Universitat Autònoma de Barcelona

Epidemiology and antibiotic resistance in Mycoplasma genitalium

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Epidemiology and antibiotic resistance in *Mycoplasma genitalium*

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Dr. Tomàs Pumarola y **Dr. Oscar Quijada-Pich**

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Dr. Tomàs Pumarola

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Beyond the visible

Miguel Fernández-Huerta

The research presented in this thesis was carried out at the Microbiology Department of the Vall d´Hebron University Hospital, Universitat Autònoma de Barcelona (UAB), Spain.

El **Dr. Tomàs Pumarola Suñé**, Jefe del Servicio de Microbiología Clínica del Hospital Universitario Vall d´Hebron, y el **Dr. Oscar Quijada Pich**, investigador postdoctoral en el Servicio de Microbiología del Hospital Universitario Parc Taulí

CERTIFICAN

que el presente trabajo de investigación titulado "Epidemiology and antibiotic resistance in *Mycoplasma genitalium*", y presentado por el Graduado en Bioquímica por la Universidad de Navarra y Especialista en Microbiología Clínica por el Hospital Universitario Vall d´Hebron, **Miguel Fernández Huerta**, ha sido realizado en el Servicio de Microbiología Clínica del Hospital Universitario Vall d´Hebron bajo su dirección y cumple las condiciones exigidas para ser presentado y defendido como Tesis Doctoral del Departamento de Genética y Microbiología ante el tribunal que corresponda.

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GLOSSARY

NGU	Non-gonococcal urethritis
PCR	Polymerase chain reaction
SLSBT	Single-locus-sequence-based typing
STI	Sexually transmitted infection
MSM	Men who have sex with men
STD	Sexually transmitted disease
LGV	Lymphogranuloma venereum genotypes
PID	Pelvic inflammatory disease
HIV	Human immunodeficiency virus
SARA	Sexually acquired reactive arthritis
NAAT	Nucleic acid amplification test
rRNA	Ribosomal RNA
MIC	Minimum inhibitory concentration
TOC	Test-of-cure
QRDR	Quinolone-resistance determining region
qPCR	Real-time (quantitative) PCR
IQR	Interquartile range
CI	Confidence interval
MSW	Men who have sex with women
POC	Point-of-care
DrasExp	Drassanes Exprés
MSMBI	Bisexual men
ST	Sequence type

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Summary

From providing the first antibiotic resistance estimates in *Mycoplasma genitalium* in Spain, to suggesting the implementation of novel treatment strategies against this sexually transmitted infection. This manuscript provides, through a comprehensive analysis of the infection in our settings, global insights regarding *M. genitalium* infection and antimicrobial resistance.

Chapter 1 updates and summarizes the clinical and epidemiological evidence of the infection to date, highlighting also some basic aspects of the physiology and pathogenesis of the bacterium. Chapter 2 "Antibiotic resistance: where are we now?" provides estimates regarding macrolide and fluoroquinolone resistance in *M. genitalium* in Barcelona, Spain, through a cohort study performed between 2016 and 2017. Additionally, the chapter reviews and describes the regional and European evolution of antibiotic resistance in *M. genitalium* in the last decade. On the other hand, chapter 3 "*Mycoplasma genitalium*: should we screen and how?" and chapter 4 "Transmission dynamics in *Mycoplasma genitalium*" are focused on asymptomatic infections; addressing the prevalence of *M. genitalium* and antimicrobial resistance among asymptomatic individuals, and revealing the transmission dynamics of the infection. Finally, chapter 5 discusses the main conclusions of this thesis work; culminating with the proposal of a novel treatment algorithm based on the findings and the evidence provided along this manuscript.

Resumen

Desde aportar las primeras estimaciones en relación a la resistencia antibiótica en *Mycoplasma genitalium*; a sugerir la implementación de nuevas estrategias terapéuticas frente a esta infección de transmisión sexual. A través de un análisis exhaustivo de la infección en nuestro entorno, este manuscrito proporciona reflexiones globales en torno a la infección por *M. genitalium* y la resistencia antimicrobiana.

El capítulo 1 actualiza y resume la evidencia clínica y epidemiológica en relación a la infección hasta la fecha, destacando también algunos aspectos básicos de la fisiología y patogénesis de la bacteria. El capítulo 2 "Antibiotic resistance: where are we now?"

proporciona estimaciones en torno a la resistencia a macrolidos y fluoroquinolonas en *M. genitalium* en Barcelona, España, a través de un estudio de cohortes realizado entre 2016 y 2017. Además, el capítulo revisa y describe la evolución regional y Europea de la resistencia antibiótica en *M. genitalium* en la última década. Por otro lado, el capítulo 3 "*Mycoplasma genitalium*: should we screen and how?" y el capítulo 4 "Transmission dynamics in *Mycoplasma genitalium*" se centran en las infecciones asintomáticas; profundizando en la prevalencia de *M. genitalium* y las resistencias antibióticas entre población asintomática, y revelando la dinámica de transmisión de la infección. Finalmente, el capítulo 5 discute las principales conclusiones de la tesis; culminando con la propuesta de un novedoso algoritmo terapéutico basado en los hallazgos y la evidencia proporcionada a lo largo de este trabajo.

CHAPTER 1: General introduction

Key messages

- *Mycoplasma genitalium* is a sexually transmitted pathogen, and a frequent cause of urethritis and further urogenital syndromes in both men and women.
- Prevalence of macrolide resistance, exceeding 50% in many countries, is around 16%-35% in Spain.
- Fluoroquinolone resistance, the second-line treatment for *M. genitalium* infection, is alarmingly emerging in Spain.
- Testing for *M. genitalium* among asymptomatic individuals is currently not recommended.

1. Introduction

Mycoplasma genitalium was first isolated in 1981 from urethral swabs of men with non-gonococcal urethritis (NGU) [1]. Since its discovery, *M. genitalium* has been well recognized as a frequent cause of urethritis and several other urogenital syndromes in both men and women [2]. In spite of the disturbing pathogenic potential of the bacterium, emergence of antibiotic resistance is an important matter of concern in *M. genitalium*. Due to the current widespread of macrolide resistance worldwide and the emergence of quinolone resistance in many countries [3], *M. genitalium* may soon become untreatable [4]. Although diagnosis has considerably improved since the use of the first polymerase chain reactions (PCRs) [5], *M. genitalium* is still underdiagnosed in many settings in Europe. Additionally, very few laboratories are able to provide assays capable of co-detecting *M. genitalium* and macrolide resistance markers so most clinicians are sentenced to use azithromycin despite its limited efficacy in many cases [6]. On the other hand, partner notification and infection control is especially complicated given the usual presence of multiple sexual partners, which are often anonymous. Although testing for *M. genitalium* among asymptomatic individuals might, indeed, reduce its prevalence [7], experts currently plead to avoid widespread screening because of the lack of scientific evidence and the worrying emergence of antibiotic resistance in *M. genitalium*, particularly to macrolides [8].

The main objective of this introductory chapter is to update the reader about the most relevant issues in *M. genitalium*. So, epidemiological, microbiological and clinical aspects of the infection are discussed through a comprehensive evaluation of the literature.

2. Pathogenesis

Mycoplasmas are the smallest free-living microorganisms. Unlike conventional bacteria, mycoplasmas are bounded by a triple-layered cholesterol-enriched membrane but lack a rigid cell wall. Additionally, through evolutionary reductions in genetic material, *M. genitalium* 580 kb genome is one of the shortest among bacteria. Consequently, *M. genitalium* has very restricted metabolic functions and relies on the host-cell to obtain growth factors and nutrients, which are essential in the infection and persistence stages [2]. The limited genomic complexity of *M. genitalium* is also responsible for its simple but intriguing structure, based on the existence of a complex cytoskeleton. These bacteria are predominantly flask/bottle shaped with a prominent tip structure, known as the attachment organelle, key in adhesion, motility and cell division (figure 1A) [9]. Thus, the surface-exposed proteins in this organelle P110 and

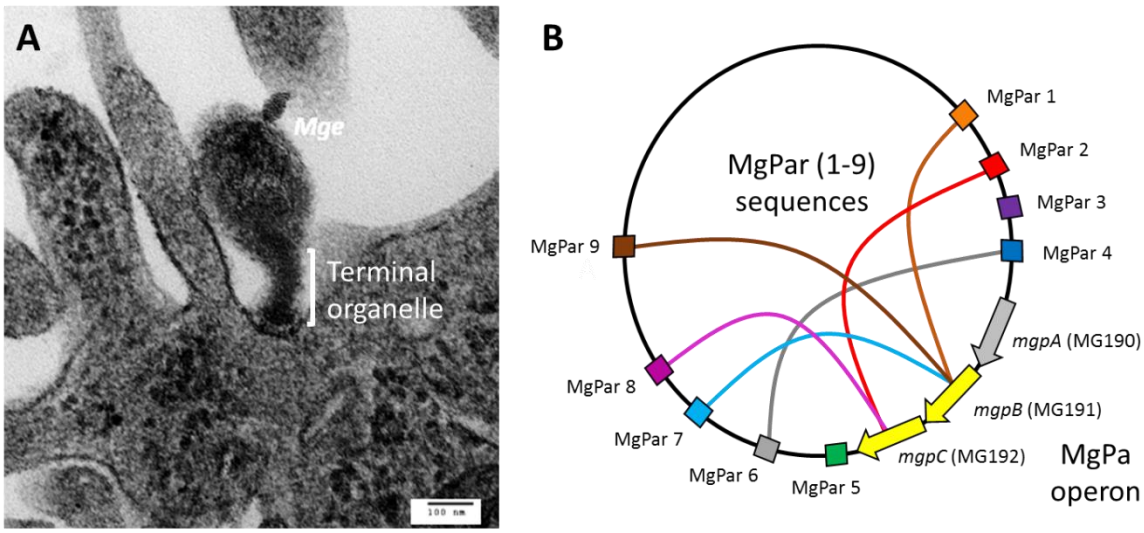

Figure 1. A: Electron micrograph exemplifying the flask shape and the characteristic terminal organelle of *M. genitalium* (*Mge*). **B:** Schematic *M. genitalium* genome showing the MgPa operon and the 9 MgPar sequences. The *mgpA* (MG190), *mgpB* (MG191) and *mgpC* (MG192) genes compose the MgPa operon. Although *mgpA* is a highly conserved gene, *mgpB* and *mgpC* contain variable regions that may suffer from a diversifier recombination with the homologous variable sites found in the MgPar sequences. While the *mgpB* and *mgpC* genes encode the major adhesins P140 (MgpB) and P110 (MgpC), respectively, the *mgpA* gene is hypothesized to account for an enzyme controlling the recombination process (image adapted from Fernández-Huerta M et al., 2019).

P140 (also known as MgpC and MgpB, respectively) represent a crucial virulence factor for *M. genitalium* [10].

Like in many other bacterial infections, the thin equilibrium between the pathogen and the host immunity determines the establishment and persistence of *M. genitalium* infection in the reproductive tract tissues (figure 2). Briefly, the bacterium is well

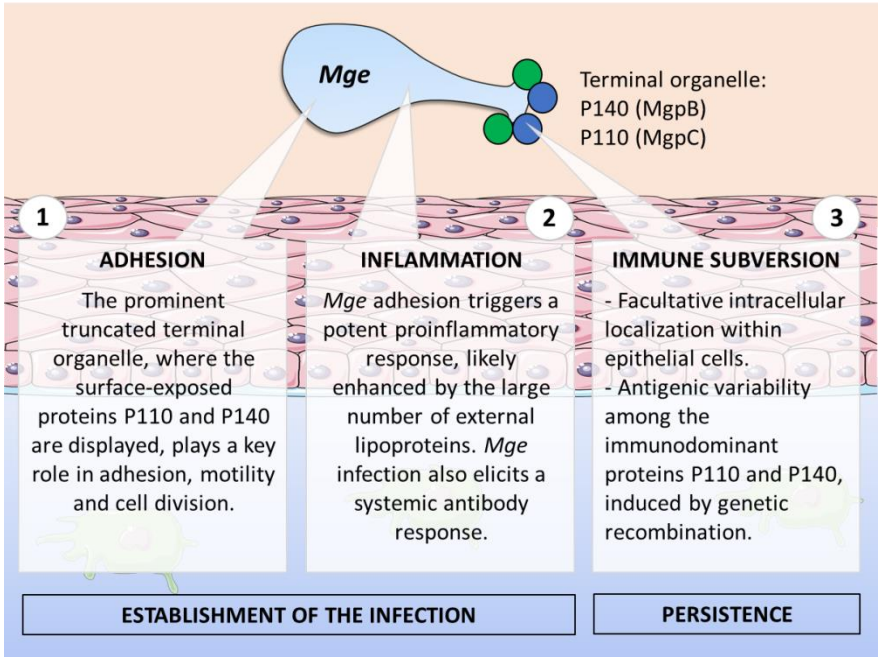

Figure 2. Stages in acute and chronic *M. genitalium* (*Mge*) infection.
M. genitalium initial adhesion and inflammation is likely key in acute processes. The posterior development of immune subversion responses is responsible for the persistence of *M. genitalium* in the reproductive tract tissues and the appearance of chronic syndromes.

associated with the inflammatory processes of urethritis in men and cervicitis in women. Adhesion of *M. genitalium* to host epithelial cells triggers acute inflammatory signals that result in leukocyte recruitment to the site of infection [11]. Due to the lack of recognized toxins or secreted factors, the large number of external lipoproteins in *M. genitalium* is likely contributing to this potent proinflammatory response. Additionally, *M. genitalium* infection also elicits a systemic antibody response in humans, primarily against proteins P110 and P140 [12]. In fact, these specific antibodies can be detected in genital tract mucosa although there is no evidence as to whether or not this response protects from subsequent exposures to *M. genitalium* [13]. Despite the seemingly clear induction of innate and adaptive immunological responses to infection, the bacterium has a remarkable ability to evade host defenses and persist leading to chronic urogenital infections, especially in women [14]. First,

facultative intracellular localization within epithelial cells constitutes an important strategy to avoid host defenses, particularly to phagocytosis by macrophages [2]. On the other hand, the *M. genitalium* genome possesses the MgPa operon (encoding for P110 and P140) but also the called MgPar sequences containing partial copies of both *mgpB* (MG191) and *mgpC* (MG192) genes (figure 1B) [15]. Recombination between the MgPar sequences and the MgPa operon produces an enormous genetic diversity and provides the means to generate antigenic variability among the immunodominant proteins P110 and P140. This striking capability of *M. genitalium* to generate extensive protein variants provides a fascinating and effective mechanism for immune system subversion.

3. Epidemiology

Prevalence

Few studies have estimated the prevalence and incidence of *M. genitalium*, especially due to the lack of accessible diagnostic tools. Additionally, the enormous methodological variability regarding the population of study, sampling procedure and laboratory techniques among reports hinder the understanding of the global burden of *M. genitalium* disease [16]. Therefore, these studies should extensively contextualize the methodology and control the putative bias, limiting deviations in epidemiological parameters among medical community. Overall, the prevalence of *M. genitalium* ranges from 1% to 4% among general population [17,18]. Nevertheless, these estimates may widely vary from 0% to 48% depending on whether the study includes high-risk or low-risk subjects [16].

