Clinical and Developmental Immunology

HIV-Associated Tuberculosis 2012

Guest Editors: K. A. Wilkinson, M. E. Torok, S. Schwander, and G. Meintjes

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Editorial Board

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Editorial
HIV-Associated Tuberculosis 2012

K. A. Wilkinson,[1,2] M. E. Torok,[3,4] S. Schwander,[5] and G. Meintjes[2]

[1] *National Institute for Medical Research, Mill Hill, London NW7 1AA, UK*
[2] *Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Rondebosch, Cape Town 7701, South Africa*
[3] *Department of Infectious Diseases, Addenbrooke's Hospital, Cambridge CB2 0QQ, UK*
[4] *Department of Medicine, Addenbrooke's Hospital, University of Cambridge, Box 157, Cambridge CB2 0QQ, UK*
[5] *Department of Environmental and Occupational Health, Center for Global Public Health, School of Public Health, UMDNJ, NJ, USA*

Correspondence should be addressed to K. A. Wilkinson, katalin.wilkinson@uct.ac.za

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Tuberculosis (TB) is an important public health problem and remains a leading cause of death in low- and middle-income countries, with an estimated 9.27 million new cases and 1.7 million deaths worldwide in 2007. HIV infection is the greatest risk factor for developing TB. The devastating association between HIV and TB means that 1.37 million of the new TB cases were amongst HIV-infected people and one out of four TB deaths was HIV related in 2007. The risk of TB is increased during all stages of HIV infection from about 10% over a lifetime (in HIV-uninfected individuals) to as high as 30% per annum in patients with advanced HIV.

This special issue addresses several important aspects of the clinical presentation and management, diagnosis, and immunology of HIV-associated TB.

Combination antiretroviral therapy (cART) has progressively decreased mortality of HIV-associated tuberculosis patients worldwide. However, despite the increasing roll out of antiretroviral therapy (ART), the number of deaths attributable to TB still remains high in some parts of the world.

The paper by T. T. Liu et al. highlights this fact and the discrepancies between TB-attributed hospital deaths and the diagnostically confirmed TB deaths, emphasizing the difficulties of diagnosing TB in a high HIV prevalence setting in routine hospital care. The paper by E. Girardi et al. examined TB treatment outcomes in HIV-infected patients diagnosed with TB in Italy. ART during TB treatment was associated with substantial reduction of death rate, while patients who were not ART naïve when they developed TB had an elevated risk of death.

HIV-infected patients are more likely to develop extrapulmonary forms of TB, such as pleural TB. While pleural TB in the immune competent host may be a self-limiting disease, it provides formidable challenges to clinicians around the world in the context of HIV. The systematic review by A. Aljohaney et al. summarises the epidemiology, the immunopathogenesis, and the challenges associated with diagnosis and treatment of pleural TB in HIV-infected patients.

The high rate of latent TB infection is the main challenge to achieving global control of TB. The introduction of IGRA has greatly facilitated diagnosis of LTBI in the developed countries; however, the difficulty in developing countries and HIV-infected individuals remains. The antibody response to tubercular glycolipid (TBGL) antigen was shown to be a promising serodiagnostic agent in Japan, and the paper by U. R. Siddiqi et al. evaluates the antibody response against this antigen in health care workers and HIV-infected individuals in the Philippines.

Chronic HIV infection leads to excessive production of proinflammatory cytokines which leads to the generation of free radicals which are in turn scavenged by free glutathione. Thus the excessive production of free radicals in HIV-infected individuals or reduced glutathione synthesis may lead to the depletion of glutathione. D. Morris et al. examined the causes for decreased glutathione in individuals with chronic HIV infection and found lower levels of intracellular glutathione in macrophages and increased levels of free radicals, interleukin-1 (IL-1), interleukin-17 (IL-17), and transforming growth factor-β (TGF-β) in plasma samples. They

also found reduced expression of enzymes responsible for glutathione synthesis. These findings may contribute to the loss of immune function observed in chronic HIV infection.

K. A. Wilkinson
M. E. Torok
S. Schwander
G. Meintjes

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Clinical Study

Frequent Detection of Anti-Tubercular-Glycolipid-IgG and -IgA Antibodies in Healthcare Workers with Latent Tuberculosis Infection in the Philippines

Umme Ruman Siddiqi,[1] Prisca Susan A. Leano,[2] Haorile Chagan-Yasutan,[1] Beata Shiratori,[1] Hiroki Saitoh,[1] Yugo Ashino,[1] Yasuhiko Suzuki,[3] Toshio Hattori,[1] and Elizabeth Freda O. Telan[2]

[1] *Division of Emerging Infectious Diseases, Graduate School of Medicine, Tohoku University, Sendai, Miyagi 980-8574, Japan*
[2] *STD AIDS Cooperative Central Laboratory, San Lazaro Hospital, Manila, Philippines*
[3] *Department of Global Epidemiology, Research Centre for Zoonosis Control, Hokkaido University, Sapporo, Japan*

Correspondence should be addressed to Toshio Hattori, hatori286@yahoo.co.jp

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Anti-tubercular-glycolipid-IgG (TBGL-IgG) and -IgA (TBGL-IgA) antibodies, and the QuantiFERON-TB Gold test (QFT) were compared in healthcare workers (HCWs, $n = 31$) and asymptomatic human immunodeficiency virus-carriers (HIV-AC, $n = 56$) in Manila. In HCWs, 48%, 51%, and 19% were positive in QFT, TBGL-IgG, and -IgA, respectively. The TBGL-IgG positivity was significantly higher ($P = 0.02$) in QFT-positive than QFT-negative HCWs. Both TBGL-IgG- and -IgA-positive cases were only found in QFT-positive HCWs (27%). The plasma IFN-γ levels positively correlated with TBGL-IgA titers ($r = 0.74$, $P = 0.005$), but not TBGL-IgG titers in this group, indicating that mucosal immunity is involved in LTBI in immunocompetent individuals. The QFT positivity in HIV-AC was 31% in those with CD4+ cell counts >350/μL and 12.5% in low CD4 group (<350/μL). 59 % and 29% were positive for TBGL-IgG and -IgA, respectively, in HIV-AC, but no association was found between QFT and TBGL assays. TBGL-IgG-positive rates in QFT-positive and QFT-negative HIV-AC were 61% and 58%, and those of TBGL-IgA were 23% and 30%, respectively. The titers of TBGL-IgA were associated with serum IgA ($P = 0.02$) in HIV-AC. Elevations of TBGL-IgG and -IgA were related to latent tuberculosis infection in HCWs, but careful interpretation is necessary in HIV-AC.

1. Introduction

Although the incidence of tuberculosis has been falling since 2002, there were still 8.8 million incident cases of TB, 1.1 million deaths from TB, and an additional 0.35 million deaths from HIV-associated TB in 2010 [1]. The high rate of latent TB infection (LTBI) is one of the factors that make it difficult to achieve global control and eliminate TB [2]. The recent introduction of the immune-based interferon-γ release assay (IGRA) made a great impact on facilitating the diagnosis of LTBI [3] and clarified the high rate of infection in TB-high-risk populations including healthcare workers (HCWs) [4]. Attempts to detect LTBI in HIV-infected individuals were also facilitated by the development of IGRA, although their higher rates of pseudonegative IGRA response due to low CD4+ T cell counts and diminished Th1 immunity cannot be ignored [5]. Trehalose 6,6-dimycolate (TDM), which constitutes a major part of the mycobacterial cell wall, was identified as the most immunogenic glycolipid and is produced predominantly by virulent MTB as well as by atypical mycobacteria [6]. Tubercular-glycolipid antigen (TBGL) consists of TDM purified from virulent mycobacterial strain H37Rv [7, 19]. The immunoglobulin-G to tubercular-glycolipid antigen (TBGL-IgG) has been proposed to be a useful marker for the serodiagnosis of active pulmonary tuberculosis (PTB) in Japan [7]. However, frequent elevated titers (17%) were also found in healthy elderly control people (age >40 years) in the same study,

and the possibility of LTBI was suggested by Maekura and colleagues [7]. Although IgA antibody to TBGL antigen (TBGL-IgA) was not evaluated earlier as a biomarker, strong association was revealed between the TBGL-IgG and-IgA titers in PTB cases [8]. Frequent positivity for TBGL-IgG (46%) and -IgA (36%) in healthy adults was also observed in our very recent study in Thailand, a TB-endemic country [9]. The TBGL-IgG-positive responses were not related to BCG vaccination [10]. Since both cellular-mediated and humoral immunity are necessary for an effective immune response against MTB, we aimed to clarify the relationship between the TBGL-IgG and -IgA responses with QuantiFERON-TB Gold In-Tube (QFT) assay system, in healthcare workers (HCWs) in a hospital of the Philippines.

Infection of human immunodeficiency virus (HIV) has substantially boosted the occurrence of tuberculosis (TB) disease worldwide [1]. The devastating association between HIV and TB is responsible for one of four TB-related deaths [11]. The East-Asian countries are predominantly TB endemic [1]. Similarly to Sub-Saharan Africa, the rapid, progressive increase of HIV infections in East-Asian countries may further accelerate TB infection in HIV/AIDS patients [12]. To clarify how HIV infection may alter immune responses in LTBI, newly diagnosed, asymptomatic, non-TB HIV-infected individuals were studied.

To understand the health condition of the individuals, we measured two TB-related biomarkers. Leptin, a cytokine-like hormone produced by bronchial epithelial cells and type II pneumocytes in addition to adipose tissue, exhibits a Th1-bias immune response [13]. Osteopontin (OPN) is a member of extracellular matrix proteins that is synthesized within the immune system by activated T cells, NK cells, dendritic cells, and macrophages. Involvement of OPN in Th1 immune responses has been reported [14]. OPN deficiency was found to be associated with the dissemination of mycobacterial disease, and its expression correlated with an effective immune and inflammatory response against mycobacteria in rodents as well as in human [15, 16]. Elevated levels of circulatory plasma OPN [17] and low levels of leptin [18] were reported to be associated with active tuberculosis; these biomarkers served as a negative evidence of active disease.

2. Materials and Methods

2.1. Study Subjects. A case-control study was conducted between March and October of 2010 in adult participants (age > 18 years) in the Philippines. Thirty-one healthy, adult healthcare workers (HCWs) without any concomitant symptoms or chest radiographic findings relevant to active TB and who had negative HIV serology were recruited from San Lazaro Hospital (SLH), Manila, Philippines. Fifty-six newly diagnosed, asymptomatic HIV carriers (HIV-AC) without any clinical symptoms relevant to tuberculosis were randomly selected from among patients receiving care at the outpatient department of the SLH. None of the subjects took any anti-HIV therapy. Subjects with AIDS-defining events, currently active tuberculosis, or any symptoms relevant to tuberculosis, other than active pulmonary diseases, underlying malignancy or metabolic disorders were excluded from the study. The exclusion criteria for active tuberculosis were based on both clinical findings and chest X-ray (CXR) findings in the HCWs. The study was approved by the ethics committee of SLH and the Tohoku University Hospital. We obtained written informed consent from all the participants. Three mL of blood was obtained directly (one mL in each tube) from each participant to perform the QFT assay. Simultaneously, plasma was separated from blood by centrifugation after treatment with EDTA and was aliquoted to CryoTubes for storage at -80°C until further utilization. All the procedures were conducted in accordance with the Helsinki declaration.

2.2. TBGL-Antibody Assay. TBGL-IgG antibody and -IgA antibodies were measured using the Determiner TBGL Antibody ELISA kit (Kyowa Medex, Tokyo, Japan), an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of anti-TBGL-IgG and -IgA in plasma. This assay employs glycolipid antigens purified from *M. tuberculosis* H37Rv (TBGL antigen) coated on a 96-well plate. The details of the assay were described in our previous study [8]. The antibody titers for TBGL-IgG and -IgA were expressed as U/mL. Positive TBGL-IgG titers were determined according to the cutoff index proposed by Kishimoto et al. [19]. The samples were classified as positive when the serum levels of anti-TBGL-IgG were ≥ 2 U/mL. An arbitrary cutoff value of ≥ 2 U/mL for TBGL-IgA was used according to the unpublished data of our previous study [8].

2.3. QuantiFERON-TB Gold In-Tube (QFT). The QFT test was performed using fresh whole blood in accordance with the manufacturer's instruction (Cellestis, Australia). The results were interpreted using specific software provided by Cellestis. The result was scored positive if the IFN-γ concentration in the tube TB-specific antigen containing was >0.35 IU/mL after subtracting the value of the nil control (IFN-γ-nc) and at least >25% of NC value. If the net IFN-γ response (TB Ag minus nil) was <0.35 IU/mL for the antigens and the response to the mitogen-positive control was >0.5 IU/mL, the response was considered as test negative. An intermediate result was recorded if the net IFN-γ response was <0.35 IU/mL for the antigen and <0.5 IU/mL for the mitogen and/or was above 8 IU/mL for the NC.

2.4. Leptin and OPN Elisa Assay. Plasma leptin levels were determined by sandwich ELISA using Quantikine Human Leptin Immunoassay kit (R&D Systems) for the quantitative determination of the human leptin concentrations in plasma according to the manufacturer's guidelines. Plasma OPN concentrations were determined using Human OPN Elisa kit (Immuno-Biological Laboratories, Takasaki, Japan) according to the manufacturer's guidelines, and values were expressed as ng/mL.

2.5. Clinical Data. We measured different laboratory markers including complete red blood cell counts, the number of white blood cells with their differential counts, levels of

hemoglobin, and serum levels of IgG and IgA. The number of CD4+ T cell counts and HIV RNA load of HIV-AC were also determined.

2.6. Statistical Analysis. The data of quantitative variables are summarized as median and range. Categorical variables were computed as frequency and percentage. The data were analyzed using Stat Flex software, version 5 (Artech Co., Ltd: http://www.statflex.net/index.html) and Statcel 2 (OMS Publishing Inc. Saitama, Japan). The ability of each single marker to discriminate HIV from HCW by receiver operating characteristic (ROC) curve and the area under curve (AUC) was also analyzed. The percentage of overall agreement between QFT and TBGL-IgG/IgA ELISA assays was calculated, and a Cohen's Kappa coefficient was used to assess the level of agreement. The significance of association for categorical variables was estimated by Fisher's exact test, whereas correlations between continuous variables were evaluated by Spearman's rank correlation coefficient. The differences in significance between continuous variables were compared by the Mann-Whitney U test. A 2-tailed P value of <0.05 was considered significant.

3. Results

3.1. Characteristics of Study Participants. A total of 31 HCWs and 56 newly diagnosed HIV-AC were enrolled in the current study. Basic demographic and clinical characteristics of the study participants are shown in Table 1. The participating HIV-AC were relatively young ($P = 0.03$) with a significant male predominance ($P < 0.0001$) compared to the HCWs. Although lymphocyte counts were comparable between the two groups, total counts of WBC, neutrophils, and monocytes were significantly lower in HIV-AC.

3.2. QFT and TBGL-Antibody Assays in HCWs. Forty-eight percent (15/31) of the HCWs showed positive reactions in the QFT assay indicating high incidences of LTBI (Table 1). The median age of the QFT-positive responders from among the HCWs were significantly higher than those of the QFT-negative group ($P = 0.002$). TBGL-IgG and TBGL-IgA were positive in 51% and 19% of HCWs, respectively (Table 1).

Eleven of 15 (73%) QFT-positive HCWs had positive TBGL-IgG responses (categorical agreement 73%), whereas 5 of 16 (31%) QFT-negative subjects had positive TBGL-IgG responses (categorical agreement 68.7%). The overall κ value was 0.42, indicating a moderate association between the two assays (overall agreement: 71%; 95% CI: 0.10∼0.73). The TBGL-IgG-positive proportions were also significantly different between QFT-positive and QFT-negative groups of HCWs ($P = 0.02$). Although the number of positive TBGL-IgA responders was small in HCWs and failed to show any significant difference ($P = 0.072$), the TBGL-IgG+IgA double-positive response was shown only by QFT-positive HCWs and none of the QFT-negative HCWs had double-positive reactions ($P = 0.043$) (Figure 1) (Table 2).

In addition, significant positive correlation was observed between the concentrations of IFN-γ-nc and TBGL-IgA titers in the QFT-positive group ($r = 0.74$, $P = 0.005$) (Figure 2), but not in the QFT-negative group. There was no such association between IFN-γ-nc and TBGL-IgG levels in HCWs, although a tendency for a positive correlation was observed in the QFT-positive HCWs ($r = 0.43$, $P = 0.11$) (Figure 2). No association was observed in the net IFN-γ concentrations in antigen-stimulated QFT-plasma with TBGL-IgG or -IgA titers (data not shown). The plasma levels of OPN and leptin were not different between QFT-positive and QFT-negative HCWs (Table 2).

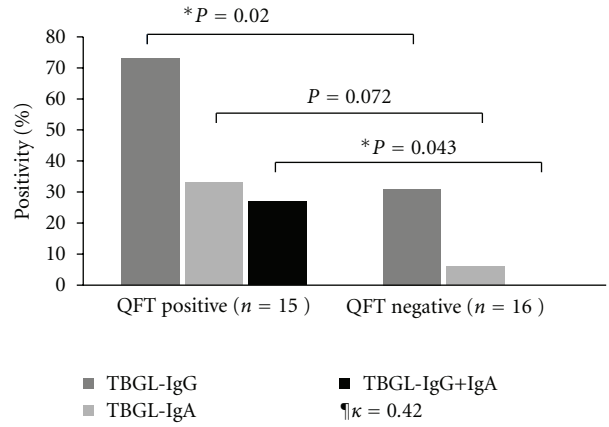

Figure 1: Positivity percentage of TBGL-IgG/IgA assay in QFT-positive/negative healthcare workers. The level of agreement between QFT and the TBGL-Ab assay was measured by Cohen's kappa (κ). ¶$\kappa = 0.42$; overall agreement 71%; 95% confidence interval: 0.1∼0.73.*Significant difference ($P < 0.05$).

3.3. QFT and TBGL-Antibody Assays in HIV-AC. As shown in Table 1, only 13 of 56 (23%) HIV carriers showed positive reactions by QFT assay. The rate of positivity was closely associated with high median CD4+ T cell counts ($P = 0.012$) and younger age ($P = 0.036$) (Table 2). Seven of 56 (12.5%) HIV-AC who had lower mitogen responses (IFN-γ concentrations: median: 1.78 IU/mL; range: 0.38∼6.73 IU/mL) than the rest (>10 U/mL) had negative responses by QFT assay. Their median CD4+ T-cell counts were 60/μL (range: 43∼425/μL) (data not shown). Thirty-three of 56 (59%) and 16 of 56 (29%) HIV-AC were attributed with positive TBGL-IgG and TBGL-IgA responses, respectively (Table 1). The positive proportions of TBGL-IgG and -IgA responses were not significantly different between QFT-positive and -negative HIV-AC (Table 2). However, 6 of 7 QFT-negative low mitogen responders in HIV-AC were positive for both TBGL-IgG and -IgA assay (data not shown). The TBGL-IgA titers were significantly higher in the TBGL-IgG-positive HIV-AC ($P = 0.041$) (Table 3). In addition, TBGL-IgA-positive HIV-AC had significantly elevated titers of TBGL-IgG ($P = 0.042$), serum IgA ($P = 0.015$), and OPN ($P = 0.03$), (Table 3). Interestingly, the TBGL-IgA-positive proportion was inversely correlated with the CD4+ T-cell counts ($P = 0.018$), and the titers were significantly higher in the HIV-AC with CD4+ T-cell count < 350/μL

Table 1: Demographic and clinical data of study participants.

Variables	HCWs ($n = 31$)	HIV-AC ($n = 56$)	P
Demographic data			
Gender: male; n (%)	16 (51.6)	55 (96.5)	<0.0001*
Age year; median (range)	35 (19~62)	28 (19~48)	0.03*
Laboratory findings†			
Hemoglobin (g/dL)	13.2 ± 2.6	13 ± 1.49	0.36
RBC (million/μL)	4.96 ± 1.6	4.43 ± .55	0.069
WBC ($10^3/\mu$L)	7.5 ± 2.5	5.9 ± 1.9	0.01*
Neutrophil ($10^3/\mu$L)	4.4 ± 2.2	3.3 ± 1.2	0.048*
Lymphocyte ($10^3/\mu$L)	2.4 ± 0.6	2.2 ± 0.9	0.82
Monocyte (/μL)	562 ± 237	338 ± 182	<0.001*
CD4+ T-cell count (/μL)	ND	443 ± 286	NA
QFT assay positive; n (%)	15 (48)	13 (23)	0.03*
TBGL-IgG positive; n (%)	16 (51)	33 (59)	0.9
TBGL-IgA positive; n (%)	6 (19)	16 (29)	0.87
IFN-γ-nc (IU/mL)	0.42 ± 0.96	0.13 ± 0.11	<0.001*
TBGL-IgG (U/mL)	3.12 ± 3.36	3.94 ± 6.63	0.14
TBGL-IgA (U/mL)	1.68 ± 2.56	3.1 ± 6.64	0.012*
Serum IgG (mg/dL)	1409 ± 212	1391 ± 224	0.49
Serum IgA (mg/dL)	246 ± 92	319 ± 138	0.058
OPN (ng/mL)	14.4 ± 11	159 ± 191	<0.00001*
Leptin (ng/mL)	18.6 ± 13.9	7.2 ± 5.4	<0.001*

Abbreviations: HCWs, healthcare workers; HIV-AC, newly diagnosed cases of asymptomatic human immune-deficiency virus infection; OPN, osteopontin; ND, not determined; NA, not applicable.
†values were presented as mean ± SD unless indicated otherwise; IFN-γ-nc: levels of IFN-γ, measured in the nonstimulated QFT-plasma samples; P values for statistical differences between HCW and HIV-AC; *significant differences ($P < 0.05$).

