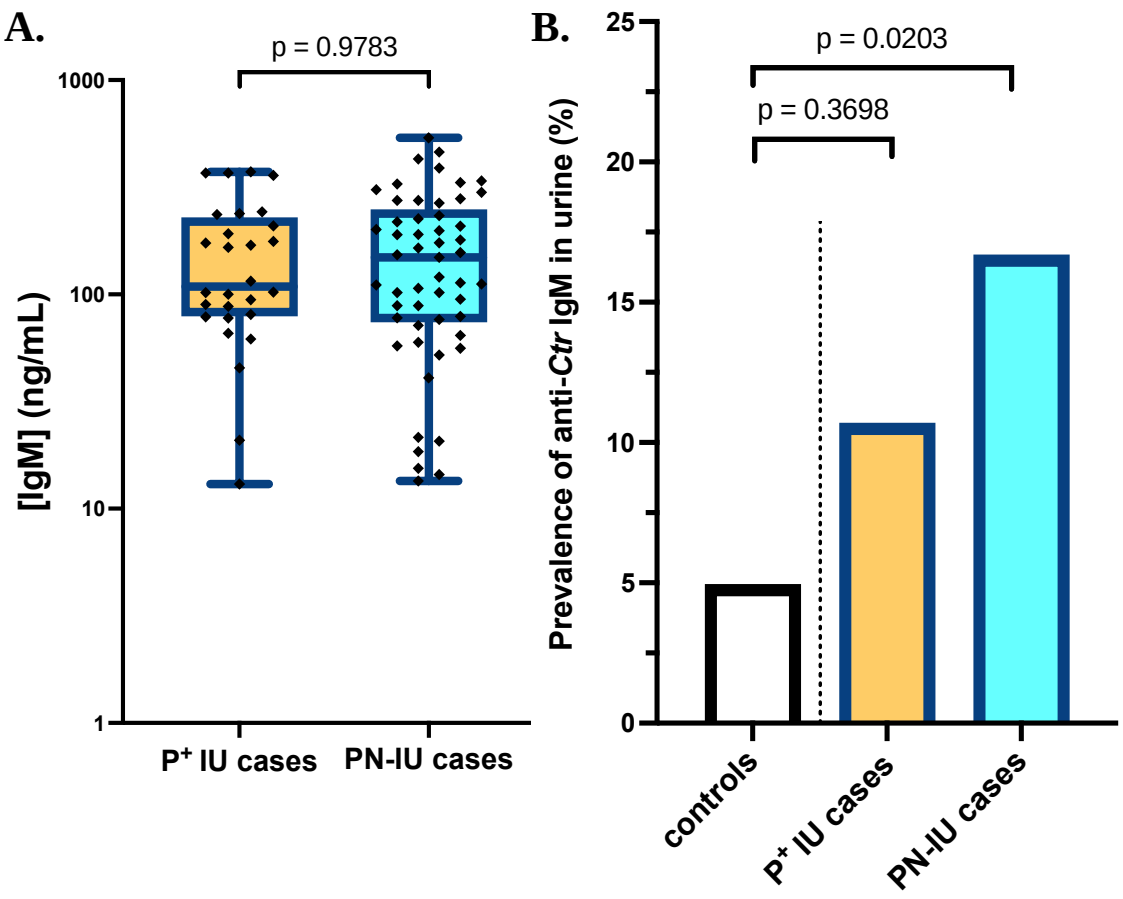

Figure 17. The prevalence of urine anti-*Chlamydia* IgM at enrollment was higher among men with pathogen-negative, but not pathogen-positive, idiopathic urethritis compared to non-NGU controls.

Idiopathic urethritis cases were classified as P^+ IU or PN-IU according to the urethral microorganisms, if any, identified by shotgun metagenomic sequencing. Concentrations of total urine IgM (see Figure 14) were similar between P^+ IU (N = 28) and PN-IU (N = 53) cases (A). The prevalence of anti-*Ctr* IgM (see Figure 16) in the urines of PN-IU cases (N = 28), but not P^+ IU cases (N = 54), differed from controls (B). Statistical significance was assessed by unpaired T-test of log-transformed IgM concentrations (A) or by Fisher's exact test for the proportions of positive responses in each IU case group versus non-NGU controls (B). *P* values for each comparison are indicated.

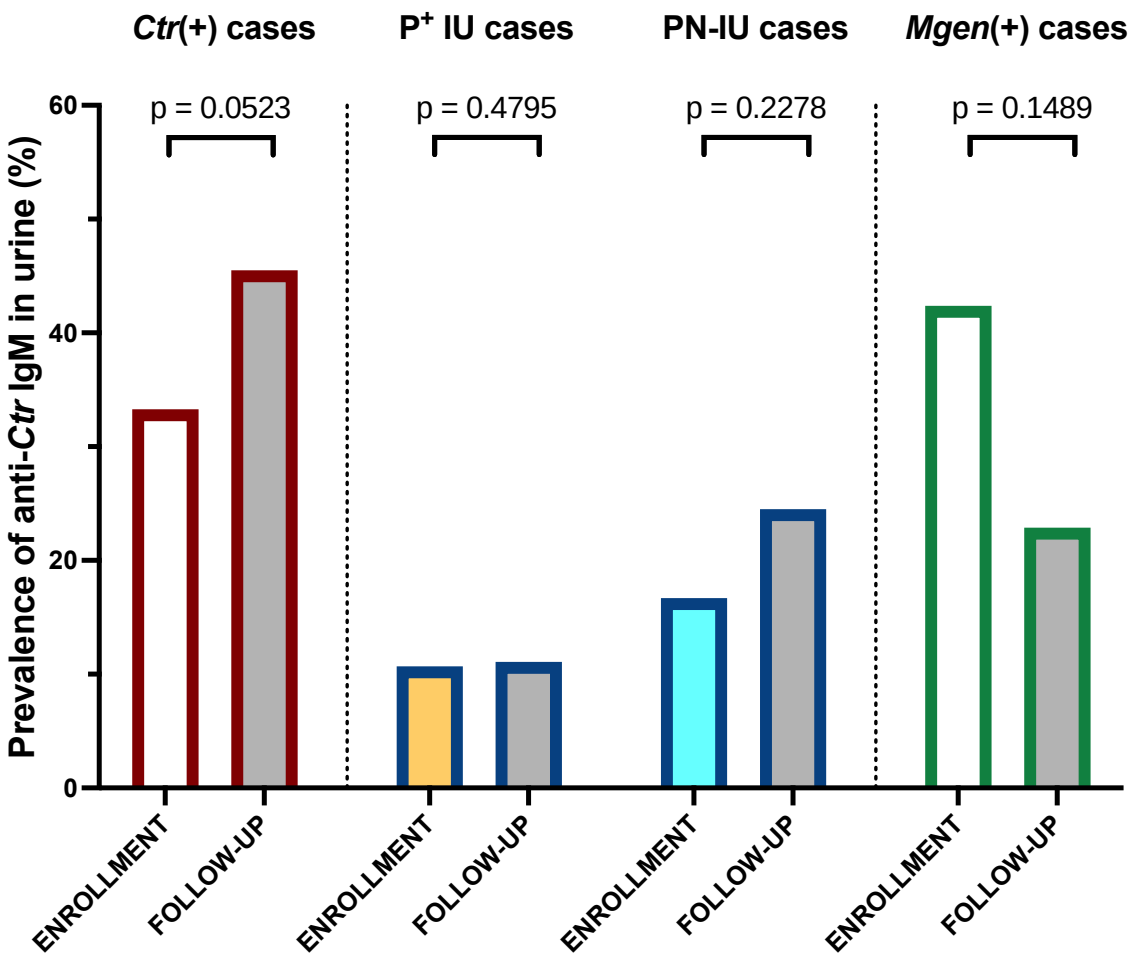

Figure 18. The prevalence of urine anti-*Chlamydia* IgM among men with NGU was similar at follow-up compared to enrollment.

Follow-up FCU specimens were obtained from participants in the IUMP study. Prevalence of anti-*Ctr* IgM in urines of NGU cases with chlamydia (N = 66), P+ IU (N = 27), PN-IU (N = 53), or *Mgen* (N = 35) was determined at a dilution of 1/2 using an indirect ELISA for antibodies to serovar D EBs. Positive detection of anti-*Ctr* IgM was delimited by sample absorbance values ≥ the mean absorbance of blank wells (background) plus 3 standard deviations. Statistical significance of the change in proportion with detectable anti-*Ctr* IgM at follow-up versus enrollment was assessed by McNemar's test; *Ctr*(+) cases, N = 59; P+ IU cases, N = 27; PN-IU cases, N= 53; *Mgen* cases, N = 30). *P* values for each comparison of anti-*Ctr* IgM prevalence at follow-up versus enrollment are indicated.

Section 6: The Detection of Anti-*Mycoplasma* Antibodies in Male Urine

Since I was able to detect anti-*Chlamydia* antibodies in the urine of men with NGU, we wondered if a similar ELISA-based approach could be used to identify urine antibodies reactive against *Mgen*. Thus, I decided to conduct a pilot study to evaluate the feasibility of this strategy in our IUMP cohort. Using microtiter plates coated with 2 ug/well of glutaraldehyde-fixed *M. genitalium* G37, I tested FCU specimens from men with NGU for anti-*Mgen* IgA, IgG, and IgM antibodies. Included in this preliminary study were all IUMP participants with *Mgen* mono-infection (N = 38), as well as the first 58 non-NGU controls, first 28 chlamydia cases, and first 60 idiopathic urethritis cases, sequentially, from our previous analysis. I also evaluated all available follow-up urine specimens from the men infected with *Mgen* (N = 37).

