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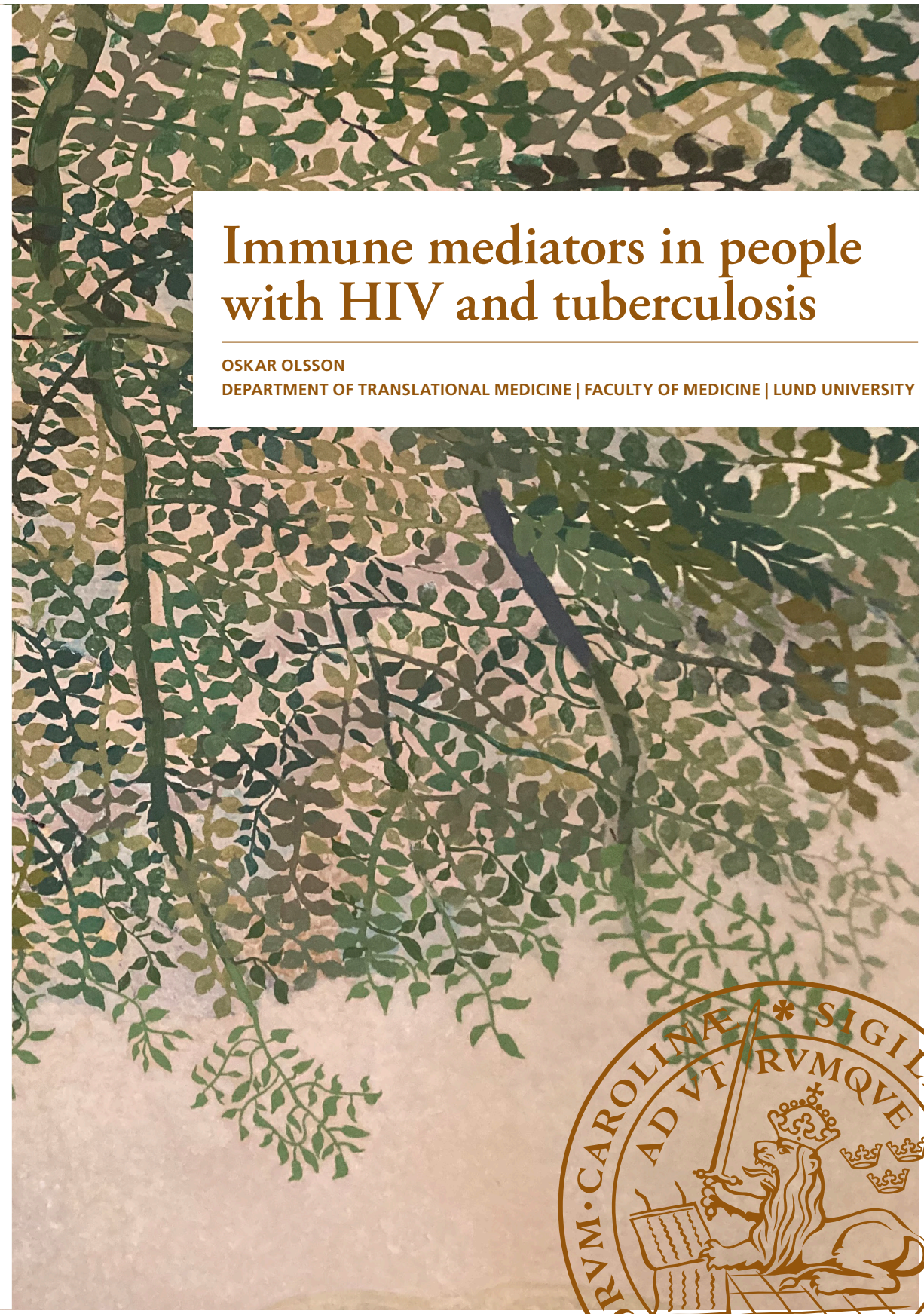

Immune mediators in people with HIV and tuberculosis

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Immune mediators in people with HIV and tuberculosis

Immune mediators in people with HIV and tuberculosis

Oskar Olsson

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine, Lund University, to be publicly defended at 1 p.m. January 19 2024, in Fernströmssalen, Forum Medicum, Sölvegatan 19, Lund

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Abstract: Tuberculosis (TB) is the most common cause of death among people with HIV (PWH). PWH often have atypical clinical manifestations of TB, and TB disease can therefore be missed. Better screening markers could identify individuals with high likelihood of TB disease. Furthermore, the mechanisms underlying the immunopathogenesis of TB disease in PWH remain incompletely understood.This thesis is based on a cohort of 812 treatment-naïve PWH recruited in Ethiopia, who were investigated with bacteriological methods for TB regardless of symptoms. TB disease was diagnosed in 137 (16.9%, HIV+/TB+), and the remainder were classified as HIV+/TB-.

In the first paper, we investigated nine markers of systemic inflammation in plasma in a subset of 260 individuals. The levels of eight of these markers were significantly increased in HIV+/TB+. Combinations were assessed with logistic multivariate models, and a combined model of C-reactive protein (CRP) and soluble urokinase plasminogen activator receptor (suPAR) was found to have superior discriminatory capacity to any of the markers alone. In the subset of individuals with CD4 count <200 cells/mm^3, this combination achieved the desired performance (>90% sensitivity and >70% specificity) of a screening marker for TB in PWH. In the second paper, we investigated the ratio between the amino acid tryptophan and its metabolite kynurenine (KT ratio) in plasma from 249 individuals. Although the KT ratio was significantly increased in HIV+/TB+, it did not show discriminatory capacity to a degree that would be clinically useful. In the third paper, we investigated expression profiles of microRNA (miRNA) and small nucleolar RNA (snoRNA) in whole blood of individuals with TB disease, with and without HIV, and uninfected controls (total n=40). We found 218 miRNAs, several of which have known roles in the TB immune response, to be differentially expressed between HIV+/TB+ and HIV-/TB+. We also found 103 snoRNA to be significantly downregulated in people with TB. These changes were partially reversed with treatment for TB and HIV.In the fourth paper, we investigated plasma ribonuclease activity in 129 PWH with and without TB. Using three different techniques, we identified increased ribonuclease activity in HIV+/TB+ individuals. We subsequently identified three different ribonuclease proteins, ribonucleases 2, 3 and T2, that were also increased in HIV+/TB+, with varying degrees of correlation to total ribonuclease activity.

In summary, this thesis includes studies on different types of blood-based biomarkers of TB disease in PWH, identifying promising performance of a combination of CRP and suPAR. Ways in which HIV and TB interact and alter immune mediators have also been investigated, and the findings point towards involvement of miRNA, snoRNA and ribonucleases.

Key words: Tuberculosis, HIV, biomarkers, RNA, ribonucleases

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Populärvetenskaplig sammanfattning

Personer med hiv drabbas i betydligt högre utsträckning än andra av tuberkulos (tbc). Globalt är tbc den vanligaste dödsorsaken hos personer med hiv. Detta är ett särskilt stort problem i Afrika söder om Sahara, och nästan 70 % av de som drabbas av samtidig hiv och tbc bor i Afrika. Att personer med hiv är extra utsatta för tbc beror framför allt på att hiv angriper och förstör en typ av vita blodkroppar, s.k. T-hjälparceller, som är viktiga för immunförsvaret mot tbc. Då risken för tbc ökar redan innan T-hjälparcellerna minskar, tror man att ytterligare mekanismer som inte är klarlagda bidrar till den ökade risken för tbc. Immunsvaret mot tbc kan ses som en känslig balansgång, där hämmat immunförsvar leder till spridning av tbc-bakterier i kroppen och förvärrad sjukdom, medan ett överaktivt immunförsvar leder till värre skador på de drabbade organen. Ett annat problem är att tbc är svårt att diagnosticera, speciellt hos personer med hiv, som ofta har tbc med spridning till andra organ än lungorna, vilket kan ge mer svårtolkade symptom. Provtagningen för tbc baseras framför allt på upphostat luftvägssekret, vilket många personer har svårt att åstadkomma. Dessutom är de flesta laboratoriemetoder som utförs för att upptäcka tbc komplicerade och tidskrävande.

Studierna i denna avhandling baseras på prover och data från en kohort av personer med hiv som undersöktes systematiskt för tbc. Samtliga personer i studien rekryterades i primärvården i Etiopien. Resultaten visade att 137 av 812 inkluderade individer också hade tbc. Vi har studerat ett flertal molekyler som representerar olika komponenter i immunförsvaret, både för att undersöka ifall dessa kan användas för att identifiera vem som har tbc, och hur de speglar immunförsvaret mot tbc hos personer med hiv.

I det första arbetet undersökte vi nio olika markörer från immunförsvaret i blodprover från totalt 260 personer med hiv, med och utan tbc. Genom att kombinera två proteiner, CRP och suPAR, kunde vi särskilja fler tbc-patienter än genom att bara analysera ett protein i taget. Särskilt bland de med mest nedsatt immunförsvar (som också är den grupp som har högst risk för tbc) visade denna kombination lovande prestanda.

I det andra arbetet undersökte vi kvoten mellan aminosyran tryptofan, och dess nedbrytningsprodukt kynurenin, i blodprover från 249 personer med hiv, med och utan tbc. Denna markör har i tidigare studier rapporterats ha hög förmåga att utskilja personer med tbc. Hos de personer vi studerade hade de med tbc förhöjd kynurenin/tryptofan-kvot, men skillnaden var inte så stor att det skulle utgöra ett användbart test inom sjukvården. Vi tror att det framför allt beror på att de personer vi studerat inte påbörjat behandling mot hiv, vilket en del deltagare i tidigare studier hade gjort. Hivbehandling påverkar nämligen också kynurenin/tryptofan-kvoten.

Det tredje arbetet handlar om små icke-kodande nukleinsyror som kallas mikro-RNA (miRNA) och små nukleolära RNA (snoRNA). Dessa liknar budbärar-RNA,

som fungerar som ritningar för alla kroppens proteiner. I stället för att koda för proteiner är dessa små molekylers roll att reglera budbärar-RNA och på så vis påverka bildandet av proteiner. Vi undersökte hur miRNA och snoRNA påverkades av hiv och tbc i blodprover hos totalt 40 personer med och utan tbc respektive hiv. Vi fann att miRNA-uttrycket vid tbc påverkades i hög grad av hiv, och då många av de miRNA som var påverkade har kända roller i immunförsvaret mot tbc, tolkar vi det som att de förändrade miRNA-nivåerna bidrar till att göra personer med hiv mer känsliga för tbc. Nivåerna av snoRNA påverkades mer av tbc, som ledde till att snoRNA-nivåerna tydligt minskade. Vi tolkar detta antingen som en mänsklig försvarsmekanism mot tbc, eller en mekanism som tbc använder för att undvika immunförsvaret. Vidare studier av dessa molekyler är viktiga för att kartlägga deras roll vid hiv och tbc, något som i förlängningen kan användas för att hitta nya diagnostiska metoder eller sätt att behandla dessa infektioner.

Det fjärde och sista arbetet handlar om ribonukleaser, enzymer som bryter ner RNA, vilka ingår i vårt försvar mot till exempel bakterier och virus. Genom att tillsätta RNA till blodprover hos personer med hiv med och utan tbc (totalt 129 personer), och studera hur dessa bryts ned, kunde vi visa att personer med tbc har ökad ribonukleasaktivitet. Detta fynd bekräftades i experiment med tre olika metoder, och vi mätte sedan tre olika specifika ribonukleaser som visade sig öka vid tbc. Detta är förmodligen en mekanism som vårt immunförsvar använder för att bekämpa tbc, men det är också möjligt att det bidrar till de skador som immunförsvaret orsakar vid tbc och hiv.

List of Papers

Paper I

Olsson O, Björkman P, Jansson M, Balcha TT, Mulleta D, Yeba H, Valfridsson C, Carlsson F, Skogmar S. Plasma Profiles of Inflammatory Markers Associated With Active Tuberculosis in Antiretroviral Therapy-Naive Human Immunodeficiency Virus-Positive Individuals. Open Forum Infect Dis. 2019;6(2):ofz015

Paper II

Olsson O, Skogmar S, Tesfaye F, Mulleta D, Jansson M, Björkman P. Kynurenine/tryptophan ratio for detection of active tuberculosis in adults with HIV prior to antiretroviral therapy. AIDS. 2022;36(9):1245–53.

Paper III

Olsson O, Tesfaye F, Søkilde R, Mazurek J, Abebe M, Yeba H, Assefa A, Skogmar S, Balcha TT, Rovira C, Björkman P, Jansson M. Expression of MicroRNAs Is Dysregulated by HIV While Mycobacterium tuberculosis Drives Alterations of Small Nucleolar RNAs in HIV Positive Adults With Active Tuberculosis. Front Microbiol. 2022;12:808250.

Paper IV

Olsson O, Søkilde R, Tesfaye F, Karlson S, Skogmar S, Jansson M, Björkman P. Tuberculosis disease is associated with elevated plasma ribonuclease activity in antiretroviral treatment-naïve people with HIV. *Manuscript.*

List of papers not in thesis

Olsson O, Winqvist N, Olsson M, Olsson P, Björkman P. High rate of latent tuberculosis treatment completion in immigrants seeking asylum in Sweden. Infect Dis. 2018;50(9):678–86.

Rothman E, **Olsson O**, Christiansen CB, Rööst M, Inghammar M, Karlsson U. Influenza A subtype H3N2 is associated with an increased risk of hospital dissemination - an observational study over six influenza seasons. J Hosp Infect. 2023;139:134–40.

Abbreviations

ART	Anti-retroviral therapy
ATT	Anti-tuberculosis therapy
AUC	Area under the curve
BMI	Body mass index
CCL5	Chemokine (C-C motif) ligand 5 (also known as RANTES)
CI	Confidence interval
CT value	Cycle threshold value
ELISA	Enzyme-linked immunosorbent assay
IGRA	Interferon-γ release assay
IL	Interleukin
IP-10	Interferon-γ induced protein 10 (also known as CXCL10)
IRIS	Immune reconstitution inflammatory syndrome
KT ratio	Kynurenine/tryptophan ratio
MUAC	Mid-upper arm circumference
miRNA	MicroRNA
Mtb	*Mycobacterium tuberculosis*
PCR	Polymerase chain reaction
PCT	Procalcitonin
PWH	People with HIV
qPCR	Quantitative polymerase chain reaction
ROC	Receiver operating characteristic
sncRNA	Small non-coding RNA
snoRNA	Small nucleolar RNA
suPAR	Soluble urokinase plasminogen activator receptor
TB	Tuberculosis
TNF-α	Tumor necrosis factor-α
TST	Tuberculin skin test
VL	Viral load
WHO	World Health Organization

Introduction

Tuberculosis

Tuberculosis (TB) is caused by the acid-fast bacilli of the *Mycobacterium tuberculosis* complex, most importantly *Mycobacterium tuberculosis* (Mtb), *Mycobacterium africanum, Mycobacterium bovis* and *Mycobacterium canettii* (1). The by far most important mode of transmission is aerosol, as small (1-5 μm) droplet nuclei containing the bacillus are able to reach the alveoli of lungs (2). Upon reaching the alveoli, the mycobacteria are taken up by dendritic cells and macrophages, eliciting a cytokine response involving the interleukins (IL) IL-1, IL-6 and IL-12, as well as tumour necrosis factor (TNF)-α. This leads to activation and recruitment of additional immune cells (3). Gradually an adaptive immune response develops, and after four weeks the adaptive immune response is evident through positive tuberculin skin-test (TST) or interferon-γ release assay (IGRA) (4), tests of cell mediated immunity that are used in clinical practice to diagnose TB infection. Locally, bacilli are transported to the regional lymph nodes by antigen presenting cells, antigen specific lymphocytes are then recruited to the primary infection site, and a granuloma takes form. The granuloma is a structure by which the immune system can decrease the number of bacilli and keep the infection contained. Mtb, however, can also survive within immune cells and remain in a dormant stage (in some cases for decades) before once again starting to multiply (3).

Traditionally, Mtb infection has been divided into two distinct states: latent TB infection, regarded as an inert condition with small numbers of dormant bacilli contained in granulomas by TB-specific immune responses, only evident through a positive TST or IGRA; and TB, with symptoms originating from affected organs and where Mtb bacteria can be cultured from that site. That view is increasingly being challenged, and TB infection is currently regarded as a continuous spectrum. Studies following TB infected individuals over time has identified changes on positron emission tomography-computered tomography (5) and on conventional chest x-ray (6) , as well as transcriptional changes (7) in peripheral immune cells, that precede development of symptoms and detectable bacteria by several months. This realization has led to the coining of the terms *incipient TB*, where TB is likely to develop, but it not possible to identify with currently existing tools, and *subclinical TB*, with abnormal radiography and/or microbiological tests, but without symptoms suggestive of disease (8). Individuals with subclinical TB may still be

important sources of transmission. Furthermore, as latent TB is only defined by the immune response, not identification of the actual bacteria, it is likely that a proportion of people with positive TB immune reactions have spontaneously resolved infection and no longer harbour viable bacteria. Besides this, tests that rely on immune response to the bacteria depend on the capacity of the individual to mount a TB-specific cellular immune response. Thus, IGRA and TST cannot adequately identify a substantial proportion of infected individuals. This is especially important in the context of HIV (9). Of the people who develop TB infection as defined by currently existing tools, about 90% will never exhibit evidence of disease. Approximately half of cases of TB disease occur within the first two years of infection (2).

Several factors can increase the likelihood of progression along the spectrum towards clinically manifest disease, such as malnutrition, immunosuppressive therapies (e.g., corticosteroids and TNF-α inhibitors), silicosis, renal failure and diabetes mellitus. Untreated HIV infection is considered to be the strongest risk factor known for TB disease progression (10).

The lungs, being the primary site of TB infection, are the organs most commonly affected by TB disease. The four classical symptoms of pulmonary tuberculosis are cough, weight loss, fever and night sweating, sometimes accompanied by haemoptysis, fatigue and chest pain. These classical symptoms are not always present, making the diagnosis of TB challenging (11). Pulmonary TB has often been clinically divided into two separate entities: primary and reactivation TB, with the former showing more systemic symptoms and non-specific radiographic findings, and the latter a more typical symptomatology, commonly with apical distribution of radiographic abnormalities, sometimes with cavitation (12). It has been demonstrated, however, that these two syndromes overlap to a large degree (13). Besides the lungs, TB disease can occur in virtually any site in the body, such as lymph nodes, bones, central nervous system, the gastrointestinal tract and genitourinary organs (2). The most common extrapulmonary manifestation of TB is lymph node TB, for which the most common extra-thoracic localisation is the cervical region (14). The most severe forms of TB disease are disseminated TB and TB meningitis, which are especially common among young children (15,16).

The diagnosis of TB disease relies on identification of Mtb bacilli, for instance, different methods of visualizing the Mtb bacillus via microscopy of samples. The most famous method is the Ziehl-Neelsen staining, based on carbol fuchsin which strongly binds to the lipid-rich, hydrophobic cell wall, staining the mycobacteria red. The sample is treated with acid alcohol, removing the colour from bacteria lacking the thick cell wall characteristic of mycobacteria (17). Several nucleic acid amplification tests for Mtb have also been developed, and the most used is the polymerase chain reaction (PCR) method Gene Xpert MTB/RIF, which specifically detects genetic elements from Mtb in addition to mutations conferring rifampicin resistance. The gold standard, most sensitive method is mycobacterial culture,

including solid Löwenstein-Jensen medium and different types of liquid media (2). In order to diagnose TB disease, it is critical to obtain representative biological samples. For pulmonary TB, this means acquisition of lower airway samples such as sputum or broncheo-alveolar lavage fluid, or ventricular lavage as Mtb bacilli are frequently swallowed and can survive in the acidic environment of the ventricle (2). In case of suspected extrapulmonary TB, aspirates or biopsies are usually required for TB bacteriological investigations.

Treatment of TB disease typically consists of combination anti-TB therapy (ATT) for several months. The most widely used regimen is an intensive phase of rifampicin, isoniazid, pyrazinamide and ethambutol for two months, followed by an additional four-month continuation phase of rifampicin and isoniazid (18). Novel or repurposed drugs and regimens are being investigated, and a shorter regimen of 4 months of rifapentine, isoniazid, pyrazinamide and moxifloxacin has been shown to be non-inferior to the standard regimen (19) and its use is now being endorsed by the World Health Organization (WHO) (20). Latent TB infection has traditionally been treated with 6-9 months of isoniazid, but newer, shorter regimens such as 3 months of weekly rifapentine and isoniazid or 1 month of daily rifapentine and isoniazid have been shown to be non-inferior and are now also recommended (21). Multi-drug resistant TB is a significant problem in many parts of the world, and in many countries in the former Soviet Union the proportion of rifampicin-resistant TB among treatment-naïve patients exceeds 20% (22).

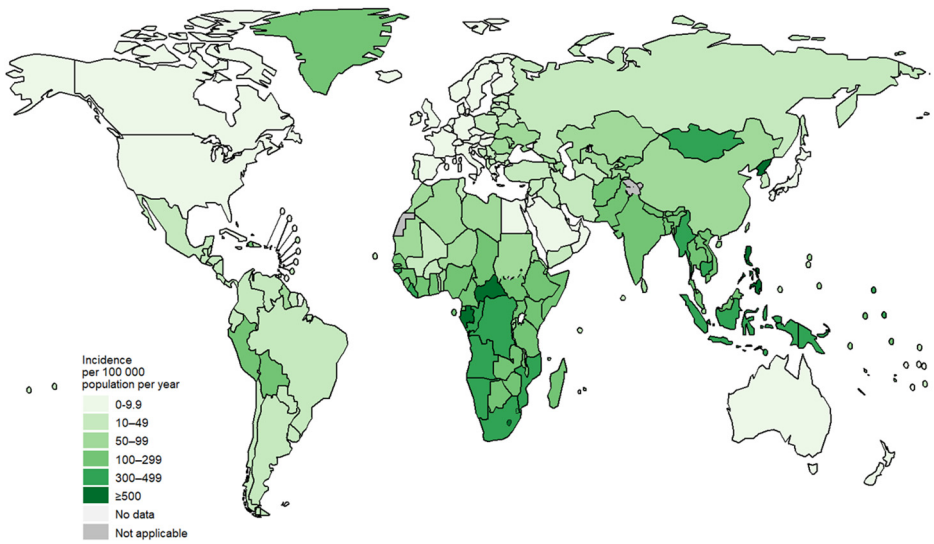

Figure 1.Global incidence of TB in 2022. Source: Global Tuberculosis Report 2023. WHO (23).

TB is unevenly distributed across the world, and the most affected regions are sub-Saharan Africa and South-East Asia (Figure 1). The estimated global disease burden has slowly decreased, but the global coronavirus pandemic has led to an increase in the estimated TB incidence for the first time in many years (23). A total of 1.3 million people are estimated to have died from TB in 2022. A global framework called *The End TB Strategy* was initiated by the WHO in 2014 (24), and the development is currently far off track from achieving the set targets in reduction of TB incidence and mortality (23). It is clear that more efforts are needed to accelerate the decline of TB mortality and morbidity, and among these are improved methods for TB case-finding.

HIV

HIV-1 and HIV-2 are single-stranded, RNA viruses that are part of the *Retroviridae* family and the *Lentivirus* genus(25). The global HIV epidemic is completely dominated by HIV-1, and all data discussed in this thesis concerns people infected with HIV-1. Thus, HIV-2 will not be discussed further, and HIV in this this text will henceforth be synonymous with HIV-1. The origin of HIV stems from zoonotic animal-to-human transmission events of the closely related simian immunodeficiency virus, present in several African monkey species (26,27). The HIV virion is around 100 nm in diameter and contains two copies of single-stranded RNA containing nine genes which are encapsulated in a capsid of the p24 protein and surrounded by a lipid envelope (28).

HIV is mainly transmitted sexually, through exposure to infected blood, or perinatally (in utero, at delivery or through breast-feeding) (29). Due to its dependence on the cellular receptors CD4, CCR5 and CXCR4, the virus preferentially infects CD4+ T cells, macrophages and dendritic cells (30). The viral glycoproteins in the envelope binds with the receptors in host cell membranes, which triggers fusion of the viral and host cell membrane. The viral genome is released into the cytoplasm and reversely transcribed into double-stranded DNA by the viral reverse transcriptase. The viral DNA is integrated into the host genome via the viral enzyme integrase. Integration can lead to productive infection, with transcription of the viral DNA and generation of new virions, but can also remain as a latent reservoir of HIV. As anti-retroviral therapy (ART) only targets actively replicating viruses, the latently integrated HIV is a major barrier against curative therapy (31).

HIV infection generates a progressive loss of CD4+ T cells, first in the gut mucosa and later in the peripheral blood (29), through mechanisms such as pyroptosis (32) and increased cellular turnover due to hyperactivation (33). These phenomena lead to gradually impaired cellular immunity. The course of this process is highly

variable (34), and some individuals maintain low viral levels and high CD4 count for many years (35). However, in the absence of ART, the majority of people with HIV (PWH) gradually lose their ability to control microbial infections, with development of severe diseases caused by microorganisms that do not cause disease in persons with normal cellular immune function, so called opportunistic infections (36).

However, the introduction of combination ART has led to major improvement of the prognosis for PWH. ART usually consists of a combination of three drugs, which target different steps of the replication cycle of HIV (28). With successful ART, HIV replication is blocked, with undetectable viral load (VL) in blood, eliminating the risk of sexual transmission (37). The degree of immune recovery is variable (38), and depends on factors such as age and stage of immunodeficiency at ART initiation (39), but with adequate drug supply and in the absence of drug resistance most ART recipients have minimal risks of opportunistic infections (36) and a life expectancy approaching that of people without HIV (40).

ART has been rolled out globally during the recent two decades. Since the launch of global ART rollout, new HIV infections have decreased by 59% since the peak in 1995 and AIDS-related deaths have decreased by 69% since the peak in 2004. Yet, many PWH have not been diagnosed, and among those diagnosed many do not receive ART. A global target is that 95% of PWH should know their status, 95% of those individuals should receive ART and 95% of them should be virally suppressed. Of the 39 million people in the world currently living with HIV, 86% know their status, of whom 89% are accessing treatment, and among these people 93% are estimated to be virally suppressed (41).

HIV and TB coinfection

As HIV depletes the cellular immune response, individuals become increasingly susceptible to TB disease. The HIV pandemic fuelled a parallel pandemic of TB, especially in the southern hemisphere, but also in high-income countries such as the USA (3). These pathogens have a special relationship, as HIV increases susceptibility to TB progression at an earlier stage than for most other opportunistic pathogens. In addition, TB disease leads to accelerated HIV replication (4). TB is the most common cause of death in PWH (3), and post-mortem studies commonly report large proportions of unidentified TB in PWH (42).

TB disease in PWH often has different clinical manifestations than in HIV-negative persons. Extrapulmonary and disseminated TB is more common among PWH, (43,44). Post-mortem studies have found disseminated disease in 90% of PWH who have died of TB (45). The reported symptoms are different, and TB has been reported to be a significant contributor to “slim disease”, a syndrome of wasting and

fever often reported in ART-naïve individuals in sub-Saharan Africa (46). One study of active TB case-finding among PWH reported half of pulmonary TB cases to be completely asymptomatic (47). There is less often upper lobe cavitation on a chest radiography in HIV-TB (48,49), but more often mediastinal lymphadenopathy or pleural effusions (13). Substantial proportions of individuals with pulmonary TB and HIV have been shown to have no or minimal changes on chest radiography (50,51).

These alterations are especially prominent in people with more advanced immunosuppression. Individuals with CD4 counts >300 cells/mm^3 have radiological manifestations similar to people without HIV, while a miliary pattern is reported in the majority of individuals with CD4 counts <100 cells/mm^3 (52). A study comparing TB presentation in PWH before and after ART implementation reported that the proportion of "typical" chest radiograph pattern increased from 25% to 45% (53). The disease is more often extrapulmonary in individuals with lower CD4 counts, and the rate of mycobacteraemia has been reported to be as high as 49% in PWH with TB disease and CD4 count<100 cells/ mm^3 (54).