Table 2: Comparison between QFT-positive and QFT-negative HCWs and HIV-AC.

Variables	HCWs			HIV-AC		
	QFT+ ($n = 15$)	QFT− ($n = 16$)	P	QFT+ ($n = 13$)	QFT− ($n = 43$)	P
Age; median (range)	45 (21~62)	23.5 (19~48)	0.002*	25 (19~45)	31 (21~35)	0.036*
Gender: male; n (%)	7 (46.6)	9 (47.4)	0.43	12 (92.3)	42 (97.67)	0.43
Work duration->10 yrs; n (%)	11(73.3)	6 (37.5)	0.098	NA	NA	NA
CD4+ count (/μL); median (range)	ND	ND	NA	611 (148~1466)	356 (13~1125)	0.012*
TBGL-IgG positive; n (%)	11 (73)	5 (31)	0.02*	8 (61.5)	25 (58.13)	0.545
TBGL-IgA positive; n (%)	5 (33)	1 (6)	0.072	3 (23)	13 (30)	0.415
TBGL-IgG+IgA positive; n (%)	4 (27)	0 (0)	0.043*	2(15.4)	10 (23.3)	0.42
IFN-γ-nc (IU/mL)†	0.3 ± 0.4	0.2 ± 0.13	0.9	0.21 ± 0.17	0.1 ± 0.07	0.0087*
Serum IgG (mg/dL)†	1450 ± 188	1368 ± 235	0.2	1306 ± 207	1414 ± 249	0.5
Serum IgA (mg/dL)†	268 ± 81	225 ± 101	0.32	330 ± 130	312 ± 138	0.68
OPN (ng/mL)†	14.5 ± 11.2	14.2 ± 11.2	0.87	115.4 ± 130	173.2 ± 203	0.43
Leptin (ng/mL)†	21.3 ± 13.3	15.9 ± 14.3	0.25	6.46 ± 4.12	7.448 ± 5.68	0.24

Abbreviations: HCWs, healthcare workers; HIV-AC, newly diagnosed cases of asymptomatic human immune-deficiency virus infection; OPN, osteopontin; ND, not determined; NA, not applicable.
†mean ± SD; IFN-γ-nc: levels of IFN-γ, measured in the nonstimulated QFT-plasma samples; P values for statistical differences between QFT-positive and QTF-negative groups; *significant differences ($P < 0.05$).

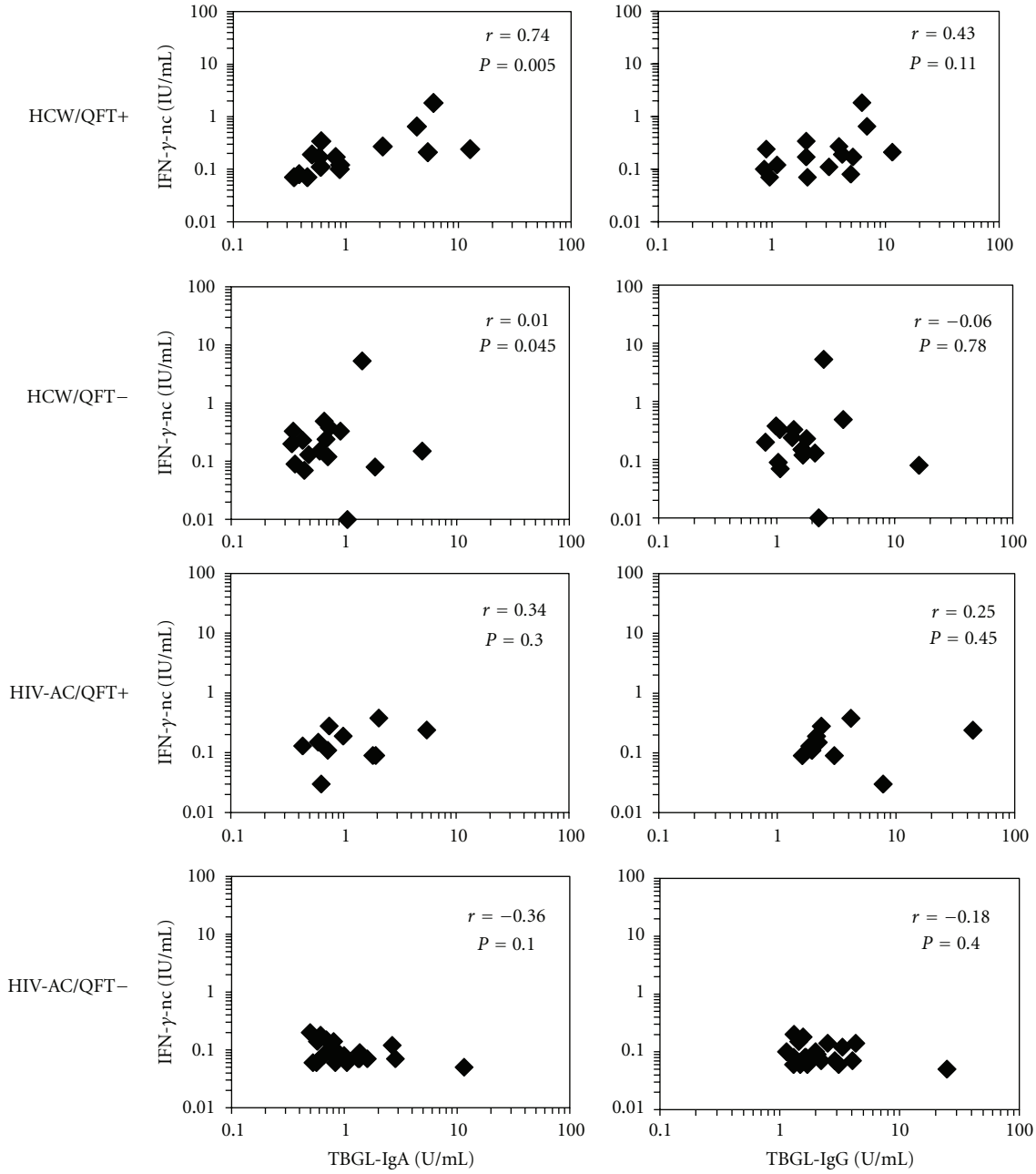

Figure 2: Correlations between TBGL-IgA or TBGL-IgG titers and IFN-γ concentrations measured in nonstimulated QFT-plasma samples (IFN-γ-nc) in QFT-positive/QTF-negative healthcare workers (HCWs) and asymptomatic HIV carriers (HIV-AC). The only significant positive correlation was observed between the IFN-γ-nc concentrations and TBGL-IgA titers in the QFT-positive HCW group ($r = 0.74$, $P = 0.005$).

(HIV-LCD) ($P = 0.048$) (Table 4). Furthermore, in the HIV-AC, a relatively higher proportion of double positive (TBGL-IgG+IgA) responders was found in the HIV-LCD group (29%) than in the HIV-HCD group (CD4+ count $\geq$ 350/μL) (16%), although the difference was not statistically significant ($P = 0.32$) (Table 4).

Moreover, the IFN-γ-nc concentrations were significantly lower in the QFT-negative HIV-AC ($P = 0.008$) (Table 2). No association was observed between the IFN-γ-nc concentrations and TBGL-IgG or -IgA titers in any group of HIV-AC (Figure 2). The plasma levels of OPN and leptin were not different between QFT-positive and QTF-negative HIV-AC (Table 2).

3.4. Comparison between the Serum Antibodies and TBGL Antibodies. The TBGL-IgG and -IgA had no correlation with

Table 3: Comparison between TBGL-IgG or TBGL-IgA-positive and -negative HIV-AC.

Variables	TBGL-IgG			TBGL-IgA		
	Positive ($n = 33$)	Negative ($n = 23$)	P	Positive ($n = 16$)	Negative ($n = 40$)	P
Age; median (range)	28 (19~48)	30 (19~41)	0.18	31.5 (19~48)	28 (19~45)	0.038*
Gender: male; *n* (%)	33 (100)	21 (91.3)	0.43	16 (100)	38 (95)	1
CD4 count (/μL); mean (range)	436 (13~1466)	450 (60~851)	0.45	346 (46~1125)	480 (13~1466)	0.06
QFT positive; *n* (%)	8 (24.2)	5 (21.7)	0.545	3 (19)	10 (25)	0.45
TBGL-IgA positive; *n* (%)	12 (36.4)	4 (17.4)	0.1	—	—	—
TBGL-IgG positive; *n* (%)	—	—	—	12 (75)	21 (52.5)	0.14
IFN-γ-nc (IU/mL)†	0.13 ± 0.09	0.1 ± 0.05	0.4	0.12 ± 0.09	0.12 ± 0.07	0.9
TBGL-IgA (U/mL)†	4.36 ± 8.4	1.28 ± 1.21	0.041*	—	—	
TBGL-IgG (U/mL)†	—	—		7.5 ± 11.6	2.5 ± 1.5	0.042*
Serum IgG (mg/dL)†	1439 ± 277	1515 ± 677	0.5	1615 ± 404	1355 ± 135	0.46
Serum IgA (mg/dL)†	277 ± 95	279 ± 74	0.37	410 ± 165	313 ± 138	0.015*
OPN (ng/mL)†	176.3 ± 199.9	136 ± 172.5	0.67	280 ± 275	115 ± 109.7	0.03*
Leptin (ng/mL)†	7.33 ± 6.16	7.18 ± 4.12	0.68	7.33 ± 6.16	7.18 ± 4.12	0.07

Abbreviations: HIV-AC, newly diagnosed cases of asymptomatic human immune-deficiency virus infection; OPN, osteopontin.
†mean ± SD; IFN-γ-nc: levels of IFN-γ, measured in the nonstimulated QFT-plasma samples; P for statistical differences between QFT-positive and QTF-negative groups; *significant differences ($P < 0.05$).

Table 4: Comparison between HIV-AC with high§ and low‡ CD4+ T-cell count.

Variables	CD4+ high§ ($n = 32$)	CD4+ low‡ ($n = 24$)	P value¶
Age; mean (range)	25.5 (19~45)	25 (22~48)	0.018*
Gender: male; *n* (%)	31 (97)	23 (98)	1.0
CD4+ count (/μL); median (range)	618 (356~1466)	201 (13~349)	<0.001*
QFT-positive; *n* (%)	10 (31)	3 (12.5)	0.12
TBGL-IgG positive; *n* (%)	16 (50)	16 (67)	0.27
TBGL-IgA positive; *n* (%)	5 (16)	11 (46)	0.018*
TBGL-IgG+ IgA positive; *n* (%)	5 (16)	7 (29)	0.32
IFN-γ-nc (IU/mL)	0.14 ± 0.12	0.13 ± 0.09	0.9
TBGL-IgG (U/mL)†	4.6 ± 8.4	3 ± 2.8	0.59
TBGL-IgA (U/mL)†	1.55 ± 2	5.16 ± 9.6	0.048*
Serum IgG (mg/dL)†	1352 ± 185	1549 ± 380	0.5
Serum IgA (mg/dL)†	265 ± 89	423 ± 149	<0.001*
OPN (ng/mL)†	119 ± 126	214 ± 246	0.19
Leptin (ng/mL)†	7.7 ± 6	6.6 ± 4.9	0.5

Abbreviation: HIV-AC, newly diagnosed cases of asymptomatic human immune-deficiency virus infection; OPN: osteopontin.
§High: CD4+ T cell count ≥350/μL; ‡low: CD4+ T-cell count <350/μL; †mean ± SD; IFN-γ-nc: levels of IFN-γ, measured in the non-stimulated QFT-plasma samples; P values for statistical differences between QFT-positive and QTF-negative groups; *significant differences ($P < 0.05$).

the serum IgG and IgA in HCW and HIV-AC except for the association between the serum IgA levels and the TBGL-IgA titers in HIV-AC ($P = 0.02$) (data not shown).

3.5. Comparison of Biomarkers between HCW and HIV-AC. The levels of IFN-γ-nc ($P < 0.001$) were significantly higher in HCWs than in HIV-AC. However, the titers of TBGL-IgA ($P = 0.012$), but not -IgG, were significantly higher in HIV-AC than in HCWs. Similarly, the serum IgA levels were also higher ($P = 0.058$). The OPN levels were significantly higher ($P < 0.0001$), and the leptin levels were considerably lower ($P < 0.001$) in the HIV-AC compared to the HCWs (Table 1).

ROC curve analysis was used to discriminate HIV from HCW groups using the net IFN-γ, leptin, and plasma levels

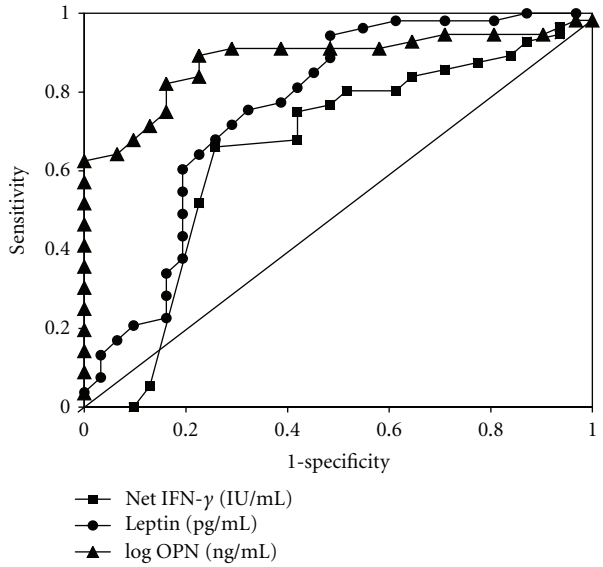

Figure 3: Receiver operating characteristic analysis for comparison of biomarkers between healthcare workers and asymptomatic HIV carriers. The result showed that the OPN plasma levels of OPN (log) exhibited the greatest ability to discriminate HIV from HCW based on the AUC (0.883), followed by leptin (0.763) and net IFN-γ (0.648).

of OPN (log) as biomarkers. As shown in Figure 3, the plasma levels of OPN (log) exhibited the greatest ability to discriminate HIV from HCWs based on the AUC (0.883), followed by leptin (0.763) and net IFN-γ (0.648). However, QFT assay as well as TBGL-IgA and -IgG did not show such profiles (data not shown).

4. Discussion

In our data, the application of QFT assay to HCWs in the Philippines demonstrated a high incidence (48%) of LTBI, which was comparable to other already published data in HCWs in TB-endemic developing countries [4]. The increased risk of LTBI among HCWs was confirmed by the recent introduction of IGRA [20, 21]. In our country, a higher incidence of LTBI in HCWs was reported in high-risk groups for TB, such as homeless areas [22], compared to other areas [23].

We aimed to clarify the relationship between the TBGL-IgG and -IgA responses and that of IFN-γ in the QFT assay in LTBI. The rate of TBGL-IgG positivity was significantly higher in the QFT-positive than QTF-negative group of HCWs. The significant association between the two assay systems indicated by the κ value in HCWs demonstrated the TBGL-IgG in LTBI. However, about 30% of QFT-positive populations from among the HCWs lacked TBGL-IgG, and 30% of those of the QFT-negative group have elevated TBGL IgG antibody, and the discordant cases were higher in TBGL-IgA. However, the reasons for such discordances between the two systems in HCWs are not clear. It is possible that the generation of antibody requires larger amounts of antigens than does the generation of T-cell responses. Although associated immunosuppressive conditions were found as risk factors for false-negative QFT responses [24], such cases were excluded from HCWs in our study.

The mechanism of the synthesis of anti-TDM antibody is not clear, though TDM is known to bind to Mincle (macrophage-inducible C-type lectin) that is present on macrophages [25], and upon the activation, on T cells [26]. It was found that Mincle is specific for the ester linkage of a fatty acid to the trehalose, which explains the strong binding of TDM, but not trehalase-treated TDM, soluble trehalose, or purified mycolate [26]. The conversion of TDM into glucose monomycolate (GMM) upon mycobacterial infection might be the mechanism by which mycobacteria escape from the Mincle-mediated immunity. However, the immune system possesses other tools to monitor and eliminate live mycobacteria through CD1 molecules expressed on the activated macrophages and dendritic cells, which are different from MHC I, II molecules. Recently, GMM but not TDM was demonstrated to interact with CD1b and may induce adaptive immunity [27]. Although it is not known whether the adaptive immune system leads to antibody synthesis, the generated antibody may recognize both TDM and GMM because the two molecules are structurally very similar.

Interestingly, the IFN-γ-nc levels that were observed to have a significant association with the TBGL-IgA titers in LTBI of HCWs. IgA is a typical marker of the mucosal immune response. An elevated serum IgA has been proposed to have a protective role in IFN-γ-positive immunocompetent LTBI individuals [28]. Frequent exposure to tubercle bacilli can possibly stimulate the mucosal immune system in TB-endemic countries. It is also known that commensal bacteria on the mucosal surface induce IgA in an NO-dependent manner [29], although it is not known whether MTB in LTBI has a similar effect in lung mucosa. Circulating glycolipid immune complexes might lead to nonspecific stimulation of T cells, but a component of TBGL, TDM, could also enhance the in vivo production of IL-12p40 and IFN-γ in mouse model [30]. IgA antibody and IFN-γ induce TNF-α and NO production, which mediated the inhibitory mechanism for *M. tuberculosis* infection in mouse model [28]. Furthermore, there is strong evidence of a synergic effect between IgA and IFN-γ in bactericidal activities against MTB infection [31]. Therefore, the association between anti-TBGL-IgA and IFN-γ may indicate protective, mucosal immune activities in LTBI in HCWs.

In HIV carriers, the QFT-positive responses were significantly lower than in HCWs and were greatly dependent on the high CD4+ T-cell counts in the present study. Much evidence suggests that the baseline CD4+ T-cell count is a determining factor for a positive QFT response in HIV infection [32]. Since HIV infection is a disease of immune deficiency, immune deprivation may be less prominent in relatively young QFT-positive cases because IFN-γ could be synthesized properly by stimulation with the appropriate signals. In contrast, the response could be altered in advance immune-deficiency state, as indicated by low CD4+ T-cell counts. Therefore, it is expected that

significant numbers of false-negative reactions are present in QFT-negative HIV carriers. The relatively low IFN-γ levels by mitogen stimulation in some of the QFT-negative responders also support this possibility. Therefore, for TB diagnosis in advanced immunosuppression, the ratio of the IFN-γ response/CD4+ T-cell count Elispot assay was suggested to improve the sensitivity of the assay [33].

It is not clear why HIV infection does not diminish the TBGL antibody titers. It is known that the CD-1 presentation pathway persists in patients with HIV, but antiglycolipid antibodies were found to have no relationship with the TST results [34] or bacillary yield [35]. Similarly, we did not find any correlation between the QFT result and anti-TBGL antibodies. It is also possible that concomitant non-TB mycobacterium infection may stimulate the TBGL antibody synthesis in HIV-AC [7]. Significant numbers of HIV carriers have antibodies to TBGL, but we could not confirm if they indicate LTBI or not.

The increases of serum IgA in advanced HIV infection and of IgG in the early stage were already reported [36]. Although specific antibody titers in HIV infection are decreased by some infectious agents including hepatitis B virus but not in hepatitis A virus, probably because of alterations in the immune systems in advanced HIV infection [37], it is not known whether nonfunctional or functional IgA was synthesized in our cases. The main limitation of the current study is the small number of study subjects and the lack of a follow-up study for estimating the risk of developing active tuberculosis.

Finally, to determine the correlations between biomarkers in infected states, we evaluated data by ROC curve analysis (Figure 3). In this study, the plasma levels of OPN were most specific to HIV and the levels were not elevated in LTBI HCWs (Figure 3, Table 2). Therefore the levels can be a good marker for active TB in non-HIV individuals, because the OPN is known as a marker of active TB [17]. In HIV-AC, the OPN plasma levels are already elevated as described here, and it was already reported that the levels further increase when they developed active TB [38, 39]. It is also known that interferon-inducible protein-10 (IP-10) and IL-18 were elevated in HIV/TB patients than in HIV patients and suggested to be helpful in monitoring the treatment for patients [38]. All these biomarkers were mainly produced by macrophages, and it was also reported that OPN is synthesized by macrophages as well as CD4+ T cells in HTLV-1-induced lymphoma [40, 41].

In this study we noted elevations of anti-TBGL antibody in LTBI in HCWs, but no link between the elevations with LTBI in HIV-AC was confirmed, probably due to the inflammatory conditions in HIV.

