# Syphilis and HIV co-infection

## Epidemiology, treatment and molecular typing of *Treponema pallidum*

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**INTRODUCTION**

Syphilis is a sexually transmitted disease caused by *Treponema pallidum*. Syphilis was endemic in Europe between the 15th and the 20th century and in the 19th century 10–20% of the population in Europe and America was thought to be infected (1). After it became known that syphilis was easily treated with penicillin the disease almost disappeared in Western countries before it surprisingly reemerged in the late 1990s (2). In Western countries, syphilis is now mainly encountered among men who have sex with men (MSM) (3). By contrast, in developing countries the disease represents an extensive problem (4;5). Above all, syphilis is a concern especially to pregnant women due to the increased risk of spontaneous abortion, stillbirth and congenital malformations of the newborns (6-8).

Denmark reached its syphilis nadir in 1999 with 22 cases of newly acquired syphilis (9;10). Increasing rates were then observed throughout a decade and peaked in 2011 with 434 cases (11). Whereas only 33% of the cases from 1994–2002 were among MSM, MSM represented 78% of the cases in 2003–2004 (12). Also in contrast to earlier, where most syphilis patients reported being infected in Eastern Europe, Africa and Asia (10), the majority of the patients now report acquiring syphilis in Denmark (13). In 2010 screening of pregnant women was re-introduced in Denmark (14) after the screening had been discontinued as a general screening in 1998 (10).

**Syphilis and HIV co-infection**

Syphilis and HIV are strongly linked with one another. The proportion of patients with concurrent HIV at the time of syphilis diagnosis has been substantial since the reemergence of the disease in Denmark (11;13;15) and peaked in 2008 where 58% of MSM diagnosed with syphilis had concurrent HIV (16). By contrast, only 32% of MSM diagnosed with syphilis in 2013 had concurrent HIV – leaving 68% susceptible to infection with HIV (13). In study I of the current thesis, it was documented that almost 10% of Danish men diagnosed with syphilis acquired HIV infection within five years after the diagnosis of syphilis (17). The marked increase in syphilis among MSM has mainly been attributed to increased sexual risk behavior in response to the improved effect of antiretroviral therapy on HIV (18;19). However, increasing use of illicit drugs may also have contributed to the increasing rates of syphilis (20). A recent Danish study (21) found that the rate of unsafe sex was increasing in HIV-infected MSM – despite this, the number of new HIV infections did not increase, indicating that cART was the reason for the decreased risk of transmission of HIV despite increased practice of unsafe sex. Grassly et al (22) proposed an alternative explanation of the increasing rates of syphilis; they suggested that the increase in syphilis was due to endogenous oscillation in disease incidence predicted by the natural dynamics of the infection. However, this theory on herd immunity has been questioned (23).

Several studies have corroborated the negative effect of syphilis on HIV infection; during syphilis infection viral load increases and CD4 cell counts decreases transiently (24;25). Also, of particular public health concern is the increased risk of transmission and acquisition of HIV during syphilis infection (26;27). Likewise, HIV has an impact on the clinical course of syphilis infection. The Syphilis and HIV study (28;29) included patients with and without HIV. Surprisingly, this multicenter, prospective study found that HIV had only a minor impact on the clinical manifestations of primary and secondary syphilis; HIV-infected patients with primary syphilis tended to present with more genital ulcers, and genital ulcers were more frequently present in HIV-infected patients with secondary syphilis compared to HIV-uninfected patients (29). Likewise, a recent study found that HIV co-infection had an impact only on the serological response in patients with primary syphilis and a CD4 cell count <500 cells/µL (30).

As a consequence of the high rates of syphilis among HIV-infected patients, an annual screening has been implemented at the departments of infectious diseases in Denmark, where patients with HIV are seen at an outpatient basis at intended intervals of 6 or 12 months.

### *Treponema pallidum*, the syphilis spirochete

*T. pallidum* subspecies *pallidum*, hereafter referred to as *T. pallidum*, is a spirochete. The spirochete family is characterized by a unique cell architecture with an outer membrane, a cell membrane and flagellar filaments in the periplasm (31). Other examples of spirochetes are the non-venereal treponemes *T. pallidum* subspecies *pertenue*, *T. pallidum* subspecies *endemicum* and *T. carateum* causing yaws, bejel (endemic syphilis) and pinta, respectively (32). The spirochete family also includes *Borrelia burgdorferi* and *Leptospira interrogans*.

*T. pallidum* is an obligate human parasite and its absolute dependence on a mammalian host for continuous viability has made it a difficult organism to study. The organism must be propagated by passage in rabbits, consequently it cannot be manipulated genetically. The genome (Nichols strain) is a circular chromosome of 1 138 006 base pairs (bp), making it one of the smaller prokaryotic genomes (33). In the absence of virulence factors such as toxins or secreted effector molecules, motility is crucial for the infectiveness and *T. pallidum* is extremely invasive and invades the skin or mucosa shortly after contact (34). The hematogenous dissemination occurs before the chancre even appears and *T. pallidum* often penetrates the blood-brain barrier during early infection (34).

The clinical manifestations during infection are caused by the inflammatory response elicited by the bacteria and bacterial constituents such as lipoproteins (34). Many theories have been proposed to explain how *T. pallidum* persists in the infected host and evades the host immune system. Strategies proposed are the extremely low metabolic rate and the antigenic variation of the outer membrane proteins of *T. pallidum* (35). It is well-established that *T. pallidum* elicits both a local and a systemic innate and adaptive immune response during early infection (36). In HIV-infected patients, cytokines have been shown to fluctuate with infection and treatment of syphilis (37). A recent study of ours investigating patients with HIV and hepatitis C virus (HCV) co-infection proposed that the reason for undetectable HCV RNA during syphilis infection was a *T. pallidum* induced cytokine secretion hindering HCV replication (38).

### Transmission of syphilis

The main route of syphilis transmission is sexual contact; however, syphilis can also be transmitted through blood, congenitally and through non-sexual contact with syphilitic lesions on skin or mucosa. The main risk of transmission occurs when mucocutaneous lesions are present, which is usually within the first year of infection (34). In the late latent stage, syphilis is not transmitted sexually (39). Patients diagnosed with syphilis should be screened for HIV and likewise, all patients who have HIV should be screened for syphilis (39;40). Sexual contacts to patients with syphilis should also be offered screening for syphilis (39). Vertical transmission of *T. pallidum* has major implications in developing countries. The risk of congenital syphilis depends on the time interval from acquisition of the infection to pregnancy and ranges from 30–80% (34;41). Based on the findings of spirochetes in the placenta and the umbilical cord, transplacental infection is most likely the major route of fetal infection (42;43).

### The clinical course of syphilis

Syphilis has been named 'The Great Imitator' due to its highly variable symptoms and multiple clinical stages. To make the diagnosis even more challenging the stages can be overlapping. From its site of primary infection, bacteria disseminate systemically through the blood to potentially any organ. The ulcer, most typically known as a chancre, appears at the inoculation site nine to 90 days after acquisition of the infection and heals spontaneously within three to eight weeks (34). In a study from the Sing Sing Prison where volunteers were inoculated intracutaneously with 10 or 100 spirochetes the mean incubation period was 24 days (44).

Untreated, the disease can progress to the secondary stage with systemic manifestations that include fever, lymphadenopathy, typical erythematous skin rashes (characteristically on palms, soles and trunk) and mucocutaneous lesions about six weeks after the primary chancre. Hepatitis, periostitis, arthritis, and glomerulonephritis is also seen in the secondary stage (39). The secondary syphilitic symptoms can occur in several different phases and between these phases the patient is in the latent stage with no clinical symptoms. During the first year of infection, about 25% of the asymptomatic patients will experience relapse to the secondary stage, hereafter relapses are far less common. For this reason the latent, asymptomatic stage is divided into an early latent stage (<1 year) and a late latent stage (>1 year or unknown duration). The term 'early syphilis' is often used and includes the primary, secondary and early latent stages of syphilis.