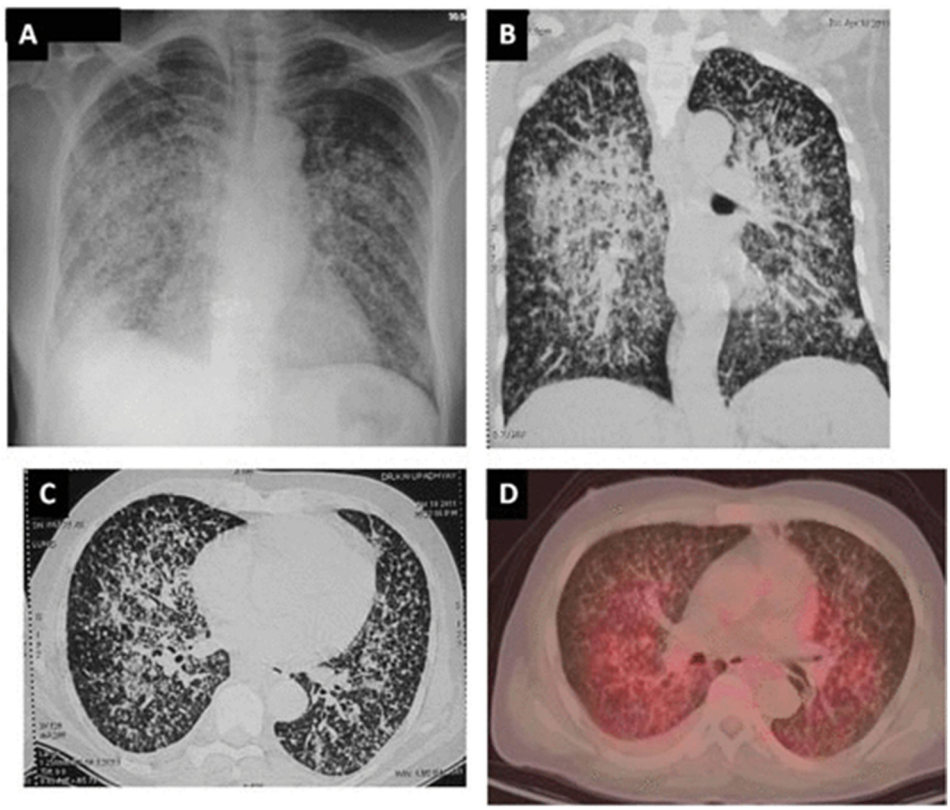

Figure 3. Miliary tuberculosis A) Chest radiograph B) and C) computered tomography D) positron emission tomography-computered tomography from the same patient, all with miliary lesions, predominantly on the right side. Source: Sharma *et al*. 2011 (55)

Not only does HIV affect the clinical and radiographic presentation of TB, HIV also affects the performance of many existing methods for TB diagnosis.

Direct microscopy of sputum samples is the method most drastically affected by HIV status (56), for instance, in a previous study of sputum diagnostic methods of TB in the cohort that this thesis is based on, the sensitivity of sputum microscopy was only 23% (57). The first generation of Gene Xpert MTB/RIF has also been reported to have impaired sensitivity in PWH in many cohorts (58). However, a multicentre study of performance the Gene Xpert MTB/RIF Ultra, the improved latest generation of this test, demonstrated comparable sensitivity of PWH and other individuals at 90%. However, the sensitivity in sputum smear-negative individuals, regardless of HIV status, was still suboptimal at 63% (59). In addition, the throughput capacity of this method currently makes it unfit for screening large numbers of PWH for TB disease. Furthermore, sputum production is often an issue when screening PWH for TB (60), and substantial numbers have extra-pulmonary disease, where sputum samples will not be conclusive (61). In addition to these issues with diagnosis of TB disease in PWH, the diagnosis of latent TB infection in PWH is hampered by the fact that both TST and IGRAs rely on cell-mediated immunity (9,62).

The burden of HIV-TB coinfection is heavily skewed towards sub-Saharan Africa (Figure 2). Of a total of 187 000 deaths worldwide from TB disease in PWH in 2022, 69% occurred in the WHO African Region. The mortality rate from TB in PWH in Africa is approximately twice as high as in TB disease in HIV negative individuals (23).

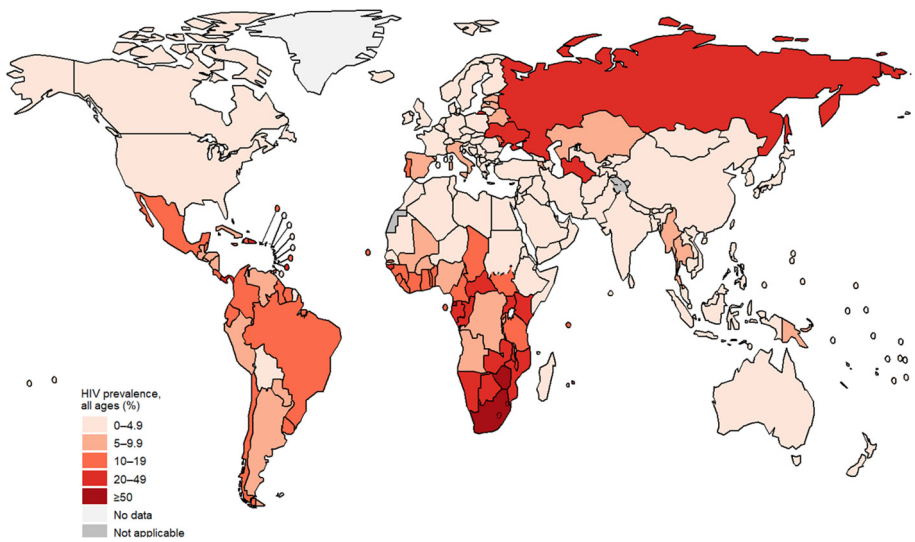

Figure 2. HIV prevalence among people with TB disease in 2022. Source: Global Tuberculosis Report 2023. WHO (23).

Immune pathogenesis in HIV and TB

In contrast to most other opportunistic infections in PWH, the risk of TB increases already within the first year after seroconversion (63). With ART and immune recovery the incidence of TB disease is drastically reduced, yet, PWH on ART living in TB-endemic setting have a higher risk of TB than people without HIV(64). This has led to research into immune mechanisms other than CD4+ T cell depletion, that render PWH particularly susceptible to TB disease.

CD4+ T cells are obviously of special interest for this interaction, as CD4+ T cell depletion is the hallmark of HIV infection, and CD4 count is well known to correlate to risk of TB disease (65). However, beyond CD4+ T cell depletion, HIV appears to affect CD4+ T cells in more subtle ways. For example, HIV appears to cause a targeted depletion of Mtb-specific CD4+ T cells before elimination of the total pool of CD4+ T cells (66).

Although the evidence is inconsistent, it seems that HIV affects the organization of the granuloma, the complex structure by which the immune system tries to contain Mtb. Most studies show that granulomas from PWH contain more bacteria, and with decreasing CD4 counts the architecture of granulomas become increasingly disorganized (67,68), a finding which is in agreement with the higher degree of TB dissemination in immunosuppressed PWH (69).

Several papers have described how HIV affects the capacity of macrophages to eliminate Mtb. As macrophages can be infected by HIV (70), and are the first cells to encounter inhaled Mtb (4), it is logical to speculate that macrophages are key cells for interactions between HIV and Mtb. Indeed, one study have indicated that alveolar macrophages from PWH, even including those receiving ART, exhibit impaired capacity of phagosomal proteolysis (71) and that macrophages infected *in vitro* with HIV have reduced capacity to control mycobacterial growth (72). Another study has demonstrated impaired autophagy in macrophages during HIV-TB through a complex mechanism involving increased hemophagocytosis (73). The relevance of macrophages in HIV-TB coinfection is not unanimously supported, however, probably reflecting different experimental conditions. For instance, macrophages from broncho-alveolar lavage of PWH did not exhibit any deficiency in limiting Mtb growth (74), and histologic studies of granulomas has revealed similar patterns in terms of macrophage distribution in people with and without HIV coinfection (75).

Other studies have demonstrated other important immune interactions, for example involving neutrophils, showing that neutrophils from PWH limit Mtb growth less efficiently, are hyperactivated and are more prone to necrotic cell death (76). Furthermore, dendritic cells from PWH show impaired antigen presentation of Mtb (77). It has been shown that PWH with TB have increased levels of myeloid-derived suppressor cells (68), and that monocytic myeloid-derived suppressor cells from

PWH exhibit a phenotype which produces less IL-12p70 and TNF-α and is more permissive to Mtb growth (78). PWH with TB also have increased proportions of natural killer cells with impaired function (79).

While many studies point towards immunosuppression as the driving force underlying the increased risk of TB progression in PWH, there are also data suggesting that HIV exerts effects on inflammatory responses, shifting the balance towards TB disease and tissue damage through proinflammatory mechanisms. The maintenance of TB in a latent state requires both potent immune responses to contain the bacilli, but also anti-inflammatory mechanisms that uphold immune balance and prevent excessive immune activation (4). For example, HIV has been found to impair the anti-inflammatory cytokine IL-10 at the site of tuberculin-skin test instillment (80). A study of Mtb-specific Th17 cells in PWH with latent and active TB has suggested that HIV mainly steers Th17 cells towards a more proinflammatory profile (81). Studies of Mtb-specific CD4+ and CD8+ T cells revealed that HIV preferentially eliminates CD4+ T cells with the immunoregulatory Th2 phenotype (82). A study of systemic cytokines in TB disease with and without HIV found almost equal numbers of inflammatory mediators upregulated and downregulated in PWH (83). The most obvious example of HIV-TB immunopathology is immune reconstitution inflammatory syndrome (IRIS), in which immune restoration after ART initiation leads to immune hyperactivation and exaggerated inflammatory reactions. Both the adaptive and the innate immune responses have been shown to be dysregulated in IRIS (4).

It also also clear that TB disease leads to increased HIV replication. Sites of TB disease have repeatedly been demonstrated to also harbour increased levels of HIV or HIV antigens (84,85). TB disease leads to activation of several intracellular pathways that accelerate HIV replication (84,86). Interestingly, a recent study showed that latent TB infection is associated with transcriptomic changes that seem to protect against HIV progression, serving as an adjuvant for HIV immunity. These changes were lost with progression to TB disease (87).

In summary, HIV affects the immune response to TB in several ways beyond CD4+ T cell depletion, probably shifting the balance both in proinflammatory and anti-inflammatory ways, with detrimental effects. In addition, TB disease fuels HIV progression. Many aspects of the immune response to TB have not been studied in the context of HIV, however.

Small non-coding RNA in HIV and TB

Small non-coding RNA (sncRNA), such as microRNA (miRNA) and small nucleolar RNA (snoRNA) are different classes of small RNA fragments that in different ways affect protein synthesis.

miRNAs are 18-22 bases long RNA fragments that bind to the 3' untranslated region of messenger RNAs (mRNAs), thereby preventing translation to encoded proteins. They have been shown to regulate many immune mechanisms, and to be involved in different disease processes, including in TB and HIV (88,89).

Mtb has been shown to be able to manipulate certain miRNAs to its own benefit, while other miRNAs appear to exert protective roles in the immune response against Mtb. Numerous studies have been conducted, both with *in vitro* models such as human macrophages, and animal models, to investigate the effect of different miRNAs on the immune response to Mtb or HIV. The roles of miRNAs in the immune defence are often complex: miR-155-5p, one of the most studied miRNAs in relation to immune responses, has been shown to increase mycobacterial survival upon infection, via inhibition of cytokines and cyclooxygenase-2 (90), but has protective effects in animal studies over longer time periods through impact on antigen presentation and development of cell-mediated immunity (91). Along the same lines, miR-155-5p has also been reported to be increased in PWH who do not respond to ART and to be linked to exhaustion and immune activation of T cells (92), however, it simultaneously suppresses HIV replication (93). miR-146a-5p, another key immune miRNA, appears to have a somewhat more straightforward role: by inhibiting the NFκB pathway it abrogates the cytokine response and bactericidal activity in Mtb infection (94) and attenuates antiviral response and fuels viral replication in HIV (95). Some of the most important miRNAs in the immune response against Mtb and HIV are listed in Tables 1 and 2, respectively.

Table 1. Examples of miRNAs and their different effects on immune responses to TB.				
	Direction of TB regulation	Target	Immune Effect	Effect on Mtb
miR-21-5p	Increased (96)	Il12p35 (97), Bcl2 (96,97), TLR4 (96,97)	Inhibited proinflammatory cytokines (96) , impaired T-cell responses (97)	Enhanced Mtb survival in macrophages (96)
miR-26a-5p	Decreased (98)	KLF4 (98)	Decreased iNOS, shift towards M2 polarisation, increased IL-10 (98)	The decrease impairs trafficking of Mtb to lysosomes (98)
miR-27a-3p	Increased (99)	Cacna2d3 (99)	Inhibited autophagy (99)	Increased Mtb load in animal model (99)
miR-27b-3p	Increased (100)	Bag2 (100)	Induced apoptosis, increased reactive oxygen species, decreased IL-1β, IL-6, TNF-α, iNOS (100)	Decreased Mtb survival in macrophages (100)
miR-29	Increased *in vivo* in most studies (101)	IFN-γ (102)	Reduced IFN-γ secretion (102)	Less efficient Mtb control animal model (102)
miR-144-3p	Increased (103)	DRAM2 (103)	Inhibited autophagy (103)	Less efficient Mtb suppression in vitro (103)
miR-146a-5p	Increased (94)	IRAK1,TRAF6 (94)	Attenuated cytokine response (94)	Impaired Mtb killing in macrophages(94)
miR-155-5p	Increased (90,91)	SHIP1, BACH1 (90,91)	Inhibition of COX-2 and IL-6 (Short term) (90) Stimulation of CD4+ T cells and IFN- γ (long term) (91)	Increased Mtb survival in macrophages (short term)(90), improved Mtb control (long term) (91)
miR-223-3p	Increased (104)	CXCL2, CCL3, IL-6 (104)	Prevents excessive neutrophil invasion (104)	Decreases Mtb bacterial load, improves survival of mice in animal model (104)

Table 2. Examples of miRNAs and their different effects in immune responses to HIV.				
	Direction of HIV regulation	Target	Immune effect	HIV effect
miR-27b-3p	Decreased (89)	Cyclin T1 (89)	Not clear (89)	Increased HIV replication (89)
miR-29b-3p and miR-33a-5p	Increased in elite controllers (105)	Not clear (105)	Not clear(105)	Inhibited HIV replication (105)
miR-34a-5p	Increased(106)	PNUTS (106)	Not clear (106)	Increased HIV replication (106)
miR-146a-5p	Increased (95)	TRAF, IRAK1 (95)	T-cell exhaustion, inhibition of TNF-α, IL-6, IL-8 (95)	Increased HIV replication (95)
miR-155-5p	Increased in ART non-responders (92)	TRIM32 (93)	T-cell exhaustion and immune activation (92)	Inhibited HIV replication (93)
miR-223-3p	Increased (107)	3' end of HIV mRNAs (108)	Not clear	Inhibited HIV replication (108)

Consequently, there are numerous miRNA pathways by which HIV and Mtb could be hypothesized to interact and accelerate disease progression reciprocally. However, whether these interactions actually occur in coinfection, and their impact on clinical presentation and outcome in vivo remains to be studied. While a role for miRNAs have been described in many immune mechanisms for these pathogens separately, there is currently limited data on how miRNAs are involved in HIV-TB coinfection. Although many studies have investigated how miRNAs are expressed in TB disease (101), there are few studies specifically investigating miRNA as biomarkers of TB in PWH (109).

The potential role of snoRNAs is even less explored in the context of HIV-TB coinfection. snoRNAs are 60-300 bases long, and their most well described function are 2' O-methylation and pseudouridylation of ribosomal RNA (rRNA). However, novel functions are emerging, such as alternative splicing of mRNA and functions more similar to miRNA, and several snoRNAs have no apparent target in rRNAs ("orphan" snoRNAs)(110). Their relevance in infectious disease immunology is beginning to be unravelled: SNORA31 has been shown to have an important role in the pathogenesis of herpes encephalitis (111) several viruses rely on snoRNAs for infectivity (110); and many different snoRNAs have been linked to macrophage polarization (112). They have so far mostly been studied in other fields of medicine, such as cancer and metabolism, but findings in those fields have revealed involvement in mechanisms that are important in the immune defence, such as apoptosis, cellular migration (113) and generation of reactive oxygen species (114). The potential roles of different snoRNAs in immune responses and host-pathogen interactions in HIV and TB are however largely unexplored.

Ribonucleases in HIV and TB

Many different proteins with ribonuclease activity have been described to be involved in immune responses. The members of the ribonuclease A family (which includes ribonucleases 1-13), are secreted extracellularly and exerts numerous immune functions. Ribonuclease 1 is increased in several inflammatory conditions (115) and has been shown to have a regulatory effect on IL-1β and IL-6 in animal studies (116). Ribonucleases 2 and 3 are both released in eosinophilic granules (115), a process linked to tissue repair and immune modulation (117). Ribonuclease 4 is contained in granules in monocytes and is secreted by T cells, suggesting immune relevance, but its exact effects are poorly understood(115). Ribonuclease 5 has been shown to inhibit neutrophil degranulation, suggesting an immunomodulatory role (118). Ribonuclease 6 and 7 both have antibacterial properties, and the latter also seems to have immunomodulatory functions (115) Besides this family, ribonuclease T2 is also secreted extracellularly, and induces chemotaxis of macrophages (119). Inside cells the monocyte chemotactic protein-induced proteins are important both for cleaving of viral RNA and by serving feedback functions on the immune response by cleaving proinflammatory mRNAs(120).

One study found increased expression of ribonuclease 2 (also known as eosinophil-derived neurotoxin), but not ribonuclease 3 (also known as eosinophil cationic protein), in people with TB disease compared to latently infected individuals (121). In addition, ribonucleases 3, 6 and 7 have been shown to have antimycobacterial effects *in vitro* (122,123).

Ribonucleases also seem to be involved in host control of HIV infection. Monocyte chemotactic protein-induced protein-1, with ribonuclease activity, has been shown to inhibit HIV in inactive CD4+ T cells, and to be downregulated in activated cells, making these more susceptible to HIV infection (124) Similarly, the absence of ribonucleases in Th17 cells has been suggested as a mechanism rendering them especially susceptible to HIV infection (125) Ribonuclease L, a key antiviral factor driven by type 1 interferons, that rapidly eliminate all RNA in the cytoplasm has been shown to be inhibited by HIV(126). In addition, members of the ribonuclease A family have been shown to inhibit HIV replication *in vitro* (127,128). Ribonucleases have also been suggested to contribute to viral latency through inhibiting replication, thereby preventing cure of HIV (129). However, the effect of these interactions between HIV and the host on the immune response to TB is not clear.

Host and microbiological biomarkers of TB in PWH

In 2015, the WHO defined priority areas for future TB diagnostics, declaring the need for new tests in different categories (130). Among these were a non-sputum triage test to be used at patients' first encounter with health care with a minimum 90% sensitivity and 70% specificity for identifying persons at risk of TB, and a diagnostic, non-sputum test with high specificity enabling direct TB diagnosis, with at least 80% sensitivity. PWH are mentioned as a prioritized target population for both of these potential tests. Although finding new markers and methods of TB identification has been an area of intensive research, only a minority of studies has focused on PWH. Promising strategies have emerged however, of which some also have shown adequate performance in cohorts including PWH.

The acute phase reactant C-reactive protein (CRP) is commonly used as an inflammation marker and has been shown to have superior performance for TB screening in PWH compared to the recommended symptom screening in large meta-analyses, and is now endorsed by the WHO for this purpose (131). However, there is heterogeneity between different cohorts in terms of optimal cut-off and since elevated CRP levels occurs in many conditions, including infections, malignancies and other inflammatory conditions, specificity varies greatly between different cohorts (132).

IL-6 has been found to be the strongest predictor of unfavourable outcomes across multiple cohorts (including PWH) among several cytokines analysed (133). Interferon-γ induced protein 10 (IP-10) has also been studied as a biomarker of TB disease in PWH. Wergeland *et al.* reported nearly perfect discriminatory potential in group of PWH, but it should be noted that their population of PWH in Norway was small (134). IP-10 was also included in a discriminatory panel for incident TB among severely immunosuppressed PWH (135). Furthermore, IP-10 was found to be the most discriminatory inflammatory marker in combination with another chemokine, CCL5, in people without HIV (136). Several other proteins involved in inflammation have been associated with TB (137,138), and studies suggest that the combinations of markers may increase the discriminatory capacity (136,138,139).

Studies from South Africa have suggested plasma kynurenine/tryptophan ratio (KT ratio) as a strong biomarker for identifying patients with TB disease among PWH (140–142) Some questions remain regarding the potential of KT ratio as a TB biomarker, however, since several participants in these studies were on antiretroviral therapy, which might affect the results. Furthermore, the performance of this marker in different forms of TB, e.g. paucibacillary pulmonary TB, has not been elucidated.

Flow cytometric analysis of the activation status of Mtb-specific CD4+ T cells has also been explored as a potential TB biomarker. In a cohort of patients with

suspected TB in Tanzania, this method showed 82% sensitivity and 93% specificity. Importantly, this performance was unaffected by HIV status (143).

Several researchers have investigated the host response through mRNA expression in whole blood or peripheral blood mononuclear cells, with construction of transcriptomic signatures for the purpose of TB case-finding. These studies have under some circumstances matched the prespecified criterion for a triage test for TB, and based on the signature "Sweeney-3" (144) the company Cepheid has developed a product for point-of-care testing (145). However, the total number of PWH in these studies was small (146), and a recent study of the new point-of-care prototype did not meet the desired performance for identifying both culture and Gene Xpert MTB/RIF positive TB cases, and for most comparisons it did not outperform CRP (145).

In addition to these markers based on the host response, new strategies for identification of pathogen-derived molecules in other specimens than sputum are being considered. Intriguingly, a study found detectable Mtb DNA using digital PCR in CD34-positive cells in peripheral blood in asymptomatic PWH in Ethiopia, suggesting that this might become a potential marker of TB infection (147). Attention has also been drawn to the possibility of identifying Mtb nucleic acids or volatile organic compounds in breath or aerosol samples as an appealing, non-invasive way of screening for TB. A review demonstrated consistently high performance, but few of these studies included PWH (148). As HIV leads to less cavitation, and fewer bacilli in sputum samples, one could speculate that breath samples might be less accurate for TB diagnosis in PWH. Several studies have found detectable cell-free DNA from Mtb in plasma in substantial numbers of individuals with TB. A review of the field found low pooled sensitivity of 48% but good specificity at 91% (149). Few participants in these studies had HIV infection, however, a recent study where a CRISPR-Cas fluorescence assay was used for detection cell-free DNA found promising results even in a cohort of the challenging population of children with HIV(150). How this method will perform in further validation studies, and how it can be used in clinical practice remains to be seen.

In summary, although many promising strategies exist, more research is needed of strategies for screening and diagnosis of TB, especially in PWH.

Aims

General aim

The overall aim of this thesis is to study how different types of immune mediators in blood of PWH reflect the host immune response to TB and how they perform as screening tests for identification of TB disease.

Specific aims

Paper I

To investigate how plasma markers of inflammation, alone and in combination, can be used to improve TB case-finding, in ART-naïve PWH.

Paper II

To determine the performance of the ratio between kynurenine and tryptophan in plasma for TB case-finding in ART-naïve PWH.

Paper III

To study how HIV alters the miRNA and snoRNA expression patterns in whole blood of people with TB disease, and how these alterations are affected by ART and ATT.

Paper IV

To explore plasma ribonuclease activity in PWH with and without TB, to study how this correlates to other immune markers and clinical parameters, and to identify host ribonuclease proteins that contribute to this phenomenon.

Materials, methods and study population

TB and HIV in Ethiopia

Ethiopia belongs to the high TB-burden countries in the world. It has had a positive trend during the recent decade, achieving the goal of the "End TB Strategy" of a 20% reduction of the incidence rate between 2015 and 2020 (23) There are approximately 610,000 PWH in Ethiopia, indicating a national average HIV prevalence of approximately 0.5% (151). However, the HIV burden is unevenly distributed across the country, with prevalence levels in urban settings several times higher than that of rural areas. A recent cross-sectional study of 1,834 women attending antenatal care in Adama, the city that the participants of these studies reside in, found a HIV prevalence of 9.3% (152). Like the situation in most countries in sub-Saharan Africa, the estimation by WHO of annual TB incidence in PWH is substantially higher than the case notifications of TB in PWH. Only two thirds of actual TB diagnoses are based on microbiological methods, illustrating the need for improved case-finding (23).

The main cohort

All individuals included in this thesis resided in and around the city of Adama, in the Oromia region, Central Ethiopia. Adama is located on the highway between Addis Abeba and Djibouti, which is an area with particularly high HIV prevalence in the country. Study participants were recruited as part of a prospective cohort study 2011-2013 at five public health centres (Adama HC, Geda HC, Dhera HC, Modjo HC and Wolenchiti HC). At these facilities, non-physician health care professionals with 3-4 years of training provide ART and HIV care.

The following criteria were applied for inclusion in the cohort: age ≥18 years, fulfilling the 2011 criteria for ART initiation (CD4 count <350 cells/mm^3 or WHO stage 4 disease) and residence in the uptake area of any of the five health centres.

Individuals who had received ART or were on treatment for TB for more than two weeks at the time of enrolment, were excluded. At inclusion, all participants were

interviewed following a study questionnaire, and physically examined, by the health centre staff. Data such as mid-upper arm circumference (MUAC) and body mass index (BMI) were collected. Blood was collected for CD4 count and other cell counts. Plasma was separated and stored in aliquots at -80ºC for analysis of HIV VL and subsequently shipped to Lund University, Sweden, for further investigation of different biomarkers. Two spontaneously expectorated morning sputum samples were collected for bacteriological analyses for TB at recruitment, and at any time later during follow-up in case of clinically suspected TB. In cases of peripheral lymphadenopathy, fine needle aspiration was performed, with material sent for TB investigation with Gene Xpert MTB/RIF and culture.

812 treatment-naïve PWH were enrolled and provided sputum samples, of whom 137 (16.9%) were found to have TB by bacteriological confirmation (positive direct microscopy, Gene Xpert MTB/RIF and/or culture). An additional 21 (2.6%) did not test positive for TB but met Ethiopian criteria of clinically diagnosed TB and were prescribed TB treatment according to national guidelines. The latter group was not included in any of the studies in this thesis, rendering 791 participants relevant for these studies. Individuals who were positive for TB with any bacteriological method were classified as HIV+/TB+ and the remainder as HIV+/TB-. For inclusion in any of the studies in this thesis, HIV+/TB- participants were also required to remain in care for 6 months after inclusion, without incident TB.

For paper I, all 130 HIV+/TB+ persons with available plasma at Lund University were included, with the addition of 130 HIV+/TB- individuals, matched on age and gender, in a nested case-control design. For paper II, all participants included in the first study with remaining plasma were included, resulting in 124 HIV+/TB+ and 125 HIV+/TB- individuals.

For paper III, two groups of participants outside this cohort were used for comparison. Alongside 13 randomly selected PWH with TB from the aforementioned cohort, a group of 14 adults who were HIV-negative and had negative Quantiferon-Gold results (Mtb-stimulated interferon-γ <0.35 IU/ml), and had no chronic diseases, symptoms of TB or other health complaints (HIV-/TB-) was included. In addition, we included 13 adults recruited at Adama and Geda HC who were diagnosed with pulmonary TB but tested negative for HIV (HIV-/TB+).

For paper IV, all individuals from the main cohort (51 HIV+/TB+, 78 HIV+/TB-) with remaining plasma samples were included.

Laboratory procedures

Initial laboratory analyses determination were performed at the Adama Regional Laboratory. To classify patients with regard to TB, two sputum samples (and, as

mentioned in some cases fine needle aspirates) were investigated with Ziehl-Neelsen stained direct microscopy, Gene Xpert MTB/RIF (Cepheid) and liquid culture using BACTEC MGIT 960 (BD Diagnostics). Whole blood collected at inclusion was analysed for CD4 count and complete blood count using flow cytometry. Plasma HIV VL was determined on stored plasma aliquots in batches.

For paper I, nine plasma proteins: CCL5, CRP, IP-10, IL-6, IL-12p70, IL-18 and IL-27, procalcitonin [PCT], and soluble urokinase plasminogen activator receptor [suPAR] were quantified using Magnetic Luminex technology (R&D Systems Inc.)

