frontiers | Research Topics

Syphilis infection: Clinical, epidemiology, basic science, and behavioral research

Edited by
Pingyu Zhou, Justin David Radolf, Lei Zhang and Joseph D. Tucker

Published in
Frontiers in Medicine
Frontiers in Immunology
Frontiers in Microbiology
Frontiers in Public Health

ISSN 1664-8714
ISBN 978-2-83251-488-7
DOI 10.3389/978-2-83251-488-7

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Syphilis infection: Clinical, epidemiology, basic science, and behavioral research

Topic editors

Pingyu Zhou — Tongji University, China

Justin David Radolf — UCONN Health, United States

Lei Zhang — Melbourne Sexual Health Centre (MSHC), Australia

Joseph D. Tucker — University of North Carolina at Chapel Hill, United States

Citation

Zhou, P., Radolf, J. D., Zhang, L., Tucker, J. D., eds. (2023). *Syphilis infection: Clinical, epidemiology, basic science, and behavioral research*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83251-488-7

Table of contents

frontiers | Frontiers in Immunology

TYPE Editorial
PUBLISHED 06 April 2023
DOI 10.3389/fimmu.2023.1182069

OPEN ACCESS

EDITED AND REVIEWED BY
Ian Marriott,
University of North Carolina at Charlotte, United States

*CORRESPONDENCE
Pingyu Zhou
zpyls@yahoo.com
Joseph D. Tucker
jdtucker@med.unc.edu
Lei Zhang
lei.zhang1@monash.edu
Justin David Radolf
JRadolf@uchc.edu

SPECIALTY SECTION
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

RECEIVED 08 March 2023
ACCEPTED 24 March 2023
PUBLISHED 06 April 2023

CITATION
Zhou P, Ye M, Tucker JD, Zhang L and Radolf JD (2023) Editorial: Syphilis infection: clinical, epidemiology, basic science, and behavioral research. *Front. Immunol.* 14:1182069. doi: 10.3389/fimmu.2023.1182069

Editorial: Syphilis infection: clinical, epidemiology, basic science, and behavioral research

Pingyu Zhou[1]*, Meiping Ye[1,2], Joseph D. Tucker[3]*, Lei Zhang[4,5,6]* and Justin David Radolf[7]*

[1]STD Institute, Shanghai Skin Disease Hospital, Tongji University School of Medicine, Shanghai, China, [2]Department of Dermatology, Xinhua Hospital, Shanghai Jiaotong University School of Medicine, Shanghai, China, [3]Departments of Medicine, Pediatrics, Genetics and Genomic Science, Molecular Biology and Biophysics, and Immunology, UConn Health, Farmington, CT, United States, [4]Artificial Intelligence and Modelling in Epidemiology Program, Melbourne Sexual Health Centre, Alfred Health, Melbourne, VIC, Australia, [5]Central Clinical School, Faculty of Medicine, Monash University, Melbourne, VIC, Australia, [6]China-Australia Joint Research Center for Infectious Diseases, School of Public Health, Xi'an Jiaotong University Health Science Center, Xi'an, China, [7]Institute of Global Health and Infectious Diseases, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States

KEYWORDS

syphilis, clinical research, epidemiology, basic science, behavioral research

Editorial on the Research Topic
Syphilis infection: clinical, epidemiology, basic science, and behavioral research

Syphilis is an ancient sexually transmitted disease caused by the spirochete *Treponema pallidum* subspecies *pallidum* (*T. pallidum*). Over the past decade, syphilis incidence has increased in many countries. Untreated syphilis can lead to serious health problems, including blindness, neurocognitive disorders, cardiovascular injury, and adverse pregnancy outcomes. Syphilis research is urgently needed to decrease morbidity and mortality. This Frontiers Research Topic focuses on diverse aspects of syphilis infection. Its purpose was to expand our knowledge of syphilis epidemiology, clinical management, public health control measures, vaccine development, and basic science.

Estimates of the incidence trends and general impact on the healthcare system are essential for syphilis prevention strategies. Fu et al. estimated global syphilis incidence in partnerhisp with the Global Burden of Disease Group. They found substantial increases in syphilis among men and people in higher-income countries. SŠmit et al. estimated that the incidence of syphilis in Germany rose from 5/100,000 person-years to 6.2/100,000 persons over a three-year period, resulting in a minimal annual cost to the German healthcare system of €20,292,110. A study from Solaimalai et al. in Southern India found that syphilis prevalence increased from 0.5% in 2015 to 2.1% in 2020 among non-pregnant persons at a single site in southern India. These data highlight the need for additional public health interventions and strategies. Tucker et al. called for the application to syphilis of COVID-19 prevention and control strategies, including new diagnostic pathways (e.g., syphilis self-testing), contact tracing services, and public engagement. Routine anal self-examination may enhance syphilis detection and control, especially among men who have sex with men (MSM). Aung et al. conducted a longitudinal study examining adherence to weekly anal self-examinations among MSM and concluded that men adhered well to weekly anal self-

examination. Tran et al. evaluated interventions that did not focus on increasing condom use or testing among MSM and found evidence that doxycycline post-exposure prophylaxis (PEP) reduces syphilis incidence.

Early diagnosis and treatment of syphilis are of great importance in determining disease prognosis. Ren et al. presented a case report of an atypical form of primary cutaneous syphilis without a chancre called Folmann Balanitis. This case underscores the diversity of clinical manifestations associated with syphilis infection. In addition, clinical diagnosis and treatment of neurosyphilis remain challenging. This issue also included two neurosyphilis reviews, one focused on China and one broader in scope. [Zhou et al., Du et al.]. Many patients treated for syphilis infection do not have a robust serological response based on changes in nontreponemal titers, but little is known about the serofast state. The serofast state is defined as the failure of nontreponemal titers to decline with disappearance of clinical symptoms after an appropriate follow-up period following treatment. Luo et al. found that serofast responses were more common among older adults compared to younger adults. Liu et al. examined rates of serological cure in patients with asymptomatic neurosyphilis. These reports underline the need for more research on neurosyphilis.

T. pallidum is an invasive bacterium that can evade the host immune response and persist for decades, hence its designation as "the stealth pathogen". The Research Topic includes articles on bacterial-host interactions and vaccine development. Houston et al. investigated the possibility of antimicrobial peptides (AMPs) production as an unrecognized defense strategy used by *T. pallidum* during infection. They demonstrated that AMPs exhibit bacteriostatic and bactericidal activity against a panel of biologically relevant bacteria. In addition, AMPs can differentially regulate the expression of two pro-inflammatory chemokines - monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8). The finding that *T. pallidum* expresses AMPs to defend against competing microbes and modulate the host immune response could be important for its ability to establish infection following transmission. Li et al. reported the potential role of m6A methylation in syphilis pathophysiogenesis, and they provided evidence that YTHDF1 negatively regulates *T. pallidum*-induced inflammation in the THP-1 macrophage cell line by promoting socs3 translation in an m6a-dependent manner. The paper by Xu et al. aimed to elucidate the role of the outer membrane lipoprotein TP0136 in activation and aggregation of human platelets. Their results suggest that Tp0136 elicits these effects by downregulating PAR1 and triggering PAR1-dependent Gq and Gi pathway activation.

There is still much research needed in order to develop an effective syphilis vaccine. Kojima et al. reviewed current technologies and approaches towards a syphilis vaccine. Additionally, Molini et al. investigated B-cell epitopes of TprC and TprD variants and demonstrated that humoral responses are primarily directed to sequences predicted to be on surface-exposed loops of TprC and TprD proteins. This supports further exploration of TprC and TprD as vaccine candidates. This important research paves the way for further multi-disciplinary syphilis research.

This collection of papers has implications for policy, programs, and research. From a policy perspective, the costing analysis demonstrates the substantial cost of syphilis within health systems. The high prevalence of syphilis in many communities underscores that more resources are needed for syphilis control. From a program perspective, some of changes inspired by COVID-19 (e.g., greater attention to partner services and self-testing) may help to enhance syphilis programs. From a research perspective, further basic science and immunological research towards a vaccine are essential. A better understanding of syphilis pathogenesis could also enhance diagnostics.

Author contributions

PZ, JT, LZ and JR contributed to the conceptualization and design of this editorial. MY drafted the manuscript. PZ, JT, LZ and JR revised it. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the National Natural Science Foundation of China (grant numbers 82072322 and 82172319).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

frontiers in Public Health

ORIGINAL RESEARCH
published: 25 November 2021
doi: 10.3389/fpubh.2021.755037

Predictors of Seronegative Conversion After Centralized Management of Syphilis Patients in Shenzhen, China

Zhenzhou Luo[1†], *Yi Ding*[1†], *Jun Yuan*[1], *Qiuhong Wu*[1], *Lishan Tian*[1], *Li Zhang*[1], *Bo Li*[1*] and *Jinsong Mou*[2*]

[1] Shenzhen Nanshan Center for Chronic Disease Control, Shenzhen, China, [2] Pingshan District Maternal and Child Healthcare Hospital of Shenzhen, Pingshan General Hospital of Southern Medical University, Shenzhen, China

OPEN ACCESS

Edited by:
Joseph D. Tucker,
University of North Carolina at Chapel Hill, United States

Reviewed by:
Ngai Sze Wong,
The Chinese University of Hong Kong, Hong Kong SAR, China
Gifty Marley,
University of North Carolina China-Project (SESH Global), China
Weibin Cheng,
Guangdong Second Provincial General Hospital, China

***Correspondence:**
Bo Li
279805496@qq.com
Jinsong Mou
moujs@foxmail.com

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: 07 August 2021
Accepted: 03 November 2021
Published: 25 November 2021

Citation:
Luo Z, Ding Y, Yuan J, Wu Q, Tian L, Zhang L, Li B and Mou J (2021) Predictors of Seronegative Conversion After Centralized Management of Syphilis Patients in Shenzhen, China. Front. Public Health 9:755037. doi: 10.3389/fpubh.2021.755037

Objective: The aim of this study was to explore the seronegative conversion status of syphilis patients after centralized management and to analyze potential determinants.

Materials and Methods: A retrospective population-based cohort study was conducted, and data for individuals who had been diagnosed with syphilis between 2011 and 2019 were retrieved from the Shenzhen Nanshan Center for Chronic Disease Control. Seroconversion statuses were summarized as percentages. Univariable and multiple Cox proportional hazard regression models were used to analyze the factors associated with seronegative conversion among syphilis patients.

Results: During the study period, 1,545 patients with syphilis participated in the syphilis convergence case management program on a voluntary basis, of whom 290 were excluded due to missing follow-up data. A total of 27.6% (346/1255) of patients with syphilis showed seronegative conversion. Multivariable analysis revealed that the following significantly determined syphilis seroconversion from positive to negative: younger age (15–19 years vs. ≥30 years: HR = 2.18), male gender (HR = 1.45), lower baseline toluidine red unheated serum test (TRUST) titer of ≤1:8 (HR = 2.23), and different disease stages, including latent syphilis (HR = 1.98), primary syphilis (HR = 7.67), and secondary syphilis (HR = 4.83).

Conclusions: Few patients with syphilis tested negative after treatment at the end of the study. Seronegative conversion in the patients was associated with age, sex, baseline TRUST titer, and syphilis stage.

Keywords: syphilis, seroconversion, Shenzhen, China, multivariable analysis, TRUST titer, centralized management, STIs

INTRODUCTION

Syphilis is a sexually transmitted infection (STI) caused by *Treponema pallidum* and is spread through contact with infectious lesions or body fluids (1). Patients typically develop cutaneous manifestations such as genital ulcers and various complications, including neurologic, renal, gastrointestinal, and hepatic diseases (2).

Despite the availability of inexpensive and effective antibiotic therapy, syphilis remains a prevalent disease in developing countries and has re-emerged as a public health threat in developed nations. Syphilis has an estimated global prevalence of 36 million cases and an incidence of over 11 million cases annually (3). In the South African adult (15–49 years) population in 2017, the estimated prevalence of syphilis among women and men was 0.50% (95% CI: 0.32–0.80%) and 0.97% (0.19–2.28%), respectively (4). In the United States, from 2013 to 2017, the national annual rate of reported primary and secondary (P&S) syphilis cases increased by 72.7%, from 5.5 to 9.5 cases per 100,000 individuals (5). A review of syphilis studies in Eastern Europe showed that even though the incidence was generally declining, high prevalence was reported in key populations, particularly sex workers and users of injectable drugs (6). In China, a range of unique biological and social forces, such as depletion of individuals with immunity, income gaps and a cultural climate that favors the re-emergence of sex work, are driving the spread of syphilis (7). With the current economic and social developments, the prevalence of sexually transmitted disease (STD) remains an important concern.

To control the disease, China has continually implemented massive syphilis control programs. However, during the past 20 years, syphilis has resurged in China (7), with a significant increase in incidence from 2003 to 2013 (8, 9). The syphilis burden has been greatest in coastal urban China areas, such as Guangdong Province (10). A meta-analysis showed that Guangdong was among the provinces of China with a very high syphilis prevalence of more than 10% (11). A significant rise in syphilis cases has been observed in Guangdong Province over the last decade (12). From January 2014 through June 2015, 82,554 syphilis cases were reported in this province. Concurrently, syphilis spatial clustering was found in the city of Shenzhen (13). A total of 6,807 syphilis cases were reported in 2013 in Shenzhen (14). The burden of syphilis in Shenzhen is large, and syphilis control should therefore be regarded as a public health priority.

Although some researchers have reported on seroconversion and its determinants after treatment (15–17), studies on syphilis seronegative conversion and associated factors in Shenzhen are limited. Generally, patients with effective treatment for syphilis have been defined as patients having a $\geq$4-fold decreased titer after treatment during follow-up. However, some patients do not exhibit seronegative conversion but rather enter a serofast state. Although seroconversion from positive to negative is recognized as the final goal of treatment for doctors and patients, some studies have found that a serofast state can also reduce the psychological burden of patients. Seronegative conversion means that syphilis has been cured completely, without risk of recurrence; thus, it is an important index of effective treatment. Therefore, this study was conducted to explore post-syphilis seronegative conversion predictors. The results can facilitate the identification of high-risk populations and promote treatment outcomes. Moreover, they can provide a scientific basis for the centralized management of syphilis. In April 2011, Nanshan District, Shenzhen, launched the Syphilis Convergence Case-Management Program to consolidate prevention, treatment, and management for better syphilis control. All health organizations in the district were asked to refer patients with syphilis seropositivity to the STD clinic of the Department of Dermatology and Venereology in Nanshan Center for Chronic Disease Control, where patients were subject to centralized management, standardized treatment, and regular serologic follow-up. By running this program, we aimed to observe the outcome of seronegative conversion after the centralized management of patients with syphilis and to explore potentially associated factors.

Abbreviations: STI, sexually transmitted infection; TRUST, toluidine red unheated serum test; HR, hazard ratio; CI, confidence interval; STD, sexually transmitted disease; TPPA, *Treponema pallidum* particle agglutination; BPG, benzathine penicillin G; RPR, rapid plasma reagin.

MATERIALS AND METHODS

Study Population

A retrospective population-based cohort study was conducted. We retrospectively analyzed the data from syphilis patients managed in the clinic of Shenzhen Nanshan Hospital for Chronic Diseases between 2011 and 2019. Patients who met the following criteria were included in the study: (1) diagnosed with syphilis, (2) had related information about treatment, and (3) completed at least one serological follow-up visit after treatment. People with any of the following conditions diagnosed by a doctor were excluded: severe heart, liver, or kidney disease; malignant disease; chronic infectious disease, such as tuberculosis, leprosy, or viral hepatitis; systemic autoimmune disease, such as lupus erythematosus, rheumatoid arthritis, or dermatomyositis; and severe mental illness.

Diagnosis, Treatment, and Follow-Up

The patients were diagnosed with syphilis infection according to epidemiological history, clinical signs and symptoms, and toluidine red unheated serum test (TRUST)/*Treponema pallidum* particle agglutination (TPPA) results. Some patients actively underwent syphilis testing when they had venereal symptoms or had engaged in high-risk behavior. Syphilis infection in other patients was discovered through passive testing, such as physical examinations, blood donation and preoperative examination. Non-penicillin-allergic participants were treated with benzathine penicillin G (BPG) in one or two courses (2.4 million units BPG weekly for 2 weeks as one course). Penicillin-allergic participants received doxycycline (100 mg taken orally twice daily for 14 days). All participants were notified of the need for follow-up for 1 year after treatment. Quantitative and qualitative testing of TRUST, indicating serological conversion negativity or lack thereof, was performed during follow-up, and the resolution of clinical signs and symptoms was evaluated.

Data Collection

Qualified professional STD physicians were installed as program investigators to collect data. They underwent unified training and became familiar with a written investigation manual prior to data collection. Face-to-face interviews were conducted with a designed questionnaire to obtain related information from the

patients at the beginning of the study. Written patient consent was obtained according to institutional guidelines.

Laboratory Testing

Blood specimens were tested for syphilis. TRUST (Rongsheng Biotech Company, Shanghai, China) and TPPA tests (Fujirebio, Tokyo, Japan) were performed on serum samples. The operational instructions were strictly obeyed, and the outcomes were identified by multiple researchers.

Statistical Analysis

The information was recorded in a database using EpiData 3.0 and analyzed using SPSS 25.0. Descriptive statistics and chi-square tests were used to analyze the differences in seronegative conversion of different syphilis subpopulations. The endpoint was defined as the outcome of the TRUST test becoming negative. If the endpoint was not reached, the patient was defined as censored. If someone was lost to follow-up, the final follow-up result was used. Univariable and multiple Cox proportional hazard analyses were performed to determine the factors associated with seronegative conversion among the patients with syphilis. Variables in the univariable analyses with a p-value <0.30 were included in subsequent multivariable regression models. Multiple regression models were built using stepwise techniques. The selection of variables in the final model was conducted using a forward-conditional method, with significance levels of ≤ 0.05 for inclusion and ≥ 0.1 for exclusion. A p-value ≤ 0.05 was considered statistically significant.

RESULTS

Characteristics of the Patients

During the study period, 1,545 patients with syphilis received routine syphilis management; 290 were excluded due to missing follow-up data. Finally, a total of 1,255 eligible patients with syphilis were included. The average observation time was 9.5 $\pm$ 3.2 months. A total of 750 (59.8%) and 505 (40.2%) patients were male and female, respectively, corresponding to a sex ratio of 1.5:1. The mean age was 35.9 $\pm$ 11.9 years, and the ages ranged from 15 to 89 years. There were 490 (39.0%) unmarried patients, 673 (53.6%) married patients, and 92 (7.3%) patients who were divorced or widowed. There were 651 (51.9%) patients with latent syphilis, 113 (9.0%) patients with primary syphilis, 249 (19.8%) patients with secondary syphilis, and 242 (19.3%) patients receiving adequate treatment.

Outcomes of Seronegative Conversion Among Syphilis Patients

Table 1 shows the outcome of seronegative conversion among the patients. According to the TRUST results, the total percentage of seronegative conversions among the patients was 27.6% (346/1255).

At the end of the study, ∼257 (34.3%) male patients with syphilis tested negative, which was more than the number of female patients ($n = 89$, 17.6%) ($p < 0.01$). Patients who adopted the syphilis test actively showed a higher frequency of TRUST seroconversion from positive to negative than those who received the test passively ($n = 281$, 31.3% vs. $n = 65$, 18.2%, $p < 0.01$).

Patients with a baseline TRUST titer of ≤1:8 had a higher frequency of seronegative conversion than those with a TRUST titer of >1:8 ($n = 214$, 30.1% vs. $n = 132$, 24.3%, $p = 0.022$). Eighty-four (74.3%) patients with primary syphilis underwent TRUST seronegative conversion, with a higher frequency of conversion observed in these patients than in patients with secondary syphilis ($n = 95$, 38.2%), patients with latent syphilis ($n = 136$, 20.9%), and patients who were receiving adequate treatment ($n = 31$, 12.8%) ($p < 0.01$). Patients with one course of BPG had a higher frequency of TRUST seronegative conversion

TABLE 1 | Outcomes of TRUST seronegative conversion among syphilis patients.

	N	TRUST seronegative conversion (n, %)	χ^2	P
Age (years)			2.605	0.272
15∼19	32	12 (37.5)		
20∼29	427	124 (29.0)		
≥30	796	210 (26.4)		
Sex			41.861	<0.01
Male	750	257 (34.3)		
Female	505	89 (17.6)		
Marital status			4.966	0.084
Unmarried	490	152 (31.0)		
Married	673	169 (25.1)		
Divorced/Widowed	92	25 (27.2)		
Method of syphilis discovery			22.226	<0.01
Active detection	897	281(31.3)		
Passive detection	358	65(18.2)		
Initial TRUST titer			5.253	0.022
≤1:8	711	214 (30.1)		
>1:8	544	132 (24.3)		
Syphilis stage			178.673	<0.01
Latent	651	136 (20.9)		
Primary	113	84 (74.3)		
Secondary	249	95 (38.2)		
After adequate treatment	242	31 (12.8)		
Treatment regimen			18.776	<0.01
Untreated	159	22 (13.8)		
One course of BPG	928	281 (30.3)		
Two courses of BPG	134	34 (25.4)		
Replacement therapy with doxycycline	34	9 (26.5)		
People living with HIV			1.978	0.159
Yes	148	48 (32.4)		
No	1,107	298 (26.9)		
Male homosexuality			3.361	0.067
Yes	174	58 (33.3)		
No	1,081	288 (26.6)		
Bisexuality				<0.01
Yes	41	22 (53.7)		
No	1,214	324 (26.7)		

(n = 281, 30.3%) than those with no treatment (n = 22, 13.8%), two courses of BPG (n = 34, 25.4%), or replacement therapy with doxycycline (n = 9, 26.5%). A higher frequency of TRUST seronegative conversion was found in bisexual patients with syphilis (n = 22, 53.7%) than in heterosexual patients ($p < 0.01$). TRUST seronegative conversion for syphilis was not significantly associated with age, marital status, HIV infection, or male homosexuality ($p > 0.05$).

Factors Associated With Seronegative Conversion Among Syphilis Patients

Univariable Cox proportional hazard regression analyses were performed to identify the factors associated with seronegative conversion. Male patients with syphilis were more likely to test seronegative than were female patients (HR = 1.88, 95% CI = 1.48–2.39; $p < 0.01$). Patients who had actively undergone the syphilis test were more likely to show seronegative conversion than those who had undergone the test passively (HR = 1.61, 95% CI = 1.23–2.11, $p < 0.01$). Furthermore, patients with a lower initial TRUST titer of ≤1:8 were more likely to test negative than those with an initial TRUST titer of >1:8 (HR = 1.34, 95% CI = 1.08–1.67, $p = 0.01$). Compared to patients who were receiving adequate treatment, patients with latent syphilis (HR = 1.84, 95% CI = 1.24–2.71, $p < 0.01$), primary syphilis (HR = 7.60, 95% CI = 5.03–11.48, $p < 0.01$), and secondary syphilis (HR = 3.18, 95% CI = 2.12–4.76, $p < 0.01$) were more likely to show seronegative conversion. Compared to untreated patients, those receiving treatment regimens, including one course of BPG (HR = 2.38, 95% CI = 1.54–3.67, $p < 0.01$), two courses of BPG (HR = 1.84, 95% CI = 1.08–3.14, $p = 0.03$), and replacement therapy with doxycycline (HR = 2.26, 95% CI = 1.04–4.90, $p = 0.04$), were more likely to test negative. Male homosexual individuals were more likely to show seronegative conversion (HR = 1.36, 95% CI = 1.03–1.80, $p = 0.03$) and bisexual syphilis patients (HR = 1.71, 95% CI = 1.11–2.64, $p = 0.01$) were associated with syphilis TRUST seroconversion from positive to negative (**Table 2**).

All variables at $p < 0.3$ in the univariable analyses were included in the multivariable Cox regression analysis. A forward selection (conditional) method was conducted to select variables, with a significance level of ≤0.05 required for inclusion and of ≥0.1 required for exclusion. The multivariable analysis suggested that patients of younger age (15–19 years vs. ≥30 years: HR = 2.18, 95% CI = 1.21–3.94, $p = 0.01$) and male gender (HR = 1.45, 95% CI = 1.12–1.88, $p = 0.01$) were more likely to test negative. Patients with a lower initial TRUST titer of ≤1:8 more easily underwent TRUST seroconversion from positive to negative than those with a TRUST titer of >1:8 (HR = 2.23, 95% CI = 1.72–2.88). Moreover, compared to patients who were receiving adequate treatment, patients at different disease stages, including latent syphilis (HR = 1.98, 95% CI = 1.34–2.93, $p < 0.01$), primary syphilis (HR = 7.67, 95% CI = 5.01–11.74, $p < 0.01$), and secondary syphilis (HR = 4.83, 95% CI = 3.09–7.54), were significantly more likely to show syphilis TRUST seroconversion (**Table 2**).

DISCUSSION

Syphilis is a persistent public health issue in many countries. Its prevalence has recently increased in some countries (18, 19). In Guangdong Province, the syphilis incidence rate increased yearly between 2005 and 2014—from 21.08/100,000 to 52.55/100,000 over this period (20). A systematic review found that 90% of syphilis patients were from resource-limited countries (21). Therefore, the Syphilis Convergence Case-Management Project was implemented to promote prevention and control. Through our research, the state of seronegative conversion and a few significant factors associated with seronegative syphilis conversion were discovered. The research results provide some basis for building an effective and synthetic model of syphilis prevention and control.

Only 27.6% of the patients showed TRUST seronegative conversion at the end of our study. A previous study reported that the rate of syphilis serological cure (17, 22) was 65–79%; however, the sample size was smaller than that in the present study. Moreover, some of the patients in our study originated from other health care facilities, and their past medical history and treatments were comparatively complicated. In addition, treatment cost, treatment adherence, and type of health care system might be correlated with seroconversion. The percentage of seronegative conversion in our study also suggests that the level of management of syphilis convergence cases, such as treatment, follow-up, and data collection, needs to be strengthened in the future.

In our study, we found that seronegative conversion was independently associated with younger age, as was also reported by Seña et al. (17), who revealed that the probability of achieving serological cure decreased with age. This association may be related to the senescence of the immune system at older age, which can be expected to influence the serological response to syphilis therapy (22). In addition, more male patients tested negative for TRUST than female patients (34.3% vs. 17.9%). The multivariable analysis also showed that male patients with syphilis were more likely to test seronegative. This difference might be related to the differences in the immune system between men and women (23), which influence the serological response. The exact mechanism underlying the association with sex is still unclear and needs further investigation.

Understanding the relationship of quantitative non-treponemal titers with disease is vital for evaluating treatment response, which can reflect the activity of the disease process or the immune response. Generally, non-treponemal antibody titers are related to disease activity. However, some studies demonstrated that patients with higher baseline rapid plasma reagin (RPR) titers were more likely to achieve serological cure (17, 22). Baker-Zander et al. (24) found that Venereal Disease Research Laboratory (VDRL)-immunized rabbits exhibited partial protection against reinfection with *T. pallidum*, which meant that high VDRL antibody titers might help control the infection. However, in our study, the patients with lower initial TRUST titers easily became seronegative. The multivariable

TABLE 2 | Results of univariable and multivariable analyses of factors associated with TRUST seronegative conversion among syphilis patients.

	Univariable analysis				Multivariable analysis			
	b	**Wald χ2**	***P***	**Hazard ratio (95% CI)**	**b**	**Wald χ2**	***P***	**Hazard ratio (95% CI)**
Age (years)		2.66	0.27			6.82	0.03	
15∼19	0.44	2.22	0.14	1.56 (0.87, 2.79)	0.78	6.71	0.01	2.18 (1.21, 3.94)
20∼29	0.10	0.75	0.39	1.10 (0.88, 1.38)	0.01	0.01	0.93	1.01 (0.80, 1.27)
≥30				1				1
Sex								
Male	0.63	26.34	<0.01	1.88 (1.48, 2.39)	0.37	7.72	0.01	1.45 (1.12, 1.88)
Female				1				1
Marital status		4.30	0.12					
Unmarried	0.10	0.22	0.64	1.11 (0.73, 1.69)				
Married	−0.13	0.37	0.54	0.88 (0.58, 1.34)				
Divorced/Widowed				1				
Method of syphilis discovery								
Active detection	0.48	11.92	<0.01	1.61 (1.23, 2.11)				
Passive detection				1				
Initial TRUST titer								
≤1:8	0.29	7.02	0.01	1.34 (1.08, 1.67)	0.80	36.77	<0.01	2.23 (1.72, 2.88)
>1:8				1				1
Syphilis stage		142.04	<0.01			125.75	<0.01	
Latent	0.61	9.30	<0.01	1.84 (1.24, 2.71)	0.68	11.73	<0.01	1.98 (1.34, 2.93)
Primary	2.03	93.00	<0.01	7.60 (5.03, 11.48)	2.04	87.72	<0.01	7.67 (5.01, 11.74)
Secondary	1.16	31.22	<0.01	3.18 (2.12, 4.76)	1.58	48.02	<0.01	4.83 (3.09, 7.54)
After adequate treatment				1				1
Treatment regimen		16.48	<0.01					
Untreated				1				
One course of BPG	0.87	15.31	<0.01	2.38 (1.54, 3.67)				
Two courses of BPG	0.61	4.94	0.03	1.84 (1.08, 3.14)				
Replacement therapy with doxycycline	0.81	4.22	0.04	2.26 (1.04, 4.90)				
People living with HIV								
Yes	0.13	0.68	0.41	1.14 (0.84, 1.54)				
No				1				
Male homosexuality								
Yes	0.31	4.57	0.03	1.36 (1.03, 1.80)				
No				1				
Bisexuality								
Yes	0.54	5.99	0.01	1.71 (1.11, 2.64)				
No				1				

model also revealed that the likelihood of TRUST seronegative conversion was increased with a decrease in the initial TRUST titer. There may be a negative association between the TRUST titer and the quantity of *T. pallidum*. The detailed mechanism requires clarification through further studies. Hence, the level of syphilis antibody titer might correspond to the levels of syphilis and immune response. Facilitating routine syphilis serological tests and follow-up in the clinic is integral to observing the efficacy of treatment and preventing adverse outcomes.

We also found that patients with primary syphilis were more likely to experience TRUST seronegative conversion than those who were receiving adequate treatment before enrollment and more so than patients with secondary and latent syphilis. The results of the current study were consistent with a previous observation that patients with secondary syphilis were more likely to experience treatment failure than those with primary syphilis (25). In addition, Tong et al. reported that the rate of serological cure decreased in the order of primary, secondary, latent, and tertiary syphilis (22). The differences in immunological functions among different disease stages may be responsible for this observation. These results suggest that enhancing early diagnosis, treatment, and city-specific syphilis screening strategies is very important and that the clinical stages of syphilis often overlap.

LIMITATIONS

Our study has several limitations. First, there may be some selection bias because several patients were excluded due to missing data. Second, the results correspond to only one district and might not be generalizable to other areas; thus, the representativeness of the sample needs to be improved. Third, some reporting bias may have been inherent in this study because some subjects may have hidden sensitive information.

CONCLUSION

The present study showed that syphilis TRUST seronegative conversion after treatment was poor. However, syphilis seroconversion from positive to negative was significantly associated with sex, baseline TRUST titer, and disease stage.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Shenzhen Nanshan Center for Chronic Disease Control. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ZL and BL conceived and designed the study. JY and QW implemented the study and conducted data collection. LT and LZ contributed to data analysis. ZL and YD wrote and drafted the manuscript. JM was involved in critical revision of the manuscript. All authors have read and approved the final manuscript.

FUNDING

The Shenzhen Healthcare Research Project (SZGW2018001) supported this study.

ACKNOWLEDGMENTS

We thank the patients for their cooperation in this study as well as all the individuals who contributed to this work. We also thank the Shenzhen Healthcare Research Project for supporting our research and Editage for English language editing.

REFERENCES

1. Wang Q, Liu JZ, Xu JH. *Sexually Transmitted Diseases Clinic Prevention and Treatment Guidelines*. 1st ed. Shanghai: Shanghai Science and Technology Press (2014).
2. Golden MR, Marra CM, Holmes KK. Update on syphilis: resurgence of an old problem. *JAMA*. (2003) 290:1510–4. doi: 10.1001/jama.290.11.1510
3. World Health Organization. *Global Incidence and Prevalence of Selected Curable Sexually Transmitted Infections - 2008*. World Health Organization (2012). Available online at: https://apps.who.int/iris/handle/10665/75181
4. Kularatne RS, Niit R, Rowley J, Kufa-Chakezha T, Peters RPH, Taylor MM, et al. Adult gonorrhea, chlamydia and syphilis prevalence, incidence, treatment and syndromic case reporting in South Africa: estimates using the Spectrum-STI model, 1990–2017. *PLoS ONE*. (2018) 13:e0205863. doi: 10.1371/journal.pone.0205863
5. Kidd SE, Grey JA, Torrone EA, Weinstock HSGJ, Torrone EA, Weinstock HS. Increased methamphetamine, injection drug, and heroin use among women and heterosexual men with primary and secondary syphilis–United States, 2013-2017. *MMWR Morb Mortal Wkly Rep*. (2019) 68:144–8. doi: 10.15585/mmwr.mm6806a4
6. Bailey H, Turkova A, Thorne C. Syphilis, hepatitis C and HIV in Eastern Europe. *Curr Opin Infect Dis*. (2017) 30:93–100. doi: 10.1097/QCO.0000000000000326
7. Chen ZQ, Zhang GC, Gong XD, Lin C, Gao X, Liang GJ, et al. Syphilis in China: results of a national surveillance programme. *Lancet*. (2007) 369:132–8. doi: 10.1016/S0140-6736(07)60074-9
8. Yang S, Wu J, Ding C, Cui Y, Zhou Y, Li Y, et al. Epidemiological features of and changes in incidence of infectious diseases in China in the first decade after the SARS outbreak: an observational trend study. *Lancet Infect Dis*. (2017) 17:716–25. doi: 10.1016/S1473-3099(17)30227-X
9. Wu Z, Zhou P. Syphilis and social upheaval in China. *N Engl J Med*. (2010) 363:1088. doi: 10.1056/NEJMc1006525
10. Tucker JD, Cohen MS. China's syphilis epidemic: epidemiology, proximate determinants of spread, and control responses. *Curr Opin Infect Dis*. (2011) 24:50–5. doi: 10.1097/QCO.0b013e32834204bf
11. Lin CC, Gao X, Chen XS, Chen Q, Cohen MS. China's syphilis epidemic: a systematic review of seroprevalence studies. *Sex Transm Dis*. (2006) 33:726–36. doi: 10.1097/01.olq.0000222703.12018.58
12. Yang LG, Tucker JD, Yang B, Shen SY, Sun XF, Chen YF, et al. Primary syphilis cases in Guangdong Province 1995–2008: opportunities for linking syphilis control and regional development. *BMC Public Health*. (2010) 10:793. doi: 10.1186/1471-2458-10-793
13. Wong NS, Chen L, Tucker JD, Zhao P, Goh BT, Poon CM, et al. Distribution of reported syphilis cases in South China: spatiotemporal analysis [Sci. rep.:9090]. *Sci Rep*. (2018) 8:9090. doi: 10.1038/s41598-018-27173-y
14. Lan L, Wu X, Zhang C, Hong F. Epidemiological analysis of syphilis in Shenzhen from 2004 to 2013. *China Trop Med*. (2015) 15:700–3. doi: 10.13604/j.cnki.46-1064/r.2015.06.018
15. Xu JJ, Zhang M, Brown K, Reilly K, Wang H, Hu Q, et al. Syphilis and HIV seroconversion among a 12-month prospective cohort of men who have sex with men in Shenyang, China. *Sex Transm Dis*. (2010) 37:432–9. doi: 10.1097/OLQ.0b013e3181d13eed
16. Zou X, Ling L, Zhang L. Trends and risk factors for HIV, HCV and syphilis seroconversion among drug users in a methadone maintenance treatment programme in China: a 7-year retrospective cohort study. *BMJ Open*. (2015) 5:e008162. doi: 10.1136/bmjopen-2015-008162
17. Seña AC, Wolff M, Martin DH, Behets F, Van Damme K, Leone P, et al. Predictors of serological cure and Serofast State after treatment in HIV-negative persons with early syphilis. *Clin Infect Dis*. (2011) 53:1092–9. doi: 10.1093/cid/cir671
18. Fenton KA, Breban R, Vardavas R, Okano JT, Martin T, Aral S, et al. Infectious syphilis in high-income settings in the 21st century. *Lancet Infect Dis*. (2008) 8:244–53. doi: 10.1016/S1473-3099(08)70065-3
19. Mutagoma M, Remera E, Sebuhoro D, Kanters S, Riedel DJ, Nsanzimana S. The prevalence of syphilis infection and its associated factors in the general

population of Rwanda: a national household-based survey. *J Sex Transm Dis.* (2016) 2016:4980417. doi: 10.1155/2016/4980417
20. Zou Y, Liu F, Chen L, Shen HC, Huang SJ, Zheng HP, et al. Epidemiological trend and disease burden of syphilis in Guangdong Province, 2005–2014. *J Sun Yat-Sen Univ Med Sci.* (2016) 37:142–7. doi: 10.13471/j.cnki.j.sun.yat-sen.univ(med.sci).2016.0025
21. Tucker JD, Bu J, Brown LB, Yin YP, Chen XS, Cohen MS. Accelerating worldwide syphilis screening through rapid testing: a systematic review. *Lancet Infect Dis.* (2010) 10:381–6. doi: 10.1016/S1473-3099(10)70092-X
22. Tong ML, Lin LR, Liu GL, Zhang HL, Zeng YL, Zheng WH, et al. Factors associated with serological cure and the serofast state of HIV-negative patients with primary, secondary, latent, and tertiary syphilis. *PLoS ONE.* (2013) 8:e70102. doi: 10.1371/journal.pone.0070102
23. Whitacre CC, Reingold SC, O'Looney PA, A. gender gap in autoimmunity. *Science.* (1999) 283:1277–8. doi: 10.1126/science.283.5406.1277
24. Baker-Zander SA, Shaffer JM, Lukehart SA, VDRL antibodies enhance phagocytosis of Treponema pallidum by macrophages. *J Infect Dis.* (1993) 167:1100–5. doi: 10.1093/infdis/167.5.1100
25. Luo Z, Zhu L, Ding Y, Yuan J, Li W, Wu Q, et al. Factors associated with syphilis treatment failure and reinfection: a longitudinal cohort study in Shenzhen, ChinaChina. *BMC Infect Dis.* (2017) 17:620. doi: 10.1186/s12879-017-2715-z

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Immunology

ORIGINAL RESEARCH
published: 28 February 2022
doi: 10.3389/fimmu.2022.818151

The Outer Membrane Lipoprotein Tp0136 Stimulates Human Platelet Activation and Aggregation Through PAR1 to Enhance G_q/G_i Signaling

Qiu-Yan Xu[1,2], *Yong-Jing Wang*[1,2], *Li-Rong Lin*[1,2], *Li-Li Liu*[1,2] *and Tian-Ci Yang*[1,2*]

[1] Center of Clinical Laboratory, Zhongshan Hospital of Xiamen University, School of Medicine, Xiamen University, Xiamen, China, [2] Institute of Infectious Disease, School of Medicine, Xiamen University, Xiamen, China

Background: Chancre self-healing, a typical clinical phenomenon of primary syphilis, is essentially wound healing. The first response to a wound is constriction of the injured blood vessels and activation of platelets to form a fibrin clot. However, the role of *Treponema pallidum* in platelet activation and clot formation remains unclear.

Objectives: We aimed to elucidate the role of the outer membrane *Treponema pallidum* lipoprotein Tp0136 in human platelet activation and aggregation and explore the related mechanism.

Methods: A series of experiments were performed to assess the effects of Tp0136 on human platelet activation and aggregation *in vitro*. The effect of Tp0136 on platelet receptors was studied by detecting PAR1 protein levels and studying related receptor sites. The involvement of the G_q/G_i signaling pathway downstream of PAR1 was explored.

Results: Tp0136 significantly accelerated the formation of human platelet clots as well as platelet adhesion to and diffusion on fibrinogen to promote platelet aggregation. Tp0136 also potentiated P-selectin expression and PF4 release to promote platelet activation and downregulated PAR1 expression. The activation and aggregation induced by Tp0136 were reverted by the specific PAR1 antagonist RWJ56110 and the human PAR1 antibody. In addition, Tp0136 significantly enhanced G_q and G_i signaling activation, thereby triggering p38 phosphorylation and Akt-PI3K activation, increasing the release of intraplatelet Ca^{2+} and attenuating the release of cytosolic cAMP. Furthermore, the specific PAR1 antagonist RWJ56110 significantly suppressed G_q and G_i signaling activation.

Conclusions: Our results showed that the *Treponema pallidum* Tp0136 protein stimulated human platelet activation and aggregation by downregulating PAR1 and triggered PAR1-dependent G_q and G_i pathway activation. These findings may contribute to our understanding of the self-healing of chancroid in early syphilis.

Keywords: Tp0136, platelet activation, platelet aggregation, protease-activated receptor 1, G_q, G_i

OPEN ACCESS

Edited by:
Pingyu Zhou,
Tongji University, China

Reviewed by:
Meiping Ye,
Shanghai Skin Disease Hospital, China
Lorenzo Giacani,
University of Washington, United States

***Correspondence:**
Tian-Ci Yang
yangtianci@xmu.edu.cn

Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: *19 November 2021*
Accepted: *08 February 2022*
Published: *28 February 2022*

Citation:
Xu Q-Y, Wang Y-J, Lin L-R, Liu L-L and Yang T-C (2022) The Outer Membrane Lipoprotein Tp0136 Stimulates Human Platelet Activation and Aggregation Through PAR1 to Enhance G_q/G_i Signaling. Front. Immunol. 13:818151. doi: 10.3389/fimmu.2022.818151

HIGHLIGHTS

1. Tp0136 enhances platelet activation and aggregation by downregulating PAR1.
2. Tp0136-downregulated PAR1 selectively stimulates PAR1-dependent G_q and G_i pathway activation.

INTRODUCTION

Syphilis, a chronic multistage disease punctuated by asymptomatic periods of latency, is caused by the spirochete *Treponema pallidum* subsp. *pallidum* (hereafter *T. pallidum*) and is primarily transmitted sexually or vertically during pregnancy (1). Syphilis is clinically manifested when spirochetes replicating at the site of inoculation induce a local inflammatory response sufficient to generate a papule that subsequently ulcerates, forming a chancre; chancres are the defining lesions of primary syphilis and are typically painless and resolve spontaneously (2). Thus far, our understanding of the mechanism of chancre self-healing in syphilis is limited.

Wound healing is one of the most complex processes in the human body, involving the spatiotemporal synchronization of multiple cell types with different roles in the phases of hemostasis, inflammation, growth, reepithelialization, and remodeling (3). The first reaction to a wound is constriction of the injured blood vessels and activation of platelets that adhere to the damaged site and aggregate to form a fibrin clot, resulting in the early stabilization of platelet thrombi that thereby initiate hemostasis (4, 5). Human platelets express protease-activated receptor 1 (PAR1), the prototypical member of the G-protein-coupled receptor family, which is activated by a variety of proteases (6). The activation of PAR1 is sufficient to trigger platelet secretion and aggregation (7), and PAR1 can couple with members of the G_q, $G_{12/13}$, and G_i families to impact a substantial of signaling pathway networks (8).

Tp0136, an outer membrane lipoprotein of *T. pallidum*, is also an adhesin that is predicted to bind to different host cells and thereby mediate the colonization of *T. pallidum* in different tissues during infection (9, 10). Our previous study found that Tp0136 promoted the migration and proliferation of fibroblasts (11) and microvascular endothelial cells (12), which could contribute to the mechanism of chancre self-healing in syphilis. In addition, high titers of anti-Tp0136 antibodies promoted the infiltration of inflammatory cells into local lesions and intensified tissue damage, thus delaying wound healing (13). While platelets have been shown to be a *T. pallidum* target (14), whether *T. pallidum* activates platelets through Tp0136 and promotes platelet aggregation to mediate the self-healing of chancre remains unclear. In the current study, we performed a series of *in vitro* experiments to elucidate the effect of Tp0136 on platelet activation and aggregation and analyzed PAR1 receptors and subsequent signaling pathways that involved in this process.

MATERIALS AND METHODS

Preparation of the Tp0136 Protein and Removed Endotoxin

Full-length Tp0136 was directly cloned into the pEXP-5-CT vector, and the Tp0136-His-Tag protein was purified by affinity chromatography using Ni-NTA as described previously (12). Endotoxin was removed from the recombinant Tp0136 protein with an EtEraser™ Endotoxin Removal Kit (Chinese Horseshoe Crab Reagent Manufactory, Ltd., Xiamen, China). Tachypleus amebocyte lysate (Chinese Horseshoe Crab Reagent Manufactory, Ltd., Xiamen, China) was used to detect endotoxin in the Tp0136 preparation, which was found to have less than 0.05 endotoxin units (EUs)/mL. A cytotoxicity assay was performed to evaluate the effect of Tp0136 on endothelial cells viability using a lactate dehydrogenase kit (NEOBIOSCIENCE Biotechnology Co., Ltd. Beijing, China). The results showed no significant cytotoxicity in Tp0136-treated cells.

Preparation of Human Platelets

Platelets from healthy volunteers were separated by the differential centrifugation of whole blood in anticoagulation tubes containing 3.8% sodium citrate as previously described (15, 16). Platelet-rich plasma (PRP) was separated from the plasma samples and centrifuged at 180 g for 15 minutes at room temperature. The samples were then centrifuged at 800 g for 20 minutes to obtain the platelet precipitates, which were then resuspended in modified Tyrode's solution (Solarbio, Beijing, China) and diluted to 2.0-3.0 × 10^8/mL for the following experiments. The studies involving human participants were reviewed and approved by the Ethics Committee of Zhongshan Hospital, Xiamen University. All volunteers provided written informed consent in accordance with the Declaration of Helsinki.

Assessment of Platelet Adhesion and Spreading

Platelet adhesion assays were performed according to Boncler et al. (15). Samples in 96-well microplates coated with 2 mg/mL fibrinogen (4°C, overnight) were blocked with 0.2% bovine serum albumin (1 hour, 37°C). The PRP samples were incubated for 15 minutes with thrombin (0.5 U/mL), Tp0136 (10 μg/mL), Tp17 (10 μg/mL) or phosphate-buffered saline (PBS) in the absence or presence of different antagonists or agonists. Then, 50 μL aliquots of PRP were added to the wells for 1 hour at 37°C. After washing, the wells were filled with a substrate solution and incubated for 1 hour. To estimate "total platelet adhesion", the PRP samples were mixed with the substrate solution and added to the uncoated wells. Platelet-deficient plasma was used as a blank control. Sodium hydroxide (2 M) was added to stop the enzymatic reaction, and the absorbance at 405 nm was read using a microplate analyzer (Thermo Scientific Multiskan FC, USA). The percentage of adherent platelets was calculated using the following formula: (sample–blank)/(total–blank) × 100.

Platelet cytoskeleton staining assays were performed as described in a previous study (17). PRP samples were treated with thrombin (0.5 U/mL), Tp0136 (10 μg/mL), Tp17 (10 μg/mL) or PBS with or without RWJ56110 (a PAR1 antagonist) (1 μM) or an anti-PAR1 antibody (1:100) and placed onto fibrinogen-coated Millicell glass slides for 1 hour at 37°C. Adherent platelets were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 5% bovine serum albumin and then stained with TRITC-labeled phalloidin at room temperature for 30 minutes. Fluorescence images were obtained on a confocal microscope (Zeiss Axio Observer LSM780, Oberkochen, Germany). The number of platelet adhesion events and the platelet spreading surface area were determined using NIH ImageJ software (NIH, Bethesda, MD, USA).

Determination of Platelet Aggregation

Agonist-induced platelet aggregation was measured using the PL-16 aggregometer (Sinnowa, Jiangsu, China) at 37°C and a constant stirring speed of 50 g to analyze platelet function. Briefly, platelets were incubated with thrombin (0.5 U/mL), Tp0136 (10 μg/mL), Tp17 (10 μg/mL) or PBS with or without different inhibitors or antagonists (RWJ56110, U73122 or PTX) for 5 minutes at 37°C and assessed on an aggregometer according to the manufacturer's protocol.

Platelet-Mediated Clot Retraction Assay

The platelet clot retraction experiment was performed as described by Ren et al. (17). After incubation with 10 μg/mL Tp0136 and Tp17 (PBS as a control), platelets were stimulated with 20 μg/mL fibrinogen and 0.5 U/mL thrombin and recorded at the indicated time point using a camera. The clot area was quantified based on the ratio of the clot area to the platelet suspension area at different time points using ImageJ software (National Institute of Mental Health, Bethesda, MD, USA).

Platelet Activation Assays

Measurement of the platelet surface molecules P-selectin (18, 19) and PF4 (20) as indices of platelet activation was performed by flow cytometry (BD FACSCanto II, NJ, USA) and the enzyme-linked immunosorbent assay (ELISA). For the P-selectin measurement, platelets were diluted to 2.0×10^8/mL with modified Tyrode's buffer and incubated for 15 minutes with thrombin (0.5 U/mL), Tp0136 (10 μg/mL) or PBS in the absence or presence of different antagonists or agonists. The aliquots (100 μL) were then stained with an APC-labeled anti-human CD41 antibody (Biolegend, Shanghai, China) as a platelet identifier and with a FITC-labeled anti-human P-selectin antibody (Biolegend, Shanghai, China) before being analyzed by flow cytometry. Data were analyzed with FlowJo (TreeStar Software, Ashland, OR, USA). The levels of PF4 in PRP samples were assessed by ELISA (Human PF4 Simple Step ELISA® Kit, Abcam, MA, USA).

cAMP Release Assays

Cyclic adenosine monophosphate (cAMP) in PRP samples was assessed by a competition-based assay (cAMP ELISA Detection Kit, GenScript, NJ, USA). The PRP samples were preincubated with iloprost (final concentration, 100 ng/mL) for 2 minutes, after which 10 μg/mL Tp0136 (PBS as a control) and RWJ56110 (1 μM) were added alone or in combination. The samples were incubated for 15 minutes at 37°C and then analyzed for cAMP content.

Determination of Ca^{2+} Fluxes

The kinetics of intracellular Ca^{2+} mobilization were assessed as previously described (21). Platelets diluted in modified Tyrode's buffer (2.0×10^8/mL) were incubated in Fluo-3-AM solution (Sigma, MO, USA) for 30 minutes at 37°C. After determining the basal Ca^{2+} levels, Tp0136 (10 μg/mL) was added to the tube in the absence or presence of RWJ56110 (1 μM), and the samples were assayed immediately. Flow cytometric analysis was then performed (BD FACSCanto II, NJ, USA).

Cell Culture, Plasmid Cloning and Transfection

Chinese hamster ovary (CHO) cells were cultured in DMEM supplemented with fetal bovine serum (10% vol/vol), penicillin (100 U/mL) and streptomycin (100 μg/mL). Full-length human-PAR1 cDNA was amplified and cloned into a pcDEF3-CMV-T7-tagged vector (MiaoLingBio, Wuhan, China) to obtain a pcDEF3/PAR1 T7-tagged wild-type plasmid that was used to generate all mutants. pcDEF3 vectors encoding the T7-tagged PAR1 mutants L38S, D39S, P40N, R41A, S42D, and F43R were generated as described previously (22). CHO cells were transiently transfected with pcDEF3/PAR1 T7-tagged wild-type or PAR1 mutants using Lipofectamine™ 3000 Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations and assessed by flow cytometry.

Western Blotting Assays

Platelets were stimulated with Tp0136 at different concentrations, and the PAR1 protein levels were measured by western blot as described previously (22). Platelets were treated with thrombin receptor activating peptide (TRAP)-6 (10 μM) or Tp0136 (10 μg/mL) in the absence or presence of RWJ56110 (1 μM) for 15 minutes at 37°C. The cell lysates were collected, and the protein levels of phosphorylated and total PI3K, Akt, and p38 were detected by western blotting (23). Antibodies against PAR1 and PI3K/Akt/p38 signaling pathway components were purchased from Cell Signaling Technology (Danvers, MA, USA) or R&D Systems (Minneapolis, MN, USA).

Data Analysis and Statistics

All results are expressed as the mean ± standard error of the mean (SEM). Multiple groups were compared by one-way analysis of variance (ANOVA). Comparisons between two groups were made using paired Student's t-tests. Differences in calcium levels over time were assessed by repeated-measures ANOVA followed by Dunnett's *post hoc* test. All calculations were performed with the GraphPad Prism 6.0 program (version 5.0, GraphPad Software Inc, San Diego, CA, USA) and IBM SPSS statistics version 26 (SPSS, Inc., Chicago, IL, USA). A two-tailed P value of <0.05 was considered statistically significant.

RESULTS

Tp0136 Promoted Human Platelet Adhesion and Aggregation

To elucidate the effect of Tp0136 on platelet adhesion and aggregation, platelets were treated with the Tp0136 protein, Tp17 protein, PBS (as a blank control) and thrombin (as a positive control). Platelet staining with TRITC-phalloidin revealed that the number of platelets adhered to the precoated fibrinogen in the Tp0136 group was higher than that in the PBS group (P <0.001), Tp17 group was no change (**Figure 1A**). The platelet spreading surface area was assessed using NIH ImageJ software, and the average diffusion area of Tp0136-treated platelets immobilized on fibrinogen was markedly increased compared with that of PBS-treated platelets (P <0.001), and Tp17-treated platelets was no statistical difference (**Figure 1B**). The effect of Tp0136 on platelet adhesion to fibrinogen was confirmed by the significantly higher adhesion of Tp0136-stimulated platelets to the fibrinogen-coated surface as determined by ELISA (P <0.001), compared with that of PBS-treated platelets, and platelet adhesion was no change stimulated by Tp17 (**Figure 1C**). The platelet aggregation assay (**Figure 1D**) revealed an increased response to Tp0136-treated platelets (P <0.001) and no increased in the Tp17-treated platelets. In addition, the result of the clot retraction assay showed that Tp0136-treated human platelets accelerated platelet aggregation

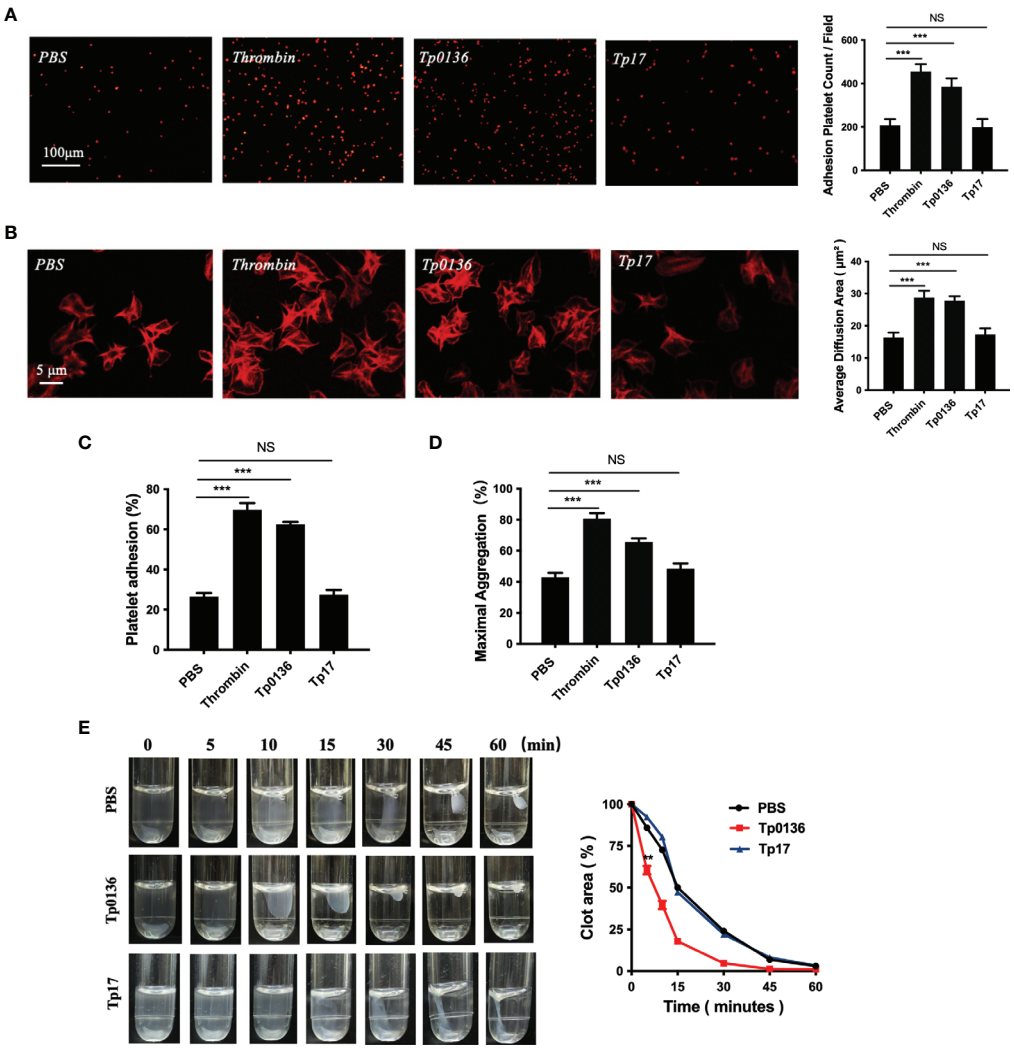


FIGURE 1 | Tp0136 promoted human platelet adhesion and aggregation. **(A, B)** Effect of Tp0136 on platelet adhesion as determined by staining with TRITC-labeled phalloidin. Statistical data were determined based on the number of platelet adhesions **(A)** and were calculated from the mean of the average surface area of individual platelets **(B)**. **(C)** Platelet adhesion as assessed by ELISA. **(D)** The maximum aggregation rate of platelets was determined by an aggregometer. **(E)** Platelet clot retraction was quantified by the ratio of the clot area to the platelet suspension area at different time points. The values are presented as the mean ± SEM of experimental triplicates and are representative of the results of three independent experiments. Values among multiple groups were compared by one-way ANOVA. Comparisons between two groups in the platelet clot retraction experiment were made using a paired t-test (NS, no significance, $^{**}P < 0.01$, $^{***}P < 0.001$).

and that the aggregation area was significantly lower than that in the PBS group after 5 minutes (P <0.01). The Tp0136-treated human platelets almost formed a very small clot after 30 minutes, while these was no difference between Tp17-treated group and PBS group, as demonstrated in **Figure 1E**. These data indicated that Tp0136 promoted human platelet adhesion and aggregation.

Tp0136 Promoted Human Platelet Activation

Given the promotional effect of Tp0136 on platelet adhesion and aggregation, we next investigated whether Tp0136 affects platelet activation. The flow cytometry results showed that the surface expression of P-selectin was increased in the Tp0136-treated platelet group by threefold compared with that in the PBS group (P <0.001) (**Figure 2A**). In addition, Tp0136 significantly promoted the secretion of PF4 (vs. PBS-treated group, P <0.01) (**Figure 2B**). These data indicated that Tp0136 promoted platelet activation.

Tp0136 Promoted Platelet Activation and Aggregation Through PAR1

Human platelets express PAR1, and the activation of PAR1 is sufficient to trigger platelet secretion and aggregation (7). To confirm the regulation of platelet PAR1 receptors by Tp0136, platelets were treated with Tp0136 at different concentrations. The protein expression of PAR1 was decreased after treatment with Tp0136 at a concentration of 5 μg/mL (P <0.01), and the best response was achieved with 15 μg/mL Tp0136 (P <0.01) (**Figure 3A**), indicating a concentration-dependent pattern. In addition, the pretreatment of platelets with RWJ56110 (a specific PAR1 antagonist) or an anti-human-PAR1 antibody ameliorated the activation and aggregation of platelets induced by Tp0136. RWJ56110 and the human-PAR1 antibody significantly reduced the expression of P-selectin on platelets (P <0.001) (**Figure 3B**), the secretion of PF4 (P <0.05) (**Figure 3C**), platelet aggregation (P<0.001) (**Figure 3D**), platelet adhesion (P <0.001) (**Figures 3E, F**) and the average platelet diffusion area (P <0.01) (**Figure 3G**). Taken together, these results demonstrate that PAR1 is essential for the Tp0136-mediated promotion of platelet activation and aggregation.

Serine proteases, such as plasmin, thrombin, and activated protein C, hydrolyze PAR1 to generate a tethered ligand that, in turn, activates PAR1 by interacting with the body of the receptor, thus triggering transmembrane signaling (7, 24, 25). In our study, the cleavage of platelet PAR1 by Tp0136 was confirmed in CHO cells expressing the T7-tagged wild-type PAR1 plasmid (**Figure 3H**). To identify the specific cleavage site of Tp0136, we performed site-directed mutations of the key residues of the PAR1 N-terminus between amino acids 38 and 43, generating the T7-tagged PAR1 mutants L38S, D39S, P40N, R41A, S42D, and F43R (**Figure 3I**), and expressed them in CHO cells. The cleavage of PAR1 by Tp0136 was suppressed in T7-S42D PAR1-transfected cells compared with T7-wild-type PAR1-transfected cells (**Figure 3J**). Thus, Tp0136 cleaves PAR1 at LDPR^{41}↓S^{42}FL to generate the S^{42}FLLRN-tethered ligand (TRAP-6), similar to that produced by thrombin (22).

Tp0136 Enhanced G_q Signaling Through PAR1 During Platelet Activation

Activated PAR1 can couple with members of the G_q and G_i families and trigger numerous intracellular signaling pathways (8). To determine whether Tp0136 activated G_q-protein-coupled pathways through PAR1 to promote platelet activation and aggregation, the expression levels of Akt, PI3K and P38 were detected. As shown in **Figure 4A**, Tp0136 triggered the phosphorylation of Akt (P <0.01), indicating PI3K activation (P <0.01), and the phosphorylation of p38 (P <0.01), confirming G_q-dependent signaling, similar to TRAP-6. To determine whether Tp0136 enhances G_q signaling through PAR1, the PAR1 antagonist RWJ56110 was utilized. Interestingly, the RWJ56110 treatment of platelets significantly reduced the activation ability of Akt, PI3K and p38 compared with that of the conditioned media-treated controls, reversing the changes caused by Tp0136 (**Figure 4A**). In addition, Tp0136 significantly elicited a G_q-triggered increase in intraplatelet calcium levels (P <0.05), as measured using the Fluo-3AM calcium indicator, and this effect was inhibited by the PAR1 antagonist

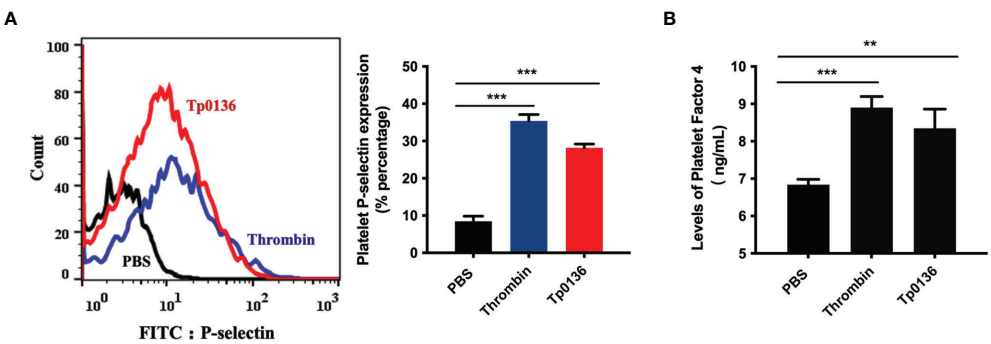


FIGURE 2 | Tp0136 promoted human platelet activation. **(A)** Expression of platelet P-selectin as measured by flow cytometry. A representative histogram is shown. Statistical data were analyzed using the X geometric mean fluorescence (left) and the percentage of gated cells (right). **(B)** PF4 expression as determined by ELISA. The values are presented as the mean ± SEM of experimental triplicates and are representative of the results of three independent experiments. Values among multiple groups were compared by one-way ANOVA (**P < 0.01, ***P < 0.001).

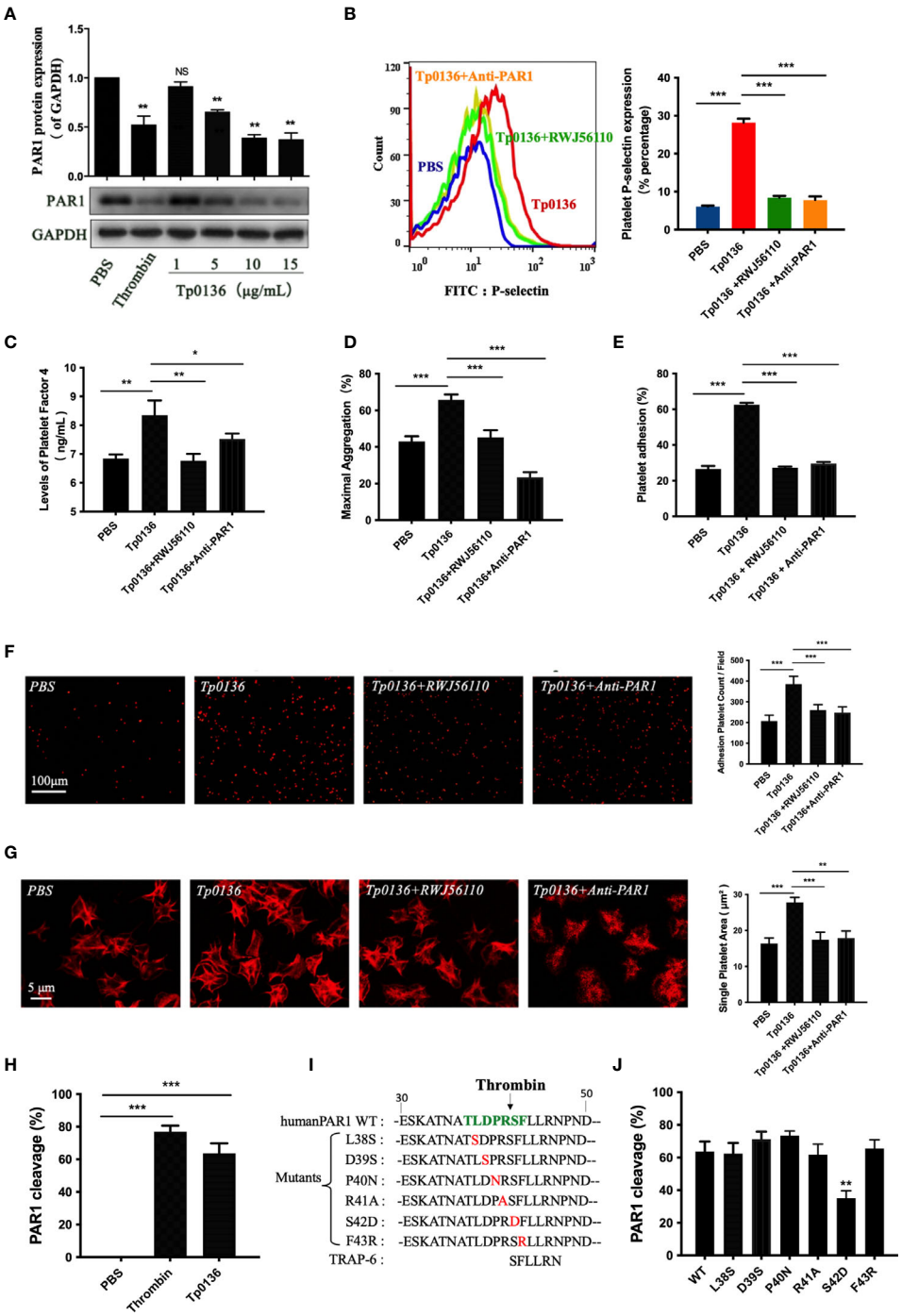


FIGURE 3 | Tp0136 promoted platelet activation and aggregation through PAR1. **(A)** PAR1 protein expression as determined by western blotting. **(B-G)** Effects of the PAR1 antagonist RWJ56110 and the anti-PAR1 antibody on platelet activation and aggregation induced by Tp0136. **(B)** P-selectin expression as measured by flow cytometry. **(C)** PF4 expression as measured by ELISA. **(D)** Maximal platelet aggregation as determined by an aggregometer. **(E)** Platelet adhesion as assessed by ELISA. **(F,G)** Platelet adhesion as assessed by staining with TRITC-labeled phalloidin. Statistical data are based on the number of platelet adhesions **(F)** and were calculated from the mean of the average surface area of individual platelets **(G)**. **(H)** Effect of Tp0136 on PAR1 as determined by flow cytometry. **(I)** Amino acid sequences of WT (wild-type) PAR1, PAR1 proteins with mutations in the extracellular domain and a PAR1 peptide agonist (TRAP-6). **(J)** Exploration of the Tp0136 protein sites that act on PAR1. The values are presented as the mean ± SEM of experimental triplicates and are representative of the results of three independent experiments. Values among multiple groups were compared by one-way ANOVA. Comparisons between two groups were made using a paired t-test (NS, no significance, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

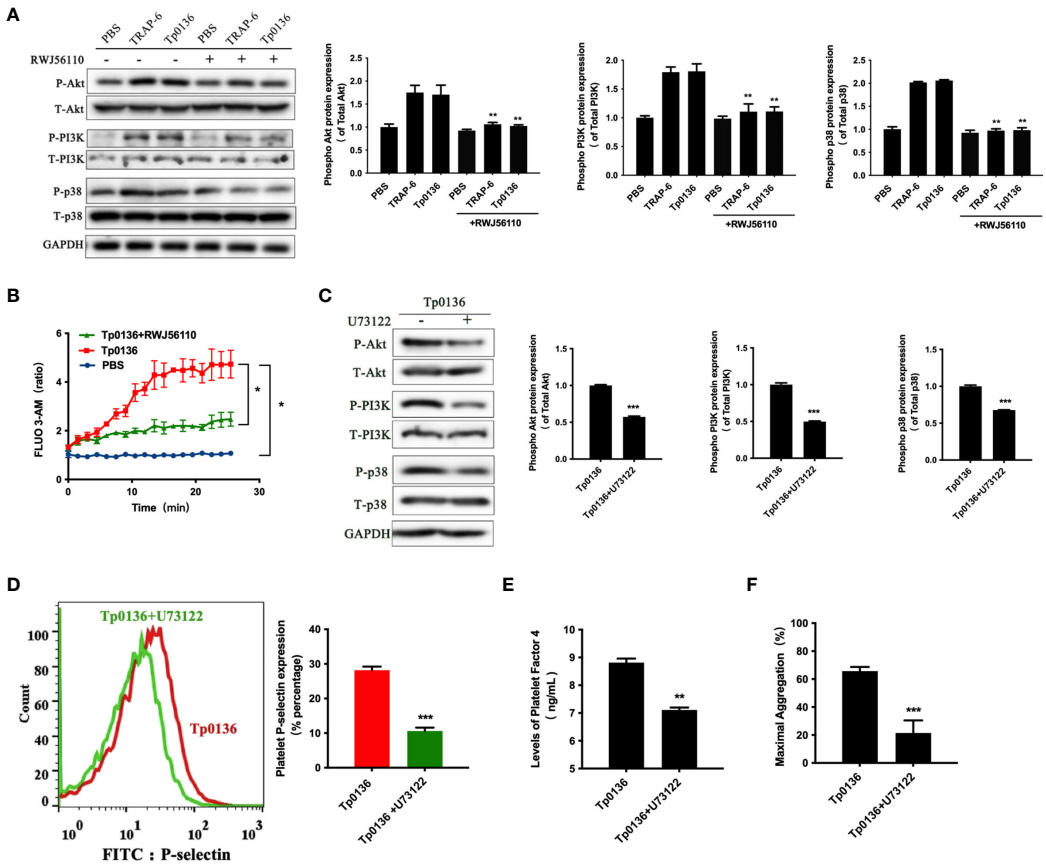


FIGURE 4 | Tp0136 enhanced G_q signaling through PAR1 during platelet activation. **(A)** The protein expression of phosphorylated/total p38/Akt/PI3K and GAPDH was assessed by western blotting. **(B)** Cytosolic free Ca^{2+} was measured by flow cytometry. **(C)** The protein expression of phosphorylated/total p38/Akt/PI3K and GAPDH was assessed by western blotting. **(D)** Expression of platelet P-selectin as measured by flow cytometry. A representative histogram is shown. **(E)** PF4 as determined by ELISA. **(F)** Maximal platelet aggregation as determined by an aggregometer. The values are presented as the mean ± SEM of experimental triplicates and are representative of the results of three independent experiments. Comparisons between the two groups were analyzed using a paired t-test. Differences in the calcium levels over time were determined by repeated-measures ANOVA followed by Dunnett's *post hoc* test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

RWJ56110 (P <0.05) (**Figure 4B**). The involvement of G^q-dependent signaling during Tp0136-induced platelet activation was significantly inhibited by U73122, a phospholipase C inhibitor (P <0.001) (**Figure 4C**). The inhibitor U73122 significantly abolished the potentiating effect of Tp0136 on P-selectin expression (P <0.001) (**Figure 4D**), the secretion of PF4 (P <0.01) (**Figure 4E**) and platelet aggregation (P <0.001) (**Figure 4F**).

Tp0136 Enhanced G_i Signaling Through PAR1 During Platelet Activation

Furthermore, cAMP was measured to determine whether the effects of Tp0136 on platelets involve G_i-dependent signaling pathways. The iloprost-induced increase in the intraplatelet cAMP concentration was affected by Tp0136 (P <0.05), and pretreatment with RWJ56110 increased the cAMP level compared to that in the group stimulated with only Tp0136 (P <0.05) (**Figure 5A**). To assess the contribution of G_i signaling to the activation of platelets by Tp0136, pertussis toxin (PTX), an inhibitor of the G_i signaling pathway, was utilized to specifically inhibit G_i. PTX significantly reversed the enhancing effect of Tp0136 on platelet surface P-selectin expression (P <0.001) (**Figure 5B**) and PF4 granular secretion (P <0.01) (**Figure 5C**) and weakened the enhancement of platelet aggregation induced by Tp0136 (P <0.01) (**Figure 5D**), showing that G_i-dependent signaling was involved in the activating effect of Tp0136 on platelets.

DISCUSSION

The well-recognized capacity of *T. pallidum*, the etiological agent of venereal syphilis, for early dissemination and immune evasion has earned it the designation of 'the stealth pathogen' (26). Patients with primary syphilis present with typical chancres, painless ulcerations, that heal spontaneously over several weeks, which gives the illusion that the person has not been infected with syphilis and thus causes the best treatment period to be missed; thereafter, *T. pallidum* enters a latent state, inducing an

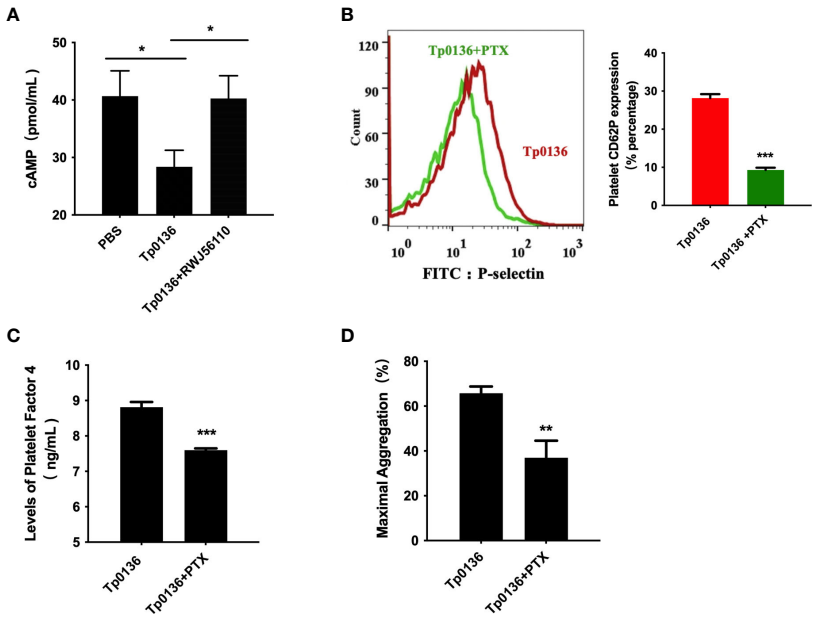


FIGURE 5 | Tp0136 enhanced G_i signaling through PAR1 during platelet activation. **(A)** cAMP as analyzed by ELISA. **(B)** P-selectin as measured by flow cytometry. A representative histogram is shown. **(C)** PF4 as measured by ELISA. **(D)** Maximal platelet aggregation as determined by an aggregometer. The values are presented as the mean ± SEM of experimental triplicates and are representative of the results of three independent experiments. Comparisons between the two groups were analyzed using a paired t-test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

insidious infection. Self-healing of chancre is essentially wound healing, which is an important multifaceted and complicated process in humans and animals that is governed by sequential but overlapping phases, including the hemostatic, inflammatory, proliferative, and remodeling phases (27). It is important to understand the first signals that activate the cellular response of injured tissue. After injury to the skin, the exposed subendothelial, collagen and tissue factors activate platelet aggregation, which results in degranulation and the release of chemotactic and growth factors to form the clot that initiates wound healing (28). In the present study, we found that Tp0136, a predicted *T. pallidum* adhesin that mediates its colonization during infection (9), enhanced platelet activation and aggregation through PAR1 and then initiated the receptor G_q and G_i signaling pathways. This process may represent the first step of platelet recruitment to syphilitic lesion sites and the initiation of wound healing. However, a recently research by Cameron group reported that *T. pallidum* directly, preferentially, and reversibly interacted with platelets, altered their movement and increased blood-brain barrier permeability, eventually facilitating their dissemination (14). Tp0136 as an important adhesion protein of *T. pallidum*, here we found that it could stimulate platelet activation and aggregation. Therefore, Tp0136 protein could play an important role in interaction with platelet mediating the dissemination of *T. pallidum*, which would need more research.

In our study, Tp0136 activated platelets and promoted platelet-fibrinogen adhesion and aggregation, resulting in the formation of platelet clots, which are naturally involved in wound healing (29). Upon activation, platelets secrete more than 300 active substances from their intracellular particles. Herein, Tp0136 activated platelets and promoted the platelet secretion of granules, such as PF4, P-selectin, and Ca^{2+}, into the surrounding cellular milieu. These secreted platelet granule components contribute to blood coagulation (30). P-selectin is an inflammatory coagulation biomarker involved in clotting (31). Our results showed that Tp0136 promoted the expression of P-selectin in platelets and promoted platelet activation and aggregation, which may have initiated the self-healing of chancres in the early stages of syphilis. Of course, this phenomenon needs to be further studied in animals (*in vivo*) infected with syphilis.

PARs are G-protein-coupled receptors that utilize a fascinating mechanism to convert an extracellular proteolytic cleavage event into a transmembrane signal; these receptors carry their own ligands, which remain hidden until unmasked by receptor cleavage (8). PAR1, the prototype of this family, is activated when thrombin cleaves its amino-terminal extracellular domain at a specific site. This cleavage reveals a new N-terminus that acts as a tethered ligand for intramolecular binding to the body of the receptor and thus affects transmembrane signaling (32). We observed that Tp0136 enhanced platelet activation and aggregation through PAR1. We next carried out site-directed mutagenesis of PAR1 and found that Tp0136 acted on the N-terminal extracellular domain of PAR1 between residues 41 and 42 ($LDPR^{41}\downarrow S^{42}FL$), which was consistent with thrombin. However, whether the Tp0136 protein has the characteristics of an active enzyme still needs to be determined.

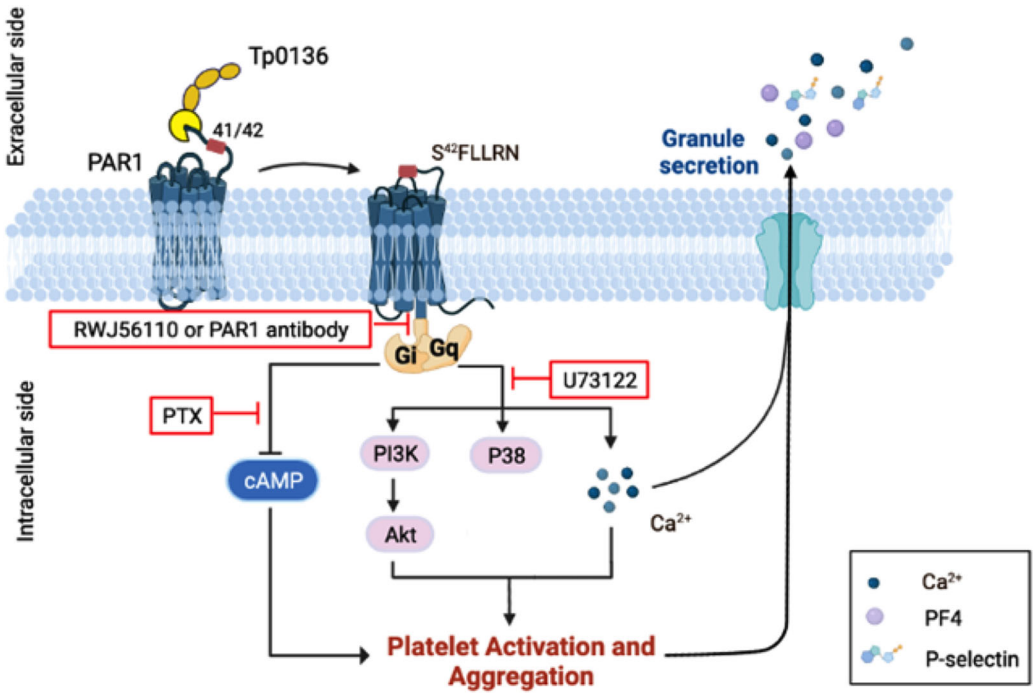


FIGURE 6 | Schematic model of the mechanism by which Tp0136 modulates platelet signaling. Tp0136 promotes human platelet activation and aggregation through PAR1 and generates an S^{42}FLLRN-tethered ligand that interacts with the receptor to induce PAR1 signaling. In particular, Tp0136 activates the G^q signaling pathway through PAR1, thereby inducing PAR1-dependent Akt-PI3K activation, p38 phosphorylation and Ca^{2+} flux, and the G^i pathway, thereby attenuating the release of cytosolic cAMP.

PAR1 can couple to members of the G_q and G_i families and thus to a host of intracellular effectors. Our results showed that Tp0136 enhanced platelet activation through PAR1, thereby initiating G_q- and G_i- activated intracellular pathways and thus predisposing platelets to be fully activated by a subsequent subthreshold stimulus. G_q generates a pathway for calcium-regulated kinases, mitogen-activated protein kinase cassettes, and other proteins that mediate cellular responses ranging from particle formation to integrin activation to platelet aggregation (33, 34). Furthermore, Akt is a serine/threonine-specific protein kinase that plays a key role in platelet aggregation, integrin signaling, particle secretion, and clot retraction (35). In our study, Tp0136 stimulated G_q activation through PAR1 in human platelets, as shown by Akt-PI3K activation and p38 phosphorylation, and increased the release of intraplatelet Ca^{2+}, a key second messenger, from intracellular stores (36, 37). As reported herein and in agreement with the findings of others (38), the treatment of platelets with U73122, a phospholipase C inhibitor, partially attenuated the upregulated expression of P-selectin and PF4 as well as the subsequent platelet adhesion and aggregation. These observations clearly underscore the critical regulatory effect of Tp0136 on the G_q-activated intracellular pathways downstream of PAR1. In addition, Tp0136 reduced the cAMP levels in iloprost-exposed platelets. Cytosolic cAMP is synthesized by adenylyl cyclase and is known as a powerful inhibitor of platelet aggregation (39). Moreover, PTX, which inhibits G_i-receptor coupling pathways, affected the potentiating activity of Tp0136 on platelet aggregation. Based on the information discussed above, Tp0136 induces Akt-PI3K activation, p38 phosphorylation and Ca^{2+} movement in platelets; however, to achieve full platelet activation, Tp0136 must be induced by regulating the intracellular cAMP levels and thereby triggering the concomitant G_i signaling pathway. A similar mechanism has been reported for other agents enhancing platelet activation, such as PGE_2 (24) and MMP-2 (22), and explains the difference between platelet primers and full platelet agonists (40).

Several limitations should be noted. First, we showed that Tp0136 activated platelets to promote their aggregation *in vitro*, and further studies, such as *in vivo* experiments, is needed to confirm our *in vitro* findings. Second, we detected changes in the protein expression of only PAR1 and related downstream signaling molecules, and further study is needed to determine whether other platelet receptors are involved in this process. Third, the effect of Tp0136 promoting platelet activation and aggregation on the development of syphilis infection remains to be further studied.

In conclusion, were herein elucidated a mechanism of platelet activation and aggregation in which Tp0136 effects platelets through PAR1 and thereby triggers downstream G_i and G_q signaling (**Figure 6**). This study suggests that Tp0136 plays a role in platelet function, and the elucidation of relevant

mechanisms represents another step toward understanding chancre self-healing in the early stages of syphilis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Zhongshan Hospital, Xiamen University. All volunteers provided written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

QY-X was first author. TC-Y was corresponding author. TC-Y and QY-X designed the study and drafted the manuscript, TC-Y and LR-L critical review and revision of the manuscript. QY-X and YJ-W performed experiments. LL-L was responsible for statistical analysis and validation. All authors agree to be accountable for the content of the work. All authors read and approved the final manuscript.

FUNDING

This work was supported by the National Natural Science Foundation of China (grant numbers 82172331, 81972028, 81973104, 81971147, 81772260, 81771312), the Key Projects for Province Science and Technology Program of Fujian Province, China (grant number 2020D017) and the Natural Science Foundation of Fujian Province, China (grant number 2021J02055). The funders played no role in the study design, data collection, or analyses, the decision to publish, or manuscript preparation.

ACKNOWLEDGMENTS

The authors thank all the contributors to this work.

REFERENCES

1. Tong ML, Lin LR, Liu LL, Zhang HL, Huang SJ, Chen YY, et al. Analysis of 3 Algorithms for Syphilis Serodiagnosis and Implications for Clinical Management. *Clin Infect Dis* (2014) 58(8):1116–24. doi: 10.1093/cid/ciu087
2. Peeling RW, Mabey D, Kamb ML, Chen XS, Radolf JD, Benzaken AS. Syphilis. *Nat Rev Dis Primers* (2017) 3:17073. doi: 10.1038/nrdp.2017.73
3. Rodrigues M, Kosaric N, Bonham CA, Gurtner GC. Wound Healing: A Cellular Perspective. *Physiol Rev* (2019) 99(1):665–706. doi: 10.1152/physrev.00067.2017
4. Clark RA. Fibrin Is a Many Splendored Thing. *J Invest Dermatol* (2003) 121 (5):xxi–xxii. doi: 10.1046/j.1523-1747.2003.12575.x
5. Versteeg HH, Heemskerk JW, Levi M, Reitsma PH. New Fundamentals in Hemostasis. *Physiol Rev* (2013) 93(1):327–58. doi: 10.1152/physrev.00016.2011
6. Sebert M, Sola-Tapias N, Mas E, Barreau F, Ferrand A. Protease-Activated Receptors in the Intestine: Focus on Inflammation and Cancer. *Front Endocrinol (Lausanne)* (2019) 10:717. doi: 10.3389/fendo.2019.00717
7. Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-Activated Receptors 1 and 4 Mediate Activation of Human Platelets by Thrombin. *J Clin Invest* (1999) 103(6):879–87. doi: 10.1172/JCI6042
8. Coughlin SR. Thrombin Signalling and Protease-Activated Receptors. *Nature* (2000) 407(6801):258–64. doi: 10.1038/35025229
9. Djokic V, Giacani L, Parveen N. Analysis of Host Cell Binding Specificity Mediated by the Tp0136 Adhesin of the Syphilis Agent Treponema Pallidum Subsp. Pallidum. *PloS Negl Trop Dis* (2019) 13(5):e0007401. doi: 10.1371/journal.pntd.0007401
10. Ke W, Molini BJ, Lukehart SA, Giacani L. Treponema Pallidum Subsp. Pallidum TP0136 Protein Is Heterogeneous Among Isolates and Binds Cellular and Plasma Fibronectin *via* Its NH2-Terminal End. *PloS Negl Trop Dis* (2015) 9(3):e0003662. doi: 10.1371/journal.pntd.0003662
11. Luo X, Gao ZX, Lin SW, Tong ML, Liu LL, Lin LR, et al. Recombinant Treponema Pallidum Protein Tp0136 Promotes Fibroblast Migration by Modulating MCP-1/CCR2 Through TLR4. *J Eur Acad Dermatol Venereol* (2020) 34(4):862–72. doi: 10.1111/jdv.16162
12. Luo X, Lin SW, Xu QY, Ke WJ, Gao ZX, Tong ML, et al. Tp0136 Targets Fibronectin (RGD)/Integrin Beta1 Interactions Promoting Human Microvascular Endothelial Cell Migration. *Exp Cell Res* (2020) 396 (1):112289. doi: 10.1016/j.yexcr.2020.112289
13. Li QL, Tong ML, Liu LL, Lin LR, Lin Y, Yang TC. Effect of Anti-TP0136 Antibodies on the Progression of Lesions in an Infected Rabbit Model. *Int Immunopharmacol* (2020) 83:106428. doi: 10.1016/j.intimp.2020.106428
14. Church B, Wall E, Webb JR, Cameron CE. Interaction of Treponema Pallidum, the Syphilis Spirochete, With Human Platelets. *PloS One* (2019) 14(1):e0210902. doi: 10.1371/journal.pone.0210902
15. Boncler M, Kehrel B, Szewczyk R, Stec-Martyna E, Bednarek R, Brodde M, et al. Oxidation of C-Reactive Protein by Hypochlorous Acid Leads to the Formation of Potent Platelet Activator. *Int J Biol Macromol* (2018) 107(Pt B):2701–14. doi: 10.1016/j.ijbiomac.2017.10.159
16. Huang WC, Lin KC, Hsia CW, Hsia CH, Chen TY, Bhavan PS, et al. The Antithrombotic Agent Pterostilbene Interferes With Integrin Alphaiibbeta3-Mediated Inside-Out and Outside-In Signals in Human Platelets. *Int J Mol Sci* (2021) 22(7):3643–58. doi: 10.3390/ijms22073643
17. Ren L, Li Q, You T, Zhao X, Xu X, Tang C, et al. Humanin Analogue, HNG, Inhibits Platelet Activation and Thrombus Formation by Stabilizing Platelet Microtubules. *J Cell Mol Med* (2020) 24(8):4773–83. doi: 10.1111/jcmm.15151
18. Berger GT, Hartwell DW. Wagner DD. P-Selectin and Platelet Clearance. *Blood* (1998) 92(11):4446–52. doi: 10.1182/blood.V92.11.4446
19. Théorêt JF, Yacoub D, Hachem A, Gillis MA. Merhi Y. P-Selectin Ligation Induces Platelet Activation and Enhances Microaggregate and Thrombus Formation. *Thromb Res* (2011) 128(3):243–50. doi: 10.1016/j.thromres.2011.04.018
20. Kaplan KL, Owen J. Plasma Levels of Beta-Thromboglobulin and Platelet Factor 4 as Indices of Platelet Activation. *Vivo Blood* (1981) 57(2):199–202. doi: 10.1182/blood.V57.2.199.199
21. Aliotta A, Bertaggia Calderara D, Alberio L. Flow Cytometric Monitoring of Dynamic Cytosolic Calcium, Sodium, and Potassium Fluxes Following Platelet Activation. *Cytomet A* (2020) 97(9):933–44. doi: 10.1002/cyto.a.24017
22. Sebastiano M, Momi S, Falcinelli E, Bury L, Hoylaerts MF, Gresele P. A Novel Mechanism Regulating Human Platelet Activation by MMP-2-Mediated PAR1 Biased Signaling. *Blood* (2017) 129(7):883–95. doi: 10.1182/blood-2016-06-724245
23. Kim S, Jin J, Kunapuli SP. Relative Contribution of G-Protein-Coupled Pathways to Protease-Activated Receptor-Mediated Akt Phosphorylation in Platelets. *Blood* (2006) 107(3):947–54. doi: 10.1182/blood-2005-07-3040

24. Fabre JE, Nguyen M, Athirakul K, Coggins K, McNeish JD, Austin S, et al. Activation of the Murine EP3 Receptor for PGE2 Inhibits cAMP Production and Promotes Platelet Aggregation. *J Clin Invest* (2001) 107(5):603–10. doi: 10.1172/JCI10881
25. Kuliopulos A, Covic L, Seeley SK, Sheridan PJ, Helin J, Costello CE. Plasmin Desensitization of the PAR1 Thrombin Receptor: Kinetics, Sites of Truncation, and Implications for Thrombolytic Therapy. *Biochemistry* (1999) 38(14):4572–85. doi: 10.1021/bi9824792
26. Radolf JD, Deka RK, Anand A, Šmajs D, Norgard MV, Yang XF. Treponema Pallidum, the Syphilis Spirochete: Making a Living as a Stealth Pathogen. *Nat Rev Microbiol* (2016) 14(12):744–59. doi: 10.1038/nrmicro.2016.141
27. Wang PH, Huang BS, Horng HC, Yeh CC, Chen YJ. Wound Healing. *J Chin Med Assoc* (2018) 81(2):94–101. doi: 10.1016/j.jcma.2017.11.002
28. Gauglitz GG, Korting HC, Pavicic T, Ruzicka T, Jeschke MG. Hypertrophic Scarring and Keloids: Pathomechanisms and Current and Emerging Treatment Strategies. *Mol Med* (2011) 17(1-2):113–25. doi: 10.2119/molmed.2009.00153
29. Heher P, Muhleder S, Mittermayr R, Redl H, Slezak P. Fibrin-Based Delivery Strategies for Acute and Chronic Wound Healing. *Adv Drug Deliv Rev* (2018) 129:134–47. doi: 10.1016/j.addr.2017.12.007
30. Golebiewska EM, Poole AW. Platelet Secretion: From Haemostasis to Wound Healing and Beyond. *Blood Rev* (2015) 29(3):153–62. doi: 10.1016/j.blre.2014.10.003
31. Blann AD, Nadar SK, Lip GY. The Adhesion Molecule P-Selectin and Cardiovascular Disease. *Eur Heart J* (2003) 24(24):2166–79. doi: 10.1016/j.ehj.2003.08.021
32. Vu TK, Hung DT, Wheaton VI, Coughlin SR. Molecular Cloning of a Functional Thrombin Receptor Reveals a Novel Proteolytic Mechanism of Receptor Activation. *Cell* (1991) 64(6):1057–68. doi: 10.1016/0092-8674(91)90261-v
33. Adam F, Kauskot A, Nurden P, Sulpice E, Hoylaerts MF, Davis RJ, et al. Platelet JNK1 Is Involved in Secretion and Thrombus Formation. *Blood* (2010) 115(20):4083–92. doi: 10.1182/blood-2009-07-233932
34. Flevaris P, Li Z, Zhang G, Zheng Y, Liu J, Du X. Two Distinct Roles of Mitogen-Activated Protein Kinases in Platelets and a Novel Rac1-MAPK-Dependent Integrin Outside-in Retractile Signaling Pathway. *Blood* (2009) 113(4):893–901. doi: 10.1182/blood-2008-05-155978
35. O'Brien KA, Stojanovic-Terpo A, Hay N, Du X. An Important Role for Akt3 in Platelet Activation and Thrombosis. *Blood* (2011) 118(15):4215–23. doi: 10.1182/blood-2010-12-323204
36. Anderson R, Theron AJ, Steel HC, Nel JG, Tintinger GR. ADP-Mediated Upregulation of Expression of CD62P on Human Platelets Is Critically Dependent on Co-Activation of P2Y1 and P2Y12 Receptors. *Pharmaceut (Basel)* (2020) 13(12) :420–35. doi: 10.3390/ph13120420
37. Koupenova M, Ravid K. Biology of Platelet Purinergic Receptors and Implications for Platelet Heterogeneity. *Front Pharmacol* (2018) 9:37:37. doi: 10.3389/fphar.2018.00037
38. Lopez JJ, Redondo PC, Salido GM, Pariente JA, Rosado JA. Two Distinct Ca2+ Compartments Show Differential Sensitivity to Thrombin, ADP and Vasopressin in Human Platelets. *Cell Signal* (2006) 18(3):373–81. doi: 10.1016/j.cellsig.2005.05.006
39. Nam GS, Nam KS. Arctigenin Attenuates Platelet Activation and Clot Retraction by Regulation of Thromboxane A2 Synthesis and cAMP Pathway. *BioMed Pharmacother* (2020) 130:110535. doi: 10.1016/j.biopha.2020.110535
40. Gresele P, Falcinelli E, Momi S. Potentiation and Priming of Platelet Activation: A Potential Target for Antiplatelet Therapy. *Trends Pharmacol Sci* (2008) 29(7):352–60. doi: 10.1016/j.tips.2008.05.002

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Medicine

ORIGINAL RESEARCH
published: 02 March 2022
doi: 10.3389/fmed.2022.851635

Incidence Trends of Five Common Sexually Transmitted Infections Excluding HIV From 1990 to 2019 at the Global, Regional, and National Levels: Results From the Global Burden of Disease Study 2019

Leiwen Fu[1†], Yinghui Sun[1†], Min Han[2†], Bingyi Wang[1†], Fei Xiao[3†], Yiguo Zhou[1], Yanxiao Gao[1], Thomas Fitzpatrick[4], Tanwei Yuan[1], Peiyang Li[1], Yuewei Zhan[1], Yong Lu[5], Ganfeng Luo[1], Junyi Duan[6], Zhongsi Hong[3], Christopher K. Fairley[7,8], Tong Zhang[6], Jin Zhao[9*] and Huachun Zou[1,10*]*

[1] School of Public Health (Shenzhen), Sun Yat-sen University, Shenzhen, China, [2] Department of Medical Administration, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangzhou, China, [3] The Fifth Affiliated Hospital of Sun Yat-sen University, Zhuhai, China, [4] Department of Internal Medicine, University of Washington, Seattle, WA, United States, [5] School of Public Health, The Key Laboratory of Environmental Pollution Monitoring and Disease Control, Ministry of Education, Guizhou Medical University, Guiyang, China, [6] Clinical and Research Center for Infectious Diseases, Beijing Youan Hospital, Capital Medical University, Beijing, China, [7] Central Clinical School, Monash University, Melbourne, VIC, Australia, [8] Melbourne Sexual Health Centre, Alfred Health, Melbourne, VIC, Australia, [9] Department of HIV/AIDS Control and Prevention, Shenzhen Center for Disease Control and Prevention, Shenzhen, China, [10] Kirby Institute, University of New South Wales, Sydney, NSW, Australia

Objective: Sexually transmitted infections (STIs) are common worldwide and pose a challenge to public health. We conducted this study to assess the annual incidence of five common STIs, including syphilis, chlamydia, gonorrhea, trichomoniasis, and genital herpes at the global, regional, and national levels.

Materials and Methods: We obtained detailed data on STIs excluding HIV from 1990 to 2019 from the Global Burden of Disease (GBD) 2019 database. Estimated annual percentage change (EAPC) was calculated to quantify trends in age-standardized incidence rates (ASR) of STIs, stratified by gender, sociodemographic index (SDI) region, and pathogenic microorganism.

Results: Globally, incident cases of STIs increased by 58.15% from 486.77 million in 1990 to 769.85 million in 2019, but the annual change in ASR was only −0.04% (95% CI −0.09 to 0.01) per year. EAPC was 0.16 (0.06 to 0.26) for syphilis, 0.09 (0.05 to 0.13) for genital herpes, 0.06 (0.03 to 0.09) for trichomoniasis, −0.21 (−0.36 to −0.06) for chlamydia, and −0.14 (−0.19 to −0.08) for gonorrhea. High SDI regions reported significant increases in ASR of syphilis and chlamydia.

Conclusions: The burden of disease from STIs remains large, though control of STIs has contributed to the decreasing incidence in most regions, especially in the low-SDI regions. Globally, over the past 20 years, the ASR has remained stable for trichomoniasis and genital herpes decreased for chlamydia and gonorrhea, and increased for syphilis.

Keywords: global burden, STIs, syphilis, chlamydia, gonorrhea, trichomoniasis, genital herpes

OPEN ACCESS

Edited by:
Pingyu Zhou,
Tongji University, China

Reviewed by:
Zhen Ning,
Shanghai Municipal Center for Disease Control and Prevention (SCDC), China
Qian-Qiu Wang,
Chinese Academy of Medical Sciences and Peking Union Medical College, China

***Correspondence:**
Huachun Zou
zouhuachun@mail.sysu.edu.cn
Jin Zhao
zhaoj@szcdc.net
Tong Zhang
zt_doc@ccmu.edu.cn

†These authors have contributed equally to this work

Specialty section:
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *10 January 2022*
Accepted: *31 January 2022*
Published: *02 March 2022*

Citation:
Fu L, Sun Y, Han M, Wang B, Xiao F, Zhou Y, Gao Y, Fitzpatrick T, Yuan T, Li P, Zhan Y, Lu Y, Luo G, Duan J, Hong Z, Fairley CK, Zhang T, Zhao J and Zou H (2022) Incidence Trends of Five Common Sexually Transmitted Infections Excluding HIV From 1990 to 2019 at the Global, Regional, and National Levels: Results From the Global Burden of Disease Study 2019. Front. Med. 9:851635. doi: 10.3389/fmed.2022.851635

INTRODUCTION

Sexually transmitted infections (STIs) are among the most common infectious diseases reported worldwide (1). More than one million people are newly infected with STIs per day (2). In 2016, the World Health Organization (WHO) estimated there were 376 million new infections of four curable STIs, including chlamydia (127 million), gonorrhea (87 million), syphilis (6 million), and trichomoniasis (156 million), respectively (2–4). Additionally, more than 500 million people are living with genital herpes (2). Although most STIs are usually not fatal; they result in a substantial burden of diseases (1). STIs such as herpes and syphilis are associated with an increased risk of HIV transmission (3, 5). STIs, including chlamydia and gonorrhea can result in reproductive tract morbidities, such as infertility and pelvic inflammatory diseases among women (6–8). Mother-to-child transmission of STIs can result in neonatal death, congenital deformities, and other adverse birth outcomes (3, 5, 9, 10). A report published by the United States Centers for Disease Control and Prevention in 2016 warned that high levels of antibiotic resistance in the USA might soon make gonorrhea untreatable, and a similar trend is now emerging in chlamydia (3, 11, 12). Although the incidence of STIs is high, prevention efforts targeting STIs have been largely ignored because safe-sex messaging related to HIV is assumed to be sufficient to control STIs transmission (11, 13). Most STIs are asymptomatic and therefore easily neglected (14, 15). Changes in sexual behaviors due to increasing availability of HIV pre-exposure prophylaxis, including decreased condom use and increased number of sexual partners, may led to a significant increase in STIs (16).

To date, there have been limited population estimates of the burden of STIs in different regions around the world (4). Two systematic reviews of estimates of STIs in 2012 and 2016 reported the global prevalence and incidence of chlamydia, gonorrhea, trichomoniasis, and syphilis in adults remained high; nearly one million new infections with curable STIs were detected every day, but varied by region and gender (4, 17). Estimates of the incidence of specific STIs at the regional level, such as herpes simplex virus and congenital syphilis, show that despite the decrease, the prevalence remains stable and poses a huge global burden (18). Previous studies have focused on cross-sectional snapshots of STI burdens in specific regions and populations but have not reported distribution patterns or trends over time. Existing monitoring data on STIs are scattered, and systematic monitoring and comparison at the national level are needed to inform the rational allocation of health resources (3).

The Global Burden of Disease Study (GBD) 2019 is a systematic effort to assess the burden of many diseases according to age, gender, and geography across the world from 1990 to 2019 (19). STIs excluding HIV have been estimated and classified into six categories: syphilis, chlamydia, gonorrhea, trichomoniasis, genital herpes, and other STIs. No previous study has quantified annual trends in the incidence of STIs, excluding HIV and specific pathogenic microorganisms over a specified time period. The first strategic direction of the WHO Global Health Sector Strategy on Sexually Transmitted Infections 2016–2021 is to collect information on STI prevalence and incidence across representative populations (20). Therefore, understanding the incidence of STIs in regions and countries is essential to advocating, funding, planning, and implementing the prevention and control of STIs. In the present study, we used the results of the GBD 2019 to estimate the burden of syphilis, chlamydia, gonorrhea, trichomoniasis, and genital herpes by determining temporal trends in incidence at the global, regional, and national levels.

MATERIALS AND METHODS

Data Source

We extracted data from the GBD 2019 using the Global Health Data Exchange query tool (http://ghdx.healthdata.org/gbd-results-tool). The GBD 2019 reports estimates of incidence, prevalence, mortality, years of life lost (YLLs), years lived with disability (YLDs), and disability-adjusted life-years (DALYs) due to 369 diseases and injuries, for two genders as well as for 204 countries and territories. Incident cases, incidence rates, and age-standardized incidence rates (ASR) of syphilis, chlamydia, gonorrhea, trichomoniasis, and genital herpes from 1990 to 2019, segregated by gender, age, location, and specific STI, were extracted using GBD's operation guide (19, 21). A Bayesian meta-regression modeling tool, DisMod-MR 2.1, was used to ensure consistency between incidence, prevalence, remission, excess mortality, and cause-specific mortality for most causes. The estimated incidence of trichomoniasis, genital herpes, syphilis; each in separate models in DisMod-MR 2.1. The incidence of chlamydia and gonorrhea was estimated in a custom process outside of Dismod, as described in a previous study (19). Available data were collected from 21 GBD regions in terms of geography, e.g., East Asia (**Table 1**), and 204 countries and territories. Age was extracted by five-year age groups for a total of 20 GBD age groups. Because the GBD database groups the age according to the interval of 5 years, it is divided into 1–4, 5–9, etc. In order to facilitate the comparison between different age groups, we did not include people under the age of one year in this study. The 204 countries and territories were categorized into five regions based on the sociodemographic index (SDI): low, low-middle, middle, high-middle, and high SDI regions. The SDI is a summary measure that estimates a location's position on a spectrum of development, which is a composite indicator of a country's lag-distributed income per capita, average years of schooling, and the fertility rate in females under the age of 25 years. The cutoff values used to determine quintiles for analysis were then computed using country-level estimates of SDI for 2019. Additional details on results from the SDI calculation are available in the GBD2019. The general methods for the GBD 2019 have been detailed in previous studies (19). Data collection for syphilis from case notification, ante-natal and community surveillance data, cross-sectional studies and claims data; for chlamydia and gonorrhea from case notification, ante-natal and community surveillance data and cross-sectional studies; for trichomoniasis from case notification and cross-sectional studies; for genital herpes from cross-sectional studies. Case definitions for all of these infections

TABLE 1 | Incident cases and age-standardized incidence rates of sexually transmitted infections in 1990 and 2019 and estimated annual percentage change from 1990 to 2019.