If left untreated the tertiary stage develops 5 to 30 years after transmission in approximately 30% of infected patients (45;46). The tertiary stage is characterized by gummatous lesions, cardiovascular syphilis and central nervous system (CNS) affection. The type of neurological manifestations depend on whether the predominant involvement is meningovascular (strokes, meningomyelitis) or parenchymatous (tabes dorsalis, general paresis of the insane) (34). Of note, neurosyphilis can occur in all stages of syphilis and includes cranial nerve dysfunction, meningitis, stroke, altered mental status and auditory or ophthalmic abnormalities (40). Neurosyphilis in early stages primarily affects meninges and cerebral or spinal cord vasculature, whereas late forms affect the spinal cord or brain parenchyma. It should be noted that cerebrospinal fluid (CSF) abnormalities are common in early syphilis, even in the absence of neurological symptoms (40).

### Diagnosis of syphilis

*T. pallidum* cannot be continuously cultivated in vitro. As a consequence, the use of direct diagnostic methods is limited. For over a century dark field microscopy of lesion exudate or tissue has been used, however, microscopy requires highly trained personnel and false-positive results can occur because *T. pallidum* is morphologically indistinguishable from commensal treponemes that can be present in body fluids (47). The mainstay for diagnosis of syphilis remains serologic testing although it should be noted that serologic tests cannot distinguish between syphilis and other treponematoses which can present a problem in regions where more treponemal infections are frequent (48;49). Since the 1990s polymerase chain reaction (PCR)-based methods for detection of *T. pallidum* have been developed (50;51) and are now routinely available in high-income countries. The targets are conserved regions of the *T. pallidum* genome. It is crucial that the target is highly specific to distinguish *T. pallidum* from other spirochetes (47). However, the success rate of the diagnostic PCR depends on the samples matrix (e.g., blood, blood fractions, CSF or lesion exudate) as well as disease stage (52;53). Where microscopy and PCR

are feasible diagnostic measures in the primary, ulcerative stage of syphilis, later stages, and above all the latent stages, are diagnosed by serological testing. Accordingly, over two thirds of the Danish patients are diagnosed by serology (13).

In Denmark, serological testing for syphilis antibodies was centralized at Statens Serum Institut (SSI) until 2011. Serological tests for syphilis fall into two categories, non-treponemal and treponemal tests. Two non-treponemal tests were used for the studies included in the current thesis: Wassermann's reaction (WR) is a complement fixation test, and the rapid plasma reagin (RPR) titre was determined by agglutination. Furthermore, three treponemal tests were used: anti-flagellum IgM (AF-M) was determined by a capture ELISA, anti-flagellum IgG (AF-G) was determined by an indirect ELISA and the fluorescent treponemal antibody absorption test (FTA-ABS) was done by immunofluorescence microscopy.

In general, the non-treponemal tests are sensitive but not highly specific. False-positive test results can occur due to viral infections, including HIV and HCV infection (54), drug addiction, malignancy, autoimmune diseases and probably also pregnancy (49). Therefore, patients with reactive non-treponemal tests should have confirmatory treponemal tests done to confirm the diagnosis. False-negative results caused by antibody in excess are called the prozone phenomenon (49). This reaction can occur during all stages of syphilis, but is most pronounced in secondary syphilis where antibody levels are at the highest level, and in the RPR test, which is dependent on the formation of an antigen-antibody lattice. The phenomenon has been associated with pregnancy and neurosyphilis (55).

The interpretation of serological test results can cause considerable confusion among clinicians, and available tests and treatment algorithms differ from country to country (56) (and now also from hospital to hospital in Denmark). In general, non-treponemal test titers decline after treatment, whereas treponemal IgG test titers will remain reactive for life. A 4-fold decline (or more) in non-treponemal titers is regarded as an appropriate serologic response, however in some patients non-treponemal antibodies can persist for a long time and these patients are referred to as 'serofast' which means that their non-treponemal test titers do not become non-reactive after an initial satisfactory response (40). Patients with a sustained 4-fold increase in non-treponemal titers combined with persisting or recurring symptoms of syphilis are considered as having a reinfection or experiencing treatment failure (40).

In 2009, PCR testing of material from ulcers was introduced in Denmark at SSI (57). Samples are tested for the presence of *T. pallidum* using two real-time diagnostic PCR assays with internal amplification controls, amplifying segments of the flaA (58) and polA (59) genes of *T. pallidum*.

The diagnosis of neurosyphilis can be a diagnostic challenge as no definitive criteria exist. When neurologic affection is suspected to be related to syphilis, the diagnosis is supported by laboratory testing of the CSF. The CSF is tested for the presence of antibodies, protein and cell count. However, it is of utmost importance to be aware that normal CSF parameters at the time of lumbar puncture do not rule out CNS infection because treponemes which recently accessed the CNS may not yet have elicited an inflammatory response (34).

**Treatment of syphilis**

Penicillin is the recommended treatment for syphilis in Europe (39) and the United States (40). Penicillin is administered parenterally in the form of benzathine penicillin G (a single dose of 2.4 million units IM for early syphilis and three doses at 1-week intervals for late latent syphilis). Procaine penicillin is used as a second-line option (one dose of 600 000 units IM once daily for 10 days for early syphilis and 21 days for late latent syphilis). As a treatment alternative, doxycycline is used (100 mg orally twice daily for 14 days for early syphilis and for 30 days for late latent syphilis) (39). Azithromycin is also used as a treatment alternative and has been proposed as a useful agent in developing countries where penicillin injections may be problematic (60). However, macrolide resistance is an increasing problem in the United States (61-64) and Shanghai (65), limiting its usefulness. During pregnancy, parenteral penicillin G is the only treatment with documented effect. In the case of penicillin allergy, the Centers for Disease Control and Prevention (CDC) recommend desensitization and subsequent treatment with penicillin (40).

In Denmark, primary, secondary and latent stage syphilis is treated with penicillin or doxycycline according to local guidelines. At the Department of Infectious Diseases at Rigshospitalet in Copenhagen patients are mainly treated with doxycycline, whereas patients at the Department of Infectious Diseases at Hvidovre Hospital and the STD clinic at Bispebjerg Hospital, likewise in the Copenhagen area, are mainly treated with benzathine penicillin G.

As many as one-quarter of patients treated for syphilis will experience the Jarisch-Herxheimer reaction (66), characterized by fever, arthralgia, myalgia and headache within the first 24 hours after initiation of therapy (40). The immunologic reaction is thought to be caused by the massive degradation of treponemes shortly after the initiation of therapy.

To monitor the treatment response, patients treated for syphilis should be followed up with serological testing and clinical assessments. The 2014 European Guideline on the Management of Syphilis recommends that patients treated for early syphilis are followed up at 1, 3, 6 and 12 months (39). Most often the non-treponemal tests are used as they most accurately reflect disease activity (40).

**Management of syphilis in HIV-infected patients**

Diagnosis and management of syphilis in patients with concurrent HIV is in general similar to that in patients without HIV (39). Nonetheless, since a case report documented clinical treatment failure in 1987 in a patient with concurrent HIV (67), the optimal antibiotic regimen for co-infected patients has been debated (68). Rapid progression to more advanced stages and a higher risk of treatment failure and neurological complications has been reported for co-infected patients (69-71). Further, serologically defined treatment failure has been documented to be more common in patients with primary and secondary syphilis and concurrent HIV (28), e.g., extremely low or high titers are more often seen among these patients (40). But in general, serologic tests are accurate and reliable also when testing HIV co-infected patients (39). The availability of cART has had a substantial impact by reversing (part of) the immunosuppression and today many HIV-infected patients may have a restored immune system, resulting in reduced rates of serologic failure (72).

Because HIV-infected patients might be at increased risk of neurologic complications (40) the need for lumbar puncture and its timing continues to be debated. Current guidelines recommend that HIV-infected patients have lumbar puncture done in

two situations: 1) neurological symptoms at time of diagnosis or after treatment, 2) patients who fail to respond to therapy (69). HIV-infected patients need closer follow-up after treatment of syphilis and the CDC recommend clinical and serological evaluation for treatment failure at 3, 6, 9, 12 and 24 months after therapy (40).

