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Aspects of Tuberculosis and HIV Coinfection in Patients at Ethiopian Health Centers

Sten Skogmar

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DOCTORAL DISSERTATION
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Abstract

HIV and tuberculosis (TB) remain the two most common infectious causes of death worldwide. During the recent decade antiretroviral treatment (ART) has become available for millions of people living with HIV (PLHIV) globally; yet more than half of PLHIV have not yet initiated ART. In order to achieve universal access, further decentralization of care is needed. This is a major challenge to health systems, since access to expertise and adapted diagnostic methods to diagnose and monitor patients on treatment in these areas are lacking. Most notably, two issues face clinicians treating PLHIV in high TB endemic areas; is this HIV-patient also infected with TB? and when should ART be started in patients with both HIV and TB? In two papers we developed clinical scoring algorithms to help answer these questions in areas with restricted access to laboratory facilities. In paper I, a clinical screening algorithm could increase the number of patients with low risk of TB, thereby limiting those in need of further investigations for TB as compared to only using the existing WHO-TB symptom screening algorithm. In paper II, a similar scoring algorithm was developed to assess the level of immunosuppression in patients with HIV and TB coinfection. For more than one third of these patients, severe immunosuppression could be excluded. According to current recommendations, this subset would therefore not be in need of immediate ART initiation during TB treatment providing time to prepare patients for life-long ART.

In a second part of the thesis, we evaluated the impact of TB on the immune system, both on peripheral CD4 cells and neopterin as a marker of immune activation. In paper III, we found that 25% of HIV negative patients with TB had low CD4 cell count (below 500 cells/mm^3). These levels increased until end of TB treatment but not to levels found in healthy controls, suggesting a sustained impact of TB on CD4 cell homeostasis. CD4 cell levels were associated with signs of severe TB (positive sputum smear microscopy, low mid upper arm circumference and bedridden status). In paper IV, neopterin levels were increased in TB patients (23.2 nmol/l) compared to controls (3.8nmol/l), particularly in HIV/TB coinfected patients (54nmol/l). Neopterin correlated to low CD4 cell count, and high neopterin levels were associated with increasing CD4 cell levels during treatment for TB. Yet, neopterin was not suitable as surrogate markers for CD4 cell count. In conclusion, this thesis shows that clinical evaluation of patients should be an integral part in the building of health care for HIV and TB in low-income settings, and that clinically based instruments may be helpful for triage and clinical decisions. Furthermore, we found that TB has an impact on both CD4 cell levels and immune activation. This further emphasizes the need to detect and treat TB in PLHIV.

Key words: HIV, TB, clinical scoring, TB screening, CD4 cell count, immunosuppression, immune activation

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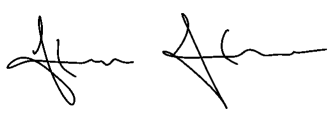
Signature  Date 2015-04-23

Aspects of Tuberculosis and HIV Coinfection in Patients at Ethiopian Health Centers

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To Anna, Ellis and Annie

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Sammanfattning på svenska

Antalet fall av tuberkulos (TB) har ökat parallellt med den globala spridningen av hiv. TB orsakar omkring 1.5 miljoner dödsfall årligen, och är den vanligaste dödsorsaken hos hivpositiva. Under det senaste årtiondet har tillgången till livräddande behandling mot hiv (antiretroviral terapi; ART) ökat kraftigt i låginkomstländer – särskilt i Afrika söder om Sahara. I dessa områden sköts i allt större utsträckning patienter med TB och hiv av sjuksköterskor i primärvården, med begränsad erfarenhet och tillgång till laboratorieresurser att diagnostisera TB och följa sjukdomsförloppet hos hiv-positiva. Bristen på tekniker för att upptäcka TB medför att många hivpositiva med TB inte får behandling, och riskerar allvarlig sjukdom eller död. I låginkomstländer behövs därför enkla men robusta metoder för att bedöma sannolikheten att en person med HIV också har TB; på så sätt kan man välja ut de patienter som verkligen är i behov att vidare testas för TB. Hos hivpositiva med TB måste man avgöra graden av skada på immunförsvaret, eftersom detta styr när ART skall inledas efter det att behandling för TB startats. Hittills har detta endast gått att bedöma med mätning av hjälparceller (CD4) i blodprov; en metod som ofta inte finns tillgänglig för patienter som får behandling i primärvården i afrikanska länder.

Vi har utvecklat nya instrument för att avgöra dessa två frågeställningar – hur kan man avgöra om en hivpositiv patient har TB eller ej; och hur kan man mäta skadan på immunförsvaret hos en hivpositiv person med TB. Båda dessa instrument är konstruerade som poängsystem baserade på symtom och resultat av enkla undersökningar. Instrumenten har utvecklats genom att jämföra dessa resultat med resultat från etablerade metoder att upptäcka TB (med bakterieodling) och mätning av hjälparceller i blod. Undersökningarna har utförts hos deltagare i två stora kohortstudier med patienter som rekryterats och undersökts vid hälsocentraler i centrala Etiopien för att spegla vården som majoriteten av patienter med hiv får idag.

För att kategorisera patienter utifrån risk för TB utgick vi från en existerande algoritm som rekommenderas av WHO (WHO-TB screening algorithm; för personer med antingen hosta, feber, viktnedgång eller nattliga svettningar rekommenderas ”vidare undersökningar” för TB). Med hjälp av vårt poängsystem, som användes i ett andra steg kunde andelen personer i behov av ytterligare undersökning för TB minskas kraftigt; detta medför i sin tur enklare och mer effektiv handläggning av patienter för sjukvården. För bedömning av graden av skada på immunförsvaret identifierades symptom och kliniska markörer som kunde användas tillsammans för att utesluta

allvarlig immunnedsättning. Denna sortering kan vara till hjälp för att välja ut de hivpositiva patienter med TB som inte behöver starta ART kort tid efter start av behandling för TB.

I avhandlingens andra del har två studier utförts som undersöker hur TB påverkar immunförsvaret, både hos patienter med och utan hiv. Det är sedan tidigare välkänt att hiv ökar risken att insjukna i TB; däremot är TBs påverkan på sjukdomsförloppet hos hivpositiva mindre studerad. I den första av dessa två studier undersökte vi hjälparcellsnivåer i blod från 1116 patienter med TB (varav 307 med samtidig hiv). Vi fann markant sänkta nivåer av hjälparceller hos TB-patienter (även utan hiv) jämfört med friska individer – 25 % hade nivåer under referensvärdet. Under behandling av TB observerade vi stigande nivåer av hjälparceller, vilket talar för att TB i sig har en påverkan på immunförsvaret. Vi jämförde också dessa data med uppgifter om patienternas sjukdomsbild. Dessa resultat visar att personer med mer allvarlig TB också hade lägre nivåer av hjälparceller.

I den andra av dessa två studier undersökte vi två molekyler i blod som mått på immunförsvarets grad av aktivering (neopterin och CRP). Syftet med denna undersökning var dels att försöka finna enklare tester för att mäta skadan på immunförsvaret, men framförallt för att bättre förstå mekanismen bakom sänkta hjälparcellsnivåer hos patienter med TB. Vi noterade att TB-patienter, särskilt hivpositiva och de med låga CD4 hade höga nivåer av immunaktivering. Detta tyder på att en överdriven aktivering av immunsystemet är orsak till förlust av hjälparceller vid TB. Eftersom sådan aktivering av immunsystemet anses vara den främsta drivkraften för sjukdomsförloppet hos hivpositiva kan våra resultat också tala för att TB kan leda till snabbare utveckling av sjukdom vid hiv – ett fynd som ytterligare visar på betydelsen av att upptäcka och behandla TB hos hivpositiva personer.

Original papers

I. Balcha T, Skogmar S, Sturegård E, Schön T, Winqvist N, Reepalu A, Jemal ZH, Tibesso G, Björk J, Björkman P. A Clinical Scoring Algorithm for Determination of the Risk of Tuberculosis in HIV-Infected Adults: A Cohort Study Performed at Ethiopian Health Centers. *Open Forum Infectious Dise*ases 2014 Oct 10; 1(3)

II. Skogmar S, Balcha TT, Jemal ZH, Björk J, Deressa W, Schön T, Björkman P. Development of a clinical scoring system for assessment of immunosuppression in patients with tuberculosis and HIV infection without access to CD4 cell testing - results from a cross-sectional study in Ethiopia. *Global Health Action* 2014 Feb 13; 7: 23105.

III. Skogmar S, Schön T, Balcha TT, Jemal ZH, Tibesso G, Björk J, Björkman P. CD4 Cell Levels during Treatment for Tuberculosis (TB) in Ethiopian Adults and Clinical Markers Associated with CD4 Lymphocytopenia. *PLoS On*e 2013 Dec 16; 8(12)

IV. Skogmar S, Schön T, Balcha TT, Jemal ZH, Sturegård E, Jansson M, Björkman P. Immune activation in tuberculosis patients with or without HIV coinfection and correlation to CD4 cell levels in peripheral blood. Manuscript.

Related papers

Balcha T, Sturegård E, Winqvist N, Skogmar S, Reepalu A, Jemal ZH, Tibesso G, Schön T, Björkman P. Intensified Tuberculosis Case-Finding in HIV-Positive Adults Managed at Ethiopian Health Centers: Diagnostic Yield of Xpert MTB/RIF Compared with Smear Microscopy and Liquid Culture. *PLoS One* 2014 Jan 22; 9(1)

Balcha T, Winqvist N, Sturegård E, Skogmar S, Reepalu A, Jemal ZH, Tibesso G, Schön T, Björkman P. Detection of Lipoarabinomannan in Urine for Identification of Active Tuberculosis among HIV-Positive Adults in Ethiopian Health Centres. *Tropical Medicine & International Health* 2014 Jun; 19(6): 734-742

Reepalu A, Balcha T, Skogmar S, Jemal ZH, Sturegard E, Medstrand P, Bjorkman P. High Rates of Virological Suppression in a Cohort of HIV-Positive Adults Receiving Antiretroviral Therapy (ART) in Ethiopian Health Centers Irrespective of Concomitant Tuberculosis. *Open Forum Infectious Diseases* 2014 Jun 19; 1(1)

Balcha T, Skogmar S, Sturegård E, Björkman P, Winqvist N. Outcome of tuberculosis treatment in HIV-positive adults diagnosed through active versus passive case-finding. *Global Health Action* 2015 Mar 27; 8: 27048

Abbreviations

AIDS	acquired Immunodeficiency Syndrome
AFB	acid-Fast Bacilli
ART	antiretroviral therapy
AZT	zidovudine
ATT	anti tuberculosis treatment
BCG	bacillus Calmette-Guérin
BMI	body mass index
CD4 cell	T-cell expressing CD4 receptor
CDC	Centers for Disease Control and Prevention
CMV	cytomegalovirus
CNS	central nervous system
DOTS	Directly Observed Treatment, Short-Course
DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
EFV	efavirenz
ELISA	enzyme-linked immunosorbent assay
EPTB	extrapulmonary tuberculosis
HIV	human immunodeficiency virus
IFN	interferon
IPT	isoniazide preventive therapy
IRIS	immune reconstitution inflammatory syndrome
LED	light-emitting diode

LNTB	lymph node tuberculosis
MDR	multi drug resistant
MTB	Mycobacterium tuberculosis
MUAC	mid upper arm circumference
NNRTI	non-nucleoside reverse-transcriptase inhibitor
NPV	negative predictive value
NRTI	nucleoside reverse transcriptase inhibitor
OD	optical density
OHL	oral hairy leukoplakia
OI	opportunistic infection
PCR	polymerase chain reaction
PEPFAR	President's Emergency Plan for AIDS Relief
PLHIV	people living with HIV
PPV	positive predictive value
RNA	ribonucleic acid
ROC	receiver operating characteristics
SIV	simian immunodeficiency virus
TB	tuberculosis
TH	T-helper [cells]
TNF	tumor necrosis factor
TST	tuberculin skin test
UNAIDS	United Nations Program on HIV/AIDS
VCT	voluntary counseling and testing
WHO	World Health Organization
ZN	Ziel-Neelsen stain

Introduction

> At the head of the most consequent symptoms, as one of the most obvious, emaciation or wasting of the habit may justly be mentioned. It is indeed true, that, even from the beginning of every species of phthisis, the usual fulness or plumpness of the patient is somewhat diminished. But after the hectic fever has subsisted for some time, this emaciation is very much increased. It is obvious, on examination of any part of the body. It evidently appears from clothes, which were before tight, becoming too wide, and it is still more certainly demonstrated by the loss of weight. But in no part is it more conspicuous than in the face. This is so much the case, that the *facies Hippocratica*, as it has been called, where every bone of the face is prominent, is, perhaps more frequently observed in pulmonary consumption than in any other disease. But when the trunk of the body is examined, the state of the spine shews projection of the bones to as great a degree, in parts remote from the face. In short, in the last stage of phtisis, the patient often becomes as it were a living skeleton."
>
> - Andrew Duncan [1]

This account of end-stage phthisis, or tuberculosis (TB), could well be a description of a patient seeking care at a primary health center in Ethiopia and other low-income settings today. The capacity of the immune system to contain the tubercle bacillus is profoundly limited by human immunodeficiency virus (HIV), which allows unrestrained proliferation of TB and development of disease within the host [2]. HIV can also mask early symptoms of TB due to the lack of adequate immune response, and patients with both TB and HIV in resource-limited settings often present with advanced disease that largely resembles the two-hundred-year-old description provided by Andrew Duncan. In this situation, with a severely debilitated immune response and widespread TB, combined with limited resources for diagnosis and monitoring of patients, mortality is high despite improved access to effective treatment of both diseases.

Since it was discovered, HIV has infected an estimated 75 million people [3] and the World Health Organization (WHO) estimates that there are presently around 35 million people are living with HIV (PLHIV) today [4]. In sub-Saharan African, HIV is one of the main reasons for the increase in TB, and TB is considered to be the most prevalent opportunistic infection and also the most common cause of AIDS related death in PLHIV [5]. A review of autopsy studies performed on HIV-positive patients

who died in sub-Saharan Africa up to 2010 showed that TB was detected in 21-54% of cases and was considered to be the main cause of death in 32-45% [6].

As with many infectious diseases HIV and TB overwhelmingly affect people living in low-resource areas, particularly in sub-Saharan Africa. This situation is especially challenging when considering the chronic nature of HIV and the long-term treatment required for TB, because both these diseases require infrastructure to provide extended treatment, as well as commitment from the health organizations launching interventions. The distribution of antiretroviral therapy (ART) to low-income areas that was initiated in 2003 has proven to be a success with millions of lives saved [7], and this has effectively quenched previous concerns that it would be impossible to provide ART in low-resource settings [8,9]. Also, the ART programs can serve as models for the management of other chronic diseases in such settings.

However, approximately half of PLHIV in need of ART are still without treatment and there are still areas with even lower coverage [7]. Furthermore, it is mainly the patients who are most readily accessible who have started ART. Those living in more remote areas have little or no access to diagnostic tools, educated staff, and other infrastructure required to provide well functioning ART. New strategies are needed to reach these patients, such as integration of ART into the primary health care system. It should also be borne in mind that the patients currently on ART may be confronted by other obstacles in the future that might compromise the effect of the current treatment, such as increasing rates of antiretroviral resistance [10], retention in care (a meta-analysis showed that after 2 years 60% were retained in care in sub-Saharan Africa [11]) as well as the inability to manage opportunistic infections.

The decentralization with an increasing transfer of ART to peripheral heath systems with limited resources was the major incentive for the present research and the development of two clinical scoring algorithms (Papers I and II). Patients treated at primary care centers were selected with the intention of creating two cohorts that best reflected the type of care the majority of these patients are receiving. Managing the complex nature when resources are limited requires simplification to achieve further decentralization. Two major problems face the clinicians caring for these patients in this context (i) considering whether an HIV patient might also have TB and in that case how it could be identified; (ii) deciding when to start ART in a patient with HIV/TB coinfection. For clinicians who are addressing the mentioned issues, clinical algorithms, used alone or in conjunction with existing diagnostic measures are potentially highly beneficial, because they require a minimal amounts of technical equipment and staff expertise.

It is well known that HIV, even in early infection markedly increases the risk of developing TB. From an immunological perspective, this is attributed to the inability to contain TB bacteria within granulomas and preferential depletion of TB-specific CD4 cells in HIV patients [12,13]. TB is also considered to influence HIV-disease

progression [14], although less is known about the mechanisms responsible for this effect. Furthermore, some studies performed primarily in West Africa and Tanzania have shown that TB is associated with a low peripheral CD4 cell count [15,16], but neither the mechanisms nor the clinical significance of this finding are fully understood. As described in Papers III and IV, the present research examined immunological aspects of HIV/TB coinfection with the aim of increasing available knowledge about the extent to which TB affects HIV disease progression.

HIV

Epidemiology of HIV

In 1981, cases of pneumocystis pneumonia and Kaposi's sarcoma and unexplained lymphadenopathy occurred in previously healthy homosexual men on the west and east coasts of the United States [17,18]. It was soon became clear that these cases represented a new concentrated epidemic characterized by pronounced cellular immunodeficiency and development of rare opportunistic infections (OI's) [19], and the condition was given the name acquired immunodeficiency syndrome, or AIDS in 1982. Reports of AIDS cases emerged in 1982 from patients with hemophilia, women and children from separate states and from Haitian immigrants suggesting that this novel epidemic was caused by a transmittable agent [19,20]. The etiological agent was identified as a previously unknown retrovirus in 1984 [21] and was given the name HIV in 1986. In 1985 ELISA-based blood tests were developed, and the genome of HIV was sequenced. Thereafter, the search began to find molecules targeting different steps in the viral life cycle for therapeutic use.

Cases of AIDS were identified in Zaire in 1984, and a study published in 1986 indicated that the condition was 15–30 times more common in that country than in the United States [22]. Late in the 1980s, it had become increasingly evident that AIDS was also widespread in other parts of Africa, where it was initially called "slim disease" [23]. The prevalence of the disease approached 30% in regions such as rural Uganda by the end of the 1980s [24] and 34% in rural South Africa at the end of the 1990s [25].

It has been estimated that HIV has infected 78 million people globally, and that 35 million are currently living with the disease [26]. There is unequal distribution of PLHIV, with more than two thirds (71%) of them (i.e., about 25 million people) living in sub-Saharan Africa [27] (see Figure 1). There were 2.1 million cases of new HIV-infections in 2013, which represents a 38% decline from 2001 and approximately half (48%) of the people infected were actually aware of their status. Approximately 1.5 million people die from this disease every year, making HIV the most common cause of death caused by an infectious agent worldwide [27].

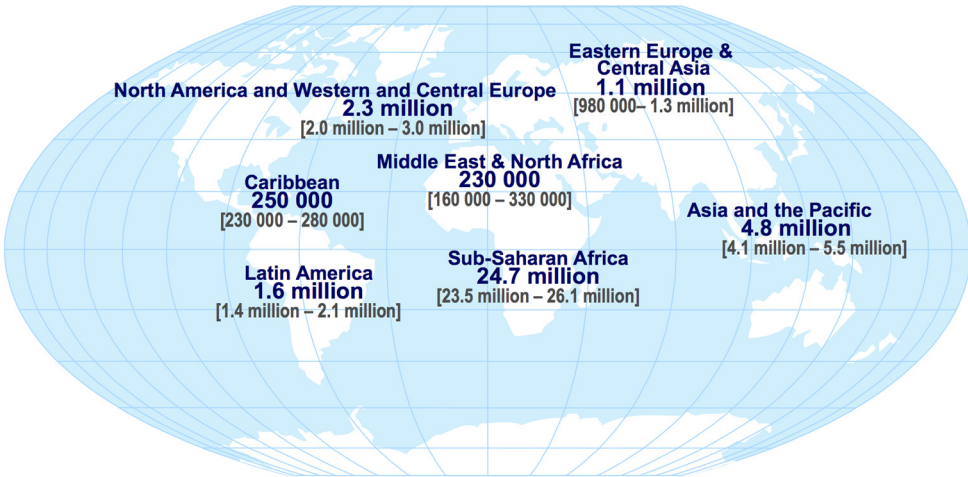

Figure 1:
Estimated prevalence of HIV in adults and childen in 2013. Credit UNAIDS 2014.

The human immunodeficiency virus

HIV is a bilipid enveloped RNA virus that exists in two major types designated HIV-1 and HIV-2. HIV-1 is spread globally and is by far the most prevalent, whereas HIV-2 is geographically restricted mainly to West Africa. Henceforth in this text, HIV will refer to HIV-1, because that is the predominant type of the virus found in Ethiopia. HIV contains genomic RNA and viral proteins, the most important of which are a viral protease, a reverse transcriptase and an integrase [28]. HIV is a retrovirus, which means that it carries its genetic information in the form of RNA that must be transcribed into DNA to enable replication. This is achieved through attachment of the virion to CD4 receptors and the chemokine receptors CXCR4 and CCR5 [29] on the surface of the host cell, which results in fusing of the membranes and injection of the RNA into the cell matrix. Inside the host cell, the RNA is transcribed into double-stranded DNA by the viral reverse transcriptase. This linear DNA then enters the nucleus and fuses with the host DNA with the help of integrase. New virus particles are transcribed through replication mediated by the host cell DNA polymerase, and, after assembly, these particles bud off the cell surface via the protease, and are ready to attach to other cells. In an HIV-infected human host, in addition to the activated circulating CD4 cells, there are also latent reservoirs of infected cells in the body in “silent” or “quiescent” CD4 cells and other immune cells, which is why HIV cannot be cured by ART. Furthermore, HIV has a rapid mutation rate, because the reverse transcriptase is prone to errors and viral generation time is short [30]. This complicates the development of

a vaccine [31] and explains the rapid emergence of drug resistance in HIV patients given monotherapy [32].

Pathogenesis

CD4+ T helper cells (CD4 cells) are the main target of HIV, although several other cell types can also be infected, such as dendritic cells and macrophages [33,34]. CD4 cells orchestrate the adaptive immune response to pathogens through cytokine mediators that elicit responses in other immune cells to clear or contain microorganisms entering the host and to orchestrate tumor immunology. Thus far, at least four distinct subtypes of CD4 cells derived from the naïve CD4 T cells have been elucidated. In the 1980s, it was found that Th1 CD4 cells predominantly direct the immune response to intracellular pathogens, and Th2 CD4 cells target extracellular helminthic infections and are important in the induction of allergic conditions and asthma. Since then, two additional major subtypes have been identified [35]: Th17 CD4 cells which mediate immune response to extracellular bacteria and fungi; T-regulatory cells (Tregs), which play a critical role in regulating the immune response to avert tissue damage.

HIV disease progression is characterized by increasing loss of CD4 cells driven by immune activation and viral replication in peripheral blood; this usually occurs gradually over the course of several years, although there is an early massive depletion in the gastro intestinal tract in acute HIV infection [36–38]. Viral replication proceeds continuously but is prominent during acute infection and late in the disease. Eventually, the loss of adaptive immunity renders the host susceptible to acquiring and succumbing to opportunistic infections (see Figure 2).

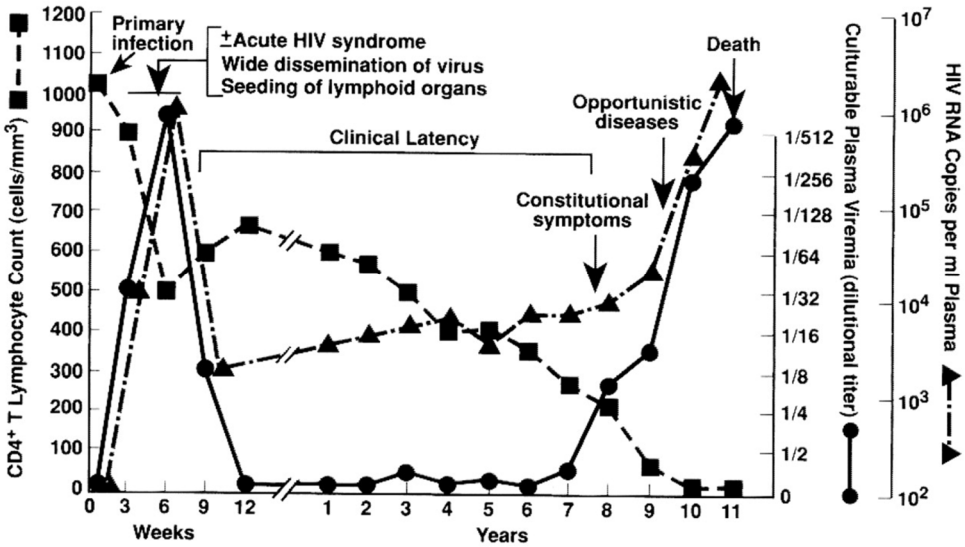

Figure 2:
Overview of CD4 cell count and viral load during the natural course of HIV infection. Reprinted from Pantaleo et al [39] with permission. Copyright Massachussetts Medical Society.

Several mechanisms behind the successive depletion of CD4 cells in HIV infection are being investigated. It appears that such depletion entails apoptosis of infected cells [40] but in most cases uninfected cells or "bystander" CD4 cells are destroyed [41,42]. In fact, only as little as 0.1%-1% of circulating CD4 cells are actually infected with HIV [43,44], and the remaining cells are thought to be destroyed by indirect means as the result of immune activation [41]. The term immune activation is a broad expression referring to a state including activation, proliferation, and death of immune cells, as well as the release of soluble molecules from such cells [45]. Immune activation is physiological when it occurs in other viral infections whereas when it arises in response to HIV, it ensures constant viral replication and depletion of immune cells. Immune activation in chronic HIV disease is thought to be multifactorial and to be induced by a number of events that occur during the course of infection [46]. HIV-replication per se causes a chronic activation. However, a more important aspect is probably microbial translocation, which involves preferential depletion of Th17 CD4 cells of the gut mucosa primarily during the acute infection; this leads to increased permeability of bacterial components from the gut to the blood and in turn contributes to chronic immune activation [47]. Furthermore, there is evidence that concurrent viral infections such as cytomegalovirus (CMV) can contribute to chronic immune activation [46,48].

Acute opportunistic infections such as TB have been shown to increase viral load both in vitro and in vivo, and a possible mechanism for this effect is related to the increased cellular immune response elicited by the concurrent infection [14]. Epidemiological

data concerning the impact of TB on HIV disease progression are divergent, although it should be noted that the studies providing such information used different outcome variables and, more importantly, they were carried out in the pre-ART era [49]. Today, it is difficult to investigate the effect of TB on the natural history of HIV due to the availability of effective treatments.

The level of immune activation in PLHIV has been assessed using several different immunophenotypic and serum markers, such as expression of CD38, CD25, CD69, and CD70 expression on T-cells, and levels of neopterin, TNF and β2-mikroglobulin in serum [50,51]. Neopterin is of particular interest, because it has been demonstrated to be strongly correlated with HIV-disease progression [52]. It is produced by macrophages upon stimulation with IFN-γ released from activated T-cells and has been shown to contribute to oxidative stress [53]. Neopterin enables cheap, stable and reliable measurement of immunosuppression [54]. Indeed, neopterin can be measured in both serum and urine and there is even a dipstick assay which makes it a suitable analyte in peripheral settings [55].

Clinical presentation

The clinical course of HIV disease is characterized by increasing susceptibility to opportunistic infections and malignancies over the course of several years. Early studies indicated that the median time from primary infection (seroconversion) to development of AIDS was approximately 10 years in Western and African materials [56,57]. However, there are large intra-individual differences, ranging from rapid progressors [58] to elite controllers [59], and also considerable individual variation in clinical spectrum, due to differences in exposure to opportunistic infections. Initial infection can be asymptomatic or present with what is called acute retroviral syndrome, which initially may present with skin rash, sore throat or oral ulcerations, fever and enlarged lymph nodes. This is followed by a longer period of up to several years when the patient is largely asymptomatic or can exhibit persistent generalized lymphadenopathy. During this period, there is continuous viral replication of the virus at a viral "set point" albeit at a lower lever than during the acute retroviral syndrome, and there is a slow decline in peripheral CD4 cells [60]. At the end stages of infection, the patient develops AIDS, the clinical syndrome characterized by a debilitated immune response and an array of opportunistic infections and malignancies.

Diagnosis of HIV-infection

Diagnosis of HIV infection is made by detection of antibodies, the detection of the p24 antigen, or viral RNA or DNA. The most common method entails antibody detection with enzyme-linked immunosorbent assay (ELISA). This technique is sensitive in chronic HIV, but antibodies against the virus do not develop until about 3-4 weeks after initial infection. Therefore, an assay detecting the p24 antigen of the virion is used to reduce the time elapsed before diagnosis by about a week. The earliest possible detection that can be achieved is by polymerase chain reaction (PCR) analysis of HIV RNA, but even this method can not identify infection until there is active replication of the virus which occurs 10-14 days after infection [61]. Rapid diagnostic tests have been developed to detect antibodies and are widely used. Ideally, if the result of these assays are positive they should be confirmed by Western Blot assay. However, in resource-limited settings, diagnosis is based on two to three rapid tests as recommended by the WHO. An independent evaluation has shown that the rapid tests used in Ethiopia have a reasonable sensitivity of 97.3% and a specificity of 98.8% [62], however there have also been recent reports of false positive rates of up to 7.7% with the Ethiopian HIV-diagnostic procedures [63].

Determination of degree of immune suppression

Immunosuppression is a heterogeneous term that refers to a clinical condition that can range from asymptomatic to severe illness, and involves the risk of contracting infections and malignancies that do not normally affect people with a healthy immune system. The clinical gold standard for assessment of immune status of a patient with HIV relies on measurement of CD4 cell count by flow cytometry. A continuous decline in CD4 cell count has proven to be strongly correlated with both death and the risk of contracting the opportunistic infections and malignancies specific to HIV disease [64,65]. Though, CD4 cell count has been difficult to obtain for many patients in resource limited settings due to a lack of flow cytometers, staff trained to operate such equipment or available reagents required to perform the analysis, although this situation is changing in some areas by the introduction of point-of-care devices [66]. To address this gap, the WHO in 1990 devised a clinical staging system (which was revised in 2005) to be used in areas with no available laboratory equipment [67]. This system has been widely applied in resource limited settings, although it has not been validated or been subjected to systematic studies. The staging system is used to clinically categorize the severity of a patient's disease over the spectrum of the impaired immune response.

Stage I HIV infection is asymptomatic or can present with persistent generalized lymphadenopathy. Clinical stage 2 is characterized by moderate weight loss and recurrent respiratory tract infections as well as skin lesions (herpes zoster, papular puritic eruptions, angular cheilitis, seborrheic dermatitis and fungal infections of the fingers) and oral lesions (recurrent oral ulcerations). In stage 3, there is severe weight loss, unexplained chronic diarrhea lasting >1 month and unexplained persistent fever for >1 month. Additional characteristics include oral lesions (oral candidiasis, oral hairy leukoplakia and acute necrotizing ulcerative stomatitis, gingivitis and periodontitis) and unexplained anemia, neutropenia and thrombocytopenia. Pulmonary tuberculosis is also a defining condition in this stage.

In stage 4, patients can exhibit severe weight loss or what is known as HIV-wasting syndrome (entailing weight loss >10%, diarrhea, and chronic weakness, as well as documented fever ≥30 days not attributable to any causes other than HIV [68]). Opportunistic infections appear, including the following: pneumocystis pneumonia; candida infection of the esophagus, trachea, bronchi, or lungs; cryptosporidiosis; visceral herpes simplex; CMV infections in organs than the liver, spleen, or lymph nodes; recurrent non-typhoidal salmonella septicaemia; visceral leishmaniasis; CNS toxoplasmosis; extrapulmonary TB and disseminated non-TB mycobacterial infection. Kaposi's sarcoma, invasive cervical carcinoma, and lymphomas (cerebral or B cell non-Hodgkin type) are also defining of stage 4 HIV disease (see Figure 3).

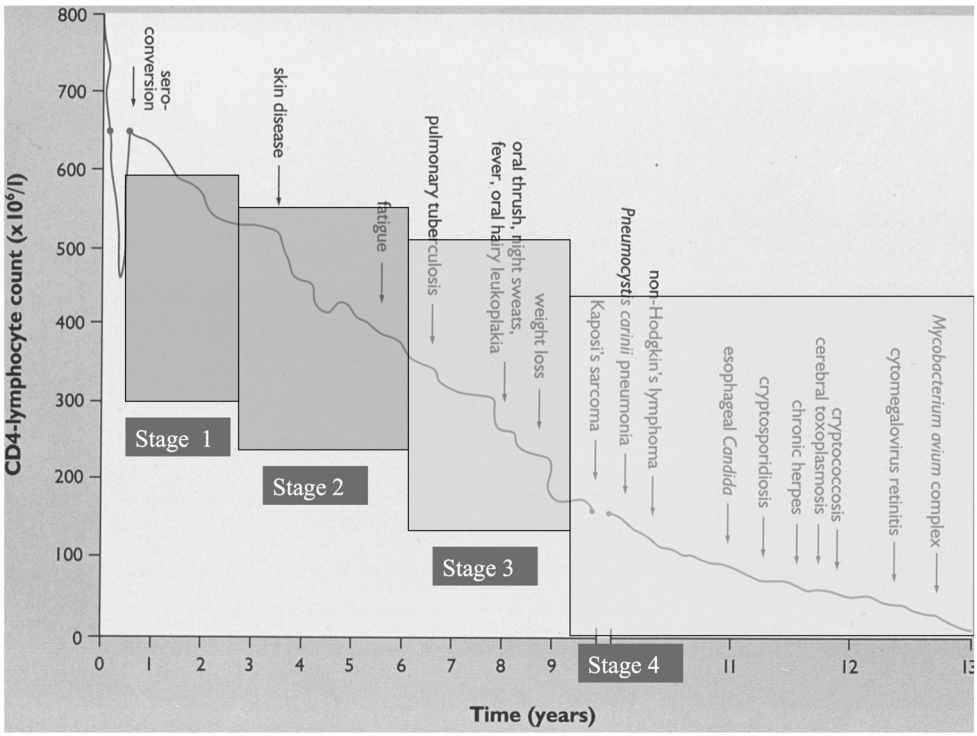

Figure 3:
Staging of HIV disease over time and associated opportunistic infections and conditions.

Antiretroviral therapy

In 1987, zidovudine (AZT) was the first drug to be approved by the US Federal Drug Administration (FDA) for treatment of HIV, and since then new drugs have been developed at a remarkable pace, with a total of 25 compounds licensed up to 2008 [69]. Initial trials using mono- and dual therapy achieved only incomplete viral suppression due to selection of drug-resistant strains, but the efficacy of triple combination regimens for HIV was demonstrated at the XI international Conference on AIDS in 1996 [70] and convincingly shown in several studies [71–74]. Triple combination therapy is based on blocking of viral transcription [75] which allows for the restoration of the immune system, and it has dramatically improved the life expectancy [76–78]. However, inasmuch as there are viral reservoirs of quiescent infected cells [79], the treatment cannot eradicate the virus, and viral replication resumes as soon as treatment is stopped. Strict adherence to treatment is also necessary (i.e., 95% of doses must be taken on time to avoid development of resistance) [80], and some drugs can interact to increase or

decrease the drug levels and thereby result in monotherapy or toxicity. For instance, it is known that in combined treatment of HIV and TB, the commonly used non-nucleoside reverse transcriptase inhibitor (NNRTI) nevirapine interacts with rifampicin to cause hepatotoxicity, and therefore it is preferable to use efavirenz instead of nevirapine [81].

The indication for initiating ART has been changed several times since triple combination therapy was introduced. In 2002, the WHO recommended that ART be started at a CD4 cell count <200 cells/mm^3 [82] in patients in resource-limited settings. In 2006, this recommendation was altered slightly to indicate that treatment should be considered and initiated during the interval of 200–350 cells/mm^3 [83]. In 2010 the guidelines were changed again to state that ART should be started in patients with a CD4 cell count of <350 cells/mm^3 irrespective of the WHO HIV stage [84]. The consolidated guidelines currently in use were formulated in 2013, and they recommend that priority be given to patients with a CD4 cell count of <350 cells/mm^3, but that treatment should be initiated in all patients with a CD4 cell count <500 cells/mm^3 [85].

