STIs/HIV and HIV/TB Interventions for Prevention and Control of Syndemics in Resource-Constrained Settings

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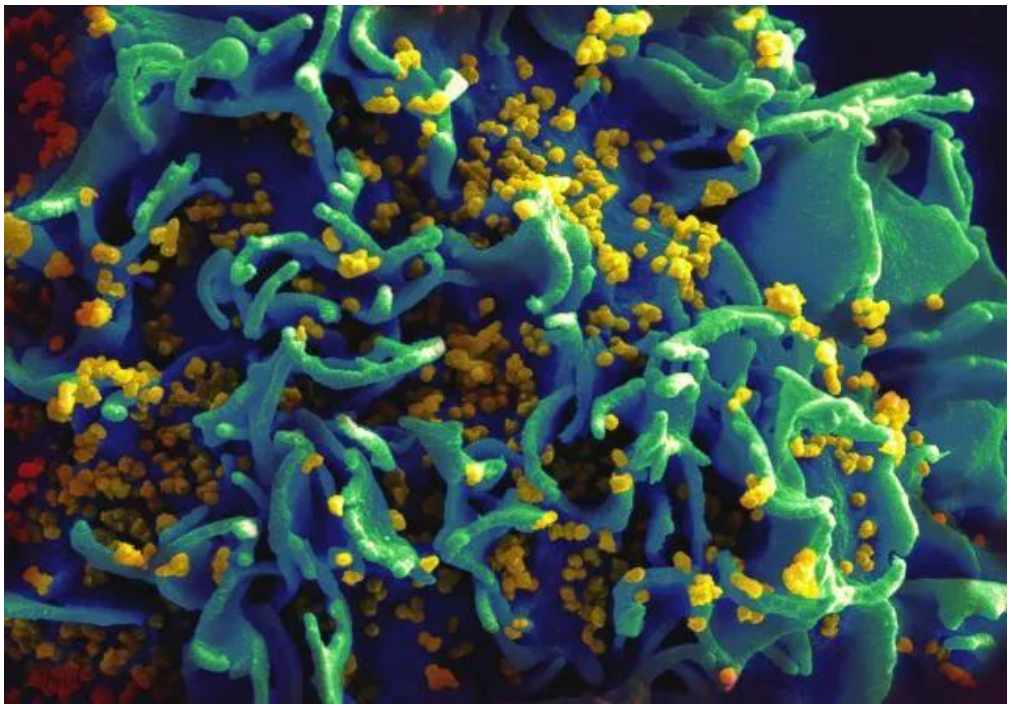

Universitat Autònoma de Barcelona

Todo da una de cal y otra de arena, todas las caras tienen su cara y su cruz, todos somos un pájaro que vuela a la vez, hacia el norte y hacia el sur, todo lo que se vuelve a contar ya es otra historia, todo lo que se rompe inventa a su enemigo, y la misma canción al cambiar de persona, no dice lo de siempre cuando dice lo mismo (Benjamín Prado)

It does not matter how slowly you go as long as you do not stop (Confucius)

PhD Thesis,

STIs/HIV and HIV/TB Interventions for Prevention and Control of Syndemics in Resource-Constrained Settings

Submitted by Juan Ignacio García,

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1 List of acronyms and abbreviations in alphabetical order

AIDS: Advanced immunodeficiency syndrome

AFB: Acid fast bacilli

AM: Alveolar macrophage

ART: antiretroviral therapy

ART: combination antiretroviral therapy

EMR: Electronic medical records

GRADE: Grading of recommendations assessment, development and evaluation

GVL: Genital viral load

HIV: Human immunodeficiency virus

HPV: Human Papillomavirus

HSV: Herpes Simplex Virus

IL: Interleukins

INF-γ: Interferon gamma

LTBI: Latent tuberculosis infection

MAC: *Mycobacterium avium* complex

MDR-TB: Multi drug resistant tuberculosis

MHC: Major histocompatibility complex

MNCHN-FP: Maternal, neonatal and child health, nutrition and family planning

Mtb: *Mycobacterium tuberculosis*

*MTB*C: *Mycobacterium tuberculosis* complex

MSM: Men who have sex with Men

MTCT: Mother-to-child transmission

NTM: Non-tuberculous Mycobacteria

OR: Odds ratio

aOR: adjusted odds ratio

PAF: Population attributable fraction

PLHIV: People living with HIV

PMTCT: Prevention of mother-to-child transmission

PNS: Guatemala HIV/AIDS and STIs Prevention and Control Program

PVL: Plasma viral load

RCT: Randomized clinical trial

RNA: Ribonucleic acid

SDGs: Sustainable Development Goals

SRHR: Sexual and reproductive health and rights

STIs: Sexually transmitted infections

TB: Tuberculosis

TB-IRIS: Tuberculosis immune reconstitution inflammatory syndrome

TGFβ: Tumour growth factor beta.

TNF: Tumour necrosis factor alpha

VCT: Voluntary counselling and testing

WHO: World Health Organization

XDR-TB: Extensively drug resistance tuberculosis

2 Thesis summary, structure and organization

This thesis research is about the burden of co-occurring epidemics affecting resource-constrained settings and the forces driving them into and excess burden of disease morbidity and mortality. This thesis presents and discusses models to effectively deliver health care according to the needs of the populations studied and the availability of resources. This thesis is organized as a compendium of 4 published papers and 2 manuscripts submitted for publication related to human immunodeficiency virus (HIV) and sexually transmitted infections (STIs) and to HIV and tuberculosis (TB). There are 2 differentiated parts: the STIs/HIV part and the HIV/TB part with HIV infection as a connection for both parts. The 2 publications for the STIs/HIV part arise from field work studies in Guatemala in 2012; the 2 publications and 2 submitted manuscripts for the HIV/TB part arise from data from Malawi, and the field work performed in Guatemala during 2013-2015 and Mozambique during 2017-2019.

The first part of the thesis analyses STIs/HIV care in Guatemala during 2005-2012 and the effectiveness of integrated, community-based STIs/HIV joint interventions to decrease prevalence trends in STIs/HIV in key populations. The importance of integrated STIs/HIV biological and behavioural surveillance for clinical management and programmatic health care performance is also discussed. Finally, it is emphasized the need of accurate data to inform and guide public health policies and interventions to effectively allocate resources for prevention and control strategies.

The second part of this thesis evaluates recommended TB/HIV collaborative activities and interventions. One line of research is the assessment of current models of TB/HIV services integration using TB incidence and mortality outcomes in people living with HIV (PLHIV). The other line of research is one of the three “I’s” of the World Health Organization’s (WHO) three “I’s” strategy, that is, intensified TB case finding; two diagnostic tests for TB screening in PLHIV are presented as well as discussion about their risks and benefits to be implemented as point-of-care (POC) tests in health care centres in resource-constrained settings. Only 3 of the published papers fulfil the requirements to be presented as a compendium of publications in this thesis research.

Resumen, estructura y organización de la tesis

Esta tesis doctoral versa sobre epidemias simultáneas y sus interacciones biológicas y epidemiológicas en espacio, población y tiempo en países con escasos recursos. Esta tesis aborda cómo distintos modelos asistenciales pueden ser implementados para cubrir las necesidades de salud de las poblaciones afectadas. Esta tesis se organiza como compendio de 6 publicaciones (4 artículos publicados y 2 en proceso de publicación) sobre infecciones de transmisión sexual (ITS) y VIH (Virus de la inmunodeficiencia humana), y sobre VIH y tuberculosis (TB). Hay dos partes diferentes: la parte ITS/VIH y la parte VIH/TB con el VIH como nexo de unión. Las 2 publicaciones de la parte de ITS/VIH se derivan del trabajo de campo realizado en Guatemala en el 2012 y las 4 publicaciones, (2 artículos publicados y 2 en proceso), de la parte de VIH/TB se derivan de estudios en Malawi y trabajo de campo realizado en Guatemala durante el 2013-2015 y Mozambique durante el 2017-2019.

La primera parte de la tesis analiza el manejo clínico y epidemiológico de ITS y VIH en Guatemala durante 2005-2012 y la efectividad de la integración a nivel comunitario de servicios asistenciales en ITS/VIH con el objetivo de disminuir la prevalencia de ITS/VIH en poblaciones de alto riesgo. Se discute la importancia de la integración de datos biológicos y de conducta sexual para, tanto la vigilancia epidemiológica como el manejo clínico de ITS/VIH, poniendo especial énfasis en la importancia de obtener datos precisos para guiar el uso efectivo de recursos en la implementación de estrategias de prevención y control.

La segunda parte de la tesis evalúa la implementación de actividades colaborativas recomendadas en TB/VIH. Por un lado, se discute el efecto de la integración de modelos asistenciales en TB y VIH y su impacto sobre la incidencia de TB y la mortalidad en personas viviendo con VIH. Por otro lado, se discute la búsqueda activa de casos de TB, [(estrategia recomendada por la organización Mundial de la Salud (OMS)], a través de la evaluación de las ventajas y desventajas de 2 pruebas diagnósticas para su implementación como pruebas diagnósticas rápidas en centros de salud de países con escasos recursos. Solamente 3 de las publicaciones presentadas cumplen los criterios establecidos para ser usados como compendio de publicaciones en esta tesis doctoral.

3 INTRODUCTION

3.1 STIs and HIV

Despite STIs control contributes to progress towards Sustainable Development Goals (SDGs), including: SDG 3.2 (By 2030, end preventable deaths of new-borns and children under 5 years); SDG 3.3 (By 2030, end the epidemics of AIDS, combat other communicable diseases); SDG 3.7 (By 2030, ensure universal access to sexual and reproductive health care), and SDG 3.8 (By 2030, achieve universal health coverage) (1), STIs still pose a huge amount of disease morbidity and mortality worldwide (2).

WHO in their 2018 STIs report, estimates that nearly 1 million people become infected every day with any of four curable STIs: chlamydia, gonorrhoea, syphilis, and trichomoniasis (3,4). In this context, the African Region and the Region of the Americas suffer the highest burden of these 4 curable STIs in men and women aged 15-49 years old (4). The burden of viral STIs is also high, with an estimated 417 million prevalent cases of herpes simplex virus (HSV) infection and approximately 291 million women infected with human papillomavirus (HPV). Estimated STIs-related mortality includes 200 000 foetal and neonatal deaths each year due to syphilis in pregnancy, and over 280 000 cervical cancer deaths each year due to HPV (4).

STIs are also responsible for a considerable burden of acute illness. Without early diagnosis and accurate therapy, their complications severely compromise women's health, fertility and productivity, and infant health and survival. Although the majority of women with STIs are asymptomatic, about 10% develop pelvic inflammatory disease (5). Other complications, especially in women, can occur years after the initial infection, causing chronic pelvic pain, ectopic pregnancy, infertility and death (6). Untreated syphilis in pregnancy can lead to perinatal transmission and miscarriage, intrauterine growth retardation, stillbirth, hydrops fetalis, prematurity and neonatal death (7,8).

The burden of STIs and the spread HIV infection and its acquired immunodeficiency syndrome (AIDS) pandemic (9) come together to increase the burden of both epidemics in a synergistical interaction (10–12).

HIV is a retrovirus belonging to the lentivirus sub family (***Figure 1***); HIV`s genome is a positive single stranded RNA which encodes for 16 proteins. This structure simplicity contrasts with its extensive genetic variability: HIV is sub divided into types, groups, subtypes, circulating recombinant forms, and unique recombinant forms (13). HIV type I and type II were originated from independent cross-species transmissions of simian immunodeficiency virus (SIV) to humans. The most plausible routes of transmission where exposure to infected blood or tissues due to hunting, butchering or even biting events (13,14). Early transmission was supposed to occur in Democratic Republic of Congo around 1920 (15), and the first documented case of HIV infection has been suggested to be from a sailor who died in 1959 (16).

Almost three decades ago, an unexplained advanced immunodeficiency syndrome was starting to affect homosexual men in urban settings (17), and still today, HIV virulence and pathogenesis is a matter of debate (18–20). The hallmark of HIV infection is the progressive loss of $CD4^+$ T cells and the host immunological abnormalities derived from the sustained and generalized activation of the immune system. The dynamics of $CD4^+$ T-cell depletion are not fully understood (21,22), but different conditions have been classified to define AIDS, including opportunistic infections, tumours and other disease processes (23). Since the approval of Zidovudine in the late 80's until the recent integrase inhibitor drugs, antiretroviral treatment (ART) has caused a dramatical increase in life expectancy in PLHIV. Despite of long-term survival of PLHIV due to ART health is not fully restored, and long-term treated PLHIV have shorter life span compared to their HIV uninfected peers. This is due by an increased frequency of complications typically associated with aging such as heart disease, cancer, liver and kidney disease, bone disease and neurocognitive decline. Theses complications have been suggested to be a consequence of a premature aging in PLHIV due to persistent inflammation and immune senescence (24–26).

ART does not cure HIV infection and latently infected cells cannot be detected and removed by the immune system. Latency occurs when proviral DNA

integrates into chromosomal DNA and fails to express viral and RNA proteins remaining hidden from immune surveillance (27).

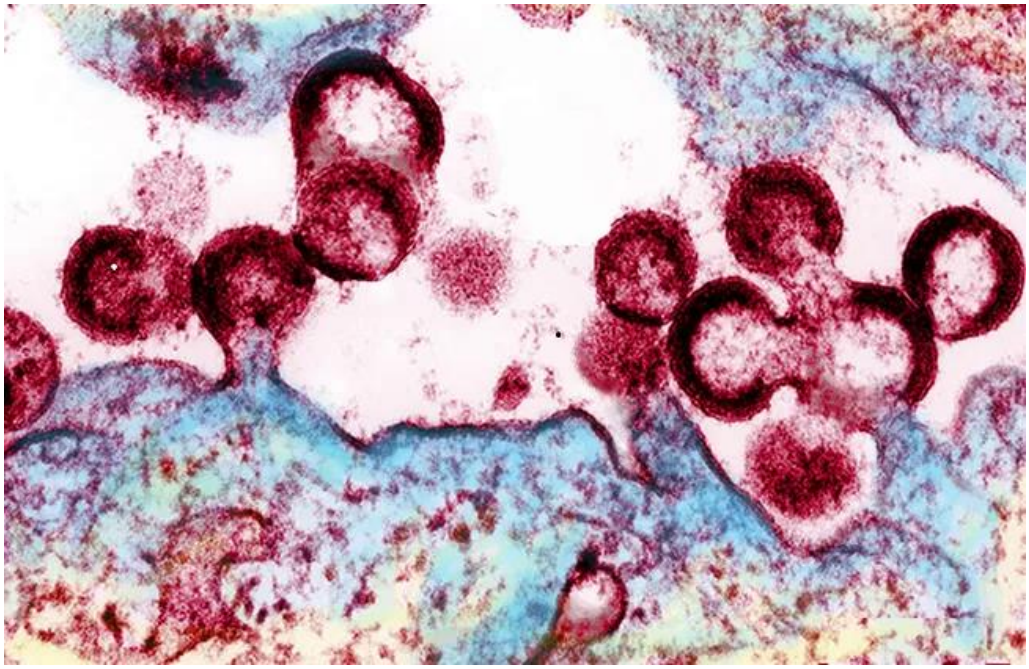


Figure 1: Budding of HIV virions (in red) from an infected $CD4^+$ T cell (in blue)

Figure 1 is courtesy of National Institute of Allergy and Infectious diseases (NIAID) and has been extracted from here: https://www.verywellhealth.com/hiv-microscopy-in-pictures-48651

Despite of ART, HIV is able to stablish a latent infection within memory $CD4^+$ T cells which act as HIV reservoirs. It has been estimated that it will take around 70 years of ART to eradicate the latent reservoir; therefore, new approaches such as the shock and kill strategy (28,29) and developing a package of curative interventions are being pursued (27,30). Recent advances in animal models, outstanding results from haematopoietic stem cells transplantation in PLHIV (31,32), and the identification of post-treatment HIV-1 controllers (33,34) have resulted in a tremendous scientific motivation towards HIV cure. In this regard, supressed viremia in the absence of ART or the complete elimination of HIV infection are currently a global priority (30).

In 2018, there were globally 37.9 million PLHIV with 770 000 deaths, and Eastern and Southern Africa accounting for 54% of the world's PLHIV. Despite high significant achievements in ART coverages and reductions in AIDS-related mortality, the annual number of new HIV infections has risen in eastern Europe and central Asia (29% increase), the Middle East and North Africa (10% increase) and Latin America (7% increase) (35). In addition, in 2018, 21.4% of PLHIV people did not know that they were living with HIV.

Ending the HIV/AIDS pandemic is embedded in almost all of the SDGs (36,37). The ambitious 90-90-90 treatment target (by 2020, 90% of all PLHIV will know their HIV status, 90% of diagnosed PLHIV will receive sustained ART), and 90% of PLHIV receiving ART will have viral suppression), launched by the Joint United Nations Program on HIV/AIDS (UNAIDS) in 2014 to end the AIDS epidemic by 2030 (38), relies on the continuum of prevention, care and treatment for HIV (39,40). Despite the use of different data sources for steps estimation, and comparability concerns due to lack of standardization (41,42), *"The Cascade of HIV Care"* is an international recognized framework to represent HIV care from HIV testing to viral suppression (43).

UNAIDS and WHO recommend the treatment as prevention strategy for the global elimination of HIV (44,45). Adherence to ART supresses viral load making individuals non-infectious; therefore, as many more PLHIV under ART less HIV transmissibility will occur in a given community or population (46,47) and more likely to achieve the estimated threshold for HIV elimination of 1 case per 1000 people per year (48). In this regard, a recent cohort study has shown that this HIV elimination threshold is feasible to achieve (49–51).

STIs and HIV are specially increased in vulnerable groups such as sex workers (SWs) and men who have sex with men (MSM). The term "sex workers" encompasses female sex workers (FSWs), male sex workers, and male-to-female transgender sex workers in a wide variety of settings (e.g. brothels, massage parlours, informal settings, and on the street). Acquisition of HIV and other STIs are important occupational hazards of sex work, and SWs and their clients might also act as a bridge population enhancing HIV and STIs transmission into

wider populations (52–54). In addition, sex work and same sex relationships are widely affected by discrimination, legal prosecution, violence, trafficking, stigmatization, and often, precarious life styles due to economic difficulties (55); these social factors are strong barriers for health seeking behaviours (56–59) which in turn negatively affects STIs/HIV treatment adherence, retention in care, and treatment outcomes (60–62).

3.2 Tuberculosis (TB)

TB caused by *Mycobacterium tuberculosis* (*Mtb*) constitutes the leading cause of death from a single infectious disease agent (63). According to WHO estimates, in 2018 there were around 10.0 million of new cases, and 1.2 million deaths attributable to TB. TB morbidity and mortality fuelled by the HIV/TB syndemic (64–66) is a major global health concern, especially in the African and Asian Regions which account for 75% of the HIV-associated TB cases worldwide (66). Currently, renewed global efforts have been put in place to end TB by 2030 (67,68) with major advances in TB mortality declines. However, if the global estimated decline in TB incidence by 1.6% per year continues, (far from the 4-5% required to reach the WHO's End TB strategy targets), only few countries are likely to meet the SDGs (69,70).

Mtb is an aerobic, non-spore-forming, and acid-fast bacilli mycobacteria belonging to the family of *Mycobacteriaceae*. The genus mycobacteria can be divided into 4 major groups based on fundamental differences in epidemiology, pathology, drug susceptibility and the ability to grow in vitro: those that belong to the *Mycobacterium tuberculosis* complex (*MTB*C), *Mycobacterium leprae*, *Mycobacterium ulcerans* and those referred to as nontuberculous mycobacteria (NTM), including the *Mycobacterium avium* complex, (MAC) (71). The species within the *MTB*C have undergone numerous taxonomic and nomenclature changes; to date, the species accepted are the following six: *Mtb, M. africanum, M. bovis*, *M. caprae, M. microti* and *M. pinnipedii* (72). (***Figure 2***)

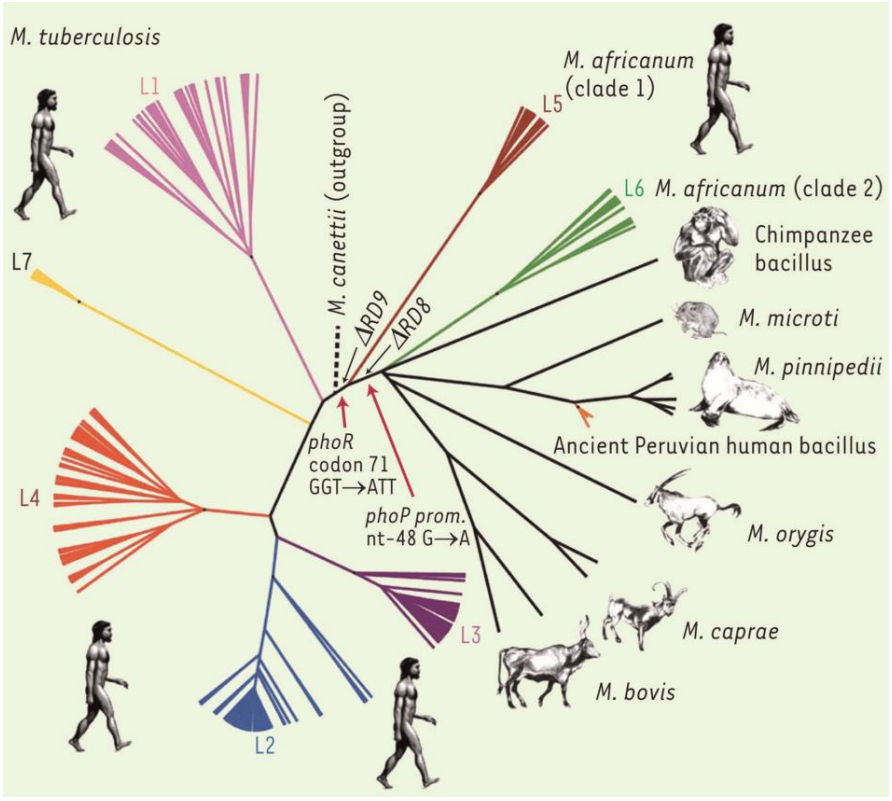


Figure 2: Phylogenetic whole genome sequence analysis of the *MTB*C: Whole-genome phylogeny of 261 strains belonging to the *MTB*C is shown. Animal and *M. africanum* specific regions of difference (RD) deletions are indicated, as well as mutations affecting the *PhoPR* virulence regulator. [Adapted from Bos et al (73), Gonzalo-Asensio et al (74), and extracted from (75,76)].

History suggests that TB appeared about 70 000 years ago (77). Recent research also suggest that sea mammals played a key role in transmitting TB to humans (73). TB remained sporadic up to the 18th century, and then, became epidemic during the industrial revolution, owing to the increased population density and the unfavourable living conditions (78). Thus, with such a long period of existence, the evolutionary trajectory of *Mycobacterium spp* has ranged from environmental, non-pathogenic species, and opportunistic pathogens that infect immunocompromised hosts, to professional pathogens (79). *Mtb* has evolved as a highly specialized obligate human pathogen resulting as a near-perfect paradigm of a host-pathogen relationship (80) in which humans and *Mtb* co-evolution might have occurred (81,82). Thus, *Mtb* has acquired a wide range of mechanisms to replicate and persist in humans, modulating the host immune response to escape from killing.

In this regard, *Mtb*'s cell envelope is a unique and complex multilayer structure that provides a barrier against antibiotics and modulates the host immune response. It is composed of 5 main layers: a- the capsule, b- the mycolic acid rich outer membrane (OM), c-the arabinogalactan layer (AG), followed by d-the core peptidoglycan (PG), and e- the plasma membrane (PM) (83,84) (***Figure 3***).

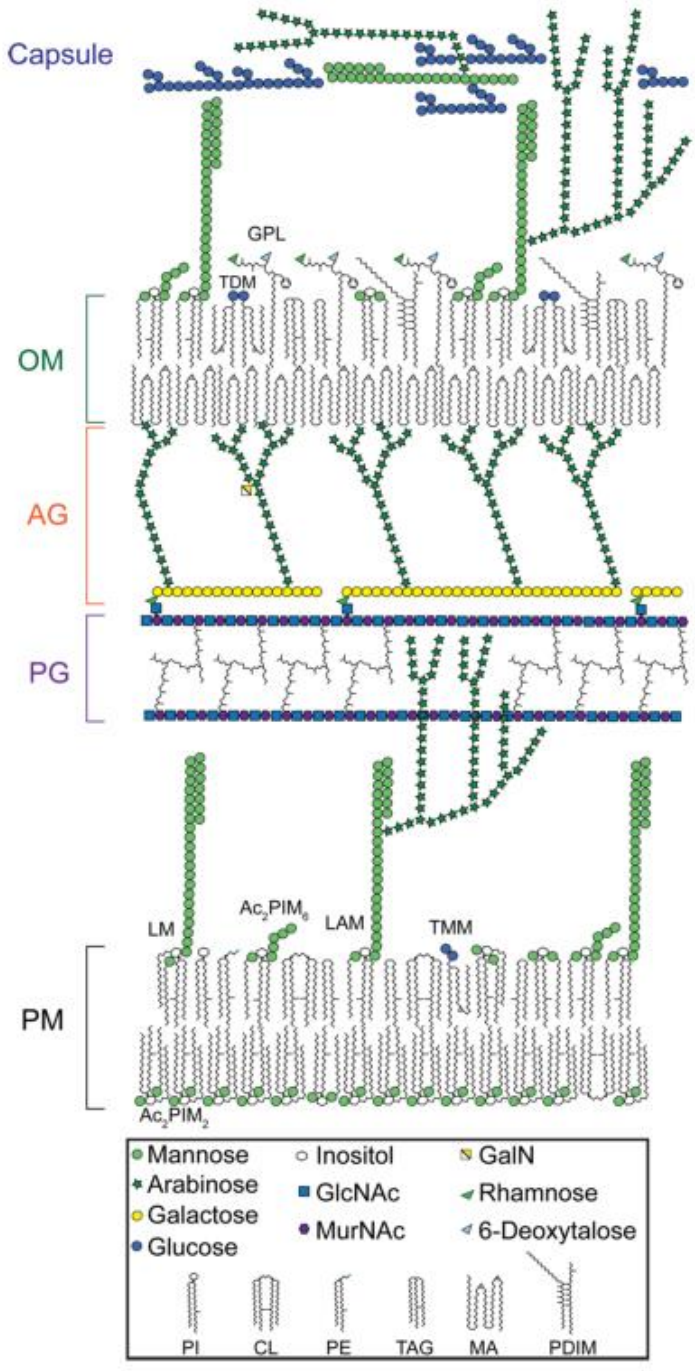


Fig. 3. MTBM cell envelope structure. The PG layer attached to the AG layer, which in turn is covalenlty attached to the mycolic acids constitutes the cell envelope core or mycolyl-arabinogalactan-peptidoglycan complex (mAGP). Intercalated within the mycolic acids layer of the mAGP there are major lipids with known roles in pathogenesis, immunomodulation, toxicity and signalling such as: Pthiocerol dimycocerosates (DIMs/PDIMs), triacylglycerols (TAGs), trehalose monomycolate (TMM), trehalose dimycolate (TDM or cord factor), diacyl-, triacyl-, pentaacyl-trehalose (DATs, TATs, PATs), sulfolipids (SLs), phenolic glycolipid (PGL, present in some strains), phosphatidyl-*myo*-inositol mannosides (PIMs with different acyl groups and mannose numbers) and their related lipoglycans, lipomannan (LM) and lipoarabinomannan (LAM) (85,86). OM: Outer membrane;

AG: Arabinogalactan layer; PG: Peptidoglycan layer; PM: Plasma membrane; GPL: Glycopeptidolipid; LM: Lipomannan; LAM: Lipoarabinomannan; TMM: Trehalose monomycolate; TDM: Trehalose dimycolate; PI: Phosphatidylinositol; CL: Cardiolipin; PE: Phosphatidylethanolamine; TAG: Triacylglycerol; PDIM: Phthiocerol dimycocerosate. Ac_2PIM_6: tetraacylated phosphatidyl-*myo*-inositol hexamannoside. Figure extracted from (83).

Infection with *Mtb* initiates when air droplets of bacteria are inhaled and *Mtb* enters the lung where it encounters the human lung mucosa followed by dendritic cells and alveolar macrophages (AM) which internalize *Mtb* by phagocytosis as a first line defence against the infection (87). Then, there are two possibilities: 1-*Mtb* infection is cleared by AM, or 2-AM fail to eliminate bacteria and *Mtb* infects or is transported by AM to the lung parenchyma stablishing the infection. *Mtb* has a wide myriad of mechanisms to survive and persist (88) hijacking host defences to its benefits such as inhibition of phagosome-lysosome function (89,90), breaking the phagosome (91), host cells death promotion (92), and inhibition of MHC class II antigen processing and presentation (93). In addition, replication of *Mtb* in AM and regional lymph nodes leads to lymphatic and hematogenous dissemination of *Mtb* that can stablish multiple organ seeding sites that might eventually give rise to extrapulmonary infection (94).

After a pro and anti-inflammatory host response (95,96), the recruitment of mononuclear cells to the parenchyma begins the formation of the signature of TB infection: The granuloma (97,98). The granuloma formation gives rise to the containment phase of *Mtb* infection and the establishment of the latent TB infection (LTBI) (88). The granuloma environment has a dual interpretation: from the host defence perspective, it is an *Mtb* prison containing the infection, but for the *Mtb* perspective it is a burden of phagocytic cells to infect and replicate within (99). The nature of the LTBI has been a matter of extensive research and is currently interpreted as a dynamic process (100). (***Figure 4***).

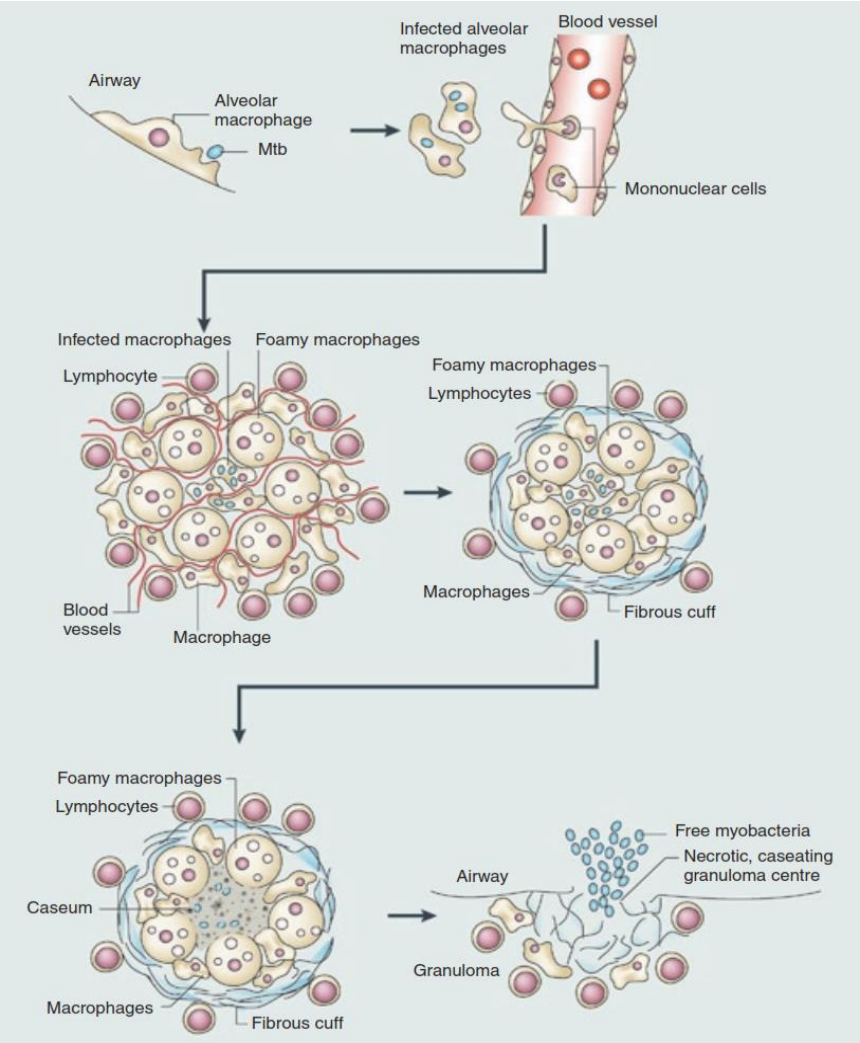


Figure 4: The granuloma formation: The granuloma consists of a kernel of infected macrophages surrounded by foamy macrophages and other mononuclear phagocytes, with a mantle of lymphocytes in association with a fibrous cuff of collagen and other extracellular matrix components that delineates the periphery of the structure. Figure extracted from (97).