In urine samples diluted 1/3, I detected anti-*Mgen* IgA antibodies in 66.0% (35/53) of non-NGU controls (Figure 19A). This raised some concern about non-specific antibody reactivity against antigens on the *Mgen* surface at this dilution. In comparison, anti-*Mgen* IgA were detected in 84.2% (32/38) of men with *Mgen*, trending towards an increased rate of detection versus controls ($p = 0.0585$). Anti-*Mgen* IgA was also frequently observed in men with chlamydia (81.5%; 22/27) and men with idiopathic urethritis (75%; 42/56), but these rates did not differ from controls.

In contrast to my observations for anti-*Ctr* antibodies, neither P^+ IU nor PN-IU participants exhibited a higher prevalence of anti-*Mgen* IgA antibodies at enrollment compared to non-NGU controls (Figure 19B). Additionally, the proportions of men with *Mgen* who had detectable anti-*Mgen* IgA in their urine did not differ between enrollment and follow-up evaluations (Figure 19C).

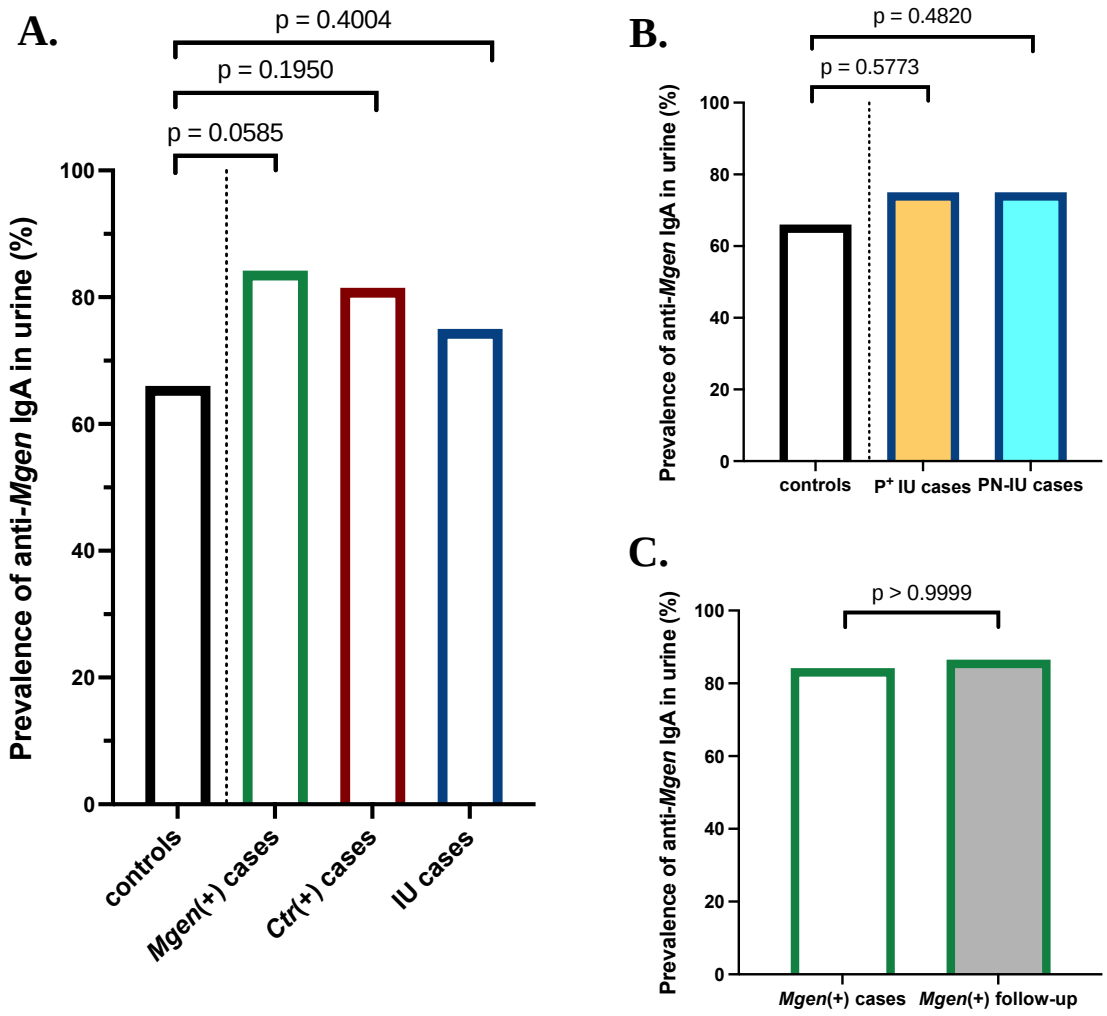

Figure 19. The prevalence of urine anti-*Mycoplasma* IgA at enrollment was similar among NGU cases compared to non-NGU controls.

FCU specimens were obtained from men who enrolled in the IUMP study. Prevalence of anti-*Mgen* IgA in the urines of non-NGU controls (N = 53) and NGU cases with *Mgen* (N = 38), chlamydia (N = 27), or idiopathic urethritis (N = 56) was determined at a dilution of 1/3 using an indirect ELISA for antibodies to *M. genitalium* strain G37. Anti-*Mgen* IgA prevalence in the follow-up specimens of *Mgen* cases (N = 37) was determined by the same method. Positive detection of anti-*Mgen* IgA was delimited by sample absorbance values ≥ the mean absorbance of blank wells (background) plus 3 standard deviations. Statistical significance was assessed by Fisher's exact test of the proportions of positive responses in each NGU case group versus non-NGU controls (A & B); statistical significance of anti-*Mgen* IgA prevalence at follow-up versus enrollment was assessed by McNemar's test (C). *P* values for each comparison are indicated.

Next, I tested urines diluted 1/10 for IgG antibodies reactive against *Mgen*. I found detectable anti-*Mgen* IgG in 76.3% (29/38) of enrollment FCU specimens from men with *Mgen* (Figure 20A). This was a significantly higher rate than I observed among non-NGU controls ($p = 0.0045$). However, anti-*Mgen* IgG was still detected in nearly half (45.1%; 23/51) of the controls. The rate of detection of anti-*Mgen* IgG did not differ between non-NGU controls and men with chlamydia (38.5%; 10/26) or with idiopathic urethritis (42.9%; 24/56).

I found that the detection rate for anti-*Mgen* IgG in enrollment FCU specimens did not differ between P^+ IU versus PN-IU participants (Figure 20B). Furthermore, the proportion of FCU specimens from men with *Mgen* infections containing detectable anti-*Mgen* IgG did not differ between enrollment and follow-up (Figure 20C).

Finally, I tested urines diluted 1/2 from men with and without NGU for IgM antibodies reactive against *Mgen*. At this dilution, I found detectable anti-*Mgen* IgM in FCU specimens of only 2 of 57 (3.5%) non-NGU controls (Figure 21A). In comparison, I detected anti-*Mgen* IgM in a higher proportion, 47.2% (17/36), of men with *Mgen* ($p < 0.0001$). Anti-*Mgen* IgM was also found to be more prevalent among men with chlamydia ($p < 0.0001$) and men with idiopathic urethritis ($p = 0.0156$) compared to controls. These antibodies were detected in the enrollment specimens of 42.9% (12/28) of men with chlamydia and 19.3% (11/57) with IU, respectively.