Transmission

Although *M. genitalium* was traditionally detected and isolated from ano-genital samples [1], sexual transmission was not postulated until the early 2000s by looking at the concordance rate of infection between sexual partners [19]. Shortly after, this explanation was evidenced using a single-locus-sequence-based typing (SLSBT) method, based on a 281 bp conserved region of the *mgpB* gene, in 79 positive specimens from 19 couples [20].

Nowadays, it is well accepted that *M. genitalium* can lead to a sexually transmitted infection (STI) (figure 3.). Despite vaginal sex may be the major route of transmission in

terms of risk [21,22], anal intercourse in men who have sex with men (MSM) is likely the most common cause of transmission due to a greater sexual promiscuity and a higher endemicity of *M. genitalium* infection in this population [23]. No molecular epidemiology approaches endorse this pattern, but several studies report higher prevalence of urethral infection among men encountering vaginal sex when compared to strict MSM [21,22]. Furthermore, the frequent subclinical course of infections in vagina and specially rectum makes both locations a common and unnoticed reservoir for *M. genitalium*. Oro-genital transmission has been proposed [24], but it is very unlikely that this route contributes to a significant *M. genitalium* spread since the carriage and viability of the microorganism in the oropharynx is negligible [20,25].

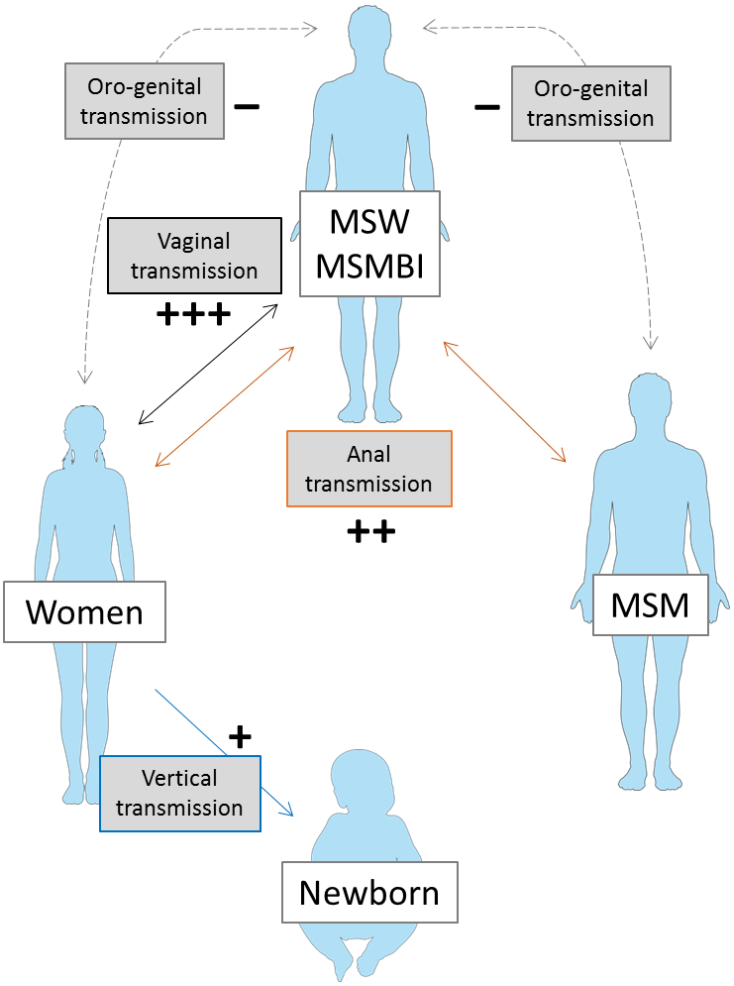

Figure 3. Putative transmission events in *M. genitalium* infection. The risk of transmission applied does not correspond to a systematic classification but to the opinion of the author.
Risk of tranmission: +++, high; ++, moderate; +, low; -, nil.
Abbreviations: MSM, men who have sex with men; MSW, men who have sex with women; MSMBI, men who have sex with men and women.

The role of *M. genitalium* in adverse pregnancy outcomes is poorly understood and the topic remains controversial. In addition, the studies addressing the association between *M. genitalium* and obstetric complications are not consistent [26,27], and mother-to-child transmission has not been systematically studied and remains unclear [28]. Nevertheless, the implication of this pathogen in spontaneous abortion or perinatal infections is a major concern which, indeed, needs to be investigated in order to implement different preventive strategies. Some

investigations have also detected *M. genitalium* in synovial fluid of individuals with arthritis [29]. However, even though hematogenous translocation of the bacterium may exist [30], *M. genitalium* joint infections should be considered very cautiously until further evidence is available [2].

The awareness around *M. genitalium* infection has exponentially improved during the last decade (figure 4), but there is still uncertainty in key natural history parameters [31]. The risk of contracting *M. genitalium* per sexual encounter, the persistence/clearance of the infection and, definitely, the basic reproductive ratio (R_0) are some unknown metrics required, for instance, if screening approaches are proposed [32,33].

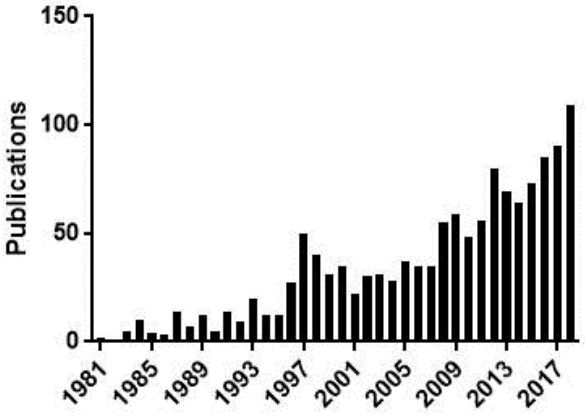

Figure 4. Number of publications in PubMed by year until 2018, using the query "*Mycoplasma genitalium*".

4. Symptoms

Since it was first identified in men with NGU [1], *M. genitalium* has been consistently associated with male urethritis [2]. Less well established, *M. genitalium* is also considered an important pathogen in female reproductive health related to several genital syndromes and complications [26,34]. Despite the significant implication of *M. genitalium* in sexually transmitted diseases (STDs), most infections, particularly in women, are asymptomatic and self-limited [35]. Thus, the virulence of the bacterium, and more likely an exacerbated host immune response, may trigger the development of symptoms in certain individuals.

Table 1 shows a summary regarding the evidence for the association between *M. genitalium* infections and disease.

Men

Urethritis, both acute and chronic, is the most common clinical manifestation of *M. genitalium* infection [2,4,16,36]. In fact, the bacterium is the second cause of NGU after *C. trachomatis*, accounting for 15% to 20% of cases. The inflammation of the urethra may occur sub-clinically or, contrary, may develop symptoms such as urethral

discharge, dysuria, penile tip irritation or urethral discomfort [37]. Although the incubation period for the development of *M. genitalium* urethritis is unknown, the slow replication rate in *M. genitalium* likely results in a prolonged period (up to 60 days or longer) between the establishment of the infection and the debut of symptoms [2,36].

Condition	**Adult men**	**Condition**	**Adult women**
- Urethritis	+++	- BV, AV[b]	+
- Balanoposthitis	+	- Cervicitis (urethritis)	++
- Epididymitis	++	- PID	++
- Infertility[a]	+	- Infertility	+
- CP	+	- APO	+
Condition		**Adults; men and women**	
- Proctitis		++	
- SARA		+	
- Conjunctivitis		+	

Table 1. Causal association between *M. genitalium* infection and disease.
The levels of evidence applied do not correspond to a systematic classification but to the opinion of the author (table adapted from Fernández-Huerta M et al., 2020).
Levels of evidence: +++, high (testing is indicated); ++, moderate (testing may be considered); +, low (testing is not indicated, it might be cautiously considered only in specialized STI units).
Abbreviations: CP, chronic prostatitis; BV, bacterial vaginosis; AV, aerobic vaginitis; PID, pelvic inflammatory disease; APO, adverse pregnancy outcomes; SARA, sexually acquire reactive arthritis.
[a]The impact of *M. genitalium* in male infertility is controversial and may be related to epididymitis-associated complications.
[b]Bacterial vaginosis is associated with vaginal discharge, pelvic inflammatory disease, infertility and adverse pregnancy outcomes. In this scenario, *M. genitalium* may play a role in bacterial vaginosis and bacterial vaginosis-related conditions.

Rectal *M. genitalium* infections among MSM are frequent ranging from 2% to 5% [21,38]. Although symptomatic proctitis caused by this pathogen may occur, especially when the bacterium is present in high loads [39], most rectal infections are asymptomatic. Rectum is so an important silent reservoir for *M. genitalium* infection in MSM, probably favoring the appearance and accumulation of antibiotic resistances within this subpopulation. Nevertheless, routine testing of *M. genitalium* in rectum is controversial (and currently not recommended) due to a de-facto screening effect that may cause harmful consequences in terms of antimicrobial resistances and antibiotic abuse [8]. Therefore, *M. genitalium* testing should only be considered in symptomatic proctitis, after the presence of other traditional anorectal pathogens such as *N. gonorrhoeae*, *C. trachomatis* (prominently lymphogranuloma venereum (LGV) genotypes), *Treponema pallidum* and herpes simplex viruses has been ruled out [6,40].

Balanitis and posthitis are the inflammation of the glans penis and the prepuce, respectively; frequently, these syndromes occur simultaneously (balanoposthitis). *M. genitalium* has been timidly associated with this condition but further evidences are required [41,42]. Despite the doubtless role of the bacterium in chronic urethritis, there is no clear association between *M. genitalium* and chronic prostatitis [2,43]. Additionally, *M. genitalium* may be a cause of epididymitis through an ascent along the urethra although data is scarce [44,45]. The link between *M. genitalium* and male infertility is controversial and may be related to epididymitis-associated complications [16].

Women

Although the implication of *M. genitalium* in female genital disorders is less consistent, the microorganism has been related to inflammatory lower urogenital tract syndromes including urethritis, cervicitis and other vaginal discharge-related conditions [2,16,34]. Usually, the abnormal vaginal discharge can be produced by bacterial vaginosis, aerobic vaginitis, yeast infections or trichomoniasis. Nevertheless, other conditions also frequently associated with sexual activity, such as cervicitis, may account for aberrant vaginal discharge [46]. Unlike *M. hominis*, the association between *M.*

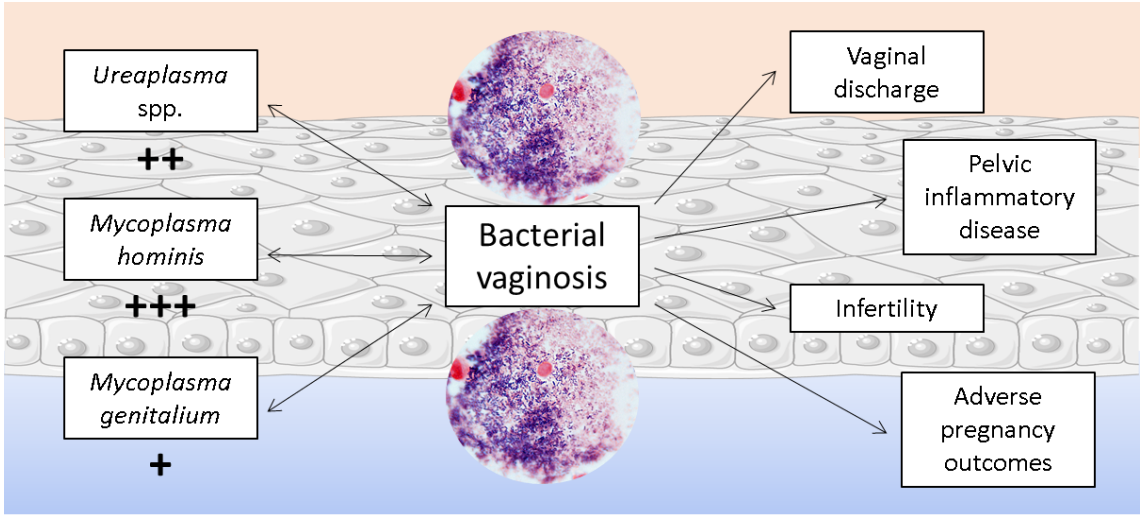

Figure 5. Association between *Ureaplasma*/*Mycoplasma* spp., bacterial vaginosis and disease.
Bacterial vaginosis is associated with abnormal vaginal discharge, pelvic inflammatory disease, infertility and adverse pregnancy outcomes. In this scenario, *Ureaplasma* spp. (*Ureaplasma urealyticum* and *Ureaplasma parvum*; previously *U. urealyticum* biovars 2 and 1, respectively), *M. genitalium*, and specially *M. hominis*, may play a role in bacterial vaginosis and bacterial vaginosis-related conditions.
The levels of evidence applied do not correspond to a systematic classification but to the opinion of the author (table adapted from Fernández-Huerta M et al., 2019).
Levels of evidence: +++, high; ++, moderate; +, low.

genitalium and the vaginal bacterial dysbiosis is controversial (figure 5) [2,34]. Contrary, the association between *M. genitalium* infection and cervicitis (usually characterized by aberrant vaginal discharge and intermenstrual/postcoital bleeding [47]) is currently accepted although certainly limited by the lack of compelling studies [2,16,34].

Typically, pelvic inflammatory disease (PID) is produced when bacteria ascend from the lower genital tract to the uterus, the fallopian tubes and the ovaries. Thus, cervicitis is frequently preceding the PID syndrome. In addition to *N. gonorrhoeae*, *C. trachomatis* and other bacteria of the vaginal flora, *M. genitalium* is recognized as a cause of PID [2,16,34,48,49]. On the other hand, the potential adverse impact of *M. genitalium* on pregnancy and infertility is also a major concern. The association between the microorganism and these obstetric complications is not consistent and is likely linked to long-term aftermaths of upper genital tract infections such as PID [2,16,34,48,49]. Of note, similar to *C. trachomatis*, *M. genitalium* infections in women are usually asymptomatic, so some of these complications may appear after a "silent" PID [48].

Although there are no studies regarding rectal *M. genitalium* infections in women; this topic likely requires the considerations previously described in men.

***M. genitalium* and HIV**

Another important aspect is the relation between *M. genitalium* infection and the human immunodeficiency virus (HIV). Although studies are scarce, the bacterial infection may increase the risk for HIV acquisition through inflammatory processes that harm the protective anogenital mucosa and facilitate the entrance of the virus while exposure [2,50,51].

Other putative clinical manifestations

The reactive arthritis (or Reiter´s syndrome) is a sterile inflammation of the joints usually enhanced by gastrointestinal or genital infections. Reactive arthritis triggered by an STI, usually infections in the lower genital tract, is referred to as sexually acquired reactive arthritis (SARA). Although *C. trachomatis* and *N. gonorrhoeae* are frequently associated with this condition; some findings suggest that *M. genitalium* and other commensal bacteria may exacerbate this potent immune response [2,52]. Although *M. genitalium* has been detected in synovial fluid of individuals with arthritis and hematogenous translocation of the bacterium may occur [29,30], joint infections

should be considered very cautiously [2]. Although conjunctivitis is well associated with SARA, one investigation reported a case of chronic conjunctivitis non-associated with reactive arthritis in which *M. genitalium* was detected in both conjunctival smear and urine (the patient also reported mild and intermittent chronic dysuria) [53]. Authors speculated self-inoculation as the most likely route of transmission. However, these cases must be considered extremely unusual.