For paper II, plasma kynurenine and tryptophan were quantified using liquid chromatography-mass spectrometry, on Nexera systems (Shimadzu), at RedGlead Discovery, Lund, Sweden.

For paper III, RNA from whole blood was isolated with the PAXgene Blood miRNA kit (Qiagen). Microarray analysis was performed for 23 samples with the Affymetrix Platform, GeneChip miRNA 4.0 Array at Swegene Centre for Integrative Biology (SCIBLU), Lund University. This analysis yielded data on mature miRNAs, miRNA precursors and snoRNAs. Some miRNAs and snoRNAs of interest were then further analysed quantitative polymerase chain reaction (qPCR) in a total of 57 samples.

For paper IV, RNA was isolated from 27 plasma samples using the miRNEasy serum/plasma advanced kit (Qiagen) according to the manufacturer's instructions. The Qiaseq miRNA QC spike-in kit (Qiagen) was included and Illumina Next-Generation Sequencing was performed at Qiagen. Subsequently, RNA from 125 samples was isolated using the same isolation kit as above, this time with the RNA Spike-in kit, for RT, according to the manufacturer's instructions. The RNA was reversely transcribed, and qPCR was performed based on SYBR Green technology (Thermo Fisher Scientific) with DNA primers designed in the software miRprimer (153) .

A subset of 78 samples were then analysed using the RNAse Alert assay (Integrated DNA technologies Inc.). Ribonucleases 3, 6 and 7 and were quantified with enzyme-linked immunosorbent assay (ELISA) kits from Cloud-Clone Corp, while ribonucleases 1, 2 and T2 were analysed with an ELISA kit from MyBioSource.

Statistical analyses

For paper I, statistical tests were performed in SPSS v. 24.0. For the remaining papers, R versions 3.5.3-4.2.3, were used. Comparisons across multiple groups were adjusted according to the Holm-Bonferroni method.

For paper I and II, differences in biomarker levels with regard to TB status were assessed with the Mann Whitney U test and receiver operating characteristics (ROC) curves. Their correlation to each other and other continuous parameters was tested using Spearman's rank correlation. To assess combinations of markers, logistic multivariate regression with stepwise removal was performed, and the final model was then assessed using ROC curves.

For paper III, microarray data was first normalized using robust multi-array normalization. Data was then log2 transformed. Probes with non-significant detection above background in more than 20% of the samples were removed from further analysis. Differential expression was assessed using the R package *limma* (154). Further explorations on expression patterns involved principal component analysis (with subsequent Hotelling's T test to assess differences between groups) and unsupervised hierarchical clustering. Subsequent qPCR data was normalized against a synthetic spike-in and differences across groups of interest were explored using the Mann-Whitney U test and Wilcoxon signed rank test, and correlations to continuous parameters was performed using Spearman's rank correlation.

For project IV, reads were mapped to miRBase v.22, and reads that could not be mapped to miRBase neither with a perfect match nor as isomiRs (maximum 2 mismatches and/or alternative starting or ending point by 2nt) were aligned to the human genome ENSEMBL GRCh38 v.97. The sequencing data was then analysed according to the *edgeR* (155) workflow. The filtering algorithm filterByExpr (requiring 10 counts in at least 70% of samples per group) determined 499 of 2632 miRNAs in miRBase to be sufficiently expressed to motivate further analysis. Data was normalized using the CalcNormFactors function. Differential expression was assessed with the exact test, and P values were adjusted according to the Benjamini-Hochberg method.

Differences in sequencing spike-ins, qPCR spike ins cycle threshold (CT) values, RNAse Alert fluorescence and ribonuclease levels were compared between HIV+TB+ and HIV+/TB- individuals using the Mann Whitney U test. Correlation between ribonucleases and continuous parameters such as VL, CD4 count, MUAC and BMI and the inflammation markers from papers II and III were assessed using Spearman's Rank Correlation.

Ethical considerations

Ethical approval was granted by the National Research Ethics Review Committee at the Ministry of Science and Technology of Ethiopia (3.10/825/05) and the Regional Ethical Review Board at Lund University, Sweden (2010/672) and (2011/558). Informed consent was obtained from all participants.

Results

Paper I

Of the nine plasma markers analysed, all were significantly increased in HIV+/TB+ participants (P<0.05), with the exception of IL12-p70 (Figure 4).

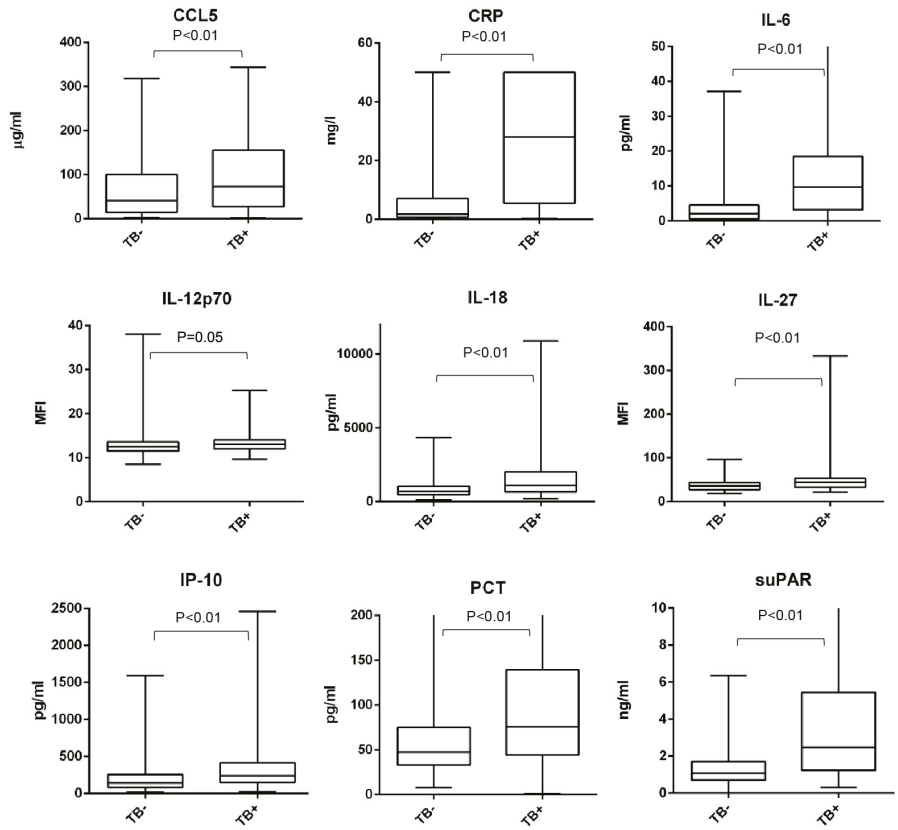

Figure 4. Boxplot of levels of the nine markers of inflammation in paper I. Boxes represent median and interquartile range. Whiskers have been cut in some cases for visual presentation. All markers except IL-12p70 showed significant differences between HIV+/TB+ and HIV+/TB- after Holm-Bonferroni correction for multiple testing. For IL-12p70 and IL-27 median flourescence intensity values are shown instead of concentrations due to unreliable conversion to concentration.

The largest magnitude of difference was seen for CRP, suPAR, IL-6 and IL-18. These generated area under the curve (AUC) values of 0.80 (95% CI: 0.75-0.86), 0.77 (95% CI: 0.71-0.83), 0.76 (95% CI 0.71-0.82) and 0.71 (95% CI 0.65-0.78)., respectively (Figure 5). It was notable, however, that a substantial proportion (24%) of the HIV+/TB+ individuals had CRP levels below the previously suggested cut-off at 5 mg/L. CRP was particularly low among asymptomatic patients (median: 1.0 mg/L), and among those with negative Gene Xpert MTB/RIF and direct microscopy (median 9.1 mg/L). In multivariate analysis, CRP and suPAR remained significantly associated with TB disease after stepwise removal, and this combination increased the AUC to 0.83 (95% CI: 0.78-0.88) in the whole patient material and to 0.93 (95% CI: 0.89-0.97) among individuals with CD4 count <200 cells/mm^3. IL-18 and IP-10 also correlated to CD4 count, irrespective of TB status. The strongest intercorrelation among the markers was found between CRP and IL-7 (ρ=0.7).

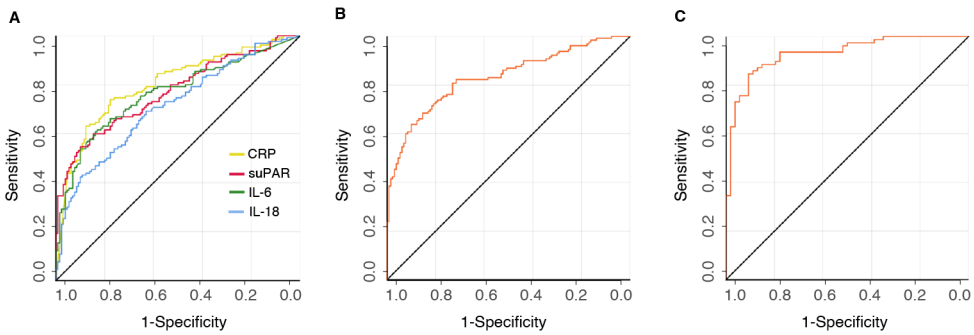

Figure 5. A) ROC curve for CRP (AUC: 0.80, 95% CI: 0.75-0.86) , suPAR (AUC: 0.77, 95% CI: 0.71-0.83), IL-6 (AUC: 0.76, 95% CI: 0.71-0.82) and IL-18 (AUC:0.71, 95% CI: 0.65-0.78) B) ROC curve for the combination of CRP and suPAR in all participants (AUC: 0.83, 95% CI: 0.78-0.88) C) ROC curve for the combination of CRP and suPAR in participants with CD4 count <200 cells/mm^3 (n=132, AUC: 0.93, 95% CI 0.89-0.97)

Paper II

The second paper focused on the ratio between tryptophan and its metabolite kynurenine, previously described to be a strong biomarker of TB in PWH (140–142). We found an increased ratio among HIV+/TB+ participants compared to HIV+/TB-, but to a lesser magnitude than previously reported (Figure 6). The increase in KT ratio was entirely due to tryptophan depletion, while kynurenine levels were similar in HIV+/TB+ and HIV+/TB-.

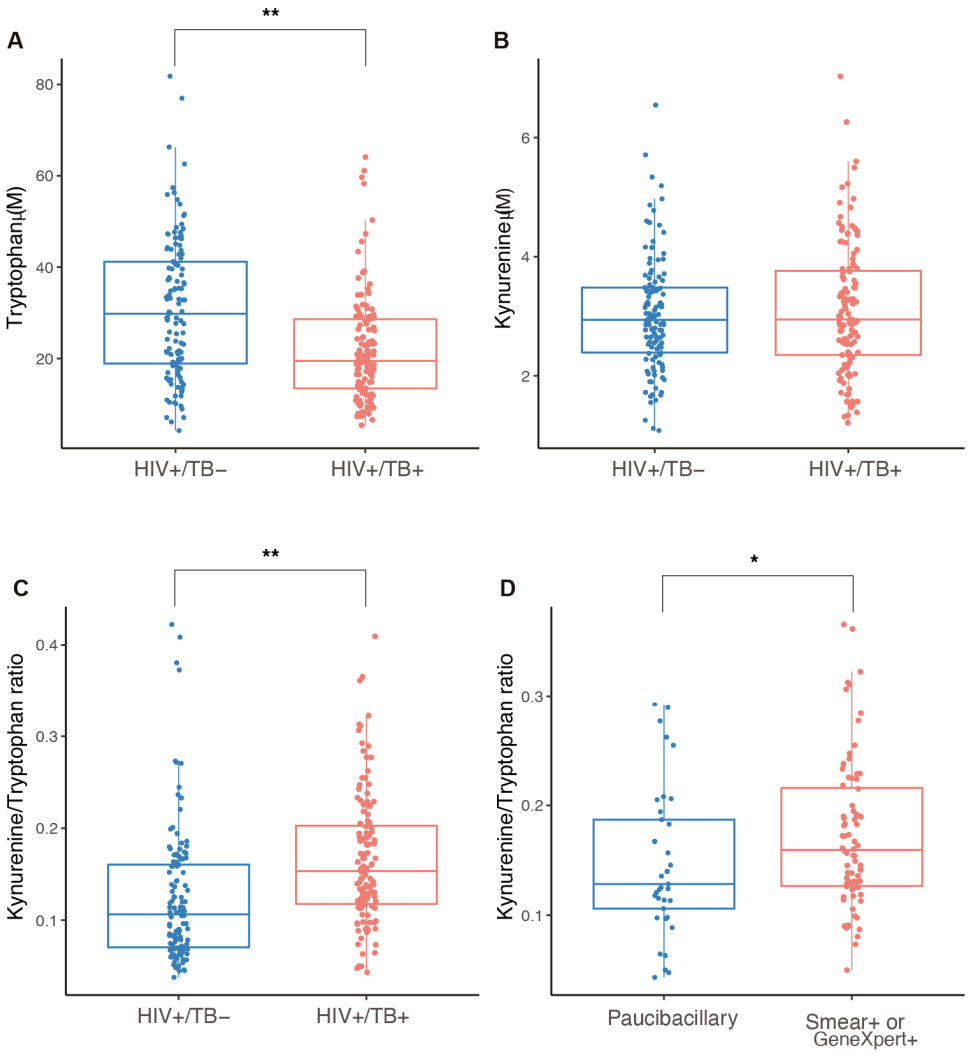

Figure 6. A) Tryptophan levels B) Kynurenine levels C) Kynurenine/tryptophan ratio in HIV+/TB+ and HIV+/TB- participants and D) Kynurenine/tryptophan ratio in HIV+/TB+ individuals with only positive culture or positive direct microscopy/GeneXpert. ** indicating P<0.01 from the Mann Whitney U test, * indicating P<0.05

The AUC value from ROC analysis was 0.70 (95% CI: 0.64-0.77) for KT ratio and 0.67 (95% CI: 0.61-0.74) for tryptophan (Figure 7). Compared to previous studies, the lower performance in this study was due to higher KT ratios in HIV+/TB- participants, leading to low specificity. KT ratio was correlated to HIV VL, and inversely correlated to CD4 count. Furthermore, it correlated to other markers of the interferon-γ axis: IL-18, IL-27 and IP-10. Combining KT ratio with the markers studied in paper II did not improve performance.

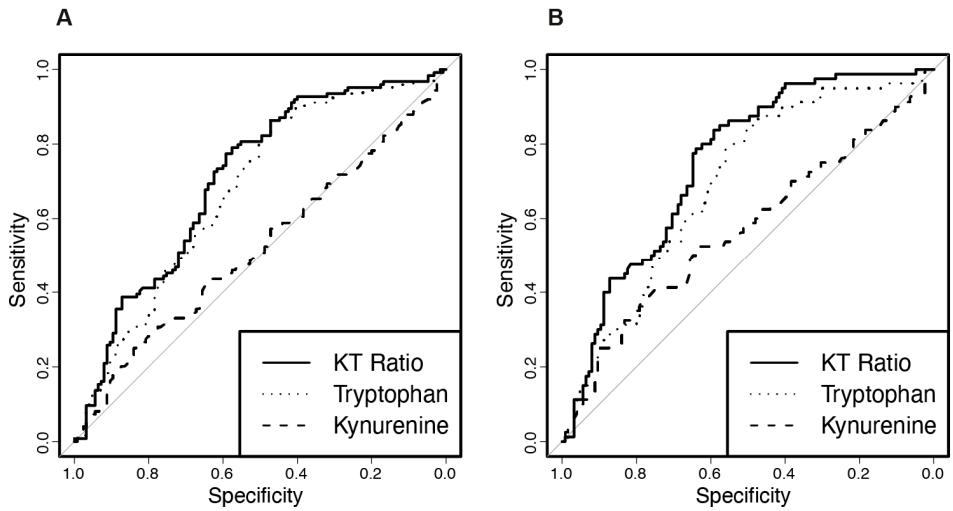

Figure 7. ROC curves for A) all participants. AUC for KT ratio: 0.70 (95% CI: 0.64-0.77), for tryptophan 0.67 (95% CI: 0.61-0.74 and for kynurenine 0.52 (95% CI: 0.45-0.59). B) Gene Xpert MTB/RIF and/or sputum spear positive participants (compared to HIV+/TB- participants) AUC for KT ratio 0.75 (95% CI: 0.68-0.81), for tryptophan 0.70 (95% CI: 0.62-0.76) and for kynurenine 0.56 (95% CI 0.48-0.65).

Paper III

In the initial microarray analysis of 23 individuals, seven individuals with HIV and TB disease (HIV+/TB+), eight HIV-negative individuals with pulmonary TB (HIV-/TB+) and eight HIV-negative healthy persons without TB infection (HIV-/TB-), 218 miRNAs differed between the groups with regard to HIV status, while people with TB mainly had decreased snoRNA levels compared to HIV-/TB- participants (Figure 8).

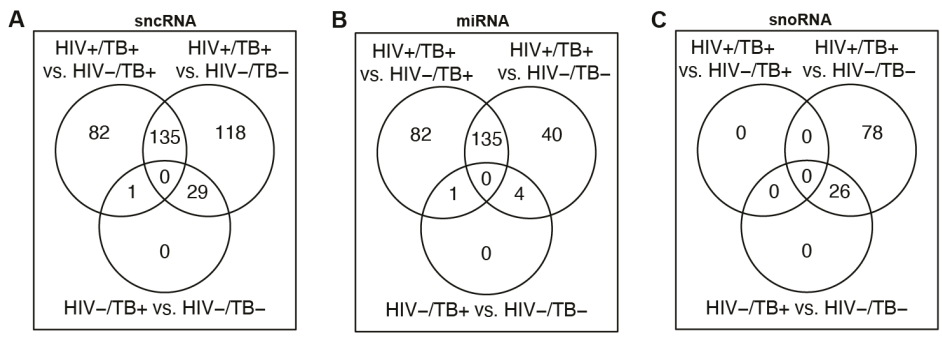

Figure 8 Venn diagrams of differentially expressed sncRNAs (FDR<0.05) A) total sncRNAs B) miRNAs C) snoRNAs between the different groups.

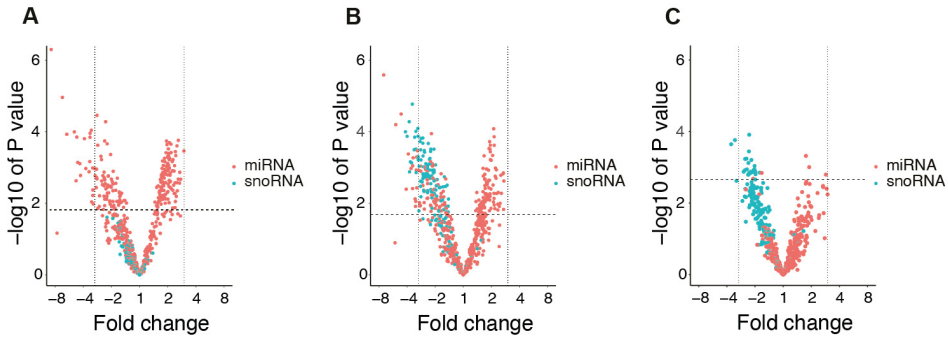

Figure 9.Volcano plot from the different comparisons A) HIV+/TB+ vs HIV-/TB+ B) HIV+/TB+ vs HIV-/TB- and C) HIV-/TB+ vs HIV-/TB- where each dot indicates a miRNA (red) or snoRNA (blue). The x axis indicates real fold change but plotted on a logarithmic axis, the y axis indicates the logaritmized P-value. The horizontal line indicates FDR <0.05 and the vertical lines indicates fold change 3 and -3, respectively.

More miRNAs were upregulated (n=122) than downregulated in HIV+/TB+ in comparison to HIV-/TB+ (n=96), however, it was evident that the downregulated miRNAs showed larger magnitude of difference (Figure 9), and a larger proportion (82% vs. 13%) were miRNAs with high annotation confidence in the miRNA database miRbase(156), most likely indicating a higher rate of true miRNAs. In hierarchical clustering analysis using the 20 most differentially expressed sncRNAs, the participants clustered according to their infection status, with one exception. In principal component plots, the separation was clearer between participants with HIV and the other groups with regard to miRNA, and between participants with and without TB, with regard to snoRNA (Figure 10). This was supported by Hotelling's T-test, where there was no significant difference between HIV-/TB+ and HIV-/TB- in miRNA expression and no difference between HIV+/TB+ and HIV-/TB+ in snoRNA expression.

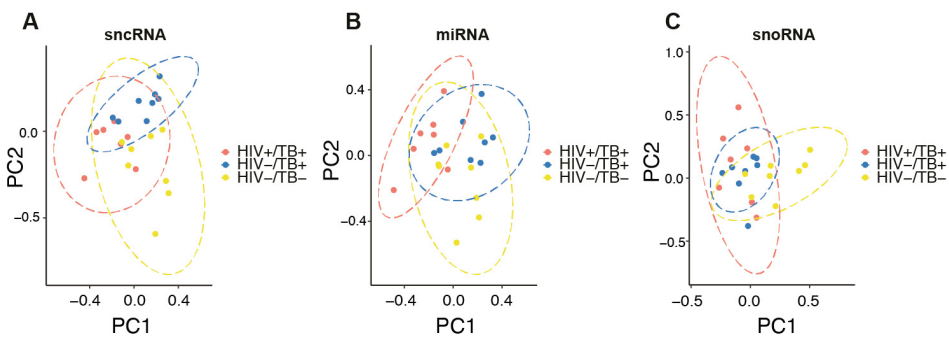

Figure 10.Principial component plots with all the participants included in the microarray, HIV+/TB+ (red), HIV-/TB+ (blue) and HIV-/TB- (yellow). Ellipses based on t-statistic, with 95% CI.

Four sncRNAs were then chosen for validation with qPCR due to differential expression in the microarray: miR-27b-3p, miR-139-3p, miR-199a-5p and the snoRNA U46. Expanding the patient material to 40 individuals, we found that the RNAs investigated were all differentially expressed in the same direction as in the microarray ($P<0.05$) (Figure 11), and the agreement between the two methods was strong (ρ 0.82-0.94).

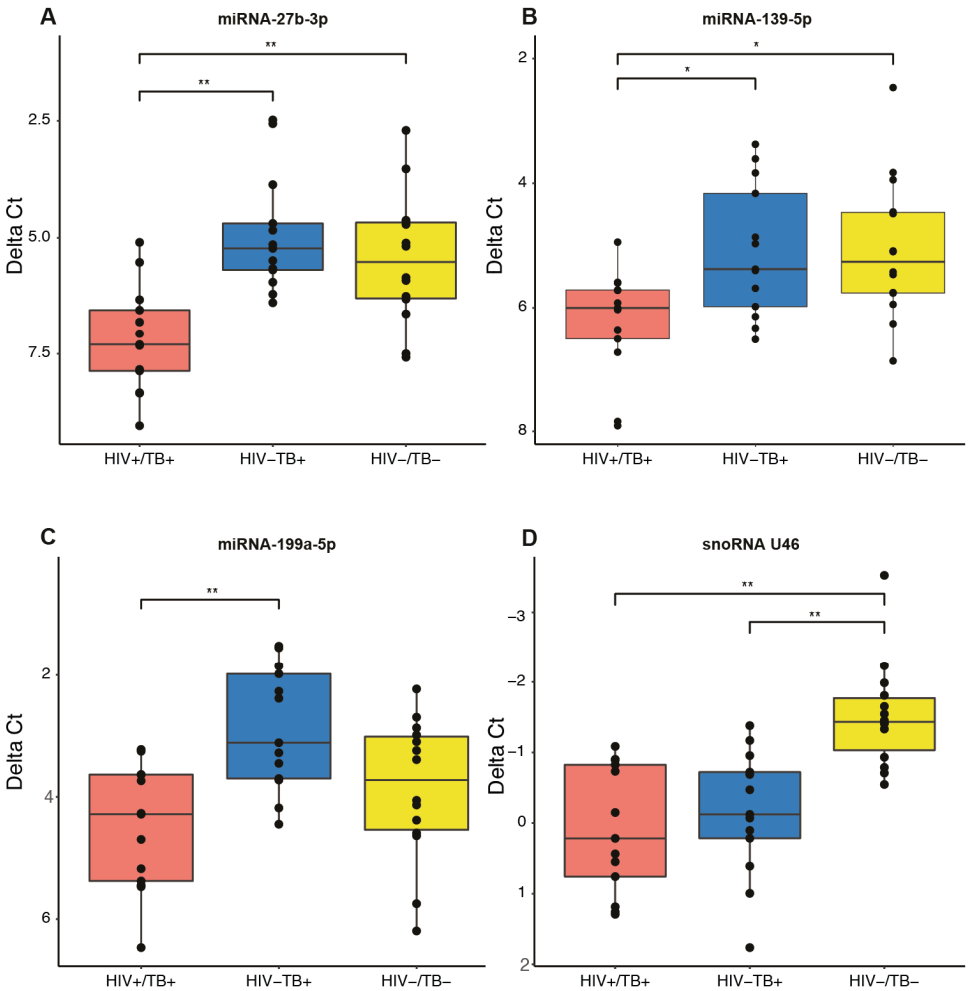

Figure 11. Levels of the four sncRNA that were validated with qPCR. Boxes indicate median and interquartile range. * indicates Holm-Bonferroni adjusted $P<0.05$ and ** Holm-Bonferroni adjusted $P<0.01$

Follow-up samples (after initiation of ATT and ART) were available for nine of the HIV+/TB+ participants, and miR-139-3p and snoRNAU46 were significantly higher compared to pre-treatment samples, approaching the levels of the uninfected individuals after approximately 6 months of treatment for TB and HIV. No clear differences were seen at follow-up for HIV-/TB+ participants, probably reflecting that the chosen snc RNA were less affected at baseline by TB disease without HIV.

Paper IV

In the fourth paper, the sequencing data revealed 7 miRNAs (miR-4435, hsa-miR-3182, miR-205-5p, miR-2116-3p, miR-181c-5p, hsa-miR-574-3p, miR-1307-5p) to be significantly differentially expressed between HIV+/TB+ and HIV+/TB- individuals (FDR <0.05). Of these, miR-205-5p and miR-181c-5p were confirmed to by qPCR to be significantly downregulated in HIV+/TB+ ($P<0.01$), but not to a degree suggesting that they would be useful as biomarkers for TB. Unexpectedly, when analysing both sequencing and qPCR data, there was evidently more degradation of the synthetic spike in RNAs in samples from HIV+/TB+ individuals. This finding, in combination with limited alteration of miRNAs in this material, led to a shift of focus in our studies to ribonuclease activity and its relation to TB and HIV.

Degradation of spike-in RNAs (i.e. lower sequencing read counts or higher qPCR CT values) were considered proxies of ribonuclease activity. There were significantly fewer ($P<0.05$, figure 12a) reads of all but one of the 52 sequencing spike-ins and they were all significantly intercorrelated (median Spearman's $\rho=0.92$). Similarly, the two spike-ins added to the qPCR reaction were significantly decreased (with higher CT values) in HIV+/TB+ participants, compared to HIV+/TB- (Figure 12b). They correlated strongly to each other ($\rho=0.97$, $P<0.01$). To confirm that the lower levels reflected increased ribonuclease activity in samples from HIV+/TB+ participants, the RNAse Alert assay was performed and confirmed higher ribonuclease activity in samples from individuals with TB ($P<0.01$, Figure 12c). This assay correlated to qPCR spike-in degradation ($\rho=0.40$, $P<0.01$, Figure 12d). RNAse Alert ribonuclease activity was also correlated to HIV VL ($\rho=0.24$, $P=0.03$), IL-27 ($\rho=0.46$, $P<0.01$), KT ratio ($\rho=0.42$, $P<0.01$), PCT ($\rho=0.56$, $P<0.01$, and inversely correlated to BMI ($\rho=-0.34$, $P<0.01$), CD4 count ($\rho=-0.39$, $P<0.01$) and MUAC ($\rho=-0.46$, $P<0.01$).