In addition to HIV infection, it is essential to treat symptomatic or asymptomatic opportunistic infections, such as *Pneumocystis jirovecci* infection, cryptococcosis and tuberculosis because ART cannot cure these coexisting conditions. Also, initial deterioration of patients can occur during ART in what has been called immune reconstitution inflammatory syndrome (IRIS), which is an inflammatory condition characterized by a paradoxical worsening of preexisting infectious conditions after the start of ART [86,87]. IRIS is believed to be due to an increase in the inflammatory response to pathogens as the immune systems recovers. The most common pathogens that may elicit this inflammatory condition are mycobacteria, herpes viruses and fungal infections such as cryptococcal meningitis [88]. TB has been recognized as a major cause of IRIS (which is then termed TB-IRIS) and TB-IRIS has been shown to be prevalent in 11-45% of patients starting ART [89]. Initiation of ART can also unmask TB with signs of TB develops within 3 months of starting ART [90]. Even if studies conducted thus far have not correlated this condition with increased mortality, it remains to be determined what the role of TB-IRIS has on treatment outcome and adherence in resource-limited settings.

Global response to HIV

In the 1990s, it became clear that HIV was treatable, but high costs still kept the majority of PLHIV in resource limited settings from access to treatment [91]. In addition, there was concern that treating HIV in low-resource areas was simply not possible due to the lack of health care infrastructure, and that a large-scale intervention

could even lead aggravate the epidemic by leading to development of drug resistant strains and "ART anarchy" [8,9]. However, by the end of the 1990s it became increasingly apparent that the prevalence of HIV in sub-Saharan Africa was reaching disastrous proportions, with a prevalence approaching one in three adults in some areas [25]. Moreover, the AIDS pandemic was beginning to have detrimental social and societal impact, thus calling for prompt action to address a decreased life expectancy, massive numbers of children without parents and the threat of impending economic collapse and rising political instability.

Through efforts of the joint United Nations Program on HIV/AIDS (UNAIDS), the Drug Access Initiative was launched in 1997 which led to the piloting access to ART in Uganda and Côte d'Ivoire in 1998 and at about the same time also in rural Haiti through the HIV Equity Program launched by the non-governmental organization Partners in Health [92]. Brazil had already initiated nationwide treatment for PLHIV in 1996 and started production of generic antiviral drugs from 2001 [93]. Encouraged by the success of these early treatment efforts, UNAIDS collaborated with the World Bank and major drug companies, to reduce costs and provide affordable treatment to more patients in resource-limited settings, mainly in sub-Saharan Africa [94].

In 2003 the WHO launched in 2003 the initiative designated "3 by 5" [95] with the objective of providing free ART to three million PLHIV living in low- and middle-income countries by 2005. This was intended to be a stepping stone towards the overall target of ensuring universal access to ART for all PLHIV, as had already been stated by Kofi Annan in his inaugural speech at the first African summit on HIV/AIDS, tuberculosis, and other infectious diseases held in 2001 in Abuja, Nigeria. The funding for the 3 by 5 initiative was established mainly through the Global Fund which was also proposed at the summit in Abuja and through the Presidential Emergency Plan for AIDS Relief (PEPFAR) initiated by the United States in 2003 [91].

The 3 by 5 initiative resulted in approximately 1.3 million people being on ART by the end of 2005, thus less than half of the projected goal. Nevertheless, this program was successful in that it proved the feasibility of universal access to ART, and it showed that this intervention averted between 250,000 and 350,000 deaths [96]. The number of people on treatment has subsequently steadily increased. In 2013 alone, 2 million people were enrolled in ART care, and in 2014, 13.6 million people, or about 37% of infected in the Africa region, were on ART [7] (see Figure 4).

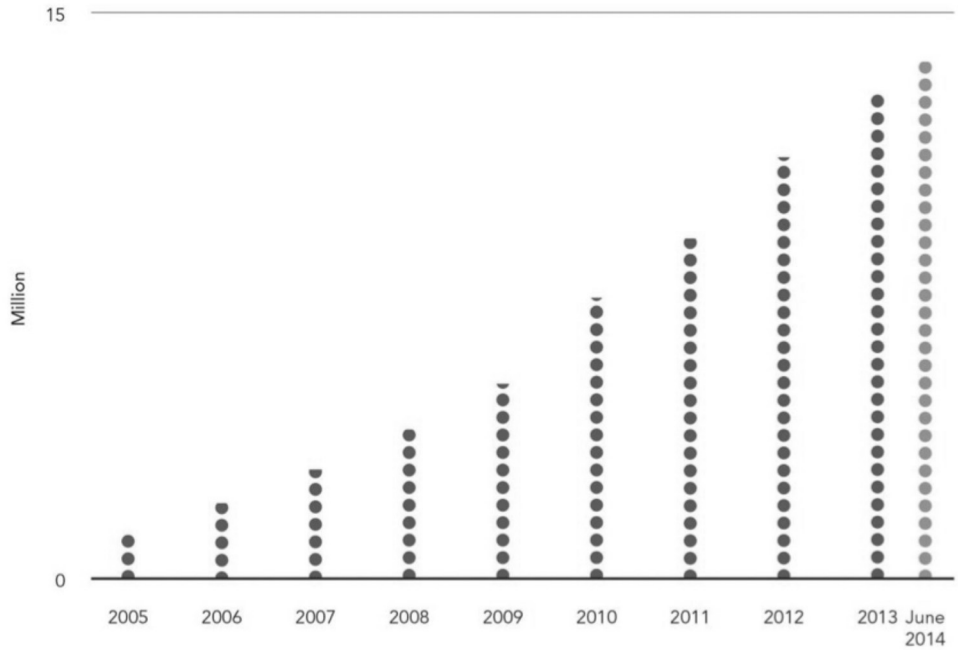

Figure 4:
People receiving ART globally between 2005 and 2014. Credit UNAIDS 2014.

This massive rollout of ART has required decentralization of the care given to these HIV patients, which means that it is increasingly being provided in primary care settings rather than at hospital clinics. Due to this increased access to ART it is necessary for the decentralization to encompass even the most remote areas. Thus the large-scale introduction has been a tremendous success in terms of numbers of patients currently receiving the treatment. Notwithstanding, this poses several challenges related to the availability of the resources needed for monitoring, for diagnosing opportunistic infections and for keeping patients on treatment.

TB

Epidemiology of TB

In contrast to HIV, TB is an ancient disease. The causative agent *Mycobacterium* tuberculosis (Mtb), has been estimated to be 3 million years old and probably originated in East Africa [97]. Evidence of infection in humans include identification of Mtb DNA in Egyptian mummies [98] and it is highly likely that a clinical condition called phthisis (or consumption) described in the Aphorisms of Hippocrates in ancient Greece was an early depiction of TB [98]. The pathogenesis of the disease was first outlined in 1819 by the French pathologist Laennec. Although controversies regarding the nature of TB as a genetic or a transmissible disease continued until 1882, when Robert Koch convincingly established that the etiology involved Mtb. This breakthrough was based on the discovery of a technique using a stain that could impregnate the waxy cell walls of the bacteria thus render them visible under a light microscope [99].

In the 19th century, TB was common in Europe and in the United States, causing up to 1,000 deaths per 100,000 persons annually [98]. A gradual decline was seen in the Western world from the 1920s, which is usually attributed to better living conditions and less crowding, and to a lesser extent also to the introduction of BCG vaccination and use of anti-mycobacterial agents after the discovery of streptomycin in 1944 [100]. However, on a global scale the rates did not decline and in fact showed a dramatic increase in the latter part of the 20th century, primarily due to the emergence of HIV. Shortly after HIV was established as the cause of AIDS, increased numbers of TB cases were seen in the United states [101,102], and a study indicated that TB had increased in 20 African nations from 1985 to1992 on average by 7.7% annually [103]. The rise in the incidence of TB in some countries with a high burden of HIV is illustrated in Figure 5.

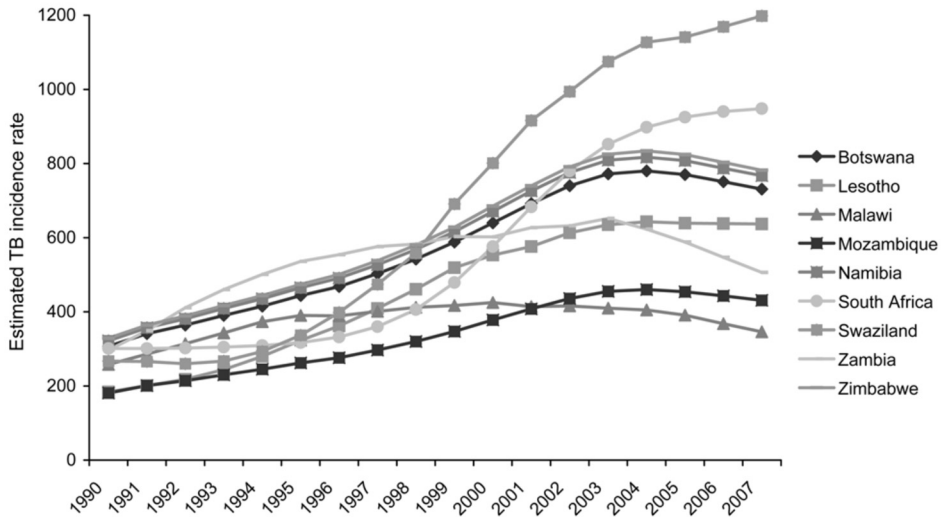

Figure 5
The incidence rate of TB has increased in countries with a high burden of HIV. Reprinted from Lawn et. al. [104] with permission of the International Union Against Tuberculosis and Lung Diseases. (Copyright The Union).

According to the WHO global tuberculosis report, the total number TB cases worldwide was 9.0 million in 2014 [5]. The same report also specified that incidence fell by approximately 1.5% yearly between 2000 and 2013 and mortality has decreased 45% between 1990 and 2013. However TB still causes 1.5 million deaths annually (360,000 of those cases known to be HIV positive in 2013), and hence TB is second only to HIV as the infectious disease with the highest mortality.

Together, the following characteristics represent a successful approach from the perspective of the TB bacteria: the slow rate at which a host is killed, the latent capabilities of the microbes within the host, and the primary focus in the host's lungs, which enables an effective route of transmission. Moreover, it is estimated that one third of the global population is currently infected with a latent form of Mtb, and hence prospects of eradication of this pathogen seem implausible.

Mycobacterium tuberculosis

Mycobacterium tuberculosis (Mtb) is an acid fast, slender rod-shaped aerobic bacterium. It has a lipid-rich cell wall that contains mycolic acid, which explains why this microbe is resistant to detergents and grows slowly in culture [105]. Mtb belongs to the genus

Mycobacterium and is grouped in the *M. tuberculosis* complex together with other genetically similar and human pathogenic strains, primarily *M. africanum* and *M. bovis* [106].

Pathogenesis

Early events in infection

Mtb is spread in aerosol form by the coughing of an infected individual [107] and enters the airways and alveoli of a new host, where neutrophils and alveolar macrophages constitute the initial line of defense [108]. It was recently reported that in many people this first innate immune defense can actually eradicate the bacteria without the development of cell-mediated immunity [109]. Bacteria that do reach the alveoli are engulfed primarily by alveolar macrophages, but also by dendritic cells. Upon entering a macrophage, Mtb is contained in a phagosome awaiting destruction in the lytic environment created by fusion of the phagosome with lysosomes. However, Mtb can prevent this fusion, which at this stage allows the bacterium to survive within the macrophage [105]. Infected macrophages secrete TNF-α, IL-12, IL-1, and IL-6, as well as various other chemokines, which function in concert to recruit and partially activate other cells, such as neutrophils, monocytes, lymphocytes, and dendritic cells [110]. Therefore, the first events in innate immunity are crucial both to accomplish initial control of the bacteria and to elicit a cell-mediated response in cases in which control has not been achieved. However, Mtb also takes advantage of the early inflammatory response as more immune cells are recruited, which is suggested to provide additional cellular niches for expansion rather than lead to eradication, and may also aid dissemination of the bacteria as infected host cells migrate from the initial focus [111]. Following the first stages of innate immunity, dendritic and neutrophils containing Mtb and Mtb-associated antigens move to regional lymph nodes to present the antigens to lymphocytes.

Adaptive immunity

Characteristic of adaptive immunity to Mtb is its delayed onset compared to other bacterial infections. Tuberculin skin test (TST) reactivity is delayed for up to 4–6 weeks in human subjects [112], and in mice Mtb antigen-specific T-cell responses are first observed in regional lymph nodes 11–14 days after aerosol inoculation [111]. The reasons for such delay is not clear, although it is possible that Mtb blocks apoptosis of neutrophils, and hence those cells fail to present antigens to T-cells [113] or upregulate

IL-10, which dampens the immune response [114]. When antigens are presented to CD4 cells by dendritic cells in the lymph nodes, they undergo clonal expansion and are recruited to the site of infection; in the presence of intracellular pathogens such as Mtb, the CD4 cells develop to the Th1 subset and secrete IFN-γ [115]. IFN-γ, in concert with TNF-α, activates macrophages to enhance their bactericidal capability [116] and thereby arrest the expansion of bacteria. Adaptive immunity results in containment of the bacteria in a characteristic structure called a granuloma. In short, in response to activation, infected macrophages fuse with each other to form giant cells that, together with fibroblasts, monocytes, and lymphocytes, surround a necrotic center in this characteristic structure (see Figure 6).

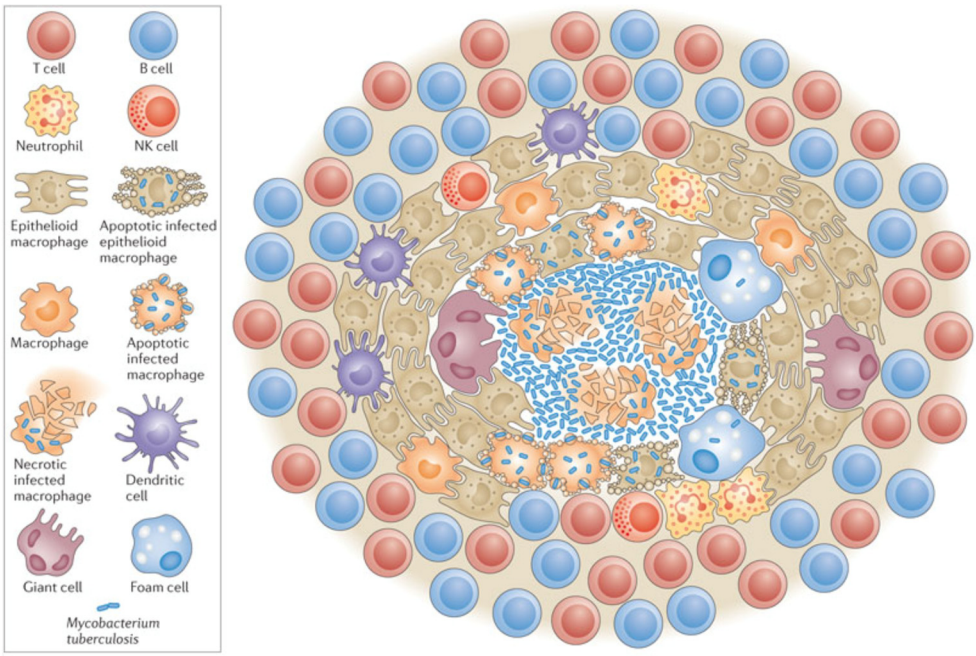

Figure 6:
Structure of a granuloma. Reprinted from Ramakrishnan et. al. [117] with permission (Copyright Nature Publishing Group).

The granuloma creates an environment in which Mtb is unable to divide due to a lack of oxygen and a low pH, but can remain dormant for extended periods of time. It may be that this "dormant state" actually entails balanced growth and death of the bacteria within granulomas, with the microbes kept at bay by an array of host responses [118]. If this fine-tuned balance is disturbed by conditions associated with a hampered immune response, such as young or old age or particularly HIV infection, it may cause

the granulomas to grow larger or disintegrate, resulting in dissemination of the bacteria into the bloodstream or the airways and causing symptoms characteristic of active TB. Thus the pathogenesis and presentation of TB rely largely on the host's immune response to Mtb infection rather than on virulence factors and toxins common to most other bacterial infections.

Interaction between HIV and TB

It is clear that HIV and TB have reciprocal interactions that benefit the proliferation of both pathogens [2]. HIV raises the risk of TB approximately tenfold. However, this cannot be entirely explained by a low CD4 cell count, because the increase is seen even during early stages of infection [119], and the risk is not completely eliminated (albeit is lowered considerably) by ART [120].

The growth and proliferation of Mtb can be augmented during several stages of HIV infection. Increased survival of Mtb has been observed in infected macrophages [121], and it is also clear that the lack of cell mediated immune system plays an essential role in the increased risk of TB seen in HIV patients. The importance of CD4 cells is illustrated by an investigation showing that both progression to active TB after exposure to Mtb and reactivation of latent TB occurred to a higher degree in macaques depleted of CD4 cells than in control animals with normal CD4 cell levels [122]. In human HIV patients with severely depressed CD4 cell counts, granulomas are ill-formed, necrotic, and multibacillary [2]. Furthermore, preferential depletion of Mtb-specific CD4 cells during HIV infection has been observed [12,123]. Conversely, TB has been found to increase the risk of both HIV-related death and other opportunistic infections [124]. This has been explained by increased viral loads [124–126] in coinfected patients, but it is also possible that other mechanisms of immune activation related to TB can contribute to this exacerbation of HIV.

Disease presentation

TB is commonly categorized as being either latent or active, although this is a simplification of a spectrum between the two forms [109,127]. Active TB can present as primary-progressive disease, which is relatively rare, develops within a few weeks after exposure, and mainly affects untreated HIV patients or children younger than 5 years of age. Reactivation of latent TB can also occur at a life-time risk of 5–10% in those infected [109]. The most common presentation of active TB in an immune competent host is pulmonary TB, which is characterized by cough and low-grade fever, and occasionally also hemoptysis and weight loss [128]. Active TB can also involve

extrapulmonary manifestations resulting from hematogenous dissemination. The symptoms and signs depend largely on the site of infection and the immune reaction triggered by the bacteria, and TB can arise in any location (e.g., pleura, lymph nodes, central nervous system, bones, and joints) and can also occur as a more diffuse spread to internal organs that is termed military TB. HIV-associated TB is characterized by atypical manifestations and patients more often present with extrapulmonary TB and have less common radiological findings consistent with TB [129], especially in patients with low CD4 cell count [130].

TB diagnosis

Despite advances in research concerning the pathology of TB, diagnosis of the active form of the disease is a complicated matter even in high-resource countries. The gold standard for a definite diagnosis is culture of the bacteria in solid or liquid medium, which represents the most sensitive method to detect TB and the only technique to enable drug sensitivity testing. However, culture is hampered by the slow growth rate of Mtb, and diagnosis can require from 2 to 8 weeks. This represents a considerable length of time when dealing with a contagious disease, and during this period there are risks of both clinical deterioration of the patient and further spread of TB [131]. Culture of the bacteria is prone to contamination [132] and requires a PF3 laboratory (biosafety level 3) as well as a steady supply of electricity and reagents, which restricts the use of this method in resource-limited settings.

The most commonly used point-of-care diagnostic tests include smear microscopy with Ziel-Neelsen (ZN) staining, a technique largely unchanged since it was first described by Robert Koch in 1882. In that it is rapid and cheap, ZN staining is widely used worldwide for detection of TB, and it is still the only method for definite diagnosis of this disease in many low-income countries, including Ethiopia. However, a limitation of this technique is that detection requires a substantial bacterial burden in sputum (10,000 bacteria/ml), which results in low sensitivity. This is especially problematic in immunocompromised individuals, because they are known to have fewer bacteria in sputum. Moreover, ZN staining cannot distinguish between tuberculous and non-tuberculous mycobacteria, and it cannot identify mycobacterial resistance. A further development of ZN staining is fluorescence microscopy, in which a smear stained with auramine or rhodamine is illuminated with LED light [133], thus allowing a larger area to be investigated and also improving the visibility of and sensitivity for mycobacteria. [133,134].

Another method is to detect Mtb DNA using PCR for partial amplification of the genetic material present in a specimen. The newly developed Gene-Xpert MTB/RIF assay offers rapid automated and real-time detection of the rpoB gene, and this method

requires minimal training to perform and was endorsed by the WHO for fast diagnosis of TB, especially in PLHIV and patients with suspected multidrug-resistant TB (MDR-TB) [135]. The Gene-Xpert MTB/RIF test has the advantages of being easy to perform and providing results in less than 2 hours. This technique also furnishes information about resistance to rifampicin, because it detects the most common resistance mutation [136], although a risk of false-positive reactions has been reported [137]. Furthermore, it should be noted that this assay is expensive, especially the cartridges that are used, and it requires an uninterrupted power supply, ambient temperature of 28 degrees, and an environment without extreme humidity or dust. These features make Gene-Xpert MTB/RIF less appealing for deployment in peripheral settings, and it has even been suggested that building sustainable primary health care would be a better investment [138].

Another widely used method is chest X-ray, which in some cases and if performed by experienced readers can add diagnostic information suggestive of TB. Unfortunately, chest X-rays of PLHIV are difficult to interpret, and thus diagnosis of TB remains elusive, especially in that patient group and therefore also particularly in peripheral low-income settings. Accordingly, TB is often diagnosed solely on clinical grounds when suspicion is high enough to initiate anti-TB treatment (ATT).

TB screening in HIV patients

Due to the complexity of identifying TB in HIV patients [130], several researchers have attempted to construct clinical algorithms based on symptom screening to identify patients at low or high risk of TB, and the largest of such studies was conducted in Southeast Asia [139]. A meta-analysis of the results of all the investigations on that topic suggested that the optimal screening tool entailed asking patients whether they had experienced weight loss, night sweating, cough, or fever of any duration [140]. This analysis covered 8,148 HIV patients, 495 of whom had TB (the majority of them [323/495] living Southeast Asia). The main intention of this set of questions was to exclude the presence of active TB; reporting none of the symptoms had a high negative predictive value (NPV) of 90–95% for TB, depending on the prevalence of the disease. This would allow for initiation of isoniazid preventive therapy (IPT) in those patients. In 2011, this algorithm was endorsed by the WHO for use in resource-limited settings to rule out TB in HIV patients [141]. However, a problem with this approach is that a large number of patients report at least one symptom, and thus the majority are in need of further TB investigation.

Treatment of TB

In 1995, when the increase in TB was being recognized worldwide, the WHO endorsed an intervention program called Directly Observed Treatment, Short-Course (DOTS). This was done after a landmark study published by Styblo [142] had shown that short-course therapy regimens could achieve high cure rates in low-resource settings. In addition to stipulating that treatment should be given under supervision in a programmatic environment, the DOTS strategy also focused on political commitment. According to some investigators [143], the wide implementation of this global health program has led to increases in treatment success rates, but follow-up data are scarce [144]. Treatment of drug sensitive TB is based on four drugs: rifampicin, isoniazid, pyrazinamide, and ethambutol [145], and it is divided into two phases: (i) an intensive phase including all four of these agents for 2 months; (ii) a continuation phase of 4 months with two of the compounds. In patients with extrapulmonary TB such treatment can be extended for up to one year, especially if there is CNS involvement. Data on the treatment of drug-resistant TB are limited, and use of such treatment is often based on expert opinion. The second-line drug regimens administered to treat MDR-TB usually include a fluoroquinolone and an injectable agent, and these regimens have serious side effects, should be administered for 18–24 months [128], and have a poor cure rate of 54–64% depending on treatment strategy [146]. Also, less than 60% of notified MDR-TB cases were started on treatment in 10 countries with a high burden of such TB in 2014 [5].

Treatment for latent TB is given to patients considered being at an increased risk of reactivation of TB (e.g., immunosuppression). The benefit of treatment was recognized as early as in the 1950s when it was noted that a 6–12 month course of treatment led to a risk reduction of up to 90% in immunocompetent [147]. IPT for 6 up to 36 month is recommended [141], although treatment with a combination of rifampicin and isoniazide could be considered.

Treatment of HIV/TB coinfected patients

Treatment of TB in HIV-positive patients follows the same guidelines as in HIV-negative patients although the underlying evidence is not as strong. The most effective approach to prevent TB infection in HIV patients is to give ART, which has been shown to provide a risk reduction of 54–92% [148]. As in HIV-negative patients, it is also recommended that IPT be given for at least 6 months to patients with positive or unknown TST results and in whom active disease has been ruled out [128], although only 30% of patients eligible for IPT were given such treatment in 2012 [5]. A meta-

analysis recently showed that this approach is beneficial in that it reduces morbidity of TB even if it has no effect on mortality in HIV/TB-coinfected patients [147]. However, as IPT is part of a scheme including screening for TB, this interventional package may still have an important impact on survival [149].

The timing of ART in TB patients has been heavily debated, starting with early concerns about drug interactions and it was recommended, if possible, to defer ART until after ATT [150]. After a series of randomized trials [151–153], it was evident that mortality was decreased if ART was introduced during treatment of TB; in a subanalysis from one of the studies, there was also a trend towards improved survival if treatment was initiated early (within 2 weeks) and in patients with severe immunosuppression (<50cells/mm^3) [153]. These observations resulted in a change in the WHO recommendations in 2013 to recommend that ART be started within 2 weeks in patients with a CD4 cell count of < 50/mm^3 and within the intensive phase (8 weeks) in patients with less severe immunosuppression [85]. However, in 2014 a large multicenter study carried out in Africa [154], showed no increase in survival in patients with CD4 cell counts of > 220/mm^3, and the authors suggest that ART could be deferred in patients above this threshold. Furthermore, Török et al. [155] found that early ART led to an increase in mortality in patients with TB meningitis.

All studies except for the study from 2014 have also confirmed increased rates of IRIS in early treatment groups. The different finding in this respect was attributed to the higher CD4 cell count inclusion criteria in this study [154]. Even if this has not been associated with increased mortality it cannot be excluded that IRIS has a negative impact on treatment outcomes of HIV/TB treatment in settings where there is less possibility of diagnosing, monitoring, and treating this condition. The randomized trials that are have been conducted since 2010 to evaluate the optimal timing of ART is summarized in Table 1.

Table 1:
Randomized trials investigating the optimal timing of ART in HIV/TB patients since 2010

Reference	Location	Included HIV/TB patients, n (CD4 threshold in cells/mm^3)	Main outcome measure	Conclusion[a]
Karim et al (SAPiT-trial) *NEJM*, 2010	South Africa	642 (<500)	Death from any cause in patients with ART during ATT vs. ART after ATT	Lower mortality in integrated therapy group compared to sequential therapy (5.4 vs. 12.1/100 PY p < 0.003) and more pronounced in low CD4 cell count < 200/mm^3, but higher IRIS (12.4% vs. 3.8%) → Suggest extension of treatment guidelines to give integrated therapy to HIV/TB patients with < 500 CD4 cells/mm^3
Blanc et al (CAMELIA-trial) *NEJM*, 2011	Cambodia	661 (<200)	Survival in patients with early ART (within 2 weeks) during ATT vs. later ART (after 8 weeks)	Lower mortality in early treatment group (within 2weeks) compared to late therapy (at 8 weeks) (8.28 vs 13.77 / 100 PY p<0.002). Low CD4 (<50cells/mm^3) was not associated with increased mortality compared to higher CD4 (51-200cells/mm^3). Significantly increased IRIS (3.76/100 PM vs. 1.53/100 PM) → significant survival benefit with early ART
Havlir et al *NEJM*, 2011	26 research sites in Africa, Asia, North America and South America	809 (<250)	Survival and no AIDS defining condition in patients with Early ART (within 2 weeks) during ATT vs. later (after 8–12 weeks)	No significant difference in mortality in early (within 2 weeks) vs late treatment (8-12 weeks) overall (12.9% vs 16.1% p=0.45), but in a subanalysis of patients with CD4 cells <50/mm^3 (n=285) compared to those with higher CD4 cell levels (n=521), there was a significant difference (15.5% vs 26.6%, p=0.02). IRIS was higher in early ART group (11% vs. 5%)
Mfinanga et al *The Lancet Infectious diseases, 2014*	South Africa, Tanzania, Uganda, Zambia	1675 (>220)	Composite of failure of TB treatment, TB-recurrence, and death within 12 months in patients with early ART (within 2 weeks) during ATT vs. later (after 6 months)	No significant difference in mortality in early (within 2 weeks) vs late treatment (after 6 months) in patients with CD4 cell counts >220/mm^3. Similar levels of IRIS (10% vs. 10%).

[a] PY =person years; PM = to person months

Ethiopia

Ethiopian health sector

Ethiopia is the second most populous country on the African continent with approximately 90 million inhabitants. The average health expenditure per capita in Ethiopia was 17.6$ compared to 8895.1$ in the united States in 2012 [156], making the per capita expenditure on health care in the US more than 500 times that of Ethiopia. Ethiopia is together with Chad, Central African Republic, Democratic Republic of Congo, Malawi, Madagascar, Eritrea and Myanmar among the countries which spent less than 25$ per capita on health care in 2012 [156]. The three most common causes of death from disease in Ethiopia 2012 were lower respiratory infections (15.3%), HIV/AIDS (7.3%) and diarrheal disease (6%) and communicable diseases accounted for 70% of life years lost [157].

Despite limited resources, general life expectancy has increased in Ethiopia from 45 to 64 years from 1990 to 2012 and mortality of children under five years of age halved from 412 000 to 205 000 between 2000 and 2012 [158]. The improvement of these parameters is largely attributed to the health extension program started in 2003, a program aiming to substantially increase the availability of primary care across the nation. This decentralized care is based on health centers and health posts in rural areas and increased coverage to basic care from 61% in 2003 to 87% in 2007 [159]. The health units of five health posts and one health center provide integrated HIV and TB services, antenatal care and urgent care. Nurses have two levels of qualifications, diploma in nursing and Bachelor of Science, and each health post is staffed by two community workers with basic training (1 year)[159]. Health centers can perform HIV-test and have their own laboratory for basic investigations such as phlebotomy, blood, sputum and stool microscopy, and urine analysis.

TB and HIV in Ethiopia

In 2014, the national prevalence of HIV in adults in Ethiopia was 1.2% (0.8% in males and 1.6% in females) [160], although there are large regional differences [161]. Expenditures on HIV prevention and treatment in the United States in 2012 was 21 billion USD [162], whereas the corresponding figure in Ethiopia was about 50 times lower at 405 million USD, 86% of which came from external sources, mainly PEPFAR [163]. At the same time, the estimated numbers of PLHIV in the two countries were close to 1.2 million [164] and 793,000 [163], respectively. Still, considerable progress has been made with the help of the national HIV programs, which have markedly increased the coverage of ART to 344,400 in 2014, and also reduced the number of new HIV infections among children by 50% between 2009 and 2012 [160]. The scale-up of ART has resulted in decentralized care (i.e., provided at primary health facilities) for HIV patients in Ethiopia. Thirty-five percent of patients received ART outside of Addis Abeba in 2005, whereas this figure had increased to 75% in 2008 [165] Also, the number of patients who received ART at health centers compared to hospitals increased almost tenfold in a single year between 2006 and 2007 (from 2.8% to 17% for chronic HIV care and from 1.1% to 11.4% for newly enrolled cases) [165].

The number of cases of TB in Ethiopia amounted to 133/100,000 persons in 2014, a decline from 152/100,000 in 2013. Approximately one third of the cases (34.0%) were smear-positive pulmonary TB, about one third (34.8%) were smear-negative pulmonary TB, and nearly one third (31.2%) were extrapulmonary TB [160]. The proportion of individuals with lymph node TB (LNTB) is greater in Ethiopia than the global average of 15% [5], and it has been speculated that this can be explained by overdiagnosis, however a study showed that 78% of patients clinically diagnosed with LNTB were culture positive for TB [166].

Data on HIV/TB coinfection in Ethiopia are rather limited. Our research team found previously undetected microbiologically confirmed TB in 17% of HIV patients eligible for ART [167]. Conversely, HIV prevalence in TB patients has been reported to be 15–30% [168,169]. It appears that the rollout of ART has resulted in a shift regarding which of the two diseases is diagnosed first. In an evaluation performed by our group (unpublished material), the proportion of patients receiving ATT before ART 2009–2013 decreased from 72% to 53% over the five-year period, whereas the proportion of patients who started ATT after ART increased (see Figure 7). This suggests that, in coinfected patients, entry into care based on positive HIV tests is increasing, and that TB is diagnosed after care has been initiated. This agrees with our finding of 17% TB in HIV-patients and points to the need of reliable TB-screening in HIV patients.

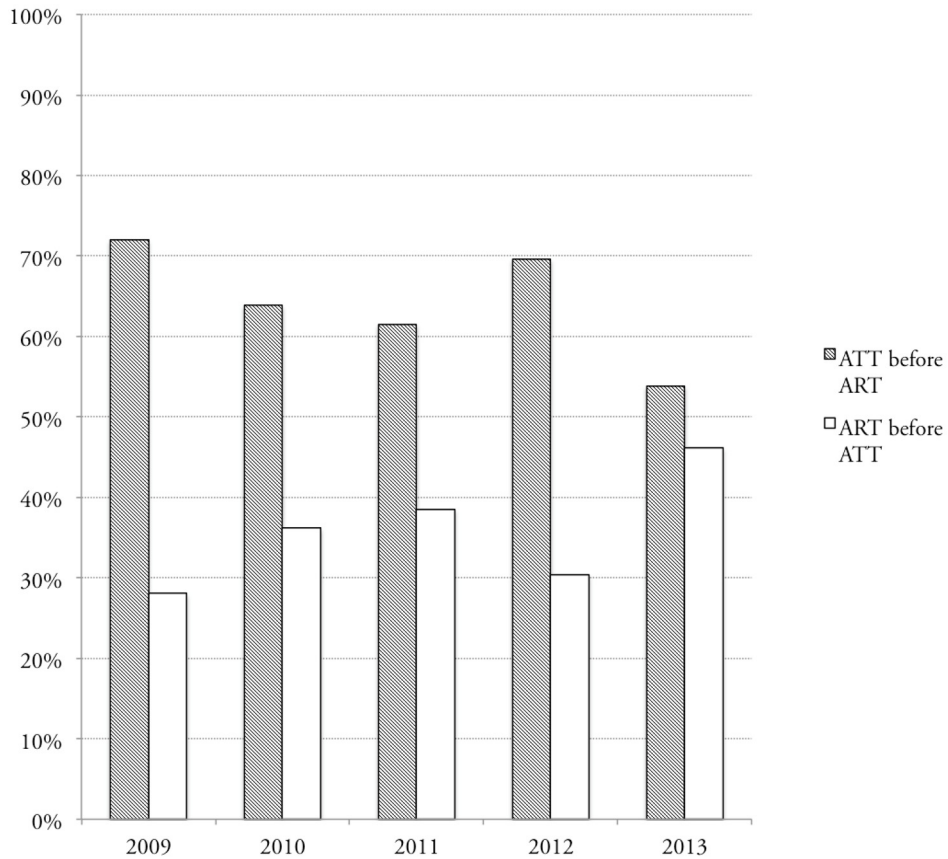

Figure 7:
Proportion of patients starting ATT before ART vs. ART before ATT at three health centers in the Oromia Region between 2009 and 2013. 2009 n=139; 2010 n=188, 2011 n=200, 2012 n=194, 2013 n=104.