Vertical transmission has not been systematically studied in *M. genitalium* [28]. Nevertheless, the role of this pathogen in spontaneous abortion or perinatal infections (e.g. neonatal conjunctivitis, neonatal respiratory diseases… etc.) is a major concern which, indeed, requires further evidence [2].

5. Diagnosis

Diagnostic tools

M. genitalium was first isolated in 1981 [1]. However, because of the limited metabolic capacity of the bacterium, the isolation of *M. genitalium*, especially in axenic mediums, is extremely fastidious and has a very limited efficiency. Consequently, the interest for *M. genitalium* culture is limited to basic research and *in vitro* antibiotic susceptibility studies.

Since the early 90s, with the development of the first PCRs for the detection of the bacterium [5], *M. genitalium* testing has been based on nucleic acid amplification tests (NAATs). Initially, most of these techniques were homemade designed using different molecular targets in the *mgpB* (MG191), the 16S ribosomal RNA (rRNA) and the *pdhD* genes [5,54-58]. Nevertheless, in the last decade, several commercial assays have been developed for the detection of *M. genitalium*. Additionally, due to the emergence of antimicrobial resistance, some of these tests have implemented the simultaneous detection of the bacterium and genotypic antimicrobial resistance-associated markers.

Because of the small size of the bacterium (0.2 µm) and the absence of cell wall, *M. genitalium* cannot be detected with regular optical-microscopy procedures [2,9]. Contrary, microscopical leukocyte counts from urethral smears or urine play an important role for diagnosis and management of urethritis [37].

Regarding the collection and preservation of samples for *M. genitalium* molecular testing, recommendations are similar to the ones for the detection of other STIs such

as *C. trachomatis* and *N. gonorrhoeae*. Of note, testing for *M. genitalium* in the oro-pharynx is not indicated since the presence and viability of the microorganism in this location is negligible.

Indications for testing in *M. genitalium*

Indications for testing and treatment in *M. genitalium* are described in table 2.

Proliles	Comments
Indicated	
- Individuals with NGU or NGNCU	MG is strongly associated with this condition. Additionally, because of the emergence of antimicrobial resistance in STIs, the detection of MG has gained special relevance.
- Individuals with cervicitis, PID, epididymitis or proctitis[a]	Although the association between MG and these syndromes has not been widely evidenced; its detection may be considered, always discarding first more frequent etiologies.
- Sexual contacts of infected partners[b]	To avoid reinfection, the detection and treatment of MG is indicated for sexual contacts (last 3 months) of infected partners[c]. If necessary, epidemiological treatment may be considered.
Non-indicated	
- Asymptomatic individuals	Unlike sexual contacts of infected partners, systematic screening of MG among asymptomatic individuals is not recommended. This includes individuals reporting sexual-risk behaviors and people at risk to acquire HIV (e.g. MSM or individuals in PrEP)[d].
- Individuals infected with NG and/or CT	Disregarding symptoms, testing for MG is not indicated among patients infected with NG and/or CT. First, the presence of these bacteria is not associated with MG infection. On the other hand, the use of azithromycin for treatment of NG and CT is being gradually restricted; consequently, its effect on antimicrobial resistance in MG will be soon limited[e].
To consider individually	
- Pregnancy	The association between MG and adverse pregnancy outcomes is very limited. Although azithromycin can be used during pregnancy, moxifloxacin is contraindicated. The use of doxycycline is controversial and there is no experience with pristinamycin. In this scenario, the risks and benefits should be individually assessed[f].
- Breastfeeding	The risk of the azithromycin in the nursling is low. On the other hand, the use of doxycycline, moxifloxacin and pristinamycin is contraindicated. In this scenario, the risks and benefits should be individually assessed.

Table 2. Clinical indications for testing and treatment in *M. genitalium*.
The conclusions in this table are those of the author and do not represent the official position of any national/international institution (table adapted from Fernández-Huerta M et al., 2019).
Abbreviations: NGU, non-gonococcal urethritis; NGNCU, non-gonococcal-non-chlamydial urethritis; MG, *Mycoplasma genitalium*; STI, sexually transmitted infections; PID, pelvic inflammatory disease; HIV, human immunodeficiency virus; MSM, men who have sex with men; PrEP, pre-exposure prophylaxis; NG, *Neisseria gonorrhoeae*; CT, *Chlamydia trachomatis*.
[a]Testing for *M. genitalium* is not systematically indicated in those syndromes were evidence regarding the role of the bacterium is low. These cases (usually balanoposthitis and chronic prostatitis) must be carefully evaluated, always discarding first (and sometimes repeatedly) more frequent etiologies.
[b]Unlike European guidelines, Australian *M. genitalium* guidelines (established by the Australasian Sexual Health Alliance - ASHA) mention that testing for *M. genitalium* should be exclusively performed in sexual contacts of symptomatically infected partners.
[c]British *M. genitalium* guidelines (promoted by the British Association for Sexual Health and HIV - BASHH) state that testing for *M. genitalium* should be offered only among usual (and recent) sexual contacts that may facilitate reinfection in the index partner.

[d]European guidelines potentially consider *M. genitalium* targetedscreenings in certain sexual-risk populations (based on local epidemiology). However, experts currently plead to strictly consider *M. genitalium* testing in symptomatic individuals and sexual contacts of infected partners.
[e]While European and Australian *M. genitalium* guidelines recommend the use of azithromycin for dual therapy in gonorrhea, British guidelines already opt for monotherapy with cephalosporins. Likely, enhanced by the epidemiology, European and Australian associations may soon modify recommendations in this sense. On the other hand, all 3 institutions offer either doxycycline or azithromycin for *C. trachomatis* treatment. Nevertheless, because of the emergence of macrolide resistance in *M. genitalium*, the use of azithromycin may be soon restricted.
[f]European *M. genitalium* guidelines potentially consider *M. genitalium* screening among pregnant women.

Chlamydial and gonococcal infections were traditionally managed only when patients presented ssymptoms. However, rapid testing and treatment STI services have been recently implemented in asymptomatic individuals to putatively reduce complications, and control STI transmission and spread. Furthermore, current international guidelines already recommend routine screening for *N. gonorrhoeae* and *C. trachomatis*, especially among sexually active young people and MSM [59,60]. Although these screening programs may, indeed, reduce the prevalence of STIs [61], they may also result in selective pressure for antibiotic resistance development not only in *N. gonorrhoeae* [62], but also in *M. genitalium* if an undetected co-infection is present and azithromycin is prescribed [8]. Testing for *M. genitalium* among asymptomatic people has been suggested [7], but experts and international guidelines currently plead to avoid widespread asymptomatic screenings due to the lack of scientific evidence, the disturbing emergence of antibiotic resistance in *M. genitalium*, particularly to azithromycin, and the consequent use of costly antibiotics (that may also cause serious side effects) [6,8,63-65]. In fact, recent studies attempt to evaluate the impact and effectiveness of these testing strategies in *M. genitalium* infection, but ultimately recommend further empirical work to improve understanding on the natural history of the infection to elucidate the real impact of these proposals [18,66]. The controversial role of *M. genitalium* in severe conditions such as PID, infertility and adverse pregnancy outcomes, and its association with HIV acquisition, may be key in order to assess the efficacy of these screening approaches.

In conclusion, except for sexual contacts of infected partners, only individuals with symptoms are suitable for systematic *M. genitalium* testing. Furthermore, depending on the syndrome, *M. genitalium* may be considered as a possible or a probable cause of symptoms and further etiologies may be assessed if required.

6. Treatment

Antibiotic resistance

M. genitalium lacks a cell wall making it resistant to several classes of antibiotics such as beta-lactams [67].

Since doxycycline demonstrated very poor efficacy eradicating *M. genitalium* infections, ranging from 30% to 40% [68,69], the macrolide azithromycin given as an extended dose along multiple days has been used and remains the recommended first-line treatment for uncomplicated infection [6]. However, the wide use of this antibiotic, mainly as a single dose for the treatment of *N. gonorrhoeae* and *C. trachomatis*, may have contributed to the emergence of macrolide resistance in *M. genitalium* worldwide [70-74]. This resistance, exceeding 50% in many countries, is strongly associated with single-point mutations in domain V of the 23S rRNA gene, mainly at positions A2071 and A2072 (A2058 and A2059, respectively, *Escherichia coli* numbering) [75,76]. Additionally, this resistance is especially prevalent in MSM population [72]. In Spain, the limited data regarding the prevalence of macrolide resistance in *M. genitalium* vary from 16% to 35% [72,77,78].

The fourth-generation fluoroquinolone moxifloxacin is the recommended treatment for macrolide resistant infections and/or complicated infections [6]. Nevertheless, fluoroquinolone treatment failures are being reported in many countries, especially in the Asia-Pacific region [3,79]. This resistance has been mainly linked to *missense* mutations in the *parC* gene, responsible for the synthesis of a topoisomerase involved in DNA replication. Unlike macrolide resistance, these mutations are not always translated into treatment failure. In this sense, very few investigations correlate these mutations with moxifloxacin *in vitro* and *in vivo* activity. In 2013, Couldwell DL et al. reported the first cases of moxifloxacin treatment failure linked to mutations at position S83I in ParC (*M. genitalium* numbering) [79]. Subsequently, Murray GL et al. observed similar findings associated with mutations S83I and S83R [3]. Finally, a recent *in vitro* susceptibility study correlated mutations S83I and D87Y with an increase in the minimum inhibitory concentration (MIC) for moxifloxacin in *M. genitalium* [80]. Furthermore, from an enzymatic-structural point of view, mutations in ParC at amino-acid positions S83 and D87 in *M. genitalium* could influence quinolone-enzyme

interactions and may confer moxifloxacin resistance [81]. In Spain, these mutations occur in around 8% of infections [72,77]. On the other hand, although the presence of mutations in GyrA, especially at positions M95 and D99 (*M. genitalium* numbering), has been linked with moxifloxacin treatment failures [79], these mutations may likely have a less-severe effect reducing the susceptibility of the bacterium for the fluoroquinolone [80,81].

Contrary to macrolides and fluoroquinolones, the molecular basis for doxycycline resistance is unknown.

Management of *M. genitalium* infection

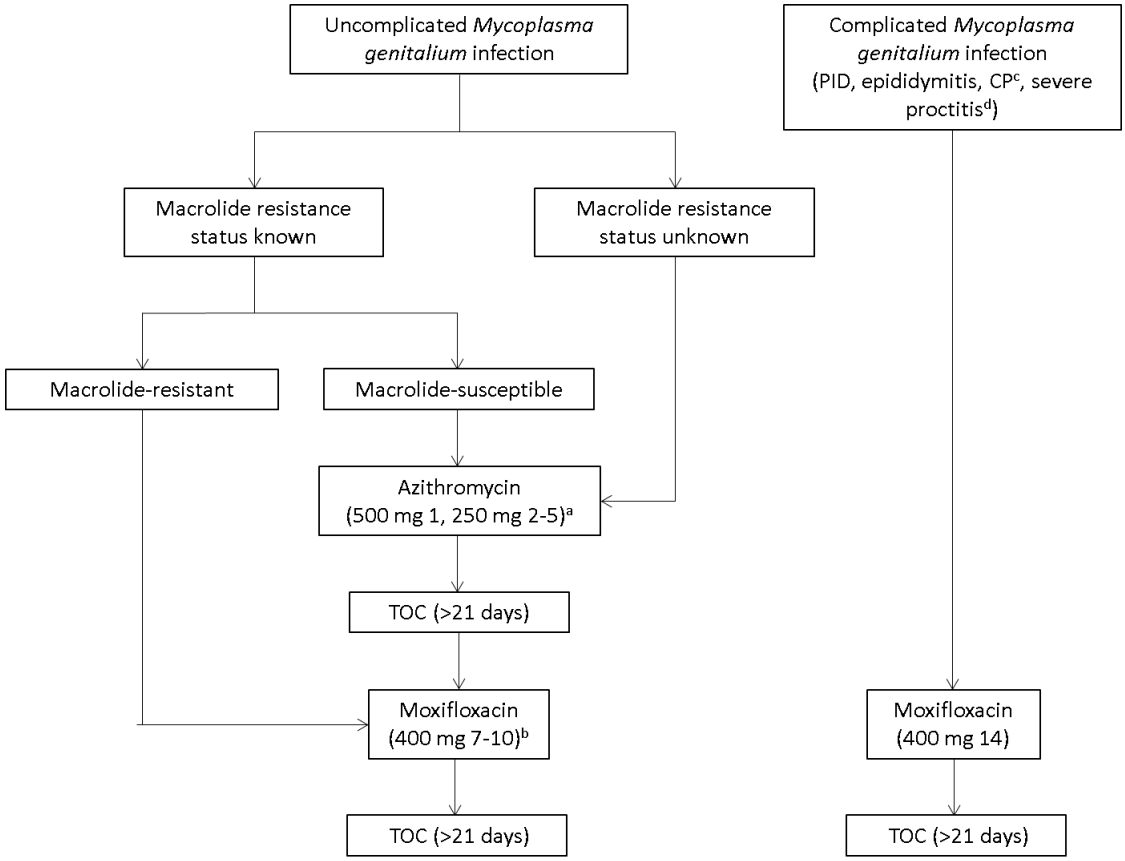

Figure 5. Antimicrobial treatment of uncomplicated *M. genitalium* infection (European management guidelines).

The European guidelines for the management of *M. genitalium* infections, published in 2016, strongly recommend the use of combined diagnostic assays for the additional detection of macrolide resistance mutations that can guide clinicians into an accurate therapy. Furthermore, due to the emergence of macrolide and fluoroquinolone resistance in *M. genitalium* worldwide, the document highlights the need for a microbiological test-of-cure, at least three weeks after treatment (image adapted from Fernández-Huerta M et al., 2019, and Unemo M et al., 2017).

Abbreviations: TOC, test-of-cure; PID, pelvic inflammatory disease; CP, chronic prostatitis.

[a]British (promoted by the British Association for Sexual Health and HIV - BASHH) and Australian (established by the Australasian Sexual Health Alliance - ASHA) *M. genitalium* guidelines recommend higher dose of azithromycin: 1 g day 1, 500 mg days 2-3 (2 g total) and 1 g day 1, 500 mg days 2-4 (2.5 g

total), respectively. Furthermore, both institutions highlight the use of doxycycline (100 mg x2, 7 days) prior to the establishment of the macrolide or the quinolone.
[b]Regarding the duration of moxifloxacin treatment: British guidelines recommend 10 days while Australian guidelines suggest 7 days.
[c]There is no clear association between *M. genitalium* and chronic prostatitis. However, if this relation is considered, clinicians should probably manage the infection as a complicated syndrome.
[d]According to British management guidelines, in cases of severe proctitis were *M. genitalium* is considered, the infection should be managed as a complicated syndrome.

Diagram in figure 5 shows the clinical management of *M. genitalium* infection according to European guidelines [6]. Because of the emergence of antibiotic resistance, test-of-cure (TOC) controls are essential in *M. genitalium* infection. Additionally, the use of combined diagnostic assays detecting not only *M. genitalium* but also genotypic resistance makers may lead to the wide implementation of the resistance-guided therapy. Optimizing antimicrobial stewardship, this approach has demonstrated very good efficacy utilizing the existing antibiotics for the treatment of *M. genitalium*. In case of fluoroquinolone resistance, European guidelines recommend doxycycline (100 mg x2, 10 days) or pristinamycin (1 g x4, 10 days). Additionally, British guidelines, (promoted by the British Association for Sexual Health and HIV - BASHH), suggest either higher dose of doxycycline (100 mg x2, 14 days) or the use of doxycycline (100 mg x2, 7 days) prior to the use of pristinamycin [65]. Minocycline (British guidelines recommend 100 mg x2, 14 days) or the fourth-generation fluoroquinolone sitafloxacin (with, presumably, higher *in vitro* activity against the bacterium) might be other therapeutic alternatives in case of multidrug resistance in *M. genitalium* [65,80,82-84].