**Molecular typing of *T. pallidum***

In general, when using molecular typing as an epidemiological tool, it is important to study more variable parts of the genome (47). If a sample is tested positive in a diagnostic *T. pallidum* PCR, the sample can be subjected to molecular typing. In line with the performance of diagnostic PCRs, the success rate of typing PCRs depends on the sample matrix (73;74). In the primary stage, treponemes circulate in the blood and should thus be detectable in blood samples. However, if the equivalent of 50–100 µL patient blood is subjected to a PCR that detects 5–10 target copies per reaction, then at least 100–1000 treponemal organisms per mL should be present in the blood sample taken, making it inherently difficult to use blood because the bacterial load is usually lower than this (47). Conversely, treponemes seem to be caught in the capillary beds of the skin and therefore ear scrapings may have high enough bacterial concentrations for detection by PCR (75).

In 1998, Pillay et al developed a subtyping system to distinguish different strains of *T. pallidum*. The method is based on heterogeneity of two genes: determination of the number of 60-bp repeats within the acidic repeat protein (arp) gene, and restriction fragment length polymorphism (RFLP) analysis of sequence differences in the *T. pallidum* repeat (tpr) subfamily II genes (tprE, tprG and tprJ) (76). This 2-component CDC subtype was recently supplemented with sequence analysis of a variable region of the tp0548 gene (77). By combining the CDC subtype and the tp0548 sequence type the discriminatory capacity was markedly improved. The new 3-component type was designated strain type by Marra et al (77) to distinguish it from the 2-component subtype.

Of note, the PCR products resulting from the amplification of the arp and tpr genes are long which is thought to compromise the sensitivity of the assays. Furthermore, the arp and tpr genes are not very specific targets because they are also present in other treponemes. However, because molecular typing is performed on samples that are confirmed positive in diagnostic *T. pallidum* specific PCR assays this usually does not present a problem (47).

## SETTING AND DATA SOURCES

Denmark has a population of 5.6 million people (78) with an estimated HIV prevalence in the adult population of 0.07% (79). The number of persons with newly diagnosed HIV, and the proportion of MSM among these, has been stable for almost 10 years with approximately 200–250 new cases per year (80). Five hundred undiagnosed HIV-infected MSM are estimated to reside in Denmark, an estimate that has remained stable over the last 10 years (81). A recent Danish study concluded that the stable incidence of new HIV diagnoses among MSM despite increased engagement in unsafe sex was a consequence of the use of cART (21).

The Danish health care system is tax-funded, including free cART. Treatment of HIV is restricted to specialized departments and patients with syphilis are seen at specialized departments at hospitals or STD clinics. All individuals in Denmark are assigned a unique 10-digit central person registration (CPR) number at birth or upon immigration. The CPR number can be used to link data from different registers upon approval from the Danish Data Protection Agency. Two nationwide registers were used for study I and study III of the current thesis. The nationwide design is a major strength because the data are analyzed at a population-level, including all patients and not just a subgroup. However, the quality of the studies depends on the validity of the data in the registers (82) and misclassification will always be of concern.

**The Danish national syphilis registration system**

Primary, secondary and early latent syphilis are notifiable diseases according to Danish legislation and the notification is the responsibility of the treating physician. The cases are registered in the Danish national syphilis registration system – a nationwide database established in 1993, based at the Department of Infectious Disease Epidemiology at SSI. In addition to the cases notified, the register also includes patients identified with syphilis by positive serologic or PCR testing. The notification information includes sex, ethnicity, sexual orientation, mode and place of acquisition, as well as HIV status (12). Data from the Danish national syphilis registration system was used for study I and study III.

**The Danish HIV Cohort Study**

The Danish HIV Cohort Study (DHCS) is a nationwide, prospective, population-based study of all Danish HIV-infected patients treated at Danish hospitals since 1 January 1995. The data are updated yearly using standardized forms and the CPR number is used to avoid multiple registrations. To assure high data quality a data assurance program has been developed. The data include demographic data, mode of acquisition, AIDS-defining events, height, weight, blood pressure, lipid status, smoking status, cART, CD4 cell counts and HIV RNA (83). Data from DHCS were used for study I.

## METHODOLOGICAL CONSIDERATIONS

The studies included in the current thesis were all observational studies. Observational studies can be longitudinal or cross-sectional. In longitudinal studies it is possible to estimate incidence rates and due to the time factor it is possible to establish cause-effect relationships and large populations can be studied for long periods at a relatively low cost. Cross-sectional studies can demonstrate associations but conclusions on causal relationships should not be drawn. On the other hand, cross-sectional studies are less resource demanding. In general, observational studies have some limitations compared to clinical trials (84). Confounding is a common problem, but if a confounder is recognized it can be adjusted for. However, not all confounders are always recognized and the number of confounders that can be controlled for is also limited. Bias, on the contrary can occur in both observational studies and clinical trials, but are often minimized in clinical trials by randomization and double-blinding (85). However, in clinical trials the study population is highly selected and may not be representative of the population of interest.

## RESULTS

**Paper I: Risk of HIV or second syphilis infection in Danish men with newly acquired syphilis in the period 2000–2010**

*Brief study outline*

Syphilis is a marker of sexual risk behavior and is of interest in patients with HIV – as a marker of unsafe sex in this population. Syphilis is also of interest in patients without HIV – to assess the risk of subsequent HIV infection. We used two nationwide registers to estimate the 5-year risk of HIV or second syphilis infection, and to determine incidence rate ratios (IRR).

From the Danish national syphilis registration system all Danish men >16 years of age diagnosed with early syphilis in the period 2000–2010 were identified. Subsequently data on HIV status were extracted from the DHCS. Kaplan-Meier analysis was used to estimate the 5-year risk of HIV or second syphilis infection, stratified by the diagnosis of syphilis before and after 1 January 2006. We used Cox regression analysis with the date of the second syphilis diagnosis introduced as the time-updated variable to determine IRR for risk of HIV diagnosis before and after a second diagnosis of syphilis. Likewise, Cox regression analysis with the date of HIV infection introduced as the time-updated variable was used to determine IRR for risk of a second syphilis infection.

During the study period the national criteria for initiation of cART were acute HIV infection, presence of an HIV-related disease, pregnancy, CD4 cell count <300 cells/µL until May 2008 and <350 cells/µL thereafter, and plasma HIV-RNA >100 000 copies/mL (until 2001).

*Principal findings*

During the 11-year study period, 1217 male patients diagnosed with early syphilis were included. After 5 years, 9.8% (95% CI 7.0–12.6%) of the population had been diagnosed with HIV. Patients with a second diagnosis of syphilis had a substantially higher risk of being diagnosed with HIV (IRR = 3.1, 95% CI 1.2–8.0). After 5 years, 14.8% (95% CI 12.1–17.4%) of the population had been diagnosed with a second episode of syphilis. HIV-infected patients had a substantially higher risk of a second syphilis diagnosis (IRR = 4.0, 95% CI 2.8–5.9). Of the HIV-infected patients diagnosed with syphilis, 33.7% had viral loads >1000 copies/mL and thereby at risk of also passing HIV to their partner.

*Considerations*

A major strength of the study was our ability to link two nationwide registers of patients diagnosed with syphilis or HIV, however, the study has some limitations. First, screening of HIV-infected patients as part of the routine testing of this group may result in underestimating the syphilis prevalence in low-risk groups. We assume that an unknown number of patients have been left out because low-risk groups have not been screened. Second, our study included both MSM and heterosexual men which might have different risk behaviors and exposures. Third, our data analysis did not account for mortality and emigration because these data were not available for the HIV-uninfected population. Finally, a 'case of syphilis' was based on serological results only and not on revision of patient files, thus reinfection or resurgence of an incompletely treated infection could potentially have been misclassified as a case.