Aim

The objective of the studies presented in this thesis (Papers I–IV) was to investigate aspects of HIV/TB coinfection in patients receiving care at primary health centers in low-income countries. Papers I and II describe work done to address aspects of management and develop two new clinical scoring systems. Papers III and IV report data concerning the impact of TB on immune markers in patients with or without HIV coinfection. The specific aims of the four studies were as follows:

- To develop a clinical algorithm for categorization of TB in HIV-infected individuals eligible to start ART.
- To develop a scoring system based on clinical variables for predicting important CD4 cell strata in HIV-positive adults with TB in order to determine the optimal time for initiating ART.
- To investigate the impact of TB on CD4 cell count and clinical characteristics of CD4 lymphocytopenia with or without HIV coinfection.
- To determine the level of immune activation in TB patients with or without HIV coinfection measured as plasma levels of neopterin, and to establish the relationship between this marker and CD4 cell count before and during ATT.
- To examine the potential role of neopterin and CRP as point-of-care markers for evaluation of immunosuppression in adults with HIV/TB coinfection.

Patients and methods

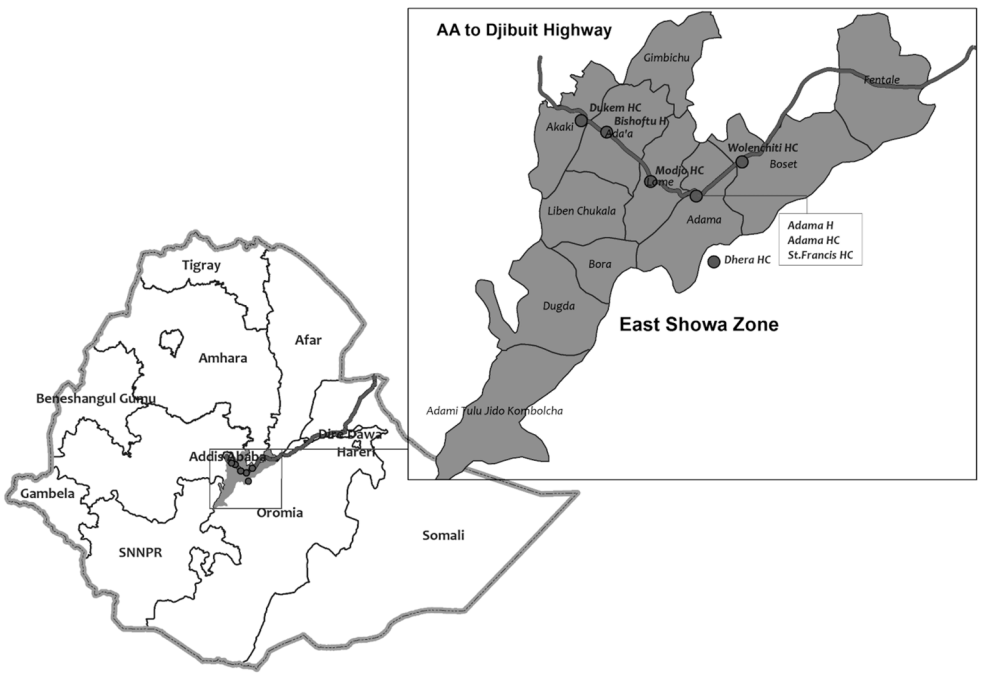

Figure 8:
Map showing the study sites

Setting

The patients evaluated in the four studies were included in two separate cohorts, one selected at HIV clinics (cohort I) and the other at TB clinics (cohort II) in the same area. All four of the present investigations were based at public health facilities in and around the city of Adama in the Oromia Region (Figure 8), which is the largest of the nine states in Ethiopia with a population of about 30 million. Adama is situated along the main high way between Addis Abeba and Djibouti, which is highly trafficked

because it is the main import and export route from the capital. Adama has a population of around 300,000 and is surrounded by agricultural areas. The prevalence of HIV is higher in the Adama region (9% PLHIV 2008)[170] than on national level (1.2%).

Patients

Participants in cohort I

The research was conducted at five HIV clinics at public health centers in and around Adama (Adama, Geda, Wolenchiti, Mojo, and Dhera). Patients were prospectively recruited at the HIV clinics where all ART-naïve HIV-positive patients receiving care at that particular facility were screened for eligibility, irrespective of symptoms or signs of active TB. Data collection began in October 2011, and new patients were included until March 2013. Follow up of these study participants is still ongoing to evaluate outcomes of ART in coinfected patients, but that aspect is outside the scope of this thesis.

The following inclusion criteria were used for cohort I: confirmed HIV infection with a CD4 cell count of < 350/mm^3 or WHO stage 4 regardless of CD4 cell count (eligibility for ART according to Ethiopian guidelines); age ≥ 18 years; residing in the uptake area of the clinic; provided written informed consent for participation in the study. Exclusion criteria were previous or current treatment with ART and TB treatment for more than two weeks.

Participants in cohort II

Cohort II was started in September 2010, and inclusion of patients continued until March 2012. Participants were selected at TB outpatient clinics at six health centers (Dukem, Mojo, Adama, Geda, Wolenchiti, and St Francis), one zonal hospital (Bishoftu), and one regional hospital (Adama Hospital). All TB care and follow-up was the responsibility of nurses at all facilities in question. Individuals with a TB diagnosis were enrolled at the start of TB treatment after eligibility screening.

Inclusion criteria for cohort II were as follows: a confirmed TB diagnosis according to Ethiopian national guidelines [171] (see sub-section headed "TB diagnosis" below); age ≥ 18 years; lived in the uptake area of the clinics; consented to participate in the studies and to undergo an HIV test. Exclusion criteria were previous or current ART and present ATT for more than two weeks or ATT within previous six months.

A group of healthy subjects was selected at a voluntary HIV counseling and testing (VCT) center at one of the participating health centers (Mojo). Individuals visiting the VCT clinic to request an HIV test were screened for eligibility. To be included, a patient had to be ≥ 18 years of age, negative for HIV by testing performed at the facility, and a resident of the catchment area of the clinic. Patients were excluded if they were undergoing active treatment for TB or had a cough of any duration, fever or night sweats during the past 2 weeks, or any acute or chronic illness. Sociodemographic data were gathered, and blood samples were collected for CD4 cell analyses and plasma was stored at –80 °C.

Methods

Methods cohort I

Participating patients were interviewed by the study nurses (with either of two levels of academic training; see previous sub-section "Ethiopian health sector") using a structured questionnaire that included items concerning sociodemographic characteristics, previous illnesses, TB history (previous or current treatment for active TB, TB in household, and previous or current IPT), and signs and/or symptoms plausibly pertaining to TB. Thereafter, the patients were subjected to a structured physical examination conducted by the study nurses with the particular objective of noting findings that could be associated with TB (i.e., BMI and mid upper arm circumference [MUAC], conjunctival pallor, oral lesions, skin abnormalities, enlarged lymph nodes, and abdominal distention or mass). The study nurses had received both collective and individual training, and they were supervised continuously by the primary investigators. Blood was drawn for analysis of CD4 cell levels and hematological parameters, and also to obtain plasma to be stored at –80 °C at the Adama regional laboratory.

Participants were provided with re-sealable plastic containers and asked to submit paired morning sputa on two occasions. Patients with enlarged lymph nodes on physical examination were referred to a pathologist for fine-needle aspiration. One of the collected sputum specimens was sent to the Adama Regional Laboratory for smear microscopy and rapid PCR (Xpert MTB/RIF assay, Cephid, Sunnyvale, CA, USA), and the other was sent to International Clinical Laboratories in Addis Ababa for liquid culture. Fine-needle aspirates were also sent for rapid PCR and culture. The results of these TB-diagnostic examinations were delivered regularly from the laboratories to the study investigators and the health center staff, and ATT was given to patients with positive results.

For all patients in cohort I, follow-up visits were scheduled once every month for the first 3 months and once at 6 months, and thereafter continued follow-up to evaluate outcome of ART (not within the scope of this thesis). At each follow-up visit, the same interview regarding symptoms and physical examination as conducted at the baseline visit were repeated by the study nurses, and new blood samples were drawn for analysis of CD4 cells and hematological parameters and to obtain plasma for storage. Patients who began ART during the course of follow-up were restarted on this follow-up protocol, and patients who developed signs suggestive of TB during follow-up were subjected to new TB investigations according to the baseline protocol.

Methods cohort II

The patients in cohort II were interviewed by the study nurses using a structured questionnaire that covered demographic characteristics, disease history (other illnesses and previous hospitalization), and common symptoms of HIV-related immunosuppression and TB (e.g., bedridden state, cough, dyspnea, fever, weight loss, anorexia, lymph node enlargement, skin rash, diarrhea, and odynophagia). A physical examination was performed that included recording of body mass index (BMI), MUAC, conjunctival pallor, oral lesions, cervical lymphadenopathy, skin rash, and herpes zoster scarring. As in cohort I, the study nurses had received both collective and individual training, and they were supervised regularly by the primary investigators. HIV testing was performed, and patients found to be positive were referred to the ART clinics at the respective health facilities, where they were considered for start of ART according to the national guidelines stating that such treatment should be initiated within 2–8 weeks of ATT or after the intensive phase, depending on CD4 cell levels or on clinical disease severity (if no CD4 cell count available) [172]. ART consisted of a combination of tenofovir (TDF), lamivudine (3TC), and efavirenz (EFV) administered according to Ethiopian guidelines. Blood samples were taken for CD4 cell count and plasma to be stored at –80 °C. The evaluation considering symptoms and signs and collection of blood were repeated at the end of the intensive phase (i.e., 2 months after start of ATT) and at the end of treatment (i.e., 6 months after start of ATT).

Laboratory procedures

HIV diagnosis

In both cohorts, HIV serostatus was determined using rapid tests according to the National Guidelines for HIV Counseling and Testing [173]. Positive results in the combined HIV1/2 rapid test (KHB, Shanghai Kehua Bio-Engineering Co., Ltd., China) were confirmed by testing with Stat-Pak (Chembio Diagnostics, USA), and discrepant results were tested using Uni-gold (Trinity Biotech, Ireland).

TB diagnosis

In cohort I, definite/bacteriologically confirmed TB was defined as a microbiological specimen positive for TB by either smear microscopy, Xpert MTB/RIF assay, or culture. In cohort II, the diagnosis of TB was based on the case definition specified by the national TB program in Ethiopia in 2008 [171], which states that pulmonary TB should be diagnosed by smear microscopy where positivity is indicated by the presence of acid-fast bacilli. Patients could also be characterized as having smear-negative pulmonary TB, if they had three negative smears and symptoms suggestive of pulmonary TB (cough for > 2weeks), and had received but not responded to a short course of broad-spectrum antibiotic and subsequently had three additional negative sputum smears. They were also to have had radiological findings suggestive of TB and a clinician's decision to start a full course of ATT. Extrapulmonary TB was defined by the following: (i) a cytological specimen from an extrapulmonary site that was suggestive of TB according to examination by a pathologist or confirmed by TB culture, or (ii) strong clinical evidence of active extrapulmonary TB and a decision by a clinician to treat with a full course of ATT [171].

CD4 cell analysis

Blood obtained from participating patients at any of the visits was subjected to CD4 cell count by flow cytometry. Whole blood samples in EDTA tubes (6–10 ml) were sent on the day of phlebotomy to the Adama Regional Laboratory, although the samples collected at Dukem or Bishoftu were sent to the laboratory at Bishoftu Hospital (cohort II). These samples were analyzed by flow cytometry using FACScount or FACSCalibur (Beckton Dickinson), which was performed by laboratory technicians at the respective laboratories. The equipment used was regularly subjected to standard monitoring and external quality assurance tests.

Neopterin and CRP

Neopterin and CRP levels in specimens collected in cohort II were determined by ELISA (IBL international, Hamburg, Germany) performed at the Adama Regional Laboratory. Frozen sera from baseline samples were selected according to the following: all available samples from HIV-positive TB patients and from HIV-negative TB patients with a CD4 cell count < 500/mm^3 were tested. In addition, consecutive samples from HIV$^-$negative TB patients with a CD4 cell count ≥ 500/mm^3 and from a subset of healthy controls were tested. For these tests, frozen sera were thawed and analyzed according to the manufacturers instructions by an experienced ELISA technician at the Adama Regional Laboratory. Assays were performed on duplicate patient samples and with kit-independent controls representing high, medium, and low values in each run. The resulting optical density readings were converted to concentrations from a standard curve using a 4-spline non-parametric reader-fit algorithm as recommended (MiraiBio Group, Hitachi Solutions, San Bruno, CA, USA).

Data analysis

Data on both cohorts were entered into case record forms by the study investigators. Thereafter data managers then entered each of the original forms into Excel and crosschecked each entry. The data were handled confidentially under study codes, and they were subsequently entered and processed using a statistical program (SPSS v.20, IBM).

The first two investigations were observational (Papers I and II), and the variables used were correlated with the respective outcomes (positive TB diagnosis [Paper I] and CD4 cell count < 100/mm^3 [Paper II]). In the first study (Paper I), the clinicians in charge diagnosed a subset of patients (n = 21) as having TB based on clinical criteria in the national guidelines (i.e., clinically suspected TB but negative microbiological results), after which the patients were started on a full course of ATT. These patients were excluded from further analysis, because their TB status could not be objectively confirmed. For seven more patients, data on all the variables of the WHO TB-screening tool were not recorded, and hence these patients were excluded from the evaluation of this screening tool and from the development of a screening algorithm.

In the third study (Paper III), we investigated the impact of TB on CD4 cell counts in patients given ATT over a period of 6 months (CD4 cells collected after the intensive phase at 2 months and at the end of treatment at 6 months), and also conducted a baseline analysis of correlation with clinical characteristics in TB patients with a low CD4 cell count. In addition, a sub-analysis of the evolution of CD4 cell levels during

ATT was performed to evaluate the characteristics of patients with either increased or decreased numbers of such cells. Patients were divided into those whose CD4 cell counts increased by ≥ 50 cells/mm^3 after 6 months, those whose CD4 cell levels decreased by 50 cells/mm^3 after 6 months, and those with no change in the counts (± 50 cells/mm^3). We used the cut-off of 50 cells/mm^3, because that level represents 10% of the lower normal range (500 CD4 cells/mm^3). For HIV patients, only those who did not start ART were included in the analysis, since being on such therapy could be a confounder.

Patients with valid results regarding both neopterin and CRP levels were included in the final study (Paper IV). Valid results were defined as a difference of ± 10% between duplicates; in cases with a larger discrepancy, the samples were reanalyzed. For the kit-independent controls, valid results were also stipulated to have a difference of ± 10% between runs. In the analysis of baseline neopterin compared with evolution of the CD4 cell count, the same definition of increase vs. decrease in CD4 cell count were used as in paper III.

Statistical methods

Continuous variables were consistently handled in a non-parametric manner. This was done because most of the variables did not have a standard distribution (as seen in histograms), and they were initially divided into quartiles for assessment of associations with outcome variables. In the first study (Paper I), we used cut-offs found in the literature in logistic regression analysis, whereas we consistently used quartile levels in the second study (Paper II).

In both those investigations, we used multiple logistic regression models to construct algorithms with variables that in combination could predict the outcome variables: the binary variable TB or not TB (Paper I), and the variable CD4 cell strata (Paper II). We chose regression models to achieve the simplest possible model with the highest predictive capacity, commonly termed a parsimonious model. The regression models were initially constructed with a univariate logistic regression analysis for each variable with the respective outcome variable. Thereafter, all variables with a significant association with a p-value of < 0.3 were entered into a combined regression model, and a procedure of backwards elimination of the least significant variable was performed until a fully significant model was reached. $P < 0.05$ was considered statistically significant. This parsimonious model was subsequently analyzed regarding predictive performance by assessing its outcome measure using receiver operating characteristics (ROC) curves. This was done both with predicted probabilities and with assigned points, 1 point for each variable (Paper I), or a weighted score based on the beta coefficients (Paper II).

ROC analysis is the most commonly used method for comparing diagnostic systems, and it can provide a measurement of a continuous diagnostic technique (e.g., a lab test or a scale) that can be compared with another dichotomous reference variable [174]. A ROC curve is obtained by depicting the true-positive rate (sensitivity) over the false-negative rate (1-specificity), and it can be used to select the optimal index test compared with a reference test. The ROC curve is a graphical representation of the tradeoff between sensitivity and specificity for the index test, and it is useful for determining the best cut-offs for clinical use [175]. Moreover, the area under the curve (AUC) is used to estimate the overall performance of the test, defined as the probability that the test will correctly identify a randomly chosen individual as having the condition. AUC values range from 0.5 (no discriminatory capacity or 50–50 chance of discrimination) and 1.0 (perfect discriminatory capacity) [176]. Interpretation of the AUC depends on the intrinsic variables in question, and a value of < 0.75 is considered to be of little diagnostic benefit [177].

We used ROC curves to compare the existing WHO algorithm (WHO-TB) to the same algorithm with an added second step (Paper I), and in a similar way we compared the performance of surrogate biomarkers and CD4 cell count (Paper IV). The approach using ROC analysis (Paper II) was used chiefly to identify the ideal balance between sensitivity and specificity for identification of CD4 cell cut-off thresholds. In both strategies, the resulting predictive capacity for the outcome measure was evaluated by calculating sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). In the study reported in Paper IV, ROC curves were used to evaluate the yield of neopterin and CRP to identify patients with a CD4 cell count of < 100/mm^3, and the curves were also compared with the clinical algorithm presented in Paper II.

Main results

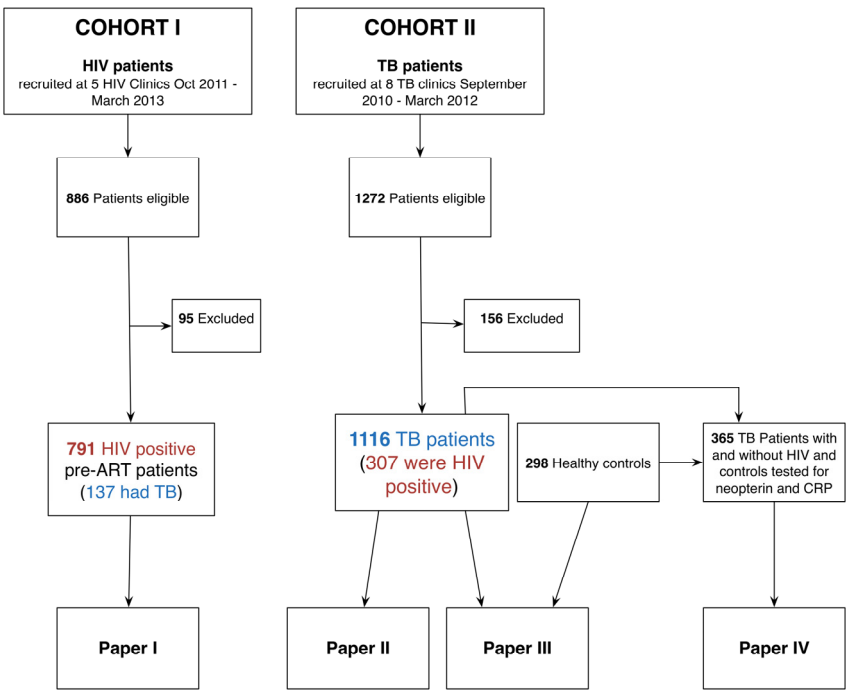

Figure 9
Overview of the patients participating in the four studies in this thesis.

Paper I

In all, 791 HIV patients (cohort I) were included in this analysis, and 137 of them had TB. Initially, 886 patients were selected for cohort I, but 95 were excluded for the following reasons: 13 had been erroneously recruited (i.e., did not fulfill the inclusion criteria), 61 did not submit sputum samples, and 21 had a clinical diagnosis of TB. TB was defined as being either confirmed by culture or positive by Xpert MTB/RIF assay. The main baseline characteristics of the cohort are presented in Table 2 (see Paper I for complete table).

Table 2:

Characteristics Characteristics			Total (n = 791) n (%)*l* (n=791)	TB (n = 137) n (%)*)	Non-TB (n = 654) n (%)*on-TB (n=654)
Demographic Characteristics	Median Age (IQR)	Years	32 (28-40)	35 (28-40)	32 (28-39)
	Gender	Male	324 (41.0)	71 (51.8)	253 (38.7)
		Female	467 (59.0)	66 (48.2)	401 (61.3)
	Illiterate	Yes	309 (39.2)	52 (38.2)	257 (39.4)
		No	479 (60.2)	84 (61.8)	395 (60.6)
	Residence	Urban	617 (79.1)	104 (78.2)	513 (79.3)
		Rural	163 (20.9)	29 (21.8)	134 (20.7)
TB related factors	Self-reported history of TB	Yes	49 (6.3)	4 (2.9)	45 (7.0)
		No	730 (93.7)	132 (97.1)	598 (93.0)
	Household members on TB treatment	Yes	13 (1.6)	2 (1.5)	11 (1.7)
		No	776 (98.4)	135 (98.5)	641 (98.3)
	Prior TB in household member	Yes	33 (4.2)	3 (2.2)	30 (4.6)
		No	753 (75.8)	134 (97.8)	619 (95.4)
HIV care	Enrolment history	New	240 (30.3)	52 (38.0)	188 (28.7)
		In care	551 (69.7)	85 (62.0)	466 (71.3)
	WHO clinical stage	1 or 2	383 (48.6)	40 (29.2)	343 (52.7)
		3 or 4	405 (51.4)	97 (70.8)	308 (47.3)
	WHO TB symptom screening	Positive	625 (79.7)	126 (92.6)	499 (77.0)
		Negative	159 (20.3)	10 (7.4)	149 (23.0)
	On IPT	Yes	19 (2.4)	1 (0.7)	18 (2.8)
		No	769 (97.6)	136 (99.3)	633 (97.2)
	On CPT	Yes	597 (75.9)	102 (75.0)	495 (76.0)
		No	190 (24.1)	34 (25.0)	156 (24.0)
	Hospitalization	Yes	11 (1.4)	3 (2.2)	8 (1.2)
		No	779 (98.6)	134 (97.8)	645 (98.8)
Laboratory characteristics	Hemoglobin (median; IQR)	g/dl	11.6 (10.2-12.7)	10.4 (9.1-11.9)	11.9 (10.5-12.9)
	CD4 cell count (median; IQR)	cells/mm^3	212 (119-321)	172 (91-269)	221 (126-328)

Baseline characteristics of the 791 HIV-positive pre-ART patients categorized according to TB diagnosis.
* n (percentage) unless IQR is specified

Seven more patients had to be excluded from analysis, because all items on the WHO screening algorithm had not been recorded for those individuals. Thus 784 were available for analysis; among those patients, 159 (20%) were negative by WHO-TB symptom screening (i.e., did not report cough of any duration, fever, night sweats, or weight loss) and 10 (6.3%) had bacteriologically confirmed TB, giving a negative predictive value (NPV) of 94%. The remaining 625 patients (80%) reported at least one of the symptoms included in the WHO-TB symptom screening, and a logistic regression analysis was performed to investigate factors associated with TB in this group. After backward elimination of non-significant variables, the following factors remained in the model (Figure 10): cough Karnofsky score ≤ 80, MUAC < 20 cm, peripheral lymphadenopathy, and hemoglobin < 10 g/dl.

The indicated patient material was used to evaluate the yield in terms of TB case findings provided by the second-step scoring system added to the WHO-TB symptom screen. Patients were categorized as having low (0–1 points), intermediate (2–3 points), or high (≥ 4 points) risk of TB (see Figure 10*)*. Twenty (7.9%) of 255 patients with low risk had TB, giving a NPV of 92% (not significantly higher than with the WHO-TB screening algorithm), and 19 of 34 in the high-risk group had TB (positive predictive value [PPV] = 54%). Thus, low risk of TB could be identified in a total of 414 patients when using the combination of the two algorithms as compared to 159 when using the WHO-TB tool alone, with a non-significant difference between the two approaches regarding the proportion of missed TB cases (7.9% vs. 6.3%, p = 0.69).

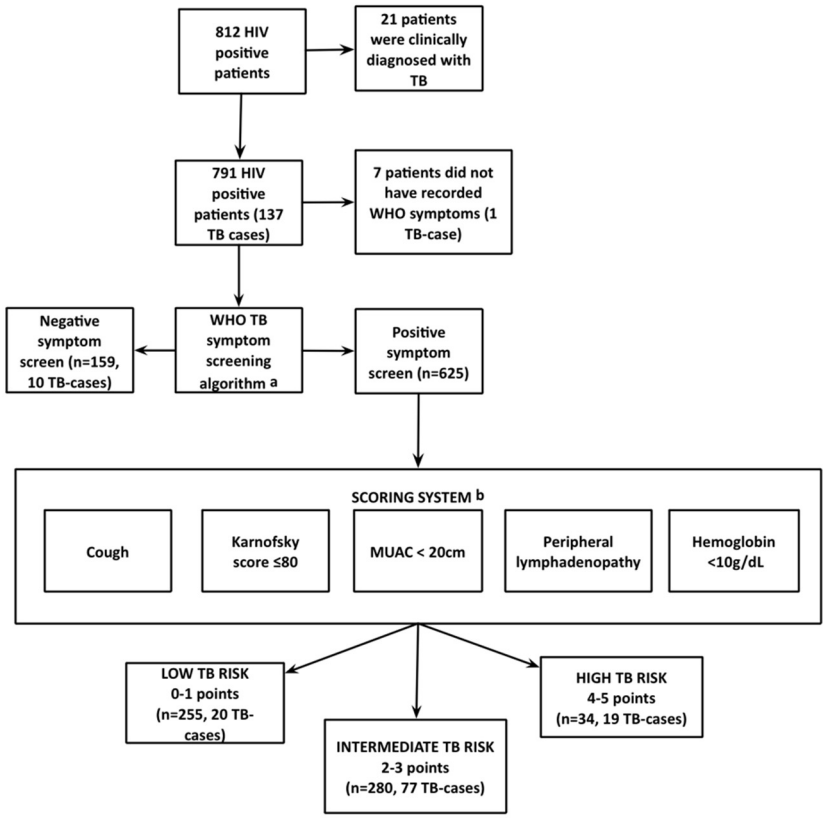

Figure 10
Flow chart illustrating the use of a second scoring step[b] added to the currently used WHO-TB symptom screening tool[a]. The combination of the two algorithms was tested on 784/791 patients (seven patients excluded because no WHO-TB symptoms were recorded). The second step was analyzed using patients for whom results on all parameters were available (569 of 625 with a positive WHO-TB screen). Each positive parameter in the second step was assigned a score of 1 point.

Paper II

Cohort II comprised 1,116 patients with TB, and 307 of those individuals were HIV positive. In a multivariable logistic regression analysis, factors associated with HIV disease were evaluated, which showed that diarrhea (> 1 week), odynophagia, conjunctival pallor, herpes zoster, oral candidiasis, skin rash, and MUAC < 20 cm were associated with HIV coinfection. In a second analysis, variables pertaining to CD4 cell strata were investigated. For patients with CD4 cell counts of < 350/mm^3, the following

symptoms and signs were found to be significantly associated with HIV coinfection: conjunctival pallor, MUAC< 20 cm, shortness of breath, oral hairy leukoplakia (OHL), oral candidiasis, and gingivitis. Variables associated with subsets of CD4 cell strata are presented in Table 3.

Table 3:
Variables significantly associated with CD4 cell count strata in multivariable analysis of 307 HIV-positive patients with TB.

	CD4 cell count <50		CD4 cell count <100		CD4 cell count <200		CD4 cell count <350			
	β	Adjusted OR (95% CI)	β	Adjusted OR (95% CI)	β	Adjusted OR (95% CI)	β	Adjusted OR (95% CI)	Total β	Rounded score
Male gender			0.8	2.3 (1.3–4.2)					0.8	1
Age ≥33 years					0.5	1.7 (1.0–2.8)			0.5	0.5
Conjunctival pallor			0.8	2.4 (1.4–4.3)					0.8	1
Shortness of breath					0.7	2.1 (1.3–3.4)	1.0	2.9 (1.6–5.3)	1.7	1.5
OHL	2.4	7.5 (2.2–25.2)							2.4	2.5
Oral candidiasis					0.6	2.1 (1.2–3.7)			0.6	0.5
Gingivitis					1.4	4.3 (1.2–15.8)			1.4	1.5
MUAC <20 cm	1.3	4.6 (2.0–10.5)	1.0	2.8 (1.6–4.9)	0.7	2.2 (1.3–3.6)	0.9	2.4 (1.2–4.5)	3.9	4
AUC		0.72		0.72		0.71		0.68		0.72

A scoring system was constructed by using the β-coefficient to weigh the relative importance of the intrinsic variables (Table 3). The performance of this system when applied to the described patient material was assessed by ROC analysis, and the results are presented in a cross-tabulation with calculated sensitivity, specificity, PPV, and NPV (Table 4).

Table 4:
Performance of the clinical scoring instrument used to predict a CD4 cell count of < 100/mm^3 for scores of 0 to > 7 points.

Score	CD4 < 100 (n)	CD4 100–350 (n)	CD4 > 350 (n)	Sensitivity (%)	Specificity (%)	Positive predictive value (PPV) (%)	Negative predictive value (NPV) (%)
0	1	10	9	100	8	25	95
1	3	17	18	99	24	27	93
2	11	31	15	95	44	30	87
3	10	26	11	81	61	33	85
4	5	17	4	68	70	37	84
5	3	12	5	61	78	41	84
6	11	15	6	57	87	47	82
7	15	10	4	43	93	53	78
>7	18	14	1	23	100	55	75
Total	77	152	73				

CD4 cell values in cells/mm^3.
Score cutoff rounded up to nearest integer. Sensitivity and PPV of being at threshold or above. Specificity and negative predictive value of being at threshold or below.
*Only patients with no missing values were included in this analysis.

For the developed scoring system, the overall capacity to identify severe immunosuppression (CD4 cell count < 100/mm^3) was AUC = 0.72 (Table 3). The system was best at excluding severe immunosuppression (< 100 CD4 cells/mm^3), which was done in 100/116 patients (NPV = 87%) with a score of ≤ 2. The PPV to identify patients with severe immunosuppression (<100 cells/mm^3) reached at most 55% with a score of > 7 (Table 4).

Paper III

In this investigation (Paper III), 200 (25%) of 809 HIV-negative TB patients had CD4 cell counts below the normal reference level (< 500 cells/mm^3). In addition, 82 (10%) of the 809 subjects had a CD4 cell count < 350/mm^3. In the control group, CD4 cell counts were within the normal range (median 896 cells/mm^3). For 472 of the HIV-negative TB patients, follow-up CD4 cell counts performed at 2 and 6 months were available and were found to have increased significantly during TB treatment at both the indicated time points, although they did not reach the levels noted in the healthy controls. CD4 cell levels in TB patients with CD4 cell counts of < 500/mm^3 increased significantly both between baseline and 2 months and between 2 and 6 months. HIV-positive TB patients who did not start ART also had increasing CD4 cell counts after 6 months but not after 2 months (see Figure 11).

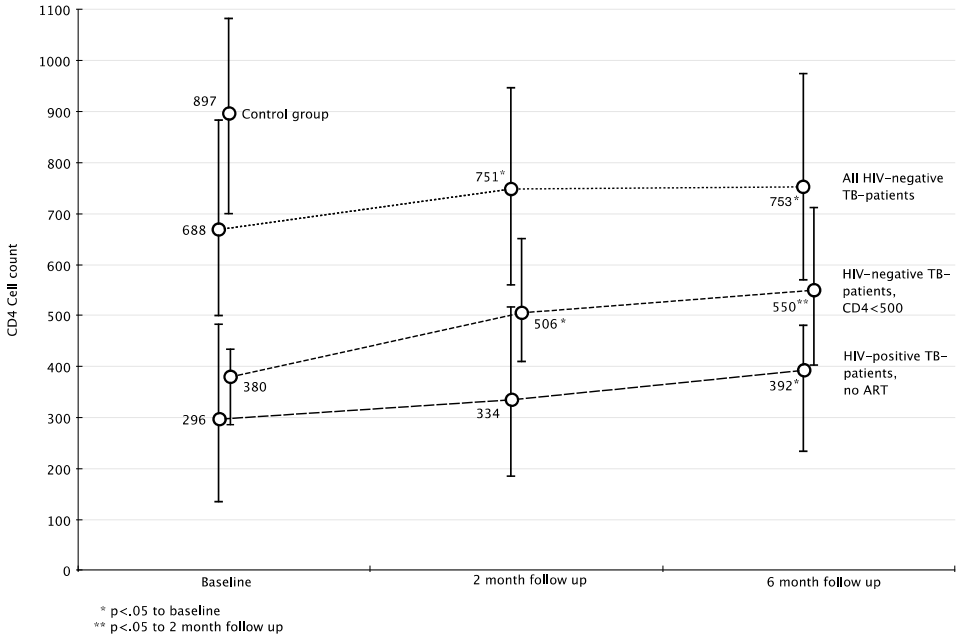

Figure 11:
Changes in CD4 cell count during ATT in patients with TB. Patients with available CD4 cell counts for both follow-up occasions were included (n = 543). Results are stratified by all HIV-negative TB patients (n = 472), HIV-negative TB patients with CD4 cell count < 500/mm^3 (n = 124), and HIV-positive TB patients who did not start ART during the follow-up period (n = 71).

In a follow-up analysis, we investigated how CD4 cell levels increased or decreased in each group. For HIV-negative patients with CD4 cell counts of < 500/mm^3, the counts increased in the majority of the subjects (n = 87), did not change in 24, and decreased in four cases. Considering HIV-positive patients, CD4 cell counts increased in 35 patients, did not change in 19, and increased in 17. There was no clear difference in clinical characteristics between these groups, but the patients who had increasing CD4 cell levels had a lower baseline CD4 cell count, and this was especially pronounced in HIV-positive patients (median 188 vs. 505 cells/mm^3). Finally, we analyzed the clinical characteristics of the HIV-negative TB patients who had low CD4 cell counts. In multivariable analysis, the following factors were associated with having a low CD4 cell count: smear-positive pulmonary TB (adjusted OR 1.6, CI 1.2–2.3), low MUAC (< 22 cm) (adjusted OR 2.2, CI 1.3–3.6), and history of a bedridden state (adjusted OR 2.3, CI 1.3–4.0).

Paper IV

In this study (Paper IV), the biomarkers neopterin and CRP were analyzed in a subset of cohort II (n = 1,116) consisting of 365 TB patients with available results, and in a control group comprising 31 healthy individuals (see flow chart in Paper IV, Figure 1). Levels of both biomarkers were elevated in the TB patients (median values: neopterin 23.2 nmol/l and CRP 33.4 µg/ml) and especially in the HIV+/TB cases (median: neopterin 54 nmol/l and CRP 36 µg/ml), as compared with controls (median levels within the normal range: neopterin 3.8 nmol/l and CRP 0.5 µg/ml). According to the assay manufacturer, normal levels are < 10 nmol/l for neopterin, and the upper limit of the normal range for CRP is 5–8 µg/ml (see Figure 12).