Also, there are some important differences between European [6], British [65], and Australian guidelines (established by the Australasian Sexual Health Alliance - ASHA) [64]. First, the extended-pattern azithromycin, the recommended first-line treatment in all guidelines, slightly varies in dose and dosage. While the European institution recommends 500 mg day 1 and 250 mg days 2-5 (1.5 g total), the British and Australian societies recommend higher dose of azithromycin: 1 g day 1 and 500 mg days 2-3 (2 g total), and 1 g day 1 and 500 mg days 2-4 (2.5 g total), respectively. On the other hand, both British and Australian guidelines recommend the use of doxycycline (100 mg x2, 7 days) prior to the use of the macrolide or the fluoroquinolone. Nevertheless, this algorithm may be similarly inferred from European guidelines by combining the

management of NGU and *M. genitalium* infection. In this sense, the synergistic effect of the sequential therapy with doxycycline has been recently assessed by Read TRH, et al. with promising results regarding the eradication of *M. genitalium* infection and the control of antibiotic resistance spread [85].

Figure 6 summarizes the clinical management of urethritis, the most common syndrome associated with *M. genitalium* infection.

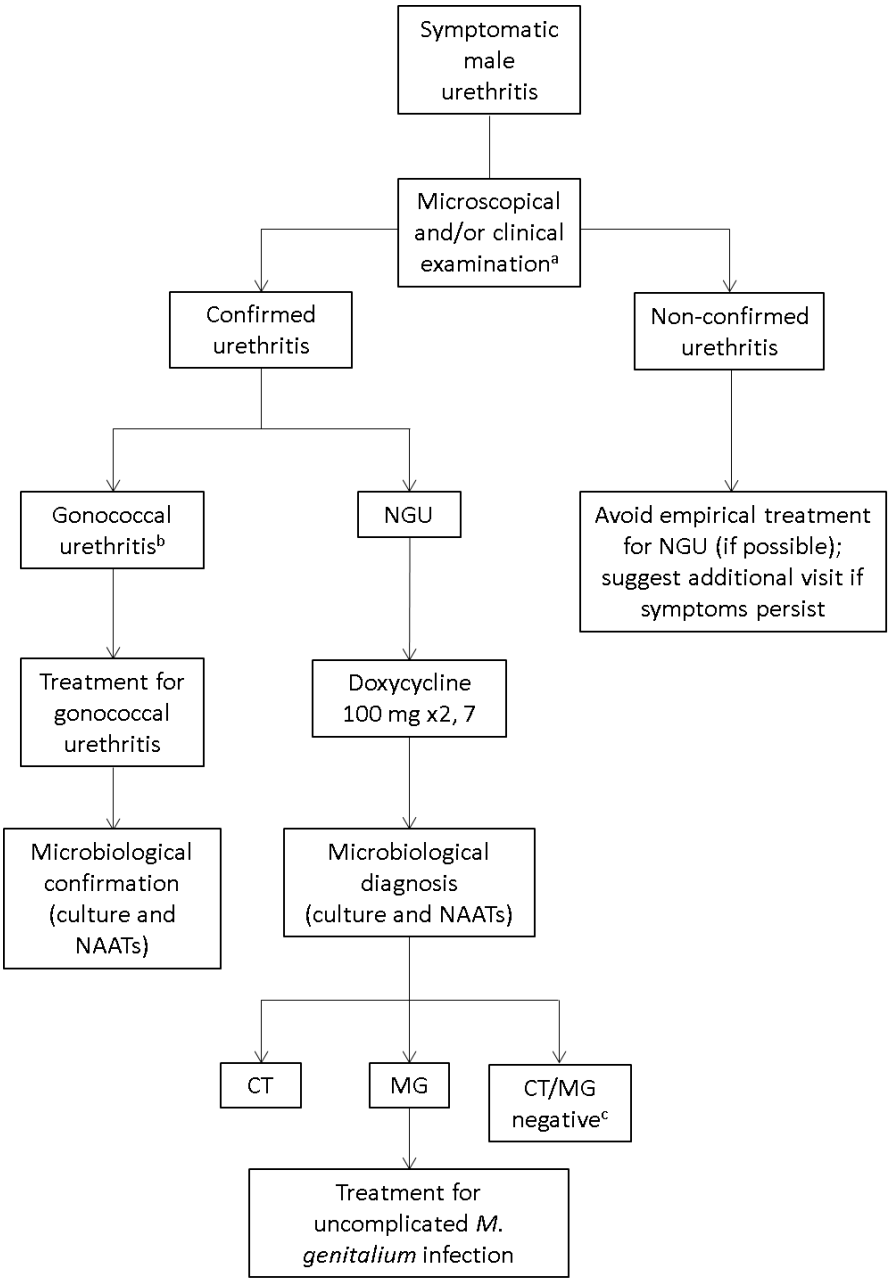

Figure 6. Therapeutic management of symptomatic urethritis.
The scheme does not include additional proceedings such as screening for further STIs, tracking of sexual contacts and other general recommendations.
The recommendations in this figure are those of the authors and do not represent the official position of any national/international institution (image adapted from Fernández-Huerta M et al., 2019).

Abbreviations: NAAT, nucleic acid amplification test; NGU, non-gonococcal urethritis; CT, *Chlamydia trachomatis*; MG, *Mycoplasma genitalium*.
[a]Inflammation should be confirmed with microscopy using gram or methylene-blue staining in urethral smears. Alternatively, the microscopical examination can be performed in first-void urines (ideally after a centrifugation process).
[b]Gonococcal urethritis may be addressed when gram-negative diplococcic are observed at microscopical examination and/or when urethral discharge is frank (this could suggest *N. gonorrhoeae* as the causative organism). When microscopy is not available, the presence of purulent urethral discharge or certain suggestive signs may indicate urethritis. On the other hand, macroscopical analyses and/or the use of test strips in first-void urines may be useful.
[c]If laboratory results are negative and symptoms persist, further less-frequent etiologies may be considered.

7. Where do we come from and where are we going in *M. genitalium* infection - *Hypothesis*

M. genitalium is now well recognized as a major cause of urethritis and further urogenital syndromes in both men and women. Additionally, this STI has demonstrated a disturbing capacity to develop resistance to the major antimicrobials against *M. genitalium*: macrolides and fluoroquinolones.

In this scenario, it is essential to develop novel management approaches to fight against *M. genitalium*, that may soon become untreatable. Since there are very few antibiotic alternatives available, any strategy that prolongs the utility of existing treatments is extremely important. On the other hand, although testing for *M. genitalium* among asymptomatic people may reduce the prevalence of this STI, it may also result enhance the selection of antibiotic resistance. Thus, current scientific evidence is insufficient to justify broad *M. genitalium* screening programs.

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OBJECTIVES

Major objective

- To estimate the overall prevalence of antibiotic resistance in *M. genitalium* in Barcelona, Spain. (CHAPTER 2)

Secondary objectives

- To collect, update and assess the evolution of antibiotic resistance in *M. genitalium* at both local and European level. (CHAPTER 2)
- To estimate the prevalence of *M. genitalium* and antibiotic resistance among asymptomatic people at risk to acquire STIs. (CHAPTER 3)
- To understand the transmission dynamics of *M. genitalium* infection and antibiotic resistance among asymptomatic populations. (CHAPTER 4)
- To establish novel approaches for the management of *M. genitalium* infection and the control of antibiotic resistance spread. (CHAPTER 5 – DISCUSSION)

CHAPTER 2:
Antibiotic resistance: where are we now?

Key messages

- The differences on the management and treatment of STIs between European countries distinctly influence antimicrobial resistance in *M. genitalium*.
- Antibiotic resistance in *M. genitalium*, especially to macrolides, has been rapidly increasing in Europe in the last decade, exceeding 50% in Northern countries.
- Macrolide and fluoroquinolone resistance in *M. genitalium* in Barcelona did not significantly increase from 2013 to 2017, being around 35%-36% and 8%-10%, respectively.

EUROPEAN COMPREHENSIVE REVIEW

***Mycoplasma genitalium* and antimicrobial resistance in Europe: a comprehensive review**

Fernández-Huerta M, Barberá MJ, Serra-Pladevall J, Esperalba J, Martínez-Gómez X, Centeno C, Pich OQ, Pumarola T, Espasa M

Int J STD AIDS. 2020 [review article]

2016-2017 COHORT: BARCELONA, SPAIN

***Mycoplasma genitalium* macrolide resistance update: rate among a 2016-2017 cohort of patients in Barcelona, Spain**

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Enferm Infecc Microbiol Clin. 2019

***Mycoplasma genitalium* and fluoroquinolone resistance detection using a novel qPCR assay in Barcelona, Spain**

Fernández-Huerta M, Esperalba J, Serra-Pladevall J, Espasa M

Enferm Infecc Microbiol Clin. 2019 [scientific letter]

1. Introduction

In addition to the pathogenic potential of *Mycoplasma genitalium* [1], this STI has demonstrated a disturbing capacity to develop resistance to the antimicrobials available against *M. genitalium*: macrolides and fluoroquinolones.

Since doxycycline demonstrated poor efficacy eradicating the infection [2], the macrolide azithromycin has been the recommended first-line treatment against *M. genitalium* [3]. Nevertheless, the wide use of this antibiotic in STIs, especially for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, has probably enhanced the emergence of macrolide resistance in *M. genitalium* worldwide [4]. Specific mutations in the 23S rRNA gene are strongly associated with this macrolide resistance [5,6]. The fourth-generation fluoroquinolone moxifloxacin is being used as a second-line antibiotic for macrolide resistant infections in Europe. Nevertheless, mutations in the quinolone-resistance determining region (QRDR) of the *parC* gene, mainly affecting specific residues, have been linked with moxifloxacin treatment failures [7-9]. In the alarming scenario of antibiotic resistance in *M. genitalium*, this STI may soon become untreatable since very few therapeutic alternatives currently exist [10].

2. Objectives

The objectives of this chapter are to:

- Collect, update and assess the evolution of antibiotic resistance in *M. genitalium* in Europe.
- Estimate the current prevalence of macrolide and fluoroquinolone resistance-associated mutations in *M. genitalium* in Barcelona, Spain.
- Assess the specific evolution of antibiotic resistance in *M. genitalium* in Barcelona, Spain from 2013 to 2017.

3. Materials and Methods

European comprehensive review

This is a comprehensive and selective review of the scientific literature published between 2012 and 2018. A PubMed search was conducted using the MeSH terms "*Mycoplasma genitalium* AND resistance". Only those relevant reports, based on

European populations, published on peer-reviewed journals and written in English were included in this review.

2016-2017 cohort: Barcelona, Spain

This is a retrospective study, conducted at the Microbiology Department of the Vall d´Hebron University Hospital between December 2016 and February 2017, among *M. genitalium*-positive specimens.

During this period, clinical samples were routinely tested for *M. genitalium* by real-time (quantitative) PCR (qPCR) using the Allplex™ STI Essential assay (Seegene, South Korea). Subsequently, *M. genitalium*-positive samples, collected during the study period, were retrospectively tested for macrolide and fluoroquinolone resistance-associated mutations with the ResistancePlus® MG (SpeeDx, Australia) and the MG+parC (beta) (SpeeDx) assays, respectively. Briefly, the ResistancePlus® MG test is a multiplex qPCR for detection of *M. genitalium* and five mutations associated with macrolide resistance (A2058G, A2059G, A2058C, A2059C, A2058T; *Escherichia coli* numbering). On the other hand, the MG+parC (beta) test detects *M. genitalium* and five mutations linked with fluoroquinolone resistance (G248T [S83I], A247C [S83R], G259T [D87Y], G259A [D87N] and G259C [D87H]; *M. genitalium* numbering [amino-acid residue affected in ParC]). Both tests were performed according to the manufacturer´s instructions. Macrolide resistant mutations in the 23S rRNA gene were confirmed by Sanger sequencing using a previously described methodology [5].

Sociodemographic and clinical characteristics of the patients were collected through a comprehensive review of the medical record. Results from a previous similar study by Barberá MJ, et al. were collected in order to evaluate the trend of antibiotic resistance in Barcelona between 2013 and 2014, and 2016 and 2017 [11].

4. Results

European comprehensive review

From 180 publications initially displayed by the PubMed search, only 25 from 12 European countries accomplished the inclusion criteria and were finally considered for the review. Figure 1A shows the 12 European countries with reported antibiotic resistance data. It is important to note the major information gap in most Central, Southern and Eastern Europe, where resistance estimates are scarce.

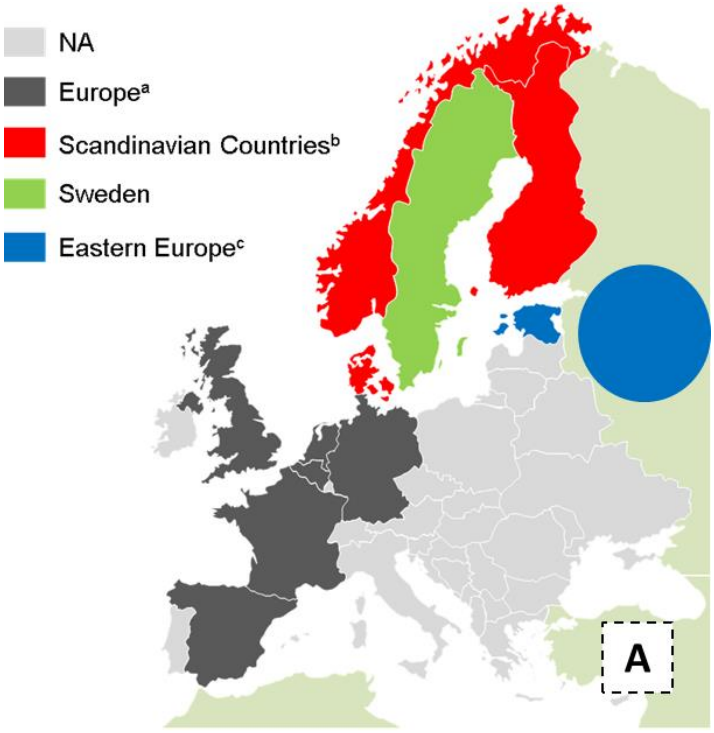

Figure 1A. Antibiotic resistance in *M. genitalium* in Europe.
European mapping of the 12 countries with antimicrobial resistance data reported since 2012. Of them, 10 (Finland, Norway, Denmark, Sweden, Germany, France, Spain, United Kingdom, Russia and Estonia) had information regarding the prevalence of both macrolide and fluoroquinolone resistance-associated mutations. The remaining 2 countries (Netherlands and Belgium) reported only macrolide resistance data.