Characteristics	1990		2019		1990–2019
	Incident cases No. × 10^3 (95% UI)	ASR per 100,000 No. (95% UI)	Incident cases No. × 10^3 (95% UI)	ASR per 100,000 No. (95% UI)	EAPC No. (95% CI)
Overall	486,771.20 (416,757.41–565,524.92)	9,323.71 (7,994.18–10,804.75)	769,851.91 (659,059.33–892,663.79)	9,535.71 (8,169.73–11,054.76)	−0.04 (−0.09 to 0.01)
Sex					
Male	271,108.35 (230,667.77–316,253.28)	10,369.15 (8,814.94–12,091.21)	426,072.07 (362,331.77–496,890.04)	10,471.63 (8,892.20–12,176.10)	−0.04 (−0.09 to 0.02)
Female	215,662.85 (185,370.23–250,535.94)	8,262.58 (7,107.40–9,594.72)	343,779.83 (293,773.14–399,266.98)	8,602.40 (7,358.00–10,001.18)	−0.04 (−0.10 to 0.02)
SDI category					
Low-SDI	50,472.93 (43,231.14–58,660.96)	11,817.48 (10,145.19–13,761.86)	107,712.82 (91,841.64–125,724.84)	10,933.50 (9,332.30–12,773.10)	−0.09 (−0.17 to −0.02)
Low-middle-SDI	89,265.65 (76,517.23–103,797.10)	8,906.49 (7649.99–10,325.71)	160,814.99 (137,884.69–186,617.47)	8,883.31 (7,608.08–10,298.21)	−0.05 (−0.08 to −0.03)
Middle-SDI	181,587.51 (155,220.87–211,757.46)	10,830.67 (9,263.26–12,636.21)	264,896.29 (226,459.24–309,655.39)	10,085.26 (8,646.52–11,806.73)	−0.15 (−0.23 to −0.08)
High-middle-SDI	112,059.93 (95,563.15–130,936.37)	9,238.83 (7,913.02–10,785.43),	148,390.88 (126,213.58–173,312.20)	9,222.42 (7,864.23–10,753.14)	−0.20 (−0.29 to −0.11)
High-SDI	53,073.66 (44,435.87–62,521.66)	5,940.18 (5,004.14–6,988.20)	65,804.44 (55,027.33–77,434.49)	6,117.57 (5,136.38–7,209.63)	0.06 (0.05 to 0.08)
Sexually transmitted infections excluding HIV					
Syphilis	8,845.22 (6,562.51–11,588.86)	160.03 (120.66–208.10)	14,114.11 (10,648.49–18,415.97)	178.48 (134.94–232.34)	0.16 (0.06 to 0.26)
Chlamydia	151,695.68 (113,998.56–199,144.01)	2,867.67 (2,150.60–3,741.43)	232,534.84 (174,269.19–303,009.11)	2,883.87 (2,161.21–3,762.80)	−0.21 (−0.36 to −0.06)
Gonorrhea	67,732.22 (51,820.12–89,251.61)	1,178.58 (912.29–1,536.00)	87,951.95 (68,461.02–112,961.84)	1,124.39 (872.97–1,441.08)	−0.14 (−0.19 to −0.08)
Trichomoniasis	205,446.49 (151,261.12–273,107.88)	4,157.14 (3,061.97–5,439.34)	354,466.58 (260,117.34–461,359.68)	4,327.29 (3,176.53–5,645.76)	0.06 (0.03 to 0.09)
Genital herpes	53,051.59 (45,029.38–61,934.04)	960.29 (822.81–1,116.81)	80,784.43 (68,810.96–94,200.33)	1,021.68 (869.15–1,191.20)	0.09 (0.05 to 0.13)
Region					
East Asia	137,725.48 (116,644.99–162,625.37)	10,607.48 (8,996.37–12,490.97)	178,904.41 (150,660.79–211,566.79)	10,476.70 (8,849.86–12,376.60)	−0.41 (−0.57 to −0.24)
Southeast Asia	49,962.40 (42,687.23–58,572.42)	11,326.64 (9,713.89–13,174.51)	82,549.05 (70,604.05–96,081.74)	11,230.92 (9,620.76–13,068.46)	−0.03 (−0.04 to −0.03)
Oceania	871.37 (746.46–1,014.18)	14,588.13 (125,49.89–16,873.11)	1,862.95 (1,576.67–2,184.08)	14,285.59 (12,118.18–16,679.43)	0.01 (−0.03 to 0.04)
Central Asia	8,196.70 (6,894.45–9,713.17)	12,383.96 (10,497.47–14,530.25)	12,178.68 (10,273.85–14,353.30)	12,235.85 (10,365.83–14,354.40)	−0.10 (−0.12 to −0.08)
Central Europe	11,629.25 (9,869.73–13,512.87)	9,173.26 (78,07.47–10,696.18)	10,679.92 (9,118.03–12,422.30)	9,126.48 (7,756.82–10,664.06)	−0.04 (−0.05 to −0.03)
Eastern Europe	23,434.74 (20,089.42–27,523.04)	9,909.11 (8,510.07–11,739.04)	21,459.59 (18,307.99–25,324.18)	9,895.41 (8,480.04–11,724.59)	−0.06 (−0.07 to −0.04)
High-income Asia Pacific	11,073.03 (9,351.29–13,025.79)	5,780.52 (4,877.69–6,788.02)	11,164.35 (9,321.58–13,155.00)	5,705.97 (4,794.87–6,714.58)	−0.02 (−0.04 to 0.00)
Australasia	1,143.33 (970.08–1,346.40)	5,247.11 (4,465.01–6,157.35)	1,521.33 (1,288.04–1,781.82)	5,018.90 (4,251.70–5,907.28)	−0.14 (−0.24 to −0.05)
Western Europe	15,705.13 (13,112.74–18,669.09)	3,768.35 (3,139.71–4,497.39)	17,190.14 (14,251.86–20,319.64)	3,729.40 (3,099.27–4,444.56)	−0.03 (−0.05 to −0.01)
Southern Latin America	2,747.36 (2367.56–3,189.59)	5,654.43 (4,850.87–6,571.61)	3,990.25 (3,429.68–4,646.06)	5,51.23 (4,849.22–6,583.30)	−0.01 (−0.02 to 0.01)
High-income North America	20,552.26 (16,813.62–24,896.24)	6,692.36 (5,494.37–8,065.15)	24,479.62 (20,010.26–29,207.73)	6,489.07 (5,320.69–7,797.60)	−0.24 (−0.28 to −0.20)
Caribbean	4,325.39 (3,700.06–5,029.65)	12,460.65 (10,649.93–14,467.67)	6,136.82 (5,240.39–7,115.89)	12,540.87 (10,720.36–14,571.42)	−0.01 (−0.02 to 0.01)
Andean Latin America	3,163.54 (2,712.04–3,676.01)	9,278.14 (7,938.01–10,763.57)	6,028.75 (5,141.35–7,050.07)	9,213.75 (7,866.87–107,48.10)	0.00 (−0.02 to 0.01)
Central Latin America	18,546.46 (15,786.31–21,785.53)	12,590.93 (10,713.35–14,664.66)	33,539.52 (28,375.32–39,254.34)	12,756.44 (10,798.14–14,937.03)	0.11 (0.08 to 0.14)
Tropical Latin America	18,910.45 (16,142.13–22,082.28)	12,809.55 (10,978.92–14,976.17)	32,029.05 (27,292.76–37,417.34)	12,955.71 (11,074.32–15,125.80)	−0.06 (−0.12 to 0.00)
North Africa and Middle East	28,845.03 (24,682.92–33,559.92)	9,552.82 (8,178.98–11,091.56)	58,679.34 (49,671.26–68,405.06)	8,946.10 (7,619.55–10,371.37)	−0.20 (−0.22 to −0.18)
South Asia	67,172.26 (57,095.03–79,685.03)	6,818.42 (5,796.52–8,055.92)	125,287.68 (106,806.37–147,313.92)	6,652.49 (5,662.30–7,819.84)	−0.11 (−0.13 to −0.09)
Central Sub-Saharan Africa	5,650.89 (4,931.90–6,496.61)	12,289.81 (10,723.69–14,164.84)	14,097.74 (12,225.69–16,294.45)	12,093.61 (10,531.43–13,910.25)	−0.07 (−0.09 to −0.05)
Eastern Sub-Saharan Africa	26,026.33 (22,217.09–30,528.73)	17,597.87 (14,939.06–20,625.45)	59,742.73 (50,627.16–70,681.29)	17,033.30 (14,330.44–20,087.35)	−0.22 (−0.27 to −0.18)
Southern Sub-Saharan Africa	10,475.91 (9,095.70–12,113.61)	21,090.11 (18,370.38–24,194.60)	16,722.29 (14,470.90–19,371.69)	19,973.12 (17,382.69–23,001.57)	−0.26 (−0.32 to −0.20)
Western Sub-Saharan Africa	20,613.89 (17,656.99–24,123.30)	13,319.18 (11,355.57–15,603.23)	51,607.69 (43,835.11–60,708.46)	13,358.91 (11,298.50–15,721.57)	−0.01 (−0.06 to 0.03)

UI, uncertainty interval; ASR, age-standardized incidence rate; CI, confidential interval; EAPC, estimated annual percentage change.

were based on laboratory findings, except late syphilis, which was ascertained from administrative data using ICD-9.093–095 and ICD-10A52 and I98.0. For chlamydia, gonorrhea, and trichomoniasis, the reference case definition was diagnosis with a nucleic acid amplification test (NAAT). For all STIs, sources were excluded if the sample population was drawn exclusively from a high-risk group (e.g., HIV-positive, men who have sex with men [MSM], or sex workers).

For all STIs excluding genital herpes, the datasets were supplemented with a manual search of national ministry of health websites, antenatal clinic surveillance reports, data from the GBD collaborator network, and case-notification data from locations where centralized reporting was mandatory. The genital herpes dataset was only supplemented by sources from the GBD collaborator network. With regard to specific STIs, details on the flow chart, definitions, input data, and modeling strategy are available in the online **Supplementary Appendix 1** of the GBD 2019 (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7567026/bin/mmc1.pdf) (19). The human development index (HDI) values of all nations were collected from the World Bank (http://hdr.undp.org/en/composite/trends).

Statistical Analysis

Incident cases of all STIs from 1990 to 2019 were summarized. According to the previous study, the estimated annual percentage change (EAPC) is a summary and widely used measure of the ASR trend over a specified interval. ASR and EAPC were calculated to estimate trends of STI incidence (22). ASR (per 100,000 persons) was calculated using the following formula:

$$\mathbf{ASR} = \frac{\sum_{\mathbf{i=1}}^{\mathbf{N}} \alpha_{\mathbf{i}} \mathbf{w_i}}{\sum_{\mathbf{i=1}}^{\mathbf{N}} \mathbf{w_i}}$$

Where α_i and w_i represent the age-specific rate and number of people (or the weight) in the i^{th} age group, respectively. N represents the number of age groups. The 95% uncertainty interval (UI) was generated from 2.5% and 97.5% quantiles extracted 1,000 times from the posterior distribution. To summarize ASR trends over a specified interval, EAPC and its 95% confidence interval (CI) were calculated using the following linear regression model (23, 24):

$$y = \alpha + \beta x + \varepsilon$$
$$EAPC = 100 \times (e^{\beta} - 1)$$

where y = ln (ASR), and x = calendar year.

An ASR was determined to represent a trend of increasing or decreasing incidence over time if both the EAPC and its 95% CI was above or below 0, respectively. To explore factors that may influence EAPC, correlation analyses were conducted comparing EAPC and ASR (1990), and HDI (2019), respectively, for each included STI. The global, regional, and national incidence rates of syphilis, chlamydia, gonorrhea, trichomoniasis, and genital herpes were described using maps, including ASR in 2019, the percentage change in incident cases, and EAPC in ASR from 1990 to 2019. Correlation analysis was used to estimate the ρ indices and p values for the association of EAPC with HDI and baseline ASR. The breakpoint was estimated by the change of ρ indices in the smoothed curve. All data were analyzed using R software 3.6.0 (R Core Team, Vienna, Austria). A P value <0.05 was considered statistically significant.

RESULTS

Incident Cases of STIs

Globally, from 1990 to 2019, the total number of combined incident cases of syphilis, chlamydia, gonorrhea, trichomoniasis, and genital herpes increased by 58.15% from 486.77 (95% CI 416.76 to 565.52) million to 769.85 (659.06 to 892.66) million (country-specific details see **Supplementary Table 7** and **Figures 1A,B**). The group aged 30–34 years had the highest number of incident cases in 2019. Except for the groups aged 10–14, 15–19, 20–24, and >85 years, men had a higher incidence of all STIs than women in 2019 (426.07 vs. 343.78 million, **Table 1**; **Figures 2A,B**). The change in STI incidence varied considerably between nations, with the most prominent increase observed in Qatar (661.68%), United Arab Emirates (552.08%), and Maldives (297.78%), and the most prominent decline observed in Georgia (−36.64%). The largest number of incident cases occurred in China (172.83 million), India (99.91 million), and Indonesia (32.61 million) in 2019.

ASR and EAPC of STIs

Globally, ASR of all STIs was 9,535.71 per 100.000 in 2019, which was significantly heterogeneous across the world, with the highest ASR in South Africa (21,759.56 per 100.000) and lowest in Belgium (3,465.76 per 100.000) (**Figure 1A**). From 1990 to 2019, ASR of all STIs globally decreased, with an EAPC of −0.04 (95% CI −0.09 to 0.01). However, this trend was not statistically significant. EAPC for men and women was −0.04 (95% CI −0.09 to 0.02) and −0.04 (95% CI −0.10 to 0.02), respectively (**Table 1**). The largest increase in ASR was seen in Mexico (0.17, 95% CI 0.12 to 0.22), while the most significant decreases were seen in Iraq (−0.47, 95% CI −0.56 to −0.38), Morocco (−0.46, 95% CI −0.52 to −0.41), and China (−0.42, 95% CI −0.59 to −0.25) from 1990 to 2019 (**Table 1**; **Supplementary Table 7**; **Figure 1C**).

In the analysis by geographic region, ASR was highest in Southern Sub-Saharan Africa (19,973.12 per 100.000 in 2019), Western Sub-Saharan Africa (17,033.30 per 100.000 in 2019), and Oceania (14,285.59 per 100.000 in 2019). Significant decreases in ASR from 1990 to 2019 were found in East Asia (EAPC = −0.41; 95% CI −0.57 to −0.24), Southern Sub-Saharan Africa (EAPC = −0.26; 95% CI −0.32 to −0.20), and High-income North America (EAPC = −0.24; 95% CI −0.28 to −0.20). In the analysis by SDI region, an increase in ASR from 1990 to 2019 was only observed in high SDI regions (EAPC = 0.06, 95% CI 0.05 to 0.08).

Incidence of STIs by Pathogenic Organism

Incident cases of each STI in 1990 and 2019 at the global and regional levels are presented in **Figures 3**, **4**. Globally in 2019, trichomoniasis incident cases outnumbered other

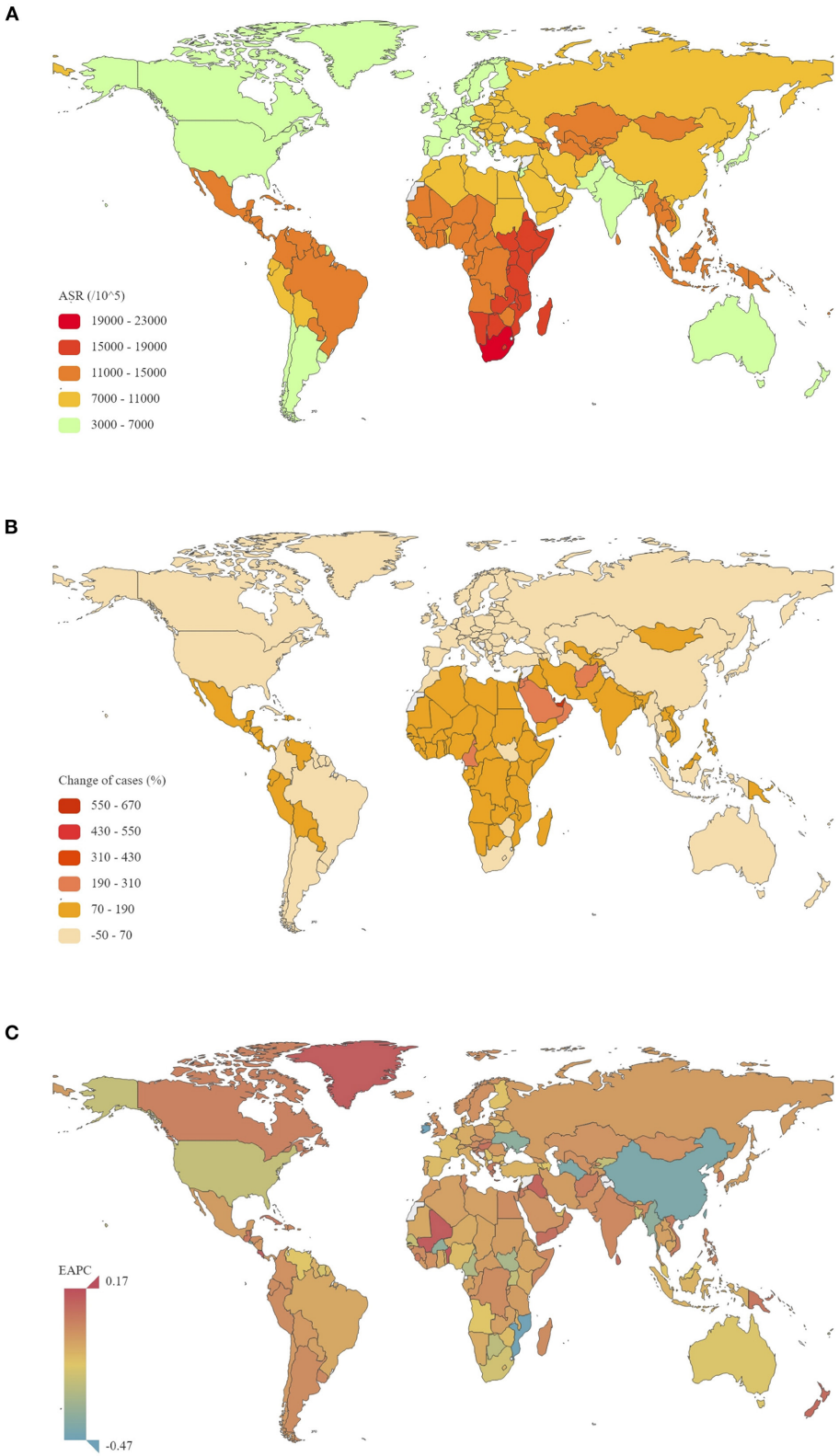


FIGURE 1 | The global disease burden of STIs excluding HIV for men and women in 204 countries and territories. **(A)** The ASR of STIs excluding HIV in 2019; **(B)** The relative change in incident cases of STIs excluding HIV between 1990 and 2019; **(C)** The EAPC in STI ASR from 1990 to 2019 ASR, age-standardized rate; EAPC, estimated annual percentage change; STIs, sexually transmitted infections; HIV, human immunodeficiency virus.

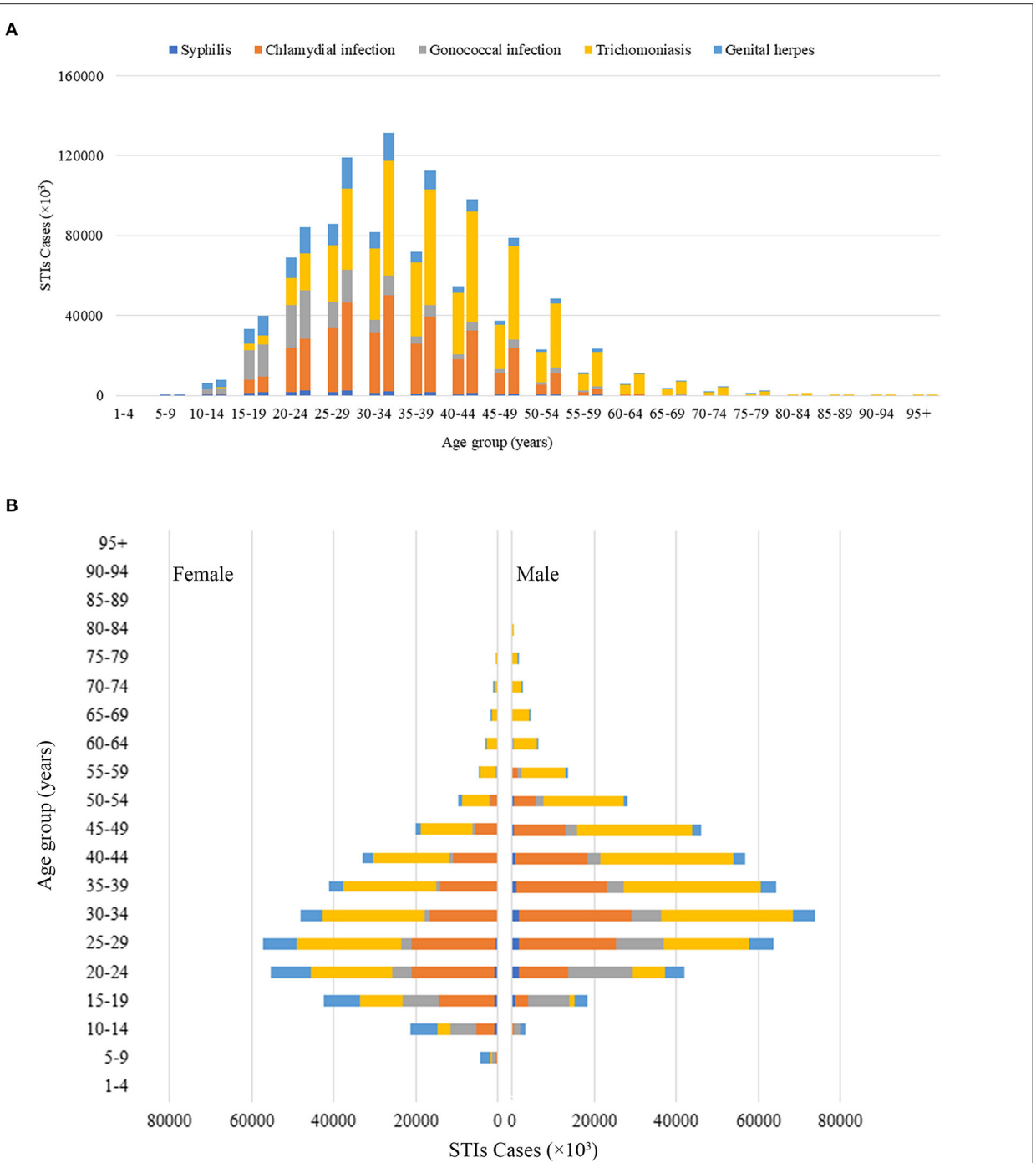


FIGURE 2 | Global STIs incident cases by 20 GBD age groups. **(A)** Global STIs incident cases by age for men and women combined in 1990 and 2019. For each group, the left column presents case data from 1990 and the right column presents data from 2019. **(B)** Difference by gender in global STI incident cases by age in 2019 GBD, Global Burden of Disease; STIs, sexually transmitted infections.

STIs, followed by chlamydia, gonorrhea, genital herpes, and syphilis, accounting for 46.04, 30.21, 11.42, 10.49, and 1.83% of total incident cases, respectively. These proportions were relatively stable at the global and regional levels over time. Among these STIs, the largest increase in the number of incident cases was trichomoniasis (72.53%) from 1990 to 2019. Globally, ASR of syphilis increased from 1990 to 2019, with an EAPC of 0.16 (95% CI 0.06 to 0.26) (**Table 1**). However, ASR decreased for chlamydia and gonorrhea, with an EAPC of −0.21 (95% CI −0.36 to −0.06) and −0.14 (−0.19 to −0.08),

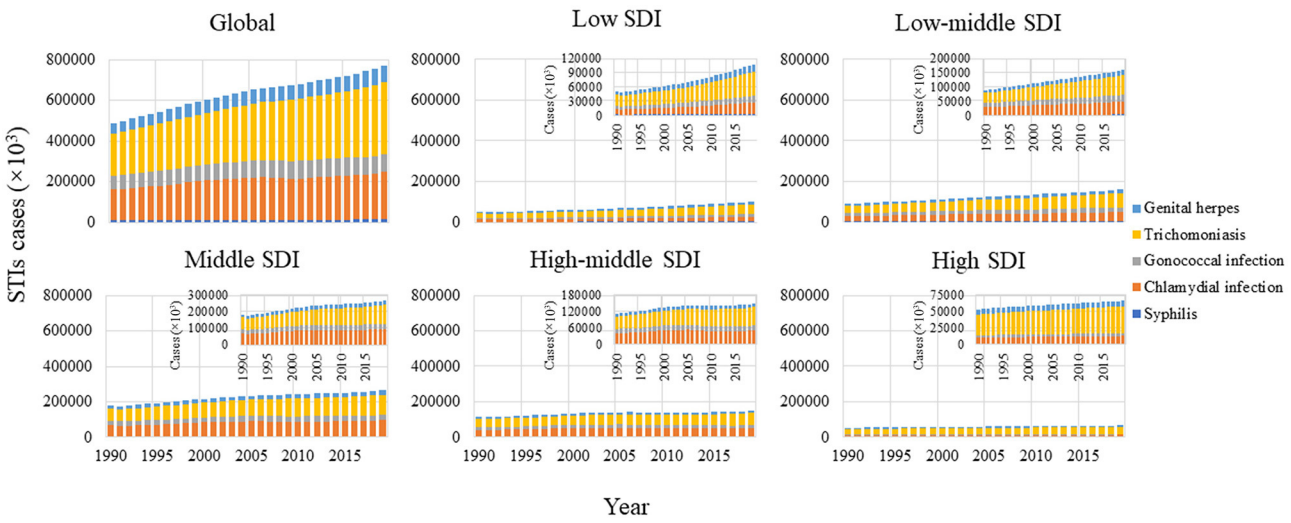


FIGURE 3 | Number of incident cases of STIs excluding HIV 1990-2019, stratified by SDI region. SDI regions with lower number of cases are magnified in the top-right of the panel SDI, sociodemographic index; STIs, sexually transmitted infections; HIV, human immunodeficiency virus.

respectively. ASR remained stable for trichomoniasis and genital herpes, with an EAPC of 0.06 (95% CI 0.03 to 0.09) and 0.09 (95% CI 0.05 to 0.13).

ASR trends for each STI were significantly heterogeneous across 5 SDI regions. The highest EAPC for syphilis was observed in high-SDI regions and the Caribbean, for chlamydia in high-SDI regions and western Sub-Saharan Africa, for gonorrhea in low-middle SDI regions and Oceania, for trichomoniasis in high-SDI regions and Oceania, and for genital herpes and in low-middle SDI regions, southern Sub-Saharan Africa, and South Asia (**Figure 5A**). Men had a larger increase in EAPC than women for syphilis (0.43 vs. −0.30) and gonorrhea (0.07 vs. −0.52). Detailed information of the incident cases, ASR, and EAPC for each STI by age, gender, region, and country or territory are presented in supplementary files.

Factors Associated With STIs Incidence

Figures 5B,C show the correlation between EAPC and ASR in 1990 and HDI in 2019 for all five STIs and for each of the 204 included countries and territories. A significant negative correlation (ρ=-0.25, P<0.001) was observed between EAPC and ASR (in 1990) for any incident STI. A significant positive relation was also detected between EAPCs and HDIs (ρ=0.26, P<0.001). Countries and territories with higher HDI experienced a more rapid increase in ASR from 1990 to 2019. These correlations for each of the five included STIs are presented in supplementary files.

DISCUSSION

To our knowledge, this is the first study to comprehensively analyze the GBD database for trends in incidence of chlamydia, gonorrhea, trichomoniasis, syphilis, and genital herpes at global, regional, and national levels from 1990 to 2019. The incident cases of these five common STIs increased by 58.15%, and ASR decreased by an average of 0.04% per year.

The trend in STI incidence over time was similar to that of other diseases whose ASR has not substantially changed (24). Global population growth appears to be the main reason for the increase in the number of incident STI cases, which increased by 25.7%, from 6.2 billion (6.0–6.3) in 2000 to 7.7 billion (7.5–8.0) in 2019, estimated by GBD 2019 Demographics Collaborators (24–26). However, trends in the incidence of these five STIs varied considerably by region and country. For example, in low and low-middle income countries, the trend in ASR for all STIs was mainly attributable to changes in syphilis. Conversely, in high-middle income regions, the decreasing trend in ASR of all STIs may be due to the decrease in the incidence of chlamydia and gonorrhea. Notably, ASR of syphilis and chlamydia was only found to be rapidly increasing in high SDI regions, a trend also reflected in the significant positive association between EAPC and HDI between 1990 and 2019. EAPC was negatively associated with baseline ASR. The baseline ASR in high-income countries was generally low, and lower the baseline ASR may explain the positive EAPCs observed in high SDI countries. It is critical to understand the exact trends in STIs with specific pathogenic microorganisms to effectively prevent STIs.

Significant increases in ASR of syphilis from 1990 to 2019 were found in male and high-SDI regions. The re-emergence of syphilis has also been reported in upper-middle-income and high-income regions, such as the United States (27, 28), Greece (29, 30), Japan (31, 32), and the UK recently (33), which is consistent with this study. Over the past decade, the incidence of syphilis among MSM has increased markedly in many countries. For example, the rate of primary or secondary syphilis among MSM in the United States increased from 11.7 cases per 100,000

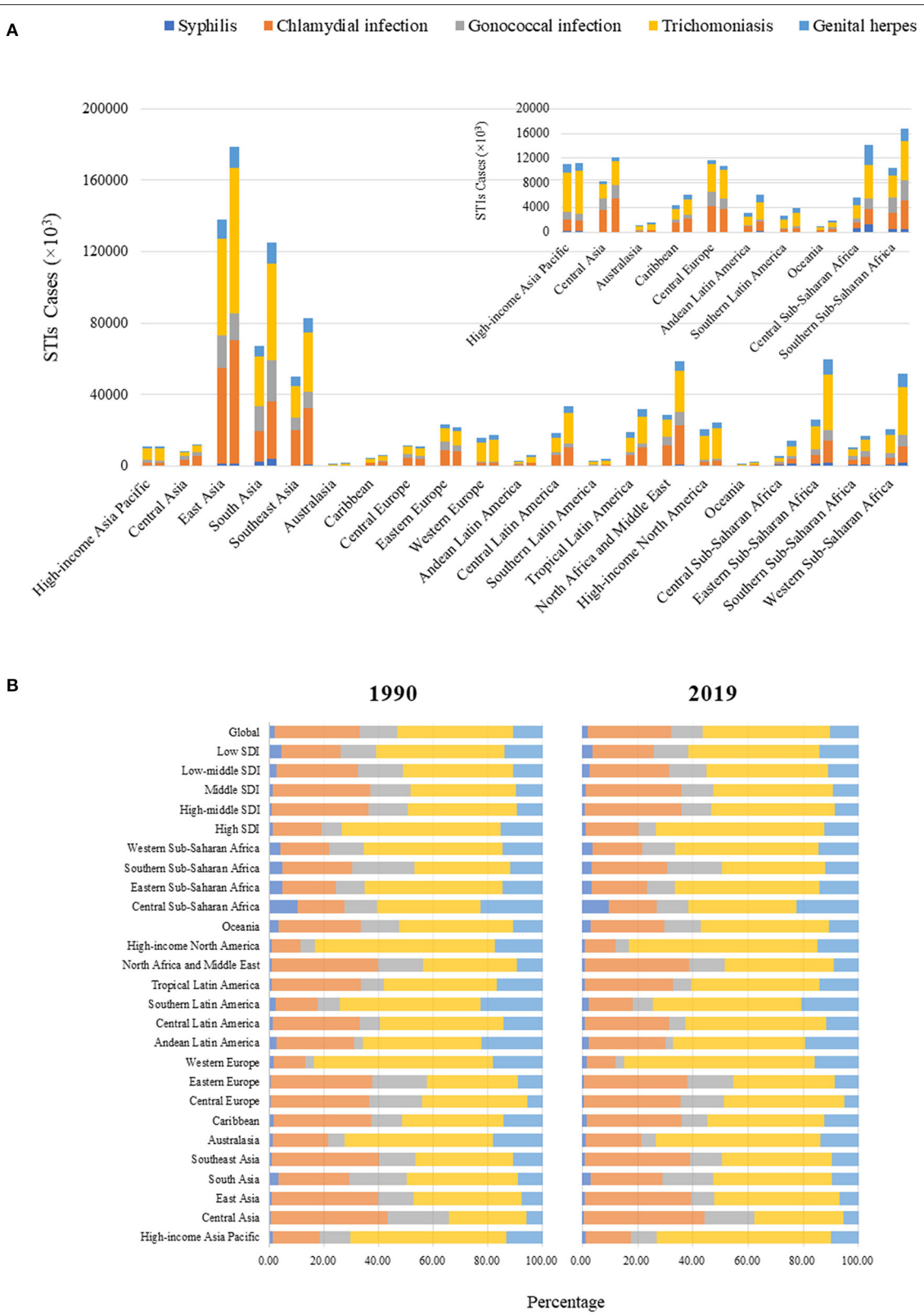


FIGURE 4 | (A) Number of incident cases of STIs excluding HIV at the regional level. The left column in each group presents cases in 1990 and the right column presents cases in 2019. Regions with lower numbers of cases are magnified in the top right of the panel. **(B)** Proportion of syphilis, chlamydia, gonorrhea, trichomoniasis, and genital herpes in all incident cases of STIs 1990-2019, at the global, SDI region, and regional levels STIs, sexually transmitted infections; HIV, human immunodeficiency virus.

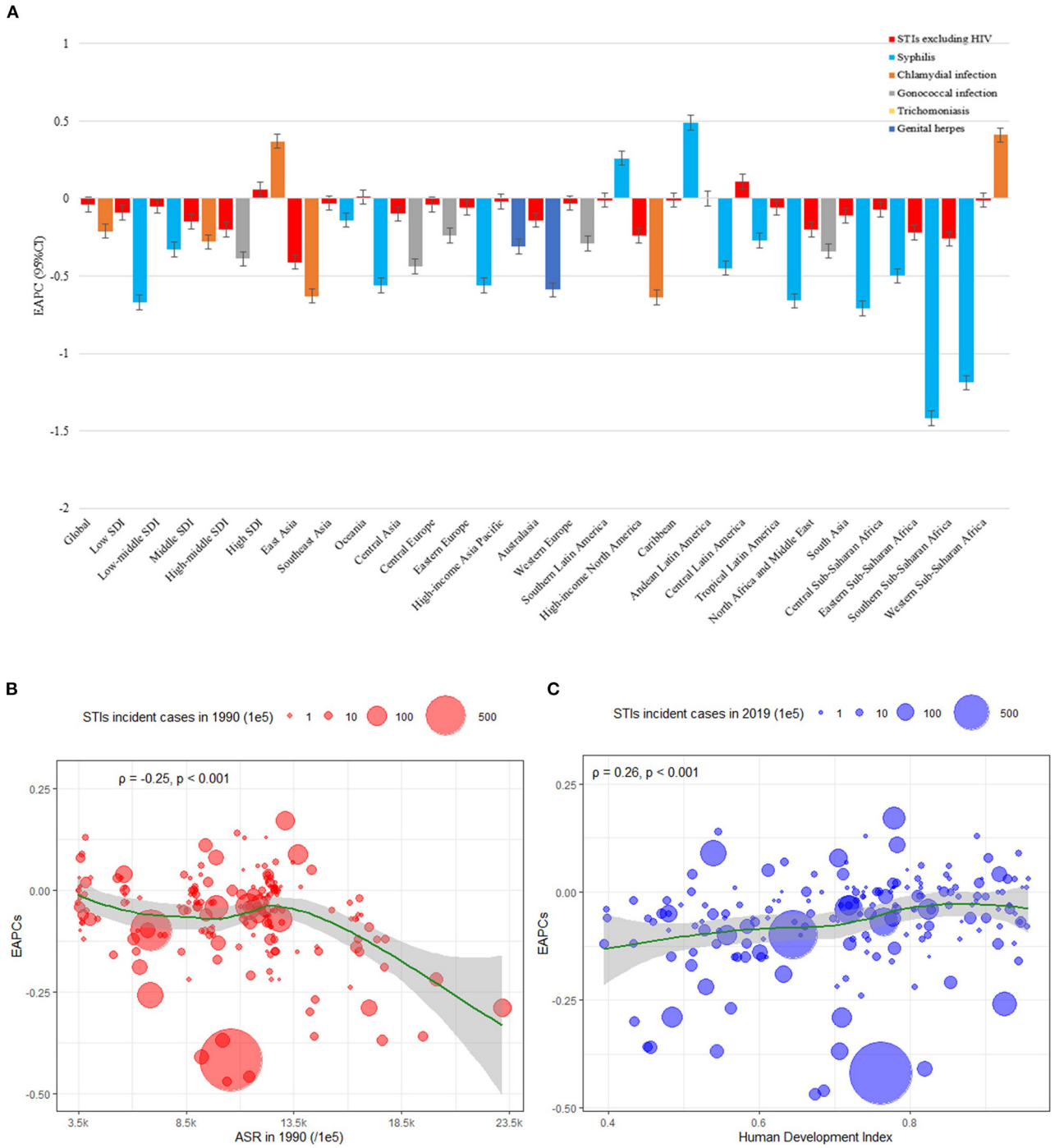


FIGURE 5 | EAPCs in STI ASR excluding HIV from 1990 to 2019, at global, regional, and national levels. **(A)** EAPC in STI ASR from 1990 to 2019, by pathogenic microorganism and by region. EAPC in each region are presented as an overall and an absolute maximum caused by pathogenic microorganism. The correlation between EAPC and **(B)** STI ASR in 1990 and **(C)** HDI in 2019. The circles represent countries with available HDI data. Circle size represents total number of STI cases. The ρ indices and p values presented in **(B)** and **(C)** were derived from Pearson correlation analysis ASR, age-standardized rate; EAPC, estimated annual percentage change; HDI, human development index; STIs, sexually transmitted infections; HIV, human immunodeficiency virus.

population in 2014 to 18.7 per 100,000 in 2018 (34). A recent meta-analysis reported that the global pooled prevalence among MSM from 2000–2020 was 7.5% (35). Among MSM who are receiving preexposure prophylaxis against HIV infection, the incidence of syphilis is particularly high due to the increase in condomless sex (36). Although the burden of syphilis in low-SDI

regions is still heavy, the high-SDI regions are areas of concern. Regular syphilis screening in the high-risk population, health education, and management of sexual partners are necessary to prevent the spread of syphilis.

Consistent with previous global estimates, we found the ASR of chlamydia and gonorrhea were highest in the middle, high middle-SDI countries and low middle, middle-SDI countries, respectively (17). Globally, the trend in ASR of chlamydia and gonorrhea decreased from 1990 to 2019. However, an increasing trend in ASR of chlamydia was observed only in countries with high SDI. Given that there are currently few data available to monitor population-based chlamydia incidence over time, the trends found in this study should be interpreted with caution. The decreasing trend of chlamydia and gonorrhea in specific populations has been reported in studies based on the Spectrum-STI model or population surveillance in some regions, such as Morocco, Washington State, Mongolia, and Western Australia (37–40). The declines in gonorrhea and chlamydia might be attributable to a combination of factors associated with the expanded HIV/STI response, including improved treatment coverage, improved reporting of cases treated, and a fall in sexual risk behaviors, possibly in part due to testing and counseling services for HIV (37). In GBD2019, the data used to estimate the incidence of chlamydia and gonorrhea are mainly derived from community surveillance data and cross-sectional research data based on the laboratory-confirmed diagnosis. Chlamydia and gonorrhea and are divided into asymptomatic and symptomatic health states, based on assumptions about the probability and duration of symptoms, including an estimate of the proportion of experiencing epididymal-orchitis (19). Chlamydia is a largely asymptomatic infection, and reported incidence is highly dependent upon rates of test uptake, particularly among asymptomatic persons at risk of infection. In high SDI countries, governments are paying more attention to screening for asymptomatic infection among sexually active young adults, which may affect estimates of the incidence of chlamydia, and partly explain the increase in incidence in these countries.

ASR of trichomoniasis and genital herpes remained stable across SDI regions from 1990 to 2019, with the highest rates being reported in lower SDI regions. Africa and Asia remain key areas for trichomoniasis and genital herpes prevention and control, which are similar to previous global surveys (17, 41). The WHO global and regional estimates for 2012 and 2016 suggested that trichomoniasis was especially common in low-income areas (17). Unlike the other four STIs, included in this study, women are more susceptible to genital herpes than men. Since many women of childbearing age have been or will be infected with HSV, the risk of transmission from mother to fetus or newborn is a major health problem (42, 43). Neonatal herpes infection is not a reportable disease, which may be why there is a high incidence of genital herpes among people aged 10–14 years in this study. Current epidemiological evidence suggests genital herpes also increases susceptibility to HIV infection and may increase HIV infectiousness in people living with HIV (44–46). However, due to the lack of data or case reports on these two STIs to investigate trends in incidence over time at national and global levels, making it is difficult to compare our findings to previously published studies.

Our study has several limitations. First, this is a secondary analysis of data extracted from the GBD 2019. The accuracy and robustness of GBD estimates depend on the quality and quantity of data used in its creation. Comparable studies are limited, so more population-based studies are needed to externally validate the findings of this study. Different sources of data in the input DisMod models, which have been clarified in the method, may hinder the comparison of the given STIs in this study. In order to sex-split data sources reported for both sexes combined, sources reporting for each sex separately were matched by age and location for each STI. Log ratios between the prevalence of each STI in females and the prevalence of each STI in males were input into MR-BRT to estimate an adjustment factor. An adjustment factor to split both sex data points into sex-specific data points was calculated for each STI, as pooled values across all ages and geographies. Second, since limited incidence data were available, estimates for a given infection and region are therefore extrapolated from a small number of data points, and ratios were used to generate estimates for some regions. The incidence in areas where key populations contribute disproportionately to sexually transmitted infection epidemics may have been underestimated despite the applied correction factor. Third, types of available tests have changed over time or are different across countries and regions. Nucleic acid amplification tests are more sensitive and specific than older techniques and are more commonly used in high SDI countries. In the absence of data on which tests were used, it is difficult to determine if changes over time or differences between locations are true or reflect differences in tests used. Fourth, many other STIs excluding HIV, are not included in the current study. Fifth, the age patterns of the incidence in this study should be interpreted with caution due to the different age patterns and different estimation processes in the input DisMod models for the incidence of different STIs. For example, GBD 2019 estimated the incidence of gonorrhea and chlamydia in a custom process outside of DisMod. Finally, the effect of the different health systems across different countries or regions was not evaluated.

The global estimates of the incidence trends of STIs are important in the first strategic direction of the WHO Global Health Sector Strategy. Currently, the Spectrum-STI estimation tool is often used to estimate the trend of national STI incidence (17, 47, 48). Compared with the GBD study, systematic reviews in the Spectrum-STI are updated, and its age group and gender data are more accurate (17). However, due to the large differences in the quality of the data included between different countries, it may affect the comprehensive comparison of global trends. The systematic literature reviews for incidence input data on STIs in GBD 2019 were completed on April 17, 2015 (19). Although the data processing and modeling strategy were different, it still needs to be updated. Despite the use of correction factors in a representative sample of the general population, such as age groups and sex ratios, the quality of studies on the

incidence of STIs needs to be improved. The comparison between GBD2017 and GBD2019 in this study shows that the difference in EAPC is essentially <0.1, and the reason for the difference can be found on the official website of GBD 2019 (http://ghdx.healthdata.org/gbd-2019). The process of generating future incidence estimates can be made more effective through continuously updated systematic reviews and continuously optimized model strategies. More population-based national monitoring data are needed to verify the accuracy of GBD estimates in STIs.

STIs remain a major public health concern globally. Efforts to combat STIs in lower-income countries are commendable. Estimates of incidence trends are essential for effective control of STIs, optimization of primary and secondary prevention strategies, including enhanced screening programs in high-risk regions, active health promotion, and construction of comprehensive STI surveillance networks. Despite the weaknesses, this study will fill a gap where actual data on STIs burden are sparse or unavailable.

DATA AVAILABILITY STATEMENT

All data are available from the Global Health Data Exchange query tool (http://ghdx.healthdata.org/gbd-results-tool).

AUTHOR CONTRIBUTIONS

HZ conceived the study and designed the protocol with LF. LF, YS, MH, and YZ performed analyses of the Global Burden of Disease data. LF, YS, MH, and BW contributed to statistical analysis and interpretation of data. LF, TY, PL, YG, and CF drafted the manuscript with all authors critically revising the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by the Natural Science Foundation of China Excellent Young Scientists Fund (82022064), Natural Science Foundation of China International/Regional Research Collaboration Project (72061137001), Natural Science Foundation of China Young Scientist Fund (81703278), the Australian National Health and Medical Research Commission (NHMRC) Early Career Fellowship (APP1092621), the National Science and Technology Major Project of China (2018ZX10721102), the Sanming Project of Medicine in Shenzhen (SZSM201811071), the High Level Project of Medicine in Longhua, Shenzhen (HLPM201907020105), the National Key Research and Development Program of China (2020YFC0840900), and the Fundamental Research Funds for the Central Universities (51000-42180001). All funding parties did not have any role in the design of the study or in the explanation of the data.

ACKNOWLEDGMENTS

We appreciate the works by the Global Burden of Disease study 2019 collaborators. The author is very grateful to Huicui Meng, Chongguang Yang, Qianglin Fang, Yawen Jiang and Siyang Liu from School of Public Health (Shenzhen), Sun Yat-sen University, and Jinqiu Yuan from the Seventh Affiliated Hospital, Sun Yat-sen University for providing the valuable opinions and suggestions for data interpretation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.851635/full#supplementary-material

REFERENCES

1. GBD 2015 Disease and Injury Incidence and Prevalence Collaborators. Global, regional, and national incidence, prevalence, and years lived with disability for 310 diseases and injuries, 1990-2015: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet*. (2016) 388:1545–602. doi: 10.1016/S0140-6736(16)31678-6
2. World Health Organization. *Sexually transmitted infections (STIs) Key facts*. (2020). Available online at: http://www.who.int/mediacentre/factsheets/fs110/en/.
3. Unemo M, Bradshaw CS, Hocking JS, de Vries HJ, Francis SC, Mabey D, et al. Sexually transmitted infections: challenges ahead. *Lancet Infect Dis.* (2017) 17:e235–e79. doi: 10.1016/S1473-3099(17)30310-9
4. Newman L, Rowley J, Vander Hoorn S, Wijesooriya NS, Unemo M, Low N, et al. Global estimates of the prevalence and incidence of four curable sexually transmitted infections in 2012 based on systematic review and global reporting. *PLoS One.* (2015) 10:e0143304. doi: 10.1371/journal.pone.0143304
5. Fuchs W, Brockmeyer NH. Sexually transmitted infections. *J Dtsch Dermatol Ges.* (2014) 12:451–63. doi: 10.1111/ddg.12310
6. Tsevat DG, Wiesenfeld HC, Parks C, Peipert JF. Sexually transmitted diseases and infertility. *Am J Obstet Gynecol.* (2017) 216:1–9. doi: 10.1016/j.ajog.2016.08.008
7. Jamison CD, Coleman JS, Mmeje O. Improving women's health and combatting sexually transmitted infections through expedited partner therapy. *Obstet Gynecol.* (2019) 133:416–22. doi: 10.1097/AOG.0000000000003088
8. Hart RJ. Physiological aspects of female fertility: role of the environment, modern lifestyle, and genetics. *Physiol Rev.* (2016) 96:873–909. doi: 10.1152/physrev.00023.2015
9. Kularatne RS, Niit R, Rowley J, Kufa-Chakezha T. Adult gonorrhea, chlamydia and syphilis prevalence, incidence, treatment and syndromic case reporting in South Africa: Estimates using the Spectrum-STI model, 1990-2017. *Physiol Rev.* (2018) 13:e0205863. doi: 10.1371/journal.pone.0205863
10. Rours GI, Duijts L, Moll HA, Arends LR, de Groot R, Jaddoe VW, et al. Chlamydia trachomatis infection during pregnancy associated with preterm delivery: a population-based prospective cohort study. *Eur J Epidemiol.* (2011) 26:493–502. doi: 10.1007/s10654-011-9586-1
11. The Lancet Infectious Diseases. Time to take sexually transmitted infections seriously. *The Lancet Infectious diseases*. (2016) 16:981. doi: 10.1016/S1473-3099(16)30277-8
12. Fernández-Huerta M, Espasa M. Mycoplasma genitalium co-infection with Chlamydia trachomatis and Neisseria gonorrhoeae among asymptomatic patients: the silent wick for macrolide resistance. *Spread.* (2019) 95:391. doi: 10.1136/sextrans-2018-053848
13. Varghese B, Maher JE, Peterman TA, Branson BM, Steketee RW. Reducing the risk of sexual HIV transmission: quantifying the per-act risk for HIV on the basis of choice of partner, sex act, and condom

use. *Sex Transm Dis.* (2002) 29:38–43. doi: 10.1097/00007435-200201000-00007
14. Farley TA, Cohen DA, Elkins W. Asymptomatic sexually transmitted diseases: the case for screening. *Preventive medicine.* (2003) 36:502–9. doi: 10.1016/S0091-7435(02)00058-0
15. Francis SC, Mthiyane TN, Baisley K. McHunu SL. Prevalence of sexually transmitted infections among young people in South Africa: a nested survey in a health and demographic surveillance site. *Jama.* (2018) 15:e1002512. doi: 10.1371/journal.pmed.1002512
16. Traeger MW, Cornelisse VJ, Asselin J, Price B, Roth NJ, Willcox J, et al. Association of HIV Preexposure prophylaxis with incidence of sexually transmitted infections among individuals at high risk of HIV infection. *Jama.* (2019) 321:1380–90. doi: 10.1001/jama.2019.2947
17. Rowley J, Vander Hoorn S, Korenromp E, Low N, Unemo M, Abu-Raddad LJ, et al. Chlamydia, gonorrhoea, trichomoniasis and syphilis: global prevalence and incidence estimates, 2016. *Bull World Health Organiz\.* (2019) 97:548–62p. doi: 10.2471/BLT.18.228486
18. Korenromp EL, Rowley J, Alonso M, Mello MB, Wijesooriya NS, Mahiané SG, et al. Global burden of maternal and congenital syphilis and associated adverse birth outcomes-Estimates for 2016 and progress since 2012. *PLoS One.* (2019) 14:e0211720. doi: 10.1371/journal.pone.0211720
19. GBD 2019 Diseases and Injuries Collaborators. Global burden of 369 diseases and injuries in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019. *Lancet.* (2020) 396:1204–22. doi: 10.1016/S0140-6736(20)30925-9
20. World Health Organization. *Global Health Sector Strategy on Sexually Transmitted Infections 2016–202*1. Geneva: World Health Organization (2016).
21. GBD 2019 Demographics Collaborators. Global age-sex-specific fertility, mortality, healthy life expectancy (HALE), and population estimates in 204 countries and territories, 1950-2019: a comprehensive demographic analysis for the Global Burden of Disease Study 2019. *Lancet*. (2020) 396:1160–203. doi: 10.1016/S0140-6736(20)30977-6
22. Hankey BF, Ries LA, Kosary CL, Feuer EJ, Merrill RM, Clegg LX, et al. Partitioning linear trends in age-adjusted rates. *Cancer Causes Control.* (2000) 11:31–5. doi: 10.1023/A:1008953201688
23. Liu Z, Jiang Y, Yuan H, Fang Q, Cai N, Suo C, et al. The trends in incidence of primary liver cancer caused by specific etiologies: Results from the Global Burden of Disease Study 2016 and implications for liver cancer prevention. *J Hepatol.* (2019) 70:674–83. doi: 10.1016/j.jhep.2018.12.001
24. Jin Z, Wang D, Zhang H, Liang J, Feng X, Zhao J, et al. Incidence trend of five common musculoskeletal disorders from 1990 to 2017 at the global, regional and national level: results from the global burden of disease study 2017. *Ann Rheum Dis.* (2020) 79:1014–22. doi: 10.1136/annrheumdis-2020-217050
25. GBD 2017 Population and Fertility Collaborators. Population and fertility by age and sex for 195 countries and territories, 1950-2017: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet.* (2018) 392:1995–2051. doi: 10.1016/S0140-6736(18)32278-5
26. Charlson FJ, Ferrari AJ, Santomauro DF. Global epidemiology and burden of schizophrenia: findings from the global burden of disease study 2016. *Schizophr Bull.* (2018) 44:1195–203. doi: 10.1093/schbul/sby058
27. Torrone EA, Bertolli J, Li J, Sweeney P, Jeffries IV WL, Ham DC, et al. Increased HIV and primary and secondary syphilis diagnoses among young men–United States, 2004-2008. *J Acquir Immune Deficiency Syndrom.* (1999) 58:328–35. doi: 10.1097/QAI.0b013e31822e1075
28. Nelson R. Congenital syphilis and other STIs rise in the USA. *Lancet Infect Dis.* (2018) 18:1186–7. doi: 10.1016/S1473-3099(18)30618-2
29. Kanelleas A, Stefanaki C, Stefanaki I, Bezrondii G, Paparizos V, Arapaki A, et al. Primary syphilis in HIV-negative patients is on the rise in Greece: epidemiological data for the period 2005-2012 from a tertiary referral centre in Athens, Greece. *J Eur Acad Dermatol Venereol.* (2015) 29:981–4. doi: 10.1111/jdv.12745
30. Tsachouridou O, Skoura L, Christaki E, Kollaras P, Sidiropoulou E, Zebekakis P, et al. Syphilis on the rise: A prolonged syphilis outbreak among HIV-infected patients in Northern Greece. *Germs.* (2016) 6:83–90. doi: 10.11599/germs.2016.1093
31. Takahashi T, Arima Y, Yamagishi T, Nishiki S, Kanai M, Ishikane M, et al. Rapid increase in reports of syphilis associated with men who have sex with women and women who have sex with men, Japan, 2012 to 2016. *Sex Transm Dis.* (2018) 45:139–43. doi: 10.1097/OLQ.0000000000000768
32. Sugishita Y, Yamagishi T, Arima Y, Hori N, Seki N. Increase in Primary and Secondary Syphilis Notifications in Men in Tokyo, 2007-2013. *Jpn J Infect Dis.* (2016) 69:154–7. doi: 10.7883/yoken.JJID.2015.312
33. Mohammed H, Blomquist P, Ogaz D, Duffell S, Furegato M, Checchi M, et al. Dunbar JK.100 years of STIs in the UK: a review of national surveillance data. *Sex Transm Infect.* (2018) 94:553–8. doi: 10.1136/sextrans-2017-053273
34. Schmidt R, Carson PJ, Jansen RJ. Resurgence of Syphilis in the United States: An Assessment of Contributing Factors. *Infect Dis.* (2019) 12:1178633719883282. doi: 10.1177/1178633719883282
35. Tsuboi M, Evans J, Davies EP, Rowley J, Korenromp EL, Clayton T, et al. Prevalence of syphilis among men who have sex with men: a global systematic review and meta-analysis from 2000-20. *The Lancet Global health.* (2021) 9:e1110–e8. doi: 10.1016/S2214-109X(21)00221-7
36. Traeger MW, Schroeder SE, Wright EJ, Hellard ME, Cornelisse VJ, Doyle JS, et al. Effects of pre-exposure prophylaxis for the prevention of human immunodeficiency virus infection on sexual risk behavior in men who have sex with men: a systematic review and meta-analysis. *Clin Infect Dis.* (2018) 67:676–86. doi: 10.1093/cid/ciy182
37. El-Kettani A, Mahiané G, Bennani A. Trends in adult chlamydia and gonorrhea prevalence, incidence and urethral discharge case reporting in morocco over 1995-2015-estimates using the spectrum-sexually transmitted infection model. *Sex Transm Dis.* (2017) 44:557–64. doi: 10.1097/OLQ.0000000000000647
38. Moore MS, Golden MR, Scholes D, Kerani RP. Assessing trends in chlamydia positivity and gonorrhea incidence and their associations with the incidence of pelvic inflammatory disease and ectopic pregnancy in Washington State, 1988-2010. *Sex Transm Dis.* (2016) 43:2–8. doi: 10.1097/OLQ.0000000000000352
39. Badrakh J, Zayasaikhan S, Jagdagsuren D, Enkhbat E, Jadambaa N, Munkhbaatar S, et al. Trends in adult chlamydia and gonorrhoea prevalence, incidence and urethral discharge case reporting in Mongolia from 1995 to 2016 - estimates using the Spectrum-STI model. *WPSAR.* (2017) 8:20–9. doi: 10.5365/wpsar.2017.8.2.007
40. Reekie J, Donovan B, Guy R, Hocking JS, Kaldor JM, Mak DB, et al. Trends in chlamydia and gonorrhoea testing and positivity in Western Australian Aboriginal and non-Aboriginal women 2001-2013: a population-based cohort study. *Sex Health.* (2017) 14:574–80. doi: 10.1071/SH16207
41. Looker KJ, Johnston C, Welton NJ, James C, Vickerman P, Turner KM, et al. The global and regional burden of genital ulcer disease due to herpes simplex virus: a natural history modelling study. *BMJ global health.* (2020) 5:e001875. doi: 10.1136/bmjgh-2019-001875
42. Management of Genital Herpes in Pregnancy: ACOG Practice Bulletinacog Practice Bulletin, Number 220. *Obstetr Gynecol.* (2020) 135:e193–202. doi: 10.1097/AOG.0000000000003840
43. Sénat MV, Anselem O, Picone O, Renesme L, Sananès N, Vauloup-Fellous C, et al. Prevention and management of genital herpes simplex infection during pregnancy and delivery: Guidelines from the French College of Gynaecologists and Obstetricians (CNGOF). *Eur J Obstet Gynecol Reprod Biol.* (2018) 224:93–101. doi: 10.1016/j.ejogrb.2018.03.011
44. Van de Perre P, Segondy M, Foulongne V, Ouedraogo A, Konate I, Huraux JM, et al. Herpes simplex virus and HIV-1: deciphering viral synergy. *Lancet Infect Dis.* (2008) 8:490–7. doi: 10.1016/S1473-3099(08)70181-6
45. Celum C, Wald A, Lingappa JR, Magaret AS, Wang RS, Mugo N, et al. Acyclovir and transmission of HIV-1 from persons infected with HIV-1 and HSV-2. *N Engl J Med.* (2010) 362:427–39. doi: 10.1056/NEJMoa0904849
46. Delany S, Mlaba N, Clayton T, Akpomiemie G, Capovilla A, Legoff J, et al. Impact of aciclovir on genital and plasma HIV-1 RNA in HSV-2/HIV-1 co-infected women: a randomized placebo-controlled trial in South Africa. *AIDS (London, England).* (2009) 23:461–9. doi: 10.1097/QAD.0b013e32831db217
47. Korenromp EL, Zhang W, Zhang X, Ma Y, Jia M, Luo H, et al. The Spectrum-STI Groups model: syphilis prevalence trends across high-risk and lower-risk populations in Yunnan, China. *Sci Rep.* (2020) 10:5472. doi: 10.1038/s41598-020-62208-3
48. Korenromp EL, Ríos C, Apolinar AL, Caicedo S, Cuellar D, Cárdenas I, et al. Prevalence and incidence estimates for syphilis, chlamydia, gonorrhea, and

congenital syphilis in Colombia, 1995–2016. *Rev Panam Salud Publica.* (2018) 42:e118. doi: 10.26633/RPSP.2018.118

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers in Immunology

ORIGINAL RESEARCH
published: 29 March 2022
doi: 10.3389/fimmu.2022.862491

B-Cell Epitope Mapping of TprC and TprD Variants of *Treponema pallidum* Subspecies Informs Vaccine Development for Human Treponematoses

Barbara Molini[1†], Mark C. Fernandez[1†], Charmie Godornes[1], Anastassia Vorobieva[2,3], Sheila A. Lukehart[1,4] and Lorenzo Giacani[1,4]*

[1] Department of Medicine, University of Washington, Seattle, WA, United States, [2] VIB-VUB Center for Structural Biology, VIB, Brussels, Belgium, [3] Structural Biology Brussels, Vrije Universiteit Brussel, Brussels, Belgium, [4] Department of Global Health, University of Washington, Seattle, WA, United States

OPEN ACCESS

Edited by:
Pingyu Zhou,
Tongji University, China

Reviewed by:
Steven J. Norris,
University of Texas Health Science Center at Houston, United States
Yi-Pin Lin,
Wadsworth Center, United States
Feijun Zhao,
University of South China, China

***Correspondence:**
Lorenzo Giacani
giacal@u.washington.edu

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: *26 January 2022*
Accepted: *07 March 2022*
Published: *29 March 2022*

Citation:
Molini B, Fernandez MC, Godornes C, Vorobieva A, Lukehart SA and Giacani L (2022) B-Cell Epitope Mapping of TprC and TprD Variants of Treponema pallidum Subspecies Informs Vaccine Development for Human Treponematoses. Front. Immunol. 13:862491. doi: 10.3389/fimmu.2022.862491

Several recent studies have focused on the identification, functional analysis, and structural characterization of outer membrane proteins (OMPs) of *Treponema pallidum* (*Tp*). The *Tp* species encompasses the highly related *pallidum*, *pertenue*, and *endemicum* subspecies of this pathogen, known to be the causative agents of syphilis, yaws, and bejel, respectively. These studies highlighted the importance of identifying surface-exposed OMP regions and the identification of B-cell epitopes that could be protective and used in vaccine development efforts. We previously reported that the TprC and TprD OMPs of *Tp* are predicted to contain external loops scattered throughout the entire length of the proteins, several of which show a low degree of sequence variability among strains and subspecies. In this study, these models were corroborated using AlphaFold2, a state-of-the-art protein structure modeling software. Here, we identified B-cell epitopes across the full-length TprC and TprD variants using the Geysan pepscan mapping approach with antisera from rabbits infected with syphilis, yaws, and bejel strains and from animals immunized with refolded recombinant TprC proteins from three syphilis strains. Our results show that the humoral response is primarily directed to sequences predicted to be on surface-exposed loops of TprC and TprD proteins, and that the magnitude of the humoral response to individual epitopes differs among animals infected with various syphilis strains and *Tp* subspecies. Rather than exhibiting strain-specificity, antisera showed various degrees of cross-reactivity with variant sequences from other strains. The data support the further exploration of TprC and TprD as vaccine candidates.

Keywords: *Treponema pallidum*, syphilis, Tpr proteins, B-cell epitope mapping, vaccine development

INTRODUCTION

The human treponematoses (syphilis, yaws, and bejel) are caused by a group of highly related pathogens classified as subspecies of the spirochete bacterium *Treponema pallidum* (*Tp*). Classically, the *pallidum* subspecies is said to causes syphilis, while the *pertenue* and *endemicum* subspecies are regarded as the causes of yaws and bejel, respectively (1), although the modes of transmission and the clinical manifestations may overlap among subspecies. These diseases are still a concern for public and global health, as they continue to result in substantial morbidity and mortality worldwide. According to the World Health Organization, the global prevalence of syphilis is ~20 million cases, with an incidence of ~6.3 million new cases every year (2). Although most of these infections occur in low- and middle-income countries, syphilis has resurged also in industrialized nations (3–7). If left untreated, syphilis can progress to affect the cardiovascular and central nervous systems of patients, potentially leading to death (8). Additionally, vertical transmission of syphilis is estimated to account for ~1/3 of stillbirths in sub-Saharan Africa (9, 10). Past public health initiatives to eliminate syphilis and congenital syphilis promoted by the CDC and WHO (11, 12) have significantly aided in reducing syphilis incidence and in generating awareness of this disease, but have not achieved their intended elimination goals. Compared to syphilis, less accurate epidemiological data are available on yaws and bejel (13). While the ongoing yaws elimination campaign in Asia and Africa using mass administration of azithromycin has demonstrated promising results (14), such efforts could be undermined by the spreading of macrolide resistant *Tp* subsp. *pertenue*, as recently demonstrated in Papua New Guinea (15). Foci of bejel have been reported in the last two decades, mostly in the Near East and Sahelian Africa (16–19), and bejel strains have recently reported to be transmitted sexually (20).

The chance of success of current and future control campaigns for all treponematoses would significantly increase if effective vaccines were available (21, 22). The most rational approach to vaccine development for these infections requires a clear understanding of the type of immune response that is protective and the identification of suitable candidate antigens to be tested in a pre-clinical animal model (21, 22). Furthermore, because there is very limited or no cross-immunity between subspecies of *Tp* and only sporadic cross-immunity between syphilis strains (23, 24), the identification of antigenic differences in potential vaccine candidates among subspecies and strains is of pivotal importance, as such differences could be key to devising a broadly protective vaccine (22). There is consensus that vaccine candidates are most likely to be found among these spirochetes' surface-exposed antigens, such as (but not limited to) integral outer membrane proteins (OMPs). Integral *Tp* OMPs will necessarily contain a membrane-embedded β-barrel domain composed of antiparallel β-strands joined together by loops that alternatively protrude toward the extracellular environment or the periplasm (25). Because *Tp* clearance from early lesions is dependent on opsonophagocytosis of *Tp* cells by activated macrophages (26, 27), the identification of surface-exposed epitopes that can be targeted by immunization to induce opsonic antibodies and promote macrophage activation is key to vaccine development. Such tasks, however, have been historically challenging due to the inability to steadily propagate the *Tp* subspecies *in vitro*, which was only recently achieved (28), and also because of the uncommon fragility and limited protein content of these spirochetes' OM (29, 30). These limitations have been partially overcome by the ability to predict *in silico* OMP-encoding genes and the structure of their encoded proteins, enabling investigation using structural and functional experimental approaches (31, 32).

Among *Tp* putative OMPs identified to date, there are several members of the *T. pallidum* repeat (Tpr) family of paralogous proteins, including TprC and TprD (encoded by the *tp0117* and *tp0131* genes in the reference Nichols strain, respectively) (33); these are reported to have OM localization and porin activity (34, 35). These paralogs have been identified as vaccine candidates by past studies in which it was demonstrated that the N-terminal conserved region of these antigens elicited strong antibody and T-cell responses during infection, and immunization with this region attenuates syphilitic lesion development upon infectious challenge (36). In this study, we examine the protein sequence variation in TprC and TprD among *T. pallidum* strains and subspecies, and predict, then confirm, the locations of B cell epitopes using antisera from infected and immunized rabbits. Variant specificity and cross-reactivity are analyzed so that epitopes with broad coverage among strains and subspecies can be identified for future evaluation as vaccine antigens.

RESULTS

Sequence Analysis of TprC and TprD Variants

Although the TprC and TprD proteins are identical in the Nichols, Chicago, and Bal73-1 strains, allelic variants of TprC and TprD exist among syphilis strains and the three *Tp* subspecies (35, 37). Among the treponemal strains used in this study (**Figure 1**), four alleles were found at the *tprD* locus, which include the reference *tprD* allele (found in the syphilis Nichols, Chicago, and Bal73-1 strains), and the *tprD2* allele (found in the syphilis strains MexicoA, Sea81-4, Bal3, and UW249) which encodes the $TprD_2$ protein (35). Also the subsp. *pertenue* SamoaD strain and subsp. *endemicum* IraqB strains harbor a $tprD_2$ allele in the *tprD* locus, but their $TprD_2$ amino acid sequences differ from the subsp. *pallidum* $TprD_2$ sequence due to five amino acid substitutions scattered throughout the length of the protein (**Figure 1**) (35). TprD2 has four unique regions that differentiate it from the reference TprD sequence. These include a large central region of 110 amino acids and three smaller regions toward the COOH-terminal end of the protein (**Figure 1**) (35). As previously reported, the *tprC* locus of MexicoA, Sea81-4, and Bal3 encodes a TprC variant with a limited number of amino acid (aa) changes (15 aa for MexicoA, 9 aa for Sea81-4, and 9 aa for Bal3) compared to the reference TprC found in Nichols, Chicago, and Bal73-1 strains (**Figure 1**) (35). The TprC protein of the *pertenue* and

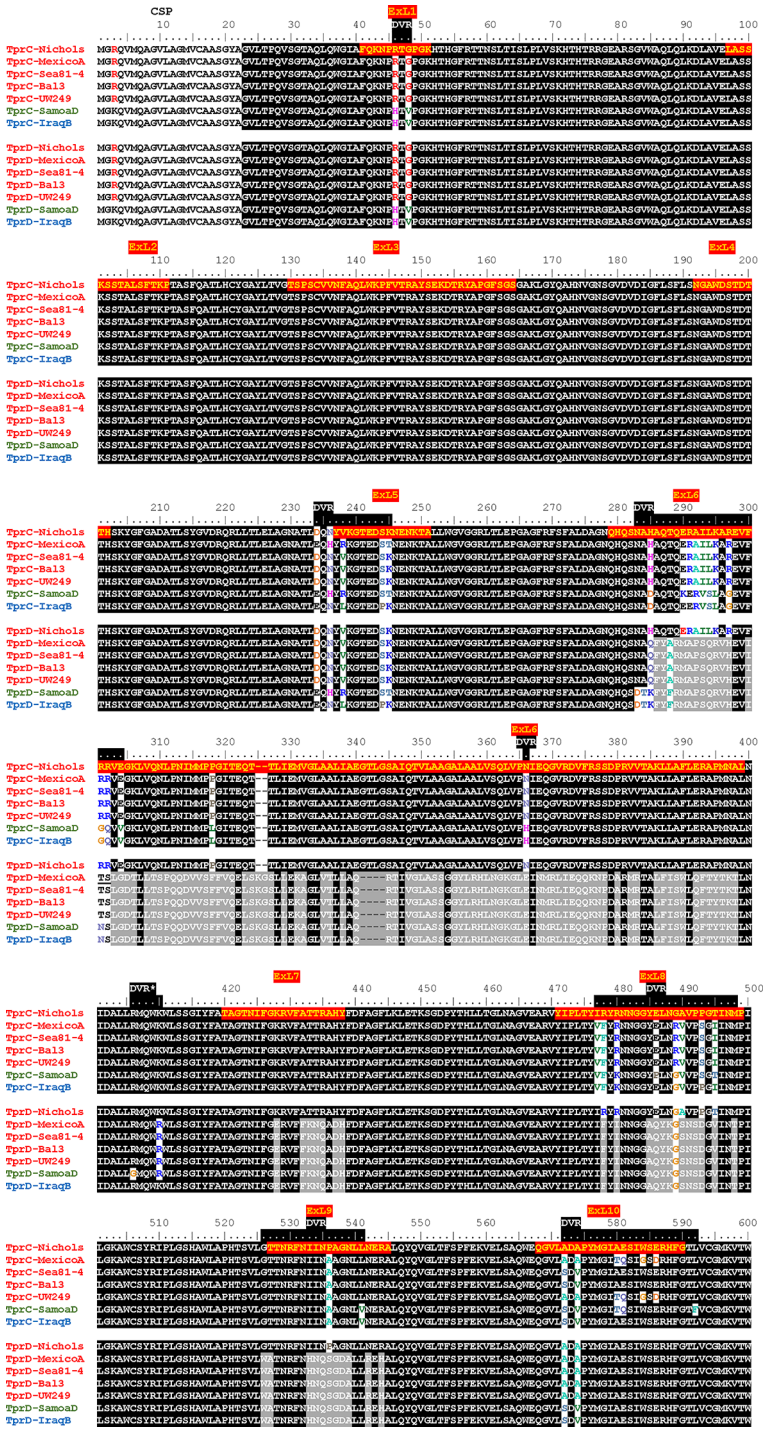


FIGURE 1 | Alignment of amino acid sequences of the TprC and TprD/D_2 variants. *Tp.* subsp. *pallidum* strains (Nichols, MexicoA, Sea81-4, Bal3, and UW249) are indicated in red font on the left of the sequence. The *Tp* subsp. *pertenue* strain (SamoaD) is in green font, and the *Tp* subsp. *endemicum* (IraqB) strain is in blue font. The Chicago and Bal73-1 TprC and TprD sequences (not shown) are identical to the Nichols strain. The MexicoA, Sea8-14, Bal3, UW249, SamoaD, and IraqB strains harbor a $TprD_2$ variant within the *tprD* locus. CSP, predicted cleavable signal peptide; ExL, External Loop. Amino acids encompassing the ExLs predicted by AlphaFold2 are highlighted in red with yellow text only in the top sequence. DVR, Discrete Variable Region. DVRs are highlighted in black along the ruler. *Indicates a DVR found in $TprD_2$ but not TprC and TprD variants.

endemicum strains studied here also shows limited amino acid changes compared to the reference TprC (31 aa for SamoaD and 26 aa for IraqB; **Figure 1**), albeit higher compared to the subsp. *pallidum* strain (35). We previously reported that TprC and TprD/D_2 sequence variation does not occur randomly, but rather is localized in discrete variable regions (DVRs; **Figure 1**) (35). When TprC and TprD variants are compared (with the exclusion of $TprD_2$), seven DVRs are found throughout the protein sequence, while 8 DVRs can be identified within the $TprD_2$ sequences (**Figure 1**). To obtain predictions of TprC and $TprD_2$ structures from their amino acid sequences (**Figure 1**) and map the DVRs on these models, we used the recently developed AlphaFold2 software (https://AlphaFold.ebi.ac.uk/) (38). These new models revealed remarkably similar structures for TprC and $TprD_2$ and identified these proteins as relatively large β-barrel integral OMPs of 20 β-strands connected by ten external loops (ExLs, protruding toward the extracellular milieu), and nine periplasmic loops (**Figure 2**). Elevated structural identity of the transmembrane region was also supported by a 0.75 backbone root-mean-square deviation (RMSD) score (**Figure 2A**). The local model quality, indicated by the Predicted Local Distance Difference Test (pLDDT) value was high in the transmembrane and periplasmic loop regions, and slightly lower in the predicted ExLs, suggesting conformational flexibility (**Figure 2B**). Except for two substitutions (aa 407 and 410 mapping to a periplasmic β-turn), all DVRs localized within a subset of the surface-exposed ExLs (**Figure 2C**). More specifically, DVRs were located in ExL1, ExL5-6 and ExL8-10 of the TprC and $TprD_2$ models; while ExL2-4 harbored conserved loops. ExL6 is also conserved between $TprD_2$ sequences from various isolates, although its shows only 60% of sequence identity to the ExL6 of other TprC and TprD variants (**Figure 1**). DALI software (39) and PDB analyses to identify structurally similar porins (**Table S1**) showed that the highest-scoring structures did not contain the exact number of β-strands predicted by AlphaFold2 for TprC and $TprD_2$ β-barrels, but slightly higher or slightly lower, but well within the models of integral OMPs with no large periplasmic domains. These results suggest that these Tpr proteins belong to a new family of porins not yet represented in the PDB database.

In silico prediction of B-cell epitopes using BepiPred2.0 (http://www.cbs.dtu.dk/services/BepiPred/), IEDB (https://www.iedb.org/), and BCpreds (http://ailab-projects1.ist.psu.edu:8080/bcpred/data.html) (**Tables S2–S5** and **Figure S1**) showed that the putative TprC and TprD ExLs were also enriched in immunogenic epitopes. Therefore, it is possible that the antigenic variability in the ExLs regions has functional significance in immunity to the *T. pallidum* subspecies.

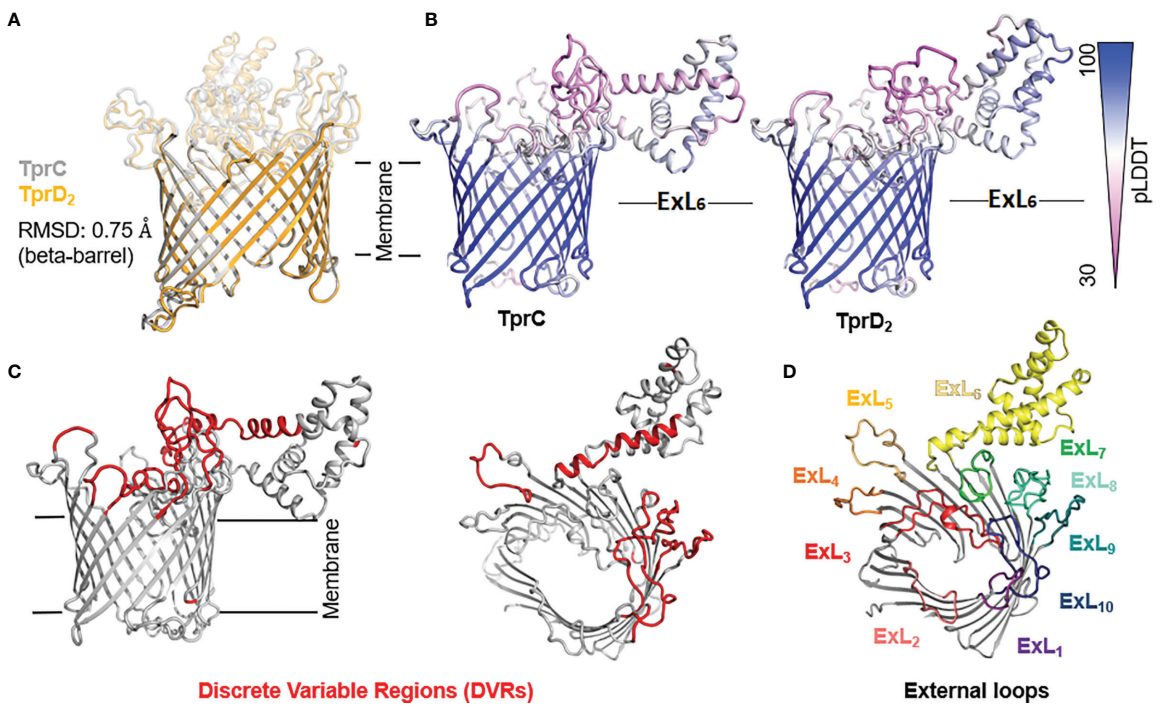


FIGURE 2 | Predicted structures of TprC and $TprD_2$ using AlphaFold2. TprC and $TprD_2$ sequences were obtained from the Sea81-4 genome (NZ_CP003679.1/CP003679.1). **(A)** Both TprC and $TprD_2$ are predicted to fold into similar 20-strands beta-barrels structures. The predicted structures are nearly identical in the transmembrane region (backbone RMSD = 0.75). **(B)** Compared to the scaffolding, slightly lower confidence in the structural models for TprC and $TprD_2$ are seen in the external loop regions based on the per-residue model confidence (pLDDT) mapped on the predicted TprC and $TprD_2$ structures, suggesting structural flexibility for these regions. Noteworthy, however, is that the largest loop (ExL7) is predicted to have a similar helical-rich structure in both TprC and $TprD_2$. Full pLDDT analysis for TprC and $TprD_2$ is reported in **Figure S3**. **(C)** The DVRs (in red on the TprC model structure) identified by aligning TprC and $TprD_2$ sequences of different strains (**Figure 1**) mostly localize in the predicted extracellular loops. **(D)** AlphaFold identified ten surface-exposed external loops (ExLs). Discrete variable regions (DVRs) based on **Figure 1** alignments are predicted to be in ExL1, ExL5-6 and ExL8-10.

To validate the B-cell epitope prediction and evaluate the cross-reactivity of these epitopes across species and strains, we performed experimental B-cell epitope mapping of the TprC, and TprD/D_2 proteins with a Geysan pepscan approach based on overlapping synthetic peptides (40) using sera from animals infected with *Tp* subsp. *pallidum*, *Tp* subsp. *pertenue*, and *Tp* subsp. *endemicum* strains. Furthermore, we compared antibody reactivity in sera from infected rabbits with that of sera from rabbits immunized with a subset of full-length refolded recombinant TprC proteins.

Humoral Responses to Homologous TprC and TprD/D_2 Peptides in Experimentally Infected Rabbits

Groups of three laboratory rabbits were infected intratesticularly (IT) with one of seven syphilis strains (Nichols, Chicago, Bal73-1, MexicoA, Sea81-4, Bal3, and UW249), one yaws strain (SamoaD), and one bejel strain (IraqB). From these animals, serum samples were obtained at day 30, 60, and 90 post-infection. Pooled sera from animals in each infection group/time point were tested in ELISA to assess reactivity to homologous overlapping synthetic peptides (20-mers overlapping by 10 amino acids) representing the TprC and TprD/D_2 variants previously identified in each strain. The full list of synthetic peptides used in this study, with amino acids encompassing predicted ExLs highlighted in red with yellow text, and percentage amino acid homology among peptides across strains is shown in **Table S6**. Peptide nomenclature is explained in **Table S6** footnote. Cumulative absorbance data from the three timepoints (sum of the mean absorbance values for day 30, 60, 90 values for each infected rabbit group) are reported in **Figures 3A–C**. Epitope mapping studies of the NH_2-terminal portion of the protein resulted in the identification of six highly reactive peptide regions (**Figure 3A**) representing sequences shared by all TprC and TprD genes in the studied subspecies *pallidum* strains: C1-C3, C6, C13-C14, C18, C20, and C25-C29. Based on AlphaFold2 structural predictions, 9 of these 13 peptides had at least 7 amino acids mapping to the predicted external loops of the protein, while only four reside in predicted transmembrane scaffolding and periplasmic loop regions (C1, C6, C20 and C25; **Figure 1A** and **Table S7**). It is noteworthy that all three B cell epitope prediction programs uniformly predicted all six of the experimentally determined epitope-containing regions of the NH_2-terminal portion of the subspecies *pallidum* TprC and D proteins (**Tables S2–S5** and **Figure S1**).

Several epitopes were also identified in the COOH-terminal region of these proteins, and corresponded to peptides the same regions in *pallidum* and non-*pallidum* subspecies: C46 and C47 homologs from Nichols (**Figure 3A** and **Table S7**), SamoaD (S-C46, S-C47; **Figure 3B** and **Table S7**) and IraqB (I-C46, I-C47; **Figure 3B** and **Table S7**), C51 homologs from Nichols (N-C51), SamoaD and iraqB (S/I-C51) (**Figures 3A, B** and **Table S7**); and C53-C55 homologs from Nichols, Bal3/Sea81-4 (**Figure 3A** and **Table S7**), and IraqB (**Figure 3B** and **Table S7**). Similarly, the C43, and D45-D47 (ExL8) peptides, mapping to the TprD$_2$ COOH-terminus were found to contain B-cell epitope(s) (**Figure 3C** and **Table S7**). Additional TprD$_2$ peptides found to be reactive were D33-D35 (ExL6), I-C39, D40-41 (ExL7), C49, and D51 (ExL9). In our 3D models of these proteins, all the reactive peptides in the COOH-terminus fall within predicted ExLs (**Tables S6–S7** and **Figure 1**), except for C43, most of I-C39 (75%), C49, and C53, which are predicted transmembrane scaffolding sequences. Of these "scaffold epitopes", only one (C43) was predicted by a B-cell prediction program (**Tables S2–S5** and **Figure S1**).

The percentage of immune sera that showed reactivity to many of the peptides was variable. For example, peptides C3, and C13 were recognized by rabbits infected with 28% of the *Tp* subsp. *pallidum* strains; peptides C1, and C27 were recognized by rabbits infected with 42% of the strains; peptides C14 and C28 were recognized by rabbits infected with 57% of the strains; C6 was recognized by rabbits infected with 71% of the strains; and peptides C2 and C18 were recognized by rabbits infected with 85% of the *Tp* subsp. *pallidum* strains (**Figure 1**). Overall, based upon the AlphaFold2 models, these results show that humoral reactivity elicited to these Tpr antigens during experimental infection is directed primarily to predicted surface-exposed regions of the TprC/D and TprD$_2$ proteins.

Reactivity to Non-Homologous TprC and TprD$_2$ Peptides

Epitope mapping using short peptides based on the TprC and TprD/D_2 sequences from multiple *Tp* strains and sera from infected animals also allowed us to investigate cross-reactivity to non-homologous peptides to determine the fine specificity of the antibody response to these antigens. Such analyses focused on peptides mapping to the proteins` COOH-terminal regions, due to the higher sequence variability in this region, compared to the more conserved NH_2-terminal region (**Figure 1**). Major variable regions include peptides C46 - C47 (mapping to the predicted ExL8), C51 (ExL9), and C55 (ExL10) (**Figure 1** and **Tables S6–S7**). Four distinct variants of each of the C46, C47, and C55 peptides, and three variants of C51, representing all sequences found in the strains studied here, were tested against all nine pools of immune sera obtained at day-90 post-experimental infection.

As shown in **Figures 4A–D**, very few sera were reactive only to their homologous peptide. For example, the Bal73-1and SamoaD antisera were primarily reactive only to their own C46 sequences (**Figure 4A**), although the Bal73-1 antiserum showed a very modest reactivity to the IraqB peptide variant (**Figure 4A**). When reactivity against the C47 peptide was analyzed, the Chicago, Bal73-1 sera reacted only to their own peptide, the IraqB antiserum reacted to all variants but the SamoaD peptide, and the Sea81-4 serum only saw the Nichols C46 variant, but not its homologous peptide. (**Figure 4B**). Only Chicago, Bal73-1, and SamoaD sera showed complete strain-specificity for the C51 peptides (**Figure 4C**), while none of the sera reactive to C55 showed complete strain-specificity (**Figure 4D**). When tested against TprD2 peptides, most antisera did not show any reactivity. There were, however, two exceptions, as the Chicago sera cumulatively showed reactivity to the D34 and D47 peptides,

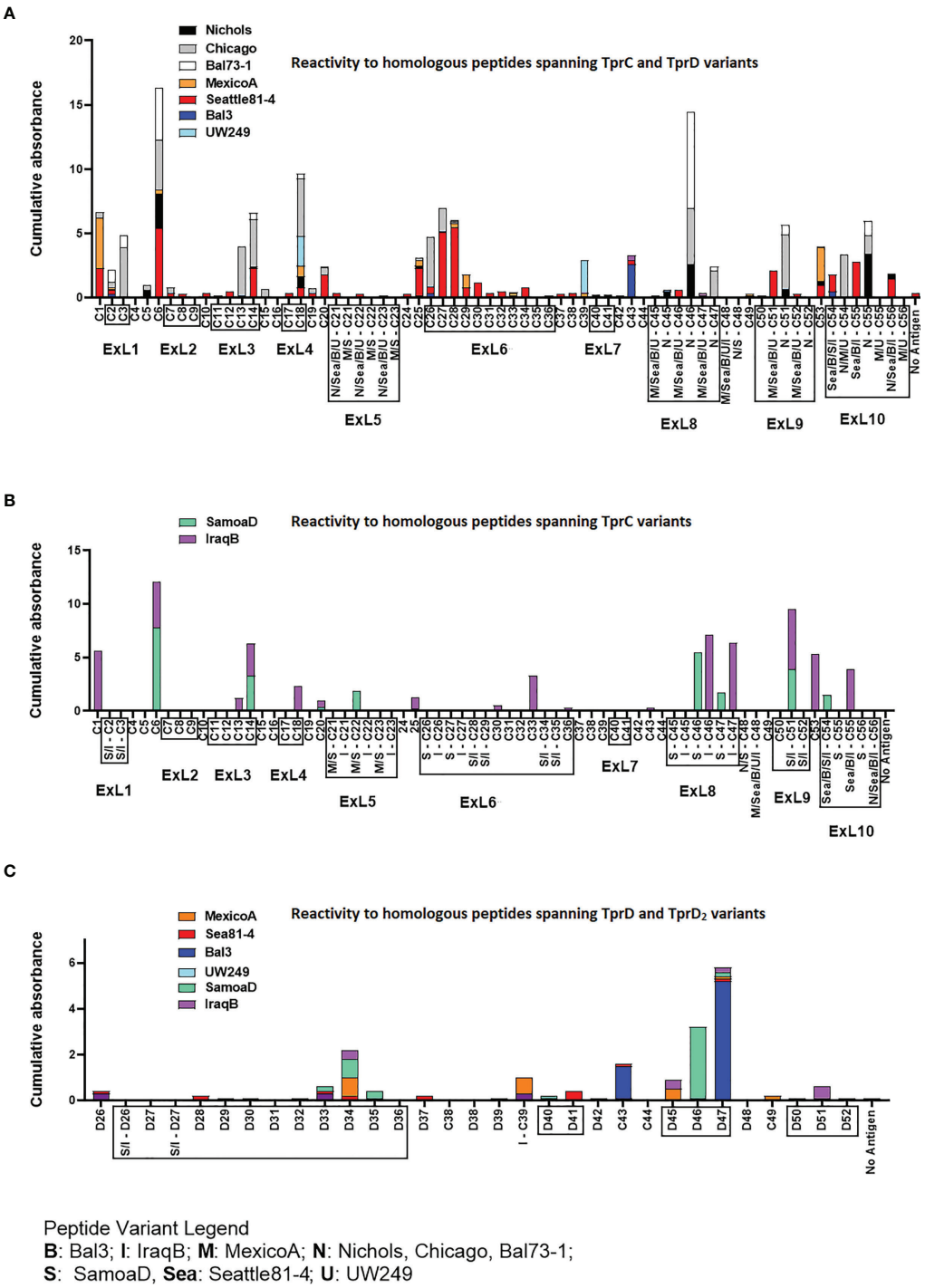


FIGURE 3 | Reactivity of sera from experimentally infected animals to homologous peptides representing the TprC, TprD and $TprD_2$ variants. **(A)** Reactivity to homologous peptides spanning TprC and TprD proteins of sera from rabbits infected with *Tp* subsp. *pallidum* (Nichols, Chicago, Bal73-1, MexicoA, Sea81-4, Bal3, and UW249B) collected at day 30, 60, and 90 post-infection. Nichols, Chicago, and Bal73-1 sequences are identical. **(B)** Reactivity to homologous peptides spanning TprC variants of immune sera from groups of rabbits infected with *Tp* subsp. *pertenue* (SamoaD) or *Tp* subsp. *endemicum* (IraqB) strains collected at day 30, 60, and 90 post-infection. **(C)** Reactivity to homologous peptides spanning TprD and $TprD_2$ variants of sera collected at day 30, 60, and 90 post-infection from all $TprD_2$-containing *Tp* subspecies and strains studied here. Cumulative Absorbance values are the sum of the mean OD values obtained from all animals in the infection group at all three time points. Boxed peptides contain at least seven amino acids (35% of the peptide length) belonging to a predicted ExL. Strain names on x axis are abbreviated as follows: N, Nichols; M, MexicoA; Sea, Sea81-4; B, Bal3; U, UW249; S, SamoaD; I, IraqB.

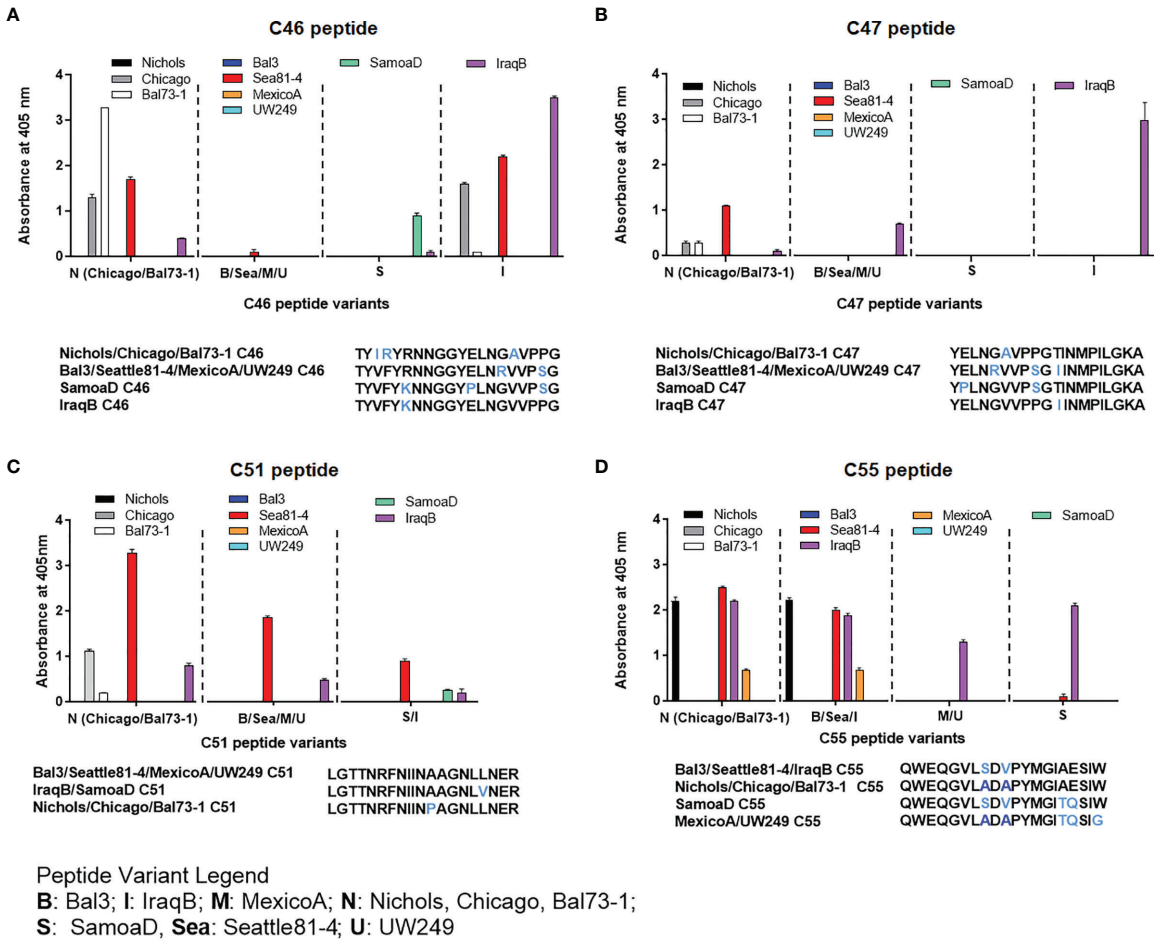


FIGURE 4 | Reactivity of sera from experimentally infected animals to homologous and non-homologous peptides C46, C47, C51, and C55. Humoral reactivity of day-90 sera from experimentally infected animals to homologous and non-homologous TprC peptides. **(A-D)** reactivity to C46, C47, C51, and C55 variants. Strain names on x axes are abbreviated as follows: N, Nichols; M, MexicoA; Sea, Sea81-4; B, Bal3; U, UW249; S, SamoaD; I, IraqB.

with OD values of 3.6 and 6.6, respectively. However, only the D47 peptide was consistently recognized at all time points, while D34 was recognized only at day 60. Overall, these data indicate that cross reactivity is possible, and perhaps suggest that immunization with a given sequence might generate cross-reactive antibodies able to overcome the obstacle of sequence diversity among TprC epitopes, a feature that is desirable in vaccine development as they may be broadly opsonic or neutralizing.

Humoral Response to TprC Peptides Following Rabbit Immunization With Full-Length Refolded Antigens

Refolded antigens, analyzed using circular dichroism (CD), were found to have a β-pleated sheet component of about 48% for all three antigen variants. Random coil was also found to be 48% of the protein structure, while only 4% was identified as alpha helices. Epitopes recognized following immunization with any of three recombinant full-length TprC variants from *Tp* subsp. *pallidum* strains (Nichols/Chicago/Bal73-1, Sea81-4/Bal3, and MexicoA) were also identified to evaluate differences with infection-induced antibody responses. Results showed that sera from animals immunized with the Nichols TprC sequence were highly reactive to peptides C1-C3, C6 and C47, and moderately reactive to peptides C5, C9, C16-18, C28, C32, C34-C35, C53 and C55 (**Figure 5A**). Of these 16 peptides, six mapped almost exclusively to putative surface-exposed loop regions (C28, C32, C34, C35, C47, and C55), five (C1, C5-C6, C16, and C53) mapped to predicted transmembrane scaffolding sequences, while five peptides (C2-C3, C9 and C17-C18) contained both surface-exposed loops and scaffold regions. Sequences of these peptides and location in the predicted protein models are reported in **Table S8**. When tested against non-homologous peptides (**Figure 5B**), the Nichols TprC-immunized sera strongly recognized the SamoaD/IraqB C2-C3 variants, and all three heterologous C47 variants (SamoaD, Iraq B, and Sea81-4), while modest reactivity was seen towards the MexicoA/UW249 C55 peptide variant, the SamoaD/IraqB C34, and both C26

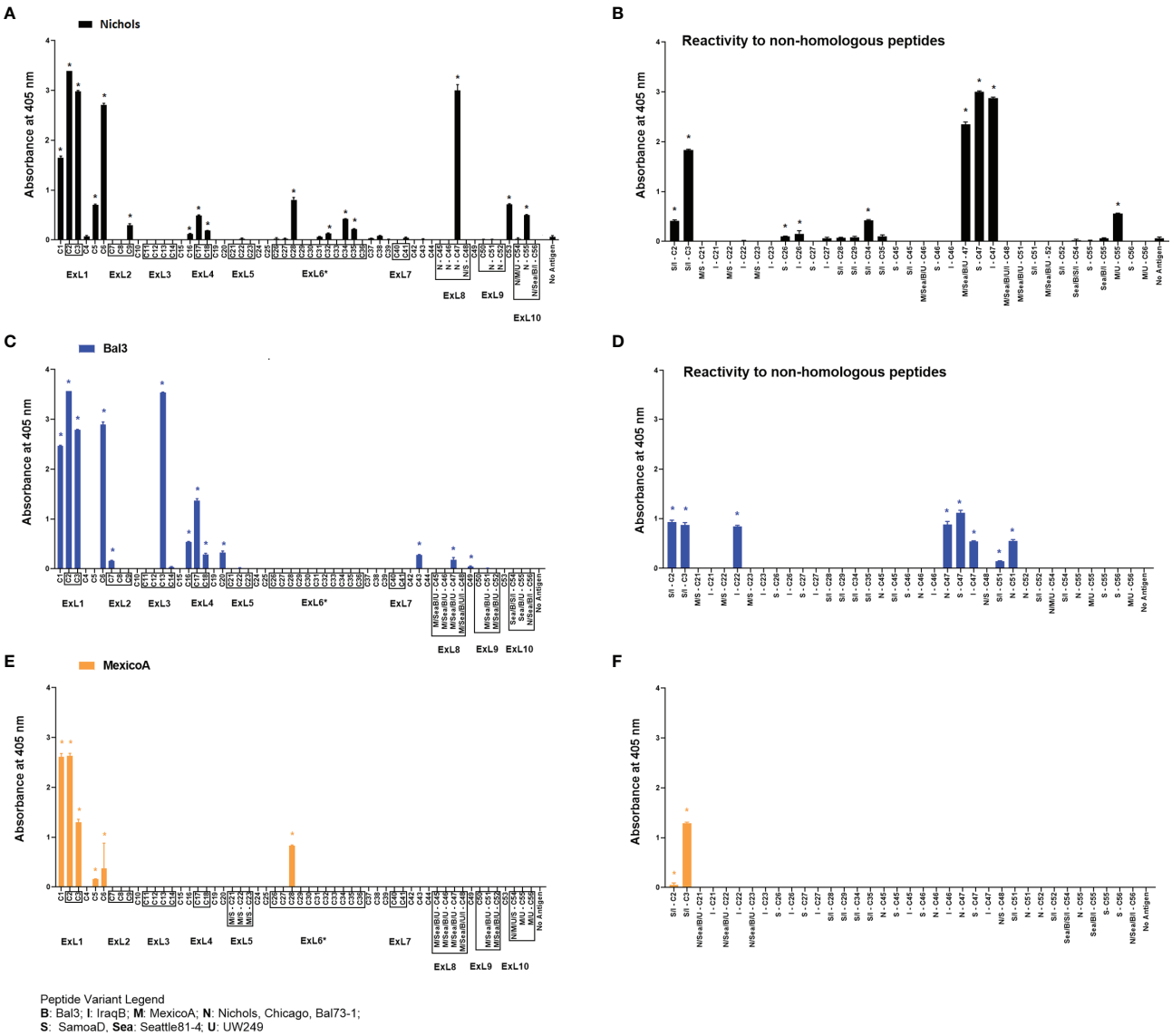


FIGURE 5 | Humoral reactivity to TprC peptides following immunization with refolded recombinant full-length TprC antigens. Reactivity to TprC homologous (left panels) and non-homologous peptides (right panels) in sera from rabbits immunized with Nichols **(A, B)**, Bal3 **(C, D)**, and Mexico A **(E, F)** variants of TprC. Asterisk (*) indicates significant reactivity compared to no antigen control. Peptides encompassing sequences predicted to be within ExLs are boxed. Peptide sequence and homology among strains are reported in **Table S6**. Strain names on x axes are abbreviated as follows: N, Nichols; M, MexicoA; Sea, Sea81-4; B, Bal3; U, UW249; S, SamoaD; I, IraqB.

variants from SamoaD and IraqB (**Figure 5B**). Immunization with the Bal3 variant of TprC elicited high reactivity to peptides C1-3, C6, and C13, and moderate reactivity to peptides C7, C16-18, C20, C43, C47, and C49 (**Figure 5C** and **Table S8**). Of these thirteen peptides, six (C2-C3, C13, C17-C18, and C47) mapped predominantly to ExLs, and seven (C1, C6, C7, C16, C20, C43, and C49) predominantly to the protein transmembrane scaffolding (**Table S8**). Cross-reactivity to non-homologous peptides was seen predominantly to the SamoaD/IraqB C2 and C3, IraqB C22, and all variants of C47 and C51 (**Figure 5D**). Antisera from rabbits immunized with the MexicoA TprC variants primarily recognized homologous peptides C1-3 and, secondarily, C5, C6, and C28 (**Figure 5E**). Of these six, one peptide mapped to the predicted ExL6 (C28), three mapped only to the transmembrane scaffolding (C1, C5-C6) and two mapped to a peptide predicted to contain portions of both (C2-C3) (**Table S8**). Cross-reactivity to the non-homologous SamoaD/IraqB C2 and C3 was also detected (**Figure 5F**). Overall, these data show that, as seen in infection-induced antibody responses, the humoral response following immunization with full-length TprC variants is mainly elicited by predicted surface-exposed sequences, rather than sequences mapping to the β-barrel

transmembrane scaffolding, and that cross-reactivity to non-homologous peptides is possible.

A side-by-side comparison of the infection- vs. immunization-induced humoral response to peptides is shown in **Figure 6**. For this comparison, the mean value of the cumulative reactivity seen in sera at day 30, 60, and 90 sera post-experimental infection is shown for each peptide. Sera from immunized animals were obtained three weeks after the last immunization. All sera were tested at the same dilution. In general, immunization-induced reactivity to most peptides appeared to be higher than that elicited by experimental infection; specific examples include C1-C3, C5, C9, C16-C17, C28, C32, C34-C35, N-C47, and C53 peptides (**Figure 6A**). For Nichols-clade *T. pallidum* strains (**Figure 6A**), which contain identical *tprC* and *tprD* loci, this was most noticeable for epitopes located in the NH_2- and COOH-terminal regions of the protein. In contrast, infection-induced antibody responses to epitopes in the central part of the protein were comparable to or higher than those induced by immunization. For TprD2-containing subsp. *pallidum* strains (**Figures 6B, C**), immunization-induced responses were limited to the NH_2-terminal portion of the protein (including ExL1-3) and virtually no immunization-

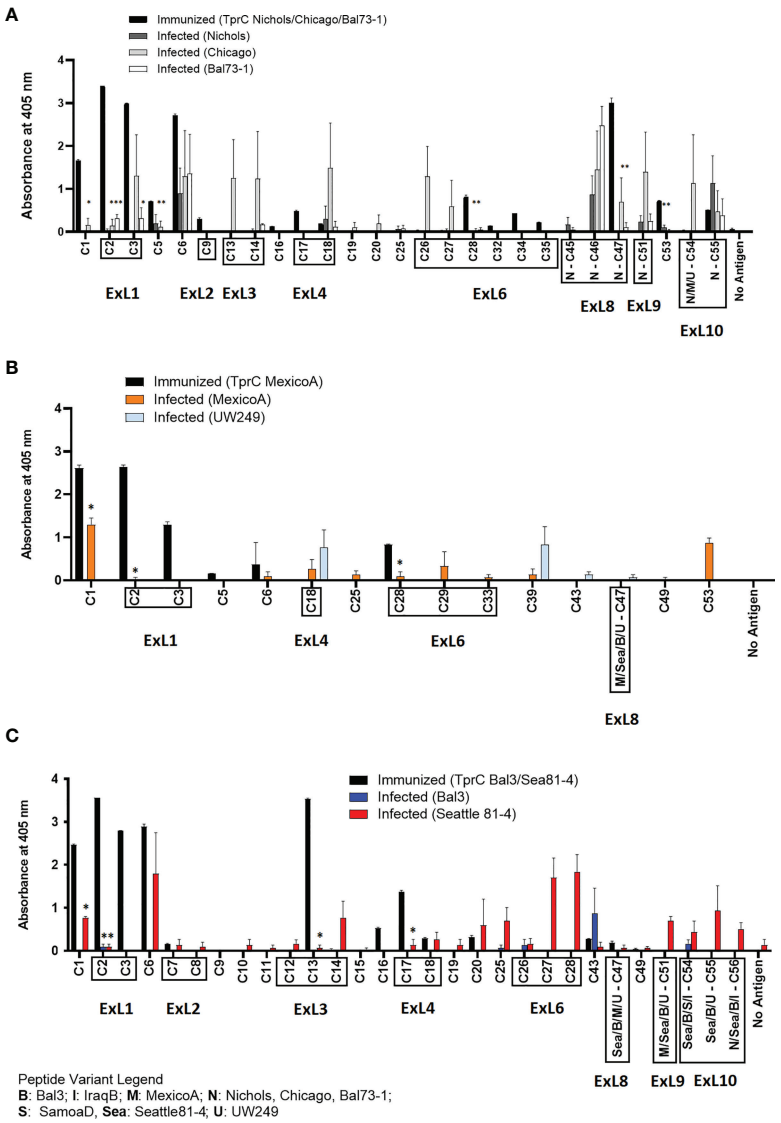


FIGURE 6 | Comparison of reactivity of sera from infected animals vs. immunized animals. **(A-C)** Reactivity to peptides following immunization with TprC variants compared to experimental infection. Data shown are means +/- SEM of 3 rabbits per group: 3 weeks post final boost (immunized) and mean +/- SEM of values for days 30, 60, 90 post-infection (infected). Asterisk (*) indicates a significant difference in reactivity compared to the reactivity value following immunization. Peptides encompassing sequences predicted to be within ExLs are boxed. Strain names on x axes are abbreviated as follows: N, Nichols; M, MexicoA; Sea, Sea81-4; B, Bal3; U, UW249; S, SamoaD; I, IraqB.

induced antibodies were detected for epitopes in the central and COOH-terminal regions, although these were recognized by infection-induced responses. Overall, these data support that, in most cases, immunization elicits a higher reactivity to TprC B-cell epitopes compared to experimental infection, particularly for those epitopes located in the NH_2-terminal portion of the protein. These data support the preferential use of the amino portion of TprC, which contains multiple conserved ExLs, for vaccine studies.

DISCUSSION

The continuing prevalence of syphilis, in the face of highly effective therapy and active control programs, highlights the need for a protective vaccine. The development of such a vaccine calls for a deeper understanding of the mechanisms of protective immunity and the antigens and adjuvants that induce protection. Our laboratories have been examining these issues for many years (22, 31, 36, 41–48). Much of that work has focused on the Tpr antigens of *T. pallidum*. In this current study, B-cell epitope mapping studies of the TprC/D and $TprD_2$ proteins of *Tp* reveal that antibodies arising during experimental infection recognize sequences predicted, using state-of-the-art modeling systems, to fall largely in the proteins' surface-exposed loops. Because opsonic antibodies are required for efficient ingestion and killing of *T. pallidum* by macrophages, surface-exposed epitopes are attractive targets as vaccine candidate antigens.

A broadly protective vaccine would need to be effective against most strains of *T. pallidum*, optimally including the agents of syphilis as well as the endemic treponematoses yaws and bejel. Because some of the external loops of Tpr C/D and $TprD_2$ demonstrate sequence heterogeneity among strains and subspecies of *T. pallidum*, we expected that these epitopes might be strain-specific, similar to the specificity demonstrated for the variable regions of TprK (43, 45, 49). For this reason, we included seven strains of *Tp* subsp. *pallidum* as well as strains from the subspecies *pertenue* and *endemicum* in our work. Unexpectedly, we saw cross reactivity of antibodies toward the variant peptides (**Figure 4**). These findings support the use of TprC/D as at least one component of broadly effective candidate vaccine. It was intriguing that in two instances (Sea81-4 serum for C47; and MexicoA serum for C55) antisera only recognized non-homologous peptides (**Figures 4B, D**). In the case of the Sea81-4 serum, reactivity was detected at day 30 post-infection, but not the at later time points. For the MexicoA serum, one could hypothesize that masking of key epitope residues occurred for the non-recognized homologous peptide during absorption to the ELISA plate which, in turn, could have reduced assay sensitivity, as discussed for peptide arrays by Cretich et al. (50).

The AlphaFold2 structural predictions for TprC/D and $TprD_2$, as well as our CD analyses of purified refolded recombinant TprC variants, support our model (35) that these Tprs are membrane-localized 20-stranded β-barrel proteins containing numerous surface-exposed loops. Very similar models for TprC were previously obtained using I-TASSER (51) (https://zhanggroup.org/I-TASSER/) (35). Interestingly, when AlphaFold2 and I-TASSER results are compared, the only difference is that I-TASSER splits ExL6 (**Figure 1**) into two external loops separated by a β-hairpin, so that I-TASSER predictions harbor 11 external loops instead of 10. AlphaFold2, on the contrary, predicts a significantly larger ExL6, mapping approximately to the proteins' central domains. AlphaFold2 is the new standard for *ab-initio* structural prediction, and in the 2020 Critical Assessment of protein Structure Prediction (CASP) global challenge, it outperformed any other structure prediction algorithm, including I-TASSER (https://predictioncenter.org/casp14/zscores_final.cgi). Furthermore, in a recent preprint (52), AlphaFold2 was shown to work well on structural prediction for membrane proteins, although the exercise focused mostly on alpha-helical membrane proteins, and additional analyses are necessary to establish the same benchmark for β-barrel proteins.

In previous work by Anand et al. (34, 53) significantly different models for the TprC/D proteins were reported, compared to those provided here. These models, however, are not supported by AlphaFold2, which finds the structure of all Subfamily I and Subfamily II Tpr family members to be very similar to the structures for TprC/D and $TprD_2$ in **Figure 2A**. Although there is not unanimous agreement on the structure of these antigens within our scientific community, our epitope mapping data support our AlphaFold2 models, predicting a predominantly β-barrel structure for TprC and TprD/D2 (34, 53). Further studies and integration of all the structural, functional, and immunological data are needed to establish a consensus on the structure of these antigens until crystallographic (or equally reliable) data become available.

This study also provides evidence that infection with different strains might lead to differences in the breadth and intensity of the humoral response against the same epitope, as reported previously for responses to longer portions of the Tpr proteins (54). It is the case, for example, of rabbits infected with the Sea81-4 strain of *Tp* that overall recognize more TprC/D peptides compared to other *Tp* subsp. *pallidum* strains. The biological basis for these differences is unclear at this time, in part due to the limitations of our understanding of *Tp* biology and syphilis pathogenesis. As the technical gap in the approaches to study this difficult pathogen narrows, and genomics, proteomics, and transcriptomics data populate public repositories, more light will be shed on the causes of differential reactivity. Overall, however, it is plausible to postulate that enhanced serological reactivity might be due to an overall increased expression of the target antigen in each strain. This hypothesis is supported by previous work where we showed the *tprC* mRNA level was higher in the Sea81-4 strain compared to other *Tp* subsp. *pallidum* strains (Nichols, Chicago, Bal73-1) used in this study (55).

Our studies further demonstrated that epitopes in TprC/D and $TprD_2$ are nearly-uniformly distributed across the length of the protein, even though the most reactive peptide epitopes are in the NH_2- and COOH-terminal regions. Previously published (36) experiments have shown that both of these regions in the Nichols TprC protein contain protective epitopes, as

immunization with these antigen fragments significantly attenuated lesion development upon infectious challenge (36), and polyclonal antisera elicited by immunization with these portions facilitated treponemal ingestion by macrophages in opsonophagocytosis assays compared to normal rabbit sera (36). Further work, however, will be necessary to identify which specific surface-exposed sequences provide targets for opsonic antibodies, which may not coincide with sero-dominant epitopes, as the pathogen gains an obvious advantage by exposing to the immune system epitopes with little or no protective value. Assuming that differentially recognized peptides are located on the antigens' protective epitopes, one could hypothesize that protective epitopes are immunologically sub-dominant during natural infection, which, *per se* could represent an additional strategy the pathogen uses to survive in the host despite the immune response that naturally develops to these antigens. If this was found to be the case, effort would need to be put into outflanking and overcoming immunodominance to target subdominant protective epitopes. Upon immunization, the immunodominance hierarchy is established at the germinal center, where B cells compete for the antigen through based on binding affinity, and subsequently undergo clonal expansion to become plasma cells or memory B cells (56). Controlling this process to drive antibody responses to increase recognition of subdominant protective epitopes would therefore be of primary importance. A possible strategy, referred to as germline targeting, relies upon the activation and expansion of rare but specific B cell lineages in naïve individuals (57, 58). For patients that are no longer naïve due to natural exposure to the pathogen, however, the same outcome would need to be achieved by manipulating established B cell immunodominance hierarchies and remodel antibody responses toward more desired targets (57, 58).