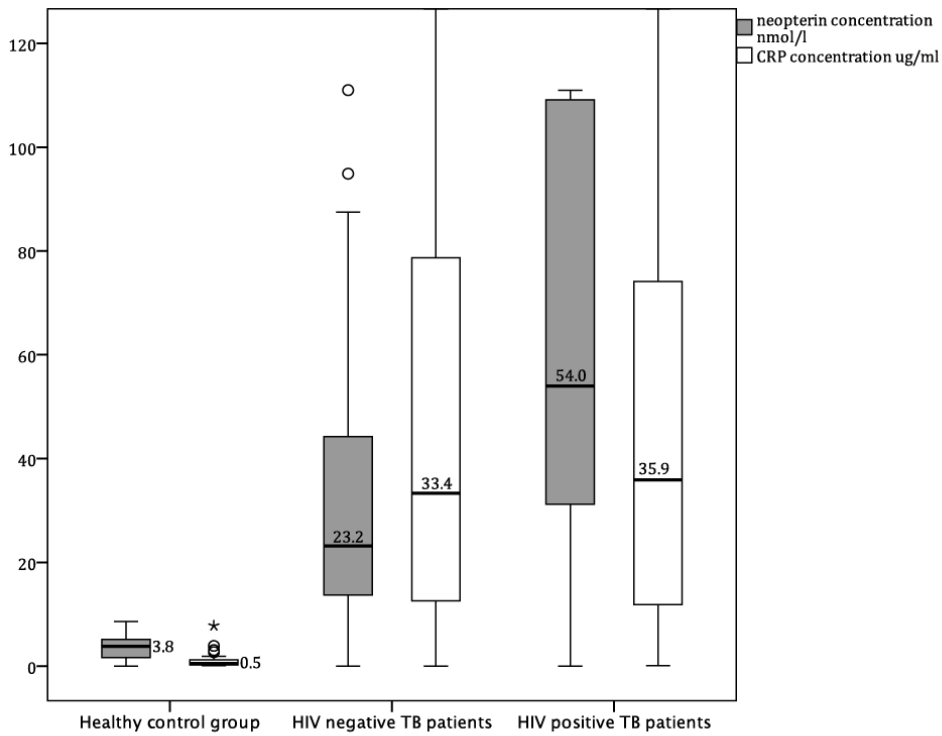

Figure 12:
Levels of neopterin and CRP in healthy controls, HIV-negative TB patients, and HIV-positive TB patients. Concentrations of neopterin (gray bars) are in nmol/l, and CRP levels (white bars) are in µg/ml. The error bars represent IQR, and the indicated values are medians. Stars and circles are outliers.

Neopterin showed a significant inverse correlation with CD4 cell count in both HIV-positive and HIV-negative patients (Spearman rank correlation –0.61, p = 0.001). The median neopterin level was 77 nmol/l in patients with < 100 CD4 cells/mm^3 but was 36 nmol/l in patients with > 350 CD4 cells/mm^3 (p = 0.001). The corresponding median levels of CRP were 47 and 17 µg/ml, respectively (p = 0.004). In an analysis of the evolution of CD4 cell count compared with baseline neopterin levels, high baseline neopterin was associated with an increase in CD4 cells. HIV-negative TB patients with increasing numbers of CD4 cells after 6 months of ATT had significantly higher levels of neopterin compared to HIV-negative TB patients without such a rise in CD4 cells (p = 0.004; see Figure 13).

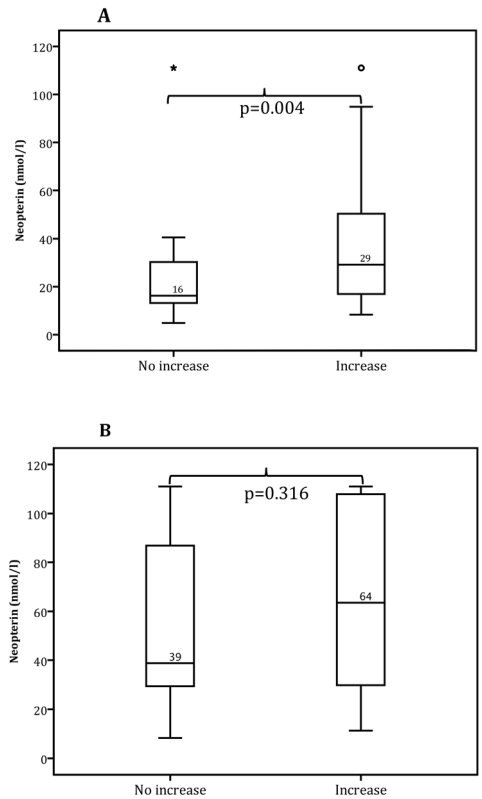

Figure 13:
Baseline neopterin in relation to changes in CD4 cell count after 6 months of ATT subdivided into two groups: no increase (CD cell count ≤ 50/mm^3 or unchanged [± 50 cells/mm^3]) and increase (CD4 cell count ≥ 50/mm^3). A. TB patients who were HIV-negative. The baseline median CD4 cell count was 405/mm^3 (n = 58) for HIV-negative TB patients whose CD4 cell levels increased and 496/mm^3 for those whose CD4 cell levels did not increase (n = 27). B. HIV-positive patients who did not start ART (n = 43; 22/43 had an increase in CD4 cell count). The baseline median CD4 cell counts were 255/mm^3 and 411/mm^3 for those with and those without increased counts, respectively (n = 21).

Measurement of neopterin and CRP levels performed poorly in predicting CD4 cell levels of <100/mm^3, and were even inferior to the previously constructed clinical scoring algorithm (Paper II) (AUC for neopterin=0.64, AUC for CRP=0.59 and AUC for the clinical algorithm = 0.75, for the same patients (Figure 14).

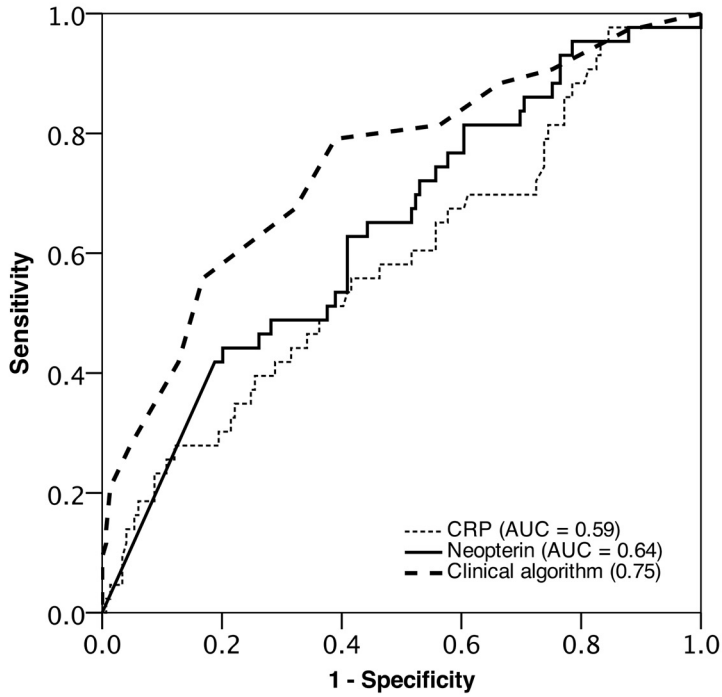

Figure 14
Head to head comparison in ROC analysis of three surrogate markers, CRP, neopterin and a clinical algorithm, for CD4 cell count<100/mm^3.

Discussion

Despite considerable efforts to reduce the burden of HIV and TB, these two conditions still represent the infectious diseases that cause the highest mortality worldwide. The increase in TB cases seen in the wake of the HIV pandemic is multifactorial and can be explained at the immunological, demographic, and social levels. Although one of these two diseases is ancient and the other relatively new, the underlying pathogens have found a common ground, each thriving in coexistence with the other. Consequently, they pose a common threat, and interventions aimed at one of the diseases must also take the other into consideration. With the increasing availability of ART, the complex care of coinfected individuals is to a greater extent being managed in primary care settings. Clinicians face the difficulties of identifying TB as well as determining the optimal time point for administering ART to maximize the chances of survival for the patients, who have advanced disease.

This thesis is based on the fact that resources in low-income countries are more restricted than those in high-income countries calling for the development of methods for management that are adapted to this situation. If resources were not limited in such areas, there would be less need for simple clinical algorithms. Admittedly, many low-resource settings are surging economically, and it is not entirely inconceivable that these regions will, within a reasonable amount of time, achieve an economic standard that enables more advanced health care. Even if that does occur, the use of clinical algorithms in health systems in emerging economies will still be warranted, because it will encourage thorough investigation of patients and concomitantly provide a checklist for the major symptoms of specific disorders and thus give clinicians the opportunity to ponder alternative diagnoses. In the present research, aspects of clinical management were investigated at the primary care level with the aim of improving health care and facilitating introduction of ART. Also, immunological aspects of TB infection in HIV patients were studied to increase the understanding of the interaction between these two diseases.

Both HIV and TB are diseases that exert their pathogenesis by altering the immune response. The mechanisms of HIV's impact on the immune system has been the subject of intense research but less is known for TB, and especially the extent of impact of TB on the immune system in patients coinfected with HIV and TB. In a second part of this research, immunological aspects of TB infection in HIV patients have been investigated in order to increase the understanding of the interaction of these diseases.

Aspects of scoring algorithms

Scoring algorithms and diagnostic scales exist in all major fields of medicine. For example, they are used for risk stratification of coronary heart disease [178], pulmonary embolism [179], depression [180], and various infectious diseases [181–184]. For some conditions (e.g., psychiatric disorders) these tools are the primary means of evaluating patients because no objective measurement exists, whereas for biological conditions they are usually a surrogate for some other diagnostic measure. The use of scales has the advantage of providing clinicians with a standardized method of evaluating patients and also enabling clinical decisions to be made without the need for more advanced examinations such as blood tests or x-rays. An evaluation using a scale can be done while the patient is visiting a care facility, which reduces the risk of loss to follow-up or clinical deterioration while waiting for a test result. Moreover, the use of scales is inexpensive, which makes these tools suitable for use in resource-limited settings where other more costly measurements are scarce or lacking entirely. However, it is also important to perform risk categorization of patients in locations where diagnostic measures exist, because that will allow reallocation of resources to patients with the greatest needs and also allows for timely treatment. Furthermore, scales provide a standardized means of examination that can be used not only to monitor individual patients, but also to compare patients during monitoring.

Clinical algorithms are difficult both to construct and to validate, as expressed by Streiner and Norman, the authors of the standard guide on this subject: *"The most common error committed by clinical researchers is to dismiss existing scales too lightly, and embark on the development of a new instrument with an unjustifiably optimistic and naïve expectation that they can do better"* [185], p. 7. Therefore, one of our studies (Paper I) aimed to evaluate and validate the existing WHO-TB screening tool in our setting, and also to attempt to improve the capacity of this tool to detect or exclude TB by adding a second step consisting of a new scoring system. In a subsequent study (Paper II), we focused on constructing a clinical scale to measure immunosuppression in HIV/TB-coinfected patients, because no such had previously been developed.

There are numerous ways of constructing scales in medicine. Some investigators have used variables based on expert opinion, and in such an approach the face validity of the intrinsic variables is an important aspect (i.e., considering whether a particular parameter really measures what it is intended to measure). In the present work, it was possible to compare the yield, that is, to concurrently validate in relation to an existing gold standard [186], as exemplified by TB confirmed by culture (Paper I) and immunosuppression measured as CD4 cell count (Paper II). Furthermore, there are many methods of finding a set of variables that best represent the condition that the scale is intended to measure, for instance, arithmetic strategies [139] and the Spiegelhalter-Knill-Jones approach [187]. We chose to use a multiple logistic regression

model as a robust technique for correlating a set of variables while at the same time adjusting for associations between variables, a procedure that is being increasingly applied for this purpose [188]. This resulted in the simplest model (i.e., the fewest variables) with the greatest capacity to predict the condition in question. In the context of decentralized care in low-income settings, a simple prediction model is highly advantageous, even if better results can be achieved with other methods using more variables.

We used different approaches to assign scores to the variables in the respective final models. In the first study (Paper I), each variable was assigned 1 point, not taking into consideration that some variables had a stronger explanatory capacity than others. In the second investigation (Paper II), we used the beta coefficients to construct a relative weight for each variable, as also done by other authors [182] and recommended by Streiner and Norman [185]. The decision not to weigh variables (Paper I) was made to maximize the simplicity of this algorithm, whereas using the relative weight (Paper II) was elected to account for the complexity of a combined score representing several CD4 cell strata. Thus, when constructing scales, it is necessary to consider not only the predictive capacity in mathematical terms, but also the biological plausibility of the intrinsic variables and the feasibility of the scale for its intended use.

The disadvantage of scoring approaches in comparison with a gold standard method is that by default they provide a simplified description of the condition of interest. Inasmuch as the scores obtained are compared with another variable, the ideal is to achieve perfect prediction of the outcome variable, but that is usually not the case. Ultimately, for the scales presented in this thesis, there is a tradeoff between the benefits of a cheaper and simpler test and having a lower predictive capacity than the gold standard. However, these scales are intended for use in settings where the gold standard approaches do not exist or are impractical, and the capacities of the respective systems should be viewed from that perspective. We made considerable efforts to mimic the situation that we believe is representative of the places where the majority of HIV and TB patients are receiving care, namely, in low-income settings at a primary health care level rather than at hospital clinics in major cities. However, the generalizability to other settings needs to be tested, because the performance of the scoring systems may differ in other populations and in areas with divergent prevalence of HIV and TB. Thus, before the scales can be used, both the reproducibility and the validity in other populations must be tested prospectively.

TB screening with a clinical algorithm

We found the prevalence of microbiologically confirmed TB to be 137/812 or 17% in HIV patients that had previously undetected TB and were eligible for ART, which emphasizes the need for more effective TB screening [167]. Reliable categorization of the risk of TB could facilitate allocation of resources for diagnostic procedures to those in greatest need and allow initiation of IPT as well as immediate ART in low-risk subjects. At present, advanced diagnostic tools to test for TB (e.g., culture) are not available to the majority of patients in Ethiopia or many other low-income settings, and even when they are at hand, there are considerable delays before the results are accessible, which leads to increased risk of mortality [104]. Furthermore, these tests are primarily used for monitoring drug sensitivity. The Xpert MTB/Rif assay has been endorsed by the WHO as a promising method for early detection of TB. It is also plausible that this test can be particularly effective for diagnosing HIV, especially in patients with a low CD4 cell count [167], and for early detection of MDR-TB, a disease that can otherwise take several months to identify. A rollout of Xpert MTB/Rif to remote areas is underway but requires significant resources. Clearly, it would be advantageous if this assay could be used to distinguish individuals with indeterminate risk of TB after screening. In addition, being determined to be at low risk of TB could enable the implementation of IPT, which has been shown to reduce morbidity of active TB in HIV patients [147].

It is presently proposed that screening for TB in HIV patients be done using the WHO-TB algorithm, in which the occurrence of four symptoms (current cough, fever, night sweats and weight loss) indicates risk of TB, and having none of those symptoms is considered to indicate low risk of TB. The results reported in Paper I confirm the projected predictive capacity of the WHO-TB screening algorithm in terms of its NPV of 90–95% at a prevalence of 10–20% TB cases [140]. This algorithm performed better in our study than in a previous evaluation by Rangaka et al. [189], which showed a NPV of 91.2%; those authors observed that a majority of investigated patients (155/654, 24%) were negative by WHO-TB screening, whereas we found that 625/784 (80%) were positive. The reason for this disparity is not clear but may be related to differences in design: only pre-ART patients with a CD4 cell count of < 350 were included in our study, and these patients may have been more immunosuppressed, with a higher risk of both TB and other OI's that could cause these symptoms. Also, our patients had not previously undergone TB screening and we used prospectively collected data on symptoms, whereas Rangaka et al. employed retrospectively collected data. Our findings imply that the majority of the patients we evaluated would require further TB investigations. To address this issue, we developed an algorithm that could be used as a second step with the WHO-TB screening to improve the potential for risk stratification of TB.

Our approach entailed applying the additional scoring items MUAC < 20 cm, Karnofsky score < 80, peripheral lymph node enlargement, and hemoglobin < 10g/dl to patients that were excluded after being deemed positive by the WHO-TB screening algorithm. Cough, which is the strongest relative predictor among the WHO-TB screening items, was also significant in the second-step scoring system. Ideally, a clinical scoring system to be used in resource-limited settings should be easy to perform and comprise items that can be collected by staff with only limited training at a primary health care level. Measurement of hemoglobin is a laboratory parameter and hence is not a clinical evaluation per se, but it was nonetheless included in our scoring system, because it can be conducted as a point-of-care test, and it is a reliable method that requires relatively inexpensive battery-run equipment [190]. CD4 cell strata was entered into the multivariate model but showed less descriptive capacity than hemoglobin. The Karnofsky performance score has been used for over 60 years to given an overall assessment of complex diseases; this tool ranks the functional status of patients from 0 (dead) to 100 (no complaints and no evidence of disease), and the score can be quickly obtained by untrained staff and is simple to evaluate [191]. Peripheral lymphadenopathy was also associated with TB in our HIV patients, which suggests that enlarged lymph nodes probably represent TB in most cases in a high TB-endemic region [166].

The use of our expanded system might reduce the number of patients that require further TB investigations. In addition, in settings with limited TB diagnostic capabilities, empirical ATT could be considered for the high-risk group, which would further reduce the number of patients in need of diagnostic workup. We argue that clinical categorization of patients according to risk of TB has not been exhausted, and adding a simple second-step algorithm would improve the current WHO standard primarily because it would further identify patients at low risk of TB. This would enable reallocation of resources to patients with a less certain diagnosis and allow faster start of IPT and ART. This could potentially lead to more patients being treated for TB and fewer patients being at risk of both clinical deterioration and community dissemination of TB.

A drawback of using the system with symptom and clinical screening is that it will inevitably miss some patients. More precisely, we found that the WHO-TB symptom screen incorrectly classified 6% of all HIV-positive patients as being at low risk of TB, and the corresponding value for the clinical scoring system was 8% ($p = 0.69$). However, this should be put in perspective that also Xpert MTB/RIF-testing, proposed as the alternative to culture, would also miss a considerable number of patients [167]. We can only speculate about what the long-term clinical outcome would be in these incorrectly classified patients, if they do not receive treatment for TB. However, the outcome was not worse in patients who were only culture positive, even if they started ATT later. Out of 10 patients with TB who were negative by WHO-TB screening ($n = 159$), only one was smear positive (10%); by comparison, 20 patients with TB

were incorrectly deemed as being at low risk of TB by the combined approach (n = 255), and four of those 20 were smear positive (20%). In Khayelitsha, South Africa, Oni and coworkers [192] observed a prevalence of 8.5% of asymptomatic TB (i.e., without the symptoms of cough, weight loss, loss of appetite, fever, and night sweating), and 56% of those patients progressed and showed symptoms within two months.

Naturally, asymptomatic patients cannot be identified with a clinical algorithm, which is a limitation of the scoring approach. Nevertheless, a benefit of a clinical scoring system is that it can easily be repeated, and thus some individuals that have TB but are missed by an original screening might subsequently develop signs or symptoms, and thus would be identified by repeated screening. Moreover, it is likely that such "asymptomatic" carriers of TB are less severely ill and less contagious compared to symptomatic carriers, as TB carriers in our study did not exhibit symptoms associated with mortality from other studies, such as low MUAC and Karnofsky score [151,193].

The overall algorithm bears resemblance to TB score [184], and especially TB Score II [194] (a simplified version of TB score I) which includes the items cough, dyspnea, chest pain, anemia, BMI <18 and <16 and MUAC <22cm and 20 cm and was developed to predict TB disease severity and unfavorable outcome and death during treatment for TB. In this study, the focus was TB risk categorization in HIV-patients, which is known to have a different clinical presentation than in HIV negative. Thus, only HIV positive patients were included which is in contrast to the TB score I and II which were developed on HIV negative as well as HIV positive TB patients. In addition to this disparity regarding the patients examined, the differences between the scales can be partly explained by the fact that some of the variables included in TB scores I and II (e.g., lung auscultation, pulse, and temperature) were not included in our scoring algorithm and vice versa (i.e., peripheral lymphadenopathy, Karnofsky score, fatigue, and CD4 cell count < 200/mm^3).

Assessing immunosuppression in HIV patients

As has been discussed, the pathogenesis of HIV is multifactorial, and the common description of HIV pathogenesis as the progressive decline in CD4 cell numbers is a simplification. In addition to CD4 cells, other types of cells (e.g., CD8+ cells, B cells, and dendritic cells) play an important role in determining the degree of immunosuppression and the increasing and characteristic susceptibility of opportunistic infections and malignancies that are seen in HIV disease [195]. Moreover, immune activation is considered to be the main driver of HIV disease progression, and promising results regarding identification of such progression have been obtained using soluble markers such as neopterin and β2 microglobulin [196].

Nevertheless, CD4 cell count is the current clinical gold standard for estimating level of immunosuppression in HIV-positive patients. Furthermore, CD4 cell count has proven to be the most reliable method available to date and also to be a convenient measurement that can convey an understanding of the disease both to patients and to the general public. Considering that CD4 cell count is as close as possible to being a gold standard assessment of immunosuppression in HIV, we chose to use this technique as the reference when constructing a score for immunosuppression in patients with HIV/TB coinfection. It is not inconceivable however, that the combination of symptoms and signs would be better compared to outcome measures such as AIDS-defining illness or death, though, it would not be ethical to study this today as there is effective treatment for both conditions.

In many resource-limited settings, CD4 cell count has not been available for HIV-positive patients due to the high cost and a lack of electricity and trained staff. New point-of-care CD4 cell testing devices are being deployed and have shown promise as a feasible alternative to laboratory flow cytometry, both in terms of cost and linkage to care [197], although large-scale use of such equipment is still being investigated. Thus CD4 cell count remains an obstacle to further decentralization, which provides a rationale for a simple yet robust algorithm as an alternative to CD4 cell analysis.

Determining when to start ART

In ART-naïve individuals with an established diagnosis of HIV/TB coinfection, a primary concern for the clinician, in addition to treating the TB, is to determine when ART should be started. This decision is based on the severity of the patient's immunosuppression, which also guides the decision to start prophylactic treatment with co-trimoxazole. The guidelines for timing of ART have changed, and the most recent version (from 2010) states that ART should be started immediately (within 2 weeks) in patients with severe immunosuppression (< 50–100 CD4 cells/mm^3), and that ART can be deferred until the end of the intensive phase of TB treatment in patients with a higher CD4 cell count (i.e., > 50-100/mm^3) [85]. Accordingly, our aim was to identify severely immunosuppressed patients (here defined as CD4 cell count < 100/mm^3), because these individuals would benefit most from early ART [151–153]. The benefits of starting early ART must be weighed against the risks of combined treatment, which mainly entail the associated risks of initial clinical deterioration due to IRIS [198] and decreased adherence to treatment due to the increased pill burden [199]. Therefore, in a recent study, it was proposed that ART be deferred for up to 6 months in patients with a CD4 cell count of > 220/mm^3 in order to minimize the mentioned risks, because it was found that deferred treatment was not associated with higher mortality [154].

Our final model poorly identified severely immunosuppressed (<100 cells/mm^3) HIV/TB-coinfected patients, with a PPV that did not reach more than 55% even at scores higher than 7. However, there was still a clear correlation between the scoring system and CD4 cell count strata, as shown in Table 4. This analysis demonstrated that the scoring system performed reasonably well in excluding severe immunosuppression (< 100 CD4 cells/mm^3), with a NPV of 87% at scores of ≤ 2, which thus might be used as a basis for deferring ART until the end of the intensive phase as stipulated in the current guidelines. This would provide much needed time to ensure the success of TB treatment by minimizing the risk of drug–drug interactions and IRIS, and to prepare patients for life-long treatment with ART.

Wasting

Early in the AIDS pandemic, wasting in HIV-positive patients was considered an AIDS-defining condition HIV [200] and was defined as weight loss of > 10% and chronic weakness for at least 30 days not attributable to a concurrent condition. However, a similar clinical presentation has long been described in TB disease as well [1]. A common denominator in two of the present studies (Papers I and II) is the finding that signs of wasting (low MUAC and to some extent low hemoglobin) were associated with TB in HIV patients and also with low CD4 cell count in HIV/TB-coinfected patients. An intercorrelation between BMI and MUAC (r = 0.61) was observed in both those investigations, but, considering that our aim was to create a parsimonious model, we included only the most strongly related of the two (i.e., MUAC in both cases) in the final models. BMI and MUAC probably reflect slightly different aspects of wasting. In short, BMI is related to the whole-body composition of fat and lean mass, whereas MUAC is an indicator of muscle reserve [201] and thus seems to be particularly affected in advanced TB.

We speculate that wasting in HIV/TB is associated with the severity of the TB, Several other studies have noted that signs of wasting are associated with worse outcome in TB patients with or without HIV, supporting the idea that severe TB can give rise to wasting. Cain et al. [151] found that low BMI was independently associated with low Karnofsky score and increased mortality in HIV/TB patients, and Gustafsson et al. [193] found an independent association between MUAC and mortality in HIV-positive patients with TB in West Africa. In TB patients with or without HIV, TB scores I and II showed that BMI, MUAC, and anemic conjunctivae were associated with adverse outcome and death during ATT [184,194]. Furthermore, Villamor et al. [202] observed that severity of TB measured as bacillary load was correlated with HIV disease, low MUAC, and low Karnofsky score. The mechanism behind this clinical

presentation caused by TB is not known, although it may involve increased production of TNF-α [203].

In addition to inducing wasting, TB has important implications for the immune system that also seem to be related to the severity of the disease, and these were examined in two of our studies (Papers III and IV).

Lymphocytopenia in TB-patients

In order to study immunological properties and the impact of TB on HIV disease, we also included HIV-negative TB cases recruited at the same clinics. As discussed above, CD4 cell count is the principal strategy for determining immunosuppression in HIV but other factors may influence these levels other than HIV, such as portal hypertension, leishmaniasis and other opportunistic infections [204]. Reports mainly from United States, west Africa and Tanzania indicated that TB causes peripheral CD4 lymphocytopenia [15,16], and some investigations performed close to our study sites described subnormal CD4 cell counts in apparently healthy Ethiopians [205–207] although they had not been evaluated for TB.

We found subnormal levels of CD4 cells in a large proportion of TB patients: counts were below the normal range in 25% and < 350 cells/mm^3 in 10%. As we found normal CD4 cell levels in our control group, we did not interpret our findings of lymphocytopenia to be attributed to factors specific to the Ethiopian setting. Rather, the low CD4 cell counts in our TB patients increased during the course of ATT, which indicates that it was indeed TB that was responsible for this observation. In our patients, a low CD4 cell count was associated with clinical parameters that are usually attributed to advanced TB, such as smear-positive disease, a bedridden state, and signs of wasting (low MUAC), which suggests advanced disease.

The group of TB patients with CD4 cell levels of < 500/mm^3 in our assessment had counts that were significantly increased after both 2 and 6 months but did not reach the levels seen in healthy controls. This indicates that the restoration of CD4 cells is a slow process that continues throughout ATT, and also implies that complete restoration, if achieved, may take even longer than the prescribed ATT period. This is in analogy with data indicating that TB influences CD4 cell restoration in HIV/TB patients, and that this effect is sustained for several years, even after TB treatment is completed [208].

We found that CD4 cell counts in HIV-positive patients who did not start ART were slightly increased after 6 months (but not after 2 months). Other studies evaluating HIV/TB patients not on ART have provided heterogeneous results. Dean et al. [209] observed an increase in CD4 cell count in such patients (n = 20), but the change was

not statistically significant. Two other investigations examined the evolution of CD4 cells during ATT in HIV patients and found that CD4 cell levels at the end of treatment were stable and comparable to the levels noted at the start of ATT. In Tanzania, Andersen et al. [16] noted that CD4 cells in HIV patients with TB (n = 500) increased after 2 months but not after 5 months. Also, Wejse et al. [210] found no significant changes in CD4 cell levels 8 months after ATT (n = 92). The overall increase seen in our evaluation must also be considered in light of the heterogeneity of CD4 cell development in the studied material. Of the 71 HIV-positive patients part of this analysis, we observed that CD4 cell levels increased in 35 subjects (> 50 cells/mm^3), did not change in 19 patients (± 50 cells/mm^3), and decreased in 17 patients (> 50 cells/mm^3). In contrast, the CD4 cell levels increased in a majority of the HIV-negative patients (87/119).

Thus, levels of CD4 cell levels in peripheral blood are depressed in many TB patients, and this may be partly reversed by ATT. We also noted that individual patients differed with respect to the evolution of CD4 cell count during ATT, and this disparity was especially pronounced in HIV-positive patients. This has implications for the interpretation of CD4 cell levels in HIV patients with a concomitant TB diagnosis. However, further research is needed to determine the extent of the practical significance of this finding, including the immunological and clinical perspectives.

A plausible immunological explanation is a reallocation of T-cells from peripheral blood to the lungs (called "homing") and other sites of disease, for which there is also support in the literature [211]. Moreover, it is possible that CD4 cells are depleted either through immunological activation or as the result of decreased production of CD4 cells caused by cytokine suppression of production of precursor cells in the bone marrow. However, we found that proportions of CD4 and CD8 cells remained unchanged. Hence, it is less likely that selective degeneration of one type of cells is the only explanation, and more likely that there is a combination of a general depression of CD4 cells caused by extensive cytokine production in cases of severe TB and reallocation of T-cells in extensive cell infiltrates in TB patients. Furthermore, since the levels of CD4 cells were not reversed entirely after ATT (seen both by us and by Andersen et al. [16]), suggests that there is also a component of exhaustion of CD4 cell levels either in the bone marrow or as a non-specific general depletion of the T-cell population. We speculated that TB-induced immune activation, which is also regarded as an intrinsic component of HIV pathogenesis, could be involved in such CD4 T-cell depletion, which was one hypothesis in Paper IV.

Immune activation in TB patients with and without HIV

Neopterin is a well-studied marker of immune activation that reflects levels of IFN-γ, representing the main pathway by which T-cells activate macrophages [53]. Neopterin is stable in peripheral blood and can be measured in plasma or urine, and rapid tests are available for detection of this purine nucleotide molecule [55]. As described in Paper IV, we analyzed neopterin along with CRP, which is a marker of systemic inflammation and can also be detected by a rapid test. Our main objective was to study the role of immune activation in TB patients with or without HIV. We also aimed to correlate neopterin and CRP levels with CD4 cell counts to ascertain whether immune activation was associated with the CD4 cell depletion observed in a previous study (Paper III), and to determine whether neopterin and CRP can be used as alternative biomarkers to predict CD4 cell level strata.

We found high levels of neopterin in patients with HIV/TB coinfection in this study (Paper IV), which currently represents the largest investigation of CD4 cell depletion in both HIV-positive and HIV-negative TB patients (see Table 5). Our results were largely in agreement with the findings of previous studies using neopterin in equivalent patients [52,212–218], which showed neopterin concentrations of 20–55 nmol/l in TB patients without HIV compared to the corresponding figure of 23.2 nmol/l in our investigation. Also, other authors have reported neopterin concentrations in the range of 37.8–90 nmol/l in HIV/TB-coinfected individuals (largely depending on CD4 cell levels), whereas we recorded a median concentration of 54 nmol/l. A limitation in our assessment was that we did not include an HIV-positive group without TB due to the design of cohort II, but previous studies have shown concentrations between 7 and 16 nmol/l in such patients (see Table 5).

Table 5:
Studies of neopterin concentrations in HIV and TB patients published from 1990 to 2015

Reference, year (country)	Mean or median neopterin level (nmol/l)			Comment
	HIV only	TB only	HIV/TB	
Fahey et al., 1990 (USA)	13.6 (n = 395)			Neopterin associated with progression of HIV disease
Ayehunie et al. 1993 (Ethiopia and Sweden)*	Asymptomatic Ethiopian HIV patients ≈ 7 (n = 20), Ethiopian AIDS patients ≈ 40 (n = 30)			Increased neopterin in HIV patients in Ethiopia compared to Sweden Not evaluated for TB or other opportunistic infections
Vanham et al., 1996 (USA)**		≈ 20 (n = 25)	≈ 70 (n = 11)	Pronounced immune activation in HIV^+/TB patients Also increased TNF-α and β-2 microglobulin
Immanuel et al., 2001 (India)		39.3 (n = 39)		ATT decreased neopterin to near normal results
Mildvan et al., 2005 (USA)	16 (n = 152)			Neopterin best predictor of clinical progression of HIV
Immanuel et al., 2005 (India)	13.5 (n = 10)	29.2 (n = 10)	90.7 (< 200 cells/mm^3) (n = 15), 37.8 (> 200 cells/mm^3) (n = 10)	Neopterin levels declined during ATT in groups but remained elevated in HIV/TB patients
Hanna et al., 2009 (India)***		55 (n= 37)	96 (< 200 cells/mm^3) (n = 24), 49 (> 200 cells/mm^3) (n = 18)	Levels declined during ATT but less in coinfected patients → underlying immune activation persisted despite ATT
Gordeuk et al., 2009 (Zimbabwe)		28 (n=15)	51 (n = 24)	Neopterin levels declined during ATT in both groups
Skogmar et al., 2015		23.2 (n = 170)	54.5 (n = 195), 77 (< 100 cells/mm^3) (n = 44)	High level of immune activation in coinfected patients High baseline neopterin correlated with increased CD4 cell count

*No exact figures given in text; figures presented here were taken from graph.
**No exact figures given; figures presented here were taken from graph and converted from ng/ml to nmol/l (nmol/l / 0.253).
***Figures converted from ng/ml to nmol/l (nmol/l / 0.253).

Immune activation is considered to be the driving force of HIV disease by leading to both continuous viral replication and successive depletion of T-helper cells. Immune activation in HIV is multifactorial and can be caused by one or more of the following: the virus itself; microbial translocation over the gut mucosa; reactivation of latent virus reservoirs (CMV); acute opportunistic infections, particularly TB. TB may appear early in HIV infection and thus has a greater potential to cause an impact on disease progression than opportunistic infections that appear later when HIV is already advanced. There is evidence that TB affects the progression of HIV disease. Although there is some controversy about the epidemiological data supporting such an impact [49], most studies have shown an elevated viral load in HIV/TB-coinfected patients in vivo [14,125,126], although one recent investigation demonstrated the opposite [219]. The detected rise in viral load may not be uniform for all types of cells, considering that experiments in vitro have actually found that Mtb has an inhibitory effect on HIV replication in macrophages [220]. Under most circumstances, however, the activation of the immune system caused by TB will result in a net increase in virus production. The high neopterin levels we observed support the assumption that TB contribute extensively to faster progression of HIV in TB-endemic settings.

Although increased viral replication is probably the main pathway by which TB affects HIV disease progression, immune activation may also exert additional effects on CD4 cells. We found the highest neopterin level (77 nmol/l) in HIV/TB-coinfected patients with a CD4 cell count of < 100/mm^3. Interestingly, we observed the same inverse relationship between low CD4 cell count and high neopterin level in HIV-negative TB patients. Together, these findings suggest that immune activation is coupled to CD4 cell depletion, not only in HIV/TB-coinfected individuals, but also in TB patients without HIV (Paper III). Thus a common mechanism that is not related to the pathogenesis of HIV may be responsible for CD4 cell depletion in both HIV-positive and HIV-negative TB patients, and such a mechanism may involve immune activation that induces apoptosis of CD4 cells [221].

Furthermore, it is possible that immune activation in TB is related to the severity of the TB infection. Our analysis of the evolution of CD4 cell count in relation to baseline neopterin level showed that a high baseline concentration of neopterin was associated with increased CD4 cell count during ATT. One interpretation of this finding is that increased neopterin levels are associated with a more severe infection that causes T-cells to activate Mtb-infected macrophages in tissues, which in turn releases neopterin. Notably, this hypothesis is further supported by results obtained by other investigators demonstrating that levels of IFN-γ in bronchoalveolar lavage fluid are correlated with the severity of TB [222]. After ATT is initiated, and there is successive clearance of the mycobacterial infection, the trigger for immune activation is removed, which permits restoration of CD4 cells in the peripheral blood and thus accounts for the association between high neopterin and increased CD4 cell levels seen in our assessments. Neopterin is thus a candidate not only for measuring HIV disease progression, but also

for quantifying the severity of TB. Furthermore, neopterin is found in much lower levels in HIV-negative than in HIV-positive TB patients, and thus it also shows promise as a marker for identifying TB in HIV patients.