*Conclusion and perspectives*

We concluded that the high risk of syphilis or HIV infection in men diagnosed with one of these STDs indicated a high frequency of unsafe sex in the Danish MSM population. The risk of being diagnosed with HIV subsequent to syphilis decreased during the later part of the study. Similar trends where syphilis outbreaks have not had a substantial impact on HIV incidence have also been reported from the United States (86). A possible explanation of the stagnation of the HIV transmission despite the increase in syphilis diagnoses could be that cART has become more widely used in recent years with viral suppression as a consequence. As one third of the HIV-infected patients in this study had viral load >1000 copies/mL our conclusion supported the initiation of cART in all HIV-infected MSM to reduce transmission of HIV.

**Paper II: Serological response to treatment of syphilis with doxycycline compared to penicillin in HIV-infected individuals**

*Brief study outline*

Penicillin is the drug of choice when treating syphilis, whereas doxycycline is used as a second-line option (40). The aim of the study was to evaluate the serological response to treatment of primary, secondary, early and late latent syphilis with intramuscularly administered penicillin or orally administered doxycycline in patients co-infected with HIV.

In this retrospective study, patients ≥18 years of age diagnosed with syphilis between 1 May 2004 and 31 October 2009 were eligible. The patients were included from three hospitals in the Copenhagen area (two departments of infectious diseases and one STD clinic). These hospitals were chosen because the vast majority of HIV-infected patients from the Copenhagen area are seen at these departments and because the vast majority of syphilis cases in Denmark is diagnosed in the Copenhagen area (87). We excluded patients who received intravenous therapy, were diagnosed with neurosyphilis or where patient files lacked information on treatment for syphilis. An individual could contribute with more than one episode, provided that treatment and appropriate treatment response was documented in the patient file. Treatment for syphilis consisted of doxycycline (100 mg orally twice daily for 14 days for early syphilis, and for 30 days for late latent syphilis) or penicillin (a single dose of intramuscular 2.4 million units of benzathine penicillin G for early syphilis and three doses at 1-week intervals for late latent syphilis).

To capture all available serological data, we obtained the serological test results from SSI where all serologic testing for syphilis was centralized during the study period. We defined serological cure as a ≥4-fold decline in non-treponemal titers following treatment of syphilis. Further, we defined serological failure as a lack of a 4-fold decline. Serological test results were allocated to a specific follow-up visit (3, 6, 9 or 12 months) if the test was done 30 days before or 30 days after the relevant point in time. The last-observation-carried-forward principle was used to handle missing values of WR and RPR.

The $X^2$ or Fisher's exact test were used to compare independent proportions and the t test and the Mann-Whitney test were used for comparison of continuous variables. The Kruskal-Wallis test was used for comparison of titers between different syphilis stages.

*Principal findings*

From 1 May 2004 to 31 October 2009, 221 cases of syphilis were diagnosed among 172 HIV-infected individuals attending three hospitals in the Copenhagen area of Denmark. The patients were diagnosed with primary, secondary, early and late latent syphilis, no patients were diagnosed in the tertiary stage. In total, 202 cases were treated with doxycycline or intramuscular penicillin and included in the study. Of these, 126 cases were evaluated at 12 months; 78 cases were treated with doxycycline and 48 cases were treated with penicillin. The two treatment groups were comparable except for proportion of patients with CD4 cell count ≤200 cells/µL and proportion on cART.

The serological outcome was assessed at 3, 6, 9, and 12 months following treatment of syphilis infection. No statistically significant differences were observed in treatment outcome between treatment groups at any point in time (all $p > 0.05$). One year after treatment, 20 cases of serological failure were observed with 12 cases (15%) and 8 cases (17%) in the doxycycline and penicillin group, respectively, resulting in a non-significant difference of 2% (95% CI, -1.08%–5.08%; $p > 0.05$).

The proportion of patients who reached serological cure was affected by syphilis stage: of the cases of primary and secondary syphilis, 100% and 89% reached serological cure at 12 months, respectively, and of the cases with early and late latent syphilis, 71% and 67% reached serological cure at 12 months, respectively ($p = 0.006$). On the contrary, the serological cure rate did not vary by CD4 cell count, HIV RNA or age (all $p > 0.05$). However, WR and RPR titers at time of diagnosis were significantly higher in patients with serological failure 12 months after treatment (WR: $p = 0.002$; RPR: $p < 0.000$).

*Considerations*

HIV-infected patients in Denmark are followed at highly specialized and centralized departments. We included patients from two departments of infectious diseases in the capital area of Denmark; these two departments follow two thirds of the total Danish HIV-infected population (88). Further, if these patients opted for treatment of syphilis at the STD clinic at Bispebjerg Hospital these episodes were also included. All patient files from the three clinics were revised to assure detailed demographic, clinical and behavioral data on all patients and to distinguish between relapse and reinfection. Further, to capture all serological data, results of serological tests were obtained from the national provider of serological syphilis testing at SSI.

As in all retrospective studies, this study was limited by its non-randomized design. The treatment given was based on local guidelines and the patients at the Department of Infectious Diseases at Rigshospitalet were mainly treated with doxycycline, whereas patients at the Department of Infectious Diseases at Hvidovre Hospital and the STD clinic at Bispebjerg Hospital were mainly treated with penicillin. Overall, the patients in the two treatment groups were comparable. However, the proportion of patients with CD4 cell count ≤200 cells/µL and proportion on cART were not distributed evenly between treatment groups. Further analyses showed that more HIV-infected individuals not receiving cART opted for treatment at the STD clinic compared to individuals on cART. This explains the lower proportion of patients on cART in the group receiving penicillin (the preferred regimen at the STD clinic). Likewise, even though the median CD4 cell count was comparable between treatment groups, a higher proportion of patients with CD4 cell counts ≤200 cells/µL were treated with penicillin. Because of the above-mentioned differences in treatment groups the serologic failure rate in the penicillin group may be overestimated since lower CD4 cell counts have been associated with increased risk of treatment failure (89).

*Conclusion and perspectives*

In line with others we found comparable rates of serologic failure in patients treated with doxycycline and penicillin (24;90). Although our study was not randomized, it seems safe to conclude that doxycycline can be used as a treatment alternative to penicillin if patients or clinicians prefer an oral treatment of syphilis. Whether doxycycline is non-inferior to penicillin can only be definitively evaluated in a clinical trial. However, it seems unlikely that such a large and expensive trial is about to be carried out. Furthermore, in this study the endpoint was serological cure 12 months after treatment. One could speculate that some of the patients who do not reach serological cure are patients without an active infection. Clinicians may prefer to treat HIV-infected patients who are asymptomatic but screen positive by serologic testing. If a clinical trial should be carried out it would be of utmost importance to exclude patients without active infection. Because the treatment of HIV-infected patients in the Danish setting is restricted to few centers and because the patients have close follow-ups, poor adherence with respect to syphilis treatment would most likely be recognized at a regular check-up. Before recommending doxycycline as a more widely used treatment option it should be assured that the clinical setup has the relevant measures to avoid lost-to-follow-up. If compliance is assumed to be a problem, the single-dose, intramuscular penicillin regimen will always be the preferred treatment. In this connection it should be remembered that doxycycline is an effective agent for treatment of multiple STDs (91). Taken together we concluded that doxycycline can be used as an efficient treatment option when treating an HIV-infected population for syphilis.

**Paper III: Molecular typing of *Treponema pallidum* in Denmark: A nationwide study of syphilis**

*Brief study outline*

Molecular epidemiology can be used for understanding the transmission of infectious diseases and thereby preventing and controlling epidemics. HIV and syphilis co-infection is common and because genital ulcers facilitate HIV transmission it is of pivotal importance to elucidate the epidemiologic determinants underlying the high rates of syphilis. This study aimed to determine the strain type diversity among all patients diagnosed in Denmark with syphilis by PCR testing of material from genital ulcers during a 4-year period.

The study was performed as a nationwide study of *T. pallidum* positive genital ulcer samples collected between May 2009 and December 2013. *T. pallidum* strain type was linked with epidemiologic data (e.g., HIV status) from the Danish national syphilis registration system. Molecular strain typing was based on characterization of three variable treponemal genes; arp, tpr and tp0548. Categorical data were compared using $\chi^2$ test or Fisher's exact test, where appropriate.