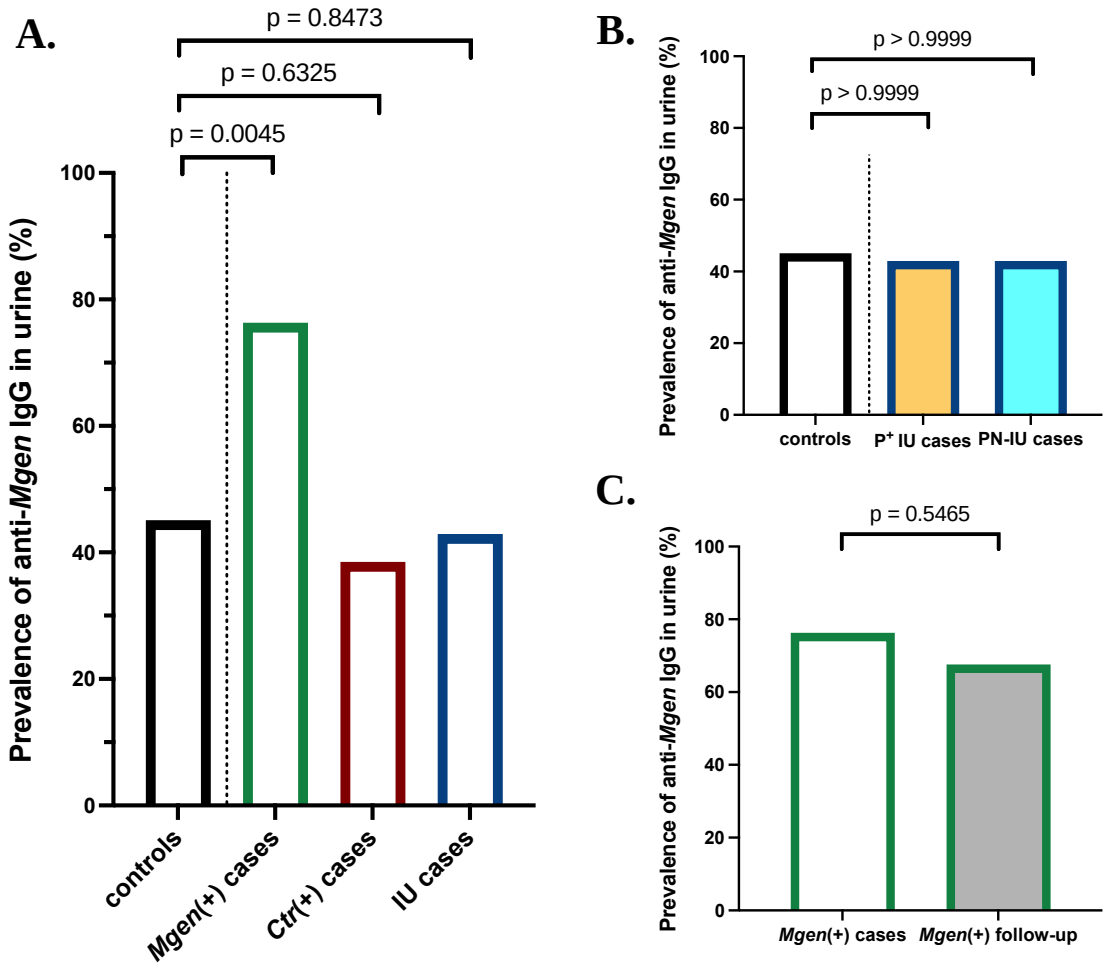

Figure 20. The prevalence of urine anti-*Mycoplasma* IgG at enrollment was higher among men with *Mgen* than non-NGU controls.

FCU specimens were obtained from men who enrolled in the IUMP study. Prevalence of anti-*Mgen* IgG in the urines of non-NGU controls (N = 51) and NGU cases with *Mgen* (N = 38), chlamydia (N = 26), or idiopathic urethritis (N = 56) was determined at a dilution of 1/10 using an indirect ELISA for antibodies to *M. genitalium* strain G37. Anti-*Mgen* IgG prevalence in the follow-up specimens of *Mgen* cases (N = 37) was determined by the same method. Positive detection of anti-*Mgen* IgG was delimited by sample absorbance values ≥ the mean absorbance of blank wells (background) plus 3 standard deviations. Statistical significance was assessed by Fisher's exact test of the proportions of positive responses in each NGU case group versus non-NGU controls (A & B); statistical significance of anti-*Mgen* IgG prevalence at follow-up versus enrollment was assessed by McNemar's test (C). *P* values for each comparison are indicated.

I found anti-*Mgen* IgM antibodies in 23.5% (8/34) of enrollment urine specimens from men with PN-IU (Figure 21B). Thus, men with PN-IU were more likely to have these antibodies in their FCU than non-NGU controls ($p = 0.0050$). However, among men with P^+ IU, I detected anti-*Mgen* IgM in only 13.0% (3/23) of FCU specimens, and this rate did not significantly differ from controls ($p = 0.1400$).

I did not observe a difference between enrollment versus follow-up in the proportions of FCU specimens from men with *Mgen* that contained detectable anti-*Mgen* IgM (Figure 21C).

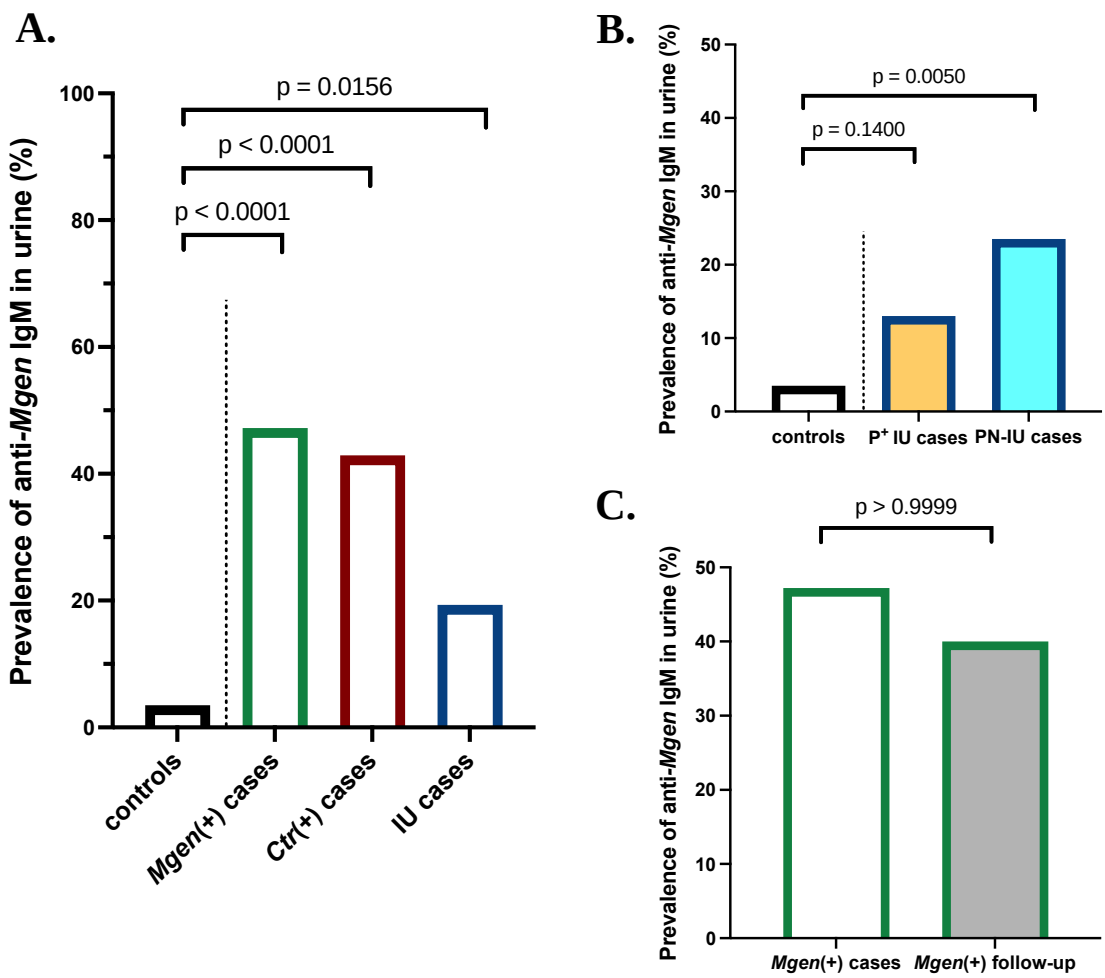

Figure 21. The prevalence of urine anti-*Mycoplasma* IgM at enrollment was higher among NGU cases than non-NGU controls.

FCU specimens were obtained from men who enrolled in the IUMP study. Prevalence of anti-*Mgen* IgM in the urines of non-NGU controls (N = 57) and NGU cases with *Mgen* (N = 36), chlamydia (N = 28), or idiopathic urethritis (N = 57) was determined at a dilution of 1/10 using an indirect ELISA for antibodies to *M. genitalium* strain G37. Anti-*Mgen* IgM prevalence in the follow-up specimens of *Mgen* cases (N = 35) was determined by the same method. Positive detection of anti-*Mgen* IgM was delimited by sample absorbance values ≥ the mean absorbance of blank wells (background) plus 3 standard deviations. Statistical significance was assessed by Fisher's exact test of the proportions of positive responses in each NGU case group versus non-NGU controls (A & B); statistical significance of anti-*Mgen* IgM prevalence at follow-up versus enrollment was assessed by McNemar's test (C). *P* values for each comparison are indicated.

CHAPTER IV – Characteristics of Chlamydia Infection Among IUMP Participants: Infecting Serovars, Urine EB Loads, and Anti-*Chlamydia* Antibodies

Section 1: Serovar Classification of *C. trachomatis* Clinical Isolates

Across my analyses of anti-*Chlamydia* IgA, IgG, and IgM antibodies in the urine of men with NGU, I detected positive responses for at least one isotype in only 73.3% (44/60) of men with chlamydia at enrollment. Positive responses were determined by indirect ELISA with immobilized *Ctr* serovar D EBs as the target antigen to capture anti-*Ctr* antibodies. *Ctr* serovars were originally classified according to differences in the recognition of strains by monoclonal antibodies. These antibodies correspond to epitopes of the major outer membrane protein (MOMP) in the EB cell envelope. Although MOMP, which is encoded by the *Ctr* gene, *ompA*, is an immunodominant antigen in serum, several other antigens, including lipopolysaccharide (LPS) and some polymorphic membrane proteins (Pmps), are immunogenic and can also elicit antibodies against EB surface antigens [120, 121]. Specifically, Crane and colleagues showed that PmpD is conserved across Ctr serovars [122]. However, I wanted to evaluate the possibility that anti-MOMP antibodies predominate the male urethral humoral response against *Ctr* and that men with NAAT-confirmed chlamydia caused by serovars dissimilar from the serovar D target antigen might not produce antibodies detectable in my assay.