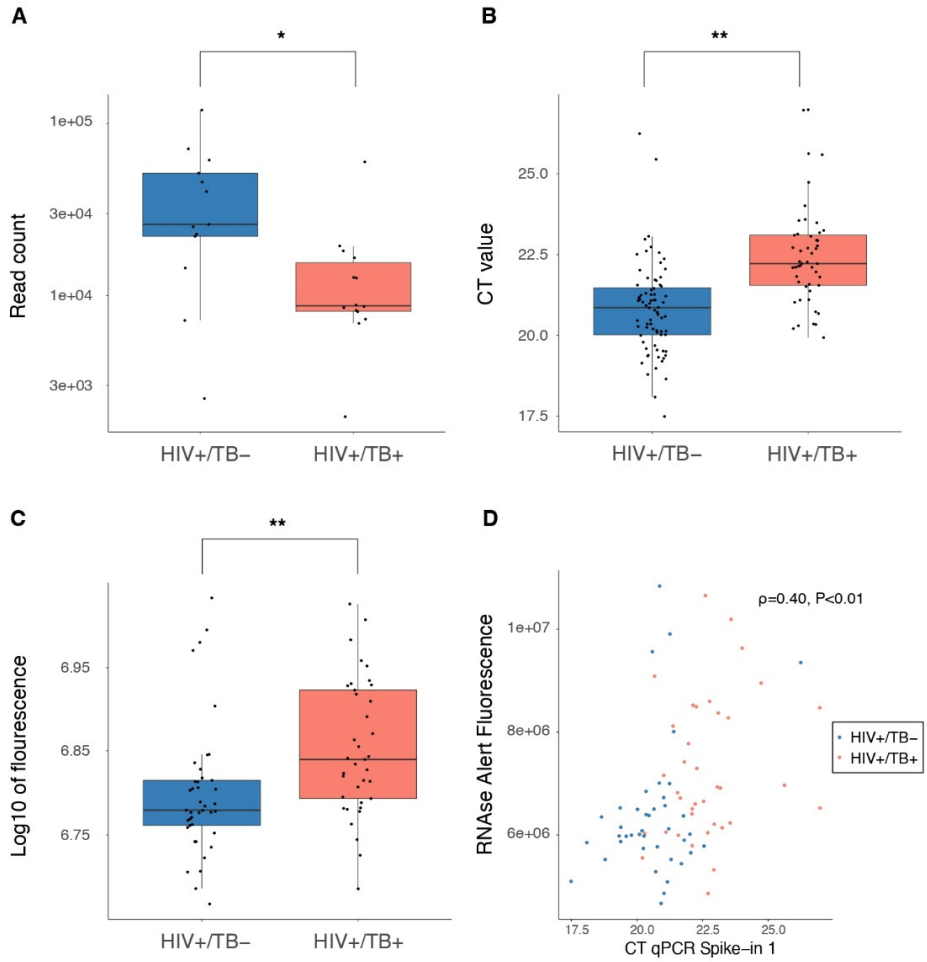

Figure 12. Ribonuclease activity in plasma of HIV+/TB+ and HIV+/TB- participants assessed by different methods. A) Read count of Sequencing Spike-in, B) CT values for qPCR Spike-in, where a higher CT value indicates later detection of the RNA, i.e. increased ribonuclease activity, and C) Log10 of the fluorescence in the RNAse Alert assay, where a higher fluorescence indicates more ribonuclease activity. * indicating P<0.05, ** indicating P<0.01, from the Mann Whitney U test. D) Correlation plot between RNAse Alert Fluorescence and the CT value for qPCR Spike-in. HIV+/TB- samples indicated by blue dots and HIV+/TB+ by red dots. Spearman's ρ and P value given in top right corner.

Further experiments investigating concentrations of individual ribonucleases in plasma confirmed higher levels of ribonuclease 2, 3 and T2, but no differences in ribonucleases 1, 6 and 7. Moreover, ribonuclease 2, 3 and ribonuclease T2 were correlated to RNAse Alert ribonuclease activity (ρ=0.42, ρ=0.28 and ρ=0.49, P<0.05). The strongest discriminatory capacity of these ribonuclease indicators was that of qPCR spike-in degradation (AUC: 0.81).

Discussion

Host biomarkers for TB

In this thesis, several potential biomarkers of TB in PWH were explored. The strength of these studies compared to many other biomarker studies is that all patients were recruited at the same facilities, and that all participants were investigated with bacteriological methods for pulmonary TB, with follow-up of participants for >6 months after enrolment, thus reducing the risk of misclassification among controls for TB disease. Unlike the majority of TB biomarker research, we have solely focused on PWH, a key population in prevention of TB mortality and morbidity.

Several other groups have explored different host biomarkers of TB. In many cases, promising findings have not been externally validated. Host markers for TB will in most cases be non-specific, making the clinical context important (157). We here describe promising discriminatory potential of a combination of CRP and suPAR, especially in the most immunosuppressed PWH, but this combination will not be indicative of TB in many other scenarios, as CRP and suPAR are unspecific markers of inflammatory activity(158,159). Similarly, KT ratio, previously reported to be highly discriminatory of TB, was here relatively high also in untreated PWH, as it is in conditions such as sepsis and cancer (160), further emphasizing that if inflammatory markers are to be employed in clinical practice, it is important to consider the context, and impact of inflammatory conditions other than TB disease.

Nonetheless, in our study the combination of CRP and suPAR achieved a high AUC of 0.93 in individuals with CD4 count <200 cells/mm^3, and both these markers are available of point-of-care test. The variation in levels of these markers between cohorts is however a concern if these markers are to be employed.

A major issue with implementing CRP as a screening test for TB is the varying cut-offs in different studies. Yoon *et al.* found a sensitivity of 89% of 10 mg/L (161), while this only generated a sensitivity of 78% in the study by Shapiro *et al*., who suggested 5 mg/L as the optimal cut-off (162). Our patient material had even lower levels of CRP, making the utility of CRP as a TB biomarker among PWH questionable. Studies of other patient materials report equal or even lower levels of CRP in HIV+/TB+ individuals (142,163). It is not clear why these variations are so large, but it may reflect other underlying inflammatory conditions, or that the degree

of discomfort that prompts people to enrol in HIV care differ between different settings. The fact that men had significantly higher levels of CRP may support the latter hypothesis. To further add to the issue of inter-cohort variation, a recent study of PWH in Zambia, with a cut-off of 10 mg/L found good sensitivity but a specificity of just 27.5%, thus ruling out very few from further investigation if this was used as a triage test (164).

The chemokine CCL5 exhibited a special pattern, somewhat different to all the other markers. Albeit not significantly, it was expressed at higher levels in HIV+/TB+ individuals with no symptoms, and in those who had paucibacillary pulmonary TB (positive liquid culture but negative direct microscopy and Gene Xpert MTB/RIF results). In line with the tendency of CCL5 to identify another group of PWH with TB compared to the other markers, it was the only marker that came close to adding significant value to the suPAR and CRP combination (P=0.06). This is similar to another study of host biomarkers of TB in people without HIV, where IP-10 was the strongest marker, and CCL5, while not being strongly discriminatory on its own, was the marker that improved the performance of IP-10 most when combinations were tested (136). A similar phenomenon was seen with CCL5 in another study of host biomarkers (165). This may suggest that CCL5 still have some benefit in these sorts of panels. In addition, the fact that it was elevated in TB but less so in patients with more severe disease, could suggest that it has a protective role. In support of this theory, deletion of CCL5 in an animal model impaired the T-cell-mediated response to TB (166) and low CCL5 levels have been shown to predict poor response to TB treatment (167). The fact that it is a natural antagonist of HIV (168) might make it particularly important in HIV-TB coinfection.

The performance of KT ratio in our study population was inferior to that previously described from South Africa. The sensitivity of the previously suggested cut-offs was still good, but since our HIV+/TB- participants also had high KT ratio levels, the specificity was poor. We hypothesize that the increased levels in our material reflect that all patients were ART-naïve, while substantial numbers in previous studies already were on ART (140–142). Consequently, KT ratio appears to be unsuitable as a TB screening marker in ART-naïve individuals, while it may have utility in people who are on ART. This remains to be externally validated, however.

Do sncRNA play a role in the interaction between HIV and TB?

The potential mechanistic role of miRNAs and snoRNAs in the immune interaction between HIV and TB is unknown. Our findings in paper III imply that this should be further explored. Several of the miRNAs that showed altered expression in PWH

in the third paper of this thesis, have been shown to have immunological functions, including responses to TB and HIV.

Depletion of miR-26a-5p decreases nitric oxide synthesis and impairs trafficking of Mtb to lysosomes (98). Similarly, miR-23a-3p is important for generation of reactive oxygen species and phagocytosis (169) and downregulation of miR-27b-3p leads to increased survival of Mtb in macrophages (100). In addition, miR-27b-3p has been shown to reduce HIV replication (89) and is higher in HIV elite controllers than (170).

On the other hand, the change seen in many miRNAs studied here would most likely have proinflammatory effects: miR-26b-5p, which was downregulated in HIV+/TB+, has an inhibitory role in the proinflammatory NF-κB (171) pathway, and miR-27b-3p has been linked to activation status of CD4+ T cells (100). miR-223-3p was significantly downregulated in HIV+/TB+ individuals, and apart from being a known HIV-suppressive factor (108), it has been shown to negatively regulate neutrophil chemotaxis, but also to prevent lung injury and reducing Mtb bacterial load (104). Given that HIV can shift the Mtb defence both in an immunosuppressive and a proinflammatory way (4), it is possible that dysregulated miRNA can have both pro- and anti-inflammatory effects in HIV-TB-coinfection.

Interestingly, the snoRNAs U32A, U33, U34 and U35A, have all been shown to be induced in cultures of macrophages and *in vivo* in mice and humans after lipopolysaccharide installation (172). A similar finding has been made in lipopolysaccharide stimulated dendritic cells for ACA24 and U15B (173), two snoRNAs that were also differentially expressed in our study participants. This response to immune stimuli consequently seems to be hampered by TB. Cells have been shown to secrete U38A and HBII-202, among many other snoRNAs, in response to IFN-α, suggesting that the reduced levels, as observed in our study, also might play a pathogenic role (174). Whether the alterations seen here reflect downregulation of TB-directed host control or are a protective immune effect against TB or Mtb virulence factors is not possible to elucidate from these data. The quite drastic downregulation in TB, irrespective of HIV coinfection, and the reversal of this phenomenon with ATT, suggest an important function of snoRNA in the immune response to TB. As several snoRNAs are “orphan” (do not have known targets within rRNAs), it is likely that these exert roles beyond rRNA modifications, which should be further explored. It could be hypothesized from the findings of snoRNA depression and ribonuclease increase is that the snoRNA depression is due to degradation from ribonucleases. Indeed, several proteins in the nucleolus have been described to have ribonuclease activity (175,176). It should be noted, however, that the experiments in paper III used another method for ribonuclease inactivation, and no degradation of synthetic spike-ins were seen. Consequently, the degradation of snoRNA in the HIV+/TB+ whole blood samples does not appear to reflect post-sampling ribonuclease degradation but a true biological phenomenon.

Do ribonucleases play a role in in HIV-TB coinfection?

We found markedly increased ribonuclease activity in PWH with TB compared to participants in whom TB disease had been excluded, a finding that has not been described before. Our different measures of ribonuclease activity were all consistent in identifying a TB-related increase, but their intercorrelation was weak, suggesting that different unidentified mechanisms are also involved. It is likely that additional host proteins with ribonuclease activity contribute to this phenomenon. In addition, Mtb encodes a number of ribonucleases as well, and while these enzymes hitherto only have been described to exert roles in bacterial RNA turnover (177), it is a tempting speculating that these could also be secreted and function as bacterial virulence factors by hampering host protein expression. It has been discovered that Mtb virulence factors can be detected also in peripheral blood from large proportions of PWH (178).

The three ribonucleases that we found to be significantly altered with regard to TB all have described immune functions that may be relevant in TB control. Ribonuclease 2 (also known as eosinophile-derived neurotoxin) is a chemoattractant for dendritic cells (179) and may consequently play a role in antigen presentation of Mtb. Ribonuclease 3 (also known as eosinophil cationic protein) can inhibit growth of mycobacteria, both through direct bactericidal mechanisms and through induction of autophagy (122,123). Ribonuclease T2 has been shown to activate TLR8 (180) which has been suggested to play a role in the immune response to TB (181).

There are limited published data on the protein levels or overall blood ribonuclease activity in TB, but a review of public databases of mRNA expression (182,183) shows cells infected with Mtb or stimulated by Mtb antigens manifest downregulation of the ribonuclease A family IDs: E-MTAB-8162, E-MTAB-7679 (184) and E-MEXP-3521. On the contrary, in circulating peripheral blood mononuclear cells or whole blood collected from individuals with TB disease, there is upregulation of most ribonuclease A family members, experiment IDs: E-GEOD-54992 (185) , GSE112104 (186), GSE19491 (187) GSE34608 (188). In data sets of PWH with moderate immunosuppression, the pattern is less clear and there is modest upregulation of a few ribonuclease A family members, experiment IDs: GSE69581 (189) and GSE37250 (190) Intriguingly, in a study of severely immunosuppressed PWH with TB the pattern was reversed, and TB was associated with depressed levels of ribonucleases 2,3 and 6, experiment ID: GSE107104 (191). It could from these data be hypothesized that the ribonuclease A- host response is lost with advanced HIV-related immunosuppression.

Future perspectives

Host biomarkers of TB

Many studies have investigated different classes of host markers in large combinations and impressive cohorts, but although CRP has shown consistent adequate performance for TB screening and has been endorsed by WHO as a rule out test for TB in PWH, most markers and panels have shown considerable inconsistency across different cohorts to be appealing for clinical use. As explained above, this also concerns CRP, due to poor specificity and uncertainty regarding optimal cutoff levels for clinical use.

One aspect that has not been fully explored is the use of combination of different classes of markers to improve performance. To study different types of markers (mRNAs, miRNAs, snoRNAs, proteins, metabolites, antibodies) in combination might add more than large panels of the same types of markers. This would however be more technically challenging both for researchers and for clinical application, especially in resource-limited settings, where most people in need of such tests live and receive care. For instance, for HIV, highly reliable tests are now widely available, based on combination of antibodies and antigens (192). One "multi-omics" study found improved performance of combining a metabolite and a miRNA for identifying TB in severely immunosuppressed PWH. However, this study tested a large number of markers in a small number of patients, making the external validity of these findings uncertain (109). In addition, the now emerging body of microbiological biomarker research opens up for combining these microbiological biomarkers with host biomarkers.

Activation states of Mtb-specific cells in flow cytometry, or large-scale mRNA signatures, might well be, if not diagnostic tests, important pieces of the diagnostic build up in complicated TB investigations, but would only be possible to use in high-income health care settings. Where the majority of the TB efforts needs to be focused, however, it seems highly unrealistic that these sorts of methods could be implemented.

We have not systematically investigated the study participants for other opportunistic infections. A study of cryptococcal screening in PWH in the same area and time period found few cases of cryptococcal antigenemia, however (193). The majority of the study participants were taking co-trimoxazole prophylaxis, making

Pneumocystis jirovecii unlikely. Nonetheless, we cannot rule out that other opportunist infections might have contributed to these findings. Another key aspect of future host biomarker studies of TB in PWH is to also include participants with other opportunistic infections.

HIV-TB pathogenesis

Many interesting studies have been performed comparing TB disease with and without HIV. Even so, we believe much work remains.

Given the findings of drastically different miRNA profiles with regard to HIV status in persons with TB disease, and that several of these miRNAs have reported functions with relevance to TB immune response, further *in vitro* studies are warranted.

Analysis of miRNA expression patterns in blood cell subsets would yield better understanding of these changes. Macrophages could either be harvested from PWH or *in vitro* infected with HIV, and be infected with Mtb to establish a coinfection model. miRNA mimics/antisense blockers could be employed to investigate whether these miRNAs alter responses such as cytokine production or Mtb degradation. In case the effects of miRNA changes in infection could be reversed, this could be transferred to animal studies. Results from such experiments might be translated into host directed therapies. Antisense miRNA treatment has been developed for chronic hepatitis C (194), proving the concept of miRNA blocking as anti-infection treatment. The delivery of mRNA to human cells through lipid nanoparticles has had tremendous success as vaccines for covid-19, further emphasizing that RNA treatments can be used in clinical practice.

A similar approach could be conducted for the snoRNAs we have identified to be drastically reduced in TB. *In vitro* infections could investigate if the same pattern can be seen after infections of macrophages. Addition of synthetic snoRNAs (or blocking of endogenous snoRNAs), preferably the orphan varieties that are more likely to have non-canonical functions, and its impact on mRNA or protein expression could possibly highlight pathways that are affected by the downregulation of snoRNAs.

With emerging drug resistance of Mtb, the possibility of host-directed therapy for modulation of inflammatory responses needs considerable research efforts. Corticosteroids are already widely in use in TB meningitis, pericarditis and IRIS(4). Much attention has been given to mammalian target of rapamycin (mTOR) inhibitors, as these both induce autophagy and modulate inflammation. A clinical trial investigating low-dose mTOR inhibitor everolimus and the phosphodiesterase inhibitor CC-11050 demonstrated safety and a benefit in lung function recovery

when added to standard-of-care TB treatment (195). It should be noted, however, that preclinical data point towards a reverse effect of mTOR inhibition on Mtb control in the context of HIV (196), illustrating the fact that HIV-TB constitutes its own immune entity and thus needs to be evaluated separately.

Many immune functions involved in HIV-TB interactions could be further explored. B-cells have often been overlooked in the field of TB. Immunoglobulins, while suggested to not be essential in determining the course of TB infection, appear to have an important role in preventing TB dissemination (197). Furthermore, sequencing studies suggest that genes related to IgA have relevance to whether TB infection develops in the first place (198). It is well known that HIV affects antibody production and B-cell function in several ways (199), and it has been shown that PWH have greater homogeneity in IgG against the mycobacterial antigen lipoarabinomannan (200), and expanded B-cell follicles have been shown to prevent Mtb reactivation in an HIV-TB coinfection animal model (201). However, antibody responses of different classes and to different antigens, how durable and functional these responses are, as well as how different subset of B-cells are affected by HIV in TB is largely unknown. Eicosanoid imbalance, with depression of the protective prostaglandin E2, to is another interesting field that is suggested to contribute to Mtb immune pathogenesis, also offering a potential for therapeutic manipulation (202). The effects of prostaglandin E2 on Mtb control appears to be radically different under different experimental conditions, however (203). How HIV affects these mechanisms is another important question that merits studies.

Many flow cytometry studies have elucidated how cell populations are altered in HIV-TB. This body of knowledge could be further deepened by sorting of cells followed by mRNA sequencing, or single cell sequencing. Single cell sequencing of peripheral blood or cells from bronchoalveolar fluid of carefully selected TB patients with and without HIV infection could be hypothesis-generating for further elaboration of the HIV-TB pathogenesis.

A key aspect of comparing clinical samples from TB samples with and without HIV, are finding participants that are comparable in other ways, such as disease severity. PWH may have less pronounced TB symptoms and frequently have smear-negative pulmonary disease, in spite of having more severe TB (in particular disseminated disease), and it is far from obvious which PWH and HIV uninfected individuals with TB really are comparable to each other. One way of at least partially solving this issue and adding another aspect to these types of studies, are including a group of PWH with TB who are well established on ART. It is likely that those individuals have a different immune phenotype of disease in comparison to severely immunosuppressed, and as the rationale for these types of comparisons are to investigate mechanisms beside CD4+ T cell depletion this group of PWH might actually be more appropriate to study.

Many other clinical aspects of HIV-TB coinfection remain to study, direct comparisons of the clinical presentations between HIV-/TB+ and HIV+/TB+ are surprisingly few. With improving availability of ART, the characterization of TB disease in PWH on ART also needs to be improved. Whether PWH with TB are important sources of TB transmission, and the characteristics and duration of incipient and subclinical TB in PWH, are other issues that should be further investigated in the future.

Conclusions

In this thesis we have explored several aspects of the immune response to TB in PWH and found increased levels of different classes of markers. Several of these also showed relation to HIV and TB severity.

CRP and suPAR showed the strongest discriminatory capacity, but different cut-offs across different settings remain an issue.

We have found that given the relatively high KT ratio in ART-naïve PWH without TB, the specificity of KT ratio for TB case-finding in ART-naïve populations is limited.

Furthermore, we describe altered miRNA profiles in regard to HIV status in people with TB disease, with implications for the immune response against TB. Furthermore, snoRNAs were generally decreased in people with TB disease, both with and without HIV.

Finally, we have noted increased ribonuclease activity in plasma from PWH with TB. This finding was confirmed with three different methods and three different ribonucleases were found to also be increased in PWH with TB.

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Paper I

Open Forum Infectious Diseases

MAJOR ARTICLE

Plasma Profiles of Inflammatory Markers Associated With Active Tuberculosis in Antiretroviral Therapy-Naive Human Immunodeficiency Virus-Positive Individuals

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Background. Diagnosis of tuberculosis (TB) in human immunodeficiency virus (HIV)-coinfected individuals is challenging. We hypothesized that combinations of inflammatory markers could facilitate identification of active TB in HIV-positive individuals.

Methods. Participants were HIV-positive, treatment-naive adults systematically investigated for TB at Ethiopian health centers. Plasma samples from 130 subjects with TB (HIV^+/TB^+) and 130 subjects without TB (HIV^+/TB^-) were tested for concentration of the following markers: CCL5, C-reactive protein (CRP), interleukin (IL)-6, IL12-p70, IL-18, IL-27, interferon-γ-induced protein-10 (IP-10), procalcitonin (PCT), and soluble urokinase-type plasminogen activator receptor (suPAR). Analyzed markers were then assessed, either individually or in combination, with regard to infection status, CD4 cell count, and HIV ribonucleic acid (RNA) levels.

Results. The HIV^+/TB^+ subjects had higher levels of all markers, except IL12p70, compared with HIV^+/TB^- subjects. The CRP showed the best performance for TB identification (median 27.9 vs 1.8 mg/L for HIV^+/TB^+ and HIV^+/TB^-, respectively; area under the curve [AUC]: 0.80). Performance was increased when CRP was combined with suPAR analysis (AUC, 0.83 [0.93 for subjects with CD4 cell count <200 cells/mm^3]). Irrespective of TB status, IP-10 concentrations correlated with HIV RNA levels, and both IP-10 and IL-18 were inversely correlated to CD4 cell counts.

Conclusions. Although CRP showed the best single marker discriminatory potential, combining CRP and suPAR analyses increased performance for TB identification.

Keywords. biomarker; CRP; HIV; *Mycobacterium tuberculosis*; sub-Saharan Africa.

Human immunodeficiency virus (HIV)-related cellular immunodeficiency increases the risk of both primary and reactivated tuberculosis (TB) and is associated with greater likelihood of extrapulmonary and disseminated disease [1]. Rates of TB coinfection among people living with HIV (PLHIV) are highest in sub-Saharan Africa [2], and TB is the leading cause of death among PLHIV globally [3]. Autopsy studies suggest that considerable proportions of HIV-related TB may be missed in TB-endemic settings [4]. Contributing factors for this are atypical disease presentation [5] and suboptimal performance of commonly available diagnostic methods for active TB, such as smear microscopy [6] and chest radiography [7]. A clinical symptom algorithm is recommended by the World Health Organization (WHO) for excluding TB among PLHIV [8]. Although this algorithm is sensitive, it has low specificity, limiting its usefulness as a screening tool [9]. Alternative strategies for screening in populations at high risk of TB could improve case finding.

These circumstances have prompted investigations of plasma biomarkers for identification of active TB. C-reactive protein (CRP) has been shown to have promising capacity as a point-of-care biomarker for TB screening among PLHIV in different settings [10–15]. Although the capacity of panels of inflammatory markers for TB identification has been investigated [16–18], this concept has not been assessed in a large PLHIV cohort systematically categorized for active TB. Because HIV leads to dysregulation of the TB immune response [19, 20], we hypothesized that HIV-positive individuals with active TB have inflammatory reactions that could be used to distinguish such cases from HIV-positive persons without TB, and that combinations of inflammatory markers would provide stronger discriminatory capacity than single markers.

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To explore this hypothesis, we designed a nested case-control study based on participants from a cohort of antiretroviral therapy (ART)-naive patients systematically investigated for active TB. We characterized plasma profiles of 9 markers of inflammation, selected on the basis of their previously reported associations with TB. We analyzed the performance for TB identification of these markers separately as well as in different combinations. Furthermore, we evaluated whether plasma levels of these markers were related to CD4 cell count and HIV-ribonucleic acid (RNA) levels, respectively.

METHODS

Study Participants

Participants for this study were identified from a prospective cohort of 812 HIV-positive adults subjected to intensified TB case finding before ART initiation at 5 public health centers in the Oromia region, Ethiopia (included between 2011 and 2013). Details of this cohort have been presented previously [9, 21]. In brief, for inclusion in the cohort, subjects were required to be ≥18 years old and meet Ethiopian criteria for ART initiation at the time of the study (CD4 cell count ≤350 cells/mm^3 and/or WHO stage 4). Individuals with previous ART experience and/or ongoing treatment for TB for ≥2 weeks, as well as those who did not provide sputum samples for TB analyses, were excluded.

At inclusion, cohort participants underwent physical examination and interview based on a structured questionnaire covering clinical and sociodemographic data. All subjects were instructed to submit 2 spontaneously expectorated morning sputum samples (irrespective of symptoms or physical findings), which were examined with Ziehl-Neelsen smear microscopy, liquid TB culture, and Xpert MTB/RIF (Cepheid, Sunnyvale, CA). Fine-needle aspirations were obtained from persons with lymphadenopathy. Venous blood was collected for analysis of CD4 cell count, complete blood count, and HIV RNA quantification. Aliquots of plasma were stored at −80°C and transported with intact cold chain to the Biomedical Center, Lund, Sweden, where laboratory analyses were undertaken after data collection and subject selection. A nested case-control design was chosen for the current study. All participants with bacteriologically confirmed TB (HIV$^+$/TB$^+$) and available plasma samples were included as cases, and, for each of these, 1 control without active TB was selected, matched for age and gender (HIV$^+$/TB$^-$). Plasma samples were available from 130 of 137 HIV$^+$/TB$^+$ subjects. Control subjects (HIV$^+$/TB$^-$) were required to have negative bacteriological results for TB and not to be diagnosed with bacteriologically confirmed nor clinically suspected TB during the first 6 months of follow-up. Participants without active TB at inclusion who were lost to follow-up or died within 6 months of follow-up were not eligible as controls, because unrecognized active TB could not be reliably excluded.

Quantification of Inflammatory Markers

Concentrations of inflammatory markers were determined using the Magnetic Luminex Assay (R&D Systems Inc., Minneapolis, MN) and Bio-Plex 200 reader (Bio-Rad Laboratories, Hercules, CA). In an initial exploratory experiment, the following 14 markers were analyzed in 20 HIV$^+$/TB$^+$ and 20 HIV$^+$/TB$^-$ subjects: CC-chemokine ligand 5 (CCL5, a.k.a. RANTES), CRP, granzyme B, interferon (IFN)-β, IFN-γ, IFN-γ-induced protein 10 (IP-10), interleukin (IL)-10, IL-12p70, IL-18, IL-27, IL-6, procalcitonin (PCT), soluble urokinase-type plasminogen activator receptor (suPAR), and tumor necrosis factor (TNF)-α. For Granzyme B, IFN-β, IFN-γ, IL-10, and TNF- α plasma levels were uniformly low or undetectable; therefore, we excluded these markers from further experiments.

C-reactive protein and CCL5 were analyzed in separate plates, diluted 1:50 and 1:200, respectively, and CRP was later diluted 1:2000 for values beyond the standard curve. The remaining markers were analyzed in 1:2 dilution in a 7-plex plate. All plates contained equal proportions of HIV$^+$/TB$^+$ and HIV$^+$/TB$^-$ samples, randomly placed on the plate. Laboratory procedures were performed according to the manufacturer's instructions.

Each plate included a standard curve of 6 standard points and 2 internal controls (1 TB$^+$, 1 TB$^-$). All samples were analyzed in duplicates. Mean intra-assay variation for concentrations was 7% coefficient of variation (%CV). Mean interassay variation for median fluorescence intensity (MFI) values for all markers except suPAR was 11.3%CV (range, 6–17) and increased to 19.6%CV (range, 11–27) after translation to concentrations. For suPAR, both MFI and concentration values showed greater variability between runs (%CV 29 and 30.5, respectively).