Protective B-cell epitopes (contrary to T-cell epitopes) are often conformational, and even when a significant portion of an epitope appears to be a short linear peptide, as in our study, it does not necessarily mean that the peptide represents the full epitope or, if it does, that the sequence will not require a certain conformation to elicit optimal bioactivity. For this reason, in the immunization studies performed in this study, we used CD-confirmed refolded recombinant antigens. The immunization-induced antibodies generally identified the same epitopes seen in infection, supporting the role of refolding in mimicking native structure, but immunization also resulted in recognition of a broader range of epitopes than seen during infection, including transmembrane scaffolding regions. This is likely because the scaffold regions are not shielded by the outer membrane in an immunization setting and are thus more easily processed for recognition. Thus, the design of vaccine immunogens is critical. Possible approaches vary from placing epitopes within chimeric antigens that could work as scaffold or, alternatively, using portions of the protein containing protective epitopes as structural elements of the antigen, or even using single β-hairpins instead of the full-length antigens. The work reported here represents an important step in evaluating TprC/D and $TprD_2$ epitopes as part of the process that will lead to an effective vaccine for syphilis.

MATERIALS AND METHODS

Strain Propagation and Experimental Infection

Outbred adult male New Zealand White rabbits ranging from 3.0-4.0 Kg were obtained from R&R Rabbitry (Stanwood, WA). Prior to entry into the study, serum from each animal was tested with both a treponemal (FTA-ABS) and a non-treponemal (VDRL; BD, Franklin Lakes, NJ) test to rule out infection with the rabbit syphilis agent *Treponema paraluiscuniculi*. Only rabbits seronegative in both tests were used for either propagation or experimental infection for sample collection. *Tp s*trains were propagated by intratesticular (IT) inoculation and harvested at peak orchitis as previously described (60). For experimental infections, groups of three rabbits were infected IT with a total of $5x10^7$ *Tp* cells per testis. In total, nine *Tp* isolates (one isolate per rabbit group) were used: seven *Tp* subsp. *pallidum* isolates (Nichols, Chicago, Bal73-1, Sea81-4, Bal3, MexicoA, and UW249), one *Tp* subsp. *endemicum* (IraqB) and one *Tp* subsp. *pertenue* (SamoaD) (**Table S9**). Briefly, on the day of infection bacteria were extracted from rabbit testes in sterile saline containing 10% normal rabbit serum (NRS), and testicular extract was collected in sterile 15-ml tubes. Extracts were centrifuged twice at 1,000 rpm (180 x g) for 10 minutes in an Eppendorf 5810R centrifuge (Eppendorf, Hauppauge, NY) to remove gross rabbit cellular debris. Treponemes were enumerated under a dark-field microscope (DFM) and percentage of motile organisms was recorded. Extracts were then diluted in serum-saline to the desired concentration ($5x10^7$/ml). Following IT injection, treponemal motility was assessed again to ensure that the time elapsed before injection into the new host did not affect pathogen viability. After IT inoculation, establishment of infection was assessed by monitoring development of orchitis during the following three weeks as well as by performing FTA-ABS and VDRL tests on sera collected at day 30 post-inoculation. Immune sera were collected from the animals at day 30, 60, and 90 post-infection. Animals were then euthanized. Extracted sera were heat-inactivated at 56°C for 30 min and stored at -20°C until use for ELISAs.

Amplification and Cloning of Full-Length *tprC* Gene Variants for Expression of Recombinant Antigens

Sequences for the *tprC* gene of *Tp* isolates (Nichols, Sea81-4, and MexicoA) were previously cloned (35). For expression, the *tprC* sequences were sub-cloned into the pET23b+ vector (Life Technologies) between BamHI and HindIII using the primers C-S (5'-cgggatccgatgg gcgtactcactccgca) and C-As (5'-gcaagcttccat gtcactttcattccac). For sub-cloning, the *tprC* ORF was amplified in a 100-µl final volume using 0.4 units of GoTaq polymerase (Promega) with approximately 10 ng of DNA template. $MgCl_2$ and dNTP final concentrations were 1.5 mM and 200 µM, respectively. Initial denaturation and final extension (72°C) were for 10 min each. Denaturation (94°C), annealing (60°C), and extension (72°C) were carried out for 1 min each for a total of 35 cycles. Amplicons were purified, digested, and ligated into the pET23b+ vector. As a result of cloning into pET23b+, 28 additional amino acids were added to the TprC ORFs (14 NH_2-terminal and

14 COOH-terminal amino acids), including the COOH-terminal 6×His tag for affinity purification. Ligation products were used to transform OneShot TOP10 chemically competent *E. coli* cells (Life Technologies) according to the provided protocol. Transformations were plated on LB-Ampicillin (100 μg/ml) agar plates for selection. For each cloning reaction, individual colonies were screened for the presence of insert-containing plasmids using primers annealing upstream and downstream of the pET23b+ vector poly-linker (T7 promoter and terminator primers). Positive plasmids were extracted from overnight liquid cultures obtained from replica colonies by using the Plasmid Mini kit (Qiagen, Germantown, MD), and two to five clones for each strain were sequenced to ensure sequence fidelity to the previously cloned templates (35). For expression of recombinant antigens, a suitable clone for each *tprC* gene variant was used to transform *E. coli* Rosetta (DE3) competent cells (Life Technologies).

Expression, Purification and Refolding of Recombinant Proteins

E. coli cells were grown overnight in LB media supplemented with ampicillin (100 μg/ml). The following day, multiple flasks containing 200 ml of auto-inducing media (61), were inoculated with 20 ml of overnight culture in a 2-liter baffled flask and grown at room temperature for 72 h at 175 rpm in a shaking incubator. Expression of recombinant antigens in induced and un-induced controls was assessed by immunoblot using a monoclonal anti-poly-histidine antibody (Millipore-Sigma, diluted 1:2000) after SDS-PAGE. Prior to purification, presence of the recombinant protein in the soluble and insoluble cellular fractions was evaluated by SDS-PAGE and immunoblot. Recombinant TprC purification was carried on under denaturing conditions. Briefly, *E. coli* cell pellets were resuspended in 5 ml/g of dry culture weight of binding buffer (50 mM NaH_2PO_4, 10 mM imidazole, pH 8.0) w/o denaturing agent, and the suspension was sonicated in ice with 100 pulses of 6 s each, with each pulse being separated by 10-s intervals. Insoluble components (containing the desired products) were precipitated by centrifugation and resuspended in 5 ml/g of culture weight of binding buffer (50 mM NaH_2PO_4, 5 mM imidazole, pH 8.0) containing 6M Guanidine-HCl denaturing agent and sonicated again as above. Insoluble components were precipitated again by centrifugation and the supernates were saved. For affinity chromatography, 5.0 ml of nickel-agarose (Ni-NTA agarose, Qiagen) was packaged into a 1.5x14 cm column (Bio-Rad, Carlsbad, CA) and washed with 3 column volumes of molecular-grade H_2O and 6 column volumes of binding buffer + denaturing agent. Cell lysate was then loaded, and the flow was adjusted to 1 ml/min. Unbound proteins were washed using 10 bed volumes of binding buffer, followed by 6 column volumes of wash buffer (50 mM NaH_2PO_4, 20 mM imidazole, pH 8.0) containing denaturing agent. Washing continued until the A_{280} of the flow through was <0.01 AU. Recombinant TprC was eluted with 15 ml of elution buffer (50 mM NaH_2PO_4, 300 mM imidazole, pH 8.0) containing denaturing agent. Eluted fractions devoid of visible contaminants by SDS-PAGE and Coomassie staining were pooled, and protein concentration was assessed by micro-bicinchoninic (BCA) assay (Thermo-Fisher). Pooled fractions were then dialyzed in PBS using a 10 kDa MWCO Slide-A-Lyzer dialysis cassette (Thermo-Fisher) over 12 hours, ensuring PBS change every ~4 hours. Precipitated protein, resulting from elimination of Guanidine-HCl during dialysis was transferred into microcentrifuge tubes and spun down at full speed. After removing the supernate, the pellet was resuspended in a volume of PBS containing 6M urea suitable to achieve a protein concentration of ~4 mg/ml, and protein concentration was then reassessed using the micro-BCA assay kit (Thermo-Fisher). Prior to immunizations, urea was eliminated using Profoldin (Hudson, MA) M7 renaturing columns for membrane proteins, which were used according to the manufacturer's protocol. M7 renaturing columns were found to provide the best yield when screened along with 19 other conditions offered by Profoldin. Lipid composition of the elute buffer included lysophosphatidylcholine (~5 mM), arginine (~150 mM), glycerol (~10%), dodecyl maltoside (0.7 mM), and Tris-HCl (0.1 mM), pH 7.5). Following buffer exchange, soluble protein concentration was evaluated using micro-BCA assay and analyzed by circular dichroism (CD) to evaluate percentage of β-sheet, alpha-helix, and random coil. CD spectra (190 to 260 nm) were acquired in triplicate at room temperature using 0.5 mg/ml of recombinant refolded TprC in a Jasco-1500 high-performance CD spectrometer. CD spectra were analyzed using the online platform Dichroweb (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) (62) and the spectra from buffer alone for background subtraction.

Rabbit Immunization

Groups of three rabbits each were immunized with one of the purified, refolded recombinant TprC variants. Rabbits were injected with 125 μg of refolded protein every 3 weeks for a total of three immunizations. Prior to injection, antigen was mixed with an equal volume of in Titermax Gold Adjuvant (Millipore-Sigma), a water-in oil emulsion containing squalene, the block co-polymer CRL-8300, and a microparticle stabilizers to obtain a final volume of 1 ml. Immunogen-adjuvant preparation was performed according to the manufacturer`s instruction, and immunizations were performed *via* four 250 μl injections (each containing 31.25 μg of protein) into 4 intramuscular sites. Three weeks after the last boost, immunized animals were deeply anaesthetized, bled through cardiac puncture, and then euthanized.

ELISA Using Synthetic Peptides

Overlapping synthetic peptides (20-mers overlapping by 10 aa) were designed to represent the sequences of all TprC and TprD/D_2 loci present in each of the seven strains examined in this study starting after the predicted signal peptide (AA 1-22; **Figure 1** and **Figure 2**). Only the C56 peptide and its variants (**Table S6**), which represent the proteins' COOH-terminus, were synthesized as 26-mers. A total of 120 peptides (**Table S6**) were produced by Genscript (Piscataway, NJ). Upon receipt, lyophilized peptides were rehydrated in sterile PBS to a stock solution of 200 μg/ml. Solubility of hydrophobic peptides was increased by adding up to 4% (v/v) DMSO per manufacturer's instruction when needed (peptides C1, C4-7, C10, C15-16, C20, C25, C38-C39, C43-44, C53; **Table S6**). Reconstituted peptides were stored at -20°C until use. For ELISA, peptides were further diluted to 10 μg/ml in PBS, and 50 μl of working dilution (500 ng total) were used to coat the

wells of a 96-well Microwell Maxisorp flat-bottom plate (Thermo-Fisher, Waltham, MA) as previously described (44). Absorbance was measured at OD_{405} using a Molecular Devices SpectraMax Plus microplate reader (Molecular Devices, San Jose, CA). A micro-BCA protein assay (Thermo Fisher) was performed in plates coated with Ag and washed to demonstrate that all peptides bound to the well surfaces in the plates (data not shown). For each serum from each group, the value of each replicate experimental wells minus background reactivity (i.e., three times the mean of the wells tested with pooled uninfected rabbit serum) was calculated and plotted. If residual value for the No-antigen control wells was present after subtraction, statistical significance was calculated with one-way ANOVA with the Bonferroni correction of multiple comparisons or t-test, with significance set at $p<0.05$. Except for figures showing cumulative absorbance, graphs represent the mean ± SEM for triplicate wells tested with pooled sera from the 3 rabbits in each group after background subtraction.

TprC/D and D2 Structure Modeling

We used the ColabFold interface (63) to construct Multiple Sequence Alignments (MSA) for the TprC and $TprD_2$ query sequences by searching UniRef30 (64), Mgnify (65) and ColabFold sequence databases with MMSeq2 (66). The MSA was used as input for structure prediction with AlphaFold2 (38) using the default settings (template=False, amber_relax=False, 3 recycles). Visualization was performed using PyMol software (https://pymol.org) (67).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Animal care was provided in accordance with the procedures described in the Guide for the Care and Use of Laboratory Animals under protocols approved by the University of Washington Institutional Animal Care and Use Committee (IACUC, PI: Sheila Lukehart). The protocol number assigned by the IACUC committee that approved this study is 2090-08.

AUTHOR CONTRIBUTIONS

BM: Performed experiments, analyzed data, and reviewed manuscript. MF: performed experiments, analyzed data, and reviewed manuscript. CG: performed experiments, reviewed manuscript. AV: generated Tpr models, reviewed manuscript. SL: experiment conceptualization, analyzed data, reviewed manuscript. LG: analyzed data and wrote manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

Research reported in this publication was supported by National Institute of Allergy & Infectious Diseases of the National Institutes of Health under award number R01AI042143 grant (to SL). Tpr models using AlphaFold2 were generated thanks to support from Open Philanthropy (to LG). This work was also partially supported also by the National Institute for Allergy and Infectious Diseases of the National Institutes of Health grant number U19AI144133 (Project 2. Project 2 leader: LG; PI: Anna Wald, University of Washington). The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

ACKNOWLEDGMENTS

The authors are grateful to Janelle Deane for aiding with some of the experimental procedures.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.862491/full#supplementary-material

REFERENCES

1. Giacani L, Lukehart SA. The Endemic Treponematoses. *Clin Microbiol Rev* (2014) 27(1):89–115. doi: 10.1128/CMR.00070-13
2. Rowley J, Vander Hoorn S, Korenromp E, Low N, Unemo M, Abu-Raddad LJ, et al. Chlamydia, Gonorrhoea, Trichomoniasis and Syphilis: Global Prevalence and Incidence Estimates, 2016. *Bull World Health Organ* (2019) 97(8):548–62p. doi: 10.2471/blt.18.228486
3. Savage EJ, Marsh K, Duffell S, Ison CA, Zaman A, Hughes G. Rapid Increase in Gonorrhoea and Syphilis Diagnoses in England in 2011. *Euro Surveill* (2012) 17(29). doi: 10.2807/ese.17.29.20224-en
4. Savage EJ, Hughes G, Ison C, Lowndes CM. Syphilis and Gonorrhoea in Men Who Have Sex With Men: A European Overview. *Euro Surveill* (2009) 14(47). doi: 10.2807/ese.14.47.19417-en
5. Simms I, Fenton KA, Ashton M, Turner KM, Crawley-Boevey EE, Gorton R, et al. The Re-Emergence of Syphilis in the United Kingdom: The New Epidemic Phases. *Sex Transm Dis* (2005) 32(4):220–6. doi: 10.1097/01.olq.0000149848.03733.c1
6. Tucker JD, Cohen MS. China's Syphilis Epidemic: Epidemiology, Proximate Determinants of Spread, and Control Responses. *Curr Opin Infect Dis* (2011) 24(1):50–5. doi: 10.1097/QCO.0b013e32834204bf
7. CDC. *2018 Sexually Transmitted Disease Surveillance*. Atlanta, GA: US Department of Health and Human Services: Centers for Disease Control and Prevention (2019).
8. LaFond RE, Lukehart SA. Biological Basis for Syphilis. *Clin Microbiol Rev* (2006) 19(1):29–49. doi: 10.1128/CMR.19.1.29-49.2006
9. Goldenberg RL, Thompson C. The Infectious Origins of Stillbirth. *Am J Obstet Gynecol* (2003) 189(3):861–73. doi: 10.1067/S0002-9378(03)00470-8

10. Moline HR, Smith JF Jr. The Continuing Threat of Syphilis in Pregnancy. *Curr Opin Obstet Gynecol* (2016) 28(2):101–4. doi: 10.1097/gco.0000000000000258
11. CDC. *The National Plan to Eliminate Syphilis in the United States*. Atlanta, GA: U.S. Department of Health and Human Services: Centers for Disease Control and Prevention (1999).
12. WHO. *The Global Elimination of Congenital Syphilis: Rationale and Strategy for Action*. Geneva: WHO Press (2007).
13. Mitjà O, Marks M, Konan DJ, Ayelo G, Gonzalez-Beiras C, Boua B, et al. Global Epidemiology of Yaws: A Systematic Review. *Lancet Glob Health* (2015) 3(6):e324–31. doi: 10.1016/s2214-109x(15)00011-x
14. Mitjà O, Houinei W, Moses P, Kapa A, Paru R, Hays R, et al. Mass Treatment With Single-Dose Azithromycin for Yaws. *N Engl J Med* (2015) 372(8):703–10. doi: 10.1056/NEJMoa1408586
15. Mitjà O, Godornes C, Houinei W, Kapa A, Paru R, Abel H, et al. Re-Emergence of Yaws After Single Mass Azithromycin Treatment Followed by Targeted Treatment: A Longitudinal Study. *Lancet* (2018) 391(10130):1599–607. doi: 10.1016/s0140-6736(18)30204-6
16. Pace JL, Csonka GW. Late Endemic Syphilis: Case Report of Bejel With Gummatous Laryngitis. *Genitourin Med* (1988) 64(3):202–4. doi: 10.1136/sti.64.3.202
17. Pace JL. Treponematoses in Arabia. *Saudi Med J* (1983) 4:211–20.
18. Julvez J, Michault A, Kerdelhue V. Serologic Studies of Non-Venereal Treponematoses in Infants in Niamey, Niger. *Med Trop (Mars)* (1998) 58 (1):38–40.
19. Galoo E, Schmoor P. Identification of a Focus of Bejel in Mauritania. *Med Trop (Mars)* (1998) 58(3):311–2.
20. Lieberman NAP, Lin MJ, Xie H, Shrestha L, Nguyen T, Huang ML, et al. Treponema Pallidum Genome Sequencing From Six Continents Reveals Variability in Vaccine Candidate Genes and Dominance of Nichols Clade Strains in Madagascar. *PloS Negl Trop Dis* (2021) 15(12):e0010063. doi: 10.1371/journal.pntd.0010063
21. Lithgow KV, Cameron CE. Vaccine Development for Syphilis. *Expert Rev Vaccines* (2017) 16(1):37–44. doi: 10.1080/14760584.2016.1203262
22. Cameron CE, Lukehart SA. Current Status of Syphilis Vaccine Development: Need, Challenges, Prospects. *Vaccine* (2014) 32(14):1602–9. doi: 10.1016/j.vaccine.2013.09.053
23. Turner TB, Hollander DH. *Biology of the Treponematoses*. Geneva: World Health Organization (1957).
24. Miller JN. Immunity in Experimental Syphilis. VI. Successful Vaccination of Rabbits With *Treponema Pallidum*, Nichols Strain, Attenuated by γ-Irradiation. *J Immunol* (1973) 110(5):1206–15.
25. Koebnik R, Locher KP, Van Gelder P. Structure and Function of Bacterial Outer Membrane Proteins: Barrels in a Nutshell. *Mol Microbiol* (2000) 37 (2):239–53. doi: 10.1046/j.1365-2958.2000.01983.x
26. Baker-Zander SA, Lukehart SA. Macrophage-Mediated Killing of Opsonized *Treponema Pallidum*. *J Infect Dis* (1992) 165(1):69–74. doi: 10.1093/infdis/165.1.69
27. Lukehart SA, Miller JN. Demonstration of the *In Vitro* Phagocytosis of *Treponema Pallidum* by Rabbit Peritoneal Macrophages. *J Immunol* (1978) 121(5):2014–24.
28. Edmondson DG, Norris SJ. *In Vitro* Cultivation of the Syphilis Spirochete Treponema Pallidum. *Curr Protoc* (2021) 1(2):e44. doi: 10.1002/cpz1.44
29. Walker EM, Borenstein LA, Blanco DR, Miller JN, Lovett MA. Analysis of Outer Membrane Ultrastructure of Pathogenic *Treponema* and *Borrelia* Species by Freeze-Fracture Electron Microscopy. *J Bacteriol* (1991) 173 (17):5585–8. doi: 10.1128/jb.173.17.5585-5588.1991
30. Radolf JD, Norgard MV, Schulz WW. Outer Membrane Ultrastructure Explains the Limited Antigenicity of Virulent *Treponema Pallidum*. *Proc Natl Acad Sci USA* (1989) 86(6):2051–5. doi: 10.1073/pnas.86.6.2051
31. Centurion-Lara A, Castro C, Barrett L, Cameron C, Mostowfi M, Van Voorhis WC, et al. *Treponema Pallidum* Major Sheath Protein Homologue Tpr K Is a Target of Opsonic Antibody and the Protective Immune Response. *J Exp Med* (1999) 189(4):647–56. doi: 10.1084/jem.189.4.647
32. Cox DL, Luthra A, Dunham-Ems S, Desrosiers DC, Salazar JC, Caimano MJ, et al. Surface Immunolabeling and Consensus Computational Framework to Identify Candidate Rare Outer Membrane Proteins of *Treponema Pallidum*. *Infect Immun* (2010) 78(12):5178–94. doi: 10.1128/IAI.00834-10
33. Fraser CM, Norris SJ, Weinstock GM, White O, Sutton GG, Dodson R, et al. Complete Genome Sequence of *Treponema Pallidum*, the Syphilis Spirochete. *Science* (1998) 281(5375):375–88. doi: 10.1126/science.281.5375.375
34. Anand A, Luthra A, Dunham-Ems S, Caimano MJ, Karanian C, LeDoyt M, et al. TprC/D (Tp0117/131), a Trimeric, Pore-Forming Rare Outer Membrane Protein of *Treponema Pallidum*, Has a Bipartite Domain Structure. *J Bacteriol* (2012) 194(9):2321–33. doi: 10.1128/JB.00101-12
35. Centurion-Lara A, Giacani L, Godornes C, Molini BJ, Brinck Reid T, Lukehart SA. Fine Analysis of Genetic Diversity of the *Tpr* Gene Family Among Treponemal Species, Subspecies and Strains. *PloS Negl Trop Dis* (2013) 16 (7):e2222. doi: 10.1371/journal.pntd.0002222
36. Sun ES, Molini BJ, Barrett LK, Centurion-Lara A, Lukehart SA, Van Voorhis WC. Subfamily I *Treponema Pallidum* Repeat Protein Family: Sequence Variation and Immunity. *Microbes Infect* (2004) 6(8):725–37. doi: 10.1016/j.micinf.2004.04.001
37. Centurion-Lara A, Sun ES, Barrett LK, Castro C, Lukehart SA, Van Voorhis WC. Multiple Alleles of *Treponema Pallidum* Repeat Gene D in *Treponema Pallidum* Isolates. *J Bacteriol* (2000) 182(8):2332–5. doi: 10.1128/JB.182.8.2332-2335.2000
38. Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, et al. Highly Accurate Protein Structure Prediction With AlphaFold. *Nature* (2021) 596(7873):583–9. doi: 10.1038/s41586-021-03819-2
39. Holm L. Using Dali for Protein Structure Comparison. *Methods Mol Biol* (2020) 2112:29–42. doi: 10.1007/978-1-0716-0270-6_3
40. Geysen HM, Meloen RH, Barteling SJ. Use of Peptide Synthesis to Probe Viral Antigens for Epitopes to a Resolution of a Single Amino Acid. *Proc Natl Acad Sci USA* (1984) 81(13):3998–4002. doi: 10.1073/pnas.81.13.3998
41. Haynes AM, Godornes C, Ke W, Giacani L. Evaluation of the Protective Ability of the Treponema Pallidum Subsp. Pallidum Tp0126 OmpW Homolog in the Rabbit Model of Syphilis. *Infect Immun* (2019) 87(8). doi: 10.1128/iai.00323-19
42. Giacani L, Lukehart S, Centurion Lara A. *Syphilis*. A,LS Barrett, editor. Cambridge, MA: Academic Press (2009).
43. Morgan CA, Lukehart SA, Van Voorhis WC. Protection Against Syphilis Correlates With Specificity of Antibodies to the Variable Regions of *Treponema Pallidum* Repeat Protein K. *Infect Immun* (2003) 71(10):5605–12. doi: 10.1128/IAI.71.10.5605-5612.2003
44. Morgan CA, Molini BJ, Lukehart SA, Van Voorhis WC. Segregation of B and T Cell Epitopes of *Treponema Pallidum* Repeat Protein K to Variable and Conserved Regions During Experimental Syphilis Infection. *J Immunol* (2002) 169(2):952–7. doi: 10.4049/jimmunol.169.2.952
45. Morgan CA, Lukehart SA, Van Voorhis WC. Immunization With the N-Terminal Portion of *Treponema Pallidum* Repeat Protein K Attenuates Syphilitic Lesion Development in the Rabbit Model. *Infect Immun* (2002) 70(12):6811–6. doi: 10.1128/IAI.70.12.6811-6816.2002
46. Cameron CE, Lukehart SA, Castro C, Molini B, Godornes C, Van Voorhis WC. Opsonic Potential, Protective Capacity, and Sequence Conservation of the *Treponema Pallidum* Subspecies *Pallidum* Tp92. *J Infect Dis* (2000) 181 (4):1401–13. doi: 10.1086/315399
47. Arroll TW, Centurion-Lara A, Lukehart SA, Van Voorhis WC. T-Cell Responses to *Treponema Pallidum* Subsp. *Pallidum* Antigens During the Course of Experimental Syphilis Infection. *Infect Immun* (1999) 67(9):4757–63. doi: 10.1128/IAI.67.9.4757-4763.1999
48. Cameron CE, Castro C, Lukehart SA, Van Voorhis WC. Function and Protective Capacity of *Treponema Pallidum* Subsp. *Pallidum* Glycerophosphodiester Phosphodiesterase. *Infect Immun* (1998) 66 (12):5763–70. doi: 10.1128/IAI.66.12.5763-5770.1998
49. LaFond RE, Molini BJ, Van Voorhis WC, Lukehart SA. Antigenic Variation of TprK V Regions Abrogates Specific Antibody Binding in Syphilis. *Infect Immun* (2006) 74(11):6244–51. doi: 10.1128/IAI.00827-06
50. Cretich M, Gori A, D'Annessa I, Chiari M, Colombo G. Peptides for Infectious Diseases: From Probe Design to Diagnostic Microarrays. *Antibodies* (2019) 8 (1):23. doi: 10.3390/antib8010023
51. Roy A, Kucukural A, Zhang Y. I-TASSER: A Unified Platform for Automated Protein Structure and Function Prediction. *Nat Protoc* (2010) 5(4):725–38. doi: 10.1038/nprot.2010.5
52. Hegedűs T, Geisler M, Lukács G, Farkas B. AlphaFold2 Transmembrane Protein Structure Prediction Shines. *bioRxiv* (2021). doi: 10.1101/2021.08.21.457196

53. Anand A, LeDoyt M, Karanian C, Luthra A, Koszelak-Rosenblum M, Malkowski MG, et al. Bipartite Topology of Treponema Pallidum Repeat Proteins C/D and I: OUTER MEMBRANE INSERTION, TRIMERIZATION, AND PORIN FUNCTION REQUIRE A C-TERMINAL β-BARREL DOMAIN. *J Biol Chem* (2015) 290(19):12313–31. doi: 10.1074/jbc.M114.629188
54. Leader BT, Hevner K, Molini BJ, Barrett LK, Van Voorhis WC, Lukehart SA. Antibody Responses Elicited Against the *Treponema Pallidum* Repeat Proteins Differ During Infection With Different Isolates of *Treponema Pallidum* Subsp. *Pallidum*. *Infect Immun* (2003) 71(10):6054–7. doi: 10.1128/IAI.71.10.6054-6057.2003
55. Giacani L, Molini B, Godornes C, Barrett L, Van Voorhis WC, Centurion-Lara A, et al. Quantitative Analysis of *Tpr* Gene Expression in *Treponema Pallidum* Isolates: Differences Among Isolates and Correlation With T-Cell Responsiveness in Experimental Syphilis. *Infect Immun* (2007) 75(1):104–12. doi: 10.1128/IAI.01124-06
56. Victora GD, Nussenzweig MC. Germinal Centers. *Annu Rev Immunol* (2012) 30(1):429–57. doi: 10.1146/annurev-immunol-020711-075032
57. Briney B, Sok D, Jardine JG, Kulp DW, Skog P, Menis S, et al. Tailored Immunogens Direct Affinity Maturation Toward HIV Neutralizing Antibodies. *Cell* (2016) 166(6):1459–70.e11. doi: 10.1016/j.cell.2016.08.005
58. Jardine JG, Ota T, Sok D, Pauthner M, Kulp DW, Kalyuzhniy O, et al. Priming a Broadly Neutralizing Antibody Response to HIV-1 Using a Germline-Targeting Immunogen. *Science* (2015) 349(6244):156–61. doi: 10.1126/science.aac5894
59. *National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals*. Washington DC: The National Academic Press (2011).
60. Lukehart SA, Marra CM. Isolation and Laboratory Maintenance of Treponema Pallidum. *Curr Protoc Microbiol* (2007) 7:12A.1.1–A.1.8. doi: 10.1002/9780471729259.mc12a01s7
61. Studier FW. Protein Production by Auto-Induction in High Density Shaking Cultures. *Protein Expr Purif* (2005) 41(1):207–34. doi: 10.1016/j.pep.2005.01.016
62. Miles AJ, Ramalli SG, Wallace BA. DichroWeb, a Website for Calculating Protein Secondary Structure From Circular Dichroism Spectroscopic Data. *Protein Sci* (2021) 31(1):37–46. doi: 10.1002/pro.4153
63. Mirdita M, Schütze K, Moriwaki Y, Heo L, Ovchinnikov S, Steinegger M. ColabFold - Making Protein Folding Accessible to All. *bioRxiv* (2021). doi: 10.1101/2021.08.15.456425
64. Suzek BE, Wang Y, Huang H, McGarvey PB, Wu CH. UniRef Clusters: A Comprehensive and Scalable Alternative for Improving Sequence Similarity Searches. *Bioinformatics* (2015) 31(6):926–32. doi: 10.1093/bioinformatics/btu739
65. Mitchell AL, Almeida A, Beracochea M, Boland M, Burgin J, Cochrane G, et al. MGnify: The Microbiome Analysis Resource in 2020. *Nucleic Acids Res* (2020) 48(D1):D570–d8. doi: 10.1093/nar/gkz1035
66. Steinegger M, Söding J. MMseqs2 Enables Sensitive Protein Sequence Searching for the Analysis of Massive Data Sets. *Nat Biotechnol* (2017) 35 (11):1026–8. doi: 10.1038/nbt.3988
67. Janson G, Zhang C, Prado MG, Paiardini A. PyMod 2.0: Improvements in Protein Sequence-Structure Analysis and Homology Modeling Within PyMOL. *Bioinformatics* (2017) 33(3):444–6. doi: 10.1093/bioinformatics/btw638

Author Disclaimer: The content is solely the responsibility of the authors and does not necessarily represent the official views of the Funders.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Immunology

ORIGINAL RESEARCH
published: 04 April 2022
doi: 10.3389/fimmu.2022.857727

YTHDF1 Negatively Regulates *Treponema pallidum*-Induced Inflammation in THP-1 Macrophages by Promoting SOCS3 Translation in an m6A-Dependent Manner

Zhijia Li, Muzhou Teng, Yinbo Jiang, Litian Zhang, Xi Luo, Yuhui Liao and Bin Yang**

Dermatology Hospital, Southern Medical University, Guangzhou, China

OPEN ACCESS

Edited by:
Pingyu Zhou,
Tongji University, China

Reviewed by:
Sarang Tartey,
IGM Biosciences, United States
Wei Xu,
Soochow University, China
Huanbin Xu,
Tulane University, United States

***Correspondence:**
Bin Yang
yangbin1@smu.edu.cn
Yuhui Liao
liaoyh8@mail.sysu.edu.cn

Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

Received: *19 January 2022*
Accepted: *16 March 2022*
Published: *04 April 2022*

Citation:
Li Z, Teng M, Jiang Y, Zhang L, Luo X, Liao Y and Yang B (2022) YTHDF1 Negatively Regulates Treponema pallidum-Induced Inflammation in THP-1 Macrophages by Promoting SOCS3 Translation in an m6A-Dependent Manner. Front. Immunol. 13:857727. doi: 10.3389/fimmu.2022.857727

Background: Previous studies have confirmed that the bacterium *Treponema pallidum* (TP) or its proteins provide signals to macrophages that induce an inflammatory response; however, little is known about the negative regulation of this macrophage-mediated inflammatory response during syphilis infection or the underlying mechanism. Recent evidence suggests the role of the RNA modification, N^6-adenosine methylation (m6A), in regulating the inflammatory response and pathogen-host cell interactions. Therefore, we hypothesized that m6A plays a role in the regulation of the inflammatory response in macrophages exposed to TP.

Methods: We first assessed m6A levels in TP-infected macrophages differentiated from the human monocyte cell line THP-1. The binding and interaction between the m6A "writer" methyltransferase-like 3 (METTL3) or the m6A "reader" YT521-B homology (YTH) domain-containing protein YTHDF1 and the suppressor of cytokine signaling 3 (SOCS3), as a major regulator of the inflammatory response, were explored in differentiated TP-infected THP-1 cells as well as in secondary syphilitic lesions from patients. The mechanisms by which YTHDF1 and SOCS3 regulate the inflammatory response in macrophages were assessed.

Results and Conclusion: After macrophages were stimulated by TP, YTHDF1 was upregulated in the cells. YTHDF1 was also upregulated in the syphilitic lesions compared to adjacent tissue in patients. YTHDF1 recognizes and binds to the m6A methylation site of *SOCS3* mRNA, consequently promoting its translation, thereby inhibiting the JAK2/STAT3 pathway, and reducing the secretion of inflammatory factors, which results in anti-inflammatory regulation. This study provides the first demonstration of the role of m6A methylation in the pathological process of syphilis and further offers new insight into the pathogenesis of TP infection.

Keywords: syphilis, *Treponema pallidum*, inflammation, m6A methylation, macrophage

INTRODUCTION

Syphilis is a sexually transmitted disease that is caused by infection with the bacterium *Treponema pallidum* (TP). The annual incidence of syphilis has increased in recent years, and its prevalence has caused serious public health problems. TP can invade the skin, mucosal membranes, and nerves, among other sites, thereby causing multi-system damage (1). As an important subpopulation of cells that are involved in the innate immune response, macrophages bind to TP lipoprotein *via* the pattern recognition receptor on the macrophage cell surface, which leads to their self-activation and initiation of the immune response (2, 3). Macrophages eradicate TP directly through phagocytosis and the secretion of a large number of inflammatory cytokines (4). In addition, the inflammatory cytokines secreted by macrophages can further stimulate CD4+ T cells to produce Th1-type cytokines, such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α, to indirectly eliminate TP (4), which also occurs in syphilitic lesions (5, 6). As these TP infection-induced immune responses can cause tissue damage, macrophages may also negatively regulate the inflammatory response upon TP stimulation through related pathways to prevent overwhelming systemic inflammation. Our previous study revealed that TP infection upregulated the expression of the microRNA miR-101-3p, which inhibited the Toll-like receptor 2 signaling pathway and reduced cytokine production (7). However, few studies have focused on the negative regulation of the inflammatory response in macrophages during syphilis infection, and the underlying mechanism remains to be clarified.

N6-adenosine methylation (m6A) is the most abundant mRNA modification, which plays an important role in the regulation of various pathophysiological processes, including the inflammatory response and immune regulation, and has thus attracted widespread attention in recent years (8, 9). m6A methylation is dynamically regulated by three regulatory factors —writers, erasers, and readers. m6A methylation participates several mRNA metabolic processes, including mRNA degradation, splicing, folding, nucleation, and translation (10). Writers, methyltransferase-like (METTL) 3 (METTL3), METTL14, and other methyltransferases form a protein complex that modifies mRNAs *via* m6A methylation in the nucleus. Moreover, demethylases (erasers), such as FTO and ALKBH5, can oxidize and remove some methyl sites (11, 12). Subsequently, the readers, including members of the YT521-B homology (YTH) domain-containing protein family (YTHDF1/2/3 and YTHDC1/2) and heterogeneous nuclear ribonucleoprotein (HNRNP) proteins, can recognize and interact with m6A sites (11, 12). For example, YTHDF1 controls mRNA degradation and promotes translation efficiency (10).

Given the importance of m6A methylation in the regulation of genes that are involved in immune and inflammatory responses, we hypothesized that this modification might also be involved in the inflammatory response of macrophages during syphilis infection. To test this hypothesis, we compared the levels of m6A methylation in macrophages cultured *in vitro* with and without TP infection, and the influence of TP infection on the changes in m6A-related proteins. We further investigated the effect of TP infection in macrophages using knockdown and overexpression experiments of m6A-related proteins and examined the changes in the gene expression profiles and impact of SOCS3 expression. SOCS3 is a negative regulator of the JAK2-STAT3 pathway, which plays an important role in inflammation and the response to infection.

MATERIALS AND METHODS

Cell Culture and Infection

THP-1 cells, a human monocyte cell line, were provided by Dr. Chuncai Gu, PhD (Nanfang Hospital, Southern Medical University, Guangzhou, China). THP-1 cells were grown in culture media, containing RPMI 1640 media (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Australia) and 1% penicillin-streptomycin. Cells were grown in a humidified 5% CO_2 atmosphere at 37°C. The culture media was then supplemented with 100 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO, USA) and incubated for 24 h to induce the differentiation of the monocytes into M0 macrophages. The obtained macrophages were then cultured in a media without penicillin-streptomycin. The cells were split into two treatment groups cultured with TP (Nichols strain, kindly provided by Yinbo Jiang, Dermatology Hospital, Southern Medical University, Guangzhou, China) at a multiplicity of infection (MOI) of 20:1 (TP:cell) or phosphate-buffered saline (PBS) as the control group. The two treatment groups were incubated for 24 h.

Small Interfering RNA (siRNA)-Mediated Knockdown and Plasmid Transfection

YTHDF1, METTL3, SOCS3, and control siRNAs (Sangon Biotech, Shanghai, China) were transfected into THP-1 cells at a final concentration of 10 nM using RNA MAX siRNA Transfection Reagent (Invitrogen) according to the manufacturer's instructions. THP-1 cells were incubated at 37°C in a CO_2 incubator for 48 h, and the transfection efficiency was evaluated using western blotting analysis as described below. The siRNA sequences are listed in **Supplementary Table S1**.

THP-1 cells were transfected with the SOCS3-expressing plasmid (Sangon Biotech, Shanghai, China) using Lipo3000 (Invitrogen).

Immunofluorescence

The Ethical Approval Board of Dermatology Hospital, Southern Medical University, China approved the use of tissue wax blocks from patients with secondary syphilis (GDDHLS-20180510). For immunofluorescence analysis of the tissues, the sections were permeabilized with 0.25% Triton X-100 for 0.5 h and blocked with 5% goat serum for 1 h. Tissue sections were then incubated with primary antibodies against CD68 (Cat. No. 66231-2-Ig, Proteintech, Wuhan, Hubei, China) and YTHDF1 (Cat. No. 17479-1-AP, Proteintech) at 4°C overnight, followed by

incubation with the secondary antibody, Alexa Fluor® 488 donkey anti-rabbit IgG (H+L) (A21206, Life Technologies) or Alexa Fluor® 594 donkey anti-mouse IgG (H+L) (A21203, Life Technologies). The coverslips were mounted onto slides using an antifade mounting medium containing DAPI. The images were captured using a fluorescence microscope.

Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of cytokines (TNF-α and IL-β) in the supernatant of the THP-1 cells were determined using ELISA kits (DAKEWE, Beijing, China) according to the manufacturer's instructions.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was performed to determine *SOCS3* mRNA levels. Total RNA was extracted from THP-1 cells using TRIzol reagent (Life Technologies), following the manufacturer's protocol. cDNA was synthesized using a PrimeScript RT Reagent Kit (Takara, Japan). The cDNA was then used as a template for qPCR with SYBR Green Master Mix (Takara, Japan), according to the manufacturer's protocol. After amplification, cycle threshold (Ct) and ΔΔCt values were obtained for quantification.

Western Blotting

Total protein from each group of cells was isolated and measured using a BCA Protein Assay Kit (EpiZyme, Shanghai). Total proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membrane was blocked with Protein-Free Rapid Blocking Buffer (EpiZyme, Shanghai) for 15 min at room temperature and incubated with the following primary antibodies overnight at 4°C: anti-METTL3 (Cat. No. 15073-1-AP, Proteintech), anti-METTL14 (Cat. No. 26158-1-AP, Proteintech), anti-YTHDF1 (Cat. No. 17479-1-AP, Proteintech), anti-SOCS3 (Cat. No. 14025-1-AP, Proteintech), anti-JAK2 (17670-1-AP, Proteintech), anti-p-JAK2 (ab32101, Abcam), anti-STAT3 (Cat. no. 60199-1-Ig, Proteintech), anti-p-STAT3 (ab267373; Abcam), and anti-GAPDH (Cat. No.60004-1-Ig, Proteintech), as a loading control. After washing thrice, the PVDF membrane was incubated with the secondary antibody for 2 h at room temperature. The membranes were visualized using chemiluminescence. The signal intensities were quantified using the ImageJ software (version 1.49).

Flow Cytometry

Single-cell suspensions were prepared from cultured THP-1 cells and incubated with anti-CD206 (374208) and anti-CD86 (321110) antibodies (both from Abcam) for 30 min at 4°C for cell-surface staining. Data were recorded on a BD Celesta flow cytometer and analyzed using the FlowJo software (V10, Treestar).

m6A Quantitation

The m6A levels were measured using an m6A RNA Methylation Assay Kit (Colorimetric) (Abcam, No. ab185912) according to the manufacturer's instructions.

RNA-Sequencing

Total whole-cell RNA from the control and TP-infected cells was extracted using the TRIzol reagent (Invitrogen) and quantified using a Qubit® RNA Assay Kit on a Qubit® 2.0 fluorometer (Life Technologies, CA, USA). The RNA purity was assessed using a NanoPhotometer spectrophotometer (IMPLEN, CA, USA). The cDNA library was constructed using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA). Sequencing was performed using an Illumina NovaSeq 6000 platform (Novogene, Beijing, China). High-quality reads were mapped to the human reference genome (hg38) using the HISAT2 program. Differential expression analysis of the two groups was performed using the DESeq2 package in R (1.10.1). Genes with expression levels differing between groups at an adjusted P value <0.05 were considered to be significantly differentially expressed. Sequencing was performed in three independent biological replicates.

m6A RNA Immunoprecipitation (MeRIP)-qPCR

Total RNA was extracted using TRIzol reagent (*Invitro*gen) and fragmented using a fragmentation buffer. The fragmented mRNA (100 ng) was then used as an input control, and the remainder of the RNA was incubated with magnetic ChIP protein A/G magnetic beads to isolate methylated RNA according to the manufacturer's instructions (Magna MeRIP m6A Assay, Millipore, 17-10499). MeRIPed RNA was analyzed using RT-qPCR.

RIP-qPCR

The procedure for RIP-qPCR was adapted from a previous study (13). THP-1 cells transfected with siNC, siMETTL3, or siYTHDF1 were washed twice with PBS, collected, and resuspended in immunoprecipitation lysis buffer. 10% of the cell lysate were saved as input, and the remaining sample was incubated with IgG antibody, YTHDF1 antibody, and protein G beads (Invitrogen) overnight at 4°C. After washing three times with wash buffer, the co-precipitated RNAs were extracted using TRIzol reagent and ethanol-precipitation with glycogen. Fold enrichment was determined by RT-qPCR.

Statistical Analysis

Data were expressed as the mean ± standard deviation, and statistical significance was determined using an unpaired Student *t*-test for the two groups and ANOVA for multiple groups with SPSS 19.0 and GraphPad Prism 5.0. Statistical significance was set at $P < 0.05$.

RESULTS

Effect of TP on m6A Methylation in Macrophages *In Vitro* and Secondary Syphilitic Lesions

To explore whether m6A methylation is related to the inflammatory response of macrophages in syphilis infection,

we first investigated m6A levels in TP-infected macrophages. Compared to the control, TP stimulation upregulated the m6A levels (**Figure 1A**). We also investigated the expression patterns of the m6A writers (METTL3 and METTL14) and readers (YTHDF1) in TP-infected macrophages. After stimulation with TP for 24 h (MOI = 20:1), the expression levels of METTL3 and YTHDF1 were significantly elevated compared with those in the control group (**Figures 1B, C** and **Supplementary Figure S1**). Similarly, immunofluorescence analysis showed upregulated expression of METTL3 and YTHDF1 in CD68+ macrophages from secondary syphilitic lesions compared with those of paired healthy skin tissue samples (**Figures 1D, E**). These data suggested that m6A methylation may participate in the pathological process of macrophages in syphilis.

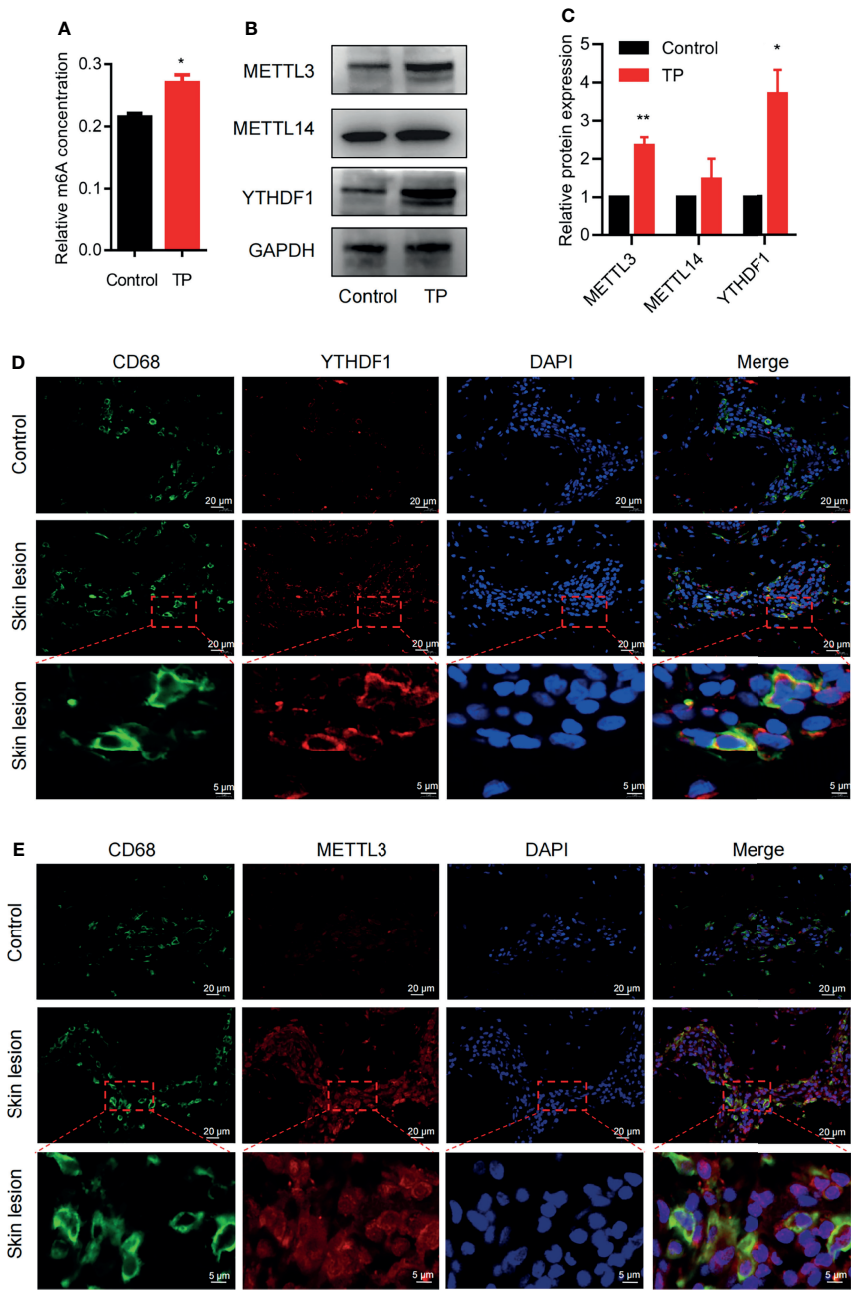


FIGURE 1 | m6A methylation: the m6A writer METTL3 and the reader YTHDF1 are upregulated in TP-infected macrophages. **(A)** Colorimetric quantification of m6A methylation in RNA from THP-1 cells with or without TP infection. **(B, C)** Western blot analysis of METTL3, METTL14, and YTHDF1 in THP-1 cells with or without TP infection. GAPDH was used as a control. *P < 0.05, **P < 0.01. **(D, E)** Immunofluorescence analysis of YTHDF1 or METTL3 in CD68+ macrophages in secondary syphilitic lesions and paired non-lesional skin tissue. Scale bar: 20 μm or 5 μm.

YTHDF1 Regulates Macrophage Polarization and the Inflammatory Response When Exposed to TP

Given the upregulation of YTHDF1 protein and RNA in TP-infected macrophages, we next verified its role in macrophage polarization. YTHDF1 silencing mediated by siRNA transfection markedly inhibited at least 80% of the YTHDF1 protein expression (**Supplementary Figure S2**). Flow cytometry showed that YTHDF1 knockdown also upregulated the M1 markers (CD86 and iNOS) and downregulated the expression of M2 markers (CD206 and ARG1) (**Figures 2A, B** and **Supplementary Figure S3**), which demonstrated that YTHDF1 inhibited M1 polarization and induced M2 polarization. Moreover, TP infection significantly upregulated the secretion of inflammatory cytokines (TNF-α and IL-1β) by macrophages, which was further enhanced by YTHDF1 knockdown (**Figures 2C, D**).

YTHDF1 and METTL3 Mediate the Protein Expression of SOCS3 in an m6A-Dependent Manner

The RNA-sequencing results clearly showed that the mRNA expression patterns between the control and TP-infected macrophages significantly differed. Analysis of mRNA expression profiles showed that 3,110 genes were significantly dysregulated (log2-fold change > 0, $P \leq 0.05$) in the TP-infected cells (859 upregulated genes and 1,251 downregulated genes) compared with those of the control group (**Supplementary Figure S4**). Kyoto Encyclopedia of Genes and Genomes analysis revealed that the upregulated genes in TP-infected macrophages were enriched in cytokine production and inflammatory signaling pathways such as the TNF signaling pathway, along with pathways associated with infection, and endocytosis (**Figure 3A**). We also investigated the top 15 upregulated genes (**Figure 3B**), which suggested that *SOCS3* may be associated with the negative regulation of the inflammatory response during TP infection. Furthermore, we predicted the association of YTHDF1 and SOCS3 with binding and perturbation using the m6A2Target tool[1] (**Figure 3C**). Given that YTHDF1 functions by binding to m6A-methylated mRNA to promote its translation (9, 14), the potential m6A sites on *SOCS3* mRNA were analyzed using m6Avar, demonstrating high confidence for position 172 of *SOCS3* mRNA as an m6A site (**Figure 3D**).

Indeed, the results of MeRIP-qPCR confirmed that TP infection increased m6A levels in *SOCS3* mRNA (**Figure 4A**). Furthermore, RIP-qPCR analysis revealed that *SOCS3* mRNA is a target of YTHDF1 protein in TP-infected THP-1 cells (**Figure 4B**). To ascertain whether *SOCS3* mRNA is a substrate for YTHDF1, we further examined the influence of YTHDF1 on the translation of SOCS3. Western blot analysis demonstrated that the SOCS3 expression level was elevated in the THP-1 cells after stimulation with TP (**Figure 4C**). YTHDF1 knockdown did not affect *SOCS3* mRNA expression (**Figure 4D**); however, knockdown of YTHDF1 significantly decreased the protein expression of SOCS3 (**Figures 4E, F**). Treatment with cycloleucine, an m6A methylation inhibitor, negatively regulated SOCS3 expression in the cells (**Figures 4G, H**). Furthermore, SOSC3 protein levels were elevated in THP-1 cells with YTHDF1 overexpression compared to those in control cells but were reduced after cycloleucine pretreatment (**Figure 4I**). In addition, we investigated whether METTL3 affects the m6A modification of SOCS3 mRNA. Western blotting showed that siRNA effectively repressed the expression of METTL3 (**Supplementary Figure S5**). Further MeRIP-qPCR analysis showed that, compared with that in the control group, the m6A modification level of *SOCS3* mRNA was decreased in the METTL3-knockdown group (**Figure 4J**). Besides, the m6A levels in the *SOCS3* mRNA were elevated in the THP-1 cells with METTL3 overexpression compared to those in control cells but were reduced after cycloleucine pretreatment (**Figure 4K**). Silencing *METTL3* impaired the protein expression of SOCS3 (**Figures 4L, M**). Protein levels of SOSC3 were elevated in the THP-1 cells with METTL3 overexpression compared to those in control cells but were reduced after cycloleucine pretreatment (**Figure 4N**). Collectively, these results indicate that METTL3 promotes the m6A modification of *SOCS3* mRNA, and that YTHDF1 recognizes the m6A-modified *SOCS3* mRNA to promote its translation.

SOCS3 Negatively Regulates the Secretion of Cytokines by Macrophages Upon TP Infection *via* JAK2/STAT3 Signaling

Previous studies have demonstrated that SOCS3 is an important negative regulator of JAK2–STAT3 signaling, which plays an indispensable role in regulating the inflammatory response (9, 14). Based on the above findings, we investigated whether SOCS3 negatively affects the inflammatory response by inhibiting JAK2–STAT3 signaling in macrophages infected with TP. Compared with those of control cells, the secretion levels of inflammatory cytokines (TNF-α and IL-1β) increased in SOCS3-knockdown cells infected with TP (**Figures 5A, B**). Furthermore, silencing of *SOCS3* promoted the activation of JAK2–STAT3 signaling (**Figures 5C, D**). The overexpression of SOCS3 partially rescued the activation of JAK2–STAT3 signaling caused by YTHDF1 knockdown (**Figure 5E**).

DISCUSSION

Syphilis is a chronic, sexually transmitted disease that seriously endangers human health. The clinical manifestations of syphilis are extremely complex and involve almost all organs and systems in the body. In the early stages, syphilis infects the skin and mucosal membranes, and in the later stages, it causes irreversible damage to vital organs, including the nerves, bones, and cardiovascular system (15). Effective control and prevention of syphilis have become a serious social and public health problem of global concern (16). Studying the immune mechanisms of the

[1] http://m6a2target.canceromics.org/

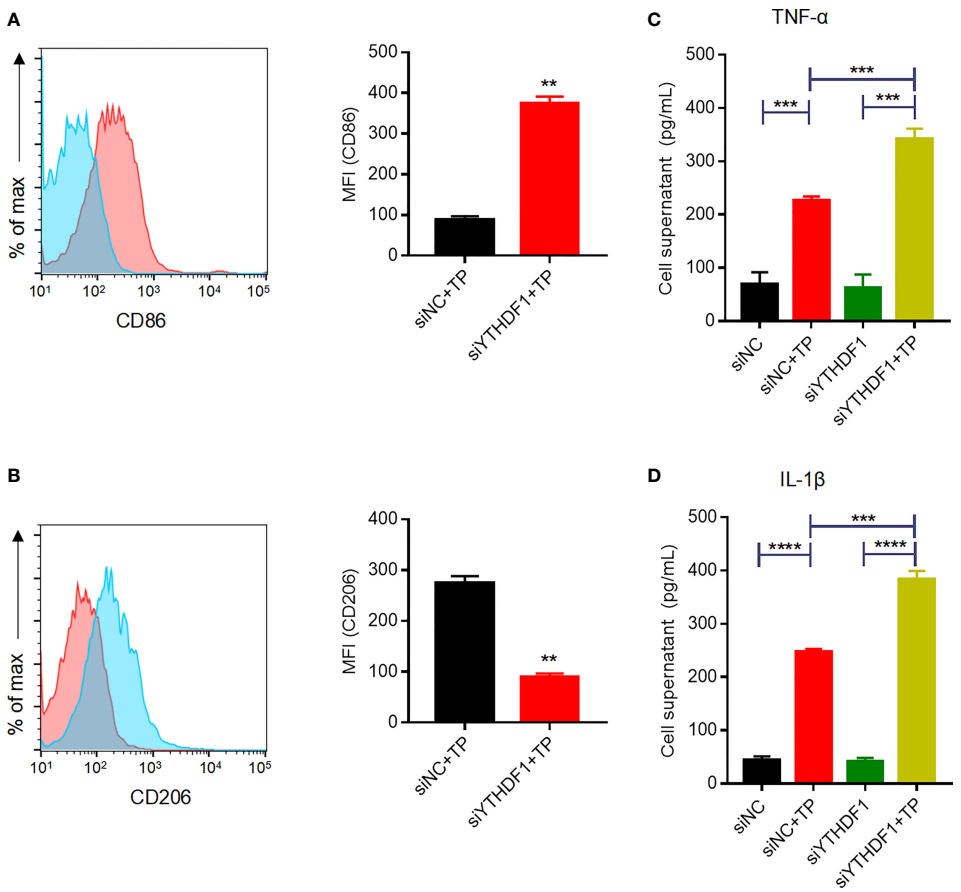
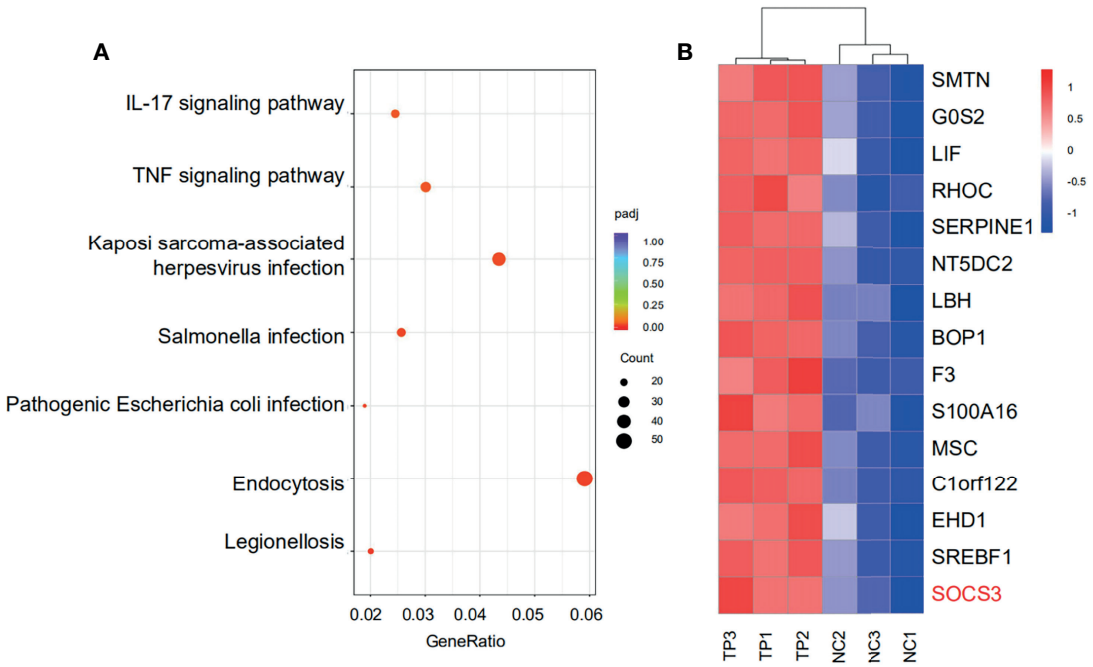


FIGURE 2 | YTHDF1 regulates M2 macrophage polarization and suppresses the secretion of inflammatory cytokines in THP-1 cells infected with TP. **(A, B)** Flow cytometry profile of the expression of M1 and M2 markers (CD86 and CD206) in THP-1 cells infected with TP. **(C-D)** The concentrations of inflammatory cytokines (TNF-α and IL-1β) in the supernatants of THP-1 cells treated with TP. Data are shown as the mean ± S.D. **P < 0.01, ***P < 0.001,****P < 0.0001.

human body after TP infection may help to find a strategy to eliminate syphilis.

When microorganisms invade, rapid cytokine production in the host is beneficial for their elimination; however, an exaggerated inflammatory response may cause severe systemic inflammation and organ dysfunction, such as the persistent inflammatory factor storm that occurs in patients severely infected with SARS-CoV-2 (17, 18). Understanding how the body controls the intensity of the secretion of inflammatory factors is a key focus of scientists studying microbial infections (19). As the main effectors of innate immunity, macrophages play an essential role in phagocytosis and clearance during microbial infection. Xu et al. (20) reported that there is a large amount of mononuclear macrophage infiltration in the skin lesions of patients with syphilis and rabbit models, and the level of the macrophage activator IFN-γ was significantly increased, along with the upregulation of the macrophage activation markers CD68, CD80, and CD86 in secondary syphilitic lesions (21). Previous studies have also shown that macrophages secrete IL-1β and promote TP phagocytosis *via* P2X7R (3). In addition, macrophages upregulate IL-6 and IL-8 expression *via* the TLR5 and MAPK/NF-κB signaling pathways when infected with TP (3). Thus, it has been confirmed that TP and its proteins induce an inflammatory response in macrophages. However, the specific negative intracellular regulators mediating these effects have not yet been thoroughly investigated. The negative effects of lipopolysaccharide (LPS), a gram-negative bacterial endotoxin that causes severe systemic inflammation and elicits numerous negative regulatory mechanisms, such as those involving A20, IRAKM, MyD88s, and SOCS1, have been investigated in a range of different hosts (19, 22). We speculate that many negative regulatory mechanisms participate in the inflammatory response caused by TP infection and the prevention of severe inflammation.

Increasing evidence has shown that m6A modification is related not only to normal physiological processes but also to immune processes involved when the body encounters pathogens. During viral and bacterial infection, m6A modifications regulate the inflammatory response and metabolic processes (23, 24). Xu et al. (25) demonstrated that LPS can significantly upregulate the



C

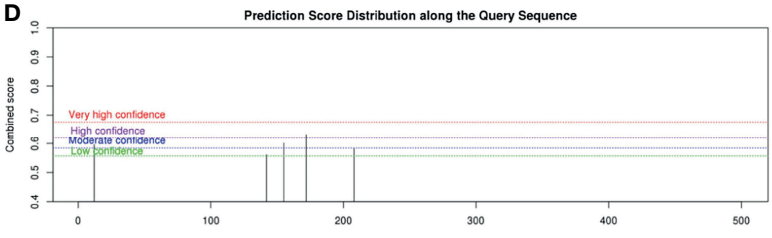
Binding

Species	Cell Line	Class	WER Name	Target Gene	Interaction	Method	Downstream Effect
Human	HeLa	Reader	YTHDF1	SOCS3	Protein-RNA	RIP-seq	translation

Perturbation

Species	Cell Line	Class	WER Name	Target Gene	Interaction	Downstream Effect
Human	HeLa	Reader	YTHDF1	SOCS3	Protein-RNA	translation
Human	A549	Writer	METTL3	SOCS3	No evidence	expression

D



#	Position	Sequence context	Structural context	Local structure visualization	Score(binary)	Score(knn)	Score(spectrum)	Score(combined)	Decision
1	12	----- ----- -ATGG GGGTG GAACT CATGA TGGAA TTGGA GCCTT	N/A	N/A	0.586	0.487	0.624	0.556	m^6A site (**Moderate** confidence)
2	142	ACGAG AACCA GACCA TGCAG GAACT CTGAT CGTGG ACATT TCAAC	N/A	N/A	0.642	0.789	0.421	0.561	m^6A site (**Low** confidence)
3	155	CATGC AGGAA CTCTG ATCGT GGACA TTTCA ACCTC CAGAA CTGTG	N/A	N/A	0.685	0.789	0.463	0.601	m^6A site (**Moderate** confidence)
4	172	CGTGG ACATT TCAAC CTCCA GAACT GTGAT CCAAA ATGCA TATGT	N/A	N/A	0.709	0.722	0.518	0.633	m^6A site (**High** confidence)
5	208	TGCAT ATGTA TCTTT GGAAG AAACT CTGAA GTAAA GGCCG GAATA	N/A	N/A	0.543	0.344	0.667	0.582	m^6A site (**Low** confidence)

FIGURE 3 | SOCS3 may be the target of the m6A reader YTHDF1 in TP-infected macrophages. **(A)** KEGG pathway analysis of biological processes associated with inflammation and infection in the RNA-seq dataset. **(B)** Heat map of the top 15 significantly upregulated mRNAs in TP-infected macrophages compared with control cells. **(C)** The association of binding and perturbation between YTHDF1 and SOCS3 was predicted by m6A2Target. **(D)** The potential m6A sites on SOCS3 mRNA were analyzed by m6Avar.

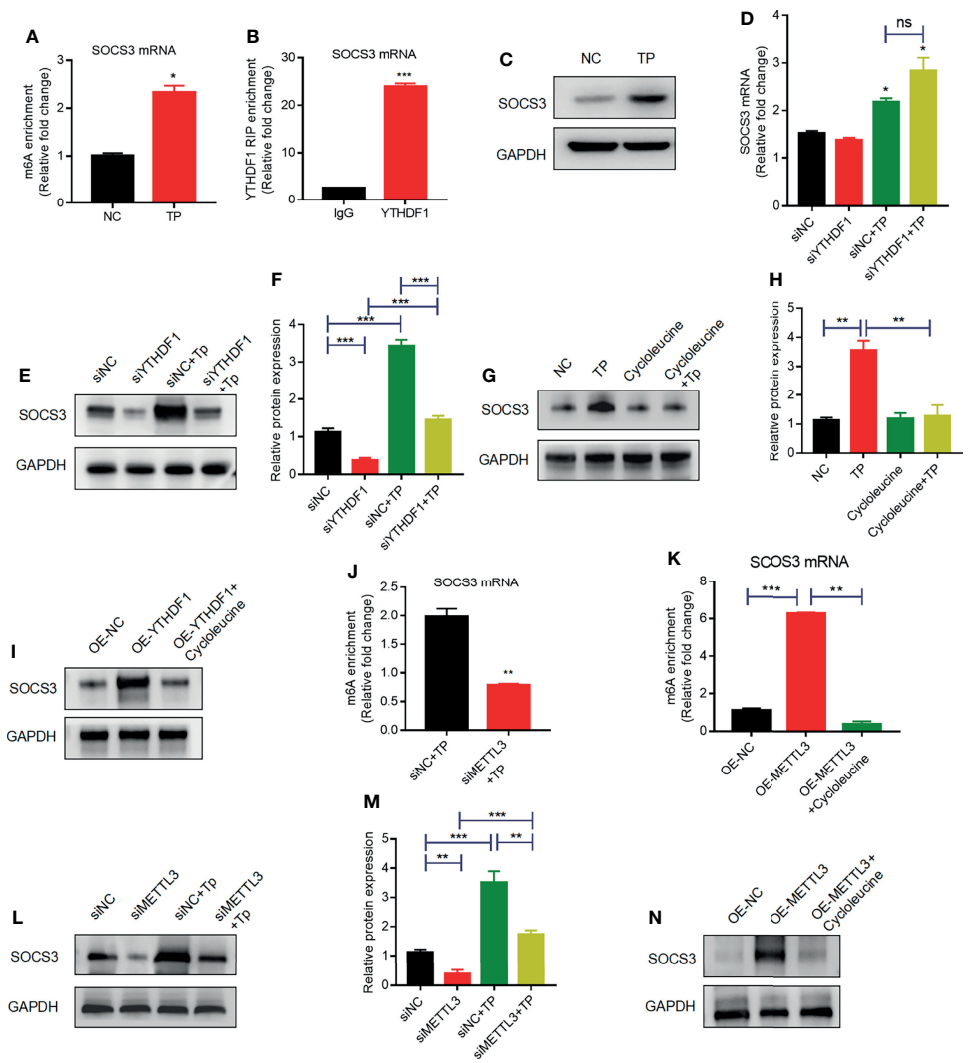


FIGURE 4 | YTHDF1 and METTL3 regulate SOCS3 translation in an m6A-dependent manner. **(A)** MeRIP-qPCR analysis of the m6A levels of SOCS3 mRNA in THP-1 cells with or without TP infection. Total RNA was isolated from the NC group or TP-infected group. Methylated RNA was immunoprecipitated and then RT-qPCR analysis of the immunoprecipitated RNA was performed. **(B)** RIP analysis of the interaction of SOCS3 mRNA with YTHDF1 protein in THP-1 cells with or without TP infection. Enrichment of SOCS3 with YTHDF1 was assessed by RT-PCR and normalized to the input. **(C)** Western blot analysis of SOCS3 in THP-1 cells with or without TP infection. GAPDH was used as a control. **(D)** RT-PCR analysis of SOCS3 mRNA in THP-1 cells with or without TP infection following transfection with siNC or siYTHDF1. *P < 0.05 compared with siNC group. **(E, F)** Western blot analysis of SOCS3 in THP-1 cells with or without TP infection following transfection with siNC or siYTHDF1. GAPDH was used as a control. **(G, H)** Western blot analysis of SOCS3 in THP-1 cells with or without TP infection and with or without cycloleucine treatment. **(I)** Western blot analysis of SOCS3 in THP-1 cells transfected with control or YTHDF1 plasmid. **(J)** MeRIP-qPCR analysis of the m6A levels of SOCS3 mRNA in TP infected-THP-1 cells with or without METTL3 knockdown. **(K)** MeRIP-qPCR analysis of the m6A levels of SOCS3 mRNA in THP-1 cells transfected with control or YTHDF1 plasmid. **(L, M)** Western blot analysis of SOCS3 in THP-1 cells with or without TP infection following transfection with control or METTL3 knockdown. **(N)** Western blot analysis of SOCS3 in THP-1 cells transfected with control or METTL3 plasmid. *P < 0.05, **P < 0.01, ***P < 0.001; ns, no significance.

expression and biological activity of METTL3 in macrophages, which induces inflammation *via* NF-κB. Additionally, they showed that the overexpression of METTL3 significantly inhibited the proliferation and reduced the inflammation in macrophages (25). Many other infection mechanisms that involve in m6A modification have been explored, such as those involving *Salmonella typhimurium* (23), vesicular stomatitis virus (24), and the gut microbiota (26). In this study, we provide the first evidence that the m6A modification level was enhanced in macrophages *in vitro* after TP infection and that the writer, METTL3, and the reader, YTHDF1, were upregulated both *in vitro* and in secondary syphilitic lesions. We inferred that METTL3 and YTHDF1 might be involved in the pathological processes of TP-stimulated macrophages. Indeed, YTHDF1 silencing experiments showed that YTHDF1 could inhibit the M1 polarization of macrophages and secretion of inflammatory

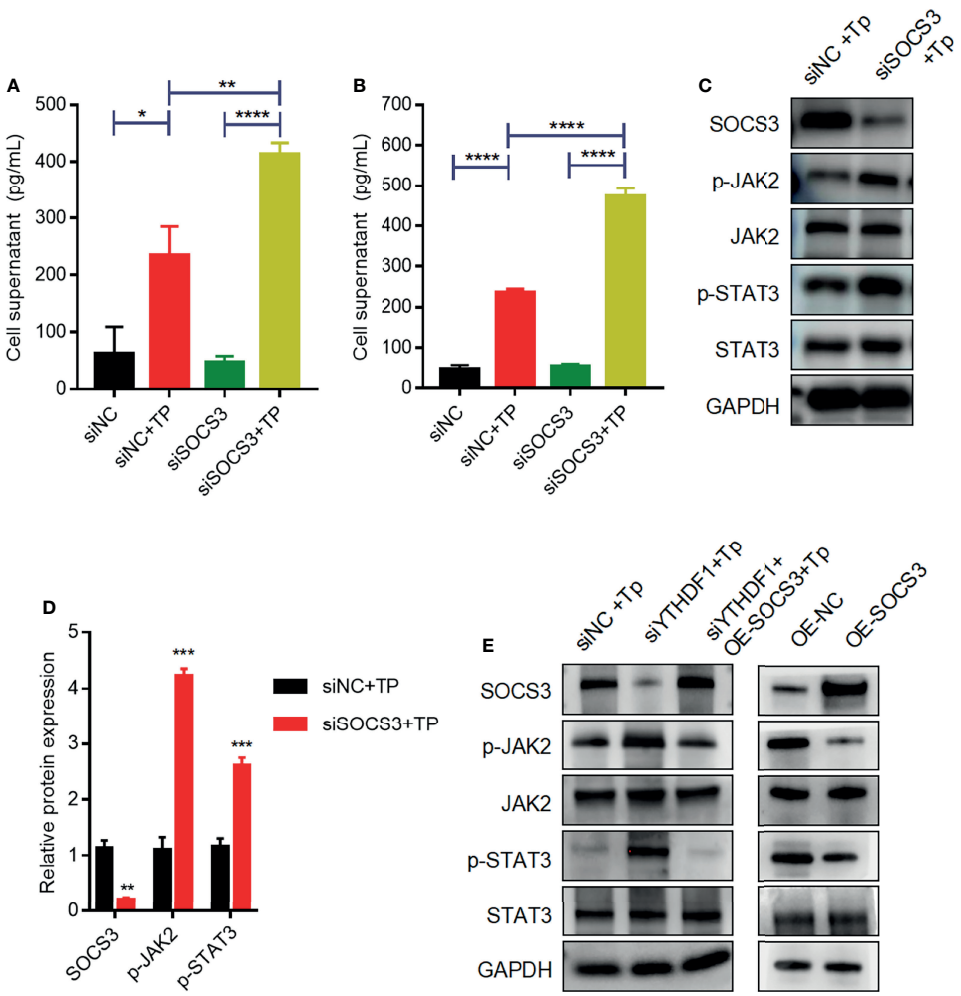


FIGURE 5 | SOCS3 suppresses inflammation in TP-infected macrophages *via* the JAK2-STAT3 pathway. **(A, B)** The concentrations of inflammatory cytokines (TNF-α and IL-1β) in the supernatants of THP-1 cells with or without TP infection following transfection with siNC or siSOCS3. **(C, D)** Western blot analysis of SOCS3, p-JAK2, JAK2, p-STAT3, and STAT3 in THP-1 cells transfected with siNC or siSOCS3. **(E)** Western blot analysis of SOCS3, p-JAK2, JAK2, p-STAT3, and STAT3 in piPSCs with or without YTHDF1 knockdown and transfected with control or SOCS3 plasmid. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

factors, suggesting that YTHDF1 may be one of the key factors negatively regulating the inflammatory response of macrophages.

The SOCS family is typically induced during inflammation and infection, which stimulates the expression of the members and negatively regulates cytokine production through various signaling pathways (27, 28). SOCS3 is a negative regulator of the JAK-STAT pathway, which plays an important negative regulatory role in inflammatory diseases and cellular immune responses (29). The effect of SOCS3 on the immune response is achieved by inhibiting the signal transduction of LPS, IL-6, and other related mediators. The SH2 domain of SOCS3 competitively binds to JAK2 and blocks the activation of STAT3, thereby inhibiting the inflammatory factors, including STAT3 and TNF-α (29). Previous studies have shown that SOCS3 has a negative regulatory effect on autoimmune inflammation in macrophages and that the absence of SOCS3 can cause subacute inflammation in mice (30). In addition, SOCS3 is considered an important mediator of gastrointestinal inflammation, and the knocking out of SOCS3 in gastric epithelial cells can promote the occurrence of gastritis (31). In a mouse model of colitis, the methyltransferase METTL3 was shown to modify the mRNA levels of *Socs1* and *Socs3* mRNAs by m6A methylation, which reduced the attenuation of both mRNAs and increased their translation, thereby negatively regulating IL-7/STAT5 signaling and the inflammatory response in T cells (32). In this study, we found that m6A modification regulates SOCS3 translation in a METTL3/YTHDF1-orchestrated manner when macrophages are stimulated by TP. Mechanistically, we propose that METTL3 promotes the m6A methylation of *SOCS3* mRNA, and then YTHDF1 recognizes and binds to the m6A-containing mRNA of *SOCS3* and promotes SOCS3 protein expression. Previous studies have demonstrated that the JAK2–STAT3 pathway is involved in the expression and secretion of the

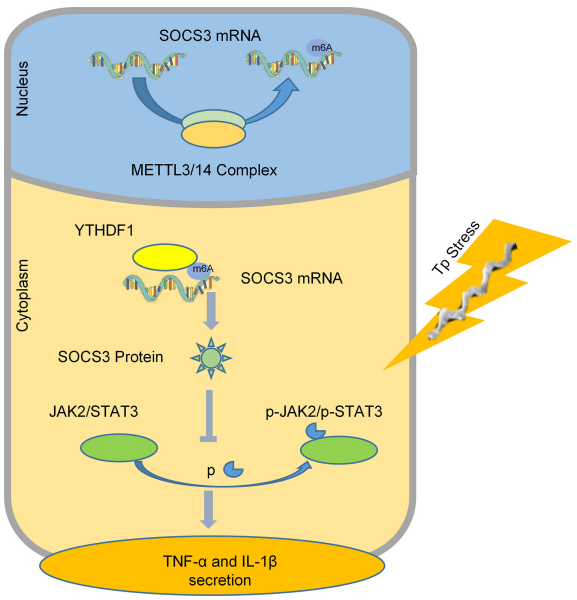


FIGURE 6 | A schematic diagram revealing the proposed mechanism by which YTHDF1 mediates the protein expression of SOCS3 in an m6A-dependent manner and regulates the inflammatory response in macrophages infected with TP.

inflammatory cytokines TNF-α and IL-1β (33, 34). Our results further suggest that YTHDF1 and SOCS3 negatively regulate the inflammatory response by regulating the JAK2–STAT3 pathway.

In summary, we found that after macrophages are stimulated by TP, YTHDF1, an m6A methylation reader is upregulated and it recognizes and binds to the m6A methylation site of *SOCS3* to promote its translation. SOCS3 inhibits the JAK2/STAT3 pathway and reduces the secretion of inflammatory factors, which achieves an anti-inflammatory effect (**Figure 6**). This study helps to explain why TP infection does not cause an excessive outburst of inflammation. Moreover, this study is the first to focus on the impact of m6A methylation in the pathological process of syphilis, providing new insights for exploring the mechanism of immune damage along with associated prevention and control strategies for syphilis, and improving our understanding of the pathogenesis of TP infection.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA787979.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethical Approval Board of Dermatology Hospital, Southern Medical University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ZL and MT: Experiments, Data curation, Writing - original draft. YJ, LZ, and XL: Preparation of TP. YL and BY: Supervision, Project administration, Writing - review and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Natural Science Foundation (NSFC) 81772240.

ACKNOWLEDGMENTS

The authors thank Editage for the English language review.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.857727/full#supplementary-material

REFERENCES

1. Keuning MW, Kamp GA, Schonenberg-Meinema D, Dorigo-Zetsma JW, van Zuiden JM, Pajkrt D. Congenital Syphilis, the Great Imitator-Case Report and Review. *Lancet Infect Dis* (2020) 20:e173–9. doi: 10.1016/S1473-3099(20)30268-1
2. Xu M, Xie Y, Jiang C, Xiao Y, Kuang X, Wen Y, et al. Treponema Pallidum Flagellins Elicit Proinflammatory Cytokines From Human Monocytes *via* TLR5 Signaling Pathway. *Immunobiology* (2017) 222:709–18. doi: 10.1016/j.imbio.2017.01.002
3. Xu SL, Lin Y, Liu W, Zhu XZ, Liu D, Tong ML, et al. The P2X7 Receptor Mediates NLRP3-Dependent IL-1beta Secretion and Promotes Phagocytosis in the Macrophage Response to Treponema Pallidum. *Int Immunopharmacol* (2020) 82:106344. doi: 10.1016/j.intimp.2020.106344
4. Lin LR, Xiao Y, Liu W, Chen YY, Zhu XZ, Gao ZX, et al. Development of Tissue Inflammation Accompanied by NLRP3 Inflammasome Activation in Rabbits Infected With Treponema Pallidum Strain Nichols. *BMC Infect Dis* (2018) 18:101. doi: 10.1186/s12879-018-2993-0
5. Carlson JA, Dabiri G, Cribier B, Sell S. The Immunopathobiology of Syphilis: The Manifestations and Course of Syphilis Are Determined by the Level of Delayed-Type Hypersensitivity. *Am J Dermatopathol* (2011) 33:433–60. doi: 10.1097/DAD.0b013e3181e8b587
6. Sell S, Norris SJ. The Biology, Pathology, and Immunology of Syphilis. *Int Rev Exp Pathol* (1983) 24:203–76.
7. Huang T, Yang J, Zhang J, Ke W, Zou F, Wan C, et al. MicroRNA-101-3p Downregulates TLR2 Expression, Leading to Reduction in Cytokine Production by Treponema Pallidum-Stimulated Macrophages. *J Invest Dermatol* (2020) 140:1566–75. doi: 10.1016/j.jid.2019.12.012

8. Han Z, Wang X, Xu Z, Cao Y, Gong R, Yu Y, et al. ALKBH5 Regulates Cardiomyocyte Proliferation and Heart Regeneration by Demethylating the mRNA of YTHDF1. *Theranostics* (2021) 11:3000–16. doi: 10.7150/thno.47354
9. Hu L, Wang J, Huang H, Yu Y, Ding J, Yu Y, et al. YTHDF1 Regulates Pulmonary Hypertension Through Translational Control of MAGED1. *Am J Respir Crit Care Med* (2021) 203:1158–72. doi: 10.1164/rccm.202009-3419OC
10. Xu Z, Peng B, Cai Y, Wu G, Huang J, Gao M, et al. N6-Methyladenosine RNA Modification in Cancer Therapeutic Resistance: Current Status and Perspectives. *Biochem Pharmacol* (2020) 182:114258. doi: 10.1016/j.bcp.2020.114258
11. Zhao BS, Roundtree IA, He C. Post-Transcriptional Gene Regulation by mRNA Modifications. *Nat Rev Mol Cell Biol* (2017) 18:31–42. doi: 10.1038/nrm.2016.132
12. Huang H, Weng H, Sun W, Qin X, Shi H, Wu H, et al. Recognition of RNA N(6)-Methyladenosine by IGF2BP Proteins Enhances mRNA Stability and Translation. *Nat Cell Biol* (2018) 20:285–95. doi: 10.1038/s41556-018-0045-z
13. Liu T, Wei Q, Jin J, Luo Q, Liu Y, Yang Y, et al. The M6a Reader YTHDF1 Promotes Ovarian Cancer Progression *via* Augmenting EIF3C Translation. *Nucleic Acids Res* (2020) 48:3816–31. doi: 10.1093/nar/gkaa048
14. Zong X, Xiao X, Shen B, Jiang Q, Wang H, Lu Z, et al. The N6-Methyladenosine RNA-Binding Protein YTHDF1 Modulates the Translation of TRAF6 to Mediate the Intestinal Immune Response. *Nucleic Acids Res* (2021) 49:5537–52. doi: 10.1093/nar/gkab343
15. Hook ER. Syphilis. *Lancet* (2017) 389:1550–7. doi: 10.1016/S0140-6736(16)32411-4
16. Peeling RW, Mabey D, Kamb ML, Chen XS, Radolf JD, Benzaken AS. Syphilis. *Nat Rev Dis Primers* (2017) 3:17073. doi: 10.1038/nrdp.2017.73
17. Fu Y, Cheng Y, Wu Y. Understanding SARS-CoV-2-Mediated Inflammatory Responses: From Mechanisms to Potential Therapeutic Tools. *Virol Sin* (2020) 35:266–71. doi: 10.1007/s12250-020-00207-4
18. Channappanavar R, Fehr AR, Vijay R, Mack M, Zhao J, Meyerholz DK, et al. Dysregulated Type I Interferon and Inflammatory Monocyte-Macrophage Responses Cause Lethal Pneumonia in SARS-CoV-Infected Mice. *Cell Host Microbe* (2016) 19:181–93. doi: 10.1016/j.chom.2016.01.007
19. Du J, Liao W, Liu W, Deb DK, He L, Hsu PJ, et al. N(6)-Adenosine Methylation of Socs1 mRNA Is Required to Sustain the Negative Feedback Control of Macrophage Activation. *Dev Cell* (2020) 55:737–53. doi: 10.1016/j.devcel.2020.10.023
20. Lafond RE, Lukehart SA. Biological Basis for Syphilis. *Clin Microbiol Rev* (2006) 19:29–49. doi: 10.1128/CMR.19.1.29-49.2006
21. Cruz AR, Ramirez LG, Zuluaga AV, Pillay A, Abreu C, Valencia CA, et al. Immune Evasion and Recognition of the Syphilis Spirochete in Blood and Skin of Secondary Syphilis Patients: Two Immunologically Distinct Compartments. *PloS Negl Trop Dis* (2012) 6:e1717. doi: 10.1371/journal.pntd.0001717
22. Kinjyo I, Hanada T, Inagaki-Ohara K, Mori H, Aki D, Ohishi M, et al. SOCS1/JAB Is a Negative Regulator of LPS-Induced Macrophage Activation. *Immunity* (2002) 17:583–91. doi: 10.1016/s1074-7613(02)00446-6
23. Wu C, Chen W, He J, Jin S, Liu Y, Yi Y, et al. Interplay of m(6)A and H3K27 Trimethylation Restrains Inflammation During Bacterial Infection. *Sci Adv* (2020) 6:a647. doi: 10.1126/sciadv.aba0647
24. Liu Y, You Y, Lu Z, Yang J, Li P, Liu L, et al. N (6)-Methyladenosine RNA Modification-Mediated Cellular Metabolism Rewiring Inhibits Viral Replication. *Science* (2019) 365:1171–6. doi: 10.1126/science.aax4468
25. Wang J, Yan S, Lu H, Wang S, Xu D. METTL3 Attenuates LPS-Induced Inflammatory Response in Macrophages *via* NF-kappaB Signaling Pathway. *Mediators Inflamm* (2019) 2019:3120391. doi: 10.1155/2019/3120391
26. Wang X, Li Y, Chen W, Shi H, Eren AM, Morozov A, et al. Transcriptome-Wide Reprogramming of N(6)-Methyladenosine Modification by the Mouse Microbiome. *Cell Res* (2019) 29:167–70. doi: 10.1038/s41422-018-0127-2
27. Klepsch O, Namer LS, Kohler N, Kaempfer R, Dittrich A, Schaper F. Intragenic Regulation of SOCS3 Isoforms. *Cell Commun Signal* (2019) 17:70. doi: 10.1186/s12964-019-0379-6
28. Gao Y, Zhao H, Wang P, Wang J, Zou L. The Roles of SOCS3 and STAT3 in Bacterial Infection and Inflammatory Diseases. *Scand J Immunol* (2018) 88:e12727. doi: 10.1111/sji.12727
29. Durham GA, Williams J, Nasim MT, Palmer TM. Targeting SOCS Proteins to Control JAK-STAT Signalling in Disease. *Trends Pharmacol Sci* (2019) 40:298–308. doi: 10.1016/j.tips.2019.03.001
30. Zhang X, Wang Y, Yuan J, Li N, Pei S, Xu J, et al. Macrophage/microglial Ezh2 Facilitates Autoimmune Inflammation Through Inhibition of Socs3. *J Exp Med* (2018) 215:1365–82. doi: 10.1084/jem.20171417
31. Zhang H, Wang Y, Li S, Tang X, Liang R, Yang X. SOCS3 Protects Against Neonatal Necrotizing Enterocolitis *via* Suppressing NLRP3 and AIM2 Inflammasome Activation and P65 Nuclear Translocation. *Mol Immunol* (2020) 122:21–7. doi: 10.1016/j.molimm.2020.03.019
32. Li HB, Tong J, Zhu S, Batista PJ, Duffy EE, Zhao J, et al. m(6)A mRNA Methylation Controls T Cell Homeostasis by Targeting the IL-7/STAT5/SOCS Pathways. *Nature* (2017) 548:338–42. doi: 10.1038/nature23450
33. Li Q, Cheng Y, Zhang S, Sun X, Wu J. TRPV4-Induced Müller Cell Gliosis and TNF-α Elevation-Mediated Retinal Ganglion Cell Apoptosis in Glaucomatous Rats *via* JAK2/STAT3/NF-κB Pathway. *J Neuroinflamm* (2021) 18:271. doi: 10.1186/s12974-021-02315-8
34. Yin L, Dai Q, Jiang P, Zhu L, Dai H, Yao Z, et al. Manganese Exposure Facilitates Microglial JAK2-STAT3 Signaling and Consequent Secretion of TNF-A and IL-1β to Promote Neuronal Death. *Neurotoxicology* (2018) 64:195–203. doi: 10.1016/j.neuro.2017.04.001

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Public Health

ORIGINAL RESEARCH
published: 26 April 2022
doi: 10.3389/fpubh.2022.883564

Epidemiology, Management, Quality of Testing and Cost of Syphilis in Germany: A Retrospective Model Analysis

Renata Šmit[1,2†], Nathalie Wojtalewicz[2†], Laura Vierbaum[2], Farzin Nourbakhsh[1], Ingo Schellenberg[2,3], Klaus-Peter Hunfeld[1,2‡] and Benedikt Lohr[1,2‡]*

[1] Northwest Medical Centre, Medical Faculty, Academic Teaching Hospital, Institute for Laboratory Medicine, Microbiology and Infection Control, Goethe University, Frankfurt, Germany, [2] INSTAND e.V. Gesellschaft zur Foerderung der Qualitaetssicherung in Medizinischen Laboratorien e.V, Duesseldorf, Germany, [3] Center of Life Sciences, Institute of Bioanalytical Sciences (IBAS), Anhalt University of Applied Sciences, Bernburg, Germany

OPEN ACCESS

Edited by:
Joseph D. Tucker, University of North Carolina at Chapel Hill, United States

Reviewed by:
Lorenzo Giacani, University of Washington, United States
Natasa Krsto Rancic, University of Niš, Serbia

***Correspondence:**
Renata Šmit
renata_smit@t-2.net

†These authors share first authorship
‡These authors share senior authorship

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

Received: 25 February 2022
Accepted: 31 March 2022
Published: 26 April 2022

Citation:
Šmit R, Wojtalewicz N, Vierbaum L, Nourbakhsh F, Schellenberg I, Hunfeld K-P and Lohr B (2022) Epidemiology, Management, Quality of Testing and Cost of Syphilis in Germany: A Retrospective Model Analysis. Front. Public Health 10:883564. doi: 10.3389/fpubh.2022.883564

Background: A multi-dimensional model can be a useful tool for estimating the general impact of disease on the different sectors of the healthcare system. We chose the sexually transmitted disease syphilis for our model due to the good quality of reported data in Germany.

Methods: The model included gender- and age-stratified incident cases of syphilis (in- and outpatients) provided by a German statutory health insurance company, as well as seroprevalence data on syphilis in first-time blood donors. Age standardized rates were calculated based on the standard German population. The test quality was assessed by extrapolating the number of false-positive and false-negative results based on data from Europe-wide external quality assessment (EQA) schemes. The model analysis was validated with the reported cases and diagnosis-related group (DRG)-statistics from 2010 to 2012. The annual direct and indirect economic burden was estimated based on the outcomes of our model.

Results: The standardized results were slightly higher than the results reported between 2010 and 2012. This could be due to an underassessment of cases in Germany or due to limitations of the dataset. The number of estimated inpatients was predicted with an accuracy of 89.8 %. Results from EQA schemes indicated an average sensitivity of 92.8 % and an average specificity of 99.9 % for the recommended sequential testing for syphilis. Based on our model, we estimated a total average minimal annual burden of €20,292,110 for syphilis on the German healthcare system between 2010 and 2012.

Conclusions: The linking of claims data, results from EQA schemes, and blood donor surveillance can be a useful tool for assessing the burden of disease on the healthcare system. It can help raise awareness in populations potentially at risk for infectious diseases, demonstrate the need to educate potential risk groups, and may help with predictive cost calculations and planning.

Keywords: syphilis, healthcare utilization database, blood donor database, Germany, retrospective model analysis, EQA, economic model

INTRODUCTION

Syphilis is a systemic disease caused by the bacteria *Treponema pallidum*. The pathogen can be transmitted via transplacental transmission, sexual contact with infectious lesions, and blood transfusions (1). Untreated or undetected infections can lead to severe health outcomes (f. e. neurosyphilis) and can even compromise pregnancy outcomes (including stillbirth and congenital syphilis) (2); thus it represents a serious health concern.

While treatment of syphilis is assessable and cost effective, the diagnosis of syphilis is challenging because traditional tools like cultivation and gram staining are not available (3). In addition, the clinical symptoms often indicate more than one possible differential diagnosis result (4, 5). Since the disease tends to manifest inconspicuously, an infection often remains undetected (6), resulting in an underassessment of infections. Currently, most syphilis cases are diagnosed through serological testing (7) and, in Germany, all laboratories are obliged to anonymously report treponemal pallidum positive serological test results to the Robert Koch Institute (RKI) (8). This offers a good insight into incident cases. Syphilis antibodies detected in blood donor samples must also be reported to the RKI (9). Transmission via transfusion has not happened for over 15 years in Germany (10). However, blood donor data is a useful tool in providing information on the seroprevalence of syphilis in the population, making it suitable for monitoring the effects of public health programs (11, 12).

Nevertheless, these sources offer only limited information on some aspects, like treatment patterns and loss of productivity due to sick leave. Claims data from statutory health insurance companies can close this information gap since they reflect real-life healthcare provisions better than clinical trials (13).

Combining different datasets into a model analysis is a helpful tool for developing recommendations and guidelines and for initiating effective public health measures (13).

The aim of this study is to provide a robust, multi-dimensional model analysis of the possible impact of syphilis on the German healthcare system in order to support healthcare decision-making by linking various health-related data sources.

It is the first study to combine cross-validated data from a German statutory health insurance company with information on seroprevalence derived from blood donor screening data from 2010 to 2012 and to conduct an evaluation based on actual data and diagnosis-related group (DRG) statistics, reported during this period. The claims data and blood donor data are normalized to the German population in the observed period of time as a retrospective model analysis to estimate the diagnostic and economic burden on the German healthcare system. The normalization of both datasets to population levels should act as an indicator of any underestimation as a result of either underreporting or underassessment of syphilis cases in Germany.

Abbreviations: ASR, age standardized rates; DAK-G, Deutsche Angestellten Krankenkasse-Gesundheit; DRG, diagnosis-related group; EBM, Einheitlicher Bewertungsmassstab; EQA, external quality assessment; GoAe, Gebuehrenordnung fuer Aerzte; RKI, Robert Koch Institute.

Furthermore, data from Europe-wide external quality assessment (EQA) schemes are used to access the current data on the quality of *in vitro* serological testing of *Treponema pallidum* and its impact on the German healthcare system. These EQA schemes are conducted by INSTAND, one of the three organizations in Germany designated as a reference institution by the German Medical Association.

MATERIALS AND METHODS

Analysis of Health Insurance Datasets

The basic dataset consisted of health insurance data from the German statutory health insurance company *Deutsche Angestellten Krankenkasse-Gesundheit* (DAK-G) from 2010 to 2012 and covered around 5.8 million people insured during the study period. The relevant international classification of disease (ICD-10-German Modification) for syphilis was used: syphilis (A50. - congenital syphilis, A51.x - early syphilis, A52.x - late syphilis, A53.x - other or unspecified syphilis). Data were available up to December 31, 2012 (**Supplementary Tables 1**, **2**). All analyses were based on anonymized subject-specific data. The personal data were exclusively handled by DAK- G in accordance with legal data protection requirements. Information on comorbidities was not included in this model, since we wanted to focus on the sole impact of syphilis. The quality of the data was checked for completeness, correct usage of inclusion criteria, and plausibility prior to analysis according to existing standards (14, 15). Incident cases of syphilis diagnosed on an inpatient and outpatient basis in 2010, 2011, and 2012 were analyzed and extrapolated to the German population. Incident cases were defined as follows: diagnostic code A50.x, A51.x, A52.x or A53.x, identifier "G" indicating a confirmed diagnosis (16) and the concurrent treatment with a suitable antibiotic (J01CE08, J01AA02, J01DD04) in the corresponding quarter of the year. Informed consent is not required for these analyses in Germany.

We extracted patient data (subject specifier, gender, year of birth, code for current residence, date of begin and end of insurance) and treatment procedures (inpatient, outpatient, medication). Additionally, data on productivity loss were included to assess possible indirect costs using the human capital method. Reported sick leave time of inpatients right before or after the hospitalization was attributed to the inpatient cohort.

Epidemiological Data

The reported *Treponema pallidum*-positive lab results are accessible in a simplified form via the German database SurvStat@RKI 2.0. The number of syphilis cases for 2010, 2011, and 2012 was retrieved from the database by age (5-year interval), gender and region (17). Residual titers of past infections, suspected double reporting, as well as suspected cases of insufficiently treated syphilis (*syphilis non satis curate*) were excluded from this dataset by RKI prior to the analysis (18).

The seroprevalence of syphilis in blood donors was kindly provided by RKI and is based on blood donor surveillance data (9). The number of positive blood donors was calculated based on the reported seroprevalence rates and the corresponding number of blood samples. We only analyzed data on new blood donors as

repeat blood donors are repeatedly screened. Thus they are less likely to be infected and are considered a low-risk population for blood-borne diseases (19). It should be noted that the statistical power of this small study population would be insufficient for further extrapolations.

German EQA Schemes for *Treponema* Pallidum

Between 2010 and 2012, six EQA surveys for syphilis were conducted by the German Society for Promoting Quality Assurance in Medical Laboratories (INSTAND) in cooperation with the central reference laboratory at the Institute for Laboratory Medicine, Microbiology & Infection Control at the Northwest Medical Center, Frankfurt/Main (Germany) and with the six reference laboratories of the Bacteriologic Infection Serology Study Group of Germany (BISSGG). Previous reports summarize the organization, structure and detailed evaluation procedures of the German EQA program for bacteriologic infection serology (20–22).

Participants can report qualitative and quantitative results together with additional information on the test kit provider, lot number and laboratory equipment used. In this study, the accuracy of the qualitative as well as the quantitative results were evaluated for TPPA, TPHA, VDRL, FTA-abs IgG and FTA-abs IgM. All EQA samples are derived either from patients with a confirmed diagnosis of syphilis or from healthy blood donors, where absence of *Treponema pallidum* antibodies was confirmed prior to the EQA survey.

Statistical Analysis

The data on the insured individuals and the epidemiological data were stratified by gender, age (<25, 25–34, 35–44, 45–54 and >54) and, in the case of the health insurance dataset, 5-digit postal codes. The data were standardized to the general population of Germany for the corresponding years. German population data for 2010, 2011, and 2012 were obtained from the official reports published by the Federal Statistical Office (www.destatis.de). Age-distributed annual incidences were calculated. For the model analysis, age standardized incidence rates (ASR) from the health insurance dataset and age standardized seroprevalence rates from the blood donor dataset were calculated for 2010, 2011, and 2012 to allow comparisons to be drawn with the incidence of reported cases. 95 %-confidence intervals were calculated based on the assumption of a Poisson distribution of the reported cases (23). Congenital syphilis was excluded from the standardization of the population level due to the low numbers and the thorough screening system for pregnant women in Germany.

To calculate the averages for sensitivity, specificity and accuracy (pass rate) from the German EQA survey data (**Supplementary Table 3**), the reported diagnoses of sample donors were used as the "gold standard". Average net sensitivity and average net specificity were calculated by sequential testing (two-stage screening) using TPHA/TPPA or ELISA as the first test and FTA-abs IgG, IgG Blot, IgG ELISA methods or TPHA/TPPA as the second test. This test algorithm is currently recommended in Germany (16, 24). Average sensitivity and average specificity were used to calculate false positives, false negatives, true positives, and true negatives based on the standardized incidence or, in the case of blood donors, the seroprevalence. Positive predictive values (PPV) and negative predictive values (NPV) were calculated using Bayes' theorem (25).

Cost Analysis

For this study, we calculated direct medical costs for inpatient and outpatient treatment, screening, and confirmatory testing, as well as indirect costs from loss of productivity for 1 year. Indirect costs from loss of productivity were calculated using existing German standards (Hanover consent). Our estimates of indirect costs were based on average earnings (€ 3,014) (26) and the median number of productivity days lost for German syphilis patients aged between 18 and 64. Short-term (< 3 days) absence from work without a doctor's note was not included due to lack of data. The serological testing costs for statutory health insurance patients (∼ 90 % of the German population) and the blood donor population were calculated using the diagnostic claims code "*Einheitlicher Bewertungsmassstab*" (EBM) (27), while the costs for the serological tests for the privately insured patients (∼10 % of the population) were calculated based on "*Gebuehrenordnung fuer Aerzte*" (GoAe) (28). The percentage of antibiotics prescribed in our insurance dataset were used to calculate medication costs (**Supplementary Table 4**). We were unable to calculate treatment costs for patients coded with unspecified syphilis (A53.x), since we had no information about the detailed dosage and duration of therapy. Therefore, these patients were excluded from the cost analysis. Total costs calculated from the claims data were extrapolated to the German population.

RESULTS

Summary of Reported Cases of Syphilis From all Datasets

Table 1 shows the general distribution of syphilis cases in all datasets, including basic characteristics of the corresponding populations. The average ages of the DAK-G cohort and the German population were comparable, while the first-time blood donor cohort was notably younger. The insurance dataset consisted of a higher proportion of women to men than the other two datasets.

The RKI data showed higher male-to-female ratios in the reported incident syphilis cases than the other two datasets, while the average age of syphilis patients was highest in the DAK-G dataset. The age-stratified distribution of the syphilis cases from the individual datasets are presented in **Figures 1**–**3**.

The reported cases show the highest number of cases for men in the age group 35–44 years; the highest number of cases for women is between 25 and 34 years. In contrast to the reported cases, the number of incident cases in the DAK-G dataset was highest in the older age groups, especially for women, where most cases were observed in the age group > 54 years. The largest proportion of all seropositive new donors was seen in the age group 35–44 for both sexes.

TABLE 1 | Basic characteristics of the datasets used for this model analysis for 2010 to 2012.

	2010	2011	2012	Average
(a)				
Population	81,751,602	80,327,900	80,523,746	80,867,749
Male to female ratio	1:1.0	1:1.1	1:1.1	1:1.0
Average age men	42	42	42	42
Average age women	44	45	45	45
No. of syphilis cases	4,077	4,633	5,012	4,574
Incidence rate / 100,000 person-years	5.0	5.8	6.2	5.7
Male to female ratio	10.0:1	11.8:1	12.5:1	11.5:1
Average age cases men	40	40	40	39
Average age cases women	39	39	37	39
	2010	**2011**	**2012**	**Average**
(b)				
Population	6,119,470	5,800,795	5,683,710	5,867,922
Male to female ratio	1:1.5	1:1.5	1:1.5	1:1.5
Average age men	41	41	41	41
Average age women	48	48	48	48
No. of syphilis cases	438	359	317	371
Incidence rate / 100,000 person-years	7.2	6.2	5.6	6.3
Male to female ratio	2.7:1	3.3:1	3.1:1	3.2:1
Average age cases men	48	47	48	48
Average age cases women	66	62	65	65
	2010	**2011**	**2012**	**Average**
(c)				
No. of samples from first-time donors	561,642	542,492	496,771	533,635
Male to female ratio	n.l.	1:1.0	1:1.0	1:1.0
Average age men	n.l.	n.l.	n.l.	26
Average age women	n.l.	n.l.	n.l.	26
No. of anti-Treponema positive samples	236	223	221	227
Seroprevalence / 100,000 blood samples	42.1	41.1	44.4	42.5
Male to female ratio	2.2:1	1.7:1	2.1:1	1.9:1
Average age cases men	1 37	37	37	37
Average age cases women	1 43	41	44	43

[a] *Reported data on total German population (www.destatis.de) and reported syphilis cases (RKI) (17).*
[b] *DAK-G dataset.*
[c] *First-time blood donors (blood donor surveillance, hosted by the RKI) (12, 29). Missing data was coded n.l. (no information).*

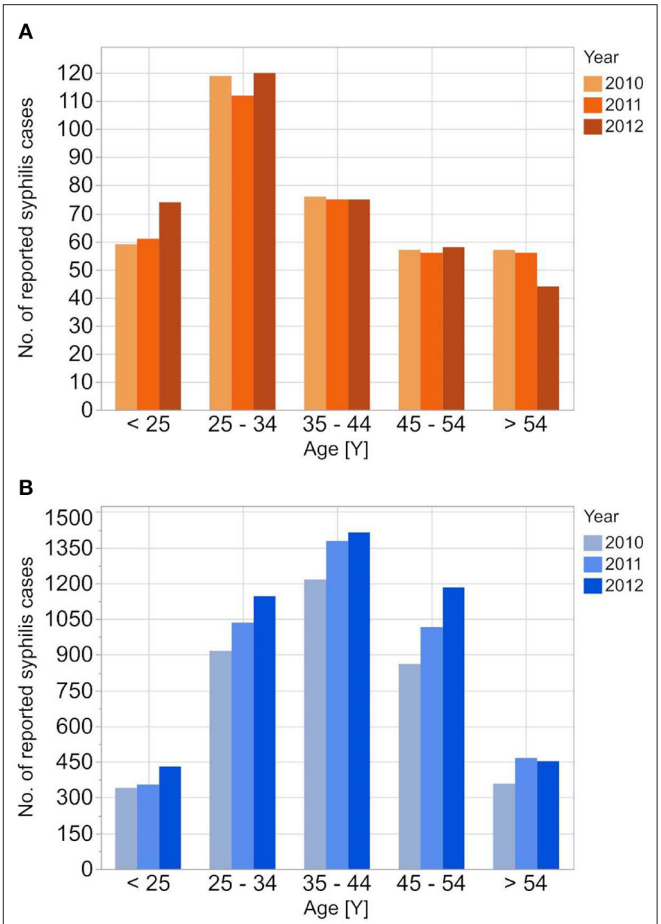


FIGURE 1 | Age stratified reported incidences of syphilis reported to RKI from 2010 to 2012 for the female **(A)** and the male **(B)** population.

The insurance cohort included more detailed information about the diagnosis as well as the form of therapy. Of all incident diagnoses, 87 % were outpatients and 13 % inpatients. Most cases were coded with either early syphilis (33 %), late syphilis (34 %) or other/unspecified syphilis (30 %), while 3 % of the incident diagnoses were coded congenital syphilis.

Extrapolation to Population Level

The syphilis incidence of the insurance and the donor datasets was extrapolated to the population level by calculation of age standardized rates (ASR), since the age structure of both study populations differs from that of the reference population.

The ASR for syphilis incidence based on the insured population dropped from 7.4 [CI: 6.7–8.1] incident cases per 100,000 standardized person-years in 2010 to 5.9 [CI: 5.2–6.5] incident cases per 100,000 standardized person-years in 2012.

In the case of first-time donors, the estimated seroprevalence rate rose from 73.7 [CI: 57.4–89.9] cases per 100,000 standardized person-years to 82.9 [CI: 64.5 - 101.3] cases per 100,000 standardized person-years (**Table 2**, **Figure 4**).

Geographical Distribution of Estimated Cases

Since the insurance dataset contained the patient's current postal code, it was possible to extrapolate a geographical distribution

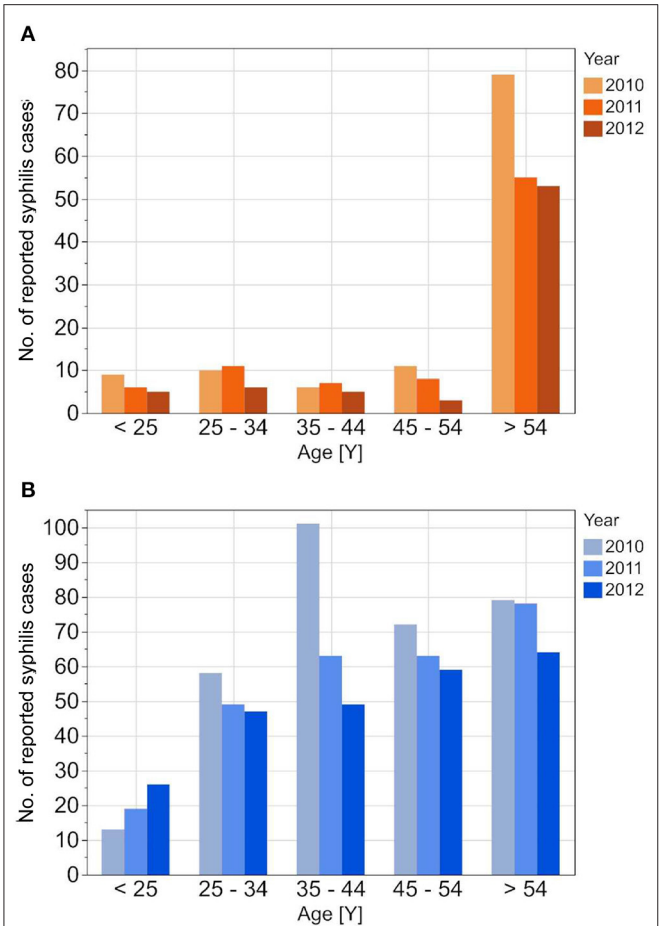


FIGURE 2 | Age stratified reported incidences of syphilis in DAK-G insurance data set from 2010 to 2012 for the female **(A)** and the male **(B)** insured population.