Thus, we decided to perform *Ctr ompA* genotyping to determine the infecting serovars in men with chlamydia whose urine specimens I tested for anti-*Ctr* antibodies (Chapter 3). Additionally, given the epidemiologic interest in establishing the current distribution of *Ctr* serovars among the local population, we expanded our *ompA* sequencing analysis to include all IUMP participants with a positive NAAT result for

chlamydia at presentation (N = 114), including those with co-infections or who were lost to follow-up.

Among this expanded group, we found that more than half of all men who enrolled in the IUMP study and were NAAT-positive for chlamydia were infected with serovar E (32.5%) or serovar D (21.1%) strains (Figure 22A). Serovars J/Ja (14.9%) and I/Ia (9.7%) accounted for another quarter of the overall serovar distribution. The genital chlamydia serovars F, G, and K were also detected in IUMP participants, but serovar H was not. Interestingly, we identified two men with chlamydia NGU who were infected with strains typically associated with other chlamydial diseases. Specifically, although the *Ctr* serovars B and L2 usually cause ocular infections (trachoma) and LGV, respectively, we detected these serovars in urine specimens from one participant, each.

We found that the distribution of chlamydial serovars among men included in my analyses of pathogen-reactive urine immunoglobulin was, by random chance, quite similar to the distribution of the overall IUMP cohort (Figure 22B).

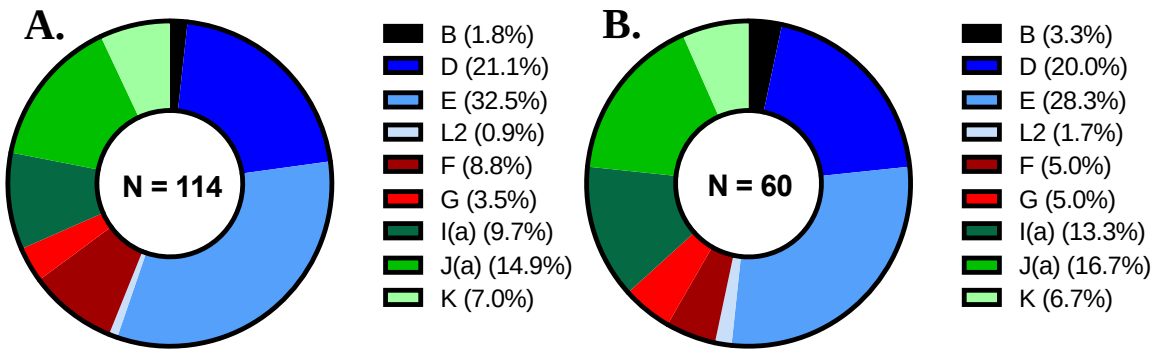

Figure 22. Distributions of *C. trachomatis* serovars by relative abundance among IUMP participants with chlamydia.

The MOMP-encoding *Ctr* gene, *ompA*, was amplified by PCR in urine obtained from chlamydia-positive IUMP participants. Purified PCR products were sequenced by a commercial vendor, and *ompA* genotypes (serovars) were assigned by aligning the *ompA* sequence(s) for each isolate with published strains using Nucleotide BLAST. Relative abundances of each *Ctr* serovar we identified are shown: (A) for all IUMP participants who tested positive for *Ctr* at enrollment by NAAT; and (B) for the subset of men meeting all criteria (including *Ctr* mono-infection) for inclusion in my antibody ELISA experiments.

I found that men infected with each *Ctr* serovar, except serovar G, had detectable anti-*Ctr* IgA, IgG, or IgM antibodies in their enrollment FCU specimens that reacted against serovar D EBs (Figure 23). Thus, it appears that the urethral immune response against *Ctr* infection elicits antibodies against surface-exposed epitopes that are not strictly serovar-specific. I also determined that not all men who were infected with serovar D had detectable anti-*Ctr* antibodies in their FCU specimens. However, the numbers of observations for each *ompA* genotype were too low to determine conclusively whether there were significant differences in the detection of anti-*Ctr* antibodies between serovars. Furthermore, because the population of men who present for care at the Bell Flower Clinic is generally at high risk for STI acquisition, the observation that many men expressed antibodies reactive against serovar D EBs could alternatively reflect an

elevated likelihood of previous chlamydia infection by a serovar D, or closely related serovar E, strain. These were the two most frequently observed serovars in the IUMP cohort, suggesting that they are also the most commonly transmitted among individuals in our catchment area.

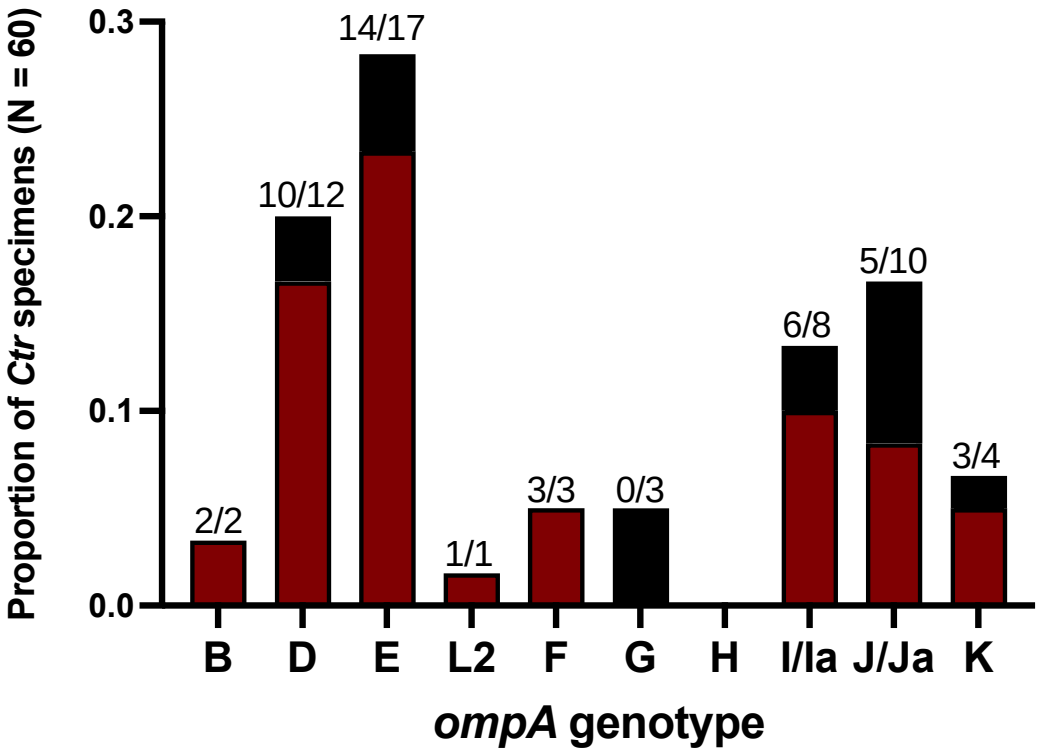

Figure 23. Men infected with various *C. trachomatis* serovars exhibited reactive antibodies in their urine against serovar D EBs.

First-catch urine specimens were obtained from participants who enrolled in the IUMP study. The urines of men with NAAT-positive chlamydia (N = 60) were tested for detectable anti-*Chlamydia* antibodies (see Chapter III) and used to determine the infecting serovars. Black bars represent the relative abundance of each *Ctr* serovar identified in this population (also depicted in Figure 22B). Superimposed red bars demonstrate the proportion of men infected with each serovar whose urine contained at least one isotype of anti-*Chlamydia* (serovar D) antibodies.

Section 2: Relationship Between *C. trachomatis* Serovars & EB Loads

Loads of *Ctr* EBs in the urines of urines of men with positive chlamydia NAAT results were determined previously by quantitative PCR (q-PCR). Given that previous reports have indicated different degrees of histopathology between serovars in chlamydia-infected women [123], I wondered if there would be differences in organism burden associated with different serovars of *Ctr* infection in men.

I found that men infected with *Ctr* serovar F had significantly higher EB loads in their urine specimens compared to men infected with serovar E ($p = 0.0099$) or serovar K ($p = 0.0065$) isolates (Figure 24A). Furthermore, when I combined *ompA* genotypes into their respective serogroups, I found that organism loads among men infected with intermediate serogroup isolates (serovars F & G) were higher than those observed among serogroup B (serovars D, E, B, & L2) ($p = 0.0084$) or serogroup C (serovars I(a), J(a), & K) ($p = 0.0131$) infections (Figure 24B). However, *Ctr* EB loads did not differ between the different serovars (Figure 24C) or serogroups (Figure 24D) of *Ctr* strains among men with chlamydia who met the inclusion criteria for my evaluation of anti-*Ctr* urine antibodies in Chapter 3.