Although we did find a correlation between neopterin and CD4 cell count, it was not strong enough to support the use of neopterin as a surrogate marker of CD4 cell levels. Using an approach similar to that described in Paper II, we evaluated neopterin and CRP (the latter chiefly reflecting systemic inflammation) regarding their ability to predict CD4 cell strata. Considering patients with < 100 CD4 cells/mm^3 as having a greater immunosuppression benefit from early ART, this group was used as outcome measure in this assessment. In a comparison including neopterin and CRP and carried out as a ROC curve analysis, none of the biomarkers under consideration performed better than the algorithm combining clinical signs and symptoms (see Figure 14).

In summary, it is clear that TB contributes significantly to immune activation in HIV/TB coinfection, and this effect was found to be related to low CD4 cell count in the largest investigation of such coinfected patients to date. This gives novel insight into how immune activation constitutes a common pathogenic mechanism in both conditions, and it underlines the urgent need to identify and treat TB in HIV patients. Based on our findings, we propose neopterin as a candidate marker for TB in HIV-infected patients and possibly also as a marker for disease severity.

Conclusions

- Compared to using only the existing WHO-TB symptom screening, performing only a simple clinical investigation could double the number of patients as being at low risk of TB, thus not prioritized for further TB investigations (20% vs. 53%).
- A simple scoring system covering symptoms and signs could be used to rule out severe immunosuppression (< 100 CD4 cells/mm^3) in more than a third (38%) of HIV/TB-coinfected patients to guide clinicians' decisions about when to initiate ART in TB patients without CD4 cell testing.
- TB was found to be associated with a low CD4 cell count (< 500/mm^3) in a large proportion (25%) of the studied patients, and this was related to symptoms and signs associated with severe disease, such as smear positivity, being bedridden, and signs of wasting. The CD4 cell count was partially restored during TB treatment but did not reach the level seen in TB/HIV-uninfected controls even after 6 months of treatment, suggesting that TB has a prolonged impact on the immune system.
- TB induces significant immune activation, measured as neopterin level, and this was especially prominent in HIV/TB-coinfected individuals compared to controls. This observation gives new insight into how TB can be a potent driver of HIV disease, because progression of HIV is related to the degree of immune activation, and it also provides further incentive to find and treat TB in areas endemic to both diseases.
- Neopterin and CRP correlated to CD4 cell count in TB patients, but were not deemed as suitable surrogate markers for CD4 cell count in HIV/TB coinfected.

Future perspectives

Clinical algorithms for HIV/TB coinfection

Like most research, the present investigations have given rise to more new questions than answers. First of all, external validation is needed before implementation of the algorithms can be considered. The models must be validated in prospective clinical outcome studies and also in other settings, because they may perform differently in different populations. In principle, the models could be used in sequence according to the model proposed In Figure 16, which would minimize the need for diagnostic workup. However this strategy has to be tested, preferably using hard outcomes such as mortality. TB is the most important opportunistic infection in HIV patients in terms of mortality, but this principle of algorithmic management could also be applied to other common opportunistic infections and AIDS-related conditions (e.g., hepatitis B and C, and cervical cancer). Development of similar constructs could be considered for other conditions that have a marked impact on management of HIV in decentralized settings, as exemplified by the proposed algorithm for ART failure [223].

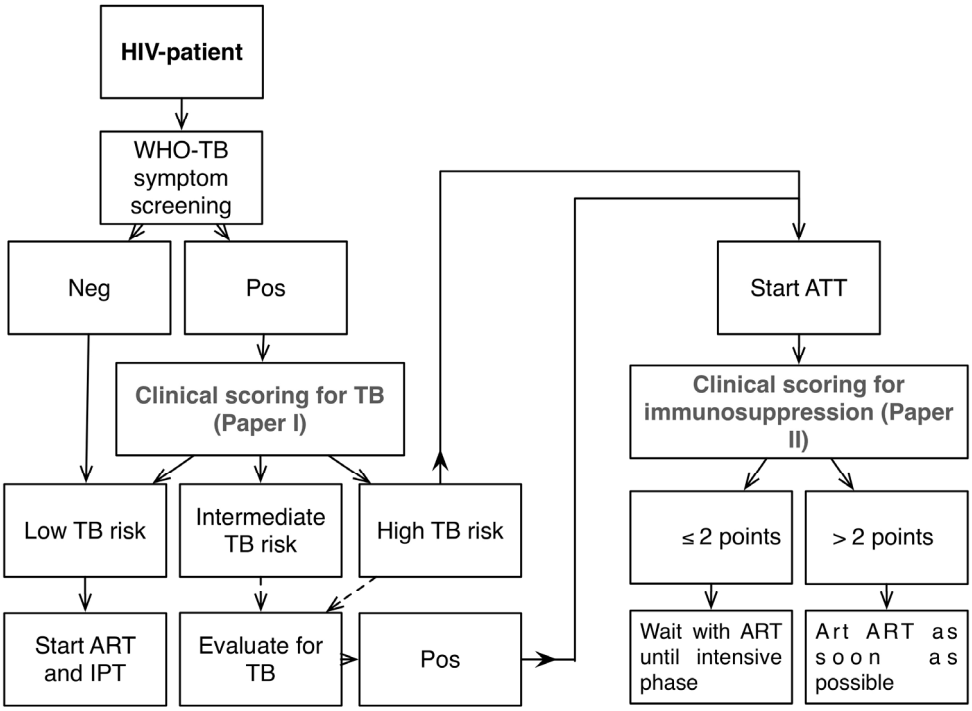

Figure 15:
Proposed model for an integrated approach of two scoring systems in HIV patients in TB-endemic areas.

An interesting aspect for further research would be to consider the patients that are missed by the first scoring algorithm, that is, those who are asymptomatic but have culture- or PCR-confirmed TB (according to both the WHO symptom screening and the clinical screening). It is known that some patients are asymptomatic, but it is unclear what this represents. One possibility is that signs (clinical or laboratory) or symptoms are actually present but have simply not been investigated. If the patients in this group are truly asymptomatic "carriers" of TB, what is the natural course of their disease? (which is impossible to study from an ethical perspective). If given only ART, would they clear the disease or progress to active TB? How contagious are they? Repeated scoring could be an alternative method to identify these patients. Moreover, the 625 patients with HIV and eligible for ART but without active TB in our studies constitute an interesting subgroup. These individuals lived in a TB-endemic area but did not have active TB despite exposure to considerable risk factors. What immunological characteristics enabled these patients to withstand TB under such conditions?

Immunological aspects of TB

The finding that TB gives rise to a low peripheral CD4 cell count poses new questions regarding the underlying mechanism of the disease in humans, which has not been satisfactorily elucidated. Are CD4 cells in peripheral blood actually destroyed, or are they merely reallocated to the site of disease as suggested by some data and animal models? Is it only TB-specific CD4 cells that are decreased in peripheral blood, or is there a general depletion in the CD4 T-cell pool? Does the depletion have any clinical relevance other than requiring consideration when interpreting CD4 cell results? For instance, could CD4 cell count be used as a marker for severity of TB, and does a low count in TB patients confer a risk of other infections?

The finding of high levels of immune activation in HIV/TB-coinfected patients provides insight into a potential mechanism by which TB can accelerate the progression of HIV disease. However, it is not clear whether this phenomenon is temporary and is mitigated by ATT, or if it has a sustained impact. Furthermore, neopterin may represent a different aspect of HIV immunopathology than CD4 cells do. Perhaps neopterin, much like viral load, is more an indicator of the velocity of disease progression than a marker for the "current state" of the immune system. Finally, considering that other studies have shown that HIV as a single infection gives rise to neopterin levels of approximately 7–16 nmol/l (see Table 5), whereas HIV/TB coinfection leads to markedly higher levels (median 54 nmol/l), it seems that neopterin shows promise as a marker for TB case finding in HIV patients, which also merits further investigation.

Disease severity

Although it has not been a focus of research as TB has been regarded largely as positive or negative, there are compelling reasons why determination of disease severity could be of value in the clinical setting. First, it may be appropriate to use different treatments for patients with severe disease and those who are asymptomatic but have positive microbiological findings. Second, a system for classifying TB severity might aid research aimed at developing diagnostic tools. Third, rating disease severity could provide beneficial information regarding the success of treatment or the risk of relapse if the parameters do not improve.

As discussed in this thesis, the pathogenesis of TB depends more on the host's response to the disease than on the disease itself. The clinical presentation of TB is probably determined by a mixture of the bacterial load, the virulence of the bacteria, and the immune response of the host. There is no reliable way to measure any of these variables, and, even more importantly, there is no method to characterize the relative contribution of each component to the total disease severity. The present results suggest

that, in addition to clinical parameters, CD4 cell count and measurement of neopterin can provide information to determination of TB disease progression, which is of interest for further research. A simplified visual presentation of the proposed relationship is presented in Figure 17.

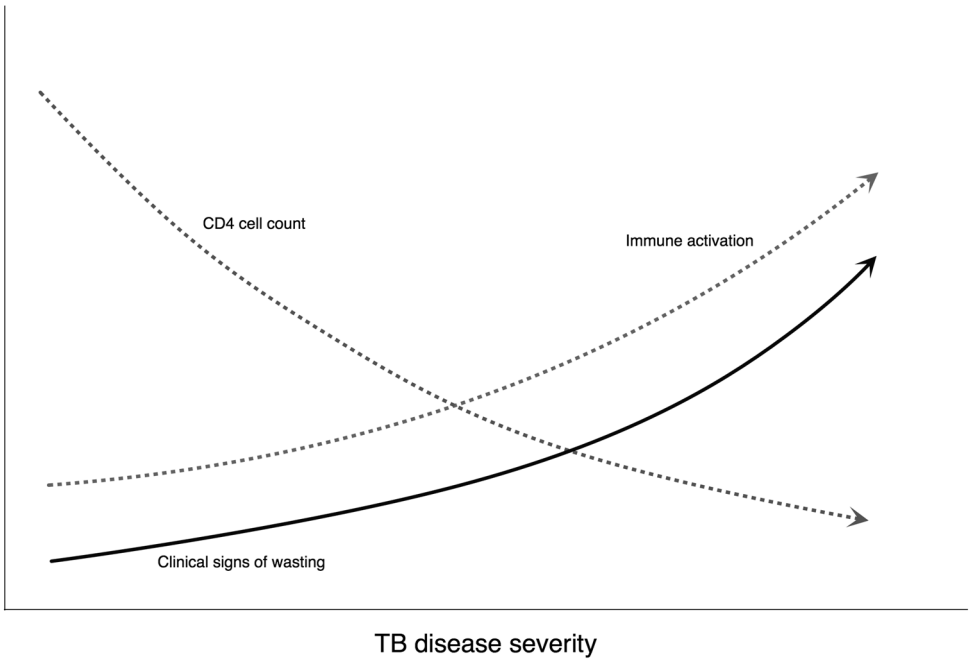

Figure 16
Schematic diagram of the proposed relationship between the progression of TB and CD4 cell count, immune activation, and wasting in HIV-positive TB patients.

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Paper I

MAJOR ARTICLE

A Clinical Scoring Algorithm for Determination of the Risk of Tuberculosis in HIV-Infected Adults: A Cohort Study Performed at Ethiopian Health Centers

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Background. The World Health Organization (WHO) tuberculosis (TB) symptom screening instrument (WHO-TB) can identify human immunodeficiency virus (HIV)-infected individuals at low risk of tuberculosis (TB); however, many patients report WHO-TB symptoms and require further TB investigations. We hypothesized that further clinical scoring could classify subjects with a positive WHO-TB screening result (WHO-TB$^+$) for the likelihood of TB.

Methods. HIV-infected adults eligible to initiate antiretroviral therapy (ART) were recruited and prospectively followed at 5 Ethiopian health centers. Irrespective of symptoms, all participants underwent sputum bacteriological testing for TB. Symptoms, physical findings, hemoglobin, and CD4 cell count results were compared between subjects with and those without bacteriologically confirmed TB. Variables associated with TB in WHO-TB$^+$ individuals were used to construct a scoring algorithm with multiple logistic regression analysis.

Results. Among 812 participants, 137 (16.9%) had TB. One hundred fifty-nine persons (20%) had a negative WHO-TB screen, 10 of whom had TB (negative predictive value [NPV], 94% [95% confidence interval {CI}, 90%–97.5%]). For WHO-TB$^+$ subjects, the following variables were independently associated with TB, and were assigned 1 point each in the clinical scoring algorithm: cough, Karnofsky score $\leq$80, mid-upper arm circumference <20 cm, lymphadenopathy, and hemoglobin <10 g/dL. Among subjects with 0–1 points, 20 of 255 had TB (NPV, 92% [95% CI, 89%–95%]), vs 19 of 34 participants with $\geq$4 points (positive predictive value, 56% [95% CI, 39%–73%]). The use of WHO-TB alone identified 159 of 784 (20%) with a low risk of TB, vs 414 of 784 (53%) using WHO-TB followed by clinical scoring ($P < .001$). The difference in proportions of confirmed TB in these subsets was nonsignificant (6.3% vs 7.2%; $P = .69$).

Conclusions. Clinical scoring can further classify HIV-infected adults with positive WHO-TB screen to assess the risk of TB, and would reduce the number of patients in need of further TB investigations before starting ART.

Clinical Trials Registration. NCT01433796.

Keywords. HIV; tuberculosis; scoring; health center; Ethiopia.

Tuberculosis (TB) and human immunodeficiency virus (HIV) have important reciprocal interactions. Hence, HIV testing for subjects with TB, as well as TB screening in people living with HIV (PLHIV), are both strongly recommended [1]. Simple methods with high

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accuracy are available for HIV screening in TB patients; however, the situation is markedly different for the reverse case—identification of TB in PLHIV [2, 3].

HIV infection affects the clinical presentation of TB; respiratory manifestations may be absent, and nonspecific constitutional symptoms that are also common in advanced HIV disease are often predominant [4, 5]. In addition, the performance of routine diagnostic methods for TB is inferior in HIV-coinfected subjects, with lower sensitivity for sputum smear microscopy [6, 7] and reduced specificity for chest radiography [8]. Consequently, active TB may be missed in PLHIV. Administration of antiretroviral therapy (ART) to patients with unrecognized active TB is associated with increased risk of death and immune reconstitution inflammatory syndrome (IRIS) during ART [9, 10]. Importantly, exclusion of active TB is required before initiating isoniazid preventive therapy (IPT). IPT has been shown to reduce mortality and TB incidence among PLHIV [11]. Despite this, rates of IPT prescription to PLHIV remain low [12], mostly due to difficulties in excluding active TB.

Currently, the World Health Organization TB symptom screening algorithm (WHO-TB) is recommended for TB screening in PLHIV [13]. This screening tool is based on the self-reported presence of any of 4 symptoms found to be associated with active TB in PLHIV (weight loss, fever, night sweats, and cough of any duration). Because WHO-TB has a high negative predictive value (NPV), it is mainly used as a "rule-out" test for TB [4]. Thereby, inappropriate IPT prescription to subjects with active TB can be avoided, and persons who may start ART without additional TB testing can be identified. However, the positive predictive value (PPV) of WHO-TB for detection of TB is low. Because the proportion of PLHIV reporting WHO-TB symptoms can be as high as 80% [4, 14], many PLHIV will require further TB investigations prior to initiation of ART and IPT.

The need for TB investigations for large proportions of PLHIV is a major challenge for health care systems in high-prevalence regions with constrained resources. In such settings, a clinical algorithm for further categorization of individuals who have positive WHO-TB screening results (WHO-TB$^+$) with regard to their likelihood of TB could be of great benefit.

We hypothesized that clinically based scoring might identify WHO-TB$^+$ patients with a high or low likelihood of TB. This would reduce the proportion of patients who need further TB investigations, and allow for timely initiation of therapy for both HIV and TB. In this study, we have constructed a scoring algorithm based on data from a cohort of PLHIV recruited in Ethiopian health centers, with bacteriologically confirmed TB for reference. We have compared the performance for TB categorization among PLHIV using this clinical scoring algorithm as a second-step screening for WHO-TB$^+$ individuals to that of screening based solely on WHO-TB.

METHODS

Study Design and Participants

Participants for this cohort study were consecutively recruited at 5 health centers in the Oromia region of Ethiopia from October 3, 2011 to March 1, 2013 [14], representing all public health centers providing ART in an uptake area of about 600 000 inhabitants. All ART-naive HIV-infected persons receiving care at the study sites were screened for eligibility, irrespective of symptoms or clinical suspicion of TB. The following inclusion criteria were applied: age ≥18 years, residence in the uptake area, submission of at least 1 pair of sputum samples, and written informed consent. Patients with previous ART and those who had received anti-TB treatment (ATT) for >2 weeks prior to screening were excluded.

The study was approved by the National Research Ethics Review Committee, Ministry of Science and Technology, Addis Ababa, Ethiopia, and the Regional Ethical Review Board, Lund University, Lund, Sweden. Written informed consent (in the presence of an impartial witness for illiterate participants) was obtained before inclusion.

Procedures

Demographic information and medical history were collected using a structured questionnaire, followed by recording of disease symptoms and findings from physical examination (including Karnofsky performance score, body mass index [BMI], and mid-upper arm circumference [MUAC]). All study procedures were performed by health center nonphysician clinicians who were certified ART providers; additionally, all persons involved in the study procedures received detailed training on the study protocol. Blood samples were obtained for hemoglobin and CD4 cell count.

At inclusion, participants were requested to submit 2 pairs of spontaneously expectorated morning sputum samples. One randomly selected sample of each pair was delivered to International Clinical Laboratories in Addis Ababa for liquid culture (using the BACTEC mycobacterial growth indicator tubes 960 system, BD Diagnostics). The remaining sample was submitted to Adama Regional Laboratory for smear microscopy (using Ziehl-Neelsen staining) and Xpert MTB/RIF testing (using a 4-module GeneXpert instrument, Cepheid). Fine-needle aspirates for liquid culture and Xpert MTB/RIF testing were obtained from subjects with enlarged peripheral lymph nodes (>1 cm). Samples were delivered under cold-chain system to the respective laboratory within 6 hours of collection.

Participants were identified with study-specific codes. Project data managers continuously entered all data into a study database and conducted regular cross-checking of all entries. The investigators performed weekly monitoring throughout the duration of the study. Laboratory staff were blinded to clinical data.

Adama Regional Laboratory is an external quality assurance center for all public laboratories in the Oromia region. International Clinical Laboratories is accredited by the Joint Commission International.

Results were regularly delivered from the laboratories throughout the duration of the study. ATT was initiated for participants with positive bacteriological test results for TB. ATT was also prescribed for patients with negative microbiological results who fulfilled clinical criteria for TB diagnosis according to Ethiopian guidelines [15]. Participants were followed for 6 months, with monthly visits for the first 3 months and 1 visit at 6 months. For patients with clinically suspected TB during this follow-up period, repeated investigations according to the procedures described above were recommended.

Statistical Analysis

Participants were categorized as TB and non-TB cases. TB cases were defined as subjects with bacteriologically confirmed pulmonary and/or extrapulmonary TB (at baseline or within 3 months after enrollment). Participants with negative bacteriological results who did not receive empirical ATT within 3 months of enrollment, were defined as non-TB cases. Participants with clinically diagnosed TB (defined as subjects who were prescribed ATT without microbiological confirmation) at baseline or within 3 months of enrollment were excluded from statistical analysis.

All variables considered to have potential association with TB based on biological plausibility (including disease symptoms, Karnofsky performance score, physical findings, biometrical data, hemoglobin, and CD4 cell count) were entered into univariate logistic regression analysis. Univariate and multivariate analyses were performed for all study participants and separately for the subset with positive WHO-TB results. Continuous variables were initially separated by quartiles depending on their distribution. For the subsequent analysis, these items were dichotomized at clinically meaningful cutoff levels, after controlling that the distribution of these variables in the study population was not discordant with such a dichotomization.

To achieve the simplest possible algorithm with the best possible predictive capacity for TB, stepwise backward multivariate logistic regression analysis was performed, including variables with a P value <.3 in univariate analysis. In each step, the least significant variable was excluded until a parsimonious model, only including significant variables ($P < .05$), was reached. Each significant variable from the final logistic multivariate analysis of WHO-TB$^+$ subjects was assigned 1 scoring point. The resulting scoring system was applied to the cohort to estimate its predictive capacity for TB. Proportions, PPV, and NPV were determined for 3 levels: low, intermediate, and high risk for TB. All statistical analyses were performed using IBM SPSS software, version 20.0.

RESULTS

Patient Characteristics

Of 873 eligible persons, 812 submitted at least 1 sputum sample for TB testing. Among these, 137 had bacteriologically confirmed TB; 31 (22.6%) of these were smear-positive, 96 (70.1%) Xpert-positive, and 123 (89.8%) culture-positive. All smear-positive cases were also positive by Xpert and/or culture. TB was detected in lymph node aspirates from 10 subjects; 8 of these were also positive by sputum testing. Twenty-one subjects with negative bacteriological results at baseline had clinically diagnosed TB, and were excluded from study analyses (8 at inclusion and 13 within 3 months of enrollment). Between 3 and 6 months after enrollment, 1 additional case of TB was diagnosed (bacteriologically confirmed). Characteristics of the 791 included subjects are shown in Table 1.

Factors Associated With TB

Each WHO-TB symptom (cough, fever, weight loss, and night sweats) was independently associated with TB in univariate analysis. Additionally, the following variables showed significant associations with TB: loss of appetite, fatigue, chest pain, dyspnea, Karnofsky score ≤80, conjunctival pallor, peripheral lymphadenopathy, BMI <18.5 kg/m^2, MUAC <20 cm, CD4 count <200 cells/μL, and hemoglobin <10 g/dL (Supplementary Material).

In multivariate analysis, the following variables retained statistically significant associations: cough, Karnofsky score ≤80, MUAC <20 cm, hemoglobin <10 g/dL, and peripheral lymphadenopathy.

Factors Associated With TB in WHO-TB$^+$ Subjects

For construction of the clinical scoring algorithm, 159 WHO-TB$^-$ participants were excluded, as well as an additional 7 WHO-TB$^+$ persons with incomplete registration of the 4 WHO-TB symptoms. For the remaining 625 subjects included in this analysis, all WHO-TB variables were significantly associated with TB, as well as the additional variables identified for the whole cohort described above.

The following variables retained statistically significant associations with TB in the parsimonious multivariate model: reported cough, Karnofsky score ≤80, MUAC <20 cm, peripheral lymphadenopathy, and hemoglobin <10 g/dL (Table 2). These variables were each assigned 1 point in the clinical scoring algorithm.

The distribution of participants with regard to the results for WHO-TB and the variables included in the follow-up clinical scoring algorithm is shown in Figure 1.

Performance of the WHO-TB Algorithm and Follow-up Clinical Scoring for TB Categorization

The respective performance of the WHO-TB symptom screening algorithm alone and combined with the clinical scoring

Table 1. Baseline Characteristics of Study Participants With Regard to Bacteriologically Confirmed Tuberculosis

Characteristics	Total (N = 791)	TB (n = 137)	Non-TB (n = 654)	*P* Value
Demographic characteristics				
Age, y, median (IQR)	32 (28–40)	35 (28–40)	32 (28–39)	.105
Sex				
Male	324 (41.0)	71 (51.8)	253 (38.7)	.005
Female	467 (59.0)	66 (48.2)	401 (61.3)	
Residence				
Urban	617 (79.1)	104 (78.2)	513 (79.3)	.815
Rural	163 (20.9)	29 (21.8)	134 (20.7)	
Permanent home				
Yes	601 (76.8)	97 (71.3)	504 (77.9)	.117
No	182 (23.2)	39 (28.7)	143 (22.1)	
TB-related factors				
Self-reported history of TB				
Yes	49 (6.3)	4 (2.9)	45 (7.0)	.082
No	730 (93.7)	132 (97.1)	598 (93.0)	
Household members on TB treatment				
Yes	13 (1.6)	2 (1.5)	11 (1.7)	1.00
No	776 (98.4)	135 (98.5)	641 (98.3)	
Prior TB in household member				
Yes	33 (4.2)	3 (2.2)	30 (4.6)	.246
No	753 (75.8)	134 (97.8)	619 (95.4)	
Behavioral factors				
Smoking				
Yes	35 (4.4)	10 (7.3)	25 (3.8)	.105
No	756 (95.6)	127 (92.7)	629 (96.2)	
Alcohol[a]				
Yes	192 (24.3)	37 (27.0)	155 (23.7)	.443
No	599 (75.7)	100 (73.0)	499 (76.3)	
Khat[b]				
Yes	60 (7.6)	16 (11.7)	44 (6.7)	.052
No	730 (92.4)	121 (88.3)	609 (93.3)	
HIV care				
Enrollment history				
New	240 (30.3)	52 (38.0)	188 (28.7)	.041
In care	551 (69.7)	85 (62.0)	466 (71.3)	
WHO clinical stage				
1 or 2	383 (48.6)	40 (29.2)	343 (52.7)	<.001
3 or 4	405 (51.4)	97 (70.8)	308 (47.3)	
WHO TB symptom screening				
Positive	625 (79.7)	126 (92.6)	499 (77.0)	<.001
Negative	159 (20.3)	10 (7.4)	149 (23.0)	
On IPT				
Yes	19 (2.4)	1 (0.7)	18 (2.8)	.224
No	769 (97.6)	136 (99.3)	633 (97.2)	
On CPT				
Yes	597 (75.9)	102 (75.0)	495 (76.0)	.826
No	190 (24.1)	34 (25.0)	156 (24.0)	
Hospitalization[c]				
Yes	11 (1.4)	3 (2.2)	8 (1.2)	.416
No	779 (98.6)	134 (97.8)	645 (98.8)	

Table 1 continued.

Characteristics	Total (N = 791)	TB (n = 137)	Non-TB (n = 654)	*P* Value
Laboratory characteristics				
Hemoglobin, g/dL, median (IQR)	11.6 (10.2–12.7)	10.4 (9.1–11.9)	11.9 (10.5–12.9)	<.001
CD4 count, cells/μL, median (IQR)	212 (119–321)	172 (91–269)	221 (126–328)	<.001

Abbreviations: CPT, cotrimoxazole preventive therapy; HIV, human immunodeficiency virus; IPT, isoniazid preventive therapy; IQR, interquartile range; TB, tuberculosis; WHO, World Health Organization.

[a] Defined as consumption of any type and amount of alcohol ≥2 times weekly.

[b] Defined as the regular chewing of leaves from the endemic plant *Catha edulis*, which is considered to have mild narcotic properties.

[c] Self-reported history of hospitalization during the 2 weeks preceding enrollment.

algorithm is shown in Figure 2. The area under the curve of the combined strategy (WHO-TB and the clinical scoring algorithm) was slightly better than WHO-TB alone, both for the whole population (0.75 vs 0.70) and for WHO-TB positive participants (0.74 vs 0.67).

Using WHO-TB screening alone, 159 (20%) participants were categorized as being at low risk of TB; 10 (6%) of these had TB (NPV, 94% [95% confidence interval {CI}, 90%–97.5%]). These TB cases were characterized by lower age, higher MUAC, higher BMI, and higher CD4 cell count than WHO-TB$^+$ cases.

Among the 625 WHO-TB$^+$ participants, 569 (91%) had recordings for all the variables included in the clinical scoring algorithm (Figure 1). For the 56 (9%) subjects with ≥1 of these variables missing, the main reason was absence of results for MUAC and/or hemoglobin (Supplementary Material). These patients were excluded from this analysis; they did not differ significantly from those included with regard to sex, age, or WHO-TB symptoms.

Based on the distribution of clinical scoring points, subjects were divided into 3 categories with regard to the likelihood of TB (Figure 1). In participants with 0–1 scoring points, 20 of 255 had TB (NPV, 92% [95% CI, 89%–95%]); low-risk group).

Table 2. Multivariate Analysis of Variables Associated With Tuberculosis (TB) in Subjects With Positive World Health Organization TB Symptom Screening

Variables[a]	Univariate OR[b]	Multivariate Model OR[c]
Cough	3.4 (2.1–5.5)	2.1 (1.3–3.3)
Karnofsky score ≤80	2.8 (1.6–5.0)	2.8 (1.4–5.7)
MUAC <20 cm	3.5 (2.2–5.7)	2.0 (1.2–3.5)
Peripheral lymphadenopathy	9.6 (4.0–23)	7.3 (2.7–19)
Hemoglobin <10 g/dL	3.7 (2.4–5.8)	2.9 (1.7–4.7)

Abbreviations: MUAC, mid-upper arm circumference; OR, odds ratio.

[a] Derived from stepwise backward elimination of least significant variables.

[b] Adjusted for age group (18–25, 26–35, 36–45, 46–55, >55 years, respectively) and sex.

[c] Model including all variables and adjustment for age group (18–25, 26–35, 36–45, 46–55, >55 years, respectively) and sex.

Among subjects with ≥4 scoring points, 19 of 34 had TB (PPV, 56% [95% CI, 39%–73%]; high-risk group). For participants with 2–3 points, 77 of 280 had TB (intermediate-risk group).

Using WHO-TB alone, 159 of 784 (20%) subjects would be excluded from further TB investigations. By applying the clinical scoring algorithm to WHO-TB$^+$ individuals, an additional 255 subjects with 0–1 scoring points could be exempted from further TB testing (in total, 414/784 [53%]; $P < .001$). The proportions of subjects who would be falsely classified as TB-negative using these strategies are 10 of 159 (6.3%) and 30 of 414 (7.2%), respectively ($P = .69$).

The TB detection rate of all diagnostic methods (smear, Xpert MTB/RIF, and culture) increased with increasing scoring points (Table 3).

DISCUSSION

We developed a clinical scoring algorithm for further categorization of PLHIV with positive WHO-TB symptom screening with regard to their likelihood of TB, and compared the performance of this combined diagnostic strategy to that of using WHO-TB alone in a cohort of PLHIV eligible to start ART. The clinical scoring algorithm could identify subsets of WHO-TB$^+$ subjects with a low likelihood of TB; such a strategy would reduce the need of further TB investigations before ART initiation.

We could confirm the projected performance of the WHO-TB symptom screening algorithm for ruling out active TB among PLHIV [4]. Our investigation was performed at Ethiopian public health centers, a setting that is typical for where most TB/HIV-coinfected patients globally receive care. This external validation of WHO-TB provides support for its use in primary health care in sub-Saharan Africa. As demonstrated in the original study [4] and in other reports [16], the WHO-TB algorithm has its principal use in identifying subjects with a low likelihood of TB; the NPV was 94% in our population, with an overall TB prevalence of 17%. However, the PPV was low; overall, around 80% of our study participants were WHO-TB$^+$, and would require further TB investigations according to existing recommendations.

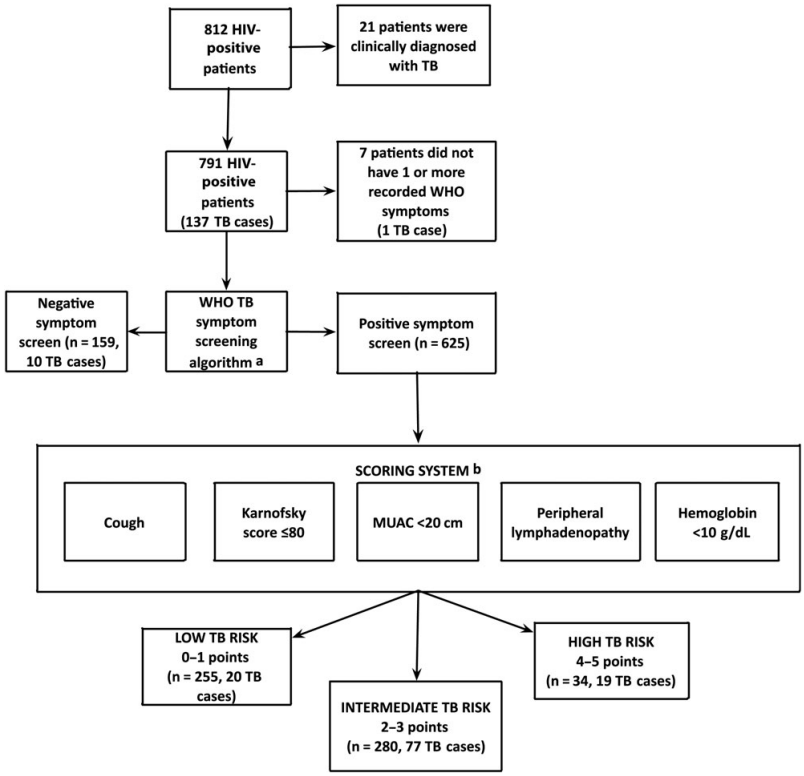

Figure 1. Flow chart of participants included for the development of the clinical scoring algorithm. [a]World Health Organization tuberculosis symptom screening instrument (WHO-TB) includes fever, cough, and night sweats of any duration and weight loss. [b]Fifty-six subjects had ≥1 missing scoring system variable and were excluded in the analysis. Abbreviations: HIV, human immunodeficiency virus; MUAC, mid-upper arm circumference.

For the development of the clinical scoring algorithm, we used multiple logistic regression [17], with bacteriologically confirmed TB as criterion for concurrent validation [18]. Although regression models are widely used for risk stratification in different fields of medicine [19], alternative methods might be considered, such as algorithmic approaches or criteria based on expert opinion [8]. A parsimonious logistic regression model has advantages of simplicity and robustness for measuring the degree of correlations, while accounting for the influence of confounding variables. For these reasons, such a model was considered to be best suited for our objectives, in particular for the construction of an algorithm intended for use in decentralized care in low-income countries.

Currently, there is a lack of practical diagnostic methods for TB case finding among PLHIV in resource-limited settings [20]. This constitutes a substantial problem for health systems, with both direct financial costs and potential negative effects with delayed ART initiation and administration of IPT for persons who first have to undergo testing for TB [2, 21]. The need for TB investigations that require laboratory resources and technical facilities is an important obstacle for efficient provision of HIV services and IPT at the health center level.

The disease manifestations in HIV-associated TB are often vague and overlap with those of HIV/AIDS [4, 5]. A prominent feature is pronounced wasting ("slim disease"); subjects with TB coinfection have lower BMI, MUAC, and hemoglobin level [22]. We hypothesized that markers of wasting could be useful to assess the risk of TB among PLHIV eligible to start ART. The presence of TB was associated with cough, low Karnofsky performance score, low MUAC, anemia, and peripheral lymph

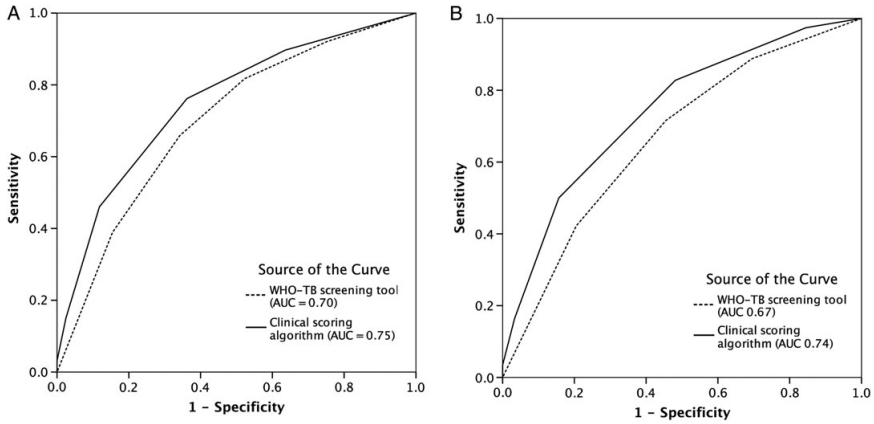

Figure 2. Receiver operating characteristic area under the curve (AUC) showing the performance for tuberculosis identification using 2 different algorithms: World Health Organization tuberculosis (WHO-TB) symptom screening alone and WHO-TB symptom screening followed by clinical scoring for WHO-TB$^+$ subjects. A, Predictive performance for all patients. B, Predictive performance for WHO-TB$^+$ patients.

node enlargement in multivariate analysis; these variables were included in the scoring system.