The long-term trend of macrolide resistance in *M. genitalium* in Europe is represented in figure 1B. Only large studies (in which more than 50 cases are evaluated) were included. Also, the study from the United Kingdom is excluded since samples were collected from persistent infections and antibiotic resistance is likely overrepresented. Thus, 18 investigations from 8 countries were finally analyzed. Data were grouped by region in Western/Southern (France, Spain and Netherlands), Scandinavian (Denmark and Norway), and Eastern (Russia and Estonia) European

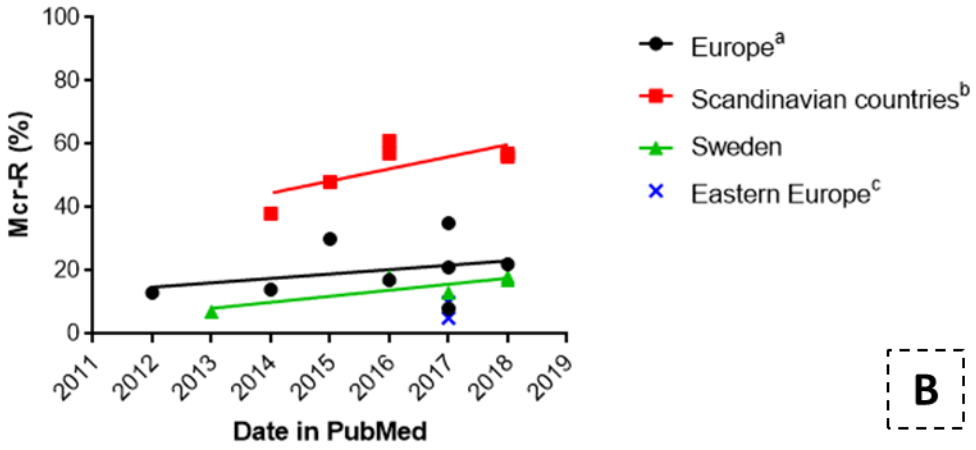

Figure 1B. Evolution of macrolide resistance in *M. genitalium* in Europe.
The estimates are plotted by their year of publication in PubMed.
Abbreviations: NA, not available; Mcr-R, macrolide resistance.
[a]The term "Europe" includes the Western/Southern countries France, Spain, and Netherlands.
[b]Includes Denmark and Norway, but not Sweden.
[c]Includes Russia and Estonia.

countries. Sweden was studied separately due to a well-described distinctive epidemiology in terms of macrolide resistance in *M. genitalium*. No longitudinal data could be collected from Eastern Europe.

The limited data regarding the *M. genitalium* prevalence of fluoroquinolone resistance-associated mutations in *parC* in Europe were insufficient to get longitudinal trends. However, estimates from 12 investigations conducted in 10 different countries (Finland, Norway, Denmark, Sweden, Germany, France, Spain, United Kingdom, Russia and Estonia) resulted in a median prevalence of 5% (interquartile range (IQR), 5%-6%).

2016-2017 cohort: Barcelona, Spain

During the study period, 1191 samples were tested for *M. genitalium*. Of them, 122 from 106 individuals resulted positive for *M. genitalium*. Finally, 89 *M. genitalium*-positive samples collected from 86 patients were included in the subsequent analyses. The prevalence of macrolide resistance was 36.1% (95% confidence interval, 25.9%-47.4%). Furthermore, macrolide resistance in *M. genitalium* was significantly more frequent among MSM compared to men who have sex with women (MSW) and women; $p < 0.001$. On the other hand, fluoroquinolone resistance was detected in 10.0% (95% CI, 4.4%-18.8%) of cases. In this sense, macrolide resistance was significantly more prevalent among *parC* mutants; $p = 0.036$.

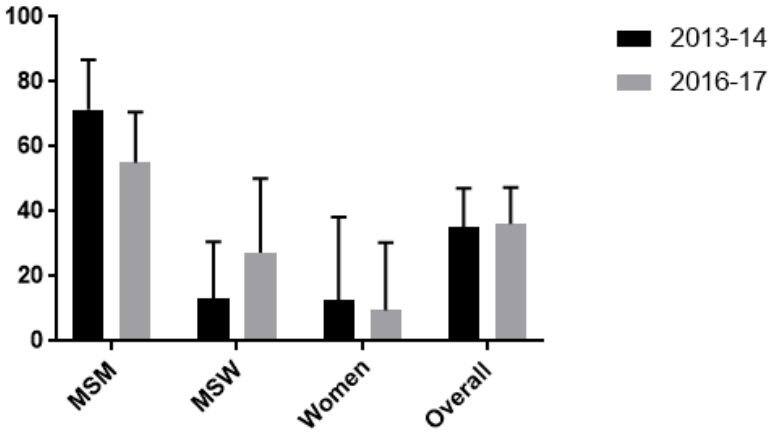

Figure 2. Rate of macrolide resistance during periods 2013-2014 and 2016-2017 in Barcelona, Spain.
Results are displayed overall and stratified by sex/sexual behaviour. Abbreviations: MSM, men who have sex with men; MSW, men who have sex with women.

The prevalence of macrolide resistance over time was evaluated in our settings by comparing findings in the current cohort with previous ones from 2013 and 2014 (figure 2) [11]. There were no significant differences between periods.

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CHAPTER 3: *Mycoplasma genitalium*: should we screen and how?

Key messages

- The prevalence of *M. genitalium* infection among asymptomatic people visiting a point of care service for rapid STI screening was 7.4%.
- The prevalence of *M. genitalium* and antibiotic resistance was much higher among men who have sex with men compared with heterosexual men and women.
- Screening for *M. genitalium* among asymptomatic individuals is currently not recommended.

MGENRES

Prevalence of *Mycoplasma genitalium* and macrolide resistance among asymptomatic people visiting a point of care service for rapid STI screening: a cross-sectional study

Fernández-Huerta M, Barberá MJ, Esperalba J, Fernández-Naval C, Vall-Mayans M, Arando M, Serra-Pladevall J, Broto C, Zarzuela F, Rando A, Pumarola T, Espasa M

Sex Transm Infect. 2019

Multicenter clinical evaluation of a novel multiplex real-time PCR (qPCR) assay for detection of fluoroquinolone resistance in *Mycoplasma genitalium*

Fernández-Huerta M, Bodiyabadu K, Esperalba J, Bradshaw CS, Serra-Pladevall J, Garland SM, Fernández-Naval C, Jensen JS, Pumarola T, Ebeyan S, Lundgren M, Tan LY, Espasa M, Murray GL

J Clin Microbiol. 2019

COINFECTION STUDY

***Mycoplasma genitalium* co-infection with *Chlamydia trachomatis* and *Neisseria gonorrhoeae* among asymptomatic patients: the silent wick for macrolide resistance spread**

Fernández-Huerta M, Espasa M

Sex Transm Infect. 2019 [scientific letter]

1. Introduction

STIs are a major problem worldwide. Despite the efforts executed by the medical community, the number of reported infections caused by *Chlamydia trachomatis* and *Neisseria gonorrhoeae* continues to rise annually; particularly among high-risk subpopulations such as MSM [1]. Although these infections were traditionally managed only when patients presented with symptoms, rapid testing and treatment STI services have been recently implemented in asymptomatic individuals to putatively reduce complications and control the spread of these STIs. These screening strategies may indeed reduce the prevalence of STIs, nevertheless, they may also result in selective pressure for antibiotic resistance in *N. gonorrhoeae* and *Mycoplasma genitalium* [2,3].

In this sense, testing for *M. genitalium* among asymptomatic individuals has been suggested [4]; but experts currently plead to avoid widespread asymptomatic screenings due to the lack of scientific evidence and the disturbing emergence of antibiotic resistance in *M. genitalium* [3,5,6]. In fact, recent studies attempted to evaluate the impact and effectiveness of these screening strategies but ultimately recommended further empirical work to improve understanding on *M. genitalium* and elucidate the real impact of these approaches [7,8].

2. Objectives

The objectives of this chapter are to:

- Estimate the prevalence of *M. genitalium* among asymptomatic people visiting a point-of-care (POC) service for rapid STI screening.
- Estimate the prevalence of antibiotic resistance among asymptomatically *M. genitalium*-infected individuals.
- Evaluate the performance of the novel MG+parC (beta) (SpeeDx, Australia) for detection of *M. genitalium* and fluoroquinolone resistance-associated mutations.
- Estimate the co-infection rate between *M. genitalium*, *C. trachomatis* and *N. gonorrhoeae* among asymptomatic people.

3. Materials and Methods

MGENRES

This is a cross-sectional study (MGENRES) conducted between October 2017 and January 2018 among asymptomatic people attending to Drassanes Exprés (DrasExp). DrasExp is a publicly funded POC service for rapid STI screening belonging to the Vall d´Hebron University Hospital in Barcelona, Spain. Therefore, testing for HIV, syphilis and the bacterial STIs *N. gonorrhoeae* and *C. trachomatis* is routinely offered to asymptomatic men and women.

Thus, participants prospectively enrolled in the study were additionally tested for *M. genitalium* and macrolide resistance using the ResistancePlus® MG (SpeeDx) test at the Microbiology Department of the Vall d´Hebron University Hospital. Pharyngeal swabs were not screened for *M. genitalium*. Patients testing positive for *M. genitalium* were invited to attend the STI Unit for a resistance-guided therapy. Macrolide resistance-associated mutations in the 23S rRNA gene detected were retrospectively confirmed by Sanger sequencing using a previously described methodology [9].

Subsequently, fluoroquinolone resistance-associated mutations in *parC* were studied using the MG+parC (beta) assay. Sanger sequencing of the *parC* gene was also performed. PCR amplicons were generated with primers MG-*parC*124-F (5´-AAACCAGTACAAAGACGGATCT-3´) and MG-*parC*478-R (5´-GAGGTTAGGCAGTAAGGTTG G-3´). For samples that primarily failed to amplify, internal primers MG-*parC*-A and MG-*parC*-B were used to perform a nested-PCR [10].

Briefly, the ResistancePlus® MG test is a multiplex qPCR for detection of *M. genitalium* and five mutations associated with macrolide resistance (A2058G, A2059G, A2058C, A2059C, A2058T; *Escherichia coli* numbering). On the other hand, the MG+parC (beta) test detects *M. genitalium* and five mutations linked with fluoroquinolone resistance (G248T [S83I], A247C [S83R], G259T [D87Y], G259A [D87N] and G259C [D87H]; *M. genitalium* numbering [amino-acid residue affected in ParC]). Both tests were performed according to the manufacturer´s instructions.

Characteristics of the study cohort were collected from a questionnaire that users must complete before screening.

The MGENRES study led to publications: i) Fernández-Huerta M, Barberá MJ, Esperalba J, et al. "Prevalence of *Mycoplasma genitalium* and macrolide resistance among asymptomatic people visiting a point of care service for rapid STI screening: a cross-sectional study" *Sex Transm Infect* 2019, and ii) Fernández-Huerta M, Bodiyabadu K, Esperalba J, et al. "Multicenter clinical evaluation of a novel multiplex real-time PCR (qPCR) assay for detection of fluoroquinolone resistance in *Mycoplasma genitalium*" *J Clin Microbiol* 2019.

Coinfection study

This is a retrospective study performed in *C. trachomatis*/*N. gonorrhoeae*-positive samples from asymptomatic individuals attending to DrasExp between November 2016 and September 2017. Positive specimens were tested for *M. genitalium* and macrolide resistance using the ResistancePlus® MG (SpeeDx, Australia) test at the Microbiology Department of the Vall d´Hebron University Hospital.

Characteristics of the study cohort were collected from the questionnaire that users must complete before screening.

4. Results

MGENRES

During the study period, 890 individuals attending to DrasExp were tested for *M. genitalium* and macrolide resistance. Of them, 66 participants were infected with *M. genitalium* and 34 harboured macrolide resistance. Results are displayed in table 1.

		***Mycoplasma genitalium* prevalence**
	Macrolide resistance prevalence	
Population	**Resistant MG N.; % (95% CI)**	**Total MG N.; % (95% CI)**	**Total individuals Tested (N.)**
Women	1; 10.0 (0.3-44.5)	10; 4.5 (2.2-8.1)	222
- Vagina	1; 14.3 (0.4-57.9)	7[a]; 3.2 (1.3-6.4)	222
- Rectum	0; 0.0 (0.0-60.2)	4; 6.9 (1.9-16.7)	58
MSW[b]	1; 10.0 (0.3-44.5)	10; 5.6 (2.7-10.0)	179
MSM	32; 69.6 (54.2-82.3)	46; 9.4 (7.0-12.3)	489
- Urethra	8[c]; 88.9 (51.8-99.7)	9[c]; 1.8 (0.8-3.5)	489
- Rectum	27; 67.5 (50.9-81.4)	40; 8.5 (6.1-11.4)	471
Total	34; 51.5 (38.9-64.0)	66; 7.4 (5.8-9.3)	890

Table 1. Prevalence of *M. genitalium* and macrolide resistance among asymptomatic individuals.
Percentages of macrolide resistance are calculated from the *M. genitalium* infections reported.
Abbreviations: MG, *Mycoplasma genitalium*; CI, confidence interval; MSW, men who have sex with women; MSM, men who have sex with men.
[a]One woman had infections in both vagina and rectum.
[b]All infections in MSW occurred in urethra.
[c]Three MSM had infections in both urethra and rectum.

The prevalence of urethral *M. genitalium* infection among MSW was significantly higher than the one among MSM; $p = 0.011$. Furthermore, those MSM engaging also heterosexual behaviours had a significantly higher prevalence of urethral infection (5.8%; 95% CI 1.2%-15.9%) compared with MSM with no female sexual partners (1.4%; 95% CI, 0.5%-3.0%); $p = 0.026$. In fact, there were no differences in urethral infection prevalence between MSW and bisexual men (MSMBI); $p = 0.960$). The prevalence of *M. genitalium* infection ($p = 0.012$) and macrolide resistance ($p < 0.001$) was markedly higher among MSM compared to MSW and women. Additionally, *M. genitalium* infection was found in 8.5% (95% CI, 4.5%-14.4%) of participants testing positive for *C. trachomatis* and *N. gonorrhoeae*.

On the other hand, the prevalence of fluoroquinolone resistance-associated *parC* mutations was 8.8% (95% CI, 2.9%-19.3%). The characteristics of the 57 individuals studied for quinolone resistance are described in table 2.

Characteristics of patients	ParC mutant MG N.; % (95% CI)	Non-mutant MG N.; % (95% CI)
N. 57	5[a]; 8.8 (2.9-19.3)	52[b]; 91.2 (80.7-97.1)
Women		
- Women	0; 0.0 (0.0-52.2)	10; 19.2 (9.6-32.5)
- MSW	0; 0.0 (0.0-52.2)	9; 17.3 (8.2-30.3)
- MSM	5; 100.0 (47.8-100.0)	33; 63.5 (49.0-76.4)
HIV status		
- Positive	1; 20.0 (0.5-71.6)	9; 17.3 (8.2-30.3)
- Negative	4; 80.0 (28.4-99.5)	43; 82.7 (69.7-91.8)
Macrolide resistance status		
- Resistant	4; 80.0 (28.4-99.5)	26; 50.0 (35.8-64.2)
- Susceptible	1; 20.0 (0.5-71.6)	26; 50.0 (35.8-64.2)
Location		
- Genital	1; 20.0 (0.5-71.6)	22[c]; 42.3 (28.7-56.8)
- Rectum	4; 80.0 (28.4-99.5)	34; 65.4 (50.9-78.0)

Table 2. Prevalence of *M. genitalium* and macrolide resistance among asymptomatic individuals.
Abbreviations: MG, *Mycoplasma genitalium*; CI, confidence interval; MSW, men who have sex with women; MSM, men who have sex with men; HIV, human immunodeficiency virus.
[a]Mutants included 2 G248T (S83I) and 3 G259T (D87Y).
[b]Non-mutant infections included 2 with *missense* mutations in position G248A (S83N)
[c]Four patients (3 MSM and 1 woman) had both genital and rectal *M. genitalium* infections.