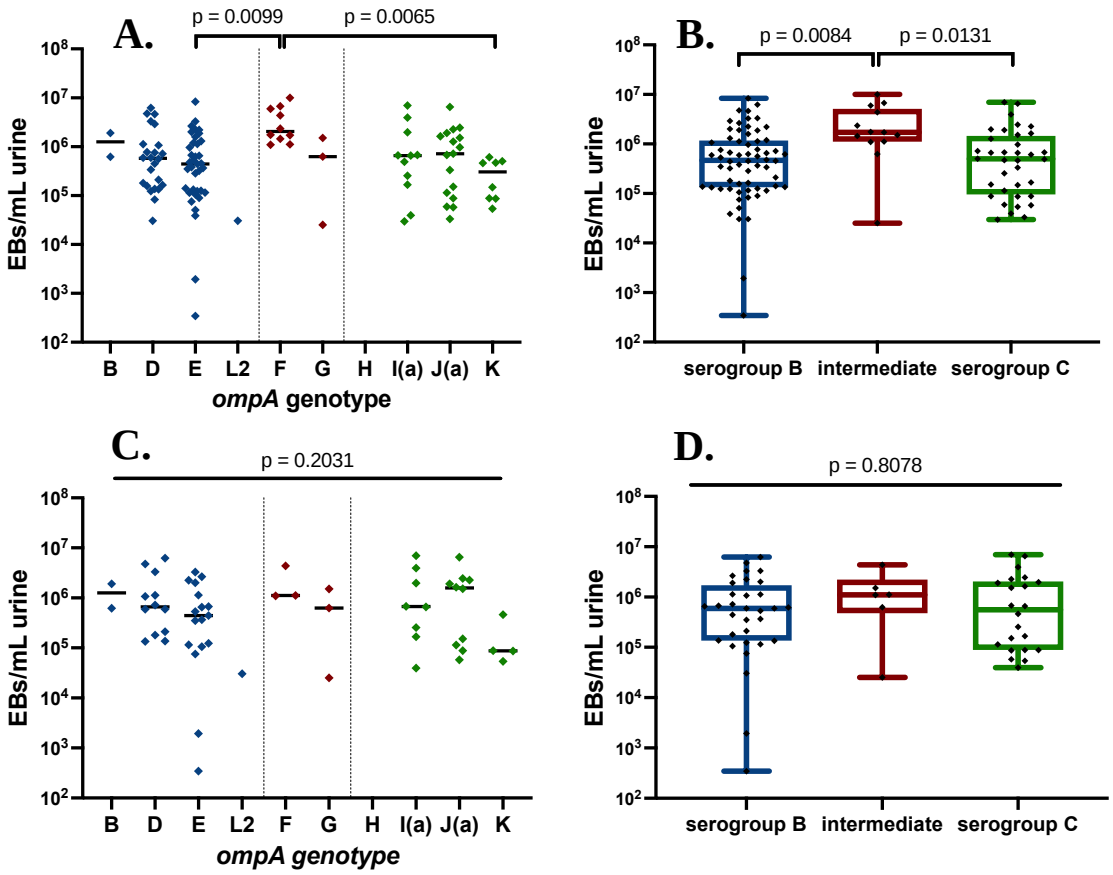

Figure 24. Estimated loads of *C. trachomatis* EBs in male urine differed by serovar (*ompA* genotype) and serogroup of infecting strain.

Urine specimens obtained from IUMP participants with chlamydia were categorized by *Ctr* serovar (see Figure 22B). Estimated concentrations of *Ctr* EBs were determined previously using q-PCR. The urine EB loads observed among men infected by each serovar are shown: (A – B) all IUMP participants (N = 114) who tested *Ctr*-positive at enrollment; (C – D) the subset of *Ctr*-positive men (N = 60) who were included in my antibody ELISA experiments. Statistical significance of the observed differences in EB loads across serovars and/or serogroups of *Ctr* infection was assessed by the Kruskal-Wallace test with Dunn's *post-hoc* test for multiple comparisons. *P* values for each significant comparison are indicated.

Section 3: Relationships Between Anti-*Chlamydia* Antibodies & EB Loads

Strikingly, when I compared the urine EB loads among chlamydia-infected men who did (N = 44) or did not (N = 16) have detectable anti-*Ctr* antibody responses for at least one immunoglobulin isotype, I found that EB loads, on average, were significantly

lower among the latter group ($p = 0.0005$) (Figure 25). This could indicate that a certain threshold level of infection must be attained in order for chlamydia-infected men to mount a urethral humoral response. However, given that organism loads are determined on a per-mL of urine basis, this finding may be confounded by sample dilution among participants who supplied larger quantities of urine. Alternatively, since we did not directly observe the production of FCU specimens, we cannot rule out the possibility that some men may not have provided true "first-catch" urine samples.

Finally, I wanted to evaluate whether there was an association between anti-*Chlamydia* antibody concentrations and infectious EB loads in the urines of men with chlamydia. Concentrations of anti-*Ctr* IgA ($N = 34$), anti-*Ctr* IgG ($N = 33$), and anti-*Ctr* IgM ($N = 20$) in the enrollment FCU specimens of IUMP participants with chlamydia were estimated using standard curves of human total immunoglobulin reference standards. Among these men, I did not observe a significant linear relationship between EB loads and the urine concentrations of any anti-*Ctr* immunoglobulin isotype: IgA ($R = 0.035$; $p = 0.8427$); IgG ($R = -0.104$; $p = 0.5662$); IgM ($R = -0.193$; $p = 0.4150$) (Figure 26). However, these results reflect EB loads and anti-*Ctr* antibody concentrations at a single point in time per individual, and it remains unclear how these continuous variables might change in relation to one another.

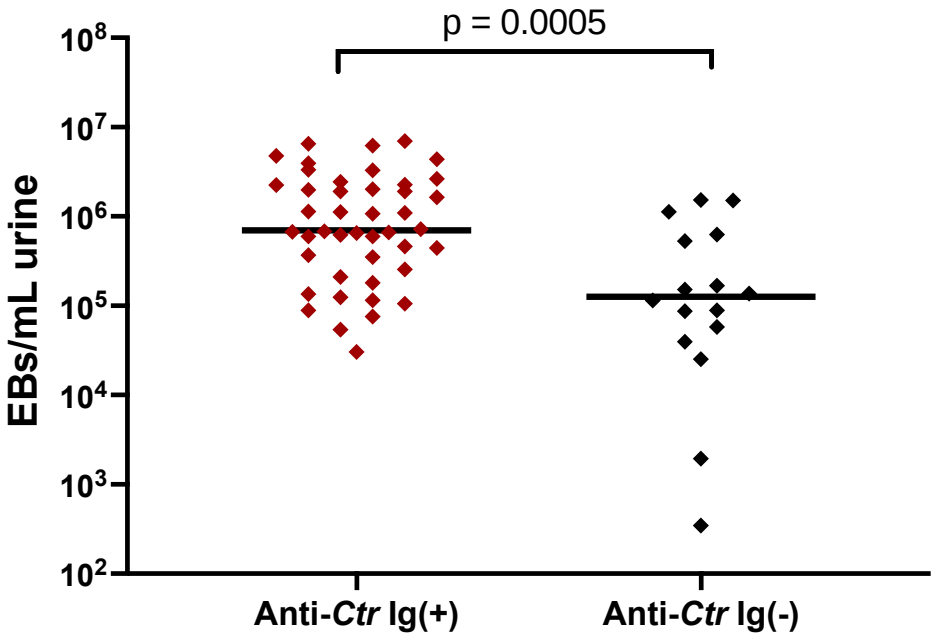

Figure 25: The detection of anti-*Chlamydia* antibodies was associated with higher EB loads in the urine of men with chlamydia.

Urine specimens from men with NAAT-confirmed chlamydia (N = 60) were tested for anti-*Chlamydia* IgA, IgG, and IgM (see Chapter III). Estimated loads of *Ctr* EBs were determined previously using q-PCR. EB loads were compared between men with chlamydia who did (N = 44) or did not (N = 16) exhibit detectable antibodies against *Ctr* (any isotype) in their urine. Statistical significance of the observed difference in EB loads was assessed using the non-parametric Mann-Whitney U test. The *P* value is indicated.

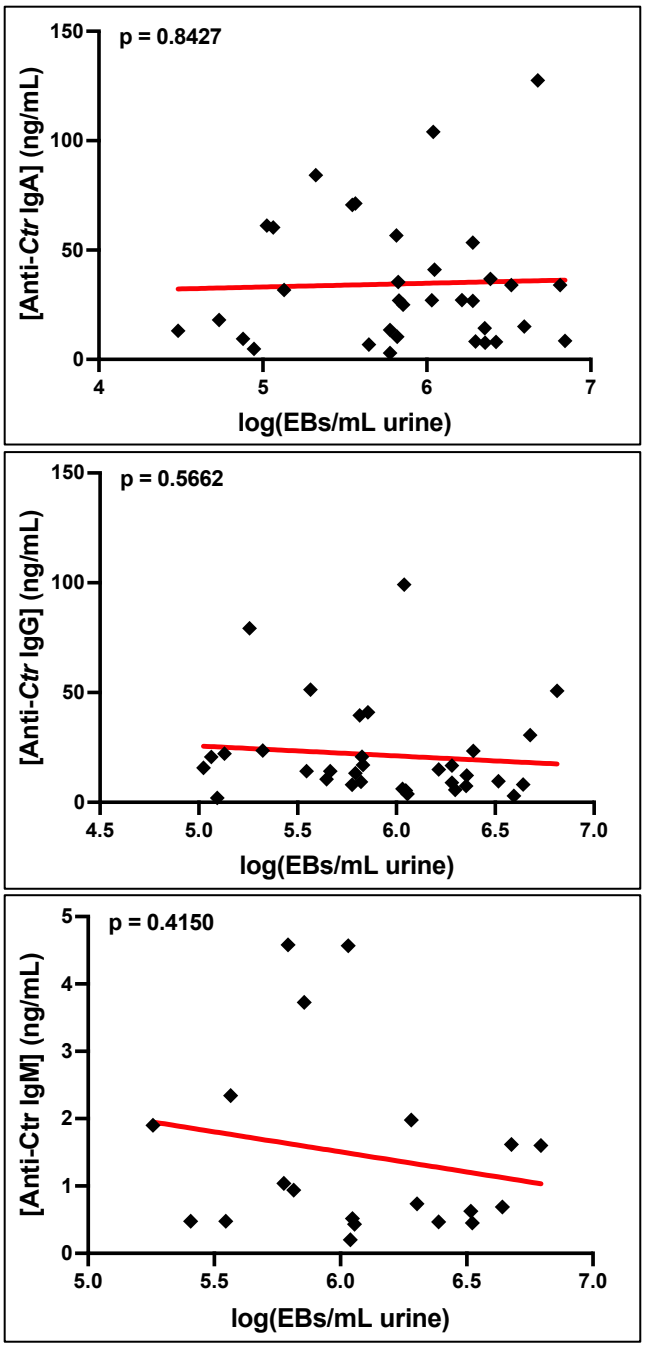

Figure 26. Estimated concentrations of anti-*Chlamydia* antibodies at enrollment did not correlate with EB loads in the urine of men with chlamydia.