*Principal findings*

During the 4-year period, 278 samples positive by *T. pallidum* PCR testing were obtained from 269 patients. The majority of the patients were men (94%) and most of the male patients were MSM (86%). Further, the majority (93%) of the patients reported acquiring syphilis in Denmark. Of the 278 positive samples, 71% were typeable with all three PCR assays. Among the fully typeable samples, 22 strain types were identified. The most common type was 14d/g, accounting for 54% followed by 14d/f (18%) and 14l/g (5%). The remaining strain types were rare. Nineteen percent of the patients were HIV-infected at time of diagnosis and all of these reported that they were MSM. The patients with concurrent HIV were diagnosed with nine different full strain types and we did not find a difference in strain type by HIV status ($p = 0.197$).

*Considerations*

Our study included all Danish patients with PCR-positive syphilis within the 4-year study period. By use of the Danish national syphilis registration system we had epidemiological data on all patients. It is a major strength that all PCR testing for syphilis in Denmark is centralized at SSI. Further, the study was based in a setting where all health-care services are publicly funded and this universal access probably results in fewer undiagnosed cases. We used the 3-component strain typing system and could demonstrate a high discriminatory capacity. However, when concluding on our results it is a major limitation that we were not able to perform typing on samples from the two thirds of the patients in

Denmark who were diagnosed by serological testing during the study period (13).

Our main interest was to investigate the strain types in HIV-infected patients to assess if these patients belonged to separate sexual networks. Therefore, it is a major limitation that the majority of the HIV-infected patients are diagnosed by serological testing (this group is screened yearly in contrast to low-prevalence groups).

Besides the distribution of HIV, our interest was to investigate if imported cases resulted in circulating strains. Again, the interpretation was limited by the unknown strain types in the patients diagnosed by serological testing. However, based on the available samples, the imported cases did not result in circulating clones. We reached a relatively high number of typeable samples and our success rate of 71% is comparable to others who have used the 3-component strain type system with success rates of 41%, 63% and 77%, respectively (92-94).

*Conclusion and perspectives*

We concluded that strain type 14d/g was the predominant strain type in Denmark, but that a high degree of heterogeneity exists. The majority of the patients had acquired syphilis in Denmark and the imported cases of syphilis did not result in circulating clones. Furthermore, HIV-infected patients were diagnosed with a wide spectrum of different strains. Currently the method of choice for *T. pallidum* typing consists of three PCR assays which is time-consuming and not applicable for routine testing. Recently a sequenced-based molecular typing system was proposed (95) and compared to the traditional CDC typing system in a group of patients with two or more parallel samples (i.e. taken at the same time). However, in the majority of the patients they found discrepancies within the arp and tpr loci using the CDC typing system (95). This was rather surprising because under experimental conditions, Pillay et al (76) have shown that the CDC subtype was stable with repeated rabbit passages of the Nichols strain. Marra et al have also confirmed the stability of the strains by demonstrating that neither the Sea 81-4 nor the Chicago C strain changed with repeated rabbit passages (77). Whether this inconsistency is caused by differences between human infections and experimental infections of rabbits or is due to the fact that skin and blood represent two immunologically distinct compartments has yet to be explored. Nonetheless, sequence-based typing systems for *T. pallidum* look promising (95-97).

**CONCLUSION AND PERSPECTIVES FOR FUTURE RESEARCH**

The purpose of the current thesis was to improve the understanding of the highly complex epidemiologic relationship between HIV and syphilis. Given that syphilis facilitates transmission and acquisition of HIV and because the two infections have similar modes of transmission, increasing rates of HIV were expected to follow the increasing rates of syphilis. However, such an increased spread of HIV has not been observed. Study I of the current thesis demonstrated that syphilis and HIV co-infection was common in Denmark and that the majority of the HIV-infected syphilis patients were on cART. The use of cART, resulting in lower viral loads, might explain why the HIV incidence has not increased despite the assumption that the increasing rates of syphilis is a marker of higher rates of unsafe sex. In line with this, a recent Danish study (21) concluded that the increased use of cART explained the discordance in the rates of new syphilis and HIV infections. Study I suggested that the use of cART should be even more used as one third of the HIV-infected patients diagnosed with syphilis (indicating unsafe sex) had viral loads >1000 copies/mL. The rates of HIV co-infection among MSM who are diagnosed with syphilis have been declining in Denmark. One possible mechanism could be that MSM with syphilis and HIV co-infection have unprotected sex with HIV-uninfected MSM, but only transmit syphilis because they are efficiently treated for their HIV infection (98). From 2008 to 2012, 65% (range, 54%–76%) of all new HIV-infected patients in Denmark were on cART within a year of diagnosis (88).

In study III we investigated whether HIV-infected patients with syphilis belonged to separate sexual networks; however, we did not find evidence of the assumption of serosorting. On the contrary, we observed that HIV-infected patients were diagnosed with a wide range of strain types. From study I we knew that HIV-infected men had a substantially higher risk of a second diagnosis of syphilis compared to the HIV-uninfected syphilis patients. Whether this higher risk of syphilis in the HIV-infected population reflects that HIV-infected patients have unsafe sex based on serosorting (though no verified evidence of this in study III) or whether a core group with high-risk behavior is responsible for the increased risk in the HIV-infected population is unknown. For example, in study III a few medium-sized clusters consisted of MSM only, indicating localized transmission networks. Rather than a general increase in sexual risk behavior among all MSM, the re-establishment of a risk-taking core group of MSM may have enabled a higher level of endemicity, causing continuous syphilis circulation (99).

Study III provided new insights on the epidemiology of syphilis in the Danish population using molecular typing. However, to fully use the potential of the molecular typing as an epidemiological tool, a study including both patients diagnosed by serological testing as well as PCR testing would be highly relevant although technically difficult. By inclusion of all patients it could be definitively investigated how the HIV-infected population interact with the HIV-uninfected population.

Study II demonstrated that the majority of the HIV-infected patients were diagnosed in the secondary and early latent stage. Diagnoses in the latent stages reflect that the annual syphilis screening of all HIV-infected patients is justified and even more frequent screening might result in earlier diagnoses and less transmission. Also, as long as it is not fully elucidated how syphilis spreads to low-prevalence groups, it seems prudent that syphilis screening was re-introduced in the general screening of pregnant women. Future studies should continue to focus on timely diagnosis of both HIV and syphilis. Moreover, study II demonstrated that syphilis was easily treated with both penicillin and doxycycline and that clinical treatment failure was not a common problem. Further, with the increased use of cART, the clinical manifestations of syphilis in HIV-infected patients might differ less from the HIV-uninfected patients in the future. Future studies focusing on the clinical manifestations of syphilis in HIV-infected patients in the cART era would be highly appreciated.

In conclusion, the rate of syphilis has stabilized in recent years but it is still too early to conclude whether syphilis is under control in Denmark. A spread to low-risk groups is of concern, especially if these patients are seen by clinicians who are not familiar with the symptomatology of syphilis. However, given the efficient treatment options, the targeted screening of HIV-infected patients, pregnant women and persons attending community-based testing (100) and STD clinics, control of the infection seems within reach. Avoiding new HIV infections is the major challenge and here cART may play a prominent role.

**SUMMARY**

The studies included in this PhD thesis examined the interactions of syphilis, which is caused by *Treponema pallidum*, and HIV. Syphilis reemerged worldwide in the late 1990s and hereafter increasing rates of early syphilis were also reported in Denmark. The proportion of patients with concurrent HIV has been substantial, ranging from one third to almost two thirds of patients diagnosed with syphilis some years. Given that syphilis facilitates transmission and acquisition of HIV the two sexually transmitted diseases are of major public health concern. Further, syphilis has a negative impact on HIV infection, resulting in increasing viral loads and decreasing CD4 cell counts during syphilis infection. Likewise, HIV has an impact on the clinical course of syphilis; patients with concurrent HIV are thought to be at increased risk of neurological complications and treatment failure.