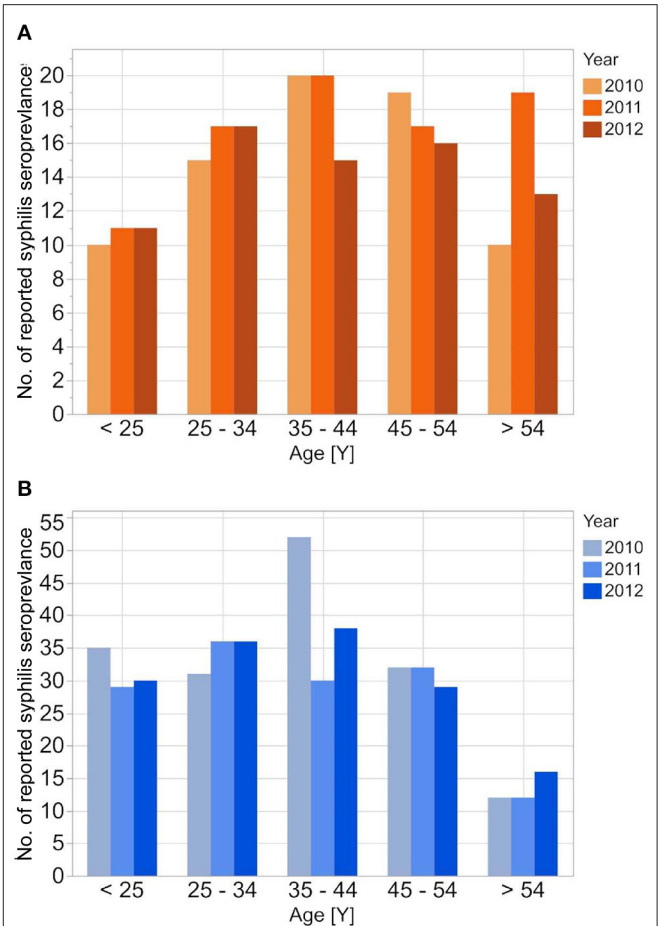


FIGURE 3 | Age stratified reported seroprevalence of syphilis in first time blood donors from 2010 to 2012 for female **(A)** and male **(B)** donors.

TABLE 2 | ASR for the insurance data set and the first-time blood donor cohort from 2010 to 2012.

Dataset	Year	ASR/100.000 person-years	95 %-CI
DAK-G	2010	7.4	6.7 - 8.1
	2011	6.3	5.6 - 6.9
	2012	5.9	5.2 - 6.5
First-time blood donors	2010	73.7	57.4 - 89.9
	2011	78.0	60.8 - 95.2
	2012	82.9	64.5 - 101.3

of the expected syphilis cases based on the extrapolated and standardized insurance data (**Figure 5A**).

The highest standardized incidence was estimated for Berlin (> 12 expected cases per 100,000 standardized person-years). Hamburg, Bremen, North Rhine-Westphalia, Hesse and Rhineland-Palatinate showed between 6 and 12 expected cases per 100,000 standardized person-years. All other federal states showed a lower estimated incidence rate. This distribution is in line with the Germany-wide pattern of the actual incidence cases reported to RKI (**Figure 5B**).

Results From Germany's Proficiency Testing Program

We analyzed the data from six Europe-wide EQA surveys for *Treponema pallidum* antibodies conducted between 2010 and 2012. In this period, an average of 1,033 laboratory results were reported per year (2010: 892; 2011: 1,191; 2012: 1,014). The mean accuracy rate of the qualitative test results (96.4 %; range 78.0–100 %) was slightly higher than that of the quantitative test results (94.0 %, range 74.5–100 %). The accuracy rate for the different detection methods used in the EQA schemes is displayed in **Figure 6.**

In terms of the qualitative results, the cardiolipin detection had the highest average accuracy (98.6 %, range 91.7–100 %) and the IgG-ELISA had the lowest (94.0 %, range 78.0–97.6 %). In the case of the quantitative results, Cardiolipin tests also showed the

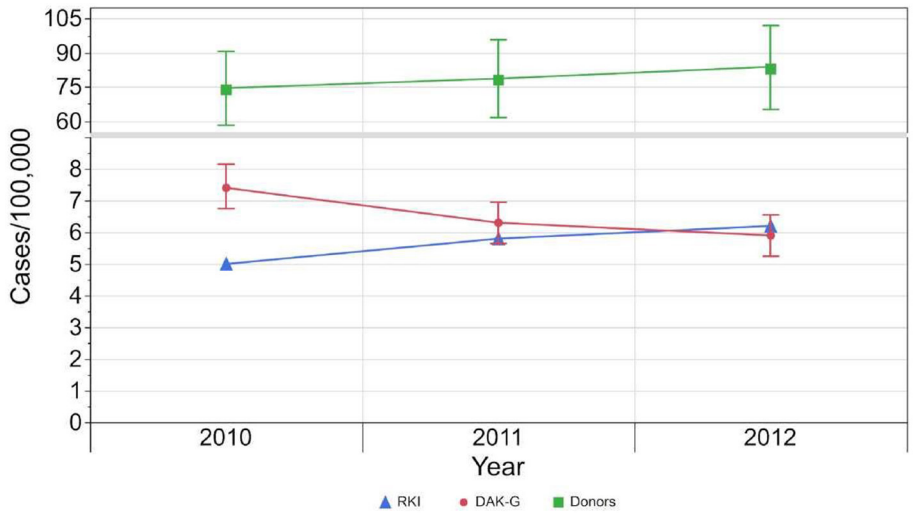


FIGURE 4 | Development of incident cases reported to RKI (blue), standardized incident cases based on health insurance data set (red) and standardized seroprevalence (green) per 100,000 standardized population from 2010 to 2012. The 95 %-confidence intervals of the estimated results per 100,000 standardized person-years are indicated by the whiskers.

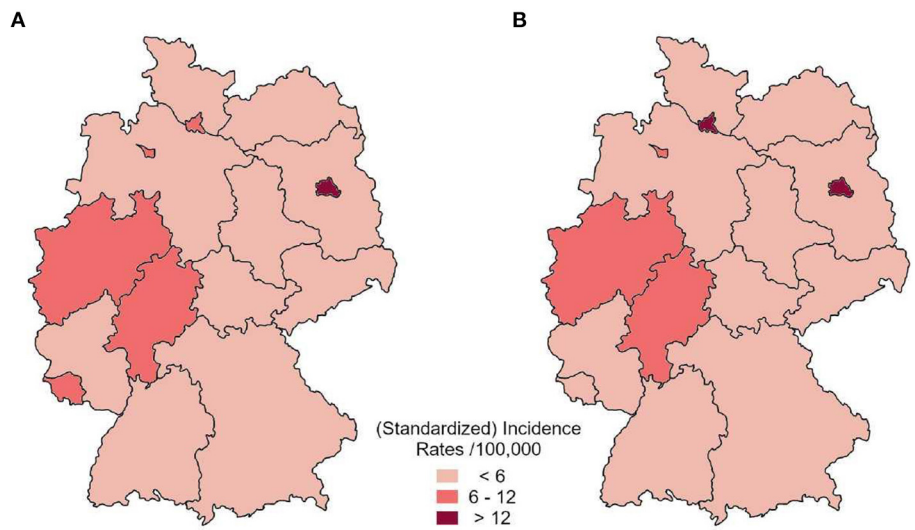


FIGURE 5 | Distribution of average annual standardized incidence by federal state based on the health insurance dataset **(A)** in comparison to reported incident cases to RKI **(B)** between 2010 and 2012.

highest average accuracy (98.0 %, range 88.5–100 %) and the IgG-FTA-abs detection methods showed the lowest average accuracy rate (90.6 %, range 74.5–100 %).

All serum samples used in these EQA schemes were derived from single donors with a clear clinical history (20–22), which enables the basic performance parameters for the different test methods to be calculated for the model. The observed average sensitivity for the recommended sequential testing for syphilis via qualitative test methods was 92.8 % and the combined specificity was 99.9 %.

Assessment of Possible Effect of Test Quality on Estimated Cases

Our estimated incidence rate for syphilis (6.5/100,000 standardized person-years) would result in an estimated 5,256 syphilis cases for the German reference population. In our model, the average sensitivity (92.8 %) and specificity (99.9 %) of sequential screening for syphilis antibodies in the EQA results leads to in 4,880 true positive and 64,195 false positive cases. The specificity 80,798,298 true negative and 377 false negative test results in the German reference population.

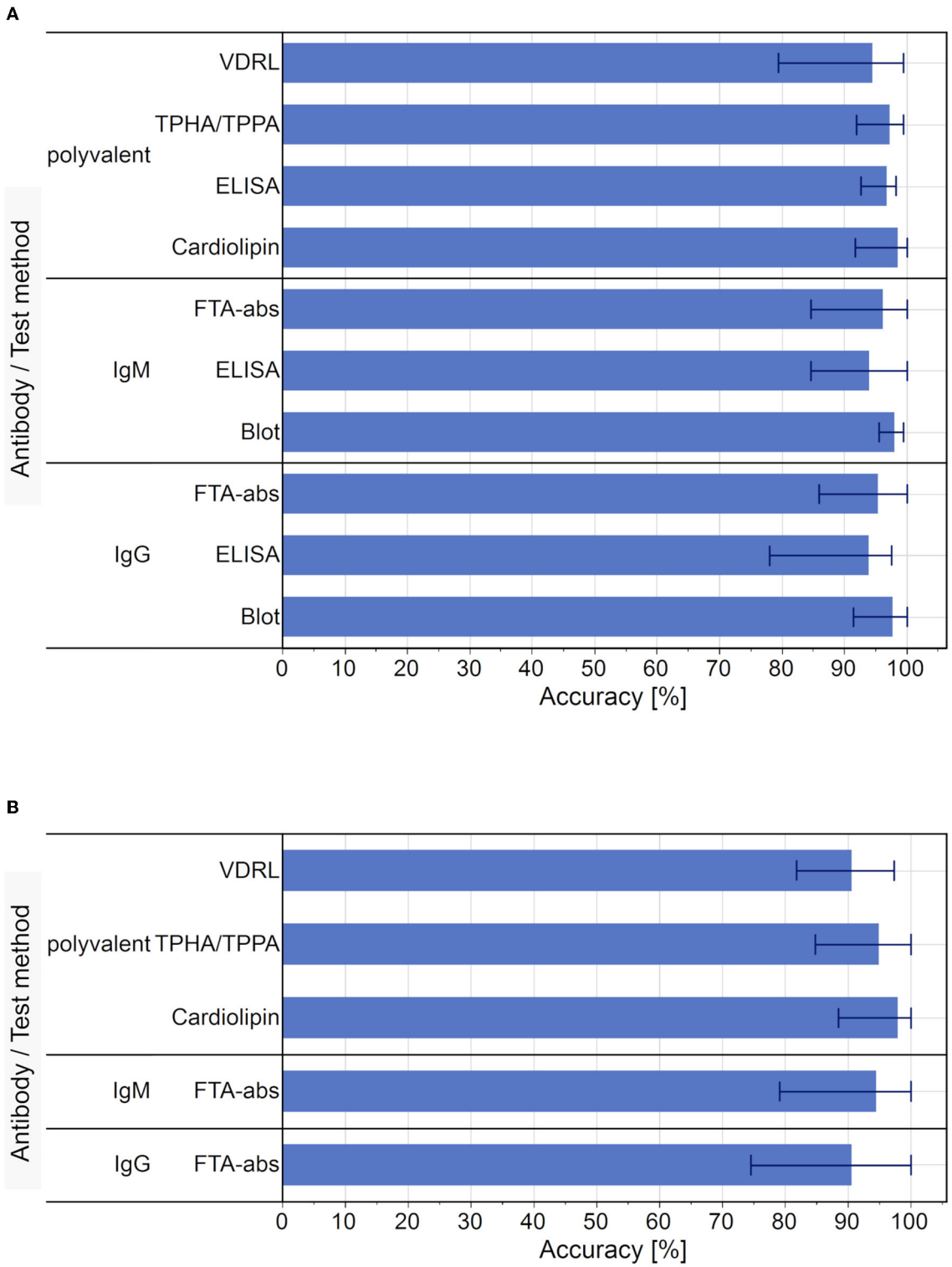


FIGURE 6 | Average accuracy rate of test results for each test used in the EQA surveys, 2010 to 2012. **(A)** Average accuracy of qualitative test results. Bar markers indicate the range of results. **(B)** Average accuracy of quantitative test results. Bar markers indicate the range of results. Blot and ELISA tests were not performed as part of the quantitative diagnostic testing for syphilis.

These quality parameters of the test algorithm and the estimated average incidence for syphilis in Germany would result in a PPV of 7.1 % and an NPV of > 99.9 % if the test algorithm were used for population-wide screening purposes.

When the estimated seroprevalence based on first-time blood donors (78.2/100,000 standardized person-years) was used to calculate both PPV and NPV for a possible population-based screening for seroprevalence of syphilis antibodies, the PPV would increase to 47.7 % while the NPV would remain at > 99.9 %.

Economic Evaluation of the Insured Population

Based on the insurance dataset, we calculated the average annual direct and indirect costs for syphilis in our insurance population.

Between 2010 and 2012, an average of 72,234 tests were performed each year (TPHA/TPPA & ELISA: 93 %, VDRL test: 7 %, FTA-abs IgG test & blots: 1 %). As the average cost for testing statutory insured outpatients was €4.96 per test, the average annual costs for screening and confirmatory testing amounted to €358,087.

In the insured population, an average of 312 incident cases of syphilis, without syphilis connata were diagnosed in an outpatient setting. 108 cases were treated as early and 106 as late syphilis; both groups were treated with benzathine penicillin, doxycycline and ceftriaxone in accordance with German treatment guidelines (12). Since the exact treatment regime of the average 98 patients with unspecified syphilis (A53.x) was unknown, they were excluded from this analysis. The average annual per-patient cost of benzathine penicillin, doxycycline or ceftriaxone in outpatients was calculated at €33.24, €9.40 and €184.70 respectively for early syphilis and €99.72, €18.80 and €258.58 respectively for late syphilis. This led to an estimated average annual cost for early and late outpatient treatment of €3,584 and €3,455 respectively. The average annual number of inpatients was 49 patients with an individual median length of stay of 6 days per year (min. 1 day, max. 55 days) average annual cost of €118,503.

236 outpatient and 40 inpatient cases in the age group of 18 to 64 years were included in the calculation of productivity loss on the basis of an average cost of €100.40 per sick day in accordance with the Hanover consent. During 2010 and 2012, inpatients were absent from work for a median of 6 days per year and patient (min. 1 day, max. 446 days), while 4.7 % of all observed outpatients were absent from work for a median of 15 days per year and patient (min. 1 day, max. 546 days), resulting in average annual indirect costs of €24,096 for inpatients and €16,705 for outpatients.

Taken together, the average annual burden for our insurance population was €524,430 for the observed period (**Table 3**).

TABLE 3 | Economic burden of syphilis in the insurance study population. Missing data was coded n.I. (no information).

Average annual expected costs (€)	
Direct costs	
Treatment, outpatients (*N* = 312)	
Early (A51.x) (*N* = 108)	3,584
Late (A52.x) (*N* = 106)	3,455
Unspecified (A53.x) (*N* = 98)	n.I.
Total treatment, outpatients	7,039
Screening and confirmatory testing, outpatients (*N* = 72,234)	
Total screening and confirmatory testing, outpatients	358,087
Inpatient care (*N* = 49)	118,503
Total direct costs	***483,630***
Indirect costs (*N* = 276)	
Productivity losses[a], outpatients (*N* = 236)	16,705
Productivity losses[a], inpatients (*N* = 40)	24,096
Total indirect costs	***40,801***
Total cost of syphilis	***524,430***

[a] *Productivity losses are costs due to the incapacity to work.*

Extrapolated Economic Evaluation of Syphilis in Germany

The expected average number of 5,256 incident cases of syphilis per year is based on the German standard population and our estimated incidence rate of 6.5/100,000 standardized person-years. Using the inpatient-outpatient ratio from our insurance population as a reference, this would result in an average of 4,573 outpatients and 683 inpatients annually when extrapolated to the German standard population. This would produce expected treatment costs for outpatients of €99,960. The treatment of the expected inpatients would lead to an average annual economic burden to healthcare of €1,652,466.

Based on our insurance data, it was estimated that an average of 1,006,727 tests would be performed, leading to a total average annual cost for screening and confirmatory testing of €5,787,886 for outpatients. Since about 10 % of the German population is covered by private health insurance, the total estimated costs for laboratory tests consist of €1,296,279 for privately insured persons and €4,491,607 for persons covered by statutory health insurance. The differences in costs were based on the different fee catalogs of the statutory and private health insurance companies in Germany (27, 28).

Based on average population data, 67,2% of all patients were estimated to be of working age (age group 18 to 64 years), leading to a total of 3,074 expected outpatients and 460 expected inpatients. As for social costs, we estimated the total average indirect costs to be €494,375 annually.

Thus, the total average cost of syphilis for the entire German population is estimated to be €8,034,688 annually during our study period (**Table 4**).

With respect to the cost of screening and testing in the blood donor population, 2,472,587 tests were performed on blood donors in Germany, leading to a further €12,257,422 in screening and confirmatory testing costs.

Taken together, the cost for blood sample screening as well as the number of estimated costs based on health insurance data in our model would result in an estimated annual cost of €20,292,110.

TABLE 4 | Expected economic burden of syphilis in Germany. Missing data was coded n.I. (no information).

Average annual expected costs (€)	
Direct costs	
Antibiotic treatment, outpatients (*N* = 4,573)	
Early (A51.x) (*N* = 1,534)	50,894
Late (A52.x) (*N* = 1,505)	49,066
Unspecified (A53.x) (*N* = 1,534)	n.I.
Total treatment, outpatients	99,960
Screening and confirmatory testing, outpatients (*N* = 1,006,727)	
Statutory insured (*N* = 906,054)	4,491,607
Privately insured (*N* = 100,673)	1,296,279
Total screening and confirmatory testing, outpatients	5,787,886
Inpatient care (*N* = 683)	1,652,466
Total direct costs	***7,540,312***
Indirect costs (*N* = 3,534)	
Productivity losses[a], outpatients (*N* = 3,074)	217,623
Productivity losses[a], inpatients (*N* = 460)	276,752
Total indirect costs	***494,375***
Total cost of syphilis	***8,034,688***

[a]*Productivity losses are costs due to the incapacity to work.*

DISCUSSION

In this paper, we present a multi-dimensional retrospective model analysis that combines several data sources to estimate the expected burden of syphilis on the German healthcare system. This includes an economic estimation, which we evaluated based on data for the years 2010 to 2012.

Since all syphilis-positive diagnoses must be reported anonymously to the RKI, we have an overview of incident cases in Germany. From 2010 to 2012 the incidence rose from 5/100,000 person-years to 6.2/100,000 persons with an average of 5.7 cases/100,000 inhabitants (17). Meanwhile, the incidence rate in our insurance dataset declined from 7.2/100,000 cases to 5.6/100,000 cases. This contrasting development of incidences was most likely due to the differences in the two populations as well as the composition and number of members of the German statutory healthcare providers (e.g., socio-economic status, gender distribution) (13).

Both populations furthermore differed in age structure and therefore we used the ASR approach to calculate the expected incidence rate for the German population between 2010 and 2012 based on our insurance dataset. The estimated incidence rates were 7.4 [CI: 6.7–8.1] incident cases per 100,000 standardized person-years in 2010, which dropped to 5.9 [CI: 5.2–6.5] incident cases per 100,000 standardized person-years in 2012, with an average of 6.5 [CI: 5.8–7.2] incident cases per 100,00 standardized person-years in this study period.

The higher incidence rate in our insurance cohort in comparison to the reported cases could be an indication of a slight underreporting of syphilis cases in Germany. This could be due to the fact that only positive laboratory results have to be reported (8), not the clinical diagnosis in general, which is why certain data could be missing in the RKI dataset. Furthermore, the RKI data do not include cases of residual titers of past infections, suspected double reporting, or suspected cases of insufficiently treated syphilis (*syphilis non satis curate)* (18). Taken together, a slightly higher incidence within the claims data was to be expected. This highlights the usefulness of the inclusion of health insurance data in these models, since all the excluded cases can also impact the healthcare system–be it further testing or treatment costs in the case of *syphilis non satis curate*.

The unusually high number of syphilis cases among the older female insurance population could have several reasons: it could be an artifact within the dataset, or the age group might consist of a bigger proportion of women at a higher risk of being infected with syphilis, or it could be a remnant of the surviving World War II generation. Since *Treponema pallidum*-specific antibodies show a lifelong persistence regardless of treatment status (30), a rising seroprevalence of *Treponema pallidum*-specific antibodies is not uncommon in aging populations (31–33).

Despite the possible limitations of our dataset, we were able to identify regional hotspots of syphilis infections in the different federal states in Germany in line with the actual reported data. In particular, the higher number of syphilis cases in this region could have been an indicator of a rise in cases since the reported incidence in the federal state of Rhineland-Palatinate rose from 3.8/100,000 person-years to 5.0/100,000 person-years (+ 31.6 %) from 2012 to 2013 (17).

Since the realization that *Treponema pallidum* could be transmitted via blood, screening tests for syphilis are routinely performed (34) and it is still mandatory for a blood donor sample to be negative for *Treponema pallidum* antibodies before it can be released for donation (35). In our study period, the mean seroprevalence for *Treponema pallidum* specific antibodies in first-time blood donors was 42.5/100,000 donations (0.04 % of all samples) and therefore notably higher than the reported incidence rates. These numbers were still considerably low in comparison to other countries, like the USA (36) or less industrialized countries (32, 37, 38). Data from blood donor surveys are a suitable tool for monitoring infectious disease (11, 12) and might reflect the German population better than our insurance cohort alone. We concentrated our model on first-time blood donors since they are less monitored than repeat donors, are often unaware of their behavioral risks (38, 39), and are less likely to be affected by selection bias (36).

After ASR normalization, the seropositive rate rose to an average seroprevalence rate of 78.2 [CI: 61.1-95.4]/100,000 person-years. Since a thorough anamnesis of current and past infections, including syphilis, is mandatory in Germany (35), one could assume that the observed seroprevalence indicates a high factor of underassessment in the German population. But since the anamnesis is mostly done by questionnaires, it is prone to errors or information bias. Other publications have already highlighted the fact that donors sometimes give false or insufficient information about their health history and possible risk factors (40–42). In Germany, a notable number of male seropositive donors stated that their way of potentially becoming infected was having sex with other men (MSM), a group that was originally excluded from blood donations at the time due to their

high risk for STIs (12, 29). Therefore, the data might include people from risk groups as well as people who have already recovered from a syphilis infection. As the assessment is made by directly testing for syphilis, which detects lifelong acquired anti-*Treponema* antibodies, it is not possible to distinguish between a cured or an active infection, as discussed above with respect to the older insurance cohort.

We used the data on laboratory diagnosis, therapy and days absent from work from the insurance dataset to estimate the possible direct and indirect economic burden of syphilis for Germany in our study period. The average annual costs in our insurance cohort amounted to €524,430 in our study period. When extrapolated to the corresponding German population, this would amount to an estimated total annual economic burden for the diagnosis and treatment of syphilis of €8,034,688.

Combining this with the costs for screening all blood donor samples (first-time and repeated), the total estimated economic burden of syphilis based on our model would amount to €20,292,110. In comparison to the average annual total healthcare costs from 2010 to 2012 as 29,730 Mio. € (https://www.gbe-bund.de/), the individual contribution of syphilis might be small, but one should keep in mind that the general incidence of syphilis in Germany was quite low at that time. A rising incidence could change the importance of this infectious disease within a few years, so these model analyses are a useful tool to estimate the expected economic burden.

In our study we used data from 2010 to 2012, because these data were available for our research. Notably, the average number of reported cases from RKI in the period from 2013 to 2019 was almost doubled in comparison with the average number of reported cases in the period from 2010 to 2012, with a peak of 7880 cases in 2019. This 1.5-fold increase in cases would result in €2.6 treatment costs for in- and outpatients. In addition the test costs would rise to 8.7 mio. Forthermore, from the societal point of view, we projected the total indirect costs to be 681,285 €. Thus, the total average cost of syphilis for the entire German population is projected to be nearly €12 Mio, without including blood donor testing costs.

To test the extrapolations of our insurance cohort, we compared the number of expected syphilis inpatients with the number of actual reported cases. Despite the limitations of our insurance cohort, the extrapolation of an average of 683 expected inpatients per year underestimated the actual number of patients reported under the DRG by 10.8 % (on average 766 cases per year) (43).

Finally, we combined the extrapolated data from the insurance dataset with the accuracy of the test systems observed in the evaluation of international EQA surveys conducted by INSTAND between 2010 and 2012. The EQA schemes for syphilis antibodies are performed with sample material from single donors with a clear medical history (20–22), which makes bias due to matrix effects unlikely.

The various test systems showed high accuracies for qualitative (96.4 %, range: 78.0 %- 100 %) und quantitative (94.0, range 74.5–100 %) test results, which are slightly higher than previously reported results (44). Using the known diagnosis for the EQA sample as a gold standard, we calculated the average sensitivity and specificity within the observed period to be 92.8 and 99.9 %, respectively.

The use of the reverse test algorithm for *Treponema pallidum* on the basis of the reference population would result in 64,195 false-positive results and 377 false-negative results. While the false-negative results could lead to a further rise in syphilis cases due to transmission, the falsepositive results would result in overtreatment.

Our study has some limitations. First, the insurance dataset differed from the German reference population with respect to age and male-to-female ratio. German statutory healthcare providers differ in terms of composition (e.g., socio-economic status, gender distribution) and number of members (13); therefore, the use of data from just one insurance institution could not be representative enough and data from further insurance providers should be included in future models to strengthen the validity.

We were unable to adjust for possible differences in socio-economic status of both populations because the information was missing in the DAK-G dataset. A low socio-economic status is a risk factor for syphilis infection (31, 36) and an unnoted difference between both datasets could contribute to the observed differences in the estimated and reported incidence rates. We did not include costs derived from possible coinfections, like HIV, even though an active syphilis infection is a known risk factor for the transmission of HIV (45, 46). This impact could be quite notable due to the high costs of anti-retroviral therapy (47), but we wanted to develop a model focusing on syphilis, which should be more easily transferable to other infectious diseases. Furthermore, we were unable to include the impact of short-term absence from work (> 3 days) since this information was missing as well. The limitation of the blood-donor dataset includes a possible underestimation of the seroprevalence based on this exclusion criteria for risk groups (12). Since we were missing detailed information about the treatment regime for outpatients coded with unspecified syphilis (A53.x), we could not include them in our economic evaluation of the direct costs. This leads to a slight underestimation of direct treatment costs for outpatients.

The strength of our model concerning the linkage of real-life population data, based on claims data from a large statutory health insurance company in Germany, with surveillance data from blood donors and the observed test quality based on international EQA schemes. Futhermore, our results show a good condordance with corresponding DRG data.

These results show the usefulness of claims data in estimating the (economic) burden of a disease on the corresponding healthcare system, especially since they often include data other than health surveys, including most prominently a treatment algorithm. Our model was able to predict the expected number of syphilis inpatients with an accuracy of 89.2 %. The assessment of the 'real-life' test quality, as estimated based on our EQA schemes, is a useful tool to further estimate the possible impact of overtreatment and possible underestimation based on test quality.

CONCLUSIONS

The linkage of claims data, results from EQA schemes and screening information, such as blood donor surveillance, can be a useful tool for assessing the burden of certain diseases like syphilis on the healthcare system–including the financial burden and indirect economic burden due to absence from work. This model might help to raise the awareness of health care professionals in special geographic regions.

Furthermore, it can help to raise awareness in possible at-risk populations and may help to estimate the minimum future budgets. The use of old datasets is useful to test the strengths and weaknesses of such model analysis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Goethe University Frankfurt (Main), which approved the usage of samples from voluntary blood donors to be used for the EQA schemes. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

RŠ, FN, BL, and K-PH contributed to conception and design of the study. RS, NW, LV, and BL performed the statistical analysis. RS and NW wrote the first draft of the manuscript. RŠ and NW share the first authorship. KPH and BL contributed equally to this work and share the last authorship. All authors contributed to manuscript revision, read and approved the submitted version.

FUNDING

The research was performed as part of an employment of the authors and by the INSTAND grant VF-01-KPH-2017.

ACKNOWLEDGMENTS

The authors would like to thank the DAK-G as well as the RKI for providing the data for this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.883564/full#supplementary-material

REFERENCES

1. WHO. *WHO Guidelines for the Treatment of Treponema pallidum (Syphilis).* Geneva: World Health Organization 2016. (2016).
2. ECDC. *Syphilis and congenital syphilis in Europe -A review of epidemiological trends (2007-2018) and options for response*. Stuckholm: European Centre for Disease Prevention and Control. (2019).
3. Cohen SE, Klausner JD, Engelman J, Philip S. Syphilis in the modern era: an update for physicians. *Infect Dis Clin North Am.* (2013) 27:705–22. doi: 10.1016/j.idc.2013.08.005
4. Peeling RW, Hook EW. 3rd. The pathogenesis of syphilis: the Great Mimicker, revisited. *J Pathol.* (2006) 208:224–32. doi: 10.1002/path.1903
5. Barnett R. Syphilis. *Lancet.* (2018) 391:1471. doi: 10.1016/S0140-6736(18)30833-X
6. Peeling RW, Mabey D, Kamb ML, Chen XS, Radolf JD, Benzaken AS. Syphilis. *Nat Rev Dis Primers.* (2017) 3:17073. doi: 10.1038/nrdp.2017.73
7. Ghanem KG, Ram S, Rice PA. The Modern Epidemic of Syphilis. *N Engl J Med.* (2020) 382:845–54. doi: 10.1056/NEJMra1901593
8. Verbraucherschutz BfJu. *Gesetz zur Verhuetung und Bekaempfung von Infektionskrankheiten beim Menschen (Infektionsschutzgesetz - IfSG)*. Available online at: Bundesministeriun fuer Justiz und Verbraucherschutz. (2020).
9. Verbraucherschutz BdJuf. *Gesetz zur Regelung des Transfusionswesens (Transfusionsgesetz - TFG).* Available online at: Bundesministerium der Justiz und fur Verbraucherschutz (accessed 25 Nov, 2020).
10. Robert-Koch-Institut. *Blutsicherheit, haeufig gestellte Fragen*. (2014). Available online at: https://www.rki.de/DE/Content/Infekt/Blut/Blutsicherheit/FAQ_node.html
11. Use CfMPfH. *Guideline on Epidemiological Data on Blood Transmissible Infections*. London: European Medicines Agency. (2010).
12. Offergeld R, Ritter S, Hamouda O. HIV, HCV, HBV and syphilis surveillance among blood donors in Germany 2008-2010. *Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz.* (2012) 55:907–13. doi: 10.1007/s00103-012-1516-1
13. Neubauer S, Kreis K, Klora M, Zeidler J. Access, use, and challenges of claims data analyses in Germany. *Eur J Health Econ.* (2017) 18:533–6. doi: 10.1007/s10198-016-0849-3
14. Sorensen HT, Sabroe S, Olsen J, A. framework for evaluation of secondary data sources for epidemiological research. *Int J Epidemiol.* (1996) 25:435–42. doi: 10.1093/ije/25.2.435
15. Swart E, Gothe H, Geyer S, Jaunzeme J, Maier B, Grobe T, et al. Gute praxis sekundardatenanalyse (GPS): leitlinien und empfehlungen. *Das Gesundheitswesen.* (2015) 77:120–6. doi: 10.1055/s-0034-1396815
16. Schoefer H, Enders M, Esser S, Feiterna-Sperling C, Hagedorn HJ, Magistro G, et al. Diagnosis and treatment of syphilis : Update of the S2k guidelines 2020 of the German STI Society (DSTIG) in cooperation with the following specialist societies: DAIG, dagnä, DDG, DGA, DGGG, DGHM, DGI, DGN, DGPI, DGU, RKI]. *Hautarzt*. (2020) 71:969–99. doi: 10.1007/s00105-020-04672-6
17. Robert-Koch-Institut. *SurvStat@RKI 2.0: RKI*. Available online at: https://survstat.rki.de.
18. Koch-Institut R. *Infektionsepidemiologisches Jahrbuch meldepflichtiger Krankheiten fur*. Berlin (2019). Available online at: https://www.rki.de/DE/Content/Infekt/Jahrbuch/jahrbuch_node.html
19. Baiao AM, Kupek E, Petry A. Syphilis seroprevalence estimates of Santa Catarina blood donors in (2010). *Rev Soc Bras Med Trop.* (2014) 47:179–85. doi: 10.1590/0037-8682-0032-2014
20. Mai M, Müller I, Hunfeld K. *Qualität bakteriologisch-infektionsserologischer Verfahren in Deutschland: Auswertung der infektionsserologischen Ringversuche 2011 - Beitrag der Qualitätssicherungskommission der Deutschen Gesellschaft für Hygiene und Mikrobiologie*. (2014). doi: 10.3205/lab000014
21. Maneg D MI, Hunfeld KP. Ergebnisse des bakteriologischinfektionsserologischen INSTAND-Ringversuchs 2010: Eine zusammenfassende Analyse - Beitrag der Qualitatssicherungskommission der

Deutschen Gesellschaft fur Hygiene und Mikrobiologie (DGHM). *GMS Ger Med Sci*. (2014) 5. doi: 10.3205/lab000012
22. Mai MMI, Walch D, Hunfeld KP. Zur Qualitat bakteriologischinfektionsserologischer Verfahren in Deutschland: Auswertung der infektionsserologischen Ringversuche (2012). Beitrag der Qualitatssicherungskommission der DGHM. *GMS Ger Med Sci*. (2016) 7. doi: 10.3205/lab000022
23. Rothman KJS, Greenland TL. *Lash Modern Epidemiology 3rd ed.* Philadelphia: Lippincott Williams & Wilkins. (2008).
24. Robert-Koch-Institut. *Syphilis - Diagnostik: RKI.* (2020). Available online at: https://www.rki.de/DE/Content/Infekt/EpidBull/Merkblaetter/Ratgeber Syphilis.htmljsessionid=24B20208998A8BF846E0245C34BEDBF4.internet121 #doc23 8263 6bodyText9.
25. Altman DG, Bland JM. Diagnostic tests 2: Predictive values. *Bmj.* (1994) 309:102. doi: 10.1136/bmj.309.6947.102
26. Average annual wages in Germany 2000–2017. *Statista*. Available online at: https://www.statista.com/statistics/416207/average-annual-wages-germany-y-on-y-in-euros/
27. Bundesvereinigung K. *Einheitlicher Bewertungsmassstab (EBM).* Available online at: https://www.kbv.de/.
28. Krankenversicherung VdP. *Gebuehrenordnung fuer Aerzte (GoA).* Available online at: https://www.pkv.de/.
29. Preubel K, Ritter S, Houareau C, Offergeld R. *HIV-, HCV-, HBV- und Syphilis-Infektionen bei Blutspendern 2011-2016, Auswertung der epidemiologischen Daten nach § 22 TFG.].* Robert Koch-Institut. (2019).
30. Gottlieb SL, Pope V, Sternberg MR, McQuillan GM, Beltrami JF, Berman SM, et al. Prevalence of syphilis seroreactivity in the United States: data from the National Health and Nutrition Examination Surveys (NHANES) 2001-2004. *Sex Transm Dis.* (2008) 35:507–11. doi: 10.1097/OLQ.0b013e3181644bae
31. Jitapunkul S. Syphilitic seroreactivity among the Thai population aged 50 years and above: value of mass screening. *Southeast Asian J Trop Med Public Health.* (2000) 31:349–53.
32. Bisseye C, Eko Mba JM, Ntsame Ndong JM, Kosiorek HE, Butterfield RJ, Mombo LE, et al. Decline in the seroprevalence of syphilis markers among first-time blood donors in Libreville (Gabon) between 2004 and (2016). *BMC Public Health.* (2019) 19:167. doi: 10.1186/s12889-019-6489-7
33. Wu X, Guan Y, Ye J, Fu H, Zhang C, Lan L, et al. Association between syphilis seroprevalence and age among blood donors in Southern China: an observational study from 2014 to (2017). *BMJ Open.* (2019) 9:e024393. doi: 10.1136/bmjopen-2018-024393
34. Seidl S. *Syphilis screening in the (1990s).* Wiley Online Library. (1990). doi: 10.1046/j.1537-2995.1990.30991048779.x
35. Richtlinie zur Gewinnung von Blut und Blutbestandteilen und zur Anwendung von Blutprodukten (Richtlinie Hamotherapie). (2017).
36. Kane MA, Bloch EM, Bruhn R, Kaidarova Z, Murphy EL. Demographic determinants of syphilis seroprevalence among U.S. blood donors, 2011-2012. *BMC Infect Dis.* (2015) 15:63. doi: 10.1186/s12879-015-0805-3
37. M'Baya B, Jumbe V, Samuel V, M'Bwana R, Mangani C. Seroprevalence and trends in transfusion transmissible infections among voluntary non-remunerated blood donors at the Malawi Blood Transfusion Service-a time trend study. *Malawi Med J.* (2019) 31:118–25. doi: 10.4314/mmj.v31i2.3
38. Pessoni LL, Aquino EC, Alcantara KC. Prevalence and trends in transfusion-transmissible infections among blood donors in Brazil from 2010 to (2016). *Hematol Transfus Cell Ther.* (2019) 41:310–5. doi: 10.1016/j.htct.2019.03.009
39. Zou S, Stramer SL, Dodd RY. Donor testing and risk: current prevalence, incidence, and residual risk of transfusion-transmissible agents in US allogeneic donations. *Transfus Med Rev.* (2012) 26:119–28. doi: 10.1016/j.tmrv.2011.07.007
40. Gardella C, Marfin AA, Kahn RH, Swint E, Markowitz LE. Persons with early syphilis identified through blood or plasma donation screening in the United States. *J Infect Dis.* (2002) 185:545–9. doi: 10.1086/338829
41. Liu S, Luo L, Xi G, Wan L, Zhong L, Chen X, et al. Seroprevalence and risk factors on Syphilis among blood donors in Chengdu, China,from 2005 to (2017). *BMC Infect Dis.* (2019) 19:509. doi: 10.1186/s12879-019-4128-7
42. Offergeld R, Hamouda O, Burger R. Epidemiological data - an important part of the hemovigilance system. *Transfus Med Hemother.* (2010) 37:125–30. doi: 10.1159/000314212
43. Destatis SB. *Tabelle 23131-0002 Krankenhauspatienten: Deutschland, Jahre, Geschlecht, Altersgruppen, Hauptdiagnose ICD-10 2010-2012.* Available online at: https://www-genesis[[sb]]$\pm$[[/s]]destatis[[sb]]$\pm$[[/s]]de/genesis/online?operation=abruftabelleBearbeiten&levelindex=1&levelid=1617972010763&auswahloperation=abruftabelleAuspraegungAuswaehlen&auswahlverzeichnis=ordnungsstruktur&auswahlziel=werteabruf&code=23131-&auswahltext=&nummer=3&variable=3&name=GES025&werteabruf=Werteabruf#abreadc rumb
44. Muller I, Brade V, Hagedorn HJ, Straube E, Schorner C, Frosch M, et al. Is serological testing a reliable tool in laboratory diagnosis of syphilis? Meta-analysis of eight external quality control surveys performed by the german infection serology proficiency testing program. *J Clin Microbiol.* (2006) 44:1335–41. doi: 10.1128/JCM.44.4.1335-1341.2006
45. Spinola SM, Orazi A, Arno JN, Fortney K, Kotylo P, Chen CY, et al. Haemophilus ducreyi elicits a cutaneous infiltrate of CD4 cells during experimental human infection. *J Infect Dis.* (1996) 173:394–402. doi: 10.1093/infdis/173.2.394
46. Wasserheit JN. Epidemiological synergy. Interrelationships between human immunodeficiency virus infection and other sexually transmitted diseases. *Sex Transm Dis.* (1992) 19:61–77. doi: 10.1097/00007435-199219020-00001
47. Stoll M, Claes C, Schulte E. Graf von der Schulenburg JM, Schmidt RE. Direct costs for the treatment of HIV-infection in a German cohort after the introduction of HAART. *Eur J Med Res.* (2002) 7:463–71.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Microbiology

ORIGINAL RESEARCH
published: 03 May 2022
doi: 10.3389/fmicb.2022.888525

Identification and Functional Characterization of Peptides With Antimicrobial Activity From the Syphilis Spirochete, *Treponema pallidum*

*Simon Houston[1], Ethan Schovanek[1], Kate M. E. Conway[1], Sarah Mustafa[1], Alloysius Gomez[1], Raghavendran Ramaswamy[1], Ayman Haimour[1], Martin J. Boulanger[1], Lisa A. Reynolds[1] and Caroline E. Cameron[1,2]**

[1] Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC, Canada, [2] Division of Allergy and Infectious Diseases, Department of Medicine, University of Washington, Seattle, WA, United States

OPEN ACCESS

Edited by:
Justin David Radolf,
UCONN Health, United States

Reviewed by:
Diane Edmondson,
University of Texas Health Science Center at Houston, United States
Darrin Akins,
University of Oklahoma Health Sciences Center, United States

***Correspondence:**
Caroline E. Cameron
caroc@uvic.ca

Specialty section:
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Microbiology

Received: 03 March 2022
Accepted: 08 April 2022
Published: 03 May 2022

Citation:
Houston S, Schovanek E, Conway KME, Mustafa S, Gomez A, Ramaswamy R, Haimour A, Boulanger MJ, Reynolds LA and Cameron CE (2022) Identification and Functional Characterization of Peptides With Antimicrobial Activity From the Syphilis Spirochete, Treponema pallidum. Front. Microbiol. 13:888525. doi: 10.3389/fmicb.2022.888525

The etiological agent of syphilis, *Treponema pallidum* ssp. *pallidum*, is a highly invasive "stealth" pathogen that can evade the host immune response and persist within the host for decades. This obligate human pathogen is adept at establishing infection and surviving at sites within the host that have a multitude of competing microbes, sometimes including pathogens. One survival strategy employed by bacteria found at polymicrobial sites is elimination of competing microorganisms by production of antimicrobial peptides (AMPs). Antimicrobial peptides are low molecular weight proteins (miniproteins) that function directly via inhibition and killing of microbes and/or indirectly via modulation of the host immune response, which can facilitate immune evasion. In the current study, we used bioinformatics to show that approximately 7% of the *T. pallidum* proteome is comprised of miniproteins of 150 amino acids or less with unknown functions. To investigate the possibility that AMP production is an unrecognized defense strategy used by *T. pallidum* during infection, we developed a bioinformatics pipeline to analyze the complement of *T. pallidum* miniproteins of unknown function for the identification of potential AMPs. This analysis identified 45 *T. pallidum* AMP candidates; of these, Tp0451a and Tp0749 were subjected to further bioinformatic analyses to identify AMP critical core regions (AMPCCRs). Four potential AMPCCRs from the two predicted AMPs were identified and peptides corresponding to these AMPCCRs were experimentally confirmed to exhibit bacteriostatic and bactericidal activity against a panel of biologically relevant Gram-positive and Gram-negative bacteria. Immunomodulation assays performed under inflammatory conditions demonstrated that one of the AMPCCRs was also capable of differentially regulating expression of two pro-inflammatory chemokines [monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8)]. These findings demonstrate proof-of-concept for our developed AMP identification pipeline and are consistent with the novel concept that *T. pallidum* expresses AMPs to defend against competing microbes and modulate the host immune response.

Keywords: antimicrobial peptides, syphilis, *Treponema pallidum*, bacteriostatic, bactericidal

INTRODUCTION

The spirochete bacterium, *Treponema pallidum* ssp. *pallidum* (hereafter *T. pallidum*), is the causative agent of syphilis, a chronic, multistage infection that is transmitted sexually or in utero. Following infection, *T. pallidum* traverses endothelial barriers and undergoes rapid and widespread dissemination via the circulatory system to infect every organ and tissue, including immunologically privileged sites such as the eyes (Marra et al., 1991; Muller et al., 2007), testes (Sell et al., 1980), and central nervous system (Collart et al., 1971; Lukehart et al., 1988). Despite host-initiated innate and adaptive immune responses, *T. pallidum* is able to persist within the host for decades (Lafond and Lukehart, 2006). The remarkable ability of *T. pallidum* to evade the immune system and establish and maintain persistent infection has earned it the designation of the "stealth" pathogen (Radolf, 1994).

During infection of a host, *T. pallidum*, which has a slow generation time of 30–33 h (Magnuson and Eagle, 1948; Cumberland and Turner, 1949), are introduced into anatomical sites that are abundant in species of microbiota, including the genital tract, skin, rectum, and oral cavity (Lafond and Lukehart, 2006), features that may put *T. pallidum* at a growth disadvantage in a polymicrobial environment. In addition, *T. pallidum* can present as a co-infection with other viral, fungal, parasitic, and bacterial pathogens, including the sexually transmitted pathogen, *Neisseria gonorrhoeae* (Bala et al., 2011; World Health Organization, 2019; Pinho-Bandeira et al., 2020; Coelho et al., 2021). Inhibition and elimination of competing microorganisms via production of antimicrobial peptides (AMPs) allows the microbiota and pathogenic bacteria found at polymicrobial sites to gain a competitive advantage (Meade et al., 2020). The ability of *T. pallidum* to establish an infection and survive in anatomical locations with a complex polymicrobial profile raised the question of whether this bacterium could use AMP production to eliminate microbial competition.

AMPs are a structurally and functionally diverse class of low molecular weight proteins produced by all branches of life (Kumar et al., 2018). Typically comprised of 10–150 amino acids, they often form amphipathic alpha helices facilitated by their net positive charge and high hydrophobic content (Kumar et al., 2018). AMPs have a direct mechanism of action that occurs via electrostatic interactions between positively-charged AMPs and negatively-charged microbial surfaces (Kumar et al., 2018). The amphipathic secondary structure of AMPs promotes membrane integration and pore formation, resulting in membrane destabilization and cell lysis as well as inhibition of essential intracellular functions such as DNA and protein synthesis (Kumar et al., 2018; Mishra et al., 2018). A second, indirect effect of AMPs can be alteration of the host immune response, including modulation of inflammatory cytokine production, immune cell recruitment and activation (Hilchie et al., 2013). The immunomodulatory effects of eukaryotic AMPs have been well documented, and recent studies have shown bacterial AMPs can have similar immunomodulatory activities (Kindrachuk et al., 2013; Malaczewska et al., 2019) that can promote bacterial survival and host infection via subversion and evasion of the host immune response (Li et al., 2014).

An important characteristic of AMPs is the presence of functionally essential regions that correspond to the shortest stretch of amino acids (often ∼10–20 residues) that retain antimicrobial effects (Chang et al., 2015). Identification of these key regions, defined as antimicrobial peptide critical core regions (AMPCCRs), allows for the design and development of discrete peptides with antimicrobial activity that are derived from, and more tractable than, their larger precursor proteins (Torrent et al., 2009, 2012).

More than two decades have passed since the first *T. pallidum* whole genome sequence was published (Fraser et al., 1998). Since then, many laboratory and clinical strains of *T. pallidum* ssp. *pallidum* have been sequenced, yet only three genes have been annotated as homologs of known AMP-related genes in other bacteria. These are *tp0688* [*Bacillus anthracis mccF,* encoding the Microcin C7 self-immunity protein (Gonzalez-Pastor et al., 1995)], *tp0522* [*Escherichia coli cvpA,* encoding the Colicin V Production protein that is required for production and secretion of the AMP Colicin V (Gilson et al., 1990)], and *tp0405* [*E. coli mcbG*, encoding the Microcin B17 self-immunity protein (San Millan et al., 1985)]. Recent *T. pallidum* genome sequencing also identified a novel 91-amino acid miniprotein (TPANIC_RS05485) which has been annotated as a putative CPBP (CAAX Protease and Bacteriocin-Processing) family intramembrane metalloprotease. Evidence suggests some members of this family may be involved in bacterial AMP processing (Pei et al., 2011). One reason that may partially account for the low number of AMP-related genes detected within *T. pallidum* to date is that the bacterium is phylogenetically distinct, with approximately 300 genes/30% of the genome predicted to encode proteins with no known orthologs or assigned functions (Fraser et al., 1998; Petrosova et al., 2013).

The present study shows that approximately one quarter of *T. pallidum* genes of unknown function are predicted to encode miniproteins of 150 amino acids or less. Bioinformatic analyses show a portion of these miniproteins possess characteristics consistent with known AMPs. These findings, when considered in the context of the success of this bacterium at establishing infection at polymicrobial anatomical sites, prompted us to investigate whether AMP production is an unexplored pathogenic mechanism used by *T. pallidum* to defend against competing microbes and the host. Herein, we investigated this potential treponemal defense strategy using a combination of bioinformatics, structure modeling, antimicrobial susceptibility testing, and immunomodulation assays. Our investigations have provided experimental confirmation of AMP activity within two *T. pallidum* miniproteins, consistent with the novel concept that *T. pallidum* expresses AMPs to establish and maintain infection at polymicrobial sites in the human host.

MATERIALS AND METHODS

Bacterial Strains and Culture

Bacterial strains used in this study were: *E. coli* ATCC 9723H, *Pseudomonas aeruginosa* ATCC 10148, *Staphylococcus aureus* ATCC 6538P (penicillin resistant), *Streptococcus pyogenes*

(hospital isolate, strain unknown), *Mycobacterium smegmatis* MC^2155, *N. gonorrhoeae* ATCC 700825 (streptomycin resistant), and *Salmonella enterica* subsp. *enterica* serovar Typhimurium SL1344 (streptomycin resistant). *E. coli*, *P. aeruginosa*, *S. aureus*, *M. smegmatis*, and *S. enterica* were cultured aerobically at 37°C in Mueller Hinton broth (MHB) (Sigma-Aldrich, MO, United States) and on nonselective Mueller Hinton agar (MHA) plates. *S. pyogenes* was cultured in 5% carbon dioxide at 37°C in MHB supplemented with 5% lysed horse blood (Quad Five, MT, United States) (MHB + 5% HB) and on nonselective MHA plates supplemented with 5% defibrinated sheep blood (Quad Five, MT, United States) (MHA + 5% SB). *N. gonorrhoeae* was cultured in 5% carbon dioxide at 37°C in gonococcal (GC) chocolate broth medium and on nonselective GC agar plates [GC medium base (BD Difco, MD, United States) supplemented with 1% BBL™ hemoglobin (BD Biosciences, MD, United States) and 1% IsoVitaleX (BD Biosciences, MD, United States)]. Prior to antimicrobial susceptibility assays (as described below), *N. gonorrhoeae* cultures were subcultured on nonselective GC agar plates (as described above), to ensure bacterial viability. All bacterial stocks were stored in 20% glycerol at −80°C.

Treponema pallidum Propagation and *in vitro* Culture

Treponema pallidum subsp. *pallidum* (Nichols strain) was propagated in, and extracted from, New Zealand White rabbits as described elsewhere (Lukehart and Marra, 2007), and stored in liquid nitrogen. Frozen treponemal stocks were then used for *in vitro* culture and sub-culture of *T. pallidum* in the presence of Sf1Ep (NBL-11) cottontail rabbit epithelial cells (ATCC CCL-68) [American Type Culture Collection (ATCC), Rockville, MD, United States]. Continuous axenic culture of *T. pallidum* in the absence of mammalian cells has not been achieved, and it is believed that the direct adherence of *T. pallidum* to Sf1Ep cells is required for the long term replication of *T. pallidum in vitro* (Edmondson et al., 2018). Dissociation of *T. pallidum* from Sf1Ep cells was accomplished using trypsin-free dissociation buffer [2 mL: 64% cell culture grade water (Sigma Aldrich), 10% modified EBSS (Earle's Balanced salt solution, 10×), 1% non-essential amino acids (Thermo Fisher Scientific), 0.15% sodium bicarbonate (Sigma Aldrich), 0.728% 100 mM sodium pyruvate (Sigma Aldrich), 0.136% 0.5M EDTA (Thermo Fisher Scientific), 0.16 mg dithiothreitol (DTT) (Sigma Aldrich)] followed by a low speed centrifugation step (220 × *g*) to separate *T. pallidum* from the rabbit cells, as previously described (Edmondson et al., 2018; Edmondson and Norris, 2021).

Bioinformatics Pipeline: *In silico* Analysis of *T. pallidum* Whole Proteomes

The flow diagram shown in **Figure 1** outlines all major steps that comprised our bioinformatics pipeline for the identification of potential AMPs and AMPCCRs in the *T. pallidum* proteome. As the first step in this approach, the whole proteome of *T. pallidum* (Nichols strain NC_021490) was obtained from the National Center for Biotechnology Information (NCBI) Genome database[1] and manually searched in order to identify all functionally-unannotated miniproteins containing 150 amino acids or less (**Figure 1A**). All *in silico* proteomic analyses performed on the *T. pallidum* strain reported in the current study were based on the NCBI whole proteome annotation from July 2021.

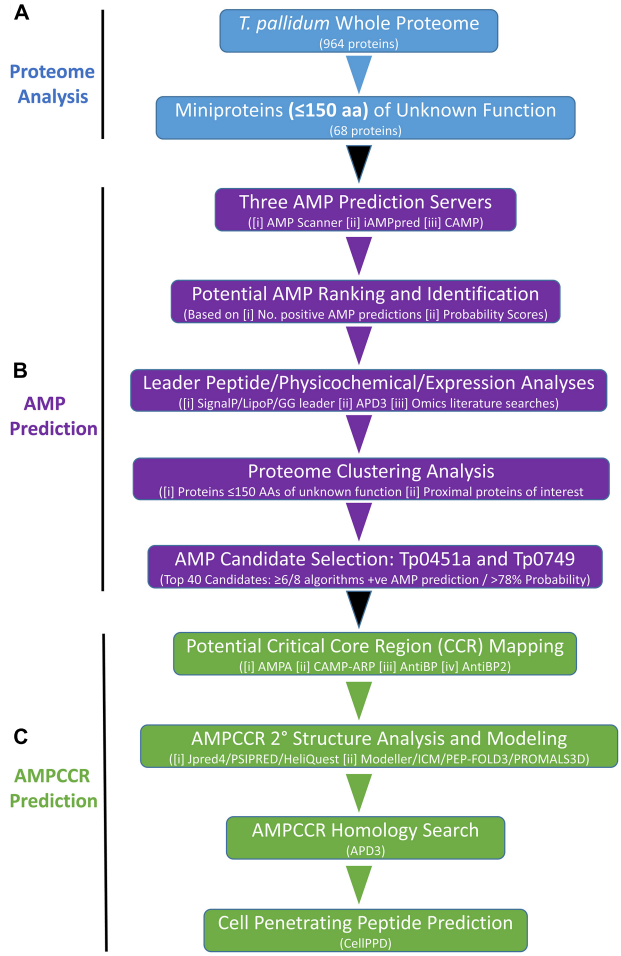


FIGURE 1 | Bioinformatics pipeline for the identification of potential AMPs and AMPCCRs in the proteome of *T. pallidum*. **(A)** Proteome Analysis: The whole proteome (from NCBI genome database, July 2021 annotation) of *T. pallidum* was searched for proteins with no assigned function and 150 amino acids or less. **(B)** AMP Prediction: Complete amino acid sequences of 68 *T. pallidum* proteins (≤150 amino acids) of unknown function were submitted to three AMP prediction servers which allowed for ranking from most likely AMP to least likely AMP. Additional leader peptide, physicochemical, expression, and proteome clustering analyses facilitated the identification of two potential AMPs (Tp0451a and Tp0749) for further analyses. **(C)** AMPCCR prediction: A combination of critical core region (CCR) mapping, structural, homology, and cell penetrating peptide analyses were then performed to further confirm the initial AMP predictions generated by the three prediction servers and to identify putative antimicrobial peptide critical core regions (AMPCCRs) within the identified predicted AMPs, Tp0451a and Tp0749.

Bioinformatics Pipeline: AMP Prediction

The full-length amino acid sequences of all functionally-unannotated miniproteins identified in the above analyses

[1]https://www.ncbi.nlm.nih.gov/genome/?term=treponema+pallidum

were submitted to three AMP prediction servers: (i) AMP Scanner Version 2 (Deep Neural Learning Method for predicting antibacterial activity only)[2] (Veltri et al., 2018), (ii) iAMPpred [Support Vector Machine (SVM) algorithms for predicting antibacterial, antiviral, and antifungal activities; machine learning method based on amino acid composition, physicochemical, and structural features][3] (Meher et al., 2017), and (iii) CAMP [SVM, Random Forest (RF), Artificial Neural Network (ANN), and Discriminant Analysis (DA) algorithms for predicting antimicrobial activity; machine learning method based on different physicochemical properties of proteins][4] (Waghu et al., 2016; **Figure 1B**). All *T. pallidum* miniproteins of unknown function were ranked from most likely AMP to least likely AMP based on the number of different server algorithms (out of eight total) that produced positive AMP predictions. Corresponding mean probability scores (1 [most likely prediction score]-0 [least likely prediction score]) were then used to rank all miniproteins within each positive AMP prediction class (**Figure 1B**).

Bioinformatics Pipeline: Leader Peptide, Physicochemical, and Proteome Clustering Analyses of Putative *T. pallidum* AMPs

To determine if any of the miniproteins of unknown function from *T. pallidum* contain potential Sec-dependent leader peptides (Sec/SP1 peptides), we used the signal peptide prediction servers, SignalP 5.0[5] (Almagro Armenteros et al., 2019) and LipoP 1.0[6] (Juncker et al., 2003). Manual searches of the *T. pallidum* miniproteins for the conserved double-glycine/glycine-alanine leader peptide motif (M[R/K]ELX$_3$E[I/L]X$_2$[I/V]XG[G/A]) that has been observed in AMPs from Gram-negative bacteria (Michiels et al., 2001; Dirix et al., 2004) were performed to identify proteins that contain Glycine-Glycine and/or Glycine–Alanine pairs within the first 31 residues of the N-terminus (**Figure 1B**). A multiple sequence alignment of the N-termini of each of the Glycine-Glycine and/or Glycine-Alanine-containing proteins was generated using Clustal Omega[7] (Madeira et al., 2019). WebLogo[8] (Crooks et al., 2004) was then used to generate a sequence logo for the identification of sites within the N-terminal residues of these proteins that exhibit homology to the conserved double-glycine/glycine-alanine leader peptide motif found in AMPs from Gram-negative bacteria (Michiels et al., 2001; Dirix et al., 2004). Physicochemical properties, including hydrophobicity, net charge, and presence/absence of cysteine residues, of the *T. pallidum* miniproteins of unknown function were analyzed using the APD3 database Antimicrobial Peptide Calculator and Predictor[9] (Wang et al., 2016; **Figure 1B**). Tertiary structure modeling of functionally-unannotated proteins of interest (>150 amino acids) found to be located in close proximity to *T. pallidum* miniproteins identified above, was performed using the protein structure modeling server, Phyre2[10] (Kelley et al., 2015).

Bioinformatics Pipeline: Tp0451a and Tp0749 AMPCCR Prediction

Two identified AMP candidates [Tp0451a (accession number WP_014342798) and Tp0749 (accession number WP_010882194)] were further analyzed using our in-house bioinformatics pipeline (**Figure 1C**). This analysis was performed to help confirm the initial AMP predictions generated by the three prediction servers and to identify potential antimicrobial peptide critical core regions (AMPCCRs) within Tp0451a and Tp0749. This pipeline was comprised of the following four stages:

(i) **Multi-server AMPCCR mapping**

Full-length amino acid sequences of Tp0451a and Tp0749 were submitted to four servers: AMPA[11] (Torrent et al., 2012), CAMP antimicrobial region prediction server (CAMP-ARP)[12] (Waghu et al., 2016), AntiBP[13] (Lata et al., 2007), and AntiBP2[14] (Lata et al., 2010). Potential AMPCCRs were identified in each protein based on high probability scoring regions (15–23 amino acid stretches) that were predicted by three or more servers.

(ii) **Structure analyses and modeling**

Secondary structure analyses of potential AMPCCRs were performed using the structure prediction servers Jpred 4[15] (Drozdetskiy et al., 2015) and PSIPRED 4.0[16](Jones, 1999) and the alpha helix screening and physicochemical characterization server, HeliQuest[17] (Gautier et al., 2008). Structure modeling of potential AMPCCRs based on PSIPRED secondary structure predictions were performed using the de novo peptide structure prediction server, PEP-FOLD 3[18] (Thevenet et al., 2012; Shen et al., 2014) and Swiss Model[19] (Bienert et al., 2017; Waterhouse et al., 2018). All models were then vetted through the comparative protein structure modelling server, Modeller[20] (Webb and Sali, 2016) and the homology modeling program, ICM (Molsoft L.L.C., CA, United States)[21] (Cardozo et al., 1995), for their lowest normalized discrete optimized protein energy value (zDOPE) and GA341 score closest to 1. Comparative homology modeling using structure-based alignment was performed using

[2]https://www.dveltri.com/ascan/
[3]http://cabgrid.res.in:8080/amppred/
[4]http://www.camp.bicnirrh.res.in/predict
[5]https://services.healthtech.dtu.dk/service.php?SignalP-5.0
[6]https://services.healthtech.dtu.dk/service.php?LipoP-1.0
[7]https://www.ebi.ac.uk/Tools/msa/clustalo/
[8]https://weblogo.berkeley.edu/logo.cgi
[9]https://aps.unmc.edu/prediction
[10]http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index
[11]http://tcoffee.crg.cat/apps/ampa/do
[12]http://www.camp.bicnirrh.res.in/predict_c/
[13]https://webs.iiitd.edu.in/raghava/antibp/submit.html
[14]https://webs.iiitd.edu.in/raghava/antibp2/submit.html
[15]http://www.compbio.dundee.ac.uk/jpred/
[16]http://bioinf.cs.ucl.ac.uk/psipred/
[17]http://heliquest.ipmc.cnrs.fr/
[18]https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD
[19]https://swissmodel.expasy.org/
[20]https://salilab.org/modeller/
[21]https://www.molsoft.com/index.html

PROMALS3D[22] (Pei et al., 2008). Together, these structure prediction analyses were used to support the multi-server AMPCCR predictions through the identification of structural folds known to be important for AMP function and for facilitating subsequent peptide design via the prediction of intact secondary structure elements.

(iii) **AMPCCR homology analyses**

Amino acid homology searches using the APD3 database (see text footnote 9) (Wang et al., 2016) were employed to determine similarity and identity of predicted *T. pallidum* AMPCCRs with known and experimentally-validated AMPs and for the identification of short orthologous AMP sequences that would be otherwise missed using the NCBI BLAST tools[23] (Altschul et al., 1990);

(iv) **AMPCCR cell penetration prediction**

AMPCCR cell penetrating abilities, a key functional feature of AMPs, were predicted using the CellPPD Protein Scanning Tool[24] (Gautam et al., 2013) (peptide fragment length = 10; prediction method = SVM based with scores ranging from −1.0 to +1.0). To increase prediction stringency, the SVM threshold for positive cell penetrating peptide predictions was increased from the default threshold (0) to ≥0.1.

RNA Extraction and RT-PCR

RNA was isolated and purified from *in vivo*-harvested *T. pallidum* subsp. *pallidum* (Nichols strain) using Invitrogen TRIzol™ reagent (Thermo Fisher Scientific, MA United States) and the RNeasy mini kit (Qiagen, ON, Canada), according to the manufacturer's instructions. RT-PCR was performed (after genomic DNA digestion/removal) using the orientation-specific RT-PCR sense (5'-aatgtcggctaccatcgctc) and antisense (5'-acgtgctctgccaattactgc) primers for *tp0451a* and the Invitrogen SuperScript™ IV First-Strand Synthesis System (Thermo Fisher Scientific), according to the manufacturer's instructions. The negative control RT-PCR reaction did not include reverse transcriptase. PCR products were electrophoresed on agarose gels and visualized with ethidium bromide staining.

Peptide Synthesis

For the experimental validation of antimicrobial and immunomodulatory activity, four putative AMPCCR peptides (**Table 1**) from two *T. pallidum* miniproteins that were identified using our AMP bioinformatics prediction pipeline (Tp0451a_N, Tp0451a_C, Tp0749_N, and Tp0749_C), a cysteine-to-serine substituted version of Tp0749_C (Tp0749_C_C61S), and a cysteine-to-serine substituted version of Tp0451a_C (Tp0451a_C_C85S), were synthesized without chemical modifications using the PepPower™ solid state peptide synthesis (SSPS) platform at GenScript (NJ, United States). The known AMP, human cathelicidin LL-37 (Turner et al., 1998), the known bullfrog (*Rana [Lithobates] catesbeiana*) AMP, RaCa-2 (Li et al., 2022), a scrambled version of LL-37 (sLL-37), and a peptide (Tp0751_p5) from the *T. pallidum* adhesin Tp0751 (Cameron et al., 2005) (**Table 1**) were also synthesized via the same SSPS platform at GenScript, and used as positive (LL-37 and RaCa-2) and negative (sLL-37 and Tp0751_p5) controls in antimicrobial susceptibility and immunomodulation assays, as described below.

Antimicrobial Susceptibility Assay—Broth Microdilution

The broth microdilution technique (Wiegand et al., 2008) was used to determine if the *T. pallidum* peptides are capable of exhibiting bacteriostatic [minimal inhibitory concentration (MIC) measurements] and/or bactericidal [minimal bactericidal concentration (MBC) measurements] activity. Bacterial suspensions were prepared by transferring bacterial colonies into MHB and resuspending using a vortex mixer to ensure complete suspension of any bacterial aggregates. Turbidity of the colony suspensions was adjusted spectrophotometrically to the required optical densities to achieve a turbidity equivalent to that of a 0.5 McFarland standard (1–2 × 10^8 CFU/mL) followed by dilution in MHB to achieve the standardized microbial inoculum of approximately 5 × 10^5 CFU/mL. Total viable counts (TVC) were routinely performed on all inoculum suspensions to ensure correct bacterial cell densities. The standardized bacterial suspensions were then incubated with two-fold serial dilutions of each peptide (dissolved in 11 μL of ultrapure sterile water; final peptide concentration range of 256 μg/mL–0.5 μg/mL) in Greiner polypropylene round bottom 96-well microtiter plates (Sigma-Aldrich, MO, United States). Each peptide was tested once per experiment, with a range of 3–9 independent experiments performed per peptide. Negative growth/sterility control wells contained bacterial growth media (100 μL) and the peptide solvent (11 μL of ultrapure sterile water). Positive growth control wells contained the standardized number of bacterial cells (100 μL of ∼ 5.0 × 10^5 CFU/mL) and peptide solvent (11 μL of ultrapure sterile water). Plates were incubated at 37°C for 16–24 h and MICs were determined using the unaided eye to identify the lowest concentration of AMP that inhibited visible growth of the tested bacterial species. If the sterility control well was turbid, the test was not considered valid. MBCs were determined by plating the entire content of the wells containing the peptide/bacteria mixture representing the MIC and the entire contents of the preceding wells containing two-fold and four-fold more concentrated AMP dilutions onto nonselective agar plates. Plates were incubated for 24 h at 37°C and MBCs were calculated as the percentage of bacteria killed at the different AMP concentrations tested (decrease in TVCs from the MBC plates compared to the initial bacterial suspension of ∼5 × 10^5 CFU/mL).

Antimicrobial Susceptibility Assay—*Neisseria* Modified Agar Dilution Method

The potential antimicrobial activity of the *T. pallidum* peptides against *N. gonorrhoeae* was determined using a modified agar

[22]http://prodata.swmed.edu/promals3d/promals3d.php

[23]https://blast.ncbi.nlm.nih.gov/Blast.cgi

[24]https://webs.iiitd.edu.in/raghava/cellppd/submit_prot.php

TABLE 1 | Chemically synthesized peptides used in the current study.

Peptide name	Peptide source and description	Amino acid sequence
Tp0451a_N	Tp AMPCCR (Tp0451a N-terminal peptide)	GCGSHCNCNVGYHRSLHCYGNELHGKQCGFSRCG
Tp0451a_C	Tp AMPCCR (Tp0451a C-terminal peptide)	IGRARAITHTWGIWCRWGKVWRRS
Tp0749_N	Tp AMPCCR (Tp0749 N-terminal peptide)	PFMQVITWARLYHKNQKRYEKIKK
Tp0749_C	Tp AMPCCR (Tp0749 C-terminal peptide)	KGIVAERILKPCVRRKVNGKFRS
Tp0451a_C_C85S	C-to-S substituted version of Tp0451a_C	IGRARAITHTWGIW**S**RWGKVWRRS
Tp0749_C_C61S	C-to-S substituted version of Tp0749_C	KGIVAERILKP**S**VRRKVNGKFRS
LL-37 (+ve)	Known human cathelicidin AMP	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
RaCa-2 (+ve)	Known bullfrog AMP	FFPIIARLAAKVIPSLVCAVTKKC
sLL-37 (-ve)	Scrambled version of LL-37	RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVKIL
Tp0751_p5 (-ve)	*T. pallidum* peptide from adhesin Tp0751	AMRIALWNRATHGEQGALQHLLAG

Tp, T. pallidum; AMPCCR, antimicrobial peptide critical core region (identified in the current study); -ve, negative control peptide; +ve, positive control peptide.

dilution method. In this assay, *N. gonorrhoeae* colonies from GC chocolate agar plates were resuspended in MHB and the turbidity of the suspension was adjusted, as described above, to achieve the standardized microbial inoculum of approximately 5×10^5 CFU/mL. A two-fold serial dilution of the peptides (11 μL in sterile ultrapure water) was then prepared in wells 1–10 of a 96-well sterile polypropylene plate to obtain a dilution series corresponding to 10 times the required testing concentrations (2,560, 1,280, 640, 320, 160, 80, 40, 20, 10, 5 μg/mL). The bacterial suspension (100 μL) was dispensed into the wells containing the peptides. A negative growth/sterility control (well 12) contained bacterial growth media (100 μL) and the peptide solvent (11 μL of ultrapure sterile water). A positive growth control (well 11) contained the standardized number of bacterial cells (100 μL of $\sim 5.0 \times 10^5$ CFU/mL) and peptide solvent (11 μL of ultrapure sterile water). The 96-well plate was then incubated at 37°C in an atmosphere of 5% CO_2 for 3 h to allow for peptide binding and antimicrobial activity. After the incubation period, an aliquot (20 μL) from wells 1–12 was removed and spotted onto the surface of a GC chocolate agar plate. *N. gonorrhoeae* spotted plates and TVC plates were incubated at 37°C in an atmosphere of 5% CO_2 for 18–24 h. Following the incubation period, MICs were determined by identifying the lowest concentration of peptide that completely inhibited visible growth on the agar plate. To determine the bactericidal activity of peptides, total viable counts (TVCs) were also prepared on GC chocolate agar plates for the 3 h-incubated peptide/bacteria mixtures. These counts were compared with TVCs from the corresponding positive growth wells to give the percentage of bacteria killed by each of the peptides following the 3-h incubation.

Antimicrobial Susceptibility Assay – *T. pallidum*

An antimicrobial susceptibility assay was developed to assess the activity of the four treponemal peptides (Tp0451a_N, Tp0451a_C, Tp0749_N, and Tp0749_C), an equimolar mix of Tp0451a_N and Tp0451a_C, and the negative (Tp0751_p5) and positive (LL-37) control peptides, against *T. pallidum*. *In vitro*-grown *T. pallidum* (100μL; 1.0–1.2 × 10^6 Tp/mL), prepared as described above, were incubated with each peptide at three concentrations (4, 16, 64 μg/mL) or the Tp0451a_N/Tp0451a_C mix (21.6 μM; ∼85 μg/mL Tp0451a_N and ∼64 μg/mL Tp0451a_C) at 34°C in an atmosphere of 93.5% nitrogen, 5% carbon dioxide, and 1.5% oxygen. Darkfield microscopy was used to monitor *T. pallidum* viability by counting motile treponemes at 0, 1, 2, and 4 h post co-incubation. For each viability measurement, at least 50 treponemes were observed for each sample at each time point.

THP-1 Monocyte Culture and Macrophage-Like Differentiation

Human THP-1 (ATCC TIB-202) monocytes (American Type Culture Collection, VA, United States) were cultured and maintained in 5% CO_2 at 37°C in RPMI-1640 medium (Gibco, Life Technologies, ON, Canada) supplemented with 10% (v/v) fetal bovine serum (FBS) (Fisher Scientific, ON, Canada), 0.05 mM 2-mercaptoethanol (BME) (Sigma-Aldrich, ON, Canada), penicillin (100 units), and streptomycin (0.1 mg/mL) (Sigma-Aldrich, ON, Canada) (hereafter referred to as "complete RPMI-1640 medium"). Cells were passaged at a density of 8×10^5 cells/mL to a maximum of 15 passages and re-seeded at 3×10^5 cells/mL for maintenance. For differentiation into plastic-adherent macrophage-like cells, THP-1 monocytes were seeded at a density of 3×10^5 cells/mL in T75 tissue culture flasks (Fisher Scientific, Ottawa, ON, Canada) and stimulated with a low concentration (25 ng/mL) of phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich, ON, Canada) in complete RPMI-1640 medium for 48 h in 5% CO_2 at 37°C. The PMA-mediated differentiation method results in the generation of cells with phenotypic characteristics that are similar to human peripheral blood mononuclear cell (PBMC) monocyte-derived macrophages; they are adherent, larger, more phagocytic, are less proliferative, and exhibit cell surface markers that are characteristic of macrophages (Chanput et al., 2014).

Following incubation with PMA, light microscopy was used to ensure the differentiated cells were adherent, and exhibited morphological changes consistent with PBMC monocyte-derived macrophages. Non-adherent cells were then removed by washing with sterile, calcium- and magnesium-free phosphate-buffered saline (PBS) (ThermoFisher Scientific, MA United States). Plastic-adherent macrophage-like cells were detached by a three- to five-minute treatment with trypsin-EDTA (0.05%) (ThermoFisher Scientific, MA, United States) and physical agitation. The macrophage-like cells were then

centrifuged at 1,500 rpm using a Sorvall Model STR04 centrifuge (ThermoFisher Scientific, MA United States) for 5 min at room temperature and seeded in PMA-free complete RPMI-1640 media, as described below.

AMP Stimulatory and AMP/IL-32γ Co-stimulatory Immunomodulation Assays

THP-1 monocytes and THP-1 macrophage-like cells were seeded into the wells of 12-well plates (Thermo Fisher Scientific, ON, Canada) in complete RPMI-1640 medium (1 mL) at a density of 0.5×10^6 cells/mL. Following seeding and prior to stimulation, macrophage-like cells were rested overnight. For AMP alone stimulation, monocytes or macrophages were exposed for 24 h at 37°C in 5% CO_2 to either (i) no stimulation (negative control for baseline cytokine production), (ii) lipopolysaccharide [LPS; from *S. enterica* serovar Typhimurium (Sigma-Aldrich, ON, Canada)] (positive control for cytokine production; final concentration 1.0 μg/mL), or (iii) the test peptides (control peptides and potential *T. pallidum* AMPs listed in **Table 1**; final concentration 25 μg/mL). For AMP/IL-32γ co-stimulation, 20 ng/mL IL-32γ (R&D Systems, MN, United States) in fresh complete RPMI-1640 medium was added to the rested macrophages. IL-32γ stimulation was immediately followed by co-stimulation by the addition of the test peptides at a final concentration of 25 μg/mL. Macrophage cells left unstimulated or stimulated with IL-32γ alone were used as negative and positive controls, respectively. Cells were stimulated for 24 h at 37°C in 5% CO_2. Following stimulation, monocytes and macrophage cells were centrifuged at 1,500 rpm using a Sorvall Model STR04 centrifuge (ThermoFisher Scientific, MA, United States) for 5 min at room temperature and the cell-free supernatant was stored at -80°C prior to quantification of cytokine levels, as described below.

THP-1 Monocyte and Macrophage Cytokine Expression Analyses

The BD™ Cytometric Bead Array (CBA) system (BD Biosciences, CA, United States) was used to quantify the expression of tumor necrosis factor (TNF), MCP-1, IL-6, IL-8, IL-10, and IL-1β according to manufacturer's instructions. For statistical analyses, data were analyzed for normality using a D'Agostino-Pearson omnibus normality test and a Shapiro–Wilk normality test. An ordinary one-way ANOVA followed by Dunnett's multiple comparisons test was used to assess differences between three or more groups of normally distributed data. A Kruskal–Wallis test followed by Dunn's multiple comparisons test was used to assess differences between three or more groups of data that were not normally distributed.

RESULTS

Identification of *T. pallidum* Miniproteins of Unknown Function

Although the size of AMPs can vary greatly, ranging from approximately five amino acids to several hundred amino acids, a search of the AMP database, APD3[25] (Wang et al., 2016), indicated that 97% of the 3324 AMPs listed at the time of analysis fall within the 10–150 amino acid size range. With this knowledge, we sought to identify potential *T. pallidum* AMPs by manually searching the whole proteome of *T. pallidum* and filtering for proteins containing 150 amino acids or less. This resulted in the identification of 151 miniproteins (≤150 amino acids) (**Supplementary Table 1**), representing ∼16% of the *T. pallidum* proteome. We then filtered for miniproteins with no assigned function or weak/incomplete annotated functions and for miniproteins with a potential AMP-related function, resulting in the identification of 68 proteins (**Supplementary Table 2**), representing 7% of the *T. pallidum* proteome. Genes corresponding to four of these 68 proteins (Tp0039, Tp0130, Tp0451a, and Tp0867) were not included in the latest annotation of the *T. pallidum* proteome (Nichols strain NC_021490), however, all four genes have been shown to be expressed at the transcript level (Smajs et al., 2005 and current study) justifying their inclusion in this study (**Supplementary Table 2**). Sixty-seven of the 68 miniproteins of unknown function that were identified in *T. pallidum* were annotated in the published proteome from July 2021 as either "hypothetical proteins," "DUF (Domains of Unknown Function) domain-containing proteins," or as proteins with motifs/domains that do not provide enough insight to confidently indicate potential protein functions (e.g., helix-turn-helix domain-containing proteins, DNA- or RNA-binding proteins, zinc ribbon domain-containing protein) (**Supplementary Table 2**). One of the 68 miniproteins was annotated as a putative CPBP family intramembrane metalloprotease (TPANIC_RS05485), some of which may be involved in AMP processing (Pei et al., 2011). However, this 91-amino acid treponemal protein is at least two-four-fold smaller than other bacterial CPBP proteins, is only predicted to contain one transmembrane segment (unlike the four or more present in known CPBP proteins), and does not contain the four conserved sequence motifs required for proteolytic activity that are found in other CPBP proteins (Pei et al., 2011). In light of these findings, Tp_RS05485 was included in the list of 68 miniproteins for further bioinformatics analyses (**Supplementary Table 2**).

Prediction Analyses for the Identification and Ranking of Putative *T. pallidum* AMPs

Full-length amino acid sequences of all 68 miniproteins identified in the analyses described above were submitted to three AMP prediction servers. Prediction data were then used to rank the 68 treponemal proteins from most likely AMP (ranking 1/68) to least likely AMP (68/68) depending on (i) how many of the eight server algorithms produced positive AMP predictions and (ii) the mean probability scores from each of the AMP predictions for each protein. In summary, 45 high-priority *T. pallidum* AMP candidates predicted by at least four of the eight algorithms were assigned mean probability scores of at least 0.505 (50.5% probability) (**Figure 2**

[25] https://aps.unmc.edu/

and **Supplementary Table 3**). AMP prediction results for all 68 *T. pallidum* miniproteins and corresponding probability scores from each of the AMP prediction servers are listed in **Supplementary Table 3**.

Leader Peptide Analyses of *T. pallidum* Miniproteins

Many bacterial AMPs are synthesized as inactive preproteins with N-terminal leader/signal peptides whose presence and cleavage are required for export and activation, respectively. These include signal peptides recognized by the general secretory (Sec) pathway [Sec-dependent signal peptides (Sec/SP1 peptides)] (Leer et al., 1995; Chiorean et al., 2018) and Sec-independent double-glycine/glycine-alanine (GG/GA) leader peptides that have been documented in AMPs from both Gram-positive and Gram-negative bacteria (Havarstein et al., 1994; Oman and van der Donk, 2010). The signal peptide prediction servers, SignalP and LipoP, were used to search for the presence of Sec-dependent SP1 signal peptides, which predicted the presence of Sec/SP1 signal peptides in only four of the 68 miniproteins (**Supplementary Table 4**). Manual searches of the 68 *T. pallidum* AMP candidates for the conserved double-glycine/glycine-alanine leader peptide motif identified 24 proteins that contain Glycine-Glycine and/or Glycine-Alanine pairs within the first 31 residues of the N-terminus (**Supplementary Figure 1**). WebLogo analysis of the 24 proteins identified an N-terminal region with similarity to the double-glycine/glycine-alanine leader peptide motif from AMPs from Gram-negative bacteria, suggesting the presence of a similar secretion/activation recognition signal in *T. pallidum* candidate AMPs (**Supplementary Figure 2**).

Physicochemical Analyses of *T. pallidum* Miniproteins

Physicochemical properties known to be important for AMP function were calculated using the APD3 online AMP calculator and predictor tool. Consistent with the high content of arginine and lysine residues in AMPs, the top 22-ranking potential *T. pallidum* AMPs were found to have mean net charges at pH 7.0 of 5.56 (8 proteins with 8/8 positive AMP predictions) and 7.91 (14 proteins with 7/8 positive AMP predictions) (**Figure 3A** and **Supplementary Table 4**). Although no trend was observed between AMP likelihood rankings and the percentage of hydrophobic amino acids found within this group of proteins, the mean hydrophobic residue content of all 68 miniproteins was high (43.1%), with 66/68 proteins comprised of more than 30% hydrophobic residues (**Figure 3B** and **Supplementary Table 4**). Also observed was a trend in AMP likelihood rankings and the number / percentage of cysteine residues per protein. In general, cysteines were found to be more common in higher-ranking predicted AMPs (**Figure 3C** and **Supplementary Table 4**). In comparison to an average cysteine content of ∼1.9% found in all *T. pallidum* proteins [calculated from the Nichols strain (NC_021490) proteome], the top eight-ranking predicted AMPs (8/8 positive AMP predictions) contained on average almost three-fold more cysteines (mean cysteine residue content = 5.31%). Interestingly, the cysteine-rich nature of these *T. pallidum* miniproteins is shared with distinct classes of eukaryotic (Simmaco et al., 1994; Fahrner et al., 1996; Shafee et al., 2016) and prokaryotic (Baindara et al., 2017; Sugrue et al., 2020) AMPs, and thus may represent an important physicochemical property for protein structure and/or function.

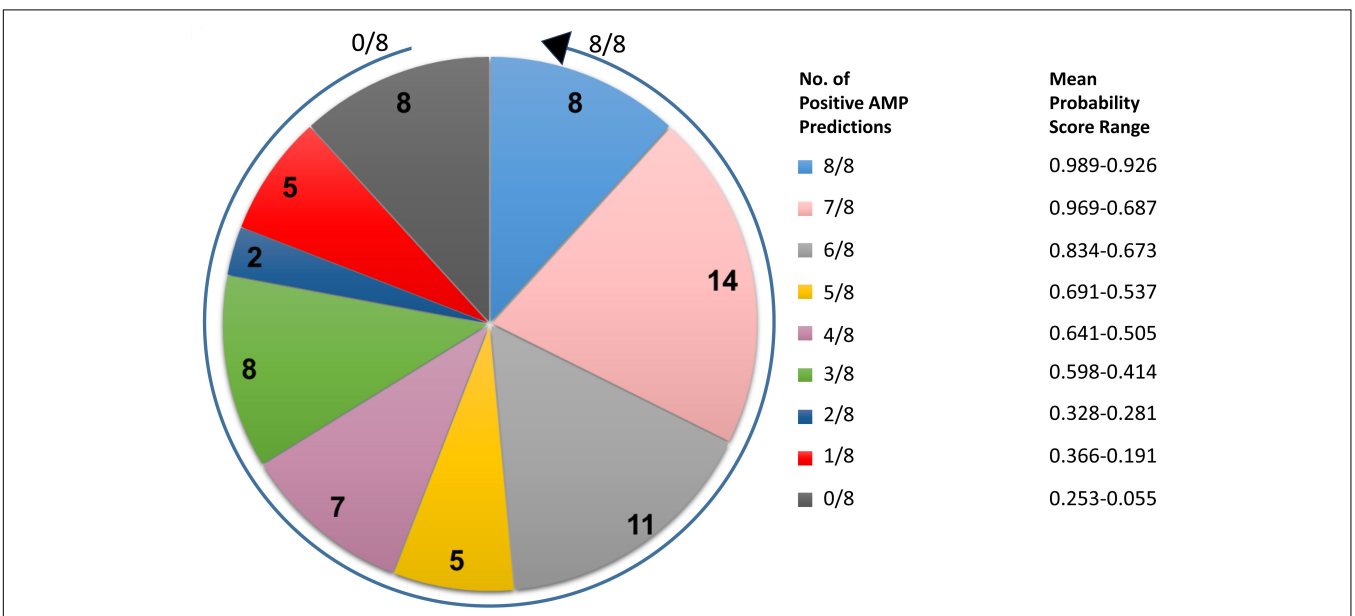


FIGURE 2 | Distribution of the number of positive AMP predictions and corresponding mean probability score ranges for *T. pallidum* miniproteins of unknown function. Pie chart indicating the number of positive AMP predictions following submission of the full-length amino acid sequences of 68 *T. pallidum* miniproteins (≤150 amino acids) to three AMP prediction servers (eight AMP prediction algorithms total). The arrow begins at the 0/8 positive AMP prediction class and finishes at the 8/8 positive AMP prediction class. The corresponding mean probability score range for all proteins from each positive AMP prediction class is also shown.

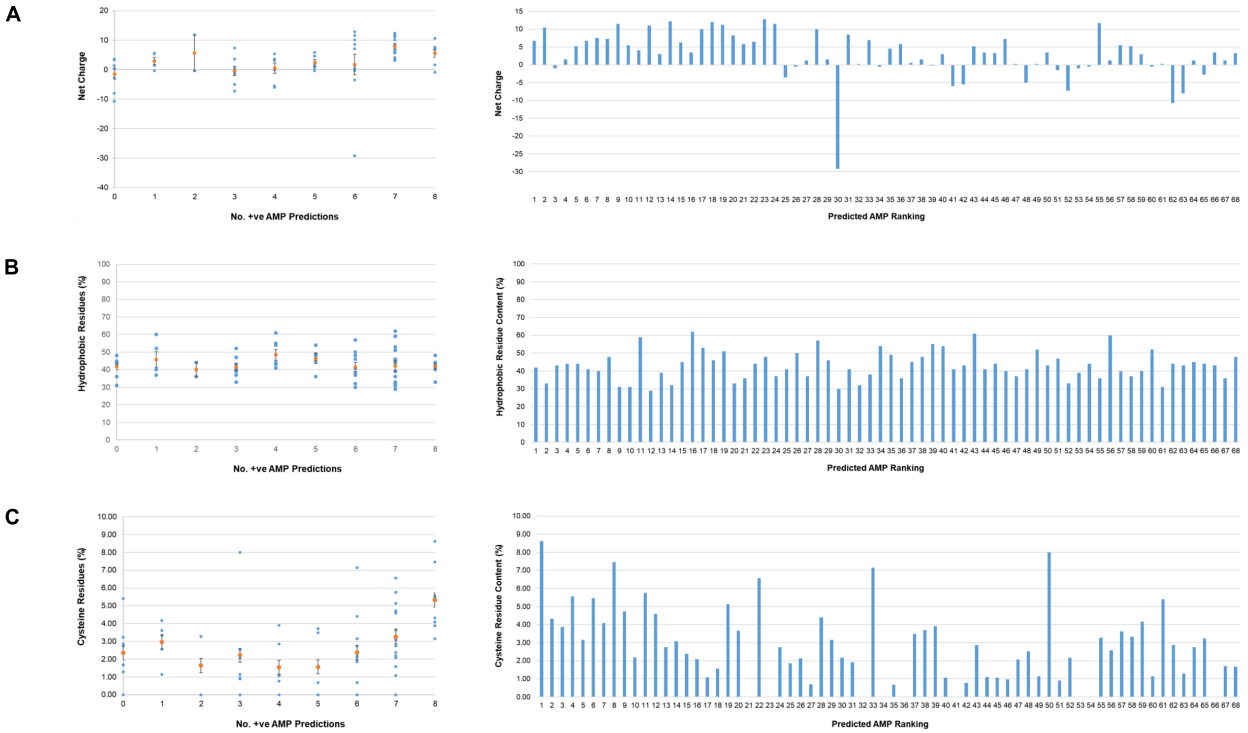


FIGURE 3 | Physicochemical properties of *T. pallidum* miniproteins of unknown function. Physicochemical properties known to be important for AMP function were calculated for the 68 *T. pallidum* proteins (≤150 amino acids) of unknown function using the APD3 online AMP calculator and predictor tool. Left: Scatter plots depicting **(A)** net charges, **(B)** hydrophobic residue content, and **(C)** cysteine residue content (orange circles represent the mean values, +/- standard error) of all proteins from each positive AMP prediction class. Right: Bar graphs showing **(A)** net charge, **(B)** hydrophobic amino acid content, and **(C)** cysteine residue content of all 68 miniproteins (≤150 amino acids) from predicted AMP ranking 1–68. No. +ve AMP Predictions equates to the number of AMP prediction programs that assigned the miniprotein as an AMP.

Expression Analyses of *T. pallidum* Miniproteins

Most of the 68 *T. pallidum* miniproteins from the current study are annotated in the published *T. pallidum* proteome as "hypothetical" proteins. However, DNA microarray-based analysis of the *T. pallidum* transcriptome following experimental rabbit infection (Smajs et al., 2005) demonstrated that 56/68 genes encoding these functionally uncharacterized proteins are expressed at the transcriptional level (**Supplementary Table 5**). Transcripts from most of the other 12 genes were not searched for in the study (Smajs et al., 2005) as they were not annotated in the *T. pallidum* genome at the time the study was performed. In addition, peptides from 15/68 miniproteins were detected in mass spectrometry-based proteomics studies of rabbit infections (McGill et al., 2010; Osbak et al., 2016), including the protein with the highest level of expression in the Osbak and colleagues study, Tp0214 (**Supplementary Table 5**). The use of trypsin for *T. pallidum* protein digestion in the two mass spectrometry studies may have contributed to the low number of miniproteins detected in these experiments. Given that miniproteins are small and contain high numbers of lysine and arginine residues, trypsin treatment, which results in cleavage after lysine and arginine residues, would be expected to cleave the miniproteins into many small peptides. Many of these peptides would be below the size detection limit, a major limiting factor for protein identification in mass spectrometry studies.

Proteome Clustering of *T. pallidum* Miniproteins

Bacterial genomic analyses show genes with related functions tend to form gene clusters (Tamames et al., 1997). To determine the spatial arrangement of all 68 miniproteins (≤150 amino acids) of unknown function within the *T. pallidum* proteome, each protein was arranged from the lowest (Tp0004) to the highest (Tp1032) locus tag number and clusters comprised of at least two miniproteins separated by five or less intervening proteins were identified. Forty-three of the 68 miniproteins (63%) were found to be located within one of 17 clusters, with 23 of the 43 proteins located in clusters comprised of at least three miniproteins of unknown function (**Supplementary Table 6** and **Figure 4A**). Twenty of the top 30-ranking predicted AMPs (67%) were found to be located within 13 miniprotein clusters, with 11 of the 20 proteins located in clusters containing at least three miniproteins of unknown function (**Supplementary Table 6** and **Figure 4B**). The top-ranking predicted AMP (Tp_RS02215) was found in a three-miniprotein cluster including the 8th-ranking predicted AMP (Tp0451a). Interestingly, analysis of the surrounding proteome

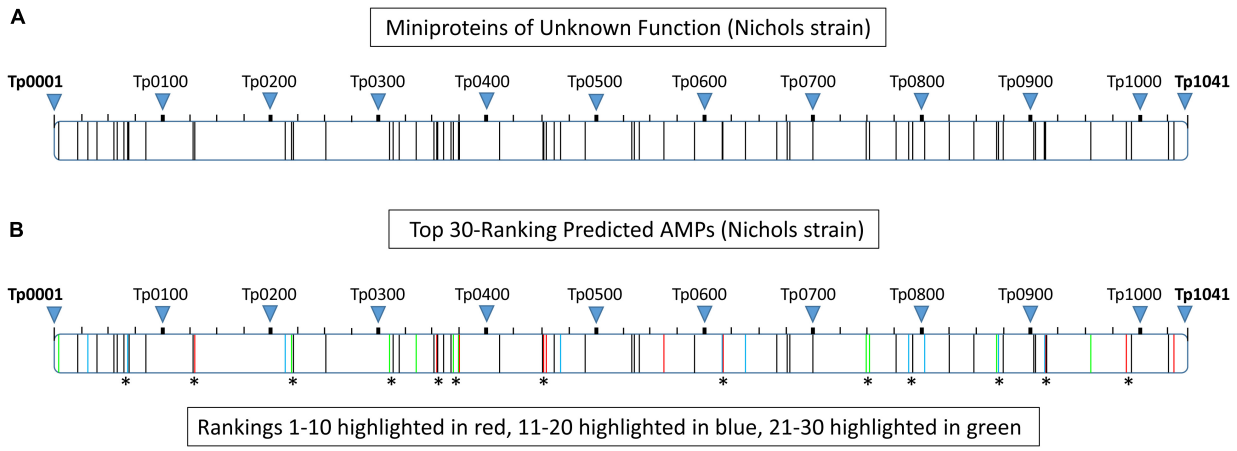


FIGURE 4 | Proteome clustering of *T. pallidum* (Nichols strain) miniproteins of unknown function. Each of the *T. pallidum* (Nichols strain) proteins (≤150 amino acids) of unknown function were arranged from the lowest (Tp0004) to the highest (Tp1032) locus tag number within the proteome (Tp0001–Tp1041) and clusters comprised of at least two miniproteins separated by five or less intervening proteins were identified. **(A)** Schematic depicting the spatial arrangement of all miniproteins (≤50 amino acids) of unknown function within the *T. pallidum* (Nichols strain) proteome. The location of each protein within the proteome is represented by a vertical black line. **(B)** Schematic showing the spatial arrangement of the 30 top-ranking predicted AMPs within the *T. pallidum* (Nichols strain) proteome. The location of proteins corresponding to rankings 1–10, 11–20, and 21–30 are shown in red, blue, and green, respectively. Asterisks denote the location of 20 of the top 30-ranking predicted AMPs that are found to be located within 13 miniprotein clusters.

identified two annotated proteins of note including the outer-membrane inner-leaflet-associated lipoprotein, Tp0453, which contains multiple outer membrane-inserting amphipathic alpha helices that result in membrane bilayer destabilization and enhanced permeability (Hazlett et al., 2005; Luthra et al., 2011). Also included in this region is Tp0454; structure modeling of Tp0454 using Phyre2 predicted tertiary structure similarity to several response regulators (**Supplementary Table 6**), including the DNA-binding response regulator, PhoP, from the PhoP-PhoQ two-component system that is a central regulator for AMP resistance in Gram-negative bacteria (Brodsky and Gunn, 2005). Proteome functional annotation analyses and Phyre2 structure modeling of open reading frames located close to other putative AMPs identified several additional potential homologs and structural orthologs with potential functions that are consistent with AMP secretion, activation, transport, and self-immunity (**Supplementary Table 6**), including the ORFs Tp0405 and Tp0688 that have been previously annotated as self-immunity proteins (Fraser et al., 1998). The close spatial arrangement of the miniproteins, in particular the high-ranking predicted AMPs, together with the observed proximity of potential AMP accessory proteins in the proteome of *T. pallidum* is consistent with the concept that functionally-related genes have a tendency to form clusters within bacterial genomes (Tamames et al., 1997).

AMP Candidate Selection: Tp0451a and Tp0749

Two AMP candidates, Tp0451a (accession number WP_014342798) and Tp0749 (accession number WP_010882194), were selected for the identification of potential AMPCCRs, the important minimalistic functional regions of AMPs. Tp0451a was selected as it (i) is one of the top-ranking predicted AMPs (8/8 positive AMP predictions, mean probability score of 92.6%) (**Supplementary Table 3**), (ii) possesses classical AMP properties (high content of positively-charged and hydrophobic amino acid residues) (**Supplementary Table 4**), (iii) is clustered in the proteome with several other potential AMPs/related proteins, as described above, and (iv) *tp0451a* is expressed at the transcript level, as described below. Although Tp0749 is a lower-ranking predicted AMP (ranked 24/68, 6/8 positive AMP predictions, mean probability score of 78.4%) (**Supplementary Table 3**), it was selected for further analyses as it (i) is highly positively-charged and contains high hydrophobic content, consistent with pore-forming AMPs (**Supplementary Table 4**), (ii) was identified in preliminary bioinformatics analyses as having clearly defined potential critical core regions, indicative of future success in AMPCCR design and synthesis, (iii) is known to be expressed at the transcript level (Smajs et al., 2005), unlike several of the higher-ranking predicted AMPs (**Supplementary Table 5**), (iv) is the second highest expressed ORF at the transcript level in the top-30 ranking *T. pallidum* miniproteins (Smajs et al., 2005) (**Supplementary Table 5**), and (v) of particular importance, it is one of only six minproteins within the top-30 ranking predicted AMPs whose expression has been detected at the protein level in experimental rabbit infections (Osbak et al., 2016) (**Supplementary Table 5**). To date, protein expression of all eight miniproteins from the top-eight ranking predicted AMPs (8/8 positive AMP predictions, mean probability score range 98.9–92.6%) has not been demonstrated in rabbit models of infection (McGill et al., 2010; Osbak et al., 2016), and only three of the top-eight ranking predicted AMPs have been shown to be expressed at the transcript level (Smajs et al., 2005). The strong experimental evidence confirming expression of Tp0749 at both the RNA and protein levels in rabbit infections increased the prioritization of this predicted

treponemal AMP over higher-ranking predicted AMPs for further bioinformatics and functional characterization studies.

Reverse Transcription-PCR Analysis of *tp0451a*

To confirm expression of *tp0451a*, we analyzed RNA isolated from *T. pallidum* by reverse transcription PCR (RT-PCR) using sense and antisense primers. When reverse transcriptase was present (RT+), the primer pair amplified a 198 base pair product, matching a similarly sized amplicon generated from *T. pallidum* genomic DNA (**Supplementary Figure 3** lanes 2 and 4, respectively). In comparison, only a very faint amplicon was detected when reverse transcriptase was omitted from the RT-PCR reaction (RT-) (**Supplementary Figure 3** lane 3), indicating that the 198 base pair product from the RT+ reaction was amplified from RNA and not contaminating DNA. Together with the previous finding that showed expression of Tp0749 at the protein level (Osbak et al., 2016), this result allowed us to proceed with investigations into potential AMP activity in *T. pallidum* by focusing on two miniproteins, Tp0451a and Tp0749, that are known to be expressed at either the transcript or protein level.

Bioinformatic Identification of Potential Critical Core Regions in Two Putative *T. pallidum* AMPs

The first step for mapping AMPCCRs in Tp0451a and Tp0749 involved using four prediction servers [AMPA (one algorithm), CAMP (three algorithms), AntiBP (three algorithms), and AntiBP2 (one algorithm)] to identify the amino acid boundaries of potential antimicrobial active regions (critical core regions, CCRs) within the two *T. pallidum* proteins based on clusters of high probability scoring regions predicted by at least three of the four servers. For both Tp0451a and Tp0749, two potential active regions were identified in the N-and C-termini of each protein (**Figures 5A,B**).

In the second step of our pipeline, secondary structure analyses and structure modeling of the two proteins and their identified potential active regions were performed. For Tp0451a, Jpred 4, and PSIPRED analyses predicted a predominantly coiled/beta strand structure that corresponded to the N-terminal predicted active region and an alpha helical structure that corresponded to the C-terminal predicted active region (**Figure 5A**). HeliQuest analysis showed that the predicted C-terminal alpha helix exhibited amphipathic properties (**Figure 5A**), a common structural characteristic in AMPs that is important for membrane integration and pore formation. Structure modeling using a combination of PEP-FOLD 2, Swiss Model, Modeller and Molsoft ICM was unable to generate high confidence models for either the N- or C-terminal regions that correspond to the predicted antimicrobial active regions. However, a robust model was generated for the intervening central region (residues E36-I71) (**Figure 5A** and **Supplementary Figure 4**). In agreement with the secondary structure predictions, this central region was modeled as two hydrophobic alpha helices. The structure prediction analyses were consistent with the multi-server AMPCCR mapping predictions by defining potential structural elements, one of which is important for AMP function, that corresponded to high-scoring predicted active regions. Together, these findings allowed for the identification of two potential critical core regions within the N- (Tp0451a_N) and C-terminus (Tp0451a_C) of Tp0451a (**Figure 5A**).

For Tp0749, both Jpred 4 and PSIPRED analyses predicted alpha helices that corresponded to the N- and C-terminal predicted active regions (**Figure 5B**). Consistently, a high confidence (92%) alpha helix was modeled for the N-terminal region that was predicted to exhibit antimicrobial activity (**Figures 5B**, **6A**). Importantly for potential AMP function, this modeled N-terminal alpha helix was also shown to be amphipathic with one face of the helix rich in positively-charged residues and the opposing face rich in hydrophobic residues (**Figures 6A,B**). In addition, a structure-based alignment using PROMALS3D of the Tp0749 N-terminal alpha helix model and a solved structure from the known AMP, human cathelicidin LL-37 (PDB:5NMN), predicted structural similarity between the two peptides (RMSD—0.31 Å over 19 Cα atoms) and conservation of 3/6 positively charged residues involved in binding target cell membrane lipids (Sancho-Vaello et al., 2017) (**Figure 6C**). In agreement with secondary structure predictions, a lower confidence (69%) partially amphipathic alpha helix model was also modeled for the C-terminal predicted antimicrobial active region (**Figures 5B**, **6D,E**). The combined approach of multi-server AMPCCR mapping, secondary structure prediction, and modeling allowed for the identification of two potential critical core regions located in the N- (Tp0749_N) and C- (Tp0749_C) terminus of Tp0749 (**Figure 5B**).

To help further resolve the potential active regions identified above, the four candidate AMPCCRs were then analyzed for similarity with known, experimentally-validated AMPs from the APD3 database and for their predicted cell penetrating capabilities using CellPPD. Amino acid sequence-based homology searches identified similarities of each of the four AMPCCRs with established AMPs with homologies ranging between 37 and 44% (**Table 2**). CellPPD analysis also predicted that three of the four potential AMPCCRs with predicted amphipathic alpha helices (Tp0451a_C, Tp0749_N, and Tp0749_C) (**Figures 5A, B**, **6**) contain peptide stretches that may have the ability to penetrate cell membranes (**Table 3**), a key functional feature of AMPs. The positive control peptide (LL-37) was also predicted to contain cell penetrating peptide regions, unlike the negative control peptide, Tp0751_p5 (**Table 3**). Together, these results bolstered the initial AMP predictions generated by the four prediction servers and identified two potential AMPCCRs within each of Tp0451a and Tp0749. These putative functionally-active core regions were prioritized for peptide synthesis and AMP functional characterization studies.

In vitro Antimicrobial Activity of *T. pallidum* AMPCCR Candidates

To evaluate the potential antimicrobial activity of the four predicted *T. pallidum* AMPCCRs identified via our

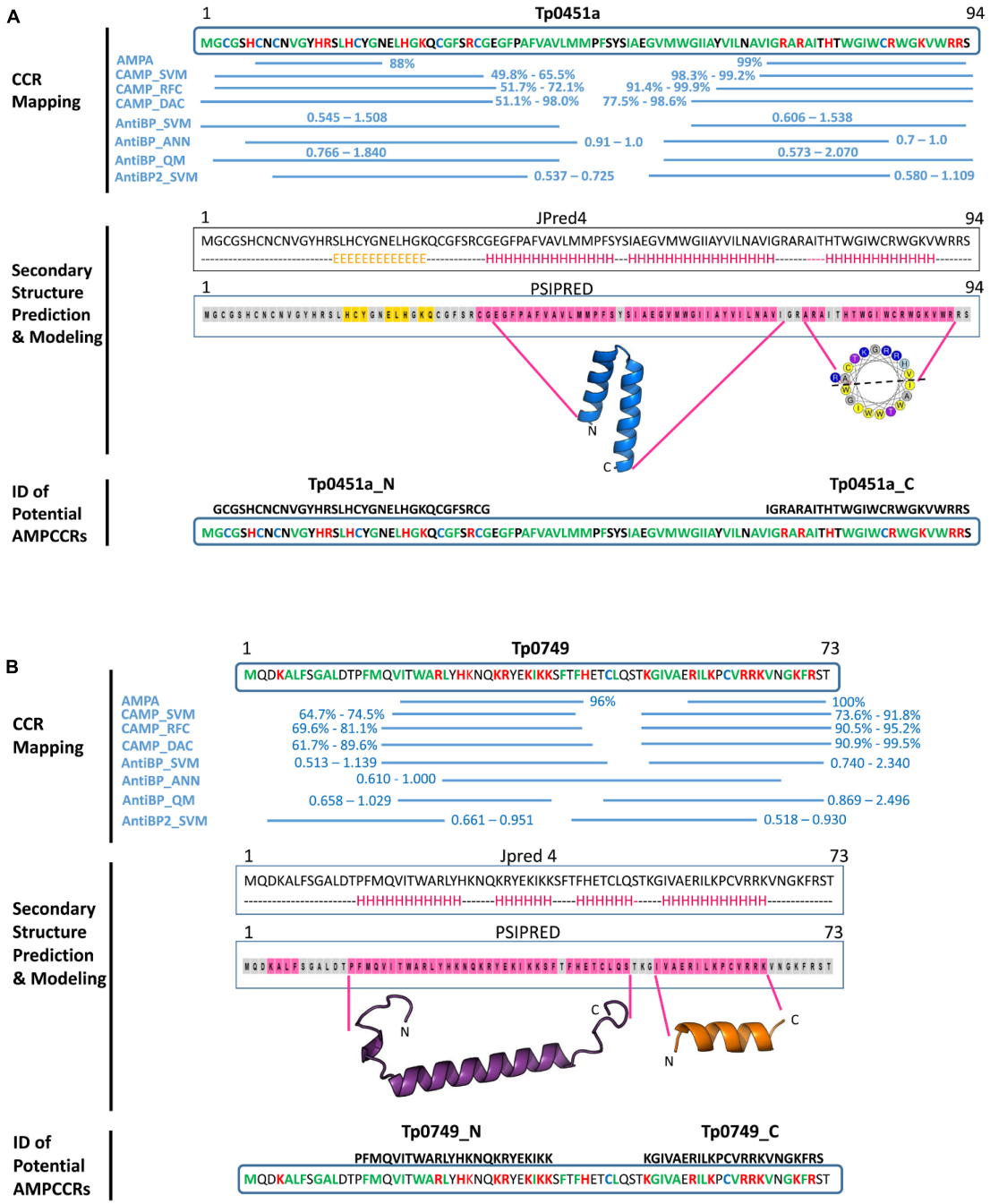


FIGURE 5 | *In silico* identification of potential *T. pallidum* AMPCCRs. The critical core regions of two candidate AMPs, **(A)** Tp0451a and **(B)** Tp0749, were predicted using our bioinformatics pipeline. The first step of the pipeline involved CCR mapping **(A,B**, top): four prediction servers [AMPA (one algorithm), CAMP (three algorithms), AntiBP (three algorithms), and AntiBP2 (one algorithm)] were used to identify the amino acid boundaries of potential antimicrobial active regions (critical core regions, CCRs). High probability/scoring regions predicted by at least three of the four servers are shown with their corresponding probabilities (AMPA and CAMP algorithms) or scores (AntiBP and AntiBP2 algorithms). Hydrophobic residues: green; Positively-charged residues: red; Cysteines: blue. In the second step of the pipeline, secondary structure analyses and modeling were performed (**A** and **B** panels, middle): secondary structure analyses of the full-length proteins were performed using Jpred 4 (H: alpha helix; E: beta strand; dashed line: coiled) and PSIPRED (pink highlight: alpha helix; orange highlight: beta strand; gray highlight: coiled). HeliQuest was used to generate helical wheel diagrams for potential alpha helices (yellow: hydrophobic residues; purple: serine or threonine; blue: positively charged residues: gray: glycine or alanine). Structure modeling using Modeller generated a confident model for the central region of Tp0451a (residues E36-I71), but confident models were not generated for the N- or C-terminal regions. Structure modeling using a combination of PEP-FOLD-2, Swiss-Model, Molsoft ICM, and Modeller generated models for the N- and C-terminal regions of Tp0749 (residues P14-S48 and I52-K65, respectively). Together, these findings allowed for the identification of two potential critical core regions within the N-terminus (Tp0451a_N and Tp0749_N) and C-terminus (Tp0451a_C and Tp0749_C) of Tp0451a and Tp0749 (**A** and **B** panels, bottom).