5. Conclusion

We have found the elevation of TBGL-IgG titers in LTBI in HCWs. In addition, the association between TBGL-IgA and IFN-γ in HCWs was found, and it was hypothesized that the mucosal immunity is involved in LTBI in HCWs. We could not find any relationships between QFT and TBGL in HIV-AC. Low CD4+ cell count was associated with inflammatory conditions as represented by high OPN in HIV-AC, which may be the reason for ambiguous results.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

US and PL did experiments using the samples and contributed equally as the first author. HCY and BS contributed to the experimental system and statistical analysis. HS and YA and YS contributed to the planning and proposal of the work. TH and ET coordinated the work.

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Clinical Study

Impact of Previous ART and of ART Initiation on Outcome of HIV-Associated Tuberculosis

Enrico Girardi,[1] Fabrizio Palmieri,[1] Claudio Angeletti,[1] Paola Vanacore,[1] Alberto Matteelli,[2] Andrea Gori,[3] Sergio Carbonara,[4] and Giuseppe Ippolito[1]

[1] *Clinical Epidemiology Unit, Department of Epidemiology and Clinical Department, National Institute for Infectious Diseases L. Spallanzani, Via Portuense 292, 00149 Rome, Italy*
[2] *Institute of Infectious and Tropical Diseases, University of Brescia, 25123 Brescia, Italy*
[3] *Division of Infectious Diseases, Department of Internal Medicine, "San Gerardo" Hospital, University of Milano-Bicocca, 20900 Monza, Italy*
[4] *Clinic of Infectious Diseases, University of Bari, 70124 Bari, Italy*

Correspondence should be addressed to Enrico Girardi, enrico.girardi@inmi.it

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Background. Combination antiretroviral therapy (cART) has progressively decreased mortality of HIV-associated tuberculosis .To date, however, limited data on tuberculosis treatment outcomes among coinfected patients who are not ART-naive at the time of tuberculosis diagnosis are available. *Methods*. A multicenter, observational study enrolled 246 HIV-infected patients diagnosed with tuberculosis, in 96 Italian infectious diseases hospital units, who started tuberculosis treatment. A polytomous logistic regression model was used to identify baseline factors associated with the outcome. A Poisson regression model was used to explain the effect of ART during tuberculosis treatment on mortality, as a time-varying covariate, adjusting for baseline characteristics. *Results*. Outcomes of tuberculosis treatment were as follows: 130 (52.8%) were successfully treated, 36 (14.6%) patients died in a median time of 2 months (range: 0–16), and 80 (32.6%) had an unsuccessful outcome. Being foreign born or injecting drug users was associated with unsuccessful outcomes. In multivariable Poisson regression, cART during tuberculosis treatment decreased the risk of death, while this risk increased for those who were not ART-naive at tuberculosis diagnosis. *Conclusions*. ART during tuberculosis treatment is associated with a substantial reduction of death rate among HIV-infected patients. However, patients who are not ART-naive when they develop tuberculosis remain at elevated risk of death.

1. Introduction

The wider access to combined antiretroviral therapy (cART) had a profound impact on HIV-associated tuberculosis.

Prospective studies conducted in high-burden and low-burden countries have clearly shown that incidence of tuberculosis is strikingly reduced in persons receiving cART [1–5], and more recently a randomized trial has shown that early initiation of cART was associated with a significant reduction of the incidence of extra pulmonary tuberculosis [6]. Nevertheless, incidence of tuberculosis may remain higher than that observed in non-HIV-infected persons even in cART treated patients [2, 7].

A number of observational studies have also demonstrated that starting cART during treatment for tuberculosis reduces mortality in ART-naive patients in spite of the increased risk of immune reconstitution syndrome [8]. The effect of early initiation of cART in HIV-infected patients with tuberculosis has been also confirmed in randomized clinical trials [9, 10].

However, little information is available on the outcome of tuberculosis occurring in patients who are not ART-naive at the time of tuberculosis diagnosis.

In a multicenter study conducted in Italy in the context of wide availability of cART, we found that more than one-third of cases of HIV-associated tuberculosis occurred in patients

who were already on antiretroviral treatment [11]. In this paper we analyze the outcome of treatment of tuberculosis among patients enrolled in that study.

2. Patients and Methods

2.1. Study Design and Subjects Selection. Design of this multicenter, prospective, observational study has been previously described [11]. Briefly, 154 Italian infectious diseases hospital units were invited to participate in the study and 96 (62,3%) agreed to enroll patients. These units are located in 18 of Italy's 20 regions, and all of them are located in public hospital and specialize in HIV care.

Individuals (in and out-patients) 18 years of age or older, with confirmed HIV infection, diagnosed with tuberculosis in the participating units during a 15-month period, were included in the study. All patients were given an identification code to guarantee confidentiality and each of the participating centers sought ethical clearance according to local regulations.

2.2. Data Collection. At baseline, the following data were collected for each enrolled subject: age, sex, country of birth, place of residence, education, employment, date of first positive HIV test, mode of HIV infection, history of active tuberculosis, results of chest radiographs and clinical evaluation and microbiological examinations for mycobacterial infection and disease. Data on concomitant AIDS defining diseases, CD4+ lymphocyte count and current and/or previous antiretroviral therapy were also recorded. Followup data were obtained from hospital wards and outpatient clinic records, and included information on antiretroviral therapy, tuberculosis treatment outcome, and vital status at the end of the study period.

All data were collected onto coded standardised forms. All forms were checked by scientific staff at the coordinating center for logical errors.

Cultures for mycobateria were performed on radiometric method (BACTEC; Becton Dickinson, Microbiology Systems, Sparks, MD, USA) and/or Löwenstein-Jensen medium.

2.3. Definitions and Outcome Variables. A case of tuberculosis was defined as a physician diagnosis of tuberculosis in a person who has bacteriological evidence of active disease (isolation of *Mycobacterium tuberculosis* from a clinical specimen and/or demonstration of *M. tuberculosis* from a clinical specimen by nucleic acid amplification) and/or signs and symptoms compatible with tuberculosis (e.g., an abnormal, unstable chest radiographs, or clinical evidence of current disease), completed diagnostic evaluation and decision by physician to treat with a full course of antituberculosis chemotherapy. On the basis of microbiological, clinical, radiological or histological findings, tuberculosis was classified as pulmonary, both pulmonary and extra pulmonary, extra pulmonary only.

A new case of tuberculosis was defined as a patient who has never had treatment for tuberculosis, or who has taken antituberculosis drugs for less than one month. Previously treated cases were defined as patient who had received at least 1 month of antituberculous therapy in the past. Drug-resistant (DR) tuberculosis was defined as caused by a *M. tuberculosis* strain resistant to one or more first-line antituberculosis drugs but not to both isoniazid and rifampin, while multidrug-resistant (MDR) tuberculosis included cases resistant to at least both. Patients were defined aware of HIV serostatus if patients they had their first HIV positive test performed al least 3 months before tuberculosis diagnosis.

For the purpose of the present analysis, patients were defined ART-naive if they had never received antiretrovirals or they received antiretrovirals for less than one month before diagnosis of tuberculosis. Patients were defined on ART at the time of tuberculosis diagnosis if they received antiretrovirals for at least one month in the three months preceding tuberculosis diagnosis.

Tuberculosis treatment outcomes were defined according WHO definitions [12]. For the purpose of the analysis, outcomes were also grouped in successful outcome (including patients cured and with completed treatment) and unsuccessful outcome (including patients transferred out, defaulted and treatment failure).

2.4. Statistical Analysis. Descriptive statistical methods were used to provide a general profile of the study population. The χ^2 or Fisher's Exact Test, as appropriate, were used to compare proportions. Odds ratios (ORs) with the associated 95% confidence intervals (CI) were calculated to measure the association between variables and treatment outcome. By fitting a polytomous logistic regression, we analyzed association of baseline characteristics associated with death and unsuccessful outcome of tuberculosis treatment, compared to successful outcome.

To investigate the impact of cART on mortality rate, a Poisson regression model was used. Results of this analysis are presented as mortality rate ratios (MRRs) with the associated 95% CI. Patients were included from the initiation of antituberculosis treatment until completion of treatment, death or loss to followup, whichever comes first; cART was included in the analysis as a time-dependent variable together with potential confounders. Analyses were performed with STATA software (Stata Corp. Stata Statistical Software. College Station, TX, USA).

3. Results

3.1. Study Population. We considered for inclusion in the analysis 271 HIV-infected patients with who were diagnosed with tuberculosis during the study period. Among these patients, 25 (9.22%) did not start tuberculosis treatment, 5 because they were transferred-out and 20 who were lost to follow up immediately after diagnosis. The remaining 246 patients entered the present analysis.

Table 1 shows characteristics of patients at tuberculosis diagnosis. The majority of patients (80.2%) were males and the median age was 36.9 (range 21.27–76.03) years. Diagnosis of tuberculosis was confirmed by culture in 160 patients

Table 1: Characteristics of 246 HIV-infected patients at tuberculosis diagnosis and outcome of treatment.

Variable	*n* (%)	Successful outcome *n* (%)	Death *n* (%)	Unsuccessful outcome *n* (%)	*P* value
Sex					
Male	199	97 (48.7)	32 (16.1)	70 (35.2)	0.035
Female	47	33 (70.2)	4 (8.5)	10 (21.3)	
Place of origin					
Born in Italy	162	84 (51.9)	29 (17.9)	49 (30.2)	0.11
Foreign born	84	46 (54.8)	7 (8.3)	31 (36.9)	
Age at TB diagnosis (years)					
<40	162	87 (53.7)	18 (11.1)	57 (35.2)	0.084
≥40	84	43 (51.2)	18 (21.4)	23 (27.4)	
Education (years)					
0–8	146	75 (51.4)	21 (14.4)	50 (34.2)	0.850
9–18	51	26 (51.0)	9 (17.6)	16 (31.4)	
Unknown	49	29 (59.2)	6 (12.2)	14 (28.6)	
History of imprisonment					
Yes	24	12 (50.0)	4 (16.7)	8 (33.3)	0.91
No/unknown	222	118 (53.2)	32 (14.4)	72 (32.4)	
Mode of HIV infection					
Injecting drug use	119	49 (41.2)	25 (21.0)	45 (37.8)	0.001
Other	127	81 (63.8)	11 (8.7)	35 (27.6)	
Housing					
Private	209	116 (55.5)	33 (15.8)	60 (28.7)	0.014
Community/homeless	37	14 (37.8)	3 (8.1)	20 (54.1)	
CD4 lymphocyte count (cells/mmc)					
0–199	156	81 (51.9)	26 (16.7)	49 (31.4)	0.18
200–350	42	20 (47.6)	8 (19.0)	14 (33.3)	
>350	48	29 (60.4)	2 (4.2)	17 (35.4)	
AIDS					
Yes	132	65 (49.2)	27 (20.5)	40 (30.3)	0.019
No	114	65 (57.0)	9 (7.9)	40 (35.1)	
Site of diseases					
Pulmonary	197	101 (51.3)	28 (14.2)	68 (34.5)	0.413
Extra pulmonary	49	29 (59.2)	8 (16.3)	12 (24.5)	
History of TB treatment					
New cases	206	113 (54.9)	28 (13.6)	65 (31.6)	0.279
Previously treated cases	40	17 (42.5)	8 (20.0)	15 (37.5)	
Drug susceptibility test[#]					
Susceptible TB	99	54 (54.5)	15 (15.2)	30 (30.3)	0.387
DRTB	22	13 (59.1)	2 (9.1)	7 (31.8)	
MDR TB	4	1 (25.0)	2 (50.0)	1 (25.0)	
Awareness of HIV seropositivity					
Yes	176	79 (44.9)	31 (17.6)	66 (37.5)	<0.001
No	70	51 (72.9)	5 (7.1)	14 (20.0)	
cART naive					
Yes	150	84 (56.0)	16 (10.7)	50 (33.3)	0.091
No	96	46 (47.9)	20 (20.8)	30 (31.2)	
Concomitant diseases at TB diagnosis					
Yes	60	30 (50.0)	15 (25.0)	15 (25.0)	0.033
No	186	100 (53.8)	21 (11.3)	65 (34.9)	

[#] Calculated on 125 patients with drug susceptibility test performed.

(74,7%); 125 patients had results of antimycobacterial drugs susceptibility testing, of whom 22 (17.6%) had drug-resistant tuberculosis and 4 (3.2%) multidrug-resistant tuberculosis.

The median time from first date of documented HIV seropositivity was 36.9 months (range: 0–201.3), and 96 (39%) were not ART-naive at the time of tuberculosis diagnosis. Of these patients, 34 received antiretroviral therapy for a median of 13.5 months (range 1–86), but not in the three months preceding diagnosis of tuberculosis, and their last ART regimen included a protease inhibitor (PI) in 20 patients and a nonnucleoside reverse transcriptase inhibitor (NNRTI) in 11 patients.

At baseline the median value of CD4 lymphocytes was 120.5/mmc (range 0–1111), and viral load median value, calculated in 241 patients, was 4.94 log copies/mL. At least one concomitant AIDS defining illness disease was recorded in 60 (24.4%) patients.

3.2. Tuberculosis Treatment Outcome. We recorded tuberculosis treatment outcomes for the 246 patients included in the analysis. A successful outcome was recorded for 130 patients (52.8%), among them 75 (30.5%) were cured and 55 (22.4%) completed treatment. Eighty patients (32.5%) had unsuccessful outcomes: 44 (17.9%) were lost to follow up in a median time of 1 month, and 25 (10.2%) were defaulters, 9 (3.7%) were transferred-out, and 2 (0.8%) were failures. Thirty-six patients (14.6%) died a median time of 2 months after tuberculosis treatment initiation.

Table 1 shows the distribution of treatment outcomes according to baseline patients' characteristics.

In multivariable polytomous logistic regression analysis (Table 2), not being ART-naive was associated with an increase of the probability of unsuccessful outcomes. Being foreign born was associated with a threefold increase of the risk of unsuccessful outcomes (OR 3.38, 95% CI 1.38–8.29, $P = 0.008$), which was also more likely for injecting drug users. Risk of death was also associated with being injecting drug users as well as to a lower CD4 cells count at the time of tuberculosis diagnosis and MDR tuberculosis.

3.3. Use of cART during Tuberculosis Treatment and Risk of Death. Among the patients enrolled, 151 (61.4%) received cART and tuberculosis treatment concurrently. Of these patients 62 were already on cART when tuberculosis was diagnosed and 89 started cART during tuberculosis treatment, 56 (62.9%) in the initial phase and 33 (37.1%) in the continuation phase and included a PI in. Patients who were already on cART at initiation of TB have been receiving antiretrovirals for a median of 24 months (range 3–108) before diagnosis of tuberculosis and their last cART regimen included a PI in 35 cases and an NNRTI in 23. An additional 21 patients were not ART-naive but not on ART at tuberculosis diagnosis. ART administered during tuberculosis treatment included a PI in 75 cases (49.7%).

We performed a further analysis in order to estimate the impact of use of cART during tuberculosis treatment on death rate of HIV-infected patients with tuberculosis.

During 161.2 person-years (PY) of observation, 36 deaths occurred with an overall mortality rate of 22.3 per 100 PY (95% CI: 16.1–31.0). Among the patients who died, 17 were not ART-naive, 7 were ART-naive and started cART during tuberculosis treatment and 12 patients never started cART.

In multivariable analysis (Table 3), the use of cART during tuberculosis treatment significantly reduced the risk of death (IRR 0.14, 95% CI 0.06–0.30, $P < 0.001$), whereas being not ART-naive at tuberculosis diagnosis caused a more than four-fold increase in the same risk (IRR 4.04, 95% CI 1.09–14.96, $P = 0.037$). Risk of death was also associated with a lower CD4 cell count, age ≥ 40 at diagnosis, and MDR tuberculosis.

4. Discussion

In this multicentre study conducted in a low tuberculosis incidence country, a successful outcome of tuberculosis treatment was documented in slightly more than 50% of HIV-infected patients; the death of the patient during treatment was recorded in almost 15% patients. When we analyzed the impact of cART before and during tuberculosis treatment on the risk of death, we found the cART use during tuberculosis treatment reduced the probability of dying, while this risk was increased in those who were not ART-naive at tuberculosis diagnosis.

The overall success rate of tuberculosis treatment observed in the present study is lower than that reported in other European studies in general population. A survey conducted in 10 European countries [13] found an overall proportion of successful outcome of tuberculosis treatment of 69% with a range between 60% and 88% in different countries and a death rate of 1%. In a systematic review of European surveys [14], an overall success rate of 74.4% was recorded with a death rate of 6.9%. These discrepancies however were not unexpected. First of all a high proportion of patients in our study population were intravenous drug users (48%) or foreign born (34%), and both these characteristics have been associated with a greatly reduced probability of successful outcome of tuberculosis treatment. For example in a Spanish study [15] intravenous drug users and foreign born persons had a six-fold or higher increase of the risk of interrupting treatment. This association was observed also in our analysis, in which the death outcome was also more likely for intravenous drug users.

However, the main difference with surveys of tuberculosis treatment outcome in general population is the increased proportion of death, and HIV infection per se may most likely account for the observed discrepancies in death rates. Indeed the above-referenced Spanish multicentre survey [15] found a six-fold increase in death rate among HIV-infected patients with tuberculosis compared to non-HIV infected patients.

We further explored the association between the risk of death and the use of cART. In our study population, almost 50% of patients continued or started cART during the initiation phase of tuberculosis treatment and an additional 14% initiated cART during the continuation phase. Overall,

Table 2: Multivariable odds ratios* of unsuccessful outcome of tuberculosis treatment and death according to baseline characteristics for 246 HIV-infected patients with tuberculosis.

Variable	Unsatisfactory outcome of TB treatment		Death	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Sex				
Male	1.00		1.00	
Female	0.38 (0.16–0.90)	0.029	0.35 (0.10–1.27)	0.112
Place of origin				
Born in Italy	1.00		1.00	
Foreign born	3.66 (1.48–9.06)	0.005	1.98 (0.52–7.45)	0.314
Age at TB diagnosis (years)				
<40	1.00		1.00	
≥40	1.04 (0.52–2.11)	0.909	2.34 (0.94–5.84)	0.068
Education (years)				
0–8	1.00		1.00	
9–18	1.00 (0.44–2.27)	0.996	0.81 (0.27–2.37)	0.698
Unknown	0.83 (0.35–1.96)	0.671	0.69 (0.20–2.35)	0.550
History of imprisonment				
Yes	1.00		1.00	
No/unknown	1.93 (0.65–5.74)	0.238	2.17 (0.53–8.90)	0.282
Mode of HIV infection				
Injecting drug use	1.00		1.00	
Other	0.42 (0.19–0.93)	0.033	0.25 (0.08–0.74)	0.012
Housing				
Private	1.00		1.00	
Community/homeless	2.21 (0.93–5.30)	0.074	0.44 (0.09–2.30)	0.333
CD4 lymphocyte count (increase: 50 cells)	0.96 (0.88–1.04)	0.326	0.83 (0.71–0.97)	0.020
AIDS				
Yes	1.00		1.00	
No	0.90 (0.45–1.82)	0.778	0.44 (0.16–1.19)	0.106
Site of diseases				
Pulmonary	1.00		1.00	
Extra pulmonary	0.66 (0.29–1.50)	0.320	1.15 (0.42–3.18)	0.782
History of TB treatment				
New cases	1.00		1.00	
Previously treated cases	0.96 (0.39–2.36)	0.930	0.71 (0.22–2.32)	0.572
Drug susceptibility test				
Susceptible TB	1.00		1.00	
DR TB	1.03 (0.33–3.21)	0.962	0.44 (0.07–2.69)	0.376
MDR TB	0.83 (0.03–20.52)	0.911	37.10 (1.98–693.79)	0.016
Not available	1.41 (0.71–2.78)	0.322	1.24 (0.50–3.07)	0.649
Awareness of HIV seropositivity and HIV treatment history				
Not aware	1.00		1.00	
Aware and cART naive	5.11 (2.04–12.80)	0.001	2.95 (0.77–11.33)	0.115
Not cART naive	3.31 (1.29–8.46)	0.013	4.04 (1.09–14.96)	0.037
Concomitant diseases at TB diagnosis				
Yes	1.00		1.00	
No	1.38 (0.59–3.26)	0.461	0.59 (0.23–1.55)	0.286

* From polytomous logistic regression model with successful outcome as reference category.

Table 3: Univariable and multivariable mortality rate ratio (MRR)* for 246 HIV-infected patients with tuberculosis.