Replication of *Mtb* in AM and regional lymph nodes leads to lymphatic and hematogenous dissemination of *Mtb* that can stablish multiple organ seeding sites that might eventually give rise to extrapulmonary TB (101,102). Briefly, the immune recognition of *Mtb* by AM and dendritic cells discussed earlier is built on the crucial role of the cytokine production in a complex process of regulation and cross-regulation of proinflammatory and anti-inflammatory cytokines (101,103–105). While interleukins (IL), IL-12, IL-15, TNF-α, and INF-γ are fundamental for granuloma formation and infection containment, IL-4, IL-10 and TGFβ produce antagonist effects counteracting protective immunity against *Mtb* (106).

INF-γ plays a key protective role in antigen-specific T-cell immunity (107,108). In the adaptative immune response to *Mtb*, mycobacterial antigen presentation by macrophages and dendritic cells occurs via major histocompatibility complex molecules II (MHC-II) to antigen-specific $CD4^+$ T cells and via MHC-I to antigen-specific $CD8^+$ T cells (102).

After many decades of debate, it seems that the binary perspective of either LTBI or active TB disease has been challenged as an oversimplification (109). Currently, *Mtb* infection is seen as a range and continuum of bacterial activation states in a dynamic balance between host responses and *Mtb* replication moving from LTBI, sub-clinical TB disease or active TB disease (100,110). (**Figure 5**).

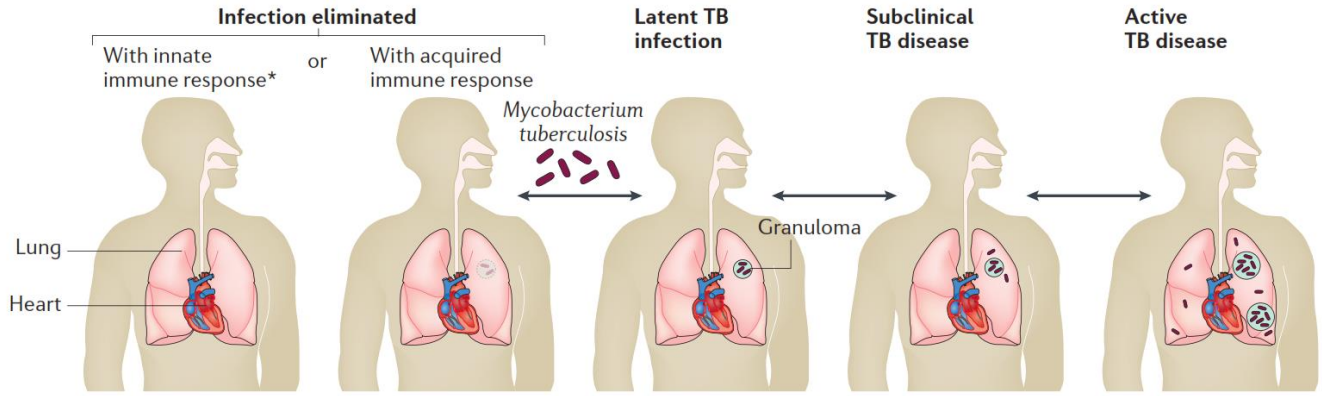


Figure 5: The spectrum of TB. From infection to disease. Extracted from (110).

TB is a disease of poverty (111), and despite *Mtb* is not considered a highly infectious pathogen (112), an average infectious individual might infect 3-10 person per year (113). There are major known risk factors for developing active TB such as undernutrition and indoor air pollution (114), type 2 diabetes mellitus (115), excessive alcohol use (116), tobacco smoke (117,118), and aging (119,120); but the strongest risk factor for developing active TB is HIV-1 infection (121).

3.3 The theory of Syndemics

The syndemic concept is based on the synergistic interaction of two or more co-existent epidemics that result in an excess burden of disease exceeding the sum of the disease burden when considered epidemics separately (11,122). The term syndemic refers not only to the temporal and geospatial co-occurrence of two or more diseases, but also to the consequences of the biological interaction among the health conditions present (66,123,124). Therefore, it appears as an alternative approach to identify and understand the conception of disease events within a biocultural approach taking into account relevant social, political, economic, and environmental forces in play (122).

In biomedicine, disease is conceptualized as a discrete entity, clinically identifiable, and separate from other diseases and from the social groups and social contexts in which they are found (125,126), despite that diseases are not entities but explanatory models (127). Thus, the theory of syndemics goes further on reconceptualising the biomedical conception of disease based on distinct and discrete entities regardless its social context, to a more complex biomedical concept where relevant psychosocial, political and economic forces play a key role in the understanding of the burden of disease. As stated by Singer and Clair: " A syndemic is a set of intertwined and mutually enhancing epidemics involving disease interactions at the biological level, that develop and are sustained in communities and populations because of harmful social conditions and injurious social connections" (128–130).

The theory of syndemics is principally a theory about population health, with disease interactions occurring at both the individual and population level, as a complex multi-level phenomenon. There are many examples of pathogen-pathogen interactions supporting the evidence of syndemic models of disease as complex biosocial processes (131). There are also different methodological approaches aiming to understand to which extent there is empirical support to the concept of disease interaction (128,132).

The syndemic approach brings a more holistic perspective in the understanding of diseases in public health and the responses needed for prevention and control in diverse populations; thus, the syndemic theory has fundamental implications

in how health care delivery models, disease surveillance systems and public health interventions and policies, could effectively manage syndemic diseases taking into account, structural, socio-demographic, behavioural and biomedical interventions in vulnerable populations and specific settings (130).

A hypothesized model of syndemics is proposed in ***figure 6***.

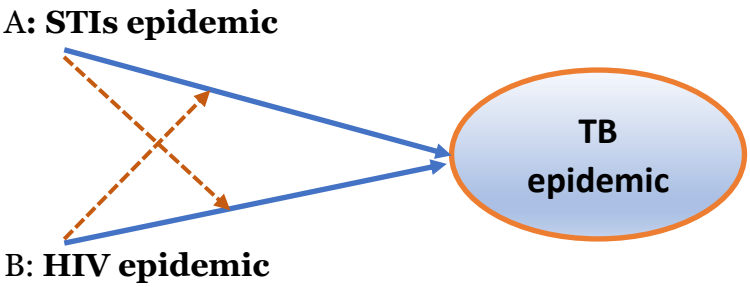


Figure 6: Syndemic proposal hypothesis

As shown in ***figure 6***, the STIs epidemic and the HIV epidemic (A and B) are not mutually causal but show synergy: the dashed arrows indicate that STIs epidemic has a greater effect on the TB epidemic in the presence of the HIV epidemic, and the HIV epidemic has a greater effect on the TB epidemic in the presence of the STIs epidemic; thus, the combined effect of both STIs and HIV epidemics on the TB epidemic exceeds the sum of their independent effects were no synergy is present. Figure adapted from (133).

There are three fundamental concepts underlying the theory of syndemics: a-disease interaction (biological interaction) b-disease concentration (epidemiological interaction), and c-social forces that give rise to them (122).

These biological and epidemiological interactions will be presented under the social, political and economic forces giving rise to them for both the STIs/HIV and the HIV/TB syndemics. Furthermore, different health care delivery models that should be put in place to address both syndemics will be discussed from a Public Health perspective.

3.3.1 The STIs/HIV syndemic

3.3.1.1 The Biological interaction

The biological interaction reflects the molecular and physiological interactions occurring in a host infected with 2 or more pathogens. In the case of STIs/HIV a large and growing body of research has focused on the presence of an STI in: a) increasing susceptibility to HIV acquisition and b) increasing the risk of HIV transmission, compared to the absence of the STI (***Figure 7).***

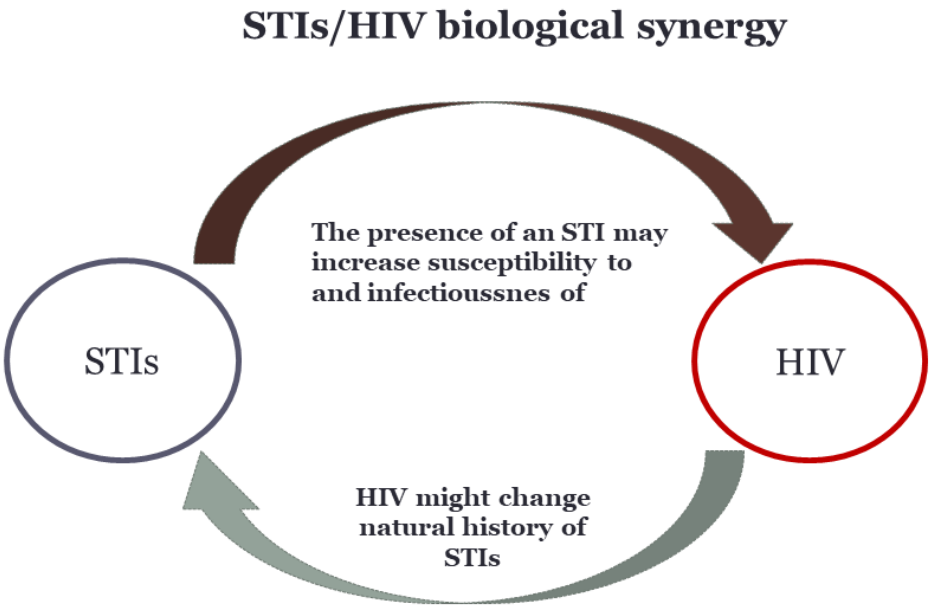


Figure 7: STIs/HIV biological synergy

The nature of the biological interactions of STIs and HIV in promoting HIV acquisition and transmission are based on mucosal disruption, HIV target cell recruitment and activation, alteration of cytokine production, enhanced HIV replication, and increased HIV shedding and viral load in plasma and genital secretions (134,135).

HIV ribonucleic acid (RNA) levels in blood plasma and genital secretions are the most significant and best described determinants of sexual transmission of HIV (136). Despite a good correlation among plasma RNA levels [i.e. (plasma viral

load (PVL)] and genital viral load (GVL), a current hypothesis is that suppressed PVL might not accurately predict HIV transmission due to “isolated” mucosal HIV shedding (PVL negative but GVL positive) (137). In this regard, it has been shown that HIV shedding in genital tract and increased GVL is associated with STIs and cervical inflammation (138). Thus, inflammation and ulcers are expected to lower the barriers to HIV infection through disruption of the mucosal epithelia and increased inflammatory cytokine concentrations, facilitating HIV infection by recruiting and activating HIV targeted cells (138–140).

The effect of genital tract infections on HIV-1 (141,142) shedding and HIV RNA detection was studied in a systematic review and meta-analyses, showing that the odds of HIV-1 detection in the genital tract was increased most substantially by urethritis (OR 3.1, 95% CI: 1.1–8.6) and cervicitis (OR 2.7, 95% CI: 1.4–5.2). The odds of HIV-1 detection were also increased significantly in the presence of cervical discharge (OR 1.8, 95% CI: 1.2–2.7), gonorrhoea (OR 1.8, 95% CI: 1.2–2.7), chlamydial infection (OR 1.8, 95% CI: 1.1–3.1), and vulvar-vaginal candidiasis (OR 1.8, 95% CI: 1.3–2.4), concluding that genital tract infections are likely to be important in promoting sexual transmission and mother-to-child transmission of HIV-1 (143,144).

Herpes Simplex Virus type 1 or 2 (HSV-1/HSV-2) infections in PLHIV with sever immunosuppression commonly present as chronic, necrotic and confluent mucocutaneous ulcerations (135,145). HSV-2 associated ulcerations break in the mucosa in the genital area and create portals for the entry of HIV. HSV-2 lesions contain substantial numbers of T CD4+ lymphocytes, which are target cells for HIV. Laboratory studies have shown that these $CD4^+$ T lymphocytes are likely to facilitate the acquisition of HIV in HIV-negative, HSV-2 positive individuals when they are exposed to HIV (146). *In vivo* and *in vitro* studies assessing the effect of HSV-2 on HIV transmission demonstrate that HIV-infected $CD4^+$ T cells are recruited to HSV-infected lesions and that HSV regulatory proteins (ICP0, ICP4, VP16) may upregulate HIV replication, thus increasing the frequency and titre of mucosal HIV shedding (147).

Treatment of non-ulcerative STIs, such as gonorrhoea, chlamydia, and trichomoniasis in PLHIV have shown substantial declines in GVL (148–150). A recent HIV seroconversion study concluded that among MSM infected with rectal *Neisseria gonorrhoeae* or *Chlamydia trachomatis*, a history of 2 additional prior rectal infections was associated with an 8-fold increased risk of HIV infection (151).

PVL correlates with GVL in both male and female genital secretions; however this correlation is modest suggesting that local factors and HIV RNA compartmentalization could influence the risk of HIV transmission (152,153). Studies in GVL in semen have demonstrated that local shedding can be persistent and at levels corresponding to "transmissible" virus (154). This is supported by the fact that GVL has been reported to correlate with HIV transmission risk independently of PVL (155). Thus, it might be possible that HIV transmission occurs despite undetectable PVL due to ART but sustained GVL due to HIV shedding in genital secretions, giving an important role of STIs in HIV transmission and influencing the HIV treatment as prevention strategy.

However, several studies have shown that there is no evidence of HIV transmission in women and men with sustained undetectable PVL (156,157), suggesting that ART prevents HIV transmission independently of STIs. Indeed, a recent systematic review and meta-analysis shows that the increase of PVL due to an STI co-infection in PLHIV under ART is less than 1 log10 difference, and thus unlikely to decrease the effectiveness of the HIV treatment as prevention strategy (158). It has become clear that STIs influence HIV-1 shedding despite of effective ART. However its role in population transmission of HIV might be very limited (159,160).

Overall, the biological interactions between STIs and HIV mutually reinforce the burden of STIs and HIV infections. Thus, an epidemiological interaction would be plausible to be observed showing an overlapping burden of both infections.

3.3.1.2 The epidemiological interaction

As Hayes *et al*, stated "the increased risk of HIV acquisition associated to both curable STIs and HSV-2 has been robustly demonstrated in observational studies. A recent meta- analysis of 31 longitudinal studies demonstrated a 4-fold increased risk of HIV infection with any laboratory-documented STI (aOR 3.9, 95%CI: 2.8-5.3). Adjusted effect estimates were higher for any genital ulcer disease (aOR 2.8, 95%CI: 2.3-3.3) than for any non-ulcerative STI (aOR 1.7, 95%CI: 1.4-2.0) and consistently fell in the 2 to 3-fold range for herpes, syphilis, chancroid, gonorrhoea and chlamydia" (134,161). "These relative risks have been measured over extended follow-up periods during which STIs had been only present for a limited period of time; this fact may imply considerably higher STI cofactor effects on HIV acquisition per sexual contact" (162). A recent cohort study in HIV-discordant heterosexual Zambian couples shows that population attributable fractions (PAFs) found due to ulcerative and non-ulcerative STIs on HIV transmission and acquisition risk, confirm the role of STIs in HIV transmission and acquisition caused by non- specific genital inflammation in both HIV discordant male-to-female and female-to-male sexual intercourse (144,163).

In the past two decades, there have been numerous studies assessing the impact of a variety of biomedical, behavioural and structural interventions on STIs/HIV prevention and control. These interventions have focused mainly in most at risk populations in the so called "high-risk strategy" (164) and are also based on the principles of 2nd generation surveillance for HIV (165), international guidelines (53), and combination HIV prevention strategies (166).

Several of these research studies have shown increases in condom use, declines in STIs prevalence and HIV incidence when implementing integrated biomedical, behavioural and structural interventions tackling STIs and HIV prevention and control in resource-constrained settings (54,167–172). These interventions have the potential to impact on STIs/HIV transmission at the population level, as vulnerable populations such as FSWs and their clients and MSM, are considered as bridging populations in the transmission of STIs and HIV to the general population (173–175). Indeed, numerous systematic reviews and meta-analyses

have measured the impact of population-based biomedical (8,176), behavioral (177–179), and structural (180,181) interventions on the prevention and control of STIs/HIV. Despite some discordant results and limited evidence for generalizability, interventions in STIs prevention and control have the potential to decrease HIV infection at individual and population levels.

Moreover, several randomized clinical trials (RCTs) have been used to measure the effectiveness of improved STIs control interventions on the impact of HIV incidence. To date, there have been 9 RCTs. Six RCTs assessed treatment of STI as an intervention to reduce HIV incidence and the remaining 3 RCT studied suppressive therapy of HSV2 to reduce HIV acquisition (2 RCTs) and transmission (1 RCT).

Four out of the 6 mentioned RCTs attempted to capture the effects of STIs on both HIV acquisition and transmission through a population-wide intervention (134). These trials are commonly named as the Mwanza (182), Rakai (183), Masaka (184), and Manicaland (185) trials because of the geographical settings where these were performed.

Only the Mwanza trial (182), which examined the effect of improved STI services using STIs syndromic management, found a reduction of HIV incidence of 40% in the general population. Differences in effect between trials have been extensively reviewed (12,134,186), and taking into account that these differences might have not occurred by chance, the following explanations are important to understand these differences:

a- Mwanza trial was conducted in a population with an expanded HIV epidemic compared to Rakai, Masaka and Manicaland trials that were experiencing a mature HIV epidemic. In mature epidemics, the cumulative probability of HIV transmission is high even in the absence of STIs (187);
b- There were differences in control group interventions among Mwanza and the other 3 trials. While in Mwanza trial the existing STI services were of poor quality (188), control groups in the other 3 trials received more active STI services, including syndromic management services. Condom social marketing and voluntary counselling and testing (VCT) were also delivered

in Rakai and Masaka trials. However, differences in the intensity of control interventions among trials do not explain fully, differences in HIV incidence impact found;

c- Differences in STIs prevalence were found among Mwanza, Rakai and Masaka trials. Gonorrhoea and Syphilis prevalence at baseline were higher in Mwanza compared to Rakai and Masaka (134,187). In addition, these trials of STI control to prevent HIV infection might have failed to provide appropriate study populations and settings (189).

As presented in the biological interaction of STIs and HIV, there is much evidence supporting the hypothesis that HSV-2 infection increases both susceptibility to and transmissibility of HIV. However, 3 RCTs measuring the impact of HSV-2 suppressive therapy on HIV acquisition (190,191) and HIV transmission (192) did not demonstrate a protective effect on HIV incidence. It seems probable that the subclinical HSV-2 inflammation causing recruitment of HIV target cells and increased HIV shedding in spite of treatment, might have reduced the benefits of acyclovir (193).

Conversely, a meta-analyses of 31 observational studies (194) showed that PAFs for HIV infection due to HSV-2 were substantial, concluding that control strategies for HSV-2 need to be incorporated into control of STIs as a strategy for HIV prevention. Indeed, a recent systematic review in India suggests that almost half of HIV infections are caused by HSV-2 infections assuming causality (195), and another modelling study in India confirms that HSV-2 contributes substantially to HIV in this context with 36% (95% CI: 22-62%) of HIV infections in FSWs being caused by HSV-2, mostly through HSV-2 asymptomatic shedding (196).

3.3.1.3 Health care delivery models and integration

3.3.1.3.1 Decentralization of HIV services.

In 2003, the WHO launched the "Treating 3 Million by 2005: Making it happen" strategy, aiming to scale up ART coverage through HIV services decentralization and simplified and standardized tools for delivering ART in resource-constrained

settings in Sub-Saharan Africa (197). This strategy has achieved remarkable milestones in the fight against HIV/AIDS (198).

Risks and benefits of implementation of decentralized HIV services have been evaluated in numerous research studies providing good evidence of increased ART enrolment and retention in care (199–204) despite of risks in losses to follow up due to HIV care delivery in less sophisticated levels (203).

However, different models of ART decentralization are to be used as decentralization also needs to adapt to specific health care settings in order to strengthen HIV linkage and retention in care, integration services, and disease surveillance.

3.3.1.3.2 STIs/HIV services integration: A bi-directional approach

The rapid scale-up of ART programs has posed significant challenges to health systems in high-burden, resource-limited settings; the optimal model for ART decentralization delivery depends on the local context, including the burden of HIV infection and the health-care delivery system. WHO proposed an ART decentralization approach using the following models (205):

1- Delivering ART in maternal and child health-care settings
2- Delivering ART in TB treatment settings and TB treatment in HIV care settings
3- Delivering ART in settings providing opioid substitution therapy
4- Delivering STIs and family planning services within HIV care settings

However, it has been challenging to define and agree definitions for terms “Integration” and “Linkage”. Although, WHO has defined integration “as the management and delivery of health services, so that clients receive a continuum of preventive and curative services according to their needs over time and across different levels of the health system”(206), the little consensus achieved represents different definitions, models, measurements and assessment approaches to integration (207,208).

In addition, the urgent response to combat the spread of the HIV/AIDS pandemic, resulted in a huge HIV-specific funding put in place as separate, parallel, and vertical models to provide HIV-only services. In resource-constrained settings, the integration focus has been based on integrating specific disease programs, including malaria, leprosy, TB and HIV aiming to improve health care delivery access, efficiency and cost effectiveness (208,209). However, at the international level, the importance of integrating maternal sexual and reproductive health, neonatal, child health, nutrition and family planning services with HIV/AIDS services is well recognized as a key strategy to improve prevention and control of HIV/AIDS (205,210–212) and meet the 2015 Millennium Development Goals (213) and the 2030 SDGs (214).

In this regard, different integration approaches have been used and systematic reviews and meta-analyses have been published. On one hand, the integration of STIs and Sexual and Reproductive Health and Rights (SRHR) into pre-existing HIV care and treatment services (215,216), and on the other hand, the integration of HIV treatment and care services into pre-existing STIs and SRHR (202,207,217,218). Overall, findings suggest that HIV/AIDS services integration with other services such as maternal, neonatal and child health, nutrition and family planning services (MNCHN-FP) is feasible and promising towards improving health-related outcomes.

Nonetheless, prevention of mother-to-child transmission (MTCT) of syphilis and HIV, and STIs/HIV service integration has been shown to be challenging due to the following factors: a- lack of health policy guidance (219), b- inadequate training of health care workers (212,220), c- increased waiting times in health care settings (221,222), d- discordant results in assessing patients' satisfaction of integrated services (223), e- difficulties in health care workers ability for task shifting, f- lack of space and insufficient supplies (224), and finally, g- context dependent social factors (225), and h- variability of models and approaches used (225,226). Currently, significant evidence gaps remain and rigorous research comparing outcomes within different grades of services integration properly adapted to specific health care settings are needed to inform health programs and

health policy makers (207) in the context of the SDGs and the 90-90-90 strategy (37,227).

Moreover, evidence of quality standards of ART monitoring in the era of decentralized HIV care needs to be improved (228) and STIs/HIV services integration is a good opportunity to integrate biological and behavioural surveillance of STIs and HIV/AIDS which are urgently needed. As it is stated in guidelines and surveillance principles for HIV, "Countries with routine STIs screening programs in any population should work on strengthening reporting systems so that STIs data can be integrated into HIV surveillance systems" (165,229,230).

Nonetheless, local, national and international implementation is limited to high income countries (231–234), and surveillance systems differ in terms of coverage, comprehensiveness and representativeness, and are also often limited to specific high-risk populations (231,235–237). In resource-constrained settings, however, implementation of integrated STIs/HIV surveillance is challenging (238), and electronic medical records (EMR) capturing socio-demographic, clinical and laboratory data are still scarce when compared to paper-based data; EMR implementation projects have strengthened HIV surveillance and continuum of care gaps analysis (239–243).

Many countries have launched their Strategic plans for HIV/AIDS and STIs prevention and control programs attempting to integrate STIs/HIV management into a public health perspective (244–252). However, operational implementation has been very limited and evidence-based data on monitoring and evaluation of integrated STIs/HIV surveillance is lacking. Overall, the benefits of STIs/HIV services integration are clear and supported by national and international health organizations (205,211,225,253) and governments (220,223,254). However, operational research for the implementation of STIs/HIV services integration needs funding, political commitment and adequate implementation policies.

At this point, biological and epidemiological interactions among STIs/HIV have been presented alongside with different approaches for STIs/HIV services integration in terms of programmatic health care performance and public health perspectives. In the following section, the HIV/TB syndemic is presented stablishing parallelisms and study links with the STIs/HIV syndemic previously introduced.

3.3.2 HIV/TB syndemic

3.3.2.1 The Biological interaction

Both HIV and *Mtb* infections produce chronic inflammation and chronic immune activation. As said before, macrophages are de primary target cells for *Mtb* infection and they have a key role in triggering a protective host immune response. HIV also infects macrophages and therefore, both pathogens could be found infecting the same cell (255). HIV-1 infected macrophages might have a deleterious effect on the macrophage immune response to *Mtb* (256).

3.3.2.1.1 TB exacerbating HIV infection to AIDS progression

TB exacerbation of HIV disease has been reported through increasing the risk of additional opportunistic infections (257,258), and as an independent risk factor for AIDS progression (259). However, the effects of *Mtb* infection and TB disease on HIV infection and progression to AIDS still need to be completely elucidated. Nonetheless, increased replication of HIV at sites of *Mtb* infection has been demonstrated (260,261), and the host immune activation due to *Mtb*, also increases HIV-1 transcription (262). In addition, while TNF-α production has a key role in controlling *Mtb* growth, it also activates HIV replication in macrophages (263). It has also been proposed that people with TB disease generate a microenvironment that facilitates HIV infection increasing the expression of co-receptors CXCR4 and CCL5 and down-regulation of CCL5 (264). Finally, it has been suggested that the Lipoarabinomannan (LAM) antigen (265) may activate the replication of HIV in pro-virus carrying cells by inducing TNF-α and IL-6 production through the NF-kB pathway (266–268).

3.3.2.1.2 HIV infection increases the risk of progression of Mtb infection to TB disease and the reactivation of LTBI

The depletion of $CD4^+$ T cells caused by HIV/AIDS is the main feature driving the dramatical increase in susceptibility of *Mtb* primary infection or reinfection and also the risk of TB reactivation in patients with LTBI (267). $CD4^+$ T cells and TNF-α are fundamental in maintaining the granuloma structure to contain *Mtb* bacilli; thus, it has been hypothesized that HIV-1 may disrupt granuloma formation, by killing resident $CD4^+$T cells and dysregulating macrophage function (269–271). HIV-1 has been reported to inhibit macrophage phagocytosis *ex vivo* (272) and *in vivo* (273) promoting *Mtb* growth (274). It has also been suggested that HIV-1 *Nef* proteins reduce *Mtb* associated macrophage apoptosis (275–277) and also inhibit innate immune intracellular signalling pathways required for *Mtb* killing (278). Interestingly, Vitamin D has been shown to improve HIV-1 and *Mtb* infection control through rescuing macrophage autophagy and apoptosis impaired functions (279,280).

As a result, it has been shown that clinical manifestations of TB disease in PLHIV are commonly seen as a systemic disease involving multiple organs that lack well defined granulomas. In addition, cavitary lesions are rarely encountered in PLHIV with less than 200 $CD4^+$ T cells/mm^3 (267). A substantial increased risk in TB and its extra-pulmonary dissemination is strongly correlated with $CD4^+$ T cells depletion (281) and the proportion of extrapulmonary TB has been reported to be higher in PLHIV compared to their non-HIV infected peers (282–284). These features presented are in concordance with patterns of chest X rays observed in PLHIV with pulmonary TB disease (285) that show an increased incidence of intrathoracic lymphadenopathies, miliary patterns, and less cavitary disease and pleural effusions (286–290).

The presence of less cavitary lesions and fewer necrotic granulomas in PLHIV with pulmonary TB disease, supports the hypothesis that T-cell mediated immunity contributes substantially to cellular necrosis and tissue damage (281,285). Direct evidence supporting this hypothesis is the TB immune reconstitution inflammatory syndrome (TB-IRIS). TB-IRIS in PLHIV under ART occurs because of the restoration of *Mtb*-specific immune responses. Specifically,

it might occur in two different forms: 1- a previously subclinical *Mtb* infection remains undiagnosed and subsequent immune recovery following ART initiation triggers the presentation of *Mtb* with the development of unusual inflammatory features (unmasked form) and 2-a pre-existing diagnosed *Mtb* infection deteriorates clinically as host immunopathological inflammatory responses are re-stablished (paradoxical form) (291,292).

An important consequence of the biological interaction of HIV and TB is the increased challenge in diagnosing TB in PLHIV compared to the diagnosis of TB in the HIV negative population. Among other factors, this is due to the paucibacillary, and more disseminated nature of TB disease as well as the differential radiological and clinical presentations observed. Some of the diagnostic tools available to diagnose TB in PLHIV will be presented and discussed later.

3.3.2.2 The epidemiological interaction

At least 1 out of 3 of the estimated 37.9 PLHIV are infected with LTBI. Globally, it is also estimated that people living with HIV are 29 times (26 – 31) more likely to develop active TB disease than those without HIV, and TB is the most common opportunistic infection and the leading cause of death among PLHIV (293). This increased risk of developing TB in PLHIV was shown to be detectable as soon as HIV seroconversion occurs (294). Globally, HIV prevalence rates are associated with higher TB incidence rates in generalized and concentrated HIV epidemics (124,295–297). In addition, HIV infection has been suggested to increase the transmission of multi drug resistant TB (MDR-TB) (298,299).

The biosocial aspects of both TB (300–303) and HIV (304–307) associated stigma and discrimination are key factors affecting health care seeking behaviours, and TB and HIV treatment outcomes (308). Furthermore, social development and economic losses due to both HIV and TB are considerable and strengthens the vicious circle among poverty and disease (44,111,309).

Thus, the overlapping epidemics of HIV and TB are a major concern for both TB and HIV control and prevention within elimination programs (310–312)

3.3.2.3 TB/HIV collaborative activities

In 2003 the WHO launched its first guidelines for implementing collaborative TB/HIV program activities due to the unprecedented scale of the HIV-associated TB epidemic (313). Soon after, an interim policy guide on collaborative TB/HIV activities was also released to inform policy makers what and how should be done to decrease the joint burden of TB and HIV (314). A core set of TB and HIV indicators was first proposed and then updated in the following Monitoring and Evaluation guides (315,316), alongside with the update of the interim policy guide published in 2012.

The objectives of collaborative TB/HIV activities are:
(A) To establish and strengthen the mechanisms of collaboration and joint management between HIV programs and TB control programs for delivering integrated TB and HIV services preferably at the same time and location.

(B) To reduce the burden of TB in PLHIV, their families and communities by ensuring the delivery of the WHO Three I's strategy [intensified case-finding (ICF), isoniazid preventive therapy (IPT) and infection control (IC)], for HIV/TB and the early initiation of ART in line with WHO guidelines.

(C) To reduce the burden of HIV in patients with presumptive and diagnosed TB, their families and communities by providing HIV prevention, diagnosis and treatment. Adapted from (314) and (317).

The updated guide in 2012 outlines the recommended 12 TB/HIV collaborative activities presented in ***table 1*** below.