Statistical Analysis

Comparison of marker levels between HIV$^+$/TB$^+$ and HIV$^+$/TB$^-$ subjects was evaluated using the Mann-Whitney U test. $P < .05$ (adjusted for multiple testing using the Holm-Bonferroni method) were considered significant. Receiver operating characteristics (ROC) curves were constructed for each marker. In addition, marker levels were correlated to CD4 cell count and HIV RNA levels using Spearman's rank-order correlation. To adjust for lower CD4 cell counts among HIV$^+$/TB$^+$ subjects, markers were dichotomized in 2 groups, with regard to median levels for the respective marker, and logistic regression was performed for each marker in respect to TB, with and without adjustment for CD4 cell count.

To assess the best combination of inflammatory markers to distinguish between HIV$^+$/TB$^+$ and HIV$^+$/TB$^-$, backward stepwise logistic multivariate analysis was performed using continuous variables. From this multivariate analysis, predicted probabilities were calculated. A ROC curve describing sensitivity and specificity of the combination of markers that constituted the best model was then fitted. This model was subsequently assessed in an analysis of subjects with CD4 cell count <200/mm^3. All statistical analyses were conducted using SPSS version 24.0 (IBM, Armonk, NY).

Ethics Statement

Ethical approval was obtained from both the National Research Ethics Review Committee at the Ministry of Science and Technology of Ethiopia (3.10/825/05) and the Regional Ethical Review Board at Lund University, Sweden (2010/672). Written informed consent was obtained from all participants at inclusion in the cohort study.

RESULTS

Participant Characteristics

The characteristics of study participants at the time of sampling (130 HIV^+/TB^+ and 130 HIV^+/TB^-) are presented in Table 1. All patients had pulmonary TB, in addition 12 subjects had bacteriologically confirmed concomitant TB lymphadenopathy. The HIV^+/TB^+ subjects were more likely to be malnourished (lower body mass index and mid-upper arm circumference). In addition, they had lower median CD4 cell count (173 vs 224 cells/mm^3), lower hemoglobin levels (10.4 vs 12 g/dL), and higher viral load (5.3 vs 5.0 $\log_{10}$ RNA copies/mL). In both groups, a majority reported positive WHO-TB symptom score results (presence of ≥1 symptom; cough, weight loss, night sweating, and/or fever [93% of HIV^+/TB^+, 79% of HIV^+/TB^-]).

Individual Inflammation Markers in Respect to Active Tuberculosis

Plasma concentrations of investigated markers are shown in Table 2, and differences are graphically presented in Figure 1. With the exception of IL-12p70, HIV^+/TB^+ subjects had significantly higher levels of all markers compared with HIV^+/TB^- (all $P < .01$). The difference between HIV^+/TB^+ and HIV^+/TB^- were most pronounced for CRP (median concentration, 27.9 vs 1.8 mg/L; $P < .001$), suPAR (median concentration, 2.6 vs 1.1 ng/mL; $P < .001$), and IL-6 (median concentration, 9.7 vs 2.1 ng/L; $P < .001$). The IL-27 and IL-12-p70 concentrations could not be extrapolated due to large proportions of values below the standard curve; therefore, these markers were excluded from further analyses.

We compared levels of inflammatory markers with regard to bacteriological results (reflecting bacterial load in sputum) and presence of TB symptoms. Levels of CRP, IL-6, IL-18, IP-10, PCT, and suPAR were significantly higher in Xpert MTB/RIF-positive HIV^+/TB^+ subjects, compared with Xpert MTB/RIF-negative HIV^+/TB^+ subjects, and CRP and IL-6 were significantly higher in smear-positive HIV^+/TB^+ subjects, compared with smear-negative HIV^+/TB^+ subjects. The HIV^+/TB^+ subjects without TB symptoms had low levels of the markers investigated (Table 2). Adjustments for CD4 cell count in logistic regression did not significantly change the odds ratio for any marker (Table 3).

Correlation of Inflammation Markers to Other Parameters

Because the incidence of active TB increases with HIV-related immunosuppression, we determined whether biomarker levels were independently associated with TB or with surrogate markers of HIV disease progression. For this purpose, we performed logistic regression with adjustments for CD4 cell count. This did

Table 1. Characteristics of 130 HIV-Positive Participants With TB Coinfection (HIV^+/TB^+) and 130 HIV-Positive Matched Controls Without TB Coinfection (HIV^+/TB^-) Selected From a Cohort of 812 ART-Naive HIV-Positive Patients Investigated for Active TB[a]

	TB^+		TB^-		
Characteristics	Included	Excluded	Included	Excluded	Total
Female	63/130 (49)	3/7 (43)	60/130 (46)	341/524 (65)	467/791 (59)
Age, years	35 (28–42)	30 (29–37)	35 (28–41)	31.5 (28–38)	32 (28–40)
CD4 count, cells/mm^3	173 (94.5–271)	91 (88–197)	224 (146–328)	220 (121–327)	211 (119–320)
Hemoglobin, g/dL	10.4 (9.1–11.8)	10.9 (8.5–14.2)	12 (10.9–13.2)	11.8 (10.3–12.7)	11.6 (10.2–12.7)
HIV RNA, $\log_{10}$ copies/ mL	5.3 (4.7–5.6)	5.4 (5.2–5.8)	5 (4.5–5.5)	5 (5.5–5.5)	5.1 (4.5–5.6)
On TB treatment at enrollment	15/130 (12)[b]	0/7 (0)	0 (130)	0/524 (0)	15/791 (2)
Bacteriological results					
Sputum smear positive	29/129 (23)	2/7 (29)	0/130	0/518 (0)	31/784 (4)
Sputum Xpert MTB/RIF positive	90/129 (70)	6/7 (86)	0/130	0/518 (0)	96/783 (12)
Sputum culture positive	118/129 (92)	6/7 (86)	0/130	0/523 (0)	124/788 (17)
Lymph node culture positive	4/5 (80)	0/0 (0)	0/3 (0)	0/1 (0)	4/9 (44)
Lymph node Xpert MTB/RIF positive	11/12 (92)	0 (0)	0/1 (0)	0/7 (0)	13/20 (65)
Clinical findings					
WHO-TB symptom screening positive[c]	120/129 (93)	6/7 (86)	101/128 (79)	398/520 (77)	625/784 (80)
MUAC cm	21 (19.4–23)	22 (18.8–22.5)	23 (21–25)	23 (21–24.5)	22.5 (20.5–24)
BMI kg/m^2	17.7 (16.1–19.7)	17.6 (16.7–20.7)	19.5 (17.8–22)	19.1 (17.6–21.1)	19 (17.4–21)

Abbreviations: ART, antiretroviral therapy; BMI, body mass index; HIV, human immunodeficiency virus; MUAC, mid-upper arm circumference; RNA, ribonucleic acid; TB, tuberculosis; WHO, World Health Organization.

[a]Twenty-one subjects with TB diagnosed on clinical criteria are not presented in this table. For continuous variables, median and interquartile range are presented; for categorical variables, absolute numbers and percentage of total are presented.

[b]Median number of days on treatment before enrollment: 7.

[c]Presence of either cough, weight loss, night sweating, or fever.

Table 2. Levels of Investigated Inflammatory Markers (Median, IQR in Brackets) in HIV$^+$ Subjects in Whom TB Was Excluded (HIV$^+$/TB$^-$) and HIV$^+$ Subjects With Bacteriologically Confirmed TB (HIV$^+$/TB$^+$) With Regard to Distribution of Bacteriological Results and Presence of Clinical Symptoms

		HIV$^+$/TB$^+$ (n = 130)					
Inflammatory Marker	HIV$^+$/TB$^-$ (n = 130)	All	Smear^{+a} (n = 29)	Gene Xpert^{+b} (n = 90)	Culture$^+$/ AFB$^-$/Gene Xpert$^-$ (n = 38)	WHO Score^{+c} (n = 120)	WHO Score$^-$ (n = 9)
CCL5, µg/mL	40.8 (14.9–98.9)	73.1(27.9–154.7)	59.8 (12.2–12.7)	73.1 (26.5–130)	91.9 (34.9–17.4)	70.3 (27.3–153.5)	91.2 (40.6–141.4)
CRP, mg/L	1.8 (0.6–6.9)	27.9 (5.9–50)	50 (31.7–50)	49.5 (15.3–50)	9.1 (1.4–49.7)	38.9 (10–50)	1 (0.6–3.8)
IL-6, pg/mL	2.1 (0.5–4.5)	9.7(3.2–18.4)	14.1 (7.4–32.4)	12.3 (4.7–22.6)	3.6 (1–10.5)	10.4 (3.7–18.7)	1.1 (0.6–2.9)
IL12p70, MFI[d]	12.5 (11.5–13.5)	13 (12–14)	13 (11.5–15)	13.3 (12–14.5)	12.65 (11.5–13.5)	13 (12–14)	12.3 (11.1–13.4)
IL-18, pg/mL	672 (469–1 023)	1098 (665–1 956)	1432 (830–2 982)	1356 (832–2394)	831 (521–1301)	1107 (670–2 106)	648 (436–1 216)
IL-27, MFI[d]	35.4 (27.0–43.4)	44.0 (32.9–52.9)	46.0 (35.0–59.3)	46.3 (34.7–54.3)	40.9 (29.1–49.3)	45.7 (33.3–53.6)	32.7 (25.4–46)
IP-10, pg/mL	143 (83–251)	241 (154–414)	319 (185–509)	295.3 (167–466)	208 (101–254)	253 (167– 418)	103 (50.1– 197)
PCT, ng/mL	0.04 (0.03–0.07)	0.08 (0.04–0.14)	0.11 (0.08–0.2)	0.08 (0.05–0.19)	0.05 (0.03–0.09)	0.08 (0.05–0.15)	0.04 (0.03–0.04)
suPAR, ng/mL	1.1 (0.7–1.7)	2.6 (1.3–5.5)	3 (1.7–10.1)	3.2 (1.5–6.7)	1.8 (1–2.6)	2.7 (1.3–5.8)	1.3 (0.9–2)

Abbreviations: CCL5, CC-chemokine ligand 5; CRP, C-reactive protein; HIV, human immunodeficiency virus; IL, interleukin; IP-10, interferon-γ-induced protein-10; IQR, interquartile range; MFI, median fluorescence intensity; PCT, procalcitonin; suPAR, soluble urokinase-type plasminogen activator receptor; TB, tuberculosis; WHO, World Health Organization.

[a]Positive Zieh-Neelsen-stained direct microscopy.

[b]Positive Gene Xpert MTB/RIF.

[c]Presence of either cough, fever, weight loss, or night sweating.

[d]For these markers, it was not possible to extrapolate reliable concentrations; hence, MFI values are presented instead.

not significantly change the odds ratio for any marker (Table 3). In addition, Spearman's rank-order correlations were performed between plasma levels of each marker and CD4 cell count and HIV-RNA levels, respectively. When the results from all 260 study participants were analyzed, all markers, except CCL5, exhibited significant inverse Spearman rank correlations to CD4 cell count (ρ: −0.34 to −0.41, all $P < .01$). The same markers were significantly inversely correlated to CD4 cell count in separate analysis of HIV$^+$/TB$^+$ subjects (ρ: −0.22 to −0.52, all $P < .05$). Among HIV$^+$/TB$^-$ subjects, IL-18 (ρ: −0.29, $P < .01$) and IP-10 (ρ: −0.35, $P < .01$) were significantly inversely correlated to CD4 cell count, whereas other markers did not show such correlation.

When assessed for correlation with HIV-RNA levels, IL-6 (ρ: 0.2, $P < .01$), IL-18 (ρ: 0.27, $P < .01$), IP-10 (ρ: 0.43, $P < .01$), and suPAR (ρ: 0.2, $P < .01$) exhibited significant correlation when all subjects were included. In HIV$^+$/TB$^+$ subjects, IL-18 (ρ: 0.34, $P < .01$), IP-10 (ρ: 0.42, $P < .01$), and suPAR (ρ: 0.27, $P < .01$) correlated to HIV RNA, whereas only IP-10 exhibited significant correlation among HIV$^+$/TB$^-$ subjects (ρ: 0.4, $P < .01$) (Tables 1 and 12; Supplementary Material). C-reactive protein and PCT were higher in male subjects ($P < .01$; median 10 vs 4 mg/L and 74 vs 44 pg/mL, respectively). Marker levels were not significantly altered in the 15 subjects who had received TB treatment before collection of samples.

Discriminatory Potential of Inflammation Markers for Tuberculosis

To explore the discriminatory potential for TB identification, ROC curves were constructed for both individual markers and combinations of markers. Individual area under the curve (AUC) values were highest for the following markers: CRP (AUC = 0.80; 95% confidence interval [CI], 0.75–0.86), suPAR (AUC = 0.77; 95% CI, 0.71–0.83), IL-6 (AUC = 0.76; 95% CI, 0.71–0.82), and IL-18 (AUC = 0.71; 95% CI, 0.65–0.77). For all AUC of all markers, see Table 3. In subjects with CD4 cell count <200 cells/mm^3, the corresponding AUC values were as follows: CRP 0.89 (95% CI, 0.84–0.95), suPAR 0.87 (95% CI, 0.81–0.93), IL-6 0.87 (95% CI, 0.8–0.91), and IL-18 0.71 (95% CI, 0.63–0.80). For the remaining markers, the AUC values were <0.7. The ROC curves of MFI are shown in Supplementary Figure 1.

Tuberculosis Discriminatory Potential of C-Reactive Protein and Soluble Urokinase-Type Plasminogen Activator Receptor, Individually and in Combination

Although CRP had the best discriminatory potential among the markers investigated, 24% of HIV$^+$/TB$^+$ subjects had CRP levels <5 mg/L, and 29% had levels <10 mg/L. Hence, using previously suggested cutoffs of 10 or 5 mg/L in this population would result in a sensitivity of 71% or 76%, respectively. Because a high proportion of HIV$^+$/TB$^+$ subjects in this population had CRP levels <5 mg/L, a cutoff with the requested 90% sensitivity would be as low as 1.2 mg/L. Figure 2 shows the distribution of CRP among HIV$^+$/TB$^+$ subjects. Median CRP levels were low in asymptomatic subjects (1.0 mg/L) and in subjects in whom TB was only detected by liquid culture (smear and Xpert negative; 9.1 mg/L). In logistic stepwise regression analysis, 2 markers, CRP and suPAR, remained independently associated with TB (both $P < .001$). The AUC of this combination was 0.83 (CI, 0.78–0.88). In subjects with CD4 cell count <200 cells/mm^3, the AUC for this combination was 0.93 (CI, 0.87–0.97). For ROC curves of CRP and suPAR alone and in combination, see Figure 3.

DISCUSSION

In this nested case-control study, based on a large cohort of ART-naive PLHIV subjected to intensified TB case finding,

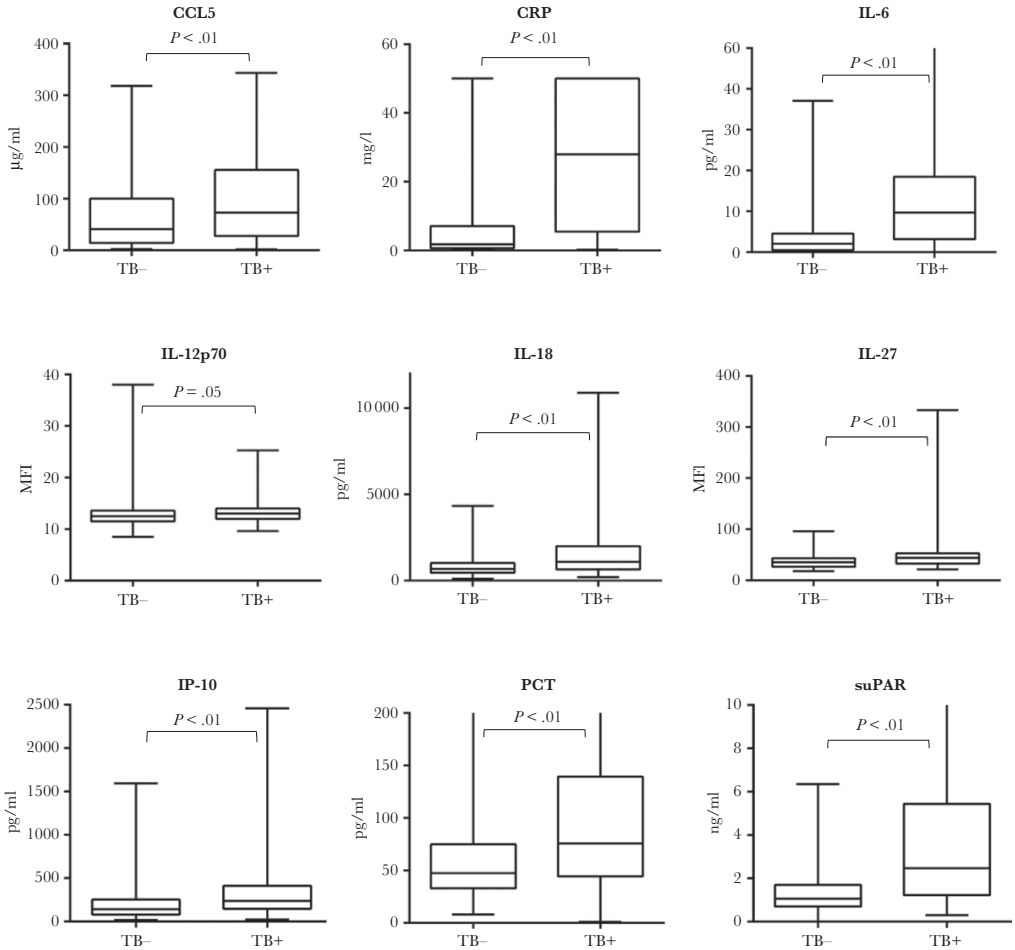

Figure 1. Levels of 9 markers of inflammation in plasma from 130 human immunodeficiency virus (HIV)$^{+}$/tuberculosis (TB)$^{+}$ and 130 HIV^{+}TB^{-} subjects. Boxes represent median and interquartile range. Whiskers have been graphically cut for soluble urokinase-type plasminogen activator receptor (suPAR), interleukin (IL)-6, and procalcitonin (PCT). Mann-Whitney *U* test *P* values are indicated in each graph. All markers remained significantly associated with TB after Holm-Bonferroni correction, except IL-12p70. For IL-12p70 and IL-27, the graphs represent mean fluorescence intensity (MFI) due to unreliable conversions to concentrations.

we hypothesized that profiles of inflammatory markers could distinguish individuals with active TB. Indeed, plasma levels of most inflammatory markers investigated were significantly elevated in participants with active TB compared with those without TB, with the most prominent differences found for CRP, suPAR, and IL-6.

A potential confounder for associations with TB in HIV-positive individuals is the degree of immunosuppression, which is linked to the risk of active TB. For this reason, we correlated levels of inflammatory markers to CD4 cell counts. Although most investigated markers showed inverse correlation to CD4 cell count, the relationships to TB coinfection remained significant after adjustment for this factor. Most notably, we observed that the associations with CD4 cell count differed with regard to TB coinfection. Whereas inverse correlation with CD4 cell count was observed for several markers among HIV^{+}/TB^{+} subjects, only IP-10 and IL-18 showed such an association in HIV^{+}/TB- patients. Both of these markers have previously been associated with HIV disease progression [22, 23]. The finding of lower CD4 cell counts in HIV^{+}/TB^{+} subjects may be explained

Table 3. Odds Ratios for HIV$^+$/TB$^+$ Versus HIV$^+$/TB$^-$ for Marker Levels Above Median Level of the Total Study Population, Compared to Levels Below Median, With and Without Adjustments for CD4 Cell Count[a]

Inflammatory Marker	Crude OR	95% CI	Adjusted OR	95% CI	AUC	95% CI	AUC in Subjects With CD4 <200 Cells/mm^3	95% CI
CCL5	2.1	1.3–3.5	2.1	1.2–3.4	0.62	0.55–0.68	0.62	0.52–0.71
CRP	8.6	4.9–15.1	8.0	4.5–14.1	0.80	0.75–0.86	0.89	0.84–0.95
IL6	5.4	3.2–9.3	5.0	2.9–8.5	0.76	0.71–0.82	0.87	0.8–0.93
IL-18	3.6	2.1–6.1	3.1	1.8–5.4	0.71	0.65–0.77	0.71	0.63–0.8
IP-10	2.9	1.7–4.8	2.5	1.5–4.2	0.67	0.6–0.73	0.65	0.55–0.75
PCT	3.1	1.9–5.2	2.8	1.7–4.8	0.68	0.61–0.74	0.73	0.64–0.81
suPAR	4.4	2.6–7.4	3.9	2.3–6.7	0.77	0.71–0.83	0.87	0.81–0.93

Abbreviations: AUC, area under the curve; CI, confidence intertval; CRP, C-reactive protein; HIV, human immunodeficiency virus; IL, interleukin; IP-10, interferon-γ-induced protein-10; OR, odds ratio; PCT, procalcitonin; suPAR, soluble urokinase-type plasminogen activator receptor; TB, tuberculosis.

[a]Area under the curve values are for all markers in whole material and in subjects with CD4 <200 cells/mm^3.

by the increasing risk of TB in relation to decreasing CD4 count [24], but this could also be due to mechanisms related to TB disease per se, such as redistribution of CD4 cells [25, 26].

In agreement with previous reports, we found that CRP, suPAR, and IL-6 had the strongest associations with active TB [10–13, 16, 27–29]. Interleukin-6 has been suggested as a potential biomarker for TB [27, 28, 30]. However, in our study, IL-6 did not add discriminative capacity in multivariate analysis, which might be due to covariance with CRP (Spearman's ρ: 0.7).

Currently, CRP appears to be the most promising biomarker for TB screening [29]. In addition, point-of-care assays for CRP testing are available. Its performance for this purpose has been validated among both TB suspects and PLHIV in cohorts from South Africa and Uganda [10–12, 15, 29]. Our results, showing an overall AUC of 0.80, support the discriminative capacity of CRP for TB identification. In a recent prospective cohort study, Yoon et al [13] found an AUC of 0.81 for a cutoff of 10 mg/L, with 90.2% sensitivity and 69.6% specificity. Although we did not aim to define a cutoff level for TB identification, a threshold value of 10 mg/L had considerably reduced sensitivity in our population. Median CRP levels in HIV$^+$/TB$^+$ subjects in our cohort were lower than those reported by Yoon et al [13] (median CRP for HIV$^+$/TB$^+$ 28 mg/L compared with 52 mg/L, respectively); with 29% having CRP levels <10 mg/L.

In agreement with Lawn et al [10], we observed that CRP levels varied with regard to bacteriological TB results, reflecting bacterial load in sputum. Persons with paucibacillary TB (Xpert negative, culture positive) had lower median CRP levels (9.1 mg/L compared with 50 mg/L in Xpert-positive patients). We have previously reported that HIV$^+$/TB$^+$ individuals with negative Xpert and smear microscopy results had less advanced disease characteristics, and they were often asymptomatic [31]. However, despite ART initiation before establishment of TB diagnosis and delayed initiation of TB therapy, these patients did not have inferior treatment outcomes. Similar observations have been made by other researchers among HIV-positive persons living in TB-endemic settings [32, 33]. It is plausible that these cases represent early active TB, and that the inflammatory response characteristic of HIV/TB coinfection is not fully evolved. Accurate identification of early asymptomatic active TB is challenging by clinical examination as well as radiology and bacteriological methods. Our findings also show low levels of inflammatory markers (including CRP) in the small number of patients with asymptomatic TB (9 of 130), implying that CRP screening may be inadequate in this situation.

Similar to our study, Shapiro et al [15] recently reported inadequate performance for CRP screening using a cutoff of 10 mg/L. However, these investigators found a sensitivity of 90.5% using a cutoff level of 5 mg/L. The corresponding sensitivity for this cutoff in our population was <80%. These results suggest that CRP, although strongly associated with active TB, might not optimally identify all TB$^+$/HIV$^+$ cases as a single biomarker.

An alternative option for TB identification among PLHIV might be the use of combinations of biomarkers. Apart from CRP, suPAR showed strong independent discriminative capacity. Besides associations with increased mortality in several conditions, including both HIV [34, 35] and TB [36], elevated suPAR levels have been associated with TB coinfection in PLHIV [36, 37]. Although both CRP and suPAR individually had discriminative performance in our population, a combination of both these markers showed higher discriminatory capacity than when either marker was used separately. The best performance was found in subjects with severe immunosuppression (CD4 <200 cells/mm^3; AUC = 0.93). These results imply a potential for improved identification of TB using a combination of biomarkers, especially in this subset of patients who represent those at highest risk of TB. The combination might be constructed as a composite score; alternatively, the combination could be used as a 2-step process, in which subjects with low CRP levels are further investigated with suPAR. Further studies are required (1) to define optimal cutoff levels and (2) to validate the proposed combination of CRP and suPAR prospectively in different populations before it is implemented in clinical care.

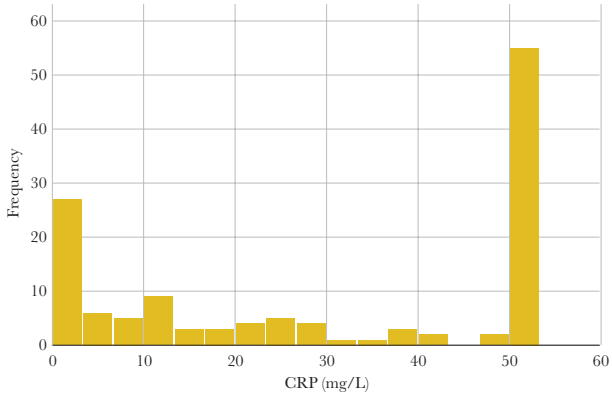

Figure 2. C-reactive protein levels (mg/L) subjects with human immunodeficiency virus and tuberculosis coinfection, note that the highest detectable level in our assay was 50 mg/L.

Our results support the concept of combining biomarkers for TB identification, and further studies, based on relevant clinical populations, are indicated.

Although other markers investigated did not show adequate discriminatory capacity as biomarkers for TB screening, we did observe significant differences related to TB coinfection. Two of those markers, IP-10, a chemokine secreted after IFN-γ stimulation [38], and IL-18, which promotes IFN-γ secretion by T cells [39], were both associated with active TB and with CD4 cell count. Previous studies have linked levels of IP-10 and IL-18 to accelerated HIV disease progression [22, 23]. Whereas IP-10 has been evaluated as a TB biomarker in both HIV-negative and HIV-positive subjects [30, 40, 41], the association between IL-18 and active TB in PLHIV has not previously been investigated to our knowledge.

Three additional markers (CCL5, PCT, and IL-27) were significantly elevated in HIV^+/TB^+ subjects, but their performance for TB identification was relatively low. Whereas most studies indicate that TB only leads to slightly elevated PCT [42, 43], raised levels of PCT have been associated with poor prognosis in TB patients [43]. Thus, PCT may serve as a prognostic marker in cases with established TB diagnosis. Although IL-27 may promote differentiation of IFN-γ-producing Th1 cells, it appears to favor bacterial survival in the setting of mycobacterial infection [44], possibly by suppressing T-cell migration into infected tissue [45]. It has been suggested as a biomarker for TB in pleural effusions [46], and it has previously been shown to be elevated in plasma from TB subjects [45, 47].

We observed differences in the distribution of CRP and PCT with regard to gender, with significantly higher median levels in men than women. Similar findings with regard to CRP in TB have been reported in a study by Lawn et al [10], where male gender was associated with CRP >50 mg/L. This phenomenon might reflect later presentation and more advanced TB and HIV disease at inclusion among men. Indeed, the male subjects had higher viral load, lower CD4 cell count, and higher proportion sputum smear and Xpert MTB/RIF-positive results, albeit none of these findings were significant. Chavez et al [48] described different cytokine profiles between males and females with TB. It is possible that different biomarkers are optimal for TB screening in men and women.

A major strength of this study was access to samples from a large prospective cohort of ART-naive PLHIV, in which all subjects had been systematically investigated for active TB. In particular, this allowed for inclusion of HIV-positive controls in whom active TB was excluded at inclusion and who were followed for 6 months without displaying clinical manifestations suggestive of active TB. Hence, we consider the risk of misclassification to be minimal. Furthermore, we explored a range of potential biomarkers for TB, including several that have not previously been investigated for the purpose of TB identification among PLHIV. We also correlated the levels of these biomarkers to CD4 cell counts and HIV-RNA levels, to determine whether the associations with TB were explained by more advanced HIV disease and not directly related to TB coinfection.