Variable	Univariable analysis		Multivariable analysis	
	MRR (95% CI)	*P* value	MRR (95% CI)	*P* value
Sex				
Male	1.00		1.00	
Female	0.52 (0.18–1.47)	0.217	0.46 (0.15–1.38)	0.163
Place of origin				
Born in Italy	1.00		1.00	
Foreign born	0.50 (0.22–1.15)	0.102	1.36 (0.42–4.41)	0.608
Age at TB diagnosis				
<40	1.00		1.00	
≥40	1.96 (1.02–3.77)	0.043	2.73 (1.26–5.93)	0.011
Education (years)				
0–8	1.00		1.00	
9–18	1.14 (0.52–2.50)	0.736	0.92 (0.39–2.21)	0.855
Unknown	0.81 (0.33–2.02)	0.659	1.00 (0.36–2.76)	0.999
History of imprisonment				
Yes	1.00		1.00	
No/unknown	0.80 (0.28–2.26)	0.675	2.05 (0.64–6.60)	0.282
Mode of HIV infection				
IDU	1.00		1.00	
Other	0.37 (0.18–0.75)	0.006	0.39 (0.15–0.99)	0.047
Housing				
Private	1.00		1.00	
Community/homeless	0.71 (0.22–2.32)	0.573	0.29 (0.07–1.17)	0.082
CD4 lymphocyte count (per 50 cells increase)	0.85 (0.75–0.96)	0.010	0.83 (0.72–0.95)	0.006
AIDS				
Yes	1.00		1.00	
No	0.40 (0.19–0.85)	0.017	0.47 (0.20–1.13)	0.091
Site of diseases				
Pulmonary	1.00		1.00	
Extra pulmonary	0.94 (0.43–2.07)	0.888	1.36 (0.59–3.11)	0.471
History of TB treatment				
New cases	1.00		1.00	
Previously treated cases	1.53 (0.70–3.35)	0.292	0.78 (0.32–1.94)	0.597
Drug susceptibility test				
Susceptible TB	1.00		1.00	
DR TB	0.48 (0.11–2.12)	0.336	0.34 (0.07–1.68)	0.184
MDR TB	3.91 (0.90–17.12)	0.070	35.50 (5.50–229.19)	<0.001
Not available	0.85 (0.43–1.71)	0.658	1.34 (0.60–3.00)	0.471
Awareness of HIV seropositivity and HIV treatment history				
Not aware	1.00		1.00	
Aware and ART-naive	2.64 (0.92–7.60)	0.072	1.73 (0.52–5.72)	0.370
Not ART-naive	3.41 (1.28–9.08)	0.014	4.27 (1.27–14.29)	0.019
cART (time dependent)				
No	1.00		1.00	
Yes	0.26 (0.13–0.52)	<0.001	0.14 (0.06–0.30)	<0.001
Concomitant diseases at TB diagnosis				
Yes	1.00		1.00	
No	0.43 (0.22–0.83)	0.012	0.63 (0.29–1.35)	0.231

*Estimated by a Poisson regression model.

the use of cART was associated with a greater than six-fold reduction of the risk of death. This effect is of the same order of magnitude of that observed both in high and low tuberculosis incidence countries [8]. Thus our data concur with available evidence suggesting the importance of starting cART early during tuberculosis treatment [16].

A relevant finding was the increased risk of death for individuals who were not ART-naive when diagnosed with tuberculosis, which remained significant when we adjusted in the analysis for current cART use as well as for other factors associated with the risk of death such as a low CD4 cells count, older age at tuberculosis diagnosis, and multidrug resistance. The reasons for this association remains to be elucidated, nevertheless some hypothesis can be put forward. First of all, some patients had interrupted cART treatment before tuberculosis was diagnosed and this may be a marker of poor adherence to cART or previous cART failure or cART toxicity. Thus, even if cART is resumed during tuberculosis treatment, a reduced effect could be expected in these patients. Moreover, treatment interruptions per se have been associated with an increased risk of death [17]. Occurrence of tuberculosis during cART may also be a marker of progression of HIV disease also when CD4 cell count and HIV viral load are taken into consideration, and indeed tuberculosis in cART-treated patients has been identified as an independent predictor of other HIV-associated clinical events and death [18].

Tuberculosis occurring in cART-treated patients may in some instances be due to the so-called unmasking, which is defined as clinical manifestation of preexisting tuberculosis infection that is due to ART-induced immune restoration and which may sometimes result in severe or even fatal disease [19]. We do not have clinical details to evaluate severity of tuberculosis in our patients. However, tuberculosis unmasking usually occurs during the first few weeks of cART [19, 20], while in the present study most of the patients who were not ART-naive were treated for several months before tuberculosis diagnosis.

In this study we do not have details of antiretroviral treatment history of patients and in particular we cannot determine if those who were not ART-naive had virological treatment failures and/or antiretroviral resistance at the time of tuberculosis diagnosis, and thus we could not estimate the impact of these factors on patients' outcome. The high proportion of patients who abandoned treatment may also have affected the analysis of factors associated with death. A further limitation is that the study was conducted on patients treated relatively early in the cART era, and thus the conclusions on the effect on cART may not necessarily be applicable to the newer cART regimens.

In conclusion these study shows an alarmingly high proportion of unsuccessful outcome of tuberculosis in HIV-infected persons who inject drugs or are migrants and stresses the need of intervention aimed at keeping these patients into care.

Tuberculosis occurred frequently in patients who were not ART-naive, and these patients had an increased risk of death compared to those who were ART-naive, also after taking into account the use of ART during tuberculosis treatment. Tuberculosis occurring in patients who already received ART may represent in the future an important issue to be addressed in high tuberculosis incidence countries in which scaling up of ART is currently underway.

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Members of Gruppo Italiano di Studio Tubercolosi e AIDS (GISTA)—Società Italiana di Malattie Infettive e Tropicali (SIMIT) study group and participating institutions.

Regional coordinators: S. Babudieri (Sassari, "SS. Annunziata"); M. Bassetti, R. Rosso (Genova, "S. Martino"); S. Carbonara (Bari, "Policlinico"); S. Carradori (Ferrara, "Arcispedale S. Anna"); B. M. Celesia (Catania, "Ascoli-Tomaselli"); A. Chirianni (Napoli, "Cotugno"); G. Di Perri (Torino,"Amedeo di Savoia"); P. Fabietti (Vasto, "Presidio Ospedaliero di Vasto"); A. Gabbuti (Firenze,"S.M. Annunziata"); V. Guadagnino (Catanzaro,"Policlinico Mater Domini"); E. Iemoli (Milano,"Sacco"); V. Manfrin (Vicenza, "S. Bortolo"); A. Matteelli (Brescia, "Spedali Civili"); M.B. Pasticci (Perugia, "Policlinico Monteluce"); B. Salassa (Torino, "Amedeo di Savoia"); F. Zacchini (Grosseto, "O. Misericordia").

Participating persons and institutions: Ancona: F. Burzacchini, A. Giacometti (Umberto I); Aosta: A. Tassara, A. Traverso (Ospedale Generale Regionale); Arezzo: P. Giorni, (S. Maria Sopra i Ponti); Ascoli Piceno: M. Gobbi (C. G. Mazzoni); Avezzano: R. Mariani (SS. Filippo e Nicola); Aviano: G. Di Gennaro (Centro di Riferimento Oncologico); Barcellona Pozza di Gotto: L. Panella, (S. Andrea); Bisceglie: R. Losappio (V. Emanuele II); Bologna: C. Beltrami P. Costigliola, (S. Orsola), G. Fasulo (C. A. Pizzardi); Bolzano: O. Moling (Ospedale Generale Regionale); Brescia: D. Bertelli, S. Tedoldi (Spedali Civili); Brindisi: D. Potenza (A. di Summa); Cagliari: P. E. Manconi (Policlinico); Casa S. Erice:S. Zichichi (S. Antonio Abate); Caserta: C. Nacca (Gruppo C); Catania: F. Fatuzzo, R. La Rosa, (V. E. Ferrarotto S. Bambino), S. Galvagna (Garibaldi); Cesena: C. Grosso (M. Bufalini); Chieti: J. Vecchiet (SS. Annunziata); Como: E. Longoni (S. Anna); Cosenza: L. Guaglianone (Ospedale di Cosenza); Cremona: A. Pan (Istituti Ospitalieri); Crotone: S. Pellicano' (S. Giovanni di Dio); Cuneo: G. Raineri (S. Croce e Carle); Foggia: S. Ferrara (Ospedali Riuniti); Frosinone: E. Anzalone, A. Gallo (Umberto I); Gallipoli: S. Cataldini (Sacro Cuore di Gesu'); Genova: F. Dodi (S. Martino); Lamezia Terme: A. Petronio (Ospedale Civile); Latina: G. Salone (S. Maria Goretti); Lecce: E. P. De Luca Andrioli (Vito Fazzi); Legnago: G. De Checchi (Ospedale Civile); Legnano: M. Villa (Ospedale Civile); Livorno: R. Pardelli (Ospedali Riuniti); Lucca: A. Tardio (Piana di Lucca); Macerata: C. Urbani † (Ospedale Generale Provinciale); Mantova: G. Gattuso (C. Poma); Massa: L. Matini (Presidio Ospedaliero di Massa C.); Matera: G. Casciano (Ospedale Provinciale); Messina: F. Loschiavo (Policlinico); Milano: I. Errante (Ca' Granda-Niguarda); G. Giani, P. Meraviglia, C. Molteni (Sacco); Modena: G. Guaraldi (Policlinico); Monza: S. Foresti

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Clinical Study

Inaccuracy of Death Certificate Diagnosis of Tuberculosis and Potential Underdiagnosis of TB in a Region of High HIV Prevalence

Theresa T. Liu,[1] Douglas Wilson,[2] Halima Dawood,[2] D. William Cameron,[1] and Gonzalo G. Alvarez[1,3]

[1] *Division of Infectious Diseases, The Ottawa Hospital, University of Ottawa, Ottawa, ON, Canada K1H 8L6*
[2] *Department of Medicine, Edendale and Grey's Hospitals, Pietermaritzburg, KwaZulu-Natal 3200, South Africa*
[3] *Division of Respirology, The Ottawa Hospital, University of Ottawa, Ottawa, ON, Canada K1H 8L6*

Correspondence should be addressed to Theresa T. Liu, theresa.liu@queensu.ca

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Despite the South African antiretroviral therapy rollout, which should reduce the incidence of HIV-associated tuberculosis (TB), the number of TB-attributable deaths in KwaZuluNatal (KZN) remains high. TB is often diagnosed clinically, without microbiologic confirmation, leading to inaccurate estimates of TB-attributed deaths. This may contribute to avoidable deaths, and impact population-based TB mortality estimates. *Objectives*. (1) To measure the number of cases with microbiologically confirmed TB in a retrospective cohort of deceased inpatients with TB-attributed hospital deaths. (2) To estimate the rates of multi-drug resistant (MDR) and extensively drug resistant (XDR) TB in this cohort. *Results*. Of 2752 deaths at EDH between September 2006 and March 2007, 403 (15%) were attributed to TB on the death certificate. 176 of the TB-attributed deaths (44%) had a specimen sent for smear or culture; only 64 (36%) had a TB diagnosis confirmed by either test. Of the 39 culture-confirmed cases, 27/39 (69%) had fully susceptible TB and 27/39 (69%) had smear-negative culture-positive TB (SNTB). Two patients had drug monoresistance, three patients had MDR-TB, and one had XDR-TB. *Conclusions*. Most TB-attributed deaths in this cohort were not microbiologically confirmed. Of confirmed cases, most were smear-negative, culture positive and were susceptible to all first line drugs.

1. Introduction

Tuberculosis (TB) is a global problem, with approximately 9 million new cases per year; of these cases, 1.1 million cases per year are due to coinfection with TB and human immunodeficiency virus (HIV) [1]. Approximately 82% of the global cases of HIV-associated TB are in the African Region, and South Africa accounts for a significant proportion of cases in the region [1]. In 2010, South Africa had the third largest absolute number of TB cases in the world, with an incidence of 0.4–0.59 million [1], or 998 cases/100,000 population [2]. 73% of new TB cases in South Africa in 2007 were HIV positive [2]. Tuberculosis is the leading cause of death in South Africa by clinically determined death notification forms [3]. Edendale Hospital (EDH) is an 860-bed district and regional hospital in Pietermaritzburg, KwaZuluNatal (KZN), South Africa, and is the regional TB referral centre. It serves a mainly Black African population of approximately 1.6 million [4]. HIV prevalence rates in regional antenatal clinics vary between 40 and 50% [5], and the rate of TB disease in KZN is estimated as between 773–1008/100,000 [3]. The high TB mortality rate in this region is likely associated with the high HIV prevalence and/or the presence of drug-resistant TB known to be in the region. Recently, an outbreak of XDR-TB (extensively drug-resistant TB) occurred in a rural hospital approximately 100 km from EDH [6]. The median survival time in this outbreak was two weeks after obtaining sputum samples. Almost all of these patients were HIV positive [6]. Information on the patients who died indirectly or directly as a result of TB at EDH may reveal

high proportions of specific patterns of drug resistance, specifically MDR-TB (multidrug-resistant TB) and XDR-TB, which are associated with high mortality. A recent post-mortem study at EDH revealed that the accuracy of limited autopsy diagnosis of TB is approximately 47%; furthermore, 42% of patients who died who previously were not suspected to have TB were actually TB culture positive on limited autopsy [7]. The present study set out to measure the number of patients with a TB diagnosis by sputum smear and culture in TB-attributed hospital deaths and to estimate the rates of MDR- and XDR-TB in TB-attributed hospital deaths.

2. Methods

A retrospective cohort study of all adult inpatients admitted to the EDH wards that died with a diagnosis of TB during a seven-month period between September 2006 and March 2007 was undertaken. A list from the EDH mortuary of patients who died during the study period with a diagnosis of TB recorded on their death certificate was recorded and cross-referenced with the EDH laboratory AFB (acid fast bacilli) smear registry and culture and drug susceptibility registry. The study period was chosen due to the availability of data from the hospital mortuary and mycobacteriology laboratories. Physicians filling out the death certificates could include up to three diagnoses. Ethics approval for the audit was obtained from the Edendale Hospital Ethics Committee. One of the authors (T.I.Liu) collected and cross-referenced the data manually. The mortuary lists of TB-attributed deaths and the AFB smear registry were on paper. The TB culture and susceptibility data was computerized, and was accessed remotely through the mycobacteriology reference laboratory at Inkosi Albert Luthuli Central Hospital.

3. Inclusion Criteria

All adult inpatients (>18 years of age) at EDH were eligible for inclusion in the study if they died in hospital during the study period, and if TB was documented by a hospital physician on their death certificate as being a direct or indirect cause of death. Death certificate data was only available from the mortuary for patients who died on an adult inpatient ward at EDH.

4. Definitions

AFB smear results were recorded as negative, scanty, 1+, 2+, or 3+, as defined by the South African National TB Control Programme Practical Guidelines [8]. Sputum samples are not routinely sent for culture in this resource limited setting, unless the patient has been on TB treatment in the past, is persistently smear-positive on treatment, or is persistently symptomatic despite two previous negative smears and a course of broad-spectrum antibiotics, or if recurrent/relapsed TB disease is suspected [8]. Smear and culture methodology are in accordance with international guidelines using florescent microscopy, liquid media for initial culture, and Middlebrook 7H10 solid media for drug susceptibility testing. Positive TB cultures that were sent for susceptibility testing were classified as pansensitive to all first line drugs, monoresistant to a single agent, multidrug resistant (MDR), or extensively drug resistant (XDR). MDR-TB is defined as resistance to at least isoniazid (INH) and rifampin (RIF). XDR-TB is defined as resistance to INH and RIF as well as resistance to the fluoroquinolones and any one of the second-line injectable antituberculous drugs (amikacin, kanamycin, or capreomycin), as per the October 2006 revised definition by the World Health Organization (WHO) Global XDR-TB Task Force [9].

Table 1: Clinical and demographic characteristics.

Total number of TB deaths	$n = 403$
Sex, female (%)	144 (36%)
Sex, male (%)	122 (30%)
Sex not documented on death certificate	137 (34%)
Average age at death, years (standard deviation)	36 (14)
Pulmonary TB (%)	227 (56%)
EPTB (%)	118 (29%)
Miliary/disseminated	30 (7%)
Meningitis	59 (15%)
Peritonitis	20 (5%)
Spine	5 (1.2%)
Lymphadenitis	3 (0.7%)
Pericardium	1 (0.2%)
TB source unknown	58 (14%)
Documented HIV positive*	22 (5.4%)
Presumed HIV positive**	55 (13.6%)
HIV status unknown	326 (81%)

* Documented HIV positive via CD4 or HIV viral load records; ** Presumed HIV positive due to death certificate diagnosis of HIV, retroviral disease (RVD), or AIDS-defining opportunistic infection.

5. Results

5.1. Demographics. The average age at death was 36 years (standard deviation 14). Approximately one-third of subjects were male, one-third was female, and for the remaining one-third, gender was unavailable (Table 1).

5.2. In Hospital TB Deaths. Of 2752 inpatient deaths in the seven-month study period from September 2006 to March 2007, 403 deaths (14.6%) were attributed to TB according to the death certificate. An average of 58 TB deaths per month occurred during the study period.

5.3. Site of Disease (Tables 1 and 2). Pulmonary TB (PTB) was the most common site of infection, with 227/403 (56%) cases (Tables 1 and 2). 118 (29%) had extrapulmonary TB (EPTB). Meningitis, miliary/disseminated, and peritoneal TB were the most common types of EPTB. 58 subjects (14%) did not have a specific site of TB infection documented on the death certificate and did not have any supportive AFB smear or culture results to help identify disease site.

Table 2: Number of TB-attributed inpatient deaths, by disease site and diagnostic test.

Diagnosis	Number (% of total deaths)	AFB smear done	AFB smear positive	Culture done	Culture positive	Smear or culture positive	Totals (% of total deaths)
PTB	227 (56)	61	28	59	29	47	227 (56)
EPTB							
Disseminated/miliary	30 (7.4)	11	1	9	4	5	
TB meningitis	59 (14.6)	10	0	13	2	2	
TB abdomen	20 (4.9)	3	1	7	1	1	118 (29)
TB spine	5 (1.2)	1	0	1	0	0	
TB pericardium	1 (0.2)	0	0	1	0	0	
TB source unknown	58 (14)	8	1	9	2	3	58 (14)
MOTT (*Mycobacteria* other than TB)	2 (0.5)	0	0	2	2	2	2 (0.5)

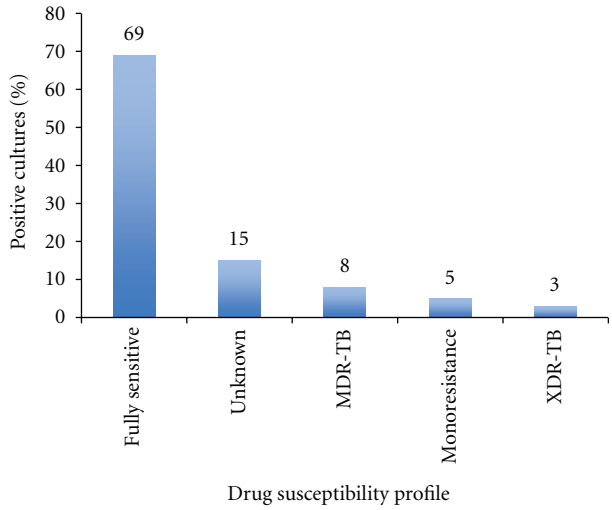

Figure 1: Drug susceptibility profile of positive cultures.

5.4. TB Culture and Susceptibility. 176/403 (44%) of the patients had a result registered for AFB smear or culture, and of those, only about 64/176 (36%) had a TB diagnosis confirmed with either test (Table 2). Of the 39 culture-confirmed cases, 27 (69%) were smear negative, of which 12 were sputum samples, 7 lymph node or abscess aspirates, 6 pleural or peritoneal fluid samples, and 1 CSF sample. Of the 39 culture-confirmed cases, 27/39 (69%) had fully susceptible TB (Figure 1). Two patients had drug monoresistance, three patients had MDR-TB, and one had XDR-TB. Six patients (15.4%) had culture-positive TB with no susceptibility results available. Of the six cases of drug-resistant TB of any kind, only two had been previously designated as MDR-TB suspects (one had culture-confirmed MDR-TB, and the other had rifampin monoresistance). Of the fifteen MDR-TB suspects, only one patient had confirmed MDR-TB. The one case of XDR-TB was not previously suspected to have had drug-resistant TB. In two patients, *Mycobacteria* other than TB (MOTT) was isolated (one grew MOTT alone, and another grew both TB and MOTT).

6. Discussion

The present study demonstrates that TB mortality remains high in this region, and that the majority of patients that died with a diagnosis of TB did not have a microbiologically confirmed diagnosis by AFB smear or culture. Of those with a confirmed diagnosis, the majority were smear negative, culture positive, and susceptible to all first line drugs; only one case of XDR-TB was identified. The findings likely reflect the known high prevalence of HIV in the region, which has increased the SNTB rates resulting in diagnostic delays in the community. Many HIV-positive patients have AFB smear-negative TB disease, resulting in diagnostic delays, and worsening of their clinical condition to the point that they require hospital admission. Following admission, confirmation of TB is further delayed by the inability to obtain an adequate diagnostic specimen from moribund patients, the patients' inability to produce sputum, and the lack of availability of appropriate invasive diagnostics. Mycobacteria cultures may not be sent on inpatients already established on antituberculosis therapy at a primary care clinic and admitted with a superimposed condition (such as community acquired pneumonia). Once a specimen is obtained, the time to confirmation of TB is further delayed by the time required for laboratory culture and sensitivity testing. All of these reasons contribute to low rates of microbiologic confirmation of a clinical TB diagnosis in this setting.