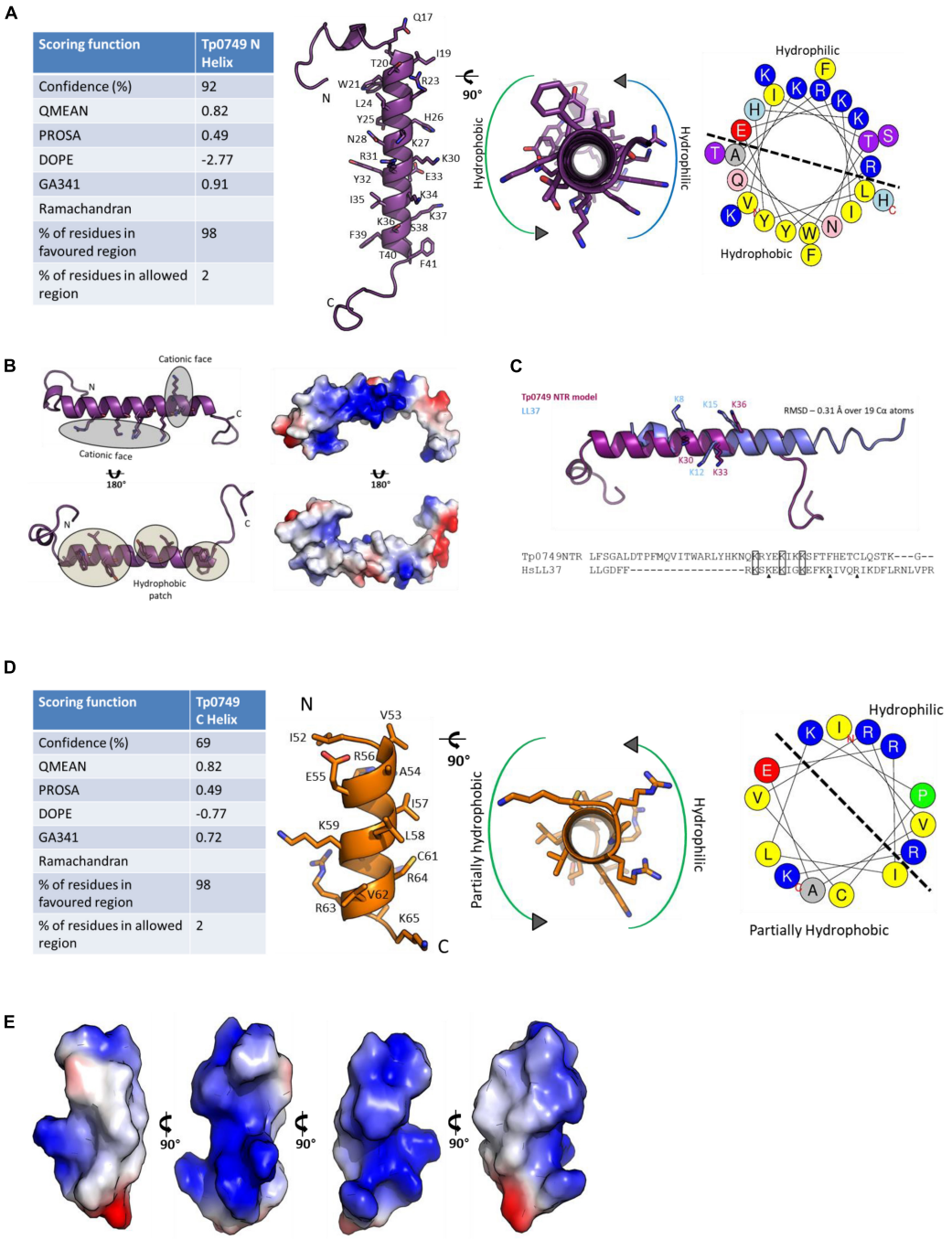


FIGURE 6 | Structure modeling of the candidate *T. pallidum* AMP, Tp0749. **(A**, Left): Table showing the scoring functions of the Tp0749 N-terminal model (residues P14-S48) generated by a combination of PEP-FOLD-2, Swiss-Model, Molsoft ICM, and Modeller. (Middle) Model ribbon structure of Tp0749 residues P14-S48 and rotated view showing amphipathicity. (Right) Helical wheel schematic of Tp0749 (P14-S48) generated using HeliQuest. Dashed line separates the hydrophilic/polar and hydrophobic/non-polar faces of the predicted alpha helix. **(B)** Ribbon and surface/charge distribution images of the Tp0749 (P14-S48) model showing one face of the alpha helix rich in positively-charged/polar residues (blue) and the opposing face rich in hydrophobic/non-polar residues (white). Red: negatively-charged/polar residues. **(C)** PROMALS3D was used to generate a structure-based comparative model of the Tp0749 N-terminal alpha helix model (P14-S48) using the structure of the known AMP, human cathelicidin LL-37 (PDB:5NMN) as a template. RMSD—0.31 Å over 19 Cα atoms and 3/6 positively charged residues involved in binding target cell membrane lipids are conserved (indicated by rectangles; triangles show non-conserved residues). **(D)** Left: Table showing the scoring functions of the Tp0749 C-terminal model (residues I52-K65) generated by using a combination of PEP-FOLD-2, Swiss-Model, Molsoft ICM, and Modeller. (Middle) Model ribbon structure of Tp0749 residues I52-K65 and rotated view showing partial amphipathicity (red: negatively charged/polar residue side chains; blue: positively charged/polar residue side chains; yellow: cysteine residue side chain). (Right) Helical wheel schematic of Tp0749 (I52-K65) generated using HeliQuest. Dashed line separates the hydrophilic/polar and partially hydrophobic/non-polar faces of the predicted alpha helix. **(E)** Surface and charge distribution views of Tp0749 (I52-K65) (red: negatively charged/polar residues; blue: positively charged/polar residues; white: hydrophobic/non-polar residues).

TABLE 2 | Known AMPs with the highest similarity to four *T. pallidum* candidate AMPCCRs.

Tp AMPCCR	Similar known AMP	AA Similarity (%)	Source	Activity
Tp0451a_N	Beta Defensin 6 (Yamaguchi et al., 2001)	37.20	Mammals	Anti-Gram-negative
Tp0451a_C	ecPis3 (Zhuang et al., 2017)	37.03	Fish	Anti-Gram-negative Anti-Gram-positive Antifungal Antiparasitic
Tp0749_N	Brevinin-1CHb (Conlon et al., 2011)	37.03	Amphibians	Anti-Gram-negative Anti-Gram-positive Antifungal
Tp0749_C	P15s (Oyama et al., 2017)	44.44	Mammals	Anti-Gram-negative

The four candidate AMPCCRs from T. pallidum were analyzed for amino acid sequence similarity with established, experimentally-validated AMPs using homology searches in the APD3 database. Tp, Treponema pallidum; AA, amino acid.

TABLE 3 | *Treponema pallidum* candidate AMPCCRs with predicted cell penetrating abilities.

Tp AMPCCR	Cell penetrating predictions	SVM Score
Tp0451a_N	GCGSHCNCNVGYHRSLHCYGNELHGKQCGFSRCG	Non-CPP
Tp0451a_C	IGRARAITHTWGI**WCRWGKVWRR**S	0.38
Tp0451a_C	IGRARAITHTW**GIWCRWGKVW**RRS	0.12
Tp0749_N	PFMQVITWARLYH**KNQKRYEKIK**K	0.36
Tp0749_N	PFMQVITWARLYHK**NQKRYEKIKK**	0.33
Tp0749_N	PFMQ**VITWARLYHK**NQKRYEKIKK	0.10
Tp0749_C	KGIVAE**RILKPCVRRK**VNGKFRS	0.46
Tp0749_C	**KGIVAERILK**PCVRRKVNGKFRS	0.21
Tp0749_C	KGIVAERILKPC**VRRKVNGKFR**S	0.20
Tp0749_C	KGIVAERILK**PCVRRKVNGK**FRS	0.19
Tp0749_C	KGIV**AERILKPCVR**RKVNGKFRS	0.15
LL-37 (+ve)	LLGDF**FRKSKEKIGK**EFKRIVQRIKDFLRNLVPRTES	0.31
LL-37 (+ve)	LL**GDFFRKSKEK**IGKEFKRIVQRIKDFLRNLVPRTES	0.16
LL-37 (+ve)	LLGDFFRKSKEKIGKEFK**RIVQRIKDFL**RNLVPRTES	0.15
LL-37 (+ve)	LLGDFFRKSKEKIGKEF**KRIVQRIKDF**LRNLVPRTES	0.13
LL-37 (+ve)	LLGDFFRKSKEKIGKE**FKRIVQRIKD**FLRNLVPRTES	0.11
LL-37 (+ve)	LLGDFFRKSKEKIG**KEFKRIVQRI**KDFLRNLVPRTES	0.10
Tp0751_p5 (–ve)	AMRIALWNRATHGEQGALQHLLAG	Non-CPP

The four candidate AMPCCRs from T. pallidum and positive (LL-37) and negative (Tp0751_p5) control peptides were analyzed for predicted cell penetrating capabilities using CellPPD (10 amino acid peptide scan). Predicted 10-amino acid cell penetrating peptides are highlighted in bold. Corresponding Support Vector Machine (SVM) scores [0.1 (lowest probability) – 1.0 (highest probability)] for each predicted cell penetrating peptide (in bold) are shown. Tp, Treponema pallidum; non-CPP, no cell penetrating peptides predicted.

bioinformatics pipeline, synthetic peptides were produced and antimicrobial susceptibility assays were performed to test for bacteriostatic and bactericidal activities against a panel of biologically and clinically relevant Gram-negative and Gram-positive bacteria. In broth microdilution assays, all four candidate AMPCCRs were active against *M. smegmatis* (**Table 4**). Consistent with our bioinformatics pipeline analyses, the three *T. pallidum* peptides with predicted amphipathic alpha helices (Tp0451a_C, Tp0749_N, and Tp0749_C) (**Figures 5A,B**, **6**) and highest AMPCCR mapping scores (**Figures 5A,B**) all exhibited robust anti-mycobacterial activity, unlike the lower-scoring Tp0451a_N. The lack of activity in the latter peptide aligns with the observation that this was the only peptide that lacked predicted (amphipathic) helical structure (**Figure 5A**). With the exception of Tp0451a_N, the treponemal peptides exhibited anti-mycobacterial activity that was similar to the positive control AMPs, LL-37 and RaCa-2. Tp0451a_N showed no antimicrobial activity against any of the other tested bacteria, whereas Tp0451a_C showed considerable bacteriostatic and bactericidal potency toward the Gram-positive bacterium, *S. pyogenes*, to a level that exceeded that observed with the positive control AMPs, LL-37 and RaCa-2. None of the other three treponemal peptides exhibited anti-streptococcal activity and all four treponemal peptides were inactive against *S. enterica* and *S. aureus*. Together, these findings demonstrate that each of the four treponemal peptides identified in our AMP discovery bioinformatics pipeline are capable of exhibiting both bacteriostatic and bactericidal activity.

TABLE 4 | Antimicrobial susceptibility testing of *T. pallidum* candidate AMPCCRs using broth microdilution.

Peptide	*E. coli* ATCC 9723H		*P. aeruginosa* ATCC 10148		*S. enterica* SL1344		*S. aureus* ATCC 6538P		*S. pyogenes* Unknown strain; Clinical Isolate		*M. smegmatis* MC^2155	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Tp0451a_N	–	–	–	–	–	–	–	–	–	–	34.7–69.5	69.5
Tp0451a_C	–	–	–	–	–	–	–	–	5.4–10.8	5.4–43.3	1.4–2.7	1.4–5.4
Tp0749_N	–	–	–	–	–	–	–	–	–	–	0.3–1.3	1.3
Tp0749_C	12.0	48.2–96.4	24.1–48.2	96.4	–	–	–	–	–	–	1.5–3.0	1.5–3.0
Tp0451a_C_C85S	10.9–21.8	10.9–43.6	–	–	–	–	–	–	5.4–10.9	10.9–43.6	5.4	10.9
Tp0749_C_C61S	24.2–48.5	–	97.0	–	–	–	–	–	–	–	12.1–24.2	24.2–48.5
Tp0751_p5 (–ve)	–	–	–	–	–	–	–	–	–	–	–	–
sLL-37 (–ve)	–	–	≥57	–	–	–	–	–	–	–	14.2–28.5	≥57
LL-37 (+ve)	1.8–7.1	1.8–7.1	3.6–14.2	3.6–14.2	1.8–28.5	1.8–28.5	7.1–28.5	7.1–57.0	28.5–57.0	28.5–57.0	0.45–1.8	0.45–1.8
RaCa-2 (+ve)	3.1–12.4	6.2–24.7	49.4–98.9	≥98.9	6.2–49.4	12.4–98.9	3.1–6.2	3.1–12.4	6.2–49.4	6.2–49.4	1.5–3.1	1.5–6.2

All peptides were tested between three and nine times in independent experiments against a panel of six clinically and biologically relevant Gram-negative, Gram-positive, and Mycobacterium species. MIC and MBC ranges (μM) are shown for each peptide. Strains are indicated for each bacterium with the exception of S. pyogenes (clinical isolate, unknown strain). -ve, negative control peptide; +ve, positive control peptide; –, no AMP activity.

Based on our six-member panel of bacteria, we found Tp0749_C to be the most broad-spectrum AMP of the four treponemal peptides. In addition to its anti-mycobacterial properties, it also showed moderate bacteriostatic activity towards *E. coli*, moderate-low bacteriostatic activity against *P. aeruginosa*, and low bactericidal activity against both *E. coli* and *P. aeruginosa* (**Table 4**). The positive control peptide, LL-37, was more active against *E. coli* and *P. aeruginosa* than Tp0749_C. The positive control peptide, RaCa-2, was more active against *E. coli* than Tp0749_C but exhibited lower anti-pseudomonal activity. The other three treponemal peptides showed no activity against these two Gram-negative bacteria. The negative control peptide, Tp0751_p5, a 24-mer peptide from the *T. pallidum* adhesin Tp0751 with similar physicochemical properties to AMPs, was inactive against all six bacteria in all experiments. Surprisingly, the negative control peptide, sLL-37, a scrambled version of LL-37, exhibited a low degree of antimicrobial activity against *M. smegmatis* and minimal bacteriostatic activity against *P. aeruginosa* (**Table 4**). Since sLL-37 retains many of the same physicochemical properties, including overall charge and amino acid composition, as LL-37, but is predicted to lack the helical content found in native LL-37, this may explain the low-level activity observed with the scrambled version of this peptide. Indeed, a similar low level of antimicrobial activity for sLL-37 has been demonstrated previously in an independent study (Gordon et al., 2005). Together, the Tp0451a and Tp0749 results suggest that *T. pallidum* is capable of producing AMPs that target Gram-negative bacteria, Gram-positive bacteria, and mycobacteria, and established proof-of-concept for our AMP discovery bioinformatics pipeline.

To evaluate if cysteine residues are important for *T. pallidum* AMP function, cysteine-to-serine substituted versions of Tp0451a_C (Tp0451a_C_C85S) and Tp0749_C (Tp0749_C_C61S) were synthesized and tested for antimicrobial activity using our panel of six bacteria and broth microdilution assays. These peptides were chosen as they have broad-spectrum AMP activity, and only contain one cysteine residue each. As shown in **Table 4**, compared to the unmodified, cysteine-containing AMPCCR, Tp0451a_C, the antimicrobial activity of Tp0451a_C85S against *M. smegmatis* was reduced two- to eight-fold, whereas the cysteine substitution had no effect on anti-streptococcal activity. Interestingly, the cysteine substituted version of Tp0451a_C exhibited moderate antimicrobial activity against *E. coli*, unlike the unmodified version. Compared to the unmodified, cysteine-containing AMPCCR, Tp0749_C, the bacteriostatic activity of Tp0749_C_C61S against *E. coli* and *P. aeruginosa* was reduced two- to four-fold, and reduced eight-fold against *M. smegmatis* (**Table 4**). Furthermore, the low bactericidal activity of Tp0749_C against *E. coli* and *P. aeruginosa* was abolished in Tp0749_C_C61S and the strong bactericidal activity against *M. smegmatis* was reduced 16-fold. Consistent with the high abundance of cysteines in the top-ranking predicted AMPs, these findings show that the cysteine residues are important for the AMP activity.

To investigate the potential antimicrobial activity of the *T. pallidum* AMPCCRs against the frequently co-infecting sexually transmitted pathogen, *N. gonorrhoeae*, an antimicrobial susceptibility assay based on agar dilution was developed and performed to test for bacteriostatic and bactericidal activities. Although growth was visibly inhibited by Tp0451a_C, none of the four treponemal AMPCCRs completely inhibited *Neisseria* growth on the agar plates at any of the peptide concentrations. The visible inhibition of growth by Tp0451a_C prompted us to test whether any of the treponemal peptides were bactericidal against *N. gonorrhoeae* by comparing TVCs of the 3 h-incubated peptide/bacteria mixtures with TVCs from the corresponding positive control growth wells to give the percentage of bacteria killed by the peptides. In agreement with our modified agar dilution results, we found Tp0451a_C to be the only treponemal peptide capable of exhibiting bactericidal activity against *N. gonorrhoeae*, with *Neisseria*-killing activity consistently observed at peptide concentrations of 64 μg/mL and higher (**Table 5**). The loss of anti-*Neisseria* activity in the cysteine substituted version of Tp0451a_C (Tp0451a_C_C85S) further

TABLE 5 | Antimicrobial susceptibility testing of *T. pallidum* candidate AMPCCRs against *N. gonorrhoeae* using a modified agar dilution method.

Peptide	256 μg/mL	128 μg/mL	64 μg/mL	32 μg/mL
	N. gonorrhoeae killing (%)			
Tp0451a_N	0	0	nt	nt
Tp0451a_C	97.6–100	94.4–100	90.5–100	0–93.0
Tp0749_N	0	0	nt	nt
Tp0749_C	0	0	nt	nt
Tp0451a_C_C85S	0	0	0	0
Tp0749_C_C61S	0	0	nt	nt
Tp0751_p5 (-ve)	0	0	0	0
sLL-37 (-ve)	0	0	0	0
LL-37 (+ve)	100	100	100	100
RaCa-2 (+ve)	100	100	100	100

The bactericidal activity of treponemal peptides was analyzed by comparing TVCs following a 3-h incubation period in the presence of N. gonorrhoeae with the TVCs from the positive growth control samples (N. gonorrhoeae, no peptides present). All peptides were tested in three to six independent experiments against N. gonorrhoeae (ATCC 700825, streptomycin resistant). The percentage killing of N. gonorrhoeae from all experiments is shown for each of the peptides. nt, not tested.

suggested the importance of cysteine residues in treponemal AMP function. These findings suggest that *T. pallidum* may express proteins that are capable of killing *Neisseria* during co-infections involving these two sexually transmitted pathogens.

AMPCCR Susceptibility Testing of *T. pallidum*

Treponema pallidum exhibits vigorous motility which can be used as an indication of bacterial viability. To assess the activity of the treponemal peptides against *T. pallidum*, *in vitro*-cultured *T. pallidum* was incubated with each peptide at three different concentrations. Treponeme viability was then determined by counting motile treponemes using darkfield microscopy. As shown in **Supplementary Figure 5**, only Tp451a_C showed antimicrobial activity against *T. pallidum* at 64, 16, and 4 μg/ml peptide concentrations, when compared to the negative control peptide, Tp0751_p5. The positive control peptide, LL-37, and Tp0749_N also showed a low level of inhibitory activity against *T. pallidum* at 64 μg/ml. These findings demonstrate that, similar to other bacteria (Beis and Rebuffat, 2019; Smits et al., 2020), *T. pallidum* is susceptible to the antimicrobial activity of some of the AMPs it produces, at least when added exogenously.

Immunomodulatory Capabilities of *T. pallidum* AMPCCRs

Based upon the well-established propensity for AMPs to suppress or induce cytokine production and the persistent nature of *T. pallidum* infection, we assessed potential immunomodulatory functions of the *T. pallidum* AMPCCRs through examining their capacity to influence cytokine production (IL-1β, IL-6, IL-8, IL-10, MCP-1, and TNF) from a human monocyte/macrophage cell line. Initially, we quantified cytokine production from monocytes (THP-1 cells) stimulated with the treponemal peptides under non-inflammatory conditions. Stimulation with treponemal peptides resulted in the production of several cytokines from monocytes, but the levels of cytokine were low (**Supplementary Figure 6**). No statistically significant differences in cytokine production between monocytes stimulated with Tp0751_p5 (peptide with no antimicrobial activity in the present study), and the treponemal AMPCCR, Tp0451a_C, was detected. Exposure of each of the peptides to macrophages (differentiated THP-1 cells) failed to induce a significant difference in cytokine expression when compared to the unstimulated control (**Supplementary Figure 7**). In summary, no treponemal AMPCCR-specific effect on cytokine secretion by undifferentiated monocytes or macrophages was observed in these non-inflammatory immunomodulation assays.

Previously, it has been shown that LL-37 modulates macrophage cytokine production under conditions where macrophages have been highly activated by exposure to the pro-inflammatory cytokine IL-32γ (Choi et al., 2014). IL-32γ also plays key roles in pathogen defense and persistence of chronic infection (Bai et al., 2010; Dos Santos et al., 2017; Li et al., 2018). To assess whether the *T. pallidum* AMPCCRs identified in the current study also modulate cytokine expression levels under cytokine-induced pro-inflammatory conditions, macrophages were activated with IL-32γ and immediately additionally stimulated with one of our control peptides (LL-37, sLL-37, and Tp0751_p5), or one of the four treponemal AMPCCRs (Tp0451a_N, Tp0451a_C, Tp0749_N, and Tp0749_C), or not exposed to a peptide (IL-32γ only) or IL-32γ (no stimulation). IL-32γ elicited robust production of the chemokine MCP-1 from macrophages compared to non-IL-32γ stimulated cells, demonstrating that the cells were successfully activated, and notably, both LL-37 and the treponemal AMPCCR Tp0451a_C were found to be the only peptides that significantly downregulated ($p < 0.0001$ for both peptides) IL-32γ-induced expression of MCP-1, in three independent experiments (**Figure 7A**). Additionally, LL-37 and Tp0451a_C were able to modulate macrophage-production of the chemokine IL-8 following IL-32γ stimulation: IL-32γ stimulation resulted in lower levels of IL-8 release compared to unstimulated cells, and co-stimulation of macrophages with IL-32γ and LL-37 or Tp0451a_C caused significantly higher levels of IL-8 release compared to from macrophages stimulated with IL-32γ alone ($p < 0.0001$ for both peptides in three independent experiments (**Figure 7B**). Co-stimulation of macrophages with each of the peptides failed to significantly affect the expression levels of IL-1β, IL-6, TNF, or IL-10 (**Supplementary Figure 8**). In addition to the antimicrobial activities described above, these findings demonstrate that the *T. pallidum* AMPCCR Tp0451a_C is also capable of immunomodulatory activities in certain inflammatory contexts.

DISCUSSION

In this study, we investigated the potential for *T. pallidum* to express AMPs as a previously unrecognized strategy to defend against competing bacteria and the host immune response. To conduct these studies, we developed a bioinformatics

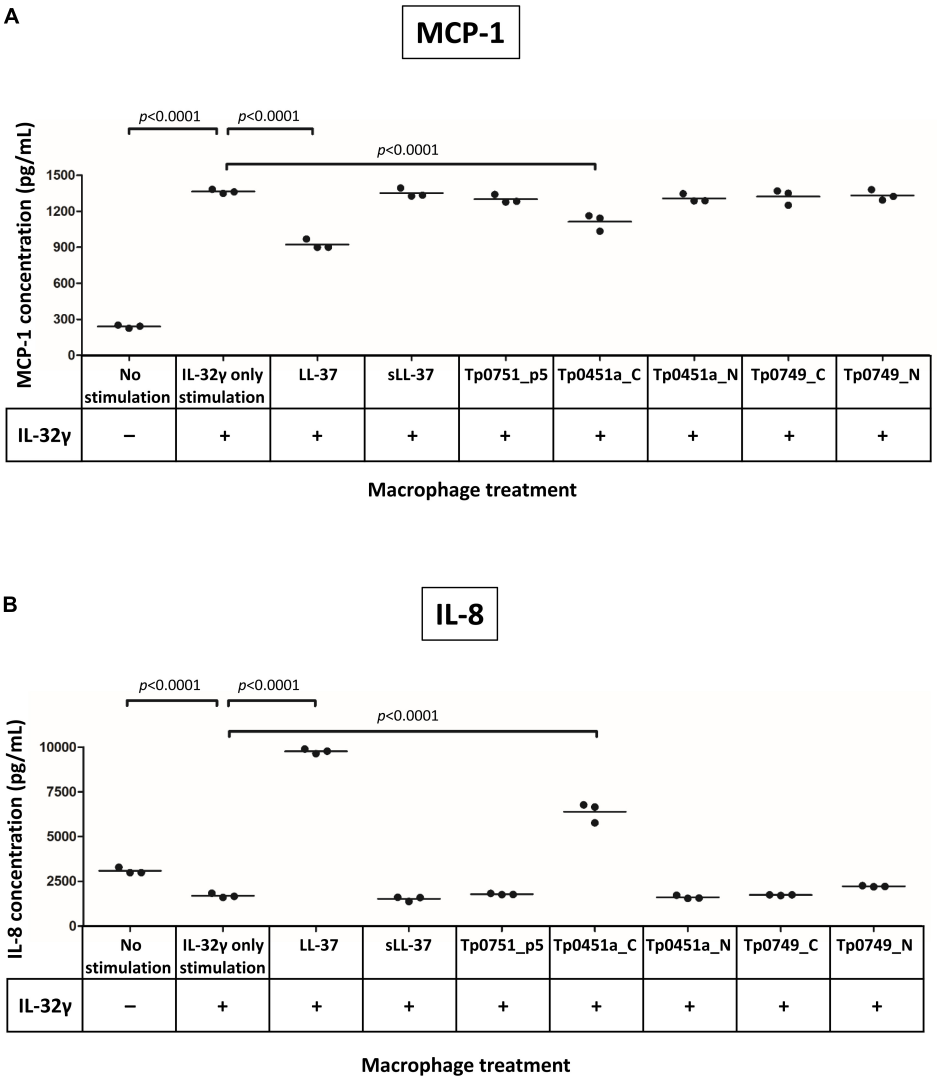


FIGURE 7 | Analysis of the immunomodulatory capacities of the *T. pallidum* AMPCCRs. Human THP-1 cells were differentiated to macrophages and then stimulated with or without IL-32γ. Cells were then immediately exposed to either LL-37, sLL-37, Tp0751_p5, Tp0451a_C, Tp0451a_N, Tp0749_C, or Tp0749_N and analyzed for expression of **(A)** MCP-1 and **(B)** IL-8. Each data point is representative of cells from one well of a 12-well plate. Data shown is representative of three independent experiments. A Dunnett's multiple comparisons test was used for normally distributed data and a Dunn's multiple comparisons test was used for data that was not normally distributed. For statistical analyses, mean values from each peptide were compared to the mean of the unstimulated control (IL-32γ only stimulation); *p*-values (Dunnett's multiple comparisons test) indicating statistically significant differences observed in three independent experiments are indicated.

pipeline for the identification of candidate *T. pallidum* AMPs. By using AMPCCR mapping, followed by a combination of structure, modeling, homology, and cell penetration prediction analyses, potential AMPCCRs were identified in two *T. pallidum* proteins, Tp0451a and Tp0749. Together, these findings enabled the design and synthesis of four putative AMPCCRs to evaluate these *T. pallidum* proteins for antibacterial and immunomodulatory functions.

Using antimicrobial susceptibility assays, we demonstrated bacteriostatic and bactericidal activity for the four predicted *T. pallidum* AMPCCRs. All four peptides were active against the model *Mycobacterium* sp., *M. smegmatis*, three of which (Tp0451a_C, Tp0749_N, and Tp0749_C) exhibited robust anti-mycobacterial activity that was similar or more potent than the activity of many antimycobacterial AMPs from other organisms (Abedinzadeh et al., 2015; Gupta et al., 2015; Helbing et al., 2019). *Mycobacterium smegmatis* is generally considered as nonpathogenic, however, it has been shown on rare occasions to cause disease in humans (Wallace et al., 1988; Newton et al., 1993; Pennekamp et al., 1997; Hong et al., 2003; Ergan et al., 2004). Interestingly, *M. smegmatis* was originally isolated from syphilitic chancres and gummas (Bloom, 1885) and is known to be present in normal genital (smegma) secretions. *Mycobacterium smegmatis* is neither a true Gram-negative nor Gram-positive due to its unusual cell envelope ultrastructure and composition, which is similar in all known mycobacteria species, including

the important human pathogen *Mycobacterium tuberculosis* (Brennan and Nikaido, 1995; Cook et al., 2009). Although few cases have been documented, *T. pallidum*/*M. tuberculosis* coinfections in HIV patients have been reported (Latif et al., 2020). Given the cell envelope conservation observed within mycobacteria, the *T. pallidum* proteins demonstrated in this study to possess AMP activity may also be capable of targeting *M. tuberculosis* during situations of co-infection.

One of the treponemal AMPCCRs (Tp0451a_C) exhibited bacteriostatic and bactericidal activity against a clinical isolate of the Gram-positive extracellular bacterial pathogen, *S. pyogenes*. At the time of writing, searches of the AMP database, APD3 (see footnote 26) (Wang et al., 2016), identified only 71 known AMPs (out of a total of 3324 known AMPs) with antimicrobial activity against *S. pyogenes*, including only 12 from other bacteria, most of which are non-pathogenic members of the microbiota. In line with these results, few reports have been published describing this rare antimicrobial activity, making Tp0451a_C a new addition to this select group of *S. pyogenes*-targeting AMPs. Furthermore, the anti-*S. pyogenes* activity of Tp0451a_C was found to be generally comparable (Cogen et al., 2010; Uhlmann et al., 2016; Ma et al., 2020) or more potent (Sornwatana et al., 2018; Li et al., 2022) than known AMPs from other organisms. The relatively small number of AMPs that show activity against *S. pyogenes* is attributed to the expression of several proteins involved in AMP resistance, including the streptococcal cysteine protease SpeB (Schmidtchen et al., 2002) which is involved in the proteolytic degradation and inactivation of AMPs, the M1 protein, streptokinase, and the streptococcal inhibitor of complement, the latter three of which are involved in resistance against defensins and/or LL-37 (Frick et al., 2003; Lauth et al., 2009; Hollands et al., 2012). Our findings show that the AMP-activity identified in Tp0451a_C is capable of circumventing the resistance mechanisms developed by pathogenic *S. pyogenes*. Importantly, *S. pyogenes* colonizes and infects several host sites that *T. pallidum* also encounters, particularly during *T. pallidum* transmission. These shared host sites include the skin and the mucosal membranes of the oropharynx, rectum, and genitals (Stamm et al., 1978; Mead and Winn, 2000; Lafond and Lukehart, 2006; Sobel et al., 2007; Minami et al., 2010; Verstraelen et al., 2011; Nelson et al., 2016; Norimatsu and Ohno, 2020). Taken together, these findings suggest that *Streptococcus*-killing AMPs may comprise a defense strategy that facilitates survival during infection by protecting *T. pallidum* against this clinically relevant, competing microbe in key host infection sites such as the genital mucosa, via direct inhibition and killing.

Tp0451a_C was also demonstrated to be the only treponemal peptide capable of exhibiting bactericidal activity against the frequently co-infecting sexually transmitted pathogen *N. gonorrhoeae* (Bala et al., 2011). This finding is consistent with the concept that *T. pallidum* and *N. gonorrhoeae* localize to the same environmental niches within the urogenital region, particularly during transmission and primary stage syphilis when treponemes are localized within the chancre at the initial site of infection. In light of the increasing global public health threat posed by *N. gonorrhoeae* due to the rapid emergence of multiple drug resistant strains and with an estimated 78 million new cases per year (Alirol et al., 2017), our finding of a *Neisseria*-active AMP is relevant to the goal of developing novel therapeutics against this pathogen.

Most AMPs from bacteria exhibit narrow antimicrobial spectra; they are produced in order to defend themselves and their environmental niche from a few genus/species-specific competing species that are often also closely related to the AMP-producing bacterium (Cleveland et al., 2001; Cotter et al., 2005; De Vuyst and Leroy, 2007). This is most likely because closely related bacterial species often reside within the same environmental niches. However, identification of broad spectrum AMPs has become increasingly common, particularly amongst non-pathogenic Gram-positive bacteria (Kemperman et al., 2003a,b; Zendo, 2013; Todorov et al., 2019). Here we found Tp0749_C and Tp0451a_C to exhibit the most broad-spectrum activity of the four treponemal peptides tested. Interestingly, Tp0749_C exhibited bacteriostatic and bactericidal activity against a non-pathogenic strain of the Gram-negative bacterium, *E. coli*. Non-pathogenic *E. coli* strains are normal, prevalent residents of the human lower gastrointestinal tract, including the rectum (Zhang et al., 2002; Tenaillon et al., 2010). The fact that syphilis chancres commonly occur in the rectum and anal canal of men-who-have-sex-with-men infected with *T. pallidum* (Lafond and Lukehart, 2006), suggests (i) the likelihood of a close association between non-pathogenic *E. coli* and *T. pallidum* during primary stage syphilis in these individuals and (ii) the possibility that *T. pallidum* AMPs are expressed that target non-pathogenic *E. coli* in this host infection site. It is also likely that *T. pallidum* encounters pathogenic / uropathogenic strains of *E. coli* during infection. Although these strains were not tested in our antimicrobial susceptibility assays, previous studies have shown that AMPs from other organisms exhibit similar levels of antimicrobial activity against both non-pathogenic and pathogenic / uropathogenic *E. coli* strains (Fedders et al., 2010; Aghazadeh et al., 2019; Mardirossian et al., 2019; Moazzezy et al., 2020; Li et al., 2022; Lin et al., 2022). Tp0749_C also exhibited antimicrobial activity against the Gram-negative bacterium, *P. aeruginosa*. *P. aeruginosa* is an opportunistic pathogen that is the causative agent of both severe acute and chronic infections in immunocompromised individuals, and is one of the major etiological agents of urinary tract infections (Ronald, 2002; Shigemura et al., 2006). Given that *T. pallidum* / HIV co-infections are common (Shockman et al., 2014; Burchell et al., 2015), and the potential for co-localization of *T. pallidum* and *P. aeruginosa* to the same environmental niches within the urogenital region in immunocompromised patients, these findings support the concept that *T. pallidum* may produce anti-pseudomonal AMPs as a defense mechanism during infection. Together, T0451a and Tp0749 were capable of inhibition and/or killing of five of the seven diverse bacterial species tested, including three Gram-negative bacteria, a Gram-positive bacterium, and a mycobacterium. This finding is consistent with the ability of *T. pallidum* to invade and disseminate to any host organ or tissue during the different stages of syphilis and the variable, and often complex, polymicrobial environments that exist within different sites of infection.

As part of our physicochemical analyses of *T. pallidum* miniproteins, we determined that the rare amino acid cysteine appears frequently in high-ranking predicted AMPs. Substitution of the single cysteine residue in Tp0451a_C with the structurally similar residue serine reduced its antimicrobial activity against *N. gonorrhoeae* and *M. smegmatis*, but resulted in a gain of antimicrobial activity against *E. coli*. Substitution of the single cysteine residue in Tp0749_C with serine greatly reduced the antimicrobial activity against *E. coli* and *P. aeruginosa*, and even more so against *M. smegmatis*. This unusual physicochemical property is shared with specific classes of AMPs, in particular certain eukaryotic AMPs, including defensins which typically contain six to twelve cysteines (Shafee et al., 2016), protegrins, which are active against several sexually transmitted pathogens and often contain four cysteines (Tamamura et al., 1995; Fahrner et al., 1996; Qu et al., 1996; Yasin et al., 1996), and brevinins which usually contain two cysteine residues (Simmaco et al., 1994). More recently, increasing numbers of cysteine-rich and defensin-like AMPs are being discovered in bacteria (Baindara et al., 2017; Sugrue et al., 2020). The even number of cysteine residues in these AMPs allows for the formation of intra-molecular disulfide bonds, which are important for AMP stability (Dias Rde and Franco, 2015). Literature and PDB searches indicate that most cysteine-containing AMP structures that have been solved are monomers that contain even numbers of cysteines / intra-molecular disulfide bonds. However, some AMPs with an odd number of cysteines form inter-molecular disulfide bonds resulting in dimers that can increase activity and stability compared to monomeric forms (Campopiano et al., 2004; Min et al., 2017). The AMPCCRs, Tp0451a_C and Tp0749_C, should only be capable of forming inter-molecular disulfide bonds / multimers, as they only contain one cysteine each. However, full-length Tp0451a_C contains seven cysteine resides and Tp0749 contains two cysteine residues, suggesting the additional possibility of intra-molecular disulfide bond formation. Similar to the aforementioned cysteine-rich AMPs, our findings are in agreement with the hypothesis that cysteines are important for the demonstrated treponemal AMP activity, possibly via the formation of inter- and/or intra-molecular disulfide bonds and miniprotein multimerization.

Due to the antimicrobial findings described herein, the fact that many AMPs have the ability to modulate immune functions (Lai and Gallo, 2009; Kindrachuk et al., 2013; Dicks et al., 2018; Malaczewska et al., 2019), and the stealth nature of *T. pallidum*, we investigated the immunomodulatory capabilities of the four treponemal peptides under both non-inflammatory and cytokine-induced inflammatory conditions, through assaying cytokine production from a human monocyte/macrophage cell line. Given that IL-32γ plays key roles in pathogen defense and persistence of chronic infection (Bai et al., 2010; Dos Santos et al., 2017; Li et al., 2018), and that AMP-mediated immunomodulatory activities have been shown to modulate IL-32γ-induced cytokine production (Choi et al., 2014), IL-32γ was selected as a biologically relevant co-stimulatory agent for our assays to mimic the pro-inflammatory environment present during *T. pallidum* infection (Lafond and Lukehart, 2006). Interleukin-32 is a recently discovered pro-inflammatory cytokine that functions in the persistence of inflammation via induction of other pro-inflammatory cytokines and in the control of infectious and chronic diseases (Khawar et al., 2016; Ribeiro-Dias et al., 2017). It has been found to be elevated in human infections, where it can have a protective (*Mycobacterium tuberculosis*, HIV) or detrimental (*Helicobacter pylori*) effect on the host (Rasool et al., 2008; Bai et al., 2010; Peng et al., 2014). Although the up-regulation and involvement of IL-32 isoforms in *T. pallidum* infection has yet to be demonstrated, the inflammation associated with *T. pallidum* infection is consistent with this possibility. Furthermore, IL-32 is upregulated in HIV infection (Rasool et al., 2008), a pathogen that is frequently involved in co-infections with *T. pallidum*.

Under IL-32γ-induced pro-inflammatory conditions in macrophages, we found that co-stimulation with Tp0451a_C resulted in decreased production of the pro-inflammatory chemokine MCP-1 and increased production of the pro-inflammatory chemokine, IL-8, compared to macrophages not exposed to peptides. MCP-1 is produced by a variety of cell types, several of which are relevant to *T. pallidum* infection, including monocytes, macrophages, epithelial, and endothelial cells (Yoshimura et al., 1989a,b; Cushing et al., 1990; Standiford et al., 1991). Monocyte chemoattractant protein-1 is the main chemoattractant for monocytes and macrophages to sites of inflammation where recruited monocytes undergo transformation into macrophages (Deshmane et al., 2009). During infection, clearance of *T. pallidum* is dependent on macrophages via antibody-mediated opsonophagocytosis and subsequent killing by macrophages (Lukehart and Miller, 1978; Baker-Zander and Lukehart, 1992; Baker-Zander et al., 1993). However, in the absence of antibiotic intervention, treponemes are never fully cleared from the host. The ability of Tp0451a to down-regulate expression of pro-inflammatory cytokines, including MCP-1, may contribute to treponemal survival via local suppression of the inflammatory response and dampening of macrophage recruitment.

In the present study, we also found Tp0451a_C capable of significantly increasing production of IL-8 in co-stimulated macrophages. This pro-inflammatory cytokine is a potent neutrophil chemoattractant that can induce neutrophil morphology changes and degranulation, and neutrophil migration to sites of infection and inflammation (Baggiolini and Clark-Lewis, 1992; Kolaczkowska and Kubes, 2013; Arango Duque and Descoteaux, 2014). Similar to the findings described herein, IL-8 has been shown to be up-regulated in macrophages stimulated with *T. pallidum* peptides derived from the major immunogenic lipoprotein, TpN47 (Sellati et al., 1998). If IL-8 up-regulation, by Tp0451a_C or other treponemal proteins, is recapitulated at the level of viable whole *T. pallidum*, the following consequences may be envisioned that could promote *T. pallidum* pathogenesis. Since neutrophils are largely ineffective against *T. pallidum* during early infection (Lafond and Lukehart, 2006), IL-8 production may function as an early infection immune diversion mechanism. Alternatively, an increase in IL-8 expression may redirect the immune response towards competing neutrophil-sensitive microorganisms in polymicrobial host sites, such as uropathogenic *E. coli*

(Svanborg-Eden et al., 1987). As IL-8 is also a pro-angiogenic chemokine (Li et al., 2003), up-regulation may contribute to the vascular inflammation and increased angiogenesis observed during secondary syphilis (French, 2007; Gao et al., 2019) and may promote treponemal accessibility to the host's circulatory system via proliferation of endothelial cells and formation of new blood vessels, similar to the mechanism proposed for the IL-8-inducing *T. pallidum* protein TpF1 (Pozzobon et al., 2016).

Here we employed a combination of bioinformatics, antimicrobial susceptibility testing, and immunomodulation assays that allowed for the experimental identification of the first proteins with AMP activity from a spirochete bacterium. However, there are limitations with our approach. First, due to difficulties with expression, full-length versions of the treponemal proteins were not used in antimicrobial susceptibility or immunomodulation experiments. Confirmation of AMP activity with the full-length proteins would be optimal, and may uncover an enhanced AMP activity due to the additive effects of the AMPCCRs. Second, in our antimicrobial susceptibility assays, we only tested for antibacterial activity and thus may have missed antifungal, antiviral, and/or antiparasitic activities. Third, although most AMPs are smaller than 150 amino acids, our bioinformatics pipeline omitted larger proteins from our analyses thereby excluding potential colicin-like bacteriocins. Fourth, the expression status of approximately three-quarters of all *T. pallidum* miniproteins of unknown function remain to be determined, due in part to the experimental difficulty with identifying small, positively-charged proteins via mass spectrometry-based proteomic analyses. Furthermore, the identification of potentially secreted *T. pallidum* proteins from *in vivo* and *in vitro* cultures by mass spectrometry and other methodologies is not possible due to the overwhelming abundance of contaminating rabbit/host proteins and the complexity of the culture medium, respectively. Fifth, similar to other AMP studies, the *in vitro* nature of the antimicrobial susceptibility assays, which were based on established, standardized protocols (Wiegand et al., 2008), are not representative of the complex host environments encountered during infection. Therefore, it remains to be determined if the concentrations of *T. pallidum* peptides that resulted in bacteriostatic and bactericidal activities are representative of the concentrations that are expressed and secreted into diverse host sites during infection.

Finally, and most significantly, similar to other recently discovered AMPs, our functional characterization of the treponemal proteins with AMP activity has yet to determine if/how they are processed/activated, and the mechanisms involved in self-immunity and export. Most bacterial AMPs that have been functionally characterized are synthesized as inactive protein precursors comprised of a N-terminal leader peptide/signal peptide, that is required for export, and a C-terminal proprotein that contains the critical core regions (Torrent et al., 2009, 2012; Chang et al., 2015; Perez et al., 2018). Following specific protease-mediated cleavage of the leader / signal peptide, the mature, functionally-active AMP is exported from the AMP-producing bacteria to the external environment via ATP-binding cassette (ABC) transporters (Beis and Rebuffat, 2019). In the present study, we showed that several *T. pallidum* candidate AMPs contain N-terminal amino acid sequences with similarity to the Gram-negative Sec-independent double-glycine/glycine-alanine (GG/GA) leader peptides, suggesting that a subset of *T. pallidum* AMPs may also use this recognition signal to direct secretion and allow for activation of precursor AMP forms. In addition, proteome functional annotation analyses and Phyre2 structure modeling of open reading frames located close to several potential AMPs identified (1) putative homologs and structural orthologs of proteases with potential roles in AMP leader peptide cleavage and activation and (2) ABC transporters that may mediate export of the mature active treponemal AMPs to the external host environment during infection. Notably, an emerging class of bacterial AMPs with varying characteristics, referred to as leaderless AMPs, do not undergo post-translational processing or modification, are produced without an export-mediating N-terminal leader/signal peptide, and are fully active immediately following expression (Perez et al., 2018). Leaderless AMPs are the most poorly understood group of bacterial AMPs, and the molecular mechanisms underlying self-immunity, secretion, and export remain largely unknown (Perez et al., 2018). Although no common secretion or export mechanism has been discovered for leaderless AMPs, an ABC transporter protein (LmrB) has been shown to be involved in the immunity and export of a leaderless AMP from *Lactococcus lactis*, LsbB (Gajic et al., 2003). Additionally, an ABC transporter protein (DDHIJ) from *Enterococcus faecalis* has been implicated in the active export of the leaderless AMP, Enterocin DD14 (Ladjouzi et al., 2020). It is possible that Tp0451a and Tp0749 belong to, or are related to, the leaderless group of bacterial AMPs. In this situation, the two *T. pallidum* AMPs would be expressed without processing/proteolytic cleavage, followed by export via one or more of the many ABC transporter proteins located within the *T. pallidum* proteome (Fraser et al., 1998). This mechanism would result in the N- and C-terminal AMPCCRs identified in the current study to remain fully intact and associated with each other, potentially allowing for enhanced, broad spectrum AMP activity due to the additive effects of the AMPCCRs. This is consistent with the knowledge that AMP activity is often localized to more than one discrete peptide region, or domain (AMPCCRs), within the full-length protein (Todorov, 2009; Torrent et al., 2012; Snyder and Worobo, 2014).

In the present study, we showed that the viability of *T. pallidum* was reduced following incubation with Tp0451a_C. This result was not surprising, given that AMPs from other bacteria are highly toxic to the AMP-producing strain, necessitating the expression of self-immunity proteins and dedicated ABC transporters that protect the AMP-producing bacteria during expression within the cell through extrusion outside and away from the cell (Smits et al., 2020). The unique composition of the *T. pallidum* cell envelope may confer a degree of protection against AMPs, as suggested in a previous study that investigated the activity of the full-length mammalian AMP LL-37 against *T. pallidum* (Cox et al., 2003). Specifically, the *T. pallidum* outer membrane has low anionic phospholipid content compared to other Gram-negative bacteria, contains

few outer membrane proteins including porins and negatively-charged proteins, lacks LPS, and contains cholesterol. Thus, *T. pallidum* possesses a cell envelope that is reminiscent of the host cell membrane with regards to a neutral surface charge and the presence of the AMP inhibitory molecule, cholesterol (Feigin et al., 1995; Matsuzaki et al., 1995; Glukhov et al., 2005). Together, these may help prevent the electrostatic binding of cationic AMPs to the *T. pallidum* surface, which is consistent with our findings in the current study that showed only minor or no decrease in *T. pallidum* viability following incubation with all but one of the AMPs that were tested.

In conclusion, this study has established proof-of-concept for our AMP discovery bioinformatics pipeline via the experimental identification of proteins with AMP activity in *T. pallidum*. The ability of *T. pallidum* to produce proteins with dual antimicrobial and immunomodulatory activities may contribute to treponeme survival by eliminating competing microbes via direct inhibition and killing effects, and by modulating the host immune response to promote both indirect inhibition and killing of competing bacterial species and immune evasion. This research has the potential to enhance our understanding of the unique pathogenesis of *T. pallidum* and reveal novel defense and survival mechanisms with broad applicability to bacterial pathogens, including other pathogenic spirochetes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the local institutional review board at the University of Victoria, and conducted in strict accordance with standard accepted principles as set forth by the Canadian Council on Animal Care, National Institutes of Health and the United States Department of Agriculture in a facility accredited by the Canadian Council on Animal Care and the American Association for the Accreditation of Laboratory Animal Care.

AUTHOR CONTRIBUTIONS

SH, CC, LR, KC, ES, RR, and MB contributed to the experimental design. SH, KC, ES, AG, SM, AH, and RR conducted the experiments. SH, KC, RR, ES, LR, MB, and CC were involved in the analysis and interpretation of the data. CC and LR acquired financial support for the project. SH wrote the first draft of the manuscript with contributions from KC. ES, RR, AG, SM, AH, CC, LR, and MB reviewed the manuscript before submission for accuracy and intellectual content. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the National Institutes of Health, National Institute of Allergy and Infectious Diseases (R37AI051334; CC), the Natural Sciences and Engineering Research Council of Canada (327196; CC and LR), and the Canadian Institutes of Health Research (388288, LR).

ACKNOWLEDGMENTS

Escherichia coli ATCC 9723H, *P. aeruginosa* ATCC 10148, *S. aureus* ATCC 6538P, and *S. pyogenes* (hospital isolate, strain unknown) were supplied by the Department of Biochemistry and Microbiology, University of Victoria, BC. *Mycobacterium smegmatis* MC^2155 was kindly supplied by Francis Nano, University of Victoria, BC, and *S. enterica* subsp. *enterica* serovar Typhimurium SL1344 was kindly supplied by Lisa Reynolds, University of Victoria, BC. We thank Caren Helbing (University of Victoria) and Inanc Birol (University of British Columbia) for permitting the use of RaCa-2 peptide as a positive control in antimicrobial susceptibility assays.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.888525/full#supplementary-material

REFERENCES

Abedinzadeh, M., Gaeini, M., and Sardari, S. (2015). Natural antimicrobial peptides against *Mycobacterium tuberculosis*. *J. Antimicrob. Chemother.* 70, 1285–1289. doi: 10.1093/jac/dku570

Aghazadeh, H., Memariani, H., Ranjbar, R., and Pooshang Bagheri, K. (2019). The activity and action mechanism of novel short selective LL-37-derived anticancer peptides against clinical isolates of *Escherichia coli*. *Chem. Biol. Drug. Des.* 93, 75–83. doi: 10.1111/cbdd.13381

Alirol, E., Wi, T. E., Bala, M., Bazzo, M. L., Chen, X. S., Deal, C., et al. (2017). Multidrug-resistant gonorrhea: a research and development roadmap to discover new medicines. *PLoS Med.* 14:e1002366.

Almagro Armenteros, J. J., Tsirigos, K. D., Sonderby, C. K., Petersen, T. N., Winther, O., Brunak, S., et al. (2019). SignalP 5.0 improves signal peptide predictions using deep neural networks. *Nat. Biotechnol.* 37, 420–423. doi: 10.1038/s41587-019-0036-z

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410.

Arango Duque, G., and Descoteaux, A. (2014). Macrophage cytokines: involvement in immunity and infectious diseases. *Front. Immunol.* 5:491. doi: 10.3389/fimmu.2014.00491

Baggiolini, M., and Clark-Lewis, I. (1992). Interleukin-8, a chemotactic and inflammatory cytokine. *FEBS Lett.* 307, 97–101. doi: 10.1016/0014-5793(92)80909-z

Bai, X., Kim, S. H., Azam, T., Mcgibney, M. T., Huang, H., Dinarello, C. A., et al. (2010). IL-32 is a host protective cytokine against *Mycobacterium tuberculosis* in differentiated THP-1 human macrophages. *J. Immunol.* 184, 3830–3840. doi: 10.4049/jimmunol.0901913

Baindara, P., Kapoor, A., Korpole, S., and Grover, V. (2017). Cysteine-rich low molecular weight antimicrobial peptides from *Brevibacillus* and related genera for biotechnological applications. *World J. Microbiol. Biotechnol.* 33:124. doi: 10.1007/s11274-017-2291-9

Baker-Zander, S. A., and Lukehart, S. A. (1992). Macrophage-mediated killing of opsonized *Treponema pallidum*. *J. Infect. Dis.* 165, 69–74. doi: 10.1093/infdis/165.1.69

Baker-Zander, S. A., Shaffer, J. M., and Lukehart, S. A. (1993). Characterization of the serum requirement for macrophage-mediated killing of *Treponema pallidum* ssp. *pallidum*: relationship to the development of opsonizing antibodies. *FEMS Immunol. Med. Microbiol.* 6, 273–279. doi: 10.1111/j.1574-695X.1993.tb00339.x

Bala, M., Mullick, J. B., Muralidhar, S., Kumar, J., and Ramesh, V. (2011). Gonorrhoea & its co-infection with other ulcerative, non-ulcerative sexually transmitted & HIV infection in a regional STD centre. *Indian J. Med. Res.* 133, 346–349.

Beis, K., and Rebuffat, S. (2019). Multifaceted ABC transporters associated to microcin and bacteriocin export. *Res. Microbiol.* 170, 399–406. doi: 10.1016/j.resmic.2019.07.002

Bienert, S., Waterhouse, A., De Beer, T. A., Tauriello, G., Studer, G., Bordoli, L., et al. (2017). The SWISS-MODEL repository-new features and functionality. *Nucleic Acids Res.* 45, D313–D319. doi: 10.1093/nar/gkw1132

Bloom, J. N. (1885). The bacillus of syphilis by DR. Sigmund Lustgarten (Translation of lustgarten s. ueber stezifische bacillen in syphilitischen. wiener medizinische wochenschrift 1884; 34:1389). *Lancet* 1, 609–610. doi: 10.1016/s0140-6736(02)17741-5

Brennan, P. J., and Nikaido, H. (1995). The envelope of mycobacteria. *Annu. Rev. Biochem.* 64, 29–63. doi: 10.1146/annurev.bi.64.070195.000333

Brodsky, I. E., and Gunn, J. S. (2005). A bacterial sensory system that activates resistance to innate immune defenses: Potential targets for antimicrobial therapeutics. *Mol. Interv.* 5, 335–337. doi: 10.1124/mi.5.6.4

Burchell, A. N., Allen, V. G., Gardner, S. L., Moravan, V., Tan, D. H., Grewal, R., et al. (2015). High incidence of diagnosis with syphilis co-infection among men who have sex with men in an HIV cohort in Ontario. *Canada. BMC Infect. Dis.* 15:356. doi: 10.1186/s12879-015-1098-2

Cameron, C. E., Brouwer, N. L., Tisch, L. M., and Kuroiwa, J. M. Y. (2005). Defining the interaction of the *Treponema pallidum* adhesin Tp0751 with laminin. *Infect. Immun.* 73, 7485–7494. doi: 10.1128/IAI.73.11.7485-7494.2005

Campopiano, D. J., Clarke, D. J., Polfer, N. C., Barran, P. E., Langley, R. J., Govan, J. R., et al. (2004). Structure-activity relationships in defensin dimers: a novel link between beta-defensin tertiary structure and antimicrobial activity. *J. Biol. Chem.* 279, 48671–48679. doi: 10.1074/jbc.M404690200

Cardozo, T., Totrov, M., and Abagyan, R. (1995). Homology modeling by the ICM method. *Proteins* 23, 403–414. doi: 10.1002/prot.340230314

Chang, K. Y., Lin, T. P., Shih, L. Y., and Wang, C. K. (2015). Analysis and prediction of the critical regions of antimicrobial peptides based on conditional random fields. *PLoS One* 10:e0119490. doi: 10.1371/journal.pone.0119490

Chanput, W., Mes, J. J., and Wichers, H. J. (2014). THP-1 cell line: an in vitro cell model for immune modulation approach. *Int. Immunopharmacol.* 23, 37–45. doi: 10.1016/j.intimp.2014.08.002

Chiorean, S., Vederas, J. C., and Van Belkum, M. J. (2018). Identification and heterologous expression of the sec-dependent bacteriocin Faerocin MK from *Enterococcus faecium* M3K31. *Probiotics Antimicrob. Proteins* 10, 142–147. doi: 10.1007/s12602-017-9374-7

Choi, K. Y., Napper, S., and Mookherjee, N. (2014). Human cathelicidin LL-37 and its derivative IG-19 regulate interleukin-32-induced inflammation. *Immunology* 143, 68–80. doi: 10.1111/imm.12291

Cleveland, J., Montville, T. J., Nes, I. F., and Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *Int. J. Food Microbiol.* 71, 1–20. doi: 10.1016/s0168-1605(01)00560-8

Coelho, E. C., Souza, S. B., Costa, C. C. S., Costa, L. M., Pinheiro, L. M. L., Machado, L. F. A., et al. (2021). *Treponema pallidum* in female sex workers from the brazilian marajo archipelago: prevalence, risk factors, drug-resistant mutations and coinfections. *Trans. R. Soc. Trop. Med. Hyg.* 115, 792–800. doi: 10.1093/trstmh/traa127

Cogen, A. L., Yamasaki, K., Sanchez, K. M., Dorschner, R. A., Lai, Y., Macleod, D. T., et al. (2010). Selective antimicrobial action is provided by phenol-soluble modulins derived from *Staphylococcus epidermidis*, a normal resident of the skin. *J. Invest. Dermatol.* 130, 192–200. doi: 10.1038/jid.2009.243

Collart, P., Franceschini, P., and Durel, P. (1971). Experimental rabbit syphilis. *Br. J. Vener. Dis.* 47, 389–400.

Conlon, J. M., Mechkarska, M., Coquet, L., Jouenne, T., Leprince, J., Vaudry, H., et al. (2011). Characterization of antimicrobial peptides in skin secretions from discrete populations of *Lithobates chiricahuensis* (Ranidae) from central and southern Arizona. *Peptides* 32, 664–669. doi: 10.1016/j.peptides.2011.01.018

Cook, G. M., Berney, M., Gebhard, S., Heinemann, M., Cox, R. A., Danilchanka, O., et al. (2009). Physiology of mycobacteria. *Adv. Microb. Physiol.* 55, 81–182, 318–189.

Cotter, P. D., Hill, C., and Ross, R. P. (2005). Bacteriocins: developing innate immunity for food. *Nat. Rev. Microbiol.* 3, 777–788. doi: 10.1038/nrmicro1273

Cox, D. L., Sun, Y., Liu, H., Lehrer, R. I., and Shafer, W. M. (2003). Susceptibility of *Treponema pallidum* to host-derived antimicrobial peptides. *Peptides* 24, 1741–1746. doi: 10.1016/j.peptides.2003.07.026

Crooks, G. E., Hon, G., Chandonia, J. M., and Brenner, S. E. (2004). WebLogo: a sequence logo generator. *Genome Res.* 14, 1188–1190. doi: 10.1101/gr.849004

Cumberland, M. C., and Turner, T. B. (1949). Rate of multiplication of *Treponema pallidum* in normal and immune rabbits. *Amer. J. Syph.* 33, 201–212.

Cushing, S. D., Berliner, J. A., Valente, A. J., Territo, M. C., Navab, M., Parhami, F., et al. (1990). Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc. Natl. Acad. Sci. U.S.A.* 87, 5134–5138. doi: 10.1073/pnas.87.13.5134

De Vuyst, L., and Leroy, F. (2007). Bacteriocins from lactic acid bacteria: Production, purification, and food applications. *J. Mol. Microbiol. Biotechnol.* 13, 194–199. doi: 10.1159/000104752

Deshmane, S. L., Kremlev, S., Amini, S., and Sawaya, B. E. (2009). Monocyte chemoattractant protein-1 (MCP-1): an overview. *J. Interferon Cytokine Res.* 29, 313–326. doi: 10.1089/jir.2008.0027

Dias Rde, O., and Franco, O. L. (2015). Cysteine-stabilized alphabeta defensins: from a common fold to antibacterial activity. *Peptides* 72, 64–72. doi: 10.1016/j.peptides.2015.04.017

Dicks, L. M. T., Dreyer, L., Smith, C., and Van Staden, A. D. (2018). A review: the fate of bacteriocins in the human gastro-intestinal tract: do they cross the gut-blood barrier? *Front Microbiol.* 9:2297. doi: 10.3389/fmicb.2018.02297

Dirix, G., Monsieurs, P., Dombrecht, B., Daniels, R., Marchal, K., Vanderleyden, J., et al. (2004). Peptide signal molecules and bacteriocins in Gram-negative bacteria: a genome-wide in silico screening for peptides containing a double-glycine leader sequence and their cognate transporters. *Peptides* 25, 1425–1440. doi: 10.1016/j.peptides.2003.10.028

Dos Santos, J. C., Heinhuis, B., Gomes, R. S., Damen, M. S., Real, F., Mortara, R. A., et al. (2017). Cytokines and microbicidal molecules regulated by IL-32 in THP-1-derived human macrophages infected with New World *Leishmania* species. *PLoS Negl. Trop. Dis.* 11:e0005413. doi: 10.1371/journal.pntd.0005413

Drozdetskiy, A., Cole, C., Procter, J., and Barton, G. J. (2015). JPred4: a protein secondary structure prediction server. *Nucleic Acids Res.* 43, W389–W394. doi: 10.1093/nar/gkv332

Edmondson, D. G., and Norris, S. J. (2021). In vitro cultivation of the syphilis spirochete *Treponema pallidum*. *Curr. Protoc.* 1:e44. doi: 10.1002/cpz1.44

Edmondson, D. G., Hu, B., and Norris, S. J. (2018). Long-term in vitro culture of the syphilis spirochete *Treponema pallidum* subsp. *pallidum*. *mBio* 9:e01153–18. doi: 10.1128/mBio.01153-18

Ergan, B., Coplu, L., Alp, A., and Artvinli, M. (2004). *Mycobacterium smegmatis* pneumonia. *Respirology* 9, 283–285. doi: 10.1111/j.1440-1843.2004.00570.x

Fahrner, R. L., Dieckmann, T., Harwig, S. S., Lehrer, R. I., Eisenberg, D., and Feigon, J. (1996). Solution structure of protegrin-1, a broad-spectrum antimicrobial peptide from porcine leukocytes. *Chem. Biol.* 3, 543–550. doi: 10.1016/s1074-5521(96)90145-3

Fedders, H., Podschun, R., and Leippe, M. (2010). The antimicrobial peptide Ci-MAM-A24 is highly active against multidrug-resistant and anaerobic bacteria pathogenic for humans. *Int. J. Antimicrob. Agents* 36, 264–266. doi: 10.1016/j.ijantimicag.2010.04.008

Feigin, A. M., Teeter, J. H., and Brand, J. G. (1995). The influence of sterols on the sensitivity of lipid bilayers to melittin. *Biochem. Biophys. Res. Commun.* 211, 312–317. doi: 10.1006/bbrc.1995.1812

Fraser, C. M., Norris, S. J., Weinstock, G. M., White, O., Sutton, G. G., Dodson, R., et al. (1998). Complete genome sequence of *Treponema pallidum*, the syphilis spirochete. *Science* 281, 375–388.

French, P. (2007). Syphilis. *BMJ* 334, 143–147.

Frick, I. M., Akesson, P., Rasmussen, M., Schmidtchen, A., and Bjorck, L. (2003). SIC, a secreted protein of *Streptococcus pyogenes* that inactivates antibacterial peptides. *J. Biol. Chem.* 278, 16561–16566. doi: 10.1074/jbc.M301995200

Gajic, O., Buist, G., Kojic, M., Topisirovic, L., Kuipers, O. P., and Kok, J. (2003). Novel mechanism of bacteriocin secretion and immunity carried out by lactococcal multidrug resistance proteins. *J. Biol. Chem.* 278, 34291–34298. doi: 10.1074/jbc.M211100200

Gao, Z. X., Liu, L. L., Lin, L. R., Tong, M. L., Liu, F., and Yang, T. C. (2019). *Treponema pallidum* induces the secretion of HDVSMC inflammatory cytokines to promote the migration and adhesion of THP-1 cells. *Front. Cell. Infect. Microbiol.* 9:220. doi: 10.3389/fcimb.2019.00220

Gautam, A., Chaudhary, K., Kumar, R., Sharma, A., Kapoor, P., Tyagi, A., et al. (2013). In silico approaches for designing highly effective cell penetrating peptides. *J. Transl. Med.* 11:74. doi: 10.1186/1479-5876-11-74

Gautier, R., Douguet, D., Antonny, B., and Drin, G. (2008). HELIQUEST: a web server to screen sequences with specific alpha-helical properties. *Bioinformatics* 24, 2101–2102. doi: 10.1093/bioinformatics/btn392

Gilson, L., Mahanty, H. K., and Kolter, R. (1990). Genetic analysis of an MDR-like export system: the secretion of colicin V. *EMBO J.* 9, 3875–3884. doi: 10.1002/j.1460-2075.1990.tb07606.x

Glukhov, E., Stark, M., Burrows, L. L., and Deber, C. M. (2005). Basis for selectivity of cationic antimicrobial peptides for bacterial versus mammalian membranes. *J. Biol. Chem.* 280, 33960–33967. doi: 10.1074/jbc.M507042200

Gonzalez-Pastor, J. E., San Millan, J. L., Castilla, M. A., and Moreno, F. (1995). Structure and organization of plasmid genes required to produce the translation inhibitor microcin C7. *J. Bacteriol.* 177, 7131–7140. doi: 10.1128/jb.177.24.7131-7140.1995

Gordon, Y. J., Huang, L. C., Romanowski, E. G., Yates, K. A., Proske, R. J., and Mcdermott, A. M. (2005). Human cathelicidin (LL-37), a multifunctional peptide, is expressed by ocular surface epithelia and has potent antibacterial and antiviral activity. *Curr. Eye Res.* 30, 385–394. doi: 10.1080/02713680590934111

Gupta, K., Singh, S., and Van Hoek, M. L. (2015). Short, synthetic cationic peptides have antibacterial activity against *Mycobacterium smegmatis* by forming pores in membrane and synergizing with antibiotics. *Antibiotics* 4, 358–378. doi: 10.3390/antibiotics4030358

Havarstein, L. S., Holo, H., and Nes, I. F. (1994). The leader peptide of colicin V shares consensus sequences with leader peptides that are common among peptide bacteriocins produced by Gram-positive bacteria. *Microbiology* 140, 2383–2389. doi: 10.1099/13500872-140-9-2383

Hazlett, K. R., Cox, D. L., Decaffmeyer, M., Bennett, M. P., Desrosiers, D. C., La Vake, C. J., et al. (2005). Tp0453, a concealed outer membrane protein of *Treponema pallidum*, enhances membrane permeability. *J. Bacteriol.* 187, 6499–6508. doi: 10.1128/JB.187.18.6499-6508.2005

Helbing, C. C., Hammond, S. A., Jackman, S. H., Houston, S., Warren, R. L., Cameron, C. E., et al. (2019). Antimicrobial peptides from *Rana [Lithobates] catesbeiana*: gene structure and bioinformatic identification of novel forms from tadpoles. *Sci. Rep.* 9:1529. doi: 10.1038/s41598-018-38442-1

Hilchie, A. L., Wuerth, K., and Hancock, R. E. (2013). Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. *Nat. Chem. Biol.* 9, 761–768. doi: 10.1038/nchembio.1393

Hollands, A., Gonzalez, D., Leire, E., Donald, C., Gallo, R. L., Sanderson-Smith, M., et al. (2012). A bacterial pathogen co-opts host plasmin to resist killing by cathelicidin antimicrobial peptides. *J. Biol. Chem.* 287, 40891–40897. doi: 10.1074/jbc.M112.404582

Hong, T., Butler, W. R., Hollis, F., Floyd, M. M., Toney, S. R., Tang, Y. W., et al. (2003). Characterization of a novel rapidly growing *Mycobacterium* species associated with sepsis. *J. Clin. Microbiol.* 41, 5650–5653. doi: 10.1128/JCM.41.12.5650-5653.2003

Jones, D. T. (1999). Protein secondary structure prediction based on position-specific scoring matrices. *J. Mol. Biol.* 292, 195–202. doi: 10.1006/jmbi.1999.3091

Juncker, A. S., Willenbrock, H., Von, H. G., Brunak, S., Nielsen, H., and Krogh, A. (2003). Prediction of lipoprotein signal peptides in Gram-negative bacteria. *Protein Sci.* 12, 1652–1662. doi: 10.1110/ps.0303703

Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N., and Sternberg, M. J. (2015). The Phyre2 web portal for protein modeling, prediction and analysis. *Nat. Protoc.* 10, 845–858. doi: 10.1038/nprot.2015.053

Kemperman, R., Jonker, M., Nauta, A., Kuipers, O. P., and Kok, J. (2003a). Functional analysis of the gene cluster involved in production of the bacteriocin circularin A by *Clostridium beijerinckii* ATCC 25752. *Appl. Environ. Microbiol.* 69, 5839–5848. doi: 10.1128/AEM.69.10.5839-5848.2003

Kemperman, R., Kuipers, A., Karsens, H., Nauta, A., Kuipers, O., and Kok, J. (2003b). Identification and characterization of two novel clostridial bacteriocins, circularin A and closticin 574. *Appl. Environ. Microbiol.* 69, 1589–1597. doi: 10.1128/AEM.69.3.1589-1597.2003

Khawar, M. B., Abbasi, M. H., and Sheikh, N. (2016). IL-32: a novel pluripotent inflammatory interleukin, towards gastric inflammation, gastric cancer, and chronic rhino sinusitis. *Mediators Inflamm.* 2016:8413768. doi: 10.1155/2016/8413768

Kindrachuk, J., Jenssen, H., Elliott, M., Nijnik, A., Magrangeas-Janot, L., Pasupuleti, M., et al. (2013). Manipulation of innate immunity by a bacterial secreted peptide: *Lantibiotic Nisin* Z is selectively immunomodulatory. *Innate Immun.* 19, 315–327. doi: 10.1177/1753425912461456

Kolaczkowska, E., and Kubes, P. (2013). Neutrophil recruitment and function in health and inflammation. *Nat. Rev. Immunol.* 13, 159–175. doi: 10.1038/nri3399

Kumar, P., Kizhakkedathu, J. N., and Straus, S. K. (2018). Antimicrobial peptides: diversity, mechanism of action and strategies to improve the activity and biocompatibility in vivo. *Biomolecules* 8:4. doi: 10.3390/biom8010004

Ladjouzi, R., Lucau-Danila, A., Benachour, A., and Drider, D. (2020). A Leaderless two-peptide bacteriocin, Enterocin DD14, is involved in its own self-immunity: evidence and insights. *Front. Bioeng. Biotechnol.* 8:644. doi: 10.3389/fbioe.2020.00644

Lafond, R. E., and Lukehart, S. A. (2006). Biological basis for syphilis. *Clin. Microbiol. Rev.* 19, 29–49. doi: 10.1128/CMR.19.1.29-49.2006

Lai, Y., and Gallo, R. L. (2009). AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends Immunol.* 30, 131–141. doi: 10.1016/j.it.2008.12.003

Lata, S., Mishra, N. K., and Raghava, G. P. (2010). AntiBP2: improved version of antibacterial peptide prediction. *BMC Bioinformatics* 11 Suppl 1:S19. doi: 10.1186/1471-2105-11-S1-S19

Lata, S., Sharma, B. K., and Raghava, G. P. (2007). Analysis and prediction of antibacterial peptides. *BMC Bioinformatics* 8:263. doi: 10.1186/1471-2105-8-263

Latif, N., Janani, M. K., Sudharshan, Selvamuthu, P., and Dutta Majumder, P. (2020). Triple trouble: A case of retinochoroiditis in a patient with syphilis, tuberculosis, and human immunodeficiency virus infection. *Indian J. Ophthalmol.* 68, 1995–1997. doi: 10.4103/ijo.IJO_2170_19

Lauth, X., Von Kockritz-Blickwede, M., Mcnamara, C. W., Myskowski, S., Zinkernagel, A. S., Beall, B., et al. (2009). M1 protein allows Group A streptococcal survival in phagocyte extracellular traps through cathelicidin inhibition. *J. Innate Immun.* 1, 202–214. doi: 10.1159/000203645

Leer, R. J., Van Der Vossen, J. M., Van Giezen, M., Van Noort, J. M., and Pouwels, P. H. (1995). Genetic analysis of Acidocin B, a novel bacteriocin produced by *Lactobacillus acidophilus*. *Microbiology* 141, 1629–1635. doi: 10.1099/13500872-141-7-1629

Li, A., Dubey, S., Varney, M. L., Dave, B. J., and Singh, R. K. (2003). IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis. *J. Immunol.* 170, 3369–3376. doi: 10.4049/jimmunol.170.6.3369

Li, C., Sutherland, D., Hammond, S. A., Yang, C., Taho, F., Bergman, L., et al. (2022). AMPlify: Attentive deep learning model for discovery of novel antimicrobial peptides effective against WHO priority pathogens. *BMC Genomics* 23:77. doi: 10.1186/s12864-022-08310-4

Li, M. F., Zhang, B. C., Li, J., and Sun, L. (2014). Sil: a *Streptococcus iniae* bacteriocin with dual role as an antimicrobial and an immunomodulator that inhibits innate immune response and promotes *S. iniae* infection. *PLoS One* 9:e96222. doi: 10.1371/journal.pone.0096222

Li, W., Deng, W., and Xie, J. (2018). The biology and role of interleukin-32 in tuberculosis. *J. Immunol. Res.* 2018:1535194. doi: 10.1155/2018/1535194

Lin, B., Hung, A., Li, R., Barlow, A., Singleton, W., Matthyssen, T., et al. (2022). Systematic comparison of activity and mechanism of antimicrobial peptides against nosocomial pathogens. *Eur. J. Med. Chem.* 231:114135. doi: 10.1016/j.ejmech.2022.114135

Lukehart, S. A., and Marra, C. M. (2007). Isolation and laboratory maintenance of *Treponema pallidum*. *Curr. Protoc. Microbiol.* Chapter 12, 12A.1.1–12A.1.18.

Lukehart, S. A., and Miller, J. N. (1978). Demonstration of the in vitro phagocytosis of *Treponema pallidum* by rabbit peritoneal macrophages. *J. Immunol.* 121, 2014–2024.

Lukehart, S. A., Hook, E. W., Baker-Zander, S. A., Collier, A. C., Critchlow, C. W., and Handsfield, H. H. (1988). Invasion of the central nervous system by *Treponema pallidum*: implications for diagnosis and treatment. *Ann. Intern. Med.* 109, 855–862. doi: 10.7326/0003-4819-109-11-855

Luthra, A., Zhu, G., Desrosiers, D. C., Eggers, C. H., Mulay, V., Anand, A., et al. (2011). The transition from closed to open conformation of *Treponema pallidum* outer membrane-associated lipoprotein TP0453 involves membrane sensing and integration by two amphipathic helices. *J. Biol. Chem.* 286, 41656–41668. doi: 10.1074/jbc.M111.305284

Ma, L., Xie, X., Liu, H., Huang, Y., Wu, H., Jiang, M., et al. (2020). Potent antibacterial activity of MSI-1 derived from the magainin 2 peptide against drug-resistant bacteria. *Theranostics* 10, 1373–1390. doi: 10.7150/thno.39157

Madeira, F., Park, Y. M., Lee, J., Buso, N., Gur, T., Madhusoodanan, N., et al. (2019). The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Res.* 47, W636–W641. doi: 10.1093/nar/gkz268

Magnuson, H. J., and Eagle, H. (1948). The minimal infectious inoculum of *Spirochaeta pallida* (Nichols strain), and a consideration of its rate of multiplication in vivo. *Amer. J. Syph.* 32, 1–18. doi: 10.1007/978-3-319-23534-9_1

Malaczewska, J., Kaczorek-Lukowska, E., Wojcik, R., Rekawek, W., and Siwicki, A. K. (2019). In vitro immunomodulatory effect of Nisin on porcine leucocytes. *J. Anim. Physiol. Anim. Nutr.* 103, 882–893. doi: 10.1111/jpn.13085

Mardirossian, M., Sola, R., Beckert, B., Collis, D. W. P., Di Stasi, A., Armas, F., et al. (2019). Proline-rich peptides with improved antimicrobial activity against *E. coli*, *K. pneumoniae*, and *A. baumannii*. *Chem. Med. Chem.* 14, 2025–2033.

Marra, C., Baker-Zander, S. A., Hook, E. W. D., and Lukehart, S. A. (1991). An experimental model of early central nervous system syphilis. *J. Infect. Dis.* 163, 825–829. doi: 10.1093/infdis/163.4.825

Matsuzaki, K., Sugishita, K., Fujii, N., and Miyajima, K. (1995). Molecular basis for membrane selectivity of an antimicrobial peptide, Magainin 2. *Biochemistry* 34, 3423–3429. doi: 10.1021/bi00010a034

McGill, M. A., Edmondson, D. G., Carroll, J. A., Cook, R. G., Orkiszewski, R. S., and Norris, S. J. (2010). Characterization and serologic analysis of the *Treponema pallidum* proteome. *Infect. Immun.* 78, 2631–2643. doi: 10.1128/IAI.00173-10

Mead, P. B., and Winn, W. C. (2000). Vaginal-rectal colonization with group A streptococci in late pregnancy. *Infect. Dis. Obstet. Gynecol.* 8, 217–219. doi: 10.1155/S1064744900000302

Meade, E., Slattery, M. A., and Garvey, M. (2020). Bacteriocins, potent antimicrobial peptides and the fight against multi drug resistant species: resistance is futile? *Antibiotics* 9:32. doi: 10.3390/antibiotics9010032

Meher, P. K., Sahu, T. K., Saini, V., and Rao, A. R. (2017). Predicting antimicrobial peptides with improved accuracy by incorporating the compositional, physico-chemical and structural features into Chou's general PseAAC. *Sci. Rep.* 7:42362.

Michiels, J., Dirix, G., Vanderleyden, J., and Xi, C. (2001). Processing and export of peptide pheromones and bacteriocins in Gram-negative bacteria. *Trends Microbiol.* 9, 164–168. doi: 10.1016/s0966-842x(01)01979-5

Min, H. J., Yun, H., Ji, S., Rajasekaran, G., Kim, J. I., Kim, J. S., et al. (2017). Rattusin structure reveals a novel defensin scaffold formed by intermolecular disulfide exchanges. *Sci. Rep.* 7:45282. doi: 10.1038/srep45282

Minami, M., Wakimoto, Y., Matsumoto, M., Matsui, H., Kubota, Y., Okada, A., et al. (2010). Characterization of *Streptococcus pyogenes* isolated from balanoposthitis patients presumably transmitted by penile-oral sexual intercourse. *Curr. Microbiol.* 61, 101–105. doi: 10.1007/s00284-010-9581-x

Mishra, A. K., Choi, J., Moon, E., and Baek, K. H. (2018). Tryptophan-rich and proline-rich antimicrobial peptides. *Molecules* 23:815. doi: 10.3390/molecules23040815

Moazzezy, N., Asadi Karam, M. R., Rafati, S., Bouzari, S., and Oloomi, M. (2020). Inhibition and eradication activity of truncated alpha-defensin analogs against multidrug resistant uropathogenic *Escherichia coli* biofilm. *PLoS One* 15:e0235892. doi: 10.1371/journal.pone.0235892

Muller, M., Ewert, I., Hansmann, F., Tiemann, C., Hagedorn, H. J., Solbach, W., et al. (2007). Detection of *Treponema pallidum* in the vitreous by PCR. *Br. J. Ophthalmol.* 91, 592–595. doi: 10.1136/bjo.2006.110288

Nelson, G. E., Pondo, T., Toews, K. A., Farley, M. M., Lindegren, M. L., Lynfield, R., et al. (2016). Epidemiology of invasive group A streptococcal infections in the United States, 2005-2012. *Clin. Infect. Dis.* 63, 478–486. doi: 10.1093/cid/ciw248

Newton, J. A. Jr., Weiss, P. J., Bowler, W. A., and Oldfield, E. C. III (1993). Soft-tissue infection due to *Mycobacterium smegmatis*: report of two cases. *Clin. Infect. Dis.* 16, 531–533. doi: 10.1093/clind/16.4.531

Norimatsu, Y., and Ohno, Y. (2020). Streptococcus pyogenes balanoposthitis. *ID Cases* 21:e00832. doi: 10.1016/j.idcr.2020.e00832

Oman, T. J., and van der Donk, W. A. (2010). Follow the leader: the use of leader peptides to guide natural product biosynthesis. *Nat. Chem. Biol.* 6, 9–18. doi: 10.1038/nchembio.286

Osbak, K. K., Houston, S., Lithgow, K. V., Meehan, C. J., Strouhal, M., Smajs, D., et al. (2016). Characterizing the syphilis-causing *Treponema pallidum* ssp. *pallidum* proteome using complementary mass spectrometry. *PLoS Negl. Trop. Dis.* 10:e0004988. doi: 10.1371/journal.pntd.0004988

Oyama, L. B., Girdwood, S. E., Cookson, A. R., Fernandez-Fuentes, N., Prive, F., Vallin, H. E., et al. (2017). The rumen microbiome: an underexplored resource for novel antimicrobial discovery. *NPJ Biofilms Microbiomes* 3:33. doi: 10.1038/s41522-017-0042-1

Pei, J., Kim, B. H., and Grishin, N. V. (2008). PROMALS3D: a tool for multiple protein sequence and structure alignments. *Nucleic Acids Res.* 36, 2295–2300. doi: 10.1093/nar/gkn072

Pei, J., Mitchell, D. A., Dixon, J. E., and Grishin, N. V. (2011). Expansion of type II CAAX proteases reveals evolutionary origin of gamma-secretase subunit APH-1. *J. Mol. Biol.* 410, 18–26. doi: 10.1016/j.jmb.2011.04.066

Peng, L. S., Zhuang, Y., Li, W. H., Zhou, Y. Y., Wang, T. T., Chen, N., et al. (2014). Elevated interleukin-32 expression is associated with *Helicobacter pylori*-related gastritis. *PLoS One* 9:e88270. doi: 10.1371/journal.pone.0088270

Pennekamp, A., Pfyffer, G. E., Wuest, J., George, C. A., and Ruef, C. (1997). *Mycobacterium smegmatis* infection in a healthy woman following a facelift: case report and review of the literature. *Ann. Plast. Surg.* 39, 80–83. doi: 10.1097/00000637-199707000-00014

Perez, R. H., Zendo, T., and Sonomoto, K. (2018). Circular and leaderless bacteriocins: biosynthesis, mode of action, applications, and prospects. *Front. Microbiol.* 9:2085. doi: 10.3389/fmicb.2018.02085

Petrosova, H., Pospisilova, P., Strouhal, M., Cejkova, D., Zobanikova, M., Mikalova, L., et al. (2013). Resequencing of *Treponema pallidum* ssp. *pallidum* strains Nichols and SS14: correction of sequencing errors resulted in increased separation of syphilis treponeme subclusters. *PLoS. One* 8:e74319. doi: 10.1371/journal.pone.0074319

Pinho-Bandeira, T., Ricoca Peixoto, V., Dias, S., and Sá Machado, R. (2020). Factors associated with coinfection and reinfection by chlamydia, gonorrhoea and syphilis in Portugal. *Eur. J. Public Health* 30:ckaa165.539. doi: 10.1093/eurpub/ckaa165.539

Pozzobon, T., Facchinello, N., Bossi, F., Capitani, N., Benagiano, M., Di Benedetto, G., et al. (2016). *Treponema pallidum* (syphilis) antigen TpF1 induces angiogenesis through the activation of the IL-8 pathway. *Sci. Rep.* 6:18785. doi: 10.1038/srep18785

Qu, X. D., Harwig, S. S., Oren, A. M., Shafer, W. M., and Lehrer, R. I. (1996). Susceptibility of *Neisseria gonorrhoeae* to protegrins. *Infect. Immun.* 64, 1240–1245. doi: 10.1128/iai.64.4.1240-1245.1996

Radolf, J. D. (1994). Role of outer membrane architecture in immune evasion by *Treponema pallidum* and B*orrelia burgdorferi*. *Trends Microbiol.* 2, 307–311. doi: 10.1016/0966-842x(94)90446-4

Rasool, S. T., Tang, H., Wu, J., Li, W., Mukhtar, M. M., Zhang, J., et al. (2008). Increased level of IL-32 during human immunodeficiency virus infection suppresses HIV replication. *Immunol. Lett.* 117, 161–167. doi: 10.1016/j.imlet.2008.01.007

Ribeiro-Dias, F., Saar Gomes, R., De Lima Silva, L. L., Dos Santos, J. C., and Joosten, L. A. (2017). Interleukin 32: a novel player in the control of infectious diseases. *J. Leukoc. Biol.* 101, 39–52. doi: 10.1189/jlb.4RU0416-175RR

Ronald, A. (2002). The etiology of urinary tract infection: traditional and emerging pathogens. *Am. J. Med.* 113 Suppl 1A, 14S–19S.

San Millan, J. L., Hernandez-Chico, C., Pereda, P., and Moreno, F. (1985). Cloning and mapping of the genetic determinants for microcin B17 production and immunity. *J. Bacteriol.* 163, 275–281. doi: 10.1128/jb.163.1.275-281.1985

Sancho-Vaello, E., Francois, P., Bonetti, E. J., Lilie, H., Finger, S., Gil-Ortiz, F., et al. (2017). Structural remodeling and oligomerization of human cathelicidin on membranes suggest fibril-like structures as active species. *Sci. Rep.* 7:15371. doi: 10.1038/s41598-017-14206-1

Schmidtchen, A., Frick, I. M., Andersson, E., Tapper, H., and Bjorck, L. (2002). Proteinases of common pathogenic bacteria degrade and inactivate the antibacterial peptide LL-37. *Mol. Microbiol.* 46, 157–168. doi: 10.1046/j.1365-2958.2002.03146.x

Sell, S., Gamboa, D., Baker-Zander, S. A., Lukehart, S. A., and Miller, J. N. (1980). Host response to *Treponema pallidum* in intradermally-infected rabbits: evidence for persistence of infection at local and distant sites. *J. Invest. Dermatol.* 75, 470–475. doi: 10.1111/1523-1747.ep12524230

Sellati, T. J., Bouis, D. A., Kitchens, R. L., Darveau, R. P., Pugin, J., Ulevitch, R. J., et al. (1998). Treponema pallidum and *Borrelia burgdorferi* lipoproteins and synthetic lipopeptides activate monocytic cells via a CD14-dependent pathway distinct from that used by lipopolysaccharide. *J. Immunol.* 160, 5455–5464.

Shafee, T. M., Lay, F. T., Hulett, M. D., and Anderson, M. A. (2016). The defensins consist of two independent, convergent protein superfamilies. *Mol. Biol. Evol.* 33, 2345–2356. doi: 10.1093/molbev/msw106

Shen, Y., Maupetit, J., Derreumaux, P., and Tuffery, P. (2014). Improved PEP-FOLD approach for peptide and miniprotein structure prediction. *J. Chem. Theory Comput.* 10, 4745–4758. doi: 10.1021/ct500592m

Shigemura, K., Arakawa, S., Sakai, Y., Kinoshita, S., Tanaka, K., and Fujisawa, M. (2006). Complicated urinary tract infection caused by *Pseudomonas aeruginosa* in a single institution (1999-2003). *Int. J. Urol.* 13, 538–542. doi: 10.1111/j.1442-2042.2006.01359.x

Shockman, S., Buescher, L. S., and Stone, S. P. (2014). Syphilis in the United States. *Clin. Dermatol.* 32, 213–218.

Simmaco, M., Mignogna, G., Barra, D., and Bossa, F. (1994). Antimicrobial peptides from skin secretions of *Rana esculenta*. Molecular cloning of cDNAs encoding esculentin and brevinins and isolation of new active peptides. *J. Biol. Chem.* 269, 11956–11961. doi: 10.1016/s0021-9258(17)32666-2

Smajs, D., Mckevitt, M., Howell, J. K., Norris, S. J., Cai, W. W., Palzkill, T., et al. (2005). Transcriptome of *Treponema pallidum*: gene expression profile during experimental rabbit infection. *J. Bacteriol.* 187, 1866–1874. doi: 10.1128/JB.187.5.1866-1874.2005

Smits, S. H. J., Schmitt, L., and Beis, K. (2020). Self-immunity to antibacterial peptides by ABC transporters. *FEBS Lett.* 594, 3920–3942. doi: 10.1002/1873-3468.13953

Snyder, A. B., and Worobo, R. W. (2014). Chemical and genetic characterization of bacteriocins: antimicrobial peptides for food safety. *J. Sci. Food. Agric.* 94, 28–44. doi: 10.1002/jsfa.6293

Sobel, J. D., Funaro, D., and Kaplan, E. L. (2007). Recurrent group A streptococcal vulvovaginitis in adult women: family epidemiology. *Clin. Infect. Dis.* 44, e43–e45. doi: 10.1086/510678

Sornwatana, T., Arpornsuwan, T., Roytrakul, S., and Wetprasit, N. (2018). M-Brucin, an antibacterial peptide against *Staphylococcus epidermidis* and *Streptococcus pyogenes*. *J. App. Pharm. Sci.* 8, 27–32.

Stamm, W. E., Feeley, J. C., and Facklam, R. R. (1978). Wound infections due to group A streptococcus traced to a vaginal carrier. *J. Infect. Dis.* 138, 287–292. doi: 10.1093/infdis/138.3.287

Standiford, T. J., Kunkel, S. L., Phan, S. H., Rollins, B. J., and Strieter, R. M. (1991). Alveolar macrophage-derived cytokines induce monocyte chemoattractant protein-1 expression from human pulmonary type II-like epithelial cells. *J. Biol. Chem.* 266, 9912–9918. doi: 10.1016/s0021-9258(18)92905-4

Sugrue, I., O'connor, P. M., Hill, C., Stanton, C., and Ross, R. P. (2020). *Actinomyces* produces defensin-like bacteriocins (actifensins) with a highly degenerate structure and broad antimicrobial activity. *J. Bacteriol.* 202:e00529–19. doi: 10.1128/JB.00529-19

Svanborg-Eden, C., Hagberg, L., Hull, R., Hull, S., Magnusson, K. E., and Ohman, L. (1987). Bacterial virulence versus host resistance in the urinary tracts of mice. *Infect. Immun.* 55, 1224–1232. doi: 10.1128/iai.55.5.1224-1232.1987

Tamames, J., Casari, G., Ouzounis, C., and Valencia, A. (1997). Conserved clusters of functionally related genes in two bacterial genomes. *J. Mol. Evol.* 44, 66–73. doi: 10.1007/pl00006122

Tamamura, H., Murakami, T., Horiuchi, S., Sugihara, K., Otaka, A., Takada, W., et al. (1995). Synthesis of protegrin-related peptides and their antibacterial and anti-human immunodeficiency virus activity. *Chem. Pharm. Bull.* 43, 853–858. doi: 10.1248/cpb.43.853

Tenaillon, O., Skurnik, D., Picard, B., and Denamur, E. (2010). The population genetics of commensal *Escherichia coli*. *Nat. Rev. Microbiol.* 8, 207–217. doi: 10.1038/nrmicro2298

Thevenet, P., Shen, Y., Maupetit, J., Guyon, F., Derreumaux, P., and Tuffery, P. (2012). PEP-FOLD: an updated de novo structure prediction server for both linear and disulfide bonded cyclic peptides. *Nucleic Acids Res.* 40, W288–W293. doi: 10.1093/nar/gks419

Todorov, S. D. (2009). Bacteriocins from *Lactobacillus plantarum* – production, genetic organization and mode of action: produção, organização genética e modo de ação. *Braz. J. Microbiol.* 40, 209–221. doi: 10.1590/s1517-83822009000200001

Todorov, S. D., De Melo Franco, B. D. G., and Tagg, J. R. (2019). Bacteriocins of Gram-positive bacteria having activity spectra extending beyond closely-related species. *Benef. Microbes* 10, 315–328. doi: 10.3920/BM2018.0126

Torrent, M., Di Tommaso, P., Pulido, D., Nogues, M. V., Notredame, C., Boix, E., et al. (2012). AMPA: An automated web server for prediction of protein antimicrobial regions. *Bioinformatics* 28, 130–131. doi: 10.1093/bioinformatics/btr604

Torrent, M., Nogues, V. M., and Boix, E. (2009). A theoretical approach to spot active regions in antimicrobial proteins. *BMC Bioinformatics* 10:373. doi: 10.1186/1471-2105-10-373

Turner, J., Cho, Y., Dinh, N. N., Waring, A. J., and Lehrer, R. I. (1998). Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. *Antimicrob. Agents Chemother.* 42, 2206–2214. doi: 10.1128/AAC.42.9.2206

Uhlmann, J., Rohde, M., Siemens, N., Kreikemeyer, B., Bergman, P., Johansson, L., et al. (2016). LL-37 Triggers formation of *Streptococcus pyogenes* extracellular vesicle-like structures with immune stimulatory properties. *J. Innate Immun.* 8, 243–257. doi: 10.1159/000441896

Veltri, D., Kamath, U., and Shehu, A. (2018). Deep learning improves antimicrobial peptide recognition. *Bioinformatics* 34, 2740–2747. doi: 10.1093/bioinformatics/bty179

Verstraelen, H., Verhelst, R., Vaneechoutte, M., and Temmerman, M. (2011). Group A streptococcal vaginitis: an unrecognized cause of vaginal symptoms in adult women. *Arch. Gynecol. Obstet.* 284, 95–98. doi: 10.1007/s00404-011-1861-6

Waghu, F. H., Barai, R. S., Gurung, P., and Idicula-Thomas, S. (2016). CAMPR3: a database on sequences, structures and signatures of antimicrobial peptides. *Nucleic Acids Res.* 44, D1094–D1097. doi: 10.1093/nar/gkv1051

Wallace, R. J. Jr., Nash, D. R., Tsukamura, M., Blacklock, Z. M., and Silcox, V. A. (1988). Human disease due to *Mycobacterium smegmatis*. *J. Infect. Dis.* 158, 52–59. doi: 10.1093/infdis/158.1.52

Wang, G., Li, X., and Wang, Z. (2016). APD3: the antimicrobial peptide database as a tool for research and education. *Nucleic Acids Res.* 44, D1087–D1093. doi: 10.1093/nar/gkv1278

Waterhouse, A., Bertoni, M., Bienert, S., Studer, G., Tauriello, G., Gumienny, R., et al. (2018). SWISS-MODEL: homology modelling of protein structures and complexes. *Nucleic Acids Res.* 46, W296–W303. doi: 10.1093/nar/gky427

Webb, B., and Sali, A. (2016). Comparative protein structure modeling using MODELLER. *Curr. Protoc. Bioinformatics* 54, 5.6.1–5.6.37.

Wiegand, I., Hilpert, K., and Hancock, R. E. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.* 3, 163–175. doi: 10.1038/nprot.2007.521

World Health Organization (2019). *Sexually Transmitted Infections (STIs)*. Available online at: https://www.who.int/news-room/fact-sheets/detail/sexually-transmitted-infections-(stis). [Accessed August 16, 2021].

Yamaguchi, Y., Fukuhara, S., Nagase, T., Tomita, T., Hitomi, S., Kimura, S., et al. (2001). A novel mouse beta-defensin, mBD-6, predominantly expressed

in skeletal muscle. *J. Biol. Chem.* 276, 31510–31514. doi: 10.1074/jbc.M104149200

Yasin, B., Harwig, S. S., Lehrer, R. I., and Wagar, E. A. (1996). Susceptibility of *Chlamydia trachomatis* to protegrins and defensins. *Infect. Immun.* 64, 709–713. doi: 10.1128/iai.64.3.709-713.1996

Yoshimura, T., Robinson, E. A., Tanaka, S., Appella, E., and Leonard, E. J. (1989a). Purification and amino acid analysis of two human monocyte chemoattractants produced by phytohemagglutinin-stimulated human blood mononuclear leukocytes. *J. Immunol.* 142, 1956–1962.

Yoshimura, T., Yuhki, N., Moore, S. K., Appella, E., Lerman, M. I., and Leonard, E. J. (1989b). Human monocyte chemoattractant protein-1 (MCP-1). Full-length cDNA cloning, expression in mitogen-stimulated blood mononuclear leukocytes, and sequence similarity to mouse competence gene JE. *FEBS Lett.* 244, 487–493. doi: 10.1016/0014-5793(89)80590-3

Zendo, T. (2013). Screening and characterization of novel bacteriocins from lactic acid bacteria. *Biosci. Biotechnol. Biochem.* 77, 893–899. doi: 10.1271/bbb.130014

Zhang, L., Foxman, B., and Marrs, C. (2002). Both urinary and rectal *Escherichia coli* isolates are dominated by strains of phylogenetic group B2. *J. Clin. Microbiol.* 40, 3951–3955. doi: 10.1128/JCM.40.11.3951-3955.2002

Zhuang, Z. R., Yang, X. D., Huang, X. Z., Gu, H. X., Wei, H. Y., He, Y. J., et al. (2017). Three new piscidins from orange-spotted grouper (*Epinephelus coioides*): phylogeny, expression and functional characterization. *Fish Shellfish Immunol.* 66, 240–253. doi: 10.1016/j.fsi.2017.04.011

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Medicine

SYSTEMATIC REVIEW
published: 13 May 2022
doi: 10.3389/fmed.2022.894841

Neurosyphilis in China: A Systematic Review of Cases From 2009–2021

Fang-Zhi Du[1], Hai-Ni Zhang[1], Jing-Jing Li[2], Zhi-Ju Zheng[1], Xu Zhang[1], Rui-Li Zhang[3] and Qian-Qiu Wang[1]**

[1] Department of Clinical Prevention and Control of STD, Institute of Dermatology, Chinese Academy of Medical Science and Peking Union Medical College, National Center for STD Control, China Centers for Disease Control and Prevention, Nanjing, China, [2] Department of Dermatology, The Fifth People's Hospital of Suzhou, Suzhou, China, [3] Department of Dermatology, The Second Affiliated Hospital of Nanjing Medical University, Nanjing, China

Considered the increased threaten of neurosyphilis in China, a review on cases reported in the literature to describe the clinical epidemiological characteristics of neurosyphilis cases, may be beneficial to the early detection and management strategies of neurosyphilis for clinicians. We searched the literature on Chinese neurosyphilis cases published from January 1, 2009 to December 31, 2021, described their clinical epidemiological characteristics and calculated the prevalence of neurosyphilis amongst other associated diseases, according to the individual study criteria. A total of 284 studies including 7,486 neurosyphilis cases were included. No meta-analysis was performed due to the heterogeneity of the data. Among 149 case reports and 93 retrospective case series studies, the main clinical manifestation of 3,507 neurosyphilis cases was cerebral parenchymal syphilis (57.3%), followed by asymptomatic neurosyphilis (16.7%), meningovascular syphilis (13.6%), meningitis syphilis (7.7%) and ocular syphilis (2.8%), etc. In addition, the initial diagnosis was incorrect in 53.2% patients, and the most frequent misdiagnoses were mental disorders (31.0%), stroke (15.9%), cognitive impairment (9.0%), etc. The positive or abnormal rates of cerebrospinal fluid non-treponemal and treponemal tests, white blood cell counts and protein concentrations were 74.2%, 96.2%, 61.5%, and 60.9%, respectively. Aqueous penicillin was the first choice for treatment in 88.3% cases, and 81.7% and 50.0% patients had response in the improvement of symptoms and serological effective in CSF, respectively. Among 26 studies on neurosyphilis patients amongst other associated diseases, the prevalence of neurosyphilis amongst central nervous system infectious diseases, syphilis-associated neurological symptoms, serofast status, coinfected with human immunodeficiency virus were 10.6%–30.1%, 23.2%–35.5%, 9.8%–56.1%, and 8.9%, respectively. In summary, the lack of early detection of neurosyphilis cases remains a clinical challenge. The high rate of misdiagnosis and high prevalence of neurosyphilis amongst associated diseases strongly remind clinicians to focus on the early detection among suspected cases. Besides, the standard treatment regimen and long-term follow-up, which complied with guideline should be provided. Further prospective studies are urgent to better delineate the clinical epidemiological characteristics of neurosyphilis in China.

Keywords: neurosyphilis, clinical epidemiological characteristics, prevalence, systematic review, China

OPEN ACCESS

Edited by:
Joseph D. Tucker, University of North Carolina at Chapel Hill, United States

Reviewed by:
Aldo Di Carlo, San Gallicano Hospital, Italy
Irina Khamaganova, Pirogov Russian National Research Medical University, Russia

***Correspondence:**
Qian-Qiu Wang
wangqianqiunj@126.com
Rui-Li Zhang
reallyvictor@126.com

Specialty section:
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *26 March 2022*
Accepted: *25 April 2022*
Published: *13 May 2022*

Citation:
Du F-Z, Zhang H-N, Li J-J, Zheng Z-J, Zhang X, Zhang R-L and Wang Q-Q (2022) Neurosyphilis in China: A Systematic Review of Cases From 2009–2021. Front. Med. 9:894841. doi: 10.3389/fmed.2022.894841

INTRODUCTION

Neurosyphilis, historically caused by *Treponema pallidum* (*T. pallidum*) infection, was reported increasing with the expansion of syphilis screening in China. *T. pallidum* invades the central nervous system (CNS) and may cause severe and irreversible neurologic sequelae in patients if left untreated (1). According to the latest report, the number of newly reported cases of syphilis was 438,199 (32.2 per 100,000) in 2016 and increased by an annual average of 8.6% from 2007 to 2016 in China; moreover, the number of reported cases of tertiary syphilis increased by 8.0% annually from 2007 to 2016 (2). Previous studies showed that the epidemiology of neurosyphilis largely paralleled that of syphilis (3, 4), and most tertiary syphilis cases were diagnosed as neurosyphilis (5), which indicated an increasing incidence of neurosyphilis in China.

Neurosyphilis has puzzled dermatologists, neurologists and psychiatrists in clinical settings for over two centuries because of its atypical symptoms and lack of a golden criteria of diagnosis. Early injury to CNS in neurosyphilis patients affects the mesenchyma, such as the meninges and blood vessels, manifesting within months to the several years after primary infection as meningismus, blindness, stroke, etc., while late injury affects the brain and spinal cord parenchyma within years to decades and presents as general paresis and tabes dorsalis (6, 7). Therefore, the rates of misdiagnosis and missed diagnosis in clinical settings are relatively high because of the diverse and atypical symptoms (8, 9). It is necessary for clinicians to be aware of the most common misdiagnosed diseases and specific clinical features of neurosyphilis when making a differential diagnosis.

Clinical recognition of neurosyphilis depends on the comprehensive assessment of clinical characteristics and cerebrospinal fluid (CSF) findings. However, no single specific and sensitive test for neurosyphilis exists. CSF pleocytosis and elevated protein concentrations are frequently observed in patients with neurosyphilis. Reactive CSF serologic tests are required for the diagnosis of neurosyphilis, and the Venereal Disease Research Laboratory (VDRL) test for CSF is thought to be the gold standard for specificity in the absence of blood contamination, but its sensitivity is still debated (10). The rapid plasma reagin (RPR) test, toluidine red unheated serum test (TRUST), fluorescent treponemal antibody adsorption (FTA-ABS) test and treponema pallidum particle agglutination (TPPA) test for CSF have all been assessed to have variable sensitivity and specificity in diagnosing neurosyphilis (11–13). Besides, no VDRL kits in China gets the approved of State Food and Drug Administration (SFDA) to date. Together the above all, the RPR and TRUST were recommended as the alternative tests by the 'China National Guidelines for the Diagnosis and Treatment of Syphilis, Gonorrhea and Chlamydia Trachomatis Infection (2020)' (14). The diagnostic criteria for neurosyphilis differed between studies due to the lack of a gold standard test. Consequently, the methodological quality of neurosyphilis diagnosis among studies is unknown and need to be evaluated.

Although many cases of neurosyphilis in China were reported, their clinical features and management information have not reviewed comprehensively to date. In this paper, all cases of neurosyphilis reported in China in the past 13 years were reviewed, and their clinical epidemiological characteristics were presented, which will be helpful to clinicians to early detection and management of neurosyphilis.

MATERIALS AND METHODS

The research and reporting methods of this review were consistent with the preferred reporting items for systematic reviews (PRISMA) (**Supplementary Table S1**).

Search Strategy

We searched the PubMed, EMBASE, and some Chinese Journal databases including China national knowledge infrastructure (CNKI) and WanFang databases for studies on neurosyphilis in China and limited the search to studies published between 1 January 2009 to 31 December 2021.We searched for ((“CSF” OR “lumbar puncture” OR “meningitis” OR “meningovascular” OR “stroke”) AND “syphilis”) OR (“neurosyphilis” OR “tabes dorsalis” OR “general paresis”) AND (“China”) in the PubMed and EMBASE databases and searched for “neurosyphilis” in the CNKI and WanFang databases (15). No language restrictions were set.

Inclusion/Exclusion Criteria

We selected unduplicated references and excluded reviews if the studies did not address neurosyphilis, did not describe the clinical features of neurosyphilis patients, reported patients already described in a different paper, did not include Chinese patients, did not report new primary material or could not be downloaded. We did not limit inclusion based on the diagnostic criteria used for neurosyphilis.

Selection Process

The selection was conducted by two independent reviewers working in parallel, first excluded duplication literature, then screening for title and abstract followed by the full text according to the inclusion/exclusion criteria. After the screening of 50 references, a validation of the screening process was conducted by comparing the screening results and discussing them within the team. Throughout the screening process, discrepancies were discussed and a third reviewer was consulted if consensus was not reached. The reasons for exclusion were documented only for full text publications (**Figure 1**).

Data Extraction

We extracted variables including available demographic information of neurosyphilis patients, duration of the study, inclusion criteria, diagnostic criteria for neurosyphilis, clinical syndromes and misdiagnosis, neuroimaging findings, and the number of human immunodeficiency virus (HIV) -infected patients in each study. We also extracted information on the treatment and follow-up of patients in these studies.

Statistical Analysis

For case reports and retrospective case series, we analyzed the sex ratio, age distribution, clinical spectrum, misdiagnosis rate,

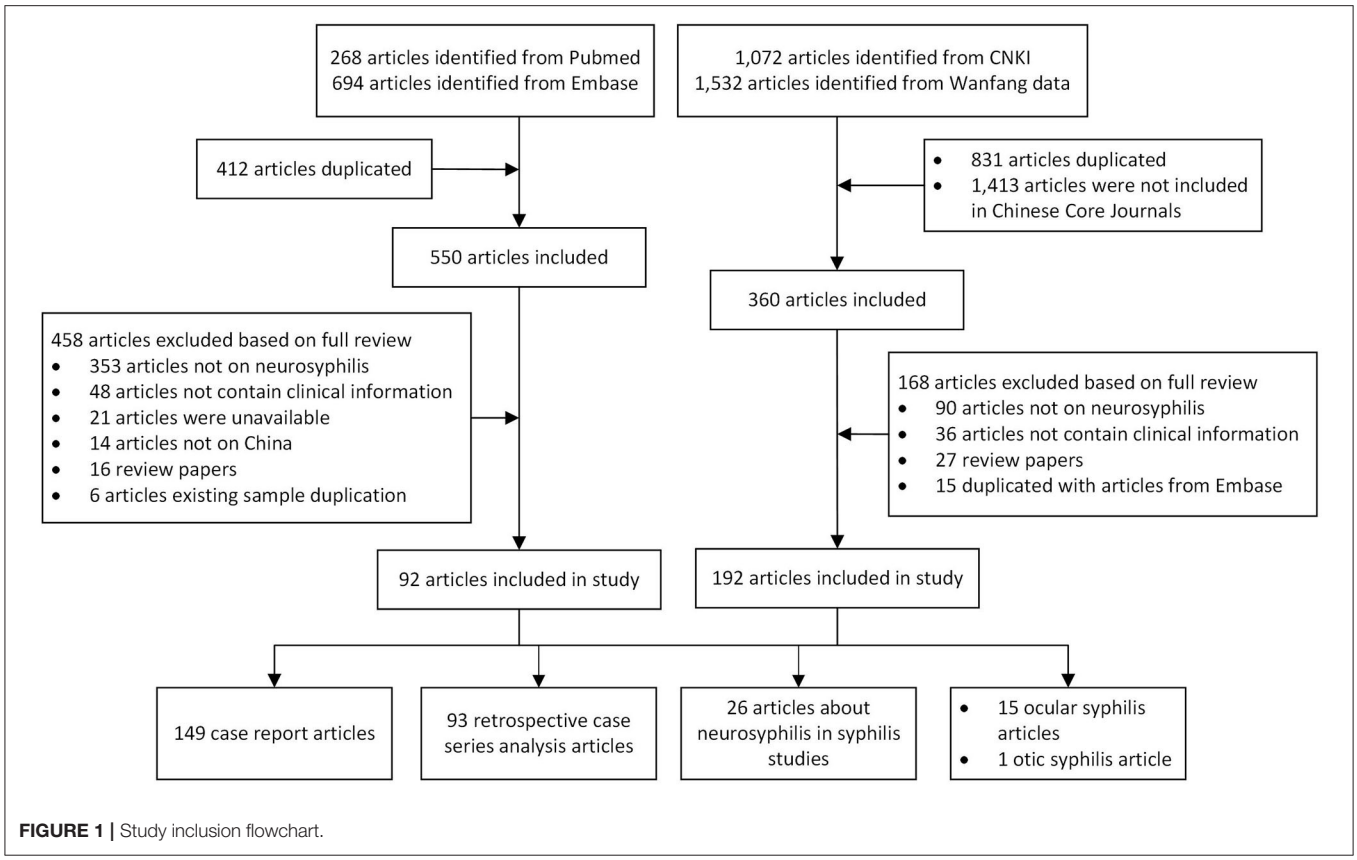


FIGURE 1 | Study inclusion flowchart.

treatment, and the proportion of anomalies of CSF tests and neuroimaging findings. For studies on neurosyphilis in patients with syphilis or HIV, we calculated the proportion of study participants who were diagnosed with neurosyphilis according to the individual study criteria. No meta-analysis was performed due to the heterogeneity of the data.

The chi-squared test was used to compare categorical variables. The Wilcoxon signed-rank test was used to compare related continuous variables with skewed distributions. A *P*-value of < 0.05 was considered statistically significant. All statistical analyses were conducted in SPSS 21.0 (IBM Corp, Armonk, NY).

RESULTS

A total of 962 articles were identified in the PubMed and EMBASE databases, and 412 duplicated articles were excluded. Among the remaining 550 articles, 458 articles were excluded based on full-text reviews. A total of 2,604 articles were identified from the CNKI and Wanfang databases, and 831 duplicated articles and 1,413 articles that were not included in Chinese Core Journals were excluded. Among the remaining 360 articles, 192 articles were excluded based on full-text reviews. Finally, 284 articles that met our inclusion criteria were selected (**Figure 1**).

Clinical Characteristics of Cases

The 149 case reports included 180 patients, consisting of 128 males and 52 females. The average age was 47.7 ± 12.9 (range from 12 to 79 years old), and the age of most patients ranged from 41 to 60 (56.7%, 102/180). The 93 retrospective case series included 3,327 patients (2,520 males, 783 females and 24 unknown), and neurosyphilis was more likely to occur in males than in females ($|Z| = 8.24$, $P < 0.001$) (**Supplementary Table S2**).