Concentrations of anti-*Chlamydia* IgA, IgG, and IgM in the enrollment urine specimens of IUMP participants with chlamydia were estimated by quantitative indirect ELISA. Estimated concentrations of *Ctr* EBs were determined previously using q-PCR. Statistical significance of the relationships between anti-*Chlamydia* antibody concentrations and EB loads was assessed by simple linear regression: IgA, N = 34 (top); IgG, N = 33 (middle); IgM, N = 20 (bottom). *P* values for each comparison are indicated.

CHAPTER V – Discussion and Future Directions

Nongonococcal urethritis (NGU) is a prevalent, but not completely understood, genitourinary syndrome that affects millions of men in the United States each year. Whereas gonococcal urethritis is urethral inflammation caused specifically by infection with *Neisseria gonorrhoeae*, NGU encompasses all other causes of urethritis, including both infectious and potentially non-infectious etiologies. The sexually transmitted bacterial pathogens, *Chlamydia trachomatis* (*Ctr*) and *Mycoplasma genitalium* (*Mgen*) are the most frequently identified causes of NGU in men. These pathogens are important public health concerns because transmission of the microorganisms to female sexual partners puts these partners at risk for pelvic inflammatory disease and long-term complications including impaired fertility and ectopic pregnancy. However, for reasons that remain poorly understood, many individuals who acquire chlamydia or infections with *Mgen* do not develop morbidity that prompts them to seek medical care and antibiotic treatment.

In addition to the identification of asymptomatic individuals who require treatment, another particular challenge of effectively managing NGU is that many cases are idiopathic with no clear microbial etiology. Nucleic acid amplification testing (NAAT) is a sensitive and specific strategy for identifying cases of urethritis caused by *N. gonorrhoeae* and *Ctr*. However, testing for other urethral pathogens is not commonly performed in most healthcare settings. Through the Idiopathic Urethritis Men's Project (IUMP), the Nelson laboratory has focused its effort on better understanding the causes of idiopathic NGU and whether the composition of the urethral microbiome might affect susceptibility to sexually transmitted infections and/or the development of urethral

pathology. Surprisingly, nearly 2/3 of men who enrolled in the IUMP study with symptomatic idiopathic urethritis did not appear to be infected with *any* pathogen. Thus, we hypothesized that these urethritis cases were caused by a common etiologic agent of NGU that had already cleared spontaneously prior to resolution of the corresponding inflammation and symptoms.

Although nucleic acid tests are highly sensitive for a few NGU-causing pathogens, results may not be available for several days. Thus, there exists an urgent need for point-of-care diagnostic tests to identify infected individuals more readily. However, the development of such tools has been delayed by an incomplete understanding of the immune response(s) raised against these urethral pathogens. Moreover, expert opinion suggests that population-level prevention of the sequelae associated with chlamydia infections will require an efficacious vaccine against *Ctr*. Work performed in mouse models of chlamydia infection suggests that the vaccination of male animals, in addition to females, could have a synergistic effect in preventing transmission and/or immune-mediated inflammatory damage of the upper genital tract [124]. However, few studies have specifically investigated immune responses against *Ctr* infection in men, and it remains unclear how best to immunize men against chlamydia.

Despite the prevalence of urogenital chlamydia in men, we are aware of only a single study that has evaluated cytokine and adaptive immune responses in infected men [85]. Pate and colleagues observed elevated levels of total Igs and *Chlamydia*-specific Igs in urethral swab specimens from men with chlamydia compared to uninfected controls. My findings generally corroborate these results, although it is noteworthy that the magnitude of the increase in total IgG that I observed among men with chlamydia was

somewhat lower than these authors. This could suggest that local production of IgG has a particular role in the inflamed or chlamydia-infected urethra.

Using a modified ELISA approach developed for the analysis of *Chlamydia*-specific antibodies in cervicovaginal secretions, cytobrush specimens, and sera [104], I analyzed first-catch urine (FCU) specimens collected from IUMP study participants at enrollment and follow-up. I found that total urine Igs were increased in men with NGU compared to controls and that concentrations of total IgA, but not IgG or IgM, remained elevated at one-month follow-up in men with chlamydia. I also found that IgA, IgG, and IgM antibodies that could recognize *Ctr* EBs were significantly more prevalent in men with chlamydia compared to controls. The prevalence of these anti-*Chlamydia* antibodies was lower among men with idiopathic urethritis (IU) compared to chlamydia. However, a retrospective analysis of IU-group participants revealed that men with pathogen-negative (PN), but not pathogen-positive (P^+), IU more frequently had anti-*Ctr* antibodies in their urine specimens compared to non-NGU controls. Additionally, among men with chlamydia, I found that the presence of detectable urine antibodies against *Ctr* was at least partially serovar-independent and was instead strongly associated with elevated urine loads of chlamydial EBs.

Taken together, my results provide proof-of-principle that *Chlamydia*-specific antibodies can be detected in male first-catch urine specimens. Thus, we propose that the longitudinal collection of urine specimens represents a simple, non-invasive approach to repeatedly sampling urethral immune responses. My results also demonstrate an intriguing association between PN-IU cases and the detection of anti-*Chlamydia* urine antibodies. This finding suggests that idiopathic urethritis is related to the spontaneous

clearance of an incident chlamydia infection. In the remainder of this chapter, I will summarize and discuss the implications of these findings and how the approaches used here can be applied to future studies of men with urethritis. In particular, I will describe how the method I adapted for this investigation can be further adjusted for use in natural history studies. Such studies will be essential to better define the course(s) of urethral (local) and systemic anti-*Chlamydia* immune responses in men. I will also discuss limitations of the current study and how these might be addressed in future work.

Discussion

Over fifty years ago, Burdon observed that urine voided through the urethra contained higher total concentrations of IgA, but not IgG, compared to urine aspirated directly from the bladder [125]. This result suggested that IgA antibodies produced locally in the urethra and secreted into the urethral lumen could be displaced by the flow of urine in appreciable quantities. It also suggested that local production of IgG in the genitourinary tract is not a major contributor to overall IgG concentrations in the urine of healthy men.

Later, extensive dissection and immunohistochemical (IHC) staining of urogenital tissues obtained from cadavers uncovered various cell populations and innate immune effectors that are likely to have roles in the defense against pathogens. Pudney and Anderson identified antibody-secreting plasma cells producing IgA, IgM, and IgG in the urethral lamina propria and showed that polymeric Ig receptor (pIgR) is highly expressed in the urethral epithelium [126]. Antibodies produced locally in the urethra require an active mechanism of transport across the polarized urethral epithelium to the lumen

where they can be collected by urethral swab or in voided urine. pIgR can serve this function by facilitating the transcytosis of polymeric IgA and IgM (but not monomeric IgG) antibodies. More recently, Gupta and colleagues demonstrated that the neonatal Fc receptor (FcRn)—so-called because it was discovered first in neonatal rats [127]—is also expressed in tissues of the human penile urethra [128]. These receptors, which had previously been identified in the female genital tract [129], can facilitate the transport of IgG across mucosal epithelia [130]. Thus, there is at least one mechanism, in addition to serum transudation, that allows for locally produced IgG to contribute to the humoral immune defense of these sites.

In the first part of this study, I investigated total immunoglobulins in the urines of men with and without nongonococcal urethritis The results of a previous study generally support the accuracy of my urine-based ELISA approach. In the first-catch urine specimens of non-NGU controls included in this study, I observed median total IgA and IgG concentrations of 1.576 ug/mL and 4.917 ug/mL, respectively. These values compare favorably with the results of a previous study that used a similar approach to evaluate urine immunoglobulin concentrations [131]. Among 24 healthy men, Kuriyama reported a mean urine IgA concentration of 107 ug/dL (1.07 ug/mL) and a mean urine IgG concentration of 371 ug/dL (3.71 ug/mL). However, this study also reported a mean IgM concentration of 390 ng/mL which was somewhat higher than I observed among the IUMP healthy controls (56.2 ng/mL). Even among men with acute chlamydia who might be expected to have been recently infected, I observed a median urine IgM concentration of only 203.3 ng/mL. However, it is unclear whether this discrepancy reflects a difference in methodology or a true difference in study populations.