Although we assessed a panel of TB-associated immune markers and explored the possibility of combing these, other markers involved in the host response to TB infection might increase the discriminatory capacity. With regard to the laboratory analyses, high interassay variability occurred for some markers, especially suPAR. However, MFI values were consistent between runs, and the results in concentrations correspond well to the MFI results in terms of discriminatory capacity (Supplementary Figure 1). In addition, samples were handled randomly in plates containing both HIV^+/TB^+ and HIV^+/TB^- samples, making false-positive findings less likely. We did not

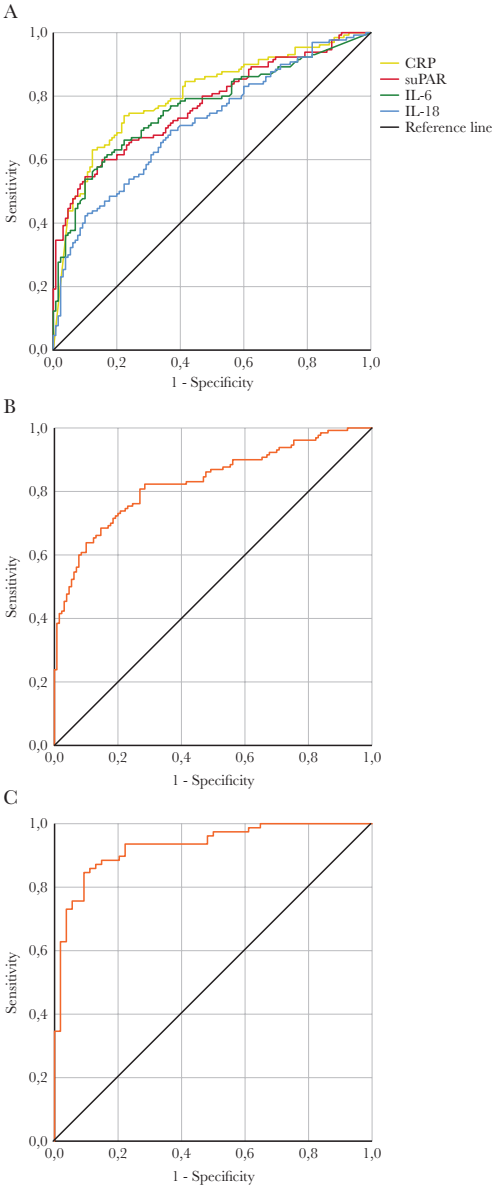

Figure 3. (A) Receiver operating characteristics (ROC) curves for C-reactive protein (CRP) (area under the curve [AUC], 0.80; 95% confidence interval [CI], 0.75–0.86), soluble urokinase-type plasminogen activator receptor (suPAR) (AUC, 0.77; 95% CI, 0.71–0.83), interleukin (IL)-6 (AUC, 0.76; 95% CI, 0.71–0.82), and IL-18 (AUC, 0.71; 95% CI, 0.65–0.78). (B) The ROC curve for the combination of CRP and suPAR in all subjects (n = 260; AUC, 0.83; 95% CI, 0.78–0.88). (C) The ROC curve for the combination of CRP and suPAR in subjects with CD4 cell count <200 cells/mm^3 (n = 132; AUC, 0.93; 95% CI, 0.89–0.97).

aim to define cutoff levels for clinical use in this exploratory study of potential biomarkers. This will be necessary to translate these results into clinical practice.

CONCLUSIONS

We found significant differences in the inflammatory profiles in HIV-positive ART-naive individuals with regard to concomitant active TB. Whereas CRP had the strongest discriminatory potential for TB identification as a single marker, 29% of subjects with bacteriologically confirmed active TB had CRP below the previously proposed cutoff level of 10 mg/L The best discriminative performance was observed for a combination of CRP and suPAR, suggesting a potential for combinations of biomarkers for TB screening among PLHIV.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

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Paper II

OPEN

Kynurenine/tryptophan ratio for detection of active tuberculosis in adults with HIV prior to antiretroviral therapy

Oskar Olsson[a,b], Sten Skogmar[a,b], Fregenet Tesfaye[a,c], Daba Mulleta[d], Marianne Jansson[e] and Per Björkman[a,b]

Objective: The aim of this study was to assess the performance of kynurenine/tryptophan ratio for tuberculosis (TB) case-finding among antiretroviral therapy (ART)-naive people with HIV (PWH), and to investigate other factors associated with kynurenine/tryptophan ratio in this population.

Design: A nested case–control study based on a cohort of 812 ambulatory PWH in the Oromia region, Ethiopia.

Methods: At enrolment, all participants submitted sputum samples for bacteriological TB investigations. Concentrations of kynurenine and tryptophan in plasma were quantified using liquid chromatography-mass spectrometry. Receiver operator characteristic curves were constructed to assess diagnostic performance (area under the curve; AUC) for kynurenine, tryptophan, and kynurenine/tryptophan ratio. Sensitivity, specificity, and predictive values were calculated. Kynurenine/tryptophan ratios were correlated to plasma levels of nine inflammation mediators, plasma HIV RNA levels, $CD4^+$ cell count, BMI, and mid-upper arm circumference (MUAC).

Results: We included 124 individuals with HIV-TB coinfection (HIV+/TB+) and 125 with HIV mono-infection (HIV+/TB-). Tryptophan levels were lower in HIV+/TB+ than in HIV+/TB- (median 19.5 vs. 29.8 μmol/l, $P < 0.01$), while kynurenine levels were similar between these groups (median 2.95 vs. 2.94 μmol/l, $P = 0.62$). Median kynurenine/tryptophan ratio was 0.15 in HIV+/TB+, significantly higher compared with HIV+/TB- (0.11; $P < 0.01$), with AUC 0.70 for TB detection. Kynurenine/tryptophan ratio was positively correlated to plasma HIV RNA levels, IP-10, IL-18, and IL-27, and negatively correlated to $CD4^+$ cell count, BMI, and MUAC (all $P < 0.01$).

Conclusion: Among ART-naive PWH, kynurenine/tryptophan ratio has modest potential for TB discrimination, limiting its utility for TB case-finding in this population.

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Introduction

People with HIV (PWH) living in low-and middle-income countries are at a high risk of active tuberculosis (TB), which remains the leading cause of death among PWH globally [1]. Several factors are responsible for the persistently high mortality attributed to HIV-TB coinfection, not least the lack of robust methods for TB screening that can be used in HIV care in resource-limited settings [2]. Detection of active TB is challenging, as TB often has atypical clinical presentation in HIV-coinfected individuals [3]. The WHO TB screening algorithm, which is recommended for TB screening in PWH [4], has unsatisfactory performance [5], illustrating the need for optimized methods. Several host biomarkers have been investigated for this purpose, but hitherto no marker has shown consistently high sensitivity and specificity across different cohorts, and no biomarker for screening has been introduced in clinical practice [6].

The metabolic degradation of tryptophan is involved in the pathogenesis of various diseases, and the kynurenine/tryptophan ratio (KT ratio) has attracted attention as a potential biomarker for different conditions [7], including TB [8]. Active TB leads to increased conversion of tryptophan to kynurenine [8,9]. This is mediated by the host enzyme indoleamine 2,3-dioxygenase 1 (IDO-1), although other mechanisms may also be involved [7]. IDO-1 activity is triggered by proinflammatory stimuli, such as interferon-γ (IFN-γ) [10], a key component in the host defence against TB [11]. Recently, investigators from South Africa have reported excellent performance using kynurenine/tryptophan ratio as a biomarker for identification of pulmonary TB in PWH [8]. Furthermore, the kynurenine/tryptophan ratio has also been assessed for TB screening among pregnant women with HIV [12], and has been found to have discriminatory capacity between active and latent TB among PWH [13]. However, high proportions of participants in these studies were receiving antiretroviral therapy (ART), and ART is known to affect plasma kynurenine/tryptophan ratio [12,14,15]. The performance of the kynurenine/tryptophan ratio in ART-naive individuals is of particular importance, as PWH who have not yet started ART are at greatest risk of developing active TB [16]. Furthermore, the capacity of the kynurenine/tryptophan ratio for TB identification in individuals with paucibacillary forms of TB has not been studied.

We have previously explored host inflammatory mediators as markers for active TB in a cohort of ART-naive Ethiopian adults with HIV, in which all participants were investigated with bacteriological sputum analyses for TB at enrolment [17]. Here, we aimed to determine the performance of kynurenine/tryptophan ratio for TB case-finding in participants identified from this cohort. We also correlated kynurenine/tryptophan ratio to levels of several plasma inflammation mediators, as well as to plasma HIV RNA levels and $CD4^+$ cell count, as markers of HIV disease severity, and to BMI and mid-upper arm circumference (MUAC) as markers of malnutrition.

Materials and methods

Study participants

Participants for this study were identified from a prospective cohort of 812 PWH, recruited 2011–2013 with a follow-up for up to 4 years at five public health centers in and around the city of Adama, Ethiopia [18,19]. HIV-positive adults (age ≥ 18 years) were enrolled into the cohort if the following inclusion criteria were met: written informed consent, $CD4^+$ cell count 350 cells/μl or less and/or WHO stage 4, and ability to produce sputum for bacteriological TB investigations. Persons with previous ART experience and/or antituberculosis therapy (ATT) for more than 2 weeks prior to enrolment were excluded.

At inclusion, data on socioeconomic condition, medical history, and current symptoms were collected, and physical examination was performed. All cohort participants submitted two spontaneously expectorated morning sputum samples, which were analyzed with smear microscopy, liquid TB culture, and Gene Xpert MTB/RIF (Xpert) [19]. In case of peripheral lymphadenopathy, these analyses were also performed on fine-needle lymph node aspirates. Venous blood was obtained in EDTA tubes for $CD4^+$ cell count determination using flow cytometry (FACS Calibur; Becton Dickinson, Franklin Lakes, New Jersey, USA), and plasma was separated for HIV RNA quantification (Abbott Real-Time HIV-1 assay; Abbot Molecular Inc., Chicago, Illinois, USA) or Abbott m2000 RealTime System Automated molecular platforms (Abbott Molecular Inc., Des Plaines, Illinois, USA). Plasma aliquots were stored at −80°C, at the study laboratory in Adama and subsequently transported to Lund University, Sweden. Levels of chemokine (C-C motif) ligand 5 (CCL5), C-reactive protein (CRP), interleukins (IL)-6, -12p70,- 18, -27, IFN-γ induced protein-10 (IP-10), procalcitonin, and soluble urokinase-type plasminogen activator (suPAR) had previously been quantified using Luminex technology [17].

Participants were selected using a nested case–control design, with matching based on age and sex, as described in a previous project on inflammatory markers [17]. To be able to correlate kynurenine/tryptophan ratio to inflammatory markers, all participants from the previous study, with available stored plasma samples, were included in this study. This led to inclusion of 124 participants with HIV-TB coinfection (HIV+/TB+) and 125 PWH without TB (HIV+/TB-). In order to prevent misclassification due to prevalent active TB not identified at inclusion, HIV+/TB- participants were also required to remain in

care for at least 6 months after inclusion, with neither confirmed nor suspected TB during this time period; in addition, persons with clinically diagnosed TB (without bacteriological confirmation) at any time point were excluded from the current study.

Determination of tryptophan and kynurenine concentration

Tryptophan and kynurenine levels were determined using liquid chromatography-mass spectrometry (LC-MS) at RedGlead Discovery Laboratories, Lund, Sweden. In brief, protein precipitation was achieved by diluting plasma 1 : 3 in 10% trifluoracetic acid, followed by centrifugation at 10 000 g for 10 min and separation of the supernatant, which was then diluted 1 : 4 in water. A standard curve was constructed by spiking charcoal stripped plasma with the analytes of interest. Liquid chromatography was conducted using a Nexera 40 CXR autosampler, Nexera degassing unit, Nexera column oven, and Nexera LC-20AD pump (all Shimadzu, Kyoto, Japan). Mass spectrometry was conducted on SCIEX QTRAP4500 (SCIEX, Framingham, Massachusetts, USA) and analyzed with Analyst software 1.6.3. These analyses were performed after completion of inclusion and TB classification of all participants. The laboratory personnel performing these analyses were unaware of TB status of participants. Kynurenine/tryptophan ratio was obtained by dividing plasma concentration of kynurenine with concentration of tryptophan.

Data analysis and statistics

All data analysis was conducted in R version 4.0.1. Graphs and figures were constructed in the *ggplot2* package. The Mann–Whitney test was used for group comparisons of kynurenine, tryptophan, kynurenine/tryptophan ratio, and other continuous variables, while Pearson's chi-squared test was used to compare categorical variables. Receiver operator characteristic (ROC) curves were used to assess discriminatory capacity for all TB cases, as well as for TB cases detected by Xpert and/or smear-microscopy separately. These results were presented as area under the curve (AUC). Sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) were calculated for different cut-offs. Predictive values were calculated on the basis of the bacteriologically confirmed TB prevalence of 16.9% that was found in the entire cohort. Correlation to other plasma inflammation markers, $CD4^+$ cell count, plasma HIV RNA levels, BMI, and MUAC was assessed using the nonparametric Spearman's rank-order correlation. For the correlation analysis between kynurenine/tryptophan ratio and other inflammation markers, the Holm-Bonferroni method of adjusting for multiple testing was applied.

Ethics statement

Ethical approval was obtained from the National Research Ethics Review Committee at the Ministry of Science and Technology of Ethiopia (3.10/825/05) and the Regional Ethical Review Board at Lund University, Sweden (2010/672).

Results

Study participant characteristics

The majority of study participants were men (132/249; 53%), and the median age for both HIV+/TB+ and HIV+/TB- was 35 years (Table 1). HIV+/TB+ had lower median $CD4^+$ cell counts and higher plasma HIV RNA levels than HIV+/TB- (171 vs. 224 cells/μl [$P < 0.01$] and 5.3 vs. 5.0 $\log_{10}$ copies/ml, respectively [$P = 0.03$]). Among the 124 HIV+/TB+ individuals, 113 had pulmonary TB, nine had concomitant pulmonary and lymph node TB, and two individuals had isolated TB lymphadenitis. Among the 122 cases of pulmonary TB, 27 (22%) were smear-positive, 79 (65%) were Xpert positive (including 26/27 smear-positive cases) and 111 (90%) were culture positive (of whom 68/111 [61%] were Xpert positive).

Tryptophan and kynurenine levels in participants with and without active tuberculosis

Tryptophan levels were lower in HIV+/TB+ than in HIV+/TB- (median 19.5 vs. 29.8 μmol/l, $P < 0.01$; Fig. 1a), while kynurenine levels were similar between these groups (median 2.95 vs. 2.94 μmol/l, $P = 0.62$; Fig. 1b). The kynurenine/tryptophan ratio was higher in HIV+/TB+ compared with HIV+/TB- (median 0.15 and 0.11, respectively, $P < 0.01$; Fig. 1c). Persons with Xpert-positive pulmonary TB had higher median kynurenine/tryptophan ratios than those for whom TB was only detected by culture (0.16 vs. 0.13; $P = 0.047$; Fig. 1d).

In the total study population, the AUC for identification of active TB using kynurenine/tryptophan ratio was 0.70, which was slightly higher than the AUC for tryptophan alone (AUC: 0.67; Fig. 2a). For the subset of HIV+/TB+ individuals who were Xpert-positive and/or smear positive in sputum, the AUC of kynurenine/tryptophan ratio (in comparison to HIV+/TB-) was 0.75 and for tryptophan alone 0.70 (Fig. 2b).

Different kynurenine/tryptophan ratio threshold levels were assessed for performance for TB identification. A previously suggested kynurenine/tryptophan ratio cut-off of 0.08 [8] resulted in 94% sensitivity, 32% specificity, negative predictive value of 96%, and positive predictive value of 22%. Increasing the cut-off to 0.1 led to 82% sensitivity, 47% specificity, negative predictive value of 93%, and positive predictive value of 24%. A cut-off of 0.12 resulted in 73% sensitivity, 61% specificity, negative predictive value of 92%, and a positive predictive value of 28% (Table 2). Thus, these findings indicate that the kynurenine/tryptophan ratio only has a modest

Table 1. Characteristics of 249 ART-naive adult people with HIV classified for active tuberculosis.

	HIV+/TB+	HIV+/TB-	*P*
Female	61/124 (49)	56/125 (45)	0.49
Age (years)	35 (28–42)	35 (28–41)	0.84
MUAC[a] (cm)	21 (20–23)	23 (21–25)	<0.01
BMI[b] (kg/m^2)	17.7 (16.3–19.7)	19.3 (17.7–21.9)	<0.01
Hemoglobin (g/dl)	10.4 (9.1–11.6)	11.9 (10.9–13.1)	<0.01
CD4$^+$ cell count (cells/μl)	171 (94–267)	224 (147–329)	<0.01
HIV RNA (log_{10} copies/ml)	5.3 (4.7–5.6)	5.0 (4.5–5.5)	0.03
WHO-TB symptom screening[c]	115/124 (93)	97/125 (78)	<0.01
On TB treatment at enrolment[d]	15/130 (12)	0 (0)	
Sputum smear positive[e]	27/123 (22)	0/124 (0)	
Sputum Xpert positive	79/123 (64)	0/124 (0)	
Sputum culture positive	111/123 (90)	0/124 (0)	
Lymph node culture positive	3/4 (75)	0/3 (0)	
Lymph node Xpert positive	10/11 (91)	0/1 (0)	

Categorical values presented as fractions of total with percentage in brackets, continuous variables presented as median values with interquartile range in brackets. *P* value indicating Pearson's Chi-squared test for categorical variables and the Mann–Whitney *U* test for continuous variables.
[a]Mid-upper arm circumference.
[b]BMI.
[c]Presence of cough, weight loss, night sweating, or fever.
[d]Median number of days on treatment prior to enrolment: 7.
[e]Of smear-positive participants, all but one were also Xpert positive.

performance for TB case-finding in ART-naive PWH, as the specificity for the previously suggested cut-off is low.

Kynurenine/tryptophan ratio in relation to HIV RNA levels, CD4$^+$ cell count, and BMI

To assess the impact of HIV-related factors on kynurenine/tryptophan ratio, we correlated kynurenine/tryptophan ratio to plasma HIV RNA levels and CD4$^+$ cell count (Fig. 3). RNA levels and kynurenine/tryptophan ratio showed a positive correlation in the total population ($\rho = 0.35$, $P < 0.01$), as well as in HIV+/TB- and HIV+/TB+ analyzed separately ($\rho = 0.27$ and 0.37, respectively, both $P < 0.01$; Fig. 3a). kynurenine/tryptophan ratio was inversely correlated to CD4$^+$ cell count ($\rho = -0.36$, $P < 0.01$), also when assessed separately in HIV+/TB- ($\rho = -0.31$, $P < 0.01$) and HIV+/TB+ ($\rho = -0.26$, $P < 0.01$; Fig. 3b).

Furthermore, we assessed the impact of malnutrition on kynurenine/tryptophan ratio. Kynurenine/tryptophan ratio was inversely correlated to BMI ($\rho = -0.41$, $P < 0.01$), in both HIV+/TB+ individuals ($\rho = -0.36$, $P < 0.01$) and HIV+/TB- individuals ($\rho = -0.30$, $P < 0.01$; Fig. 3c). In addition, MUAC, commonly used as a marker of wasting, was inversely correlated in both HIV+/TB+ individuals ($\rho = -0.35$, $P < 0.01$) and HIV+/TB- individuals ($\rho = -0.38$, $P < 0.01$; Fig. 3d). As can be observed in Supplementary figure 1, http://links.lww.com/QAD/C482, the correlation between kynurenine/tryptophan ratio and these parameters is mainly explained by tryptophan depletion. Taken together, these analyses suggest that the correlations between kynurenine/tryptophan ratio and markers of HIV progression and malnutrition may contribute to its low specificity for TB identification in ART-naive PWH with poor nutrition status.

Kynurenine/tryptophan ratio in relation to inflammatory cytokines and acute-phase reactants mediators

In order to explore the potential relationship between kynurenine/tryptophan ratio and systemic inflammation, we assessed correlations to levels of inflammatory cytokines and acute-phase reactants, which previously have been investigated for their capacity as biomarkers for active TB among PWH [17]. For both HIV+/TB- and HIV+/TB+, the strongest correlation with kynurenine/tryptophan ratio was found for IP-10 ($\rho = 0.58$ and 0.47, respectively, both $P < 0.01$; Supplementary Figure 2a and 2b, http://links.lww.com/QAD/C482). Similarly, IL-18 and IL-27 were significantly correlated to kynurenine/tryptophan ratio, in the HIV+/TB+ group ($\rho = 0.37$ and $\rho = 0.31$, respectively) as well as in the HIV+/TB- group ($\rho = 0.41$ and $\rho = 0.3$; all $P < 0.01$).

CRP ($\rho = 0.24$), IL-6 ($\rho = 0.32$), procalcitonin ($\rho = 0.35$), and suPAR ($\rho = 0.35$) levels were positively correlated to kynurenine/tryptophan ratio in HIV+/TB+ participants ($P < 0.01$; Supplementary figure 2a, http://links.lww.com/QAD/C482), whereas no significant correlations were found in HIV+/TB- individuals (Supplementary figure 2b, http://links.lww.com/QAD/C482). CCL5 and IL-12p70 were not correlated to kynurenine/tryptophan ratio in either of these groups. In summary, correlations between kynurenine/tryptophan ratio and inflammation markers were noted in both the HIV+/TB- and HIV+/TB+ groups.

Discussion

In this nested case–control study of Ethiopian HIV-positive adults subjected to intensified TB case-finding

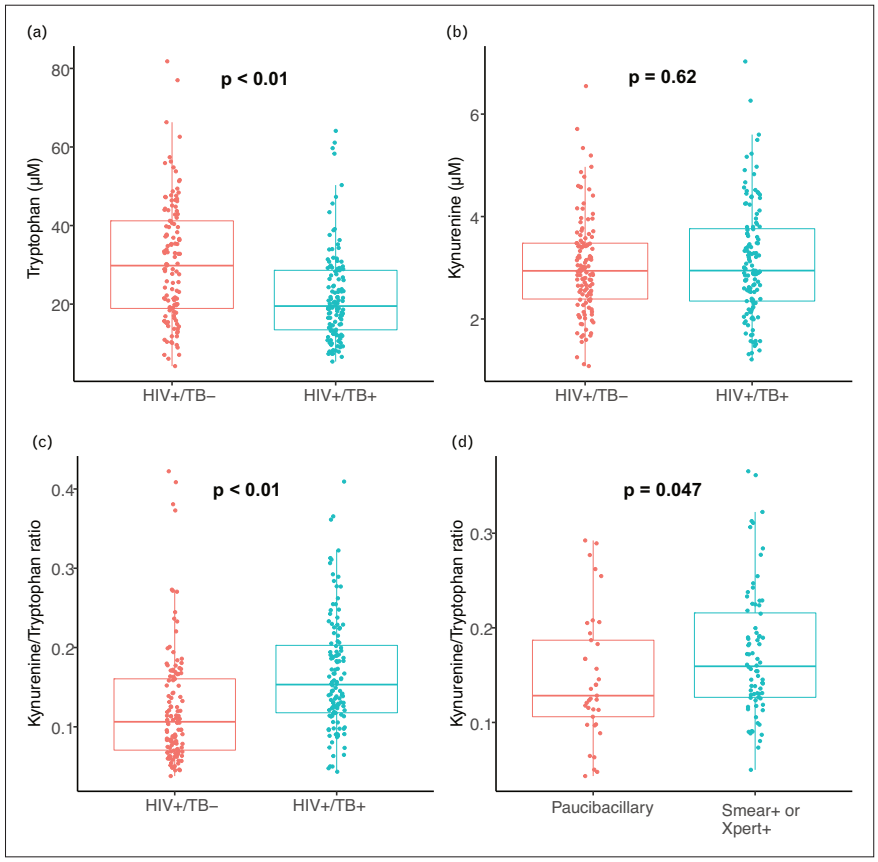

Fig. 1. (a) Tryptophan levels in HIV+/TB+ and HIV+/TB- participants. (b) Kynurenine levels in HIV+/ TB+ and HIV+/TB- participants. (c) Ratio between kynurenine and tryptophan in HIV+/TB+ and HIV+/ TB participants. (d) Ratio between kynurenine and tryptophan in HIV+/TB+ participants, divided into paucibacillary (negative Xpert and smear microscopy) and Xpert and/or smear microscopy positive (no patients with lymph node TB shown).

before ART initiation, individuals with bacteriologically confirmed TB had significantly higher kynurenine/tryptophan ratio than that observed in persons without active TB. However, overall discriminatory capacity for TB was rather low, as the control group of HIV mono-infected individuals also had high levels of kynurenine/tryptophan ratio. This yielded low specificity and positive predictive value for TB.

The finding of elevated kynurenine/tryptophan ratio in PWH with active TB is in accordance with previous data from South Africa [8]. However, in contrast to findings reported by Adu-Gyamfi *et al.* [8], the performance of kynurenine/tryptophan ratio for TB case finding was modest in our study population. Whereas kynurenine/tryptophan ratios among persons with TB were of a similar magnitude as those observed in the South African study (0.15 vs. 0.135), participants with HIV mono-infection had higher kynurenine/tryptophan ratio in our cohort (0.11 vs. 0.02). Given our stringent criteria for exclusion of active TB, it is unlikely that the relatively high kynurenine/tryptophan ratios were due to unrecognized TB. Instead, our data imply that the high kynurenine/tryptophan ratios in this group are explained by higher HIV plasma RNA levels and lower $CD4^+$ cell counts, which in turn can be related to the fact that all participants in our study were ART-naive at the time of sampling. In contrast, around 40% of participants in the South African cohort were sampled during ART.

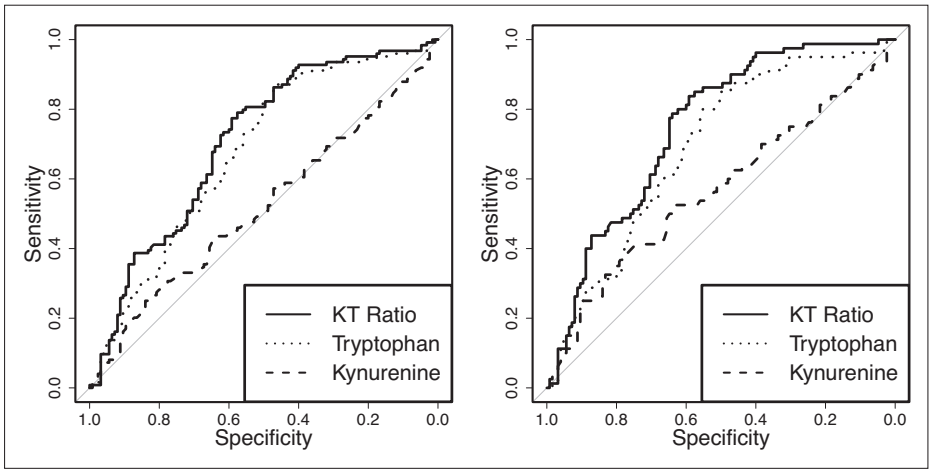

Fig. 2. Receiver operator characteristic curves for kynurenine, tryptophan, and the ratio between kynurenine and tryptophan. (a) Performance for all TB cases. (b) Performance for sputum Xpert and/or smear microscopy positive individuals.

In order to assess the potential value of kynurenine/tryptophan ratio for TB screening among PWH, it is important to consider that HIV infection *per se* can induce conversion of tryptophan to kynurenine [20]. Several studies of ART-naive PWH in sub-Saharan Africa have reported kynurenine/tryptophan ratios similar to, or higher, than those found in our participants. For example, in South African and Ugandan ART-naive PWH, the median kynurenine/tryptophan ratios were 0.16 [21] and 0.13 [22], respectively. Furthermore, several studies show significant reductions of kynurenine/tryptophan ratio in individuals on ART [12,14,15], although kynurenine/tryptophan ratios appear to remain elevated in PWH receiving ART compared with those of HIV-negative individuals, also in those starting ART early after seroconversion [23].

Different mechanisms may be responsible for the high kynurenine/tryptophan ratios observed in ART-naive PWH. HIV-triggered systemic inflammation is likely to play an important role; for example, interferon-γ induces IDO-1 activity, resulting in increased conversion of tryptophan to kynurenine [24]. In order to explore these interactions, we correlated kynurenine/tryptophan ratios to plasma levels of different inflammatory mediators. The contribution of the interferon-γ pathway to elevated kynurenine/tryptophan ratio is supported by the significant correlations found for IL-18, IL-27, and IP-10, all of which are mediators in this pathway [25–27].