Among the 255 WHO-TB$^+$ patients with 0–1 scoring points (45% of the WHO-TB$^+$ group), the risk of TB was low (20/255 [8%]; NPV = 92%). For such individuals, both ART and IPT might be initiated without further TB investigations. Conversely, for the 34 subjects with ≥4 scoring points, the likelihood of TB was 56%, whereas subjects with 2–3 points had an intermediate risk of TB (27%). For the latter group, further diagnostic TB testing is clearly indicated. This should also be considered for those with higher scores, although in settings with restricted access to TB diagnostic resources, it could be reasonable to initiate empirical treatment for active TB before starting ART in such patients [23].

The collection of the scoring variables requires neither great expertise from care providers nor advanced technical resources, apart from measurement of hemoglobin. An important advantage of a scoring system is that it can be used to indicate both a low and high likelihood of having a certain condition.

Table 3. Performance of the World Health Organization Tuberculosis (WHO-TB) Screening Instrument[a] Alone and WHO-TB Screening Followed by the Clinical Scoring Algorithm[b] for Categorization for the Likelihood of TB in HIV-Infected Adults at Ethiopian Health Centers

Categorization for Likelihood of TB	No. of Positive Variables	No. of Subjects	TB Cases	Smear-Positive[c]	Xpert-Positive[c]	Culture-Positive[d]
WHO TB screening algorithm[c] (n = 784)	0	159	10 (6)	1 (10)	4 (40)	9 (90)
	1–2	309	38 (12)	8 (21)	23 (61)	36 (95)
	3–4	316	88 (28)	22 (25)	69 (78)	77 (88)
Clinical scoring algorithm[e] (n = 569)	0–1	255	20 (8)	4 (20)	11 (55)	17 (85)
	2–3	280	77 (28)	16 (21)	58 (75)	69 (90)
	4–5	34	19 (56)	7 (37)	16 (84)	17 (89)

Data are presented as No. (%) unless otherwise specified.

Abbreviations: HIV, human immunodeficiency virus; TB, tuberculosis; WHO, World Health Organization.

[a] The WHO-TB symptom screening algorithm includes 4 symptoms (fever, weight loss, night sweats, and/or cough of any duration); persons reporting any of these symptoms are categorized as WHO-TB$^+$.

[b] The clinical scoring algorithm is intended for use in WHO-TB$^+$ subjects, and includes 5 variables (cough, Karnofsky score ≤80, mid-upper arm circumference <20 cm, hemoglobin <10 g/dL, and peripheral lymphadenopathy). Each of these variables confers 1 point in the score (possible range, 0–5 points).

[c] Seven hundred eighty-four persons with all 4 recorded symptoms were included in the analysis; 136 had TB.

[d] Percentage was calculated from bacteriologically confirmed TB cases with the same range of variables or clinical scores.

[e] Including WHO-TB$^+$ subjects (n = 625). Five hundred sixty-nine persons with recordings for all variables were included in the analysis; 116 had TB.

A proportion of TB cases were missed by the WHO-TB screening algorithm and the clinical scoring algorithm (10 WHO-TB$^-$ patients and 20 WHO-TB$^+$ persons with 0–1 points). This may be explained by the occurrence of subclinical TB, which has been reported in settings with high TB prevalence [24]. Such cases might not be possible to identify by clinically based screening. It is likely that patients with TB not detected through our scoring strategy have less advanced disease with better prognosis [14, 25]. However, this approach could lead to inadvertent administration of IPT to subjects with active TB, and might also increase the risk of unmasking TB-IRIS for those who initiate ART. Although TB cases with initial negative scoring results might be identified through repeated scoring, most such persons would probably have started ART during this interval. Because exclusion of active TB using WHO-TB has been shown to be inferior in ART recipients, we do not propose use of the clinical scoring algorithm for such subjects [16].

Because several scoring variables reflect the severity of wasting, it is likewise plausible that subjects identified by clinical scoring are those with most advanced disease. For example, low MUAC has been shown to predict mortality in HIV/TB-coinfected subjects [26]. Interestingly, several components in the clinical scoring algorithm are similar to those included in the TBscore I and II [27, 28]. This scoring system was constructed to estimate TB disease severity and prognosis, but could also be useful for TB case finding [29].

Other techniques, independent of clinical manifestations, exist for TB case finding among PLHIV. In particular, rapid polymerase chain reaction technology (Xpert MTB/RIF) shows promise in the field of TB diagnostics, and has been validated for intensified case finding in PLHIV eligible to start ART [14, 33]. Yet, widespread access to Xpert MTB/RIF testing may be difficult to achieve [30]. Furthermore, this test fails to detect TB in approximately one-third of culture-confirmed cases [14, 25]. The role of chest radiography for TB screening among PLHIV has been assessed in different settings; its value for the confirmation of TB in PLHIV is low, especially in the absence of qualified interpretation [8, 31]. Another option for TB screening could be point-of-care biomarker testing; for instance, a level of C-reactive protein <10 mg/L has been shown to have a high NPV for exclusion of TB among individuals with suspected TB [32].

This study shows that the potential for clinically based categorization for the risk of TB among PLHIV has not been exhausted. Through prospective follow-up, we could estimate the proportion of TB cases presenting in patients not diagnosed at inclusion. Most of these cases occurred in subjects starting ART, and probably represent occult TB prevalent at baseline, a phenomenon that may lead to misclassification of subjects in cross-sectional studies. In our population, incident TB during 6-month follow-up was rare, illustrating the benefit of intensified TB case finding at ART initiation. All health centers providing integrated TB and HIV care in a defined uptake area of central Ethiopia participated in the study, and we consider the risk of inclusion bias into the cohort to be low.

This study has certain limitations. Before being considered for routine use, external validation of the scoring system is required. The performance of the clinical scoring algorithm is likely to be reduced in regions with lower TB endemicity, and the distribution and correlations between clinical variables and prevalent TB could be different in other settings. We did not assess interobserver variability for recording of variables, another factor that could affect performance. Our diagnostic protocol focused on pulmonary TB, and required submission of morning sputum samples. A proportion of subjects (61/873 [7%]) did not submit such samples, and were not included in the study; we consider this proportion to be small and unlikely to affect the results. Our protocol did not include investigations for extrapulmonary TB apart from peripheral lymphadenitis; hence, it is possible that some cases of extrapulmonary TB may have been missed.

In conclusion, by performing clinical scoring for HIV-infected adults with positive WHO-TB screening, the proportion of persons in need of further TB investigations could be significantly reduced. This classification strategy would be particularly useful in TB-endemic settings with restricted resources. By the combined use of these 2 clinically based instruments, a decision on the likelihood of active TB could be reached for a majority of PLHIV with access to health center–based ART, allowing for early initiation of treatment for both HIV and TB.

Supplementary Material

Supplementary material is available online at *Open Forum Infectious Diseases* (http://OpenForumInfectiousDiseases.oxfordjournals.org/).

Notes

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Potential conflicts of interest. All authors: No potential conflicts.

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Paper II

Global Health Action

ORIGINAL ARTICLE

Development of a clinical scoring system for assessment of immunosuppression in patients with tuberculosis and HIV infection without access to CD4 cell testing – results from a cross-sectional study in Ethiopia

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Background: Currently, antiretroviral therapy (ART) is recommended for all HIV-positive patients with tuberculosis (TB). The timing of ART during the course of anti-TB treatment is based on CD4 cell counts. Access to CD4 cell testing is not universally available; this constitutes an obstacle for the provision of ART in low-income countries.

Objective: To determine clinical variables associated with HIV co-infection in TB patients and to identify correlations between clinical variables and CD4 cell strata in HIV/TB co-infected subjects, with the aim of developing a clinical scoring system for the assessment of immunosuppression.

Design: Cross-sectional study of adults with TB (with and without HIV co-infection) recruited in Ethiopian outpatient clinics. Clinical variables potentially associated with immunosuppression were recorded using a structured questionnaire, and they were correlated to CD4 cell strata used to determine timing of ART initiation. Variables found to be significant in multivariate analysis were used to construct a scoring system.

Results: Among 1,116 participants, the following findings were significantly more frequent in 307 HIV-positive patients compared to 809 HIV-negative subjects: diarrhea, odynophagia, conjunctival pallor, herpes zoster, oral candidiasis, skin rash, and mid-upper arm circumference (MUAC) <20 cm. Among HIV-positive patients, conjunctival pallor, MUAC <20 cm, dyspnea, oral hairy leukoplakia (OHL), oral candidiasis, and gingivitis were significantly associated with <350 CD4 cells/mm^3. A scoring system based on these variables had a negative predictive value of 87% for excluding subjects with CD4 cell counts <100 cells/mm^3; however, the positive predictive value for identifying such individuals was low (47%).

Conclusions: Clinical variables correlate with CD4 cell strata in HIV-positive patients with TB. The clinical scoring system had adequate negative predictive value for excluding severe immunosuppression. Clinical scoring systems could be of use to categorize TB/HIV co-infected patients with regard to the timing of ART initiation in settings with limited access to laboratory facilities.

Keywords: *HIV*; *tuberculosis*; *Ethiopia*; *scoring system*; *CD4 cell*; *timing of ART*

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Tuberculosis (TB) is the leading opportunistic infection (OI) globally in people living with HIV and is estimated to be responsible for 400,000 deaths per year in HIV-positive persons (1). In contrast to most other OIs, TB can occur over the whole spectrum of immunodeficiency, although the prevalence increases dramatically with decreasing CD4 cell count (2). Recently, several studies have demonstrated significant survival benefits in HIV-positive subjects initiating antiretroviral therapy (ART) before the completion of anti-tuberculosis

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treatment (ATT) (3–5). Consequently, the WHO has revised its guidelines, and currently ART is recommended for all TB/HIV co-infected individuals, irrespective of CD4 cell counts (6).

Importantly, the benefit of starting ART early during the intensive phase of ATT is related to CD4 cell levels; a reduction in mortality with 'immediate ART' (initiated within the first 2 weeks of ATT) has been shown for patients with severe immunosuppression, but not for those with less advanced HIV disease (3–5, 7). Hence, current WHO guidelines recommend ART initiation within 2 weeks after starting ATT in patients with CD4 cell count <50 cells/mm^3, and within 8 weeks for subjects with less advanced immunosuppression (6). Although early initiation of ART during ATT reduces the risk of death among severely immunosuppressed persons, this strategy may also lead to more complications during treatment, such as an increased incidence of immune reconstitution inflammatory syndrome (IRIS) (8–10) and a higher risk of severe adverse events in patients with tuberculous meningitis (11).

Assessment of immunosuppression in HIV-positive patients is thus still necessary to determine the timing of ART initiation. This relies on the measurement of CD4 cell levels, in combination with the WHO clinical staging system. However, the performance of the WHO staging system for identification of subjects eligible to initiate ART is suboptimal (12). In particular, the WHO staging system cannot be used to categorize patients with concomitant TB with regard to the degree of immunosuppression, since TB is included as a staging variable. Consequently, CD4 cell measurement is the sole reliable method for the assessment of immunosuppression among subjects with TB/HIV co-infection.

Although CD4 cell technology has been introduced in many locations along with scaling up of ART, this method remains unavailable for many persons living with HIV and may not be cost-effective in low-income countries (13). In order to achieve greater ART coverage in resource-limited settings, greater proportions of patients will receive both ATT and ART within the primary health care system. The need for CD4 cell testing constitutes an important obstacle for efficient decentralization of HIV care to peripheral health levels. Therefore, alternative robust methods that may be used for the assessment of immunosuppression and that do not require laboratory facilities would facilitate management of HIV-infected individuals with TB.

We hypothesized that clinical parameters associated with HIV-related immunosuppression correlate with low CD4 cell counts in HIV-positive patients with TB. Such correlations would allow for the construction of a clinically based scoring system that could be used to categorize patients according to the degree of immunosuppression, and could help to identify subjects who need to start ART early during the course of ATT. Since the clinical manifestations of HIV/AIDS and TB are similar, we also determined the distribution of clinical variables in HIV-negative patients with TB, in order to identify parameters primarily related to HIV infection.

Patients and methods

Patients

Individuals diagnosed with TB were consecutively screened for eligibility in TB outpatient clinics at six health centers, one zonal hospital (Bishoftu), and one regional hospital (Adama) in the Oromia Regional State of Ethiopia, between September 2010 and March 2012. The following inclusion criteria were applied: 18 years or older, TB diagnosis according to Ethiopian National Guidelines (14), and consent to HIV testing. Patients with previous or current ART, ATT for more than 2 weeks for a current episode of TB, or those who had received ATT within the preceding 6 months were excluded, as well as subjects not residing in the catchment area of the respective clinics.

Patients in whom acid-fast bacilli were detected in sputum were classified as smear-positive cases. The diagnosis of smear-negative pulmonary TB was established in patients with symptoms compatible with TB, who had repeatedly negative sputum smear microscopy, no response to broad-spectrum antibiotic therapy, and chest X-ray lesions suggestive of TB. For a diagnosis of peripheral lymph node TB, a compatible fine-needle aspirate cytology result was required. Other forms of extrapulmonary TB were diagnosed using targeted investigations, depending on disease manifestation.

HIV serostatus was determined using rapid tests, according to Ethiopian National Guidelines (15). Positive results by KHB (Kehua Bio-engineering Co, Shanghai, China) were immediately followed by testing with Statpack (HIV 1/2 Stat-Pak, Chembio Diagnostic Systems Inc., 3661 Horseblock Rd, Medford, New York, USA) for confirmation of HIV infection. Further testing was performed by Unigold (Uni-Gold TM HIV, Trinity Biotech Plc., Bray, Co. Wicklow, Ireland) when discordant results were obtained with these two assays. Patients diagnosed with HIV infection were referred to HIV clinics in the same health facility for further management and were considered for ART initiation in accordance with Ethiopian national guidelines (16). These guidelines recommend starting ART either after 2–8 weeks or after completion of the intensive phase of ATT, depending on CD4 levels or clinical disease severity, in case CD4 cell counts are not possible to obtain.

Methods

TB clinic nurses, who received detailed and repeated training by the research group members on the study protocol, performed all study investigations. These nurses had two

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different levels of qualification (diploma in nursing and bachelor of science). All nurses involved in the study had received training required for being employed in a public Ethiopian TB clinic. A full-day training session with all staff from the participating clinics was arranged by the study team every 6 months for the duration of the study. These training sessions encompassed details of the structured questionnaire and instructions on how to perform patient interviews and physical examination, with particular focus on recognition of various symptoms and clinical findings. The investigators performed once weekly monitoring of the study procedures throughout its duration. This included crosschecking of all relevant data using the clinic registers and records as data sources. In association with these visits, training with each study investigator was repeated. Data clerks entered the data recorded on the questionnaires into a database continuously, with repeated crosschecking of all entries.

A structured questionnaire was used for the collection of study data. This questionnaire focused on disease history and symptoms that were considered to be common or typical of HIV-related immunosuppression, such as bedridden state, hospitalization, cough, dyspnea, fever, weight loss, anorexia, lymph node enlargement, skin rash, diarrhea, and odynophagia.

The degree of wasting was estimated both by calculating the body mass index (BMI; based on measurement of height and weight at the time of inclusion) and by measurement of mid-upper arm circumference (MUAC; using a designated measuring tape to the nearest 0.5 cm). The physical examination included the following items: conjunctival pallor, oral candidiasis, oral hairy leukoplakia (OHL), gingivitis, cervical lymphadenopathy, skin rash (without further specification), and herpes zoster scar.

Blood was drawn into EDTA tubes for analysis of CD4 cell and hemoglobin levels in conjunction with this visit. These blood samples were transported to one of two laboratories (Adama Regional Laboratory or Bishoftu Zonal Hospital Laboratory). CD4 cell count was determined within 24 hours using flow cytometry according to the instructions of the manufacturer (FACSCount or FACSCalibur, BD Biosciences, respectively). Continuous internal and external quality assurance monitoring was done in both laboratories every 3 months.

Results of these blood tests were released to the study investigators within 1 week after patient inclusion and collection of clinical data; consequently, study staff were blinded to CD4 cell counts.

Statistical analysis

The study was conducted using a cross-sectional design. Participants were first grouped according to HIV serostatus. HIV-positive patients were then further divided into subgroups based on different CD4 cell count levels used for deciding when to initiate ART (<50, <100, <200, and <350 cells/mm^3). Continuous variables were categorized based on the distribution in the study population; for BMI and MUAC, the material was separated into quartiles whereas age was managed as a bivariate variable.

Following an initial logistic regression univariate analysis, variables with p-values <0.3 were entered into a stepwise logistic regression multivariate analysis. Variables found to be significant (p-value of <0.05) for each separate CD4 cell stratum in the respective final regression models were used as items in the final scoring system. The beta coefficients (i.e. the log-transformed odds ratios) from these models were added together to assign a weighted scoring point. The final scores were rounded to the nearest 0.5 integer. In the final model, MUAC was included as a binary variable based on the distribution from the previous step.

The resulting score consisted of variables that had associations with CD4 cell count of <350 cells/mm^3; Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for patients with CD4 cell counts below 100 cells/mm^3. Only patients with complete data and no missing values were used in this analysis. Receiver operator characteristic (ROC) analysis was performed and area under the curve (AUC) was calculated for the score at the selected CD4 cell cutoff levels. All analyses were done using IBM SPSS statistics version 20.

Ethical considerations

Patients provided written informed consent of their confidential participation in the study. For illiterate subjects, an independent witness certified that they had received information about the study and their acceptance of inclusion. The study received ethical approval by the National Ethics Review Committee at the Ministry of Science and Technology of Ethiopia and by the Ethical Committee at Lund University, Sweden.

Results

Patient characteristics

During the inclusion period, 2,135 patients were registered in the study clinics. Among these, 1,116 were included, 307 (27.5%) of whom were HIV-positive. A flow chart of eligible subjects and participants is presented in Fig. 1. Characteristics with regard to HIV serostatus are shown in Table 1. Patients co-infected with HIV were slightly older than HIV-negative subjects (median age 32 vs. 29 years, respectively) but gender distribution was similar in both groups. Most patients reported urban residence. Approximately two thirds of the TB cases were pulmonary, with a higher proportion of smear-negative disease in HIV-positive subjects. Peripheral lymphadenitis

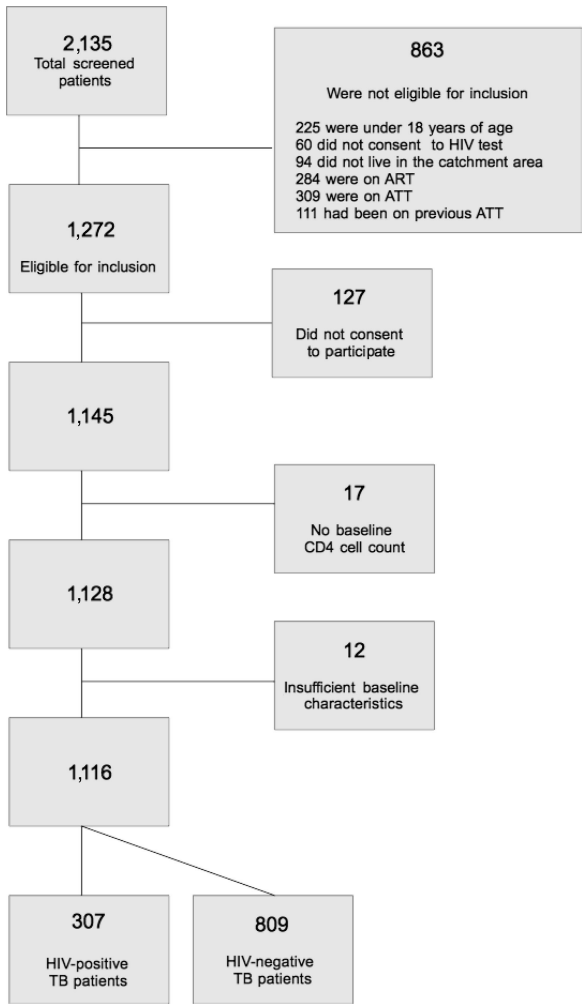

Fig. 1. Flow diagram of study participants.

was the most frequent cause of extrapulmonary TB, both for HIV-positive and HIV-negative persons. However, no significant differences between HIV-positive and negative subjects were found with regard to type of TB manifestation.

Clinical variables associated with HIV co-infection

The following clinical variables were found to be significantly associated with HIV infection in multivariate analysis: oral candidiasis, herpes zoster scar, skin rash, MUAC <20 cm, diarrhea, odynophagia, and conjunctival pallor (in order of decreasing degree of association; Table 1). The median CD4 cell count for HIV + /TB patients was 173 cells/mm^3 and 671 cells/mm^3 for HIV-/TB patients ($p<0.001$). Previous TB was less commonly reported by HIV-positive patients.

Correlation of clinical variables to CD4 cell count strata in HIV-positive patients

The frequencies of various clinical variables were determined for different CD4 cell strata (Table 2). The degree of wasting (estimated either by BMI and MUAC) was

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Table 1. Background characteristics and comparison between 307 HIV-positive- and 809 HIV-negative-TB patients

		HIV-positive TB-patients ($n=307$)	HIV-negative TB-patients ($n=809$)	OR (95% CI)*
Background data	Median age (years; range)	32 (18–70)	29 (18–80)	–
	Male gender	156 (50.8)	432 (53.4)	–
	Urban residence	274/306 (89.5)	669/807 (82.9)	–
	Rural residence	32/306 (10.5)	138/807 (17.1)	–
	Smear-positive PTB	96 (31.3)	309 (38.2)	–
	Smear negative PTB	96 (31.3)	214 (26.5)	–
	Peripheral lymphadenitis	91 (29.6)	205 (25.3)	–
	Other location of TB	34 (11.0)	91 (11.3)	–
	Median CD4 cell count (IQR)	173 (95–336)	671 (500–883)	–
	Median CD4/CD45% (IQR)	12 (8–18) ($n=209$)	37 (31–43) ($n=518$)	–
Symptoms associated with HIV diagnosis	Oral candidiasis	92 (30.0)	11 (1.4)	5.9 (3.5–9.9)
	Herpes zoster	24/306 (7.8)	4 (0.5)	4.3 (1.6–11.2)
	Skin rash	38 (12.4)	4 (0.5)	2.7 (1.2–5.9)
	Diarrhea	66/305 (21.6)	34/808 (4.2)	1.9 (1.1–3.1)
	MUAC $\geq$22 cm	105 (30)	409 (50.6)	REF
	MUAC 20–22 cm	92 (30)	239 (29.5)	1.5 (1.0–2.1)
	MUAC 19–20 cm	42 (13.7)	71 (8.8)	2.2 (1.4–3.6)
	MUAC $<$19 cm	68 (22.1)	90 (11.1)	2.5 (1.6–3.9)
	Odynophagia	117 (38.1)	102 (12.6)	1.6 (1.1–2.4)
	Conjunctival pallor	118 (38.4)	93/806 (11.5)	1.6 (1.1–2.4)
	Previous history of TB	7 (2.3)	40 (4.9)	0.3 (0.1–0.7)

Presented as *n* (%) unless otherwise stated. CD4 cell values in cells/mm^3.
No significant associations (95% CI) were found for age, gender residence, and type of TB.
*Multivariate associations between HIV diagnosis and the variables.

associated with more advanced immunosuppression (CD4 cell count $<$100 cells/mm^3, $p<0.001$). As BMI and MUAC showed colinearity, MUAC, which showed the stronger correlation of the two, was included in the final model as a binary variable based on the distribution from the previous step. However, reported history of weight loss showed no such correlation ($p=0.15$). In multivariate regression analysis, male gender, age $>$32 years, conjunctival pallor, shortness of breath, OHL, oral candidiasis, and MUAC $<$20 cm were independently associated with CD4 cell counts $<$350 cells/mm^3 (Table 3). CD4 cell counts $<$50 cells/mm^3 were associated with OHL and MUAC $<$20 cm.

A clinical scoring system for immunosuppression based on clinical variables

Based on the multivariate analysis, a weighted clinical score was developed by the addition of the beta coefficients of variables with significant independent associations with CD4 cell strata (Table 3). The maximum score that could be obtained with this system was 12.5 points. The clinical variables were assigned the following scoring points: MUAC $<$20 cm 4, OHL 2.5, gingivitis 1.5, shortness of breath 1.5, conjunctival pallor 1, male gender 1, age $>$32 years 0.5, oral candidiasis 0.5 points. Among these variables, MUAC $<$20 cm showed the strongest correlation with CD4 cell strata, whereas OHL, despite being a relatively rare finding, was associated with the lowest CD4 cell strata, and thus rendered a high score value.

Performance of the clinical scoring system

The distribution of the clinical scoring points for the study population is shown in Table 4. The overall AUC of the model for identification of patients with CD4 cell counts $<$100 cells/mm^3 was 0.721. The resulting scoring system could exclude most patients without advanced immunosuppression (CD4 cell counts below 100 cells/mm^3); 101/116 (NPV 87%) patients with scores $\leq$2 had CD4 cell counts above this threshold level. There was a clear trend for lower CD4 levels in patients with higher scores, however the PPV remained low even at higher points (47% for a score of 6 points or more) for identification of patients with CD4 $<$100 cells/mm^3.

Discussion

In this population of HIV-positive adults with TB consecutively recruited in Ethiopian out-patient clinics, several clinical variables were found to correlate with CD4 cell strata used to decide the timing for initiating

Table 2. Frequency of symptoms and signs in 307 TB patients co-infected with HIV

		Total, $n=307$	CD4 <100, $n=79$	CD4 100–350, $n=155$	CD4 >350, $n=73$	P*
Background information	Male gender	156 (50.5)	51 (64.6)	71 (45.8)	34 (46.6)	0.022
	Previous TB	7 (2.3)	1 (1.3)	3 (1.9)	3 (4.1)	0.714
Diagnosis of TB	Smear positive PTB	99 (32.2)	29 (36.7)	46 (29.7)	24 (32.9)	0.621
	Smear negative PTB	94 (30.6)	27 (34.2)	46 (29.7)	21 (28.8)	0.422
	Lymphnode TB	87 (28.3)	20 (25.3)	44 (28.4)	23 (31.5)	0.326
	Other location of TB	36 (11.7)	8 (10.1)	22 (14.2)	6 (8.2)	0.760
Symptoms	Bedridden state	81 (26.4)	31 (39.2)	32 (20.6)	18 (24.7)	0.018
	Blood-stained sputum	47/305 (15.4)	15/78 (19.2)	20/154 (13.0)	12 (16.4)	0.906
	Cough	190/306 (62.1)	55/78 (70.5)	100 (64.5)	35 (47.9)	0.001
	Diarrhea	66/305 (21.6)	22/78 (28.2)	32/154 (20.8)	12 (16.4)	0.048
	Diarrhea, recurrent	43/302 (14.2)	16/77 (20.8)	21/153 (13.7)	6/72 (8.3)	0.015
	Fever	253 (82.4)	68 (86.1)	128 (82.6)	57 (78.1)	0.063
	Hospitalized	30/305 (9.8)	14 (17.7)	10/153 (6.5)	6 (8.2)	0.005
	Loss of appetite	249 (81.1)	71 (89.9)	127 (81.9)	51 (69.9)	0.001
	Night sweats	256 (83.4)	70 (88.6)	126 (81.3)	60 (82.2)	0.043
	Odynophagia	117 (38.1)	35 (44.3)	56 (36.1)	26 (35.6)	0.173
	Shortness of breath	148/305 (48.5)	46/78 (59)	82/154 (53.2)	20 (27.4)	0.000
	Significant weight loss	254/306 (83.0)	67 (84.8)	131/154 (85.1)	56 (76.7)	0.150
Clinical findings	Cervical lymph node enlargement	58/306 (19.0)	9/78 (11.5)	32 (20.6)	17 (23.3)	0.015
	Conjunctival pallor	118 (38.4)	44 (55.7)	53 (34.2)	21 (28.8)	0.004
	Gingivitis	24/305 (7.9)	9/78 (11.5)	13/154 (8.4)	2 (2.7)	0.010
	Herpes zoster	24/306 (7.8)	6 (7.6)	14/154 (9.1)	4 (5.5)	0.685
	Oral candidiasis	92 (30.0)	32 (40.5)	45 (29)	15 (20.5)	0.003
	Oral hairy leukoplakia	15/306 (4.9)	9 (11.4)	4/154 (2.6)	2 (2.7)	0.005
	Skin rash	38 (12.4)	10 (12.7)	19 (12.3)	9 (12.3)	0.669
Grouped continuous variables	BMI					0.001
	BMI $\geq$19.5	73 (23.8)	12 (15.2)	39 (25.2)	22 (30.1)	
	BMI 17.5–19.49	82 (26.7)	23 (29.1)	39 (25.2)	20 (27.4)	
	BMI 16–17.49	83 (27.0)	19 (24.1)	47 (30.3)	17 (23.3)	
	BMI $<$16	69 (22.5)	25 (31.6)	30 (19.4)	14 (19.2)	
	MUAC					$<$0.001
	MUAC $\geq$22	105 (34.2)	15 (19)	57 (36.8)	33 (45.2)	
	MUAC 20–22	92 (30.0)	19 (24.1)	48 (31.0)	25 (34.2)	
	MUAC 19–20	42 (13.7)	17 (21.5)	19 (12.3)	6 (8.2)	
	MUAC $<$19	68 (22.1)	28 (35.4)	31 (20.0)	9 (12.3)	
	Age ($\geq$33)	153 (49.8)	47 (59.5)	75 (78.4)	31 (42.5)	0.031

Presented as *n* (%) unless otherwise stated. CD4 cell values in cells/mm^3.
*Correlation of variable to CD4. Binary variables tested with Mann–Whitney U test, Continuous variables tested with Spearman's rank correlation coefficient.

ART during ATT. Based on these findings, a clinical scoring system was developed for the categorization of patients with regard to these CD4 cell strata.

Determining the degree of immunosuppression in HIV infection relies on the measurement of CD4 cell levels in peripheral blood, which is well recognized to have a strong predictive value for HIV disease progression (17). As a consequence, CD4 cell counts are used in all guidelines for recommendations on when to initiate ART. The need for CD4 cell testing constitutes an important obstacle for further roll-out of ART, especially at primary health care level in low-income countries. In such facilities, regular and reliable access to laboratory facilities may not be possible to achieve. Therefore, alternative robust methods for assessing the degree of immunosuppression are needed; a scoring system completely based on clinical data would be the most attractive option. In current guidelines, the WHO staging system is recommended in settings without access to CD4 cell testing. However, validation data for this system for the purpose

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Table 3. Significant variables in multivariable analysis

	CD4 cell count <50		CD4 cell count <100		CD4 cell count <200		CD4 cell count <350			
	β	Adjusted OR (95% CI)	β	Adjusted OR (95% CI)	β	Adjusted OR (95% CI)	β	Adjusted OR (95% CI)	Total β	Rounded score
Male gender			0.8	2.3 (1.3–4.2)					0.8	1
Age ≥33 years					0.5	1.7 (1.0–2.8)			0.5	0.5
Conjunctival pallor			0.8	2.4 (1.4–4.3)					0.8	1
Shortness of breath					0.7	2.1 (1.3–3.4)	1.0	2.9 (1.6–5.3)	1.7	1.5
OHL	2.4	7.5 (2.2–25.2)							2.4	2.5
Oral candidiasis					0.6	2.1 (1.2–3.7)			0.6	0.5
Gingivitis					1.4	4.3 (1.2–15.8)			1.4	1.5
MUAC <20 cm	1.3	4.6 (2.0–10.5)	1.0	2.8 (1.6–4.9)	0.7	2.2 (1.3–3.6)	0.9	2.4 (1.2–4.5)	3.9	4
AUC		0.72		0.72		0.71		0.68		0.72

Presented according to CD4 cell count strata.

of ART initiation are scarce (18, 19). The fact that TB in itself is used as a WHO staging criterion hampers its use in TB/HIV co-infected patients. Furthermore, the timing of ART during the intensive phase of ATT in settings without access to CD4 cell testing has not been the subject of specific investigation, and the WHO guidelines do not specify how to manage patients in this situation.

Scoring systems are used in various fields of medicine for determining the likelihood of certain diagnoses (e.g. pulmonary embolism (20)), but also for classification of disease severity (e.g. NYHA scoring system for heart failure (21)). An advantage of scoring systems as compared to screening algorithms is that they allow for a two way identification of thresholds (both identification of severe disease and exclusion of disease), which make such systems especially suitable for prediction of continuous variables like the degree of immunosuppression. Furthermore, a clinically based scoring system may be repeated during follow up of individual patients, which might allow for identification of subjects with disease progression.

Scoring systems could potentially be of great use in decentralized HIV care. For example, Lynen et al. have presented a clinically based scoring system for estimating the likelihood of virological treatment failure in Cambodian patients receiving ART, intended for use in settings without access to viral load testing (22). To our knowledge, clinical correlates of CD4 cell counts in HIV/TB co-infection has hitherto only been investigated in a small

Table 4. Distribution of scoring points among 302* HIV-positive patients with TB, and performance for identification of patients with <100 CD4 cells/mm^3

Score	CD4 < 100 (n)	CD4 100–350 (n)	CD4 > 350 (n)	Sensitivity (%)	Specificity (%)	Positive predictive value (PPV) (%)	Negative predictive value (NPV) (%)
0	1	10	9	100	8	25	95
1	3	17	18	99	24	27	93
2	11	31	15	95	44	30	87
3	10	26	11	81	61	33	85
4	5	17	4	68	70	37	84
5	3	12	5	61	78	41	84
6	11	15	6	57	87	47	82
7	15	10	4	43	93	53	78
>7	18	14	1	23	100	55	75
Total	77	152	73				

CD4 cell values in cells/mm^3.

Score cutoff rounded up to nearest integer. Sensitivity and PPV of being at threshold or above. Specificity and negative predictive value of being at threshold or below.

*Only patients with no missing values were included in this analysis.

hospital-based study of patients with concomitant TB in South Africa (23). These authors found that CD4 cell counts <200 cells/mm^3 were associated with a BMI <18 kg/m^2, low Karnofsky score and low hemoglobin concentration.

Since no scoring system for the assessment of immunosuppression in HIV/TB patients has previously been proposed, we aimed to determine the feasibility of our hypothesis by construction of an optimized system. We used CD4 cell count to categorize subjects with regard to the degree of immunosuppression. Our study thus investigates the concurrent validity of the scoring system to predict CD4 cell strata; we did not aim to explore the potential predictive capacity of the system for outcomes such as incident opportunistic diseases or mortality.

We used logistic regression to identify variables with correlation to CD4 cell strata, and used those showing significant associations as items in the scoring system. Other statistical methods could be considered for this purpose, for instance an algorithmic approach (24); however, we think that such a strategy would have been too complex for this exploratory study. In order to find the best possible algorithm based on our data, we decided to weight variables significantly associated with CD4 cell count strata using beta coefficients. Beta coefficients have been used by other researchers for the purpose of assigning variables a relative weight (25–28). Although the value of weighting variables for the development of scoring systems has been debated (29, 30), Streiner et al. found a benefit of weighting in analyses containing <40 items, which is the case for this study (31).

Our scoring system is based on variables that can be collected by health professionals with limited training. Considering that it is based on clinical parameters, this scoring system has an adequate NPV for CD4 cell counts ≥ 100 cells/mm^3 for subjects with scores of 2 or less. This would correctly classify 87% of persons with less advanced immunosuppression, for whom ART initiation after 2 weeks of ATT ('immediate ART') is not indicated. Although there was a clear trend of higher scores with decreasing CD4 cell counts, the PPV was below 50%, making it unsuitable for direct identification of patients with advanced immunosuppression. Yet, subjects with a high score (6 or greater) were unlikely to have CD4 cell counts above 350 cells/mm^3 (11 out of 95 patients, 12%).