Table 1: Recommended TB/HIV collaborative activities

A. Establish and strengthen the mechanisms for delivering integrated TB and HIV services
A.1. Set up and strengthen a coordinating body for collaborative TB/HIV activities functional at all levels
A.2. Determine HIV prevalence among TB patients and TB prevalence among people living with HIV

A.3. Carry out joint TB/HIV planning to integrate the delivery of TB and HIV services A.4. Monitor and evaluate collaborative TB/HIV activities
B. Reduce the burden of TB in people living with HIV and initiate early antiretroviral therapy (the Three I's for HIV/TB)
B.1. Intensify TB case-finding and ensure high quality antituberculotic treatment B.2. Initiate TB prevention with Isoniazid preventive therapy and early antiretroviral therapy B.3. Ensure control of TB Infection in health-care facilities and congregate settings.
C. Reduce the burden of HIV in patients with presumptive and diagnosed TB
C.1. Provide HIV testing and counselling to patients with presumptive and diagnosed TB C.2. Provide HIV prevention interventions for patients with presumptive and diagnosed TB C.3. Provide co-trimoxazole preventive therapy for TB patients living with HIV C.4. Ensure HIV prevention interventions, treatment and care for TB patients living with HIV C.5. Provide antiretroviral therapy for TB patients living with HIV

Table adapted from (317)

Each of these activities (***table 1***) have a set of recommendations; the quality of the evidence and the strength of each recommendation is assessed using the Grading of Recommendations Assessment, Development and Evaluation (GRADE) methodology (318). The 2015 updated guide on Monitoring and Evaluation of TB/HIV collaborative activities summarizes all the indicators to be monitored and classified under 3 groups: 1- Core indicators for global and national monitoring and reporting; 2- Core indicators for only national-level monitoring and reporting; and 3- Optional indicators for use at national level. The following indicators have been considered as priority indicators for health management and program performance (316):

- **A.1: Proportion of registered new and relapse TB patients with documented HIV status.**
- **A.2: Proportion of registered new and relapse TB patients with documented HIV-positive status.**

- **A.3: Proportion of people living with HIV newly enrolled in HIV care with active TB disease.**
- **A.4: Proportion of HIV-positive new and relapse TB patients on ART during TB treatment.**

Evaluation of the implementation of TB/HIV collaborative activities has been shown to be challenging in terms of policy uptake through national guidelines, unified political commitment, inherent health system barriers, and lack of flexibility and integration of National TB and HIV control Programs (319–323). Nonetheless, significant increases have been achieved in HIV testing in people with TB, TB screening in PLHIV, ART uptake and proper timing of ART initiation in people with TB, and coverage of cotrimoxazole and isoniazid preventive therapy, resulting in millions of lives saved in the last decade (63,124,324,325).

This thesis is focused to discuss objectives A and C of collaborative TB/HIV activities lpresented previously, and specifically, to address the following questions:

a- *What are the best models to integrate TB and HIV services and what are the benefits in terms of prevention and control that they could achieve?* (activity A.3).

b- *To what extent is intensified TB case finding implemented in rural and resource-constrained settings and what are the best diagnostic tools put in place to diagnose TB in PLHIV?* (activity B.1).

3.3.2.3.1 Integration of HIV and TB services

a- What are the best models to integrate TB and HIV services and what are the benefits in terms of prevention and control that they could achieve? (Activity A.3).

Different delivery models of integrated TB/HIV care have been suggested (326). (***Figure 8***).

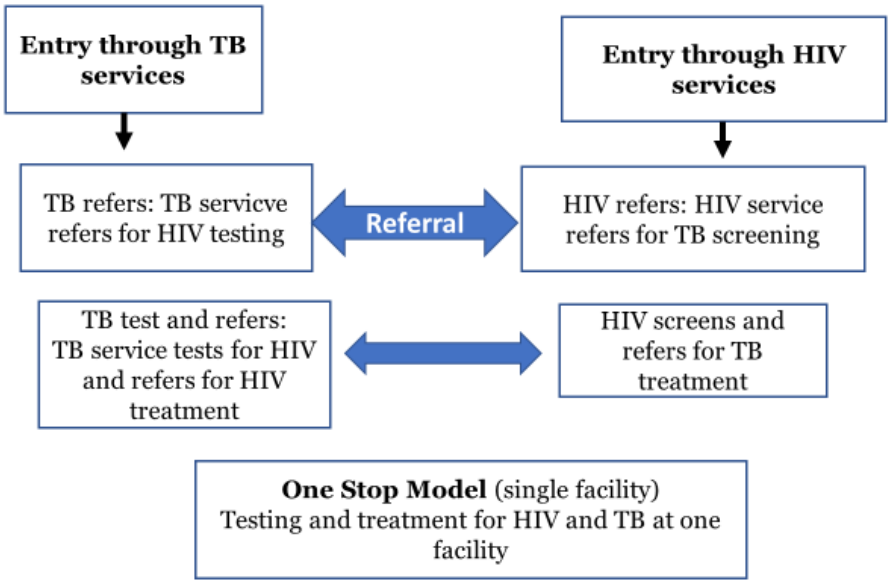


Figure 8: Models of TB/HIV service integration.
Figure adapted from (326).

TB/HIV integration models can be classified based on the degree of TB/HIV services integration at the point of service delivery related to the continuum of care: referral, partially integrated, or fully integrated. The aim of TB/HIV integrated health services is to organize and manage health delivery as a continuum of care providing people the care they need, creating a synergy between HIV and TB programs to address health care efficiency, fragmentation, duplication, quality of care and consumer satisfaction (327–329). (***Figure 9 and Table 2***).

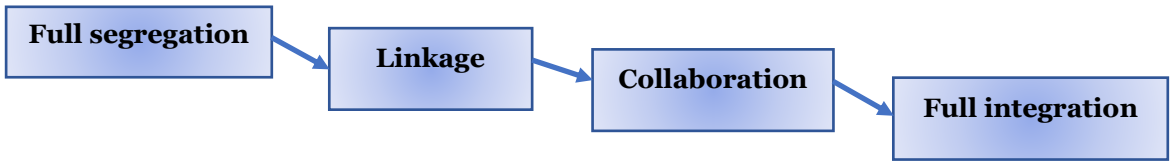


Figure 9: Continuum of integration of health services based on TB/HIV service delivery models. Figure adapted from (291)

Table 2: Relationship between the level of integration and different service delivery models.

Level of integration	Delivery models	World Health Organization descriptions
Linkage	Referral	Entry via TB service and referral for HIV testing and care
		Entry via HIV service and referral for TB screening, diagnosis and treatment
Collaboration	Partially integrated	Entry via TB service and referral for HIV care after HIV testing
		Entry via HIV service and referral after TB screening
Full integration	One stop service	TB and HIV care services provided at a single facility

Table adapted from (327)

Apparently, it makes sense that the "full integration or single facility" (later named the One Stop Service) delivery model, were TB and HIV screening and treatment is provided at one co-located facility, could be the most appropriate. Potential benefits of fully or partially integrated models rely on optimization of resources to overcome service duplication, programs collaboration, staff training and task shifting, decreased schedule visits and time spent at hospital, decreased transport costs and traveling distances, and confidentiality issues. All together, these factors might help to increase TB and HIV retention in care, treatment adherence and TB and HIV treatment outcomes (206,313,316,330)

However, the feasibility and effectiveness of the integration model to be delivered is influenced by different key factors such as HIV and TB prevalence, existing health care delivery referrals and linkages, health care staff availability and structural and logistic issues (331–333).

Several published studies have evaluated the effects of partially or fully HIV and TB services integration on a variety of TB and HIV outcomes, processes, and implementation progress through monitoring of TB/HIV integration indicators. For example, increased ART uptake of TB patients and increased TB screening in PLHIV has been shown after implementation of TB/HIV services integration in Sub-Saharan Africa (334–338) and India (325,339). In addition, TB/HIV service integration has been reported to improve TB treatment outcomes in Ghana (327,340) and South África (341,342). However, high mortality rates despite of integration are still observed (327), as well as delayed initiation of ART in people with TB (343), despite that ART initiation delay has been suggested as a consequence of non-integrated (segregated) TB/HIV services (344,345). In addition, integration might hinder TB infection control procedures in overcrowded health care centres and hospitals, posing a real threat on prevention of TB transmission dynamics (317,346).

Overall, TB/HIV integration services has shown good evidence in improving and facilitating synergies between TB and HIV programs and simplifying patient's costs and circuits in health care settings. However, research gaps still remain, and more studies assessing the impact benefits of TB/HIV services integration on TB and HIV treatment outcomes and mortality are needed. Operational research questions such as how to implement and monitor best fitting TB/HIV service integration models should be addressed (336,347–349).

3.3.2.3.2 Intensified TB case finding

b- To what extent is intensified TB case finding implemented in PLHIV in rural and resource-constrained settings and what are the best diagnostic tools put in place to diagnose TB in PLHIV? (activity B.1).

Effective clinical and microbiological tools and algorithms are fundamental to diagnose TB in PLHIV due to the increased difficulties in its TB diagnosis compared to the non-HIV infected population. HIV infection compromises the validity of clinical and microbiological tools available such as those based in TB

symptom screening (310,350–353), chest radiography (99,354), sputum-smear microscopy (354–357), and molecular diagnosis (358–362) specially, in rural health constrained settings in high TB/HIV burden countries (354,363,364). TB culture is the gold standard for diagnosis of TB, but its long turn-around time, the need of expensive equipment and trained stuff, and laboratory requirements has made it challenging to routinely use it in rural health care settings in high TB and HIV burden countries (363,365)

Nonetheless, rapid scale up of novel molecular diagnostic technologies such as GeneXpert® *MTB*/RIF (Xpert) (366–369) and point-of-care (POC) tests such as the lateral flow urine LAM Ag detection assay (LAM test) (370–373) linked to strengthened clinical algorithms (374–376) have improved substantially the diagnosis and prognosis of HIV associated TB. In this regard, and after extensive research on diagnostic accuracy and TB yield studies, the LAM test has arisen as an easy to perform, fast and non-invasive TB diagnostic test, that has shown to be very useful in the diagnosis of TB in PLHIV with immunosuppression. In addition, a positive LAM test result has been reported to predict mortality in adults and children living with HIV (377–386), and therefore it is able to identify PLHIV at high-risk of death. Thus, the low cost LAM test, is a promising tool to increase early TB treatment initiation and therefore, improve TB treatment outcomes in PLHIV (387–389).

It is not well understood how LAM enters the urine (390) but its presence is indicative of haematogenous dissemination, renal involvement in PLHIV with advanced HIV disease (384), and disseminated TB and mycobacteriuria (391,392). LAM is a major antigenic lipoglycan found in *Mtb*'s cell surface, and when "capped" with short mannose oligosaccharides (known as ManLAM) is involved in interaction with host receptors and survival during infection (356–359) (***Figure 10***). All pathogenic species of the genus Mycobacterium are known to possess ManLAM (397–399), which is responsible of the phagosome maturation arrest through inhibition of lysosomal fusion and acidification (400). The immunomodulatory properties of ManLAM (401,402) depend on its degree of acylation, the length of the D-mannan and D-arabinan cores, the length of the mannose caps, as well as the presence of other acidic constituents such as

succinates, lactates and/or malates, and also the presence of one 5-methylthioxylosyl residue per molecule (265).

Despite that the sensitivity of the LAM test is higher in PLHIV with low CD4 values, the overall LAM POC test sensitivity is still too low (52%) to be used as a standard diagnostic test alone (373). Therefore, new innovative solutions to improve LAM test sensitivity are urgently needed.

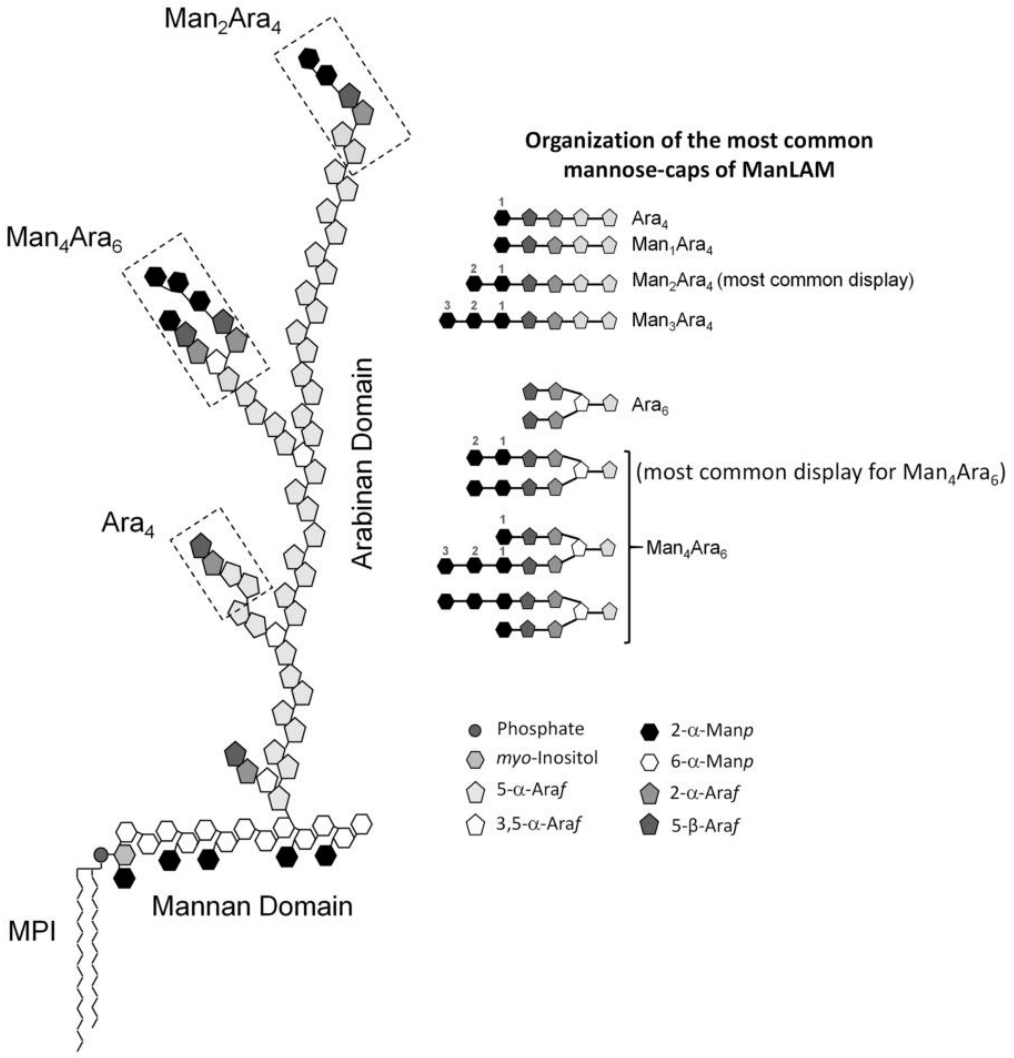


Figure 10: Schematic visualization of the ManLam structure: Representation of ManLAM structure and its different mannose-cap motifs. ManLAM has an MPI anchor, and α-mannan and α -arabinan domains. The α -arabinan domain is decorated by α (1→2) mannose caps of different length providing uniqueness to this molecule present in all pathogenic *Mycobacterium* spp. described to date. For the mannose caps of ManLAM the most common displays found are Man_2Ara_4 and Man_4Ara_6. In the case of Man_4Ara_6 is commonly found as branched Ara_6 substituted with two α (1→2) mannoses per branch. Numbers in mannose caps indicate the number of mannoses per each mannose-cap arrangement presented. Figure and legend extracted from (403).

As said before, the most widely used diagnostic test for TB disease is sputum smear microscopy, but its low sensitivity especially in PLHIV, makes alternative and easy to perform tests worth it to investigate. Both Löwenstein-Jensen (L-J) and BACTEC Mycobacterial Growth indicator tube (MGIT) cultures require specialized equipment and high costs, so they are not available in district health care centres in resource-constrained settings (404,405). Therefore, the development of rapid and low-cost culture methods is a priority in the global fight against TB.

Solid media techniques and microscopic observation drug susceptibility assays (MODS) have been used in mycobacterial laboratories for TB and MDR-TB diagnosis (406,407). Specifically, microcolony detection on thin layer agar (TLA) has been in use for a long time with the added advantage as an alternative rapid method for diagnosis of *Mtb* and MDR-TB (408–410). A modified 7H11 agar medium supplemented with oleic acid albumin dextrose complex (OADC) has been reported as an accurate, simple and economic test for drug susceptibility testing of MDR-TB and XDR-TB (410).

Interestingly, the evolution and re-shaping of these tests have the potential to be adapted as POC tests both in diagnosing human TB and MDR-TB and bovine TB. As an example, the Colour Plate Agar-based culture test (TB-CX) has been reported to be an accurate, economic and rapid test to diagnose *MTB* in a median time of 12 days in Ethiopia as well as the potential to do so for human MDR-TB (411,412) (***Figure 11***).

Therefore, rapid and easy to use agar-based TB diagnostic tests might be useful as POC tests in rural health care settings in resource constrained-settings were TB culture or molecular diagnostic tests are not available.

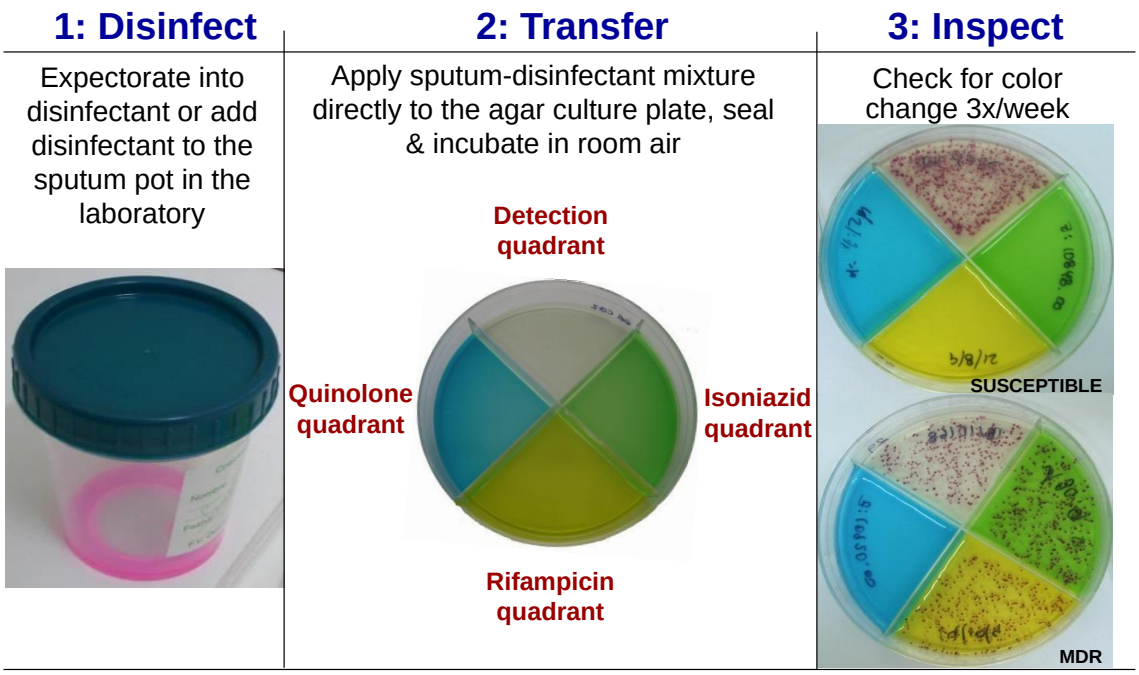


Figure 11: The TB-CX test. Figure adapted from (413)

4 RESEARCH QUESTIONS AND OBJECTIVES

The purpose of this thesis research is:

- To provide scientific evidence on new diagnostic technologies and "*best fitting"* health care models aimed to improve interventions in prevention and control of the STIs/HIV and HIV/TB syndemics.

4.1 STIs/HIV Research questions and objectives

STIs/HIV Research questions:

A: Is the implementation of a population-based HIV cohort feasible using an electronic medical record in a resource-constrained setting?

B: What are the characteristics of the populations attending STIs/HIV services in Central America?

C: Is an integrated approach to prevent and treat STIs/HIV feasible and effective to improve health care management, and prevention and control of STIs/HIV in Guatemala?

STIs/HIV general objectives (GO):

1) To systematically collect longitudinal socio-demographic, clinic and epidemiologic data of PLHIV attending hospitals in Guatemala (responds to A)

2) To describe the populations attending specialised HIV care units (2a) and STI clinics (2b) in Guatemala (both 2a and 2b respond to B)

3) To describe the HIV cascade of care in Guatemala (responds to B)

4) To assess the effectiveness of an integrated STIs/HIV health care delivery model for prevention and control of STIs/HIV in Escuintla department, Guatemala (responds to C)

STIs/HIV operational objectives (OpO) derived from GO:

1) To design and implement an electronic medical record to be used in the 14 specialised HIV care units in Guatemala (derived from GO1)

2) To describe the socio-demographic, clinic and epidemiologic characteristics of PLHIV attended in the 14 specialised HIV care units (derived from GO2a)

3) To describe the socio-demographic, sexual behaviour, and HIV transmission knowledge characteristics of people attending 3 STI sentinel clinics in Escuintla Department (derived from GO2b)

4) To estimate percentages of PLHIV with ART eligibility and those in ART treatment (derived from GO3)

5) To estimate retention in HIV care at 12 and 24 months (derived from GO3)

6) To estimate HIV and STIs prevalence and describe its trends in people attending STI clinics within an integrated model of STIs/HIV health care delivery (derived from GO4)

4.2 HIV/TB Research questions and objectives

HIV/TB Research questions:

a) What is the level of integration and clinical performance of HIV and TB services in resource-constrained settings?

b) To what extent are intensified TB case finding strategies implemented in PLHIV from Central America and Sub Saharan Africa?

c) Is it possible to improve TB POC tests performance and introduce them in clinical settings to screen for TB in Guatemala?

HIV/TB general objectives (GO):

1. To describe the level of HIV and TB services integration in Guatemala (1a) and Mozambique (1b) (responds to a)

2. To evaluate the clinical performance of the "One Stop" HIV/TB model attending PLHIV with TB disease in Guatemala (2a) and Mozambique (2b) (responds to a)

3. To evaluate the implementation of intensified TB case finding strategies in low to high TB and HIV burden countries in resource-constrained settings (responds to b)

4. To determine the feasibility, performance and diagnostic accuracy of innovative diagnostic tools for TB (responds to b and c)

HIV/TB operational objectives (OpO) derived from GO:

1) To perform evaluation visits of the 14 UAIs in Guatemala and the 14 health centres in Manhiça district, Mozambique (derived from GO1b)

2) To estimate TB incidence and mortality rates and their risk factors in cohort studies of PLHIV (derived from GO2 and GO3)

3) To evaluate different enzymatic treatments aiming to improve LAM test sensitivity in the laboratory (derived from GO4)

4) To estimate the diagnostic accuracy of the LAM and TB CX tests in diagnosing TB in PLHIV in clinical settings (derived from GO4)

5 METHODS

Overview:

This thesis research is organized as a compendium of 6 research studies, 4 of them already published, and 2 of them currently submitted for publication. These studies are related to the research fields of STIs, HIV and TB. The 2 published research studies for the STIs/HIV part arise from the field work performed in Guatemala in 2012 and the 4 research studies (2 published and 2 submitted for publication) for the HIV/TB part arise from data from Malawi, and the field work performed in Guatemala during 2013-2015 and Mozambique during 2017-2019.

Local, national and international ethics committees did approve all research studies involving study participants. All the studies were conducted under the direction of the National Tuberculosis and STIs/HIV Control Programs of countries where the studies took place. All study participants were offered to participate and if agreed, a written consent was mandatory in order to participate in the study. Data from study participants were anonymized using a unique alphanumeric code. All diagnostic and laboratory procedures for STIs, HIV and TB were performed following National guidelines from each country where studies took place, and are specifically detailed in the published research studies and manuscripts used in this thesis (***Annex***); the LAM test and the TB-CX test were performed following published procedures (410,414–417). All statistical analyses were performed using Stata (StataCorp. 2015. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP).

Study design of research studies

For the STIs/HIV part, cohort studies were implemented to address research questions; the MANGUA project was developed as a prospective cohort study using an EMR to capture data on PLHIV (***table 3***). In the case of the UALE project within the STI clinics, an EMR was also developed. Questionnaires used were in paper-based format and data entry personnel were responsible of digitalize the information collected into the EMR developed on a daily basis.

Table 3: **Variables collected in the MANGUA cohort study**

Socio-demographic and behavioural variables
MANGUA study code, date of enrolment, gender, date of birth, country of origin, province of residence, ethnicity, religion, marital status, educational level, employment status, name of UAI, previous treatment, casualty date, causality cause, cause of death, informed consent signed, vulnerability group, transmission group, probable year of infection, sexual orientation, condom use.
Laboratory variables
Biochemistry and haematology variables, HIV rapid test, HIV ELISA test, serologies for toxoplasma, cytomegalovirus, histoplasma, cryptococcal HBV, HCV, HSV, and syphilis. Sputum-smear microscopy for tuberculosis.
Clinical variables
WHO clinical stage, date of visit, type of visit, date of next scheduled visit, CD4 count, CD8 count, HIV RNA viral load, AIDS related events, AIDS defining events, other co-infections.
Treatment variables
ART type, prophylaxis type, other treatments, initiation and stop date*, reason for regime change*, adverse effects*.

HBV: Human hepatitis B virus; HCV: Human hepatitis C virus; HSV: Human herpesvirus simplex type I and II. *for each type of treatment.

In the HIV/TB part, cohort studies were also implemented. In all studies, specific questionnaires were designed to collect data either in paper-based format or in electronic case report forms (eCRF). Data was then manually or electronically uploaded into storage platforms such as ODK (Open Data Kit) and RedCap (Research Electronic Data Capture) or local databases, respectively.

In the case of Guatemala, PLHIV with TB symptoms were recruited and prospectively followed up until death, loss to follow up, or TB diagnosis. In the case of Mozambique, a retrospective approach was used; PLHIV with

presumptive TB that were initially ruled out for TB were followed up for 2 years to ascertain mortality and TB disease outcomes. Embedded in the Guatemalan cohort study, LAM and α-LAM diagnostic accuracy studies were performed and 2 by 2 tables used. Diagnostic accuracy studies were designed as cross-sectional studies embedded into a prospective cohort study where consecutive patients were recruited to perform the screening tests needed to be evaluated.

Study analysis and statistical methods

The MANGUA cohort was analysed as a descriptive cohort and it has not been used yet to ascertain association relationships between exposures and outcomes. Therefore, only descriptive statistics were calculated. The HIV cascade of care depicted in MANGUA is cumulative and cross-sectional. In the case of the UALE cohort, there were key variables with high percentages of missing data and follow up visits were limited; therefore, a cross-sectional analytical approach was preferred.

Descriptive statistics such as percentages, means, medians and IQR (interquartile) range were used to describe categorical and continuous variables. In addition, socio demographic and clinical variables were compared to check for associations using Fischer's exact test, Pearson X^2 test, student's t-test, U Mann-Whitney, and the Wilcoxon Rank-Sum test. P values <0.05 were considered statistically significant.

Research studies in paper 2, manuscript 3 and manuscript 4 assessed the association between exposures and outcomes and also quantified the strength of the associations found to decide if the observed associations were likely to be causal. Causality is one of the most important concepts in epidemiology, and it is key to determine the likelihood that alternative explanations such as chance, bias and confounding account for the true relationship between exposures and the outcomes. Therefore, all multivariable analyses were addressed to overcome the mixed effects of other factors different from those exposures studied that could account for the explanation of the outcome of interest, i.e. Adjustment for specific confounding factors was done, and adjusted and unadjusted estimates with 95% confidence intervals (CI) were calculated. No interaction neither effect modification was studied.

In order to proceed to the multivariable analysis, variables showing an association in the univariate analysis with p value < 0.2 were tested in the multivariable analysis.

Specifically, estimates on STIs and HIV prevalence and its trends, and risk factors for STIs/HIV were studied using multivariable Poisson regression. Adjusted and unadjusted prevalence ratios instead of odds ratios were preferred to increase precision of estimates and facilitate interpretation of results as previously suggested (418–420). The extension of the Mantel-Haenzsel test for trend adjusting for age was used when the Poisson test for linear trend was not of sufficient rank to be performed. In both cohort studies from the HIV/TB part, TB incidence and mortality rates, and mortality predictors were estimated using Cox proportional hazards regression; person years (PYs) of follow up were calculated and Kaplan-Meier survival curves were estimated. Like in the STIs/HIV part, unadjusted and adjusted estimates with 95% CI were calculated when assessing the relationship between exposures and outcomes.

In the case of the diagnostic accuracy studies, 2x2 tables were used and sensitivity, specificity, and positive and negative predictive values were calculated and likelihood ratios were estimated and Fischer's exact test was used to compare sensitivity and specificity between groups.

6 RESULTS

Overview:
The results section is presented through the results obtained from the 4 published research papers and the 2 submitted manuscripts. Results are divided in 2 parts: The STIs/HIV part and the HIV/TB part.

Results from the **STIs/HIV part** are linked to the **4 general objectives** presented:

- **Paper 1**, titled: **The MANGUA project: A Population-Based HIV cohort in Guatemala**, responds to general objectives 1, 2a, and 3 below:

  - ✓ **GO1**: To systematically collect longitudinal socio-demographic, clinic and epidemiologic data of PLHIV attending hospitals in Guatemala
  - ✓ **GO2a**: To describe the populations attending specialised HIV care units in Guatemala
  - ✓ **GO3:** To describe the HIV cascade of care for Guatemala

- **Paper 2**, titled: **The UALE project: a cross-sectional approach for trends in HIV/STI prevalence among key populations attending STI clinics in Guatemala,** responds to general objectives 2b and 4 below:

  - ✓ **GO2b**: To describe the populations attending STI clinics in Guatemala
  - ✓ **GO4:** To assess the effectiveness of an integrated STIs/HIV health care delivery model for prevention and control of STIs/HIV in Escuintla department, Guatemala

Results from the **HIV/TB part** are also linked to the **4 general objectives** presented:

- **The situational analysis of TB and HIV services integration in rural health care centres in Manhiça district, Southern Mozambique** performed by the PhD candidate responds to general objective 1b:

- **GO1b:** To describe the level of HIV and TB services integration in Mozambique

- **Manuscript 3,** titled: ***Mortality and risk of tuberculosis among people living with HIV in whom tuberculosis was initially ruled out*** responds to general objectives 2b and 3

  - **GO2b:** To evaluate the clinical performance of the "One Stop" HIV/TB model attending PLHIV with TB disease in Mozambique.
  - **GO3:** To evaluate the implementation of intensified TB case finding strategies in high TB and HIV burden countries in resource-constrained settings

- **Manuscript 4,** titled: **Diagnostic accuracy of LAM and α-Mannosidase treated urine LAM test to improve tuberculosis diagnosis among a cohort of people living with HIV in Guatemala** responds to GO1a, GO2a, GO3, and GO4:
  - **GO1a:** To describe the level of HIV and TB services integration in Guatemala
  - **GO2a:** To evaluate the clinical performance of the "One Stop" HIV/TB model attending PLHIV with TB disease in Guatemala
  - **GO3:** (see above)
  - **GO4:** To determine the feasibility, performance and diagnostic accuracy of innovative diagnostic tools for TB

- **Paper 5**, titled: **Improved Alere Determine Lipoarabinomannan Antigen Detection Test for the Diagnosis of Human and Bovine Tuberculosis by Manipulating Urine and Milk** responds to **GO4**

- **Paper 6**, titled: **Low-cost diagnostic test for susceptible and drug-resistant tuberculosis in rural Malawi** responds to **GO4**.

Note: Results from the situational analysis of TB and HIV services integration manuscripts 3 and 4 and paper 6 are found in the Annex section.