Anti-*Chlamydia* IgA, IgG, and IgM antibodies were commonly observed in men with chlamydia. Surprisingly, IgM antibodies reactive against *Ctr* serovar D EBs were also commonly observed among men infected with *M. genitalium*. This may be the result of two different factors. First, anti-*Chlamydia* IgM was relatively rare in FCU specimens and was tested at a dilution of just 1/2. Furthermore, IgM antibodies are the first isotype produced during the immune response to a pathogen and have relatively lower affinity for specific epitopes than IgA or IgG antibodies. Thus, it is possible that infection with *M. genitalium* naturally generates IgM antibodies that recognize antigens on the cell surface that are similar to those present on the EB cell envelope.

All men with NGU who enrolled in the IUMP study were treated empirically with antibiotics according to the CDC-recommended standard of care [7]. Azithromycin has good efficacy against *Ctr* and most of the men who presented with chlamydia in this study achieved microbiologic cure at follow-up. Strikingly, we observed similar rates of anti-*Chlamydia* IgA antibody detection in these men at enrollment compared to follow-up. This finding suggests that IgA reactive against *Ctr* may persist in the urethra for some time following the resolution of an infection. In contrast, both total IgG concentrations and the detection of anti-*Chlamydia* IgG antibodies were reduced at follow-up among this group compared to enrollment. Thus, unlike anti-*Chlamydia* IgA which may be suggestive of previous chlamydia infection, IgG appears to be indicative of a current infectious process. Thus, it is not surprising that anti-*Mycoplasma* IgG antibodies remained prevalent at follow-up among men with *M. genitalium* infection as many of these individuals were observed to experience azithromycin treatment failures.

Previous studies of chlamydial pathogenesis have occasionally studied the associations between anti-*Ctr* antibodies, *Ctr* serovar, and organism burden, but clear relationships between these variables have not been established. In the second part of this study, I evaluated these parameters in FCU specimens from well-characterized men with NAAT-confirmed chlamydia. First, I determined the serovar and serogroup distributions of *Ctr* isolates from men in the IUMP cohort. This revealed that more than half of the men who enrolled in the IUMP study were infected with *Ctr* serovars E and D and that serovars J/Ja, I/Ia, and F were also common. Interestingly, serovars E and D, followed by F, I/Ia, and J/Ja were also the most commonly observed genotypes in a previous study of women that was conducted in the same geographic area [132]. This finding suggests that the serovar distributions of chlamydia infections within a population remain stable over time. Other studies that have determined the genotypes of *Ctr* isolates also reported that serovar E strains were the most commonly observed among populations of women in the southeastern United States [133] and men in Italy [134] and Greece [135].

After determining the infecting serovars of men with chlamydia included in my antibody studies, I evaluated whether the detection of anti-*Chlamydia* antibodies using the ELISA approach I developed was significantly influenced by serovar or serogroup of infection. Given the results of other studies that have used similar methods to detect anti-*Chlamydia* antibodies [104, 133], I expected that antibodies generated during chlamydia infection could recognize serovar-independent epitopes on the EB surface. As hypothesized, I found that antibodies against *Ctr* of at least one isotype were present in urine specimens from men infected with almost all of the serovars we identified. However, the extent to which these results may have been affected by the selection of

serovar D EBs as the capture antigen for my ELISA assay remains unclear. In this study, the number of observations per serovar was too low to effectively identify differences in the rate of anti-*Chlamydia* antibody detection across serovars. Nonetheless, future users of this ELISA approach should consider the use of pooled EBs with multiple representative serovars.

I also observed differences in urine EB loads among men infected with different chlamydial serovars. In particular, I found that loads were higher in men infected with serovar F strains compared to serovars E and K. Previous studies have generated mixed results concerning the relationship between chlamydial genotypes, EB loads, and pathogenesis. Eckert and colleagues observed that individuals infected with serogroup B strains had a higher infectious burden than those infected with serogroup C strains [136]. In contrast, Gomes *et al*. observed that chlamydial loads were similar across serovars and also similar between individuals with symptomatic versus asymptomatic infections [137].

Intriguingly, I found that the detection of anti-*Chlamydia* antibodies in the FCU specimens of men with chlamydia was significantly associated with EB loads. This difference remained significant across each individual antibody isotype and suggests that some threshold level of *Ctr* antigen must be present in order to mount an adaptive immune response against this pathogen. However, carefully designed natural history studies of chlamydia infection that allow for comparisons between EB infectious loads and the generation of antibodies over time will be particularly instructive for understanding the development of urethral immune responses. In particular, longitudinal studies with regular sampling intervals will be needed to identify which factors affect the risk of chlamydia acquisition or spontaneous clearance, which might predict the

development of urethritis symptoms and/or urethral antibodies, and which are affected by the use of standard or non-standard antibiotic therapies.

Taken together, the results of this dissertation suggest that we may be arriving at an important inflection point in terms of the clinical management of nongonococcal and idiopathic urethritis. The development of point-of-care diagnostic tests to identify etiologic agents of NGU could transform the ways that healthcare providers use antibiotics for urethritis in both primary care and STI clinic settings. Furthermore, the work described here indicates that such tests for chlamydia may be viable using first-catch urine and should, at least in men, prioritize the detection of ant-*Ctr* IgG as a biomarker for acute or on-going infection. Moreover, the analysis of male first-catch urine specimens could present exciting new opportunities for performing longitudinal clinical investigations in men.

Ultimately, the findings of this project also support the hypothesis that many IU cases could actually represent urethral inflammation that persists following spontaneous clearance of chlamydia. Thus, this work strongly suggests that the sexual partners of men with IU could be at substantially increased risk for asymptomatic or "silent" chlamydia infections. Given the consequences such asymptomatic infections can have in women, we believe there is now compelling evidence to support the urgent need for novel study designs that investigate chlamydia transmission within sexual partnerships. Such studies will be essential to more fully interrogate the hypothesis that pathogen-negative idiopathic urethritis is not caused by an unknown or emerging pathogen, but by one of the more prevalent STI on the planet.

APPENDIX – Antibiotic Susceptibility Testing of *Haemophilus* spp. Isolates

Introduction:

Haemophilus influenzae is a Gram-negative, facultatively anaerobic bacterium that colonizes the human nasopharynx and respiratory tract [138]. *H. influenzae* infection commonly causes otitis media and sinusitis in children and exacerbations of chronic obstructive pulmonary disease (COPD) in susceptible adults [138, 139]. More recently, *H. influenzae* and other closely related *Haemophilus* spp. have been associated with nongonococcal urethritis following unprotected oral sex [140, 141]. We previously cultured 11 *Haemophilus* spp. isolates from the urethral specimens of six IUMP participants with acute urethritis (five at enrollment & one at follow-up). Whole-genome sequencing and phylogenetic analysis revealed that four of these isolates were strains of *H. influenzae* or *H. quintini*. The remaining seven isolates mapped to the poorly characterized clade *Haemophilus* sp. paraurethrae. We wanted to determine if the first- (azithromycin, doxycycline) or second-line (moxifloxacin) standard-of-care antibiotics for NGU would be effective at resolving infections with *Haemophilus* organisms.

Methods:

Antibiotic zones of inhibition for each *Haemophilus* spp. isolate were determined by disk diffusion. Testing was performed according to the procedure described in the Clinical and Laboratory Standards Institute (CLSI) document M100 (32nd edition) [142] with minor modifications for material availability. Briefly, frozen stocks of IUMP *Haemophilus* spp. isolates were streaked for single-colony isolation on chocolate agar

and incubated at 35˚C in a 5% CO_2 environment. Following incubation, individual colonies (~ 4-5 per isolate) were suspended in 2 mL PBS and adjusted to an OD_{625nm} between 0.08 and 0.13 (equivalent to 0.5 McFarland turbidity). Freshly prepared *Haemophilus* spp. suspensions in PBS (60 µL per isolate) were spread over the surface of 100-mm *Haemophilus* Test Medium (HTM) plates (Remel) using sterile inoculating loops. Inoculated HTM plates were allowed to dry for 15 minutes at room temperature before applying paper antibiotic diffusion disks containing azithromycin (15 µg), moxifloxacin (5 µg), or tetracycline (5 µg). (Note that the 5 µg tetracycline disks used here differ from the 30 µg CLSI standard.) Finally, the HTM plates were inverted and incubated at 35˚C/5% CO_2 for 16-18 hours. Zones of inhibition were determined manually by measuring, to the nearest millimeter, the diameter of cleared area surrounding each antibiotic diffusion disk. Susceptibility of each *Haemophilus* spp. isolate to the three antibiotic compounds was assessed according to the "Zone Diameter Breakpoints" described in CLSI M100 (Table 2E).

Results:

All stored *Haemophilus* spp. isolates were culturable on chocolate agar within two attempts. Across two replicates of antibiotic resistance testing, I observed a total of five distinct colony morphologies which I designated A – E. Descriptions of each morphology are as follows: (A) white/off-white, slightly mucoid colonies; (B) dull grey, non-mucoid colonies; (C) dull grey, mucoid colonies; (D) small white colonies interspersed with grey mucoid colonies; and (E) dull grey, slightly mucoid colonies. Colony morphology types on chocolate agar for each *Haemophilus* spp. isolate are listed in Table 3. Of the 10

isolates that were cultured on chocolate agar for re-testing (replicate #2), six were observed to have the same colony morphology type. The colony morphologies of four isolates differed between replicates.