This scoring instrument might thus be used for identification of subjects with less advanced immunosuppression (CD4 ≥ 100 cells/mm^3). According to current guidelines, such persons are eligible to start ART during the intensive phase of ATT, but do not qualify for immediate ART. Thus, categorization based on our scoring system would permit a longer time interval between the initiation of these two treatments for such patients. This strategy may be of benefit in order to assess the tolerability of ATT and treatment adherence before ART initiation. A CD4 cell count <100 cells/mm^3 was used to define severe immunosuppression. This range is slightly broader than that used to identify subjects eligible for immediate ART in the current WHO guidelines (50 cells/mm^3). Indeed, there is no exact threshold for reduced mortality with immediate ART; for instance, Blanc et al. demonstrated increased survival with this strategy for patients with CD4 cell counts <200 cells/mm^3 (5). Among our patients, 38% (115/302) had scores of 2 or less, showing that more than one third of co-infected patients might be classified in this way.

However, 15 out of 115 subjects (13%) with CD4 cell counts <100 cells/mm^3 would not be recognized as candidates for 'immediate ART'. Whether this would confer an increased risk of death among such individuals cannot be determined from our study design. Since current guidelines recommend care-providers to consider starting ART during the first 2 months of ATT for all TB patients, this misclassification would probably not lead to great delays in ART initiation. Furthermore, since our clinical scoring system also correlates with markers known to be associated with poor outcome among TB patients, the absence of such manifestations suggest that this subgroup of patients might have a lower risk of mortality (despite low CD4 cell counts) (32).

Although early initiation of ART during ATT leads to decreased overall mortality in patients with advanced immunosuppression, this approach may also have certain negative consequences. The risk of IRIS is clearly increased with early initiation of ART (8), and the outcome of TB-associated IRIS in patients managed in health centers is not well known. Furthermore, the incidence of severe adverse events is increased in patients with tuberculous meningitis (11), which is common in HIV-infected persons (33). Correct identification of this condition is difficult even in high-resource settings. In our material only one TB patient was diagnosed with meningitis. There is a definite need for targeted studies on TB in HIV-positive patients with advanced immunosuppression receiving care in health centers, especially with regard to the risk of adverse events and IRIS.

The strongest association with low CD4 cell counts was found for MUAC <20 cm. By itself, this parameter had an AUC of 0.62 for identification of patients with CD4 cell counts <100 cells/mm^3. BMI showed intercorrelation with MUAC, but MUAC proved to be a stronger predictor for low CD4 cell counts. MUAC is a reliable and easy way of measuring the degree of wasting and can also be performed in bedridden patients (32), and has furthermore been shown to be highly predictive of mortality in persons with TB. It is possible that these parameters for wasting would show different correlations to CD4 cell counts in other settings and populations.

In order to determine the contribution of active TB to the distribution of clinical variables among co-infected

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subjects, we included HIV-negative TB patients from the same uptake area. We observed significant differences in clinical presentation with regard to HIV serostatus. Clinical parameters associated with HIV infection were typical features of HIV-related immunosuppression such as oral candidiasis, herpes zoster, gingivitis and diarrhea, low MUAC, odynophagia and conjunctival pallor, most of which are part of the WHO staging system. Interestingly, several WHO staging variables did not correlate to CD4 cell count strata (e.g. weight loss, fever, diarrhea). This supports previous reports showing a low performance for identification of subjects with low CD4 cell levels using the WHO staging system (34).

To our knowledge, this study is the largest investigation of associations between clinical variables and degrees of immunosuppression in TB/HIV co-infected subjects. Since most participants were recruited in health centers the study population is representative of TB/HIV co-infected patients in Ethiopia. The collection of data was performed by regular clinic staff with limited medical training, which might have decreased the accuracy of detected symptoms and signs. However, this also shows the feasibility of the clinical scoring approach in routine practice. The risk of bias in data collection with regard to CD4 cell levels was eliminated since these results were released after completion of the scoring examination.

Limitations

This proof-of-concept study has several limitations. In order to test our main hypothesis – the feasibility of constructing a clinical scoring system to assess immunosuppression in HIV/TB co-infected subjects – we chose a model optimized for the study population. This might have reduced the validity of the scoring system in other settings; testing of the scoring system in other geographical areas is necessary. In addition, assessment of the predictive validity and interobserver reliability is required before implementation in clinical practice can be considered.

We chose to define threshold levels for MUAC based on the distribution of values in our study population instead of using standard levels. Although this provided more relevant data for our purpose, these levels may have to be revised and modified for use in other settings. Furthermore, we included age and gender in our scoring system. These parameters could differ between populations. Different reasons may exist for the associations between these variables and CD4 cell strata. Previous studies have shown variations in CD4 cell levels with regard to age and gender (35), suggesting that inclusion of these variables is justified. We also constructed an algorithm excluding age and gender (data not shown); this model had a slightly worse performance (AUC 0.69 vs. 0.72).

For the diagnosis of TB, Ethiopian national guidelines were used, and microbiological confirmation was not required for inclusion. Although some patients may have been incorrectly diagnosed with TB, this reflects the normal situation in sub-Saharan Africa, where access to microbiological diagnostic methods for TB remains restricted.

Conclusions

Several clinical variables that can be recorded by primary health care professionals with limited training were associated with the degree of immunosuppression among Ethiopian patients with TB and HIV co-infection. A scoring system based on these parameters for categorization of patients with regard to the optimal time point for ART initiation during ATT in settings with restricted access to laboratory facilities is feasible; however, further development and validation of this algorithm is needed.

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Paper III

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CD4 Cell Levels during Treatment for Tuberculosis (TB) in Ethiopian Adults and Clinical Markers Associated with CD4 Lymphocytopenia

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Abstract

Background: The clinical correlations and significance of subnormal CD4 levels in HIV-negative patients with TB are unclear. We have determined CD4 cell levels longitudinally during anti-tuberculosis treatment (ATT) in patients, with and without HIV co-infection, and their associations with clinical variables.

Method: Adults diagnosed with TB (maximum duration of ATT for 2 weeks, and with no history of antiretroviral therapy (ART) in HIV-positive subjects) were included consecutively in eight out-patient clinics in Ethiopia. Healthy individuals were recruited for comparison at one of the study health centers. Data on patient characteristics and physical findings were collected by trained nurses following a structured questionnaire at inclusion and on follow-up visits at 2 and 6 months. In parallel, peripheral blood CD4 cell levels were determined. The evolution of CD4 cell levels during ATT was assessed, and the association between clinical characteristics and low CD4 cell levels at baseline was investigated using regression analysis.

Results: In total, 1116 TB patients were included (307 HIV-infected). Among 809 HIV-negative patients, 200 (25%) had subnormal CD4 cell counts ($<$500 cells/mm^3), with $<$350 cells/mm^3 in 82 (10%) individuals. CD4 cell levels increased significantly during the course of ATT in both HIV+ and HIV- TB-patients, but did not reach the levels in healthy subjects (median 896 cells/mm^3). Sputum smear status, signs of wasting (low mid upper arm circumference (MUAC)), and bedridden state were significantly associated with low CD4 cell counts.

Conclusion: A high proportion of Ethiopian TB patients have subnormal CD4 cell counts before starting treatment. Low CD4 cell levels are associated with smear positive disease and signs of wasting. The continuous increase of CD4 cell counts during the course of ATT suggest a reversible impact of active TB on CD4 cell homeostasis, which may be considered in interpretation of CD4 cell counts in HIV/TB co-infected subjects.

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Introduction

The majority of persons co-infected with TB and HIV (79% of 1.1 million patients in 2011) live in sub-Saharan Africa [1]. HIV-infected individuals have a high risk of developing active TB following infection, and also have increased mortality. Initiation of ART during the course of ATT has been shown to reduce mortality in co-infected persons, especially in those who are severely immunosuppressed [2–4], and is recommended in current WHO guidelines [5].

Absolute CD4 cell levels are the main markers for disease severity in patients with HIV, as well as the best markers yet for disease progression [6]. The time for initiation of ART is based on these levels, also for patients with concomitant TB [5]. The reference range of CD4 cell counts is broad, and these counts can be affected by several factors [7]. Some studies have observed a lower range of CD4 cell counts in apparently healthy subjects in regions of sub-Saharan Africa than the reference range in Caucasian populations [8,9], suggesting the existence of geographical variations. Furthermore, low CD4 cell counts in HIV-negative patients with TB have been described from different settings, suggesting that TB by itself could have an impact on CD4 cell homeostasis; however, the mechanism, clinical correlations or significance of this phenomenon are not well understood [10–12].

We have recently found that low CD4 cell count strata are strongly correlated to signs of wasting among HIV-positive

Ethiopian adults with TB (unpublished data). In order to estimate the contribution of TB per se to the associations between clinical parameters and CD4 cell counts, we have prospectively followed these variables in TB-patients, with and without HIV co-infection recruited in Ethiopian health centers.

Materials and Methods

Ethics statement

All subjects provided written informed consent prior to inclusion into the study. No interventions interfering with standard care were done during the study period, with the exception of CD4 cell count analysis of HIV-/TB individuals. The study was approved by the National Ethics Review Committee at the Ministry of Science and Technology of Ethiopia and by the Ethical Review Board at Lund University, Sweden.

Study design and setting

This prospective cohort study was conducted in outpatient TB clinics in the Oromia region, Ethiopia. Adult patients with TB were recruited at six health centers and two hospitals between September 2010 and September 2012. A reference group of healthy HIV-negative subjects were recruited consecutively from a voluntary HIV counseling and testing (VCT) facility in one of the health centers.

Diagnosis and treatment of patients with TB

Patients were diagnosed with TB according to Ethiopian National Guidelines [13]. Three sputum smears were obtained from each patient. Smear positive pulmonary TB was defined by the detection of acid-fast bacilli (AFB) in at least one out of three sputum smears. The diagnosis of smear negative pulmonary TB required two series of negative sputum smears, non-response to broad-spectrum antibiotics, as well as clinical presentation and chest X-ray compatible with pulmonary TB. For a diagnosis of peripheral lymphnode TB, fine-needle aspirate cytology consistent with the diagnosis was required. Other forms of extrapulmonary TB were diagnosed using targeted investigations, depending on organ manifestation.

ATT was provided as directly observed treatment (DOTS) with daily visits to the clinic during the intensive phase treatment. From September to December 2010, an eight-month course of rifampicin, isoniazid, pyrazinamide and ethambutol was given for two months, followed by isoniazid and ethambutol for 6 months. This regimen was then changed to a standard short-course regimen (two-month treatment with all four drugs with a four-month continuation phase of rifampicin and isoniazid).

HIV testing

HIV testing is recommended for all patients with confirmed or suspected TB in Ethiopia, and is performed using rapid tests by TB clinic staff through provider-initiated testing. During the study period, KHB tests (Kehua Bio-engineering Co, Shanghai, China) were used for screening. Positive results with this test were confirmed with Statpack (HIV 1/2 Stat-Pak, Chembio Diagnostic Systems, New York, USA); if this test showed a negative result, further testing was done with Unigold (Uni-Gold TM HIV, Trinity Biotech, Wicklow, Ireland). In case of a positive test, patients were referred to an ART clinic in the same facility for enrolment in HIV care and consideration of ART initiation.

Methods

The following inclusion criteria were applied: age 18 years or older, TB diagnosed according to national guidelines, residence in the clinic catchment area and consent to HIV testing. Exclusion criteria were: having received ATT for more than 2 weeks for the current episode of TB at the time of inclusion, previous ATT within the preceding 6 months, or ART of any duration. TB-clinic nurses, who received detailed and repeated training by the research group members on the study protocol, performed all study investigations.

The study protocol included questions on disease history and symptoms (bedridden state, hospitalization, cough, dyspnea, fever, weight loss, anorexia, lymph node enlargement, skin rash, diarrhea and odynophagia). The physical examinations focused on findings potentially associated with immunosuppression, including conjunctival pallor, oral candidiasis, oral hairy leukoplakia (OHL), gingivitis, cervical lymphadenopathy, skin rash (without further specification) and herpes zoster scar. MUAC and BMI were used as markers for wasting and were collected, using scales and wall mounted measuring sticks for BMI calculation and dedicated measuring bands for measurement of MUAC provided to the health centers.

Follow-up examination was performed following the same procedure after 2 and 6 months of ATT. Treatment outcome was defined according to WHO based national guideline criteria, i.e. treatment completion, cure, death, treatment failure, default and transfer out [5]. The date of ART initiation was noted for those HIV patients who started such treatment during the follow-up period.

Consenting healthy individuals were recruited at a VCT clinic located in one of the study health centers. These subjects were required to be 18 years of age or older, have a negative HIV rapid test and have no known chronic illness or any symptoms suggestive of TB or acute disease. They were recruited and interviewed by a trained peer counselor using a structured questionnaire, with measurement of BMI and MUAC.

Blood samples were obtained for CD4 cell count analysis at baseline, and were repeated at 2 and 6 months for all TB-patients. CD4 cell count flow cytometry was performed at two central laboratories (Adama regional laboratory and Bishoftu hospital laboratory) using FACSCount and FACSCaliber (Becton Dickinson). Regular monitoring and external quality assurance tests of the machines were performed regularly. For the purpose of this study, HIV testing was repeated after 6 months in patients testing negative for HIV at baseline if initial CD4 cell counts were below 350 cells/mm^3.

Data collection and statistical analysis

Data was collected on paper forms and was entered into a Microsoft Excel® database and crosschecked before transfer to IBM SPSS® V.20, which was used as a base for all statistical analysis. Baseline characteristics were reported as frequencies, percentages or median values. Wilcoxon signed rank test was used for statistical test for significance between observations at different time points. Two threshold levels were used for definition of low CD4 cell counts in HIV-negative TB patients (HIV-/TB) subnormal CD4 cell counts (below 500 cells/mm^3; since this is the lower normal reference value), and CD4 lymphocytopenia (below 350 cells/mm^3;the current recommended threshold for starting ART in HIV+ patients).

A univariate analysis of all variables was performed. For this analysis, BMI and MUAC were categorized according to the median of the HIV-negative patients. Variables with a p-value of less than 0.3 were entered into a multivariable regression analysis, adjusting for age and gender.

For TB patients, we analyzed development of CD4 levels during ATT. For HIV-positive TB patients (HIV+/TB), only those who did not start ART during the follow-up period were included for this

analysis to avoid the effect of ART on CD4 cell count evolution. A change of at least 50 cells/mm^3 between observation time points was used to define increasing or decreasing CD4 cell counts.

Results

Baseline characteristics

Out of 2135 patients registered in the clinics during the study period, 1116 (52%) were included, whereas 1019 had at least one exclusion criterion. Among these, 225 (22%) were under 18 years of age, 127 (12%) did not consent to participation, 60 (6%) did not consent to HIV-testing, 94 (9%) resided outside the catchment area, and 309 (30%) had received more than 2 weeks of ATT at the time of screening for eligibility. Among HIV-positive subjects, 284 were on ART at the time of TB diagnosis. Seventeen participants were excluded since they did not provide blood for baseline CD4 cell count. Furthermore, for 81 HIV-/TB patients, follow up CD4 samples at either 2 or 6 months were missing and those patients were only included in the baseline analysis.

Of the 1116 patients included, 307 (28%) were HIV positive and 166 (54%) of these started ART during the 6-month follow up period. Baseline characteristics of the study participants are presented in *table 1*. The HIV positive and negative subjects were comparable in distribution of age, gender, residence and occupational distribution. There was a non-significant increased frequency of smear positive pulmonary TB among HIV-/TB patients, and conversely a higher frequency of smear negative pulmonary disease in HIV+/TB patients. The healthy individuals tended to be younger and have rural residence, but the gender distribution was similar.

Among HIV+/TB patients the median CD4 cell count was 173 cells/mm^3 (IQR 95-336). The median CD4 cell count was lower in the HIV-/TB patients compared to reference subjects, 671 cells/mm^3 (IQR 500–883.5) vs. 896 cells/mm^3 (IQR 700–1083). Two-hundred HIV-/TB patients (25%) had CD4 cell counts below 500 cells/mm^3, and 82 (10%) had CD4 cell counts lower than 350 cells/mm^3.

Evolution of CD4 cell counts during ATT

Among 472 HIV-/TB patients with follow-up results for CD4 cell counts during ATT, the median counts increased from 688 cells/mm^3 (IQR 497–917) to 753 cells/mm^3 (*figure 1*). Patients without follow-up CD4 cell count results (n = 337) had similar median baseline CD4 cell counts (666 cells/mm^3; IQR 508–859).

The rise in CD4 cell counts was even more pronounced in HIV-/TB patients with low baseline CD4 cell counts; in HIV-/TB patients with CD4 cell counts less than 500 cells/mm^3 these counts increased from a median of 380 to 550 cells/mm^3 after 6 months. In contrast to the other subgroups (*figure 1*), these patients also manifested a significant increase throughout treatment (both between baseline and 2 month follow up as well as between 2 and 6 month follow up). For HIV+/TB patients the increase was significant from baseline to either 2 month or 6-month follow up.

Although the majority of HIV-/TB patients with CD4 cell counts below 500 cells/mm^3 had increasing CD4 cell levels during treatment (n = 87), 24 of these subjects did not show this pattern, and in 8 patients these levels even decreased. The characteristics of these subgroups of HIV-/TB patients are presented in *table 2 and 3*. No clear differences in the distribution of such characteristics were observed between these subgroups.

For HIV+/TB patients who did not start ART (n = 71), overall median CD4 cell counts increased, but for these subjects the pattern was more heterogeneous than that found in HIV-/TB patients. In 35 cases CD4 cell counts had increased after 6 months, while these counts had decreased for 17 patients, and remained at similar levels for 19 patients. HIV+/TB patients with increasing CD4 cell counts had lower baseline levels (188 cells/mm^3) than those with decreasing CD4 cell counts (505 cells/mm^3).

Clinical characteristics of HIV-/TB patients with low CD4 cell counts

The correlation between clinical parameters and low CD4 cell counts at baseline (less than 500 cells/mm^3 or less than 350 cells/mm^3) is presented in detail in *table 4*. Patients with baseline CD4 cell counts below 500 cells/mm^3 were significantly more likely to

Table 1. Baseline characteristics of 1116 patients with TB with and without HIV and 298 healthy individuals.

		All TB patients (n = 1116)	HIV+/TB patients (n = 307)	HIV-/TB patients (n = 809)	Healthy individuals (n = 298)
Median Age (years; IQR)		28 (22–28)	32 (28–40)	29 (22 – 42)	23 (20 – 28)
Male gender, n (%)		724 (51.2)	156 (50.8)	432 (53.4)	136 (54.4)
Occupation	Sales and services	96 (8.6)	35 (11.4)	61 (7.5)	19 (6.6)
	Manual labor	335 (30)	113 (36.8)	222 (27.4)	216 (74.5)
	Agriculture	208 (18.6)	43 (14.0)	165 (20.4)	53 (18.3)
	Unemployed	477 (42.7)	116 (37.8)	361 (44.6)	2 (0.7)
Residence, n (%)	Urban	943 (84.7)	274 (89.5)	669 (82.9)	182 (65.5)
	Rural	170 (15.3)	32 (10.4)	138 (17.1)	96 (34.5)
Type of TB, n (%)*	Smear positive	405 (36.3)	99 (32.2)	306 (37.8)	-
	Smear negative	310 (27.8)	94 (30.6)	216 (26.7)	-
	Lymph node TB	296 (26.5)	87 (28.3)	209 (25.8)	-
	Other location of TB	125 (11.2)	36 (11.7)	89 (11.0)	-
Previous TB		47 (3.3)	7 (2.3)	40 (4.9)	-
Median CD4 cell count (IQR)		639 (376 – 890.3)	173 (95 – 336)	671 (500 – 883.5)	897 (700 – 1083)
Median CD4 percentage (IQR)		35 (25–41)	12 (8 – 18)	37 (30.9 – 43.0)	37 (33 – 43)

*20 patients had both a diagnosis of pulmonary and extrapulmonary TB.

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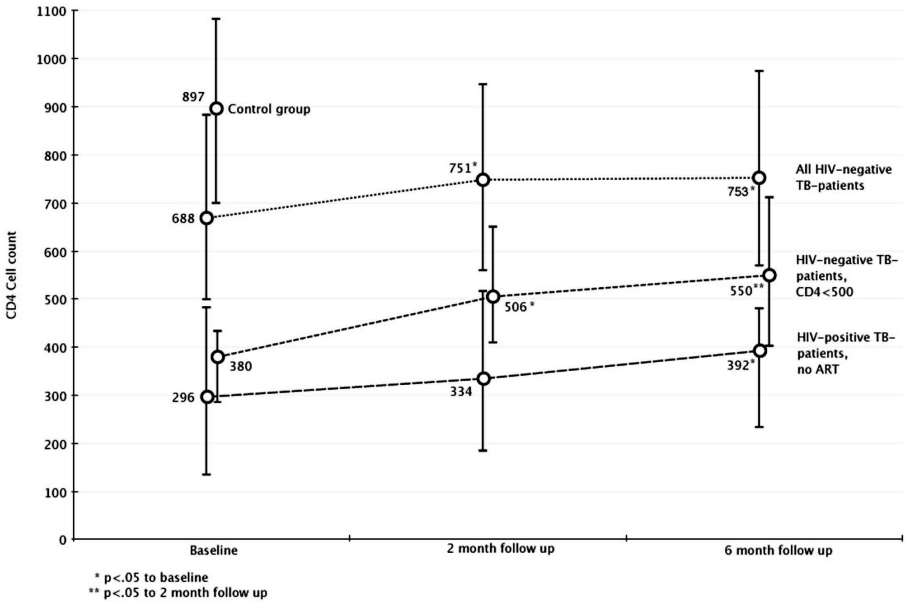

Figure 1. Evolution of CD4 cell counts in HIV negative and positive patients with TB during treatment for TB. The figure shows median CD4 levels and the bars represent IQR. Only patients with follow up CD4 are included in this analysis, and for the control group only baseline CD4 was measured. For HIV+ patients, only patients who did not start ART and had follow up CD4 were included (n = 71).
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have smear positive pulmonary TB (adjusted OR 1.6, IQR 1.2-2-3). Reversely, the prevalence of smear negative pulmonary TB was lower in this group (adjusted OR 0.6, IQR 0.4–0.9). A similar, although not statistically significant, relationship was observed for patients with CD4 cell counts below 350 cells/mm^3.

MUAC, being a surrogate marker for wasting, was significantly associated with low CD4 cell counts in multivariate analysis, with a more than twofold chance of having a MUAC below 22 cm with baseline CD4 cell counts below 350 cells/mm^3 (adjusted OR 2.2, IQR 1.3–3.6). There was a trend between lower BMI and decreasing CD4 cell counts, although this did not reach statistical significance. Furthermore, patients with a history of bedridden state during their current illness were more likely to have low CD4 cell counts (OR 2.3, IQR 1.3–4.0).

Table 2. Characteristics of HIV-/TB patients and CD4 cell count of less than 500, divided by either increase, decrease or stagnant CD4 cell count after 6 months ATT.

	Increase after 6 month (n = 87)			Decrease after 6 month (n = 8)			No change after 6 month (n = 24)		
	Baseline	2 Month	6 month	Baseline	2 Month	6 month	Baseline	2 Month	6 month
Age	29			36			24		
Male gender, n (%)	54 (62.1)			7 (87.5)			14 (58.3)		
Smear positive TB, n (%)	39 (44.8)			3 (37.5)			9 (37.5)		
Smear negative TB, n (%)	15 (17.2)			3 (37.5)			6 (25)		
Lymph node TB, n (%)	23 (26.4)			0			6 (25)		
Other location of TB, n (%)	10 (11.5)			2 (25)			3 (12.5)		
Median BMI kg/height^2	18.4	19.1	19.8	17.7	19.0	18.8	17.2	18.4	18.7
Median MUAC (cm)	21.5	22.0	23.0	21.0	21.8	22.0	20.0	22.0	22.5
Median CD4 cells/mm^3	372	557	605	424.5	448	342.5	400	435	406
Median Percentage	29.6	-	32.4	32.0	-	37.0	33.5	-	30.5

*Increase or decrease defined as >50 cells/mm^3. Only patients who had follow up CD4 cell counts were included in the analysis
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Table 3. Characteristics of HIV+/TB patients not starting ART divided by either increase, decrease or stagnant CD4 cell count after 6 months ATT.

	Increase after 6 month (n = 35)			Decrease after 6 month (n = 19)			No change after 6 month (n = 17)		
	Baseline	2 month	6 month	Baseline	2 month	6 month	Baseline	2 month	6 month
Age	35 (28–38)			31			32		
Male gender, n (%)	19 (54.3)			8 (42.1)			8 (47.1)		
Smear positive pulmonary TB, n (%)	9 (25.7)			7 (36.8)			4 (23.5)		
Smear negative pulmonary TB, n (%)	13 (37.1)			7 (36.8)			5 (29.4)		
Lymph node TB, n (%)	5 (14.3)			6 (31.6)			6 (35.3)		
Other location of TB, n (%)	5 (14.3)			2 (10.5)			2 (11.8)		
Median BMI kg/height2	17.8	18.5	19.6	18.5	19.7	19.6	18.4	18.8	19.9
Median MUAC (cm)	20	21	22.5	22	23	23	21	22	24
Median CD4 cells/mm^3	188	288	417	505	505	407	243	235	249
Median Percentage	11.0		15.0	23.0		18.0	13.0		12.0

Increase or decrease defined as >50 cells/mm^3. Only patients who had follow up CD4 cell counts were included in the analysis
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Outcome of ATT

Thirteen deaths were observed among HIV-/TB patients. These subjects did not show significant differences in clinical features or CD4 cell counts as compared to other patients; their median BMI and MUAC values were slightly lower (18.1 kg/m^2 and 21 cm, respectively) as well as their CD4 cell counts (559 cells/mm^3).

Discussion

In this cohort of Ethiopian adults with TB the prevalence of low CD4 cell counts among HIV-negative persons before initiation of

Table 4. Correlation of clinical parameters to CD4 cell cut off levels in HIV-/TB patients.

		CD4 cell count <500 cells/mm^3, n = 200			CD4 cell count <350 cells/mm^3, n = 82		
	Frequency all HIV-/TB patients	Frequency	OR unadjusted	OR adjusted*	Frequency	OR unadjusted	OR adjusted*
Previous TB	40	3	0.2 (0.1–0.8)	0.2 (0.1–0.7)	2	0.5 (0.1–1.9)	0.3 (0.1–1.9)
Smear positive PTB	306	94	1.7 (1.2–2.3)	1.6 (1.2–2.3)	36	1.3 (0.8–2.1)	1.3 (0.8–2.0)
Smear negative PTB	216	41	0.6 (0.4–0.9)	0.6 (0.4–0.9)	20	0.9 (0.5–1.5)	0.8 (0.5–1.4)
Lymphnode TB	209	42	0.7 (0.5–1.0)	0.8 (0.5–1.2)	18	0.8 (0.5–1.4)	0.9 (0.5–1.5)
Cervical lymph node enlargement	120	18	0.5 (0.3–0.8)	0.6 (0.3–1.0)	10	0.8 (0.4–1.5)	0.9 (0.5–1.8)
Cough	509	142	1.6 (1.1–2.3)	1.4 (1.0–2.0)	58	1.5 (0.9–2.4)	1.3 (0.8–2.2)
Bloodstained sputum	162	49	1.4 (1.0–2.1)	1.3 (0.9–1.9)	24	1.8 (1.1–2.9)	1.6 (1.0–2.8)
Shortness of breath	342	92	1.2 (0.9–1.7)	1.2 (0.9–1.6)	38	1.2 (0.7–1.9)	1.1 (0.7–1.8)
Fever	618	159	1.3 (0.9–1.9)	1.3 (0.9–1.9)	67	1.4 (0.8–2.6)	1.4 (0.8–2.6)
Night sweats	627	161	1.2 (0.8–1.9)	1.2 (0.8–1.8)	68	1.4 (0.8–2.6)	1.4 (0.8–2.5)
Conjunctival pallor	93	27	1.3 (0.8–2.1)	1.1 (0.7–1.9)	13	1.5 (0.8–2.9)	1.4 (0.7–2.7)
Diarrhea	34	11	1.5 (0.7–3.1)	1.6 (0.7–3.3)	3	0.9 (0.3–2.8)	0.9 (0.3–2.9)
Diarrhea, recurrent	29	13	2.6 (1.2–5.5)	2.3 (1.1–5.0)	6	2.4 (1.0–6.1)	2.2 (0.9–5.6)
Loss of appetite	558	143	1.2 (0.8–1.7)	1.2 (0.8–1.7)	62	1.4 (0.9–2.4)	1.4 (0.9–2.5)
Odynophagia	102	27	1.1 (0.7–1.8)	1.1 (0.7–1.8)	15	1.6 (0.9–3.0)	1.6 (0.9–3.0)
Significant weight loss	554	144	1.3 (0.9–1.8)	1.2 (0.8–1.7)	65	1.8 (1.0–3.2)	1.7 (1.0–3.1)
Lower than median BMI (<18.5 kg/m^2)	388	105	1.3 (0.9–1.8)	1.2 (0.8–1.6)	41	1.1 (0.7–1.7)	1.0 (0.6–1.6)
Lower than median MUAC (<22 cm)	400	125	2.0 (1.5–2.8)	1.9 (1.4–2.7)	55	2.3 (1.4–3.7)	2.2 (1.3–3.6)
Hospitalized	15	5	1.5 (0.5–4.5)	1.7 (0.6–5.1)	3	2.3 (0.6–8.3)	2.5 (0.7–9.3)
Bedridden state	102	41	2.3 (1.5–3.6)	2.1 (1.4–3.3)	19	2.3 (1.3–4.1)	2.3 (1.3–4.0)

*Adjusted for age and gender.Only parameters with significance of less than 0.3 in univariate analysis are present in the table. Variables that did not reach this level of significance were: other location of TB, gingivitis, herpes zoster, oral candidiasis, oral hairy leukoplakia and skin rash.
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ATT was substantial; 25% had CD4 cell counts below 500 cells/mm^3 and 10% had CD4 cell counts lower than 350 cells/mm^3, which is the currently recommended lower threshold level for starting ART in HIV-positive subjects. By correlating CD4 cell strata with clinical variables, we could show that low CD4 cell counts are associated with TB disease severity, such as sputum smear positivity, lower MUAC and bedridden state.

Low CD4 cell levels in HIV-/TB patients have been reported previously; in a study on hospitalized TB patients from Senegal, Kony et. al. found CD4 cell counts below 300 cells/mm^3 in 14% [11]. Fifty-three (7%) HIV-/TB cases from our cohort had CD4 cell levels below this threshold level. In line with our findings on associations with clinical severity reported here, the lower prevalence of CD4 lymphocytopenia in our population could be related to more advanced disease in hospitalized subjects. In contrast to previous findings from factory workers in Wonji, a district in the vicinity of our study area in Ethiopia [9], we did not find subnormal CD4 cell counts among healthy individuals.

The continuous increase of CD4 cell counts during treatment for TB strongly suggests that TB per se contributes to subnormal CD4 cell levels in peripheral blood. Similar findings have been reported by other researchers in HIV-/TB patients both in the African setting and in other geographical locations [11,14–17]; however, the reasons for this phenomenon, or its clinical significance, are not well understood.

In parallel with increasing median CD4 cell counts during ATT, we observed continuous improvements in signs of wasting (both BMI and MUAC), which implies that CD4 cell depletion and wasting are related to similar underlying factors in TB. Associations between signs of wasting and CD4 cell levels have been explored previously, with discrepant results. In Argentinian HIV-/TB patients, Pilheu et al found lower CD4 cell count in subjects reporting significant weight loss (>20%) than in those with better general condition [17]. In contrast, other researchers failed to show an association between BMI and CD4 cell counts in HIV-/TB patients (14, 16). In a study on CD4 cell levels among healthy Ethiopian factory workers, Abuye et al reported lower BMI in subjects with subnormal CD4 cell counts [8].

For the investigation of correlations between CD4 cell counts and clinical variables we used both BMI and MUAC as markers for wasting and malnutrition. In multiple regression analysis, MUAC <22 cm was correlated with low CD4 cell counts (both below 500 and 350 cells/mm^3) in HIV-/TB patients. BMI did not turn out to be significant, although there was a trend towards lower CD4 cell count with decreasing BMI. This suggests that MUAC may be a more suitable marker for wasting in this patient group than BMI. In a study from Guinea Bissau MUAC showed a stronger correlation to mortality in HIV+/TB patients than BMI [18].

Several studies have shown a relationship between malnutrition and mortality in TB patients [19–21]. We did not, however, find associations between ATT outcome and low baseline CD4 cell counts in our HIV-/TB patients, probably due to the low frequency of adverse outcomes. There was also no clear association between signs of wasting and poor outcome.

Interestingly, we have previously found relationships between CD4 cell strata and wasting (particularly MUAC) in HIV+/TB patients from this cohort (unpublished data), a finding that might be useful to identify subjects with severe immune suppression in settings without access to CD4 cell testing. The fact that similar associations between CD4 cell counts and wasting exist in HIV-/TB suggests that TB may contribute to the wasting syndrome commonly found in co-infected patients, as well as contributing to CD4 cell depletion. Our finding that CD4 cell counts increased during ATT in HIV+/TB patients not initiating ART further supports this, though the mechanism of the interaction between wasting and low CD4 cell count remains a matter of debate [22,23].

In contrast to HIV+/TB, HIV-/TB patients with low CD4 cell counts had normal CD4 cell percentage, suggesting that the observed decreases in absolute CD4 cell counts are related to peripheral blood lymphocytopenia. Possible explanations for this could be pooling of T-cells at the site of infection [24,25], a direct cytokine mediated suppressive effect on the production of peripheral lymphocytes [26], or effects related to hypermetabolism and malnutrition secondary to TB infection [23,27].

The recovery rate of CD4 cell counts in HIV-/TB patients with subnormal baseline levels was slow and continuous, and did not reach the levels found in healthy individuals from the same geographical area even at the completion of ATT. This is in agreement with findings from HIV+/TB patients, showing an impact of TB on CD4 cell counts for several years after completion of ATT [28].

In a study from Tanzania, an increase was demonstrated between baseline and 2 months. Between baseline and 5 month follow up CD4 was unchanged for those not receiving ART [14]. Increasing CD4 cell levels were also observed in HIV+/TB patients not starting ART in our cohort. When divided into subgroups of those with increasing and decreasing CD4 cell count, the result was heterogeneous. This may be expected since HIV in itself lowers CD4 cell counts, thus cancelling a potential effect of ATT on CD4 cell count in some patients. The number of HIV+/TB patients not starting ART during ATT was low (n = 71), which limits the interpretation of these findings. However, our data suggest that severe TB (as measured by the degree of wasting) can contribute to CD4 lymphocytopenia in co-infected subjects, and that it may be partly reversed by ATT.

Our study design has allowed us to correlate clinical variables with CD4 cell counts longtitudinally during ATT in patients with TB, both with and without HIV co-infection. This study also has some limitations. Firstly, TB diagnosis was based on Ethiopian guidelines, and did not include microbiological confirmation other than smear microscopy. Consequently, some subjects with smear-negative pulmonary TB and extrapulmonary disease may have had diagnoses other than TB. We were unable to estimate this proportion; however, in a previous study, 78% of patients diagnosed with lymph node TB according to the Ethiopian Guidelines were found to have positive TB cultures from lymph node aspirates [29], suggesting that the fraction of participants with diagnoses other than TB was small. Furthermore, the methods used for TB diagnosis among our patients reflect the actual situation in most resource-limited settings. We cannot exclude the existence of other factors not screened for in this study that may have had an impact on CD4 cell counts. Finally, HIV diagnosis relied on rapid tests (evaluated by WHO in 2004 [30]), which could potentially have produced false negative results from subjects in HIV seroconversion phase. For this purpose, we repeated HIV testing in patients with CD4 cell counts below 350 cells/mm^3 at the end of ATT. No HIV seroconversions were detected.