For clarification purposes, and despite of some content redundancy, ***tables 4*** and ***table 5*** below, connect research questions, objectives and results from papers and manuscripts.

Table 4: Relationship among research questions, objectives and results for the STIs/HIV part

Research questions (RQ)	General objectives (GO)	Operational objectives (OpO)	Results
STIs/HIV part			
A: Is the implementation of a population-based HIV cohort feasible using an electronic medical record in a resource-constrained setting?	1) To systematically collect longitudinal socio-demographic, clinic and epidemiologic data of PLHIV attending hospitals in Guatemala (responds to A)	1) To design and implement an electronic medical record to be used in the 14 specialised HIV care units in Guatemala (derived from GO1)	**Paper 1:** The MANGUA project: A Population-Based HIV cohort in Guatemala
B: What are the characteristics of the populations attending STIs/HIV services in Central America?	2) To describe the populations attending specialised HIV care units (2a) and STI clinics (2b) in Guatemala (responds to B) 3) To describe the HIV cascade of care for Guatemala (responds to B)	2) To describe the socio-demographic, clinic and epidemiologic characteristics of PLHIV attended in the 14 specialised HIV care units (derived from GO2a) 3) To describe the socio-demographic, sexual behaviour, and HIV transmission knowledge characteristics of people attending 3 STI sentinel clinics in Escuintla Department (derived from GO2b) 4) To estimate percentages of PLHIV with ART eligibility and those in ART treatment (derived from GO3) 5) To estimate retention in HIV care at 12 and 24 months (derived from GO3)	**Paper 1** and **Paper 2:** The UALE project: a cross-sectional approach for trends in HIV/STI prevalence among key populations attending STI clinics in Guatemala
C: Is an integrated approach to prevent and treat STIs/HIV feasible and effective to improve health care management, and prevention and control of STIs/HIV in Guatemala?	4) To assess the effectiveness of an integrated STIs/HIV health care delivery model for prevention and control of STIs/HIV in Escuintla department, Guatemala (responds to C)	6) To estimate HIV and STIs prevalence and describe its trends in people attending STI clinics within an integrated model of STIs/HIV health care delivery (derived from GO4)	**Paper 2**

Table 5: Relationship among research questions, objectives and results for the HIV/TB part

Research questions (RQ)	General objectives (GO)	Operational objectives (OpO)	Results
HIV/TB part			
a) What is the level of integration and clinical performance of HIV and TB services?	1. To describe the level of HIV and TB services integration in Guatemala (1a) and Mozambique (1b) (responds to a) 2. To evaluate the clinical performance of the "One Stop" HIV/TB model attending PLHIV with TB disease in Guatemala (2a) and Mozambique (2b) (responds to a)	1) To perform evaluation visits of the 14 UAIs in Guatemala and the 14 health centres in Manhiça district, Mozambique (derived from GO1b) 2) To estimate TB incidence and mortality rates and their risk factors in cohort studies of PLHIV (derived from GO2 and GO3)	**Field work evaluation:** The situational analysis of TB and HIV services integration in rural health care centres in Manhiça district, Southern Mozambique. **Manuscript 3:** Mortality and risk of tuberculosis among people living with HIV in whom tuberculosis was initially ruled out. **Manuscript 4:** Diagnostic accuracy of LAM and α-Mannosidase treated urine LAM test to improve tuberculosis diagnosis among a cohort of people living with HIV in Guatemala
b) To what extent are intensified TB case finding strategies implemented in PLHIV?	3. To evaluate the implementation of intensified TB case finding strategies in high TB and HIV burden countries in resource-constrained settings (responds to b) 4. To determine the feasibility, performance and diagnostic accuracy of innovative diagnostic tools for TB (responds to b and c)	2) To estimate TB incidence and mortality rates and their risk factors in cohort studies of PLHIV (derived from GO2 and GO3) 3) To evaluate different enzymatic treatments aiming to improve LAM test sensitivity in the laboratory (derived from GO4)	**Manuscript 3**, **manuscript 4** and **paper 3:** Improved Alere Determine Lipoarabinomannan Antigen Detection Test for the Diagnosis of Human and Bovine Tuberculosis by Manipulating Urine and Milk
c) Is it possible to improve TB POC tests performance and introduce them in clinical settings to screen for TB?	GO4: (see above)	4) To estimate the diagnostic accuracy of the LAM and TB CX tests in diagnosing TB in PLHIV in clinical settings (derived from GO4)	**Manuscript 4 and paper 6:** Low-cost diagnostic test for susceptible and drug-resistant tuberculosis in rural Malawi

6.1 STIs/HIV RESULTS

6.1.1 Paper 1: The MANGUA project: A Population-Based HIV cohort in Guatemala.

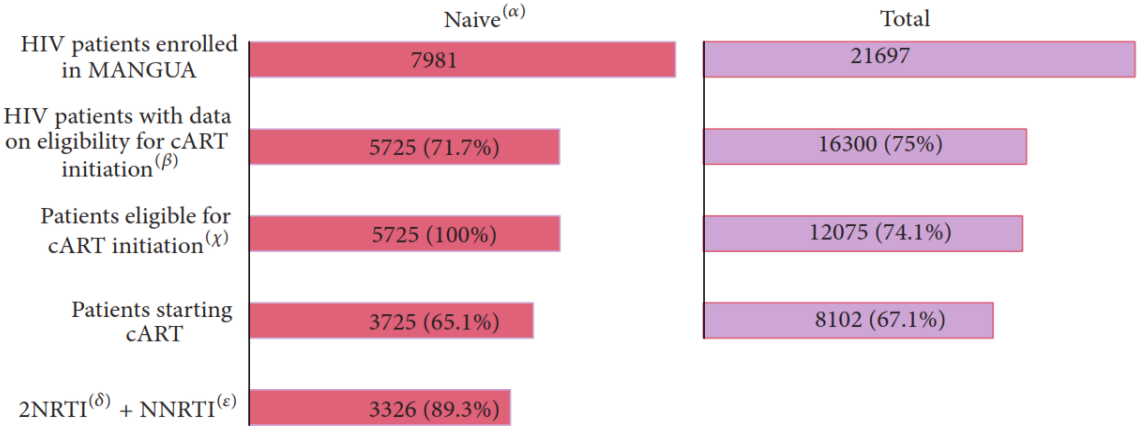
García JI, Samayoa B, Sabidó M, Prieto LA, Nikiforov M, Pinzón R, Santa Marina de León LR, Ortiz JF, Ponce E, Mejía CR, Arathoon E, Casabona J, and The MANGUA Study Group (The MANGUA study group is composed with all clinicians attending PLHIV in the UAIs of Guatemala).

Published in Journal: AIDS Research and Treatment. Received 15 February 2015; Revised 13 May 2015; Accepted 19 May 2015. http://dx.doi.org/10.1155/2015/372816

Background: Guatemala has a concentrated HIV epidemic with an estimated HIV prevalence in high-risk groups of 5% in female sex workers (FSW) and 11% in MSM. Around 20-30% PLHIV are unaware of their HIV infection. Currently, Guatemala has a population of 17.26 million inhabitants with a density of 158 people per km² of land area. When this study was performed (2012), Guatemala had a density of 142.5 people per km².

Objective: To estimate and describe the socio-demographic, clinic and epidemiological characteristics of PLHIV in Guatemala.

Main results: The MANGUA "cascade-like" of HIV care



(α) We excluded 975 patients that were retreated or reenrolled since we lack data on treatment regimen

(β) Patients with data on baseline CD4 cell count or on AIDS-related events available

(χ) CD4 cell count <350 cell/mm^3 or WHO clinical stage 3 or 4. Number of patients eligible for cART initiation over HIV patients with data on eligibility for cART initiation with data on treatment eligibility

(δ) Nucleoside reverse transcriptase inhibitor

(ε) Nonnucleoside reverse transcriptase inhibitor

As of October 1st, 2012, 21.697 HIV-positive adults ≥16 years of age were enrolled. Coverage of collected data was of 70-75%. Briefly, 75% of HIV patients enrolled had data on eligibility for ART initiation (CD4 cell counts or WHO clinical stage 3 or 4).In addition, 74.1% of patients with data on eligibility for ART initiation, had signs of advanced HIV infection or disease at baseline (CD4 cell counts <350 cells/mm^3 or WHO clinical stage 3 or 4). Finally, only 67.1% of eligible HIV patients for ART initiation started ART.

- ✓ Median CD4 T cells at enrolment was of 131 IQR (46 – 279) and 63.7% of HIV positive patients had < 200 CD4 T cells (66.9% in males Vs 33.1% in females).
- ✓ Median time in days from HIV diagnosis to ART initiation was of 75 days (27 – 308).
- ✓ Overall, for the period 2001–2011, the average retention in care proportion was of 73.8% and 61.5% at 12 and 24 months, respectively

Note: Retention in care was defined as patients known to be alive and receiving c ART at the end of the follow-up period. Attrition was defined as discontinuation of ART for any reason, including death, loss to follow-up, and stopping ARV medications while remaining in care (421).

6.1.2 Paper 2: The UALE project: a cross-sectional approach for trends in HIV/STI prevalence among key populations attending STI clinics in Guatemala

García JI, Sabidó M, Nikiforov M, Smith A, Hernández G, Ortiz R, Ardani L, Cajas A, Camey E, Torrelles JB, Wang SH, Campbell CNJ, Folch C, Casabona J; and The UALE Study Group

Published in Journal: BMJ Open. Received 6 March 2018, revised 19 June 2018, and accepted 23 July 2018. https://doi.org/10.1136/bmjopen-2018-022632

Background: STI clinics located in Escuintla Department, Guatemala attending FSW*, MSM* and HRH* populations in a setting with high migration dynamics and sex work industry due to seasonal sugar cane harvest. Integrated biological and behavioural surveillance of STIs and HIV.

Objective: To describe and compare trends in prevalence, sexual behaviour and HIV transmission knowledge data related to STIs and HIV in patients attending three STI clinics over an 8-year period in Escuintla Department, Guatemala

*FSW: Female sex workers, MSM: Men who have sex with men; HRH: High risk heterosexuals.

Main results:

- Socio-demographic:

- ✓ There were 4027 clinic attendees: 79.8% FSW, 5.7% MSM, and 14.5% HRH
- ✓ 61% of clinic attendees had a single visit (from which 56.4% were FSW, 91.1% were MSM and 57.2% were HRH. The remaining patients had a median number of visits of 3.6 IQR (2 – 4).
- ✓ 29.2% of FSW, 29.7% of MSM and 69.7% of HRH reported having a regular partner.

- Sexual behaviour and HIV transmission knowledge

- ✓ FSW
  - Decreasing trends in condom use in last sexual intercourse (CLSI) with regular partners. APr (Adjusted trends in proportions) of 0.95 (0.90 – 0.99) p value=0.029
- ✓ HRH
  - Decreasing trends in CLSI with regular and occasional partners with APr of 0.94 (0.8 – 0.99) p value= 0.042, and of 0.79 (0.63 – 0.98) p value=0.033, respectively.

- STIs/HIV prevalence

Overall results:

- HIV prevalence was of 2.10% in FSW, 8.17% in MSM and 4.12% in HRH.
- Gonorrhoea prevalence was of 9.28% in FSW, 22.76% in MSM, and 14.91% in HRH. Prevalence in FSW and HRH did not decrease over the study period.
- Chlamydia prevalence was of 6.80% and 6.91% in FSW and HRH, respectively. Trichomonas prevalence was of 12.26% and 12.38% in FSW and HRH, respectively.

- ✓ FSW
  - Decreasing trends in HIV [APR* of 0.78 (0.71 – 0.86) p value < 0.001], Syphilis [APR of 0.81 (0.75 – 0.87) p value < 0.001] and Chlamydia [0.88

(0.84 – 0.93) p value < 0.001] infections, and increasing trends in Trichomonas infections [1.14 (1.10 – 1.19) p value < 0.001]

*APr: Adjusted prevalence ratios

- ✓ HRH
  - Increasing trends in Chlamydia and Trichomonas infections with APR of 1.34 (1.10 – 1.77) p value= 0.006 and of 1.48 (1.27 – 1.71) p value < 0.001, respectively.

- <u>Risk factors for STIs/HIV</u>

- ✓ FSW
  - Those illiterate FSW or with incomplete primary school had an APR for HIV infection of 1.57 (1.08 – 2.28) p value= 0.019 compared to FSW with superior education, and an APR for Chlamydia infection of 1.27 (1.05 – 1.55) p value= 0.016 compared to FSW with superior education.
- ✓ MSM
  - Those MSM without a reported regular partner had an APR for Syphilis infection of 3.90 (1.28 – 11.76) p value= 0.016 compared to MSM with a reported regular partner.
- ✓ HRH
  - Those HRH without a reported regular partner had an APR for Gonorrhoea infection of 1.64 (1.07 – 2.50) p value= 0.022 compared to HRH with a reported regular partner.

6.2 HIV/TB RESULTS

6.2.1 The One Stop Model

6.2.1.1 Situational analysis of TB/HIV services in Manhiça district, Southern Mozambique. (PhD candidate field work evaluation).

Note: Results of this field work evaluation are found in Annex I

6.2.1.2 Manuscript 3: Mortality and risk of tuberculosis among people living with HIV in whom tuberculosis was initially ruled out.

García JI, E.Mambuque, D. Nguenha, F. Vilanculo, C. Sacoor, G. Sequera, M.Fernández-Quevedo, M. Leroux-La Pierre, H. Chiconela, LA. Faife[†], D. Respeito[2], B. Saavedra, T. Nhampossa, E.López-Varela, AL. García-Basteiro

Manuscript submitted to Scientific Reports: www.nature.com\scientificreports

Note: Results of this submitted paper are found in Annex I

6.2.1.3 Manuscript 4: Diagnostic accuracy of LAM and a-Mannosidase treated urine LAM test to improve tuberculosis diagnosis among a cohort of PLHIV in Guatemala

García JI, Meléndez J, Alvarez de Leon R, Mejía-Chew C, Kelley HV, Sidiki S, Castillo A, Mazariegos C, Forno D, Ayala N, Balada-Llasat JM, López-Téllez C, Mejía-Villatoro CR, Wang SH, Torrelles JB and Ikeda J.

Manuscript submitted to International Journal of Infectious Diseases.

Note: Results of this submitted paper are found in Annex I

6.2.2 Intensified case finding through innovative TB diagnostic tools:

6.2.2.1 The ALERE Determine TB LAM antigen test (LAM test)

6.2.2.1.1 Paper 5: Improved Alere Determine Lipoarabinomannan Antigen Detection Test for the Diagnosis of Human and Bovine Tuberculosis by Manipulating Urine and Milk

García JI, Kelley HV, Melendez J, Alvarez de Leon RA, Castillo A, Sidiki S, Yusoof KA, Nunes E, Tellez-Lopez CA, Mejia-Villatoro CR, Ikeda J, Garcia-Basteiro AL, Wang SH, and Torrelles JB.

Published in Journal: Scientific Reports.https://www.nature.com/articles/s41598-019-54537-9 Received: 14 March 2019; Accepted: 14 November 2019; Published: 29 November 2019.

Background: LAM test sensitivity is still low to be used as a POC test alone in the diagnosis of TB in PLHIV. Increasing the LAM test sensitivity would be an outstanding achievement with high impact benefits to diagnose TB in PLHIV and the HIV negative population. Hospitals and rural health care settings in resource-constrained settings were sputum-smear microscopy is routinely used, but TB culture and molecular diagnostic tests are not available, will benefit most with the potential to substantially increase TB diagnosis and TB treatment initiation. To do so, we have hypothesized that LAM association with other molecules such as phospholipids and immunoglobulins could make it less likely to be recognized by antibodies. Therefore, specific biochemical treatments of LAM-spiked urine or milk might be able to improve its sensitivity and overall performance.

Objective: To determine which biochemical treatments of LAM-spiked urine or milk might be able to improve LAM test sensitivity.

Main results: α-mannosidase treatment of LAM-spiked urine for human TB and combined lactase/caseinase treatment of LAM-spiked milk for bovine TB enhanced 10-fold the detection levels of LAM test in the laboratory.

6.2.2.1.2 Manuscript 4: Diagnostic accuracy of LAM and α-Mannosidase treated urine LAM test to improve tuberculosis diagnosis among a cohort of people living with HIV in Guatemala.

(See previous page).

6.2.2.2 The Color Plate Agar-based culture test (TB-CX test)

6.2.2.2.1 Paper 6: Low-cost diagnostic test for susceptible and drug-resistant tuberculosis in rural Malawi

Zhang A, Jumbe E, Krysiak R, Sidiki S, Kelley HV, Kipruto-Chemey E, Kamba C, Mwapasa V, **García JI**, Norris A, Pan X, Evans C, Wang SH, Kwiek JJ, and Torrelles JB

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Note: Results of this published paper are found in Annex

7 DISCUSSION

There were 4 specialised HIV units in Guatemala in 2004 which increased to 14 in 2010; ART scale up and decentralization in the country was linked to the implementation of an EMR designed to capture key longitudinal data on PLHIV. The rationale behind the development of the EMR was based on previous implementation experiences (422) and following a standardized methodology (423) aiming to improve HIV/AIDS surveillance systems, to harmonize the reporting of national HIV indicators, and to provide a nation-wide platform for scientific research in HIV/AIDS (424,425). Nowadays, the "Ministerio de Salud Pública y Asistencia Social de Guatemala" uses the MANGUA EMR as the national tool to monitor the HIV epidemic in the country. Transition from paper-based to electronic medical records has proven to be feasible to implement and effective to provide key HIV information for surveillance, clinical management and prevention and control interventions.

The estimated HIV prevalence in adults of 15-47 years old in Guatemala in 2012 was of 0.7% with 95% CI (0.4 – 1.5) (426). The HIV epidemic in Guatemala was classified as "concentrated". However, the accuracy of the "concentrated" classification (165), should be interpreted with caution for the following reasons: a- Representativity and generalizability of Guatemala's population-based surveillance data, due among others, to a low coverage of HIV screening in pregnant women, (and poor implementation of PMTCT of HIV), despite of a substantial increase from 15% in 2008 to 42% in 2015 (171,427,428); b- UNAIDS data estimations using mainly "paper-based" aggregated national data from different sub-level health data registries, instead of standardized EMR based data (239,243,429,430); and c- PLHIV unaware of their HIV infection could had been equal or higher than the estimated 30% for Europe, due to weaker surveillance data on number of HIV diagnosis, AIDS cases, and deaths in PLHIV (431–434).

UNAIDS estimations for PLHIV in 2012 for Guatemala were of 58 000 with 95% CI (36 000 - 130 000). MANGUA estimated coverage of diagnosed PLHIV is of 85%. Taking into account UNAIDS estimates and the 21 697 PLHIV registered in the MANGUA cohort in 2012, results give an estimated 57% of PLHIV whom do not know their HIV status. In this scenario it seems difficult to address HIV transmission dynamics and an effective control of the HIV pandemic in the country (435).

The HIV *cascade-like* of care estimated from the MANGUA cohort was one of the first HIV care cascades described in Central America. The term "cascade-like" is used to be precise and differentiate the actual column bars in the current cascades from the one depicted using the MANGUA cohort. The latter do not provide data on linkage or retention in care nor data on viral load suppression (436,437). MANGUA was designed as a population-based cohort but its cross-sectional analysis lacks of the modern "longitudinal continuum" component assessing for changes over time (43,438). However, the following key HIV care gaps were identified: Only 67.1% of eligible HIV patients for ART initiation started ART. In addition, there was a 28.3% gap on ART eligibility, so ART treatment initiation in these PLHIV with no data on ART eligibility was unknown. Another significant result is that almost 11% of naïve HIV patients did not start an ART regimen based on 2NRTI (Nucleoside reverse transcriptase inhibitor) + 1NNRTI (Nonnucleoside reverse transcriptase inhibitor).

On the other hand, ART eligibility criteria did compromise early ART initiation as understood today within the test and treat strategy (439,440). As per national guidelines, eligibility was based on WHO clinical stage 3 or 4, and/or CD4 cell values ≤350 cells/µl. Thus, incidence of common opportunistic infections could have had a substantial impact on morbidity and mortality outcomes. An immediate question to ask is how many lives could have been saved if the test and treat strategy would had been implemented at that time (441–443) or how many lives could be saved during the unknown lag time from the test and treat strategy adoption to real implementation (444–448). In this regard, an important factor to take into account, is the delay from publication of national and international guidelines to real implementation in day to day clinical practices in resource-constrained settings. As an example, the average lag time of WHO 2009 guidelines adoption was of 24 months in the 33 Sub-Saharan countries accounting for 97% of the HIV burden (449).

As per 2016, it was estimated that there were 46 271 PLHIV in Guatemala, of whom 28 844 were alive and knew their HIV status (62%), 18 611 were linked to care (40%), 17 733 were on ART (38%), and 11 938 had supressed viral load (26%) (450). These data are far behind from the recommended 90-90-90 targets; only a few high income countries have been able to achieve these targets (451–453). Currently, the breakpoints "from estimated PLHIV to diagnosis" and "from retention in care to ART" in the continuum of care are of major concern (40,454). In addition, different definitions are used of key elements within the continuum such as linkage to and retention in care,

and different viral load thresholds are also used to define viral suppression. Thus, comparability of cascade among countries is difficult and challenging, making an standardised reporting methodology urgently needed (455–457).

Either way, globally, a tremendous effort in HIV prevention, control and care was put in place to combat the spread of the HIV/AIDS pandemic. United Nations, pharmaceutical companies, committed (and some corrupted) governments, global health and philanthropic organizations depicted the operational implementation of the ART scale up worldwide in an unremarkable joint action plan (458).

The challenge was, as usual, how to do it (459). ¿How to deliver funds and resources properly in a wide range of settings from high to low income countries? ¿How to coordinate with new-born National HIV/AIDS Control Programs, and how to empower them to achieve a sustainable long-term operational capacity to deal with their own HIV and TB epidemics? The fact is that the answer on how to do it was not clearly agreed, and the response was mainly based on urgently funding vertical, separated and specialized HIV-only units instead of using a more horizontal, integrated and diversified HIV-other running health care program approach (206,216,254,460).

HIV is an STI, and STIs have been affecting humans since ancient times (461,462). Deliver HIV prevention, treatment, and care through a pre-stablished health care program, (e.g. Family planning, sexual and reproductive health, maternal, antenatal, and child health care), seems a synergistic and efficient approach. This integration approach could be bi-directional, that is to say, to use newly HIV units to strengthen or newly deliver sexual and reproductive health care programs depending on the availability and particularities of health care settings involved (208,221,222,332).

The benefits of integration rely on efficacy through organization and optimization of resources, flexibility and task shifting. In addition, integration could reduce transport costs (increasing health care accessibility), promote confidentiality, reduce visit schedules and pharmacy visits, with the potential increase in user's satisfaction, and thus, retention in care (113,120).

Importantly, integration is best seen as a continuum rather than a binary process: integrated vs non-integrated. Thus, different degrees of integration are envisaged according to the health needs and the organization of health care settings to provide a continuum of preventive and curative services over time (206,225). ***Figure 12*** depicts the linkage and the theoretical patient flow within the integrated STIs/HIV services in

Escuintla department, Guatemala, in a collaborative effort between the non-governmental organization (NGO) *Fundació Sida I Societat* (FSiS) and the Guatemalan Ministry of Health, through the National HIV and STIs Prevention and Control Program (PNS).

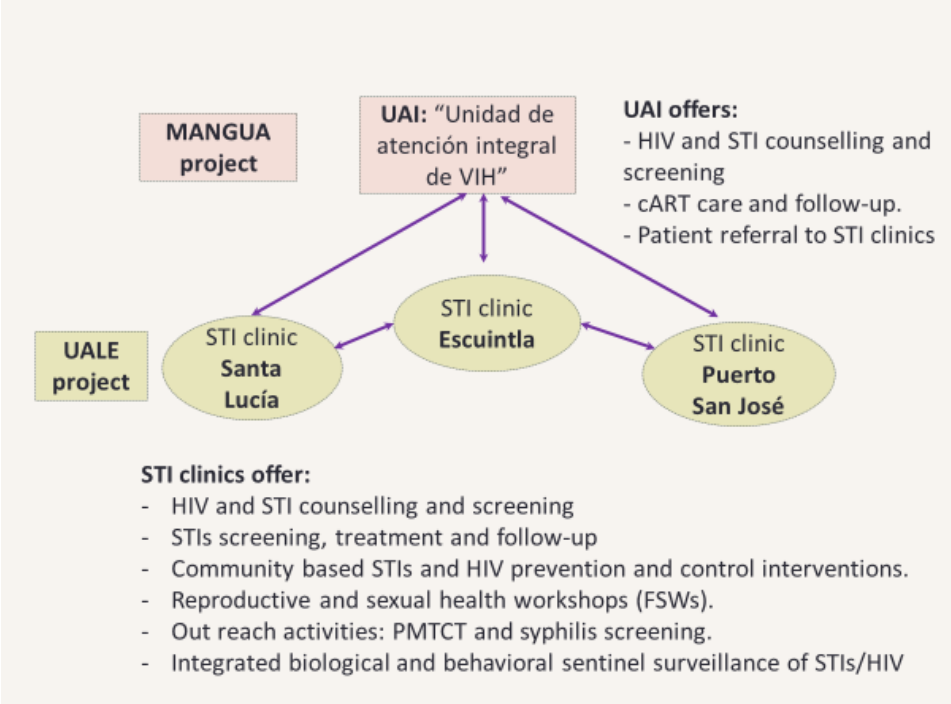

Figure 12: Bi-directional linkages for UAI and STI clinics attendees and health services provided.

Briefly, during 2005-2012, there were 3 STI clinics running. The Escuintla STI clinic was the reference STI clinic and it is co-located within the Escuintla National Hospital were the specialized HIV unit (Escuintla UAI) was created to decentralize ART in the department. A total of 16 UAI were implemented in Guatemala during the 2005-2014 ART decentralization process. Escuintla UAI offers pre and post HIV counselling, ART initiation and follow up, STIs screening and referral to Escuintla STI clinic if needed. Updates on data from the MANGUA cohort and STIs clinic were monthly reported to the PNS. Monitoring & Evaluation visits to UAIs were also performed every three months with an interdisciplinary team composed of members from the PNS and FSiS.

The main limitation of our model is that the integrated biological and behavioural surveillance database running in the STI clinics was not linked to MANGUA database from the Escuintla UAI; therefore, STIs and HIV data from STI clinics needs to be matched with data from the MANGUA cohort, making it a challenge and labour intense process. Moreover, our model is a specific setting-based model run initially by an

external NGO. Thus, generalizability of results and replicability in other settings is a potential concern.

The main strengths of this integrated STIs/HIV care model are:

1- Co-localization with the HIV UAI unit. Different building but same place. This means that in the same hospital visit, different STIs/HIV screenings and laboratory procedures might be done, increasing time spent at hospital but decreasing transfers and transport costs, and thus, accessibility.
2- Specialized national health care workers and peers support within a potent information, education and communication (IEC) network through civil society, non-governmental NGOs, and local institutions, for community-based prevention and control interventions.

Importantly, the question of "How to implement a STIs/HIV integrated intervention approach in a resource-constrained setting?" was carefully assessed and evaluated to decide best delivery approaches. A methodological approach for project implementation was followed, aiming to achieve long term sustainability through a gradual capacity transfer to local and national authorities in a defined period of time (423). Several research studies were performed as a baseline situational analysis to deal with sexual health and violence and migration patterns in females sex workers (463–465).

It is important to highlight the point of "sustainability and capacity transfer" contextualized in our study as a local-evidence based approach for implementation. This approach might differ if compared to global health approaches to be urgently implemented. The concept of *'philanthrocapitalism'*, and the complex relationships among governments, institutional global health organizations, and donors, rises awareness of potential funding biases and conflicts of interests. In addition, the high dependence of external funds of STIs, HIV, TB and Malaria National Control Programs in resource-constrained settings, might long term hinder their own development and empowerment to control their own epidemics and use their own data (466–468).

Overall, our results from HIV incidence (469,470) and STIs/HIV prevalence trends studies using an integrated STIs/HIV care model showed a positive impact in the decline of HIV incidence and STIs/HIV prevalence over an 8-year period of uninterrupted STI clinics activities (471). This correlates with other research studies in African and American settings strongly suggesting the effectivity of STIs/HIV

integrated intervention programs at individual, community and population levels (84–86,88,93,96,416,417). Moreover, our model has served to provide accurate STIs/HIV surveillance data in at high risk populations in an effort to routinely collect STIs/HIV biological and behavioural data to inform district and national health authorities (238).

On the other hand, TB/HIV services integration has had a different historical perspective. *Mtb* disease appeared soon as the most common and major killer opportunistic infection in PLHIV (475–478). Therefore, TB screening in PLHIV and HIV screening in people with presumptive TB are the basis for diagnosis, prevention and control; moreover, they are key to monitore and evaluate HIV and TB activities, as well as joint TB and HIV efforts in health care settings. Importantly, TB and HIV together, double the sociological impact of stigma and discrimination and also highly affect the poorest and the most vulnerables. These key socio-economic determinants of HIV/AIDS and TB diseases are fundamental in the Syndemic theory but currently, a worldwide neglected issue from a Public health perspective.

TB incidence and mortality rates observed in our research studies in PLHIV from Guatemala and Mozambique are strickinginkly high, and higher when compared to other Sub-Saharan settings (479,480) although similar to others (481). Low TB case notification rates reported and TB misdiagnosis and its undiagnosed TB associated mortailty might partially explain these outcomes (482–486). In this regard, the combination of WHO's 4-symptom screening rule for TB in PLHIV and chest radiography might not be effectively implemented in Sub-Saharan settings (353). Results from an study in Kenya assessing the performance of symptom-based screening for TB in PLHIV show that clinical screening missed 25% of laboratory-confirmed TB cases (487). In addition, the implementation of IPT in high TB burden settings is challenging (488,489), and specially in children (490). The benefits of IPT have been widely documented (491) and IPT is one of the pillars to end TB. However, operational implementation in resource-constrained settings is scarce and derived morbidity and mortality outcomes may undermine TB control efforts. In this regard, a survey at 47 sites in 26 countries assessing the implementation HIV/TB services integration, intensified TB case finding, IPT, and TB infection control shows a low level implementation of these strategies (492).

Overall, these results rise serious concerns about current TB and HIV prevention and control interventions towards TB and HIV elimination milestones and targets

(493,494). However, there are some limitations in our research studies coming from the fact that the evaluation of One Stop models has been only based on TB incidence and mortality outcomes; importantly, no TB treatment oucomes neither TB and HIV treatment adherence and attrition were measured, and there is no clear specification on how patient flows through the health care model occurred, specially in Guatemala settings. It is important to include patient's satisfation outcome measures in this evaluation studies in order to gain better insights in how to implement integrated models with a risk and benefits assessment (208,333,495–497).

Moreover, in Mozambique setting, selection bias might have occurred taking into account that almost 42% and 21% of study participants were initially excluded due to demographic criteria and unknown HIV status, respectively. Demographic exclusion was mainly based due to previous or current migration status, and taking into account the estimated 35-40% HIV prevalence in the district, we might assume that PLHIV who migrate is high, with the implicits risks of migration affecting HIV retention and care and ART adherence (498–501).

On the other hand, it is hypothesized that known TB risk factors such as poverty and poor socio-economic development, HIV/TB stigma and discrimination intertwined with fragile and overwhelmed health care systems might hinder achievements in TB "cured" or "treatment completed" treatment outcomes, and therefore, their potential "herd effect" benefits (112). Specifically, these inherent factors on HIV/AIDS and TB disease may hinder TB and HIV treatment outcomes despite of optimization of integrated TB/HIV health care delivery, suggesting that still today, poverty, stigma and poor health care seeking behaviours (300,308,502,503), TB misdiagnosis and mortality (201,486,504,505), HIV associated MDR-TB (506), and migration patterns and high percentages of patient's lost to follow up (65,498–500,507), are behind the global widespread of the HIV/TB syndemic.