As shown in **Table 1**, among the 3,200 patients with clearly defined clinical subtypes, 533 (16.7%) has asymptomatic neurosyphilis, 726 (22.7%) had mesenchymal syphilis (245 (7.7%) had meningitis syphilis, 435 (13.6%) had meningovascular syphilis and 46 had undefined syphilis), 1,832 (57.3%) had parenchymal neurosyphilis (1,537 (48.0%) had general paresis, 115 (3.6%) had tabes dorsalis, 54 (1.7%) had syphilitic gumma and 126 had undefined syphilis), 90 (2.8%) had ocular syphilis, 42 (0.6%) had mixed-type syphilis, and 210 had undefined syphilis. The initial diagnosis was correct in 774 (46.8%) patients and incorrect in 847 (53.2%) patients among 173 studies, the correct diagnostic rate ranged from 17.0 to 100% (95%CI, 59.1–76.2%). Among the 847 misdiagnosed patients, 578 patients showed the misdiagnosed diseases, including mental disorders (179 patients, 31.0%), stroke (92 patients, 15.9%), cognitive impairment (52 patients, 9.0%), encephalitis (46 patients, 8.0%), ophthalmic diseases (40 patients, 6.9%), Alzheimer's disease (AD) (22 patients, 3.8%), brain tumors (20 patients, 3.5%), epilepsy (13 patients, 2.3%), myeleterosis (12 patients, 2.1%), peripheral neuropathy (11 patients, 1.9%), demyelinating disease or multiple sclerosis (10 patients, 1.7%), Parkinson's disease

TABLE 1 | Clinical characteristics of neurosyphilis cases from 149 case reports and 93 retrospective case series.

Clinical characteristics				Number of cases	Proportion (%) *
		Asymptomatic		533	16.66%
		Mesenchymal syphilis	Meningitis	245	7.66%
			Meningovascular	435	13.59%
			Not mentioned	46	1.44%
Clinical subtypes	196 studies mentioned	Parenchymal syphilis	General paresis	1,537	48.03%
			Tabes dorsalis	115	3.59%
			Syphilitic gumma	54	1.69%
			Not mentioned	126	3.94%
		Ocular syphilis		90	2.81%
		Mixed		19	0.59%
		Not mentioned		97	-
	46 studies (28 case reports) not mentioned			210	-
Diagnosis	correct diagnosis			744	46.76%
	173 studies misdiagnosed diseases mentioned	Mental disorders		179	30.97%
		Stroke		92	15.92%
		Encephalitis		46	7.96%
		Cognitive impairment		52	9.00%
		ophthalmic diseases		40	6.92%
		Brain tumor		20	3.46%
		Alzheimer's disease		22	3.81%
		Epilepsy		13	2.25%
		Myeleterosis		12	2.08%
		Peripheral neuropathy		11	1.90%
		Parkinson's disease		9	1.56%
		Demyelinating disease and multiple sclerosis		10	1.73%
		Ataxia		4	0.69%
		Hydrocephalus		6	1.04%
		menopausal symptom		4	0.69%
		Encephalatrophy		3	0.52%
		Others		55	9.52%
		Not mentioned		269	-
	69 studies (21 case reports) not mentioned			1,916	-

**The denominator is the sum of the cases who mentioned the clinical characteristics.*

(PD) (nine patients, 1.6%), hydrocephalus (six patients, 1.0%), ataxiaor menopausal symptom (four patients, 0.7%, respectively), encephalatrophy (three patients, 0.5%), and other diseases (55 patients, 9.5%).

Laboratory Characteristics and Auxiliary Examination of Cases

Among 180 patients from 149 case reports studies, nontreponemal tests (VDRL/RPR/TRUSTs) were performed in 83.3% (150/180) of the patients, and 82.0% (123/150) of them patients had positive results. Treponemal tests (TPPA/TPHA) were performed in 78.3% (141/180), and 95.0% (134/141) of them had positive results. As shown in **Table 2**, patients were more likely to have positive results on the treponemal tests than non-treponemal tests ($\chi^2 = 11.97$, $P = 0.001$). However, there were no differences in the abnormal CSF white blood cell counts (WBC) rate (74.6%, 82/110) and abnormal CSF protein (PRO) rate (78.9%, 86/109) between these two groups ($\chi^2 = 0.58$, $P = 0.52$). We also found that 24 patients were diagnosed with neurosyphilis without reactive CSF serologic tests. (**Supplementary Table S1**).

Among the 93 retrospective case series studies, non-treponemal tests (VDRL/RPR/TRUST) were performed in 88.8% (2,491/2,805) of the patients, and 73.7% (1,747/2,370) of them had positive results, the positive rates ranged from 0 to 100% (95%CI, 69.5–81.2%). Treponemal tests (FTA-ABS/TPPA/TPHA) were performed in 91.0% (2,552/2,805) of the patients, and 96.2% (2,339/2,431) of them had positive results, the positive rates ranged from 66.7 to 100% (95%CI, 90.8–97.3%). Patients were more likely to have a positive result on a treponemal test than a nontreponemal test ($|Z = 5.78$, $P < 0.001$). CSF-WBC were abnormal among 61.0% (1,654/2,712) patients, the abnormal rates ranged from 22.2% to 100% (95%CI, 59.0–69.7%). CSF-PRO test was abnormal among 60.2% (1,665/2,765) patients, the abnormal rates ranged from 11.1 to 100% (95%CI,

TABLE 2 | Laboratory findings of neurosyphilis cases from 149 case reports and 93 retrospective case series.

Laboratory findings			Number of cases	Positive or abnormal proportion (%) *	χ^2	$\|Z\|^{\dagger}$	*P*
CSF serological test	Case reports	VDRL/RPR/TRUST-positive	123	82.00%	11.97		0.001
		FTA-ABS/TPPA/TPHA-positive	134	95.04%			
		WBC count abnormal	82	74.55%	0.58		0.523
		PRO level abnormal	86	78.90%			
	Retrospective case series	VDRL/RPR/TRUST-positive	1,747	73.71%		5.78	<0.001
		FTA-ABS/TPPA/TPHA-positive	2,339	96.22%			
		WBC abnormal	1,654	60.99%		1.1	0.267
		PRO level abnormal	1,665	60.22%			
Neuroimaging examination (MRI/CT)	215 studies mentioned	Abnormal	1,750	81.89%			
		Normal	387	18.11%			
		Not mentioned	475				
	27 studies (12 case reports) not mentioned	897					
EEG	50 studies mentioned	Abnormal	458	77.50%			
		Normal	133	22.50%			
		Not mentioned	494				
	192 studies (129 case reports) not mentioned	2,422					
HIV	135 studies mentioned	Positive	228	8.34%			
		Negative	2,505	91.66%			
	107 studies (73 case reports) not mentioned	774					

*CSF, cerebrospinal fluid; VDRL, venereal disease research laboratory; RPR, rapid plasma reagin; TRUST, toluidine red unheated serum test; FTA-ABS, fluorescent treponemal antibody adsorption; TPPA, treponema pallidum particle agglutination; TPHA, treponema pallidum hemagglutination assay; WBC, white blood cell; PRO, protein; MRI, magnetic resonance imaging; CT, computed tomography; EEG, electroencephalography; HIV, human immunodeficiency virus. *The denominator is the sum of the cases who mentioned the laboratory findings.*

†The Wilcoxon signed-rank test was used to compare the difference in positive rate between CSF non-treponemal tests and treponemal tests, as well as the abnormal rate between CSF WBC and PRO level.

65.1–75.8%). There were no differences in the rates of abnormal CSF-WBC and CSF-PRO results among studies ($|Z| = 1.1$, $P = 0.27$). The diagnostic criteria for neurosyphilis were not provided in two studies including 51 patients, and the results of CSF serologic tests were not mentioned in 12 studies including 474 patients. (**Supplementary Table S3**).

The numbers of patients with HIV infection were described in 135 studies, including 2,733 patients, and the coinfection rate was 8.34% (228/2,733). In addition, imaging examination results were presented in 215 studies; 81.8% (2,137/2,612) of the patients underwent neuroimaging examinations (skull/spine/orbital magnetic resonance imaging (MRI) or computed tomography (CT)), and 81.9% (1,750/2,137) of them had abnormal results (**Table 2**), the abnormal rates ranged from 25.0 to 100% (95%CI, 80.5–88.2%). Forty-two studies presented electroencephalography (EEG) findings; 54.47% (591/1,085) of the patients underwent EEG, and 77.50% (458/591) of them had abnormal results, the abnormal rates ranged from 11.5% to 100% (95%CI, 70.6–87.8%) (**Supplementary Table S3**).

Treatment and Prognosis

Of the 242 studies, treatment of neurosyphilis was described in 201 studies including 1,738 patients. As shown in **Table 3**, the treatment drugs were described for 1,634 patients. Aqueous penicillin was the first choice for treatment (88.3%, 1,442/1,634), and ceftriaxone (7.4%, 121/1,634) and doxycycline (0.5%, 8/1,634) were the alternative choices for those who were allergic to penicillin. The remaining 63 (3.9%) patients were not treated with the drugs recommended by the National Guidelines, such as benzathine penicillin (3.2%, 53/1,634), minocycline, azithromycin, traditional Chinese medicine (TCM), etc. Thirty

TABLE 3 | The treatment and follow-up of neurosyphilis cases from 149 case reports and 93 retrospective case series.

Treatment and prognosis				Number of cases	Proportion (%) *
Treatment drugs		Aqueous penicillin		1,442	88.25%
		Ceftriaxone		121	7.41%
		Doxycycline		8	0.49%
	201 studies mentioned	Benzathine penicillin		53	3.24%
		Other drugs		10	0.61%
		Refused treatment		10	-
		Referred		38	-
		Not mentioned		56	-
	41 studies (11 case reports) not mentioned			1,769	-
		Symptoms	improved	650	68.06%
			recovery	130	13.61%
			no-response (persisted, deteriorated, or recurrence)	175	18.32%
Follow-up and Prognosis	181 studies mentioned	Serum non-treponemal test dropped at least 2 titers or turn to negative		195/288	67.71%
		CSF non-treponemal test dropped at least 2 titers or turn to negative		89/178	50.00%
		CSF WBC decreased		145/188	77.13%
		CSF PRO decreased		153/202	75.74%
		Neuroimaging improved		35/36	97.22%
		Death		9	-
	20 studies (9 case reports) not mentioned		679	-	

*CSF, cerebrospinal fluid; WBC, white blood cell; PRO, protein. *The denominator is the sum of the cases who mentioned the treatment and prognosis.*

eight patients were referred to other hospitals and 10 patients refused treatment.

The follow-up and prognosis of patients were described in 181 studies. The longest follow-up time was 6 years, while the efficacy of treatment among 19.3% (29/150) patients were evaluated before discharge and only 17.3% (26/150) patients follow-up at least 1 year. Most patients (52%, 78/150) follow-up for 3 to 6 months. As shown in **Table 3**, during the follow-up period, 81.7% (780/955) patients had an improvement or recovery of clinical symptoms (range from 0 to 100% in 47 cases series studies, 95%CI: 75.0–88.3%, **Supplementary Table S2**). Unfortunately, the persistence, deteriorate or recurrence of symptoms were occurred among 18.3% (175/955) patients. The results of nontreponemal test in serum or CSF turned negative or dropped by at least two titers in 67.7% (195/288) (range from 0 to 100% in 14 cases series studies, 95%CI: 33.0%−73.3%, **Supplementary Table S2**) and 50% (89/178) (range from 0 to 100% in 10 cases series studies, 95%CI: 23.2–73.7%, **Supplementary Table S2**) of patients, respectively. The CSF WBC or PRO decreased or return to normal range in 77.1% (145/188) (range from 42.9 to 100% in 12 cases series studies, 95%CI: 64.5–94.8%, **Supplementary Table S2**) and 75.7% (153/202) (range from 45.5 to 100% in 10 cases series studies, 95%CI: 64.8–93.4%, **Supplementary Table S2**) of patients, respectively. In addition, the rate of improvement or recovery on neuroimaging findings was 97.2% (35/36) mentioned in 34 case report studies. Nine patients died during follow-up (**Supplementary Tables S2, S3**).

Among 121 cases report studies, 131 and 13 patients were mentioned treated by using aqueous penicillin or ceftriaxone, and the response to clinical symptoms after treatment were mentioned in 107 and 12 cases, respectively. We found that there is no difference in the rate of improvement or recovery of clinical symptoms between patients treated by using aqueous penicillin or ceftriaxone (87.9% (94/107) vs. 83.3% (10/12), $P = 1.0$). Data about the syphilitic serological response rate in serum and CSF was too few for comparison.

Neurosyphilis Amongst Patients With Other Associated Diseases

Seven studies reported cases of neurosyphilis amongst CNS diseases (**Table 4**, **Supplementary Table S3**). The diagnostic criteria of neurosyphilis were not stated in five studies, and the results of CSF serological tests were presented in only one study.

TABLE 4 | Studies reporting cases of neurosyphilis with CNS diseases.

ID	Study	Province	Year	Neurosyphilis	Prevalence of neurosyphilis	Study design	Study Duration	Inclusion criteria	CSF criteria for diagnosis of neurosyphilis	% HIV positive
1	Dai LL [18]	Beijing	2014	2	3.33%	retrospective	2009-2011	HIV/AIDS patients (≥13 years old) with a complaint of new or recurrent neurological or psychiatric symptoms/signs	Not Stated	100.00%
2	Guan LQ [19]	Shanghai	2016	36	10.62%	retrospective	2010-2015	HIV with CNS lesions	Positive result on CSF-VDRL, or FTA-ABS tests with abnormal CSF-WBC	100.00%
3	FF Yu[20]	Tianjin	2021	11	22.92%	retrospective	2017-2020	HIV with CNS diseases	Not Stated	100.00%
4	Lv LX [21]	Tianjin	2015	1	Syphilis: 0.22% CNS diseases: 0.0028%	retrospective	2011-2014	Inpatient with nervous system disease	Not Stated	Not Stated
5	Qin LH [22]	Guangxi	2014	1	1.43%	retrospective	2003-2012	HIV with neuropathy	Not Stated	100.00%

CNS, central nervous system; CSF, cerebrospinal fluid; HIV, human immunodeficiency virus; AIDS, acquired Immune Deficiency Syndrome; VDRL, venereal disease research laboratory; FTA-ABS, fluorescent treponemal antibody adsorption; WBC, white blood cell.

Twenty one studies reported cases of syphilis with neurosyphilis (**Table 5**). The performance of LP was mentioned in all studies, however, the CSF serologic tests were not performed among 238 patients in three studies.

CNS Diseases

A case-control study in patients with CNS infection showed that 30.1% (84/279) of patients were diagnosed as neurosyphilis with evidence of positive reaction on CSF serological tests (16). Another study in patients with cognitive impairment showed that neurosyphilis was prevalent in 17.1% (6/35) of the patients with CNS infectious diseases; however, the diagnostic criteria of neurosyphilis were not provided (17). Other three studies reported neurosyphilis amongst HIV-infected patients with CNS disorders. There were 60, 339, and 48 patients recruited, and 2, 36 (10.6%) and 11(22.9%) of them were diagnosed with NS, respectively (18–20). However, only one study (19) provided evidence in positive results on the CSF-VDRL test or FTA-ABS test and abnormal CSF-WBC for the diagnosis of neurosyphilis.

Other Nervous System Diseases

One study reported syphilis serological test results among 36,151 patients hospitalized for nervous system disorders; only one neurosyphilis patient was identified among 449 patients with reactive syphilis serological tests (21). Another study recruited 70 HIV-infected patients with nervous system disorders, and only one patient (1.4%) was diagnosed with neurosyphilis (22). These two patients were diagnosed without any evidence on CSF tests.

Syphilis Patients With Neurological Symptoms or Who Received Lumbar Puncture

Three retrospective studies reported neurosyphilis among syphilis patients with neurological symptoms. The prevalence of neurosyphilis was 23.7% (100/422), 33.2% (123/370), and 35.5% (156/440) (23–25), respectively. All neurosyphilis patients were diagnosed according to reactive CSF serological tests. Four retrospective studies reported patients with suspected neurosyphilis who underwent lumbar puncture (LP). The prevalence of neurosyphilis in these studies ranged from 13.6 to 61.2% (26–29), respectively. However, only 67.72% (426/630) of the patients were diagnosed with neurosyphilis according to reactive CSF serological tests; the remaining 204 patients had only abnormal routine CSF test results.

Serofast Status and RPR/TRUST Titer Fluctuations After Treatment

Six retrospective studies reported 1,171 serofast syphilis patients and 44 patients with RPR/TRUST titers that fluctuated continuously (30–35). A total of 405 (33.3%) patients were diagnosed with neurosyphilis according to reactive results in CSF serological tests and/or abnormal results on CSF routine tests. The prevalence of neurosyphilis in these studies ranged from 9.8 to 56.1%. Unfortunately, 99 neurosyphilis patients were diagnosed without CSF serological evidence, and one study did not provide the results of CSF serological tests.

Individuals With HIV and Syphilis Coinfection

A retrospective study recruited 157 patients with HIV and syphilis coinfection, and 8.9% (14/157) of them were diagnosed

TABLE 5 | Studies on neurosyphilis among patients with syphilis.

ID	Study	Province	Year	Neurosyphilis	Prevalence of Neurosyphilis	Study design	Study Duration	Inclusion criteria	CSF Criteria for diagnosis of neurosyphilis	% HIV +
1	Li K [23]	Shanghai	2013	100	23.70%	retrospective case-control	2009–2012	Syphilis LP performed	CSF-VDRL (87 cases) and TPPA (13 cases) reactivity	0.00%
2	Xiao Y [24]	Fujian	2017	123	33.24%	retrospective	2008–2014	HIV-negative with neurological symptoms	CSF-RPR (123 cases) reactivity	0.00%
3	Zhang L [25]	Guang dong	2010	156	35.45%	clinical trial	2007–2010	Syphilis diagnosed in a dermatology clinic	VDRL/FTA-ABS (156 cases) reactivity	Not Stated
4	Zhu L [26]	Shanghai	2014	210	13.63%	retrospective	2009–2012	Syphilis in patients ≥18 years old	Positive CSF-TPPA in the absence of contamination with blood or CSF-VDRL reactivity (210 cases)	0.00%
5	Shi M [27]	Shanghai	2016	191	22.90%	retrospective	2009–2013	HIV-negative syphilis	(1) CSF-VDRL reactivity or nonreactivity (117 cases) and (2) an elevated CSF-protein level (>50 mg/dL) or WBC (>10 cells/L) in the absence of other known causes of the abnormalities	0.00%
6	Ma CD [28]	Jiangsu	2013	7	26.92%	retrospective	2007–2011	Inpatients with syphilis	CSF-RPR (7 cases) and TPHA (13 cases) reactivity	Not Stated
7	Lin DH [29]	Fujian	2017	222	61.16%	retrospective	2005–2013	Syphilis LP performed	CSF-RPR (92 cases) or TPPA reactivity and WBC count or PRO level abnormality	0.00%
8	Li SL [30]	Fujian	2012	115	56.10%	retrospective	2006–2009	Serofast syphilis	CSF-TRUST (58 cases) and TPPA (93 cases) reactivity	0.00%
9	Cai SN [31]	Beijing	2017	139	34.58%	retrospective	2008–2016	Serofast syphilis	Presence of one or more CSF abnormalities (pleocytosis, elevated protein concentration, or CSF-RPR reactivity (40 cases))	0.00%
10	Zheng TH [32]	Guang dong	2016	6	9.84%	cost-benefit analysis	2013	Serofast syphilis	CSF-RPR (6 cases) reactivity and an abnormal CSF-WBC	Not Stated
11	He WQ [33]	Guang dong	2015	12	26.09%	retrospective case-control	Not Stated	Serofast syphilis	CSF-FTA-ABS (5 cases) or CSF-TPHA (11 cases) reactivity	0.00%
12	Ye YJ [34]	Zhejiang	2018	127	27.79%	clinical trial	2012–2015	Serofast syphilis	CSF-VDRL (67 cases), CSF-RPR (73 cases), CSF-TPPA (252 cases), or CSF-FTA-ABS (244 cases) reactivity	0.44%
13	Chen XS [35]	Guang dong	2011	6	13.64%	retrospective	2002–2009	Syphilis patients whose RPR titer increased by 2 times or more without reinfection after standardized treatment	CSF-RPR and TPPA (1 case) and CSF-RPR and TPPA and VDRL (5 cases) reactivity	Not Stated
14	Wang YJ [36]	Taiwan	2012	14	8.92%	retrospective	2000–2009	HIV and syphilis coinfection	CSF-WBC >20 cells/μL (7 cases) or elevated VDRL titers in CSF samples (7 cases)	100.00%
15	XX Sun[37]	Henan	2020	51	50.00%	retrospective	2014-2017	CSF abnormal and HIV-coinfection	CSF TPPA (51 cases) and RPR (15 cases) reactivity and abnormal CSF-WBC and PRO	100.00%
16	Zhu L [38]	Shanghai	2019	7	26.92%	retrospective	2008–2018	Malignant syphilis	CSF-VDRL (7 cases) reactivity	14.29%

(Continued)

TABLE 5 | Continued

ID	Study	Province	Year	Neurosyphilis	Prevalence of Neurosyphilis	Study design	Study Duration	Inclusion criteria	CSF Criteria for diagnosis of neurosyphilis	% HIV +
17	Tang WM [5]	Guang dong	2017	1615	0.54%	retrospective	2009–2014	Syphilis LP performed	CSF-VDRL reactivity or a CSF WBC > 20 cells/μL	Not Stated
18	Wang H [39]	Sichuan	2011	24	1.25%	retrospective	2006–2010	Inpatient status	CSF-TRUST (24 cases) reactivity and an abnormal CSF-WBC and CSF-PRO level	Not Stated
19	J Yan[40]	Beijing	2021	416	78.79%	retrospective	2013-−2019	patients >18 years old; laboratory-confirmed syphilis in Department of Neurology or HIV infection	reactive CSF TPPA or TRUST, CSF WBC ≥5 cells/μL for HIV-negative patients and >20 cells/μL for HIV-positive, or elevated protein (>500mg/L); if CSF TPPA or TRUST was not reactive, no evidence of other diseases of the CNS could cause CSF pleocytosis or elevated protein.	73.08%
20	J Cao[41]	Xinjiang	2021	10	13.70%	retrospective	2016–2019	Serofast syphilis, or coinfection with HIV, or with neurological symptoms, or serum titer 4 times fluctuation	CSF TPPA (10 cases) reactivity, or CSF TRUST (5 cases) reactivity and CSF WBC or PRO abmormal	10.00%
21	YH Hua[42]	Jiangsu	2021	121	23.63%	retrospective	2016–2019	Serofast syphilis, or with neurological symptoms, or serum titer fluctuation	CSF TPPA (121 cases) reactivity, or CSF TRUST (77 cases) reactivity and CSF WBC (59 cases) or PRO (68 cases) abmormal	Not Stated

CSF, cerebrospinal fluid; LP, lumbar puncture; VDRL, venereal disease research laboratory; TPPA, treponema pallidum particle agglutination; HIV, human immunodeficiency virus; RPR, rapid plasma reagin; FTA-ABS, fluorescent treponemal antibody adsorption; WBC, white blood cell; PRO, protein; TRUST, toluidine red unheated serum test; CNS, central nervous system.

TABLE 6 | Diagnostic criteria in 93 retrospective case series and 26 studies on neurosyphilis in patients with other diseases.

Criteria for the diagnosis of neurosyphilis	Number of studies
VDRL/RPR/TRUST and/or FTA-ABS/TPPA/TPHA positivity and abnormal CSF-WBC or PRO levels	100
VDRL/RPR/TRUST and/or FTA-ABS/TPPA/TPHA positivity and no evidence of CSF-WBC or PRO level abnormalities	8
Only TPPA/TPHA positivity, with no evidence of CSF-WBC or PRO level abnormalities	2
RPR/TRUST positivity, or TPPA/TPHA positivity, or CSF-WBC or PRO level abnormalities	3
Not mentioned	6

NS, neurosyphilis; VDRL, venereal disease research laboratory; RPR, rapid plasma reagin; TRUST, toluidine red unheated serum test; FTA-ABS, fluorescent treponemal antibody adsorption; TPPA, treponema pallidum particle agglutination; TPHA, treponema pallidum hemagglutination assay; CSF, cerebrospinal fluid; WBC, white blood cell; PR, protein.

with neurosyphilis according to reactive CSF-VDRL (seven cases) or CSF-WBC >20 cells/mL (seven cases) (36). Another study recruited 102 syphilis patients coinfected with HIV and with abnormal CSF routine tests, and half of patients were diagnosed with neurosyphilis according to reactive CSF-TPPA with/without reactive CSF-RPR (15 cases) (37).

Malignant Syphilis

A retrospective study reported 26 malignant syphilis patients with typical, serious skin lesions and high non-treponemal tests titers (38). Seven (26.9%) patients were diagnosed with neurosyphilis according to the reactive CSF-VDRL test among these patients. The study found that the proportion of malignant syphilis patients who developed concurrent neurosyphilis was higher than common syphilis patients (13.1%).

Other Studies

A retrospective study reported an 1.3% prevalence of neurosyphilis in 1,927 inpatients with positive syphilis screening results (39). Another study reported an 82.1% prevalence of neurosyphilis in 1,968 tertiary syphilis and only 27.1% of neurosyphilis patients received standard treatment (5). Recently, a study reported a 78.8% prevalence of neurosyphilis in 528 laboratory-confirmed syphilis in department of neurology or HIV infection patients (40). Another two studies reported 13.7% and 23.6% prevalence of neurosyphilis in 73 and 512 patients with serofast status, or coinfection with HIV, or with neurological symptoms, or serum titer fluctuation after treatment, respectively (41, 42).

Ocular Syphilis and Otic Syphilis

Four case reports included 6 ocular syphilis patients, and 11 retrospective case series included 244 patients (**Supplementary Table S4**) which comprised 163 males and 81 females, indicating that ocular syphilis tended to occur more frequently in males than in females ($|Z| = 2.56$, $P = 0.01$). LP was not performed in two case reports and four retrospective case series; 91.9% (136/148) of the patients in the remaining nine studies received LP, and 56.1% (78/139) (ranged from 9.1 to 91.7%) of them were diagnosed with neurosyphilis according to the reactive CSF serological tests.

A retrospective case series analysis reported 6 syphilis patients with recurrent refractory vertigo and sensorineural deafness. Only one patient with CNS symptoms underwent LP and was diagnosed with neurosyphilis according to the reactive CSF serological tests. (**Supplementary Table S4**).

Diagnostic Criteria

The CSF diagnostic criteria differed among 93 retrospective case series studies and 26 studies on neurosyphilis amongst other diseases (**Table 6**), 42 of which mentioned that neurosyphilis was diagnosed according to US CDC or/and European guidelines, and 25 of which mentioned diagnosed according to Chinese guidelines. It was worthy to note that most studies (84.0%, 100/119) met diagnostic criteria established by the Chinese National Guidelines (14), which included VDRL/RPR/TRUST and/or the FTA-ABS/TPPA/TPHA positivity for CSF samples, as well as abnormal CSF-WBC or CSF-PRO levels. Unfortunately, eight studies (6.7%) did not present the results of routine CSF tests. Two studies diagnosed neurosyphilis with only TPPA/TPHA positivity in CSF. Three studies defined neurosyphilis as reactive CSF-RPR/TRUST or CSF-TPPA/TPHA or abnormal CSF-WBC or CSF-PRO levels. The diagnostic criteria were not mentioned in six studies.

DISCUSSION

This is the first review of the literature on the clinical epidemiological characteristics of neurosyphilis in China from 2009 to 2021, as well as neurosyphilis in patients with other associated diseases. The inconsistent diagnostic criteria in these studies and the heterogeneity among case sources led to limited conclusions.

Although the incidence of neurosyphilis in China is unknown, the number of reported cases increased over 13 years according to the results of this review (**Supplementary Figure S1**), indicating an increasing health threat requiring neurosyphilis prevention and control. The regional distribution of cases showed a concentration in the eastern coastal areas, including Guangdong, Fujian, Beijing, Zhejiang, Shanghai, etc. (**Supplementary Figure S2**). YS Tao et al. found that the incidence of early syphilis in inland provinces has increased over time and has been higher than that in eastern coastal provinces since 2010 (2). Considering that the prevalence of neurosyphilis is similar to that of syphilis, more attention probably be paid to the detection and research of neurosyphilis in eastern coastal areas than inland areas. Therefore, more

training in the management of neurosyphilis in inland areas is needed in the future.

Among all the studies, we found that neurosyphilis, as well as ocular syphilis, tended to occur most frequently in middle-aged males, consistent with previous reports (27, 43). We also found that parenchymal syphilis was the main manifestation of neurosyphilis, and general paresis with progressively impaired memory, mental abnormalities and occasional seizures was the most common symptom. This clinical spectrum is consistent with those in previous reports in China but is very different from those associated with cases in Western countries (7). In the penicillin era, early forms, such as meningitis and meningovascular, are more common than late forms in Western countries because treatment can effectively prevent the progression of neurosyphilis (44), as indicated in the most recent reports (45). This result suggests that the early detection and treatment of neurosyphilis in China probably be insufficient. In addition, with the rapid expansion of syphilis screening among all populations, an increasing number of patients infected with syphilis have been diagnosed with neurosyphilis, which has probably progressed to the late stage at the time of diagnosis. It is worth noting that many patients present with atypical, ill-defined neurological complaints (46, 47), causing great challenges in the diagnosis of neurosyphilis for clinicians. Unfortunately, the results showed that the misdiagnosis rate of neurosyphilis was more than 50%, and the most common misdiagnosed diseases were neurodegenerative diseases (including stroke, cognitive impairment, Alzheimer's disease, Parkinson's disease, epilepsy, etc.), mental disorders, encephalitis and ophthalmic diseases. Two studies reporting patients with reactive CSF serological tests and diagnosed with neurosyphilis showed that the prevalence rates of neurosyphilis among patients with CNS-associated infectious diseases and CNS disorders and HIV coinfection were 30.1 and 10.6% (16, 19), respectively. In addition, among patients with syphilis infection, the prevalence of neurosyphilis ranged from 23.2 to 35.5% among patients with neurological symptoms (23–25), 13.6 to 67.7% among patients with suspected neurosyphilis who underwent LP (26–29), and 9.8 to 56.1% among syphilis patients with serofast status (30–35); additionally, the rates were 8.9, 26.9 and 43.0% in individuals coinfected with HIV, malignant syphilis and ocular syphilis (36, 37), respectively. The results suggest that clinicians in neurology, psychiatry and ophthalmology departments should pay much attention to detecting suspected neurosyphilis. Serum syphilis tests can be performed in patients with neurological, psychiatric, or ocular symptoms caused by unknown etiologies, especially patients with CNS-associated infectious diseases or HIV infection, to examine the status of syphilis infection. Moreover, CSF serological testing is recommended for syphilis patients with neurological symptoms, serofast status, coinfection with HIV or the presence of serious skin lesions.

Penicillin G has been demonstrated to be effective for the serological and clinical cure of neurosyphilis since 1940 (48, 49), and it has always been the first-line drug for the treatment of neurosyphilis. Ceftriaxone can be used as an alternative regimen in patients with penicillin allergy, and no difference was found in the efficacy of the two drugs in previous studies (50, 51). The data from case reports studies in this paper also showed that there was no difference in the recovery rate of clinical manifestations of patients treated with penicillin or ceftriaxone. It was worthy to note that approximately 4% of patients did not receive treatment regimens recommended by the national guidelines according to the available data in this review, which will be detrimental to preventing the progression of CNS injury. In addition, neurosyphilis patients need long-term follow-up for clinical management according to the recovery of disease, however, the follow-up results within 6 months were reported in more than half of cases. Hence, medical institutions need to strengthening the management of follow-up and increase the compliance, such as call patients regularly to urge return visits, provide higher quality of health care for patients, provide multidisciplinary therapy, psychological guidance, etc. In addition, we found that 81.7% patients had an improvement of symptoms, and 77.1% patients had a decrease of CSF WBC counts during the follow-up period, however, only 66.7% and half of patients had responses to serological tests of syphilis in serum and CSF, respectively. Therefore, more sensitive and specific biomarkers for assessment of prognosis of neurosyphilis are needed, especially if effective biomarkers can be found in serum in patients unwilling to undergo lumbar puncture during follow-up.

From the results of the case reports and retrospective case series, the treponemal test positivity rate was over 95%, which was significantly higher than the non-treponemal test positivity rate (∼75%), and abnormal CSF-WBC counts and CSF protein levels were observed in nearly 70% of NS patients, supporting the importance of CSF serological testing for diagnosis (52). Although the CSF-TPPA testis diagnostically sensitive, considering that TP-IgG can cross the intact blood brain barrier (BBB), reactive treponemal testing of CSF samples is not specific for the diagnosis of neurosyphilis (53). The specificity of the CSF-TPPA test has been debated and ranges from 49 to 84% in some studies (54, 55), and its recommendation as a diagnostic indicator varies according to different guidelines (14, 56, 57). Moreover, the cases in most studies were retrospectively analyzed and included reactive treponemal test in the inclusion criteria, which probably biased positivity rate toward 100% (53). Therefore, CSF nontreponemal tests combined with treponemal tests are needed to reduce the possibility of misdiagnosis.

Neurosyphilis patients frequently have abnormal neuroimaging findings due to CNS inflammation and impairment. Neuroimaging may also show progression in patients who have no neurological symptoms or signs and in patients who receive standardized treatment (58). In addition, neurosyphilis patients may have specific EEG signal characteristics that are different from those of non-neurosyphilis patients (59), and the EEG-Lempel-Ziv complexity (LZC) value may be used as a diagnostic index and the reference index to assess neurosyphilis cure (60). According to our review, abnormal neuroimaging or EEG findings were observed in approximately 80% of patients, and the neuroimaging findings of 97% cases improved or return to normal on follow-up, supporting the recommendation of neuroimaging and EEG examinations for the differential clinical diagnosis and neurosyphilis follow-up (61).

Progression to neurosyphilis is more common in patients coinfected with HIV (62). We found that 8.9% of patients with neurosyphilis were infected with HIV, but most studies have used HIV infection as an exclusion or inclusion criterion, resulting in inconsistent rates.

Although the diagnostic criteria in most of the included studies followed international or national guidelines, unlike previous reports from Africa (15), approximately one-tenth of the studies provided insufficient evidence for a neurosyphilis diagnosis. Hence, standardized diagnostic criteria and protocols are urgently needed to ensure accurate diagnostic results and research conclusions.

This review had several limitations. First, there were some missing clinical information extracted from many studies, especially the data of treatment and follow-up, may result in information bias. Second, the great heterogeneity between studies according to the different diagnostic criteria of neurosyphilis may result in limited conclusions. Third, we did not review the literature published before 2009 due to the lack of clinical diagnosis and treatment guidelines for neurosyphilis in China before 2008, may lead to selection bias.

CONCLUSIONS

In summary, through the review of clinical epidemiology data on neurosyphilis in China, we found that the reported cases of neurosyphilis in the literature presented an increasing trend. More than half of the cases were in the late stage of neurosyphilis, suggesting that early detection of neurosyphilis was inadequate. The most common misdiagnosed diseases and the high prevalence of neurosyphilis amongst patients with CNS-associated infectious disease, CNS disease comorbid with HIV, syphilis with neurological symptoms, serofast status, coinfection with HIV or presesntations comprising serious skin lesions should remind clinicians to pay much attention to the early detection of neurosyphilis among these cases. Meanwhile, standardized treatment and long-term follow-up of neurosyphilis according to the national guidelines should be strengthened to promote the recovery of patients. During follow-up, neuroimaging and EEG examinations should be performed, as they play a positive role in auxiliary diagnosis and the observation of curative effects. Moreover, the combination of CSF non-treponemal tests with treponemal tests should be implemented for diagnosis to reduce misdiagnosis. Inadequate diagnostics remain a great obstacle to progress in understanding this disease. Future well-designed prospective studies are needed to better delineate the incidence and clinical epidemiological of neurosyphilis in China.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

F-ZD, Q-QW, and R-LZ wrote the draft and revised it. F-ZD, H-NZ, J-JL, and Z-JZ selected literature. F-ZD and XZ extracted data from literature. F-ZD performed the statistical analysis. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Union Innovation Team Project of the Chinese Academy of Medical Sciences (2016-I2M-3021), the National Natural Science Foundation of China (81772209 and 81601804), and the Nanjing Incubation Program for National Clinical Research Center (2019060001).

ACKNOWLEDGMENTS

Many thanks to all participants of the study for their cooperation.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.894841/full#supplementary-material

REFERENCES

1. Gonzalez H, Koralnik IJ, Marra CM. Neurosyphilis. *Semin Neurol.* (2019) 39:448–55. doi: 10.1212/CON.0000000000000250
2. Tao Y, Chen MY, Tucker JD, A. nationwide spatiotemporal analysis of syphilis over 21 years and implications for prevention and control in China. *Clin Infect Dis.* (2020) 70:136–9. doi: 10.1093/cid/ciz331
3. Berger JR, Dean D. Neurosyphilis. *Handb Clin Neuro.* (2014) 121:1461–72. doi: 10.1016/B978-0-7020-4088-7.00098-5
4. Unemo M, Bradshaw CS, Hocking JS, de Vries HJC, Francis SC, Mabey D, et al. Sexually transmitted infections: challenges ahead. *Lancet Infect Dis.* (2017) 17:e235–e79. doi: 10.1016/S1473-3099(17)30310-9
5. Tang W, Huang S, Chen L, Yang L, Tucker JD, Zheng H, et al. Late neurosyphilis and tertiary syphilis in Guangdong province, china: results from a cross-sectional study. *Sci Rep.* (2017) 7:45339. doi: 10.1038/srep45339
6. Ropper AH. Neurosyphilis. *N Engl J Med.* (2019) 381:1358–63. doi: 10.1056/NEJMra1906228
7. Wu F, Wang QQ. Advances in neurosyphilis. *Int J Dermatol Venereol.* (2015) 41:268–71. doi: 10.3760/cma.j.issn.1673-4173.2015.04.019
8. Zhang HL, Lin LR, Liu GL, Zeng YL, Wu JY, Zheng WH, et al. Clinical spectrum of neurosyphilis among HIV-negative patients in the modern era. *Dermatology.* (2013) 226:148–56. doi: 10.1159/000347109
9. Wang YH, Shi HS, Le H, Zhong XM, Chen XR, Ling L, et al. Clinical and neuropsychological characteristics of general paresis

misdiagnosed as primary psychiatric disease. *BMC Psychiatry.* (2016) 16:230–6. doi: 10.1186/s12888-016-0925-3
10. Musher DM. Editorial commentary: polymerase chain reaction for the tpp47 gene: a new test for neurosyphilis. *Clin Infect Dis.* (2016) 63:1187–8. doi: 10.1093/cid/ciw518
11. Marra CM, Tantalo LC, Maxwell CL, Ho EL, Sahi SK, Jones T. The Rapid plasma reagin test cannot replace the venereal disease research laboratory test for neurosyphilis diagnosis. *Sex Transm Dis.* (2012) 39:453–7. doi: 10.1097/OLQ.0b013e31824b1cde
12. Marra CM, Tantalo LC, Maxwell CL, Dougherty K, Wood B. Alternative cerebrospinal fluid tests to diagnose neurosyphilis in HIV-infected individuals. *Neurology.* (2004) 63:85–8. doi: 10.1212/01.wnl.0000131902.69113.34
13. Marra CM, Maxwell CL, Dunaway SB, Sahi SK, Tantalo LC. Cerebrospinal fluid treponema pallidum particle agglutination assay for neurosyphilis diagnosis. *J Clin Microbiol.* (2017) 55:1865–70. doi: 10.1128/JCM.00310-17
14. National Center for STD Control, China centers for disease control and prevention. guidelines for the diagnosis and treatment of syphilis, gonorrhea and chlamydia trachomatis infection (2020). *Chin J Dermatol.* (2020) 53:168–79. doi: 10.35541/cjd.20190808
15. Michael Marks, Joseph N Jarvis, William Howlett, Mabey DCW. Neurosyphilis in Africa: a systematic review. *PLoS Negl Trop Dis.* (2017) 11:e0005880. doi: 10.1371/journal.pntd.0005880
16. Xiao Y, Chen MJ, Shen X, Lin LR, Liu LL, Yang TC, et al. Metabolic disorders in patients with central nervous system infections: associations with neurosyphilis. *Eur Neurol.* (2019) 81:270–7. doi: 10.1159/000503626
17. Wang S, Zhang J, Liang J, Song H, Ji X. Treatable causes of adult-onset rapid cognitive impairment. *Clin Neurol Neurosurg.* (2019) 187:105575. doi: 10.1016/j.clineuro.2019.105575
18. Dai LL, Mahajan SD, Guo CP, Zhang T, Wang W, Li T, et al. Spectrum of central nervous system disorders in hospitalized HIV/AIDS patients (2009–2011) at a major HIV/AIDS referral center in Beijing, China. *J Neurol Sci.* (2014) 342:88–92. doi: 10.1016/j.jns.2014.04.031
19. Guan LQ, Lu HZ, Shen YZ, Liu L, Qi TK, Song W, et al. Spectrum of central nervous system disorders in the first hospitalized HIV/AIDS patient. *Chin J AIDS STD.* (2016) 22:510–3. doi: 10.13419/j.cnki.aids.2016.07.07
20. Yu FF, Zhang DF, Huang XJ, Zhao SD, Ma P. Clinical analysis of AIDS complicated with central nervous system disease in 48 cases. *Shandong Medical Journal.* (2021) 61:81–4. doi: 10.3969/j.issn.1002-266X.2021.11.021
21. Lv LX, Kan PC, Zhu Y, Xue J, Zhang B. Analysis of antibody detection of Treponema pallidum in 36,151 inpatients with nervous system diseases. *Chin J Infect Dis.* (2015) 33:426–7. doi: 10.3760/cma.j.issn.1000-6680.2015.07.013
22. Qin LH, Mo XA, Huang W, Qiu D, Luo WJ, Wu LS, et al. Clinical analysis of 70 cases of AIDS complicated with neuropathy. *Chin J Nerv Ment Dis.* (2014) 40:109–12. doi: 10.3936/j.issn.1002-0152.2014.02.011
23. Li K, Wang CN, Lu HK, Gu X, Guan Z, Zhou P. Regulatory T cells in peripheral blood and cerebrospinal fluid of syphilis patients with and without neurological involvement. *PLoS Negl Trop Dis.* (2013) 7:e2528. doi: 10.1371/journal.pntd.0002528
24. Xiao Y, Tong ML, Liu LL, Lin LR, Chen MJ, Zhang HL, et al. Novel predictors of neurosyphilis among HIV-neative syphilis patients with neurological symptoms: an observational study. *BMC Infect Dis.* (2017) 17:310. doi: 10.1186/s12879-017-2339-3
25. Zhang L, Tian X, Lin LY, Song WZ, Liang YH Bi C, et al. Analysis on seral and brain spinal fluid positive samples from 2,145 Syphilis case. *Chin J Derm Venereol.* (2010) 24:939–40.
26. Zhu L, Gu X, Peng RR, Wang CN, Gao ZX, Zhou PY, et al. Comparison of the cerebrospinal fluid (CSF) toluidine red unheated serum test and the CSF rapid plasma reagin test with the CSF venereal disease research laboratory test for diagnosis of neurosyphilis among HIV-negative syphilis patients in China. *J Clin Microbiol.* (2014) 52:736–40. doi: 10.1128/JCM.02522-13
27. Shi M, Peng RR, Gao Z, Zhang S, Lu H, Guan Z, et al. Risk profiles of neurosyphilis in HIV-negative patients with primary, secondary and latent syphilis: implications for clinical intervention. *J Eur Acad Dermatol Venereol.* (2016) 30:659–66. doi: 10.1111/jdv.13514
28. Ma CD, Ding KY, Shen HJ, Zhao ZG, Zhang Y. Clinical analysis of 14 cases of syphilis with cerebrospinal fluid abnormality. *Chin J Derm Venereol.* (2013) 27:166–7.
29. Lin DH Li SL, Lin HL, Lin ZF, Zhang HL. The Role of serum reagin titer in lumbar puncture of neurosyphilis. *Chin J Derm Venereol.* (2017) 31:994–7. doi: 10.13735/j.cjdv.1001-7089.201701046
30. Li SL, Lin ZF, Zhang HS, Lin HL, Tong ML, Liu GL, et al. Relationship between syphilis serofast reaction and neurosyphilis. *Chin J Nosocomiol Vol.* (2012) 22:2235–8.
31. Cai SN, Long J, Chen C, Wan G, Lun WH. Incidence of asymptomatic neurosyphilis in serofast Chinese syphilis patients. *Sci Rep.* (2017) 7:15456. doi: 10.1038/s41598-017-15641-w
32. Zheng TH, Zeng TS, Feng TJ, Wu XB, Qiu LX. Cost-benefit analysis of neurosyphilis screening in serofast populations. *Chin J Heal Statis.* (2016) 33:829–32.
33. He WQ, Wang HL, Zhong DQ, Lin LY, Qiu XS, Yang RD. Treponemal antibody in CSF and cellular immunity in peripheral blood of syphilitic patients with persisting positive rapid plasma regain. *Int J Clin Exp Pathol.* (2015) 8:5775–80.
34. Ye YJ, Liu LF, Xu AE. Epidemiological analysis of 457 cases of refractory syphilis. *Chin J Health Lab Tec.* (2018) 28:1250–3.
35. Chen XS, Chi FH, Fan RQ Li HY, Deng JQ. Analysis of clinical, serology and cerebrospinal fluid in 52 cases of syphilis inpafients. *Chin J Derm Venereol.* (2011) 25:616–7.
36. Wang YJ, Chi CY, Chou CH, Ho CM, Lin PC, Liao CH, et al. Syphilis and neurosyphilis in human immunodeficiency virus-infected patients: a retrospective study at a teaching hospital in Taiwan. *J Microbiol Immunol Infect.* (2012) 45:337–42. doi: 10.1016/j.jmii.2011.12.011
37. Sun XX, Xu HQ, Liu XL, Tian F. Clinical analysis of 102 syphilis cases coinfected with HIV and with abnormal cerebrospinal fluid. *J Clin Derm.* (2020) 49:200–2. doi: 10.16761/j.cnki.1000-4963.2020.04.003
38. Zhu L, Shi M, Peng RR, Gu X, Guan Z, Xu H, et al. Neurosyphilis is more common in malignant syphilis: a case series and review of the literature. *Int J STD AIDS.* (2019) 30:779–85. doi: 10.1177/0956462419826710
39. Wang H, Zhang HW Li DY. Analysis of syphilis infection in inpatients of general hospital. *Chin J Derm Venereol.* (2011) 25:618–20.
40. Yan J, Luo L, Han J, Yan D, Zhang B, Zhang Z, et al. Comparing non-invasive predictors of neurosyphilis among syphilis patients with and without HIV co-infection based on the real-world diagnostic criteria: a single-center, retrospective cohort study in China. *AIDS Res Hum Retroviruses.* (2021). doi: 10.1089/AID.2021.0085. [Epub ahead of print].
41. Cao J, Zhang LJ, Fan JW, Palida ABLZ, Ayiguli YSP. Analysis of clinical characteristics, serology and cerebrospinal fluid of 73 hospitalized patients with syphilis. *J Clin Derm.* (2021) 50:725–7. doi: 10.16761/j.cnki.1000-4963.2021.12.006
42. Hau YH Li ZH, Yu XY, Yan N. Clinical analysis of 121 neurosyphilis patients. *J Clin Derm.* (2021) 50:648–50. doi: 10.16761/j.cnki.1000-4963.2021.11.003
43. Vadboncoeur J, Labbé AC, Fortin C, Serhir B, Rabia Y, Najem K, et al. Ocular syphilis: case series (2000–2015) from two tertiary care centres in Montreal, Canada. *Can J Ophthalmol.* (2020) 55:30–7. doi: 10.1016/j.jcjo.2019.05.009
44. Chilver-Stainer L, Fischer U, Hauf M, Fux CA, Sturzenegger M. Syphilitic myelitis: rare, non-specific, but treatable. *Neurology.* (2009) 72:673–5. doi: 10.1212/01.wnl.0000342460.07764.5c
45. Borges CR, Almeida SM, Sue K, Koslyk JLA, Sato MT, Shiokawa N, et al. Neurosyphilis and ocular syphilis clinical and cerebrospinal fluid characteristics: a case series. *Arq Neuropsiquiatr.* (2018) 76:373–80. doi: 10.1590/0004-282X20180054
46. Hooshmand H, Escobar MR, Kopf SW. Neurosyphilis. A study of 241 patients. *JAMA.* (1972) 219:726–9. doi: 10.1001/jama.219.6.726
47. Joyce-Clarke N, Molteno AC. Modified neurosyphilis in the Cape Peninsula. *S Afr Med J.* (1978) 53:10–14
48. Mahoney JF, Arnold RC, Sterner BL, Harris A, Zwally MR. Landmark article Sept 9, 1944: Penicillin treatment of early syphilis: II. By JF Mahoney, RC Arnold, BL Sterner, A Harris and MR Zwally. *JAMA.* (1984) 251:2005–10. doi: 10.1001/jama.251.15.2005
49. Ghanem Kgreview. Neurosyphilis: a historical perspective and review. *CNS Neurosci Ther.* (2010) 16:e157–68. doi: 10.1111/j.1755-5949.2010.00183.x

50. Buitrago-Garcia D, Martí-Carvajal AJ, Jimenez A, Conterno LO, Pardo R. Antibiotic therapy for adults with neurosyphilis. *Cochrane Database Syst Rev.* (2019) 5:CD011399. doi: 10.1002/14651858.CD011399.pub2
51. Bettuzzi T, Jourdes A, Robineau O, Alcaraz I, Manda V, Molina JM, et al. Ceftriaxone compared with benzylpenicillin in the treatment of neurosyphilis in France: a retrospective multicentre study. *Lancet Infect Dis.* (2021) 21:1441–7. doi: 10.1016/S1473-3099(20)30857-4
52. Wu KQ, Zhang SF, Bao CH, Zou X, Gu X, Wang CN, et al. Circulating microRNAs as potential biomarkers in the diagnosis of neurosyphilis. *Int J Dermatol Venereol.* (2021) 4:16–25. doi: 10.1097/JD9.0000000000000127
53. Park IU, Tran A, Pereira L, Fakile Y. Sensitivity and specificity of treponemal-specific tests for the diagnosis of syphilis. *Clin Infect Dis.* (2020) 71:S13–20. doi: 10.1093/cid/ciaa349
54. Dumaresq J, Langevin S, Gagnon S, Serhir B, Deligne B, Tremblay C, et al. Clinical prediction and diagnosis of neurosyphilis in HIV-infected patients with early Syphilis. *J Clin Microbiol.* (2013) 51:4060–6. doi: 10.1128/JCM.01989-13
55. Lu Y, Ke W, Yang L, Wang Z, Lv P, Gu J, et al. Clinical prediction and diagnosis of neurosyphilis in HIV-negative patients: a case-control study. *BMC Infect Dis.* (2019) 19:1017. doi: 10.1186/s12879-019-4582-2
56. Centers for Disease Control and Prevention. Sexually transmitted diseases treatment guidelines. (2018). Available online at: https://www.cdc.gov/std/syphilis/default.htm
57. Janier M, Unemo M, Dupin N, Tiplica GS, Potočnik M, Patel R. 2020 European guideline on the management of syphilis. *J Eur Acad Dermatol Venereol.* (2021) 35:574–88. doi: 10.1111/jdv.16946
58. Shang XJ, He CF, Tang B, Chang XL Ci C, Sang H. Neuroimaging features, follow-up analyses, and comparisons between asymptomatic and symptomatic neurosyphilis. *Dermatol Ther (Heidelb).* (2020) 10:273–83. doi: 10.1007/s13555-020-00361-3
59. Zhang XQ, Zhao GM, Su HT. Clinical and imaging findings of neurosyphilis: a case report. *Chin Med J Metallurgical Ind.* (2009) 26:496.
60. Jiang MJ, Zhang HJ Li WR, Wu WQ, Huang YM, Xu DM, et al. Analysis of EEG Lemple-Ziv complexity and correlative aspects before and after treatment of anti-syphilis therapy for neurosyphilis. *Neurol Res.* (2019) 41:199–203. doi: 10.1080/01616412.2018.1520438
61. Liu H, Zhao ZB, You NX. Diversity in clinical manifestations and imaging features of neurosyphilis: obstacles to the diagnosis and treatment (report of three cases). *Int J Neurosci.* (2018) 128:785–90. doi: 10.1080/00207454.2017.1412963
62. Hobbs E, Vera JH, Marks M, Barritt AW, Ridha BH, Lawrence D. Neurosyphilis in patients with HIV. *Pract Neurol.* (2018) 18:211–8. doi: 10.1136/practneurol-2017-001754

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

frontiers | Frontiers in Public Health

TYPE Original Research
PUBLISHED 26 July 2022
DOI 10.3389/fpubh.2022.908591

OPEN ACCESS

EDITED BY
Lei Zhang,
Melbourne Sexual Health Center (MSHC), Australia

REVIEWED BY
Lorenzo Giacani,
University of Washington, United States
Muhammad Morshed,
University of British Columbia, Canada

*CORRESPONDENCE
John AJ Prakash
johnajpp@gmail.com

SPECIALTY SECTION
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Public Health

RECEIVED 30 March 2022
ACCEPTED 30 June 2022
PUBLISHED 26 July 2022

CITATION
Solaimalai D, Gupta A, George L, Manesh A, Karthik R, Sathishkumar D, Peter CVD, Varghese GM, Pulimood SA, Kannangai R and Prakash JA (2022) Upward trends of syphilis in the non-pregnant adults: A six-year report on clinical and epidemiological profile of syphilis from a tertiary care center, India. *Front. Public Health* 10:908591. doi: 10.3389/fpubh.2022.908591

Upward trends of syphilis in the non-pregnant adults: A six-year report on clinical and epidemiological profile of syphilis from a tertiary care center, India

Dhanalakshmi Solaimalai[1], Ankan Gupta[2], Leni George[2], Abi Manesh[3], Rajiv Karthik[3], Dharshini Sathishkumar[2], C. V Dincy Peter[2], George M Varghese[3], Susanne A Pulimood[2], Rajesh Kannangai[4] and John AJ Prakash[1]*

[1]Department of Clinical Microbiology, Christian Medical College, Vellore, India, [2]Department of Dermatology, Christian Medical College, Vellore, India, [3]Department of Infectious Diseases, Christian Medical College, Vellore, India, [4]Department of Clinical Virology, Christian Medical College, Vellore, India

Since 2000, a resurgence of syphilis has been noted in many developed and developing countries, especially among men who have sex with men (MSM). Incidence and prevalence of syphilis in pregnant women have been reduced drastically by mandatory screening in early pregnancy. Insufficient data in other populations especially from developing countries limit targeted public health interventions. This study aimed to describe the clinical and epidemiological profile of serologically confirmed syphilis cases among the non-pregnant high-risk group reporting to a tertiary care center in Southern India. A retrospective study was carried out in a tertiary care center in Southern India for 6 years from 2015 to 2020. A total of 265 serologically confirmed syphilis patients were included. A statistically significant increase in positivity from 0.52 to 2.1% was observed in this study (2015 to 2020). Among risk factors, high-risk behavior with multiple heterosexual partners was the commonest (51.3%), followed by marital partners who tested positive (9.4%) and MSM (7.5%). The majority of the patients were diagnosed at the latent stage (79%), followed by secondary syphilis (10%) and tertiary syphilis (8%). A quarter of patients (23%) were coinfected with HIV. Serological non-responsiveness was more common among HIV infected (47 vs. 24%). Sixteen had neurosyphilis and six had ocular involvement. HIV co-infection complicated 50% (8/16) of neurosyphilis patients. Syphilis is still prevalent, especially in high-risk groups including those are attending STI clinics. Further prospective multicentric studies are needed to identify and implement public health measures.

KEYWORDS

syphilis, STI, MSM, HIV, neurosyphilis, seroprevalence

Introduction

Syphilis is one of the four curable and preventable sexually transmitted infections (STI) apart from chlamydia, trichomoniasis, and gonorrhea. The incidence and prevalence of syphilis had decreased after the introduction of the HIV prevention program. However, the increased prevalence has been noted in many developing countries and western countries, especially among specific subgroups since 2000 (1). The recent systematic analysis showed a very high pooled global prevalence of 7.5% (2000 to 2020) among men who have sex with men (MSM) compared to 0.5% among men in the general population estimated in 2016 (2, 3). The Chinese notifiable infectious diseases surveillance registry reported a three-fold increase in syphilis cases over 10 years (from 135,210 in 2005 to 441,818 in 2014) (4).

The WHO periodically releases the global estimates of four curable STI, providing evidence for policymakers to monitor, evaluate and improve STI prevention programmes. According to that, more than one million new curable STI are acquired every day worldwide and 7.1 million new cases of syphilis were estimated in 2020 (5). The WHO released a global health sector strategy for 2016 to 2021, with a goal of a 90% reduction in syphilis incidence worldwide and 50 or fewer cases of congenital syphilis per 100,000 live births in 80% of countries (6). This prioritized eliminating congenital syphilis by implementing mandatory screening and treating syphilis among pregnant women.

The National AIDS Control Organization (NACO) in India launched a national strategy for the elimination of parent-to-child transmission of syphilis in February 2015 based on the global initiative by WHO in 2007. To eliminate parent-to-child transmission of syphilis and HIV by 2020, the Government of India has taken a policy of universal screening of pregnant women for syphilis and HIV during the first visit (1st trimester) as part of the essential antenatal care package. Testing is done at all levels of healthcare facilities such as medical colleges, district hospitals, primary health centers and subcentres at free of cost (7, 8). At a tertiary care center in north Tamil Nadu, a reduced seroprevalence rate among pregnant women from 0.4% (1998-99) to <0.1% (2011-15) was observed due to an effective intervention after mandatory screening in the early pregnancy (9, 10). However, there is no clear epidemiological data to support specific subgroup screening from India (11). Therefore, this study aimed to describe clinical and epidemiological profiles of serologically confirmed syphilis cases among the non-pregnant high-risk group reporting to a tertiary care center in Southern India.

Materials and Methods

We conducted a retrospective study in our 2600-bed tertiary care center spanning 6 years (from January 2015 to December 2020). The Clinical Microbiology laboratory is a large volume laboratory (ISO 15189: 2012 accredited) that receives approximately 20,000 samples per year for syphilis serology from the following groups of individuals: antenatal women, neonates of suspected congenital syphilis, PLHIV patients before starting cART, patients with suspicion of syphilis based on skin or genital rash and patients seeking STI diagnosis and treatment.

For this study, we included adult non-pregnant patients (>18 years) with serologically confirmed syphilis which is defined as Venereal Disease Research Laboratory (VDRL) reactive and *Treponema pallidum* haemagglutination (TPHA) positive or TPHA alone positive. The VDRL assay was performed using the VDRL antigen (Institute of Serology, Calcutta, India) as described previously (9). The TPHA assay (Omega Diagnostics, Scotland, UK) was performed and interpreted according to the manufacturer's instructions. In our center, the traditional algorithm is followed for the screening of syphilis. First, the patient is screened with a VDRL test. If VDRL is reactive then confirmed with TPHA (specific test). However, both the tests were performed simultaneously for the patients who came to our center for confirmation of syphilis (treated outside).

Pregnant women and children (<17 years) were excluded from the study. The following clinical and epidemiological data for each patient were recorded from the patients' electronic medical records: risk factors, clinical presentation, stage of the disease, treatment, follow-up details, other associated STIs, and cerebrospinal fluid (CSF) parameters for neurosyphilis cases.

The stage of the disease at diagnosis was assigned by the treating clinicians as per the standard criteria (12). Neurosyphilis cases were diagnosed by the combination of serological tests for syphilis plus CSF analysis of elevated cells $>5/mm^3$ with lymphocytic predominance with or without elevated protein (>45 mg/dL). The symptomatic patients were classified into early (meningitis, meningo-vascular, neuro-ocular, ocular) and late (general paralysis of insane) neurosyphilis based on the clinical presentation, CSF parameters, eye examination and MRI brain by Infectious Disease Physicians. Asymptomatic cases were diagnosed based on CSF analysis that was performed for pre-ART/ co-infection work-up among HIV-infected patients (13).

Serological response to the treatment was defined as a four-fold reduction in VDRL titer between the initial titer and subsequent testing. The time interval of retesting is at 6, 12 and 24 months on those reporting for follow-up. A fourfold reduction in titer at any time during the 24 months of follow-up is considered as definitive evidence of cure (12). The old infection was not counted if the patient came for follow-up.

Data were summarized using mean and standard deviation (SD) for continuous variables and frequency along with percentage for categorical variables. All categorical associations were tested using chi-square statistics. The analysis was done using Microsoft Excel and a p-value < 0.05 was considered statistically significant.

10.3389/fpubh.2022.908591

Our Institutional Review Board and the ethical committee approved the study (IRB Min No 13417, dated 23.9.2020).

Results

During the study period, a total of 1,12,689 samples were tested for syphilis serology. Among these 86,691 (77%) samples were collected from antenatal mothers and 25,998 (23%) from other patients. Totally 265 non-pregnant patients satisfied the inclusion criteria and were analyzed. The demographic details of the study patients are described in Table 1. Male preponderance was noted (218/265, 82.3%). The year-wise analysis of mean age was performed and observed that slight decrease in mean age from 38.5 in 2015 to 35 in 2020 (Table 1).

The confirmed cases increased gradually from 2015 to 2019. We observed a sudden increase in the number of cases in the year 2019. There was a decline in 2020 due to the COVID 19 pandemic and a smaller number of samples tested during that period. However, the positivity increased from 1.86% in 2019 to 2.1% in 2020. The pooled percentage of patients who presented with genital ulcers or other genital symptoms prior to 2020 (2015 to 2019) was 18% (41/228) compared to13.5% (5/37) in 2020. This reflects the true increase in positivity, though samples tested were less during the COVID 19 pandemic.

The samples tested during the study period (2015 to 2020) were 5,169, 5,291, 5,628, 4,390, 3,756, and 1,764, respectively. The year-wise distribution of confirmed cases and seroprevalence is shown in Figure 1. A statistically significant increase in positivity from 0.52 to 2.1% was observed in this study ($p = 0.0015$).

Amongst the risk factors, a history of multiple sex partners (heterosexual) was the most common risk factor (51.3%), followed by marital partners who tested positive (9.4%) and MSM (7.5%) (Table 1).

TABLE 1 The demographic details.

Parameters	**Observation**	
Male/female ratio	**4.63 (218/47)**	
Married/ unmarried	**3.5 (206/59)**	
Mean age (SD)	**36 (±10.2)**	
Annual mean age and sex ratio	**Mean age**	**Male/female ratio**
2015	38.5	2.4 (19/8)
2016	39	5.4 (27/5)
2017	36.4	4.6 (37/8)
2018	35	2.9 (40/14)
2019	34.5	10.7 (64/6)
2020	35	5.2 (31/6)
Age-wise distribution and sex ratio	***n* (%)**	**Male/female ratio**
17 to 24	29 (11%)	2.6 (21/8)
25 to 34	109 (41%)	5.4 (92/17)
35 to 44	74 (28%)	5.2 (62/12)
45 to 54	37 (14%)	4.3 (30/7)
55 to 64	12 (4.5%)	5 (10/2)
>65	4 (1.5%)	3 (3/1)
Year-wise positivity	**Number of samples tested**	**Positivity *n*(%)**
2015	5169	27 (0.52%)
2016	5291	32 (0.6%)
2017	5628	45 (0.8%)
2018	4390	54 (1.2%)
2019	3756	70 (1.86%)
2020	1764	37 (2.1%)
Risk factors	***n* (%)**	
Multiple sex partners	136 (51.3%)	
Without HIV	84 (61.8%)	
PLHIV	52 (38.2%)	
MSM	20 (7.5%)	
Without HIV	11 (55%)	
PLHIV	9 (45%)	
Partner tested positive	25 (9.4%)	
Denied of any HRB	48 (18%)	
Data not available	36 (13.6%)	
Associated STIs and other infections	***n* (%)**	
HIV	61 (23%)	
Herpes simplex virus	6 (2.3%)	
Hepatitis B virus	6 (2.3%)	
Hepatitis C virus	2 (0.8%)	
Neisseria gonorrhea	1 (0.4%)	

PLHIV, People living with human immunodeficiency virus; MSM, Men have sex with men; HRB, high risk behavior.

Stage of the Disease

Among 265 patients, 54 were partially treated elsewhere before presentation in our center. These 54 patients were excluded for further analysis due to insufficient data regarding the stage of disease at diagnosis and treatment history. The stage of the disease at the time of diagnosis is illustrated (Figure 2) for 211 remaining patients. The majority of the patients were diagnosed at the latent stage (166, 79%), followed by secondary syphilis (22, 10%) and tertiary syphilis (17, 8%).

We looked at the various clinical settings where latent syphilis was diagnosed (Figure 3). The latent syphilis cases were diagnosed most commonly during an active screening among those presenting with symptoms suggestive of STI with or without a high-risk behavior ($n = 60$) and PLHIV ($n = 45$). Passive screening among blood donors, organ donors and medical evaluation (for a foreign work visa) identified 32 patients (Figure 3).

During the study period, a total of 17 patients were diagnosed with tertiary syphilis. Sixteen patients were diagnosed

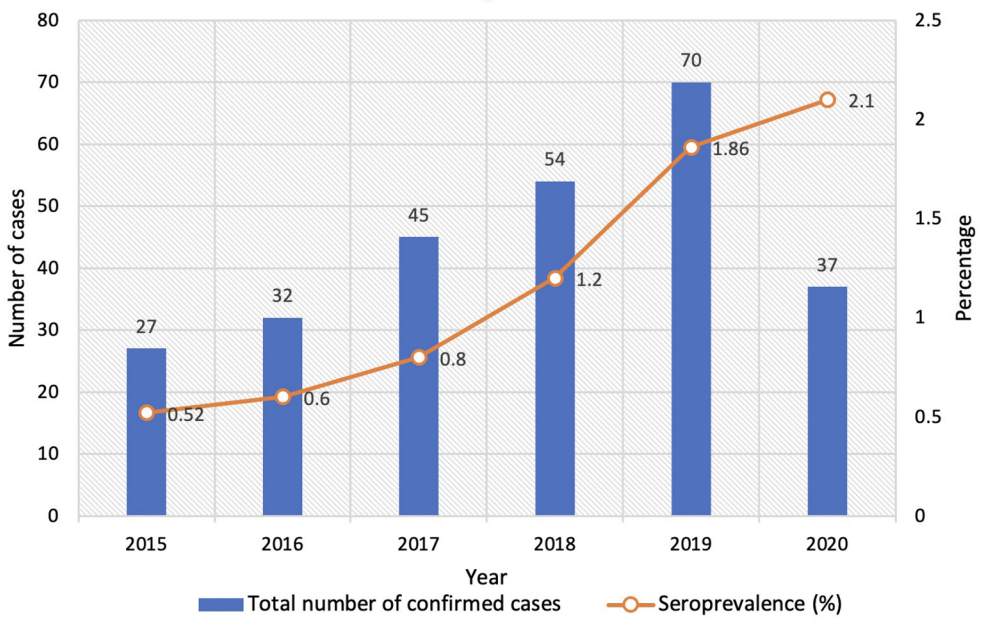


FIGURE 1
Year-wise distribution of cases and seroprevalence.

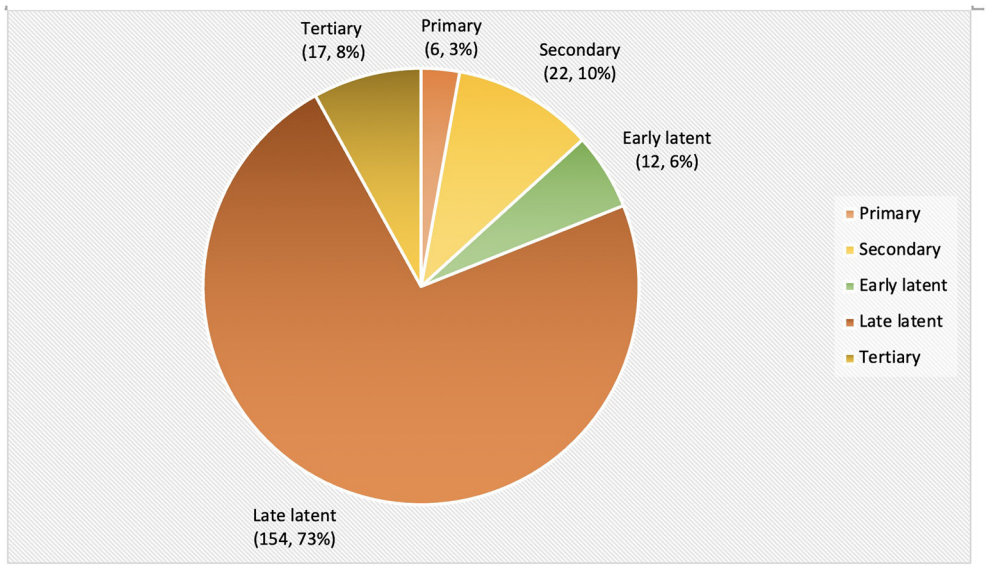


FIGURE 2
The stage of syphilis at diagnosis.

with neurosyphilis and one with gummatous syphilis. Neuro-ocular syphilis ($n = 6$) was the most common, followed by asymptomatic neurosyphilis and meningeal ($n = 4$ each), and general paresis of insane ($n = 2$). CSF analysis showed a characteristic pleocytosis with lymphocyte predominance and elevated protein among all early neurosyphilis cases. However, CSF analysis was normal in late neurosyphilis cases (general paresis of insane) (Table 2). HIV co-infection was identified in 50% (8/16) of patients. All except two patients were treated either with intravenous aqueous crystalline penicillin G or Inj. Ceftriaxone for 2 weeks. Nine out of 16 patients had at least one follow-up visit with us after treatment and five among them showed serological response i.e., either a four-fold reduction in titer or non-reactive serum VDRL.

10.3389/fpubh.2022.908591

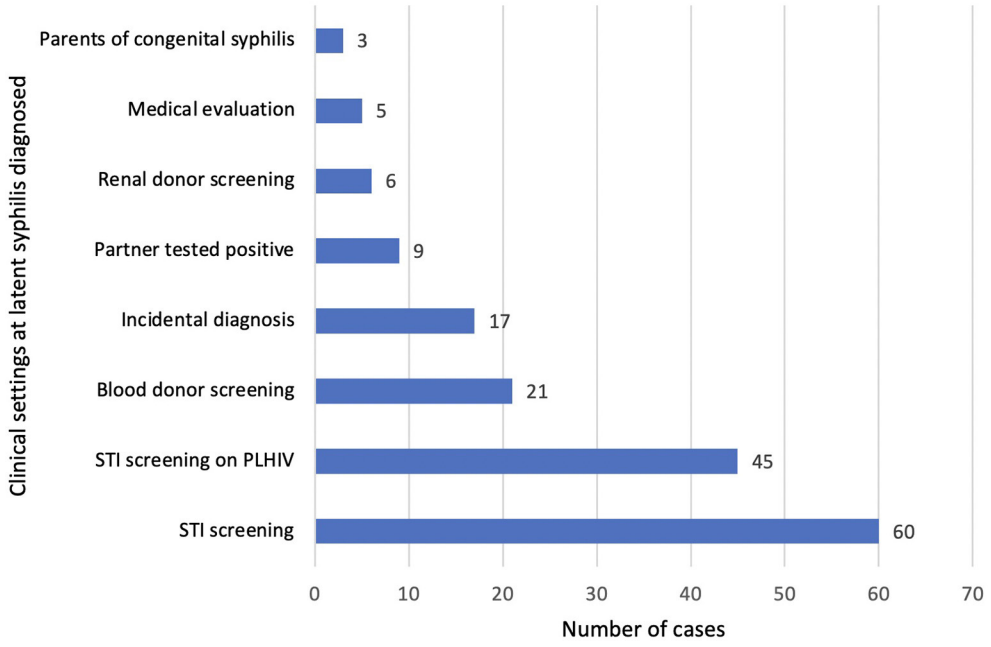


FIGURE 3
Diagnosis of latent syphilis.

TABLE 2 Serum and CSF parameters of neurosyphilis patients.

	Early neurosyphilis				Late neurosyphilis
Total cases ($n = 16$)	**Meningeal/ meningo-vascular neurosyphilis ($n = 4$)**	**Asymptomatic neuro syphilis ($n = 4$)**	**Neuro-ocular syphilis ($n = 4$)**	**Ocular syphilis ($n = 2$)**	**general paresis of insane ($n = 2$)**
Mean age	37	39	39	37	52
Gender male *n*(%)	2 (50)	3 (75)	4 (100)	0	2 (100)
HIV positive *n*(%)	3 (75)	4 (100)	1 (25)	0	0
Serum TPHA positive - *n*(%)	4 (100)	4 (100)	4 (100)	2(100)	2 (100)
Serum VDRL reactive - *n*(%)	4 (100)	4 (100)	3 (75)	0	1 (50)
CSF VDRL/TPHA reactive - *n*(%)	3 (75)	3 (75)	2* (67)	0	1 (50)
CSF WBC >5 cell/mm^3	3 (75)	3 (75)	3* (100)	0	0
CSF Protein >45 mg/dL -*n* (%)	3 (75)	4 (100)	3* (100)	0	2 (100)

*CSF analysis not done in one patient.

Treatment and follow-up

In our hospital, 164 (77.7%) out of 211 diagnosed patients were treated appropriately for the stage of the disease with benzathine penicillin and 47 (22.3%) patients lost follow-up after diagnosis.

With the available follow-up data among 52 patients, 7 (13.5%), 16 (30.8%), 10 (19.2%), 6 (11.5%) and 13 (25%) patients had a follow-up visit till 3 months, 6 months, 12 months, 18 months and $\geq$ 24 months, respectively. Re-infection or second infection was considered in a patient who was treated adequately and had the same or rise in VDRL titer in a follow-up visit. Those patients were treated again and not counted as a new infection in the study. Moreover, very few patients ($n = 2$) were identified as re-infection in this study.

TABLE 3 Serological response to the treatment.

	HIV positive	HIV negative	*p*-value
Total number of patients (*n* = 52)	15	37	0.21 (Chi-square statistic = 1.56)
Four-fold reduction of titer observed *n* (%)	8 (53.3%)	28 (75.7%)	

p-value <0.5 is significant.

Serological non-response to the treatment was higher among HIV co-infected patients (46.7%) compared to HIV-negative patients (24.3%). However, this is statistically not significant (Table 3).

Discussion

Syphilis is not uncommon in developing countries despite mandatory testing in pregnant women. There is a paucity of data from other groups (1). We observed a steady increase in syphilis positivity over 6 years in our study group (0.52 to 2.1%). A previous Indian study reported a comparable increase from 0.95 to 1.76% in 6 years (14), while another HIV care clinic-based study demonstrated an increase from 0.7 to 1.3% (15). Given the public health perspective, this finding mandates the need for constant surveillance among high-risk groups.

Amongst the risk factors, a history of multiple sex partners (heterosexual) was the most common risk factor (51.3%), followed by marital partners who tested positive (9.4%) and MSM (7.5%). In western countries, MSM is the most common risk factor (16). Interestingly, evidence of high-risk sexual behavior was unavailable for approximately one-third of our patients. This may be due to the healthcare provider's concern about the patient's objection to the sensitive question regarding sexual behaviors (17). The Centers for Disease Control and Prevention (CDC) and US Preventive Services Task Force (USPSTF) recommend screening of syphilis in asymptomatic non-pregnant adults and adolescents who are at risk. The CDC also recommends more frequent screening at 3 to 6 months intervals in sexually active MSM and at least annually in people with HIV (18, 19).

Early diagnosis and treatment is a must to prevent the transmission and progression of the disease. Our study observed that nearly one-fourth of the diagnosed patients were lost to follow-up from our center. This has implications like disease progression and transmission of disease to the sexual partners. We also observed two untreated latent syphilis patients who progressed to neurosyphilis. This indicates the importance of follow-up and completion of treatment at any stage of the disease (20).

Syphilis and HIV co-infection is viewed as a potentially dangerous combination since both diseases negatively impact treatment response to each other. Previous studies observed serological non-response to syphilis treatment, cognitive impairment, and virological failure of ART among syphilis and HIV co-infected patients (21–23). Our study observed that nearly one-fourth of the study population had co-infection with HIV. The co-infection rates are varied in the literature, ranging from 6.4 to 34% (24–26). Serological non-responsiveness was identified in 47% of HIV patients compared to 24% in non-HIV patients among the available follow-up details in 52 patients, although the finding was not statistically significant.

Neurosyphilis was diagnosed in 13% (8/61) of HIV-co-infected patients. In concordance with other studies, ocular manifestations were the most common presentation among neurosyphilis patients (14, 27). Neuro-ocular syphilis was commonly identified in HIV-negative patients (5/6), similar to a study by Borges et.al (27).

We observed that most of the patients (166/211, 79%) were diagnosed at the latent stage of the disease. This is comparable to a study from China that identified 51.9% of latent syphilis, 9% of primary syphilis, and 19.8% of secondary syphilis for a period of 9 years from 2011 to 2019 (28). In contrast, other studies have demonstrated primary and secondary syphilis as the common presentation among STI clinic attendees (29, 30).

This study has limitations. First, as data was collected retrospectively from the patients' electronic medical records, some subjects had incomplete data for some of the variables. Second, though the patients were from wide geographic areas, the results obtained are from a single tertiary care center. Third, the follow-up of patients after diagnosis and treatment was limited since the hometown of many patients was far from our center. However, this study provides information that syphilis is still prevalent and suggests that there is a rise in syphilis cases amongst those at risk. Further, this is not a random sampling as those with risk factors and reporting to a tertiary care center were tested for syphilis (targeted sampling) and therefore our reports are subject to bias.

Still, the data generated from this study provides information regarding the age group affected and the nature of transmission predominantly among heterosexuals. This will help us make a policy regarding the possible preventive measures to be taken.

In summary, we report in a large hospital-based study, a sustained increase in cases of syphilis among non-pregnant patients over a six-year period. We also show that the drivers are very different in the Indian context - transmission predominantly happens via multiple heterosexual contacts and the contribution of MSM remains low. We also highlight a considerable proportion of our patients are HIV coinfected - highlighting the close association and need for complementary efforts to prevent syphilis in our population. Appropriate

management of syphilis according to the stage will reduce morbidity and further transmission.

Conclusion

This hospital-based study suggests that syphilis is not uncommon. Heterosexual transmission is the commonest risk factor and the PLHIV group is also significantly affected. To adequately control syphilis, first, awareness needs to be increased among the general public about the disease especially its association with HIV. Second, the screening and follow-up of high-risk patients need to be intensified. Healthcare providers must elicit the sexual history to identify the high-risk patients for the screening (17, 31). Moreover, the rapid point of care tests for syphilis will improve the identification of high-risk patients (31). Third, all the diagnosed patients at any stage should be treated appropriately to prevent the transmission and progression of the disease. Healthcare setup should have a system to identify the missing patients for treatment and follow-up.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Review Board and Ethical Committee, Christian Medical College and Hospital, Vellore, India. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

DSo, JP, AG, LG, AM, RKar, DSa, CP, GV, SP, and RKan contributed to the concept and design of the study. DSo, JP, AG, LG, AM, RKar, DSa, and CP contributed in data acquisition. DSo drafted the manuscript. DSo and JP involved in analysis. JP, GV, SP, and RKan involved in interpretation. JP contributed in supervision. GV, SP, and RKan involved in data analysis. JP, AG, LG, AM, RKar, DSa, CP, GV, SP, and RKan contributed in critically reviewing the manuscript. All authors contributed to the article and approved the submitted version.

Acknowledgments

Authors would like to acknowledge the Institutional review board for approval of the study.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Kojima N, Klausner JD. An update on the global epidemiology of syphilis. *Curr Epidemiol Rep.* (2018) 5:24–38. doi: 10.1007/s40471-018-0138-z

2. Tsuboi M, Evans J, Davies EP, Rowley J, Korenromp EL, Clayton T, et al. Prevalence of syphilis among men who have sex with men: a global systematic review and meta-analysis from 2000–20. *Lancet Glob Health.* (2021) 9:e1110–8. doi: 10.1016/S2214-109X(21)00221-7

3. Rowley J, Vander Hoorn S, Korenromp E, Low N, Unemo M, Abu-Raddad LJ, et al. Chlamydia, gonorrhoea, trichomoniasis and syphilis: global prevalence and incidence estimates, 2016. *Bull World Health Organ.* (2019) 97:548-562P. doi: 10.2471/BLT.18.228486

4. Zhang X, Hou F, Li X, Zhou L, Liu Y, Zhang T. Study of surveillance data for class B notifiable disease in China from 2005 to 2014. *Int J Infect Dis.* (2016) 48:7–13. doi: 10.1016/j.ijid.2016.04.010

5. Sexually transmitted infections (STIs) Available online at: https://www.who.int/news-room/fact-sheets/detail/sexually-transmitted-infections-(stis) [cited 2022 Mar 1].

6. WHO-RHR-16.09-eng.pdf. Available online at: http://apps.who.int/iris/bitstream/handle/10665/246296/WHO-RHR-16.09-eng.pdf;jsessionid=8780B688B66F418EF067D8E6A7254210?sequence=1 [cited 2022 Mar 1].

7. Srinivas V, Turlapati PL, Bhola AK, Singh AK, Rajan S, Gupta RS, et al. Towards elimination of parent-to-child transmission of syphilis in India: a rapid situation review to inform national strategy. WHO South-East Asia. *J Public Health.* (2015) 4:197. doi: 10.4103/2224-3151.206690

8. Ministry of Health and family Welfare Government of India. *Standard operating procedures for HIV & Syphilis. Screening of pregnant women at VHSND Sites.*

9. Mathai E, Mathai M, Prakash JA, Bergström S. Audit of management of pregnant women with positive VDRL tests. *Natl Med J India.* (2001) 14:202–4.

10. Ebenezer ED, Benjamin SJ, Sahni RD, Prakash JAJ, Chelliah H, Mathews JE, et al. retrospective study of the prevalence and outcomes of syphilis in pregnancy in a 5-year period. *Int J Gynaecol Obstet Off Organ Int Fed Gynaecol Obstet.* (2018) 140:42–6. doi: 10.1002/ijgo.12336

11. Seale A, Broutet N, Narasimhan M. Assessing process, content, and politics in developing the global health sector strategy on sexually transmitted infections 2016–2021: implementation opportunities for policymakers. *PLoS Med.* (2017) 14:e1002330. doi: 10.1371/journal.pmed.1002330

12. Ghanem KG, Ram S, Rice PA. The modern epidemic of syphilis. *Campion EW, editor N Engl J Med.* (2020) 382:845–54. doi: 10.1056/NEJMra1901593

13. Ha T, Tadi P, Dubensky L. *Neurosyphilis*. In: StatPearls. Treasure Island (FL): StatPearls Publishing (2022) [cited 2022 Mar 9]. Available online at: http://www.ncbi.nlm.nih.gov/books/NBK540979/

14. Sethi S, Mewara A, Hallur V, Prasad A, Sharma K, Raj A. Rising trends of syphilis in a tertiary care center in North India. *Indian J Sex Transm Dis AIDS.* (2015) 36:140. doi: 10.4103/0253-7184.167137

15. Kulkarni V, Parchure R, Darak S. Let's not let the guard down! – Early indications of syphilis resurgence? *Indian J Dermatol Venereol Leprol.* (2019) 85:246. doi: 10.4103/ijdvl.IJDVL_728_17

16. Syphilis Statistics - STD information from CDC (2021) [cited 2022 Mar 30]. Available online at: https://www.cdc.gov/std/syphilis/stats.htm.

17. Clement ME, Hicks CB. Syphilis on the rise: what went wrong? *JAMA*. (2016) 315:2281. doi: 10.1001/jama.2016.7073

18. *STI Screening Recommendations*. (2021). Available online at: https://www.cdc.gov/std/treatment-guidelines/screening-recommendations.htm [cited 2022 Mar 13].

19. US Preventive Services Task Force (USPSTF), Bibbins-Domingo K, Grossman DC, Curry SJ, Davidson KW, Epling JW, et al. Screening for syphilis infection in non-pregnant adults and adolescents: US preventive services task force recommendation statement. *JAMA*. (2016) 315:2321–7. doi: 10.1001/jama.2016.5824

20. Ghanem KG, Erbelding EJ, Wiener ZS, Rompalo AM. Serological response to syphilis treatment in HIV-positive and HIV-negative patients attending sexually transmitted diseases clinics. *Sex Transm Infect*. (2007) 83:97–101. doi: 10.1136/sti.2006.021402

21. Marra CM, Deutsch R, Collier AC, Morgello S, Letendre S, Clifford D, et al. Neurocognitive impairment in HIV-infected individuals with previous syphilis. *Int J STD AIDS.* (2013) 24:351–5. doi: 10.1177/0956462412472827

22. Fan L, Yu A, Zhang D, Wang Z, Ma P. Consequences of HIV/Syphilis Co-Infection on HIV Viral Load and Immune Response to Antiretroviral Therapy. *Infect Drug Resist.* (2021) 14:2851–62. doi: 10.2147/IDR.S320648

23. Neto PLF, Fonseca RR de S, Avelino ME de S, Vilhena EM, Barbosa M dos A de AP, Lopes CAF, et al. Prevalence and factors associated with syphilis in people living with HIV/AIDS in the State of Pará, Northern Brazil. *Front Public Health*. (2021) 9:646663. doi: 10.3389/fpubh.2021.646663

24. Riley LT, Johnson KL, Stewart J, Byers P. Syphilis and HIV Co-infection in Mississippi: implications for control and prevention. *AIDS Behav*. (2020) 24:1064–8. doi: 10.1007/s10461-019-02562-0

25. Dai W, Luo Z, Xu R, Zhao G, Tu D, Yang L, et al. Prevalence of HIV and syphilis co-infection and associated factors among non-commercial men who have sex with men attending a sexually transmitted disease clinic in Shenzhen, China. *BMC Infect Dis.* (2017) 17:86. doi: 10.1186/s12879-017-2187-1

26. Eticha BT, Sisay Z, Alemayehu A, Shimelis T. Seroprevalence of syphilis among HIV-infected individuals in Addis Ababa, Ethiopia: a hospital-based cross-sectional study. *BMJ Open.* (2013) 3:e002566. doi: 10.1136/bmjopen-2013-002566

27. Borges CR, Almeida SM de, Sue K, Koslyk JLA, Sato MT, Shiokawa N, et al. Neurosyphilis and ocular syphilis clinical and cerebrospinal fluid characteristics: a case series. *Arq Neuropsiquiatr*. (2018) 76:373–80. doi: 10.1590/0004-282x20180054

28. Luo Z, Ding Y, Yuan J, Wu Q, Tian L, Zhang L, et al. Predictors of seronegative conversion after centralized management of syphilis patients in Shenzhen, China. *Front Public Health.* (2021) 9:755037. doi: 10.3389/fpubh.2021.755037

29. Jain A, Mendiratta V, Chander R. Current status of acquired syphilis: a hospital-based 5-year study. *Indian J Sex Transm Dis AIDS.* (2012) 33:32. doi: 10.4103/0253-7184.93814

30. Arando M, Caballero E, Curran A, Armengol P, Barberá MJ, Vall-Mayans M. The epidemiological and clinical characteristics of the epidemic of syphilis in Barcelona. *Actas Dermo-Sifiliográficas Engl Ed.* (2019) 110:841–9. doi: 10.1016/j.adengl.2019.03.027

31. Schmidt R, Carson PJ, Jansen RJ. Resurgence of Syphilis in the United States: an assessment of contributing factors. *Infect Dis Res Treat.* (2019) 12:117863371988328. doi: 10.1177/1178633719883282

frontiers | Frontiers in Immunology

TYPE Review
PUBLISHED 28 July 2022
DOI 10.3389/fimmu.2022.952284

OPEN ACCESS

EDITED BY
Justin David Radolf,
UCONN Health, United States

REVIEWED BY
Nikhat Parveen,
The State University of New Jersey, United States
David Šmajs,
Masaryk University, Czechia

*CORRESPONDENCE
Noah Kojima
nkojima@ucla.edu

SPECIALTY SECTION
This article was submitted to Microbial Immunology, a section of the journal Frontiers in Immunology

RECEIVED 24 May 2022
ACCEPTED 30 June 2022
PUBLISHED 28 July 2022

CITATION
Kojima N, Konda KA and Klausner JD (2022) Notes on syphilis vaccine development. *Front. Immunol.* 13:952284. doi: 10.3389/fimmu.2022.952284

Notes on syphilis vaccine development

Noah Kojima[1]*, Kelika A. Konda[1,2] and Jeffrey D. Klausner[3]

[1]Department of Medicine, University of California Los Angeles, Los Angeles, CA, United States, [2]Centro de Investigación Interdisciplinaria en Sexualidad Sida y Sociedad, Universidad Peruana Cayetano Heredia, Lima, Peru, [3]Departments of Medicine and Population and Public Health Sciences, Keck School of Medicine of the University of Southern California, Los Angeles, CA, United States

The quest for a syphilis vaccine to provide protection from infection or disease began not long after the isolation of the first *Treponema pallidum* subspecies *pallidum* (*T. pallidum*) strain in 1912. Yet, a practical and effective vaccine formulation continues to elude scientists. Over the last few years, however, efforts toward developing a syphilis vaccine have increased thanks to an improved understanding of the repertoire of *T. pallidum* outer membrane proteins (OMPs), which are the most likely syphilis vaccine candidates. More has been also learned about the molecular mechanisms behind pathogen persistence and immune evasion. Published vaccine formulations based on a subset of the pathogen's OMPs have conferred only partial protection upon challenge of immunized laboratory animals, primarily rabbits. Nonetheless, those experiments have improved our approach to the choice of immunization regimens, adjuvants, and vaccine target selection, although significant knowledge gaps remain. Herein, we provide a brief overview on current technologies and approaches employed in syphilis vaccinology, and possible future directions to develop a vaccine that could be pivotal to future syphilis control and elimination initiatives.

KEYWORDS

syphilis, *treponema pallidum*, vaccine, vaccines, *treponema pallidum* antibodies

Introduction

Syphilis, caused by the spirochete *Treponema pallidum* subspecies *pallidum* (*T. pallidum*), remains a significant global and public health problem. The disease is still endemic in low- and middle-income countries and resurgent in high-income ones. In the United States, the number of syphilis cases in 2020 was the highest since 2000 (1). Furthermore, congenital syphilis transmission is the most common infection associated with fetal loss or stillbirth in low-income settings, with an estimated 1.4 million pregnant women infected every year globally, resulting in an estimated 305,000 prenatal or perinatal deaths, and 215,000 infants born prematurely and/or with clinical signs of syphilis (2, 3). Past public health initiatives to eliminate syphilis and congenital

syphilis promoted by the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO), have significantly reduced syphilis incidence, but have not achieved their intended elimination goals. Thus, new tools are needed to aid the currently available diagnostic tests and therapeutic options.

The only successful study of syphilis vaccination was reported by Dr. James Miller in 1973 (4). In his study, Miller used an extensive immunization regimen, injecting rabbits intravenously 60 times over 37 weeks with γ-irradiated *T. pallidum* cells. Immunization was followed by intradermal homologous challenge with the same *T. pallidum* strain. Immunized rabbits displayed complete protection that persisted for at least one year, as shown by the lack of development of chancres at the challenge sites and the absence of clinical/serological evidence of syphilis.

T. pallidum proteomic array to study humoral immunity to syphilis

Evidence that repeated exposure to *T. pallidum* is conductive to partially protective immunity was also provided by human inoculation experiments conducted by Magnuson *et al.* in the 1950's (5), which showed that a previous syphilitic infection of sufficiently long duration would produce significant protection against subsequent challenge. Others have found that in patients with repeated episodes of syphilis, reinfections led to less severe skin manifestations than in patients at their first syphilis episode (6). The evidence that previous syphilis attenuates the clinical manifestations associated with reinfection has been recently reiterated by Marra et al. (7) The study of how the immunological response to *T. pallidum* antigens differs in patients diagnosed with active syphilis but with and without a history of the disease could provide a basis for vaccine development. By increasing the understanding and describing the differences in immune responses between those two groups, researchers hope to identify unique aspects of the adaptive humoral response to inform vaccine development. If this protective immunity could be replicated through vaccination, the result could be a vaccine that leads to attenuation of early symptoms and, hence, decreased infectivity and transmission of *T. pallidum* (8). Therefore, a vaccine that can only induce partial protection, but can prevent early symptoms, and possibly pathogen dissemination could result in reduced syphilis spread and be of substantial public health benefit for populations at increased risk of syphilis.

Among the newest tools to study syphilis immunology is a *T. pallidum* proteomic array developed by Antigen Discovery Inc. (ADI) in collaboration with the Giacani laboratory (9). That array is conceptually not dissimilar from that described by Brinkman et al. in the early 2000's based on the Nichols strain genome. However, the ADI array covers 99% of the proteome of two strains of *T. pallidum* (Nichols and SS14) and is amenable to high throughput analyses with mere microliters of serum. The array is currently being evaluated with longitudinal serum samples from long-term infected rabbits, and longitudinal serum samples from rabbits that were infected, treated with benzathine penicillin G (BPG) weeks after infection, and then re-infected after residual antibiotic was cleared. Furthermore, the array is being used to evaluate reactivity to ~150 clinical serum samples provided by our group and collected in Peru. Comparison of the differential reactivity to *T. pallidum* antigens (with emphasis on putative vaccine candidates) in serum from patients with and without a history of syphilis at diagnosis will help pinpoint protective antigens to be tested in pre-clinical vaccination/challenge experiments. Additionally, the ADI system for cell-free synthesis of *T. pallidum* antigens could be adapted to the investigation of *T. pallidum* antigens inducing a robust cellular response during infection. Those antigens could be added to a vaccine formulation to promote T cell activation and IFN-γ production to activate macrophages.

The usefulness of an instrument like the ADI proteomic array becomes far more remarkable when a vast array of well-characterized clinical samples is available. The institution of centralized biospecimen repositories with serum and lesion swab specimens would greatly benefit research efforts to understand syphilis immunology and genetic diversity in this pathogen.

Genetic diversity and vaccine development

The publication of the Nichols strain genome sequence in 1998 by Fraser et al. (10), opened the gateway to reverse syphilis vaccinology. The conserved region of the variable antigen TprK (encoded by the *tp0897* gene) was one of the first vaccine candidates identified with that approach and it is still included in several modern experimental vaccine formulations (11, 12). Some preliminary work has been conducted to improve expression of surface-exposed integral outer membrane proteins like TprK and Tp0435 genes on engineered non-infectious *Borrelia burgdorferi* strains (13). Partial protection was observed in rabbit models that underwent immunization with *B. burgdorferi* that expressed TprK, while those that were immunized with Tp0435 did not have the same protection. Analysis of the humoral response to TprK antigen suggested there was reactivity to conformational epitopes to the antigen.

Dr. Caroline Cameron pioneered the application of genome-wide-bioinformatics analyses to predict *T. pallidum* open reading frames (ORFs) encoding putative outer membrane proteins (OMPs) to identify adhesins that would interact with receptors among the host extracellular membrane components

(14). The result of that study was the identification of the Tp0751 adhesin. Lithgow et al. showed that Tp0751-immunized animals had a significantly reduced *T. pallidum* organ burden upon infectious challenge compared with unimmunized animals (15). Although not unanimously accepted by the research community (16), Tp0751 remains a current vaccine candidate worthy of further exploration. Additional *in silico* OMP-mining work further contributed to defining the putative repertoire of *T. pallidum* surface-exposed integral membrane proteins (17), which today consists of 27 proteins —the current best candidates for syphilis vaccine development, which are made up of components of the BAM complex, Lpt complex, 9-stranded beta-barrels, FadL-like protein, components of the efflux systems, and *T. pallidum* repeat proteins (18).

As complete and partial genomes from historical and modern isolates accumulated over the last two decades, it became increasingly clear that substantial genomic diversity was concentrated within the genes of *T. pallidum* encoding for surface-exposed proteins (19–21). This evidence has profound implications for developing a broadly protective vaccine and emphasizes the necessity to obtain high-quality, near-complete genomes that can be used to assess OMP variability. The sequence of several *T. pallidum* OMP-encoding genes has been notoriously challenging to elucidate through whole genome sequencing due to the presence of repetitive sequences. Several ongoing initiatives are addressing the necessity to obtain more *T. pallidum* genomes to refine vaccine development. Upon completing those efforts, the vaccine research community will benefit from a vast array of genomes, primarily obtained directly from patient samples and from many diverse geographical areas spanning all continents, including areas where syphilis is endemic.

Modern approaches that combine *T. pallidum* DNA enrichment with pathogen-specific probes (19, 22), specific genome amplification before high-throughput sequencing and technologies capable of sequencing Kb-long DNA molecules will ensure the availability of complete high-quality genomes for comparative genomics analyses (23–28). Deposition of reads and assembled genomes in public data repositories will enable more researchers to participate in vaccine development. A syphilis vaccine will likely need to be tailored to the genetic pedigree of strains circulating locally. On the upside, *T. pallidum* modern strains continue to share over 99% of genomic identity if we exclude the hypervariable gene *tprK*, which undergoes intra-strain gene conversion to foster *T. pallidum* persistence (29–31). Evidence supports two major clades of this pathogen circulating worldwide, the SS14-like and Nichols-like clade (28). The recent strain sequencing work by Lieberman *et al.* with *T. pallidum* isolates from Peru, Ireland, USA, Papua New Guinea, Madagascar, Italy, Japan, and China were included in the 196 near-complete genomes sequenced from eight countries and six continents (19).

Omics and other approaches to the rescue

As the genomics gap is being closed at unprecedented speed for *T. pallidum*, the application of other "-omics," mainly transcriptomics and proteomics, will provide complementary information to help identify vaccine candidates (32, 33). Only limited work to date has focused on analyzing the *T. pallidum* transcriptome and proteome (32–38). Yet those studies are pivotal to understand the timing and level of expression of potential immunogens. In addition to being poorly expressed, selected OMP-encoding genes have been reported to undergo stochastic modulation of gene expression through phase variation, which might contribute to changing the pathogen surface antigenic profile (39, 40). To date, a microarray study describing transcriptome of Nichols strain was conducted and found that the RNA transcript of *T. pallidum* profiles between *in vitro* culture and rabbit infection were similar (33). A better understanding of gene regulation and gene expression could lead to exclusion of specific vaccine candidates whose transcription might be turned off with no detriment to the pathogen.

To date, transcriptional profiles of *T. pallidum* have been obtained from rabbit or *in vitro*-propagated strains (33). Although those studies are valuable in improving our understanding of gene expression in *T. pallidum*, an equally important endeavor would be to assess gene expression in spirochetes from patient samples. Transcriptomics of clinical samples can be challenging: the small amount of clinical material obtained from clinical samples often precludes sequencing of the entire genome. Producing high quality whole genome sequencing data needs advanced molecular techniques for selected whole genome amplifications and bait enrichment of libraries to enable gathering meaningful data from clinical samples. At the same time, determination of levels of paralogous genes will be also very challenging as many tpr genes share identical sequences and some undergo recombinations.

An analysis of the *T. pallidum* transcriptome using bacterial cells present in lesions from individuals diagnosed with early syphilis, without strain propagation in rabbits, could also help better understand the immunology of natural infection. That work would inform whether specific gene expression patterns correlate to the development of the immune response and disease manifestations during early syphilis and, more generally, which vaccine candidates are expressed during different stages of the infection.

Successful genetic engineering of *T. pallidum* was reported in 2021 (41). Genetic manipulation of *T. pallidum* has the potential to pinpoint vaccine candidates. Efforts have been made to ablate the *tprK* ORF with no avail, which led to the hypothesis that *TprK* is an essential *T. pallidum* gene. That hypothesis is also supported by the evidence that extensive *tprK* sequencing never yielded a variant carrying an early termination due to a premature stop codon or a frameshift mutation, despite the

extensive recombination events that involve this hypervariable gene. A vaccine design based on an OMP necessary for pathogen viability could be preferred to a design based on a non-essential gene. Current experiments to assess the "essential" OMP repertoire are ongoing in the Giacani laboratory through genetic engineering. New molecular tools for *T. pallidum*, such as transposon-mediated insertional mutagenesis, GFP-expressing *T. pallidum* cells, and a *T. pallidum* strain expressing constitutively spell out Cas9, are also being evaluated to accelerate the discovery of genes that, albeit not essential, might be necessary for *T. pallidum* virulence.

Protein structure and vaccine development strategies

Immunization with recombinant treponemal proteins would greatly benefit from increased knowledge of the native structure of the candidate immunogens. However, no conclusive experimental data exist on the structure of *T. pallidum* OMPs. For about half of these molecules, the level of homology with other bacterial proteins has been sufficient to obtain high-confidence models using a battery of mainstream computational and bioinformatic tools (18, 42). On the contrary, there is an ongoing debate concerning the structure of Tpr antigens because the structure that is inferred from functional assays differs from that hypothesized based on structural data from protein fragments. Refining the structural models for all *T. pallidum* OMPs is therefore pivotal for vaccine development. High-confidence models will allow the excision of surface-exposed epitopes to be mounted on a carrier that is easier to produce than a recombinant OMP, contains fewer amino acid sequences that are not instrumental to developing a protective response, but maintain the structural characteristics of the native epitopes to allow the development of antibodies to conformational epitopes. Carriers such as viral-like particles, small beta-barrel antigens, liposomes, and outer membrane vesicles are all options worth trying.

Adjuvants

Which adjuvant to use in a syphilis vaccine is also an issue that requires additional experimentation. Currently as vaccine research focuses on rabbit models, experimentation is conducted using ribi, titermax, or SAS, none of which are approved for human use. Experimentation has made clear that an adjuvant necessary to induce a Th1 response that will lead to INF-γ activated macrophages is crucial to an effective vaccine (43–46). Any vaccine formulation that includes adjuvants not suitable for humans will eventually have to be retested with adjuvants approved for human use. The reliance on a rabbit model for vaccine discovery leads to inevitable but necessary gaps in the potential for vaccine formulations to advance to human trials.

Vaccine efficacy and target populations

To provide a favorable risk to benefit ratio, vaccines need to be safe for users and effective in preventing disease. For other vaccinations, a threshold of 50% efficacy rate had been determined to be adequate by large governing bodies (47). Given the need for syphilis vaccines in a global setting, heat-stable vaccines would greatly benefit distribution.

Furthermore, syphilis epidemiology is different in high-income versus low- and middle-income countries. In high-income countries, syphilis predominantly affects men who have sex with men (MSM), while in low- and middle-income countries, where the disease is endemic, syphilis impacts the general population. In implementing a syphilis vaccine, especially one that is only partly effective, it would be sensible to have different distribution strategies between high-income and low- and middle-income countries. In high-income countries, immunization should target those at increased risk for syphilis such as MSM and sex workers. In low- and middle-income countries, vaccination of the general population with a focus on protecting those of reproductive age to decrease risk of congenital syphilis would be recommended. With recent increases in congenital syphilis in the United States, vaccination of women of reproductive age may also be worthwhile.

Cost analysis/Mathematical modeling

A mathematical model was created for a hypothetical syphilis vaccine assuming an efficacy of 80%. That study focused on vaccination in Africa, using different estimates of the prevalence of HIV infection in the general population (1.5%, 10%, and 15%). Syphilis vaccination reduced syphilis incidence for all the studied scenarios. However, focusing solely on young women or only on high-risk populations, was not as impactful on syphilis prevalence over time as mass vaccination (48). Additional work is needed to understand better how vaccines with differing efficacy and/or the reduction of clinical symptoms of syphilis can reduce transmission.

Concluding remarks

Despite the low costs associated with syphilis testing and treatment, syphilis control has remained elusive.

The years of life lost due to congenital syphilis are substantial (49). A vaccine able to reduce syphilis incidence, especially congenital syphilis, could significantly improve public health and lower the estimated 3.6 million disability-adjusted life years that are currently lost annually due to this serious infection.

Author contributions

NK, KK, JK: writing and editing. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by US National Institutes of Health though the National Institutes of Health/ National Institute of Allergy and Infectious Diseases, grant number 5R01AI139265.

Acknowledgments

The authors are grateful to Dr. Lorenzo Giacani (University of Washington) for his suggestions to improve this manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Sexually transmitted disease surveillance 2020. (2022).

2. Newman L, Kamb M, Hawkes S, Gomez G, Say L, Seuc A, et al. Global estimates of syphilis in pregnancy and associated adverse outcomes: analysis of multinational antenatal surveillance data. *PloS Med* (2013) 10(2):e1001396. doi: 10.1371/journal.pmed.1001396

3. Cerqueira LRP, Monteiro DLM, Taquette SR, Rodrigues NCP, Trajano AJB, Souza FM, et al. The magnitude of syphilis: from prevalence to vertical transmission. *Rev Inst Med Trop Sao Paulo* (2017) 59:e78. doi: 10.1590/S1678-9946201759078

4. Miller JN. Immunity in experimental syphilis. VI. successful vaccination of rabbits with treponema pallidum, nichols strain, attenuated by -irradiation. *J Immunol* (1973) 110(5):1206–15.