Almost ten percent of Danish men with syphilis acquired HIV infection within 5 years after they were diagnosed with syphilis during an 11-year study period. Interestingly, the risk of HIV declined during the later part of the period. Moreover, HIV-infected men had a substantial increased risk of re-infection with syphilis compared to HIV-uninfected men. As one third of the HIV-infected patients had viral loads >1000 copies/mL, our conclusion supported the initiation of cART in more HIV-infected MSM to reduce HIV transmission. During a 5-year study period, including the majority of HIV-infected patients from the Copenhagen area, we observed that syphilis was diagnosed in the primary, secondary, early and late latent stage. These patients were treated with either doxycycline or penicillin and the rate of treatment failure was similar in the two groups, indicating that doxycycline can be used as a treatment alternative – at least in an HIV-infected population. During a 4-year study period, the T. pallidum strain type distribution was investigated among patients diagnosed by PCR testing of material from genital lesions. In total, 22 strain types were identified. HIV-infected patients were diagnosed with nine different strains types and a difference by HIV status was not observed indicating that HIV-infected patients did not belong to separate sexual networks.

In conclusion, concurrent HIV remains common in patients diagnosed with syphilis in Denmark, both in those diagnosed by serological testing and PCR testing. Although the rate of syphilis has stabilized in recent years, a spread to low-risk groups is of concern, especially due to the complex symptomatology of syphilis. However, given the efficient treatment options and the targeted screening of pregnant women and persons at higher risk of syphilis, control of the infection seems within reach. Avoiding new HIV infections is the major challenge and here cART may play a prominent role.

**ABBREVIATIONS**

| | |
|---|---|
| AIDS | Acquired immune deficiency syndrome |
| AF-M | Anti-flagellum IgM |
| AF-G | Anti-flagellum IgG |
| arp | Acidic repeat protein |
| bp | Base pair |
| cART | Combination antiretroviral treatment |
| CD4 | Cluster of differentiation 4 |
| CDC | Centers for Disease Control and Prevention |
| CNS | Central nervous system |
| CI | Confidence interval |
| CPR number | Central person registration number |
| CSF | Cerebrospinal fluid |
| DHCS | Danish HIV Cohort Study |
| FTA-ABS | Fluorescent treponemal antibody absorption |
| HCV | Hepatitis C virus |
| HIV | Human immunodeficiency virus |
| IM | Intramuscular |
| IRR | Incidence rate ratio |
| MSM | Men who have sex with men |
| PCR | Polymerase chain reaction |
| RFLP | Restriction fragment length polymorphism |
| RPR | Rapid plasma reagin |
| SSI | Statens Serum Institut |
| STD | Sexually transmitted disease |
| *T. pallidum* | *Treponema pallidum* |
| tpr | *T. pallidum* repeat |
| WR | Wassermann's reaction |

**REFERENCES**

1. Radolf JD, Lukehart SA. Pathogenic Treponema. Molecular and cellular biology. Caister Academic Press;2006.
2. 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 Apr;32:220-6.
3. Jebbari H, Simms I, Conti S, Marongiu A, Hughes G, Ward H, et al. Variations in the epidemiology of primary, secondary and early latent syphilis, England and Wales: 1999 to 2008. Sex Transm Infect 2011 Apr;87:191-8.
4. Hawkes S, Matin N, Broutet N, Low N. Effectiveness of interventions to improve screening for syphilis in pregnancy: a systematic review and meta-analysis. Lancet Infect Dis 2011 Sep;11:684-91.
5. Kuznik A, Lamorde M, Nyabigambo A, Manabe YC. Antenatal syphilis screening using point-of-care testing in Sub-Saharan African countries: a cost-effectiveness analysis. PLoS Med 2013 Nov;10(11):e1001545.
6. Gloyd S, Chai S, Mercer MA. Antenatal syphilis in sub-Saharan Africa: missed opportunities for mortality reduction. Health Policy Plan 2001 Mar;16:29-34.
7. Rydzak CE, Goldie SJ. Cost-effectiveness of rapid point-of-care prenatal syphilis screening in sub-Saharan Africa. Sex Transm Dis 2008 Sep;35:775-84.
8. 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 Mar 1;91:217-26.
9. Holk K, Mordhorst CH. Syfilis diagnosticeret i Danmark i 1991. EPI-NEWS 1992;9.
10. Axelsen N. Syphilis 1998-1999. EPI-NEWS 2000;34.
11. Søborg B, Cowan S, Hoffmann S. Syphilis 2011. EPI-NEWS 2012;37.
12. Cowan S. Syphilis in Denmark-Outbreak among MSM in Copenhagen, 2003-2004. Euro Surveill 2004 Dec;9:25-7.
13. Cowan S, Hoffmann S. Syphilis 2013. EPI-NEWS 2014;34.
14. Christiansen AH, Cowan S. General HBV, HIV & syphilis screening of pregnant women. EPI-NEWS 2010;27-33.
15. Axelsen N, Mazick A, Cowan S. Syphilis 2004. EPI-NEWS 2005;16.
16. St-Martin G, Cowan S, Hoffmann S, Jensen JS. Syphilis 2008. EPI-NEWS 2009;23.
17. Salado-Rasmussen K, Katzenstein TL, Gerstoft J, Cowan SA, Knudsen TB, Mathiesen L, et al. Risk of HIV or second syphilis infection in Danish men with newly acquired syphilis in the period 2000-2010. Sex Transm Infect 2013 Aug;89:372-6.
18. Sterne JA, Hernan MA, Ledergerber B, Tilling K, Weber R, Sendi P, et al. Long-term effectiveness of potent antiretroviral

therapy in preventing AIDS and death: a prospective cohort study. Lancet 2005 Jul 30;366(9483):378-84.
19. Lohse N, Hansen AB, Pedersen G, Kronborg G, Gerstoft J, Sorensen HT, et al. Survival of persons with and without HIV infection in Denmark, 1995-2005. Ann Intern Med 2007 Jan 16;146(2):87-95.
20. Zetola NM, Klausner JD. Syphilis and HIV infection: an update. Clin Infect Dis 2007 May 1;44(9):1222-8.
21. Cowan SA, Gerstoft J, Haff J, Christiansen AH, Nielsen J, Obel N. Stable incidence of HIV diagnoses among Danish MSM despite increased engagement in unsafe sex. J Acquir Immune Defic Syndr 2012 Sep 1;61(1):106-11.
22. Grassly NC, Fraser C, Garnett GP. Host immunity and synchronized epidemics of syphilis across the United States. Nature 2005 Jan 27;433(7024):417-21.
23. Breban R, Supervie V, Okano JT, Vardavas R, Blower S. Is there any evidence that syphilis epidemics cycle? Lancet Infect Dis 2008 Sep;8(9):577-81.
24. Kofoed K, Gerstoft J, Mathiesen LR, Benfield T. Syphilis and human immunodeficiency virus (HIV)-1 coinfection: influence on CD4 T-cell count, HIV-1 viral load, and treatment response. Sex Transm Dis 2006 Mar;33(3):143-8.
25. Jarzebowski W, Caumes E, Dupin N, Farhi D, Lascaux AS, Piketty C, et al. Effect of early syphilis infection on plasma viral load and CD4 cell count in human immunodeficiency virus-infected men: results from the FHDH-ANRS CO4 cohort. Arch Intern Med 2012 Sep 10;172(16):1237-43.
26. Mabey D. Interactions between HIV infection and other sexually transmitted diseases. Trop Med Int Health 2000 Jul;5(7):A32-A36.
27. Farhi D, Dupin N. Management of syphilis in the HIV-infected patient: facts and controversies. Clin Dermatol 2010 Sep;28(5):539-45.
28. Rolfs RT, Joesoef MR, Hendershot EF, Rompalo AM, Augenbraun MH, Chiu M, et al. A randomized trial of enhanced therapy for early syphilis in patients with and without human immunodeficiency virus infection. The Syphilis and HIV Study Group. N Engl J Med 1997 Jul 31;337(5):307-14.
29. Rompalo AM, Joesoef MR, O'Donnell JA, Augenbraun M, Brady W, Radolf JD, et al. Clinical manifestations of early syphilis by HIV status and gender: results of the syphilis and HIV study. Sex Transm Dis 2001 Mar;28(3):158-65.
30. Knaute DF, Graf N, Lautenschlager S, Weber R, Bosshard PP. Serological response to treatment of syphilis according to disease stage and HIV status. Clin Infect Dis 2012 Dec;55(12):1615-22.
31. Izard J, Limberger R. Structural and genomic features of treponemal architecture. In: Radolf JD, Lukehart SA, editors. Pathogenic Treponema. Molecular and cellular biology. Caister Academic Press; 2006. p. 9-18.
32. Marks M, Solomon AW, Mabey DC. Endemic treponemal diseases. Trans R Soc Trop Med Hyg 2014 Oct;108(10):601-7.
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 Jul 17;281(5375):375-88.
34. Radolf JD, Hazlett KRO, Lukehart SA. Pathogenesis of syphilis. In: Radolf JD, Lukehart SA, editors. Pathogenic Treponema. Molecular and cellular biology. Caister Academic Press; 2006. p. 197-236.
35. Giacani L, Brandt SL, Puray-Chavez M, Reid TB, Godornes C, Molini BJ, et al. Comparative investigation of the genomic regions involved in antigenic variation of the TprK antigen among treponemal species, subspecies, and strains. J Bacteriol 2012 Aug;194:4208-25.
36. Salazar JC, Cruz AR, Pope CD, Valderrama L, Trujillo R, Saravia NG, et al. Treponema pallidum elicits innate and adaptive cellular immune responses in skin and blood during secondary syphilis: a flow-cytometric analysis. J Infect Dis 2007 Mar 15;195:879-87.
37. Knudsen A, Benfield T, Kofoed K. Cytokine expression during syphilis infection in HIV-1-infected individuals. Sex Transm Dis 2009 May;36:300-4.
38. Salado-Rasmussen K, Knudsen A, Krarup HB, Katzenstein TL, Gerstoft J. Undetectable hepatitis C virus RNA during syphilis infection in two HIV/HCV-co-infected patients. Scand J Infect Dis 2014 Sep;46:617-23.
39. Janier M, Hegyi V, Unemo M, Tiplica GS, Potocnik M, French P, et al. 2014 European Guideline on the Management of Syphilis. IUSTI 2014.
40. MMWR. Sexually Transmitted Diseases Treatment Guidelines. Centers for Disease Control and Prevention 2010.
41. Schulz KF, Cates W, Jr., O'Mara PR. Pregnancy loss, infant death, and suffering: legacy of syphilis and gonorrhoea in Africa. Genitourin Med 1987 Oct;63:320-5.
42. Qureshi F, Jacques SM, Reyes MP. Placental histopathology in syphilis. Hum Pathol 1993 Jul;24:779-84.
43. Sheffield JS, Sanchez PJ, Wendel GD, Jr., Fong DW, Margraf LR, Zeray F, et al. Placental histopathology of congenital syphilis. Obstet Gynecol 2002 Jul;100:126-33.
44. Magnuson HJ, Thomas EW, Olansky S, Kaplan BI, De Mello L, Cutler JC. Inoculation syphilis in human volunteers. Medicine (Baltimore) 1956 Feb;35:33-82.
45. Clark EG, Danbolt N. The Oslo study of the natural history of untreated syphilis; an epidemiologic investigation based on a restudy of the Boeck-Bruusgaard material; a review and appraisal. J Chronic Dis 1955 Sep;2:311-44.
46. Gjestland T. The Oslo study of untreated syphilis; an epidemiologic investigation of the natural course of the syphilitic infection based upon a re-study of the Boeck-Bruusgaard material. Acta Derm Venereol Suppl (Stockh) 1955;35(Suppl 34):3-368.
47. Bruisten SM. Protocols for detection and typing of Treponema pallidum using PCR methods. Methods Mol Biol 2012;903:141-67.
48. Baker-Zander SA, Lukehart SA. Molecular basis of immunological cross-reactivity between Treponema pallidum and Treponema pertenue. Infect Immun 1983 Nov;42:634-8.
49. Larsen SA, Steiner BM, Rudolph AH. Laboratory diagnosis and interpretation of tests for syphilis. Clin Microbiol Rev 1995 Jan;8:1-21.
50. Burstain JM, Grimprel E, Lukehart SA, Norgard MV, Radolf JD. Sensitive detection of Treponema pallidum by using the polymerase chain reaction. J Clin Microbiol 1991 Jan;29:62-9.
51. Grimprel E, Sanchez PJ, Wendel GD, Burstain JM, McCracken GH, Jr., Radolf JD, et al. Use of polymerase chain reaction and rabbit infectivity testing to detect Treponema pallidum in amniotic fluid, fetal and neonatal sera, and cerebrospinal fluid. J Clin Microbiol 1991 Aug;29:1711-8.
52. Gayet-Ageron A, Ninet B, Toutous-Trellu L, Lautenschlager S, Furrer H, Piguet V, et al. Assessment of a real-time PCR test to diagnose syphilis from diverse biological samples. Sex Transm Infect 2009 Aug;85:264-9.
53. Marfin AA, Liu H, Sutton MY, Steiner B, Pillay A, Markowitz LE. Amplification of the DNA polymerase I gene of Treponema