Conversely, LAM and TB CX tests results obtained have the potential to improve TB screening in PLHIV and reduce among others, TB misdiagnosis and associated mortality, and help to alleviate TB diagnostic challenges in crowded, rural health care centres in resource-constrained settings. However, implementation of well-designed studies is needed to validate our results. Performance of the LAM test with α-Mannosidase pre-treatment of the urine needs to be carefully evaluated in cohort studies in PLHIV and compared to LAM test alone and Xpert and liquid cultures. The recent WHO endorsement of the LAM test as a complementary test for TB diagnosis in

PLHIV (373,508) needs to be urgently implemented and evaluated, specially, in those high TB/HIV burden settings that could benefit most of same day LAM test results. Thus, clear LAM test algorithms linked to clinical evaluation and referral are fundamental to put in place alongside with availability of LAM test with solid supply chains.

The TB CX test might be a good alternative to AFB, MGIT and culture to diagnose drug resistant TB, and importantly, to perform drug resistant TB treatment monitoring in a monthly basis up to 9-12 months as required. It may provide the adding value of both increasing the sensitivity and accuracy of the bacilli/not bacilli outcome and the ascertainment if MDR or XDR TB development is taking place during TB treatment, or if the patient is responding well to TB treatment. Our research studies have small sample sizes and results are also setting-dependent with limited external validity and generalizability. Therefore, further studies are needed to accurately measure the diagnostic accuracy and the potential adding value of the TB CX test in health care centers of resource-constrained settings with a standardized assessment methodology. In addition, it would be interesting to evaluate using modelling tools the potential impact of LAM and TB CX tests on increased sensitivity on TB case notifications, and time to TB diagnosis and days averted prior to TB initiation.

Overall, implementation of TB/HIV collaborative activities should be urgently scale up using a set of basic indicators for program performance and TB/HIV surveillance. The degree of integration of the HIV/TB services is setting dependent and should be evaluated carefully to gain flexibility and effectiveness in patient flows while being sustainable and friendly for health care workers and users. Based on our TB incidence and mortality results, the "One stop" HIV/TB service model depicted seems not enough to contain the HIV/TB syndemic. Globally, low TB detection rates in high TB and HIV burden countries are clear symptoms that fragile and underfunded health care systems persist. On the other hand, efforts should be also re-focused in how to alleviate poverty, stigma and discrimination which undermines health care access seeking behaviors, increasing TB and HIV diagnostic delays, TB/HIV transmission, and TB morbidity and mortality. Furthermore, the effect of high migration patterns in HIV and TB treatment outcomes should be carefully assessed in order to design interventions to reduce attrition in highly mobile populations (451–455).

We have discussed STIs, HIV, and TB relationships within an intertwined range from molecular to public health perspectives. Currently, global efforts with ambitious action

plans and targets to eliminate STIs, HIV and TB are taking the scene. This global politic commitment needs to be translated into an effective operational plan under standardized M&E tools, and following the principles of equity and inclusion. STIs, HIV and TB are diseases that severely affect the most vulnerable populations who also live in low-income countries with fragile health care systems.

If we do not seriously take into account social and structural determinants of STIs, HIV/AIDS and TB disease, biomedical interventions will not be enough to achieve targets towards TB and HIV/AIDS elimination.

7.1 Conclusions

1) ART decentralization using electronic medical records provides key longitudinal data for HIV/AIDS surveillance and monitoring and evaluation purposes.

2) Ascertainment of the HIV epidemic status and its driving forces in resource constrained settings might be challenging due to inaccurate data and estimations.

3) Implementation of an integrated STIs/HIV care delivery model linked to an HIV specialized unit is feasible and effective for STIs/HIV clinical management and surveillance purposes.

4) STIs/HIV prevalence trends declined in key populations over an 8-year period using an integrated STIs/HIV care model in sentinel STI clinics.

5) Safer sexual behaviours should be reinforced and a deep understanding of the HRH group characteristics is needed to gain insights into potential bridging connections from FSWs and MSM to the general population

6) One Stop HIV/TB service delivery models are feasible to implement

7) Effectiveness of One Stop HIV/TB service delivery models aiming to improve TB and HIV treatment outcomes should be scaled up and evaluated.

8) TB incidence and mortality rates in PLHIV under One Stop HIV/TB services should decline in Guatemala and Mozambique settings studied in order to reach End TB and HIV 90-90-90 targets.

9) Undiagnosed and/or misdiagnosed TB and its associated mortality in PLHIV poses a burden of hidden TB transmission at a community level.

10) Novel, rapid, accurate and low-cost diagnostic tests for TB screening in PLHIV are urgently needed.

11) Further clinical studies to assess LAM test sensitivity using α-mannosidase treated urine and evaluations of the CX TB test for TB diagnosis and treatment follow up have the potential to increase TB diagnosis and TB treatment initiation.

In this regard, the following local and global recommendations are suggested.

7.2 Recommendations

1- To develop an integrated platform to collect biological and behavioural STIs/HIV data in MANGUA to be used in the 3/16 UAIs currently running in Guatemala as a pilot study to evaluate a potential nation-wide scale up.

2- To integrate rapid STIs diagnostic tests during pre and post HIV counselling units in rural health care settings in high HIV burden countries.

3- To urgently scale up HIV-1/2 and LAM tests with portable CD4 equipment's at a district level in rural health care settings of the 14 high TB, HIV/TB and MDR-TB burden countries*.

*Note: As an example, Mozambique has 11 provinces with approximately 13 districts per province and around 5 health care centres per district, for a total of 11 x 13 x 5 = 715 estimated health care centres in the country.

4- Worldwide scale up of an integrated HIV/TB cascade of care in clinical settings with basic and standardized indicators for clinical performance and disease surveillance.

5- Implementation of pilot studies to eliminate health care inequalities by 2025 through De-capitalization of health management in resource-constrained settings.

6- To be aware of the Post-colonialism effect in Latin America and Sub-Saharan Africa, that is, people from high income countries explaining people from low income countries how to deal with their own epidemics, while using their settings and data for research purposes and clinical trials, and with limited impact on country development and health benefits.

7- To stablish an action plan to gradually implement alternative funding options for STIs/HIV/TB in order to empower National STIs/HIV and TB prevention and control programs avoiding "charity" and external funding dependency

8- To link universal basic income trials to STIs/HIV and TB incidence research studies in high TB, HIV/TB and MDR-TB burden countries.

9- To stablish an action plan to strengthen information, education and communication interventions to mitigate TB and HIV stigma and discrimination.

8 Annex

I. Co-directors certificate

CEEIS Cat
Centre d'Estudis Epidemiològics
sobre les Infeccions de Transmissió
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RE: A l'escola de doctorat de la UAB i al coordinador del programa de doctorat de metodología de la investigación biomédica i Salut Pública

Jo, Jordi Casabona i Barbarà, director científic del Centre d'estudis epidemiològics sobre infeccions de transmissió sexual, VIH i sida de Catalunya, i co-director dels estudis de doctorat de Juan Ignacio García,

DECLARO

Que he llegit i evaluat la tesi doctoral de títol:

STIs/HIV and HIV/TB interventions for prevention and control of syndemics in resource-constrained settings

I aprovo que aquesta tesi doctoral sigui presentada sota el format de compendi de publicacions

Cordialment,

Jordi Casabona

Generalitat de Catalunya
Departament de Salut

TEXAS BIOMEDICAL RESEARCH INSTITUTE

Jordi B. Torrelles, PhD

Professor
Lead, Population Health Program
Director, Biosafety Level 3 Program

December 4, 2019

Subject: Ms. Juan Ignacio García Ph.D. Thesis Defense (Autonomous University of Barcelona)

To Whom It May Concern,

My name is Jordi B. Torrelles, a Professor, and Lead of the Population Health Program, and Director of the Biosafety Level 3 Program at Texas Biomedical Research Institute in San Antonio, Texas, United States of America.

As a co-Director of Mr. Juan Ignacio García doctoral studies, I am writing to declare that I read and evaluated the thesis entitled "*STIs/HIV and HIV/TB Interventions for Prevention and Control of Syndemics in Resource-Constrained Settings*" submitted by Mr. Juan Ignacio García.

Following the guidelines provided and after my careful reading, I agree that the quality of this thesis is optimal for Mr. Juan Ignacio García's verbal defense to fulfill the PhD Program in Methodology of Biomedical Research and Public Health, within the Faculty of Medicine, Department of Pediatrics, Obstetrics & Gynecology, and Preventative Medicine, at the Autonomous University of Barcelona.

If you have any questions or comments related to the above stated, please do not hesitate to contact me. My office phone number and email address are below.

Sincerely,

Jordi B. Torrelles, PhD
Professor & Lead, Population Heatlh Program
Director, Biosafety Level 3 Program
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II. PhD candidate situational analysis, manuscript abstracts 3 and4, and published paper 6.

Situational analysis of TB and HIV services integration in rural health care centres in Manhiça district, Southern Mozambique.

Main results:

The level of TB and HIV services integration in Manhiça district, Southern Mozambique, is depicted in ***figure 6***. Sputum smear microscopy is available in all TB units from health care centres except for Mirona and Chibututuine. Chest radiography and Xpert technology are only available at Manhiça and Xinavane Hospitals.

PLHIV attending HIV units are screened for TB using the WHO's 4S symptoms screening in every scheduled visit. People attending the TB unit are screened for TB and for HIV. PLHIV with TB symptoms are generally screened using chest radiography, sputum-smear microscopy, and Xpert, depending on the availability of these techniques in health care centres. PLHIV with TB disease and without ART are scheduled to start ART therapy within 2-8 weeks after TB treatment initiation as per NTCPs guide and WHO policy recommendations. TB treatment and ART in PLHIV are offered and followed up in the TB unit until TB treatment finishes; regimens are often administered as fixed dose combinations (FDC) as: 2 months of (EHRZ) and 4 months of (HR) in a single dose. Those previously treated relapsed cases are treated with 2 months of (EHRZ) and 4 months of (HR) + Streptomycin. Since august 2018, the PNCT strategy is to treat previously treated relapsed cases as drug-susceptible TB cases. The standard MDR-TB drug scheme is 6 months of Km-Lfx-Eto-Cs-E-Z and 18 months of Lfx-Eto-Cs-E-Z.

Note: E=Ethambutol, H=Isoniazid, R=Rifampicin, Z=Pyrazinamide, Km= Kanamycin, Lfx=Levofloxacin, Eto=Ethionamide, and Cs=Cycloserine.

Table 6: Level of TB/HIV services integration in the 14 health centers within Manhiça district and their TB/HIV health care delivery models

		Level of TB/HIV integration	
n	**Health care centers in Manhiça district**	**Full integration: One stop service**	**Linkage: Referral for HIV testing and/or TB screening and care.**
1	3 de Fevereiro CS	not available	available: referral to Xinavane CS
2	Calanga CS	available	
3	Chibucutzo CS	available	
4	Chibututuine PS	not available	available: referral to Manhiça CS
5	Ilha Josina CS	available	
6	Maluana CS	available	
7	Manchiana CS	available	
8	Manhiça CS*	available	
9	Maragra CS	available	
10	Mirona CS	not available	available: referral to Chibucutzo CS
11	Munguine CS	available	
12	Nwamatibjana CS	available	
13	Taninga CS	available	
14	Xinavane CS*	available	
* These 2 health care centres have Xpert *MTB*/RIF technology and 2nd line regimens to treat MDR people with TB. CS: Centro de saúde			

Table adapted from (349)

Manuscript 3: Mortality and risk of tuberculosis among people living with HIV in whom tuberculosis was initially ruled out.

Juan Ignacio García, Edson Mambuque, Dinis Nguenha, Faustino Vilanculo, Charfudin Sacoor, Guillermo Sequera, Manuel Fernández-Quevedo, Maxime Leroux-La Pierre, Helio Chiconela, Luis A. Faife[†], Durval Respeito[2], Belén Saavedra, Tacilta Nhampossa, Elisa López-Varela, Alberto L. García-Basteiro

Background: Mozambique is one of the 14 countries included in the high TB, TB/HIV and MDR-TB burden list, with an estimated TB incidence rate of 551 (356-787) cases per 100.000 in 2018 (64). Case detection rates are presumably low, and it has been estimated that around half of the TB cases are not diagnosed

or reported to the health authorities (63,514). As per 2016, the estimated population of Manhiça district was of 186,241 inhabitants living in an area of 2373 Km^2.TB case notification rate was 552 per 100 000 in 2012. HIV prevalence in the district is high, peaking at 39.9% in individuals aged 18-47, estimated from community based cross-sectional studies (495,515,516). TB and HIV treatment is offered free of charge, and for those PLHIV with TB disease, both TB and HIV treatments are provided at the National TB Control Program (PNCT) units following the One Stop Model for TB/HIV implemented in 2012 (326).

Objective: This research study is a retrospective review of a cohort of PLHIV with presumptive TB in whom TB was initially ruled out as per TB symptom screening and Xpert, and who were followed up for 2 years to estimate TB incidence and mortality rates and its risk factors.

Of the 1031 presumptive TB study participants, approximately 40% did not have a valid demographic unique code, and 21% had unknown HIV status, and thus, were excluded from the analysis. Finally, 382 PLHIV with TB symptoms in whom TB was initially ruled out, were followed up for 2 years to estimate TB incidence and mortality rates.

Incidence of TB among HIV-positive presumptive TB cases.

Overall, 37 HIV-positive TB cases were diagnosed with TB within the 2 year follow up period. The TB incidence rate in the cohort was 5.4/100 PYs (95% CI: 3.9 – 7.5). The TB incidence rates in males and females were 5.4/100 PYs (95% CI: 3.2 – 8.9) and 5.4/100 PYs (95% CI: 3.6 – 8.3), respectively. A total of 16 TB cases (16/37, 43.2%) were diagnosed of TB during the first year of follow up.

All-case mortality rate among HIV positive presumptive TB cases.

There were 44 deaths (12.7%) among the 345 HIV positive presumptive TB cases without a reported TB diagnosis after the 2-year follow up period. The overall mortality rate was 6.8/100 PYs (95% CI:5.0 - 9.2). The mortality rate in males and females was 7.2/100 PYs (95% CI: 4.6 - 11.3) and 6.6/100PYs (95% CI: 4.4 - 9.7), respectively. Over one third of all deaths (16/44, 36.4%) occurred within the first 12 months of follow up.

Manuscript 4: Diagnostic accuracy of LAM and α-Mannosidase treated urine LAM test to improve tuberculosis diagnosis among a cohort of people living with HIV in Guatemala

García JI, Meléndez J, Alvarez de Leon R, Mejía-Chew C, Kelley HV, Sidiki S, Castillo A, Mazariegos C, Forno D, Ayala N, Balada-Llasat JM, López-Téllez C, Mejía-Villatoro CR, Wang SH, Torrelles JB and Ikeda J.

Submitted to International Journal of Infectious Diseases.

Background: TB is the most common AIDS defining illness (ADI) in Guatemala (517). From 2010 to 2016, new HIV infections increased 23% and ADIs have doubled (518). In 2017, Guatemala reported 3,505 cases of TB (estimated incidence of 25 per 100,000 population), and although only 7.2% affected PLHIVIV, 16% of them died (63). New unpublished data from the Guatemalan Ministry of Health suggest that one third of Guatemala's territorial departments (8 of 23) have the highest HIV burden contributing yearly to more than half of the incident TB cases.

This research study is a prospective longitudinal study of PLHIV with clinical or radiological symptoms of TB attended in two "Unidades de Atención Integral para el VIH" (UAI) in two departments in Guatemala: Guatemala City and Quetzaltenango. These UAI function as a One Stop TB/HIV service delivery model (349) for integral management of HIV/AIDS with co-located TB and HIV services.

Objectives:

1- To estimate TB incidence and TB mortality and its risk factors in 2 UAIs functioning as a One Stop TB/HIV service delivery model.
2- To evaluate the diagnostic accuracy of the Alere Determine™ TB LAM Ag test and an enzymatic treatment of urine using α-mannosidase to improve LAM test sensitivity in 2 UAIs in Guatemala.

Main results for Objective 1: TB incidence in our cohort of PLHIV subjects with TB symptoms was of 21.4/100 PYs with 95% CI of (16.6 - 27.6). TB risk was of 16.3% with 95% CI of (12.9 - 20.5). Mortality rate was of 11.1/100 PYs with 95% CI (8.2 - 15.0). Mortality risk was of 14.9% with 95%CI of (11.6 - 19.1). Almost 74%

of PLHIV participants in our study died during the first 6 months after enrolment and 21/44 (47.7%) subjects who died were under 2nd line regimens, and 9/53 (17.0%) subjects who died were not on ART or their ART status was unknown.

The MDR/XDR-tuberculosis coloured agar-based test (CX test)

Paper 6: Low-cost diagnostic test for susceptible and drug-resistant tuberculosis in rural Malawi

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Background: Malawi is in the list of the 20 high TB/HIV burden countries in the world. In addition, rural settings in Malawi where molecular TB diagnostics are not currently available, need easy-to-use tests that do not require additional processing or equipment. While AFB smear is the most common and often only TB diagnosis test performed in rural settings, it is labour intensive, has less-than-ideal sensitivity, and cannot assess TB drug susceptibility patterns nor for diagnosis neither for TB treatment follow up.

Objective: To determine the feasibility of a MDR or XDR-TB colored agar-based culture test (TB CX test), which can detect *Mtb* growth and evaluate for drug susceptibility to isoniazid, rifampicin and a fluoroquinolone (i.e ciprofloxacin) in approximately 14 days.

Main results: Our results showed a high level of concordance between the AFB smear (12 positive) and TB CX test (13 positive); only one sample presented a discordant result (AFB negative and TB CX test positive) later confirmed positive with Xpert. The average time to a positive TB CX test was 10 days. Of the positive samples, the TB CX test detected no cases of drug resistance, results that were later confirmed by Xpert.

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11 Full published articles used in this Thesis research (3 main and 1 secondary)

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Research Article

The MANGUA Project: A Population-Based HIV Cohort in Guatemala

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Introduction. The MANGUA cohort is an ongoing multicenter, observational study of people living with HIV/AIDS in Guatemala. The cohort is based on the MANGUA application which is an electronic database to capture essential data from the medical records of HIV patients in care. *Methods.* The cohort enrolls HIV-positive adults ≥16 years of age. A predefined set of sociodemographic, behavioral, clinical, and laboratory data are registered at entry to the cohort study. *Results.* As of October 1st, 2012, 21 697 patients had been included in the MANGUA cohort (median age: 33 years, 40.3% female). At enrollment 74.1% had signs of advanced HIV infection and only 56.3% had baseline CD4 cell counts. In the first 12 months after starting antiretroviral treatment 26.9% ($n = 3938$) of the patients were lost to the program. *Conclusions.* The implementation of a cohort of HIV-positive patients in care in Guatemala is feasible and has provided national HIV indicators to monitor and evaluate the HIV epidemic. The identified percentages of late presenters and high rates of LTFU will help the Ministry to target their current efforts in improving access to diagnosis and care.

1. Introduction

Currently, in Guatemala the HIV epidemic is concentrated in most-at-risk populations, with prevalence between 4 and 15% among commercial sex workers and 11.5–18.3% among men who have sex with men. HIV prevalence among other vulnerable groups is 18% in people with tuberculosis (TB), 13% in prison populations, and 3.3% among youth at social risk [1–3]. During the period 2001–2005, with the support from Médecins Sans Frontières, four hospitals were providing antiretroviral treatment (ART) in Guatemala. In 2004, in alignment with the World Health Organization (WHO) 3-5 strategy and with financial aid from the Global Fund to fight AIDS, tuberculosis, and Malaria (the Global Fund),

Table 1: Standardized variables collected in MANGUA.

Sociodemographic and behavioral variables	MANGUA study code, date of enrollment, gender, date of birth, country of origin, province of residence, ethnicity, religion, marital status, educational level, employment status, name of UAI, previous treatment, casualty date, casualty cause, cause of death, informed consent signed, vulnerability group, transmission group, probable year of infection, sexual orientation, and condom use.
Laboratory variables	Biochemistry and hematology variables, HIV rapid test, HIV ELISA test, serologies for toxoplasma, cytomegalovirus, histoplasma, cryptococcus, HBV, HCV, HSV, and syphilis. Sputum-smear microscopy for tuberculosis.
Clinical variables	WHO clinical stage, date of visit, type of visit, date of next scheduled visit, CD4 count, CD8 count, HIV RNA viral load, AIDS-related events, AIDS-defining events, and other coinfections.
Treatment variables	ART type, prophylaxis type, other treatments, initiation and stop date*, reason for regime change*, and adverse effects*.

HBV: human hepatitis B virus; HCV: human hepatitis C virus; HSV: human herpes virus simplex types I and II. UAI: *Unidad de atención integral*; ELISA: enzyme-linked immunosorbent assay; RNA: ribonucleic acid; and ART: antiretroviral treatment.
*For each type of treatment.

the *Ministerio de Salud Pública y Asistencia Social* (MSPAS) started to implement specialized hospital-based HIV units—*Unidades de Atención Integral* (UAIs)—across the country; therefore, extending ART coverage and increasing access to care for people living with HIV/AIDS (PLHIV) [4–6]. UAIs are administered by minimal essential staff: a physician, a nurse, a laboratory technician, and a social worker. Currently 16 UAI offer combination antiretroviral therapy (cART) free of charge (Figure 1).

In a collaborative effort between the National AIDS Program (PNS) of the MSPAS and the *Fundació Sida i Societat* (FSiS), a Catalan non-governmental organization working in global health, the HIV electronic medical record (EMR) application, MANGUA, was developed in 2006 to be implemented in all the UAIs of Guatemala. The purpose of the MANGUA application was to facilitate clinical management and provide data for national HIV indicators to the MSPAS. The MANGUA application is registered in Catalonia, Spain, and was designed and piloted with the support of the HIV department at the *Clínica Familiar* Luis Ángel García in Guatemala City. The use of the MANGUA application rapidly expanded along with the roll-out and scale-up of UAIs. MANGUA is a database application structured in query language (SQL server 2008 R2, Microsoft, Redmond, WA 98052). It was developed as an EMR to capture demographic, behavioral, clinical, and laboratory information for HIV-positive individuals enrolled in health care services in the UAIs [1, 7].

The MANGUA cohort is an ongoing multicenter, prospective observational study based on the data captured in the MANGUA application; its main objective is to generate outputs to improve both public health and clinical practice related to HIV/AIDS in Guatemala.

2. Methods

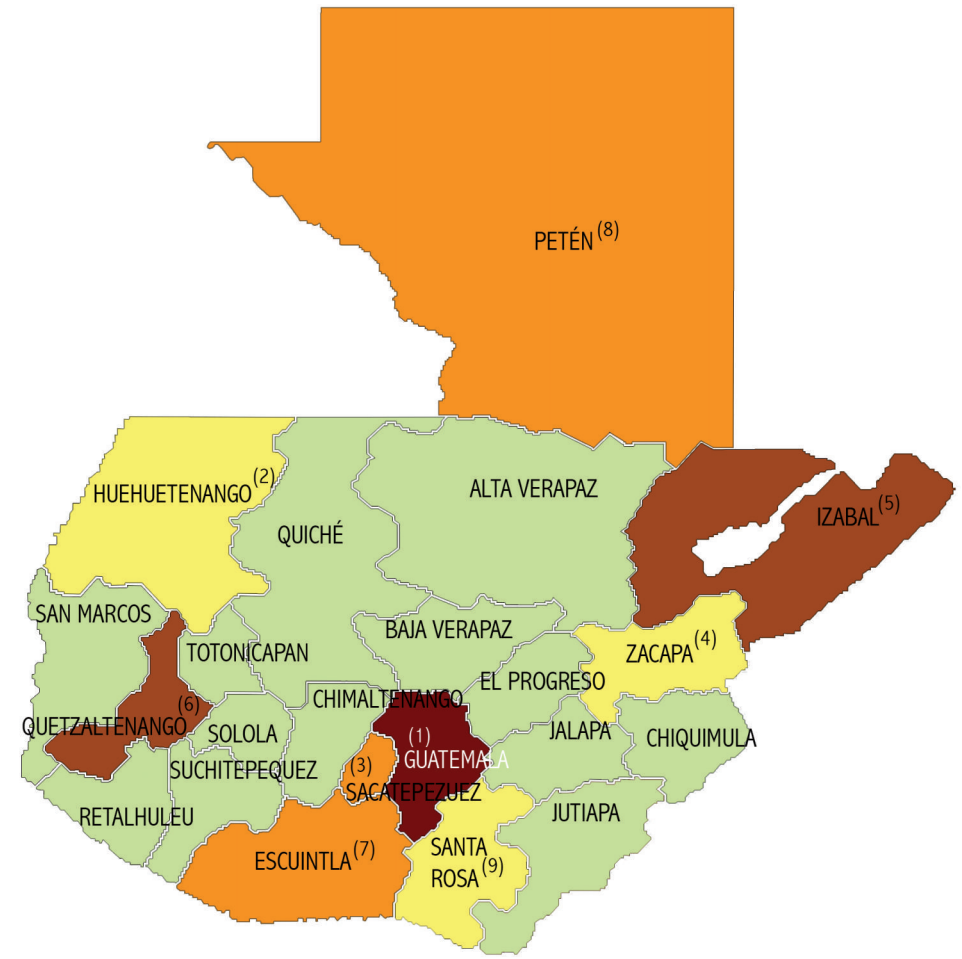
Currently, 14 of the 16 current UAIs participate in the cohort. 21 697 HIV-positive adults ≥16 years of age are enrolled. Data are collected by the medical staff of the UAIs following the national ethical guidelines. The cohort includes 74.3% of the cumulative number of HIV-positive patients reported to the national health authorities in 2012 ($n = 29\,211$) [5]. Patients are eligible to enter the MANGUA cohort when there is confirmation of a positive HIV rapid test either with an enzyme-linked immunosorbent assay (ELISA) or a second highly specific rapid test. Discordant results are confirmed by Western blot. Patient informed consent is obtained before performing any HIV test. Participants are recruited at the UAI at HIV diagnosis, referred from primary and secondary level health care centers, or voluntary counseling and testing sites. HIV testing is repeated at the UAI for confirmation. Local policy regarding informed consent for HIV testing is applied.

Data were retrospectively collected from 2001 to 2006, and then, prospectively collected from 2006 onwards. In each UAI there is at least one data entry manager who registers routine health care data obtained from paper medical records into the MANGUA application. Retrospective clinical data dates back to the time of the patient's initial HIV diagnosis or when the clinician first visited the patient. The database is regularly updated to contain follow-up status.

Data are anonymized and sent periodically to *Unidad de Apoyo Técnico* (UAT) at the FSiS, and to the National Health Care Database (SIGSA), where data are checked for quality before returning to the UAI. All data are stripped of names and other personal identification and a unique MANGUA study code is assigned to each participant to protect confidentiality.

Every two months an interdisciplinary quality control team composed of UAT staff and data managers from PNS visits each UAI to perform source-to-database data audits. At these visits the quality control team selects at random a proportional sample of participants' medical records, such as clinical and laboratory reports, and MANGUA data are reviewed against this original documentation [8].

To guarantee the continual improvement of data validity, UAT data managers generate lists of potential errors (i.e., initiation of ART before HIV diagnosis, etc.) and check for inconsistencies and missing data [1, 9]. A MANGUA data dictionary and user manual was developed and distributed among UAI personnel. Descriptive analyses are approved by the PNS and MSPAS. A predefined set of sociodemographic, behavioral, clinical, and laboratory data are recorded at first entry to the cohort study and subsequent visits (Table 1).



Number of patients by department

- 5001–12500
- 1001–5000
- 501–1000
- 0–500
- Departments without UAI

Number of UAIs and number of patients attended at each UAI by Guatemalan department

(1) Guatemala, 4 UAIs: Clínica Familiar Luis Ángel García, San Juan de Dios Hospital ($n = 3887$). Infectious Diseases Clinic, Roosevelt Hospital ($n = 7270$). Hospital del Instituto Guatemalteco de Seguridad Social (IGSS) ($n = 1273$). Sanidad Militar ($n \approx 60$. UAI not participating in MANGUA cohort. Data not included)
(2) Huehuetenango, 1 UAI: Hospital Departamental de Huehuetenango Dr. Jorge Vides Molina ($n = 75$)
(3) Sacatepéquez, 2 UAIs: Hospital Nacional Pedro Bethancourt, Antigua ($n = 285$). Hospicio de San José ($n = 363$)
(4) Zacapa, 1 UAI: Hospital Nacional Carlos Arana Osorio ($n = 415$)
(5) Izabal, 2 UAIs: Hospital Nacional de la Amistad Japón-Guatemala de Puerto Barrios ($n = 1891$). Hospital Infantil Elisa Martinez ($n \approx 60$. UAI not participating in MANGUA cohort. Data not included)
(6) Quetzaltenango, 3 UAIs: Juan José Ortega de Coatepeque National Hospital, Coatepeque ($n = 3252$). Hospital Regional de Occidente San Juan de Dios ($n = 226$). Clínica Isaac Cohen Alcache, Hospital Nacional Rodolfo Robles ($n = 1174$)
(7) Escuintla, 1 UAI: Hospital Nacional Regional de Escuintla ($n = 816$)
(8) Petén, 1 UAI: Hospital Nacional de San Benito Petén ($n = 523$)
(9) Santa Rosa, 1 UAI: Hospital Nacional de Cuilapa ($n = 247$)
UAI: *Unidad de atención integral*

Figure 1: Number of UAIs and number of patients attended at each UAI by Guatemalan department.

Table 2: Patient characteristics at enrollment in the MANGUA cohort.

(*n*, % of available data from variable)	Total		Men		Women		*P* value*
	n	%	*n*	%	*n*	%	
Gender (*n* = 21 697, 100%)							
Age in years (*n* = 21 629, 99.7%)							
Median, IQR	33 (27–42)						
16–24	3660	16.8	1616	44.5	2013	55.5	<0.0001
25–49	15079	69.1	9182	61.4	5779	38.6	
≥50	3073	14.1	2106	68.6	964	18.6	
Ethnic group (*n* = 12 172, 56.1%)							
Ladino	9842	80.9	5881	59.8	3961	40.2	0.91
Indigenous	2330	19.1	1395	59.9	935	40.1	
Transmission route (*n* = 12 592, 58%)							
Heterosexual	11709	92.5	6593	56.4	5102	43.6	<0.0001
Homosexual	907	7.2	844	98.1	16	1.9	
Injection drug users	40	0.3	34	91.9	3	8.1	
Advanced HIV at enrollment** (*n* = 10892, 50.2%)	4168	38.3	2778	66.7	1390	33.3	<0.0001
CD4 cells/mL at enrollment (*n* = 12219, 56.3%)							
Median, IQR	131 (46–279)						
≥350	2129	17.4	956	44.9	1173	55.1	<0.0001
200–349	2311	18.9	1203	52.1	1108	47.9	
<200	7779	63.7	5208	66.9	2571	33.1	
cART status at enrollment (*n* = 8919, 41.1%)							
Naive	7981	89.5	4793	60.1	3188	39.9	0.01
Previous treatment	938	10.5	550	58.6	388	41.4	
Median (IQR) time in days between HIV diagnosis and cART initiation in days (*n* = 13195, 60.8%)	75 (27–308)	NA***	70 (28–247)	NA	86 (26–388)	NA	0.97

* *P* values were calculated taking into account that transexuals (*n* = 152) were added to men for statistical convenience.
$P < 0.05$ was considered as statistically significant.
** Clinical stages 3 and 4 from WHO.
*** Not applied.
IQR: interquartile range; cART: combination antiretroviral therapy.

Classification of AIDS-defining events is based on WHO criteria [10]. At each quarterly or semiannual follow-up visit, laboratory and clinical data are obtained and updated into the MANGUA database.

2.1. Statistical Analysis. Data were analyzed using STATA software version 11, (StataCorp LP, College Station, TX, USA). Sociodemographic and clinical characteristics at enrollment were compared using Pearson χ^2 test for categorical variables and Student's *t*-test for continuous variables.

3. Results

As of October 1st, 2012, 21 697 patients had been included in the MANGUA cohort (median age: 33 years, 40.5% female). The cohort has 45 853 person-years of follow-up, with a median follow-up time of 1.1 years. Percentages of patients with available data on the following variables are as follows: transmission route (58%), cART status at enrollment (41.1%), advanced HIV status at enrollment (50.2%), and CD4 cell counts at enrollment (56.3%). Among these patients with available data most, 92.9% (11 709/12 592), were infected by heterosexual relations. At enrollment, 89.3% (8169/9144) were naive and 74.1% (12 075/16 300) had signs of advanced HIV infection (CD4 cell counts < 350 cells/mm^3 or WHO clinical stage 3 or 4) [10]. Only 56.3% (12 219/21 697) had baseline CD4 cell counts, of whom 63.7% (7782/12 222) had CD4 cell counts <200 cells/mm^3 (Table 2).

During follow-up, 67.4% (14 616/21 697) started ART of which 98% started cART. At cART initiation, the median CD4 cell count was 126 cell/mm^3 (Interquartile range [IQR]: 47–240). The treated cohort has 41 741 person-years of follow-up and a median follow-up time of 2.3 years.

Retention analysis was based on the definition described elsewhere [11, 12]. Retention proportions of living HIV-positive adults on active cART were calculated at 12 and 24 months after cART initiation. Pre- and postdecentralization periods should be differentiated. During the 2001–2005 predecentralization period the average retention was 84% and 79.7% at 12 and 24 months, respectively, with 2095 patients starting cART. For the 2006–2011 postdecentralization period, the average retention was 65.3% and 55.5% at 12 and 24 months, respectively, with 11 265 patients starting cART.

Overall, for the 11-year period 2001–2011, the average retention proportion was 73.8% and 61.5% at 12 and 24 months, respectively.

Attrition analysis was adapted from definitions described elsewhere [11, 13] and was defined as episodes of discontinuation of cART at the end of a follow-up period for the following reasons: death, loss to follow-up (LTFU), or stopping ART for any other reason while remaining in care. LTFU included the following two situations [14]. The first situation applies when patients are permanently LTFU for a 12-month period during which any additional patient visits disqualified them as permanently LTFU. In this situation, a differentiation was made between patients with no follow-up, defined as those that did not return to the clinic after the first visit scheduled for cART initiation, and patients with LTFU after starting cART, defined as those who start cART and are lost to follow-up for at least 180 days after the last visit scheduled. The second situation applies to patients LTFU who later reentered follow-up, defined as those who start cART and return for at least one visit after which the patient is not seen in the clinic for ≥180 days after the last visit scheduled but reappears at the clinic from at least day 181 onwards.

In order to calculate the global LTFU rate, the analysis took into account that a single patient might have had more than one episode of LTFU. Thus, during 41 741 person-years of follow up, 8572 episodes of LTFU (including cART stops for other reasons), were recorded with a rate of 20.5 per 100 person-years, very close to the rates seen elsewhere [14].

Attrition rates at 6- and 12-month follow-up period were studied; the 6-month interval was chosen to be consistent with the LTFU definition used; the 12-month interval was used to analyze the evolution of LTFU episodes and to be able to compare LTFU outcomes with retention rates. In the first 6 months after starting cART, 19% ($n = 2785$) of patients were lost to the program: 575 patients (20.6%) did not return to the clinic after cART initiation (no follow-up), 1568 (56.3%) were LTFU (including cART stops for other reasons), and 642 (23.1%) died. In the first 12 months after starting cART 26.9% ($n = 3938$) of patients were lost to the program: 618 patients (15.7%) did not return to the clinic after cART initiation (no follow-up), 2510 (63.7%) were LTFU (including cART stops for other reasons), and 810 (20.6%) died.

Future measurements will include an analysis of LTFU risk factors, late diagnosis, and incidence of opportunistic infections by CD4 cell counts. It is also necessary to measure death outcomes in patients starting cART and the incidence of side effects of cART.

4. Discussion

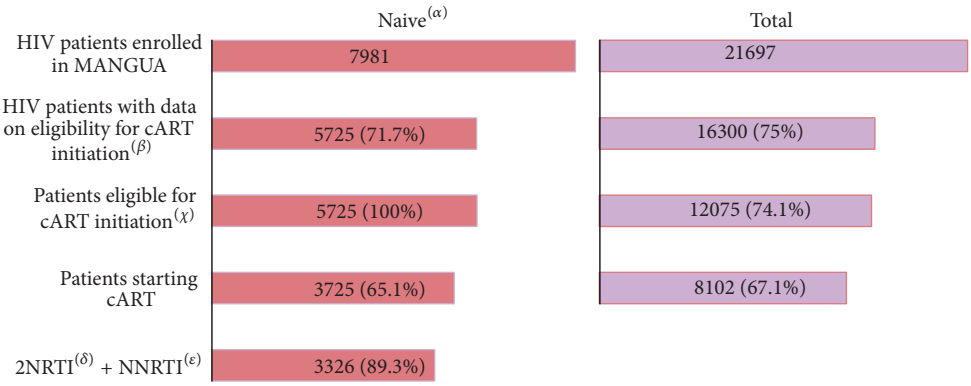
This is the largest cohort of HIV-positive people compiled in Central America and the cohort has a wide representation of the total HIV population registered throughout Guatemala. The MANGUA cohort has a nationwide, population-based design, with long-term follow-up. National scale-up of cART has resulted in a considerable number of patients enrolled in HIV treatment and care for HIV prevention and control in a country constrained by limited resources.

The MANGUA application was implemented soon after the scale-up of UAIs; in 2004 only 4 UAIs existed in the country; in 2008, 12 UAIs were already functioning. Scale-up of UAIs was possible because of international financial aid, mainly from the Global Fund, and the commitment of the MSPAS to decentralize HIV care in Guatemala. This scenario facilitated the acceptability of an EMR by UAI professionals, because it allowed systematization and harmonization of data collection and analysis. Comprehensive training activities were targeted to professionals, physicians, nurses, and data entry personnel, on how to use the MANGUA application. The implementation of the MANGUA cohort has been successful and has proven to be useful for both clinicians and the PNS through the provision of data for key HIV indicators to monitor the HIV epidemic in Guatemala. The MANGUA application has been integrated within the SIGSA web information system, which is crucial to ensure sustainability and strengthen HIV monitoring and surveillance in Guatemala.

Nevertheless, only approximately half of the patients registered in the cohort have data available on CD4 cell counts at baseline, partly due to under-notification and the fact that only some UAIs have available CD4 cell count analyzers. Among those with baseline data on CD4 cell counts, as much as 82.6% do not present for HIV testing until advanced symptoms of HIV infection (CD4 cell counts $< 350/mm^3$). Late presenters are often more ill, have a higher mortality risk, and are less likely to respond to treatment after initiation [15–18].

According to the Guatemalan National Guidelines on HIV Diagnosis and Treatment, adults with WHO clinical stage disease 3 or 4 or CD4 cell counts < 350 cells/mm^3 are eligible for cART [19]. However, 65.1% of those eligible patients do not start cART treatment and many of them are LTFU (Figure 2).

Delayed cART initiation has been reported to be the major cause of death and associated morbidity in HIV/AIDS patients [20, 21]. This fact, together with late presentation for HIV diagnosis and care and high LTFU, remains a significant challenge to efficiently prevent morbidity and mortality outcomes. It is worrisome that 30.1% (2582/8572) of patients considered LTFU return to the hospital after having interrupted ART for at least 6 months, which increases their risk of developing drug resistance or death [22–24]. Efforts should be focused on identifying reasons for LTFU with strategies to minimize it, such as creating interdisciplinary teams to investigate its underlying causes, proposing solutions that have shown to be effective in other contexts [25, 26], and expanding treatment access points in order to reduce time spent travelling to the clinic.



(α) We excluded 975 patients that were retreated or reenrolled since we lack data on treatment regimen

(β) Patients with data on baseline CD4 cell count or on AIDS-related events available

(χ) CD4 cell count <350 cell/mm^3 or WHO clinical stage 3 or 4. Number of patients eligible for cART initiation over HIV patients with data on eligibility for cART initiation with data on treatment eligibility

(δ) Nucleoside reverse transcriptase inhibitor

(ε) Nonnucleoside reverse transcriptase inhibitor

Figure 2: Spectrum of cART initiation in patients enrolled in MANGUA cohort.

Retention analysis reveals that the postdecentralization period has less retention proportions than the predecentralization period. This might be explained by different factors; first, the number of HIV-positive patients starting cART in the postdecentralization period is 5.4 times higher than in the predecentralization period; and second, recently created UAIs might have less capacity to provide integrated care to HIV-positive patients, lacking the experience to effectively implement adherence and follow-up interventions.

5. Conclusions

Today, the MANGUA HIV cohort is a powerful tool for monitoring and evaluation as well as for research on HIV-positive patients in respect to (i) epidemiologic patterns of the HIV epidemic, (ii) delayed access to cART and burden of opportunistic infections, (iii) risk factors for LTFU and late diagnosis, and (iv) long term cART outcomes.

The strengths of this cohort include its size and the representativeness of data collected from almost all registered HIV patients in Guatemala gathered from 14 of the 16 UAIs. As a main strength, data within MANGUA covers the spectrum of engagement in care, including late HIV diagnosis, suboptimal linkage to and retention in HIV care, insufficient use of antiretroviral therapy, and suboptimal adherence to therapy [27].

In Guatemala, the AIDS surveillance system is composed of several sources of information that are not yet linked. As a result, the system is highly heterogenic and complex, making it difficult to extract data for decision making [28]. Information on quality of the AIDS surveillance system is lacking, that is, whether it is representative of the whole country and updated in a timely manner and its level of completeness and accuracy. The MANGUA database is contributing to the harmonization of HIV national surveillance data. In 2012, it gained recognition from national stakeholders and UNAIDS Guatemala as the official HIV application system. Some limitations should be noted. The MANGUA cohort has not yet reached 100% of registered HIV-positive patients declared to the MSPAS (29 211). Moreover, neither the MSPAS nor MANGUA has the ability to register HIV-positive patients from private health care centers throughout Guatemala; thus, selection bias in the MANGUA cohort might be a limitation.

The cohort is dependent on the quality of data entered by data entry managers from written medical records; therefore, long standing and skilled staff are required to maintain the quality of the clinical data, which is not always feasible. We attempt to validate clinical data and provide feedback to the UAIs in order to minimize errors and improve data recording. Nonetheless, data omissions, duplicated patient records, and incomplete and out-of-date laboratory and sociodemographic information might occur. In fact, there is a high proportion of missing data in key variables such as CD4 cell counts at baseline, treatment status at enrollment, transmission route of infection, and clinical stage category. This limits the ability to draw conclusions from the entire cohort because of data representativeness and selection bias due to differences in characteristics of patients with data available compared to patients without complete data available. In most UAIs it is not possible to maintain computerized records; clinical data are collected for administrative purposes rather than for clinical ones. Although the MSPAS is currently working through this, there is no standardized paper-based instrument shared in all UAIs to collect the essential variables recorded in MANGUA cohort, which would help in homogenizing data collection.

The MANGUA cohort is not linked with the national registry of deaths; consequently, there is little information on AIDS-related deaths, and mortality rates may be underestimated. In addition, TB and HIV services are not well integrated in Guatemala. TB screening of HIV-positive patients is poorly performed in UAIs and patients are referred to the primary health care center for TB preventive chemotherapy and treatment. Thus, data entry managers from UAIs lack information on TB treatment outcomes on HIV patients, hampering data entry into the MANGUA application from the UAIs, and underestimating TB associated incidence among HIV-positive people.

Nevertheless, the two main limitations are: first, the MANGUA application is not used directly by medical staff as an EMR in most UAIs, rather data are collected initially on paper and then digitalized. The process of informatization of the UAIs in the country may, on a medium-term basis, contribute to correct this situation. Second, high rates of LTFU may introduce selection bias due to differences in follow-up. UAI decentralization needs to be consolidated to partly overcome this problem, the stabilization of the staff in peripheral UAIs being one of its main challenges, which will assure the continuum of care and facilitate adherence to treatment.

The MANGUA cohort aims to put Guatemalan HIV/AIDS research on a par with other international collaborations in the field. This will be achieved through the development of a nationally and internationally recognized policy-relevant program of research in HIV therapy and public health and through the establishment of training and research opportunities to graduate students, postdoctoral fellowships, and clinicians across the country.

Our efforts to improve research dissemination to physicians and PLHIV as well as to improve knowledge and translational research will contribute to global recognition of the MANGUA cohort. Over the past 6 years, we have expanded our capacity to recruit participants, collect, extract, and analyze data, and engage clinicians, policy stakeholders, and funders. We are committed to share and promote MANGUA research findings to help shape programs and policy that will improve the lives of PLHIV in Guatemala and elsewhere.

Disclosure

The members of the MANGUA Cohort Study Group include the following: Executive Committee: Eduardo Arathoon, Carlos Mejía, Zonia Pinzón, Enrique Ponce, Elsy Camey, and Jordi Casabona; Scientific Committee: Elsy Camey, Juan Ignacio García, Meritxell Sabidó, Luis A. Prieto, Mikhail Nikiforov, Luis Ardani, and Jordi Casabona, from Fundació Sida i Societat, Escuintla, Guatemala; Eduardo Arathoon, Blanca Samayoa, and Erwin Argueta, from Clínica Familiar Luis Ángel García, San Juan de Dios Hospital, Asociación de Salud Integral, Guatemala City, Guatemala; Carlos Rodolfo Mejía, Rodolfo Pinzón, Johana Samayoa, Mirci Romero, and Ericka Boror, from Infectious Diseases Clinic, Roosevelt Hospital, Guatemala City, Guatemala; Ernesto Ponce and Pedro Rizzo, from the National Programme for Prevention and Control of STI and HIV/AIDS (PNS), Guatemala City, Guatemala; José Fernando Ortiz and Edwin Leslie Cambranes Morales, from Hospital del Instituto Guatemalteco de Seguridad Social (IGSS), Guatemala City, Guatemala; Vilma Reyes, from Hospital Departamental de Huehuetenango Dr. Jorge Vides Molina, Huehuetenango, Guatemala; Carolina Zacarías from Hospital Nacional Pedro Bethancourt, Antigua, Sacatepéquez, Guatemala; Maudi González from Hospicio de San José, Sacatepéquez, Guatemala; Claudia Mazariegos from Hospital Nacional Carlos Arana Osorio, Zacapa, Guatemala; Carlos Eduardo Chajón and Marco Antonio Tobar from Hospital Nacional de la Amistad Japón-Guatemala de Puerto Barrios, Izabal, Guatemala; Luis Roberto Santa Marina de León, Gladys Sajche, and Ingrid Azucena from Juan José Ortega de Coatepeque National Hospital, Coatepeque, Quetzaltenango, Guatemala; Diana Mazariegos from Hospital Regional de Occidente San Juan de Dios, Quetzaltenango, Guatemala; César Augusto López-Téllez and Janet Ikeda from Clínica Isaac Cohen Alcache; Hospital Nacional Rodolfo Robles, Quetzaltenango, Guatemala; Aura Méndez from Hospital Nacional Regional de Escuintla, Guatemala; Mario Baldizón from Hospital Nacional de San Benito Petén; Petén, Guatemala; Alba Contreras from Hospital Nacional de Cuilapa, Santa Rosa, Guatemala.

Conflict of Interests

All authors declare that they have no conflict of interests.

Authors' Contribution

All authors have made substantial contributions to design, analysis, and interpretation of data and have been involved in drafting and revising the paper critically for important intellectual content and have given final approval of the version to be published.

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BMJ Open

The UALE project: a cross-sectional approach for trends in HIV/STI prevalence among key populations attending STI clinics in Guatemala

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ABSTRACT

Objective To describe and compare trends in prevalence, sexual behaviour and HIV transmission knowledge data related to sexually transmitted infections (STI) and HIV in patients attending three STI clinics over an 8-year period in Escuintla Department, Guatemala.

Methods STI clinic attendees were classified into transmission groups as follows: female sex workers (FSW), men who have sex with men (MSM) and 'high-risk heterosexuals' (HRH). Annual cross-sectional analysis and multivariable Poisson regression adjusted for sociodemographic variables were used for prevalence comparisons and adjusted prevalence trends for HIV/STI outcomes and used for adjusted trends in proportions in sexual behaviour and HIV transmission knowledge outcomes. Endocervical swabs were obtained to detect trichomonas, chlamydia and neisseria infections. Serologies for syphilis and HIV were performed using rapid tests. For reactive HIV samples, positivity was confirmed by an ELISA. All reactive syphilis samples were further confirmed for diagnosis of active syphilis disease.

Results From a total of 4027 clinic attendees, 3213 (79.78%) were FSW, 229 (5.69%) were MSM and 585 (14.53%) were HRH. The proportion of FSW, MSM and HRH who had a single visit was 56.42%, 57.23% and 91.10%, respectively. Overall, HIV prevalence was 2.10% in FSW, 8.17% in MSM and 4.12% in HRH. Prevalence trends in HIV and syphilis decreased in FSW. Prevalence trends in gonorrhoea did not decrease over time neither in FSW nor in HRH. Chlamydia and trichomonas infections in HRH showed an increase prevalence trend. In FSW, trends in condom use in last sexual intercourse with regular and occasional clients were above 93%.

Conclusions FSW show a decreasing trend in HIV, syphilis and chlamydia prevalence. Gonorrhoea prevalence in FSW and HRH did not decrease over time. HRH is a hard to engage population with low follow-up rates and high potential to act as a bridge population.

Strengths and limitations of this study

- This is one of the large datasets collected in Central America in HIV/sexually transmitted infections (STI) prevalence in key populations attending sentinel STI clinics.
- HIV/STI trends are calculated from an 8-year study period using prevalence ratios with Poisson regression analysis rather than odds ratios.
- The cross-sectional approach used to analyse data compared with a cohort study may be less powerful regarding the strength of the evidence of associations found.
- Selection bias issues and missing values for some variables may affect interpretation and generalisability of some results.

INTRODUCTION

Despite continuous efforts for prevention and control of sexually transmitted infections (STI), these still pose a high burden of disease in both low-income and high-income countries.[1 2] Guatemala is one of the Central American countries with higher prevalence of STI and HIV.[3 4] Of the estimated 55 000 people living with HIV, approximately less than half of them have been diagnosed.[5] Guatemala has a concentrated HIV epidemic driven by sexual transmission with high prevalence in core groups such as female sex workers (FSW) and men who have sex with men (MSM), at 5% and 11%, respectively.[6 7] FSW and MSM are classified as core groups because of social and biological factors, and thus, are targeted for effective STI and HIV prevention and control interventions.[8]

Multicomponent packages for STI and HIV prevention have been shown to be effective when properly designed and delivered at individual and community levels. However, strong evidence from intervention evaluations is crucial, and accurate surveillance systems are needed.[9 10] Patients attending

STI clinics provide valuable HIV and STI biological and behavioural data for surveillance purposes; therefore, surveillance systems in STI clinics should be strengthened and integrated into health information systems to better provide nationwide insights into transmission dynamics of HIV and STI.[11]

The UALE project (UALE means 'health' in Latin) started in 2005 as an intervention for STI and HIV prevention and care originally addressed to FSW in Guatemala.[12] This project was designed with the following principles: (1) prevention and treatment of STI as a strategy to prevent HIV infection, (2) integrated biological and behavioural surveillance of HIV/STI and (3) effective integrated HIV/STI management and care. It included three STI clinics located in Escuintla's Department, which has one of the highest prevalence of HIV and STI among the 22 Departments of Guatemala.[6] Contributing factors of high HIV and STI prevalence are its position along a major thoroughfare for migration and trafficking and the widespread sex work industry due, in part, to the influx of seasonal labourers during the sugar cane harvest.

The UALE project includes three main components: biomedical, behavioural and structural, as previously described. The biomedical component provides clinical capacity for early diagnosis, treatment and follow-up for STI offering HIV and STI care access to all populations seeking sexual and reproductive health. The behavioural component relies on communication for behavioural change; it consists of community-based activities to promote safer sexual practices, including proper condom use, improved negotiation skills, partner notification strategies and sexual and reproductive health empowerment activities addressing issues related to stigma and discrimination. The structural component aims to decrease the criminalisation and stigmatisation of sex work through sensitisation and collaboration with the owners of commercial sex establishments, the police and with governmental organisations and non-governmental organisations (NGO) for advocacy meetings and community health promotion.

The aims of this study were the following: (A) describe sociodemographic characteristics of patients at entry to the clinics, (B) determine trends in sexual behaviour and HIV transmission knowledge, (C) determine trends and compare STI/HIV prevalence and (D) describe sociodemographic determinants for STI/HIV infection, over a period of 8 years using data from sentinel STI clinics in Guatemala.

METHODS

Study design and data collection

Annual cross-sectional analysis was performed using opportunistic programmatic data gathered from STI clinics in the UALE project, in collaboration with the Guatemalan Ministry of Health and the NGO *Fundació Sida i Societat*. STI clinic attendees were recruited from January 2005 to December 2012. Sociodemographic, biological, behavioural and HIV transmission knowledge data were collected at baseline registration and during consecutive visits. The study was conducted in three STI clinics located in the municipalities of Santa Lucía Cotzumalguapa, Puerto de San José and Escuintla, in the department of Escuintla. All clinics offered uninterrupted HIV and STI counselling and testing on a voluntary and anonymous basis from 07:00 to 15:00. After obtaining written informed consent from participants, trained health educators conducted pretest HIV counselling and collected data on sociodemographic characteristics and sexual behaviours by means of a structured questionnaire. Subsequently, a medical doctor performed a complete clinical examination, as described elsewhere.[9] Follow-up visits were performed on a 6-month basis, although a drop-in service was available on request. Samples were collected by a nurse practitioner who also performed both the HIV and syphilis testing by finger prick. Blood was drawn by venepuncture from participants with a reactive HIV or positive syphilis rapid test result; participants were counselled posttest and asked to return to the STI clinic after 7 days to receive their confirmatory test result. Subjects with an HIV positive confirmatory test were referred and accompanied by a community health worker to the HIV referral hospital for enrolment in the HIV care and treatment programme. STI treatment was provided for those patients with positive results according to national guidelines.

All analytical tests were performed at the Escuintla STI clinic microbiology laboratory following national standard operating procedures as previously described.[9] The presence of *Trichomona vaginalis* and yeasts were examined by wet mount preparation microscopy of secretions collected from the posterior vaginal fornix. An endocervical swab was tested by enzyme immunoassay (Chlamydia Ag Card; Ulti med products, GmbH, Ahrensburg, Germany) to detect *Chlamydia trachomatis*. A second endocervical swab was cultured on modified Thayer-Martin medium for the diagnosis of *Neisseria gonorrhoeae*. Serologies for syphilis and HIV were performed using rapid tests. For reactive HIV samples, positivity was confirmed by ELISA using Bioelisa HIV-1+2 (Biokit, Lliçàd'Amunt, Spain). All reactive syphilis samples were further confirmed for diagnosis of active syphilis disease using *Treponema pallidum* haemagglutination test (Immutrep TPHA; Omega Diagnostics, Alva, UK) and the Venereal Infections Research Laboratory test (VDRL; MurexBiotech Limited, Dartford, UK). Active syphilis was diagnosed when both the VDRL and the TPHA tests were positive, regardless of the VDRL titers and treatment history.

Measures and definitions

FSW were defined as women who reported having sex in exchange for money during the preceding 12 months. MSM were defined as men who self-identified as homosexuals or who reported having sex with penetration with a same-sex partner during the last 12 months. Those clinic attendees who reported being heterosexual and did not

self-identified neither as FSW nor MSM were grouped as 'high-risk heterosexuals' (HRH), previously described elsewhere.[13] 'Regular clients' were defined as those men from which FSW reported having sex in exchange of money more than twice in the last year; 'occasional clients' were defined as those men from which FSW reported having sex in exchange of money once or twice in the last year. HIV transmission-related knowledge was assessed by asking attendees to identify HIV transmission routes as previously defined.[9 10] Prevalence of any STI was defined as the proportion of patients with a positive test for an STI among patients tested for the same STI during one specific year, as previously suggested.[4] Only the first positive test occurred in any specific year was included, and recurrence of STI other than HIV for any specific year was not considered. Patients were classified as new patients when first attending any of the three STI clinics. Patients attended were defined as any clinic attendee with at least one complete visit in any specific year. Trends in sexual behaviour and HIV transmission knowledge variables were ascertained taking into consideration the answer given in the latest risk questionnaire performed in the specific year.

Data analysis

Data were anonymised using an alphanumeric unique code for each participant, and analysed using STATA V.12.1 (Stata-Corp, College Station, TX). χ^2 Fisher's exact test and χ^2 test for linear trends were used in the univariate analysis. Median comparisons were performed using Kruskal-Wallis test. Prevalence trends were studied for HIV, syphilis, gonorrhoea, chlamydia and trichomonas infections. Proportions and adjusted proportion (APr) test for trends were studied for sexual behaviour and HIV transmission knowledge outcomes. Adjusted prevalence ratios (APRs) and APR tests for trends instead of odds ratios were used for HIV and STI prevalence outcomes to increase precision of adjusted estimates and facilitate interpretation of results as previously suggested.[14 15] Poisson regression analysis with robust variance for cluster estimations was used in the multivariable analysis. In the calculations of APR test for trends, year of visit was used as an independent categorical variable. Sociodemographic variables with an association with the outcome of interest with a p value of <0.20 in the univariate model were further studied for associations and adjustment for confounding in the multivariable model. The covariables used for adjustment in the multivariable Poisson regression were age, sex, STI clinic origin, having a regular partner, country of birth, education and marital status. The extension of the Mantel-Haenzsel test for trend adjusting for age was used when the Poisson test for linear trend was not of sufficient rank to be performed. Annual and overall HIV and STI prevalence were also compared among transmission groups using χ^2 test and residual analysis for multiple comparisons. Complete case analysis was used to handle missing data, and no interaction neither effect modification measures were considered in the multivariable model.

Patient involvement

Within the UALE project pilot implementation phase, patients were asked about their priorities and preferences in order to generate the questionnaires to be used. Prestudy meetings were held with civil society associations to better inform about the research agenda and the outcomes and benefits of the study. Results of the study will be disseminated through the clinics using friendly scientific language and translated into Spanish and Maya.

RESULTS

A total of 4027 new attendees were registered during the 8-year study period. Of these, 3213 (79.78%) were FSW, 229 (5.69%) were MSM and 585 (14.53%) were classified as HRH. There were a total of 8109 visits by 4027 persons. Sixty-one per cent of clinic attendees had a single visit; the remaining 1553 patients had a mean number of 3.6 visits per person (IQR, (2–4) max 14). The peak number of new patients per year occurred in 2008 (646), falling to 301 in 2012. The proportion of follow-up visits increased throughout the project from 16.80% in 2005 to 61% in 2012. Majority of HRH (91.10%) had only a single visit. The proportion of FSW and MSM who had a single visit was 56.42% and 57.23%, respectively. Sociodemographic characteristics at baseline registration were studied for new patients attending the STI clinics (table 1). FSW have the highest proportion of illiteracy or incomplete primary school education (52.20%) and the MSM group has the lowest (19.80%). Having a regular partner was common in HRH (69.70%) and less common in FSW (29.20%) and MSM (27.70%). HRH is composed mainly of general heterosexual population, partners of FSW, not self-identified FSW and MSM, truck drivers, military staff and immigrants working in the sugar cane industry.

APr linear trends for sexual behaviour and HIV transmission knowledge variables were evaluated for transmission groups (tables 2 and 3). In most years, over 95% of FSW reported condom use in their last sexual intercourse (CLSI) with both regular and occasional clients. However, condom use was lower with their regular partners ranging from 19.60% in 2006 to 22% in 2012. For FSW, having unprotected sex as a way to transmit HIV showed proportions over 95% since 2008. Conversely, vertical transmission knowledge of HIV showed lower proportions, despite an increasing linear trend from 9.20% in 2006 to 41.30% in 2010. For MSM, proportions of CLSI with occasional partners ranged from 57.1% in 2007 to 58.10% in 2012. The proportion of HRH reporting CLSI with regular partners was lower than 20%. Furthermore, proportion of CLSI with occasional partners showed a decreasing trend during the study period (from 35% to 14.30%, respectively).

In table 4, prevalence and prevalence trends were analysed for HIV and STI in clinic attendees. For FSW, a decreasing prevalence trend for HIV, syphilis and chlamydia was observed along the study period. Prevalence of trichomonas decreased from 14% in 2005 to 9.90% in

Table 1 Sociodemographic characteristics at baseline registration of new STI clinic attendees by transmission group 2005–2012

	Sample size, n	Median (IQR)	SexFemale (%)	Country of birth Guatemala (%)	Marital status Cohabitated* (%)	Education Illiterate† (%)	Workstatus Unemployed (%)	Having a regular partner Yes (%)
FSW								
	n = 3 213	24.90 (20.64–29.98)	100	66.03	11.38	52.18	NA	29.18
MSM								
	n = 229	22.40 (19.13–29.4)	0	93.45	4.37	19.82	38.21	27.75
HRH								
	n = 585	24.70 (20.34–32.12)	54.19	98.63	57.71	36.60	56.41	69.71

*Including marriage and free union.
†This category also includes incomplete primary school.
FSW, female sex workers; HRH, high-risk heterosexuals; MSM, men who have sex with men; NA not apllicable

2009, and since then, an increased prevalence trend was observed (15.50% in 2012). For MSM, annual HIV and STI prevalence are presented. However, due to sample size limitations, trends could not be estimated. Gonorrhoea prevalence had an unexpected high increase in year 2012 in all transmission groups (FSW: 21.20%, MSM: 35% and HRH: 25%). For HRH, prevalence trends of HIV and syphilis could not be estimated due to sample size limitations. Gonorrhoea prevalence trends in HRH did not decrease over time, and HRH also showed an increased prevalence trend in chlamydia and trichomonas infections reaching a peak at the end of the study period in 2012 with prevalence of 13.40% and 23.90%, respectively.

Annual and overall HIV and STI prevalence were also compared among transmission groups. Globally, for the period 2007–2012, differences were observed in HIV among MSM (8.17%) in comparison with FSW (2.10%) and HRH (4.12%). Differences in gonorrhoea prevalence during the 2007–2012 study period were observed. The highest prevalence was in MSM (22.76%), and both MSM and HRH (14.91%) had higher gonorrhoea prevalence compared with FSW (9.28%). Overall prevalence for chlamydia in FSW and HRH were 6.80% and 6.91%, respectively. Overall prevalence for trichomonas in FSW and HRH were 12.26% and 12.38%, respectively. In table 5, sociodemographic risk factors for STI in clinic attendees were analysed by transmission groups. Sociodemographic risk factors for HIV and STI among FSW were older age and having a limited education level. The risk of chlamydia and trichomonas infections was higher in Guatemalan-born FSW compared with Central American-born FSW. Having a regular partner was a 'protective factor' for gonorrhoea infection in the case of HRH.

DISCUSSION

Our results present the evolution of HIV/STI prevalence, sexual risk behaviours and HIV transmission knowledge over 8 years of the UALE programme. HIV and syphilis prevalence consistently declined in FSW. The overall HIV prevalence was higher in MSM compared with FSW and HRH. Interestingly, no differences in HIV prevalence between FSW and HRH were observed, as reported previously.[13] Annual and global HIV, gonorrhoea and chlamydia prevalence in FSW was higher compared with other recent studies[16] but similar to those reported in HIV national and international reports.[6] Our results indicate that most STI diagnoses were detected at first visit; in the case of HIV, 78.10% of all cases detected occurred in patient's baseline visit at the clinics, so the contribution of new patients to the estimated burden of HIV was high. Gonorrhoea prevalence was higher in MSM and HRH compared with FSW. Gonorrhoea prevalence trends did not decline over time in any of the transmission groups studied. The unexpected high increase in year 2012 for all transmission groups could be partly explained by the decline in the activity of 2 of the 3 clinics in years 2011 and 2012, due to funding restrictions that might

Table 2 Proportions and proportion trends in sexual behaviour and HIV transmission knowledge in FSW attended per year

	Sexual behaviour					HIV transmission knowledge	
	Condom use in last sexual intercourse with:						
	Regular partner	Regular client	Occasional client	Place of sex work	Number of clients in the last week	By unprotected sex	Vertical transmission
	Yes (%)	Yes (%)	Yes (%)	Bar (%)	≥5 clients (%)	Yes (%)	Yes (%)
FSW							
Year (n*)							
2005 (543)†	–	–	–	–	–	–	–
2006 (507)	19.61	93.42	96.63	97.63	34.16	90.74	9.25
2007 (755)	37.78	96.73	95.79	94.35	62.11	93.67	61.64
2008 (871)	24.17	98.92	88.24	94.88	52.36	97.07	35.71
2009 (854)	22.51	94.98	82.16	92.03	53.64	98.57	31.67
2010 (837)	20.95	96.90	95.45	91.50	51.20	98.66	41.29
2011 (735)	29.41	96.19	97.79	87.95	49.18	99.03	–
2012 (525)	21.99	95.13	97.14	89.67	47.98	99.03	–
APr test for trend‡	0.95 (0.90 to 0.99)	1 (0.99 to 1)	1.01 (1 to 1.01)	0.98 (0.98 to 0.99)	0.99 (0.98 to 1.01)	1.02 (1.02 to 1.03)	1.21 (1.18 to 1.24)
P values	0.029	0.887	<0.0001	<0.0001	0.863	<0.0001	<0.0001
Variable missing data (%)	10.12	21.22	9.69	1.21	2.65	<0.50	1.10

*Sample size of FSW attended in the specific year.
†The sexual behaviour questionnaire was not fully implemented in 2005.
‡Adjusted proportion (APr) test for trend with 95% CI using a Poisson binomial model with year as an ordered categorical variable adjusted for sociodemographic variables with a p value <0.2 in the univariate test, (age, sex, STI clinic origin, having a regular partner, country of birth, education and marital status).
FSW, female sex workers.

have caused a cumulative effect of patients attending the remaining STI clinic. Increasing prevalence trends in chlamydia and trichomonas infections were observed in HRH that might be also partly explained for the decline in STI clinics activities. However, the true disease burden of gonorrhoea and chlamydia might be underestimated if we take into account that only first STI episodes per year were analysed, and therefore, reinfection rates were not studied despite previous reported reinfection rates for gonorrhoea in FSW in the 7%–10% range. Older age and illiteracy or incomplete primary school were sociodemographic risk factors for HIV in FSW as previously reported.[13] Proportions of CLSI with regular and occasional clients in FSW remained high, mostly over 90%. In contrast, CLSI with regular partners was lower, mainly below 30%, and decreased over time as suggested by others.[12] Evidence shows that low condom use among FSW is the norm in private life, mainly because it may serve as a psychological distinction between work and private lives and because of feelings of trust and intimacy with regular partners.[17] Nevertheless, FSW condom use negotiation skills might be influenced either by violence associated with rejecting unprotected sex with regular partners or due to economic pressures during commercial sex with clients as previously suggested.[18 19] HRH had the lowest proportions and trends of CLSI with regular and occasional partners among transmission groups studied; this is in accordance to previous studies showing that HRH had higher than expected HIV and STI prevalence and sexual risk behaviours.[13 20] HRH is a difficult group to reach; it is composed mainly of general heterosexual population, partners of FSW, not self-identified FSW and MSM, truck drivers, military staff and immigrants working in the sugar cane industry, among other hard-to-reach populations. Of concern is that HRH might be acting as a bridge population as 17% of them reported having sex with FSW and multiple partners in previous studies.[13] The fact that 91% of HRH attendees had only one visit to the clinics, and most importantly, the fact that prevalence for HIV, chlamydia and trichomonas did not differ among FSW and HRH, makes the characterisation of this group a challenging and worthwhile effort to better understand the driving forces of the HIV/STI epidemiology in Escuintla. FSW, MSM and HRH had suboptimal follow-up rates, as more than 56.40%, 57.20% and 91% of them, respectively, had only one visit. Adherence to the programme seems to be associated to risk perception, being HRH and FSW the groups with the lowest and highest follow-up rates, respectively. Reasons to explain this fact could rely on stigma and discrimination driving healthcare seeking behaviours.[21] Structural interventions addressing sexual violence, stigma and discrimination and how they negatively impact health seeking behaviours need to be better identified and targeted for effective behavioural change outcomes.[22 23] HIV transmission knowledge by unprotected sex seems to be well known among all transmission

Table 3 Proportions and proportion trends in sexual behaviour and HIV transmission knowledge in MSM and HRH attended per year

	Sexual behaviour			HIV transmission knowledge	
	Condom use in last sexual intercourse with:				
	Regular partner Yes (%)	Occasional partner Yes (%)	Age at first sex* median (IQR)	By unprotected sex Yes (%)	Vertical transmission Yes (%)
MSM†‡					
Year (n§)					
2007 (16)	–	57.14	10 (9–13)	93.75	62.50
2008 (59)	–	78.38	12 (11–15)	100	42.37
2009 (87)	–	57.14	12 (12–15)	97.70	13.95
2010 (83)	–	62.50	13 (12–15)	97.60	37.35
2011 (77)	–	76.09	12 (14–15)	100	–
2012 (57)	–	58.06	11 (14–15)	100	–
APr test for trend¶	–	0.96 (0.79 to 1.18)**	NA††	1 (1 to 1.01)	0.95 (0.81 to 1.12)
P value	NA††	0.733	0.013	0.187	0.584
Variable missing data (%)	–	8.53	5.80	<0.5	<0.5
HRH†					
Year (n§)					
2007 (111)	13.84	35	15 (14–17)	90.90	69.10
2008 (123)	13.33	37.93	16 (14–17)	95.04	58.68
2009 (142)	14.11	36.58	15 (14–17)	93.62	70.92
2010 (87)	9.52	37.50	16 (14–17)	93.90	64.63
2011 (72)	8	14.28	15 (13–16)	97.22	–
2012 (66)	18.60	14.28	16 (14–17)	100	–
APr test for trend*	0.94 (0.88 to 0.99)	0.79 (0.63 to 0.98)¶	NA††	1 (0.99 to 1.01)	0.91 (0.83 to 1)
P value	0.042	0.033	0.345	0.187	0.056
Variable missing data (%)	14.06	22.95	<0.5	<0.5	<0.5

§Sample size of MSM and HRH attended in the specific year.
*Sex with penetration.
†The sexual behaviour questionnaire was not fully implemented in years 2005–2006.
‡Sexual behaviour data are from last male partner either occasional or regular.
¶Adjusted proportion (APr) test for trend with 95% CI using a Poisson binomial model with year as an ordered categorical variable and adjusted for those sociodemographic variables with a p value <0.2 in the univariate test (age, sex, STI clinic origin, having a regular partner, country of birth, education and marital status).
**Mantel-Haenszel extension for linear trend adjusted for age was used when the Poisson model test for clustered data was not of sufficient rank to be performed.
††Not applicable.
HRH, high-risk heterosexuals; MSM, men who have sex with men; STI, sexually transmitted infections.

groups studied. However, a community-based intervention may be necessary to educate about vertical transmission of HIV, which seems to be known as a lesser extent. Recent studies show the effectiveness in increasing testing coverage for HIV and syphilis in rural areas using outreach interventions.[24] Unfortunately, our HIV transmission knowledge results are aligned with recent data suggesting that Guatemala has one of the lowest percentages of antiretroviral coverage in pregnant women, and thus altogether suggests that vertical transmission of HIV is still a big concern.[3]

Limitations

Selection bias may affect external validity if those FSW, MSM or HRH attending the clinics differ from those not attending the clinics, and therefore, different from those FSW, MSM and HRH in the area served by the clinics. Possible reasons could be that FWS, MSM and HRH with less economic resources might be more likely to attend free-of-charge STI clinics than those with more resources who might attend private health centres to avoid potential stigmatisation. Sexual behaviour indicators reporting CLSI may not adequately represent condom use dynamics. We

Table 4 Prevalence trends of HIV/STI in clinic attendees by transmission group

	HIV		Syphilis		Gonorrhoea		Chlamydia		Trichomonas	
Year (n*)	n	Prevalence (%)	n	Prevalence (%)	n	Prevalence (%)	n	Prevalence (%)	n	Prevalence (%)
FSW										
2005	538	6.13	14	14.29	462	17.53	379	17.41	470	14.04
2006	472	3.60	418	7.89	501	11.38	483	9.73	511	6.65
2007	737	4.07	744	9.81	755	7.81	750	9.47	765	5.10
2008	834	2.04	836	5.26	822	12.77	821	8.65	820	9.15
2009	842	1.31	839	4.53	835	9.10	831	3.73	844	9.95
2010	717	2.51	742	3.64	840	2.86	825	3.64	843	13.29
2011	593	0.84	617	3.89	724	5.94	719	10.57	724	22.51
2012	504	1.19	520	2.88	518	21.24	522	4.79	523	15.49
APR test for trend†		0.78 (0.71 to 0.86)		0.81 (0.75 to 0.87)		0.96 (0.92 to 1)		0.88 (0.84 to 0.93)		1.14 (1.1 to 1.19)
P values		<0.0001		<0.0001		0.08		<0.0001		<0.0001
MSM‡										
2007	15	13.33	16	12.50	12	8.33	13	0.00	NA‡	NA‡
2008	44	18.18	48	20.83	50	44	45	2.22	NA‡	NA‡
2009	86	3.49	90	4.44	90	26.67	88	2.27	NA‡	NA‡
2010	56	12.50	61	4.92	79	12.66	74	0.00	NA‡	NA‡
2011	53	3.77	63	7.94	78	7.69	74	0.00	NA‡	NA‡
2012	52	5.77	60	0.00	60	35.00	55	0.00	NA‡	NA‡
APR test for trend‡§		NA‡		NA‡		NA‡		NA‡	NA‡	NA‡
P values		NA‡		NA‡		NA‡		NA‡	NA‡	NA‡
HRH‡										
2007	98	4.08	98	8.16	89	7.87	90	4.44	89	3.37
2008	109	3.67	109	4.59	106	17.92	102	2.94	100	8.00
2009	122	4.92	122	2.46	130	19.23	128	5.47	124	5.65
2010	57	7.02	58	1.72	85	9.41	81	11.11	74	21.62
2011	37	2.70	37	0	72	8.33	71	7.04	72	20.83
2012	62	1.61	66	0	68	25.00	67	13.43	67	23.88
APR test for trend‡§		NA‡		NA‡		1.07 (0.94 to 1.21)		1.34 (1.10 to 1.77)		1.48 (1.27 to 1.71)
P values		NA‡		NA‡		0.288		0.006		<0.0001

*Sample size of clinical attendees in the specific year
†Adjusted prevalence ratio (APR) test for trend with 95% CI using a Poisson binomial model with year as an ordered categorical variable adjusted for sociodemographic variables with a p value <0.2in the univariate test (age, sex, STI clinic origin, having a regular partner, country of birth, education and marital status).
‡For MSM and HRH, data from STI outcomes was not fully implemented in 2005–2006.
§Not applicable due to sample size limitations.
FSW, female sex workers; HRH, high-risk heterosexuals; MSM, men who have sex with men; STI, sexually transmitted infections.

Table 5 Sociodemographic risk factors for HIV/STI by transmission group

Transmission group	HIV/STI			APR (95% CI)*	P values
FSW	HIV	Age	≤30	1	–
			>30	1.86 (1.30 to 2.65)	0.001
		Education	Superior	1	–
			Illiterate or incomplete primary school	1.57 (1.08 to 2.28)	0.019
	Syphilis	Age	≤30	1	–
			>30	1.86 (1.46 to 2.37)	<0.0001
	Chlamydia	Education	Superior	1	–
			Illiterate or incomplete primary school	1.27 (1.05 to 1.55)	0.016
		Country of birth	Other Central American country	1	–
			Guatemala born	1.51 (1.20 to 1.90)	<0.0001
	Trichomonas	Country of birth	Other Central American country	1	–
			Guatemala born	1.38 (1.16 to 1.63)	<0.0001
MSM	Syphilis	Having a regular partner	Yes	1	–
			No	3.90 (1.28 to 11.76)	0.016
HRH	Gonorrhoea	Having a regular partner	Yes	1	–
			No	1.64 (1.07 to 2.50)	0.022

*Adjusted prevalence ratio (APR) test with 95% CI using a Poisson binomial model with year as an ordered categorical variable and adjusted for sociodemographic variables with a p value < 0.2 in the univariate test, (age, sex, STI clinic origin, having aregular partner, country of birth, education and marital status).
FSW, female sex workers; HRH, high-risk heterosexuals; MSM, men who has sex with men; STI, sexually transmitted infections

also observed potential data discrepancies between STI prevalence trends and condom use data obtained; these discrepancies could be partly explained by self-reporting information bias in clinic attendees. Moreover, high proportions of missing data observed in some sexual behaviour variables might also affect accurate interpretation of data. Prevalence trends in MSM and HRH were not analysed due to sample size limitations, and trends in sexual behaviour should be interpreted with caution. Syphilis results in MSM and HRH for years 2011 and 2012 might be not accurate as sample size was small and previous positive patients might not had been tested again. Further research is needed to accurately describe MSM and HRH sexual behaviour and HIV and STI prevalence data.

CONCLUSIONS

This is one of the largest clinical datasets for FSW and MSM collected in Guatemala. The multicomponent interventions implemented by the UALE programme are characterised as employing the principles of a combination prevention model to reduce the burden of HIV and STI. Evidence from additional studies within the UALE programme support its effectiveness in prevention and control of HIV/STI and the usefulness of integrated biological and behavioural surveillance and integral care management of HIV/STI.[24–26] In FSW, HIV, syphilis and chlamydia prevalence declines were observed, and proportions of CLSI with regular and occasional clients above 90% were also observed, despite lower proportions with regular partners. However, MSM and HRH need to be targeted more effectively with behaviour change communication to decrease HIV and STI prevalence, along with improved healthcare access and retention in care. HRH is a difficult group to target with low follow-up rates and high potential to act as a bridge population. A deeper analysis of the multifactorial risk relationships between subpopulations with high risk of HIV and STI and HRH is needed to identify uncovered bridging connections. Continued interventions to engage civil society, NGO, police/military and bar owners with information, education and communication of HIV/STI are critical to reduce stigma and discrimination.[25 27] Lack of funds to continue providing integral HIV/STI care in target groups in clinical settings might diminish prevention and control strategies in a high prevalent region for HIV and STI and affect the quality of programmatic data for surveillance purposes.[26]

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Patient consent Not required.

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Data sharing statement Due to the sensitive nature of the questions asked in this study, survey respondents were assured raw data would remain confidential and would not be shared.

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Improved Alere Determine Lipoarabinomannan Antigen Detection Test for the Diagnosis of Human and Bovine Tuberculosis by Manipulating Urine and Milk

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Tuberculosis (TB) disease still kills 1-person every 21-seconds. Few TB diagnostic tests are considered truly appropriate for point of care settings. The WHO-endorsed immunodiagnostic Alere Determine Lipoarabinomannan Ag-test (LAM-test) detects *Mycobacterium tuberculosis* complex LAM in urine, and its use is recommended for TB diagnosis among HIV co-infected individuals with low CD4 T-cell counts. Here we found that a simple 15-minute enzymatic treatment at room temperature of LAM-spiked urine with α-mannosidase (for human TB), and LAM-spiked milk with combined lactase and caseinase (for bovine TB), enhanced 10-fold the detection levels of the LAM-test and thus, improved the detection of LAM by the LAM-test in urine and milk that otherwise could be missed in the field. Future separate clinical research studies specifically designed to address the potential of these findings are required.

Tuberculosis (TB) is the leading cause of death by a single infectious disease[1]. The *Mycobacterium tuberculosis* (*M.tb*) complex cell wall has many cell wall components that are being evaluated for diagnosis purposes. One of them is the lipoglycan so called mannose-capped lipoarabinomannan (LAM)[2–4], which is found in urine of active TB patients[2–8]. To date, the only WHO supported TB point-of-care (POC) test is the Alere Determine Lipoarabinomannan (LAM) Ag test (LAM-test)[9]. This test is based on polyclonal antibodies of unknown specificity recognizing LAM in urine, as an indicator of TB disease[7,10]. The LAM-test best performance is in HIV-positive individuals with CD4 T cells counts below 50/mm^3 of blood (52% sensitivity/98% specificity)[7,10,11].

To improve the sensitivity of this test, we closely looked at the structure of the *M.tb* complex mannose-capped LAM or ManLAM (reviewed in[4]), ManLAM (from here on called LAM) is composed of a GPI-anchor, arabinan and mannan domains, and mannose caps[4]. The number of caps, length and branching of the arabinan and

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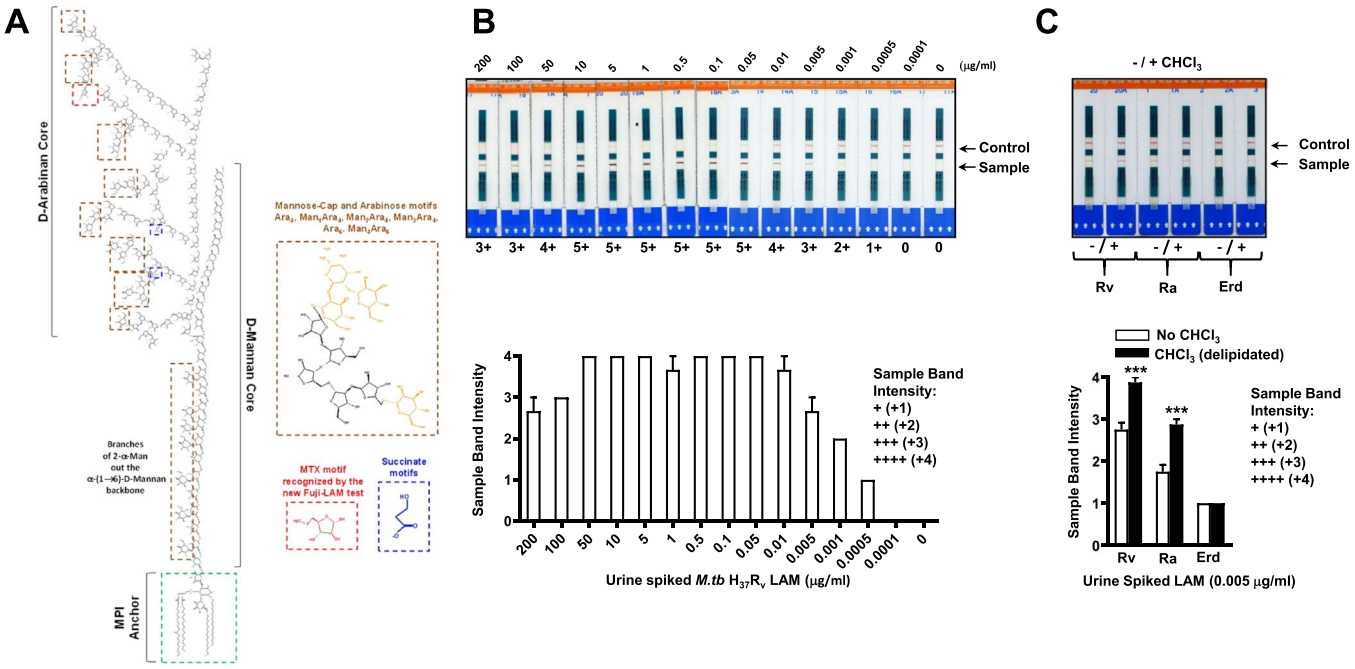

Figure 1. (**A**) Structure of mannose-capped lipoarabinomannan (ManLAM) present in all *M.tb* complex strains. ManLAM (depicted here as LAM) is a heterogeneous molecule comprised of a GPI-anchor, which can contain from 1–4 fatty acids, an α-(1 → 6) mannan core with multiple branches of a single mannose, an α-(1 → 5) arabinan core with multiple branches of different length at the C3 position of some arabinoses. The non-reducing end of some of these arabinan branches are decorated with 2-α-mono-, di- and tri-mannosaccharide caps. A 5-methyl-thio-xylose (MTX) is present per LAM molecule, being the epitope recognized by the new FujiLAM test. LAM also contains succinate motifs, which biological function is still unclear but participate in determining the spatial conformation of LAM. (**B**) Alere Determine LAM Ag test (LAM-test) performed in *M.tb* $H_{37}R_v$ LAM spiked urine determining that the lowest amount that this test can detect LAM in urine is 0.0005 μg/ml of urine (500 pg). (**C**) A quick delipidation step for LAM spiked urine using chloroform ($CHCl_3$) improves the detection of LAM by the LAM-test. Student's *t* test, treatment *vs*. non-treatment, n = 3–8, using LAM spiked urine from different human donors; $^{***}p < 0.0005$.

mannan domains, number of succinates, and the number and nature of fatty acids in the GPI-anchor makes this an extremely heterogeneous molecule[4]. A unique methylated thio-xylofuranose (MTX) is attached to a single mannose-cap of LAM[4], being described in susceptible and drug resistant *M.tb* strains (Fig. 1A)[4]. MTX detection is the basis for the new (Foundation for Innovative New Diagnostics (FIND) supported POC Fujifilm/SILVAMP TB-LAM (FujiLAM) test, being tested in field diagnostic validation trials[12–14].

Based on the high LAM structural complexity[4], and the low sensitivity of the LAM-test[7,10,11], we rationalized that the complex LAM molecule could be associated with other molecules (phospholipids, immunoglobulins, creatinine, etc.) in urine and/or milk that could made it less likely to be recognized by antibodies[15,16]. Thus, we biochemically treated LAM-spiked urine or milk to improve the LAM-test performance for the diagnosis of human and bovine TB. Out of several treatments tested (organic solvent delipidation, Proteinase-K, non-specific esterase, phospholipase, phosphatase, urease, creatinase, α-mannosidase, caseinase, and/or lactase treatments), our results showed that α-mannosidase treatment of LAM-spiked urine (for human TB) and combined lactase/caseinase treatment of LAM-spiked milk (for bovine TB) enhanced 10-fold the detection levels of the LAM-test in laboratory settings.

Results

Improving LAM-test detection in LAM-spiked urine in the laboratory setting. First, using urine spiked with purified *M.tb* $H_{37}R_v$ LAM, we determined that the minimal amount of LAM that the LAM-test detects in urine is 0.0005 μg (or 500 pg, Fig. 1B). We further observed that at higher LAM concentrations (50–200 μg of LAM/ml of urine), the detection of soluble LAM by the LAM-test was worse. We also observed that the optimal recognition of LAM by the LAM-test ranged from 0.05–10 μg of LAM/ml of urine (Fig. 1B). Importantly, in repeated experiments, we found consistent results.

Seeking methods to improve the LAM-test in a POC setting, we evaluated several field feasible options; the first, urine delipidation removing inherent lipids that could interfere with the LAM-test detection, and second, urine enzymatic treatment with different hydrolytic enzymes. Our results indicate that extracting with chloroform lipids present in urine spiked with 0.001 μg (or 1 ng) of LAM, we were able to increase the band intensity detection of the LAM-test (Fig. 1C), inferring that LAM molecules were detected better. This result indicates that natural lipids present in urine interfere with the LAM-test performance.

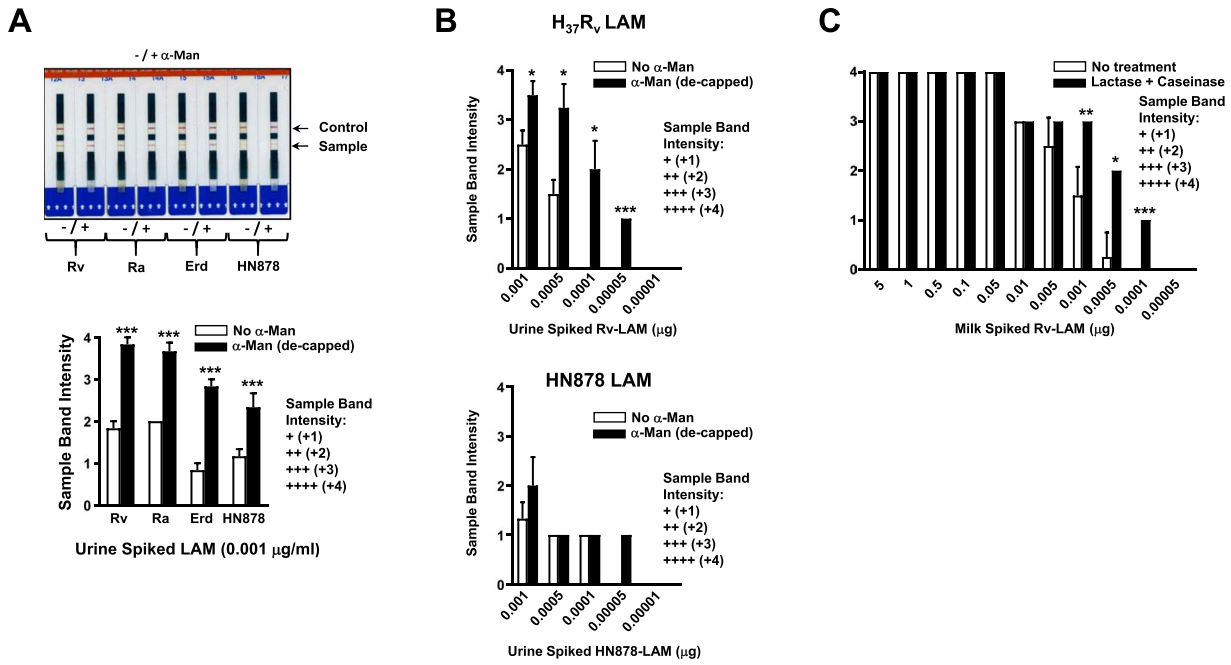

Figure 2. (**A**) Alere Determine LAM Ag test (LAM-test) performed in *M.tb* $H_{37}R_v$, $H_{37}R_a$, Erdman (Erd) or HN878 LAM-spiked urine treated with α-mannosidase to remove the mannose-caps of LAM. (**B**) A quick α-mannosidase treatment step for LAM-spiked urine (from two different *M.tb* strains, $H_{37}R_v$ (upper graph) and HN878 (lower graph) allows the detection of this molecule in urine by the LAM-test at lower concentrations. (**C**) Lactase and caseinase treatment of LAM-spiked milk also allows the detection of this molecule in milk by the LAM-test at lower concentrations. Student's *t* test, treatment *vs*. non-treatment, n = 3–6, using LAM-spiked urine/milk from different human/animal donors; $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$.

Importantly, out of all the enzymatic treatments tested, when urine spiked with 0.001 μg of LAM from different *M.tb* strains was treated with 0.1 IU of α-mannosidase at room temperature, results showed that this very simple step done before performing the LAM-test significantly increased the intensity of the LAM-test detection band (Fig. 2A). This α-mannosidase treatment removes terminal 2-linked mannose residues in both, the mannose caps and from the single 2-mannose branched mannan-core of LAM (Fig. 1A). We observed this improvement in LAM-test detection in urine spiked with structurally diverse LAMs obtained from different *M.tb* strains. (Fig. 2A). Further, using *M.tb* $H_{37}R_v$ LAM spiked urine, α-mannosidase treatment increased LAM-test detection levels to be as low as 0.00005 μg (or 50 pg) of LAM/ml of urine, a 10-fold detection improvement when compared to the LAM-test without α-mannosidase treatment of urine, which could only detect as low as 0.0005 μg/ml of urine (Figs. 1B and 2B). Interestingly, although the LAM-test was not consistent in detecting LAM from different strains of *M.tb*, at lower levels (0.0005 μg), it was capable of detecting structurally different LAMs from different *M.tb* strains (Fig. 2B). As expected, controls consisting of α-mannosidase treatment of non-spiked LAM urine were LAM-test negative (data not shown).

Improving LAM-test detection in LAM-spiked milk. We further assessed the efficiency of the LAM-test to detect LAM in milk, an easy specimen to obtain in the field, for the purpose of determining if the LAM-test could be used for the bovine TB diagnosis in a POC setting. Unexpectedly, no improvement was observed with LAM-spiked milk treated with α-mannosidase (data not shown). Indeed, the LAM-test was able to detect LAM in milk and detection levels were 10-fold improved (from 0.001 μg/ml to 0.0001 μg/ml) after LAM-spiked milk was treated with both caseinase and lactase (Fig. 2C).

Discussion

Currently, there are three tests for the detection of LAM (whole or fragmented) in human samples, the LAM-test[7], the Lionex test[17], and the new FujiLAM test[18]. Of these, currently only the LAM-test is WHO supported for the diagnosis and screening of active TB in people living with HIV[9,19]. In order to improve the efficacy of this test in detecting LAM in urine, we accounted for the LAM structural characteristics and how urine acts as a buffer. Without further sample manipulation, the LAM-test could detect as low as 0.0005 μg of LAM/ml of urine. Interestingly, we encountered that large amounts of LAM in urine interfered with the reading in a way that the intensity of the visible band on the LAM-test strip had lower intensity at the LAM concentrations ranging from 100–200 μg/ml. This could be due to micelle formation of LAM in urine affecting the LAM epitopes disposition of being recognized by the LAM-test polyclonal antibodies. In this regard, LAM contains up to 4 fatty acids and thus, LAM micelle formations in aqueous buffers have been described[20]. Nonetheless, LAM-test higher band intensities were observed in concentrations between 50 and 0.01 μg/ml of urine. In this regard, other studies

have demonstrated a semi-quantitative relationship between LAM concentration and band intensity of the LAM test[7,21–23], and others suggested that a darker LAM band intensity relates to a greater bacillary load of *M.tb*[24–26]. No detection of LAM was observed in concentrations below 0.0005 μg/ml of urine.

A quick step involving the removal of lipids allowed us to detect LAM in urine better. These results were observed in urine spiked with virulent *M.tb* $H_{37}R_v$ and the attenuated *M.tb* $H_{37}R_a$ LAM types; however, this was not observed for the *M.tb* Erdman LAM. This indicates the importance of the LAM structure from different *M.tb* strains (reviewed in[4,27]) and their spatial conformation when dissolved in urine, as well as the interactions of LAM molecules with other lipids present in urine in determining how well the LAM-test detects this molecule.

After exposure of urine to several enzymatic treatments (Proteinase-K, non-specific esterase, phospholipase, phosphatase, urease, creatinase) failed to improve the LAM-test (data not shown), we opted to determine if the removal of the mannose-caps from LAM using α-mannosidase was able to further enhance the detection of this molecule by the LAM-test in urine. Our results indicate that a 15-minutes treatment of urine with 0.1 IU of α-mannosidase in the lab setting enhanced the recognition of LAM by the LAM-test. This result was observed for the LAMs of all *M.tb* strains tested. Thus, removal of the mannose caps of LAM may uncover specific arabinan epitopes of this molecule and probably also changes its spatial conformation, increasing the affinity of the LAM-test polyclonal antibodies to recognize and bind LAM, increasing by 10-fold the detection levels of this test.