5. Magnuson HJ, Thomas EW, Olansky S, Kaplan BI, De Mello L, Cutler JC. Inoculation syphilis in human volunteers. *Med (Baltimore)* (1956) 35(1):33–82. doi: 10.1097/00005792-195602000-00002

6. Courjon J, Hubiche T, Dupin N, Grange PA, Del Giudice P. Clinical aspects of syphilis reinfection in HIV-infected patients. *Dermat* (2015) 230(4):302–7. doi: 10.1159/000369617

7. Marra CM, Maxwell CL, Sahi SK, Tantalo LC, Dunaway SB, Lukehart SA. Previous syphilis alters the course of subsequent episodes of syphilis. *Clin Infect Dis Mar 1* (2022) 74(4):e1–5. doi: 10.1093/cid/ciab287

8. Cameron CE. Syphilis vaccine development: Requirements, challenges, and opportunities. *Sex Transm Dis Sep* (2018) 45(9S Suppl 1):S17–9. doi: 10.1097/OLQ.0000000000000831

9. Li L, Bannantine JP, Campo JJ, Randall A, Grohn YT, Schilling MA, et al. Identification of sero-diagnostic antigens for the early diagnosis of johne's disease using MAP protein microarrays. *Sci Rep Nov 26* (2019) 9(1):17573. doi: 10.1038/s41598-019-53973-x

10. Fraser CM, Norris SJ, Weinstock GM, White O, Sutton GG, Dodson R, et al. Complete genome sequence of treponema pallidum, the syphilis spirochete. *Sci* (1998) 281(5375):375–88. doi: 10.1126/science.281.5375.375

11. Centurion-Lara A, Castro C, Barrett L, Cameron C, Mostowfi M, Van Voorhis WC, et al. Treponema pallidum major sheath protein homologue tpr K is a target of opsonic antibody and the protective immune response. *J Exp Med* (1999) 189(4):647–56. doi: 10.1084/jem.189.4.647

12. Morgan CA, Lukehart SA, Van Voorhis WC. Immunization with the n-terminal portion of treponema pallidum repeat protein K attenuates syphilitic lesion development in the rabbit model. *Infect Immun* (2002) 70(12):6811–6. doi: 10.1128/IAI.70.12.6811-6816.2002

13. Parveen N, Fernandez MC, Haynes AM, Zhang RL, Godornes BC, Centurion-Lara A, et al. Non-pathogenic borrelia burgdorferi expressing treponema pallidum TprK and Tp0435 antigens as a novel approach to evaluate syphilis vaccine candidates. *Vaccine* (2019) 37(13):1807–18. doi: 10.1016/j.vaccine.2019.02.022

14. Cameron CE. Identification of a treponema pallidum laminin-binding protein. *Infect Immun* (2003) 71(5):2525–33. doi: 10.1128/IAI.71.5.2525-2533.2003

15. Lithgow KV, Hof R, Wetherell C, Phillips D, Houston S, Cameron CE. A defined syphilis vaccine candidate inhibits dissemination of treponema pallidum subspecies pallidum. *Nat Commun* (2017) 8:14273. doi: 10.1038/ncomms14273

16. Luthra A, Montezuma-Rusca JM, La Vake CJ, LeDoyt M, Delgado KN, Davenport TC, et al. Evidence that immunization with TP0751, a bipartite treponema pallidum lipoprotein with an intrinsically disordered region and lipocalin fold, fails to protect in the rabbit model of experimental syphilis. *PloS Pathog* (2020) 16(9):e1008871. doi: 10.1371/journal.ppat.1008871

17. Cox DL, Luthra A, Dunham-Ems S, Desrosiers DC, Salazar JC, Caimano MJ, et al. Surface immunolabeling and consensus computational framework to identify candidate rare outer membrane proteins of treponema pallidum. *Infect Immun Dec* (2010) 78(12):5178–94. doi: 10.1128/IAI.00834-10

18. Hawley KL, Montezuma-Rusca JM, Delgado KN, Singh N, Uversky VN, Caimano MJ, et al. Structural modeling of the treponema pallidum outer membrane protein repertoire: a road map for deconvolution of syphilis pathogenesis and development of a syphilis vaccine. *J Bacteriol* (2021) 203(15): e0008221. doi: 10.1128/JB.00082-21

19. Lieberman NAP, Lin MJ, Xie H, Shrestha L, Nguyen T, Huang ML, et al. Treponema pallidum genome sequencing from six continents reveals variability in vaccine candidate genes and dominance of nichols clade strains in Madagascar. *PloS Negl Trop Dis Dec* (2021) 15(12):e0010063. doi: 10.1371/journal.pntd.0010063

20. Pla-Diaz M, Sanchez-Buso L, Giacani L, Smajs D, Bosshard PP, Bagheri HC, et al. Evolutionary processes in the emergence and recent spread of the syphilis agent, treponema pallidum. *Mol Biol Evol* (2022) 39(1). doi: 10.1093/molbev/msab318

21. Kumar S, Caimano MJ, Anand A, Dey A, Hawley KL, LeDoyt ME, et al. Sequence variation of rare outer membrane protein beta-barrel domains in clinical strains provides insights into the evolution of treponema pallidum subsp. pallidum, the syphilis spirochete. *mBio* (2018) 9(3). doi: 10.1128/mBio.01006-18

22. Beale MA, Marks M , Cole MJ, Lee MK, Pitt R, Ruis C, et al. Global phylogeny of treponema pallidum lineages reveals recent expansion and spread of contemporary syphilis. *Nat Microbiol* (2021) 6(12):1549–60. doi: 10.1038/s41564-021-01000-z

23. Thurlow CM, Joseph SJ, Ganova-Raeva L, Katz SS, Pereira L, Chen C, et al. Selective whole-genome amplification as a tool to enrich specimens with low treponema pallidum genomic DNA copies for whole-genome sequencing. *mSphere* (2022) 2:e0000922. doi: 10.1128/msphere.00009-22

24. Arora N, Schuenemann VJ, Jager G, Peltzer A, Seitz A, Herbig A, et al. Origin of modern syphilis and emergence of a pandemic treponema pallidum cluster. *Nat Microbiol* (2016) 2:16245. doi: 10.1038/nmicrobiol.2016.245

25. Sun J, Meng Z, Wu K, Liu B, Zhang S, Liu Y, et al. Tracing the origin of treponema pallidum in China using next-generation sequencing. *Oncotarget* (2016) 7(28):42904–18. doi: 10.18632/oncotarget.10154

26. Beale MA, Marks M, Sahi SK, Tantalo LC, Nori AV, French P, et al. Genomic epidemiology of syphilis reveals independent emergence of macrolide resistance across multiple circulating lineages. *Nat Commun* (2019) 10(1):3255. doi: 10.1038/s41467-019-11216-7

27. Pinto M, Borges V, Antelo M, Pinheiro M, Nunes A, Azevedo J, et al. Genome-scale analysis of the non-cultivable treponema pallidum reveals extensive within-patient genetic variation. *Nat Microbiol* (2016) 2:16190. doi: 10.1038/nmicrobiol.2016.190

28. Grillova L, Oppelt J, Mikalova L, Novakova M, Giacani L, Niesnerova A, et al. Directly sequenced genomes of contemporary strains of syphilis reveal recombination-driven diversity in genes encoding predicted surface-exposed antigens. *Front Microbiol* (2019) 10:1691. doi: 10.3389/fmicb.2019.01691

29. Addetia A, Lin MJ, Phung Q, Xie H, Huang ML, Ciccarese G, et al. Estimation of full-length TprK diversity in treponema pallidum subsp. pallidum. *mBio* (2020) 11(5). doi: 10.1128/mBio.02726-20

30. Lin MJ, Haynes AM, Addetia A, Lieberman NAP, Phung Q, Xie H, et al. Longitudinal TprK profiling of *in vivo* and *in vitro*-propagated treponema pallidum subsp. pallidum reveals accumulation of antigenic variants in absence of immune pressure. *PloS Negl Trop Dis Sep* (2021) 15(9):e0009753. doi: 10.1371/journal.pntd.0009753

31. Giacani L, Molini BJ, Kim EY, Godornes BC, Leader BT, Tantalo LC, et al. Antigenic variation in treponema pallidum: TprK sequence diversity accumulates in response to immune pressure during experimental syphilis. *J Immunol* (2010) 184(7):3822–9. doi: 10.4049/jimmunol.0902788

32. Smajs D, McKevitt M, Howell JK, Norris SJ, Cai WW, Palzkill T, et al. Transcriptome of treponema pallidum: gene expression profile during experimental rabbit infection. *J Bacteriol* (2005) 187(5):1866–74. doi: 10.1128/JB.187.5.1866-1874.2005

33. De Lay BD, Cameron TA, De Lay NR, Norris SJ, Edmondson DG. Comparison of transcriptional profiles of treponema pallidum during experimental infection of rabbits and *in vitro* culture: Highly similar, yet different. *PloS Pathog* (2021) 17(9):e1009949. doi: 10.1371/journal.ppat.1009949

34. Veith PD, Glew MD, Gorasia DG, Chen D, O'Brien-Simpson NM, Reynolds EC. Localization of outer membrane proteins in treponema denticola by quantitative proteome analyses of outer membrane vesicles and cellular fractions. *J Proteome Res* (2019) 18(4):1567–81. doi: 10.1021/acs.jproteome.8b00860

35. Houston S, Lithgow KV, Osbak KK, Kenyon CR, Cameron CE. Functional insights from proteome-wide structural modeling of treponema pallidum subspecies pallidum, the causative agent of syphilis. *BMC Struct Biol* (2018) 18(1):7. doi: 10.1186/s12900-018-0086-3

36. McGill MA, Edmondson DG, Carroll JA, Cook RG, Orkiszewski RS, Norris SJ. Characterization and serologic analysis of the treponema pallidum proteome. *Infect Immun* (2010) 78(6):2631–43. doi: 10.1128/IAI.00173-10

37. Osbak KK, Van Raemdonck GA, Dom M, Cameron CE, Meehan CJ, Deforce D, et al. Candidate treponema pallidum biomarkers uncovered in urine from individuals with syphilis using mass spectrometry. *Future Microbiol* (2018) 13:1497–510. doi: 10.2217/fmb-2018-0182

38. Osbak KK, Houston S, Lithgow KV, Meehan CJ, Strouhal M, Smajs D, et al. Characterizing the syphilis-causing treponema pallidum ssp. pallidum proteome using complementary mass spectrometry. *PloS Negl Trop Dis* (2016) 10(9):e0004988. doi: 10.1371/journal.pntd.0004988

39. Giacani L, Brandt SL, Ke W, Reid TB, Molini BJ, Iverson-Cabral S, et al. Transcription of TP0126, treponema pallidum putative OmpW homolog, is regulated by the length of a homopolymeric guanosine repeat. *Infect Immun* (2015) 83(6):2275–89. doi: 10.1128/IAI.00360-15

40. Giacani L, Lukehart S, Centurion-Lara A. Length of guanosine homopolymeric repeats modulates promoter activity of subfamily II tpr genes of treponema pallidum ssp. pallidum. *FEMS Immunol Med Microbiol* (2007) 51(2):289–301. doi: 10.1111/j.1574-695X.2007.00303.x

41. Romeis E, Tantalo L, Lieberman N, Phung Q, Greninger A, Giacani L. Genetic engineering of treponema pallidum subsp. pallidum, the syphilis spirochete. *PloS Pathog* (2021) 17(7):e1009612. doi: 10.1371/journal.ppat.1009612

42. Molini B, Fernandez MC, Godornes C, Vorobieva A, Lukehart SA, Giacani L. B-cell epitope mapping of TprC and TprD variants of treponema pallidum subspecies informs vaccine development for human treponematoses. *Front Immunol* (2022) 13:862491. doi: 10.3389/fimmu.2022.862491

43. Hawley KL, Cruz AR, Benjamin SJ, La Vake CJ, Cervantes JL, LeDoyt M, et al. IFNgamma enhances CD64-potentiated phagocytosis of treponema pallidum opsonized with human syphilitic serum by human macrophages. *Front Immunol* (2017) 8:1227. doi: 10.3389/fimmu.2017.01227

44. Van Voorhis WC, Barrett LK, Koelle DM, Nasio JM, Plummer FA, Lukehart SA. Primary and secondary syphilis lesions contain mRNA for Th1 cytokines. *J Infect diseases* (1996) 173(2):491–5. doi: 10.1093/infdis/173.2.491

45. Arroll TW, Centurion-Lara A, Lukehart SA, Van Voorhis WC. T-Cell responses to treponema pallidum subsp. pallidum antigens during the course of experimental syphilis infection. *Infect Immun* (1999) 67(9):4757–63. doi: 10.1128/IAI.67.9.4757-4763.1999

46. Leader BT, Godornes C, VanVoorhis WC, Lukehart SA. CD4+ lymphocytes and gamma interferon predominate in local immune responses in early experimental syphilis. *Infect Immun* (2007) 75(6):3021–6. doi: 10.1128/IAI.01973-06

47. Kaplan RM, Milstein A. Influence of a COVID-19 vaccine's effectiveness and safety profile on vaccination acceptance. *Proc Natl Acad Sci U S A* (2021) 118(10). doi: 10.1073/pnas.2021726118

48. Champredon D, Cameron CE, Smieja M, Dushoff J. Epidemiological impact of a syphilis vaccine: a simulation study. *Epidemiol Infect* (2016) 144(15):3244–52. doi: 10.1017/S0950268816001643

49. GBD 2015 Mortality and Causes of Death Collaborators. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the global burden of disease study 2015. *Lancet* (2016) 388(10053):1459–544. doi: 10.1016/S0140-6736(16)31012-1

frontiers | Frontiers in Medicine

TYPE Brief Research Report
PUBLISHED 01 August 2022
DOI 10.3389/fmed.2022.941041

OPEN ACCESS

EDITED BY
Pingyu Zhou,
Tongji University, China

REVIEWED BY
Mei Shi,
Shanghai Dermatology Hospital, China
Ligang Yang,
Southern Medical University, China

*CORRESPONDENCE
Ei T. Aung
eaung@mshc.org.au
Eric P. F. Chow
eric.chow@monash.edu

SPECIALTY SECTION
This article was submitted to Infectious Diseases - Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

RECEIVED 11 May 2022
ACCEPTED 11 July 2022
PUBLISHED 01 August 2022

CITATION
Aung ET, Fairley CK, Ong JJ, Phillips TP, Tran J, Chen MY, Maddaford K and Chow EPF (2022) Adherence to weekly anal self-examination among men who have sex with men for detection of anal syphilis. *Front. Med.* 9:941041. doi: 10.3389/fmed.2022.941041

Adherence to weekly anal self-examination among men who have sex with men for detection of anal syphilis

Ei T. Aung[1,2]*, Christopher K. Fairley[1,2], Jason J. Ong[1,2], Tiffany P. Phillips[1,2], Julien Tran[1,2], Marcus Y. Chen[1,2], Kate Maddaford[1] and Eric P. F. Chow[1,2,3]*

[1]Melbourne Sexual Health Centre, Alfred Health, Melbourne, VIC, Australia, [2]Central Clinical School, Faculty of Medicine, Nursing and Health Sciences, Monash University, Melbourne, VIC, Australia, [3]Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, The University of Melbourne, Melbourne, VIC, Australia

Background: Men who have sex with men (MSM) practicing exclusively receptive anal sex are more likely to present with secondary than primary syphilis, implying primary anorectal lesions may be missed. If men could detect anorectal lesions by regular anal self-examination, the duration of infectiousness could be reduced. This study aimed to examine adherence to weekly anal self-examination.

Method: We conducted a longitudinal feasibility study examining the adherence to weekly anal self-examinations among MSM attending a sexual health clinic in Melbourne, Australia between December 2020 and June 2021. Adherence to weekly anal self-examinations over 12 weeks was assessed from a logbook and 4-weekly surveys. Participants who identified abnormalities in their anus were recommended to seek medical review.

Results: Of the 30 men who completed the study, anal self-examination was performed at least weekly for 308 of 360 person-weeks (86% of the weeks, 95% CI: 82–89). The mean adherence was 3.6 (95% CI: 3.3–3.9) examinations per 4-weeks per person in Weeks 1–4, 3.5 (95% CI: 3.1–3.8) in Weeks 5–8 and 3.3 (95% CI: 2.9–3.7) in Weeks 9–12 ($P_{trend} = 0.06$). Six men (20%, 6/30) were seen for medical review after they identified abnormalities, whilst eight men (27%, 8/30) reported abnormalities, but did not seek medical review. No participants were diagnosed with syphilis during the study period.

Conclusion: We conclude that men adhered well to weekly anal self-examination. Therefore, it is feasible to trial this as a routine practice among MSM. Future studies should investigate possible reductions in adherence over time and ways to increase medical review for abnormalities that men find.

KEYWORDS

anal self-examination, syphilis, men who have sex with men (MSM), adherence, anal syphilis, weekly exam, feasibility

Introduction

High and rising rates of syphilis among gay, bisexual, and other men who have sex with men (MSM) are occurring despite substantial public health interventions and strategies to improve syphilis control (1–4). These public health interventions include widespread testing, contact tracing, contact treatment and behavioral interventions such as promoting condoms. The limited success in syphilis control with the existing public health interventions and strategies highlights the need for additional interventions.

Regular syphilis screening is one option for potentially improving syphilis control. The current guidelines for screening of human immunodeficiency virus (HIV) and sexually transmitted infections (STIs) in Australia recommend 3 monthly screening (including syphilis serology) for sexually-active MSM and also for MSM taking HIV pre-exposure prophylaxis (PrEP) (5). An Australian study found a substantial proportion of infectious syphilis cases (58% of primary syphilis and 44% of secondary syphilis) were diagnosed between the 3-monthly routine clinic visits among MSM taking PrEP (6). The findings indicate that even 3-monthly screening is insufficient to detect all cases and a substantial number of cases occur between these visits. Therefore, additional measures for the early detection of syphilis are warranted.

One strategy to shorten the duration of infectious of syphilis cases has been to improve the patients' recognition of syphilis symptoms and encourage early presentation to health care. However, recent research has indicated some primary syphilis lesions may be in anatomical positions that make their detection difficult. A study examining the sexual position and staging of syphilis reported that MSM who practiced exclusively receptive penile-anal sex were four times more likely to present with secondary syphilis than those who practiced exclusively insertive penile-anal sex, suggesting primary anorectal lesions were often missed leading to secondary syphilis (7). If there was a way of detecting the missed ano-rectal primary syphilis lesions, then progression to secondary syphilis would be prevented. Preventing secondary syphilis is important not only because there is substantial shedding of *T. pallidum* in this stage (8) but also because secondary syphilis is associated with systemic illnesses and complications such as ocular syphilis and neurosyphilis. We hypothesized that if men examine their anus regularly (e.g., once a week), men might be able to detect primary anorectal lesions and therefore seek medical care and receive timely diagnosis and treatment before progressing to secondary syphilis, thereby reducing infectiousness and further transmission.

Regular anal self-examination is a new concept for detecting primary syphilis but has been investigated among MSM living with HIV to detect anal cancer at an early stage (9). These studies have shown that anal self-examination is acceptable for the detection of anal cancer (9, 10). Furthermore, qualitative, and quantitative studies have shown that MSM are willing to perform anal self-examinations for detecting anal syphilis (11, 12). Before we examine the effectiveness of anal self-examination for early anal syphilis detection, we first need to examine whether weekly anal self-examination is feasible as a regular practice among MSM.

We designed this feasibility study to explore adherence to weekly anal self-examination. The primary aim of this study was to investigate the adherence to weekly anal self-examination, and the secondary aim was to examine the proportion of men returning for medical review when they detect abnormality in the anus during anal self-examination.

Materials and methods

Anal self-examination

Anal self-examination in our study was defined as inserting a finger into one's anus, feeling around the anal canal (360°), and using a mirror to check the anus and surrounding area for any abnormalities.

Study population and recruitment

This was a cohort study conducted at the Melbourne Sexual Health Center (MSHC) between 1st December 2020 and 17th June 2021. The last participant was recruited on 10th March 2021 and the data collection of the last participant was on 17th June 2021. MSHC is a public sexual health clinic in Victoria, Australia, which provided approximately 50,000 consultations in 2019. To be eligible in this study, men must be aged 18 years or above, identified as cis-male who had had receptive penile-anal sex with another man in the past 12 months and planned to reside in Victoria, Australia in the next 3 months. Men who only had insertive penile-anal sex or men who had been performing weekly anal self-examination were not eligible. We aimed to recruit 30 MSM including ten men living with HIV, ten men taking PrEP, and ten men not living with HIV and not taking PrEP.

Eligible men were identified by clinicians and were referred to the research team. One of the research team members (EA, KM, ER) met with the potential participant on the same day or arranged another appointment if they were unavailable on the day. A member of the research team obtained written informed consent from participants before the start of the study. We sent the study website link *via* SMS to the participants on the day of enrolment. We explained the process of anal self-examination using anal self-examination instruction (Supplementary Figure 1) and provided the instruction sheet to the participants. The study website contained information about

anal self-examination, an animated video about the instruction on how to perform anal self-examination, contact details of the research team if the participant found any abnormalities during anal self-examination, a downloadable logbook and anal self-examination instructions.

Study protocol

Baseline

At recruitment, demographics (e.g., age, gender), sexual practices (e.g., sexual orientation and position of anal sex), and medical history (e.g., HIV status, PrEP use, past history of syphilis and previous experience of anal self-examination) were collected using a self-administered questionnaire. Participants were asked to perform weekly anal self-examination over 12 weeks and record their activity on a logbook (electronic or paper-based) (Supplementary Table 1).

Follow-up

Participants were asked to complete another three surveys at Weeks 4, 8, and 12. The follow-up surveys collected data on adherence to anal self-examination, and abnormal findings and problems encountered during anal self-examination. The Week 12 survey also collected the willingness to perform anal self-examination in the future. In order to differentiate anal self-examination from At the end of Week 12, the participants were also asked to return the logbook and complete the last survey. Participants were given an AU$50 (∼US$22) gift card for remuneration at the end of the study.

Adverse event or abnormal findings

Participants were advised to contact the research team *via* email or phone if they identified any abnormal findings in their anus that concerned them. Once the participant contacted the research team, an appointment was arranged at a time suitable for the participant to see a study doctor, which was usually about within 1–3 days. The study doctor examined the participant's anus to review the abnormal findings and the participant also had a serological test for syphilis on the day. A polymerase chain reaction (PCR) test for *Treponema pallidum* was also performed if any anal lesions were present. If the participants could not attend MSHC for review, the participants then opted to have a review with a general practitioner, and they would inform the research team of the review and diagnosis.

Syphilis diagnoses

Syphilis test results from the participants over the 12-week study period were also extracted from the electronic medical records at the Melbourne Sexual Health Center. In addition, we collected syphilis test results data 12 weeks after they completed the final Week 12 visit.

Outcomes

The primary outcome was self-reported adherence to anal self-examination (i.e., whether men performed weekly anal self-examination) over a 12-week period. The secondary outcomes were (1) adverse events where the participants identified abnormal findings during anal self-examination or (2) diagnosed with syphilis during the study period. The adverse events were defined as any abnormal findings from anal self-examination. There were two categories of adverse events: (1) participants were concerned about abnormal findings and requested a medical review, and (2) participants were not concerned about abnormal findings and did not request a medical review. In the group of men who requested a medical review due to abnormal findings, the events were reported to the research team *via* email or phone contact, or the events were noted from the medical records at MSHC when they returned for review. If the participants sought medical review with their general practitioners instead, the participants would inform the research team of the outcomes. In the group of men who had abnormal findings but did not request a medical review, the events were noted from the surveys and followed up by email or phone contact with the participants.

Ethics approval

Ethical approval was obtained from the Alfred Hospital Ethics committee, Melbourne, Australia (Project 603/20).

Sample size

We designed this study to provide reasonably precise 95% confidence intervals (CI) around the primary aim of adherence to weekly examinations over 12 weeks. With 360 weeks of observation, we estimated the 95% CI will be ± 5% of the mean proportion (13).

Statistical analysis

The primary outcome of the adherence to weekly anal self-examination was the proportion of the number of weeks expressed in person-time. It was defined as the number of weeks where the participants had performed anal self-examination divided by the total number of weeks in the study period for total study participants. The outcome was expressed in person-weeks. The 95% CI of the proportion were calculated using the

TABLE 1 Demographic characteristics and sexual practices among 30 participants at baseline.

Demographic characteristics and sexual practices	Number of participants, Percentage (%)	
Age (median, interquartile range) (years)	32 (IQR: 27–41)	
Gender		
Men	30	100%
Sexual orientation		
Men who have sex with men	30	100%
Men who have sex with men and women	0	0%
HIV and PrEP		
Living with HIV	8	27.0%
Taking PrEP	11	37.0%
Not taking PrEP & not living with HIV	11	37.0%
Anal sex position in the past 12 months		
Receptive penile-anal sex only	14	47.0%
Receptive and insertive penile-anal sex	16	53.0%
Past syphilis infection		
Yes	9	30.0%
One infection	7	23.0%
More than one infection	2	7.0%
No	21	70.0%
Condom use in the past 3 months^	$N = 29$	
Always	3	10.0%
Never	9	31.0%
Sometimes	17	59.0%
No anal sex	0	0.0%
Ever inserted their fingers in their anus previously		
Yes	25	83.0%
No	5	16.0%
Previous abnormalities reported by men who had inserted their fingers in their anus *	$N = 25$	
Yes[†]	9	36.0%
No	16	64.0%
Reasons for inserting their fingers in their anus among those who had performed previously*§	$N = 25$	
To check for symptoms of STI	15	58.0%
On recommendation by health professionals or friends/family/partners	4	15.0%
To check for abnormalities	3	12.0%
Pleasure/masturbation	2	8.0%
Hygiene	2	8.0%
Anal cancer screening	1	4.0%
Median sexual partners for receptive anal sex in the past 3 months*	4 [IQR: 1–7]	
Median frequency of anal self-examination* (per 4 weeks)	1 [IQR: 0.3–4]	
Mean frequency of anal self-examination* (per 4 weeks)	1 [SD ± 1.1]	

*Refers to the men who had previously performed anal self-examination prior to the enrolment in the study and the description in brackets were as described.
^The total number does not equal to 30 due to missing data.
§Participants could provide more than one reason.
†Reported abnormalities included hemorrhoids, anal fissure, lump, dry skin, anal STI symptoms- bleeding, ulcer, discharge, pain.
IQR, interquartile range.
SD, standard deviation.

binomial exact method. We calculated the mean with 95% CI of the frequency of anal self-examination every 4 weeks per person and we examined the temporal trend on anal self-examination per 4-week using linear regression analysis.

The secondary outcomes were summarized using descriptive statistics by reporting the proportion of men who reported abnormal findings out of the total number of participants. The adverse events were calculated for the proportion of men who reported abnormal findings and requested medical review (either at MSHC or with their general practitioners), and for the proportion of men who reported abnormal findings and did not request medical review.

Kaplan-Meier survival curves were constructed to present the cumulative proportion of men who first developed adverse outcomes and received medical review. All statistical analyses were conducted using STATA 16 (StataCorp LLC, Texas, USA).

Results

There were 36 men recruited from December 2020 to March 2021, and the last participant finished the study in June 2021. Of

TABLE 2 Sexual practices and anal self-examination including motivators and views of ways to improve adherence to anal self-examination.

	Number	Percentage (%)
Number of sexual partners for receptive anal sex in the past one month, median (IQR)	2 (1–4)	
Condom use in the past 3 months[*‡]	$N = 28$	
Always	4	14.0%
Sometimes	9	32.0%
Never	13	46.0%
No anal sex	2	7.0%
Anal self-examination positions used by participants[†‡]	$N = 28$	
Standing	6	21.0%
Squatting	7	25.0%
Standing & squatting	4	14.0%
Lying on the back	3	11.0%
Lying on the side	1	4.0%
Lying on the back & squatting	2	7.0%
Lying on the back & lying on the side	1	4.0%
Using more than 2 positions	4	14.0%
Items used in anal self-examination[†‡]	$N = 59$	
Lubricants	17	29.0%
Soap	14	24.0%
Water	18	31.0%
Gloves	2	3.0%
Mirrors	6	10.0%
None of the above	2	3.0%
Location to perform anal self-examination[†‡]	$N = 38$	
Shower	21	55.0%
Bed	5	13.0%
Bathroom/toilet	12	32.0%
Reasons for non-adherence[†‡]	$N = 16$	
Busy with work or study or life	11	69.0%
Forgot to perform anal self-examination	10	63.0%
Had not had anal sex	5	31.0%
No symptoms of STI	6	38.0%
Uncomfortable or difficult to perform	3	19.0%
Participants' views on the use of reminder system[*‡]	$N = 30$	
Weekly reminder system *via* SMS or a smartphone app or using phone as a reminder	11	34.0%
3-monthly SMS reminder	7	22.0%
Smartphone app (frequency not specified)	6	19.0%
Reminder not required	6	19.0%
Logbook	1	3.0%
Monthly reminder (method not specified by participant)	1	3.0%
Motivators to perform anal self-examination regularly[*‡]	$N = 30$	
Having symptoms of STI	6	22.0%
Increased sexual activity ^	6	22.0%
Reminder	4	15.0%
Improvement in knowledge such as knowing what to look for and differentiating normal and abnormal	4	15.0%
Medical advice and recommendations or proven effective to be used as a screening for anal syphilis	3	11.0%
Does not need any motivation to perform regularly	4	15.0%

*Some missing data.
†Multiple options and total may exceed 100%.
^ Increased sexual activity refers to increased sexual partners, engaging in high-risk anal sex such as condomless anal sex.
‡Data were collected at Week 12.

the 36 men, four never completed the baseline surveys and were classified as lost to follow-up. One completed only the baseline survey, and one withdrew from the study at Week 9. A total of 30 men who completed baseline and follow-up surveys were included in the final analysis (Supplementary Figure 2). All 30 men tested negative for active syphilis at baseline.

Table 1 presents the demographic characteristics and sexual practices of 30 participants. The age of the participants ranged from 19 to 55 years (median 32 years, IQR: 27-41). Among the 25 men (83%) who had previously inserted their fingers in their anus, the median frequency of performing this was once per 4 weeks (IQR: 0.3–4).

Adherence to performing weekly anal self-examination

All 30 men were followed for 12 weeks and therefore provided 360 person-weeks of follow up. Anal self-examination was performed in 308 of 360 person-weeks (86% of the follow-up weeks, 95% CI: 82–89). Of the 30 men, 47% ($n = 14$) performed weekly anal self-examination over 12 weeks and 53% ($n = 16$) did not perform weekly anal self-examination.

The mean frequency of anal self-examination per 4 weeks changed from 3.6 (95% CI: 3.3–3.9) times in Weeks 1–4, to 3.5 (95% CI: 3.1–3.8) times in Weeks 5–8, and 3.3 (95% CI: 2.9–3.7)

times in Weeks 9–12; however, this change was not statistically significant ($P_{trend} = 0.06$) (Supplementary Table 2).

Reasons for not performing anal self-examination every week and motivators

Among the 16 men who did not perform anal self-examination every week, the commonly reported reasons were busy with work, study, or life, and forgetting to perform their anal self-examinations (Table 2). They reported having STI-related symptoms, increased sexual activity, and receiving a reminder as the top three motivators to perform anal self-examination regularly (Table 2).

Preferences and intention to perform anal self-examination

At the end of the study, men reported standing, squatting or a combination of the two positions as the most common positions (81%, $n = 17$). The shower (55%, $n = 21$) was the most common location to perform anal self-examination. Most men (60%, $n = 35$) used water or lubricants to aid with anal self-examination (Table 2 and Supplementary Figure 3).

More than half (53%, $n = 16$) of participants felt that weekly anal self-examination was too frequent, while 37% ($n = 11$) reported that weekly anal self-examination was acceptable.

Most men (63%, $n = 17$) expressed their intention to continue performing anal self-examination if it were recommended in the future, while 23% ($n = 7$) were unsure if they would continue. A small number of men (7%, $n = 2$) chose not to continue to perform anal self-examination. Among those men who decided to continue their examinations, the mean preferred frequency was two times (95% CI: 1.4–2.6) per month.

Abnormal findings during anal self-examination

Of the fourteen men (47%, 14/30, 95% CI: 28–66%) who identified some abnormalities in their anus, six men (43%, 6/14, 95% CI: 18–71%) sought medical review (Figure 1); four at MSHC and two at their general practitioners. One of them presented twice with the same abnormalities and was diagnosed with recurrent herpes simplex (Table 3). Half of the men (50%, 3/6) who requested medical review reported abnormalities in Weeks 1–4, while the other half (50%, 3/6) reported abnormalities in Weeks 9–12.

Eight men (27%, 8/30 men, 95% CI: 12-46%) reported abnormalities in surveys but did not seek medical review

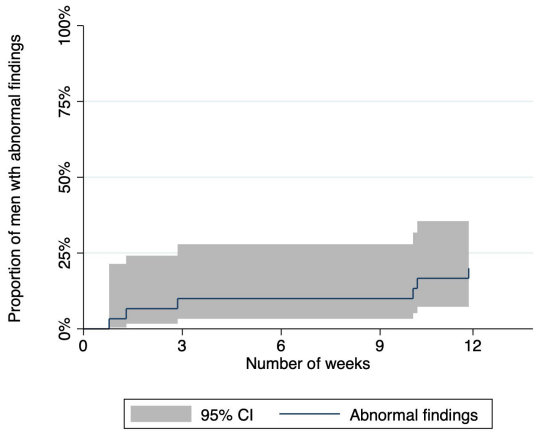


FIGURE 1
Graph showing the proportion of men who reported abnormal findings and requested medical review during the 12-week study period.

(Supplementary Table 3). The symptoms reported were pain, lump, itch, dry skin, and blisters with anal pain being the most commonly reported symptoms (Supplementary Table 3).

Syphilis diagnosis

None of the 30 men in the study were diagnosed with active syphilis at baseline (recruitment), at the completion of the study and also 3 months after completing the study (from reviewing the syphilis test results at MSHC).

Discussion

This is the first study to examine adherence to weekly anal self-examination as a potential means of detecting anal syphilis in MSM. We found a high level of adherence (86% of the follow-up weeks) among the participants indicating that men might perform anal self-examination regularly if it were recommended. Almost half of the participants reported abnormalities in their anus but only about 40% of them sought medical review. Future studies with a longer follow-up period will be needed to assess the long-term adherence to anal self-examination and its sensitivity to detect anal syphilis. Nonetheless, finding abnormalities through anal self-examination demonstrate that it was feasible for men to perform anal self-examination and detect abnormalities.

There have been very limited studies examining the use of anal self-examination for early detection of anal syphilis and none examining adherence to these examinations (11, 12, 14, 15). Previous studies have involved both qualitative and quantitative surveys of MSM and found anal self-examinations to be highly acceptable (11, 12, 15). A survey of 574 MSM

TABLE 3 Reported abnormalities and clinical diagnoses among six men who presented for medical review after identifying an abnormality.

Participant	Number of weeks first reported abnormality	Description of abnormality	Location of medical review	Diagnosis	Syphilis serology	Syphilis PCR
3	12	Pain, bleeding	MSHC	Anal tear	Negative	Negative
12	11	Pain, bleeding, itch	GP	Rectal chlamydia	Negative	Not done
17	1	Lump	MSHC	Possible anal wart	Negative	Negative
23	2	Lump	GP	No abnormality found*	Negative	Not done
25	11	Itch, rash	MSHC	No abnormality found*	Negative	Negative
30	3, 12	Discomfort/pain	MSHC	Recurrent HSV-2	Negative	Negative

MSHC, Melbourne Sexual Health Center.
GP, General Practitioner.
HSV-2, Herpes Simplex Virus Type II.
*No abnormality found: Clinicians could not find any abnormalities at review; therefore, no diagnosis was given at the reviews.

reported up to 68% of men had never performed anal self-examinations but were willing to perform them in the future (12). Consistent with these findings, we found that only a small proportion of MSM (7%) indicated that they did not want to continue performing anal self-examinations. Most of the 20 MSM in the qualitative interview study expressed their willingness to perform anal self-examination in the future if it were recommended by a health professional (11). Taken together with these findings, there is substantial evidence to support anal self-examination having a potential role in the detection of syphilis MSM.

Our study identified some issues that should be considered when designing future studies. Our findings suggest adherence to anal self-examinations may decline with time although this was not statistically significant. However future studies should consider incorporating SMS reminders in future studies on anal self-examination as most men in our study chose SMS messaging as a preferred reminder system. This finding was consistent with other studies showing SMS reminders increased the odds of adherence to intervention (16, 17). We also found that half of the abnormalities were reported at the start of the study suggesting men were identifying pre-existing abnormalities rather than new abnormalities that developed during the study period. This could potentially lead to an overestimation of the true incident abnormalities.

In our study, we found a high proportion of participants reporting abnormalities during self-examination yet none of the men had syphilis. Importantly, only about half of them sought medical review and we did not know the reasons why other participants did not seek medical review. We also did not know if anal syphilis was to occur, whether it would have an abnormality that could be detected by men during anal self-examination. Understanding these issues is going to be critical in determining whether syphilis can be detected earlier and whether it would be cost-effective or not.

Limitations

There are several limitations to our study. First, clients attending sexual health clinics tend to be more health-conscious about their sexual health and therefore, there might be a selection bias with participants more likely to be adherent than the general MSM population. So, we may have overestimated adherence in our study, although high levels of acceptance were found in questionnaire studies (12). Second, the sample size was not sufficient for the estimates of our secondary outcomes such as the proportion reporting abnormalities. Third, self-reported bias in the adherence to anal self-examination might have occurred. There was a possibility of over-reporting and overestimating the adherence. Fourth, the study was conducted during the COVID-19 pandemic and lockdown periods resulting in some men reducing their sexual practices and changing the frequency of performing anal self-examination. In the surveys, men reported increased sexual activity and condomless sex would motivate them to perform anal self-examination more frequently. Therefore, COVID-19 pandemic restrictions could have affected their adherence (18–20).

Conclusions

Overall, we conclude that men adhered well to weekly anal self-examination and therefore, they are feasible to trial as a routine practice among MSM.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving human participants were reviewed and approved by the Alfred Hospital Ethics Committee, Melbourne, Australia (Project 603/20). The patients/participants provided their written informed consent to participate in this study.

Author contributions

CF, EC, and EA designed and coordinated the study. JO, TP, and MC contributed to the development of the study protocol. EA, KM, and ER recruited the participants. EA conducted follow-ups, distributed the survey, coordinated the medical reviews, and drafted the 1st manuscript. EC and EA performed the analysis of the data and revised the manuscript. All authors reviewed and edited the manuscript and read and approved the final manuscript.

Funding

This study was supported by an Australian National Health and Medical Research Council (NHMRC) Investigator Grant (GNT1172873) and there was no direct involvement by NHMRC in our study.

Acknowledgments

We would like to thank Mark Chung from MSHC for his contribution to developing the study website and animated videos of anal self-examination instruction, Jason Kenna from MSHC for providing voice cover for the video, and Prof Alan Nyitray for assisting us in developing anal self-examination instruction by sharing resources. We are also very grateful to our clinicians who recruited participants for our study especially Andrew Buchanan, Tina Schmidt, and Elena Rodriguez and lastly, all the participants for participating in this study.

Conflict of interest

EC was supported by an Australian NHMRC Emerging Leadership Investigator Grant (GNT1172873). CF was supported by an Australian NHMRC Leadership Investigator Grant (GNT1172900). JO was supported by an Australian NHMRC Emerging Leadership Investigator Grant (GNT1193955). EA was supported by Australian Government Research Training Program (RTP) scholarship from Monash University and Research Entry Scholarship from the Chapter of Sexual Health Medicine, Royal Australasian College of Physician. JT was supported by Australian Government Research Training Program (RTP) Scholarship from Monash University.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.941041/full#supplementary-material

References

1. Aung ET, Chen MY, Fairley CK, Higgins N, Williamson DA, Tomnay JE, et al. Spatial and temporal epidemiology of infectious syphilis in victoria, Australia, 2015-2018. *Sex Transm Dis.* (2021) 48:e178–e82. doi: 10.1097/OLQ.0000000000001438

2. Chow EPF, Grulich AE, Fairley CK. Epidemiology and prevention of sexually transmitted infections in men who have sex with men at risk of HIV. *Lancet HIV.* (2019) 6:e396–405. doi: 10.1016/S2352-3018(19)30043-8

3. Kirby Institute. *HIV, Viral Hepatitis and Sexually Transmissible Infections in Australia Annual Surveillance Report 2018.* Sydney: Kirby Institute UNSW (2018).

4. Williamson DA, Chen MY. Emerging and reemerging sexually transmitted infections. *N Engl J Med.* (2020) 382:2023–32. doi: 10.1056/NEJMra1907194

5. STIs in Gay Men Action Group (STIGMA). *Australian Sexually Transmitted Infection And HIV Testing Guidelines 2019 for Asymptomatic Men Who Have Sex With Men*. Sydney: NSW Sexually Transmissible Infections Unit (2019).

6. Peel J, Chow EPF, Denham I, Schmidt T, Buchanan A, Fairley CK, et al. Clinical presentation of incident syphilis among men who have sex with men taking HIV pre-exposure prophylaxis in melbourne, Australia. *Clin Infect Dis.* (2021) 73:e934–37. doi: 10.1136/sextrans-2021-sti.261

7. Cornelisse VJ, Chow EPF, Latimer RL, Towns J, Chen M, Bradshaw CS, et al. Getting to the bottom of it: sexual positioning and stage of syphilis at diagnosis, and implications for syphilis screening. *Clin Infect Dis.* (2020) 71:318–22. doi: 10.1093/cid/ciz802

8. Towns JM, Leslie DE, Denham I, Wigan R, Azzato F, Williamson DA, et al. Treponema pallidum detection in lesion and non-lesion sites in men who have sex with men with early syphilis: a prospective, cross-sectional study. *Lancet Infect Dis.* (2021) 21:1324–31. doi: 10.1016/S1473-3099(20)30838-0

9. Ong JJ, Temple-Smith M, Chen M, Walker S, Grulich A, Fairley CK. Exploring anal self-examination as a means of screening for anal cancer in HIV positive men who have sex with men: a qualitative study. *BMC Public Health.* (2014) 14:1257. doi: 10.1186/1471-2458-14-1257

10. Nyitray AG, Hicks JT, Hwang LY, Baraniuk S, White M, Millas S, et al. A phase II clinical study to assess the feasibility of self and partner anal examinations to detect anal canal abnormalities including anal cancer. *Sex Transm Infect.* (2018) 94:124–30. doi: 10.1136/sextrans-2017-053283

11. Aung ET, Fairley CK, Ong JJ, Bilardi JE, Chen MY, Chow EPF, et al. Exploring the attitudes of men who have sex with men on anal self-examination for early detection of primary anorectal syphilis: a qualitative study. *BMC Infect Dis.* (2021) 21:982. doi: 10.1186/s12879-021-06686-4

12. Aung ET, Fairley CK, Ong JJ, Phillips TR, Chen MY, Tran J, et al. A *Cross-Sectional Survey on Attitudes of Men Who Have Sex With Men Towards Anal Self-Examination for Detection of Anal Syphilis. Sci Rep.* 12, 8962 (2022). doi: 10.1038/s41598-022-12881-3

13. Billingham SA, Whitehead AL, Julious SA. An audit of sample sizes for pilot and feasibility trials being undertaken in the United Kingdom registered in the United Kingdom Clinical Research Network database. *BMC Med Res Methodol.* (2013) 13:104. doi: 10.1186/1471-2288-13-104

14. Taylor MM, Peterson B, Post J, Williams C, Vanig T, Winscott M. Self-examination behaviors for syphilis symptoms among HIV-infected men. *J Acquir Immune Defic Syndr.* (1999). 2010 55:284–5. doi: 10.1097/QAI.0b013e3181e13ed9

15. Aung ET, Chow EPF, Fairley CK, Phillips TR, Chen MY, Tran J, et al. Preferences of men who have sex with men for performing anal self-examination for the detection of anal syphilis in Australia: a discrete choice experiment. *Lancet Reg Health West Pac.* (2022) 21:100401. doi: 10.1016/j.lanwpc.2022.100401

16. Ibeneme SC, Ndukwu SC, Myezwa H, Irem FO, Ezenwankwo FE, Ajidahun AT, et al. Effectiveness of mobile text reminder in improving adherence to medication, physical exercise, and quality of life in patients living with HIV: a systematic review. *BMC Infect Dis.* (2021) 21:859. doi: 10.1186/s12879-021-06563-0

17. Thakkar J, Kurup R, Laba T-L, Santo K, Thiagalingam A, Rodgers A, et al. Mobile telephone text messaging for medication adherence in chronic disease: a meta-analysis. *JAMA Intern Med.* (2016) 176:340–9. doi: 10.1001/jamainternmed.2015.7667

18. Chow EPF, Hocking JS, Ong JJ, Schmidt T, Buchanan A, Rodriguez E, et al. Changing the Use of HIV pre-exposure prophylaxis among men who have sex with men during the COVID-19 pandemic in Melbourne, Australia. *Open Forum Infect Dis.* (2020) 7:275. doi: 10.1093/ofid/ofaa275

19. Chow EPF, Hocking JS, Ong JJ, Phillips TR, Schmidt T, Buchanan A, et al. Brief report: changes in PrEP Use, sexual practice, and use of face mask during sex among MSM during the second wave of COVID-19 in Melbourne, Australia. *J Acquir Immune Defic Syndr.* (2021) 86:153–6. doi: 10.1097/QAI.0000000000002575

20. Chow EPF, Hocking JS, Ong JJ, Phillips TR, Fairley CK. Sexually transmitted infection diagnoses and access to a sexual health service before and after the national lockdown for COVID-19 in Melbourne, Australia. *Open Forum Infect Dis.* (2020) 8:536. doi: 10.1093/ofid/ofaa536

frontiers | Frontiers in Medicine

TYPE Original Research
PUBLISHED 03 August 2022
DOI 10.3389/fmed.2022.938016

OPEN ACCESS

EDITED BY
Lei Zhang,
Melbourne Sexual Health Centre (MSHC), Australia

REVIEWED BY
Jun Li,
Peking Union Medical College Hospital (CAMS), China
Simon Houston,
University of Victoria, Canada

*CORRESPONDENCE
Wen-Hui Lun
ditanlun@ccmu.edu.cn

SPECIALTY SECTION
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

RECEIVED 16 May 2022
ACCEPTED 15 July 2022
PUBLISHED 03 August 2022

CITATION
Liu J, Zhao T-W, Zhou C, Yan H-W and Lun W-H (2022) Changes of serofast status in HIV negative asymptomatic neurosyphilis patients after treatment. *Front. Med.* 9:938016. doi: 10.3389/fmed.2022.938016

Changes of serofast status in HIV negative asymptomatic neurosyphilis patients after treatment

Jing Liu[1], Tian-Wei Zhao[1], Chun Zhou[2], Hui-Wen Yan[1] and Wen-Hui Lun[1]*

[1]Department of Dermatology and Venereology, Beijing Ditan Hospital, Capital Medical University, Beijing, China, [2]Department of Clinical Laboratory, Beijing Ditan Hospital, Capital Medical University, Beijing, China

Serofast status after therapy in syphilis patients is a common phenomenon. A proportion of patients who have serofast status exhibit abnormal cerebrospinal fluid test results, which can be defined as asymptomatic neurosyphilis (ANS); however, it remains unclear whether ANS patients can achieve serological cure after anti-neurosyphilis treatment as quickly as other serofast patients. In this study, non-treponemal pallidum antibody serological responses were studied in ANS and serofast control patients, and the cumulative rates of serological cure in the ANS group were 9.6, 22.1, 25.9, and 30.2% in 3, 6, 9, and 12 month after treatment, which were statistically higher than those of the serofast control group. The change gap in serological cure rates was even more pronounced within 6 months after treatment, but the majority of ANS patients had no change in serofast status at 12 months after treatment. Our study indicates that anti-neurosyphilis therapy can partially change the serofast status. As serofast status cannot easily be changed even under neurosyphilis treatment in the majority of patients, the pathogenesis of this condition needs further research.

KEYWORDS

syphilis, serofast, asymptomatic neurosyphilis, serological cure, treatment

Introduction

Syphilis is an old disease that can cause serious health problems without treatment. Since the introduction of penicillin in 1943, there have been great improvements in syphilis treatment. Theoretically, syphilis is a sexually transmitted disease that can be cured. However, in actual clinical settings, judging whether syphilis has already been cured is not an easy task (1). Non-treponemal serological response is an important laboratory indicator of whether treatment has been effective, and serological cure is defined as either a negative rapid plasma regain (RPR) or $\geq$ 4-fold decrease (twofold dilution) in titer at 6–12 months following therapy. However, approximately 15–20%

of patients with early syphilis do not meet the criteria of serological cure and are therefore referred to as being "serofast." Serofast status is defined as a < 4-fold (twofold dilution) decline in non-treponemal antibody titer at 6–12 months or as persistently low titer after treatment (2). The etiology and the optimal management of serofast status remain unclear.

Retreatment is recommended for serofast patients if follow-up cannot be ensured, which is common in clinical practice. Several studies have reported no incremental benefit for re-treating HIV-negative serofast patients (3, 4). In our previous study, a subset of serofast patients exhibited cerebrospinal fluid (CSF) abnormalities that could be defined as asymptomatic neurosyphilis (ANS) according to the appropriate guidelines (5). Neurosyphilis is caused by *Treponema pallidum* infecting the central nervous system, which can occur at any stage of syphilis. ANS is a type of neurosyphilis without any neurological symptoms, and its diagnosis relies on CSF test abnormalities, including positive venereal disease laboratory research tests (VDRL), abnormal white blood cell (WBC) counts, and elevated protein (6). At present, disputation surrounds ANS, and thus, defining ANS has been extremely difficult and controversial (2). Most definitions rely on a combination of CSF laboratory tests (such as VDRL, WBC count, and protein level), but no consensus definition exists. Some research has suggested that a negative CSF fluorescent treponemal antibody absorption (FTA-ABS) IgG test as the exclusion criterion for neurosyphilis, but this is not unanimously recognized (7).

There are many different views about ANS. On the one hand, ANS is the early stage of neurosyphilis, which can develop into symptomatic neurosyphilis; therefore, ANS should be treated in accordance with neurosyphilis. On the other hand, it is thought that abnormal changes in CSF are common in the early stages of syphilitic infection, but most patients heal naturally without intervention. Thus, debate continues as to whether asymptomatic patients need CSF testing, but there is not enough evidence to determine whether the diagnosis and treatment of ANS could contribute to prognosis in these patients.

The aim of this study was to compare changes in non-treponemal antibody serum titer in ANS patients with those in serofast patients. In addition, the study evaluated whether anti-neurosyphilis treatment provides benefit for serological cure in these patients.

Materials and methods

All patients in this study were enrolled from the clinical database of Beijing Ditan Hospital, Capital Medical University, between September 2015 and August 2018. All of the enrolled patients were HIV-negative and in serofast status. Serofast status was defined as described in the previous studies (5, 8, 9), as follows: (1) in early syphilis patients who were at least 6 months after initial recommended treatment and regular follow-up, no fourfold decline in serum RPR titer was observed even after additional treatment; (2) RPR titer exhibited a < 4-fold decline more than 1 year after treatment in late latent syphilis; and (3) patients who had a fourfold decline in serum RPR titer after initial treatment but exhibited no seroreversion or decline in non-treponemal antibody titer for more than 1 year. Lumbar puncture and CSF tests were carried out, and ANS was diagnosed as described in our previous study (5), which followed the European Guidelines on the Management of Syphilis and US CDC guidelines (2, 8). ANS was characterized by reactive CSF RPR or negative CSF RPR but CSF WBC count $> 5 \times 10^6$/L, or negative CSF RPR, CSF WBC count $\leq 5 \times 10^6$/L but CSF protein concentration > 45 mg/dL, and without any neurological signs or symptoms. Other neurological diseases were excluded based on clinical history and physical examination.

According to the CSF examination results, serofast patients were divided into two groups: serofast patients with abnormal CSF test results were classified as the ANS group and the serofast patients with normal CSF test results were classified as the serofast control group. The ANS group was treated according to the recommended neurosyphilis treatment regime (8), and the serofast control group was followed up without recommended treatment. All patients were followed every 3 months for serological RPR card test. Serologically defined treatment response was used to classify the patients as serological cure (defined as patients whose RPR titer dropped > 4-fold or RPR became negative), non-response (RPR tier was not changed; drop or increase < 4-fold), or treatment failure (RPR titer increased > 4-fold) (9, 10).

The study was approved by the Institutional Ethics Committee of Beijing Ditan Hospital, Capital Medical University.

Data analysis and statistics

Data were analyzed using IBM SPSS version 19.0. Figures were drawn using GraphPad Prism 8. Continuous variables are described using median and interquartile range (IQR), whereas categorical variables are described by numbers and percentages. Associations between categorical variables were assessed using the chi-square test. Titers were obtained 3, 6, 9, and 12 months after enrollment, and Kaplan–Meier product-limit survival curves were used to examine the rates of serologic response to treatment. All hypothesis testing was two-sided, and $P < 0.05$ was considered to be statistically significant.

TABLE 1 Demographic and clinical characteristics of the ANS and the serofast control groups.

		ANS (n = 136)	Serofast control (n = 309)	P-value
Gender	Male (n, %)	57 (41.9)	100 (32.4)	0.052
	Female (n, %)	79 (58.1)	209 (67.6)	
Age (years, median and interquartile range)		36 (28, 52)	32 (27, 43)	0.023
Disease course (months, median and quartile)		24 (12, 36)	24 (12, 36)	0.615
Syphilis stage	Latent	115 (84.6)	267 (86.4)	0.697
	Primary	1 (0.7)	4 (1.3)	
	Secondary	20 (14.7)	38 (12.3)	
RPR titer (median and interquartile range)		1:8 (1:4, 1:16)	1:8 (1:4, 1:16)	0.013
	≦ 1:2	18 (13.2)	67 (21.7)	
	1:4	28 (20.6)	79 (25.6)	
	1:8	42 (30.9)	71 (23.0)	
	1:16	26 (19.1)	57 (18.4)	
	≧ 1:32	22 (16.2)	35 (11.3)	
Initial regimen with benzathine Penicillin (n, %)		120 (88.2)	272 (88.0)	0.950
CSF RPR	Negative	118 (86.7)	309 (100)	NA
	1:1	11 (8.1)	–	
	1:2	5 (3.7)	–	
	1:4	2 (1.5)	–	
CSF WBC count/μL (n, %)	≤ 5	28 (20.6)	309 (100)	NA
	> 5	108 (79.4)	–	
CSF protein concentration mg/dL (n, %)	≤ 45	94 (69.1)	309 (100)	NA
	> 45	42 (30.9)		

NA, not available.

TABLE 2 Comparison of serological responses between the ANS group and the serofast control group.

		ANS (n, %) (n = 136)	Serofast control (n, %) (n = 309)	P-value
3 Months	Serological cure	13 (9.6)	13 (4.2)	0.027
	Serofast	122 (89.7)	293 (94.8)	0.047
	Treatment failure	1 (0.7)	3 (1.0)	1*
6 months	Serological cure	30 (22.1)	39 (12.6)	0.011
	Serofast	104 (76.4)	270 (87.4)	0.004
	Treatment failure	2 (1.5)	0 (0)	0.093*
9 months	Serological cure	35# (25.9)	52 (16.8)	0.029
	Serofast	98# (72.6)	255 (82.6)	0.012
	Treatment failure	2# (1.5)	2 (0.6)	0.589*
12 months	Serological cure	41 (30.2)	62 (20.1)	0.020
	Serofast	94 (69.1)	244 (79.0)	0.025
	Treatment failure	1 (0.7)	3 (0.9)	1*

*Fisher's exact test; #n = 135.

Results

Baseline demographics and patient characteristics

According to the recommended CSF assessment criteria for asymptomatic serofast patients, 445 HIV-negative serofast syphilis patients were enrolled in Beijing Ditan Hospital, Capital Medical University, between September 2015 and August 2018. All of the patients had received lumbar puncture for CSF testing, and the median time from first diagnosis of syphilis to lumbar puncture was 24 (IQR 12, 36) months. Through CSF examination, 136 participants (30.6%) exhibited an abnormal CSF test that could be diagnosed as ANS (ANS group), whereas 309 participants (69.4%) exhibited a normal CSF test result (serofast control group). The demographic and clinical characteristics of the two groups are listed in Table 1. The baseline data for the ANS and serofast control groups did not exhibit any statistically significant differences in sex, duration of serofast status, initial stage of syphilis, initial serum RPR titer, or initial treatment regimen. The median age of the participants in the ANS group was greater than that of patients in the serofast control group (P = 0.023). The serum RPR titer of the ANS group was higher than that of the serofast control group at the time of lumbar puncture (Wilcoxon symbol rank and test, P = 0.013).

Treatment and serological response

The patients in the ANS group were treated with aqueous crystalline penicillin G, 4 million units IV every 4 h for 14 days, followed by benzathine penicillin G, 2.4 million units IM weekly for 3 weeks. Re-treatment was not recommended in the serofast control group. All of the participants were followed every 3 months for serological tests.

The proportion of evaluable participants who exhibited serological response varied by group and time after therapy. The rates of serological cure at 3, 6, 9, and 12 months are shown in Table 2, the proportion of patients with serological cure in the ANS group was higher than that in the serofast control group and the difference was statistically significant between the two groups.

Time-dependent proportion of serofast patients

Survival analyses using the log-rank test were conducted to determine the proportion of patients with unchanged serofast

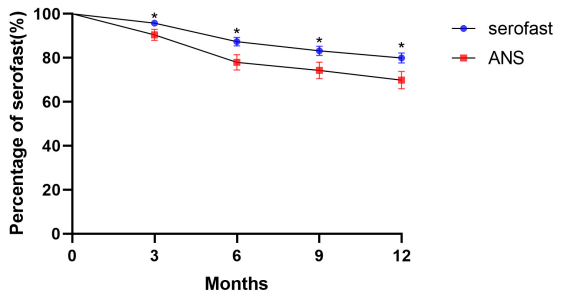


FIGURE 1
Kaplan–Meier plots of the number of patients remaining in serofast status in the ANS group after therapy as compared with the serofast control group (*$P < 0.05$).

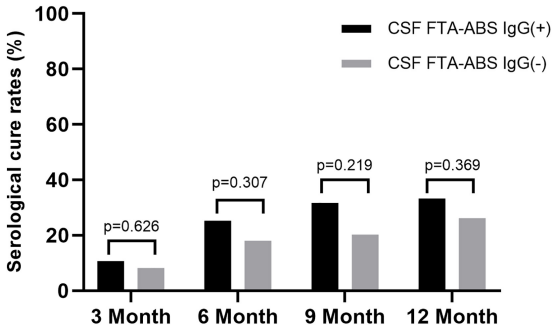


FIGURE 2
Serological cure rates based on CSF FTA-ABS in ANS group.

status. The time-dependent accumulated serological cure rates after treatment at 3, 6, 9, and 12 months are shown in Figure 1. The trend of the change in serological cure rates in both groups as shown in the talbe2 indicates that there was a significant difference between ANS and serofast control group in the serological cure rate at each follow-up time point, majority of serological cure cases appeared in the half year after treatment.

Serological cure rates based on exhibited cerebrospinal fluid fluorescent treponemal antibody absorption

Some researchers have suggested CSF FTA-ABS as an exclusion criterion for neurosyphilis diagnosis, but others disagree (7). We tested the CSF FTA-ABS IgG and IgM titers in the ANS group. All patients were negative for CSF FTA-ABS IgM, but 75 patients were positive for CSF FTA-ABS IgG and 61 were negative for CSF FTA-ABS IgG. The serological responses between the two groups were compared.

The rates of serological cure were 10.7, 25.3, 30.7, and 33.3% in the CSF FTA-ABS IgG(+) patients at 3, 6, 9, and 12 months, respectively. In contrast, the rates of serological cure were 8.2, 18, 21.3, and 26.2% at 3, 6, 9, and 12 months, respectively, in the CSF FTA-ABS IgG(-) patients. The difference in serological cure rates between the two groups was not significant (Figure 2).

Discussion

Despite the availability of effective treatments, 15–20% of persons with early syphilis are referred to as being serofast, and the incidence of serofast status in late syphilis is much higher (1–4). At present, the etiology of serofast status remains unknown. Many studies have shown that re-treatment does not significantly improve a patient's serological cure rate, and in serofast patients with early syphilis, it may not provide any significant benefit, even though re-treatment is commonly performed in clinical practice (3, 4). In our previous study, abnormal CSF test results were observed in a substantial proportion of serofast patients, which could be defined as ANS (5). CSF assessment is recommended in asymptomatic patients who experienced serological failure or are serofast (2). To our knowledge, there are no reports that describe the changes in non-treponemal serological titer in ANS patients after treatment.

In the present study, 136 serofast patients with abnormal CSF tests were treated according to the guideline for neurosyphilis therapy. As expected, a decline in RPR titer was observed as early as 3 months after treatment as compared with the control group. The rates of serologic cure gradually increased over time, from 9.6% in the third month to 22.1, 25.9, and 30.2% at 6, 9, and 12 months, respectively. A significant difference in serological cure rates as compared with the control group was observed. Our results demonstrate the benefit of serological cure in ANS patients after treatment and indicate the importance of screening for ANS in serofast patients. Treatment with a neurosyphilis-directed regimen could significantly improve the serological cure rate in this group of patients.

Some research has suggested that CSF FTA-ABS might be better suited to exclude rather than confirm a diagnosis of neurosyphilis, but there are limitations to CSF treponemal testing, as the negative predictive value of any test is dependent on the prevalence of the condition in the population in which the test is undertaken (11). Therefore, in a patient with a high pre-test probability, a negative CSF FTA-ABS result cannot exclude the diagnosis with sufficient confidence. It was observed that a negative CSF FTA-ABS is not sufficient

evidence for clinicians to decide not to treat for neurosyphilis (7). We were therefore interested to determine if there were any differences in the rates of serological cure after treatment between CSF FTA-ABS IgG(+) and FTA-ABS IgG(–) ANS patients in our study. Interestingly, the serological cure rates in the CSF FTA-ABS IgG(–) group were lower than those in the CSF FTA-ABS IgG(+) group, but the differences were not statistically significant. In both the CSF FTA-ABS IgG(+) and CSF FTA-ABS IgG(–) groups, the serological cure rates were higher than those in the serofast control group. Indeed, CSF testing was repeated in 47 of the ANS patients after 6 months. Even though all of those patients had received treatment for neurosyphilis, the CSF RPR for one of the patients turned from negative to positive (titer of 1:1). In addition, 5 patients' CSF FTA-ABS IgG results turned from negative to positive (data not shown). It is possible that a negative CSF FTA -ABS IgG test does not mean that the CSF FTA-ABS IgG test will always be negative in the future. Therefore, in the setting of CSF abnormalities, the role of a negative CSF FTA-ABS test in neurosyphilis diagnosis needs further investigation.

The survival curve chart clearly shows that the increase in the serological cure rate of syphilis is more obvious after 3 months and 6 months of neurosyphilis treatment, and the gap in the syphilis serological cure rate trend between the ANS group and the serofast control group became steady after 6 months. Although the difference in serological cure rates between the ANS group and serofast control group with neurosyphilis treatment was statistically significant, nearly 70% of cases in the ANS group retained serofast status at 12 months after treatment. In our previous study, the neuron damage biomarker of symptomatic neurosyphilis was not present in the majority of patients with ANS (12). This indicated that ANS only partly explains the serofast status. As the serofast status in the majority of patients cannot easily be changed even with neurosyphilis treatment, the pathogenesis of serofast status requires further research.

In this study, all of the participant have experienced secondary retreatment and at least one course of benzathine penicillin G, 2.4 million units IM weekly for 1–3 weeks, actually nearly 90% patients have received benzathine penicillin G anti-syphilis treatment in the initial regime and others in the secondary retreatment. So all of patients have enough treatment when they are diagnosed to be serofast status. because many studies have demonstrated that There is no evidence that patients will benefit from multiple retreatments in spite of this being commonly performed in clinical practice (3, 4), the serofast control group did not re-treat after CSF tests.

The present study has several limitations. First, this was a retrospective cohort study. CSF testing was repeated in only 34.6% (47 of 136) of patients in the 6 months after anti-neurosyphilis treatment in the ANS group. Good adherence to CSF testing during long-term follow-up is difficult in actual clinical settings. Although all ANS patients had undergone a full course of anti-neurosyphilis treatment, we could not determine the percentage of ANS patients in which the CSF returned to normal; Second, Although there was no statistical difference in the serological cure rate between the CSF FTA-ABS(+) and CSF FTA-ABS (-) groups, there was a difference in the serological cure rate between the two groups, which needs to be further studied with an enlarged sample size in the future. Third, there were some difference in baseline age and RPR titers between ANS and serofast control groups, which might reflected the facts in real world, we don't think these difference will influence the results, Since the inclusion and exclusion criteria were the same, the indicator for evaluating the treatment effect was the change in RPR titer before and after treatment, rather than a direct comparison of RPR titers. Some studies suggest that the RRP titer declines more slowly in the elderly, but in this study, although there is a difference in age between the two groups, the older patients are in the ANS group, which may affect the serum cure rate of some ANS patients, but The statistical results of the comparison between the two groups were not affected.

Conclusion

ANS is one of reasons for serofast status of syphilis patient, this study demonstrated that some ANS could achieve serological cure after anti-neurosyphilis treatment, However, a significant proportion of ANS remained serofast status after 1 year of treatment, and the reasons deserve further investigation.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Ethics statement

The studies involving human participants were reviewed and approved by the Institutional Ethics Committee of Beijing Ditan Hospital, Capital Medical University. The patients/participants provided their written informed consent to participate in this study.

Author contributions

JL and W-HL wrote the main manuscript text. T-WZ, CZ, and H-WY collected the clinical data. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Beijing Science and Technology Development projects (Z181100001718140).

Acknowledgments

We thank all of their study participants. We also thank the clinical staff in the Department of Dermatology and Venereology, Department of Infectious Disease and Department of Neurology, Beijing Ditan Hospital, Capital Medical University.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

References

1. Qin JB, Yang TB, Wang H, Feng T, Liu X. Potential predictors for serofast state after treatment among HIV-negative persons with syphilis in China: a systematic review and meta-analysis. *Iran J Public Health.* (2015) 44:155–69.

2. Janier M, Unemo M, Dupin N, Tiplica GS, Potoènik M, Patel R, et al. 2020 European guideline on the management of syphilis. *J Eur Acad Dermatol Venereol.* (2021) 35:574–88. doi: 10.1111/jdv.16946

3. Ren RX, Wang NW, Zheng HY, Li J. No improvement in serological response among serofast latent patients retreated with benzathine penicillin. *Int J STD AIDS.* (2016) 27:58–62. doi: 10.1177/0956462415573677

4. Seña AC, Wolff M, Behets F, Van Damme K, Martin DH, Leone P, et al. Response to therapy following retreatment of serofast early syphilis patients with benzathine penicillin. *Clin Infect Dis.* (2013) 56:420–2. doi: 10.1093/cid/cis918

5. Cai SN, Long J, Chen C, Wan G, Lun WH. Incidence of asymptomatic neurosyphilis in serofast Chinese syphilis patients. *Sci Rep.* (2017) 7:15456. doi: 10.1038/s41598-017-15641-w

6. Khalil G. Ghanem neurosyphilis: a historical perspective and review. *CNS Neurosci Ther.* (2010) 16:e157–68. doi: 10.1111/j.1755-5949.2010.00183.x

7. Smibert OC, Abbing S, Spelman DW, Jenney AWJ. Neurosyphilis: concordance between cerebrospinal fluid analysis and subsequent antibiotic strategy for patients undergoing evaluation of a diagnosis of neurosyphilis. *Int J Infect Dis.* (2019) 82:73–6. doi: 10.1016/j.ijid.2019.03.003

8. Centers for Disease Control and Prevention [CDC]. Sexually transmitted diseases treatment guidelines. *Morb Mort Wkly Rep.* (2010) 58:26–34.

9. Seña AC, Wolff M, Martin DH, Behets F, Van Damme K, Leone P, et al. Predictors of serological cure and serofast state after treatment in HIV-negative persons with early syphilis. *Clin Infect Dis.* (2011) 53:1092–9. doi: 10.1093/cid/cir671

10. Seña AC, Zhang XH, Li T, Zheng HP, Yang B, Yang LG, et al. A systematic review of syphilis serological treatment outcomes in HIV-infected and HIV-uninfected persons: rethinking the significance of serological non-responsiveness and the serofast state after therapy. *BMC Infect Dis.* (2015) 15:479. doi: 10.1186/s12879-015-1209-0

11. Harding AS, Ghanem KG. The performance of cerebrospinal fluid treponemal-specific antibody tests in neurosyphilis: a systematic review. *Sex Transm Dis.* (2012) 39:291–7. doi: 10.1097/OLQ.0b013e31824c0e62

12. Xu D, Cai SN, Li R, Wu Y, Liu SA, Lun WH, et al. Elevation of cerebrospinal fluid light and heavy neurofilament levels in symptomatic neurosyphilis. *Sex Trans Dis.* (2020) 47:634–8. doi: 10.1097/OLQ.0000000000001236

frontiers | Frontiers in Medicine

TYPE Perspective
PUBLISHED 22 August 2022
DOI 10.3389/fmed.2022.899200

OPEN ACCESS

EDITED BY
Marc Jean Struelens,
Université libre de Bruxelles, Belgium

REVIEWED BY
Gengfeng Fu,
Jiangsu Provincial Center for Disease Control and Prevention, China

*CORRESPONDENCE
Joseph D. Tucker
jdtucker@med.unc.edu

SPECIALTY SECTION
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

RECEIVED 18 March 2022
ACCEPTED 01 August 2022
PUBLISHED 22 August 2022

CITATION
Tucker JD, Marley G, Marks M and Mabey D (2022) Prioritizing syphilis control: Now is the time for action. *Front. Med.* 9:899200. doi: 10.3389/fmed.2022.899200

Prioritizing syphilis control: Now is the time for action

Joseph D. Tucker[1,2,3]*, Gifty Marley[1,3], Michael Marks[2,4] and David Mabey[2,4]

[1]Institute for Global Health and Infectious Diseases, University of North Carolina at Chapel Hill, Chapel Hill, NC, United States, [2]Clinical Research Department, Faculty of Infectious and Tropical Diseases, London School of Hygiene and Tropical Medicine, London, United Kingdom, [3]SESH Global, Guangzhou, China, [4]Hospital for Tropical Diseases, University College London, London, United Kingdom

Syphilis control programs and research received fewer resources and attention compared to HIV and other sexually transmitted infections (STIs) in the pre-pandemic era. The neglect of syphilis within comprehensive STI control efforts may be related to diagnostic (poor diagnostics), historical (legacies of racism in research), public health (limited partner services), and social problems (limited public engagement). At the same time, there are increasingly compelling reasons to prioritize syphilis control programs and research by harnessing lessons learned and advances during COVID-19. The closure of many STI facilities has accelerated new syphilis diagnostic pathways (e.g., syphilis self-testing), providing new ways for people to be screened outside of clinics. COVID-19 has underlined health inequities that fuel syphilis transmission, providing an opportunity to reckon with the historical legacy of racism that is linked to syphilis research. COVID-19 partner tracing efforts have also contributed to additional resources for partner services which may enhance syphilis control efforts. Finally, COVID-19 has demonstrated the importance of public engagement, making the case for greater public involvement in syphilis control and prevention programs. Urgent action is needed to prioritize syphilis control in a wide range of settings.

KEYWORDS

syphilis, advocacy, COVID-19, partner services, crowdsourcing

Introduction

Prior to COVID-19, syphilis was often neglected in global health research and programs. According to an analysis of data on infectious diseases research supported by G20 (a group of twenty countries) countries across 18-years, syphilis received the least amount of research grants per disability-adjusted life year (1). However, syphilis increases the risk of acquiring and transmitting HIV infection (2). Syphilis infection among pregnant women increases the risk of neonatal death, preterm labor, and other adverse birth outcomes (3). In addition, there is substantial stigma associated with syphilis (4). Improving syphilis services could decrease stillbirths, decrease syphilis-related stigma, decrease persistent health disparities related to at-risk groups, and improve the lives of many vulnerable individuals.

Ultimately, repeated calls to action from academic researchers and policy-makers (5) have not resulted in meaningful policy change. Yet the COVID-19 provides a new opportunity to re-think syphilis control because both are infectious diseases that require partner services, have self-testing options, and exacerbate health inequalities. This policy perspective examines the relative neglect of syphilis within comprehensive STI control systems, assessing critical historical, diagnostic, public health, and social issues before COVID-19. We also map out concrete ways that COVID-19 interventions could be used to prioritize syphilis control within health systems.

Malign neglect

The prevailing approach to syphilis control efforts within public health systems may be characterized as one of malign neglect—causing harm by doing nothing. Routine syphilis screening rates are low in many countries (6). The malign neglect of syphilis within comprehensive STI control efforts may be related to historical issues, diagnostics, partner services, and social issues (Figure 1).

The unique history of syphilis research has cast a long shadow on subsequent syphilis research. For example, in the United States, the Tuskegee trial enrolled poor, black men who were intentionally misled about the research study and denied treatment (7). An analysis of US Centers for Disease Control mortality data found that disclosure of the Tuskegee mistreatment in 1972 was correlated with greater mortality, medical mistrust, and delayed care health care seeking among black men in the United States (8). The Tuskegee mistreatment of black men and related inequalities may partly explain the higher burden of syphilis among black men at the time (9). Similar unethical syphilis research was organized by US government scientists in Guatemala (10) and these unethical trials have helped to inform human subjects training. The history of unethical syphilis research may discourage investigators from focusing on syphilis and research participants from joining syphilis studies.

Until recently, poor syphilis diagnostics have been another major barrier to expanded syphilis research in many settings. Syphilis diagnostics remained largely unchanged over the course of the 20th century (11). The most commonly used non-treponemal test (rapid plasma regain, RPR) required equipment, reagents, and training that many resource-constrained clinics lacked (12). Centralized syphilis testing at clinics made it difficult for many key populations to receive regular syphilis testing. In recent years, affordable, sensitive and specific point of care serological tests (13) have become available using the lateral flow format. This requires no equipment and has been widely used by the general public for SARS-CoV2 self-testing, opening new opportunities for syphilis screening in resource-limited settings (14).

Partner notification and testing services are essential components of a comprehensive syphilis response, but have historically been constrained by limited financial resources. Partner services include identification, testing, and treatment of sexual partners of confirmed syphilis cases. Syphilis transmission rates are extremely high (51–64% per sexual partnership) (15), underlining the importance of timely partner services. Incomplete partner services before COVID-19 have thwarted syphilis control programs. Syphilis partner services are chronically under-funded within many public health departments (16), contributing to incomplete partner service programs for early syphilis cases (17).

Finally, public engagement in syphilis research has been under-developed. Public engagement refers to a mutually beneficial interaction between specialists and non-specialists to develop solutions. Whereas, HIV control benefitted from strong public engagement in research dating back to the 1980s (18, 19), syphilis has not inspired widespread public engagement programs. There have been fewer public engagement programs to engage local communities about syphilis infection compared to HIV. Public health HIV screening programs have generally not been integrated with syphilis screening programs in many health systems (5), despite strong public engagement focused on other STIs.

COVID-19 and syphilis

Lessons learned from COVID-19 have the potential to transform sexual health service delivery systems, including syphilis-specific strategies globally. Despite the lack of direct links between COVID-19 interventions and syphilis, there are several COVID-19 developments that could indirectly help to prioritize syphilis in broader STI control research and programs. Programs and research focused on COVID-19 inequities, diagnostics, partner services, and public engagement may enhance syphilis projects during and beyond the COVID-19 pandemic.

First, COVID-19 has accelerated public health systems for decentralized diagnostic testing, including self-testing, self-sampling, and community-based testing. Self-testing involves a person conducting and interpreting their own test result. Clinic closures and travel restrictions during COVID-19 have accelerated syphilis self-testing uptake (20, 21) and expanded self-sampling pilots (22). Expanding these opportunities for syphilis testing in diverse settings could help to catalyze more syphilis control programs, policies, and research.

Second, COVID-19 has highlighted the impact of inequities in delivering health services. Many COVID-19 programs have explicitly focused on better serving the needs of ethnic and racial minorities. Racial disparities in COVID-19 vaccine uptake have

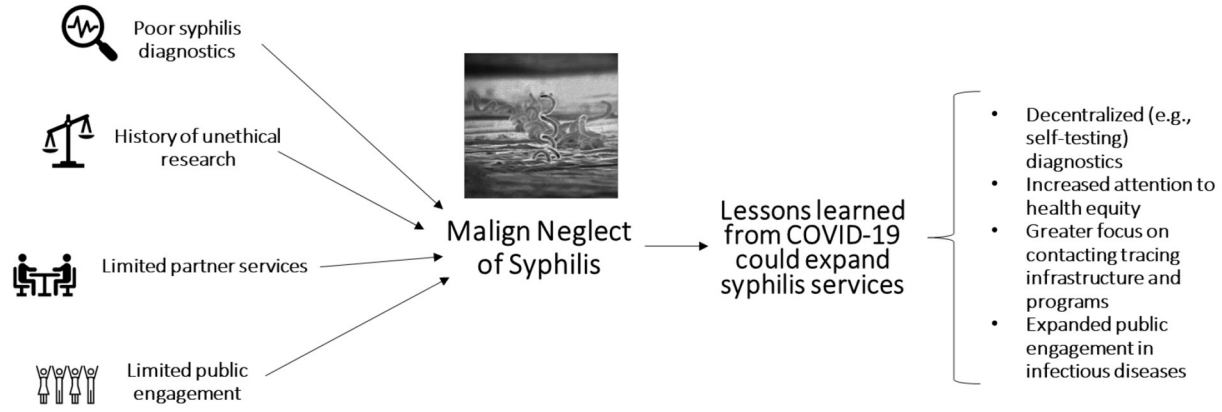


FIGURE 1
Overview of the malign neglect of syphilis within comprehensive STI control programs.

increased urgency to rebuild trust in care providers (23). While these are necessarily long-term efforts, the renewed attention on health equity could help galvanize trust in medicine among people at greater risk for syphilis.

Third, COVID-19 pandemic has increased attention to the science and logistics of contact tracing which could help syphilis contact tracing. Responses to the COVID-19 pandemic have bolstered local public health infrastructure, especially providing resources for contact tracing, rapid testing, and related components of partner services. In some settings, the pandemic response has also altered in-person partner services toward digital adaptations. For example, a syphilis outbreak investigation during COVID-19 organized by a local health department was conducted entirely online (24).

Finally, COVID-19 provides an opportunity to strengthen public engagement in infectious diseases service delivery. Community-based coalitions that include diverse groups have formed in response to COVID-19 and could be adapted for syphilis responses (25, 26). In addition, innovative methods for public engagement such as crowdsourcing have helped to inform COVID-19 programs (27) and demonstrated to be effective in randomized controlled trials (28). Crowdsourcing has a group of people solve all or part of a problem and then implement selected solutions (29). Crowdsourcing methods have been used to increase syphilis test uptake (30, 31).

Discussion

As COVID-19 restrictions are lifted and there is additional scope for sexual health programs, syphilis deserves greater attention. This greater focus has implications for research, implementation, and policy. From a research perspective, increased public health research on decentralized testing pathways that could be implemented in diverse settings is needed. Many people at greatest risk of syphilis do not attend centralized clinics where serological syphilis testing is available. From an implementation perspective, more intensive programs to support the dual elimination of HIV and syphilis among pregnant women through service integration is warranted. This aligns with the WHO call for the elimination of mother-to-child transmission of HIV and syphilis. From a policy perspective, partner services need to be more completely transitioned into the digital age. While COVID-19 has supported pilots, research and policies are needed.

This perspective also underlines the need for *action* at several levels of the public health system. Within local public health programs, syphilis self-testing could provide a new service delivery mechanism that does not require laboratory equipment or trained staff. Syphilis self-testing could be used in many remote settings and spur follow-up testing. At national ministries of health, more financial resources for syphilis control programs will be essential for strengthening control responses. Finally, at the global level, innovative programs to encourage syphilis testing and linkage to clinical care are necessary. Now is the time for action on syphilis.

Ethics statement

Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

JT wrote the first draft. All authors contributed to the manuscript and read and agreed with the final manuscript.

Funding

This research was supported by the NIH (NICHD UH3HD096929, NIAID K24AI143471, and NIAID R01AI158826).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Head MG, Brown RJ, Newell M-L, Scott JAG, Batchelor J, Atun R. The allocation of US$105 billion in global funding from G20 countries for infectious disease research between 2000 and 2017: a content analysis of investments. *Lancet Global Health.* (2020) 8:e1295–304. doi: 10.1016/S2214-109X(20)30357-0

2. Wu MY, Gong HZ, Hu KR, Zheng HY, Wan X, Li J. Effect of syphilis infection on HIV acquisition: a systematic review and meta-analysis. *Sex Transm Infect.* (2021) 97:525–33. doi: 10.1136/sextrans-2020-054706

3. Gomez GB, Kamb ML, Newman LM, Mark J, Broutet N, Hawkes SJ. Untreated maternal syphilis and adverse outcomes of pregnancy: a systematic review and meta-analysis. *Bull World Health Organ.* (2013) 91:217–26. doi: 10.2471/BLT.12.107623

4. Zhang X, Wang X, Wang H, He X, Wang X. Stigmatization and social support of pregnant women with HIV or syphilis in Eastern China: a mixed-method study. *Front Public Health.* (2022) 10:764203. doi: 10.3389/fpubh.2022.764203

5. Peeling RW, Mabey D, Fitzgerald DW, Watson-Jones D. Avoiding HIV and dying of syphilis. *Lancet.* (2004) 364:1561–3. doi: 10.1016/S0140-6736(04)17327-3

6. Tucker JD, Hawkes SJ, Yin YP, Peeling RW, Cohen MS, Chen XS. Scaling up syphilis testing in China: implementation beyond the clinic. *Bull World Health Organ.* (2010) 88:452–7. doi: 10.2471/BLT.09.070326

7. Brandt AM. Racism and research: the case of the Tuskegee Syphilis Study. *Hastings Cent Rep.* (1978) 8:21–9. doi: 10.2307/3561468

8. Alsan M, Wanamaker M. Tuskegee and the Health of Black Men*. *Q J Econ.* (2017) 133:407–55. doi: 10.1093/qje/qjx029

9. Hahn RA, Magder LS, Aral SO, Johnson RE, Larsen SA. Race and the prevalence of syphilis seroreactivity in the United States population: a national sero-epidemiologic study. *Am J Public Health.* (1989) 79:467–70. doi: 10.2105/AJPH.79.4.467

10. Reverby SM. "Normal Exposure" and Inoculation Syphilis: A PHS "Tuskegee" Doctor in Guatemala, 1946–1948. *J Policy Hist.* (2011) 23:6–28. doi: 10.1017/S0898030610000291

11. Peeling RW, Mabey D, Kamb ML, Chen X-S, Radolf JD, Benzaken AS. Syphilis. *Nat Rev Dis Prim.* (2017) 3:17073. doi: 10.1038/nrdp.2017.73

12. Satyaputra F, Hendry S, Braddick M, Sivabalan P, Norton R. The laboratory diagnosis of syphilis. *J Clin Microbiol.* (2021) 59:e0010021. doi: 10.1128/JCM.00100-21

13. Tucker JD, Bu J, Brown LB, Yin YP, Chen XS, Cohen MS. Accelerating worldwide syphilis screening through rapid testing: a systematic review. *Lancet Infect Dis.* (2010) 10:381–6. doi: 10.1016/S1473-3099(10)70092-X

14. Mabey DC, Sollis KA, Kelly HA, Benzaken AS, Bitarakwate E, Changalucha J, et al. Point-of-care tests to strengthen health systems and save newborn lives: the case of syphilis. *PLoS Med.* (2012) 9:e1001233. doi: 10.1371/journal.pmed.1001233

15. Stoltey JE, Cohen SE. Syphilis transmission: a review of the current evidence. *Sex Health.* (2015) 12:103–9. doi: 10.1071/SH14174

16. Rietmeijer CA, Kissinger PJ, Guilamo-Ramos V, Gaydos CA, Hook EW 3rd, Mead A, et al. Report From the National Academies of Sciences, engineering and medicine-STI: adopting a sexual health paradigm-A synopsis for sexually transmitted infection practitioners, clinicians, and researchers. *Sex Transm Dis.* (2022) 49:169–75. doi: 10.1097/OLQ.0000000000001552

17. Cuffe KM, Gift TL, Kelley K, Leichliter JS. Assessing partner services provided by state and local health departments, 2018. *Sex Transm Dis.* (2021) 48:429–35. doi: 10.1097/OLQ.0000000000001328

18. Taggart T, Ritchwood TD, Nyhan K, Ransome Y. Messaging matters: achieving equity in the HIV response through public health communication. *Lancet HIV.* (2021) 8:e376–86. doi: 10.1016/S2352-3018(21)00078-3

19. Caswell G, Dubula V, Baptiste S. Etya'ale H, Syarif O, Barr D. The continuing role of communities affected by HIV in sustained engagement in health and rights. *J Int AIDS Soc 24 Suppl.* (2021) 3:e25724. doi: 10.1002/jia2.25724

20. Cheng W, Wang C, Tang W, Ong JJ, Fu H, Marks M, et al. Promoting routine syphilis screening among men who have sex with men in China: study protocol for a randomised controlled trial of syphilis self-testing and lottery incentive. *BMC Infect Dis.* (2020) 20:455. doi: 10.1186/s12879-020-05188-z

21. Sri-Pathmanathan C, Nhamo D, Mamvuto T, Chapwanya G, Terris-Prestholt F, Mahaka I, et al. Syphilis self-testing to expand test uptake among men who have sex with men: a theoretically informed mixed methods study in Zimbabwe. *Sex Transm Infect.* (2021). doi: 10.1101/2020.11.30.20240788

22. Leenen J, Hoebe C, Ackens RP, Posthouwer D, van Loo IHM, Wolffs PFG, et al. Pilot implementation of a home-care programme with chlamydia, gonorrhoea, hepatitis B, and syphilis self-sampling in HIV-positive men who have sex with men. *BMC Infect Dis.* (2020) 20:925. doi: 10.1186/s12879-020-05658-4

23. Lin C, Tu P, Terry TC. Moving the needle on racial disparity: COVID-19 vaccine trust and hesitancy. *Vaccine.* (2022) 40:5–8. doi: 10.1016/j.vaccine.2021.11.010

24. Davis C, Wright SS, Babcock M, Kingdon E, Broussard D, Oyervides O, et al. Lessons learned from a centers for disease control and prevention virtual partner services technical assistance pilot project to respond to a local syphilis outbreak. *Sex Transm Dis.* (2022) 49:166–8. doi: 10.1097/OLQ.0000000000001547

25. AuYoung M, Rodriguez Espinosa P, Chen WT, Juturu P, Young MT, Casillas A, et al. Addressing racial/ethnic inequities in vaccine hesitancy and uptake: lessons learned from the California alliance against COVID-19. *J Behav Med.* (2022) 22:1–14. doi: 10.1007/s10865-022-00284-8

26. van Staden Q, Laurenzi CA, Toska E. Two years after lockdown: reviewing the effects of COVID-19 on health services and support for adolescents living with HIV in South Africa. *J Int AIDS Soc.* (2022) 25:e25904. doi: 10.1002/jia2.25904

27. Day S, Li C, Hlatshwako TG, Abu-Hijleh F, Han L, Deitelzweig C, et al. Assessment of a crowdsourcing open call for approaches to University community engagement and strategic planning during COVID-19. *JAMA Netw Open.* (2021) 4:e2110090. doi: 10.1001/jamanetworkopen.2021.10090

28. Wang C, Han L, Stein G, Day S, Bien-Gund C, Mathews A, et al. Crowdsourcing in health and medical research: a systematic review. *Infect Dis Poverty*. (2020) 9:8. doi: 10.1186/s40249-020-0622-9

29. Tucker JD, Day S, Tang W, Bayus B. Crowdsourcing in medical research: concepts and applications. *PeerJ*. (2019) 6:e6762. doi: 10.7717/peerj.6762

30. Tang W, Ritchwood TD, Wu D, Ong JJ, Wei C, Iwelunmor J, et al. Crowdsourcing to improve HIV and sexual health outcomes: a scoping review. *Curr HIV AIDS Rep*. (2019) 16:270–8. doi: 10.1007/s11904-019-00448-3

31. Iwelunmor J, Ezechi O, Obiezu-Umeh C, Gbaja-Biamila T, Musa AZ, Nwaozuru U, et al. Enhancing HIV self-testing among Nigerian youth: feasibility and preliminary efficacy of the 4 youth by youth study using crowdsourced youth-led strategies. *AIDS Patient Care STDS*. (2022) 36:64–72.s doi: 10.1089/apc.2021.0202

frontiers | Frontiers in Medicine

TYPE Systematic Review
PUBLISHED 20 September 2022
DOI 10.3389/fmed.2022.952476

OPEN ACCESS

EDITED BY
Justin David Radolf,
UCONN Health, United States

REVIEWED BY
Benedetto Maurizio Celesia,
UOC Infectious Diseases ARNAS Garibaldi, Italy
David Mabey,
University of London, United Kingdom

*CORRESPONDENCE
Julien Tran
jtran@mshc.org.au

SPECIALTY SECTION
This article was submitted to Infectious Diseases-Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

RECEIVED 25 May 2022
ACCEPTED 31 August 2022
PUBLISHED 20 September 2022

CITATION
Tran J, Fairley CK, Bowesman H, Aung ET, Ong JJ and Chow EPF (2022) Non-conventional interventions to prevent gonorrhea or syphilis among men who have sex with men: A scoping review. *Front. Med.* 9:952476. doi: 10.3389/fmed.2022.952476

Non-conventional interventions to prevent gonorrhea or syphilis among men who have sex with men: A scoping review

Julien Tran[1,2]*, Christopher K. Fairley[1,2], Henry Bowesman[1,2], Ei T. Aung[1,2], Jason J. Ong[1,2] and Eric P. F. Chow[1,2,3]

[1]Melbourne Sexual Health Centre, Alfred Health, Melbourne, VIC, Australia, [2]Central Clinical School, Faculty of Medicine, Nursing and Health Sciences, Monash University, Melbourne, VIC, Australia, [3]Centre for Epidemiology and Biostatistics, Melbourne School of Population and Global Health, The University of Melbourne, Melbourne, VIC, Australia

Objectives: We assessed nonconventional interventions that did not traditionally focus on increasing condom use and/or testing among men who have sex with men (MSM) and the evidence for these interventions.

Methods: Guided by the Participants, Concept and Context (PCC) framework, we searched five online databases from inception to 9 August 2021 for original research on interventions that do not focus on increasing condom use and/or testing to prevent gonorrhea and/or syphilis in MSM. Two researchers screened titles and abstracts to assess eligibility, reviewed articles' full text and resolved discrepancies through discussion. We charted relevant study information, and the included studies were critically appraised.

Results: Of 373 articles retrieved, 13 studies were included. These studies were conducted in Australia ($n = 3$), Belgium ($n = 2$), China ($n = 3$), the Netherlands ($n = 1$) and the US ($n = 4$). Two randomized controlled trials (RCTs) of doxycycline as pre-exposure prophylaxis (PrEP) and post-exposure prophylaxis (PEP) reduced any STI incidence (gonorrhea, syphilis, or chlamydia), but only doxycycline PEP significantly reduced syphilis incidence. Six studies of interventions that facilitated self-collection, self-examination, and self-testing, found varied evidence for gonorrhea and/or syphilis prevention. Four RCTs and one single-arm trial examined the efficacy of mouthwash, but the evidence remains inconclusive on whether mouthwash use can prevent transmission between men.

Conclusion: We found evidence for doxycycline PEP in reducing syphilis incidence, evidence on the use of mouthwash to prevent gonorrhea transmission between men remains inconclusive. More evidence is needed for interventions that do not focus on increasing condom use and/or testing to prevent gonorrhea and/or syphilis.

KEYWORDS

gonorrhea, syphilis, MSM, sexually transmitted infection, review, intervention

Introduction

Sexually transmissible infections (STIs), including chlamydia, gonorrhea, and syphilis, disproportionately affect men who have sex with men (MSM) (1–3). Globally, the World Health Organization has estimated that ∼131 million people are infected with chlamydia each year, followed by 78 million people with gonorrhea and 6 million people with syphilis (4). While the incidence rate for chlamydia remains relatively stable (5–7), the incidence rates for both gonorrhea and syphilis have increased in high-income settings since the 2010s in MSM (1, 2) and heterosexuals (8–10). This review aimed to focus on the prevention of gonorrhea and/or syphilis among MSM.

Gonorrhea, caused by *Neisseria gonorrhoeae (N. gonorrhoeae)* (2, 11), can occur at the genitals (urethra/cervix), anorectum, and oropharynx. Since the 2010s, gonorrhea incidence among MSM attending sexual health clinics has significantly increased, particularly anorectal and oropharyngeal infections (12–16). The commonly accepted route for gonorrhea transmission between MSM is from an infected genital site to the anus and oropharynx through condomless sexual contact (17), but the importance of the oropharynx has recently been raised (18).

Due to its potential of becoming increasingly resistant to the antibiotics used for its treatment, gonorrhea has emerged as a global public health concern (19). Studies have demonstrated that due to lateral gene transfer, the oropharynx is an important anatomical site for antimicrobial resistance (AMR) (20–24). While decreasing gonorrhea incidence is key to reducing AMR (25), gonorrhea prevention strategies often focus on encouraging condom use. However, with the recent significant decline in condom use for anal sex (11, 26) and even less common use for oral sex among MSM (27), halting this decline or attempting to increase condom use to prevent gonorrhea can be challenging.

In addition to the decline in condom use, there have been concerns over the effectiveness of condoms based on research suggesting that the role of the penis may not be as important for transmission between men. For instance, research has shown that substantial bacterial loads of *N. gonorrhoeae* can be cultured in the saliva of individuals diagnosed with oropharyngeal gonorrhea (28, 29). Moreover, research has found that using saliva as a lubricant for anal sex is a risk factor for anorectal gonorrhea (30) and that tongue-kissing is an independent risk factor for oropharyngeal gonorrhea in MSM (31, 32). Given that most infections at the oropharynx are asymptomatic (33), interventions that target the oropharynx may be required for gonorrhea prevention.

Syphilis, caused by *Treponema pallidum* (*T. pallidum),* continues to rise despite regular screening and contact tracing (34–38). Individuals with primary syphilis often present with lesions (or chancres) at the site of infection, and those with secondary syphilis present with relatively non-specific symptoms such as a skin rash. In contrast, individuals with early latent syphilis do not have symptoms (39). If left untreated, syphilis can lead to serious health concerns, including cardiac involvement (40), neurosyphilis (41), and ocular syphilis (41).

Previous research demonstrated that MSM who engaged in receptive anal sex only (where a partner's penis is inserted into their anus) were almost four times more likely to present with secondary syphilis than primary syphilis compared to men who did not engage in receptive anal sex (42). This suggests that a significant proportion of these men may have missed primary anorectal lesions and, therefore, progress from primary to secondary syphilis (43). Unrecognized oral and anal shedding of *T. pallidum* occurs most frequently in MSM with secondary syphilis. Therefore, progression toward this stage should be prevented to reduce the duration of infectiousness (43).

MSM who take PrEP are at a higher risk of acquiring syphilis, with an estimated incidence of 8.6 per 100 PY (39). Research has demonstrated that the recommended 3-monthly PrEP clinic appointments for syphilis screening have failed to detect a proportion of primary and secondary syphilis infections in MSM (39), suggesting that regular syphilis screening may be insufficient. Therefore, additional strategies are required to prevent onward transmissions of syphilis, especially in the context of higher syphilis incidence among HIV PrEP users (44).

There is an existing body of literature that consists of studies of interventions delivered by new media, such as websites, social media, or smartphone apps (45, 46) to increase condom use and clinic-based active recall interventions that aim to increase testing (where healthcare attendance is required) among MSM (38, 47). Therefore, the current review will not assess changes to condom use and/or testing in MSM, as an intervention outcome. Our scoping review aims to: (1) identify knowledge gaps and scope within the current body of literature on interventions that do not traditionally rely on increasing condom use and/or testing to prevent gonorrhea and/or syphilis in MSM and (2) synthesize knowledge to answer questions about the evidence for these interventions.

This scoping review focused on the following questions:

1. What non-conventional interventions that do not traditionally focus on increasing condom use and/or testing have been investigated or examined to prevent Gonorrhea and/or syphilis in MSM?

2. What is the evidence for these interventions?

As a guide to form our primary questions, we used the Participants, Concept and Context (PCC) framework, as recommended by Joanna Briggs Institute for scoping reviews (48). Here, we considered participants as MSM aged ≥16 years, including men living with HIV. Our concept focused on interventions that do not focus on condom use and/or testing to prevent gonorrhea and/or syphilis; and with regards to context,

we focused on interventions in all possible settings or in different geographical regions and cultural contexts.

Methods

We followed the methodology outlined in the manual for scoping reviews from the Joanna Briggs Institute (48).

Eligibility criteria

We included original research evaluating interventions that do not focus on increasing condom use and/or testing to prevent gonorrhea and/or syphilis in MSM. We included biomedical interventions and self-managed interventions (i.e., self-collection, self-examination, and self-testing). Self-collection involves individuals only collecting their own samples, while self-testing required individuals to collect, test, and interpret sample results themselves. We also included interventions incorporating eHealth (i.e., health communication, new technology, technology-based engagement, and/or mHealth).

Exclusion criteria

We excluded editorials, reviews, and studies of interventions that only focused on HIV and STIs that were not gonorrhea and/or syphilis, such as chlamydia, Hepatitis B, Hepatitis C, and *Mycoplasma genitalium*. We also excluded articles focusing on interventions focused on the use, promotion, or distribution of condoms, and campaigns that encourage testing without post-intervention evaluation data.

Search strategy and study selection

We searched Ovid Medline, Ovid Global Health, Embase, Scopus and Web of Science Core Collection from inception to 9 August 2021. For each main concept, we used Medline to identify relevant medical subject headings (MeSH). The detailed search terms are provided in Supplementary Table S1. The results from our database searches were imported, and duplicates were removed in Endnote. Two researchers (JT and HB) independently screened the titles and abstracts of all the retrieved articles to assess eligibility, reviewed the full text of articles, and resolved discrepancies through discussion. Searches through the reference lists of included studies were conducted to find relevant studies (see Figure 1).

Data charting and critical appraisal

For each study, we extracted information about the author, year of publication, study period, study location, study objective, study design (RCT, pre-and post-intervention, and post-intervention), study population, sample size, intervention description, and relevant findings. According to the study design, JT and ETA critically appraised the included studies against Joanna Briggs Institute checklists for RCTs (49), quasi-experimental studies and cross-sectional studies (50) for quality assurance when determining the evidence for the interventions.

Results

We identified 374 articles from our database searches, of which 13 were included (10 from our database searches and three from our searches of reference lists of the included studies). Of 13 included studies, two investigated the efficacy of doxycycline prophylaxis, five examined the efficacy of mouthwash use, and six focused on the effectiveness of self-managed interventions in MSM. Of the six self-managed interventions, two required men to self-collect their samples only, two required men to self-examine their oral, anal, and urogenital areas, and two required men to self-test. Table 1 summarizes the characteristics of the 13 included studies.

Quality assessment

Trial design was appropriate in all six RCTs included in this review. Of the included RCTs, true randomization occurred in all of six studies, while 67% concealed treatment group allocation, 67% blinded participants, 33% blinded those who delivered the treatment assignment, and 33% blinded outcome assessors to treatment assignment. Baseline data was similar in all of the six studies. There were no other differences in care or treatment received other than the intervention of interest across the compared groups in all of the six trials. Follow-up was complete in 67% of the studies and intention-to-treat analysis occurred in 50% of the studies. All studies measured their outcomes reliably and analyses were appropriately conducted, and all six studies measured their outcomes the same way in treatment groups. In the one single arm trial included, the temporal relationship of the cause and effect were clear. There was no control group, and therefore, group comparisons were not applicable. There were multiple measurements of the outcome, and these were measured in a reliable way and were appropriately analyzed.

All six studies checked against checklists for analytical cross-sectional studies clearly defined their inclusion criteria and described their subjects and setting in detail. All reliably measured their exposures. Of the six studies, 33% determined their sample using objective criteria and this criterion was

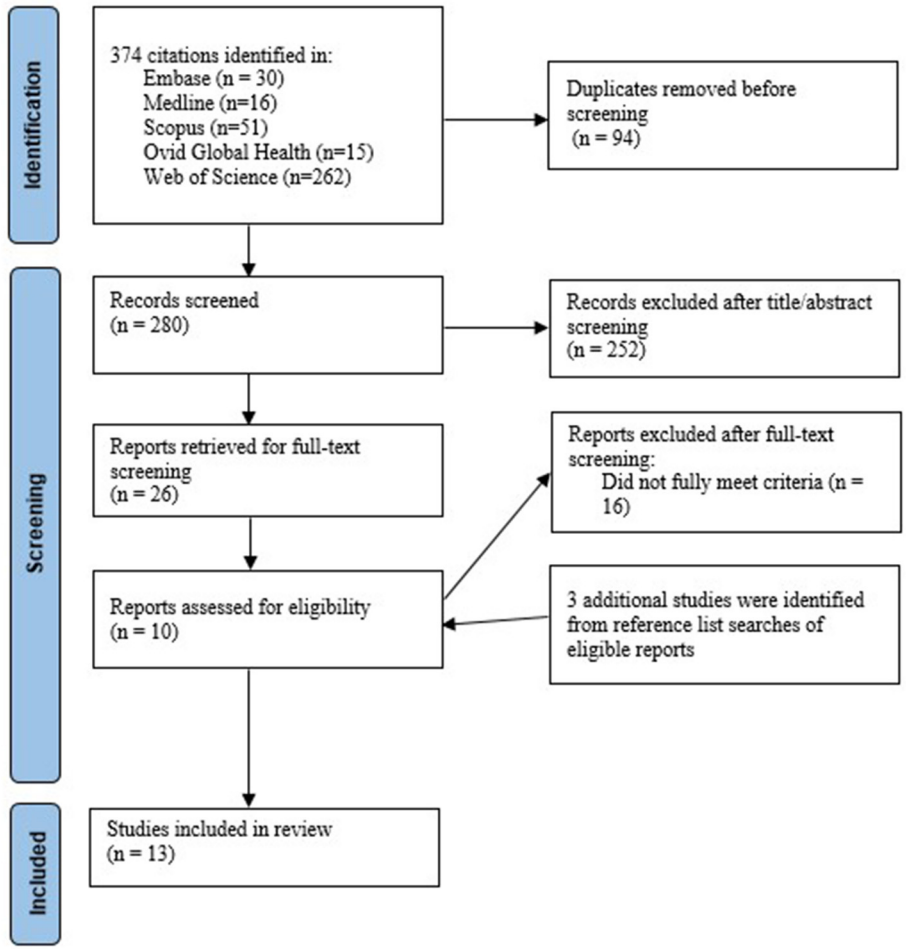


FIGURE 1
Flow chart of article selection.

not applicable to 33% of the studies. Identifying and adjusting for confounding was not applicable to all the studies. Most studies (83.3%) reliably measured and appropriately analyzed their outcomes.

Biomedical interventions

Doxycycline is a tetracycline antibiotic used to treat bacterial infections. Two RCTs have demonstrated that using doxycycline as pre- and post-exposure prophylaxis may be an effective biomedical intervention to prevent STIs, including gonorrhea and/or syphilis, in MSM. The first was a US-based, 48-week open-label RCT of 30 MSM living with HIV and who also had previous syphilis infection randomized at a 1:1 ratio. This trial found that men who received 100 g of doxycycline pre-exposure prophylaxis daily were significantly less likely to test positive for any of the selected bacterial STIs (with 73% reduction in syphilis, gonorrhea, chlamydia or a combination of these STIs) compared to men who received contingency management, where there was a financial incentive if they remained STI-free throughout the trial ($p = 0.02$) (51). When incidence for gonorrhea, chlamydia and syphilis was examined individually, there were no significant differences in incidence between the men in both groups. There were no significant differences in sexual risk behaviors between men in both groups.

In France, a 10-month open-label RCT of 232 MSM and transgender women randomized at a 1:1 ratio, found that there was a 47% relative reduction in the risk of acquiring any STIs (syphilis, gonorrhea, chlamydia, or a combination of these STIs) in individuals who received 200 g of doxycycline as post-exposure prophylaxis (PEP) within 24–72 h after sex compared to individuals who did not receive doxycycline PEP. There was a 73% relative risk reduction in acquiring syphilis in individuals who received doxycycline PEP compared to those who did not receive doxycycline PEP ($p = 0.047$). However, the relative risk of acquiring gonorrhea did not significantly

TABLE 1 Characteristics of the included studies of non-conventional interventions for gonorrhea and/or syphilis prevention in MSM.

References	Study period	Country	Study objective	Study design	Population	Sample size
Biomedical						
Bolan et al. (51)	Sep 2011–Jan 2012	USA	To determine whether daily doxycycline is efficacious in reducing STIs in high-risk groups	RCT 1:1 ratio 48-week follow-up	MSM and transgender women with syphilis history	30
Molina et al. (52)	Jul 2015–Jan 2016	France	To assess whether doxycycline as post-exposure prophylaxis can reduce STI incidence	RCT 1:1 ratio 10-month follow-up	MSM who had condomless sex and who used HIV PrEP	232
Mouthwash						
Chow et al. (53)	May 2015–February 2016	Australia	To determine whether Listerine can inhibit *N. gonorrhoeae*	RCT 1:1 ratio No follow-up	MSM	196
Chow et al. (54)	Sep 2018–Feb 2020	Australia	To examine whether a 14-day course of mouthwash twice daily is efficacious in treating oropharyngeal gonorrhea	Parallel group, open-label RCT 1:1 ratio 28-day follow-up (Day 14: follow-up visit 1; Day 28: follow-up visit 2)	MSM	12
Chow et al. (55)	March 2016–October 2018	Australia	To compare the efficacy of Listerine Zero and Biotène mouthwashes in preventing gonorrhea in MSM.	RCT 1:1 ratio 12-week follow-up	MSM	530
Van Dijck et al. (56)	Apr 2019–Mar 2020	Belgium	To assess the use of an antiseptic mouthwash to prevent STIs	RCT 1:1 ratio 6-month follow-up (2 3-month visits)	MSM	343
Van Dijck et al. (57)	NS	Belgium	To assess efficacy of a mouthwash containing chlorhexidine in eradicating *N. gonorrhoeae* from the oropharynx	Single-arm pilot trial	MSM Asymptomatic oropharyngeal gonorrhea	3
SELF-MANAGED Self-collection	2016	USA	To evaluate the effectiveness of a novel STI self-collection program in HIV treatment clinic	Pre- and post-intervention study	MSM living with HIV	1,520 during the baseline year 1,510 during intervention year
Bardee et al. (58)						
Leenen et al. (59)	March-May 2018	The Netherlands	To pilot a free home-based STI self-collection program at an HIV treatment clinic	Post-intervention study	MSM living with HIV	28

(Continued)

10.3389/fmed.2022.952476

TABLE 1 (Continued)

References	Study period	Country	Study objective	Study design	Population	Sample size
Self-examination						
Surie et al. (60)	2010–2011	USA	To increase self-examination to detect syphilis in MSM	Pre- and post-intervention	MSM	171 who had read the printed materials 735 who had not read the print materials
Taylor et al. (61)	February 2008–January 2009	USA	Evaluate intervention to increase oral and rectal self-examination for syphilis ulcers	Pre- and post-intervention study	Men living with HIV 76% MSM	689
Self-testing						
Wu et al. (62)	Jun 2017–Nov 2019	China	To evaluate a program which involved social media-based secondary distribution of HIV/syphilis self-testing kits	Pre- and post-intervention study	MSM	371

differ between individuals in both groups (52). These RCTs demonstrated that results for specific STIs varied depending on whether doxycycline was used as pre-exposure prophylaxis or post-exposure prophylaxis.

Self-managed behavioral interventions

Self-collection

Self-collection, where men only collect their own pharyngeal, rectal and urine specimens, has been found to increase detection among MSM. For instance, in a US study by Barbee et al. (58), men were given kits to self-collect their samples by following instructional posters placed on the walls of a room designated for self-collection at their local sexual health clinic during the intervention year. Baseline data on infections was collected the year prior to the intervention year. Self-collection during the intervention year detected 147 gonorrhea infections, which was 49 (31 oropharyngeal; 18 anorectal) more infections compared to the 98 infections detected during the baseline year, resulting in a 50% increase in detection. Sexual practices were not measured.

Self-collection at home can also increase STI detection among MSM. Conducted in the Netherlands, a study of 28 MSM living with HIV who were offered free home-based kits for self-collection of pharyngeal, rectal and urine specimens, and blood samples for syphilis testing at their routine care visit by healthcare professionals, found that 17.9% (5/28) were newly diagnosed with one or more STIs (59).

Self-examination

Several studies have been investigated self-examination, which requires men to examine their oral, anal, and urogenital areas, as an intervention to prevent syphilis in MSM. A US-based study involved 689 men (76% MSM) living with HIV who received posters of primary and secondary syphilis lesions before their quarterly clinic visits. Syphilis prevention messages were included at the top of each poster, for example: "Sores caused by syphilis are painless and can be found in the mouth, anus, rectum, and penis", or "Neurosyphilis can cause blindness, hearing loss, cognitive decline, stroke, and chronic headaches". At baseline and at each of their quarterly visits, the men were asked questions about unprotected oral and anal sex with their regular partner, or casual or anonymous partners, and whether they had self-examined their oral and anal areas for syphilis lesions. There were no significant differences in the men's number of unprotected oral and anal sex activities with regular, casual, or anonymous partners at baseline through to their third clinic visit. However, self-examination of oral and anal areas increased from 46% at

baseline to 72% among men with three clinic visits ($p < 0.001$) (61).

Another US-based study involved 906 MSM who were provided with brochures about syphilis symptoms, transmission, and prevention after their clinic visits. The men were asked whether they had read the brochures and those who responded "yes" were grouped as having read the brochures and those who responded "no" were grouped as not having read the brochures. The study found that men who read the brochures from a previous visit ($n = 171/906$) were significantly more likely than men who did not read the brochures ($n = 735/906$) to self-examine their oral (adjusted prevalence ratio; aPR = 1.2, 95% CI: 1.14–1.36, $p < 0.05$), anal (aPR = 1.3, 95% CI 1.15–1.52. $p < 0.05$), genital areas (aPR = 1.1, 95% CI: 1.01–1.14, $p < 0.05$) and their skin (aPR = 1.2, 95% CI 1.05–1.19) for at least once a week and were more likely to examine their partners' oral (aPR = 1.6, 95% CI 1.10–1.2.26, $p < 0.05$) and anal areas (aPR = 1.3, 95% CI 1.03–1.73, $p < 0.05$) for at least once a week (60). There were no significant differences in examining partner's genitals and skin between men in both groups.

Self-testing

Self-testing involves individuals collecting and testing their specimens and interpreting the results. One way to increase self-testing is through secondary distribution, which involves giving an individual multiple self-testing kits to distribute to people within their social networks. A study conducted in Zhuhai, China, recruited 331 MSM ("indexes") who distributed HIV/syphilis self-tests to 281 individuals within their social networks ("alters") (62). The self-tests had to be ordered through WeChat (a multifunctional social app) and were mailed out to the 331 men. Using Quick Response (QR) codes, pictures of test results were anonymously uploaded to WeChat. However, the study concluded that there were no significant differences in the reactive syphilis results between the indexes and alters.

A study by Yang et al. (63), also conducted in Zhuhai, China, assessed HIV/syphilis self-testing among social networks of sexual health influencers and non-influencers. Men were sexual health influencers if they scored >3 and sexual health non-influencers if they scored <3 on six items using a 5-point Likert-type scale. The six items assessed whether men could influence others to seek advice about HIV/STI issues and how often they discussed HIV/STI topics with other people. The study found that sexual health influencers were more likely to influence people within their social networks to upload their test results using QR codes to WeChat compared to sexual health non-influencers (adjusted rate ratio = 2.07, 95% CI: 1.59–2.69). Compared to the alters of sexual health non-influencers, sexual health influencers had more alters who were from a rural area (45.5 vs. 23.8%, $p < 0.001$), did not attend university (57.7 vs. 37.1%, $p < 0.001$), and who had multiple casual sex partners (25.2 vs. 11.9%, $p < 0.001$) in the previous 6 months (63).

Mouthwash as an intervention

In the late 2010s, mouthwash was proposed as an intervention for gonorrhea prevention and treatment by several researchers. We identified three RCTs examining the efficacy of mouthwash in preventing STIs; one RCT in Belgium (56) and two were conducted in Australia (53, 55). Additionally, two RCTs examined the efficacy of using mouthwash as treatment for oropharyngeal gonorrhea (54, 57).

The randomized, placebo-controlled, crossover trial conducted in Belgium investigated the efficacy of daily use of Listerine mouthwash and mouthwash use before and after sex among 343 MSM taking PrEP and who also had an STI in the previous 24 months. This trial found men who used Listerine did not significantly reduce STI incidence (incidence rate ratio 1.17, 95% CI 0.84–1.64) compared to men who used the placebo mouthwash. In the Listerine-placebo group, the STI incidence was 140.4 per 100 PY during the Listerine phase and 102.6 per 100 PY during the placebo phase. In the placebo-Listerine group, the STI incidence rate was 133.9 per 100 PY during the placebo phase and 147.5 per 100 PY during the Listerine phase (56). A significantly higher proportion of oropharyngeal gonorrhea cases were detected when using Listerine than when using placebo (OR 5.78, 95% CI 1.52–136.56, $p = 0.024$). However, Listerine use was not significantly associated with gonorrhea cases at any anatomical site (OR 1.48, 95% CI 0.81–2.83). There were no significant differences in syphilis cases between Listerine use and placebo.

The first ever RCT on mouthwash was conducted in Australia that involved 196 MSM with untreated oropharyngeal gonorrhea. Men were randomized at 1:1 ratio to either using Listerine Cool Mint mouthwash (containing 21.6% alcohol) or a saline solution. Men were asked to rinse and gargle 20 ml of the allocated solution for 1 min. Swabs at the tonsillar fossae and posterior oropharynx were taken before and 5 min after the men rinsed and gargled. This trial found that culture positivity on the pharyngeal surface was significantly lower in men who use Listerine mouthwash (52%) compared to men who used the saline solution (84%) ($p = 0.013$) (53).

The second mouthwash RCT was the OMEGA trial and involved 530 MSM in Australia. Men were randomized at 1:1 ratio to either using Listerine Zero (0% of alcohol) mouthwash or Biotène mouthwash (i.e., a mouthwash did not have any inhibitory effect against *N. gonorrhoeae*). Men were asked to rinse and gargle the allocated mouthwash for 60 s at least once daily over 12 weeks. This trial found that the cumulative incidence of oropharyngeal gonorrhea did not significantly differ between men in the Listerine mouthwash group and men in the Biotène mouthwash group (adjusted risk difference 3.1%,

95% CI −1.4 to 7.7) (55). However, the trial also found that a significant reduction in urethral gonorrhea (<1 vs. 4%; adjusted risk difference −4.3%, 95% CI −7.4 to −1.3) between men in the Listerine Zero group compared to the Biotène mouthwash group, but not for anorectal gonorrhea (7 vs. 4%; adjusted risk difference 2.5%, 95% CI −1.9 to 7.0). There were no significant differences in syphilis incidence between the men in both groups (adjusted risk difference −0.4%, 95% CI −2.2 to 1.3).