Coinfection study

During the study period, a total of 298 positive specimens for *C. trachomatis* and *N. gonorrhoeae*, from 249 individuals, were retrospectively tested for *M. genitalium* and macrolide resistance. Overall, *M. genitalium* was detected in 12 participants (4.8%) of whom 8 were men (7 MSM) and 4 were women. Furthermore, *M. genitalium* was markedly more prevalent in vagina (8.9%) and rectum (8.9%) compared to the

nasopharynx (0.8%) and the urethra (2.9%). There were no differences between rates of co-infection among *C. trachomatis*-infected and *N. gonorrhoeae*-infected individuals, 4.0% and 5.2%, respectively; $p = 0.649$. Finally, 7 *M. genitalium* infections, all detected in MSM, harboured resistance-mediating mutations (58.3%).

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CHAPTER 4: Transmission dynamics in *Mycoplasma genitalium*

Key messages

- Molecular epidemiology in asymptomatic *M. genitalium* infections reveals two distinct clusters that significantly correlate with sexual conduct, suggesting the presence of two independent sexual networks with little connectivity between them: heterosexuals and men who have sex with men.
- The rise of antibiotic resistance in *M. genitalium* is a multiclonal phenomenom.
- The issue of macrolide resistance in *M. genitalium* among men who have sex with men may respond to allodemics affecting also other STIs.

MGENRES

Single-locus-sequence-based typing of the *mgpB* gene reveals transmission dynamics in *Mycoplasma genitalium*

Fernández-Huerta M, Serra-Pladevall J, Esperalba J, Moreno-Mingorance A, Fernández-Naval C, Barberá MJ, Aparicio D, Pich OQ, Pumarola T, Jensen JS, Espasa M

J Clin Microbiol. 2020

1. Introduction

Sexual transmission in *Mycoplasma genitalium* was first suggested by looking at concordance of infections between sexual partners [1], and, shortly after, it was evidenced with molecular epidemiology using a SLSBT system in positive samples from infected couples [2]. This procedure was originally developed from a diagnostic PCR assay [3]. After revealing high efficiency and excellent discriminatory power, the *mgpB* (MG191) gene region targeted by the MgPa-1/MgPa-3 primer set was proposed for molecular typing purposes [4]. Subsequently, this feasible molecular typing method, based on the sequence of a 231 bp non-repeated region of the MgPa operon [5], has been robustly used in general epidemiological studies demonstrating also reproducibility and stability [2, 6, 7]. Nevertheless, little is known regarding the structure of sexual networks, and the transmission and spread of antimicrobial resistance in *M. genitalium*.

2. Objectives

The objective of this chapter is to:

- Explore the transmission dynamics of *M. genitalium* infection and the spread of antimicrobial resistance among asymptomatic individuals.

3. Materials and Methods

This is a retrospective study conducted between October 2017 and January 2018 among asymptomatic people as part of the parent study MGENRES, already described in chapter 3 [8, 9].

Thus, *M. genitalium*-positive specimens from study participants were stored at -20°C and subsequently sent to the Statens Serum Institut in Copenhaguen, Denmark, for molecular typing. SLSBT analysis of the *mgpB* gene was performed for the molecular epidemiology procedure in *M. genitalium*. Briefly, specimens were re-extracted using 20% Chelex® 100, and PCR amplification and sequencing of the 231 bp region of the *mgpB* gene was performed as previously described using the MgPa-1/MgPa-3 primer set [2, 3].

To visualize the relationships between genotype profiles, a maximum likelihood phylogeny was constructed with IQ-TREE (version 1.6.10) software using a K3Pu+F+I model. Branch support values were generated from 1000 bootstrap replicates. The phylogenetic tree was then complemented using iTOL (version 4.4.2).

4. Results

A total of 70 *M. genitalium*-positive specimens from 66 individuals were collected during the recruiting period of the parent study [8, 9]. Of them, 65 samples from 61 participants were suitable for the subsequent SLSBT analysis and sequencing was successful in 57 samples (87.7%) from 54 individuals (88.5%). Overall, 32 different sequence types (STs) were described among the 54 infected individuals included in the study. None of the STs was identical with the reference ST_G37. Despite the remarkable genetic heterogeneity, some strains from different participants revealed an identical ST.

The dendrogram in figure 1, based on the genotype profiles from the *mgpB*-based SLSBT method, was complemented with additional information: gender/sexual conduct, infection site, HIV serostatus, and the presence of macrolide and fluoroquinolone resistance-associated genotypic markers in the 23S rRNA and the *parC* genes, respectively. Overall, individuals with *M. genitalium* infection can be separated into two distinct clusters that also significantly correlate with sexual conduct. The major genotypic cluster 1, which includes reference ST_G37, is mainly comprising infections occurring in women and MSW (11/17; 64.7%), while major cluster 2 is mostly grouping infections in MSM and MSMBI (31/37; 83.8%); $p < 0.001$. Regarding macrolide resistance, mutations A2071G and A2072G in the 23S rRNA gene were randomly distributed among STs in our study. Also, mutations G248A (S83N) and G259T (D87Y) in the *parC* gene, associated with fluoroquinolone resistance in *M. genitalium*, appeared among distinct ST-clones.

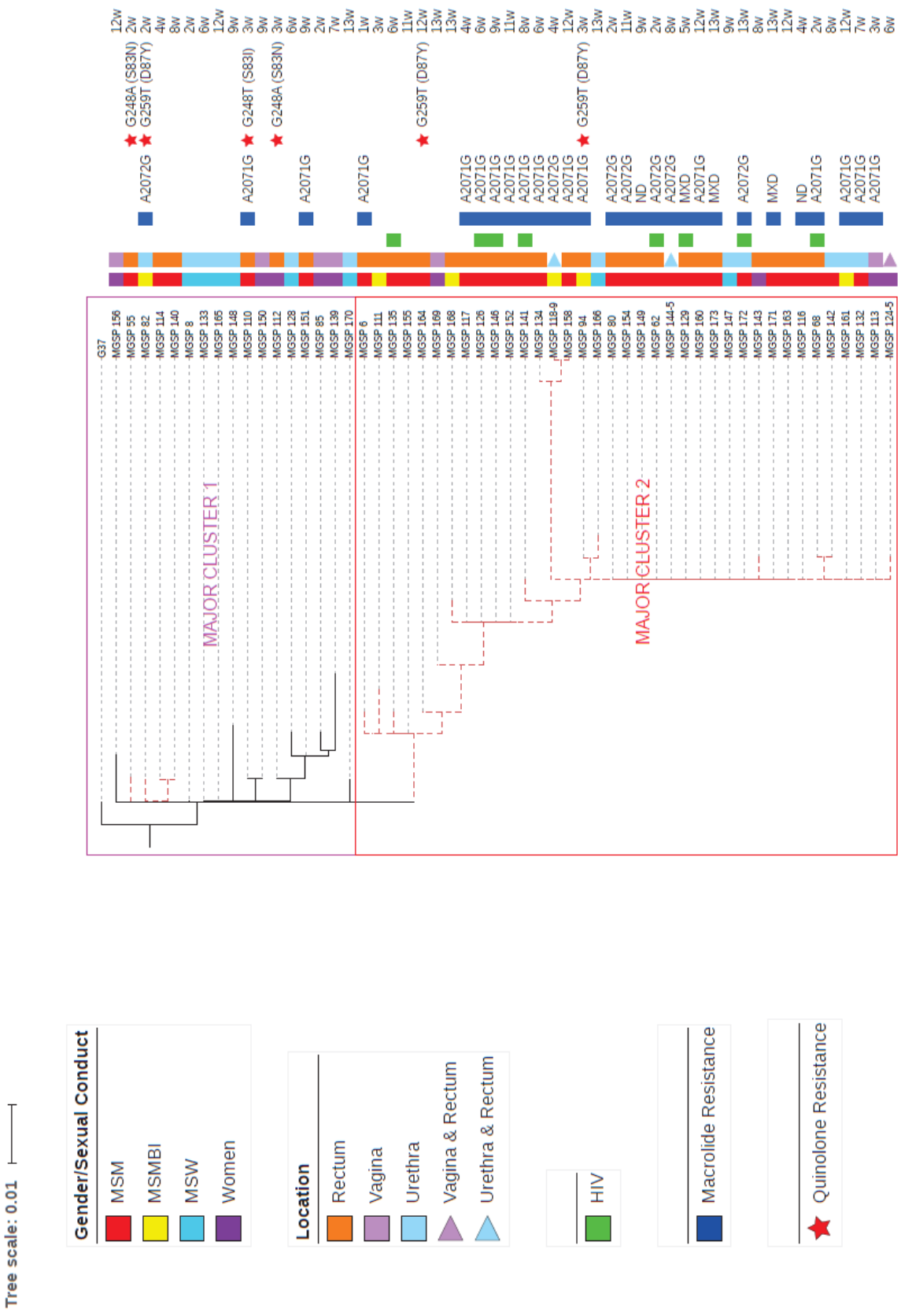

Figure 1. Clustering dendogram, using the *mgpB*-based single-locus-sequence-based typing, from *M. genitalium* strains of asymptomatically infected individuals.

The sequence of the *M. genitalium* G37 strain is used as a reference (accession number NC_000908.2). Macrolide resistance-associated mutations in the 23S rRNA gene and fluoroquinolone resistance-

associated mutations in the *parC* gene are also shown (*M. genitalium* numbering). The last column (w) accounts for the study week of specimen collection. The red dashed lines constitute clusters mostly represented by MSM.

Abbreviations: MSM, men who have sex with men; MSMBI, bisexual men; MSW, men who have sex with women; HIV, Human Immunodeficiency Virus; ND, not determined; MXD, mixed genotypes.

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CHAPTER 5: General discussion

1. *M. genitalium* and antibiotic resistance in Europe

Despite the publication of numerous guidelines by the *International Union against Sexually Transmitted Infections Europe* (IUSTI-Europe), management and treatment of STIs differ between European countries depending on national/regional recommendations usually based on local epidemiology and the available scientific evidence. Consequently, these differences may distinctly influence antimicrobial resistance in *Mycoplasma genitalium* among European regions. In fact, Sweden is already well recognized to have a particular scenario in terms of macrolide resistance [1]. Unlike most European countries, doxycycline has always been the preferred treatment for NGU and *Chlamydia trachomatis* in Sweden [2-5]. Presumably, this may account for the low prevalence of macrolide resistance in *M. genitalium* reported in Sweden [1]. Additionally, the extended dose azithromycin regimen for *M. genitalium* infection was commonly used in Sweden, likely contributing to limit also the selection and spread of resistances [4]. In the investigation presented in chapter 2, the Swedish estimates of macrolide resistance were among the lowest in Europe. Nevertheless, as similarly pointed by Anagrius C et al. [5], an important increase in resistance level has been also observed in Sweden throughout recent years. However, the management of NGU and *C. trachomatis* infection has not significantly changed and the consumption of macrolides for non-STIs has shown a decreasing trend during this period [5]. Furthermore, although clonal spread of resistant strains has been postulated [6,7], molecular epidemiology studies reveal this unlikely [5,8]. Consequently, the flow of strains from other European countries may account as a hypothesis for this increase in macrolide resistance in Sweden [5]. Also, the use of single-dose azithromycin in dual therapy against *Neisseria gonorrhoeae* could have undesirably influenced macrolide resistance in *M. genitalium*.

The situation in the other Nordic countries (Finland, Norway, and Denmark) is completely opposed. Contrary to Sweden, these countries have widely used azithromycin, mainly as a single dose, for NGU and *C. trachomatis* [1]. In chapter 2, we have reported dramatically high rates of macrolide resistance, usually exceeding 50%, with a growing trend in recent years. Although most European countries have followed very similar antimicrobial regimens, Northern countries have developed a well-defined

and efficient system for STI screening including *C. trachomatis* and *N. gonorrhoeae* [1]. Therefore, these programs may indeed reduce the prevalence of STIs [9], but they may also result in selective pressure for antibiotic resistance in *M. genitalium* [10]. This could explain the remarkable difference in macrolide resistance estimates between Northern Europe (excluding Sweden) and Western/Southern Europe, where these screening programs have not been widely implemented.

On the other hand, in Western/Southern Europe, where azithromycin has been widely utilized, macrolide resistance has also increased throughout the years. Although data in chapter 2 demonstrated high heterogeneity among countries, the prevalence of macrolide resistance ranges between the two previously described scenarios: Nordic countries and Sweden.

Lastly, in the review conducted in chapter 2, data from Eastern Europe are very limited since only one multicenter cross-sectional study was performed in Russia and Estonia between 2013 and 2016 [11]. This investigation reported the lowest rate of macrolide resistance-associated mutations in Europe ranging from 33/719 (5%) in Russia to 11/110 (10%) in Estonia. Authors attributed this finding to two factors: first, testing for *M. genitalium* is not introduced everywhere so most infections are not diagnosed and treated; and second, *C. trachomatis* screening is less frequent compared to most European countries. Both reasons are translated into a restricted use of azithromycin against STIs, and consequently this may limit the selection and spread of macrolide resistance in *M. genitalium*.

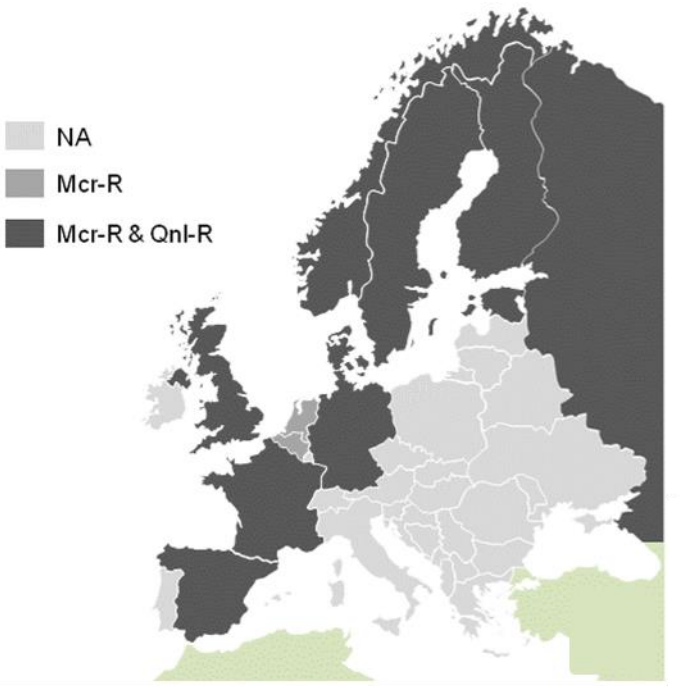

Figure 1. European mapping of countries with antimicrobial resistance data reported until 2018.
Abbreviations: NA, not available; Mcr-R, macrolide resistance; Qnl-R, quinolone resistance.

Fluoroquinolone resistance in *M. genitalium* is also emerging in Europe, although data remain scarce. In the review presented in chapter 2, 12 studies

from 10 different countries provided rates of fluoroquinolone resistance-associated mutations in *parC* resulting in an estimated prevalence median of 5%. The increase in the use of moxifloxacin, a costly antibiotic that may also cause important adverse events, may produce selective pressure for fluoroquinolone resistance in Europe. Nevertheless, unlike the issue of macrolide resistance, ParC mutants do not always lead to moxifloxacin treatment failures so further information is required to fully establish the contribution of these mutations to in vivo fluoroquinolone resistance [12].

In conclusion, the prevalence of macrolide resistance has been rapidly increasing in Europe, markedly in Northern countries (except Sweden) where estimates exceed 50%. Also, fluoroquinolone resistance-associated mutations are emerging in Europe up to a rate of 5%. The discrepancies on the management and treatment of STIs between European countries may distinctly influence antimicrobial resistance in *M. genitalium*. These findings support the need to conduct representative and well-defined surveillance on antimicrobial resistance in *M. genitalium* at both local and European level, since data remain scarce (figure 1).

2. *M. genitalium* and antibiotic resistance in Barcelona, Spain

The additional research presented in chapter 2 provides evidence regarding macrolide resistance in Spain, where epidemiological data remains limited [13-16]. In the study, the overall rate of macrolide resistance in *M. genitalium* was 36%. However, macrolide resistance was significantly more frequent among MSM (55%) compared to MSW or women (27% and 10%, respectively); findings previously reported by Barberá MJ et al. [13]. Additionally, results from this 2016 to 2017 cohort described in chapter 2 were compared with a previous report published by Barberá MJ et al. [13], in which macrolide resistance in *M. genitalium* was estimated in similar settings in Barcelona during 2013–2014, utilizing a comparable methodology. Inferences from this analysis indicate that macrolide resistance did not significantly increase during this period; neither from an overall approach nor within specific subpopulations (MSM, MSW and women).

Emerging macrolide resistance in *M. genitalium* has been widely reported in the last decade, as described in the previous section. Nevertheless, most of these cross-sectional studies focus on very specific populations at risk for STIs. So, there is limited longitudinal data regarding the overall evolution of macrolide resistance among general population in the medium term. The research suggests that macrolide resistance may be stabilizing around 35%, at least in our local epidemiology. Several factors could be influencing this situation. Firstly, there is increasing concern about *M. genitalium* and antibiotic resistance worldwide since first treatment failures appeared a decade ago [17,18]. Consequently, medical societies are fully aware of this issue leading to a significant improvement on the diagnosis and management of *M. genitalium* infections in many settings [19,20]. Secondly, since May 2016, as a response to the increasing macrolide resistance in *M. genitalium*, European guidelines changed to recommend doxycycline as the first-line empirical treatment for non-filiated NGU [21]. Thus, this statement also recommends the posterior investigation for further infectious etiologies (including *M. genitalium* and macrolide resistance, if available) in order to refine the empirical therapy. Indeed, this treatment change may have contributed to control the spread of macrolide resistance in *M. genitalium*. Finally, macrolide resistance in our settings may have reached the plateau leading its stabilization around 35%.

In conclusion, the study included in chapter 2 provides further data regarding macrolide resistance in *M. genitalium* in Spain. Additionally, the comparison between the current data and previous investigations indicates that macrolide resistance in *M. genitalium* did not significantly increase, at least between 2013 and 2017 in our study area, Barcelona. This evidence contrasts with the increasing emergence of antimicrobial resistance worldwide and could provide a second opportunity for the adequate utilization of azithromycin against *M. genitalium* based on novel approaches. Nevertheless, macrolide resistance continues to be a major concern in *M. genitalium* since therapeutic alternatives remain scarce. A strict local surveillance is evidently required to facilitate the optimization of antibiotic administration for NGU and *M. genitalium* infections, and reduce the selection and transmission of resistance. Indeed, the implementation of combined diagnostic-resistance tests for *M. genitalium* plays a key role in this purpose.

Additional results in chapter 2 provided further data regarding fluoroquinolone resistance in *M. genitalium* in Spain, where estimates remain limited. In the same study cohort, fluoroquinolone resistance-associated mutations were detected in 10% of infections, similar to previous investigations [13,14,22]. Additionally, the presence of fluoroquinolone resistance-associated mutations was strongly associated with macrolide resistance in our series. In fact, the prevalence of resistance to both classes of antibiotics was (8%) in the study population. Consequently, multi-drug resistant infections may gradually appear in our settings against which therapeutic options are very scarce.

3. *M. genitalium* infection among asymptomatic individuals: should we screen and how?

Most of the work described in chapters 3 and 4, belongs to the MGENRES project, focused on the study of *M. genitalium* infection among asymptomatic people.

Chapter 3 aims to estimate the prevalence of *M. genitalium* and antibiotic resistance among asymptomatic individuals visiting a POC service for rapid STI screening. The overall prevalence of *M. genitalium* in this cohort was 7%, with higher estimates among MSM (9%) compared to heterosexuals (5%), as already discussed in the previous section. There are few investigations on asymptomatically *M. genitalium*-infected people [23-25], all reporting lower prevalence. On the other hand, the prevalence of macrolide resistance was 52%, being seven times more prevalent among MSM when compared to MSW and women. Similar resistance rates have been recently reported in different settings in Spain [13]. The prevalence of fluoroquinolone resistance-associated mutations was 12% in this series. In accordance with results discussed in the previous section, these findings provide further evidence that fluoroquinolone resistance is emerging in Europe, particularly among MSM.

Furthermore, the work showed that MSW and bisexual men had a similar prevalence of urethral *M. genitalium* infection, much higher when compared with MSM with no female sexual partners. Several studies have reported that urethral *M. genitalium* infection is more associated with unprotected vaginal sex rather than anal sex [25,26]. Behavioural patterns but also a better environment for *M. genitalium* fitness in vagina,

resulting in higher bacterial loads, could explain this aspect. In this sense, some investigations conclude that vaginal intercourse may be a major route of transmission for *M. genitalium*, and suggest that strengthening the screening of this bacterium among bisexual men could be an important strategy to control the infection [25].

Additionally, the mother study of the MGENRES project prospectively evaluated the resistance-guided therapy approaching *M. genitalium* asymptomatic screening. Despite the limited number of infected individuals who returned for clinical assistance, no treatment failures were detected among macrolide-resistant infections using moxifloxacin, and only one probable azithromycin failure case was observed in a wild-type *M. genitalium* infection. Similar rates of macrolide resistance selection were previously reported using a resistance-guided sequential treatment approach in Melbourne, Australia [27].

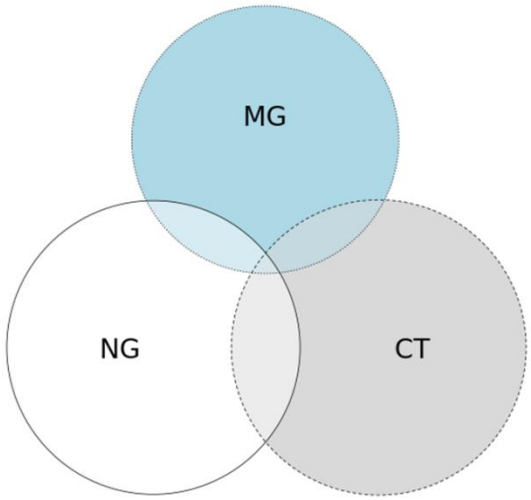

Figure 2. Venn diagram of bacterial STIs co-infection. Figure adapted from findings in chapter 3, and created with http://eulerr.co.
Abbreviations: MG, *Mycoplasma genitalium*; NG, *Neisseria gonorrhoeae*; CT, *Chlamydia trachomatis*.

Despite current available evidence is insufficient to justify *M. genitalium* screening among any defined asymptomatic population, there are some important points that should be considered. First, *M. genitalium* usually causes urethritis in men but it is also associated with very serious adverse outcomes in women ranging from cervicitis to preterm birth, spontaneous abortion, PID and infertility [19,28,29]. Second, although poorly established, the relation between *M. genitalium* and HIV suggests that the bacterial infection may facilitate HIV acquisition through disruptive and inflammatory processes in the anogenital mucosa during exposure [30]. Last, results in chapter 3 demonstrate that the prevalence of *M. genitalium* is similar to the one of *C. trachomatis* and *N. gonorrhoeae*. Given the similarities, it may be paradoxical why gonorrhoea screening, which also may select for antimicrobial resistance [31], is widely recommended while *M. genitalium* asymptomatic testing is not [19,32]. Additionally, *C. trachomatis* and *N. gonorrhoeae* screening strategies may

facilitate macrolide resistance spread in *M. genitalium* if an undetected co-infection (9% in the MGENRES cohort) is present and azithromycin is prescribed (figure 2) [10]. This last aspect is especially discussed in the manuscript entitled "*Mycoplasma genitalium* co-infection with *Chlamydia trachomatis* and *Neisseria gonorrhoeae* among asymptomatic patients: the silent wick for macrolide resistance spread" and presented in Chapter 3.

On the other hand, chapter 4 aims to explore the transmission dynamics of *M. genitalium* infection and the spread of antimicrobial resistance. Overall, infected individuals from the MGENRES cohort can be separated into two distinct genotypic clusters that also significantly correlate with sexual conduct. The first genotypic cluster mainly comprises infections occurring in women and MSW, while the other cluster mostly comprises infections in MSM and MSMBI. This characteristic clustering structure reveals transmission dynamics of *M. genitalium* infections among a general

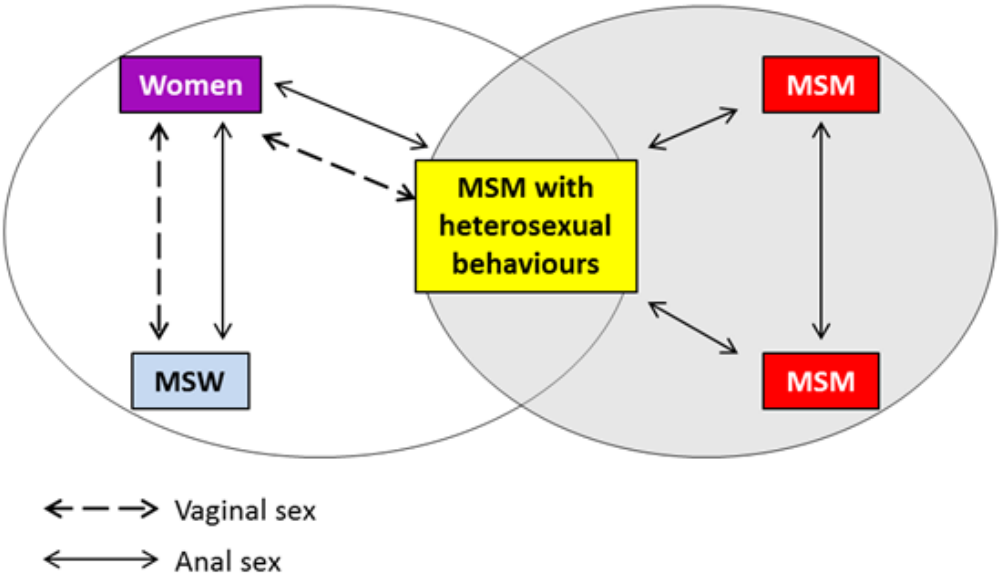

Figure 3. Hypothetical transmissibility of *M. genitalium* infection between sexual networks.
Hypothetical modeling based on the existence of two well defined sexual networks where bisexual men may disturb such structure by bridging the genotype distribution patterns.
Abbreviations: MSW, men who have sex with women; MSM, men who have sex with men.

asymptomatic population. The correlation between sexual behavior and genotype may translate into the structuration of well-defined epidemiological clusters, and may suggest the presence of two independent sexual networks with little connectivity between them (figure 3). Consequently, the spread of genetic diversity and antimicrobial resistance may be limited to each sexual transmission group. In this

hypothetical scenario, the MSMBI group might act as a bridge between the disjoint populations of women/MSW and MSM. Despite that vaginal sex may be the major route of *M. genitalium* transmission in terms of risk, as described in the previous section, anal intercourse in MSM is likely the most common cause of spread because of dense sexual networks in this subpopulation.

In the current context of the exponential rise of antimicrobial resistance in *M. genitalium* worldwide, some studies have suggested selection of certain clones as a possible cause of this resistance spread [6,7]. However, in accordance with other investigations [3,8,33,34], results in chapter 4 do not support the idea of a clonal phenomenon but demonstrates the multiclonal feature of the emergence of antibiotic resistance in *M. genitalium* to both macrolides and fluoroquinolones (figure 4).

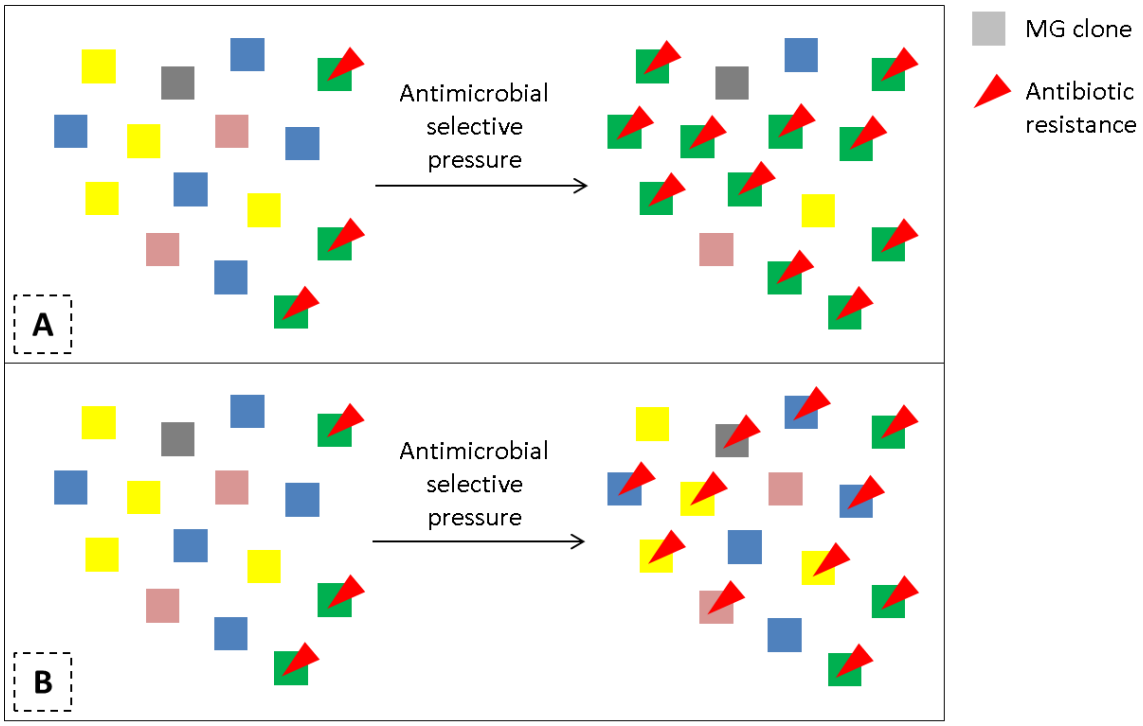

Figure 4. A: Modeling of antimicrobial resistance spread driven by a clonal phenomenon in response to selective pressure. **B:** Hypothetical polyclonal feature of antibiotic resistance in *M. genitalium*. Abbreviations: MG, *Mycoplasma genitalium*.