Finally, we determined if the LAM-test could be useful to detect LAM in milk, and thus be used as a POC test in farms for the detection of Bovine TB in cattle. Bovine TB is an increased global health problem, with areas around the globe where 15–25% of human TB cases are directly related to *Mycobacterium bovis* infection through zoonotic transmission or consumption of *M. bovis* contaminated products (*i.e.* unpasteurized milk)[28]. The LAM-test could in fact detect well LAM in milk at the lowest concentration of 0.001 μg/ml of milk. This is in contrast to the LAM concentration of 0.0005 μg that this test could detect in urine, indicating that milk contains soluble components that interfere with the detection of LAM by the LAM-test. In order to improve LAM detection in milk, we tried different methods (delipidation, Proteinase-K treatment, α-mannosidase, centrifugation to analyze the plasma milk phase, etc., data not shown); however, in our hands, only the combination of lactase and caseinase treatment resulted in an improvement of LAM detection in milk, where the LAM-test was able to detect as low as 0.0001 μg/ml (or 100 pg/ml), a 10-fold improvement relative to non-enzymatically treated milk. As a caution, in milk samples and in some instances, we observed that when the LAM-test was repeated, exact band intensity readings were not repeated. However, in our LAM-test studies an initial positive reading [1+ to 4+] never gave us a negative reading [0] or vice versa when the LAM-test was repeated. This variation in band intensity from repeated tests using LAM-spiked milk samples is a concern that somehow gets mitigated with the current reading card of the LAM-test, where the appearance of a positive band at any intensity (+1 to +4) is indicative of a positive detection.

Overall these studies attempted to improve the LAM detection levels in urine/milk by the commercially available LAM-test. Two quick steps, one in urine (α-mannosidase treatment) and one in milk (lactase/caseinase-combined treatment), both for 15-minutes at room temperature, significantly reduced the concentrations of LAM in these specimens that could be detected by the LAM-test. All these enzymatic treatments can be easily performed in the field as part of the POC LAM-test, adding only 15 minutes to the 25 min that this test already requires. Importantly, from the point of view of the added cost, currently the LAM-test cost is US$3.5 per test. The addition of the α-mannosidase urine treatment will add the cost of US$0.45 to the LAM-test; and the combined addition of lactase and caseinase milk treatment will add the cost of US$0.48 per test, allowing the detection of lower concentrations of LAM by the LAM test that otherwise will be missed.

A major limitation of this study is that the α-mannosidase treatment was tested on urine and milk spiked with purified bacterial LAM, and thus the need for separate clinical research studies specifically designed to address the potential of our findings. Our current efforts are directed to test this treatment in ongoing pilot studies in different high TB burden sites (e.g. Guatemala, Ethiopia, Mozambique). It is thought that immunological properties of LAM detected in the urine of TB patients are quite different from those of the antigen directly purified from bacteria[18], thus future field studies will determine if α-mannosidase treatment of urine allows better detection of the natural secreted form of LAM in urine from active TB patients.

These fast and easy to perform biochemical treatment could potentially also improve other POC tests, such as the novel FujiLAM, which detects the MTX present in the mannose-caps of LAM (FIND personal communication). In this instance, treatment with α-mannosidase should remove all mannose-caps from ManLAM except the one containing MTX, and thus, potentially this could be better exposed to the recognition by FujiLAM test specific MTX Abs. This hypothetical reasoning will need to be further evaluated in future studies when the FujiLAM test will be commercially available.

Further studies are required to replicate and corroborate our laboratory results in field settings with scarce diagnostic techniques and delayed diagnosis[29] using urine from human subjects with presumable symptoms of active TB, as well as using milk from cattle presumable with symptoms of bovine TB.

Methods

Human subjects and ethics statement and specimens. All experiments involving human specimens (urine) were performed in accordance with relevant guidelines and regulations. For laboratory studies, pre-existing human urine samples were obtained from anonymous healthy volunteers [Human Subjects Institutional Review Board (IRB) exempted research approved by UT-Health San Antonio IRB] in Texas.

Whole milk was obtained from unidentified healthy cows from an Ohioan farm (IACUC exempt, no animal manipulations). All specimens were spiked with known amounts of in-house purified LAMs[30,31], as indicated in figures and figure legends, from different laboratory reference *M.tb* strains ($H_{37}R_v$, $H_{37}R_a$, Erdman) and from a hypervirulent *M.tb* clinical isolate (HN878)[30].

Alere determine urine LAM Ag test (LAM-test). LAM-tests were performed as indicated by the manufacturer instructions. Urine (60 μL) was applied to LAM-test strips followed by incubation at room temperature for 25 minutes. LAM-test results were then inspected by the naked-eye. In the lab setting, the intensity of any visible band on the LAM-test was graded as positive and from +1 (minimum intensity) to +4 (maximum intensity) depending on its intensity and following the post 2014 reference card (new grades from +1 to +4) provided by the manufacturer. Readings were independently performed by two to three researchers in a blinded manner following manufacturer's instructions.

Delipidation of urine and milk. Chloroform was added to LAM-spiked urine/milk 1:1 (v/v), hand mixed and led settle for 15 minutes at room temperature until bipartition was observed. The aqueous phase containing LAM was then directly used to perform LAM-tests following manufacturer's instructions.

Enzymatic treatments of urine and milk. Proteinase-K, non-specific esterase, phospholipase, phosphatase, urease, or α-mannosidase, (each at 0.1 IU, Sigma-Aldrich, Saint Louis, MO) was added to 150 μl of LAM-spiked urine, and lactase and caseinase (both at 0.1 IU, Sigma-Aldrich) were added to 150 μl of LAM-spiked milk at the concentrations indicated in the figures and figure legends, hand mixed and further incubated for 15 minutes at room temperature. Enzymatically treated LAM-spiked urine or milk was then directly used to perform LAM-tests following manufacturer's instructions.

Statistical analysis. Experiments were performed using urine or milk from different human or animal donors, respectively. Unpaired two-tailed Student's *t*-test was used for two group comparisons (delipidation/enzymatic treatment *vs*. non-treatment). Statistical significance was determined using Prism 4 GraphPad software and reported as $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$.

Data availability

All materials, data and associated protocols will be promptly available to readers upon proper request.

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Author contributions

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Competing interests

The authors declare no competing interests.

Additional information

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Low-cost diagnostic test for susceptible and drug-resistant tuberculosis in rural Malawi

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Background: Rural settings where molecular tuberculosis diagnostics are not currently available need easy-to-use tests that do not require additional processing or equipment. While acid-fast bacilli (AFB) smear is the most common and often only tuberculosis diagnosis test performed in rural settings, it is labour intensive, has less-than-ideal sensitivity, and cannot assess tuberculosis drug susceptibility patterns.

Objective: The objective of this study was to determine the feasibility of a multidrug-resistant (MDR) or extensively drug-resistant (XDR)-tuberculosis coloured agar-based culture test (tuberculosis CX-test), which can detect *Mycobacterium tuberculosis* growth and evaluate for drug susceptibility to isoniazid, rifampicin and a fluoroquinolone (i.e. ciprofloxacin) in approximately 14 days.

Method: In this study, 101 participants were enrolled who presented to a rural health clinic in central Malawi. They were suspected of having active pulmonary tuberculosis. Participants provided demographic and clinical data and submitted sputum samples for tuberculosis testing using the AFB smear and tuberculosis CX-test.

Results: The results showed a high level of concordance between the AFB smear (12 positive) and tuberculosis CX-test (13 positive); only one sample presented discordant results, with the molecular GeneXpert MTB/RIF® test confirming the tuberculosis CX-test results. The average time to a positive tuberculosis CX-test was 10 days. Of the positive samples, the tuberculosis CX-test detected no cases of drug resistance, which was later confirmed by the GeneXpert MTB/RIF®.

Conclusion: These findings demonstrate that the tuberculosis CX-test could be a reliable low-cost diagnostic method for active pulmonary tuberculosis in high tuberculosis burden rural areas.

Introduction

In 2017, the World Health Organization (WHO) estimated that 4000 people die of tuberculosis every day. Malawi is among the 20 countries with a WHO-defined 'high' tuberculosis and HIV burden: the country reported 15 737 new and relapsed cases in 2015.[1] Of these cases, only 6% were tested with rapid tuberculosis diagnostics at the time of diagnosis (by GeneXpert MTB/RIF®, Cepheid, Sunnyvale, California, United States).[2] Most, 75%, were diagnosed as pulmonary tuberculosis by clinical symptoms; 58% of these had confirmatory culture and only 47% were provided with tuberculosis treatment. The recently-reported tuberculosis incidence rate in Malawi is 193 per 100 000 people per year, with 53% of cases occurring in people that are also HIV-positive.[1] WHO data for Malawi estimates that 0.75% of new cases and 6.4% of previously-treated cases are multidrug-resistant (MDR)-tuberculosis[1]; however, real numbers may be higher due to current limited drug susceptibility testing in the country.

With the adoption of the 'Sustainable Development Goals' and the 'End TB Strategy' in late 2015, worldwide efforts to end the global tuberculosis epidemic are ambitious and require new advancements in tuberculosis diagnostics. While acid-fast bacilli (AFB) smear is the most common

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tuberculosis diagnostic method in Malawi and many other low-income and high tuberculosis-burden countries around the globe, it is labour intensive, has less-than-ideal sensitivity, and cannot be used to assess tuberculosis drug susceptibility. Access to tuberculosis diagnostics has improved in recent years but still remains limited. In Malawi, there are approximately two smear and microscopy facilities per 100 000 people and one laboratory capable of performing tuberculosis drug susceptibility tests per 10 million people.[3] Culture-based diagnostic methods remain the gold standard for drug susceptibility testing, but can take up to 86 days to yield results.[4] In 2010, the WHO recommended GeneXpert MTB/RIF® as an initial tuberculosis diagnostic test. The GeneXpert MTB/RIF® is a polymerase chain reaction-based test that takes less than two hours to perform and simultaneously detect *Mycobacterium tuberculosis* and rifampicin resistance in the tested sample. In this test, rifampicin resistance is used as a surrogate marker for MDR-tuberculosis, which is defined as resistance to both isoniazid and rifampicin. Despite its sensitivity and utility, the GeneXpert MTB/RIF® implementation in high tuberculosis burden areas is cost prohibitive (approximately $18 per sample in Malawi), requires expensive instrumentation with weekly maintenance and monthly calibration, a sustained power source, and laboratory technicians with specialised training.[5] These drawbacks render it currently inaccessible to most areas in high tuberculosis-burden countries. There is a need for a simple, inexpensive tuberculosis diagnostic test that can be performed in rural health facilities, where access to molecular tuberculosis diagnostics may not be feasible.

The MDR/XDR-tuberculosis coloured agar-based test (tuberculosis CX-test) is a non-commercial, thin-layer agar-based tuberculosis culture method capable of simultaneously detecting *M. tuberculosis* and tuberculosis drug susceptibility in approximately 14 days. The tuberculosis CX-test contains four quadrants: one quadrant detects *M. tuberculosis* growth and the other three quadrants detect resistance to isoniazid, rifampicin and a fluoroquinolone (i.e. ciprofloxacin). The tuberculosis CX-test is simple to use: expectorated sputum is mixed with disinfectant, the mixture is cultured onto the tuberculosis CX-test, and colonies are enumerated after incubation. In a study completed in a research laboratory setting using 197 archived *M. tuberculosis* clinical isolates, the tuberculosis CX-test detected drug resistance with 98% sensitivity for isoniazid, rifampicin, and ciprofloxacin and 99% for MDR-tuberculosis, compared to drug susceptibility testing results using a liquid culture method.[6] Specificities reported for isoniazid were 100% (95% CI 82–100), 88% (95% CI 69–97) for rifampicin, 91% (95% CI 83–96) ciprofloxacin and 90% (95% CI 74–98) for MDR-tuberculosis.[6,7] A systematic review of three studies assessing the thin-layer agar-based assay technique found a pooled sensitivity of 100% (95% CI 97–100) and pooled specificity of 100% (95% CI 99–100) for the detection of rifampicin; and a pooled sensitivity of 100% (95% CI 91–100) and pooled specificity of 100% (95% CI 99–100) for the detection of isoniazid.[8,9,10,11] The tuberculosis CX-test has also been shown to be highly specific in identifying *M. tuberculosis* from atypical mycobacteria.[12] Although the tuberculosis CX-test has been shown to be accurate in research laboratories, its performance in the field and in clinical settings in high tuberculosis-burden areas has yet to be characterised.

In this study, we sought to determine the feasibility of the tuberculosis CX-test to diagnose active pulmonary tuberculosis and patterns of tuberculosis drug susceptibility to isoniazid, rifampicin, and ciprofloxacin in a rural Malawian health clinic using direct sputum specimens, where currently only AFB smear is performed and no routine cultures are sent for confirmation or drug susceptibility testing.

Methods

Ethical considerations

Institutional review board approval was obtained from The Ohio State University (study number 2014H0381) and the Malawi College of Medicine Research and Ethics Committee (study number P.09/14/1627). All participants were adults and were enrolled in the study using a written consent form. Tuberculosis CX-test results were for research only and were not used for diagnosis and they did not influence treatment outcomes. Following the Malawian Ministry of Health recommendations, GeneXpert MTB/RIF® positive samples were to be retested by the Malawian National Tuberculosis Control Program before patients were notified.

Study population

Participants were recruited from the Child Legacy International-McGuire Wellness Center in Msundwe, Malawi. Child Legacy International serves a catchment area of 68 rural villages (~18 000 people) in the central region of Malawi.[13,14,15,16,17] Participants were limited to patients suspected of having active pulmonary tuberculosis disease based on clinical symptoms (i.e. fever, chills, night sweats, shortness of breath, chest pain, cough ≥ 2 weeks, loss of weight, fatigue), who were ≥ 18 years of age, capable of providing informed written consent, and able to provide sputum. Participants answered a survey regarding demographics (i.e. age, sex) and clinical history (i.e. HIV status, tuberculosis clinical symptoms, previous diagnosis and treatment). HIV status and testing were verified by Health Passport or offered on-site to all participants, along with pre- and post-HIV counselling; HIV testing was not required for participation in this study. Participants were recruited over the course of 11 months during the period of November 2015 to October 2016.

Tuberculosis CX-test preparation

The tuberculosis CX-tests were prepared according to published methods as previously described[6] in different batches in research laboratories at The Ohio State University in the United States. Tuberculosis CX-test quality control per each batch was confirmed in an Ohio State University Biosafety Level 3 laboratory using verified susceptible, mono-isoniazid resistant, mono-rifampicin resistant and MDR *M. tuberculosis*

clinical isolates provided by the Ohio Department of Health State Laboratory. Upon tuberculosis CX-test quality control confirmation, tuberculosis CX-test batches were transported to Malawi and properly stored at 4 °C until used within four months of being made.

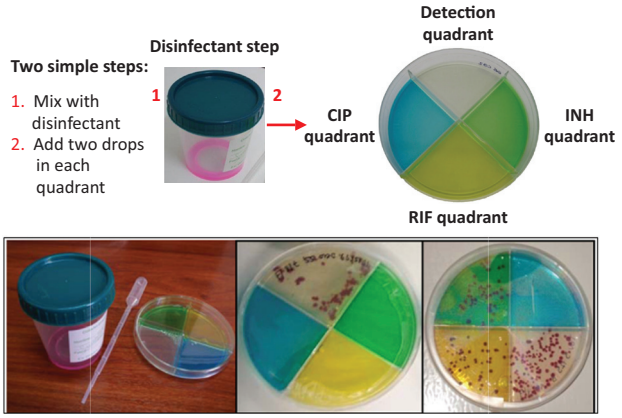
Tuberculosis CX-testing

Sputum samples were collected, numerically coded (de-identified) and equally divided by pipetting for (1) AFB smear, (2) tuberculosis CX-test, and (3) GeneXpert MTB/RIF® testing (in the case of discordant results). Sputa for AFB smear and tuberculosis CX-tests were processed immediately after collection, whereas samples for GeneXpert MTB/RIF® testing were stored at -20 °C until used.

For the AFB smear, standard procedures dictated by the Malawi Ministry of Health were followed for sputum collection, Ziehl-Neelsen technique for AFB staining, microscopy, and smear grading.[18] During Ziehl-Neelsen staining, sputum was applied to a slide and heat fixed. The slide was submerged into carbol fuchsin, heated to dry, and rinsed with water. Next, slides were submerged in a 3% solution of hydrochloric acid (de-staining step), briefly washed with water, and then counterstained with methylene blue.[19]

For the tuberculosis CX-test (Figure 1), one-third of the collected sputum was added to a 50 mL tube containing twice the volume of disinfectant (stock solution: 2 g tri-sodium phosphate, 0.05 g ammonium sulphate, 0.005 g magnesium sulphate, 0.0025 g ferric ammonium citrate, 10 mL sterile water, and 0.01 mL red food coloring, all mixed by manual shaking). Two drops of the sputum/disinfectant mixture (1:2, v/v) were then plated directly onto each quadrant of the tuberculosis CX-test.[6] The tuberculosis CX-test was then incubated at 37 °C for 28–42 days and checked three times per week for bacterial growth. Colonies from each quadrant were counted during each check. The presence of *M. tuberculosis* was microscopically defined as the presence of the typical rough colony on the detection quadrant, using a low-resolution (20X) microscope (clear quadrant). Drug resistance was defined by the detection of growth on each of the specific quadrants: isoniazid (yellow quadrant), rifampicin (green quadrant), and ciprofloxacin (blue quadrant). The drug concentration in each quadrant was as follows: isoniazid (0.2 µg/mL), rifampicin (1 µg/mL) and ciprofloxacin (2 µg/mL). As a precautionary measure, all tuberculosis CX-tests were kept in the incubator for an additional seven days before being discarded.

For discordant results between the AFB smear and the tuberculosis CX-test, coded sputum samples stored at -20 °C were transported to Lilongwe and tested with GeneXpert MTB/RIF® by the University of North Carolina Project at Lilongwe, as described previously.[1] Moreover, approximately an additional 10% of samples tested by AFB smear and the tuberculosis CX-test were selected randomly and also analysed by GeneXpert MTB/RIF® to confirm the obtained results.



TB CX-test: Sputum is collected into disinfectant (1:2 ratio) and the mixture is applied directly to the TB CX-test without further processing

TB CX-test showing a drug susceptible TB case, where *M. tb* only grew in the clear detection/ susceptible quadrant (red colonies), with no growth in the coloured antibiotic-containing quadrants

TB CX-test showing a multi-drug (MDR) TB case, where *M. tb* grew in the detection, INH and RIF containing quadrants, but the quinolone (CIP) prevented TB growth, ruling out pre-XDR-TB

CIP, ciprofloxacin; CX, coloured agar-based culture; INH, isoniazid; MDR, multi-drug resistant; *M.tb*, *Mycobacterium tuberculosis*; RIF, rifampicin; TB, tuberculosis; XDR, extensively drug resistant.

Note: This test consists of very simple steps that can be performed by non-specialised individuals. Step 1 – Disinfect: 1 volume of collected sputum is mixed with 2 volumes of disinfectant (see Materials and Methods section). Step 2 – Transfer: The mixture of sputum/ disinfectant is applied (2 drops equalling 100 µL in each quadrant) to the tuberculosis CX-test, then the test is placed in a Zip-lock bag, and incubated at 37 °C. Step 3 – Inspection of Growth (presence of red coloured colonies) and Confirmation: The tuberculosis CX-test is checked three times per week for growth. If after 14 days only colonies (red colour, indicative of *M. tuberculosis* growth) are visible in the detection quadrant (clear quadrant) then the *M. tuberculosis* strain is susceptible to isoniazid, rifampicin and ciprofloxacin. If colonies are also observed in the green quadrant (isoniazid) and yellow quadrant (rifampicin), the sample is defined as a multidrug-resistant *M. tuberculosis* strain. Colonies observed in all quadrants indicate the detection of a pre-extensively drug-resistant *M. tuberculosis* strain.

FIGURE 1: The tuberculosis CX-test.

Statistical analysis

Descriptive data regarding participant demographics and clinical characteristics were collected and summary statistics were compiled. The Wilcoxon Rank-Sum test was used to test age association with tuberculosis diagnosis. Fisher's exact test was used to identify clinical characteristics significantly associated with a positive tuberculosis diagnosis. Data analysis was performed using JMP software (version 11; SAS [https://www.jmp.com/en_us/home.html]).

Results

A total of 101 participants were enrolled in the study. Of these, five participants were excluded due to tuberculosis CX-plate contamination or the plate drying out. The average participant age was 47 years (SD = 17) (Table 1). A large percentage of participants had been already tested for HIV (97%) at enrolment. Of these, 10 (10.4%) stated they were HIV-positive, 82 (85.4%) stated they were HIV-negative, while the remaining 4 (4.2%) did not know their status or had never been tested. The majority (88.5%) had never been tested previously for tuberculosis, while eleven (11.5%) had previous tuberculosis testing but of these only eight (8.3%) were diagnosed with active pulmonary tuberculosis. Of the 10 HIV-positive participants, only two (20%) reported they were taking antiretroviral treatment. No participant reported

TABLE 1: Participant demographics and clinical characteristics.

Demographic	*N*†	*N*	%
Age	96	47.0 ± 17.4‡ (Range: 19–90)	
18–29	-	17	18
30–39	-	21	22
40–49	-	16	17
50–59	-	13	13
60–69	-	16	17
70–79	-	9	9
80–89	-	3	3
90–99	-	1	1
Tested for HIV	96	-	-
Yes	-	93	97
No	-	3	3
HIV status	96	-	-
Positive	-	10	10
Negative	-	82	85
Don't know	-	2	2
Never tested	-	2	2
Taking ART	10	-	-
Yes	-	2	20
No	-	8	80
Previously tested for TB	96	-	-
Yes	-	11	11
No	-	85	89
Previously diagnosed with TB	11	-	-
Yes	-	8	73
No	-	3	27
Taking TB medication	96	-	-
Yes	-	0	-
No	-	96	100
Symptoms§	96	-	-
Cough	-	93	97
Blood in sputum	-	29	30
Fever	-	52	54
Chills	-	37	39
Night sweats	-	53	55
Weight loss	-	68	71
Fatigue	-	60	63

ART, antiretroviral treatment; TB, tuberculosis.
†, Some questions were not answered by participants and have been excluded from this table.
‡, Age mean ± SD.
§, Some patients presented multiple symptoms.

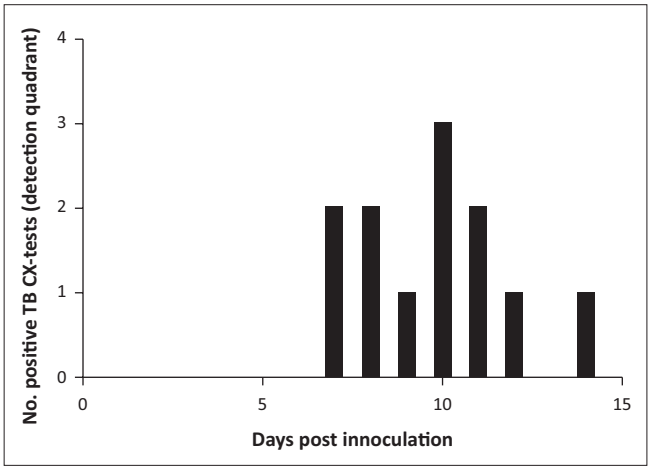
taking tuberculosis medication at the time of enrolment. The participants' most commonly-reported symptoms included dry and persistent cough (97%), weight loss (71%), fatigue (63%), night sweats (55%), fever (54%), chills (39%), and bloody sputum (30%). None of these symptoms was found to be significantly associated with a positive tuberculosis diagnosis.

Of the 96 participants, 12 (12.5%) were positive by both AFB smear and the tuberculosis CX-test, and 83 (86.5%) were negative by both AFB smear and the tuberculosis CX-test (Table 2). One sample was found to have discordant results between AFB smear (negative) and the tuberculosis CX-test (positive). In this case, the GeneXpert MTB/RIF® revealed a positive result for the presence of *M. tuberculosis* in sputum, in agreement with the tuberculosis CX-test. The tuberculosis CX-test was also comparable to the AFB smear (99% agreement on the diagnosis results), and positively identified a tuberculosis

TABLE 2: Contingency table of acid-fast bacilli smear microscopy vs. tuberculosis CX-test.

TB CX-test	AFB staining (+)	AFB staining (-)	Totals
TB CX-test(+)	12	1*	13
TB CX-test(-)	0	83	83
Totals	**12**	**84**	**96**

AFB, acid-fast bacilli; CX, coloured agar-based culture; TB, tuberculosis.
*, GeneXpert MTB/RIF® confirmed that the specimen was *M. tuberculosis* positive.



CX, coloured agar-based culture; TB, tuberculosis.

Note: Of the 96 sputum specimens studied, 12 were positive for *M. tuberculosis* by the tuberculosis CX-test. Of these, all were defined as susceptible tuberculosis by the presence of growth in the detection/susceptible quadrant. The time to a positive tuberculosis CX-test ranged from 7 to 14 days. The average time was 10 days after inoculation (SD = ±2.33). No mono-resistant, multidrug-resistant or pre-extensively drug-resistant tuberculosis was detected in the sputum specimens studied.

FIGURE 2: Time to positive tuberculosis CX-test.

diagnosis, while AFB smear yielded a single false negative result. The time to obtain a positive tuberculosis CX-test result ranged between 7 and 14 days (mean = 10, SD = ±2.33) (Figure 2). The tuberculosis CX-test detected no cases of drug resistance; however, late growth was observed in the antibiotic quadrants of three plates. Late growth is defined as growth in any of the antibiotic quadrants that takes place after growth in the detection quadrant is observed. This growth is not indicative of drug resistance. The GeneXpert MTB/RIF® testing of nine positive samples that were sent for verification confirmed the presence of *M. tuberculosis* and no rifampicin drug resistance, including the three samples that contained late growth in the rifampicin quadrant. Among patients with a positive tuberculosis diagnosis, three (23%) stated they were also HIV-positive. Younger age (18 to 39-years old) was significantly associated with a positive tuberculosis diagnosis ($Z = -2.2$, $p < 0.03$).

Discussion

Our findings demonstrate that the tuberculosis CX-test can be implemented in rural health clinics in a low-income and high tuberculosis-burden setting, with diagnosis sensitivity comparable to AFB smear. The sensitivity of AFB staining is dependent on the presence of high bacterial load in the patient's sputum and the technical skills of the microscopist. One study estimated that AFB smear microscopy fails to detect *M. tuberculosis* in a third of patients who are later diagnosed by culture, and that AFB smear microscopy is particularly insensitive in high-risk tuberculosis populations

including children and HIV-positive individuals.[5] Moreover, in areas with high incidence of nontuberculous mycobacteria infections, the AFB smear cannot distinguish clearly between nontuberculous mycobacteria and *M. tuberculosis*. However, studies that used the tuberculosis CX-test demonstrated that this test is able to differentiate *M. tuberculosis* from nontuberculous mycobacteria infections with > 99.6% specificity.[6] In this study, there was high concordance between AFB staining and the tuberculosis CX-test, with the latter offering higher sensitivity over AFB staining. Altogether, our results suggest that the tuberculosis CX-test accurately detects drug susceptible tuberculosis in low-income and high tuberculosis-burden settings.

A previous study of 702 patients at a HIV and tuberculosis treatment clinic in Lilongwe found a low (1.4%) prevalence of drug-resistant tuberculosis, with only 10 cases of culture-confirmed isoniazid resistance, and one case (0.1%) each for rifampicin resistance and MDR- tuberculosis.[20] Our results also confirmed the lack of drug-resistant tuberculosis among participants in this study and thus we are unable to report on the tuberculosis CX-test's ability to identify drug-resistant *M. tuberculosis.* Of a subset of positive samples (10%) sent for the GeneXpert MTB/RIF® testing, the tuberculosis CX-test was in agreement with the GeneXpert MTB/RIF®, in that the samples were susceptible to rifampicin. None of the participants who stated that they had taken first-line anti-tuberculosis drugs for susceptible tuberculosis had failed therapy, further suggesting the absence of clinically-significant drug-resistant tuberculosis.

Although there are several commercially-available molecular tuberculosis diagnostic tests on the market,[21] including the GeneXpert MTB/RIF®, which has exceptional sensitivity, specificity, and minimal time to results, GeneXpert tests are currently cost prohibitive in many low-income countries, and especially in countries with a high tuberculosis burden. As a result, the long-term implementation of such diagnostic tests requires ongoing financial support from governments or nongovernmental organisations such as the WHO, the United States Agency for International Development, and the World Bank, among others.

Limitations

Of the 96 valid tests carried out in this study, three (3%) showed late growth in the rifampicin quadrant several days after growth was detected in the detection quadrant; results from the GeneXpert MTB/RIF® test indicated that the three samples were all drug susceptible. There are several possible explanations for the late growth in the tuberculosis CX-test in the drug-containing quadrants days after growth was observed in the detection quadrant. This may be as a result of either a spontaneous mutation allowing bacteria to grow in the drug-containing quadrant, or could be due to breakdown of drugs after a certain period of time. Alternatively, the drug concentration in the quadrants with late growth could be suboptimal (thereby giving a bacteriostatic effect instead of a bactericidal effect), or samples could contain bacteria that are heteroresistant. Indeed, tuberculosis patients may harbour both drug susceptible and -resistant *M. tuberculosis* strains. Although these are minor possibilities, these point out the importance of reading drug resistance results on the same day that the growth is observed in the detection quadrant (drug susceptible) of the tuberculosis CX-test. In the case that growth is observed in the drug-containing quadrants within seven days after being observed in the detection quadrant, the late growth will need to be further confirmed by GeneXpert MTB/RIF® testing or other molecular techniques to rule out heteroresistance or a false negative result.

Other limitations include the small sample size and that the same personnel tabulated both the AFB smear and tuberculosis CX-test. Findings presented herein showed good correlation to AFB smear results; however, AFB microscopy (the only method used in the clinic where this study was performed) has been shown to have poor sensitivity or specificity when compared to either GeneXpert MTB/RIF® or liquid culture (Mycobacteria Growth Indicator Tube [MGIT]) tests. Importantly, the time to detection of a positive tuberculosis CX-test was between 7 and 14 days, which is at least comparable to the time to detection for positive MGIT results for *M. tuberculosis* cultures. Finally, a correlation of smear grades to time to detection of the tuberculosis CX-test would also be useful in providing further comparators of performance.

Recommendations

To determine the accuracy of diagnosis of tuberculosis drug resistance, the tuberculosis CX-test needs to be tested in countries with a high prevalence of drug resistance, where it can be compared to culture-confirmed drug susceptibility testing and GeneXpert MTB/RIF®.

Conclusions

A tuberculosis test that is easy-to-use and inexpensive does not require expensive equipment and highly-specialised staff, and is relatively quick to detect drug resistance (~14 days vs. 86 days), is currently in demand in many low-resource, high tuberculosis-burden countries. The tuberculosis CX-test, with a current production cost of $2.00 USD, could be the necessary tool to fill this gap. While our findings support the accuracy of the tuberculosis CX-test in detecting active pulmonary tuberculosis, a larger study focusing on the ability to detect drug susceptibility is needed before this test could be evaluated for implementation as a front-line drug susceptibility test. A comparison of the tuberculosis CX-test to either GeneXpert MTB/RIF® or MGIT culture would also be useful and is recommended to show the CX-test as an alternative to either of these platforms. In a setting of low drug resistance, this test is less useful than AFB smear, which is cheaper and less labour intensive. Thus, we hypothesise that the greatest utility for the tuberculosis CX-test is an environment with higher prevalence of MDR or XDR tuberculosis.

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Competing interests

The authors declare that they have no financial or personal relationships which may have inappropriately influenced them in writing this article.

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Authors' contributions

A.Z. analysed and interpreted the data and drafted the first version of the manuscript. E.J. acquired the samples, performed the experiments, contributed to data analysis and revised the manuscript. R.K. performed experiments. S.S. and H.V.K. performed the experiments and revised the manuscript. E.K.C., C.K., V.M., J.I.G., A.N., X.J.P., C.E. and S.-H.W. conceived of and designed the study and revised the manuscript. J.J.K. and J.B.T. conceived of and designed the study, performed data analysis and interpretation, and wrote the final version of the manuscript and were both co-senior authors in this manuscript.

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