Table 2. Sensitivity, specificity, negative, and positive predictive value in percentage for different cut-offs.

Performance for all HIV+/TB+ cases				
Cut-off	Sensitivity	Specificity	NPV	PPV
0.08	94	32	96	22
0.10	82	47	93	24
0.12	73	61	92	28

Apart from active TB and HIV-related factors, nutritional status may influence kynurenine/tryptophan ratio, with reduced tryptophan levels in malnourished individuals [28]. Inadequate nutrition could therefore partly explain the high kynurenine/tryptophan ratios found in both HIV+/TB- and HIV+/TB+ participants (median BMI 19.3 and 17.7, respectively), and we observed significant negative correlation between kynurenine/tryptophan ratio and BMI in both groups. However, some data suggest that systemic inflammation in advanced HIV infection confers a catabolic state [29]. This could also be a mechanism linking wasting and kynurenine/tryptophan ratio elevations in PWH. This circumstance further limits the utility of kynurenine/tryptophan ratio as a TB marker in PWH, especially in low-income countries, where wasting is a prominent feature of various HIV-related disease conditions. PWH in low-income countries have consistently been found to have higher kynurenine/tryptophan ratios than in high-income countries [21].

Somewhat surprisingly, the difference in kynurenine/tryptophan ratio reported in this study was entirely due to tryptophan depletion, while kynurenine levels were similarly high in both groups. Many studies report both tryptophan depletion and kynurenine elevation influencing altered kynurenine/tryptophan ratio [7]. However, similar to our findings, the only previous study on HIV-TB coinfection that also reported levels of kynurenine

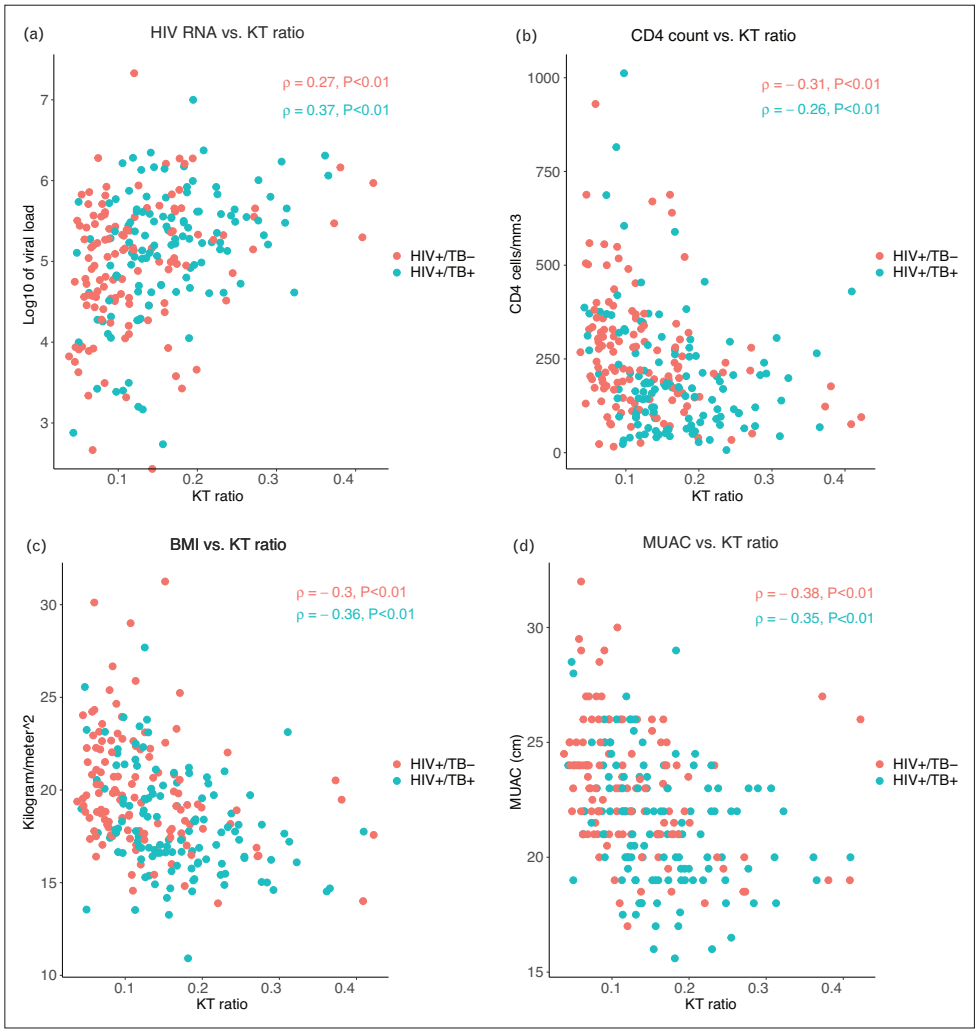

Fig. 3. Correlation between KT ratio and (a) HIV RNA levels, (b) CD4$^+$ cell count, (c) BMI, and (d) mid-upper arm circumference (MUAC). HIV+/TB- individuals illustrated by red dots, HIV+/TB+ by light blue. Spearman's rho and *P* value from Spearman's rank-order correlation indicated in each corner.

and tryptophan levels separately found a minimal difference in kynurenine between individuals with active and latent TB [13], supporting that elevated kynurenine/tryptophan ratio in HIV-TB coinfection is mainly due to tryptophan depletion. The enzyme kynurenine monooxygenase that metabolizes kynurenine is, like IDO-1, activated by interferon-γ [10]. It is possible that this enzyme activation counteracts the kynurenine increase one might expect from HIV and TB-driven IDO-activation. The immunologic effects of tryptophan metabolism on TB and HIV pathogenesis are however not entirely dependent on kynurenine, as metabolites further downstream the kynurenine pathway, such as 3′-hydroxyanthranilic acid, also exert immunosuppressive function in these conditions [30,31]. Although beyond the scope of this study, investigation of how other metabolites in the kynurenine pathway are involved in HIV-TB coinfection is warranted.

In several previous studies on kynurenine/tryptophan ratio in PWH, data on ART and TB status have not been reported in detail, factors which we believe are critical for interpretation. For this study, we selected both cases and controls from a large cohort of ART-naive HIV-positive adults recruited at Ethiopian health centers. Importantly, all participants had been subjected to bacteriological TB case-finding, including liquid culture, at enrolment, which led to detection of active TB also in persons without clinically suspected TB, as well as paucibacillary cases of pulmonary TB. Furthermore, this allowed us to determine the capacity of kynurenine/tryptophan ratio for TB identification in relation to bacillary burden, with lower performance observed for cases of pulmonary TB only detected by culture.

Investigations for active TB were mainly focused on pulmonary TB, and cases of extrapulmonary TB could potentially have been missed. To account for this, and to reduce the risk of misclassification, HIV+/TB- participants for this study were required not to have clinically suspected TB at enrolment, and to remain in follow-up for at least 6 months without confirmed nor suspected incident TB. Cohort participants were not systematically assessed for opportunistic infections other than TB, and we cannot exclude that such conditions may have affected kynurenine/tryptophan ratio.

In conclusion, ART-naive PWH with concomitant active TB had significantly higher kynurenine/tryptophan ratio than individuals without active TB. However, due to high kynurenine/tryptophan ratios in persons with HIV mono-infection, kynurenine/tryptophan ratio had low specificity for identification of active TB in PWH. Apart from association with active TB, kynurenine/tryptophan ratio showed significant correlations with HIV plasma RNA levels, $CD4^+$ cell count, MUAC and BMI, indicating an impact on kynurenine/tryptophan ratios by factors not only related to TB coinfection. Consequently, we conclude that kynurenine/tryptophan ratio does not show promise as a marker for TB screening in ART-naive PWH in sub-Saharan Africa.

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Conflicts of interest

The authors have no conflicts of interest to report.

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Paper III

frontiers in Microbiology

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Expression of MicroRNAs Is Dysregulated by HIV While *Mycobacterium tuberculosis* Drives Alterations of Small Nucleolar RNAs in HIV Positive Adults With Active Tuberculosis

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HIV infection affects the course of tuberculosis (TB), and HIV and *Mycobacterium tuberculosis* (Mtb) synergize in disease progression through complex immunological interplay. To gain further understanding of these mechanisms, we compared the microRNA (miRNA) and small nucleolar RNA (snoRNA) expression patterns in whole blood of individuals with active TB, with and without HIV coinfection (HIV+/TB+ and HIV-/TB+), and HIV and TB-negative individuals (HIV-/TB-). We found that 218 miRNAs were differentially expressed between HIV+/TB+ and HIV-/TB+, while no statistically significant difference in snoRNA expression was observed between these groups. In contrast, both miRNA (n = 179) and snoRNA (n = 103) expression patterns were significantly altered in HIV+/TB+ individuals compared to those of the HIV-/TB- controls. Of note, 26 of these snoRNAs were also significantly altered between the HIV-/TB+ and HIV-/TB- groups. Normalization toward the miRNA and snoRNA expression patterns of the HIV-/TB- control group was noted during anti-TB and antiretroviral treatment in HIV+/TB+ participants. In summary, these results show that HIV coinfection influences miRNA expression in active TB. In contrast, snoRNA expression patterns differ between individuals with and without active TB, independently of HIV coinfection status. Moreover, in coinfected individuals, therapy-induced control of HIV replication and clearance of Mtb appears to normalize the expression of some small non-coding RNA (sncRNA). These findings suggest that dysregulation of miRNA is a mechanism by

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which HIV may modify immunity against TB, while active TB alters snoRNA expression. Improved understanding of how regulation of sncRNA expression influences the disease course in coinfected individuals may have implications for diagnostics, risk stratification, and host-directed therapy. Here, we propose a novel mechanism by which HIV alters the immune response to TB.

Keywords: HIV, tuberculosis, small non-coding RNA, microRNA, small nucleolar RNA, anti-tuberculosis treatment, anti-retroviral therapy

INTRODUCTION

HIV infection is the strongest known risk factor for active tuberculosis (TB) (Ai et al., 2016). Furthermore, TB is the leading cause of death among people with HIV (PWH), causing one-third of HIV-related deaths globally (UNAIDS, 2019).[1] The pathogenesis of active TB is largely determined by interactions with different components of the immune system, and several such mechanisms have been shown to be involved in the dysregulated immune responses observed in HIV/TB coinfection (Pawlowski et al., 2012). Previous studies have described HIV-related alterations in apoptosis (Patel et al., 2007), antigen presentation (Singh et al., 2016), immune cell functionality (Kalsdorf et al., 2009), and cytokine release (Zhang et al., 1994) in the response to TB.

Small non-coding RNAs (sncRNAs), like microRNAs (miRNAs) and small nucleolar RNAs (snoRNAs), have been implicated in regulating immune responses (Okuyama et al., 2014). miRNAs can affect both innate and adaptive immune responses through post-transcriptional regulation of a vast number of protein-coding genes by suppressing mRNAs, including those encoding proinflammatory cytokines (O'Connell et al., 2010). In addition, several bacteria and viruses, including Mtb and HIV, modify inflammatory responses via miRNA dysregulation (Drury et al., 2017). Studies have demonstrated mechanisms by which HIV (Kapoor et al., 2015) and Mtb (Sahu et al., 2017) manipulate specific miRNAs to evade the immune response. In contrast, snoRNAs regulates protein synthesis through post-transcriptional chemical modifications of rRNA (Cao et al., 2018). snoRNA expression is essential for the infectivity of several viruses (Stamm and Lodmell, 2019). Moreover, snoRNA expression differs among individuals with active and latent TB infection (de Araujo et al., 2019). However, the impact of HIV coinfection on the expression of these sncRNAs in individuals with active TB has not been explored.

Thus, to investigate the role of sncRNAs in the pathogenesis of active TB in PWH, we analyzed expression patterns of miRNAs and snoRNAs in whole blood samples of individuals with active pulmonary TB, with or without HIV coinfection (HIV+/TB+ and HIV-/TB+, respectively), and in HIV-negative individuals without TB infection (HIV-/TB-). Furthermore, to explore the effects of antiretroviral (ART) and antituberculosis (ATT) treatments on sncRNA expression, we studied miRNA and snoRNA expression patterns longitudinally before and after therapy initiation.

MATERIALS AND METHODS

Selection of Study Participants and Clinical Sampling

Participants for this study were recruited and followed at public health centers providing TB and HIV care in and around the city of Adama, Oromia Region, Ethiopia, between 2011 and 2015. Three groups of participants were included in this study. First, HIV-positive adults (age $\geq$18 years) with pulmonary TB (HIV+/TB+) were selected from a cohort of PWH who had been bacteriologically characterized for active TB (Balcha et al., 2014). These participants were required to have bacteriologically confirmed pulmonary TB (by liquid culture, direct microscopy, and/or Xpert MTB/RIF assay), to not have received ATT within the preceding six months, to not currently be receiving ATT for more than two weeks, and to not have received ART. ATT was initiated when a positive bacteriological result was known and subsequently also non-nucleoside transcriptase-inhibitor-based ART, both following the Ethiopian guidelines (Federal HIV/AIDS Prevention and Control Office Federal Ministry of Health, 2008).[2] Second, HIV-negative adults with pulmonary tuberculosis (HIV-/TB+), diagnosed by sputum smear microscopy or clinical criteria, were identified at TB clinics at the same health facilities. The exclusion criteria applied were confirmed or suspected extrapulmonary TB, ATT within the previous 6 months and/or treatment for the current episode of TB for $>$ 2 weeks, and presence of a chronic disease. Third, healthy HIV-negative subjects without active or latent TB infection (HIV-/TB-) were recruited during voluntary counseling at HIV testing clinics (at the same facilities). To exclude TB infection, a negative result for QuantiFERON Gold-in-Tube test ($<$0.35 IU/mL), and the absence of symptoms suggestive of active TB were required. Blood samples were collected from all participants at the time of enrollment, and additional sampling was scheduled at six months after the initiation of ATT for HIV-/TB+ subjects and initiation of ART for HIV+/TB+ subjects.

Venous blood samples were collected in syringes and directly transferred to PAXgene® Blood RNA Tubes (BD Biosciences, San Jose, CA, United States). PAXgene tubes were subsequently frozen at −80°C and transported with an intact cold chain

[1] https://www.unaids.org/sites/default/files/media_asset/tuberculosis-and-hiv-progress-towards-the-2020-target_en.pdf

[2] https://www.who.int/hiv/pub/guidelines/ethiopia_art.pdf

TABLE 1 | Study participant characteristics[a].

	HIV+/TB+[b]	HIV-/TB+[c]	HIV-/TB-
Participants	13	13	14
Female	9 (69)	10 (77)	12 (86)
Age (years)	32 (28–45)	28 (23–35)	23 (20–27)
$CD4^+$ cell count (cells/μL)	270 (157–328)	639 (494–842)	720 (671–828)
Viral load (log_{10} copies/mL)	5.1 (4.6–5.5)	NA	NA
Hemoglobin (g/dL)	11.8 (10.7–12.4)	12.1 (11.6–12.9)	14.3 (12.6–15.1)
Lymphocyte count (cells/μL)	1,600 (1,100–2,500)	1,400 (1,300–1,650)	1,900 (1,600–2,600)
Neutrophil count (cells/μL)	3,100 (1,900–3,700)	7,000 (3,400–9,825)	3,400 (2,650–4,025)
Platelet count (cells/mL)	242 (195–267)	396 (356–470)	234 (214–265)

[a]*Categorical variables are shown as absolute numbers, with the percentage of the total number given in brackets; continuous variables are given as a median value, with interquartile range in brackets.*
[b]*Of the HIV+/TB+ participants (three were sputum microscopy positive, nine were GeneXpert positive, and one was only culture positive).*
[c]*Among the HIV–/TB+ participants, nine were sputum microscopy positive and four were diagnosed based on clinical criteria.*
NA, not applicable.

to Lund University, Sweden, for further analysis. In addition, blood samples were collected for determining total blood count (Sysmex KX-21; Sysmex Corporation, Kobe, Japan) and $CD4^+$ cell count by flow cytometry (FACS Calibur; Becton Dickinson, Franklin Lakes, NJ, United States) for all participants and for viral load determination in HIV+/TB+ patients. Plasma viral load was determined using the Abbott m 2000rt RealTime system (lower detection limit 40 RNA copies/mL; Abbott, Chicago, IL, United States).

sncRNA Expression Analysis Using Microarray

RNA was extracted from whole blood using the PAXgene Blood miRNA Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentrations and purity were assessed using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States). Samples with low RNA concentrations (< 30 ng RNA/μl) were concentrated using a SpeedVac vacuum concentrator. Before microarray analysis, RNA integrity was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies). The RNA integrity number was calculated based on 28S and 18S rRNA peaks and the absence of degradation products. Samples with RNA integrity numbers > 7 were included in the further analysis. Samples (370 ng of RNA) were analyzed for sncRNA expression on the Affymetrix Platform, GeneChip® miRNA 4.0 Array (Affymetrix, Thermo Fisher Scientific, Waltham, MA, United States) using 4603 probes for human miRNA (including mature miRNA and stem-loop precursors) and 1996 probes for human snoRNA (including snoRNA, CDBox, HAcaBox, and small Cajal-body RNA (scaRNA) at the Swegene Centre for Integrative Biology (SCIBLU), Lund University, Sweden.

Determination of miRNA and snoRNA Expression Using Quantitative PCR

For cDNA synthesis, 4 ng/μL RNA template was poly-A-tailed with *E. Coli* poly-A polymerase and reverse-transcribed using MuLV reverse transcriptase (both enzymes were obtained from New England Biolabs Inc., Ipswich, MA, United States). miRNA-39 from *C. elegans* was spiked in as an internal control. PCR was performed with cDNA at 1/20 dilution, using forward and reverse primers (for primer sequences see **Supplementary Table 1**), designed using the miRprimer software (Busk, 2014). SYBR Green dye (Applied Biosystems™, Thermo Fisher Scientific, Waltham, MA, United States) was used for quantitation. RT-qPCR was performed on the StepOnePlus™ real-time PCR system (Applied Biosystems™). The reaction mixture was heated to 95°C for 15 min and then the temperature was altered between 95°C (15 s) and 60°C (30 s) for 45 cycles. All reactions for each sncRNA were carried out in a single 96-well plate to minimize technical variation. Melting curves from 60 to 99°C were determined to ensure specificity. Each plate included two negative controls, one without template and one without reverse transcriptase. Each plate included a positive control sample of human reference RNA.

Data Analysis and Statistics

All data analyses were performed using RStudio version 1.3.959 (Boston, MA, United States). The microarray results were normalized using the robust multi-array average method (Irizarry et al., 2003), and the data were log_2 transformed. The microarray chip contained probes for 6599 human sncRNA targets. Probes that generated detection above background $P > 0.01$ in > 20% of samples were filtered out. In the cases where several probes represented the same RNA, results from the probe with the highest expression values were chosen. miRNAs that had been removed from the miRBase database were removed from further analysis. This filtering rendered inclusion of 674 sncRNAs for further analysis. Differential expression of miRNAs and snoRNAs was assessed using the R package Limma (Ritchie et al., 2015). sncRNAs with the Benjamini-Hochberg false discovery rate (FDR) was less than 0.05, were considered significantly differentially expressed. The microarray data were analyzed using principal component analysis (PCA), in which the data were median centered, and all samples were plotted on a graph with principal components 1 and 2 as the axes. Differences between the

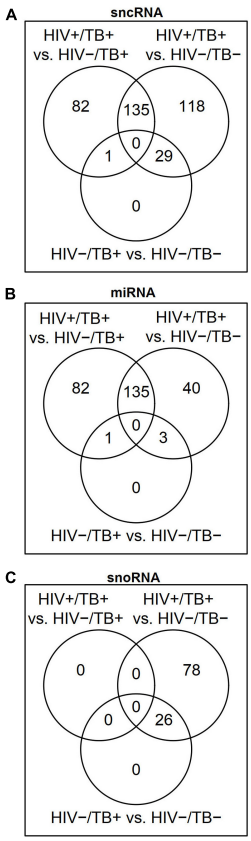

FIGURE 1 | Venn diagrams showing differentially expressed (FDR < 0.05) **(A)** small non-coding RNAs (sncRNAs), **(B)** microRNAs (miRNAs), and **(C)** small nucleolar RNAs (snoRNAs) in the HIV+/TB+, HIV-/TB+ and HIV-/TB- groups.

groups in principal components 1 and 2 were assessed using Hotelling's T-squared test. In addition, hierarchical clustering was performed, for the 20 most differentially expressed (smallest adjusted *P*-value) from each contrast, using the Pearson correlation as the distance measure, and average linkage to build clusters.

For the RT-qPCR, we used exogenous spike-in cel-miR-39 as an internal reference. Fold change values were calculated using the double delta method, and statistical significance was assessed using the Mann-Whitney U test for the comparisons between groups before treatment and Wilcoxon signed-rank test for the comparisons of paired samples before and after treatment. Holm-Bonferroni correction was applied to adjust for multiple testing (correcting for the number of RNAs that were tested in each group comparison). For easy interpretation, the *P*-values were adjusted rather than the significance threshold, and all *P*-values reported in the qPCR results section were adjusted using the Holm-Bonferroni method. To assess how different RNAs correlated with $CD4^+$ cell count, Spearman's rank correlation analysis was performed with Holm-Bonferroni correction for number of tests.

RESULTS

Participant Characteristics

A total of 40 participants were included [HIV+/TB+ (n = 13), HIV-/TB+ (n = 13), and HIV-/TB- (n = 14)], and the majority were women, irrespective of infection status (**Table 1**). At inclusion, all HIV+/TB+ subjects were ART naïve. The median baseline viral load was 5.1 $\log_{10}$ RNA copies/mL and the median $CD4^+$ cell count was 270 cells/μl in coinfected individuals. In 12 HIV+/TB+ subjects, ART was started after ATT (median 39 days, range 26–141 days), and in one of these subjects, ATT was started after ART (25 days).

Differential Expression of Both miRNAs and snoRNAs in HIV-Infected Individuals With Active Tuberculosis Revealed by Microarray Analysis

To analyze the differential sncRNA expression between HIV+/TB+, HIV-/TB+, and HIV-/TB-, we performed microarray analysis on whole blood samples obtained from seven HIV+/TB+, eight HIV-/TB+, and eight HIV-/TB- subjects. A total of 218 sncRNAs were differentially expressed [false discovery rate (FDR) < 0.05] between HIV+/TB+ and HIV-/TB+ groups (**Figure 1A** and **Supplementary Table 2**). All of these sncRNAs were either mature miRNA (n = 206) or miRNA precursors (n = 12, **Figure 1B**). In contrast, none of the snoRNAs were differentially expressed between these groups (**Figures 1C**, **2A**). Although the majority of the miRNAs differentially expressed between HIV+/TB+ and HIV-/TB+ were upregulated in HIV+/TB+ subjects, the downregulated miRNAs generally showed a larger magnitude in fold-change (**Figure 2A**). For example, 22 miRNAs with downregulated expression exhibited a greater than three-fold change, while none of the miRNAs with upregulated expression displayed such a change. Moreover, a larger proportion [79/96 (82%)] of the miRNAs with significantly downregulated expression had high annotation confidence (according to miRbase[3], the primary miRNA sequence repository), indicating lower rates of false-positive miRNA (Alles et al., 2019), compared to miRNAs with upregulated expression [16/122 (13%)]. Taken together, these results indicate that miRNA expression is significantly altered by HIV coinfection in individuals with active TB.

Besides sncRNAs that were differentially expressed between HIV+/TB+ and HIV-/TB+, 282 sncRNAs, including 179 miRNAs, were differentially expressed comparing the HIV+/TB+

[3]https://mirbase.org/

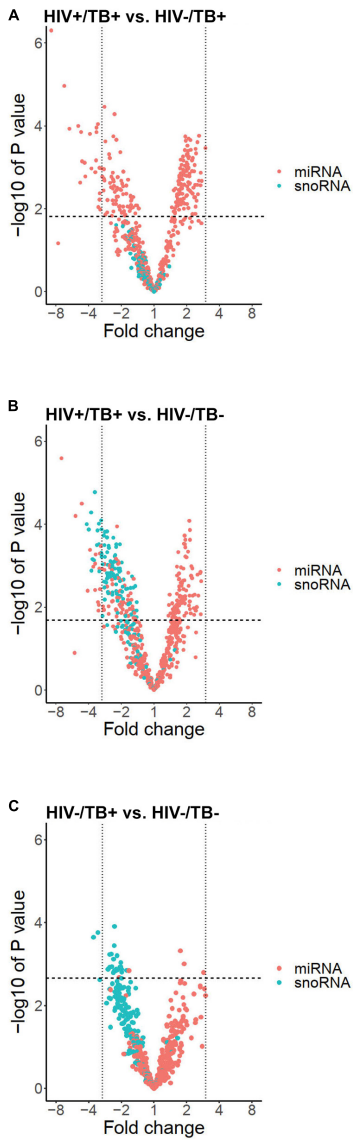

FIGURE 2 | Volcano plots illustrating expression profiles of miRNAs (red dots) and snoRNAs (blue dots) according to fold change on a logarithmic scale (x-axis) and -log of *P*-value (y-axis) in the groups **(A)** HIV+/TB+ vs. HIV-/TB+, **(B)** HIV+/TB+ vs. HIV-/ TB-, and **(C)** HIV-/TB- and HIV-/TB+. Vertical black lines indicate a fold change of three. Horizontal line indicates largest *P*-value generating FDR < 0.05.

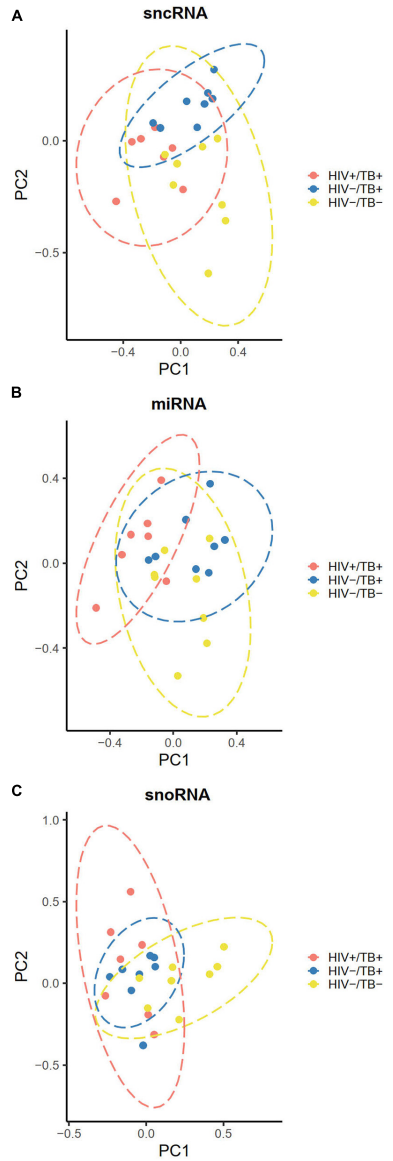

FIGURE 3 | Principal component plots illustrating clustering of the participants according to their infection status HIV+/TB+ (red), HIV-/TB+ (blue) and HIV-/TB- (yellow) based on **(A)** all sncRNAs **(B)** miRNAs **(C)** snoRNAs. Data ellipses calculated based on the t-statistic with 95% confidence intervals. Microarray data were median centered before analysis.