In conclusion, we found a high proportion of CD4 cell lymphocytopenia in Ethiopian HIV-negative adults diagnosed with TB. These levels increased during ATT, both in HIV-negative patients and in HIV+/TB patients not initiating ART, indicating an impact of TB on CD4 cell homeostasis. Decreased CD4 cell counts was associated with clinical markers of advanced TB disease, such as sputum smear positivity, low MUAC and

bedridden state. These findings suggest that similar factors cause wasting and CD4 cell depletion in peripheral blood in TB.

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Author Contributions

Conceived and designed the experiments: SS TS TTB GT PB. Performed the experiments: SS TTB ZHJ PB. Analyzed the data: SS JB. Wrote the paper: SS TS PB TTB.

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Paper IV

Immune activation in tuberculosis patients with or without HIV co-infection and correlation to CD4 cell levels in peripheral blood

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Abstract:

Background: Tuberculosis (TB) can cause CD4 lymphocytopenia in HIV-negative subjects. We investigated immune activation markers in adults with TB, with and without HIV co-infection, to assess their correlation with CD4 cell levels during the course of anti-TB treatment (ATT).

Methods: Adults with TB at Ethiopian health centers (195 HIV-positive, 170 HIV-negative) and 31 controls were tested for plasma levels of neopterin and C-reactive protein (CRP). Their relation to peripheral blood CD4 cell count was analyzed before and after ATT.

Results: Levels of both biomarkers were elevated in TB patients, with the highest median concentrations in HIV-positive cases (neopterin 54 nmol/l, CRP 36 µg/ml). Neopterin levels correlated inversely to CD4 cell count, with the highest levels in subjects with CD4 cell count <100 cells/mm^3 (median 77 nmol/l). However, neopterin had poor predictive value for identification of such subjects (area under the curve [AUC] = 0.64). High baseline neopterin was associated with increased CD4 cell count after ATT.

Conclusion: Both HIV-negative and HIV-positive TB patients displayed high neopterin levels, which correlated both to the degree of CD4 lymphcytopenia and CD4 cell evolution during ATT. These findings support the hypothesis that TB-related immune activation contributes to CD4 cell depletion in HIV infection.

Key words: Immune activation, TB, HIV, coinfection, CD4 cell count, Ethiopia

Running head: Level of immune activation attributable to TB

INTRODUCTION

Measurement of CD4 cell count in peripheral blood predicts HIV disease progression (1), and is routinely used to determine the degree of immunosuppression and the indication for antiretroviral therapy (ART) in people living with HIV (PLHIV). CD4 cell depletion also reflects a central component in the immunopathogenesis of HIV (2). However, several opportunistic infections (OI) may *per se* have an impact on the absolute CD4 cell count in peripheral blood (3). The most important example is TB (4–6), which continues to be the major OI and cause of death among PLHIV (7). Recently we reported subnormal CD4 cell levels (<500 cells/mm^3) in 25% of Ethiopian TB patients without HIV co-infection (6). Furthermore, CD4 cell levels increased in most subjects during the course of ATT, implying an impact of TB on CD4 cell homeostasis.

The mechanism of the association between TB and peripheral CD4 cell levels is not well understood. CD4-positive Th1-lymphocytes are essential for the host defense against Mycobacterium tuberculosis (Mtb), and cause macrophage activation through the secretion of proinflammatory cytokines such as interferon gamma (8). Although proinflammatory cytokines are necessary for eradication of intracellular Mtb, this response may also contribute to pathogenesis as a result of exaggerated immune activation. This is especially pronounced in HIV co-infection, with accelerated viral replication and CD4 cell destruction (9). Whether similar mechanisms are involved in CD4 cell depletion in HIV-negative subjects with TB is unknown (10).

Several plasma surrogate markers have been investigated as markers of immune activation and predictors of disease progression in HIV. Neopterin (11,12) is a catabolic product of guanosine triphosphate secreted mainly by macrophages in response to activation by pro-inflammatory cytokines such as interferon-gamma (13), and has been used assess the level of Th1-type immune activation (14). However, determination of neopterin for monitoring HIV infected subjects has not been used in regular clinical practice, and data on neopterin in TB patients with or without HIV co-infection is limited (15–19).

Another commonly used biomarker is C-reactive protein (CRP), which is triggered through the IL-6 pathway, and reflects the degree of systemic inflammatory response. Raised CRP levels were associated with low CD4 cell count (<200 cells/mm^3) in South African HIV/TB co-infected subjects (20). Increased CRP levels in HIV-positive patients before ART initiation have been found to be associated with higher risk of disease progression (21).

We hypothesized that the CD4 cell depletion in TB patients – with or without HIV co-infection - could be a consequence of immune activation. For this purpose, we investigated plasma levels of neopterin and CRP and their relation to CD4 cell levels in HIV-positive TB patients and HIV-negative TB patients with low CD4 cell count (<500 cells/mm^3), and to the pattern of CD4 cell evolution during anti-tuberculosis

therapy (ATT). In addition, we aimed to explore the potential value of these substances as surrogate markers for CD4 cell count in HIV/TB co-infection.

METHODS

Study participants

Participants for this study were selected from a prospective cohort (described in detail previously (6,22)). For the current study, the following groups of subjects were selected for analysis from the original cohort, as detailed in figure 1: 1) all HIV/TB co-infected patients (HIV^+/TB); 2) all HIV-negative TB patients (HIV^-/TB) with baseline CD4 cell levels <500 cells/mm^3; 3) a subset of consecutive HIV^-/TB patients with baseline CD4 cell levels >500 cells/mm^3 4) a subset of consecutive healthy persons as controls.

Patients were recruited and followed up at eight outpatient TB clinics (based in 6 health centers, 1 regional hospital and one zonal hospital) in the Oromia region, Ethiopia between September 2010 and September 2012. Inclusion criteria were: diagnosis of active tuberculosis, age 18 years or greater, residence in the clinic uptake area, and consent to HIV testing. Subjects having received ATT for more than 2 weeks for their current episode of TB, or who had been treated for TB within the preceding 6 months were excluded, as well as persons with current or previous ART.

Trained nurses investigated patients for symptoms and signs related to immunosuppression using a structured questionnaire (at baseline, at 2 months and at the end of ATT after 6 months). All subjects provided written informed consent to participate in the study. The study was approved by the National Ethics Review Committee at the Ministry of Science and Technology of Ethiopia and by the Ethical Review Board at Lund University, Sweden.

Active TB was defined according to Ethiopian national guidelines (23). Pulmonary TB was diagnosed in subjects with at least one positive sputum smear for acid-fast bacilli, or in sputum smear-negative patients with compatible clinical presentation and chest radiography, and lack of improvement after antibiotic therapy. Peripheral TB lymphadenitis was diagnosed with fine needle aspiration cytology; other forms of extrapulmonary TB were diagnosed using targeted investigations depending on disease manifestation.

A control group of healthy individuals was recruited at a voluntary HIV counseling and testing clinic located at one of the study health centers. HIV-negative subjects without signs or symptoms suggestive of TB or other illness were eligible for inclusion. For all participants, HIV testing was done according to national guidelines by two rapid tests (24). ART was prescribed to HIV^+ patients according to Ethiopian guidelines, (at the

time of data collection: CD4 cell count <350 cells/mm^3 for pulmonary TB and irrespective of CD4 cell count for extrapulmonary TB) (24).

Laboratory methods

At baseline, 2 month and 6 month, 8 ml blood was drawn in EDTA tubes. After performing CD4 cell flow cytometry by standard protocols at either of two testing facilities (using either FACSCount or FACSCalibur (BD) instruments) plasma aliquots were stored at -80°C until further analysis.

Plasma were analyzed using ELISA kits for neopterin and highly sensitive CRP (IBL international, Hamburg, Germany) according to the manufacturer's instructions. Assays were performed in duplicates with three kit-independent controls included in each separate run, representing high, medium and low values. Analyses were performed at the Adama Regional Laboratory, Ethiopia. The optical density (OD) readings were transposed to levels of neopterin and CRP from a standard curve from a 4-spline nonparametric reader fit algorithm as recommended (Miraibio group, Hitachi solutions, San Bruno, CA, USA). The kit-independent controls were used to assess the inter-assay variation. Mean values of duplicate samples from each individual were used. Samples with more than 10% difference were reanalyzed.

Statistical analysis

TB patients were subdivided into groups according to HIV-serostatus. HIV/TB co-infected subjects were further subdivided according to CD4 cell strata (<100 cells/mm^3, 100-350 cells/mm^3, >350 cells/mm^3). HIV-/TB patients were subdivided into those with low (<500 cells/mm^3) and normal (≥500 cells/mm^3) CD4 cell count. Increasing or decreasing CD4 cell count during the course of ATT was defined as a change of 50 cells/mm^3 more or less than the baseline value. A follow-up value within these limits was defined as unchanged.

Neopterin and CRP data were statistically analyzed by nonparametric statistical tests and divided into medians and quartiles. Differences between groups of median biomarker concentrations were tested with Mann Whitney U test and correlations were analyzed with the Spearman rank test. ROC curves and areas under the curves were calculated to evaluate biomarker predictive performance (where an AUC of 1.0 is perfect discriminatory capability and 0.5 is chance (25)) for the aforementioned CD4 cell count cut off thresholds. All statistical analyses were performed using SPSS v.20 (IBM).

RESULTS

Participant characteristics

Neopterin and CRP levels were analyzed in 365 patients (195 HIV^+/TB and 170 HIV^-/TB) from the original cohort of 1116 TB patients In addition, plasma from 31 healthy controls were analyzed for these biomarkers (Figure 1).

There was no difference between the subset of patients selected for analysis of biomarkers compared to the remaining group of HIV^+/TB patients with regard to gender, type of TB, mid upper arm circumference (MUAC) and body mass index (BMI); however, persons not included for analysis had non-significantly higher median CD4 cell count (192 vs. 150 cells/mm^3, p=0.102). HIV^- patients included for testing were also similar to non-tested HIV^- patients, but were more often smear positive (44% vs. 36%) and had significantly lower CD4 cell count (438 vs 715 cells/mm^3;p=0.001). These differences may be explained by the study design with a focus on HIV^- negative patients with low CD4 cell count (Table 1).

Plasma levels of neopterin and CRP in TB with and without HIV co-infection

To study level of immune activation in TB patients with and with or without HIV co-infection plasma samples were analyzed for levels of neopterin and CRP. The median level of neopterin in TB patients was higher (median 37.3 nmol/l, IQR 18.8 – 78.3) than in the control group (median 3.8 nmol/, IQR 1.5-5.2; p<0.001; Table 2). Median neopterin levels were significantly higher in HIV^+/TB-patients (median 54 nmol/l, IQR 32- 108) than in HIV^-/TB-patients (median 23 nmol/l, IQR 13 – 45; p<0.001). Similarly, CRP levels were increased in TB patients compared to controls (36 µg/ml [IQR 12 - 74] vs. 0.5 µg/ml [0.2 – 1.2]; p<0.001), but no significant difference was observed when comparing CRP levels in HIV^+ and HIV- patients (median 35 µg/ml [IQR 13 - 73] vs. 31 µg/ml [IQR 11 – 70] respectively, p=0.613; Table 1). The control group had median neopterin and CRP levels within the normal range (neopterin <10nmol/l, CRP <5 µg/ml).

Correlation between neopterin and CD4 cell count

In the analysis of potential associations between immune activation and CD4 lymphocytopenia we observed that plasma neopterin levels showed a significant inverse correlation to CD4 cell count (-0.61, p<0.001; Supplementary material). Significantly higher levels were noted with decreasing CD4 cell count in TB cases, both with and without HIV co-infection. The median neopterin concentration in HIV^+/TB patients with CD4 cell count <100 cells/mm^3 was 66 nmol/l (IQR 42-111) compared to 35

nmol/l (24-55) in patients with CD4 cell count >350 cells/mm^3; p<0.001). Neopterin levels in relation to CD4 cell strata are presented in table 2.

Baseline levels of neopterin and kinetics of CD4 cell count during ATT

With the aim to analyze the relation between immune activation and evolution of CD4 cell count during ATT, neopterin was measured in follow-up plasma samples. For HIV$^-$ patients, baseline median CD4 cell count for the group with increasing CD4 cell count (n=58) was 405 cells/mm^3 and 496 cells/mm^3 for the group without increasing CD4 cell count (n=27). For HIV$^+$ patients, baseline median CD4 cell count for the group with increasing CD4 cell count (n=22) was 255 cells/mm^3, and 411 cells/mm^3 for the group without CD4 cell count increase (n=21).

There was a positive correlation of 0.34 between baseline neopterin concentration for all TB patients and change in CD4 cell count after 6 months. HIV-/TB patients (n=88) with increasing CD4 cell count after 6 months of ATT (n=58) were found to have significantly higher baseline plasma neopterin levels compared to those with unchanged CD4 count (n=30) (29nmol/l vs. 16nmol/l, p=0.004). In HIV$^+$/TB patients who did not start ART (n=44) baseline neopterin was also increased in patients with increasing CD4 cell levels during ATT, but this difference? was non-significant, (Figure 3).

Neopterin and CRP as predictive markers of CD4 cell count

To investigate the predictive value of plasma levels of neopterin and CRP for CD4 cell count AUC for these markers were analyzed. The AUC for neopterin as a surrogate marker for CD4 cell count for all included patients was 0.87. For HIV$^+$/TB subjects with CD4 cell count <350 cells/mm^3 and <100 cells/mm^3 the respective figures were 0.7 and 0.64. CRP showed poor correlation to CD4 cell count at all examined levels in HIV$^+$/TB subjects. As demonstrated in the ROC analysis it had limited capacity to identify patients with CD4 count less than <350 cells/mm^3 (AUC=0.65) and even less for the lowest strata of CD4 cell count (AUC for CD4<100 cells/mm^3 0.59, figure 2).

DISCUSSION

In this study we found that TB patients exhibited elevated immune activation, as assessed by high plasma levels of neopterin. Furthermore, neopterin levels correlated inversely with CD4 cell count in these subjects. Interestingly, these findings were observed both in HIV-positive patients and in HIV-/TB cases with low CD4 cell count. These result suggest that immune activation caused by TB can lead to CD4 lymphopenia, and support previous findings that TB can accelerate HIV disease progression.

Neopterin is an established marker of immune activation that has high predictive capacity for HIV disease progression (26) Ayehunie et al found that median levels of neopterin were higher in Ethiopian than in Swedish PLHIV (27); however, these subjects were not categorized for active TB. Since TB is common among HIV-positive patients in Ethiopia (28), this difference could be explained by undiagnosed TB co-infection in the Ethiopian participants in that study; we have recently reported active TB in 17% of HIV infected patients from Central Ethiopia (29). Our findings of high neopterin levels in TB patients are similar to the limited published data on neopterin in TB. One study from the United States (25 HIV^-/TB patients, 11HIV^+/TB), three studies from India (in total 88 HIV^-/TB patients and 70 HIV^+/TB patients) and one from Zimbabwe (49 HIV^-/TB patients and 32 HIV^+/TB patients) showed increased levels of neopterin levels in TB patients (15–19); in HIV^- the range of median neopterin levels were 28-55nmol/l and for co-infected subjects 51-91nmol/l (higher levels in patients with CD4<200 cells/mm^3). In summary, although not entirely comparable, these figures are similar to our results of 23 and 54nmol/l respectively for these groups. Healthy controls from the same uptake area in our study had normal CD4 cell count, as well as plasma levels of both neopterin and CRP.

The pronounced immune activation in HIV+/TB individuals, reflected by high neopterin levels, are in agreement with the immunological interactions between HIV and TB (30). HIV disrupts the control of MTb by direct and indirect cytotoxic effects on CD4 cells (31). In PLHIV active TB is associated with higher rates of viral replication (32). Our findings on the inverse correlation between CD4 cell count and neopterin levels in HIV-/TB suggest that TB also could contribute to CD4 cell depletion in peripheral blood through immune activation, which is considered to be the major driver of HIV disease progression (33)

The degree of immune activation, measured by plasma neopterin levels in our study, may reflect the strength of Th1 cellular response. In addition, immune activation is a major contributor to disease progression and CD4 cell depletion in HIV infection (34). Whether this also occurs in HIV-/TB remains unclear. Our finding of preserved CD4/CD8 cell percentage in peripheral blood (6) indicates that this phenomenon may be less prominent in HIV-negative subjects. In active TB, activated T cells migrate from the circulation to the sites of infection, and the decreased peripheral blood CD4 cell count observed in TB could merely reflect compartmental redistribution of these cells in the organism. Thus, it is likely that the mechanisms of TB-related CD4 lymphocytopenia are different in HIV^+ and HIV- individuals. However, our data suggest that TB leads to increased immune activation, especially in HIV-positive subjects, and that TB may be an important contributor to HIV disease progression through this pathway.

The correlation between neopterin levels and CD4 cell count found in this study could be related to the severity of TB. We have previously reported that HIV-/TB cases with subnormal CD4 cell count had more advanced disease characteristics, reflected by higher rates of sputum smear positivity, bedridden state and lower median MUAC (6).

Similarly, Jones et al found an association between TB disease severity and low CD4 cell levels in HIV- patients (35).

In most HIV-/TB patients with low CD4 cell count, as well as in several HIV$^+$/TB cases, these levels increased during the course of ATT. As we had access to follow up CD4 cell count of patients during ATT we investigated the relationship between neopterin and development of CD4 cell count after ATT. Somewhat unexpectedly, we found that higher baseline levels of neopterin were associated with greater CD4 cell count increases during ATT. One explanation for this finding could be that adequate treatment of the underlying cause of immune activation (ie TB) leads to reversion of this phenomenon, which in turn would allow for restoration of CD4 homeostasis. We did not have access to lasma samples for determination ofneopterin levels during the course of ATT, but previous studies have shown that these levels decrease during TB treatment in co-infected persons, but remain elevated even after completion of ATT (17–19). This would provide further support for the importance of correct identification of TB in PLHIV, also in settings with access to ART.

Both neopterin and CRP are established biomarkers with prognostic capacity in several conditions. Both markers are available as rapid tests, which would allow for use at primary health care centers (36). Considering previous reports of relationships between these markers and CD4 cell count (14,21), we hypothesized that neopterin and CRP could be used as alternative markers for determination of immunosuppression, in particular to identify HIV$^+$/TB subjects in need of early ART during ATT. According to current guidelines, ART should be started as soon as possible during ATT in patients with severe immunosuppression (defined as <100 cells/mm^3), whereas ART could be deferred for two months in persons with higher CD4 cell count (37).

Despite the inverse correlation found between CD4 cell count and neopterin levels, the performance of neopterin as a surrogate for CD4 cell determination was poor. CRP had even lower predictive capacity for severe immunosuppression, approaching chance (0.5) for this threshold (25). Indeed, a previously constructed algorithm based on symptoms and clinical findings to predict severe immunosuppression (CD4 cell count <100 cells/mm^3) had a better AUC than either neopterin or CRP based on the same patient material (0.75 vs 0.64 and 0.58, respectively)(22), (Supplementary material, figure 2). Since CRP did not show any clear correlation with CD4 cell count in our population, it is unlikely that the combination of these two markers would increase the predictive performance and allow for use as an alternative method for immunosuppression.

To our knowledge, this study is the largest investigation of the degree of immune activation, measured by plasma neopterin, in TB patients with or without HIV co-infection in relation to CD4 cell count, and the first report that investigates both neopterin and CRP in the same population. Our participants are representative for the large burden of co-infected patients managed in primary health care in resource-limited settings. Our study also has certain limitations. TB diagnoses were based on routinely

available methods at Ethiopian health centers, and microbiological confirmation was restricted to sputum smear microscopy. Although neopterin is a well established marker for immune activation a combination of soluble and cellular biomarkers associated with immune activation (such as TNFα, proportion of HLA DR-positive lymphocytes, β2 microglubulin, and soluble CD14 receptor) might have given a more detailed description of immune activation. Finally, we did not have access to follow-up plasma samples for investigation of the evolution of biomarker plasma levels nor a control group of HIV-positive patients without TB.

In conclusion, we found enhanced immune activation in TB patients, with significantly higher levels of neopterin in HIV-co-infected individuals. High neopterin levels were also found to be inversely correlated to low CD4 cell count in TB patients, independent of HIV infection. In addition HIV-/TB patients with higher neopterin levels showed greater CD4 cell count recovery during ATT. This implies that CD4 lymphopenia in TB is linked to the degree of immune activation, and provides further support for the role of TB in HIV disease progression.

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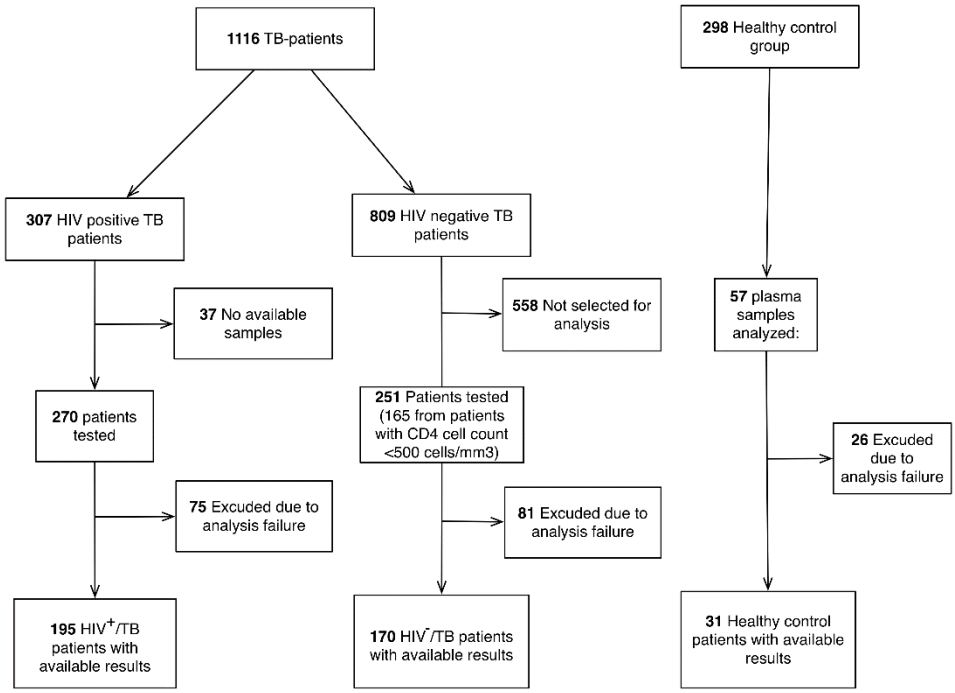


Figure 1:
Flow chart of patients available for analysis. From HIV+ patients we aimed to analyze all available samples (of which there were 37 missing) whereas for HIV-/TB patients and controls, a subset was selected according to principles outlined in the methods section. Analysis failure refers to patients with samples where the ELISA did not accurately predict either internal or external controls.

Table 1:
Baseline characteristics for patients with available Neopterin and CRP results from a cohort of 1116 patients with TB and or HIV and 298 healthy controls

	HIV^{+}/TB patients		HIV$^{-/}$TB patients [a]		
	Included (n=195)	**Not included (n=112)**	**Included (n=170)**	**Not included (n=639)**	**Healthy controls (n=31)**
Age, median (IQR)	32 (28 - 40)	33 (28-40)	30 (23-44)	28 (22-42)	22 (20-25)
Female gender, n (%)	99 (51)	52 (46)	67 (39)	310 (49)	22 (71)
Smear positive pulmonary TB, n (%)	62 (32)	37 (33)	75 (44)	231 (36)	-
Smear negative pulmonary TB, n (%)	62 (32)	32 (29)	43 (35)	173 (27)	-
Extrapulmonary TB, n (%) [b]	74 (38)	49 (44)	54 (32)	244 (38)	-
BMI kg/m^2 (IQR) [c]	17.5 (16-19.5)	17.6 (16.1 - 19.5)	18 (16.4 - 19.7)	18.2 (16.5 - 20)	21.6 (20.7 - 22.8)
MUAC cm (IQR) [d]	21 (19-22)	20 (19 - 22.4)	21 (20-23)	22 (20 - 23)	24.5 (24 - 26.5)
CD4 levels, median (IQR)	192 (106 - 344)	150 (82 - 310)	438 (323-688)	715 (569 - 925)	1022 (869 - 1118)

[a] All patients with CD4<500 cells/mm^3 were included and consecutive patients with CD4≥500cells/mm^3.

[b] Patients could have a simultaneous diagnosis of extrapulmonary and pulmonary TB (included HIV patients, n=3, included HIV- patients, n=2).

[c] BMI is the calculated Body mass index (weight/height^2).

[d] MUAC is the mid upper arm circumference measured by a measuring tape.

Table 2:
Neopterin and CRP levels analyzed by CD4 cell count strata in 365 TB patients with and without HIV and 31 controls.

CD4 cell count strata	Neopterin in HIV^{+}/TB patients[a]	Neopterin in HIV^{-} /TB patients[a]	Neopterin in Healthy controls[a]
All	54 (30 - 111) (n=195)	23.2 (14 - 44) (n=170)	3.8 (1.6 - 5.5)
>500 cells/mm3	32 (22 – 50) (n=22)	14 (10 - 22) (n=67)	-
>350 cells/mm3	36 (24 – 56) (n=48)	20 (12 – 38) (n=113)	-
100-350 cells/mm3	63 (35 – 111) (n=103)	30 (17-51) (n=54)	-
<100 cells/mm3	77 (43 – 111) (n=44)	82 (79 – 104) (n=3)	-
CD4 cell count strata	CRP in HIV^{+} /TB patients[b]	CRP HIV- /TB patients[b]	CRP in Healthy controls[b]
All	36 (12 - 74) (n=195)	33.4 (12 - 79) (n=170)	0.5 (0.2 - 1.2)
>500 cells/mm3	16 (2.8 – 41) (n=22)	19 (6,3 – 53) (n=67)	-
>350 cells/mm3	17 (5.3 – 51) (n=48)	27 (10 – 66) (n=113)	-
100-350 cells/mm3	40 (16 – 75) (n=103)	56 (21 – 90) (n=54)	-
<100 cells/mm3	47 (13 – 95) (n=44)	33 (33 - 33) (n=3)	-

[a] Neopterin levels (nmol/l), median (IQR) and number of study subject (n=)

[b] CRP levels (µg/ml), Median (IQR) and number of study subject (n=)

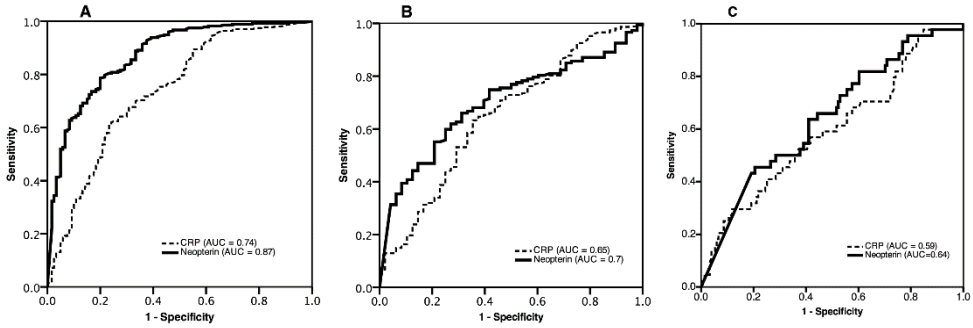


Figure 2:
ROC curves showing heads up comparison between neopterin and CRP for predicting patients under 3 different CD4 cell cut off levels <500 cells/mm^3, <350 cells/mm^3, <100 cells/mm^3.

A: CD4 cell count <500 cells/mm^3. Includes all patients, both HIV$^+$ and HIV$^-$ -patients and controls

B: CD4 cell count <350 cells/mm^3. Includes HIV$^+$ patients

C: CD4 cell count <100 cells/mm^3. Includes HIV$^+$ patients.

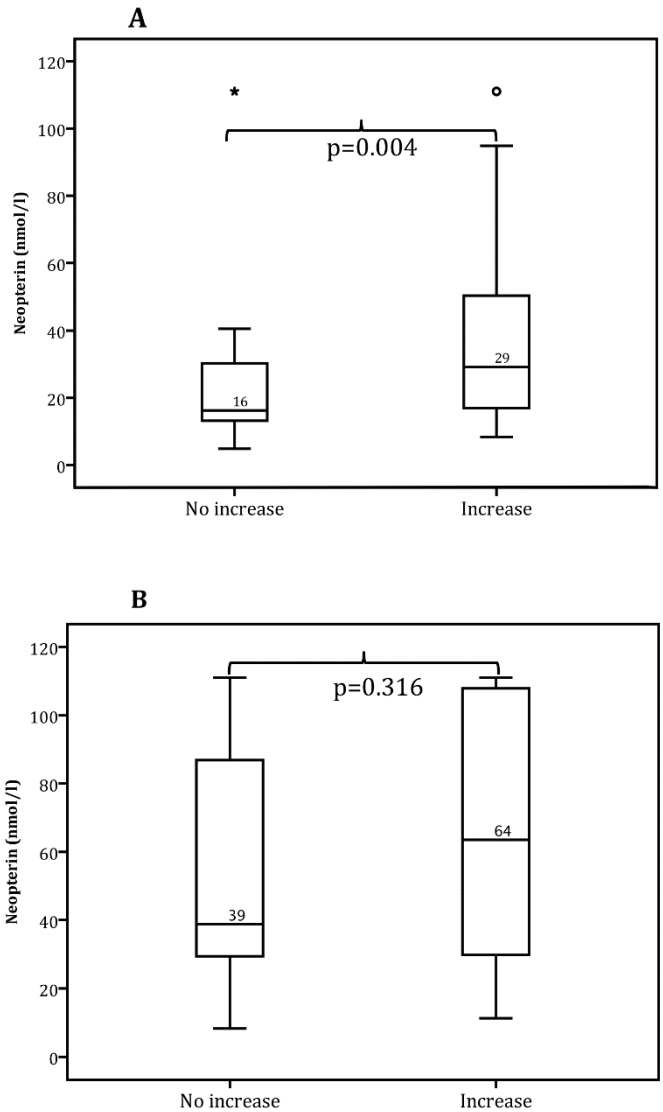
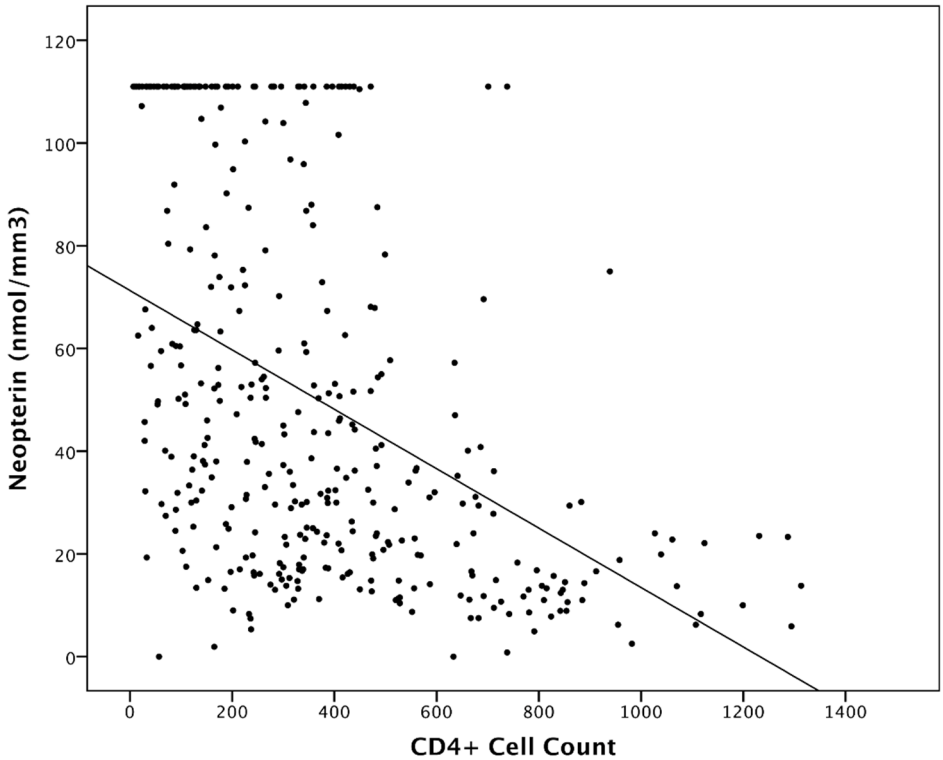
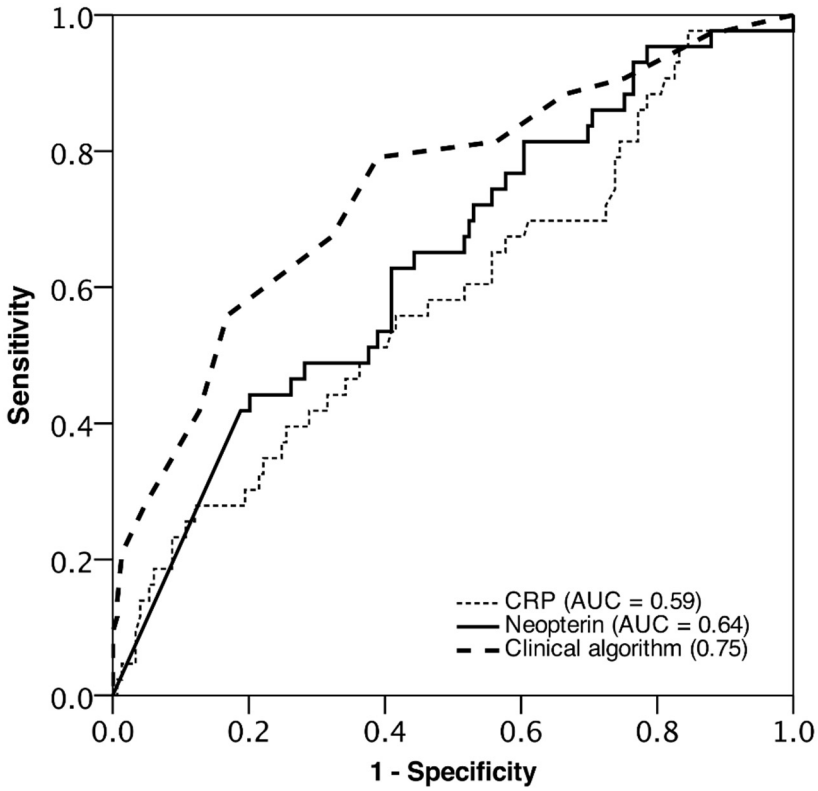


Figure 3:
Baseline neopterin in relation to development of CD4 cell count after 6 month of ATT subdivided into two groups; No increase (CD cell count ≤50 cells/mm^3 or unchanged CD4 cell count, ± 50 cells/mm^3 after 6 months ATT), and increase (CD4 ≥50 cells/mm^3 after 6 months ATT). Graph **A** displays patients who were **HIV**$^-$ (n=85 of whom 58 had an increase in CD4 cell count). Baseline median CD4 cell count for those who increased was 405 cells/mm^3 and 496 cells/mm^3 for those who did not increase (n=27). Graph **B** displays **HIV**$^+$ patients who did not start ART (n=43 of whom 22 had an increase in CD4 cell count). Baseline median CD4 cell count for those who increased was 255 cells/mm^3 and 411 cells/mm^3 for those who did not increase (n=21).

Supplementary material:



Supplementary figure 1:
Scatterplot showing the relation between neopterin levels and CD4 cell count at baseline for all TB patients (n=396). Spearman rank correlation is -0.61, p=0.001. The levels of neopterin were cut off at 111nmol/l according to the specified detection limit as specified by the manufacturer of the ELISA-kits (IBL international).



Supplementary figure 2.
Predictive capacity of neopterin and CRP for prediction of CD4 cell strata <100 cells/mm3 compared to a previously constructed clinical algorithm.

Footnote page:

Clinical trials registration number: NCT01252537

Conflicts of interest

The authors do not have commercial or other association that might pose a conflict of interest.

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