The challenge of antimicrobial resistance in MSM, particularly to macrolides, requires further discussion. As previously pointed out by several authors, macrolide resistance in *M. genitalium* is not homogeneously distributed among the general population but the prevalence in MSM is usually overwhelming [13,35,36]. Furthermore, findings in chapter 4 suggest that a significant transmission of this resistance to heterosexual men and women is unlikely. This specific phenomenon in MSM relies on different structural

and environmental characteristics such as a dense network connectivity and an antibiotic selective pressure enhanced by the wide use of azithromycin in STIs. The term “allodemics” was first introduced by Baquero F et al. to describe the polyclonal spread of extended-spectrum-beta-lactamase producing bacteria in a clinic in Spain, as a consequence of the selective pressure exerted by the use of cephalosporins in that hospital [37]. Thus, as already pointed out by Kenyon et al. [38], the phenotype of macrolide resistance in MSM may also be an allodemic phenomenon, triggered by the previously described challenges, affecting a wide range of STIs such as gonorrhea, syphilis, enteric pathogens, and *M. genitalium*. Consequently, to avoid undesirable selection and spread of macrolide resistance in *M. genitalium*, azithromycin should be gradually replaced in the syndromic management of STIs, particularly in vulnerable populations such as MSM.

In conclusion, chapter 4 provides further evidence regarding the structure of transmission dynamics and sexual networks in *M. genitalium* infection. Additionally, the investigation demonstrates the multiclonal selection and spread of antibiotic resistance for the bacterium, and point the allodemic phenomenon of macrolide resistance in STIs among MSM.

4. Management of *M. genitalium* infection, the way forward

Recently, a resistance-guided sequential treatment for *M. genitalium* infection in Melbourne, Australia, using a novel assay that reports both the detection of the bacterium and macrolide resistance-associated mutations, demonstrated good efficacy eradicating uncomplicated infections, and it was associated with 3% to 5% selection of *de novo* macrolide resistance [27,39]. In fact, Australian and British guidelines for the management of *M. genitalium* infection already recommend this strategy [40,41]. This resistance-guided sequential therapy relies on the syndromic initial use of doxycycline, which possibly lowers the *M. genitalium* bacterial load, sequenced with either azithromycin or moxifloxacin based on the etiology and the macrolide resistance status (figure 5) [40,41]. Although this two-step strategy is promising and provides evidence base for current management guidelines, there are certain points that require further discussion.

Despite the limited clinical evidence [36], the extended regimens compared with single-dose ones are now well accepted for azithromycin against *M. genitalium* infection, except for the United States [42]. Nevertheless, there are significant differences between guidelines in terms of macrolide dosage that require further consensus: (i) the International Union against Sexually Transmitted Infections Europe recommends azithromycin 500 mg day 1 and 250 mg days 2 to 5 (1.5 g total) [19]; (ii) the Australasian Sexual Health Alliance establishes azithromycin 1 g day 1 and 500 mg days 2 to 4 (2.5 g total) [40]; and (iii) the British Association for Sexual Health and HIV recommends azithromycin 1 g day 1 and 500 mg days 2 to 3 (2 g total) [41]. Besides that, the resistance-guided sequential therapy starts with doxycycline 100 mg for seven days, before the initiation of the macrolide or the fluoroquinolone [40,41]. In a recent editorial commentary [43], Vazquez F and Fernández J highlighted the fact that long antimicrobial strategies can detrimentally affect treatment adherence and augment antibiotic adverse effects. Combined rather than sequential therapies for *M. genitalium* infection may shorten the duration of the treatment and be equally effective, but this must be further evaluated. In fact, preliminary *in vitro* observations have demonstrated some evidence of synergistic effect between moxifloxacin and doxycycline, indicating that this might be, for instance, a promising combination for future trials [44]. Finally, bacterial load determinations might be also useful in the prognosis of the treatment outcomes, but further research must first standardize these technical approaches.

On the other hand, despite the limited evidence, several guidelines recommend moxifloxacin 400 mg for 14 days for the treatment of complicated *M. genitalium* infections [19,40,41]. This includes severe syndromes in which *M. genitalium* may be considered as probable or possible cause of the disease (e.g., PID, epididymitis, severe proctitis, or chronic prostatitis). Again, although prolonged antimicrobial regimens are likely improving cure rates, they difficult the adherence and favor the appearance of adverse events, potentially serious with fluoroquinolones. In this scenario, shorter courses of moxifloxacin may be of interest for the treatment of these complicated conditions, but evidence is very limited [40].

Antimicrobial alternatives in case of multidrug resistance in *M. genitalium* are scarce, but pristinamycin, sitafloxacin, or minocycline may successfully eradicate such infections [44]. Thus, the pursuit of new active agents against *M. genitalium* is still required. Of note, the treatment of *M. genitalium* infections in pregnancy, breastfeeding, or perinatal infections is beyond the scope of these lines and requires special considerations.

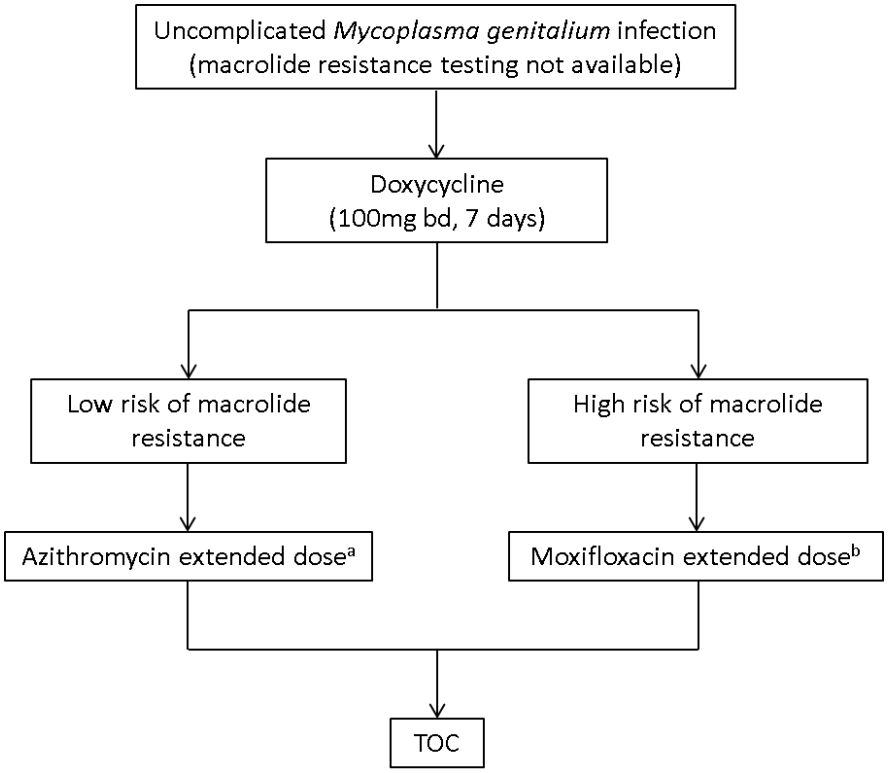

Figure 5. Risk-guided sequential management of uncomplicated *M. genitalium* infection.
In case of azithromycin treatment failure in a low-risk individual, moxifloxacin could be prescribed. Contrary, if moxifloxacin treatment failure occurs in high-risk patients, azithromycin may be used as an alternative since other options are scarce.
Abbreviations: TOC, test-of-cure.
[a]Currently, differences in azithromycin dosage recommendations exist and require further discussion.
[b]It require additional consensus whether moxifloxacin treatment should exceed seven days up to, for instance, 10 days.

Unfortunately, the diagnostic technology required for the resistance-guided therapy is not yet widely available, and very few health centers can implement this management algorithm. In response to this scenario and based on the findings reported in this manuscript, we would like to propose the implementation of a risk-guided sequential treatment in settings where resistance testing is not available (figure 5). This is, specific subpopulations with an evidenced overwhelming macrolide resistance in *M.*

genitalium, such as MSM, could use moxifloxacin as first-line treatment always preceded by 7-day doxycycline; optimizing thus antimicrobial efficacy and stewardship. In fact, Australian guidelines for the management of *M. genitalium* infection already point a similar statement, "Without access to resistance testing, it is reasonable to assume macrolide resistance in infections persisting after failure of azithromycin and in MSM" [40]. The major concern of the wide implementation of this risk-guided therapy would be that the overall increase in the use of moxifloxacin, a costly antibiotic associated also with important adverse events, may potentially produce selective pressure for fluoroquinolone resistance in *M. genitalium*. However, unlike macrolide resistance, mutations in the QRDR of the *parC* gene, mainly involving the ParC residues S83 and D87 (*M. genitalium* numbering), are not always translated into moxifloxacin treatment failures, even for the widely reported mutation S83I [12,45]. This association and the complementary contribution of *gyrA* mutations in quinolone resistance must be further elucidated. On the other hand, of note, prescribing moxifloxacin among *M. genitalium*-infected individuals with high risk of macrolide resistance would presumably occur in most of them following azithromycin regimens, rationalizing the overconsumption of the fluoroquinolone. Finally, the use of moxifloxacin among these defined subpopulations may potentially reduce the rate of treatment failure, optimizing antimicrobial stewardship and reducing the cost of successive TOCs. Thus, this prompt eradication of *M. genitalium* with the use of moxifloxacin might control the spread of antibiotic resistance to both macrolides and fluoroquinolones within dense sexual networks, such as those described in MSM.

Indeed, studies must further evaluate the impact of this risk-guided sequential treatment in terms of economic costs, individual health, and epidemiology. Furthermore, this approach should be first assessed by experts, always supported by local epidemiology; and for that, well-defined surveillance on antimicrobial resistance in *M. genitalium*, currently limited, is essential.

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CONCLUSIONS

- The prevalence of macrolide resistance in *M. genitalium*, distinctly influenced by differences in STI management recommendations among European countries, has been increasing in the last decade in Europe.
- Despite the overall rise of antimicrobial resistance in *M. genitalium*, this has not significantly increased in the last years in Barcelona, Spain.
- Although there is insufficient data to justify *M. genitalium* screening strategies, the prevalence of the infection and antibiotic resistance is notable among asymptomatic individuals, especially in men who have sex with men.
- The rise of macrolide resistance in *M. genitalium* does not respond to a clonal spread but to a multiclonal phenomenon likely influenced by allodemics.
- The implementation of a risk-guided sequential treatment may be efficient eradicating *M. genitalium* infection and optimizing antimicrobial stewardship.

LINEAS DE FUTURO

A raíz de los trabajos realizados en el Hospital Universitario Vall d´Hebron de Barcelona en el contexto de la tesis doctoral "Epidemiology and antibiotic resistance in *Mycoplasma genitalium*", podemos plantear una serie de líneas de trabajo futuras de importante relevancia microbiológica y clínica.

Vigilancia epidemiológica de resistencias antibióticas en *M. genitalium*

En el contexto actual, parece imprescindible la elaboración de un programa de vigilancia y monitorización de las resistencias antibióticas en *M. genitalium*; tanto a nivel local como a nivel nacional/internacional. En este sentido, hospitales de tercer nivel y/o centros de referencia han de recoger, analizar y divulgar los datos epidemiológicos de resistencia antibiótica en *M. genitalium*.

Validación analítica/clínica de las herramientas diagnósticas disponibles

En los últimos años, los avances tecnológicos han permitido el desarrollo de herramientas diagnósticas moleculares, algunas comerciales, capaces de detectar *M. genitalium* y mutaciones asociadas con la resistencia a macrólidos y/o fluoroquinolonas. Así, es responsabilidad de los laboratorios de microbiología mantener esta metodología bajo constante evaluación para asegurar la calidad y eficiencia diagnósticas frente a la epidemiología y los cambios genéticos naturales de la bacteria.

Estudios de susceptibilidad antibiótica *in vitro* e *in vivo*

A diferencia de la resistencia a macrólidos, la relación entre los genes *parC* y *gyrA* y la resistencia a fluoroquinolonas no es clara. En este sentido, son necesarios estudios de susceptibilidad *in vitro* en *M. genitalium* para evaluar la correlación genotipo-fenotipo en el contexto de la resistencia a fluoroquinolonas. Del mismo modo, es importante también valorar clínicamente estas mutaciones en relación a la eficacia terapéutica de las fluoroquinolonas contra la infección.

Los estudios de susceptibilidad *in vitro* pueden servir también para testar la eficacia de nuevos antibióticos y estudiar posibles efectos de sinergia antimicrobiana.

Determinantes genéticos de virulencia en *M. genitalium*

M. genitalium es reconocido como causa frecuente de uretritis y otros síndromes genitales en hombres y mujeres. No obstante, se desconoce la implicación real de esta bacteria en complicaciones como la epididimitis o la enfermedad pélvica inflamatoria. Las proteínas P110 y P140 de *M. genitalium* están implicadas en la adhesión y constituyen un importante factor de virulencia. En este sentido, es interesante profundizar en la relación existente entre estos factores proteicos y las distintas complicaciones patológicas en el ser humano; así como la inmunología detrás de estas condiciones.

Nuevas estrategias en el manejo clínico de la infección por *M. genitalium*

La terapia secuencial guiada por resistencia y/o riesgo, el uso de tratamientos combinados, o la utilización de fluoroquinolonas como antimicrobianos de primera línea, así como la optimización de las posologías empleadas en la actualidad, son algunas de las estrategias que han de evaluarse en profundidad en el contexto del manejo de la infección por *M. genitalium*. Equipos multidisciplinares han de estar preparados técnicamente para poder evaluar los riesgos y beneficios de los distintos enfoques.

Debido a la emergencia de resistencias antibióticas en *M. genitalium*, es también necesaria la búsqueda de alternativas no antibióticas para combatir la infección.

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PUBLICATION OVERVIEW

Major publications

Fernández-Huerta M, Barberá MJ, Esperalba J, et al. Prevalence of *Mycoplasma genitalium* and macrolide resistance among asymptomatic people visiting a point of care service for rapid STI screening: a cross-sectional study. Sex Transm Infect. 2019

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Fernández-Huerta M, Serra-Pladevall J, Esperalba J, et al. Single-locus-sequence-based typing of the *mgpB* gene reveals transmission dynamics in *Mycoplasma genitalium*. J Clin Microbiol. 2020

Supplemental publications

Fernández-Huerta M, Serra-Pladevall J, Pich OQ, et al. From resistance-guided to risk-guided antimicrobial therapy in *Mycoplasma genitalium*. Sex Transm Dis. 2020 [commentary]

Fernández-Huerta M, García-Pérez JN, Pich OQ, et al. *Mycoplasma genitalium*: an emerging pathogen at the STIs units. Piel. 2019. [article in Spanish] [review article]

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Fernández-Huerta M, Serra-Pladevall J, Barberá MJ, et al. *Mycoplasma genitalium* and antibiotic resistance in Spain; the need for an effective response against an emerging problem. Enferm Infecc Microbiol Clin. 2019 [article in Spanish] [letter to editor]

Other related publications:

Barberá MJ*, **Fernández-Huerta M***, Jensen JS, et al. *Mycoplasma genitalium* macrolide and fluoroquinolone resistance: prevalence and risk factors among a 2013-2014 cohort of patients in Barcelona, Spain. Sex Transm Dis. 2017

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Fernández-Huerta M, Salmerón P, Silgado A, et al. Clinical evaluation of the ResistancePlus MG FleXible test on the GeneXpert Infinity-48s instrument: a near-patient assay for simultaneous detection of *Mycoplasma genitalium* and macrolide resistance. Diagn Microbiol Infect Dis. 2020