In our cohort of 39 culture-confirmed cases among all TB-attributed deaths, 69% had isolates susceptible to all first-line drugs. Despite having effective antituberculosis medications, many people in this region are dying of pansusceptible TB, most likely due to the difficulties encountered in the diagnosis of TB in HIV-infected patients. HIV-infected patients are less likely to have AFB smear-positive disease or cavitary TB and are more likely to have disseminated or EPTB [7]. In this study, the proportion of PTB (56%) to EPTB (29%) cases was approximately 2 : 1. In a previous study at EDH in 2001, a similar ratio of PTB to EPTB cases (65% compared to 36%) was noted [4]. The expected ratio of PTB to EPTB cases ranges from 5 : 1 [10] to 9 : 1 [1]; according to the WHO 2011 Global Tuberculosis Control Report, in high burden countries in 2010, the distribution of PTB : EPTB cases was approximately 9 : 1 [1]. The disproportionately high number of EPTB cases at EDH is likely due to high rates of undiagnosed HIV/TB coinfection in admitted patients. Out of fifteen MDR suspects in our cohort, only one had confirmed MDR-TB. This finding underscores the fact that MDR-TB and XDR-TB are difficult to distinguish clinically from pansusceptible TB disease, and therefore routine

airborne infection control practices should be undertaken in all admitted TB suspects, regardless of the suspicion of drug resistance. However, given the TB burden and limited resources in this part of the world, the logistics of infection control remains a significant challenge.

A recent postmortem study by Cohen et al. that was done at EDH one year after the present study was completed showed that in a representative sample of 240 inpatient deaths between October 2008 and August 2009, 110 (47%) were culture positive on limited autopsy pooled sampling of respiratory tract secretions and core biopsies of lung, liver, and spleen [7]. 121 of these inpatient deaths (50.4%) were not on TB treatment at the time of death, and 119 (49.5%) of them were on TB treatment [7]. Of those 119 cases on TB treatment, 64 (58%) were culture positive, and 55 (46%) were culture negative [7]. Therefore 55/240 (23%) of these cases would have been attributed to TB in hospital deaths based on the death certificate, in the absence of a positive culture result. In this study, which was done at the same center as our study only one year later, the TB diagnosis rate (by culture-positive limited autopsy samples) among TB suspects was 58%, which demonstrates the significant underestimation of TB hospital attributed deaths in this region when using only the death certificate. The death certificate diagnosis rate of TB at EDH was less accurate than limited autopsy likely due to the challenges in obtaining sputum or other tissue as outlined previously, as well as the high rates of smear-negative TB associated with high HIV prevalence.

Most patients with culture-confirmed TB had drug-susceptible TB, which was perhaps surprising giving the documented prevalence of MDR- and XDR-TB in this region of South Africa [1]. A selection bias that may have affected our results is that admitted patients who died of TB may have died immediately following admission, prior to appropriate samples being sent for testing. Information on length of hospital stay prior to death was not available for our subjects. A selection bias could have resulted from these patients, since they may have had a higher rate of resistance; it is known that patients infected with XDR-TB have a shortened survival [6]. A recent study in KZN demonstrated markedly high early mortality rates for HIV/drug-resistant TB coinfection; 40% of HIV/MDR-TB and 51% of HIV/XDR-TB cases died within 30 days of sputum collection [11]. Because patients in our study may have died rapidly after admission, they may have been too debilitated to give an adequate sputum sample for culture or undergo invasive testing. The selection bias would apply to the 226 subjects that died without having a specimen sent for AFB smear or culture; however these patients would have had the same random chance of having either a sputum sample or another type of specimen sent to the laboratory prior to admission. We would have expected that patients that were at higher risk of resistance would have had sputum sent for testing, as per the South African National TB Control Program guidelines [8]. Furthermore, patients infected with MDR- or XDR-TB may have died before they even presented to hospital and thus would not be accounted for in hospital mortality registries. From the WHO 2011 Global Tuberculosis Control Report, an estimated 1.8% of incident TB cases in South Africa were MDR-TB [1]. In our study, from the 39 culture-confirmed TB deaths, we found 3 cases of MDR-TB and 1 case of XDR-TB. Although we do not know the South African MDR-TB mortality rate, this is in keeping with the published rates of incident MDR-TB cases in South Africa [1].

Another limitation of our study is that we were not able to determine the HIV status of the majority of our subjects since at the time of the study point of care testing was not available. It is well documented that HIV/TB coinfection increases the rapidity of clinical deterioration and increases the risk of death [12–15]. Stigma associated with HIV/AIDS may influence people's willingness to be tested for HIV—"retroviral disease" was a common euphemism used on the death certificates to avoid labeling patients as HIV positive. Of the four patients with either MDR-TB or XDR-TB in our study, three had documented HIV infection. EDH has since developed a program in which a designated team of lay health workers offer point of care HIV testing and assist in obtaining sputum samples to all admitted TB suspect patients [16]. Future studies should focus on streamlining which patients should get smears and cultures sent to the laboratory, given the high number of specimens processed and the limited resources of regional laboratories.

Misclassification bias may have occurred in physician-completed death certificates. All over the world, physicians often have to make a judgment call on the ultimate cause of death of the deceased. Determining the cause of death in patients with active TB disease is a challenge and thus may have resulted in misclassification. A review of 60 cases of pulmonary TB in a hospital in Manchester, Great Britain, in 1983, demonstrated that the actual cause of death in admitted inpatients with TB can be difficult to ascertain [17]. In this study, the pulmonary TB in-hospital mortality rate over a 7-year period (1974–1981) was 7%; of the 60-patients that died, 36 died while on treatment for TB. 10 patients died with unequivocal evidence of overwhelming tuberculosis infection, 6 died for unrelated reasons, and 4 died of drug-related conditions. The remaining 16 (27%) died suddenly or unexpectedly for unclear reasons—13 of these patients underwent autopsy, and 9 patients still did not have an identifiable cause of death on postmortem [17]. Despite autopsy, there were still difficulties in identifying the actual cause of death in patients with pulmonary TB. Verbal autopsies, in which fieldworkers interview close family members to complete a questionnaire asking about a patient's symptoms and course of illness prior to their death, have been validated as a useful tool in South Africa [18, 19]. In a study looking at mortality in South African gold miners with pulmonary TB, the cause of death was determined by reviewing clinical records and from the results of limited autopsies of the heart and lungs [14]. In this population, limited autopsies are mandated for purposes of compensation but have also provided further information on patterns of TB disease and drug susceptibility in South Africa.

7. Conclusions

Our study demonstrates that hospitalized TB patients continue to experience a high mortality, despite major efforts to

roll out antiretrovirals (ARVs) in this high HIV prevalence region, and that the majority of patients that died with TB identified as the cause of death on the death certificate did not have a TB diagnosis confirmed by AFB smear or culture. The majority of the TB suspects that were tested for and died of TB had a fully susceptible organism. The cohort studied detected only one XDR-TB case, despite significant XDR-TB outbreaks associated with high mortality in a nearby hospital. Death certificate diagnosis of TB was less accurate than limited autopsy diagnosis of TB at the same hospital, during a similar time period.

8. Lessons Learned

This study emphasizes the importance of active TB and HIV case finding among inpatients at EDH, such as through the work of EDH's "TB Warriors" program in coordinating laboratory testing treatment and follow-up of TB suspects [16]. In this area of high HIV endemicity, routine testing for HIV should continue to be offered to TB patients to help identify more HIV/TB coinfected people, who are at higher risk of dying. Diagnosing TB quickly and efficiently in this setting continues to be a challenge given the high prevalence of both TB and HIV, but it is limited by hospital and laboratory resources. There is also an urgent need for diagnostic tools that will rapidly diagnose TB in patients too ill to produce sputum. Clinicians should have a low threshold for sending sputum for TB culture in HIV positive patients, given the greater possibility of smear negative and/or drug-resistant TB. The earlier patients are diagnosed, the greater chance of starting both TB and HIV treatment in a timely fashion, thus reducing HIV/TB-associated mortality.

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Review Article

A Systematic Review of the Epidemiology, Immunopathogenesis, Diagnosis, and Treatment of Pleural TB in HIV- Infected Patients

A. Aljohaney,[1,2] K. Amjadi,[2] and G. G. Alvarez[2]

[1] *Department of Internal Medicine, Faculty of Medicine, King Abdulaziz University, P.O. Box 80215, Jeddah 21589, Saudi Arabia*
[2] *Divisions of Respirology and Infectious Diseases, Ottawa Hospital Research Institute, at the Ottawa Hospital, University of Ottawa, 725 Parkdale Avenue, Ottawa, ON, Canada K1Y 4E9*

Correspondence should be addressed to G. G. Alvarez, galvarez@ohri.ca

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Background. High HIV burden countries have experienced a high burden of pleural TB in HIV-infected patients. *Objective*. To review the epidemiology, immunopathogenesis, diagnosis, and treatment of pleural TB in HIV-infected patients. *Methods*. A literature search from 1950 to June 2011 in MEDLINE was conducted. *Results*. Two-hundred and ninety-nine studies were identified, of which 30 met the inclusion criteria. The immunopathogenesis as denoted by cells and cytokine profiles is distinctly different between HIV and HIV-uninfected pleural TB disease. Adenosine deaminase and interferon gamma are good markers of pleural TB disease even in HIV-infected patients. HIV-uninfected TB suspects with pleural effusions commonly have a low yield of TB organisms however the evidence suggests that in dually infected patients smear and cultures have a higher yield. The Gene Xpert MTB/RIF assay has significant potential to improve the diagnosis of pleural TB in HIV-positive patients. *Conclusions*. Pleural TB in HIV-infected patients has a different immunopathogenesis than HIV-uninfected pleural TB and these findings in part support the differences noted in this systematic review. Research should focus on developing an interferon gamma-based point of care diagnostic test and expansion of the role of Gene Xpert in the diagnosis of pleural TB.

1. Background

The World Health Organization reported 1.1 million new cases of TB among HIV-infected persons in 2009 [1]. High HIV prevalence regions have experienced a greater burden of extrapulmonary TB [1]. Extrapulmonary TB is more common in HIV-infected patients compared with patients without HIV infection [2, 3] and its incidence has doubled since the beginning of the HIV pandemic [4]. Furthermore, pleural TB is the second most common form of extrapulmonary TB after peripheral lymph nodes in HIV-infected patients [4]. Pleural TB in the context of HIV continues to provide formidable challenges to clinicians around the world. In this systematic review, we report the epidemiology, immunopathogenesis, clinical presentation, diagnosis, and management of tuberculous pleural effusions in HIV-positive patients and explore some of the important differences between them and HIV-uninfected patients as well as how these findings could be applied in resource poor settings.

2. Methods

The PRISMA guidelines for systematic reviews [34] were used to formulate this paper.

2.1. Eligibility Criteria. Studies were included if all of the following criteria were met: (1) all study types except case reports published in English, French, Spanish, and Arabic and (2) pleural tuberculosis were defined by one of the following features: (a) positive culture of the pleural fluid or pleural biopsy specimen or both for *M. tuberculosis,* (b) positive sputum culture for *M. tuberculosis* and pleural effusion, positive acid fast bacilli (AFB) stain of the pleural fluid, or pleural biopsy specimen, or (c) granuloma on pleural biopsy with no alternative diagnosis and improvement on

antituberculous therapy. (3) HIV infection documented by at least serological testing.

2.2. Search Strategy. A literature search in MEDLINE was conducted by one of the investigators (AA). Articles were limited to English, French, Spanish, and Arabic published between 1950 to June 2011. Using Boolean operator "and", we combined the following research themes (1) ("Tuberculosis, Pleural/complications" [Mesh] or "Tuberculosis Pleural/diagnosis" [Mesh] or "Tuberculosis, Pleural/ drug therapy" [Mesh] or "Tuberculosis, Pleural/enzymology" [Mesh] or "Tuberculosis, Pleural/epidemiology" [Mesh] or "Tuberculosis, Pleural/microbiology" [Mesh] or "Tuberculosis, Pleural/mortality" [Mesh] or "Tuberculosis, Pleural/pathology" [Mesh] or "Tuberculosis, Pleural/radiography" [Mesh] or "Tuberculosis, Pleural/surgery" [Mesh] or "Tuberculosis, Pleural/therapy" [Mesh]) or pleural tuberculosis [Text Word] and (2) "HIV Infections" [Mesh] or HIV infection [Text Word].

We also scanned the bibliographies of key articles to identify additional studies. The literature search was crosschecked by the university librarian to ensure reproducibility and that no other citations existed.

2.3. Study Selection and Data Collection Process. One reviewer (AA) screened the titles and abstracts of identified records. Articles were retrieved for full text review if they contained any information related to pleural tuberculosis and HIV infection. Full text review of these articles was performed by two reviewers (AA, GGA) independently using predefined case report forms. The two reviewers then met to discuss each article using the predefined case report forms. Differences were resolved by consensus. Variables that were collected included: incidence, prevalence, immunopathogenesis, diagnosis, management, and treatment of pleural TB in HIV-infected patients.

3. Results

3.1. Study Selection. Figure 1 outlines the identification, screening, eligibility, and inclusion of studies in this systematic review. The initial search strategy identified 295 potentially relevant articles and four additional studies from review of bibliographies. 63 articles were chosen based on titles and abstracts. 30 articles fulfilled the eligibility criteria and 33 articles were excluded after full text reviews. Selection and information bias, lack of uniform reporting, and inclusion of low methodological quality studies prevented a formal meta-analysis. Table 1 shows the characteristics of the 30 included studies.

4. Epidemiology of Pleural TB in HIV-Infected Patients

The prevalence rate of HIV-pleural tuberculosis was highest in the African regions compared to other regions (Table 2). The highest rate was in Zimbabwe where 85% of patients with tuberculous pleural effusions in one study were HIV-positive [16] and the lowest rate was seen in another study done in Spain at 10% [15]. Although the study populations were heterogeneous, it can be extrapolated that due to the fact that the WHO estimates that 37% of dually infected incident cases came from the African region [1], it would be expected that more pleural TB would be seen in these regions. Males were more frequently infected with HIV and there was no gender differences noted between HIV-positive compared with HIV-uninfected individuals [18, 23, 24].

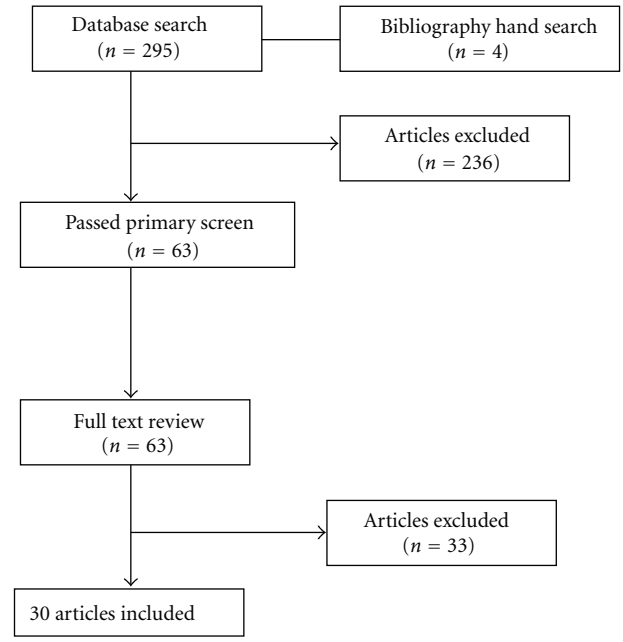

Figure 1: Identification, screening, eligibility, and inclusion of studies.

5. Immunopathogenesis of Pleural TB in HIVInfected Patients

Pleural TB has been characterized as a systemic inflammatory response which in some HIV-uninfected patients has been documented to resolve without the use of antibiotics [36]. This systemic inflammatory response likely contributes to the constellation of symptoms seen in these patients which can be more severe than in pulmonary TB [37]. The development of a pleural effusion in immunocompetent hosts is associated with an intense cell-mediated immune response with infiltration of CD4 T cells and production of high levels of proinflammatory cytokines such as gamma interferon (IFN-) and tumor necrosis factor alpha (TNF-α) [36, 38]. In contrast, the immunopathogenesis of pleural TB in HIV-infected patients is different because of the CD4 T cell depletion and subsequent reduction in antigen-specific cytokine responses [39]. HIV-infected patients have decreased numbers s of CD4+ T cells and cytokine responses to TB [40–43]. When these immunological components are lacking the key protective immune response is significantly

Table 1: Characteristics of included studies.

Study/reference	Year	No of pleural TB patients		Design	Recruitment of patients
		HIV+	HIV−		
Aderaye et al. [5]	1996	8	54	Prospective cohort	Consecutive
Batungwanayo et al. [6]	1993	28	82	Prospective cohort	Consecutive
Cordero et al. [7]	1995	12	107	Retrospective chart review	N/A
Dheda et al. [8]	2009	38	13	Prospective cohort	Consecutive
Dheda et al. [9]	2009	20	31	Prospective cohort	Consecutive
Domoua et al. [10]	2007	30	17	Prospective cohort	Consecutive
Elliott et al. [11]	1993	57	13	Prospective cohort	Consecutive
Frye et al. [2]	1997	22	169	Retrospective chart review	N/A
Gil et al. [12]	1995	10	93	Retrospective chart review	N/A
Heyderman et al. [13]	1998	63	11	Prospective cohort	Consecutive
Hodsdon et al. [14]	2001	66	29	Prospective cohort	Consecutive
Luzze et al. [15]	2001	109	33	Prospective cohort	Consecutive
Riantawan et al. [16]	1999	37	52	Prospective cohort	Consecutive
Richter et al. [17]	1994	65	47	Prospective cohort	Consecutive
Richter et al. [18]	1994	49	26	Prospective cohort	Consecutive
Trajman et al. [19]	1997	13	30	Cross-sectional retrospective	N/A
Villena et al. [20]	1996	9	41	Prospective cohort	N/A
Baba et al. [21]	2008	16	2	Retrospective case series	N/A
Baba et al. [22]	2008	145	20	Retrospective case control	N/A
Baba et al. [23]	2008	5	23	Prospective cohort	Consecutive
Bezuidenhout et al. [24]	2009	6	6	Case series	N/A
Collins et al. [25]	2007	8	0	Case series	N/A
Conde et al. [26]	2003	13	71	Prospective cohort	Consecutive
Hirsch et al. [27]	2001	16	8	Prospective cohort	Consecutive
Jones et al. [28]	2000	3	0	Case series	N/A
Elliott et al. [29]	2004	197	0	Randomized controlled Trial	Consecutive
Tshibwabwa-Tumba et al. [30]	1997	159	68	Prospective cohort	Consecutive
Toossi et al. [31]	2011	20	20	Prospective cohort	Consecutive
Siawaya et al. [32]	2009	12	11	Case series	N/A
Mayanja-Kizza et al. [33]	2009	20	0	Case series	N/A

N/A: not mentioned in the methods section of the paper.

weakened. T helper 1 type cells secrete TNFα, IFNγ, IL-2, and IL12. These cells are important in the delayed type hypersensitivity reaction and in activating macrophages in pleural TB. Furthermore, granuloma formation is mediated by CD4+ T cells of the T helper 1 type.

5.1. Histopathology. In an early study done in Tanzania [35], 36 HIV TB pleural biopsies were compared with 21 HIV-uninfected TB-pleural biopsies. Histological characterization of the tissue reaction in pleural biopsies were examined as follows: reactive (well-formed granulomas with caseous necrosis, epitheliod cells, giant cells, scarce acid fast bacilli, or undetected AFB), hyporeactive (poorly formed granulomas with noncaseous necrosis, few epithelioid cells or macrophages, and no giant cells, AFB were easily seen) and non-reactive (no true granuloma formation, noncaseous necrosis with nuclear debris and neutrophils, no giant cells and numerous AFB). Although CD4 counts were not done in this study, HIV-positive patients had significantly more pleural biopsies demonstrating the hyporeactive and nonreactive patterns than HIV-uninfected patients (14/36 versus 2/21 $P < 0.02$) and their level of immunodeficiency was worse than those with reactive patterns among the HIV-infected patients as evidenced by a greater number of AIDS

Table 2: Reported prevalence rates of HIV-pleural tuberculosis across various studies/countries.

Study/year	Country	Prevalence
Heyderman et al. 1998 [13]	Zimbabwe	85%
Batungwanayo et al. 1993 [6]	Rawanda	80%
Luzze et al. 2001 [15]	Uganda	80%
Domoua et al. 2007 [10]	Ivory Coast	63%
Richter et al. 1994 [18]	Tanzania	58%
Riantawan et al. 1999 [16]	Thailand	37%
Elliott et al. 1993 [11]	Zambia	31%
Trajman et al. 1997 [19]	Brazil	30%
Aderaye et al. 1996 [5]	Ethiopia	22%
Frye et al. 1997 [2]	USA	11%
Gil et al. 1995 [12]	Spain	10%
Cordero et al. 1995 [7]	Spain	8%

related complications. Furthermore, hyporeactivity in the HIV group seemed to show a trend towards a mortality risk (3/11 versus 1/18 deaths). In contrast, a case series of 12 patients (6 HIV-positive and 6 HIV uninfected) more necrotic granulomas were seen in HIV-positive patients with pleural TB [24].

In a case series of three HIV-pleural TB cases [28], a significant number of mesothelial cells were noted in the pleural fluid. Commonly, few mesothelial cells are seen in the pleural space in patients with pleural TB since it is believed that there is extensive chronic inflammation that covers the mesothelium preventing it from exfoliating these cells into the pleural fluid.

5.2. Cytokine Profiles in Pleural TB in HIV-Infected Patients. Cytokine profiles in the pleural fluid of pleural TB in HIV-infected patients versus TB-pleural patients showed a mostly Th1 (or proinflammatory, instead of the Th2 or anti-inflammatory) cytokine profile [31]. No differences were found in the levels or patterns of cytokines in pleural fluid between HIV-positive and negative patients except for higher IL-8 levels seen in dually infected individuals [31]. In another study, comparing HIV-uninfected and HIV-positive pleural TB patients, 29 cytokines were measured in the plasma and pleural fluid. IL-1β, IL-10, and TNFα were significantly decreased in the pleural fluid of HIV-positive pleural TB patients [32]. In addition, two proinflammatory markers, CXCL10/IP-10 and CCL3/MIP-1α, measured in the plasma were characteristic of pleural TB [32]. However, HIV infection affected the diagnostic accuracy as evidenced by a shift in cutoff values used, resulting in increased specificity at the expense of decreased sensitivity in pleural TB in HIV-infected persons compared to pleural TB in HIV-noninfected persons [32]. Other studies have shown that the levels of TNFα and MCP-1 were significantly elevated in the pleural fluid compared with autologous plasma in dually infected individuals [33]. MCP is a chemokine produced by fibroblasts and mesothelial cells and is a chemotactic agent for monocytes and lymphocytes. Both TB and HIV products can induce the production of MCP-1. Furthermore, transcription activation of HIV-1 in situ can be significantly reduced by neutralization of MCP-1; however, only when TNFα. was not neutralized suggesting a possible relationship between MCP-1/TNFα [33].

Necrotic granulomas from pleural biopsies done in coinfected patients with pleural disease showed significantly elevated TNF α-positive cells [24]. Although the authors acknowledged that this may not have equated to increased levels of the cytokine, the presence of this marker on the cells is an important difference which may explain the progression of pleural TB in HIV-infected patients in HIV-infected patients since apoptotic activity has been linked with TNFα and its continued presence is detrimental to the cellular environment [44] resulting in caseating necrosis. In another study, TNFα was elevated in HIV-pleural TB compared to HIV-uninfected pleural TB but the difference was not statistically significant [14]. In this same study, IFNγ was significantly elevated in serum and pleural fluid in HIV-pleural TB compared to HIV-uninfected pleural TB and it was suggested that CD8+ T cells could be the source which is supported by another study showing a relative increase in CD8+ T cells in HIV-positive patients with pleural TB [14]. However, mycobacterial replication was not controlled in the pleural space despite high levels of IFNγ. No difference was seen in the IL-10 levels in the pleural space between the two groups [14]. Another study [27] suggested apoptosis and levels of IFNγ are increased in HIV-infected patients with pleural TB; however, this finding was not unique to HIV TB and seen in pleural TB alone.

6. Clinical Features of Pleural TB in HIV-Infected Patients

HIV-infected patients with pleural tuberculosis were more likely to present with fever, [13, 15, 17], dyspnea [17], cough [15] and significant weight loss [15] in comparison with HIV-uninfected patients. Furthermore, systemic symptoms and signs such as fatigue, night sweats, diarrhea, lymphadenopathy, splenomegaly, and hepatomegaly were more common in HIV-infected patients [13]compared to HIV-uninfected patients [45]. Bilateral pleural effusions were also more frequently reported in HIV-positive patients [5] but the size or location of pleural effusions were comparable [5, 13, 15]. HIV-positive patients that present with pleural TB are generally sicker than non HIV-uninfected individuals as reflected by the increased frequency of systemic symptoms. Symptoms alone are limited in their ability to diagnose pleural TB in HIV-positive patients due to their non specific nature [13]. The severity of symptoms at presentation may reflect the higher degree of impairment in the immune system in HIV-positive patients which leads to more disseminated forms of the disease resulting in more advanced disease at presentation.

7. Diagnosis

7.1. Pleural Fluid. Pleural fluid examination showed increased mesothelial cells [28], decreased albumin and higher

Table 3: Reported yields of different tests in HIV-infected patients with pleural TB.

Study and year	Pleural fluid smear	*Pleural fluid culture	Pleural biopsy smear	*Pleural biopsy culture	Pleural biopsy histology	Sputum culture
Elliott al. 1993 [11]	0%	26%	NR	11%	52%	NR
Richter et al. 1994 [18]	6%	43%	20%	35%	86%	NR
Luzze et al. 2001 [15]	NR	43%	NR	NR	57%	NR
Conde et al. 2003 [26]	8%	15%	38%	77%	92%	77%
Hirsch et al. 2001 [27]	NR	87%	NR	NR	NR	NR
Kitinya et al. 1993 [35]	5%	5%	14%	14%	NR	NR
Heyderman et al. 1998 [13]	18%	NR	19%	42%	60%	NR

* Reported yields of cultures using Löwenstein-Jensen medium.
NR: not referred for test.

gamma globulinlevels in HIV-infected patients [17]. Lymphocyte predominant effusions [15], LDH, protein and glucose levels did not differ between dually infected patients with pleural TB [16].

7.2. Mycobacterium TB Identification in Pleural Fluid. Ziehl-Neelsen (ZN) stain [13, 15], liquid culture using BACTEC [14, 15] and Löwenstein-Jensen (LJ) cultures [11, 13, 15, 18, 26, 27, 41] consistently provided a higher yield in HIV-infected individuals compared to HIV-uninfected individuals. In one study [13], the more immunocompromised the patient, the higher chance of finding TB organisms in the pleural fluid and the pleura itself. A CD4 count of $< 200 \times 10^6$/L was associated with a positive pleural fluid smear (37% versus 0% $P = 0.0006$) and biopsy Ziehl-Neelsen stain (35% versus 7% $P = 0.021$)[13]. The TB yield of different tests used in tuberculouspleuritis in HIV-infected patientsis shown (Table 3).

Although many studies looking at nucleic acid amplification tests (NAAT) applied to pleural TB in HIV-infected patients have been done [45] they have shown considerable variability. However, the new gene xpert MTB/RIF assay is a significant advance in point of care molecular diagnostic biology [46] that could provide a significant improvement in diagnosing pleural TB in HIV-infected patients with the added benefit that rifampin resistance is also detected by the assay and the result can be obtained in 2 hours. Although no studies have been published in HIV-infected patients with pleural TB, a retrospective analysis of specimens sent to a national reference laboratory in Germany for mycobacteria [47] studied various specimen types including 113 pleural fluid samples for which the specificity was calculated at 98.1% and the sensitivity was not calculable.

7.3. HIV Identification in Pleural Fluid. HIV viral load in pleural fluid in dually infected patients has been demonstrated to be higher in the pleural fluid when compared to plasma [31]. Another study [33] showed that transcriptional activity of HIV-1 was significantly higher in pleural fluid mononuclear cells (PFMC) compared to peripheral blood mononuclear cells (PBMC). Increased HIV viral production was seen in the pleural space of HIV-pleural TB patients from activated HLA-DR mononuclear cells including lymphocytes and CD14+ macrophages [41]. Another study [25] suggested that HIV-positive patients with pleural TB showed higher HIV viral replication and heterogeneity which then migrated to the blood increasing systemic HIV heterogeneity. The excess viral loads seen in the pleural fluid of HIV-TB-pleural patients make it an important site to gain a better understanding of whether the increased levels of virus seen in the pleural fluid of dually infected patients may affect HIV progression in these patients. The development of targeted inhibitors of viral replication in dually infected patients could offer new insight.

7.4. Pleural Biopsy. Ziehl-Neelsen stain of pleural biopsy carries significantly higher yield in HIV-infected patients than non HIV in two studies [13, 26] and not significantly higher in other studies [17, 35]. The utility of histological examination of pleural biopsy was high in HIV-infected patients where granulomatous inflammation or caseous necrosis was detected in 52%–92%. This finding was comparable with HIV-uninfected patients [11, 13, 15, 17, 26].

7.5. Sputum Culture. Sputum culture yield in pleural tuberculosis was reported to be higher in HIV-infected patients, however this was not statistically significant [13, 26]. One of these studies [26], demonstrated that the yield of sputum cultures using sputum induction in dually infected patients with pleural TB who could not produce sputum spontaneously was high even in patients with no pulmonary findings on chest radiographs.

The use of the pleural fluid or biopsy smear to diagnose pleural TB in HIV-infected patientsremains an effective tool which is widely available and inexpensive especially in regions with limited resources. Clinicians often do not bother sending pleural fluid for smear and culture due to their low yield in the HIV-uninfected patient population; however, the evidence suggests that in dually infected patients smear and cultures should play an important role in the diagnostic process (Table 3). The pleural fluid and pleural biopsy TB culture yield is higher in HIV-infected patients. This may

suggest that a higher bacillary burden is seen in the pleural space in HIV-infected patients because TB might cross from the lung parenchyma to the pleural space with greater ease as a result of an impaired immune response in the pleural space. Furthermore, the use of bedside inoculation of pleural fluid in BACTEC liquid medium provides a better sensitivity and faster results in HIV coinfection [15]. All of these classic forms of diagnosing pleural TB are limited in the prolonged time it takes to obtain results and also from the limited laboratory access in the clinic/hospital where the patients are assessed. These diagnostic delays result in significant morbidity and mortality of patients. In regions with limited resources, a point of care test which could be done at the bedside on pleural fluid would have a significant impact on the management and outcome of these patients.

7.6. Adenosine Deaminase (ADA). Adenosine deaminase (ADA) a T lymphocyte enzyme that catalyzes the conversion of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. Two different molecular forms of ADA, ADA 1, and ADA2 have been identified [48]. ADA1 is found in all cells, with its greatest activity in lymphocytes and monocytes. ADA2 isoenzyme is found mainly in monocytes/macrophages. Most of the ADA found in tuberculous pleural fluid is ADA2, whereas most of the ADA found in other pleural fluids is ADA1. Testing ADA levels in the pleural fluid is an easy, inexpensive, and useful test to establish the diagnosis of pleural TB. ADA retains its high utility in all HIV-infected patients [16] even patients with low CD4 counts [22]. ADA improves the accuracy of diagnosis in HIV-infected patients with pleural TB [16, 17, 22]. In all HIV-infected patients regardless of CD4 counts, the sensitivity of ADA was 94% when the cutoff value of 30 u/l was used and specificity of 95% [22]. The positive likelihood ratio was 18.9 and the negative likelihood ratio was 0.06 [22]. The sensitivity wasalso highat 96% when the cutoff value used was 60 u/l in HIV-infected patients [16]. These results were comparable to HIV-uninfected patients [16].

However, it should be noted that the sensitivity and specificity of ADA vary according to the different cutoff levels and also to different TB population prevalence. ADA measurement has a limited value in regions of low TB prevalence [49] as it can also be elevated in patients with empyema, lymphoma, lung cancer, rheumatoid arthritis, systemic lupus erythematosus, brucellosis, and Q fever [50]. In a high HIV and TB prevalence region, the use of ADA at a higher cut point (47 IU/L) compared to the standard cut point (30 IU/L) in which a subgroup of HIV tested patients were studied, it was noted that ADA increases its specificity while use of the standard cut point (30 IU/L) results in loss of specificity but an increase in its sensitivity and thus improves its ability to rule out disease.

A possible explanation for the high levels of ADA even in HIV-infected patients with low CD4 counts may be related to the fact that monocytes are not significantly affected by HIV coinfection and they are the primary cells responsible for the production of isoenzyme ADA-2. Riantawan et al. [16] documented the best cutoff of ADA at 60 U/L in HIV-infected patients which provides sensitivity of 95% and specificity of 96%. Liang QL et al. [51] published a meta-analysis in mostly HIV-uninfected patients which included 63 studies documenting sensitivity and specificity of pleural ADA in the diagnosisof pleural TB to be 92 and 90%, respectively [51]. The positive likelihood ratio was 9.03, the negative likelihood ratio was 0.10, and the diagnostic odds ratio was 110.08 [51].The most widely accepted cutoff value for pleural fluid ADAis 40 U/l [50]; however, this cutoff will likely need to be higher in the HIV population.

7.7. Interferon Gamma. Interferon gamma levels in pleural tuberculosis were significantly higher in both serum and pleural fluid of HIV-positive patients when compared with HIV-uninfected patients [14]. There was no statistically significant correlation between blood CD4 cell count and the level of pleural INF-gamma [14, 21] but a positive correlation with pleural fluid viral loads was noted (correlation coefficient 0.54, $P = 0.02$) [14]. Another study documented interferon gamma sensitivity of 99% and specificity of 98% using 3.7 U/mL cutoff point which did not differ between HIV-positive and HIV-uninfected patients [20]. In a meta analysis of 27 studies evaluating the role of interferon gamma release assays (T-SPOT.TB1, QFT-G-IT), and Tuberculin Skin Test (TST) in diagnosing active tuberculosis [52], 5 studies used IGRA tests for the diagnosis of pleural TB [8, 21, 53–55]. None of these studies tested all of the patients entering the respective studies for HIV and all were limited by small sample sizes. However, one study [8] did test most of the cohort for HIV (51/67) and managed to confirm that even in a high HIV burden region, unstimulated pleural fluid interferon gamma levels measured in TB suspects were found to be highly sensitive and specific for distinguishing pleural TB from non-TB effusions [8]. In the same study it was also shown that because many HIV-positive patients likely have paucicellular pleural inflammation, adequate volumes of pleural fluid need to be obtained (>20 mL) in order to obtain adequate number of cells to analyze [8]. Interferon gamma inducible protein of 10 kDa (IP-10) and lipoarabinomannan (LAM) mycobacterial antigen-detection assay were not useful in discriminating pleural TB patients versus non-TB patients in a subset of HIV-infected patients; however, future work should focus on validating IP-10 ability to rule out pleural TB [9]. The impact of immunosupression seen in HIV infection on the diagnostic accuracy of pleural mononuclear cells and their capacity to secrete interferon gamma requires further study in larger studies to confirm the accuracy of the findings presented.

Pleural TB in HIV-infected patients remains a challenge to diagnose because often sputum smears and cultures are negative. Thoracentesis is commonly performed to examine the composition of the pleural fluid. However, biochemical, histological and microbiological examination is usually limited in low-resource countries. A single point of care test that could be applied to pleural fluid would have enormous impact on the diagnosis and management of these cases.

8. Treatment

There is no evidence to suggest that HIV-pleural tuberculosis should be treated differently than pulmonary TB. Two months of isoniazid, rifampin, pyrazinamide and ethambutol followed by 4 months of isoniazid and rifampin for susceptible organisms to all first line drugs are the international standard [56].

It is recommended that antiretroviral therapy should be delayed for 2 months unless the CD4 < 100×10^6/L to prevent immune reconstitution syndrome which could occur in one-third of patients [57]. The use of rifabutin instead of rifampin is recommended to avoid drug interaction with protease inhibitors and most nonnucleoside reverse transcriptase inhibitors, with the exception of efavirenz. Even so, rifampin-containing regimens can be prescribed if the selected antiretroviral drugs include efavirenz and two nucleoside transcriptase inhibitors (e.g., tenofovir and emtricitabine) [50]. In one randomized controlled study of 197 patients with HIV associated pleural TB, the administration of prednisolone as adjunctive therapy to standard first line TB therapy did not result in a survival benefit and although it was associated with faster resolution of the TB it was also associated with an increased risk of Kaposi sarcoma [29]. The patients in this study were significantly immunocompromised and the effects of steroids on HIV-infected patients with CD4 counts >200×10^6/L still need to be assessed. A recent Cochrane review found no clear evidence to support the use of adjunctive corticosteroids in all persons with TB-pleural effusion [58].

9. Quality of Included Studies

Many of the studies had small sample sizes and did not have consecutive recruitment of patients which may have resulted in selection and information bias. Furthermore, the study design limited the quality of several studies with only one randomized controlled trial, several prospective cohorts, some retrospective chart reviews and several case series. Another significant limitation is the fact that many of the HIV-infected patients had different levels of immunosuppression (different levels of CD4 cells) which could affect the immunopathogenesis studies.

10. Reasons for Exclusion of Studies

Many of the studies that were excluded from this analysis did not establish the diagnosis of pleural TB or HIV accurately. One common reason for exclusion was that the patients in the HIV-uninfected group were not tested for HIV and assumed to be HIV uninfected. Some of the earlier studies opted to make a clinical diagnosis of HIV instead of getting serological testing which may have not been available at the time of the study. Other studies categorized pulmonary TB in the same category as pleural TB, whereas we categorized it as extrapulmonary disease with specific criteria depicted in the inclusion criteria section. Several studies were excluded because they were case reports.

11. Future Research

Pleural TB in HIV-infected patients has a different immunopathogenesis than HIV-uninfected pleural TB and these findings in part support the diagnostic differences seen in the yield of TB in the pleura in HIV-infected patients compared to HIV-uninfected patients. More research is needed in the field of immunopathogenesis of HIV-pleural TB disease as it offers an important microcosm where the bacteria and virus interact allowing the study of this complex interaction between HIV and TB to be further elucidated. Furthermore, the immunopathogenic differences may help develop a better interferon gamma-based point of care test that could be used at the bedside on pleural fluid in low-resource countries with high HIV prevalence. The Gene Xpert MTB/RIF assay technology applied to pleural fluid is a formidable area of research which could have enormous impact on the early diagnosis of pleural TB in HIV-positive patients and should be deemed a research priority in this field.

Conflict of Interests

No conflict of interest to declare by any of the authors.

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Research Article

Unveiling the Mechanisms for Decreased Glutathione in Individuals with HIV Infection

Devin Morris,[1] Carlos Guerra,[2] Clare Donohue,[3] Hyoung Oh,[1] Melissa Khurasany,[4] and Vishwanath Venketaraman[1,5]

[1] *Graduate College of Biomedical Sciences, Western University of Health Sciences, Pomona, CA 91766, USA*
[2] *Science Department, College of Osteopathic Medicine of the Pacific, Western University of Health Sciences, Pomona, CA 91766, USA*
[3] *Pitzer College, 1050 N Mills Avenue, Claremont, CA 91711, USA*
[4] *College of Dental Medicine of the Pacific, Western University of Health Sciences, Pomona, CA 91766, USA*
[5] *Department of Basic Medical Sciences, College of Osteopathic Medicine of the Pacific, Western University of Health Sciences, 309 East Second Street, Pomona, CA 91766, USA*

Correspondence should be addressed to Vishwanath Venketaraman, vvenketaraman@westernu.edu

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We examined the causes for decreased glutathione (GSH) in individuals with HIV infection. We observed lower levels of intracellular GSH in macrophages from individuals with HIV compared to healthy subjects. Further, the GSH composition found in macrophages from HIV$^+$ subjects heavily favors oxidized glutathione (GSSG) which lacks antioxidant activity, over free GSH which is responsible for GSH's antioxidant activity. This decrease correlated with an increase in the growth of *Mycobacterium tuberculosis (M. tb)* in macrophages from HIV$^+$ individuals. In addition, we observed increased levels of free radicals, interleukin-1 (IL-1), interleukin-17 (IL-17) and transforming growth factor-β (TGF-β) in plasma samples derived from HIV$^+$ individuals compared to healthy subjects. We observed decreased expression of the genes coding for enzymes responsible for de novo synthesis of GSH in macrophages derived from HIV$^+$ subjects using quantitative PCR (qPCR). Our results indicate that overproduction of proinflammatory cytokines in HIV$^+$ individuals lead to increased production of free radicals. This combined with the decreased expression of GSH synthesis enzymes leads to a depletion of free GSH and may lead in part to the loss of immune function observed in HIV patients.

1. Introduction

According to the World Health Organization (WHO), it is estimated that approximately 33.3 million people are infected with HIV, two-thirds of which live in sub-Saharan Africa. It is also estimated that nearly 2.6 people are infected each year [1]. People diagnosed with AIDS often suffer from life-threatening diseases caused by opportunistic infections including tuberculosis (TB). In recent years, there has been a significant increase in the incidence of TB due to the emergence of multidrug and extreme-drug resistant strains of *M. tb* and due to increased numbers of highly susceptible immunocompromised individuals arising from the AIDS pandemic. It is also believed that in developing countries, as many as 40 to 80% of individuals with AIDS are at risk of developing TB [2, 3]. In previous studies, our lab has reported that the virulent strain of *M. tb* is sensitive to the antioxidant, GSH [4–9]. Our studies have shown that HIV-infected individuals have deficiencies of intracellular GSH in red blood cells (RBC's) as well as peripheral blood mononuclear cells (PBMC's), which include T cells, natural killer cells, and monocytes [8].

GSH is a tripeptide composed of glutamine, cysteine, and glycine which plays a major role in the maintenance of the intracellular redox state. GSH is also important for cellular homeostasis as well as many different cellular functions such as protein synthesis, enzyme catalysis, transmembrane transport, receptor action, intermediary metabolism, and cell maturation [10–12]. GSH is produced by nearly all cell types and exists in two forms. Reduced or free GSH is responsible for the antioxidant functions of GSH, while GSSG is the

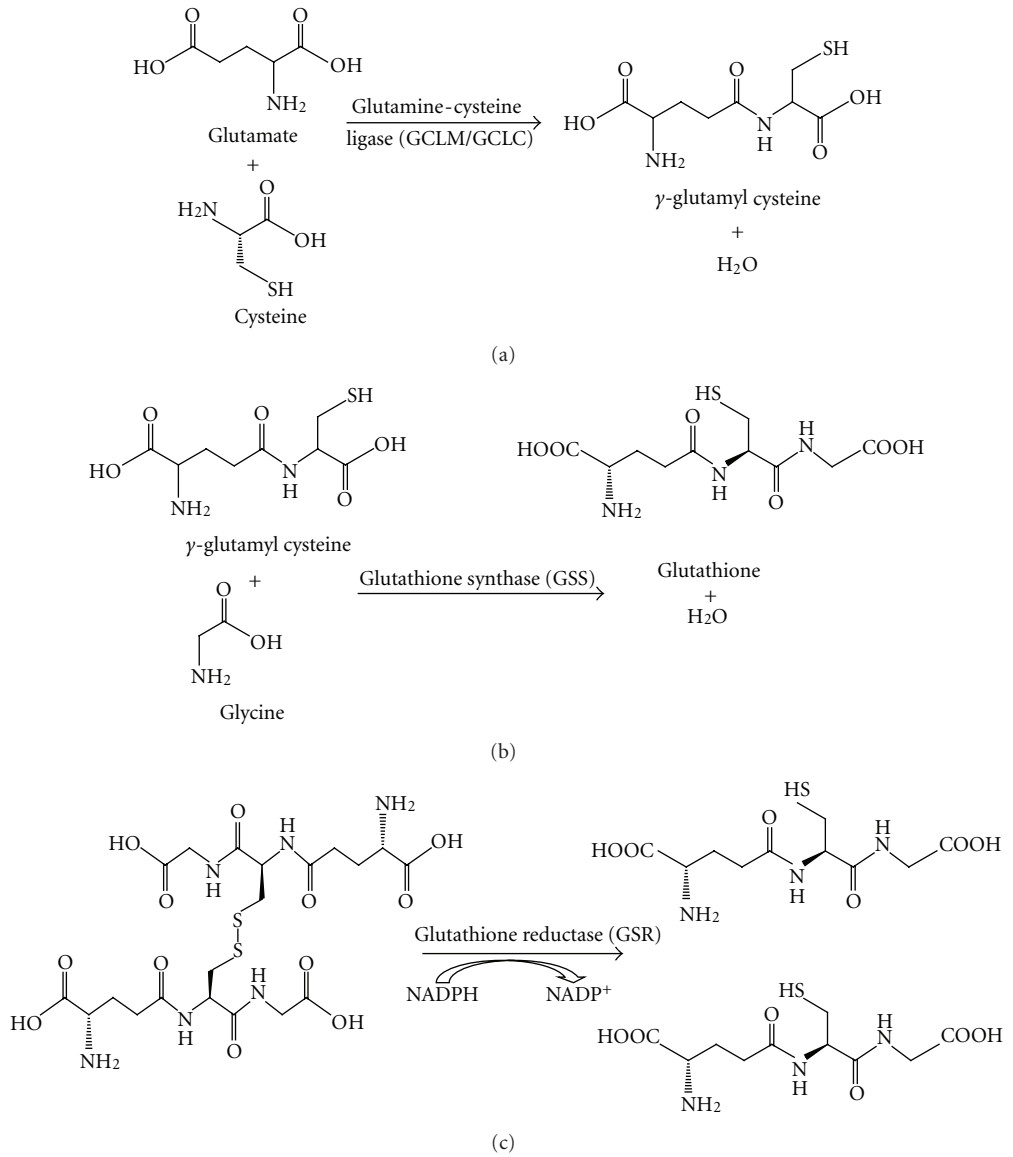

Figure 1: (a) The first step in de novo GSH biosynthesis is rate limiting. Glutamine and cysteine are linked by the homodimeric enzyme glutamine-cysteine ligase, (b) GSS catalyzes the second step in GSH biosynthesis, linking glycine and γ-glutamyl cysteine to form GSH. (c) Oxidized GSH can be converted to free GSH by GSR, utilizing NADPH as a cofactor.

byproduct of the free radical scavenging activity of GSH and lacks antioxidant function [10–12].

Free GSH is synthesized in the cell via two different mechanisms. Free GSH can be synthesized de novo through a two-step process which is mediated by two different enzymes. The first, and rate-limiting step in de novo GSH synthesis is the linking of glutamine and cysteine by glutamine-cysteine ligase (GCL) to form γ-glutamyl cysteine (Figure 1(a)). The second step is the linking of glycine to γ-glutamyl cysteine which is catalyzed by the enzyme glutathione synthase (GSS) (Figure 1(b)). Free GSH can also be synthesized via the reduction of GSSG. This reaction is catalyzed by glutathione Reductase (GSR) which utilizes NADPH as a cofactor (Figure 1(c)).

It is our hypothesis that chronic HIV infection leads to excessive production of proinflammatory cytokines such as IL-1, IL-17, and TNF-α. The chronic overproduction of proinflammatory cytokines leads to the generation of free radicals. These free radicals are scavenged by free GSH. The excessive production of free radicals in HIV-infected individuals will lead to the depletion of GSH. In addition, elevated TGF-β blocks the production of GCLC which reduces the production of new molecules of GSH. Elevated levels of IL-1 will also facilitate the loss of intracellular cysteine, which further reduces the production of new GSH molecules. We tested our hypothesis by performing in vitro studies using human monocyte-derived macrophages isolated from healthy individuals and individuals with HIV infection. Our

results signify an important mechanism that is responsible for decreasing the levels of GSH in macrophages from individuals with HIV infection resulting in enhanced multiplication and survival of intracellular *M. tb*.

2. Materials and Methods

2.1. Subjects. A total of 26 volunteers (13 healthy subjects and 13 individuals with HIV infection) were recruited for the study. Individuals with HIV infection were recruited from the Foothills AIDS project. Healthy subjects without HIV infection or a history of TB were recruited from the university faculty and staff. All HIV-infected volunteers had been diagnosed with HIV-1, were taking some form of antiretroviral treatment (ART), and had $CD4^+$ T-cell counts between 271 and 1415 cells per mm^3. Thirty five milliliters of blood was drawn once from both healthy volunteers and individuals with HIV infection after obtaining a signed informed consent. All our studies were approved by both the Institutional Review Board and the Institutional biosafety committee.

2.2. Isolation of Monocytes and In Vitro Culture for Differentiation to Macrophages. We first isolated PBMC from the whole blood of healthy and HIV-infected individuals using density gradient centrifugation with FICOLL Histopaque (Sigma). Plasma samples from healthy and HIV-infected subjects were collected for cytokine measurement. Monocytes were isolated from PBMCs by adherence to poly-L lysine (%0.005) treated-96 well-tissue culture plates. Briefly, PBMCs (1×10^5 cells/well) were added to poly-L lysine-treated tissue culture plates and incubated overnight at 37°C to facilitate monocyte adherence. Following overnight adherence, the nonadherent cells were removed, and fresh media [RPMI (Sigma) supplemented with 5% human AB serum (Sigma)] was added to the adherent cells which were then incubated for 7 days in culture conditions to allow the monocytes to differentiate into macrophages.

2.3. Assay of IL-1, TGF-β, IL-17, and Malondialdehyde (MDA) in Plasma and Macrophage Lysates. Cytokines were measured from collected plasma and macrophage culture supernatants by enzyme-linked immunosorbent assay (ELISA) (eBioscience). MDA was measured in macrophage lysates by colorimetric assay (Cayman Chemical).

2.4. Assay of GSH Levels in Macrophages from Healthy and HIV-Infected Subjects. GSH levels were measured in isolated macrophages from healthy subjects and individuals with HIV infection. Intracellular levels of GSH in macrophages were determined by spectrophotometry using an assay kit from Arbor Assays. Briefly, macrophages (3×10^5) were detached from the culture plate by treatment with trypsin (50 μL/10^5 cells) for 10 minutes in culture conditions. Detached cells were washed and resuspended in ice cold 5% 5-sulfosalicylic acid dehydrate solution (SSA). Supernatants collected after centrifugation were analyzed for total and oxidized GSH as per manufacturer's instructions. Free GSH was calculated by subtracting measured oxidized GSH concentrations from the measured total GSH concentrations, per the manufacturer's instructions. All GSH measurements were normalized with total protein levels.

2.5. Assay of Total Protein Levels in Macrophage Supernatants and Cell Lysates. Proteins in the isolated macrophage supernatants and lysates were measured by Bradford's method using a Coomassie protein assay reagent (Thermo Scientific).

2.6. Preparation of Bacterial Cells for Macrophage Infection. All infection studies were performed using the virulent laboratory strain of *M. tb*, H37Rv inside the biosafety level 3 (BSL-3) facility. *M. tb* was processed for infection as follows: static cultures of H37Rv at their peak logarithmic phase of growth (between 0.5 and 0.8 at A600) were used for infection of monocytes. The bacterial suspension was washed and resuspended in RPMI (Sigma) containing AB serum (Sigma). Bacterial clumps were disaggregated by vortexing five times with 3 mm sterile glass beads. The bacterial suspension was passed through a 5 μm syringe filter (Millipore) to remove any further clumps. The total number of organisms in the suspension was ascertained by plating. Processed H37Rv was frozen as stocks at −80°C. At the time of infection, frozen stocks of processed H37Rv were thawed and used for monocyte infection.

2.7. Infection of Macrophages with M. tb. Monocyte-derived macrophages were infected with processed H37Rv at a multiplicity of infection of 10 : 1 and incubated for 2 hours for phagocytosis. Unphagocytosed mycobacteria were removed by washing the infected macrophages three times with warm sterile PBS. Infected macrophages were cultured in RPMI + 5% AB serum. Infected cultures were terminated at 1 hour and 5 days postinfection to determine the intracellular survival of H37Rv. Culture supernatants were collected, filtered through a 0.2 μm filter, and stored at −80°C for the assay of cytokines.

2.8. Termination of Infected Macrophage Cultures and Measurement of Colony Forming Units. The termination of *M. tb* infected cultures was performed by the addition of 200 μL of distilled sterile water to each culture well. The collected cell lysates were plated on 7H11 medium (Hi Media) enriched with albumin dextrose complex (ADC), to estimate the extent of H37Rv growth in macrophages.

2.9. Quantitative PCR Analysis of GSH Synthesis Genes. RNA was isolated from macrophages from healthy and HIV-infected individuals using Trizol (Invitrogen) per the manufacturer's instructions. Isolated RNA was quantified using a NanoVue spectrophotometer (GE). Isolated RNA was reverse transcribed to cDNA using a qScript cDNA synthesis kit (Quanta Biosciences) per the manufacturer's instructions. qPCR analysis of gene expression was performed using an EvaGreen qPCR master mix (Biotium) and primers for the GSH-metabolism genes: glutamine-cysteine ligase (catalytic subunit) (GCLC), glutamine-cysteine ligase (modulatory subunit) (GCLM), glutathione synthetase (GSS), glutathione Reductase (GSR), and γ-glutamyl transferase 1 (GGT1) (Elim Biopharmaceuticals) on a StepOne Plus thermocycler

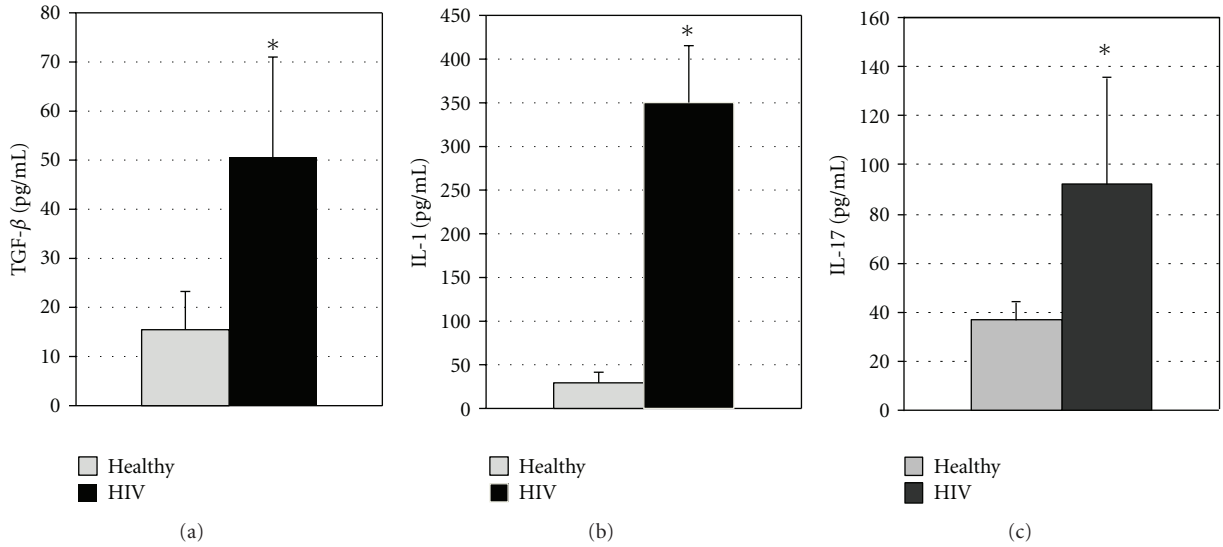

Figure 2: (a) *Assay of TGF-β in plasma from HIV-infected and healthy subjects*. Plasma samples separated from blood of healthy volunteers and HIV-infected individuals were used for measurement of TGF-β. Levels of TGF-β in the plasma samples were determined by ELISA using assay kits procured from eBioscience. Results in Figure 2 are averages of data collected from eight healthy subjects and twelve individuals with HIV infection. Results show elevated TGF-β in HIV-infected subjects ($^*P \leq 0.05$). (b) *Assay of IL-1 in plasma from HIV-infected and healthy subjects*. Plasma samples separated from blood of healthy volunteers and HIV-infected individuals were used for measurement of IL-1. Levels of IL-1 in the plasma samples were determined by ELISA using assay kits procured from eBioscience. Results in (b) are averages of data collected from eight healthy subjects and twelve individuals with HIV infection. Results show elevated IL-1 in HIV-infected subjects ($^*P \leq 0.05$). (c) *Assay of IL-17 in plasma from HIV-infected and healthy subjects.* Plasma samples separated from blood of healthy volunteers and HIV-infected individuals were used for measurement of IL-17. Levels of IL-17 in the plasma samples were determined by ELISA using assay kits procured from eBioscience. Results in (c) are averages of data collected from eight healthy subjects and eight individuals with HIV infection. Results show elevated IL-17 in HIV-infected subjects ($^*P \leq 0.05$).

(ABI) per the manufacturer's instructions. Using the ΔΔCt method, comparative gene expression analysis was performed. Target gene expression was compared to the endogenous control gene β-actin (ACTB) for each sample. Gene expression for each target gene in samples from HIV-infected subjects was compared to the expression observed in healthy controls.

3. Results

3.1. Assay of Cytokines and MDA in Plasma and Macrophage Supernatants. Measurement of IL-1 concentrations in the plasma of healthy and HIV-infected individuals revealed a significant increase in the amounts of IL-1 present in the plasma of HIV-infected individuals over those found in healthy individuals. In fact, our study demonstrates a greater than 6-fold increase in the plasma IL-1 concentrations of HIV-infected individuals (Figure 2(b)). In addition, our assay of IL-1 in the culture supernatants of macrophages from HIV-infected and healthy individuals demonstrated a significant increase in the production of IL-1 by HIV-infected over uninfected macrophages (Figure 3(a)).

We also observed a significant increase in the levels of TGF-β in the plasma of HIV-infected individuals compared to healthy subjects (Figure 2(a)). Assay of TGF-β in the supernatants of macrophage cultures also demonstrated a significant increase in the production of TGF-β by macrophages from the HIV-infected group over those from the healthy group (Figure 3(c)).

Our analysis also included an assay of IL-17 in the plasma of our HIV and control groups. We found a significant elevation in the levels of IL-17 in the plasma from the HIV-infected group in comparison to the control group (Figure 2(c)).

Our comparison of TNF-α in the supernatants of HIV-infected and healthy macrophages revealed a significant increase in TNF-α production by HIV-infected macrophages (Figure 3(b)).

In addition to the cytokines mentioned above, we also assayed the concentrations of MDA in the macrophage lysates from healthy and HIV-infected subjects. MDA is a by-product formed during lipid peroxidation. Measurement of MDA has been shown to be a reasonably accurate representation of free radical formation [13, 14]. Our assay of MDA revealed increased levels in the macrophage lysates of HIV-infected individuals over healthy individuals (Figure 3(d)). These elevated MDA levels correspond to elevated production of free radicals.

3.2. Assay of GSH in Macrophage Lysates. Our analysis of GSH in macrophage lysates demonstrated a marked decrease in the total GSH present in HIV-infected macrophages when compared to uninfected macrophages (Figure 4(a)). In addition, our analysis of the GSH present in the tested macrophage lysates demonstrated significantly low levels of

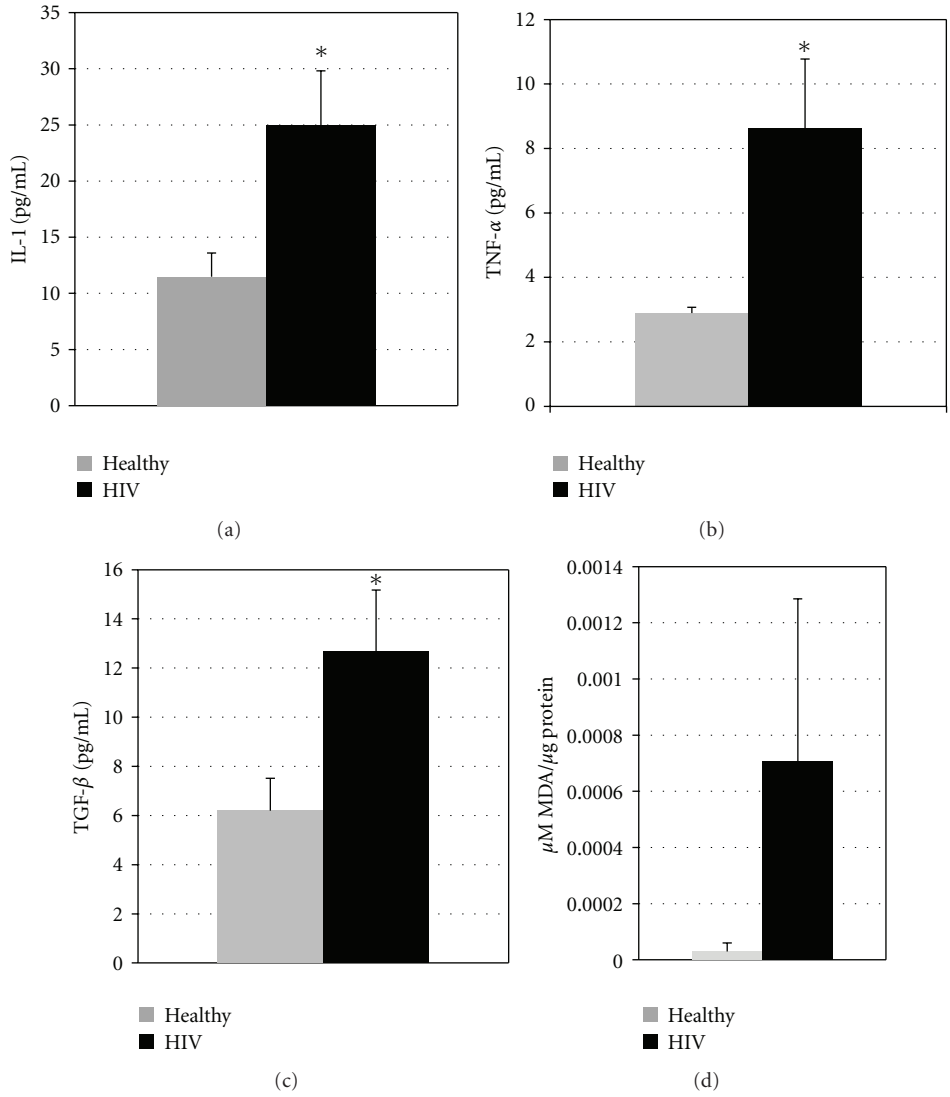

Figure 3: (a) *Assay of IL-1 in macrophage culture supernatants from HIV-infected and healthy subjects*. Supernatants from human monocyte-derived macrophages (from healthy and HIV-infected individuals) were assayed for the levels of IL-1 using assay kit from ebioscience. Data in (a) denote means ± SE from eight healthy individuals and nine individuals with HIV infection ($^*P \leq 0.05$). (b) *Assay of TNF-α in macrophage culture supernatants from HIV-infected and healthy subjects*. Supernatants from human monocyte-derived macrophages (from healthy and HIV-infected individuals) were assayed for the levels of TNF-α using assay kit from ebioscience. Data in (b) are means ± SE from eight healthy individuals and nine individuals with HIV infection ($^*P \leq 0.05$). (c) *Assay of TGF-β in macrophage culture supernatants from HIV-infected and healthy subjects*. Supernatants from human monocyte-derived macrophages (from healthy and HIV-infected individuals) were assayed for the levels of TGF-β using assay kit from ebioscience. Data in (c) represent means ± SE from eight healthy individuals and nine individuals with HIV infection ($^*P \leq 0.05$). (d) *Assay of MDA in macrophage lysates from HIV-infected and healthy subjects.* Free radical levels in macrophage lysates from healthy subjects and individuals with HIV infection were determined by measuring the levels of MDA using a colorimetric assay kit from Cayman. Results in (d) are averages of data collected from five healthy subjects and five individuals with HIV infection. These elevated MDA concentrations are indicative of elevated free radical concentrations. High standard error values observed in HIV-infected subjects (a–d) are likely due to the varying stages of HIV infection and antiretroviral treatment in the HIV-infected population.

free GSH in macrophages from individuals with HIV-infection when compared to macrophages from healthy subjects (Figure 4(b)). Of particular interest are the relative percentages of free GSH and GSSG. In macrophages isolated from HIV-positive subjects, we observed the total GSH as being composed of about 30% free GSH and 70% GSSG. In macrophages isolated from healthy individuals, we observed a GSH composition of about 60% free GSH and 40% GSSG (Figure 4(c)).

3.3. Determination of the Intracellular Survival of M. tb in Isolated Monocyte-Derived Macrophages. Our test for the intracellular survival of *M. tb* in isolated macrophages demonstrated growth of *M. tb* in the macrophages from both

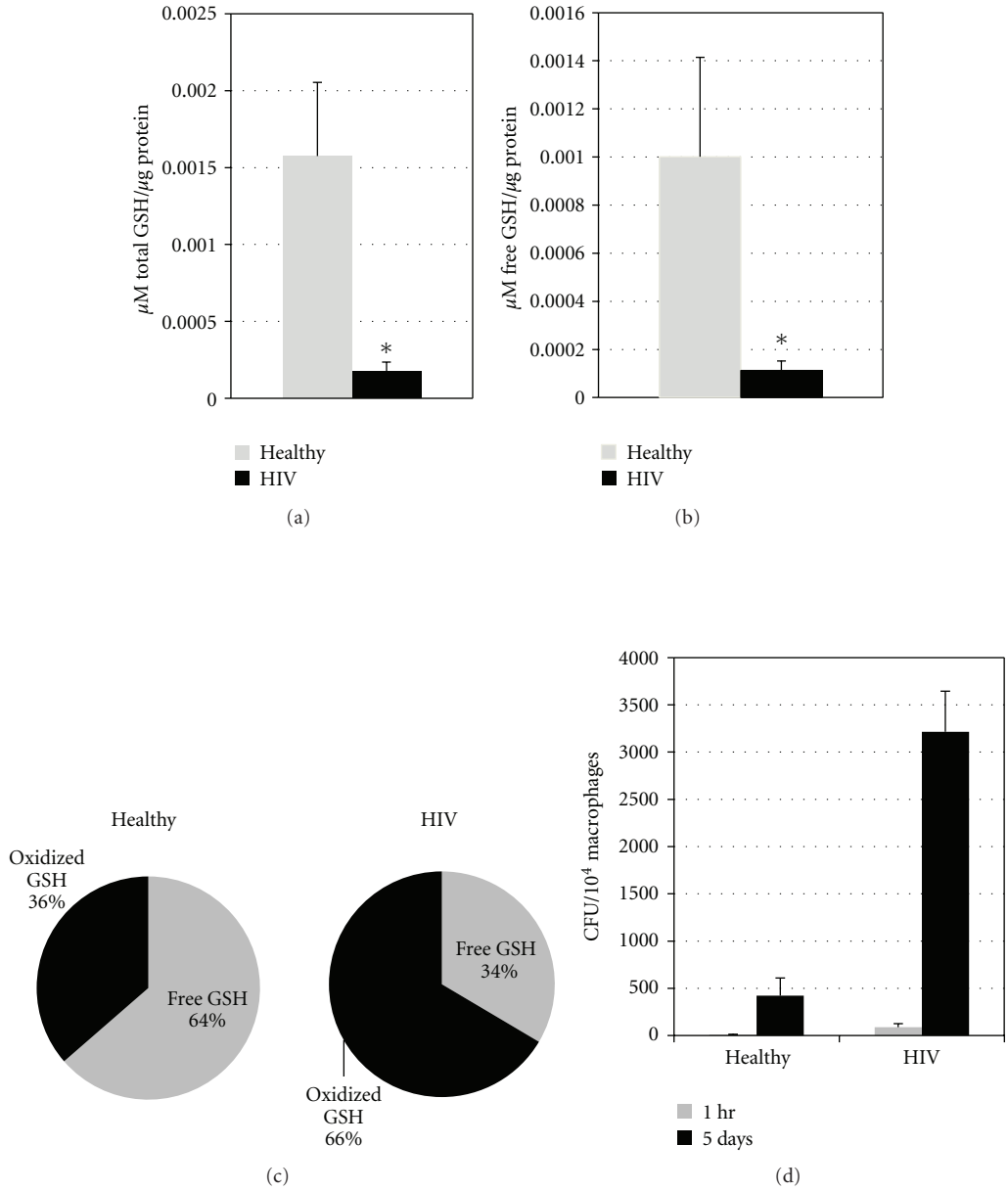

Figure 4: (a) *Assay of total GSH concentrations in macrophage lysates from HIV-infected and healthy subjects.* GSH levels were measured in isolated macrophages from healthy subjects and individuals with HIV infection by spectrophotometry using an assay kit from Arbor Assays. Briefly, an equal volume of ice cold 5% 5-SSA was added to the macrophage (3×10^5) pellet. Supernatants collected after centrifugation were analyzed for total, and oxidized GSH as per manufacturer's instructions. All GSH measurements were normalized with total protein levels. Data in (a) represent means ± SE from five different healthy and HIV-infected individuals. GSH concentrations are decreased in HIV-infected macrophages with respect to healthy macrophages ($^*P \leq 0.05$). (b) *Assay of free GSH concentrations in macrophage lysates from HIV-infected and healthy subjects.* Free GSH was calculated by subtracting measured oxidized GSH concentrations from the measured total GSH concentrations, per the manufacturer's instructions. Data in (b) denote means ± SE from five different healthy and HIV-infected individuals. GSH concentrations are decreased in HIV-infected macrophages with respect to healthy macrophages ($^*P \leq 0.05$). Free GSH concentrations are decreased in HIV-infected macrophages with respect to healthy macrophages ($^*P \leq 0.05$). (c) A comparison of the composition of the total GSH in healthy and HIV-infected subjects reveals that the majority of GSH in HIV-infected macrophages exists as oxidized GSH (~70%), whereas healthy macrophages contain a more balanced GSH makeup (~40% oxidized, ~60% free). (d) *Intracellular growth of M. tb in macrophages from healthy and HIV-infected individuals at 1 hour, and 5 days postinfection*. Human monocyte-derived macrophages (from healthy and HIV-infected individuals) were infected with the processed H37Rv at a multiplicity of infection of 10 : 1. Infected macrophages were terminated at 1 hour and 5 days postinfection to determine the intracellular survival of H37Rv inside macrophages from healthy and HIV-infected individuals. Macrophage lysates were plated on 7H11 medium enriched with ADC to estimate the growth or killing of H37Rv. Results shown in (d) are averages from $n = 4$ HIV and $n = 5$ healthy. Each experiment was performed in triplicate. Macrophages from HIV-infected subjects demonstrate a markedly decreased ability to control intracellular *M. tb* growth.

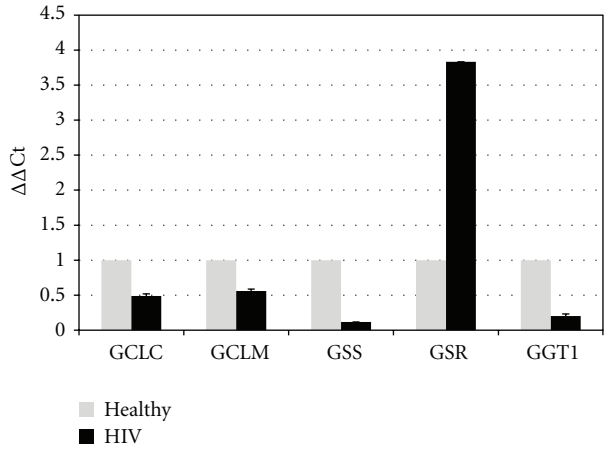

Figure 5: Relative gene expression for the specified target genes in comparison to the endogenous control ACTB. A comparison of gene expression in macrophages from healthy and HIV-infected subjects demonstrates reduced gene expression for all genes involved in the de novo synthesis of GSH in macrophages from HIV-infected subjects. A greater than 3-fold increase in GSR expression is observed in HIV-infected macrophages. Results are from $n = 3$ individuals for both healthy and HIV-infected macro-phages.

healthy and HIV-infected macrophages; however, there was several-fold increase in the growth of *M. tb* in macrophages from HIV-infected subjects. In fact, there was over 3 times the *M. tb* growth in HIV-infected macrophages when compared to healthy macrophages (Figure 4(d)). These results confirm that the ability of the macrophages collected from HIV-infected subjects to control infections is impaired.

3.4. Comparative Gene Expression Analysis of GSH Synthesis Enzymes. Comparative gene expression analysis of GCLC, GCLM, GSS, and GGT1 in macrophages from HIV-infected subjects demonstrated a significant reduction in the expression of these genes when compared to healthy macrophages. Expression of GCLC and GCLM in HIV-infected macrophages was reduced by about half. Expression of GSS in HIV-infected macrophages was reduced by about 89%. Interestingly, GSR expression in HIV-infected macrophages was increased by a factor of 3.8 over GSR expression in macrophages isolated from healthy subjects. Finally expression of GGT1 in HIV-infected macrophages was found to be reduced by about 80% compared to healthy macrophages (Figure 5).

4. Discussion

M. tb remains one of the most pernicious and an enduring pathogen of mankind. It is inferred that the first infected cell is the alveolar macrophage that internalizes the bacilli following inhalation of droplets aerosolized by infected individuals [15]. The macrophage is known to be the primary defense mechanism against microbial invasion [16, 17]. Macrophages have a phagocytic system that delivers the microbe into a compartment, that is, the site of generation of reactive oxygen intermediates, increasing acidity, hydrolytic activity, and the presence of antimicrobial peptides [15]. Importantly, we reported that GSH plays a key role in limiting intracellular growth of H37Rv in both human and murine macrophages [4–6]. Thus, GSH has direct antimycobacterial activity distinct from its role as an NO carrier, functioning as an effector molecule in innate defense against *M. tb* infection [4–6]. These results unfold a novel and potentially important innate defense mechanism adopted by human macrophages to control *M. tb* infection [5, 6]. Consistent with these observations, we have also found that GSH in combination with cytokines, such as IL-2 and IL-12, enhance the activity of natural killer cells to control *M. tb* infection inside human macrophages [7]. Importantly, data from our most recent studies indicate that GSH activates the functions of T lymphocytes to control *M. tb* infection inside human monocytes (unpublished). All these observations support the fact that GSH controls *M. tb* infection by functioning as an antimycobacterial agent as well by enhancing the functions of immune cells. Finally, we demonstrated that GSH levels are significantly reduced in PBMC isolated from individuals with HIV infection, and this decrease correlated with increased production of proinflammatory cytokines and enhanced growth of *M. tb* [8].

In this study, we examined the causes for decreased GSH in individuals with HIV infection. Furthermore, we also characterized the effects of decreased GSH in impairing the growth inhibition of *M. tb* inside macrophages.

Measurement of IL-1 and TGF-β concentrations in the plasma and macrophage supernatants of healthy and HIV-infected individuals revealed a significant increase in the amounts of these cytokines present in HIV-infected individuals over healthy individuals. IL-1 is a proinflammatory cytokine, when bound to its receptor, and transduces a signal that initiates expression of a wide variety of inflammatory genes via the NF-κB system. The subsequently transcribed genes can produce a variety of inflammatory products including chemokines, and proinflammatory cytokines, such as TNF-α, IL-6, or IL-8 [18]. IL-1 and TNF-α have been shown to be produced by either the binding of gp120 to the CD4 molecules on mononuclear phagocytes or infection with HIV [19, 20]. Several studies have demonstrated a link between increased TGF-β production and decreases in GCLC gene expression, as well as synthesis of GSH [21, 22]. In addition, we observed significant increases in the production IL-1 and TGF-β in the supernatants of macrophage cultures from the HIV-infected group compared to healthy individuals (Figures 3(a) and 3(c)).

We also observed a significant increase in the levels of IL-17 in plasma samples from individuals with HIV-infection. IL-17 is thought to play a significant role in activating and inducing antimicrobial peptides and proinflammatory cytokines like IL-6, CCL2, and TNF-α [23, 24]. Furthermore, high levels of this cytokine have been linked to a number of inflammatory diseases including rheumatoid arthritis, multiple sclerosis, and asthma. Low levels, on the other hand, are thought to cause both impaired host defense against mycobacterial infection and decreased antibacterial immunity [23, 25].

Studies on the effects of HIV on IL-17 concentrations using flow cytometry have found that HIV-infected patients have significantly increased levels of IL-17 [26]. However, Brenchley et al. [25] noted that there were significantly fewer IL-17 producing Th17 cells in the gastrointestinal tract of HIV-infected patients. In fact, the study indicated that Th17 cells were preferentially targeted during HIV infection. The decrease of IL-17 concentrations at the mucosal wall of the gastrointestinal tract could greatly increase the probability of bacterial infections, which could in turn have significant implications for the speed of HIV pathogenesis [26]. As Levy noted [27], chronic immune activation increases the production of proinflammatory cytokines (IL-6, IL-17, TNF-α, etc.). This upregulation of proinflammatory cytokines often leads to the rapid loss of $CD4^+$ T cells via apoptosis.

We observed a significant increase in the production of TNF-α in the supernatants of macrophage cultures from the HIV-infected group compared to healthy individuals (Figure 3(b)). Moreover, in a separate study, we have observed significantly increased concentrations of TNF-α in the plasma of HIV-infected subjects (unpublished). TNF-α, another proinflammatory cytokine which plays a major role in HIV infection, is produced by monocytes, macrophages, and natural killer cells in response to HIV infection [19, 20]. There is correlation between HIV viremia and TNF-α and IL-1 levels in the serum of HIV-infected patients. Excess production of TNF-α may lead to inflammatory damage and toxicity, which could lead to degradation of the host immune response independent of $CD4^+$ T cell depletion. These high levels of TNF-α contribute to fever, anorexia, and other symptoms of HIV/AIDS. As HIV causes chronic infection, this leads to the prolonged production of IL-1 and TNF-α, which stimulate the prolonged production of free radicals. This chronic state of inflammation leads in turn to chronic oxidative stress [19, 20].

Our assay of MDA revealed increased levels in the macrophage lysates of HIV-infected individuals over healthy individuals (Figure 3(d)). These elevated MDA levels correspond to elevated production of free radicals. Furthermore, in a previous study, we demonstrated significantly elevated levels of MDA in plasma isolated from HIV-infected individuals over plasma from healthy individuals (unpublished).

We observed a significant decrease in both the total and free GSH present in macrophages from individuals with HIV infection compared to macrophages from healthy subjects (Figures 4(a) and 4(b)). We also observed an increased percentage of GSSG in macrophages from individuals with HIV infection compared to macrophages from healthy subjects (Figure 4(c)).

The decrease in the levels of both total and free GSH correlated with several-fold increase in the growth of *M. tb* inside macrophages from individuals with HIV infection (Figure 4(d)). These results confirm that the ability of the macrophages derived from HIV-infected subjects to control *M. tb* infection is impaired. The immunocompromised status of the HIV-infected macrophages correlates with the increased levels of proinflammatory cytokines, TGF-β, and decreased levels of GSH.

Chronic oxidative stress is commonly observed in HIV patients, indicating a benefit for increased antioxidant supplements which may reduce DNA damage, possibly slowing the progression of infection [28]. The progression of HIV infection is characterized by a loss of $CD4^+$ T cells and decreased immunity. Prolonged free radical overload of monocytes and granulocytes in combination with the decreased production of GSH synthesis enzymes indicated by our experiments contribute to the deficiency of antioxidant mechanisms, GSH in particular. This may lead to the loss of $CD4^+$ T cells often seen in the progression of HIV [28–30].

We observed a significant reduction in the expression of GCLC, GCLM, GSS, and GGT1 genes in macrophages from individuals with HIV infection compared to healthy subjects. As most cells are unable to transport GSH or GSSG across the plasma membrane, these compounds must be first cleaved at the γ-glutamyl bond before they can be transferred into the cytosol [10, 31]. GGT1 performs this cleavage, allowing the components for new GSH molecules to be transferred into the cell [11, 12]. The reduction in GGT1 expression we observed would indicate a reduction in the raw materials necessary for de novo GSH production, also contributing to GSH deficiency. Interestingly, GSR expression in HIV-infected macrophages was increased by a factor of 3.8 over GSR expression in macrophages isolated from healthy subjects. This increased GSR expression indicates an attempt by the cell to increase free GSH concentrations through the reclamation of oxidized GSH. However; since the productivity of this enzyme is limited by the amount of oxidized GSH present and requires NADPH as a cofactor, this increase in GSR does not appear to be enough to compensate for the decrease in de novo GSH production.

To summarize, we observed a significant increase in the levels of proinflammatory cytokines such as IL-1, TNF-α, and IL-17 in individuals with HIV infection (Figures 2 and 3). This increase in the levels of proinflammatory cytokines in individuals with HIV infection correlated with increased production of free radicals (Figure 3). Additionally, we also observed a significant increase in the levels of TGF-β in both plasma and macrophage supernatants from individuals with HIV infection, and this increase correlated with reduction in the expression GCL gene in macrophages (Figures 2, 3, and 5). The results of our studies indicate that increased levels of free radicals (induced by proinflammatory cytokines) and decreased expression GCL (induced by TGF-β) may cause decrease in the levels of GSH in macrophages derived from individuals with HIV infection. Importantly, decreased levels of GSH in macrophages from individuals with HIV infection is accompanied by enhanced intracellular survival of *M. tb*.

To conclude, we observed a correlation between decreased intracellular GSH levels, increased proinflammatory cytokines, and increased free radicals. Our data supports our hypothesis that decreased intracellular GSH levels in HIV-infected individuals is a result of chronic overproduction of inflammatory cytokines (IL-1, TNF-α and IL-17), and cytokines (TGF-β) which interfere with the biosynthesis of GSH (Figure 6). Our data also supports the idea that chronic inflammation leads to increased production of free-radicals, which in turn promote production of proinflammatory cy-

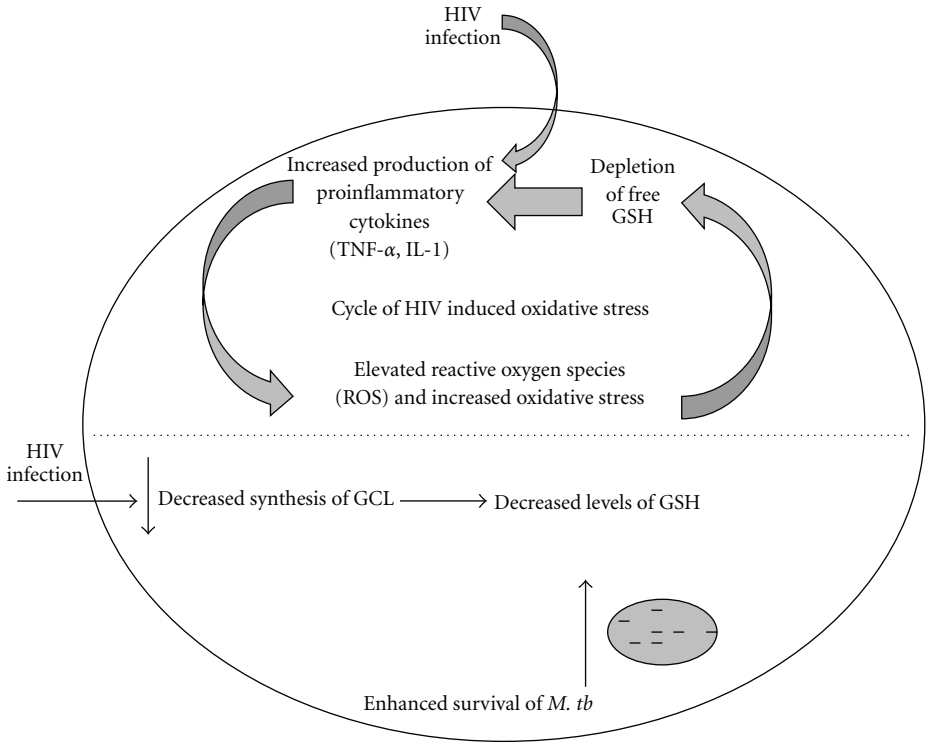

Figure 6: Model illustrating the reasons for decreased levels of GSH in macrophages from individuals with HIV infection.

tokines, and thus further depletion of intracellular GSH, and increased production of free radicals. We believe that this is a self-promoting loop of inflammation. In future studies, we hope to demonstrate that this loop can be broken by supplementing GSH. If successful, our data would indicate the possibility of efficacy for supplemental GSH therapy in individuals with HIV and *M. tb* coinfection.

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