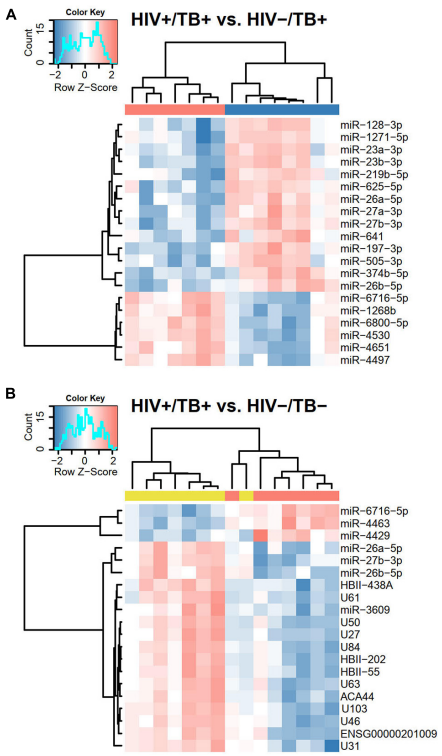

FIGURE 4 | Heatmaps based on hierarchical clustering analyses including **(A)** HIV+/TB+ and HIV-/TB+ and **(B)** HIV-/TB- and HIV+/TB+ subjects and the 20 sncRNAs that generated the smallest *P*-values in Limma analysis from the respective comparisons. Microarray data were median centered before the analysis. Pearson's correlation was used as a distance matrix. The red and blue colors indicate upregulated and downregulated sncRNAs, respectively. Color codes at the top represent infection status, HIV+/TB+ (red), HIV-/TB+ (blue), and HIV-/TB- (yellow). Probe HBII-438A also assigned to HBII-438B, probe U84 to ENSG00000263442/ENSG00000264591/ENSG00000265325/ENSG00000265607/ENSG00000266646/ENSG00000266755, probe ACA44 to ENSG00000252840, and probe U103 to U103B.

and the control HIV-/TB- subjects (**Figures 1A,B** and **Supplementary Table 2**). An overlap (n = 135) in miRNAs differentially expressed between HIV+/TB+ group and HIV-/TB+ or HIV-/TB- groups, was noted, as illustrated by the Venn diagram (**Figure 1B**). Differential expression was in the same direction for HIV+/TB+ compared to both HIV-/TB+ and HIV-/TB- for all these miRNAs. In contrast to the HIV+/TB+ vs. HIV-/TB+ comparison, snoRNA (n = 103) expression differed between HIV+/TB+ and HIV-/TB- (**Figures 1C**, **2B**). All differentially expressed snoRNAs were downregulated in HIV+/TB+ subjects (**Figure 2B**). In the HIV-/TB+ vs. HIV-/TB- comparisons, the 30 differentially expressed sncRNAs were dominated by the 27 snoRNAs (**Figures 1A–C**), which were also significantly downregulated (**Figure 2C**). This pattern suggests that active TB predominantly downregulates snoRNA expression, and that this effect is more pronounced in HIV-positive individuals.

Clustering of Individuals Based on sncRNA Expression

To explore potential clustering of participants based on sncRNA expression profiles, we performed PCA and assessed group differences using Hotelling's T-squared test. When both miRNA and snoRNA expressions were used for the PCA, significant separation of all three groups based on the sncRNA expression was observed (adjusted $P < 0.01$) (**Figure 3A**). The separation pattern was less distinct when only miRNA expression was considered for the PCA. The HIV+/TB+ group was significantly different from the other groups (adjusted $P < 0.01$), whereas the separation of the HIV-/TB- and HIV-/TB+ groups was not statistically significant (adjusted $P = 0.07$, **Figure 3B**). When PCA was carried out for snoRNAs alone, the pattern was markedly different, with no separation of the HIV+/TB+ and HIV-/TB+ groups (adjusted $P = 0.7$), while both these groups were significantly different from the HIV-/TB- group (adjusted $P < 0.01$) (**Figure 3C**).

Next, we performed hierarchical clustering of the 20 sncRNAs that generated the lowest *P*-values in pairwise comparisons. With one exception, distinct clustering according to HIV and TB infection status was observed (**Figures 4A,B** and **Supplementary Figure 1**). All 20 sncRNAs that showed most significant differential expression between HIV+/TB+ and HIV-/TB+ were miRNAs (**Figure 4A**). In contrast, in the comparison between HIV+/TB+ and HIV-/TB- (**Figure 4B**) and the comparison between HIV-/TB+ and HIV-/TB- groups (**Supplementary Figure 1**), 13 and 18, respectively, of the 20 most significantly differentially expressed sncRNAs were snoRNAs, and all of these were expressed at lower levels in the subjects with active TB. These analyses support the finding that HIV predominantly affects miRNA expression, whereas TB alters the expression of snoRNA.

Validation of Differential Expression of Small Non-coding RNAs

To validate the sncRNA expression results obtained from the microarray analysis, we performed reverse transcription quantitative polymerase chain-reaction (RT-qPCR) and expanded the number of study participants, which resulted in the inclusion of a total of 40 individuals (13 HIV+/TB+, 13 HIV-/TB+, and 14 HIV-/TB-). In this validation step, we focused on the expression of three miRNAs (miR-27b-3p, miR-139-5p, and miR-199a-5p), selected based on the microarray results (with FDR < 0.05 and $\geq$ 3-fold change in expression level), ranging from 3.1 to 8.2-fold down-regulation (**Supplementary Table 2**) comparing the HIV+/TB+ and HIV-/TB+ groups. The marked decrease of miR-27b-3p and miR-139-5p expression

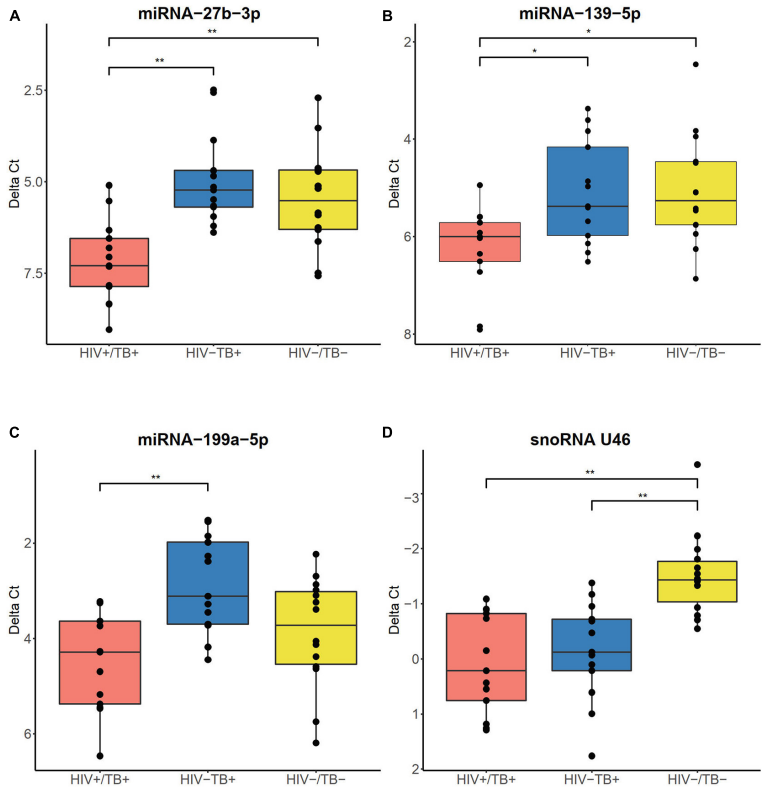

FIGURE 5 | Boxplots illustrating expression of **(A)** miR-27b-3p, **(B)** miR-139-5p, **(C)** miR-199a-5p, and **(D)** snoRNA U46, validated by RT-qPCR. Boxes show median and interquartile range, and whiskers extend to the largest values within 1.5 interquartile range from the quartile. Infection status is represented by colors, HIV+/TB+ (red), HIV-/TB+ (blue), and HIV-/TB- (yellow). Symbols on top indicate: * adjusted $P < 0.05$ and ** adjusted $P < 0.01$. *P*-values from the Mann–Whitney *U*-test with Holm-Bonferroni correction.

in the HIV+/TB+ group compared to in the other two groups (FC < 0.15 and FC < 0.4, respectively), was validated by qPCR (FC ≤ 0.3, adjusted $P < 0.01$, and FC < 0.5, adjusted $P < 0.05$, respectively, **Figures 5A,B**). The downregulation of miR-199a-5p expression in HIV+/TB+ compared to HIV-/TB+ (FC = 0.3) was also confirmed by qPCR (FC = 0.3, adjusted $P < 0.01$, **Figure 5C**). Next, we validated the expression of snoRNA U46, as a representative snoRNA downregulated in the HIV+/TB+ and HIV-/TB+ groups compared to HIV-/TB- subjects from the microarray analysis (FC = 0.3 and 0.4, respectively). This finding was further confirmed using qPCR (FC = 0.3 and 0.4, respectively, adjusted $P < 0.01$, **Figure 5D**). In addition, we performed correlation analysis between results obtained from the microarray and qPCR for the analyzed sncRNAs, and our results showed that the Spearman's rank rho was 0.90 for miRNA-27b-3p, 0.94 for miRNA-139-5p, 0.82 for miR-199a-5p, and 0.94 for snoRNA U46 (adjusted $P < 0.05$ for all; **Supplementary Figure 2**). In summary, microarray findings for the differentially expressed miRNAs and snoRNAs could be validated by qPCR.

Impact of Antituberculosis and Antiretroviral Treatment on sncRNA Expression

Next, we analyzed the expression of the four sncRNAs (miR-27b-3p, miR-139-5p, miR-199a-5p, and the snoRNA U46) in whole blood samples obtained before and after the initiation of ATT and ART in a subset of the HIV+/TB+ individuals ($n = 9$) for whom the follow-up samples were available. These samples were obtained at a median of 200 days (range 126–331 days) after the initiation of the ATT and at a median of 175 days (range 92–202 days) after starting ART. At follow-up, $CD4^+$ cell

count was significantly higher than at baseline (P = 0.03), and all subjects with available viral load determinations (n = 8/9) had undetectable levels.

In the follow-up samples, increased expression levels of miR-27b-3p were observed in seven out of nine HIV+/TB+ individuals (FC = 3.1, **Figure 6A**), however, this change was not statistically significant (adjusted P = 0.08). On the contrary, a statistically significant increase in the expression of miR-139-5p was observed during the treatment (FC = 2.5, adjusted P = 0.047, **Figure 6B**). In samples obtained after treatment initiation, the expression of neither miR-27b-3p nor miR-139-5p was significantly different in the HIV+/TB+ group compared to the HIV-/TB- control group. The change in miR-199a-5p expression during treatment varied within the HIV+/TB+ group and no clear trend was observed (**Figure 6C**). As for the impact of the treatment on snoRNA U46 expression in the HIV+/TB+ group, we observed a significantly increased expression (FC = 2.1, adjusted P = 0.047, **Figure 6D**), which also led to normalization of the expression toward that of the control group.

Follow-up samples from eight HIV-/TB+ participants were obtained after a median of 183 days (range 153–184 days) after starting the ATT. However, we did not find statistically significant changes in any of the four sncRNAs (**Supplementary Figure 3**), however, the significant alteration of snoRNA U46 was no longer evident in this group when compared to the HIV-/TB- group. In summary, miRNA and snoRNA alterations appeared to normalize during ATT and ART in the HIV+/TB+ group, while clear changes in expression of the analyzed sncRNAs were not seen in the HIV-/TB+ group, in line with less pronounced differential expression before treatment compared to the control group.

Correlation Analyses Between sncRNA and CD4$^+$ Cell Counts at Baseline and During Treatment

Next, we explored potential associations between expression levels of the sncRNAs and CD4$^+$ cell count to assess whether the differences in the sncRNA level in the whole blood between the groups could be due to changes in the whole blood composition, such as depletion of CD4$^+$ cells in the coinfected subjects. The three miRNAs measured with qPCR did not show a significant correlation between their expression levels and CD4$^+$ cell count in any of the groups, calculated by the Spearman correlation analyses and Holm-Bonferroni adjustments, neither at the baseline (**Figures 7A–C**) nor at the follow-up time points (data not shown). However, a strong correlation was observed between snoRNA U46 expression and CD4$^+$ cell count at the baseline in the HIV+/TB+ subjects (Spearman's rho 0.89, adjusted $P < 0.01$. **Figure 7D**). This correlation was not present in samples obtained after treatment initiation (data not shown). No significant correlations were observed between CD4$^+$ cell count and any of the miRNAs or snoRNA U46 in the HIV-/TB+ and HIV-/TB- groups. Taken together, these results indicate that peripheral blood CD4$^+$ cell count is not the main reason for the differences in expression of the analyzed sncRNA. Moreover, the correlation between snoRNA U46 expression and CD4$^+$ cell count was dependent on the infection status and also reversed by ART and ATT in the HIV+/TB+ group, suggesting that other HIV-related mechanisms than CD4$^+$ cell count, affects snoRNA U46 expression.

DISCUSSION

In this study, we demonstrate that HIV coinfection significantly alters miRNA expression during active TB. Our findings also reveal the downregulation of several snoRNAs in subjects with TB, regardless of their HIV infection status. Furthermore, normalization of both miRNA and snoRNA expression levels, toward the levels in the uninfected subjects were observed during ART and ATT in HIV-positive individuals with active TB.

To the best of our knowledge, a comparison of whole blood miRNA and snoRNA expression profiles in individuals with active TB and HIV coinfection has not been reported, and only a handful of studies on miRNA expression in active TB have included the PWH. A recent study, based on a multinational cohort exploring miRNA, metabolites and cytokines as biomarkers for TB in PWH, identified 11 miRNA as differentially expressed between individuals with and without TB (Krishnan et al., 2021). This study, however, was not designed to compare miRNA expression differences between HIV+/TB+ and HIV-/TB+. Two studies (one from Cameroon and one with participants from Italy, Uganda, and Tanzania) included subsets of PWH, but no direct comparisons between patients with and without HIV coinfection were included (Miotto et al., 2013; Ndzi et al., 2019). In addition, Honeyborne et al. (2015) have shown similar changes in total plasma sncRNA levels as an indicator of successful ATT regardless of HIV status, however, comparisons of individual sncRNA expressions between HIV+/TB+ and HIV-/TB+ subjects before treatment were not reported.

Our results clearly show that HIV alters the expression of a range of miRNAs in subjects with active TB. Thus, it is tempting to speculate that these alterations may directly be involved in the insufficient TB control in PWH, since miRNAs reportedly play an important role in multiple immunological responses (Drury et al., 2017). Macrophages play a key role in the interactions between HIV and Mtb (Auld and Staitieh, 2020), as both pathogens infect these cells (Gartner, 2014; Esmail et al., 2018). Moreover, HIV-infected macrophages show increased Mtb growth (Pathak et al., 2010), impaired phagocytosis (Mazzolini et al., 2010), and reduced phagosomal proteolysis function (Jambo et al., 2014). Hence, it is possible that the alterations in miRNA expression in HIV-positive individuals with active TB are linked to impaired macrophage function. Indeed, several of the dysregulated miRNAs identified in our study have known functional roles in macrophages. For example, decreased levels of miR-26a-5p, one of the 20 most downregulated miRNAs in HIV+/TB+ patients in our study, has been linked to decreased nitric oxide synthase activity and prevention of Mtb trafficking to lysosomes (Sahu et al., 2017). miR-23a-3p, found to be downregulated in HIV+/TB+ individuals, was recently shown to be downregulated in cells stimulated with mycobacteria

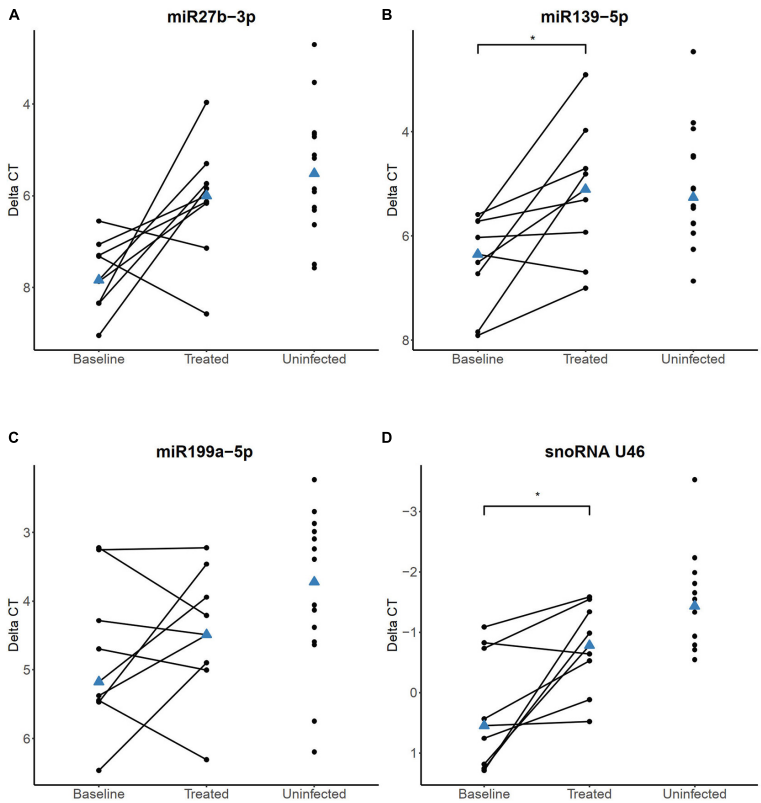

FIGURE 6 | sncRNA expression levels quantified by RT-qPCR in HIV+/TB+ study participants before and after the initiation of anti-tuberculosis treatment and antiretroviral treatment **(A)** miR-27, **(B)** miR-139-5p, **(C)** miR-199a-5p, and **(D)** snoRNA U46. Paired samples of participants longitudinally followed before and during treatment are connected with a line. HIV-/TB- control group is included in all plots for comparison. The blue triangle indicates the median value. *Adjusted $P < 0.05$. *P*-values were calculated from paired samples Wilcoxon test with Holm-Bonferroni correction.

in vitro and in peripheral blood mononuclear cells obtained from subjects with TB, leading to impaired generation of reactive oxygen species and phagocytosis (Chen et al., 2020). Furthermore, downregulation of miR-27b-3p (as is reported in these HIV+/TB+ subjects), has been associated with increased survival of Mtb in macrophages and impaired macrophage apoptosis (Liang et al., 2018). In addition, miR-199a-5p, another miRNA found to be downregulated in HIV+/TB+, was demonstrated to have a positive relationship with TLR-4 signaling and IL-6 expression in macrophages in a cystic fibrosis model (Zhang et al., 2015). These findings, taken together with our results, suggest that HIV coinfection may impair the macrophage response to TB.

Chronic immune activation is a hallmark of HIV infection and has been postulated to drive pathogenesis via increased cellular turnover and exhaustion, in addition to dysregulated homing of $CD4^+$ and $CD8^+$ cells to extra-lymphoid tissues (Moir et al., 2011). Whereas pro-inflammatory responses are required for control of mycobacterial replication, tissue damage is mainly driven by exaggerated immune reactions (Esmail et al., 2018). While the immune-related TB pathology is less prominent at sites of infection in PWH with advanced immunosuppression (manifested by deficient granuloma formation and lower frequency of pulmonary cavitation) (Esmail et al., 2018), systemic inflammation is more prominent (Skogmar et al., 2015), and some studies point toward deficient immunoregulation as a reason for deficient TB control in PWH (Tomlinson et al., 2014; Srabanti Rakshit et al., 2020). The alterations in the expression of certain miRNAs observed in our study have been linked to proinflammatory responses and may contribute

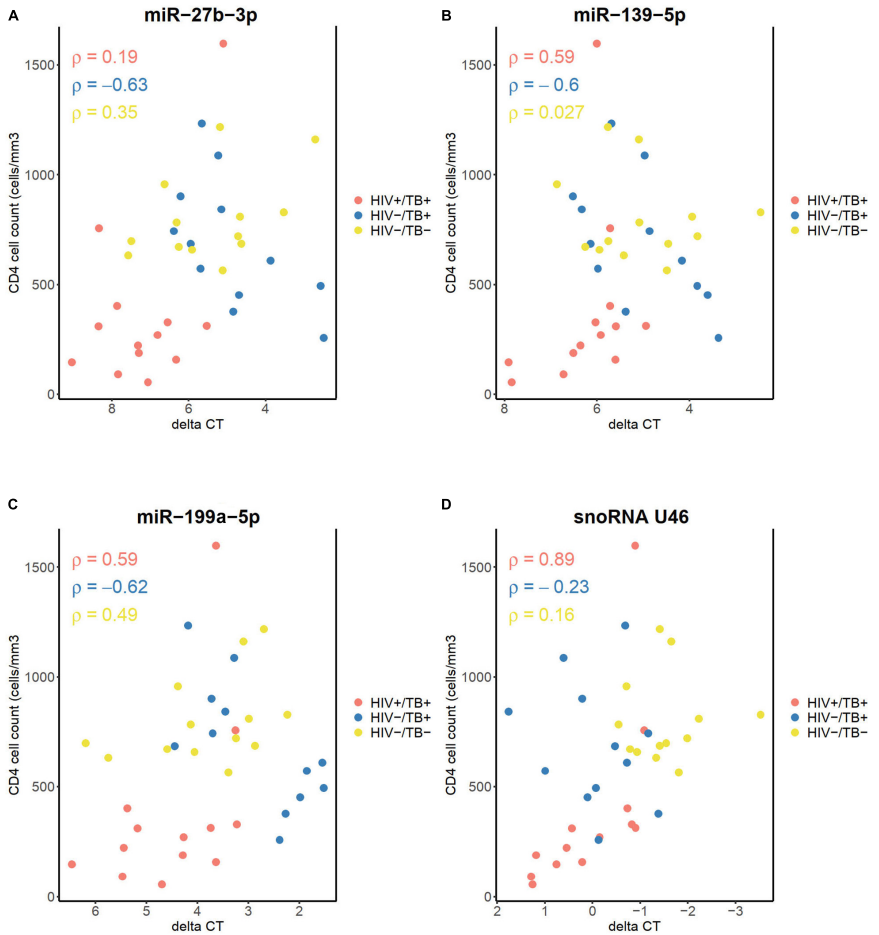

FIGURE 7 | Correlation between CD4$^+$ cell count before treatment, and the levels of **(A)** miR-27b-3p, **(B)** miR-139-5p, **(C)** miR-199a-5p, and **(D)** snoRNA U46, measured by qPCR. Infection status is represented by color, HIV+/TB+ (red), HIV-/TB+ (blue) and HIV-/TB- (yellow). Spearman's rho is indicated for each group in the matching color.

to inflammation and chronic immune activation. miR-26b-5p targets TAK1, a component of the proinflammatory NF-κB pathway, suggesting that the downregulation of this miRNA may exert proinflammatory effects (Li et al., 2018). Furthermore, downregulated expression of miR-27b-3p has been linked to the increased activation of CD4$^+$ cells (Chiang et al., 2012). Another of the most downregulated miRNAs in HIV+/TB+ individuals, miR-505-3p, reportedly negatively regulates the expression of chemokine receptors CCR3, CCR4, and CXCR1 (Escate et al., 2018), suggesting that decreased miR-505-3p levels could enhance immune cell migration and tissue invasion. Thus, the miRNAs dysregulated by HIV infection in patients with active TB could contribute to excessive inflammation in HIV-related TB disease.

Dysregulation of miRNAs is a possible mechanism underlying HIV infection pathogenicity (Chiang et al., 2012). Furthermore, studies on peripheral blood mononuclear cells have demonstrated HIV-associated downregulation of miRNA expression (Houzet et al., 2008). Interestingly, miR-27b-3p, which is downregulated in HIV+/TB+ individuals, has been

shown to suppress HIV replication (Chiang et al., 2012), and its levels are lower in individuals with viremic progression than in elite controllers (Egaña-Gorroño et al., 2014). The mechanisms by which HIV alters miRNA expression have not been fully elucidated, but it has been suggested that the viral regulatory protein Tat inhibits the capacity of the enzyme Dicer to process miRNA precursors (Sánchez-del Cojo et al., 2011).

Interestingly, our results also showed a pronounced effect on the snoRNA expression in individuals with active TB. The biological consequences of this finding are difficult to interpret, as there is limited data on the role of snoRNAs in the immune response to pathogens, including Mtb and HIV. A previous study by de Araujo et al. (2019) found one upregulated and one downregulated snoRNA in whole blood samples of patients with TB. In contrast, we observed differential expression of a range of snoRNAs in patients with active TB. The reasons for these discordant findings may depend on the methods used for identifying differentially expressed snoRNAs, i.e., sequencing vs. microarray, or the differences in method of filtering sncRNAs before analysis. Of note, snoRNA U104, whose expression level was decreased in subjects with TB in their study, was also significantly decreased in our HIV+/TB+ subjects. Furthermore, they found that snoRNA expression was downregulated in Mtb-infected peripheral blood mononuclear cells and M2 macrophages, but not in classically activated M1 macrophages. Classically activated M1 macrophages have a better capacity to limit mycobacterial growth than alternatively activated M2 macrophages (Rao Muvva et al., 2019). Whether downregulating snoRNA expression is a host defense mechanism driving the immune response toward effective clearance of infection, or whether it is induced by mycobacteria to evade host response is not clear. Interestingly, lower expression of snoRNAs U32A, U33, U34, and U35 in HIV-/TB- controls, has been linked to the diminished ability of fibroblasts to generate reactive oxygen species (Michel et al., 2011), raising the possibility that this could be a mechanism by which Mtb evades an immune mechanism. The role and mechanisms underlying the dysregulated expression of snoRNAs in TB and HIV infection warrant further studies.

Of note, we observed significant changes in sncRNA expression during treatment in TB and HIV coinfected individuals. This further supports the conclusion that the observed changes are triggered by these respective infections rather than by an unidentified confounder. Whether the observed changes in miRNA levels are related to ART or ATT cannot fully be clarified by the current study. However, since alterations in miRNA expression at baseline mainly were associated with the HIV infection, we consider it most likely that the longitudinal changes in miRNA are due HIV suppression through ART. The only significant correlation to $CD4^+$ cell count was observed to be with the snoRNA U46 expression in coinfected individuals. Importantly, this correlation was not observed during the treatment, suggesting that this correlation is associated with HIV-related mechanisms other than the depletion of absolute $CD4^+$ cell count. Since we could not reliably distinguish whether a correlation between a cell type and a sncRNA was due to actual expression of the sncRNA in that particular cell type, or by a separate effect of the HIV and/or TB infection status on cell counts and sncRNA levels, we opted against adjusting for peripheral blood cell counts in the statistical analysis. It is likely, however, that some of the sncRNA alterations noted in whole blood are related to different cell type compositions, rather than intracellular changes of sncRNA expression. Thus, further studies on the relation between sncRNA expression and specific cell populations are needed.

This study has certain limitations including the relatively small number of patients enrolled in the study. Furthermore, it should be noted that several of the differentially expressed miRNAs in the microarray were of low annotation confidence, and that microarray studies likely contain some false positive findings. However, the strong agreement between the microarray and qPCR results strengthens the conclusions inferred from the array data. The fact that the methods of TB diagnosis were different in the HIV+/TB+ and HIV-/TB+ groups is also a possible confounder, as the larger proportion of smear-positive subjects in the HIV-/TB+ group implies greater bacterial burden in the airway secretions. Still, a larger proportion of sputum smear positivity among HIV-negative individuals is expected due to the lower sensitivity of sputum smear microscopy in PWH (Esmail et al., 2018).

In summary, we observed distinct alterations in miRNA expression patterns associated with HIV coinfection among adults with active TB. These phenomena are likely to reflect dysregulated immune responses contributing to the pathogenesis of TB in PWH. In addition, the global downregulation of snoRNA in TB regardless of HIV status implies a role of these mediators in active TB. Further studies to elucidate the regulatory roles of miRNAs and snoRNAs in HIV-TB coinfection are required to understand how these mediators are involved in pathogenesis and to identify novel intervention targets.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the BioStudies repository, accession number S-BSST715 (https://www.ebi.ac.uk/biostudies/studies/S-BSST715).

ETHICS STATEMENT

The study was approved by the National Research Ethics Review Committee, Addis Abeba, the Armauer Hansen Research Institute (AHRI/ALERT) Ethics Review Committee, Addis Abeba, Ethiopia, and the Regional Ethical Review Board at Lund University. Written informed consent was obtained from all the study participants.

AUTHOR CONTRIBUTIONS

OO: laboratory work, data analysis, and writing manuscript. FT: conceptualization, laboratory work, and revising and approving

manuscript. RS and CR: planning of laboratory work, data analysis, revising, and approving manuscript. JM: conceptualization, planning of project, revising, and approving manuscript. MA, AA, and HY: laboratory work, revising, and approving manuscript. SS and PB: conceptualization, data analysis, revising, and approving manuscript. TB: data analysis, revising, and approving manuscript. MJ: conceptualization, data analysis, supervision of laboratory work, revising, and approving manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.808250/full#supplementary-material

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