pallidum from whole blood of persons with syphilis. Diagn Microbiol Infect Dis 2001 Aug;40:163-6.
54. Augenbraun M, French A, Glesby M, Sanchez-Keeland L, Young M, Greenblatt R, et al. Hepatitis C virus infection and biological false-positive syphilis tests. Sex Transm Infect 2010 Apr;86:97-8.
55. Liu LL, Lin LR, Tong ML, Zhang HL, Huang SJ, Chen YY, et al. Incidence and risk factors for the prozone phenomenon in serologic testing for syphilis in a large cohort. Clin Infect Dis 2014 Aug;59:384-9.
56. 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 Apr;58:1116-24.
57. St-Martin G, Cowan S, Hoffmann S, Jensen JS. Syphilis 2009. EPI-NEWS 2010;35.
58. Salazar JC, Rathi A, Michael NL, Radolf JD, Jagodzinski LL. Assessment of the kinetics of Treponema pallidum dissemination into blood and tissues in experimental syphilis by real-time quantitative PCR. Infect Immun 2007 Jun;75:2954-8.
59. Leslie DE, Azzato F, Karapanagiotidis T, Leydon J, Fyfe J. Development of a real-time PCR assay to detect Treponema pallidum in clinical specimens and assessment of the assay's performance by comparison with serological testing. J Clin Microbiol 2007 Jan;45:93-6.
60. Riedner G, Rusizoka M, Todd J, Maboko L, Hoelscher M, Mmbando D, et al. Single-dose azithromycin versus penicillin G benzathine for the treatment of early syphilis. N Engl J Med 2005 Sep 22;353:1236-44.
61. Lukehart SA, Godornes C, Molini BJ, Sonnett P, Hopkins S, Mulcahy F, et al. Macrolide resistance in Treponema pallidum in the United States and Ireland. N Engl J Med 2004 Jul 8;351:154-8.
62. Mitchell SJ, Engelman J, Kent CK, Lukehart SA, Godornes C, Klausner JD. Azithromycin-resistant syphilis infection: San Francisco, California, 2000-2004. Clin Infect Dis 2006 Feb 1;42:337-45.
63. The A2058G Prevalence Workgroup. Prevalence of the 23S rRNA A2058G point mutation and molecular subtypes in Treponema pallidum in the United States, 2007 to 2009. Sex Transm Dis 2012 Oct;39:794-8.
64. Grimes M, Sahi SK, Godornes BC, Tantalo LC, Roberts N, Bostick D, et al. Two mutations associated with macrolide resistance in Treponema pallidum: increasing prevalence and correlation with molecular strain type in Seattle, Washington. Sex Transm Dis 2012 Dec;39:954-8.
65. Martin IE, Gu W, Yang Y, Tsang RS. Macrolide resistance and molecular types of Treponema pallidum causing primary syphilis in Shanghai, China. Clin Infect Dis 2009 Aug 15;49:515-21.
66. Lukehart SA. Syphilis. In: Brauwald E, Faucci AS, Hauser SL, Longo DL, Jameson JL, editors. Harrison's Principles of Internal Medicine. McGraw Hill; 2004. p. 977-85.
67. Berry CD, Hooton TM, Collier AC, Lukehart SA. Neurologic relapse after benzathine penicillin therapy for secondary syphilis in a patient with HIV infection. N Engl J Med 1987 Jun 18;316:1587-9.
68. Blank LJ, Rompalo AM, Erbelding EJ, Zenilman JM, Ghanem KG. Treatment of syphilis in HIV-infected subjects: a systematic review of the literature. Sex Transm Infect 2011 Feb;87:9-16.
69. Ghanem KG. Evaluation and Management of Syphilis in the HIV-Infected Patient. Curr Infect Dis Rep 2010 Mar;12:140-6.
70. 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 Apr;83:97-101.
71. Marra CM, Maxwell CL, Smith SL, Lukehart SA, Rompalo AM, Eaton M, et al. Cerebrospinal fluid abnormalities in patients with syphilis: association with clinical and laboratory features. J Infect Dis 2004 Feb 1;189:369-76.
72. Ghanem KG, Moore RD, Rompalo AM, Erbelding EJ, Zenilman JM, Gebo KA. Antiretroviral therapy is associated with reduced serologic failure rates for syphilis among HIV-infected patients. Clin Infect Dis 2008 Jul 15;47:258-65.
73. Castro R, Prieto E, Aguas MJ, Manata MJ, Botas J, Pereira FM. Molecular subtyping of Treponema pallidum subsp. pallidum in Lisbon, Portugal. J Clin Microbiol 2009 Aug;47:2510-2.
74. Peng RR, Wang AL, Li J, Tucker JD, Yin YP, Chen XS. Molecular typing of Treponema pallidum: a systematic review and meta-analysis. PLoS Negl Trop Dis 2011 Nov;5(11):e1273.
75. Castro R, Prieto E, Aguas MJ, Manata MJ, Botas J, Santo I, et al. Detection of Treponema pallidum sp pallidum DNA in latent syphilis. Int J STD AIDS 2007 Dec;18:842-5.
76. Pillay A, Liu H, Chen CY, Holloway B, Sturm AW, Steiner B, et al. Molecular subtyping of Treponema pallidum subspecies pallidum. Sex Transm Dis 1998 Sep;25:408-14.
77. Marra C, Sahi S, Tantalo L, Godornes C, Reid T, Behets F, et al. Enhanced molecular typing of treponema pallidum: geographical distribution of strain types and association with neurosyphilis. J Infect Dis 2010 Nov 1;202:1380-8.
78. Statistics Denmark. Available from http://www.dst.dk (accessed 15 January 2015).
79. Lohse N, Hansen AB, Jensen-Fangel S, Kronborg G, Kvinesdal B, Pedersen C, et al. Demographics of HIV-1 infection in Denmark: results from the Danish HIV Cohort Study. Scand J Infect Dis 2005;37:338-43.
80. Christiansen AH, Cowan S, Fonager J, Trebbien R. HIV 2013. EPI-NEWS 2014;37.
81. Hamers FF, Phillips AN. Diagnosed and undiagnosed HIV-infected populations in Europe. HIV Med 2008 Jul;9 Suppl 2:6-12.
82. Mason K, Thygesen LC, Stenager E, Bronnum-Hansen H, Koch-Henriksen N. Evaluating the use and limitations of the Danish National Patient Register in register-based research using an example of multiple sclerosis. Acta Neurol Scand 2012 Mar;125:213-7.
83. Obel N, Engsig FN, Rasmussen LD, Larsen MV, Omland LH, Sorensen HT. Cohort profile: the Danish HIV cohort study. Int J Epidemiol 2009 Oct;38:1202-6.
84. Black N. Why we need observational studies to evaluate the effectiveness of health care. BMJ 1996 May 11;312(7040):1215-8.
85. Altman DG. Practical Statitistics for Medical Research. Chapman and Hall; 1991.
86. Trends in primary and secondary syphilis and HIV infections in men who have sex with men--San Francisco and Los Angeles, California, 1998-2002. MMWR Morb Mortal Wkly Rep 2004 Jul 9;53:575-8.
87. Axelsen N, Mazick A, Cowan S. Syphilis 2006. EPI-NEWS 2007;18.
88. The Danish HIV Cohort Study. Annual report (In Danish). Available from http://www.rigshospitalet.dk/NR/rdonlyres/185334E3-D378-4DC6-B9D9-91DF6A2DBAB8/466064/DHK_2012.pdf (accessed 15 January 2015).

89. Jinno S, Anker B, Kaur P, Bristow CC, Klausner JD. Predictors of serological failure after treatment in HIV-infected patients with early syphilis in the emerging era of universal antiretroviral therapy use. BMC Infect Dis 2013;13:605.
90. Tsai JC, Lin YH, Lu PL, Shen NJ, Yang CJ, Lee NY, et al. Comparison of serological response to doxycycline versus benzathine penicillin G in the treatment of early syphilis in HIV-infected patients: a multi-center observational study. PLoS ONE 2014;9(10):e109813.
91. Clement ME, Okeke NL, Hicks CB. Treatment of syphilis: a systematic review. JAMA 2014 Nov 12;312:1905-17.
92. Tipple C, McClure MO, Taylor GP. High prevalence of macrolide resistant Treponema pallidum strains in a London centre. Sex Transm Infect 2011 Oct;87:486-8.
93. Grange PA, Allix-Beguec C, Chanal J, Benhaddou N, Gerhardt P, Morini JP, et al. Molecular subtyping of Treponema pallidum in Paris, France. Sex Transm Dis 2013 Aug;40:641-4.
94. Cole MJ, Chisholm SA, Palmer HM, Wallace LA, Ison CA. Molecular epidemiology of syphilis in Scotland. Sex Transm Infect 2009 Oct;85:447-51.
95. Mikalova L, Pospisilova P, Woznicova V, Kuklova I, Zakoucka H, Smajs D. Comparison of CDC and sequence-based molecular typing of syphilis treponemes: tpr and arp loci are variable in multiple samples from the same patient. BMC Microbiol 2013;13:178.
96. Flasarova M, Pospisilova P, Mikalova L, Valisova Z, Dastychova E, Strnadel R, et al. Sequencing-based molecular typing of treponema pallidum strains in the Czech Republic: all identified genotypes are related to the sequence of the SS14 strain. Acta Derm Venereol 2012 Nov;92:669-74.
97. Grillova L, Petrosova H, Mikalova L, Strnadel R, Dastychova E, Kuklova I, et al. Molecular Typing of Treponema pallidum in the Czech Republic during 2011 to 2013: Increased Prevalence of Identified Genotypes and of Isolates with Macrolide Resistance. J Clin Microbiol 2014 Oct;52:3693-700.
98. Truong HM, Kellogg T, Klausner JD, Katz MH, Dilley J, Knapper K, et al. Increases in sexually transmitted infections and sexual risk behaviour without a concurrent increase in HIV incidence among men who have sex with men in San Francisco: a suggestion of HIV serosorting? Sex Transm Infect 2006 Dec;82:461-6.
99. Spielmann N, Munstermann D, Hagedorn HJ, an der HM, Houareau C, Gunsenheimer-Bartmeyer B, et al. Time trends of syphilis and HSV-2 co-infection among men who have sex with men in the German HIV-1 seroconverter cohort from 1996-2007. Sex Transm Infect 2010 Oct;86:331-6.
100. Qvist T, Cowan SA, Graugaard C, Helleberg M. High linkage to care in a community-based rapid HIV testing and counseling project among men who have sex with men in Copenhagen. Sex Transm Dis 2014 Mar;41:209-14.