Azithromycin. Susceptibility to azithromycin (AZM), the previous CDC-recommended first-line therapy for NGU, was assessed using 15 μg disks. The zone of inhibition breakpoint that delimits AZM-susceptibility in *Haemophilus* spp. is ≥ 12 mm. I identified four *Haemophilus* isolates obtained from two IUMP participants that exhibited antibiotic resistance to AZM (Table 4). Another two isolates (specimens #411 and #640) had disparate resistance phenotypes between replicates.

Tetracycline. The tetracycline-class antibiotic, doxycycline, has replaced AZM as the CDC-recommended first-line therapy for NGU. Susceptibility to tetracycline (TE) was assessed using non-standard 5 μg disks. The zone of inhibition breakpoint for TE susceptibility in *Haemophilus* spp. is ≥ 29 mm with 30 μg antibiotic disks. Here, I identified seven isolates with average zones of inhibition ≥ 19 mm that would likely reach the 29 mm threshold for TE susceptibility if 30 μg disks were used (Table 5). Two isolates obtained from IUMP participant 102-1 (specimens #631 and #640) were putatively resistant to TE with average zones of inhibition less than 10 mm. A third isolate from this participant (specimen #646) demonstrated disparate TE-resistance phenotypes between replicates; only one replicate of disk diffusion testing was performed on the fourth isolate (specimen #637). As noted in CLSI M100, however, resistance against TE does not conclusively indicate resistance against doxycycline, specifically.

IUMP Participant	Specimen #	Species	Colony Morphology	
			Replicate #1	Replicate #2
3-0	**79**	*H.* sp. paraurethrae	**A**	**A**
35-0	**289**	***H. influenzae***	**B**	**E**
	322	***H. influenzae***	**B**	**E**
38-0	**301**	***H. influenzae***	**C**	not tested
80-0	**411**	*H.* sp. paraurethrae	**C**	**C**
	416	*H.* sp. paraurethrae	**C**	**C**
87-0	**446**	***H. quintini***	**C**	**E**
102-1	**631**	*H.* sp. paraurethrae	**D**	**D**
	637	*H.* sp. paraurethrae	**D**	**D**
	640	*H.* sp. paraurethrae	**A**	**C**
	646	*H.* sp. paraurethrae	**C**	**C**

Table 3. Colony morphology on chocolate agar of IUMP *Haemophilus* spp. isolates
(See descriptions of colony morphology types on page 90.)

IUMP Participant	Specimen #	Azithromycin (AZM) Zones of Inhibition (mm)			Interpretation
		Replicate #1	Replicate #2	Average (SD)	
3-0	**79**	0	0	**0.0 (0.0)**	**resistant**
35-0	**289**	26	24	**25.0 (1.4)**	susceptible
	322	24	23	**23.5 (0.7)**	susceptible
38-0	**301**	23	n/a	**23.0 (n/a)**	susceptible
80-0	**411**	17	0	**8.5 (12.0)**	inconclusive
	416	15	15	**15.0 (0.0)**	susceptible
87-0	**446**	17	16	**16.5 (0.7)**	susceptible
102-1	**631**	0	0	**0.0 (0.0)**	**resistant**
	637	n/a	0	**0.0 (n/a)**	**resistant**
	640	0	16	**8.0 (11.3)**	inconclusive
	646	0	0	**0.0 (0.0)**	**resistant**

Table 4. Azithromycin (15 µg) zones of inhibition for *Haemophilus* spp. on HTM

		Tetracycline (TE) Zones of Inhibition (mm)			
IUMP Participant	**Specimen #**	**Replicate #1**	**Replicate #2**	**Average (SD)**	**Interpretation**
3-0	**79**	21	20	**20.5 (0.7)**	likely susceptible
35-0	**289**	24	26	**25.0 (1.4)**	likely susceptible
	322	23	25	**24.0 (1.4)**	likely susceptible
38-0	**301**	24	n/a	**24.0 (n/a)**	likely susceptible
80-0	**411**	18	20	**19.0 (1.4)**	likely susceptible
	416	18	20	**19.0 (1.4)**	likely susceptible
87-0	**446**	24	22	**23.0 (1.4)**	likely susceptible
102-1	**631**	0	8	**4.0 (5.7)**	**putative resistant**
	637	n/a	10	**10.0 (n/a)**	inconclusive
	640	8	10	**9.0 (1.4)**	**putative resistant**
	646	9	28	**18.5 (13.4)**	inconclusive

Table 5. Tetracycline (5 µg) zones of inhibition for *Haemophilus* spp. on HTM

		Moxifloxacin (MXF) Zones of Inhibition (mm)			
IUMP Participant	**Specimen #**	**Replicate #1**	**Replicate #2**	**Average (SD)**	**Interpretation**
3-0	**79**	27	28	**27.5 (0.7)**	susceptible
35-0	**289**	35	36	**35.5 (0.7)**	susceptible
	322	37	36	**36.5 (0.7)**	susceptible
38-0	**301**	33	n/a	**33.0 (n/a)**	susceptible
80-0	**411**	22	21	**21.5 (0.7)**	susceptible
	416	23	22	**22.5 (0.7)**	susceptible
87-0	**446**	31	27	**29.0 (2.8)**	susceptible
102-1	**631**	25	24	**24.5 (0.7)**	susceptible
	637	n/a	28	**28.0 (n/a)**	susceptible
	640	24	23	**23.5 (0.7)**	susceptible
	646	24	29	**26.5 (3.5)**	susceptible

Table 6. Moxifloxacin (5 µg) zones of inhibition for *Haemophilus* spp. on HTM

Moxifloxacin. Susceptibility to moxifloxacin (MXF) was assessed using 5 μg disks. MXF is recommended as second-line therapy for urethritis cases that persist after unsuccessful first-line treatment with AZM or doxycycline. The zone of inhibition breakpoint for MXF-susceptibility in *Haemophilus* spp. is ≥ 18 mm. All of the isolates I tested here were susceptible to MXF (Table 6).

Conclusions:

Haemophilus influenzae can colonize the male urethra and has been proposed as an emerging etiologic agent of nongonococcal urethritis. Eleven urethral isolates of *Haemophilus* spp. were tested for susceptibility to standard-of-care NGU antibiotics. Some of these isolates exhibited resistance to azithromycin and/or tetracycline, which suggests that microbiological cure of urethral *Haemophilus* infections may not be achieved with recommended first-line therapies for NGU. However, the second-line antibiotic, moxifloxacin, demonstrated good efficacy against all *Haemophilus* isolates tested. Collectively, these results indicate that existing standard-of-care treatment regimens for NGU are likely to resolve most *H. influenzae* infections in the urethra.

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Abstracts

1. **Ryan, JD**, *et al*. Anti-Chlamydia antibodies in first-catch urine from males with acute *Chlamydia trachomatis* mono-infection or idiopathic non-gonococcal urethritis [abstract]. Chlamydia Basic Research Society, 11th Biennial Meeting; Omaha, NE, 2023 (oral presentation)
2. **Ryan, JD**, *et al*. Investigating epithelial cell-autonomous immune responses against acute *Chlamydia trachomatis* infection [abstract]. Midwest Microbial Pathogenesis Conference; East Lansing, MI, 2021 (poster presentation)
3. **Ryan, JD**, *et al*. Epithelial cell-autonomous immune responses against acute *Chlamydia trachomatis* infection [abstract]. Indiana Clinical and Translational Sciences Institute, 2021 Annual Meeting; virtual, 2021 (oral mini-presentation)
4. **Ryan, JD**, *et al*. Pathogen-specific metabolic pathways and innate immune responses associated with *Chlamydia trachomatis* infection and other STIs [abstract]. Association for Clinical and Translational Science TS21 (annual meeting); virtual, 2021 (e-poster presentation)

Publications

1. **Ryan JD***, Toh E*, Jordan SJ, Nelson DE. Investigation of the relationship between urine *Chlamydia trachomatis* loads and anti-chlamydial antibody responses in men with chlamydial urethritis. 2023. In preparation.
2. Toh E*, Xing Y*, **Ryan JD***, Gao X, Jordan SJ, Batteiger TA, Dong Q, Nelson DE. Male idiopathic urethritis is a complex syndrome that can be elicited by novel infectious and non-infectious etiologies. 2023. In preparation.

3. **Ryan JD**, Nelson DE. A Same-Genus Screening Approach Reveals Novel Effectors and New Possibilities for Investigating Chlamydia Pathogenesis. *J Bacteriol*. 2021 Mar 22; 203(11): e00157-21.
4. Jordan SJ, Toh E, Williams JA, Fortenberry LJ, LaPradd M, **Ryan JD**, Nelson DE, Batteiger TA. No Pathogen-Specific Sign or Symptom Predicts the Etiology of Monomicrobial Nongonococcal Urethritis in Men. *Sex Transm Dis*. 2020 May; 47(5): 329-331.
5. Tsui HC, Zheng JJ, Magallon AN, **Ryan JD**, Yunck R, Rued BE, Bernhardt TG, Winkler ME. Suppression of a deletion mutation in the gene encoding essential PBP2b reveals a new lytic transglycosylase involved in peripheral peptidoglycan synthesis in Streptococcus pneumoniae D39. *Mol Microbiol*. 2016; 100(6): 1039-65.

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