While the first two RCTS from Australia investigated the efficacy of mouthwash for STI prevention, the third RCT in Australia investigated mouthwash as potential STI treatment. The OMEGA2 trial was an RCT of 12 Australian MSM with untreated oropharyngeal gonorrhea who were randomized at 1:1 ratio to either receive a 14-day course of mouthwash twice a day or standard antibiotic treatment to cure their oropharyngeal gonorrhea (54). Men were asked to abstain from sex and kissing for 14 days after enrolling in the study. Of those who returned on day 14, the cure rate for oropharyngeal gonorrhea was 20% (1/5) for those randomly assigned to the mouthwash group, while the cure rate was 100% (6/6) for the standard treatment group (54). This trial failed to demonstrate using mouthwash as an alternative treatment for oropharyngeal gonorrhea and therefore, the trial was terminated early.

An open-label single-arm trial which also investigated mouthwash as treatment for STIs was conducted in Belgium and involved in 6 MSM with asymptomatic oropharyngeal gonorrhea. The men were required to gargle mouthwash (containing 0.2% mg/mL chlorhexidine) twice daily over 6 days. Three men exited the trial before their day 7 visit. The use of mouthwash containing chlorhexidine failed to eradicate *N. gonorrhoeae* from the oropharynx of three asymptomatic men (efficacy 0%; 95% confidence interval, 0–56.1%). Therefore, this trial was terminated early.

Discussion

We identified studies of non-conventional interventions to prevent gonorrhea and/or syphilis in MSM conducted in different geographical regions and cultural contexts. While these interventions seemed to be highly acceptable to the men, there are potential issues related to terminology, transferability, and sustainability of these interventions that need to be considered if future interventions that do not focus on increasing condom use and/or testing are going to target high-risk, hard-to-reach groups and to be implemented at the population level.

The efficacy for doxycycline prophylaxis has only been demonstrated in clinical trials. Doxycycline pre-exposure prophylaxis did not reduce syphilis incidence in one study, which was most likely due to its small sample size (i.e., 15 patients per arm) (51), but doxycycline post-exposure prophylaxis significantly reduced syphilis incidence in MSM (52). Given the significance of antibiotic resistance, it is important to establish the effectiveness of doxycycline PEP so that this benefit can be evaluated within the context of the substantial increase in the use of antibiotics. An RCT "Syphilaxis" examining the efficacy of doxycycline PrEP in reducing the incidence of STIs (including gonorrhea, chlamydia, and syphilis) among MSM is underway in Australia (Identifier: NCT03709459). Additionally, this trial will also evaluate resistance in the gut microbiota among men using doxycycline PrEP. Four other RCTs are in progress or development for doxycycline prophylaxis to prevent STIs in MSM (64).

We found that there are some inconsistencies and misuse of the terminology related to self-testing. For instance, self-testing requires individuals to collect and test their own specimen and interpret the results themselves. Still, some interventions that only required individuals to self-collect their samples were labeled as self-testing. There is some evidence for interventions that use instructional materials to influence self-collection behaviors and in turn, detect gonorrhea (58). Similarly, there is also evidence for using educational materials such as syphilis prevention brochures to increase self-examination and partner-examination (60, 61), however, without gonorrhea and syphilis infections reported as associated outcomes, the evidence is insufficient.

Secondary distribution as an approach to increase the number of new self-testers among people within already established social networks holds some promise, particularly distribution by sexual health influencers (63). This approach can be adapted to other geographical regions and cultural contexts and cater to the needs of high-risk, hard-to-reach groups, such as men residing in more isolated rural areas with a high number of male casual sex partners (62). There are two RCTs underway in China; one examining the efficacy of social network distribution of syphilis self-testing in MSM (Identifier: ChiCTR2000036988) (65) and the other examining the efficacy of free syphilis self-tests in MSM (Identifier: ChiCTR1900022409) (66). The findings from these studies may help determine the transferability of these interventions.

Our review found RCTs that assessed the efficacy of mouthwash use to prevent oropharyngeal gonorrhea in MSM. One RCT demonstrated that culture positivity on the pharyngeal surface was significantly lower in men who gargled mouthwash compared to those who gargled the saline solution (53), suggesting that mouthwash use can increase gonococcal clearance. However, this was an immediate effect 5 min after the use of mouthwash and the effectiveness of consistent use or long-term use of mouthwash is unclear. Two further RCTs examining the efficacy of daily use of Listerine mouthwash for gonorrhea prevention but the results are inconclusive (55, 56). Additionally, two other RCTs also revealed that mouthwash appears to be an ineffective treatment for oropharyngeal gonorrhea compared to antibiotics (54, 55). The oropharynx plays an important role in gonorrhea transmission; and therefore, more conclusive evidence is needed

to inform preventive options that target the oropharynx at the population level.

Mathematical modeling has indicated that if upon presentation for STI testing, 30% of MSM are vaccinated with a gonococcal vaccine with 50 or 100% efficacy, gonorrhea prevalence could be reduced by 94 or 62%, respectively, within 2 years (67). There is some evidence for the cross-over protection from an outer membrane vesicle Neisseria *meningitidis* serogroup B (MeNZB) vaccine against *N. gonorrhoeae*. For instance, a retrospective case-control cohort study of 14,730 sexual health clinic attendees, found that gonorrhea incidence decreased by 31% among vaccinated individuals (68). Currently, a 24-month multi-center, double-blinded, RCT is underway to investigate the efficacy of the 4CMenB vaccine to reduced gonorrhea in MSM (Identifier: NCT04415424).

This scoping review has several limitations. First, this review may not have been able to identify and in turn, may have missed published studies of self-managed behavioral interventions that were labeled using terms other than self-screening, self-testing, self-examination, and/or self-collection. Furthermore, while the studies we assessed were conducted in various geographical regions and cultural contexts, the potential for transferability of the findings is yet to be determined due to the lack of conclusive evidence.

Conclusions

While there is some promise in several of the alternative strategies assessed, more robust evidence is needed to support their effectiveness and transferability. Recent evidence supports the effectiveness of doxycycline prophylaxis (69), but there are concerns about the development of AMR and whether the benefit outweighs the potential overuse of doxycycline and the risk of AMR. Questions have also been raised about the cost-effectiveness and sustainability of doxycycline prophylaxis. While it is currently unavailable, an effective vaccine for preventing gonorrhea in MSM and other groups who are at risk could reduce infections markedly. Several trials investigating the efficacy of the 4CMenB vaccine for gonorrhea prevention are currently underway. While the use of antibacterial mouthwash can inhibit the growth of *N. gonorrhoeae* in the oropharynx, there is no evidence that daily use of antibacterial mouthwash could prevent individuals from acquiring gonorrhea. There is no evidence for self-managed strategies, such as regular anorectal self-examination and syphilis self-testing facilitated by social media platforms, like WeChat, for syphilis control in MSM, as these strategies are currently not supported by sufficient data linked to changes in men's syphilis infections. However, investigations are currently underway to examine the effectiveness of these self-managed strategies for syphilis control among MSM.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author/s.

Author contributions

EC, JO, and JT conceived and designed the review. JT performed the search, screened, charted information for eligible studies, and wrote the first draft of the manuscript. HB assisted with screening for eligible studies. EA assisted with the critical appraisal. All authors were involved in the interpretation of findings, commented on manuscript drafts, and contributed to the final version of the manuscript.

Funding

EC and JO are supported by an Australian National Health and Medical Research Council (NHMRC) Emerging Leadership Investigator Grant (GNT1172873 for EC and GNT1104781 for JO). CF was supported by an Australian NHMRC Leadership Investigator Grant (GNT1172900). JT and EA was supported by the Australian Government Research Training Program (RTP) Scholarship.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.952476/full#supplementary-material

References

1. Tsuboi M, Evans J, Davies EP, Rowley J, Korenromp EL, Clayton T, et al. Prevalence of syphilis among men who have sex with men: a global systematic review and meta-analysis from 2000-20. *Lancet Glob Health.* (2021) 9:e1110–e8. doi: 10.1016/S2214-109X(21)00221-7

2. Kirkcaldy RD, Weston E, Segurado AC, Hughes G. Epidemiology of gonorrhoea: sa global perspective. *Sex Health.* (2019) 16:401–11. doi: 10.1071/SH19061

3. Unemo M, Bradshaw CS, Hocking JS, de Vries HJC, Francis SC, Mabey D, et al. Sexually transmitted infections: challenges ahead. *Lancet Infect Dis.* (2017) 17:e235–e79. doi: 10.1016/S1473-3099(17)30310-9

4. World Health Organization. *Global Health Sector Strategy on Sexually Transmitted Infections 2016-2021*. Geneva, (2016).

5. The Kirby Institute. *National Update on HIV, Viral Hepatitis and Sexually Transmissible Infections in Australia: 2009–2018*. Sydney, NSW: Kirby Institute (2020).

6. Newman L, Rowley J, Hoorn SV, Wijesooriya NS, Unemo M, Low N, et al. Global estimates of the prevalence and incidence of four curable sexually transmitted infections in 2012 based on systematic review and global reporting. *PLoS ONE.* (2015) 10:e0143304. doi: 10.1371/journal.pone.0143304

7. Rowley J, Hoorn SV, Korenromp E, Low N, Unemo M, Abu-Raddad LJ, et al. Chlamydia, gonorrhoea, trichomoniasis and syphilis: global prevalence and incidence estimates, 2016. *Bull World Health Organ.* (2019) 97:548–62P. doi: 10.2471/BLT.18.228486

8. Bamberger DM. Trends in sexually transmitted infections. *Mol Med.* (2020) 117:324–7.

9. Jasek E, Chow EP, Ong JJ, Bradshaw CS, Chen MY, Hocking JS, et al. Sexually transmitted infections in Melbourne, Australia from 1918 to 2016: nearly a century of data. *Commun Dis Intell Q Rep.* (2017) 41:E212–22.

10. Mohammed H, Blomquist P, Ogaz D, Duffell S, Furegato M, Checchi M, et al. 100 years of STIs in the UK: a review of national surveillance data. *Sex Transm Infect.* (2018) 94:553–8. doi: 10.1136/sextrans-2017-053273

11. Chow EPF, Grulich AE, Fairley CK. Epidemiology and prevention of sexually transmitted infections in men who have sex with men at risk of HIV. *Lancet HIV.* (2019) 6:e396–405. doi: 10.1016/S2352-3018(19)30043-8

12. Callander D, Guy R, Fairley CK, McManus H, Prestage G, Chow EPF, et al. Gonorrhoea gone wild: rising incidence of gonorrhoea and associated risk factors among gay and bisexual men attending Australian sexual health clinics. *Sex Health.* (2019) 16:457–63. doi: 10.1071/SH18097

13. Igoe D, Kelleher M, Cooney F, Clarke S, Quinlan M, Lyons F, et al. There has been a true rise in Neisseria gonorrhoeae but not in Chlamydia trachomatis in men who have sex with men in Dublin, Ireland. *Sex Transm Infect.* (2014) 90:523. doi: 10.1136/sextrans-2014-051662

14. Ling DI, Janjua NZ, Wong S, Krajden M, Hoang L, Morshed M, et al. Sexually Transmitted infection trends among gay or bisexual men from a clinic-based sentinel surveillance system in British Columbia, Canada. *Sex Transm Dis.* (2015) 42:153–9. doi: 10.1097/OLQ.0000000000000250

15. Martí-Pastor M, García de Olalla P, Barberá M-J, Manzardo C, Ocaña I, Knobel H, et al. Epidemiology of infections by HIV, Syphilis, Gonorrhea and Lymphogranuloma Venereum in Barcelona City: a population-based incidence study. *BMC Public Health.* (2015) 15:1015. doi: 10.1186/s12889-015-2344-7

16. Weston EJ, Kirkcaldy RD, Stenger M, Llata E, Hoots B, Torrone EA. Narrative review: assessment of neisseria gonorrhoeae infections among men who have sex with men in national and sentinel surveillance systems in the United States. *Sex Transm Dis.* (2018) 45:243–9. doi: 10.1097/OLQ.0000000000000740

17. Hook EW III, Handsfield HH. Gonococcal Infections in the Adult. In: Holmes KK, Sparling PK, Stamm WE, Piot P, Wasserheit JN, Corey L, et al., editors. *Sex Transm Dis*. 4th ed. New York, NY: McGraw-Hill Medical (2008). p. 627–46.

18. Fairley CK, Cornelisse VJ, Hocking JS, Chow EPF. Models of gonorrhoea transmission from the mouth and saliva. *Lancet Infect Dis.* (2019) 19:e360–e6. doi: 10.1016/S1473-3099(19)30304-4

19. Wi T, Lahra MM, Ndowa F, Bala M, Dillon J-AR, Ramon-Pardo P, et al. Antimicrobial resistance in Neisseria gonorrhoeae: global surveillance and a call for international collaborative action. *PLoS Med.* (2017) 14:e1002344. doi: 10.1371/journal.pmed.1002344

20. Lewis DA. Will targeting oropharyngeal gonorrhoea delay the further emergence of drug-resistant Neisseria gonorrhoeae strains? *Sex Transm Infect.* (2015) 91:234–7. doi: 10.1136/sextrans-2014-051731

21. Adamson PC, Klausner JD. The staying power of pharyngeal gonorrhea: implications for public health and antimicrobial resistance. *Clin Infect Dis.* (2021) 73:583–5. doi: 10.1093/cid/ciab074

22. Unemo M, Shafer WM. Antimicrobial resistance in Neisseria gonorrhoeae in the 21st century: past, evolution, and future. *Clin Microbiol Rev.* (2014) 27:587–613. doi: 10.1128/CMR.00010-14

23. Dong HV, Klausner JD. Neisseria gonorrhoeae resistance driven by antibiotic use. *Nat Rev Urol.* (2019) 16:509–10. doi: 10.1038/s41585-019-0206-2

24. Dong HV, Pham LQ, Nguyen HT, Nguyen MXB, Nguyen TV, May F, et al. Decreased cephalosporin susceptibility of oropharyngeal neisseria species in antibiotic-using men who have sex with men in Hanoi, Vietnam. *Clin Infect Dis.* (2020) 70:1169–75. doi: 10.1093/cid/ciz365

25. Centers for Disease Control and Prevention. *Antibiotic Resistance Threats in the United States*. Atlanta, GA: Centers for Disease Control and Prevention (2013).

26. Holt M, Lea T, Mao L, Kolstee J, Zablotska I, Duck T, et al. Community-level changes in condom use and uptake of HIV pre-exposure prophylaxis by gay and bisexual men in Melbourne and Sydney, Australia: results of repeated behavioural surveillance in 2013-17. *Lancet HIV.* (2018) 5:e448–56. doi: 10.1016/S2352-3018(18)30072-9

27. Phillips T, Fairley CK, Walker S, Chow EPF. Associations between oral sex practices and frequent mouthwash use in men who have sex with men: implications for gonorrhoea prevention. *Sex Health.* (2019) 16:473–8. doi: 10.1071/SH18131

28. Chow EPF, Lee D, Tabrizi SN, Phillips S, Snow A, Cook S, et al. Detection of Neisseria gonorrhoeae in the pharynx and saliva: implications for gonorrhoea transmission. *Sex Transm Infect.* (2016) 92:347. doi: 10.1136/sextrans-2015-052399

29. Chow EPF, Tabrizi SN, Phillips S, Lee D, Bradshaw CS, Chen MY, et al. Neisseria gonorrhoeae bacterial DNA load in the pharynges and saliva of men who have sex with men. *J Clin Microbiol.* (2016) 54:2485–90. doi: 10.1128/JCM.01186-16

30. Chow EPF, Cornelisse VJ, Read TRH, Lee D, Walker S, Hocking JS, et al. Saliva use as a lubricant for anal sex is a risk factor for rectal gonorrhoea among men who have sex with men, a new public health message: a cross-sectional survey. *Sex Transm Infect.* (2016) 92:532–6. doi: 10.1136/sextrans-2015-052502

31. Chow EPF, Cornelisse VJ, Williamson DA, Priest D, Hocking JS, Bradshaw CS, et al. Kissing may be an important and neglected risk factor for oropharyngeal gonorrhoea: a cross-sectional study in men who have sex with men. *Sex Transm Infect.* (2019) 95:516–21. doi: 10.1136/sextrans-2018-053896

32. Tran J, Ong JJ, Bradshaw CS, Chen MY, Kong FYS, Hocking JS, et al. Kissing, fellatio, and analingus as risk factors for oropharyngeal gonorrhoea in men who have sex with men: a cross-sectional study. *EClinicalMedicine.* (2022) 51:101557. doi: 10.1016/j.eclinm.2022.101557

33. Barbee LA, Dombrowski JC, Kerani R, Golden MR. Effect of nucleic acid amplification testing on detection of extragenital gonorrhea and chlamydial infections in men who have sex with men sexually transmitted disease clinic patients. *Sex Transm Dis.* (2014) 41:168–72. doi: 10.1097/OLQ.0000000000000093

34. Bissessor M, Fairley CK, Leslie D, Howley K, Chen MY. Frequent screening for syphilis as part of HIV monitoring increases the detection of early asymptomatic syphilis among HIV-positive homosexual men. *J Acquir Immune Defic Syndr.* (2010) 55:211–6. doi: 10.1097/QAI.0b013e3181e583bf

35. Down I, Wilson DP, McCann PD, Gray R, Hoare A, Bradley J, et al. Increasing gay men's testing rates and enhancing partner notification can reduce the incidence of syphilis. *Sex Health.* (2012) 9:472–80. doi: 10.1071/SH12023

36. Aar F, Schreuder I, Weert Y, Spijker R, Götz H, Op de Coul E, et al. Current practices of partner notification among MSM with HIV, gonorrhoea and syphilis in the Netherlands: an urgent need for improvement. *BMC Infect Dis.* (2012) 12:114. doi: 10.1186/1471-2334-12-114

37. Hengel B, Jamil MS, Mein JK, Maher L, Kaldor JM, Guy RJ. Outreach for chlamydia and gonorrhoea screening: a systematic review of strategies and outcomes. *BMC Public Health.* (2013) 13:1040. doi: 10.1186/1471-2458-13-1040

38. Zou H, Fairley CK, Guy R, Chen MY. The efficacy of clinic-based interventions aimed at increasing screening for bacterial sexually transmitted infections among men who have sex with men: a systematic review. *Sex Transm Dis.* (2012) 39:382–7. doi: 10.1097/OLQ.0b013e318248e3ff

39. Peel J, Chow EPF, Denham I, Schmidt T, Buchanan A, Fairley C, et al. Clinical presentation of incident syphilis among men who have sex with men taking HIV Pre-Exposure Prophylaxis in Melbourne, Australia. *Clin Infect Dis.* (2021) 76:e934–7. doi: 10.1136/sextrans-2021-sti.261

40. Read PJ, Donovan B. Clinical aspects of adult syphilis. *Intern Med J.* (2012) 42:614–20. doi: 10.1111/j.1445-5994.2012.02814.x

41. Rasoldier V, Gueudry J, Chapuzet C, Bodaghi B, Muraine M, Tubiana R, et al. Early symptomatic neurosyphilis and ocular syphilis: a comparative study between HIV-positive and HIV-negative patients. *Infect Dis Now.* (2021) 51:351–6. doi: 10.1016/j.medmal.2020.10.016

42. Cornelisse VJ, Chow EPF, Latimer RL, Towns J, Chen M, Bradshaw CS, et al. Getting to the bottom of it: sexual positioning and stage of syphilis at diagnosis, and implications for syphilis screening. *Clin Infect Dis.* (2020) 71:318–22. doi: 10.1093/cid/ciz802

43. Towns JM, Leslie DE, Denham I, Wigan R, Azzato F, Williamson DA, et al. Treponema pallidum detection in lesion and non-lesion sites in men who have sex with men with early syphilis: a prospective, cross-sectional study. *Lancet Infect Dis.* (2021) 21:1324–31. doi: 10.1016/S1473-3099(20)30838-0

44. Ong JJ, Baggaley RC Wi TE, Tucker JD, Fu H, Smith MK, et al. Global epidemiologic characteristics of sexually transmitted infections among individuals using preexposure prophylaxis for the prevention of HIV infection: a systematic review and meta-analysis. *JAMA Netw Open.* (2019) 2:e1917134. doi: 10.1001/jamanetworkopen.2019.17134

45. Swanton R, Allom V, Mullan B, A. meta-analysis of the effect of new-media interventions on sexual-health behaviours. *Sex Transm Infect.* (2015) 91:14–20. doi: 10.1136/sextrans-2014-051743

46. Long L, Abraham C, Paquette R, Shahmanesh M, Llewellyn C, Townsend A, et al. Brief interventions to prevent sexually transmitted infections suitable for in-service use: a systematic review. *Prev Med.* (2016) 91:364–82. doi: 10.1016/j.ypmed.2016.06.038

47. Desai M, Woodhall SC, Nardone A, Burns F, Mercey D, Gilson R. Active recall to increase HIV and STI testing: a systematic review. *Sex Transm Infect.* (2015) 91:314–23. doi: 10.1136/sextrans-2014-051930

48. Peters M, Godfrey C, McInerney P, Soares C, Khalil H, Parker D. *The Joanna Briggs Institute Reviewers' Manual 2015: Methodology for JBI Scoping Reviews.* Adelaide: Joanna Briggs Institute (2015).

49. Moola S, Munn Z, Tufanaru C, Aromataris E, Sears K, Sfetcu R, et al. Chapter 7: systematic reviews of etiology and risk. In: Aromataris E, Munn Z, editors. *JBI Manual for Evidence Synthesis.* Adelaide: Joanna Briggs Institute (2020).

50. Tufanaru C, Munn Z, Aromataris E, Campbell JLH. Chapter 3: systematic reviews of effectiveness. In: Aromataris E, Munn Z, editors. *JBI Manual for Evidence Synthesis.* Adelaide: Joanna Briggs Institute (2020).

51. Bolan RK, Beymer MR, Weiss RE, Flynn RP, Leibowitz AA, Klausner JD. Doxycycline prophylaxis to reduce incident syphilis among hiv-infected men who have sex with men who continue to engage in high-risk sex: a randomized, controlled pilot study. *Sex Transm Dis.* (2015) 42:98–103. doi: 10.1097/OLQ.0000000000000216

52. Molina JM, Charreau I, Chidiac C, Pialoux G, Cua E, Delaugerre C, et al. Post-exposure prophylaxis with doxycycline to prevent sexually transmitted infections in men who have sex with men: an open-label randomised substudy of the ANRS IPERGAY trial. *Lancet Infect Dis.* (2018) 18:308–17. doi: 10.1016/S1473-3099(17)30725-9

53. Chow EPF, Howden BP, Walker S, Lee D, Bradshaw CS, Chen MY, et al. Antiseptic mouthwash against pharyngeal Neisseria gonorrhoeae: a randomised controlled trial and an in vitro study. *Sex Transm Infect.* (2017) 93:88–93. doi: 10.1136/sextrans-2016-052753

54. Chow EPF, Maddaford K, Hocking JS, Bradshaw CS, Wigan R, Chen MY, et al. An open-label, parallel-group, randomised controlled trial of antiseptic mouthwash versus antibiotics for oropharyngeal gonorrhoea treatment (OMEGA2). *Sci Rep*. (2020) 10:19386. doi: 10.1038/s41598-020-76184-1

55. Chow EPF, Williamson DA, Hocking JS, Law MG, Maddaford K, Bradshaw CS, et al. Antiseptic mouthwash for gonorrhoea prevention (OMEGA): a randomised, double-blind, parallel-group, multicentre trial. *Lancet Infect Dis.* (2021) 21:647–56. doi: 10.1016/S1473-3099(20)30704-0

56. Van Dijck C, Tsoumanis A, Rotsaert A, Vuylsteke B, Van den Bossche D, Paeleman E, et al. Antibacterial mouthwash to prevent sexually transmitted infections in men who have sex with men taking HIV pre-exposure prophylaxis (PReGo): a randomised, placebo-controlled, crossover trial. *Lancet Infect Dis.* (2021) 21:657–67. doi: 10.1016/S1473-3099(20)30778-7

57. Van Dijck C, Tsoumanis A, De Hondt A, Cuylaerts V, Laumen J, Van Herrewege Y, et al. Chlorhexidine mouthwash fails to eradicate oropharyngeal gonorrhea in a clinical pilot trial (MoNg). *Sex Transm Dis.* (2021) 49:e38–e41. doi: 10.1097/OLQ.0000000000001515

58. Barbee LA, Tat S, Dhanireddy S, Marrazzo JM. Effectiveness and patient acceptability of a sexually transmitted infection self-testing program in an HIV care setting. *J Acquir Immune Defic Syndr.* (2016) 72:E26–31. doi: 10.1097/QAI.0000000000000979

59. Leenen J, Hoebe C, Ackens RP, Posthouwer D, van Loo IHM, Wolffs PFG, et al. Pilot implementation of a home-care programme with chlamydia, gonorrhoea, hepatitis B, and syphilis self-sampling in HIV-positive men who have sex with men. *BMC Infect Dis.* (2020) 20:9–25. doi: 10.1186/s12879-020-05658-4

60. Surie D, Furness BW, Hernandez-Kline P, Turner A, Perkins RC, Taylor MM, et al. Examining self and partners for syphilis among men who have sex with men: five US cities, 2009-2011. *Int J STD AIDS.* (2012) 23:859–61. doi: 10.1258/ijsa.2012.012016

61. Taylor MM, Peterson B, Post J, Williams C, Vanig T, Winscott M. Self-examination behaviors for syphilis symptoms among HIV-infected men. *J Acquir Immune Defic Syndr.* (2010) 55:284–5. doi: 10.1097/QAI.0b013e3181e13ed9

62. Wu D, Zhou Y, Yang N, Huang S, He X, Tucker J, et al. Social media-based secondary distribution of HIV/syphilis self-testing among Chinese men who have sex with men. *Clin Infect Dis*. (2020) 73:e2251–7. doi: 10.2139/ssrn.3498429

63. Yang N, Wu D, Zhou Y, Huang S, He X, Tucker J, et al. Sexual health influencer distribution of HIV/syphilis self-tests among men who have sex with men in China: secondary analysis to inform community-based interventions. *J Med Internet Res.* (2021) 23:e24303. doi: 10.2196/24303

64. Grant JS, Stafylis C, Celum C, Grennan T, Haire B, Kaldor J, et al. Doxycycline prophylaxis for bacterial sexually transmitted infections. *Clin Infect Dis.* (2020) 70:1247–53. doi: 10.1093/cid/ciz866

65. Wang YJ, Zhang W, Bao DP, Ong JJ, Tucker JD, Ye RX, et al. Social network distribution of syphilis self-testing among men who have sex with men in China: study protocol for a cluster randomized control trial. *BMC Infect Dis.* (2021) 21:491–99. doi: 10.5772/intechopen.87410

66. Cheng WB, Wang CY, Tang WM, Ong JJ, Fu HY, Marks M, et al. Promoting routine syphilis screening among men who have sex with men in China: study protocol for a randomised controlled trial of syphilis self-testing and lottery incentive. *BMC Infect Dis.* (2020) 20:8. doi: 10.1186/s12879-020-05188-z

67. Hui BB, Padeniya TN, Rebuli N, Gray RT, Wood JG, Donovan B, et al. A gonococcal vaccine has the potential to rapidly reduce the incidence of Neisseria gonorrhoeae infection among urban men who have sex with men. *J Infect Dis.* (2021) 225:983–93. doi: 10.1093/infdis/jiab581

68. Petousis-Harris H, Paynter J, Morgan J, Saxton P, McArdle B, Goodyear-Smith F, et al. Effectiveness of a group B outer membrane vesicle meningococcal vaccine against gonorrhoea in New Zealand: a retrospective case-control study. *Lancet.* (2017) 390:1603–10. doi: 10.1016/S0140-6736(17)31449-6

69. Luetkemeyer A, Dombrowski J, Cohen S, Donnell D, Grabow C, Brown C, et al., editors. Doxycycline post-exposure prophylaxis for prevention of STIs among MSM and TGW who are living with HIV or on PrEP. In: *The International AIDS Conference.* Montreal, QC: The International AIDS Society (2022).

frontiers | Frontiers in Medicine

REVIEW
published: 20 September 2022
doi: 10.3389/fmed.2022.800383

An Updated Review of Recent Advances in Neurosyphilis

Jia Zhou[1,2†], *Hanlin Zhang*[1,2†], *Keyun Tang*[1,2†], *Runzhu Liu*[1,2] *and Jun Li*[1,2*]

[1] *Department of Dermatology, Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences, Beijing, China,* [2] *State Key Laboratory of Complex Severe and Rare Diseases, Peking Union Medical College Hospital, Chinese Academy of Medical Sciences, Beijing, China*

Neurosyphilis is caused by *Treponema pallidum* invading the central nervous system, of which the incidence is increasing worldwide. Due to its variable clinical manifestations, diagnosis of neurosyphilis remains challenging, especially the asymptomatic form. This review focuses on recent advances in neurosyphilis, including epidemiology, clinical manifestations, laboratory findings, comorbidities, diagnosis, treatment, prognosis, and basic research. The expansion of men who have sex with men and the infection of human immunodeficiency virus mainly accounted for the increasing incidence of neurosyphilis. The rate of some historically described forms of neurosyphilis in the pre-antibiotic era declined significantly; atypical features are more prevalent. Neurosyphilis, regarded as a great mimicker for neuro-ophthalmic, audio-vestibular, and psychiatric disorders, often presents concomitantly with other diseases, including metabolic disorders. Studies on long non-coding RNAs, miRNAs, chemokines, and metabolites in peripheral blood and cerebrospinal fluid may facilitate exploring the pathogenesis and identifying novel biomarkers of neurosyphilis. The drug resistance of *Treponema pallidum* to penicillin has not been reported; ceftriaxone was proposed to be more effective than penicillin, whereas few randomized controlled trials supported this view. This study may pave the way for further research, especially the diagnosis and treatment of neurosyphilis.

Keywords: neurosyphilis, syphilis, *Treponema pallidum*, cerebrospinal fluid, ocular syphilis, otosyphilis, penicillin, ceftriaxone

OPEN ACCESS

Edited by:
Pingyu Zhou,
Tongji University, China

Reviewed by:
Ahmet Cagkan Inkaya,
Hacettepe University, Turkey
Mei Shi,
Shanghai Dermatology Hospital, China

***Correspondence:**
Jun Li
lijun35@hotmail.com

[†] *These authors have contributed equally to this work*

Specialty section:
This article was submitted to Infectious Diseases–Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

Received: *23 October 2021*
Accepted: *14 March 2022*
Published: *20 September 2022*

Citation:
Zhou J, Zhang H, Tang K, Liu R and Li J (2022) An Updated Review of Recent Advances in Neurosyphilis. Front. Med. 9:800383. doi: 10.3389/fmed.2022.800383

INTRODUCTION

Syphilis, caused by the infection of *Treponema pallidum* (*T. pallidum*), can progress to neurosyphilis at any time after the initial infection. Due to the widespread use of antibiotics, the reported cases of neurosyphilis are less than those in the pre-antibiotic era. However, there has been a resurgence in recent years (1). Supporting evidence is anticipated for the diagnosis and treatment of neurosyphilis, and new research progress has been made recently (2, 3). Therefore, this article reviews recent advances in neurosyphilis, including epidemiology, clinical manifestations, laboratory findings, comorbidities, diagnosis, treatment, prognosis, and basic research.

EPIDEMIOLOGY

In 2012, 18 million cases of syphilis were reported worldwide, among which 3,50,000 cases caused adverse pregnancy outcomes (4). The incidence continues to rise due to the expansion of susceptible populations, including men who have sex with men (MSM) and people living with

HIV (PLWH) (5). The rate of neurosyphilis in PLWH is about twofold as in the immunocompetent population (6). Although the incidence of syphilis is low among women in the USA, it increased by 30.0% in 2018–2019; as for congenital syphilis, the incidence has also been increasing since 2013. In China, from 1990 to 2017, the incidence of syphilis increased from 0.9 to 34.49 per million, second only to viral hepatitis and tuberculosis among infectious diseases. The increasing trend aligned with that of neurosyphilis (7). Male, MSM, advanced age (≥45 years), PLWH without combined antiretroviral therapy, drug use disorder, lack of antisyphilitic therapy, reinfection after a previous syphilis infection, and patients in serofast state are risk factors for neurosyphilis, especially the late forms (8–11). Elevated serum and cerebrospinal fluid (CSF) rapid plasma reagin (RPR) titer, CSF protein concentration, and increased burden of the cerebral small vessel diseases may indicate the aggravation of neurological symptoms (10, 12, 13). Specific subtypes of *T. pallidum* are more likely to induce neurosyphilis; for instance, subtype 14d is the most common neurosyphilis-related genotype in China (14). Specific genes the hosts carry also determine their susceptibility of neurosyphilis, including some single nucleotide polymorphisms on Toll-like receptor (TLR)1, TLR2, TLR6, and the interleukin 10 (IL-10) promoter (15, 16).

Some novel characteristics of neurosyphilis have also been observed nowadays. Currently, the most common form of neurosyphilis is asymptomatic neurosyphilis and meningoencephalitis, although meningovascular syphilis had the highest incidence in some cohort studies (17). The incidence of prevalent symptoms in the pre-antibiotic era, such as tabes dorsalis, dementia paralytica, and gummatous neurosyphilis declined dramatically, as the widespread use of antibiotics in other diseases might indirectly treat neurosyphilis, and timely diagnosis and treatment shortened the course of the disease (18). Recent studies also reported an increase in the incidence rate of ocular syphilis and otosyphilis (19). However, molecular typing showed little evidence of a type specifically causing ocular syphilis. Thus, this increase might just reflect a resurgence in total syphilis cases (20).

CLINICAL MANIFESTATIONS AND LABORATORY FINDINGS

Based on the disease progression, neurosyphilis can be divided into early and late stages. Manifestations that do not fall into either of the two stages are classified as atypical neurosyphilis. Early neurosyphilis can be classified as asymptomatic and symptomatic forms, and the symptomatic form includes symptomatic meningitis and meningo-vasculitis. The manifestations of late neurosyphilis include dementia paralytica and tabes dorsalis. Patients with early neurosyphilis are more likely to have ocular involvement than those of late stage (18). Cerebral small vessel disease, one of the major causes of cognitive impairment, is more prevalent in patients of late stage (12). These forms were detailed in the following sections.

Asymptomatic neurosyphilis is defined as a neurologically asymptomatic form with serological and CSF abnormalities, typically occurring in the early months of infection. Patients can be diagnosed with the following features, CSF lymphocytosis (<100 cells/μL), elevated protein concentration (<100 mg/dL), a reactive result of cerebrospinal fluid-Venereal Disease Research Laboratory (CSF-VDRL) assay, or a combination of these abnormalities. However, these existing benchmarks cannot be directly used in PLWH, for HIV itself may trigger CSF lymphocytosis and elevated protein concentration.

As a common manifestation of early neurosyphilis, symptomatic meningitis often appears within the first year of infection, in the form of headache, nausea, vomiting, and neck pain, accompanied by ocular involvement like inflammation and blurry vision. It can further lead to cranial neuropathy, meningo-vasculitis, and damage to the brain parenchyma. CSF abnormalities in early symptomatic meningitis are more severe than those in asymptomatic forms, with lymphocyte count around 200–400 cells/μL, protein concentration around 100–200 mg/dL, and CSF-VDRL tests almost always reactive (6). Neuroimaging profile usually demonstrates enhancement of CSF, meninges, or cranial nerves in this stage. Meningo-vasculitis can cause thrombosis, ischemia, and infarction. For young patients with strokes, syphilitic meningo-vasculitis should be considered, since there was one study showing that 14.09% of patients with neurosyphilis had an ischemic stroke as a primary symptom (21). In southern Brazil, the reactive result of serological tests for syphilis is common in patients with acute stroke (22). As in cases of meningo-vasculitis, CSF white blood cell (WBC) count is usually around 5–74 cells/μL, with protein concentration around 22–101 mg/dL, while CSF-VDRL tests are not always reactive (23). In patients with meningo-vasculitis, focal narrowing, dilatation, and occlusion may be seen in angiography, while neuroimaging may show evidence of infarction. It was reported that neuroimaging progression could continue in symptomatic patients even after standardized antisyphilitic therapy, which was confirmed by a study showing infarction lesions in 42.1%, mild to severe brain atrophy in 47.4%, and white matter demyelination in 15.8% of treated patients in the follow-up (24).

Dementia paralytica and tabes dorsalis are two major forms of late neurosyphilis, usually appearing 15–20 years after the initial infection. The CSF of patients with late neurosyphilis shows only mild lymphocytosis or slightly elevated protein concentration, compared to those with early neurosyphilis. Dementia paralytica presents with neurasthenic syndromes and personality disorders in the early stage, which is difficult to distinguish from Alzheimer's disease (25); however, in the middle and late stages, patients will show physical symptoms, such as Argyll Robertson pupils, optic atrophy, sensory ataxia, tremors, and dysarthria. In those paretic patients, CSF protein concentration ranged from 20 to 186 mg/dL, and CSF WBC count ranged from 0 to 98 cells/μL, with 94.9% of CSF-VDRL tests turned to be reactive (26). Signs of tabes dorsalis include areflexia, urinary retention, sexual dysfunction; 60% of patients in that stage present with Argyll Robertson pupils. In tabetic patients in a cohort study, CSF protein concentration ranged from 14 to 64 mg/dL, and CSF WBC count ranged from 2 to 145 cells/μL (26).

Atypical neurosyphilis is more frequently observed nowadays. The symptoms of early neurosyphilis can mimic most neuro-ophthalmic diseases, while the neurological involvement in the late stage can mimic psychiatric or autoimmune disorders. Rapidly progressive psychosis, such as dementia (27), cognitive impairment (28), and subacute confusion (29), was reported in some cases of late neurosyphilis, some of which were initial clinical presentations. Thus, it is essential to ask for consultation from psychiatrists. Additionally, herpesviral encephalitis, polyradiculoneuropathy, mania, extrapyramidal syndrome, amyotrophic lateral sclerosis, and cauda equina syndrome were also reported in recent cases (28). For patients without other common risk factors but with neuropsychiatric symptoms, screening for syphilis should be recommended.

COMORBIDITIES

Patients with neurosyphilis may have other diseases, such as HIV infection, posing great diagnostic and therapeutic challenges to clinicians. Recent studies focused on the current status and underlying mechanisms of the comorbidities of neurosyphilis and HIV infection. A study from Missouri reported that 7.4% of HIV-positive MSM patients were diagnosed with primary or secondary syphilis in 2016, compared with 3.1% of immunocompetent MSM patients (30). Syphilis can exacerbate HIV infection and inflammation in the central nervous system, increasing HIV viral load and decreasing CD4 + T lymphocyte count. For HIV-uninfected individuals, syphilitic skin lesions may also provide access to HIV acquisition (31). Meanwhile, HIV-associated immunosuppression can increase susceptibility to neurosyphilis. The diagnosis of neurosyphilis is also more difficult in PLWH. On the one hand, PLWH may have false positive reactions to serological tests for syphilis (32). On the other hand, HIV itself can lead to a slight increase in CSF WBC count and protein concentration (9). Besides, cases of gonorrhea, viral hepatitis, and chlamydia comorbid with syphilis were reported (33). Weathers et al. also showed that comorbid hepatitis and herpes simplex would increase the risk of syphilis (34).

Ocular syphilis and otosyphilis were discussed as comorbidities of neurosyphilis here. Ocular syphilis, occurring at any stage of syphilis, is becoming an important cause of uveitis. Furtado et al. found that the most common form of intraocular inflammation induced by ocular syphilis was posterior uveitis, followed by panuveitis (35). Diminished vision, interstitial keratitis, optic neuropathy, and retinal vasculitis are typical manifestations, and ocular hypertension and cataract were also reported (36). Mathew et al. found that 37% of patients with ocular syphilis had neurosyphilis (37). The frequency of neurosyphilis comorbid with ocular syphilis was significantly higher in PLWH than in immunocompetent patients (37). In one study on PLWH with comorbid neurosyphilis, those with ocular syphilis were more likely to have elevated CSF WBC count than those without ocular syphilis (38). Due to these possible misleading symptoms, ocular syphilis was sometimes misdiagnosed as, for example, retinal detachment or vitreous hemorrhage. Delayed management of ocular syphilis can cause poor vision in the follow-up (39). Otosyphilis is also a mimicker for many audio-vestibular disorders, occurring at any stage of syphilis. The main manifestation is sensorineural hearing loss, which will develop into conductive hearing loss. Other common manifestations include vertigo, tinnitus, and gait instability (40); accompanying meningitis and ocular involvement were also reported (41). Some studies suggest that *T. pallidum* may impair the eighth cranial nerve, temporal bone, and cochleovestibular apparatus (42). Regardless of the CSF profile, otosyphilis and ocular syphilis should be treated with the same protocols for neurosyphilis (43).

Patients with neurosyphilis might have specific metabolic characteristics. The incidence of hyperlipidemia and hypertension in patients with neurosyphilis was 21.4% and 45.9%, respectively, both significantly higher than that in patients with other neurological infections. Patients with neurosyphilis also presented with significantly higher levels of low-density lipoprotein, total cholesterol, systolic and diastolic blood pressure (44). Glycated hemoglobin A1c (HbA1c) is an indicator of diabetes and neurosyphilis, playing an unclear role in blood-brain barrier (BBB) disruption, an essential step in the progression of both diseases (45).

DIAGNOSIS

The diagnosis of neurosyphilis is based on serum and CSF tests, which can be further classified as treponemal and nontreponemal tests. Nontreponemal serum tests mainly include rapid plasma reagin (RPR) and venereal disease research laboratory (VDRL); treponemal serum tests mainly include *T. pallidum* particle agglutination assay (TPPA), *T. pallidum* enzyme immunoassay (TP-EIA), chemiluminescence immunoassay, and fluorescent treponemal antibody absorption (FTA-ABS). For patients with ocular or neurological symptoms, features of tertiary syphilis, or serofast status, lumbar puncture and CSF tests are needed for the diagnosis of neurosyphilis. CSF tests mainly include CSF WBC count, CSF protein concentration, CSF-RPR, CSF-TPPA, and CSF-FTA-ABS. As for nontreponemal tests, the sensitivity varies by the progress of neurosyphilis; for patients with late neurosyphilis, nontreponemal tests are of a high false-negative rate (46).

Conventional diagnostic methods of neurosyphilis face some challenges. The diagnosis relies heavily on CSF-VDRL, of which the specificity is high (89–96%), but the sensitivity is relatively low (12–48%) (47). Alternative methods, such as CSF toluidine red unheated serum test (CSF-TRUST), might not perfectly remedy the low sensitivity; CSF chemiluminescence immunoassay was preferred, with the sensitivity of 99.57–100%, and the specificity of 99.81–99.88% (38). Some studies suggested chemokine (C-X-C motif) ligand (CXCL) 13 as a promising indicator, with specificity from 76 to 81% and sensitivity from 78 to 83% (47–49). However, the optimal benchmark for CXCL13 remains controversial. CSF samples from cohorts are needed to establish an appropriate cut-off for the diagnosis of neurosyphilis (47). The combination of

CXCL8, CXCL10, and CXCL13 might be a better indicator with specificity of 92.9% and sensitivity of 90.4% (50). CSF WBC count, protein concentration, and clinical manifestations should all be considered along with CSF-TPPA; the sensitivity of this combined diagnostic method was up to 98% (51). Adjusting the cut-off of tests was also proposed as a solution to low specificity and sensitivity. Serum TPPA, commonly used in patients with unknown prior history of syphilis, was recommended to enhance the specificity of CSF test, when the titer cut-off was adjusted to >1:320. For those with high suspicion of neurosyphilis but without a positive result of CSF VDRL test, the CSF treponemal assay like CSF TPPA was a reasonable recommendation to reduce the false-negative rate (52). Polymerase chain reaction (PCR) and immunoblot were recommended as new automated treponemal tests. A study showed that the specificity and sensitivity of PCR ranged from 60 to 100% and 40 to 70%, respectively; as for immunoblot, the specificity varied from 91.7 to 92.0%, and the sensitivity varied from 93.8 to 100% (48). Nowadays, automated treponemal tests are often accompanied by a nontreponemal test as a following confirmatory step, which is called the reverse-sequence algorithm and has shown ideal effects in the low-prevalence populations (53). Consequently, the diagnosis should make full use of serum and CSF samples by a combination of several tests in order to obtain a convincing result.

Conventional benchmarks may not be convincing for neurosyphilis patients coinfected with HIV, because HIV may cause CSF lymphocytosis and elevated protein concentration. Due to the difficulty in diagnosing asymptomatic neurosyphilis in PLWH, lumbar puncture was recommended by some experts for all syphilis patients coinfected HIV, which might also cause unnecessary costs and risks. CDC suggested that lumbar puncture should be given to PLWH with neurological signs or symptoms, and/or serofast status, and/or tertiary syphilis, which was supported by another cohort analysis (30).

Recently, the point-of-care syphilis tests were gradually popularized, due to the epidemic of syphilis in low-and middle-income countries. Low-and middle-income countries also showed high prevalence rates, long diagnostic delays, low follow-up rates, and inaccessibility to trained clinicians and necessary facilities (54). Optimized commercial immunochromatographic strip tests, such as the CSF-Syphicheck and the DPP Chembio syphilis assay, showed satisfying diagnostic specificity and sensitivity compared to CSF-VDRL (62–64% sensitivity, 79–81% specificity in CSF-Syphicheck; 63–92% sensitivity, 85–100% specificity in DPP Chembio syphilis assay) (55, 56). Though further evaluation is needed, point-of-care tests may be widely applied in resource-limited regions and contribute to the control of sexually transmitted diseases, especially for neurosyphilis and congenital syphilis.

TREATMENT AND PROGNOSIS

Treatment guidelines for neurosyphilis released by the United States, the United Kingdom, Europe, and China are outlined in **Table 1**. They all highlighted that PLWH should be treated as immunocompetent patients, and ceftriaxone might be an alternative treatment to penicillin. A divergence of views lay on the adoption of steroids in patients with neurosyphilis, which was only recommended in some of the guidelines. Efficacy of different therapies is always a major concern, whereas it is hard to draw a definite conclusion due to those low-quality, small-size, and highly-biased trials (58, 59). A cohort study including 208 patients with neurosyphilis during 1997–2017 showed serological cure rates of 88% in the ceftriaxone group and 82% in the penicillin G group (2), while another study showed clinical cure rates of only 44% in the ceftriaxone group and 18% in the penicillin G group (59). Meanwhile, neurological sequelae were reported in 41.8% of the neurosyphilis patients in a retrospective study (60). PLWH coinfected with neurosyphilis were more likely to fail the intravenous penicillin G therapy (61, 62).

TABLE 1 | Treatment recommendations for neurosyphilis.

Guidelines	Treatment modalities
Guidelines from Centers for Disease Control and Prevention of the United States (6)	IV[#] aqueous crystalline penicillin G 3–4 million U every 4 h, or continuous infusion 18–24 million U/d for 10–14 days.
The United Kingdom National Guidelines (52)	IM* procaine penicillin G 1.8–2.4 million U/d, following with oral probenecid 500 mg every 6 h for 2 weeks; or IV penicillin G 1.8–2.4 g every 4 h for 2 weeks.
European Guidelines (43)	IV penicillin G 18–24 million U/d, as 3–4 million U every 4 h for 10–14 days; or IV ceftriaxone 1–2 g/d for 10–14 days; or IM procaine penicillin G 1.2–2.4 million U/d, following with oral probenecid 500 mg every 6 h for 10–14 days, when IV penicillin G cannot be used.
Guidelines from Chinese Center for Disease Control and Prevention (57)	IV aqueous crystalline penicillin G 18–24 million U/d for 10–14 days, if necessary, following with IM benzathine penicillin G 2.4 million U/week for 3 doses; or IM procaine penicillin 2.4 million U/d and oral probenecid 500 mg every 6 h for 10–14 days, if necessarily, following with IM benzathine penicillin G 2.4 million U/week for 3 doses. Second-line therapy option: IV ceftriaxone 2 g/d for 10–14 days. For penicillin-allergic patients, oral doxycycline 100 mg twice a day, for 1 month.

[#]*IV = intravenous.*
**IM = intramuscular.*

Penicillin is preferred worldwide as front-line therapy for neurosyphilis, and the drug resistance of *T. pallidum* to penicillin has not been reported (63). Some studies found that ceftriaxone was more effective than penicillin, but few large randomized controlled trials supported this view (2, 59, 60). Tetracycline, erythromycin, and chloramphenicol have all been applied to treat syphilis, whereas erythromycin and chloramphenicol were reported to induce intestinal dysbiosis and irreversible aplastic anemia, respectively (59). The treatment effects of penicillin and doxycycline were similar in some series (64); doxycycline is

cheaper yet forbidden in pregnant women. The only treatment modality certified for pregnant women with syphilis is parenteral penicillin G. The concurrent administration of probenecid is widely used in antibiotic therapy for neurosyphilis. A recent study proved that oral amoxicillin with probenecid could elevate the drug level in CSF (43). However, oral probenecid with intramuscular (IM) procaine penicillin should be carefully used in HIV-positive MSM (65).

The Jarisch-Herxheimer reaction (JHR) may occur within the first 24 h of neurosyphilis treatment, presenting as headache, chills, fever, rashes, and myalgias, of which the mechanism is unclear (66). Commonly used antibiotics, including penicillin, erythromycin, azithromycin, tetracyclines, clarithromycin, and levofloxacin, could all cause JHR (67). JHR usually occurs among patients with early syphilis, as the risk of JHR increases by 19% for every twofold increase in RPR titer, suggesting that higher spirochete load may cause a significantly higher risk of JHR (68). For PLWH, particularly those with encephalitis, JHR may be more severe (28). Steroids, like prednisolone, are routinely used to prevent JHR; TNF-α antibodies and acetaminophen were also used in some cases (69).

Serofast, a lack of serological response to antisyphilitic therapy, is currently a major concern for clinicians. This clinical scenario occurred in over one-third of patients, especially those with lower test titers or late latent syphilis (70). A successful response to antisyphilitic therapy is defined as a fourfold dilution decline in CSF-VDRL and the normalization of CSF WBC count, except for PLWH. Patients with serofast only show a decrease less than fourfold in non-treponemal antibody titers after a specific period of time (6 months for early-stage syphilis; 12 months for late-stage syphilis), or show low titers consistently after treatment (6). The cause of serofast is still unclear. Possible explanations of the lack of serological response to antisyphilitic therapy include that the patients have undiagnosed neurosyphilis and fail to respond to routine therapy; patients have comorbid HIV, malignancies, or immune diseases; and patients are re-infected by having sex with others (71, 72). Prediction of the serofast occurrence is still difficult, and retreatment to serofast patients can not significantly improve the cure rates (73, 74).

Several factors influence the prognosis of patients with neurosyphilis, which can also be used as indicators to assess the therapeutic effect. Although HIV coinfection may confound the diagnosis of neurosyphilis, the prognosis of patients does not differ by the HIV infection status (75). Diplopia and severe atrophy of brain parenchyma were proved to be associated with poor prognosis. Headache often contributes to an early diagnosis of disorders in the central nervous system, which is a favorable prognostic factor in patients with neurosyphilis (60); the changes in electroencephalogram and metabolites in CSF may be auxiliary indicators of the prognosis. Jiang et al. found that the electroencephalogram Lempel-Ziv complexity (EEG-LZC) value after treatment was significantly higher than that before treatment, suggesting that neuron reconnection would improve the brain function (76). In addition, CXCL13, tau, neurogranin, and metabolites in CSF could also be used to evaluate the cognitive function of patients with neurosyphilis and the effectiveness of antisyphilitic therapy (25, 77).

Reinfection of syphilis, which can start with the latent or tertiary stage (78), is more prevalent in MSM and PLWH (79, 80). A retrospective study on MSM in California from 2002 to 2006 showed that 5.9% of MSM had repeat infection of syphilis within 2 years after the first infection (80). Another 8-year surveillance in Brazil showed that 13.6% of patients had reinfection with *T. pallidum* (81). A four-fold drop in serum RPR titer of patients within 6–12 months is defined as the serological cure. However, some patients have a rise in RPR titer within the first 12 months, for which, the main reason is usually reinfection, rather than recurrence (82). Molecular studies are necessary when the corroborative history of patients is inaccessible to determine whether the rise in RPR titer is due to reinfection or recurrence (83). The mechanisms of reinfection are related to the destruction of adaptive immunity by *T. pallidum* (84). An immunodominant-evasion model further suggested that the TprK, an outer membrane protein with prolific antigenic variation, explained the ability of *T. pallidum* to escape the adaptive immunity (84).

BASIC RESEARCH

A major obstacle for the research on *T. pallidum* is that it can rarely be cultured continuously *in vitro*. Recent basic research on neurosyphilis mainly focuses on the pathogenic mechanisms of neurosyphilis, and the potential biomarkers for diagnosis or prognosis monitoring.

Research in the pathogenic mechanisms has proved that *T. pallidum* crosses the endothelial barriers through interjunctional penetration, disseminating throughout the body and invading various organs (85). This process depends largely on the outer membrane proteins of *T. pallidum*, such as Tp92, to promote the adhesion and proliferation of spirochetes, enhance the permeability of endothelial cells, and immunomodulate in the inflammatory responses (86). *T. pallidum* can activate both humoral and cellular immunity by antigens such as *T. pallidum* repeat protein (Tpr), whereas cellular immunity plays a major role in eliminating pathogens but is also significantly suppressed in the late stage of infection (87). The immune escape mechanism of *T. pallidum* has been well studied. Due to the lack of exposed lipoproteins on the outer membrane of *T. pallidum*, antigen-presenting cells are more difficult to contact the pathogen-associated molecular patterns and unable to activate the innate immune system (88). The common targets of humoral immune response are the variable region of membrane proteins such as TprK (89). However, it was reported that the continuous mutation of the TprK gene, especially the variable region, enabled *T. pallidum* to escape the immune response and caused persistent infection (90). Qin et al. reported that in syphilis patients in serofast state, unbalanced T cell subsets could suppress the cellular immunity with decreased levels of CD4+ T cells, decreased ratio of T helper (Th)1/Th2 cells, and increased levels of CD8+ T cells (91). Another study found increased levels of regulatory T cells and transforming growth factor-β in peripheral blood of the serofast patients with secondary syphilis, which was

mainly induced by the antigen TpF1 and might suppress the cellular immunity (92).

Host immune response also plays an important role in the pathogenesis of neurosyphilis, mainly in the form of T cell-mediated delayed-type hypersensitivity (87). Recent studies showed a significantly elevated levels of IL-8, CC chemokine ligand 20 (CCL-20) in serum, elevated levels of CXCL13, interferon(IFN)-γ in CSF, and elevated levels of IL-6, IL-10, IL-17 in both serum and CSF of neurosyphilis patients (49, 93–96). While the imbalance between Th17 and Th1 had been proved to aggravate inflammation in patients with secondary syphilis, how neurosyphilis developed in syphilis patients and the role host immune response played in the development of neurosyphilis remained unclear (97). The latest research proposed that CXCL13/CXCR5 mediated the accumulation of B cells and immunoglobulin G in the CSF of neurosyphilis patients (98); IL-17 not only mediated the inflammatory response, but also activated endothelial contraction and destroyed the tight junctions of the BBB (99); IL-8 and CCL-20 induced by TpF1 could induce vascular inflammation and angiogenesis (100). Liu et al. found that, compared to syphilis/non-neurosyphilis patients, patients with symptomatic neurosyphilis showed a significantly increased expression of CD8+ IFN-γ+ cell but decreased expression of CD8+ IL-17+ cell in the peripheral blood, which might also explain the pathogenesis of symptomatic neurosyphilis (96).

Recent studies also focused on the mechanisms of neurological damage caused by *T. pallidum*. *T. pallidum* can invade the central nervous system since the early stage of syphilis; however, the relationship between *T. pallidum* and the BBB remains unclear. Neurosyphilis, especially in the stage of general paresis, is similar to Alzheimer's disease. Thus, Alzheimer's-associated biomarkers are widely studied in neurosyphilis patients with cognitive impairment. Receptors expressed on myeloid cells 2 (sTREM2), neurofilament light proteins, and phosphorylated neurofilament heavy subunit were elevated in CSF of patients with neurosyphilis, suggesting that activated microglia were associated with neuron damage caused by *T. pallidum* (101, 102). β-amyloid precursor protein cleaving enzyme (BACE1) and neurogranin also increased in the CSF of patients with neurosyphilis, indicating that the synaptic destruction would appear in the stage of general paresis (25). CXCL13 was also involved in CSF pleocytosis and neurological damage (49, 103).

Studies on RNA, proteins, and metabolites in peripheral blood and CSF have facilitated the discovery of novel indicators for neurosyphilis. Long non-coding RNAs expressed in peripheral blood of patients with neurosyphilis were emphasized in recent studies. IFN-γ production was proved to be mediated by the interaction between lncRNA-ENST00000421645 and PCM1 (104). miRNAs in exosomes released by neuroglial cells could be potential biomarkers in CSF of patients with neurosyphilis for brain parenchymal damage, including miR-570-3p, miR-590-5p, miR-570-5p, and miR-21-5p (105). The elevated level of chemokines was associated with inflammatory disorders in the central nervous system (50); CXCL13, CXCL8, and CXCL10 were suggested as novel biomarkers with high sensitivity (49). Interleukin-10 in CSF was also regarded as a biomarker of neurosyphilis, especially of asymptomatic neurosyphilis (93). In addition, metabolomics studies recently identified representative metabolites in CSF of patients with neurosyphilis as new indicators, such as L-histidine, bilirubin, alpha-kamlolenic acid, prostaglandin E2, palmitoyl-L-carnitine, butyryl-L-carnitine, D-mannose, L-gulono-gamma-lactone, hypoxanthine, and N-acetyl-L-tyrosine (77, 106).

DISCUSSION

As the folk goes, "One night with Venus, a lifetime with Mercury". The everlasting stigmatization of syphilis has hindered the development of diagnostic and therapeutic management, especially in the follow-up, partner notification, and wide-range screening in susceptible populations (107). As a so-called prevalent disease in celebrities with a libertine lifestyle, syphilis was said to have infected the most influential people like Shakespeare and Columbus, and evoked further panic in Europe by the horrible symptoms of mercury poisoning during treatment (108). Mystified and misread for centuries, syphilis was ultimately controlled by the wide use of antibiotics. However, it has not been eliminated till now, due to the increase in sexual risk behaviors mainly in homosexual communities since the mid-1990s (109). Neurosyphilis, a stage that *T. pallidum* invades the central nervous system, may progress to severe cardiovascular complications and irreversible neurological damages if treatment is delayed. The alarming resurgence of syphilis also attracts attention to the incidence of neurosyphilis, particularly in MSM and PLWH.

The latest research progress on neurosyphilis in recent years is summarized. In some series, neurosyphilis was diagnosed disproportionately in young patients with ischemic stroke, which argued for the screening in low-risk populations presenting with specific abnormalities, including ischemic stroke, rapidly progressed neuropsychiatric symptoms, and recurrent ocular inflammation (110). However, the implementation of screening for sexually transmitted infections may bring humiliation to the subjects, damage their close relationships and personal image in society (53). Comorbid neurosyphilis with HIV or other sexually transmitted infections has been reported worldwide, of which the comorbidities may cause misdiagnosis and failure of conventional therapy; HIV coinfection does not affect the prognosis of patients with neurosyphilis (75). Most of the basic research focused on the pathogenesis of neurosyphilis, based on the changes of non-coding RNAs, chemokines, proteins, lymphocyte subtypes, and metabolites in CSF of patients with neurosyphilis. These potential biomarkers may be promising for diagnosis and disease monitoring, but whether they can explain the pathogenesis of neurosyphilis remains unclear. Evidence showed similarity between neurodegenerative diseases and neurosyphilis in pathogenesis and clinical manifestations, which suggested that the existing theories of Alzheimer's

disease might enlighten the research of neurosyphilis (25). The epidemic of syphilis and neurosyphilis in low- and middle- income countries is difficult to control due to the resource-limited settings there. However, point-of-care syphilis tests developed in recent years may relieve the pressure of disease control for its feasibility and inexpensiveness (56). JHR has also been reported in almost all types of commonly used antibiotics for syphilis, especially in the treatment of PLWH (68).

There are still some problems to be solved. (1) Due to the inaccessible *in-vitro* culture and unknown host response to the infection of *T. pallidum*, the development of vaccines still has a long way to go (111). (2) The false-negative and false-positive rates remain high in a single treponemal or nontreponemal test, thus combined use of various diagnostic methods is recommended, alongside neuroimaging and individuals' history of sex life. It should be noticed that not only false-negative tests may delay the treatment, but false-positive tests may also cause unnecessary management and stigmatization to patients. (3) Penicillin remains the first choice for the treatment of neurosyphilis, but there are few alternative treatment modalities for patients with cross-allergy of penicillin and ceftriaxone (59). Some conventional therapies also have side effects for PLWH (65). (4) Another challenge in the treatment is serofast. The cause of the serofast is still unclear and retreatment to serofast patients can not significantly improve the cure rates (71, 72). Prediction of the serofast occurrence is also difficult. Moreover, some serofast patients may no longer be threatened by syphilis, but other patients may still be at risk, which is hard to evaluate. (5) Lumbar puncture also remains controversial in the diagnosis of neurosyphilis, due to its difficulty of implementation and potential risks posed to patients. CDC does not recommend lumbar puncture for PLWH with syphilis yet without neurological symptoms, and only one lumbar puncture is allowed every 6 months after treatment to monitor the prognosis. Thus, patients with neurosyphilis are easily lost to follow-up. (6) Additionally, the ignorance of patients about the symptoms of early syphilis or asymptomatic neurosyphilis is common and often results in delayed diagnosis and treatment, which might account for the rising incidence of neurosyphilis. A study from Tanzania found that the average consultation delay for rural patients was almost double that for urban patients (112). Another study demonstrated that only 16.7% patients with symptomatic neurosyphilis visited dermatology department, as patients rarely paid attention to their skin conditions (24). (7) Some essential approaches for controlling neurosyphilis are still time-consuming and difficult to undertake, including public education, partner notification, and screening projects. Whereas, excessive screening and prophylactic treatment of syphilis may bring humiliation to the individuals and waste of resources to the society. Thus, when developing new screening or prevention policies, balance should be maintained carefully. For instance, prophylactic treatment is not recommended for partners of neurosyphilis patients due to the limited infectiousness of neurosyphilis, whereas serological tests are necessary for them to exclude the risk of transmission (54). Further research could concentrate on the pathogenesis, standards and guidelines, treatment modalities, JHR and serofast, follow-up, and the prevention of large-scale coinfection in MSM and PLWH.

CONCLUSIONS

This article reviews recent advances in neurosyphilis, including epidemiology, clinical manifestations, laboratory findings, comorbidities, diagnosis, treatment, prognosis, and basic research. The incidence of neurosyphilis has been increasing in recent years, mainly in MSM. The incidence of historically described forms of neurosyphilis in the pre-antibiotic era declined significantly nowadays, with atypical features becoming more common. Neurosyphilis can mimic most neuro-ophthalmic, audio-vestibular, and psychiatric disorders. Comorbidities of neurosyphilis are also prevalent and variable, and patients may present with specific metabolic characteristics. Clinical diagnostic methods remain immature, whereas recent studies on long non-coding RNA, miRNA, chemokines, and metabolites in peripheral blood and CSF may facilitate the research on the pathogenesis and new indicators of neurosyphilis. The resistance of *T. pallidum* to penicillin has not been discovered. Some studies also found that ceftriaxone was more effective than penicillin, but few large randomized controlled trials supported this view. Attention should also be paid to specific populations, such as PLWH, pregnant women, and those allergic to penicillin.

AUTHOR CONTRIBUTIONS

JZ, HZ, and KT reviewed the literature and wrote the manuscript. RL reviewed the literature. JL revised the manuscript. All authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this article, take responsibility for the integrity of the work as a whole, and have given their approval for this version to be published.

FUNDING

The Rapid Service Fee was funded by Peking Union Medical College Hospital. We acknowledge the support from Beijing Municipal Science and Technology Commission (No. Z191100006619011), the Capital's Funds for Health Improvement and Research (2020-2-4016), and CAMS Innovation Fund for Medical Sciences (CIFMS 2020-I2M-C&T-B-048).

REFERENCES

1. Ropper A. Neurosyphilis. *N Engl J Med.* (2019) 381:2377. doi: 10.1056/NEJMc1914502
2. Bettuzzi T, Jourdes A, Robineau O, Alcaraz I, Manda V, Molina J, et al. Ceftriaxone compared with benzylpenicillin in the treatment of neurosyphilis in France: a retrospective multicentre study. *Lancet Infect Dis.* (2021) 21:1441–7. doi: 10.1016/S1473-3099(20)30857-4
3. Zhang K, Chu F, Wang C, Shi M, Yang Y. Progressive stroke caused by neurosyphilis with concentric enhancement in the internal cerebral artery on high-resolution magnetic resonance imaging: a case report. *Front Neurol.* (2021) 12: 675083. doi: 10.3389/fneur.2021.675083
4. Newman L, Rowley J, Vander Hoorn S, Wijesooriya NS, Unemo M, Low N, et al. Global estimates of the prevalence and incidence of four curable sexually transmitted infections in 2012 based on systematic review and global reporting. *PLoS ONE.* (2015) 10:e0143304. doi: 10.1371/journal.pone.0143304
5. de Voux A, Kidd S, Torrone EA. Reported cases of neurosyphilis among early syphilis cases-United States, 2009 to 2015. *Sex Transm Dis.* (2018) 45:39–41. doi: 10.1097/OLQ.0000000000000687
6. Workowski KA, Bachmann LH, Chan PA, Johnston CM, Muzny CA, Park I, et al. Sexually transmitted infections treatment guidelines, 2021. *MMWR Recomm Rep.* (2021) 70:1–187. doi: 10.15585/mmwr.rr7004a1
7. Ting-ting TIAN Y-xH, Yu-qing LI, Hong-jiao QI, Mo CHEN, Mei-xia LÜ. Spatio-temporal analysis of incidence rate of syphilis in China. *J Shanghai Jiaotong Univ.* (2021) 41:648–52. doi: 10.3969/j.issn.1674-8115.2021.05.015
8. Pintado Maury I, Alves M, Fonseca T. Neurosyphilis prevalence at a Portuguese stroke unit care. *Aging Clin Exp Res.* (2019) 31:1155–61. doi: 10.1007/s40520-018-1052-4
9. Salado-Rasmussen K, Wessman M, Cowan S, Gerstoft J, Katzenstein T. Syphilitic hepatitis and neurosyphilis: an observational study of Danish HIV-infected individuals during a 13-year period. *Sex Transm Infect.* (2019) 95:416–8. doi: 10.1136/sextrans-2018-053921
10. Li W, Jiang M, Xu D, Kou C, Zhang L, Gao J, et al. Clinical and laboratory characteristics of symptomatic and asymptomatic neurosyphilis in HIV-negative patients: a retrospective study of 264 cases. *Biomed Res Int.* (2019) 2019:2426313. doi: 10.1155/2019/2426313
11. Centers for Disease Control and Prevention (CDC). Notes from the field: repeat syphilis infection and HIV coinfection among men who have sex with men–Baltimore, Maryland, 2010-2011. *MMWR Morb Mortal Wkly Rep.* (2013) 62:649–50.
12. Xiang L, Zhang T, Zhang B, Zhang C, Hou S, Yue W. The associations of increased cerebral small vessel disease with cognitive impairment in neurosyphilis presenting with ischemic stroke. *Brain Behav.* (2021) 11:e02187. doi: 10.1002/brb3.2187
13. Tan X, Zhang J, Li J, Yue X, Gong X. The prevalence of asymptomatic neurosyphilis among HIV-negative serofast patients in China: a meta-analysis. *PLoS ONE.* (2020) 15:e0241572. doi: 10.1371/journal.pone.0241572
14. Dai T, Li K, Lu H, Gu X, Wang Q, Zhou P. Molecular typing of Treponema pallidum: a 5-year surveillance in Shanghai, China. *J Clin Microbiol.* (2012) 50:3674–7. doi: 10.1128/JCM.01195-12
15. Marra CM, Sahi SK, Tantalo LC, Ho EL, Dunaway SB, Jones T, et al. Toll-like receptor polymorphisms are associated with increased neurosyphilis risk. *Sex Transm Dis.* (2014) 41:440–6. doi: 10.1097/OLQ.0000000000000149
16. Pastuszczak M, Jakiela B, Jaworek AK, Wypasek E, Zeman J, Wojas-Pelc A. Association of Interleukin-10 promoter polymorphisms with neurosyphilis. *Hum Immunol.* (2015) 76:469–72. doi: 10.1016/j.humimm.2015.06.010
17. Kissani N, Nafia S, Zahlane S, Louhab N. Neurosyphilis: a series of 178 cases at the 3rd-level hospital of Marrakesh (Morocco). *Eur J Clin Microbiol Infect Dis.* (2021) 40:2129–35. doi: 10.1007/s10096-021-04253-y
18. Landry T, Smyczek P, Cooper R, Gratrix J, Bertholet L, Read R, et al. Retrospective review of tertiary and neurosyphilis cases in Alberta, 1973-2017. *BMJ Open.* (2019) 9:e025995. doi: 10.1136/bmjopen-2018-025995
19. Singh AE. Ocular and neurosyphilis: epidemiology and approach to management. *Curr Opin Infect Dis.* (2020) 33:66–72. doi: 10.1097/QCO.0000000000000617
20. Theeuwen H, Whipple M, Litvack JR. Otosyphilis: resurgence of an old disease. *Laryngoscope.* (2019) 129:1680–4. doi: 10.1002/lary.27635
21. Liu LL, Zheng WH, Tong ML, Liu GL, Zhang HL, Fu ZG, et al. Ischemic stroke as a primary symptom of neurosyphilis among HIV-negative emergency patients. *J Neurol Sci.* (2012) 317:35–9. 2012/04/10. doi: 10.1016/j.jns.2012.03.003
22. Targa Martins R, Castilhos R, Silva da. Silva P, Costa L. Frequency of screening and prevalence of neurosyphilis in stroke population. *Cerebrovasc Dis.* (2020) 49:301–6. doi: 10.1159/000508491
23. Gonzalez H, Koralnik I, Marra C. Neurosyphilis. *Semin Neurol.* (2019) 39:448–55. doi: 10.1055/s-0039-1688942
24. Shang X, He C, Tang B, Chang X, Ci C, Sang H. Neuroimaging features, follow-up analyses, and comparisons between asymptomatic and symptomatic neurosyphilis. *Dermatol Ther.* (2020) 10:273–83. doi: 10.1007/s13555-020-00361-3
25. Zhang M, Zhong X, Shi H, Vanmechelen E, De Vos A, Liu S, et al. BACE1 and Other Alzheimer's-related biomarkers in cerebrospinal fluid and plasma distinguish Alzheimer's disease patients from cognitively-impaired neurosyphilis patients. *J Alzheimer's Dis.* (2020) 77:313–22. doi: 10.3233/JAD-200362
26. Wang C, Zhu L, Gao Z, Guan Z, Lu H, Shi M, et al. Increased interleukin-17 in peripheral blood and cerebrospinal fluid of neurosyphilis patients. *PLoS Negl Trop Dis.* (2014) 8:e3004. doi: 10.1371/journal.pntd.0003004
27. Mukku S, Safal S, Pritam R, Nashi S, Nagarathna C, Pt S, et al. Neurosyphilis presenting as rapidly progressive psychosis & dementia - a forgotten entity. *Asian J Psychiatr.* (2019) 40:103–6. doi: 10.1016/j.ajp.2019.02.010
28. Spelber D, Lahijani S. Neurosyphilis presenting as mania and psychosis after incidental treatment with cephalexin: a case report and literature review of jarisch-herxheimer reactions. *Psychosomatics.* (2020) 61:177–80. doi: 10.1016/j.psym.2019.06.001
29. G N, Moore E, Sithole N. The great imitator: neurosyphilis presenting as subacute confusion. *Br J Hosp Med.* (2021) 82:1–3. doi: 10.12968/hmed.2020.0669
30. Rotman L, Luo X, Thompson A, Mackesy-Amiti M, Young L, Young J. Risk of neurosyphilis in HIV-infected persons with syphilis lacking signs or symptoms of central nervous system infection. *HIV Med.* (2019) 20:27–32. doi: 10.1111/hiv.12677
31. Wu MY, Gong HZ, Hu KR, Zheng HY, Wan X, Li J. Effect of syphilis infection on HIV acquisition: a systematic review and meta-analysis. *Sex Transm Infect.* (2021) 97:525–33. doi: 10.1136/sextrans-2020-054706
32. Nandwani R, Evans DT. Are you sure it's syphilis? *A review of false positive serology Int J STD AIDS.* (1995) 6:241–8. doi: 10.1177/095646249500600404
33. Buder S, Schöfer H, Meyer T, Bremer V, Kohl PK, Skaletz-Rorowski A, et al. Bacterial sexually transmitted infections. *J Dtsch Dermatol Ges.* (2019) 17:287–315. doi: 10.1111/ddg.13804
34. Weathers EN, Waller JL, Nahman NS. Jr., Colombo RE, Kheda MF, Baer SL. Incidence, risk factors and distribution of syphilis in the end-stage renal disease population in the USA. *Clin Kidney J.* (2020) 13:625–30. doi: 10.1093/ckj/sfz090
35. Furtado JM, Simões M, Vasconcelos-Santos D, Oliver GF, Tyagi M, Nascimento H, et al. Ocular syphilis. *Surv Ophthalmol.* (2021) 67:440–62. doi: 10.1016/j.survophthal.2021.06.003
36. Oliver GF, Stathis RM, Furtado JM, Arantes TE, McCluskey PJ, Matthews JM, et al. Current ophthalmology practice patterns for syphilitic uveitis. *Br J Ophthalmol.* (2019) 103:1645–9. doi: 10.1136/bjophthalmol-2018-313207
37. Mathew D, Smit D. Clinical and laboratory characteristics of ocular syphilis and neurosyphilis among individuals with and without HIV infection. *Br J Ophthalmol.* (2021) 105:70–4. doi: 10.1136/bjophthalmol-2019-315699
38. Bazewicz M, Lhoir S, Makhoul D, Libois A, Van den Wijngaert S, Caspers L, et al. Neurosyphilis cerebrospinal fluid findings in patients with ocular syphilis. *Ocul Immunol Inflamm.* (2021) 29:95–101. doi: 10.1080/09273948.2019.1672193
39. Uhr JH, Dubovy SR, Gregori NZ. Syphilitic uveitis masquerading as a vitreous hemorrhage and retinal detachment. *Ophthalmol Retina.* (2021) 5:729. doi: 10.1016/j.oret.2021.04.018
40. Ramchandani MS, Litvack JR, Marra CM. Otosyphilis: a review of the literature. *Sex Transm Dis.* (2020) 47:296–300. doi: 10.1097/OLQ.0000000000001155

41. Bradshaw D, Pallawela S, Nelson M, Scott C, Day S. Otosyphilis: missed opportunities for early treatment? *Sex Transm Infect.* (2012) 88:573. doi: 10.1136/sextrans-2012-050792
42. Kivekäs I, Vasama JP, Hakomäki J. Bilateral temporal bone otosyphilis. *Otol Neurotol.* (2014) 35:e90–1. doi: 10.1097/MAO.0b013e3182a3603f
43. Janier M, Unemo M, Dupin N, Tiplica GS, Potočnik M, Patel R. 2020 European guideline on the management of syphilis. *J Eur Acad Dermatol Venereol.* (2021) 35:574–88. doi: 10.1111/jdv.16946
44. Xiao Y, Chen MJ, Shen X, Lin LR, Liu LL, Yang TC, et al. Metabolic disorders in patients with central nervous system infections: associations with neurosyphilis. *Eur Neurol.* (2019) 81:270–7. doi: 10.1159/000503626
45. Wang F, Ge H, Su X, Wang R, Zeng J, Miao J. High HbA(1c) level is correlated with blood-brain barrier disruption in syphilis patients. *Neurol Sci.* (2020) 41:83–90. doi: 10.1007/s10072-019-04031-x
46. Boog G, Lopes J, Mahler J, Solti M, Kawahara L, Teng A, et al. Diagnostic tools for neurosyphilis: a systematic review. *BMC Infect Dis.* (2021) 21:568. doi: 10.1186/s12879-021-06264-8
47. Marra C. Alternatives to the cerebrospinal fluid venereal disease research laboratory test for neurosyphilis diagnosis. *Sex Transm Dis.* (2021) 48:S54–7. doi: 10.1097/OLQ.0000000000001450
48. Smit D, De Graaf M, Meyer D, de Groot-Mijnes J. Immunoblot and polymerase chain reaction to diagnose ocular syphilis and neurosyphilis in HIV-positive and HIV-negative Patients. *Ocul Immunol Inflamm.* (2020) 28:1049–55. doi: 10.1080/09273948.2019.1698753
49. Gudowska-Sawczuk M, Mroczko B. Chemokine ligand 13 (CXCL13) in neuroborreliosis and neurosyphilis as selected spirochetal neurological diseases: a review of its diagnostic significance. *Int J Mol Sci.* (2020) 21:2927. doi: 10.3390/ijms21082927
50. Wang C, Wu K, Yu Q, Zhang S, Gao Z, Liu Y, et al. CXCL13, CXCL10 and CXCL8 as potential biomarkers for the diagnosis of neurosyphilis patients. *Sci Rep.* (2016) 6:33569. doi: 10.1038/srep33569
51. Lu Y, Ke W, Yang L, Wang Z, Lv P, Gu J, et al. Clinical prediction and diagnosis of neurosyphilis in HIV-negative patients: a case-control study. *BMC Infect Dis.* (2019) 19:1017. doi: 10.1186/s12879-019-4582-2
52. Kingston M, French P, Higgins S, McQuillan O, Sukthankar A, Stott C, et al. UK national guidelines on the management of syphilis 2015. *Int J STD AIDS.* (2016) 27:421–46. doi: 10.1177/0956462415624059
53. Forrestel AK, Kovarik CL, Katz KA. Sexually acquired syphilis: laboratory diagnosis, management, and prevention. *J Am Acad Dermatol.* (2020) 82:17–28. doi: 10.1016/j.jaad.2019.02.074
54. Judith W, WHO. *Guidelines for the Treatment of Treponema pallidum (Syphilis).* Geneva: World Health Organization (2016).
55. Ho EL, Tantalo LC, Jones T, Sahi SK, Marra CM. Point-of-care treponemal tests for neurosyphilis diagnosis. *Sex Transm Dis.* (2015) 42:48–52. doi: 10.1097/OLQ.0000000000000222
56. Gonzalez H, Koralnik I, Huhn G, Tantalo L, Ritz E, Orban Z, et al. A dual-platform point-of-care test for neurosyphilis diagnosis. *Sex Transm Dis.* (2021) 48:353–6. doi: 10.1097/OLQ.0000000000001308
57. Chinese expert consensus statement on diagnosis and treatment of syphilis. *Chin Med J.* (2020) 133:2335–7. doi: 10.1097/CM9.0000000000001035
58. Marra CM, Boutin P, McArthur JC, Hurwitz S, Simpson PA, Haslett JA, et al. A pilot study evaluating ceftriaxone and penicillin G as treatment agents for neurosyphilis in human immunodeficiency virus-infected individuals. *Clin Infect Dis.* (2000) 30:540–4. doi: 10.1086/313725
59. Buitrago-Garcia D, Martí-Carvajal A, Jimenez A, Conterno L, Pardo R. Antibiotic therapy for adults with neurosyphilis. *Cochrane Database Syst Rev.* (2019) 5:CD011399. doi: 10.1002/14651858.CD011399.pub2
60. Ozturk-Engin D, Erdem H, Hasbun R, Wang S, Tireli H, Tattevin P, et al. Predictors of unfavorable outcome in neurosyphilis: multicenter ID-IRI study. *Eur J Clin Microbiol Infect Dis.* (2019) 38:125–34. doi: 10.1007/s10096-018-3403-7
61. Gordon SM, Eaton ME, George R, Larsen S, Lukehart SA, Kuypers J, et al. The response of symptomatic neurosyphilis to high-dose intravenous penicillin G in patients with human immunodeficiency virus infection. *N Engl J Med.* (1994) 331:1469–73. doi: 10.1056/NEJM199412013312201
62. Marra CM, Longstreth WT. Jr., Maxwell CL, Lukehart SA. Resolution of serum and cerebrospinal fluid abnormalities after treatment of neurosyphilis Influence of concomitant human immunodeficiency virus infection. *Sex Transm Dis.* (1996) 23:184–9. doi: 10.1097/00007435-199605000-00005
63. Peermohamed S, Kogilwaimath S, Sanche S. Neurosyphilis. *CMAJ.* (2020) 192:E844. doi: 10.1503/cmaj.200189
64. Girometti N, Junejo M, Nugent D, McOwan A, Whitlock G. Clinical and serological outcomes in patients treated with oral doxycycline for early neurosyphilis. *J Antimicrob Chemother.* (2021) 76:1916–9. doi: 10.1093/jac/dkab100
65. Richardson D, Goldmeier D. Probenecid in the treatment of neurosyphilis in men who have sex with men: a commentary. *Sex Transm Infect.* (2021). doi: 10.1136/sextrans-2021-055278. [Epub ahead of print].
66. Butler T. The jarisch-herxheimer reaction after antibiotic treatment of spirochetal infections: a review of recent cases and our understanding of pathogenesis. *Am J Trop Med Hyg.* (2017) 96:46–52. doi: 10.4269/ajtmh.16-0434
67. Tsai MS, Yang CJ, Lee NY, Hsieh SM, Lin YH, Sun HY, et al. Jarisch-Herxheimer reaction among HIV-positive patients with early syphilis: azithromycin versus benzathine penicillin G therapy. *J Int AIDS Soc.* (2014) 17:18993. doi: 10.7448/IAS.17.1.18993
68. Kapila R, Schwartz R. Neurosyphilis and the jarisch-herxheimer reaction: a therapy concern with HIV disease. *Dermatol Ther.* (2021) 34:e14839. doi: 10.1111/dth.14839
69. Cui L, Xu Z, Hou H. Diagnosis and treatment of spinal syphilitic gumma: a case report. *Front Neurol.* (2019) 10:1352. doi: 10.3389/fneur.2019.01352
70. Marra CM, Ghanem KG. Centers for disease control and prevention syphilis summit: difficult clinical and patient management issues. *Sex Transm Dis.* (2018) 45(Suppl. 1):S10–2. doi: 10.1097/OLQ.0000000000000851
71. Ren M, Szadkowski L, Walmsley SL. Deciphering the serological response to syphilis treatment in men living with HIV. *Aids.* (2020) 34:2089–96. doi: 10.1097/QAD.0000000000002656
72. Jia X, Wang Z, Liu X, Zheng H, Li J. Peripheral blood mononuclear cell microRNA profiles in syphilitic patients with serofast status. *Mol Biol Rep.* (2020) 47:3407–21. doi: 10.1007/s11033-020-05421-7
73. Wang ZS, Liu XK Li J. Serological response to therapy following retreatment of serofast early syphilis patients with benzathine penicillin. *J Antimicrob Chemother.* (2018) 73:1348–51. doi: 10.1093/jac/dky006
74. Liu XK, Wang ZS Li J. Predictors of serofast state after treatment of patients with syphilis. *Chin Med J (Engl).* (2020) 133:2874–6. doi: 10.1097/CM9.0000000000001175
75. Dunaway S, Maxwell C, Tantalo L, Sahi S, Marra C. Neurosyphilis treatment outcomes after intravenous penicillin G versus intramuscular procaine penicillin plus oral probenecid. *Clin Infect Dis.* (2020) 71:267–73. doi: 10.1093/cid/ciz795
76. Jiang M, Zhang H, Li W, Wu W, Huang Y, Xu D, et al. Analysis of EEG Lemple-Ziv complexity and correlative aspects before and after treatment of anti-syphilis therapy for neurosyphilis. *Neurol Res.* (2019) 41:199–203. doi: 10.1080/01616412.2018.1520438
77. Qi S, Xu Y, Luo R, Li P, Huang Z, Huang S, et al. Novel biochemical insights in the cerebrospinal fluid of patients with neurosyphilis based on a metabonomics study. *J Mol Neurosci.* (2019) 69:39–48. doi: 10.1007/s12031-019-01320-0
78. Jame R, Al-Saeigh Y, Wang LL, Wang K. Justified suspicion: symptomatic syphilitic alopecia in a patient with well-controlled HIV. *Case Rep Infect Dis.* (2021) 2021:1124033. doi: 10.1155/2021/1124033
79. Jain J, Santos GM, Scheer S, Gibson S, Crouch PC, Kohn R, et al. Rates and correlates of syphilis reinfection in men who have sex with men. *LGBT Health.* (2017) 4:232–6. doi: 10.1089/lgbt.2016.0095
80. Cohen SE, Chew Ng RA, Katz KA, Bernstein KT, Samuel MC, Kerndt PR, et al. Repeat syphilis among men who have sex with men in California, 2002-2006: implications for syphilis elimination efforts. *Am J Public Health.* (2012) 102:e1–8. doi: 10.2105/AJPH.2011.300383
81. Almeida VC, Donalisio MR, Cordeiro R. Factors associated with reinfection of syphilis in reference centers for sexually transmitted infections. *Rev Saude Publica.* (2017) 51:64. doi: 10.1590/s1518-8787.2017051006432
82. Read PJ, Donovan B. Clinical aspects of adult syphilis. *Intern Med J.* (2012) 42:614–20. doi: 10.1111/j.1445-5994.2012.02814.x

83. Chang CC, Leslie DE, Spelman D, Chua K, Fairley CK, Street A, et al. Symptomatic and asymptomatic early neurosyphilis in HIV-infected men who have sex with men: a retrospective case series from 2000 to 2007. *Sex Health.* (2011) 8:207–13. doi: 10.1071/SH10060
84. Addetia A, Tantalo LC, Lin MJ, Xie H, Huang ML, Marra CM, et al. Comparative genomics and full-length Tprk profiling of Treponema pallidum subsp. pallidum reinfection. *PLoS Negl Trop Dis.* (2020) 14:e0007921. doi: 10.1371/journal.pntd.0007921
85. Thomas DD, Navab M, Haake DA, Fogelman AM, Miller JN, Lovett MA. Treponema pallidum invades intercellular junctions of endothelial cell monolayers. *Proc Natl Acad Sci U S A.* (1988) 85:3608–12. doi: 10.1073/pnas.85.10.3608
86. Zhang RL, Wang QQ. The Treponema pallidum outer membrane protein Tp92 activates endothelial cells via the chemerin/CMKLR1 pathway. *Int J Med Microbiol.* (2020) 310:151416. doi: 10.1016/j.ijmm.2020.151416
87. Carlson JA, Dabiri G, Cribier B, Sell S. The immunopathobiology of syphilis: the manifestations and course of syphilis are determined by the level of delayed-type hypersensitivity. *Am J Dermatopathol.* (2011) 33:433–60. doi: 10.1097/DAD.0b013e3181e8b587
88. Cruz AR, Ramirez LG, Zuluaga AV, Pillay A, Abreu C, Valencia CA, et al. Immune evasion and recognition of the syphilis spirochete in blood and skin of secondary syphilis patients: two immunologically distinct compartments. *PLoS Negl Trop Dis.* (2012) 6:e1717. doi: 10.1371/journal.pntd.0001717
89. Centurion-Lara A, Giacani L, Godornes C, Molini BJ, Brinck Reid T, Lukehart SA. Fine analysis of genetic diversity of the tpr gene family among treponemal species, subspecies and strains. *PLoS Negl Trop Dis.* (2013) 7:e2222. doi: 10.1371/journal.pntd.0002222
90. Reid TB, Molini BJ, Fernandez MC, Lukehart SA. Antigenic variation of TprK facilitates development of secondary syphilis. *Infect Immun.* (2014) 82:4959–67. doi: 10.1128/IAI.02236-14
91. Qin J, Yang T, Wang H, Feng T, Liu X. Potential predictors for serofast state after treatment among HIV-negative persons with syphilis in china: a systematic review and meta-analysis. *Iran J Public Health.* (2015) 44:155–69.
92. Guo N, Liu L, Yang X, Song T, Li G, Li L, et al. Immunological changes in monocyte subsets and their association with Foxp3(+) Regulatory T cells in HIV-1-infected individuals with syphilis: a brief research report. *Front Immunol.* (2019) 10:714. doi: 10.3389/fimmu.2019.00714
93. Li W, Wu W, Chang H, Jiang M, Gao J, Xu Y, et al. Cerebrospinal fluid cytokines in patients with neurosyphilis: the significance of interleukin-10 for the disease. *Biomed Res Int.* (2020) 2020:3812671. doi: 10.1155/2020/3812671
94. Yan Y, Wang J, Qu B, Zhang Y, Wei Y, Liu H, et al. CXCL13 and TH1/Th2 cytokines in the serum and cerebrospinal fluid of neurosyphilis patients. *Medicine (Baltimore).* (2017) 96:e8850. doi: 10.1097/MD.0000000000008850
95. Luo X, Zhang X, Gan L, Zhou C, Zhao T, Zeng T, et al. The outer membrane protein Tp92 of Treponema pallidum induces human mononuclear cell death and IL-8 secretion. *J Cell Mol Med.* (2018) 22:6039–54. doi: 10.1111/jcmm.13879
96. Liu L. Changes of T lymphocyte subsets in patients with HIV-negative symptomatic neurosyphilis. *Microb Pathog.* (2019) 130:213–8. doi: 10.1016/j.micpath.2019.03.008
97. Zhu A, Han H, Zhao H, Hu J, Jiang C, Xie F, et al. Increased frequencies of Th17 and Th22 cells in the peripheral blood of patients with secondary syphilis. *FEMS Immunol Med Microbiol.* (2012) 66:299–306. doi: 10.1111/j.1574-695X.2012.01007.x
98. Yu Q, Cheng Y, Wang Y, Wang C, Lu H, Guan Z, et al. Aberrant humoral immune responses in neurosyphilis: CXCL13/CXCR5 play a pivotal role for B-cell recruitment to the cerebrospinal fluid. *J Infect Dis.* (2017) 216:534–44. doi: 10.1093/infdis/jix233
99. Balasa R, Barcutean L, Balasa A, Motataianu A, Roman-Filip C, Manu D. The action of TH17 cells on blood brain barrier in multiple sclerosis and experimental autoimmune encephalomyelitis. *Hum Immunol.* (2020) 81:237–43. doi: 10.1016/j.humimm.2020.02.009
100. Guziejko K, Czupryna P, Pancewicz S, Swierzbińska R, Dunaj J, Kruszewska E, et al. Analysis of CCL-4, CCL-17, CCL-20 and IL-8 concentrations in the serum of patients with tick-borne encephalitis and anaplasmosis. *Cytokine.* (2020) 125:154852. doi: 10.1016/j.cyto.2019.154852
101. Li W, Chang H, Wu W, Xu D, Jiang M, Gao J, et al. Increased CSF soluble TREM2 concentration in patients with neurosyphilis. *Front Neurol.* (2020) 11:62. doi: 10.3389/fneur.2020.00062
102. Xu D, Cai S, Li R, Wu Y, Liu S, Lun W. Elevation of cerebrospinal fluid light and heavy neurofilament levels in symptomatic neurosyphilis. *Sex Transm Dis.* (2020) 47:634–8. doi: 10.1097/OLQ.0000000000001236
103. Lämmermann T, Kastenmüller W. Concepts of GPCR-controlled navigation in the immune system. *Immunol Rev.* (2019) 289:205–31. doi: 10.1111/imr.12752
104. Liu W, Wu K, Wang X, Lin L, Tong M, Liu L. ENST00000421645LncRNA-promotes T cells to secrete IFN-γ by sponging PCM-1 in neurosyphilis. *Epigenomics.* (2021). doi: 10.2217/epi-2021-0163
105. Chen H, Zhou Y, Wang Z, Yan B, Zhou W, Wang T, et al. Exosomal microRNA profiles from serum and cerebrospinal fluid in neurosyphilis. *Sex Transm Infect.* (2019) 95:246–50. doi: 10.1136/sextrans-2018-053813
106. Liu L. Metabolite profiles of the cerebrospinal fluid in neurosyphilis patients determined by untargeted metabolomics analysis. *Front Neurosci.* (2019) 13:150. doi: 10.3389/fnins.2019.00150
107. Hook EW 3rd. Syphilis. *Lancet.* (2017) 389:1550–7. doi: 10.1016/S0140-6736(16)32411-4
108. Heymann WR. The history of syphilis. *J Am Acad Dermatol.* (2006) 54:322–3. doi: 10.1016/j.jaad.2005.10.003
109. Jin F, Prestage GP, Kippax SC, Pell CM, Donovan BJ, Kaldor JM, et al. Epidemic syphilis among homosexually active men in Sydney. *Med J Aust.* (2005) 183:179–83. doi: 10.5694/j.1326-5377.2005.tb06989.x
110. Andreae A, Barr A, Fulton Z, Enterline D, Dicks K. Appearance of meningovascular neurosyphilis causing an acute ischemic stroke. *Sex Transm Dis.* (2021) 48:e109–10. doi: 10.1097/OLQ.0000000000001330
111. Gottlieb SL, Johnston C. Future prospects for new vaccines against sexually transmitted infections. *Curr Opin Infect Dis.* (2017) 30:77–86. doi: 10.1097/QCO.0000000000000343
112. Haule A, Msemwa B, Mgaya E, Masikini P, Kalluvya S. Prevalence of syphilis, neurosyphilis and associated factors in a cross-sectional analysis of HIV infected patients attending Bugando Medical Centre, Mwanza, Tanzania. *BMC Public Health.* (2020) 20:1862. doi: 10.1186/s12889-020-09984-9

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers | Frontiers in Medicine

TYPE Case Report
PUBLISHED 21 September 2022
DOI 10.3389/fmed.2022.958456

OPEN ACCESS

EDITED BY
Pingyu Zhou,
Tongji University, China

REVIEWED BY
Jun Li,
Peking Union Medical College Hospital (CAMS), China
Marcus G. Tan,
The University of Ottawa and The Ottawa Hospital, Canada
Xin Gu,
Shanghai Dermatology Hospital, China

*CORRESPONDENCE
An-qi Liu
anqi-liu@ntu.edu.cn

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Infectious Diseases – Surveillance, Prevention and Treatment, a section of the journal Frontiers in Medicine

RECEIVED 31 May 2022
ACCEPTED 29 August 2022
PUBLISHED 21 September 2022

CITATION
Ren X-q, Nie Q-l and Liu A-q (2022) Primary syphilis without chancre – A case report of rare syphilitic balanitis of Follmann. *Front. Med.* 9:958456. doi: 10.3389/fmed.2022.958456

Primary syphilis without chancre – A case report of rare syphilitic balanitis of Follmann

Xu-qi Ren†, Qun-ling Nie† and An-qi Liu*

Department of Dermatology, Affiliated Hospital of Nantong University, Nantong, China

Introduction: Syphilitic balanitis of Follmann (SBF) is a rare condition of primary syphilis which is characterized by any kind of balanitis with or without chancre on the penis combined with the presence of swollen inguinal lymph nodes confirmed by the finding of Treponema pallidum in the lesions or by the positive serological syphilitic testing. Timely identification of the SBF is very important in properly treating the disease stopping the spread of syphilis.

Case presentation: A 42year-old heterosexual male patient came to our clinic and complained of a painless, hard erythema nodule with a whitish scale in his coronal sulcus of the penis for about a week. The dermatologic examination revealed an infiltrative, hard erythematous lesion surrounding the coronal sulcus of the patient's penis, with mild erosion and a small amount of exudation. There was a whitish pseudomembrane-like covering on the surface of the erythematous lesion in the coronal sulcus, which is mimicked as candidal balanitis. The result of the fungus microscopic examination was negative, while the laboratory findings showed positive results in serologic syphilitic testing. The patient was diagnosed with primary syphilis and intramuscularly treated with a dose of benzylpenicillin of 2.4 million units. The patient's skin lesions disappeared completely 60 days after penicillin treatment.

Conclusion: To our knowledge, this is the first SBF case reported in China. Syphilitic balanitis of Follmann may have variable clinical appearances. We emphasize that when balanitis with risky sexual activities or with sexually transmitted diseases, the diagnosis of SBF should be kept in mind.

KEYWORDS

syphilis, primary, primary syphilis without chancre, syphilitic balanitis, rare

Introduction

The incidence of syphilis has been increasing steadily in recent years (1, 2). It is believed that the agent of syphilis, *Treponema pallidum* (TP), invades the human skin or mucosa, then spreads to our body, and forms a long-term persistent chronic infection (3). Based on the time of infection, syphilis was divided into two stages, early

and late stages. Early syphilis includes primary, secondary, and early latent syphilis. Among those, primary and secondary syphilis are very contagious. Therefore, it is very important to treat the infected people in the early stages to stop the spread of the disease.

The syphilitic chancre is usually described as a painless, solitary, superficially eroded, genitalia involved, and self-healing erythematous nodular lesion (4). Although the typical syphilitic chancres are easy to identify, a low proportion of incident, clinical primary, and secondary syphilis cases are reported in the early stage, and in the United States, only 32% of primary and secondary syphilis cases were identified in the primary stage (5, 6). The fact suggests that a considerable number of primary syphilis cases are misdiagnosed or missed, which will greatly increase the spread of the disease. One of the reasons for the misdiagnosis of primary syphilis is the "mysterious character" of syphilitic chancers (7, 8). Herein, we report a case of primary syphilis with mucocutaneous manifestations mimicking candida balanitis, without obvious chancre, a rare manifestation in primary syphilis, so-called "syphilitic balanitis of Follmann" (SBF).

Case presentation

On 8th March 2022, a 42-year-old male patient came to our clinic and complained that he had a skin lesion on his penis for nearly a week and reported that he found a hard, strip of erythema distributed in the coronal sulcus on the left side of the penis at the beginning, and then quickly covered with the whitish substance on the surface of the erythema. Thereafter, the skin lesion gradually enlarged to a circle around the coronal sulcus. The lesion was painless with a mild burning sensation.

The patient is heterosexual and had his first sexual activity at the age of seventeen and divorced 4 years ago. He also reported that he had a history of taking methamphetamine for more than 10 years and was treated in Yixing's compulsory isolation drug treatment center in Jiangsu Province, China from October 2018 to December 2020. There was no relapse after drug treatment for more than 1 year. His medical history was generally normal except that for hypertension and he had no erosion or ulcer or blister on his penis before. When he was in the drug rehabilitation center, he underwent sexually transmitted diseases (STDs) testing, including Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), Human Immunodeficiency Virus (HIV), and syphilis, which were all negative. He had unprotected sex with multiple anonymous sex partners in the last 15 months after his compulsory isolation drug treatment, and his last unprotected casual sex was with a woman he met in a bar, which occurred 3 weeks before he visited our hospital.

On admission, physical examination revealed an infiltrative, hard erythematous lesion surrounding the coronal sulcus, with mild erosion and a small amount of exudation. There was a whitish pseudomembrane-like covering on the surface of the erythematous lesion in the coronal sulcus and the glans penis was not involved (**Supplementary Figure 1**). There were no other mucosal or skin lesions observed on the head, face, oropharynx, trunk, perianal, limbs, hands, and feet. The patient's inguinal lymph nodes were normal. Considering the clinical manifestations of the patient's lesion (erythematous lesion with mild erosion, a small amount of exudation, and the whitish covering) somewhat mimicked clinical characteristics of candidal balanitis, fungi microscopic examination was performed and showed a negative result. The patient then received the blood tests for STDs, including Treponema pallidum particle agglutination assay (TPPA), Rapid plasma reagin (RPR) assay, and Human Immunodeficiency Virus (HIV); Herpes simplex virus (HSV) type 1IgG, IgM, HSV type 2 IgG, IgM, which revealed positive in TPPA and HSV1, 2IgG test, and negative in RPR, HIV, HSV1, and 2 IgM tests.

Based on the fact that the patient had high-risk sexual behaviors 3 weeks before visiting our hospital, that he had an infiltrative, hard erythematous lesion surrounding the coronal sulcus hardly with any symptoms, and that he had no STDs history, a diagnosis of primary syphilis was made and a dose of benzylpenicillin 2.4 million units was given intramuscularly according to the guideline of the STD Association, China CDC (9). One day after the first dose of benzylpenicillin treatment, the patient came back to our hospital and reported that his mild burning sensation completely disappeared, and the hard erythematous lesion tended to soften. On examination, the redness and swelling of the erythematous lesion on the coronal margin subsided, and the exudation could not be observed. The whitish covering was significantly drier than that before treatment and the hard erythematous lesion seemed to soften (**Supplementary Figure 2**). After that, the patient returned to the hospital every day for another 6 days to have the above lesion observed. The erythematous lesion and whitish covering disappeared gradually, and the test for syphilis performed on day 7 after the treatment showed the result of TPPA (+) RPR (-). Then, on the 14th follow-up, the skin lesions of the coronal sulcus seemed to have almost disappeared without sequelae other than slight erythema at the site of previous lesions (**Supplementary Figure 3**). The blood TPPA and RPR was then rechecked and showed positive in TPPA and negative in RPR. Sixty days after treatment, the patient came back to the hospital again for the follow-up of the serum treponema and non-treponemal tests, which showed positive TPPA and RPR, and the RPR titer was 1:1. Dermatological examination showed that the skin of the coronal sulcus had completely returned to normal (**Supplementary Figure 4**). No other skin or mucosal lesions were observed, and the patient reported that he had no sex after syphilis treatment.

Discussion

The syphilitic chancre is in the first stage of syphilis, appearing within 90 days from the Treponema pallidum inoculation (4). The typical syphilitic chancres could be a painless, hard, and erosive plaque or nodule or superficial ulceration with a clear margin, which is easily recognized. However, these typical clinical manifestations are seen only in about 40% of primary syphilis cases, and the remaining cases may be atypical, making the diagnosis difficult (7), especially when chancre is absent in primary syphilis.

Syphilitic balanitis of Follmann (SBF) is a rare condition of primary syphilis, which is characterized by any kind of balanitis, with or without chancre on the penis, as well as with the presence of swollen inguinal lymph nodes confirmed by the finding of Treponema pallidum in the lesions or by the positive serological syphilitic testing (8).

SBF was first described by Follmann, a dermatologist from Budapest. In 1931, 1934, and 1939 he declared that erosive balanitis could be the only manifestation of primary syphilis (10). Thereafter, the reported cases of SBF could be seen occasionally and the variable clinical manifestations of SBF were revealed. People believe that except for the absence of a chancre, SBF could be seen before, after, or at the same time as a chancre (11). SBF can present with any kind of balanitis, not just erosive balanitis.

Our case presented unusual clinical manifestations of SBF, the infiltration, erosion, exudation, pseudomembrane-like covering, and hard erythematous lesion were mainly seen in the coronal sulcus sparing the patient's glans penis, and no enlargement of inguinal lymph nodes was observed, which were not consistent with the previous reports. Although the whitish covering on the surface of the coronal sulcus and the mild erosion of the erythematous base seemed somewhat like candidal infection, an infiltrative, hard erythematous lesion and the negative fungi microscopic examination refused the diagnosis of candidal balanitis. The reported case by Babu et al. described a patient who is HIV-positive with indurated SBF (12), and recently Mainetti et al. pointed out that many clinical manifestations are related to SBF, the skin lesions can be erosive or only indurated and scaly (8), which may partially support the viewpoint that our case is SBF. The short infection period of our case may explain why inguinal lymphadenopathy was not present. A suspicion of active herpetic infection could be made because of the positive serological tests of HSV IgG in our case. However, the patient reported that he had had no erosion or ulcer, or blister on his penis previously. The quickly subsided skin lesions after one dose of benzylpenicillin treatment, the positive serum treponema test, and the positive non-treponemal test could later be evidence of SBF diagnosis.

The diagnosis of primary syphilis could be a challenge in some cases. It is reported that 20–30% of the patients with chancre may have negative results in non-treponemal serological tests (13, 14). The positive serologic tests for Treponema pallidum appeared before non-Treponema pallidum. In our case, particularly, the short infection period of our case may explain why the non-treponemal serological test was negative before treatment. Then, the penicillin treatment further delayed the emergence of non-treponemal antibodies, which may explain why RPR was negative in the second week after treatment, and RPR turned positive 60 days after the treatment.

Since the patients refused to provide the skin samples, our case lacked etiological evidence, such as dark-field examination, histopathological findings, and PCR testing, which may limit its convincingness. However, this is a rare presentation of primary syphilis, which may help physicians to know more about the syphilitic balanitis of Follmann.

Our case suggests syphilitic balanitis of Follmann, which is a special type of primary syphilis that can have many different clinical manifestations and is easy to misdiagnose, especially when chancre is absent in this kind of primary syphilis. SBF can mimic any kind of balanitis, such as balanitis xerotica obliterans, plasma-cell balanitis, balanitis related to bacteria, virus, yeast, or parasite infection, and even trauma or irritants balanitis. Therefore, when balanitis with risky sexual activity or with other STDs, the diagnosis of SBF should be kept in mind.

Conclusion

To our knowledge, this is the first SBF case reported in China. The variable clinical manifestations of syphilitic balanitis of Follmann indicate that SBF may not be as rare as believed. To exclude the diagnosis of SBF, a detailed sexual activity history should be investigated and Dark-field examination (DFE) or the polymerase chain reaction (PCR) swab testing for TP should be given, or serological tests for syphilis should be performed when patients are presented with any kind of balanitis.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the [patients/participants OR patients/participants legal guardian/next of kin] was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

X-qR was responsible for receiving the patient, taking medical histories, comprehensively analyzing the patient's condition, and diagnosing syphilitic balanitis of Follmann (SBF), which is a rare condition of primary syphilis and recorded the treatment process and followed up on the patient's treatment. Q-lN collated in collaboration all the treatment records, pictures, and follow-ups. A-qL assisted in the diagnosis of the disease and provided advice on the treatment of the disease. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.958456/full#supplementary-material

References

1. Kidd SE, Grey JA, Torrone EA, Weinstock HS. Increased methamphetamine, injection drug, and heroin use among women and heterosexual men with primary and secondary syphilis – united states, 2013–2017. *MMWR Morb Mortal Wkly Rep.* (2019) 68:144–8. doi: 10.15585/mmwr.mm6806a4

2. Ghanem KG, Ram S, Rice PA. The modern epidemic of syphilis. *N Engl J Med.* (2020) 382:845–54. doi: 10.1056/NEJMc2006129

3. The Garnett GP, Aral SO, Hoyle DV, Cates W Jr., Anderson RM. The natural history of syphilis. implications for the transmission dynamics and control of infection. *Sex Transm Dis.* (1997) 24:185–200. doi: 10.1097/00007435-199704000-00002

4. Sparling PF, Swartz MN, Musher DM, et al. Clinical manifestations of syphilis. 4th ed. In: Holmes KK, Sparling PF, Stamm WE editors. *Sexually Transmitted Diseases*. New York: McGraw Hill (2008). p. 661–84.

5. Peterman TA, Kahn RH, Ciesielski CA. Misclassification of the stages of syphilis: implications for surveillance. *Sex Transm Dis.* (2005) 32:144–9. doi: 10.1097/01.olq.0000156552.91788.25

6. Gunn RA, Klausner JD. Enhancing the control of syphilis among men who have sex with men by focusing on acute infectious primary syphilis and core transmission groups. *Sex Transm Dis.* (2019) 46:629–36. doi: 10.1097/OLQ.0000000000001039

7. Cusini M, Ramoni S, Alessi E. Syphilis and other treponematoses. In: Giannetti A, Forno C editors. *Textbook of Dermatology & Sexually Transmitted Diseases.* (Chap. Italy), Piccin (2013). p. 799–854. doi: 10.3760/cma.j.issn.0412-4030.2014.05.022

8. Mainetti C, Scolari F, Lautenschlager SJ. The clinical spectrum of syphilitic balanitis of follmann: report of five cases and a review of the literature. *Eur Acad Dermatol Venereol.* (2016) 30:1810–3. doi: 10.1111/jdv.13802

9. Ncfsccfdca P, Vgso D, Sovd A. The diagnosis and treatment guidelines of syphilis, gonorrhea, genital herpes and chlamydial trachomatis infection (2014). *Chin J Dermatol.* (2014) 47:365–72. doi: 10.3760/cma.j.issn.0412-4030.2014.05.022

10. Follmann E. Le probleme de la balanite syphilitique. La vulvo-vaginite primaire syphilitique. *Ann Dermatol Syph.* (1948) 8:470–83.

11. Lejman K, Starzycki Z. Syphilitic balanitis of follmann developing after the appearance of the primary chancre. *Case Report Br J Vener Dis.* (1975) 51:138–40. doi: 10.1136/sti.51.2.138

12. Babu CS, Vitharana S, Higgins SP. Primary syphilis presenting as balani-tis. *Int J Std Aids.* (2007) 18:497–8. doi: 10.1258/095646207781147346

13. Seña AC, White BL, Sparling PF. Novel *Treponema pallidum* serologic tests: a paradigm shift in syphilis screening for the 21st century. *Clin Infect Dis.* (2010) 51:700–8. doi: 10.1086/655832

14. Hart G. Syphilis tests in diagnostic and therapeutic decision making. *Ann Intern Med.* (1986) 104:368–76. doi: 10.7326/0003-4819-104-3-368

Frontiers in Medicine

Translating medical research and innovation into improved patient care

A multidisciplinary journal which advances our medical knowledge. It supports the translation of scientific advances into new therapies and diagnostic tools that will improve patient care.

Discover the latest Research Topics

See more →

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact