Table 4 Comparison of specificity between T-SPOT.*TB* and QFT-3G

		T-SPOT.*TB*[2]			
		Positive	Border	Negative	Total
QFT-3G[1]	Positive	1	0	0	1
	Border	0	0	14	14
	Negative	0	0	96	96
	Total	1	0	110	111

Cut-off:
1: 0.1 IU/ml below: negative, From 0.1 IU/ml to 0.35 IU/ml: border
More than 0.35 IU/ml: positive
2. Less than 4 SFC: negative, 5−7 SFC: border, More than 8 SFC: positive

はQFT検査が不得意とする集団においても診断性能は優れていることが知られていることから，承認されれば結核感染診断がより一層充実するものと考えられる。しかし，一方ではIGRAs検査はツ反と比べると歴史は浅く，その診断特性が十分把握されていない面も多々あると思われる。IGRAsは潜在性結核感染者の診断に威力を発揮するが，感染から陽性になるまでの期間，あるいは年齢や治療が及ぼす影響，免疫抑制者における反応性，乳幼児での反応性等，明確にすべき検討課題も多く残されている。特に，結核感染した個人の免疫応答の強弱により判定が変動する可能性も考えられているが，この点についての解析はほとんどされていないのが現状である。また，QFT-3G検査は採血の時から採血量や採血管の振り方，あるいは採血検体の保管温度等，注意すべきことが多々あるため，採血現場においては決してユーザーフレンドリーな検査法とは言えず，さらなる改善が期待される。

おわりに

IGRAs検査は，今や日本の結核対策にとって必須の診断法として位置付けられているため，正確な検査結果を得られるように利用者側もまた販売会社側もより一層努力する必要があると思われる。また，検査特性の不明な点についても今後さらなる研究の進展が待たれる。

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2. HIV感染者やステロイド服用患者等の免疫脆弱宿主においてIGRAs（Interferon-γ Release Assays）をどのように利用するか？

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はじめに

HIV感染者，人工透析患者，ステロイド等免疫抑制薬服用患者など結核発病のハイリスク患者から発病を防ぐためには，早い時点で結核感染を診断し，潜在性結核感染症（LTBI）を治療することが有効と考えられる。とくに，HIV感染者に対しては，WHOは3つの'I's戦略の一つとしてIsoniazid preventive therapyを提唱している。しかし，免疫脆弱宿主ではLTBI診断のツールであるInterferon-γ release assays（IGRAs）は，細胞性免疫機能低下のためにその反応が低下する恐れがある。すなわち，結核菌に感染していても偽陰性や判定不能となる可能性

がある。

免疫脆弱宿主でも結核診断におけるクォンティフェロン（QFT）の有用性は免疫適格宿主と同様であるとする報告があるが[1]，免疫脆弱宿主における研究でも免疫適格宿主も含めた検討でも末梢血リンパ球数減少例では感度が低下すると報告されている[2)3)]。また，QFTの測定原理からも細胞性免疫機能はQFTの結果に影響を及ぼすと考えられる。しかし，QFTの診断性能を免疫脆弱宿主の疾患単位や病態との関連から検討した報告は少ない。

研　究　1

IGRAs適用に関する米国CDCガイドライン（2010）においては，免疫脆弱宿主におけるデータはまだ限定的と記載されているが，HIV感染者の日和見感染予防治療に関する米国ガイドラインでは，LTBIの診断のためにIGRAsまたはツベルクリン検査を実施することと記載している[4]。

演者らは，HIV感染者107例（うち結核治療開始前の活動性結核患者は9例）を対象にクォンティフェロン® TB-2G（以下QFT-2G）検査の診断性能を，とくにCD4陽性リンパ球数（$CD4^+$）に注目して検討した。

詳細は演者らが発表した論文[5]を参照されたいが，$CD4^+$ 50/μL以上の群では結核患者6例中5例でQFT-2G陽性（ESAT-6またはCFP-10が0.35 IU/mL以上）で感度は83%，特異度99%であった。$CD4^+$ 50未満の群では判定不能例が50/μL以上の群と比較して有意に多かった（$p<0.0001$）。また，$CD4^+$が500未満では陽性コントロールに対するインターフェロンγの反応性は$CD4^+$が少ないほどより低下していた（$p=0.0001$）。HIV感染者において$CD4^+$が50以上ならばQFTの感度・特異度は高いといえるが，それでもHIV感染者においてはインターフェロンγの反応性が低下する点には留意すべきであろう。

なお，活動性結核を合併したHIV感染者におけるQFT-2Gの結果については演者らの報告を含めて国内でこれまで3本の報告（Table）[5)~7)]があり，陽性率は56～77%である。

研　究　2

2010年にQFT-2Gから移行したクォンティフェロン® TBゴールド（以下QFT-3G）による結核発病患者の診断感度はQFT-2Gの83.0%と比較して92.6%と高いことが報告されている[8]。

研究2では，免疫脆弱宿主を対象にQFT-3Gによる結核の潜在性結核感染症（LTBI）発見の可能性について検討するために，臨床的には活動性結核と診断されていない患者に対してQFT-3G検査を行った。ただし，LTBI確定のgold standardはないこと，QFT-3G検査後の観察期間が現時点で短いという限定された検討である。

Table Results of QuantiFERON®-TB Gold (QFT-2G) among active TB patients with HIV infection in Japan

Study	National survey[6]	Fujita[5]	Nagai[7]
Number of pts.	n=19	n=9	n=13
Interpretation			
Negative	37%	22%	0%
Doubtful positive	5	11	15
Positive	63	56	77
Indeterminate	5	11	8
$CD4^+$ <50 of total cases	NA	23	38

NA: not available

（1）方法

対象はHIV感染者42例（男/女：34例/8例，平均年齢48±15歳，CD4数の分布は6～1131/μL），透析患者15例（男/女：13例/2例，平均年齢67±10歳），ステロイド等免疫抑制療法患者27例（男/女：9例/18例，平均年齢61±15歳）の3グループである，結果は添付文書に従い陽性，判定保留，陰性，判定不可と分類した。

（2）結果

①HIV感染者：臨床的には活動性結核と診断されていない患者42例ではQFT-3G検査の結果，陽性例は2例（5%），判定保留（疑陽性）例は2例（5%），判定不可は2例（5%）であった。陽性あるいは判定保留の4例うち3例は過去のQFT検査（QFT-2G）は陰性であった。なお，肺結核またはLTBIの治療歴を有する例が3例含まれていたが，QFT-3Gはすべて陰性であった。結核発病3例において，QFT-3Gは陽性であった。

②透析患者：臨床的には活動性結核と診断されていない患者15例にQFT-3G検査を行った結果，陽性例は3例（20%），判定保留（疑陽性）例は3例（20%），判定不可は0例であった。結核発病3例において，2例はQFT-3G陽性，1例は判定保留（疑陽性）であった。

③免疫抑制療法患者：疾患別では関節リウマチが最も多く12例（44%）で，ステロイドは25例（93%）に投与，11例（41%）にメソトレキセートを，11例（41%）にその他の免疫抑制剤が投与されていた。

臨床的には活動性結核と診断されていない患者27例にQFT-3G検査を行った結果，陽性例は2例（7%），判定保留（疑陽性）例は2例（7%），判定不可は4例（15%）であった。結核発病2例において，1例はQFT-3G陽性，1例は判定保留（疑陽性）であった。

末梢血リンパ球数が500未満では陽性コントロール値もかなり低下していたが（Fig.），リンパ球数と陽性コントロール値が必ずしも相関しない例が存在した。

（3）考察

①HIV感染者：今回のQFT-3G検査では，演者らが

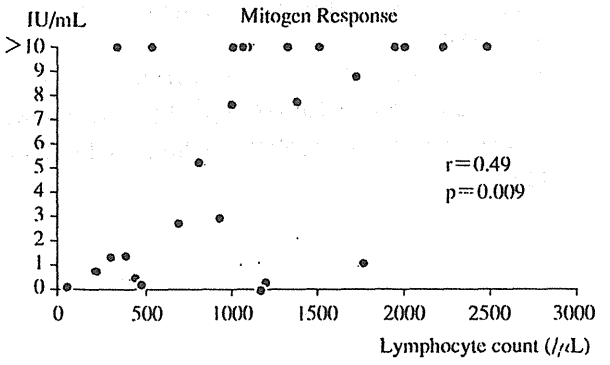

Fig. Relationship between lymphocyte count and mitogen response using QFT-GIT (QFT-3G) in patients on immunosuppressive therapy (n=27)

QFT-2Gを用いて検討した結果と比較して結核非発病例における陽性率が高いようであったが，QFT-3G検査の検討例数は少なく，この2つの検討における$CD4^+$＜50の例の割合に差があるため，さらに多数例による検討が必要である。

②透析患者：このグループでは高齢者が比較的多く，QFT-3G陽性例の中には過去の既感染者も含まれる可能性があるが，LTBI治療を推奨する方向性からは，陽性であれば年齢の影響のみと解釈せずにLTBI治療について検討したほうがよいかもしれない。QFT-2Gによる検討は井上らが活動性結核を含む131例中で24.1%が判定不能，非結核例（既往なし）でも10例が陽性であると報告しており[9]，透析患者の中にはLTBIが少なからず存在する可能性がある。

③免疫抑制療法患者：関節リウマチにおけるQFT-2GによるLTBI診断に関しては，坂野が結核既治療患者の結果から陽性のCut-off値を0.1 IU/mLまで下げても問題ないと考えられたと報告しているが[10]，結核既治療患者の成績をもってLTBI診断の値を設定してよいのかについて疑問もあり，今回は添付文書に従って判定した。

判定不能4例中2例はステロイドパルス後，1例はエンドキサンパルス後であり，このような時期にQFTを検査することは避けたほうがよいと思われた。

実地臨床において免疫抑制療法患者に対してIGRAsを検査するタイミングとしては，a）治療（免疫抑制療法）開始時，b）生物学的製剤導入時，c）定期的（年1回？），d）結核発病を鑑別診断したい時，などがあげられる。a) b) c) はLTBIのスクリーニングとしての検査である。しかし，IGRAsの感度については，末梢血リンパ球数が少なくなると低下すると報告されている。今回陽性コントロール値でみた場合、リンパ球数が1000以上あってもコントロール値がかなり低い例が存在した。従って，IGRAsの反応性に関してはリンパ球数のみならず，原疾患や治療薬による影響が生じる可能性を考慮する必要があろう。

ま と め

（1）臨床的には結核を疑っていないHIV感染者等の免疫脆弱宿主（結核発病ハイリスク者）にQFT検査を行うことで，潜在性結核感染症（LTBI）をスクリーニングできる可能性が示唆された。

（2）健常者におけるQFT-3Gの陽性率1.3%（添付文書）と比較して，免疫脆弱宿主における陽性率は高い傾向を示した。検討症例数が少ない解析結果ではあるが，結核発病リスクが高い集団なので，LTBIを発見するため症例背景を考慮しつつQFT-3G検査を行うことは意義があると思われた。

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Distinct Clinical Features in Nontuberculous Mycobacterial Disease with or without Latent Tuberculosis Infection

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Nontuberculous mycobacteria (NTM) diseases are in the face of a progressive increase even in immune-competent subjects, and the clinical features of NTM diseases are heterogenous. The decision to institute treatment of the patients should be made after a period of follow up, because therapy is often prolonged, and frequently ineffective. The reasons why some patients develop severe NTM diseases are not clear. Here we observed the involvement of latent tuberculosis infection (LTBI) in clinical and laboratory features of NTM diseases. We evaluated various tuberculosis-related inflammatory markers including osteopontin (OPN), pentraxin-3 (PTX-3), and soluble IL-2 receptor (sIL-2R) in NTM infected patients with or without LTBI. Eight NTM and 5 tuberculosis (TB) patients, and 5 healthy subjects were enrolled. Polymerase Chain Reaction (PCR) analysis confirmed the absence of tuberculosis specific gene (RD1 region), among clinical isolates from NTM patients. Interferon-γ (IFN-γ) release assay (IGRA) using Early Secreted Antigenic Target-6 (ESAT-6) and CFP-10, the RD1-encoded protein, was employed for determining LTBI. IGRA was positive in 4/8 NTM (NTM with LTBI, 50%) and 5/5 TB patients. Only 2 of 4 NTM with LTBI were under chemotherapy among all NTM patients, and others were followed up. The plasma levels of OPN, PTX3 and sIL-2R were significantly higher in NTM patients with LTBI than in those without LTBI ($P < 0.05$). The two patients under therapy showed the highest OPN levels that persisted after treatment. The increased inflammatory levels in NTM patients with LTBI indicate enhanced inflammatory reaction. Extensive therapy may be necessary in such patients.

Keywords: interferon-γ release assay; nontuberculous mycobacterial disease; latent tuberculosis infection; osteopontin; pentraxin-3
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Infection caused by nontuberculous mycobacterium (NTM), especially by *Mycobacterium avium complex* (MAC), has been increasing in both immunocompromised and immunocompetent individuals in Japan and worldwide (Prince et al. 1989). MAC may cause progressive lung diseases leading to morbidity and mortality in previously healthy patients as well as in patients with pre-existing lung diseases and immunodeficiency (Griffith et al. 2007). Recently, it was reported that progressive lung disease due to MAC is associated with specific variable number of tandem repeat (VNTR) genotypes (Kikuchi et al. 2009) or with the clinical features of cavity formation (Ito et al. 2012). Lung disease due to NTM occurs commonly in structural lung diseases, such as chronic obstructive pulmonary disease (COPD), bronchiectasis, cystic fibrosis, pneumoconiosis, prior tuberculosis, pulmonary alveolar proteinosis, and esophageal motility disorders (Griffith et al. 2007). The detection of both *Mycobacterium tuberculosis* (MTB) and NTM by PCR in a patient was reported in Japan (Takeda et al. 2008) and most patients with MTB and MAC co-infection reported in USA were foreigners (Khan et al. 2010). NTM infection in Japan is frequently observed in elderly people, and elderly people are more frequently latently infected with MTB (LTBI) than young people in Japan and the Philippines (Siddiqi et al. in press), as confirmed by IFN-γ release assay (IGRA) and in Thailand, as confirmed by anti-tuberculous glycolipid (TBGL) assay (Siddiqi et al. 2012). Recently, several studies in different countries showed that the incidence of TB infections examined by IGRA (in most of the cases IGRA ELISA was used) was very low in NTM patients in MTB non-endemic countries (Van Leeuwen et al. 2007; Adams et al. 2008). In contrast,

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significant percentages of NTM were positive by IGRA in Taiwan and Korea (Wang et al. 2007; Ra et al. 2011). Given the variety of clinical courses of NTM, the effect of LTBI on the clinical and/or laboratory features of NTM should be evaluated.

It is well known that CD4 cells and Th1-mediated signaling molecules and their pathways are important in the defense against NTM infection (Griffith et al. 2007). Recently, it was found that osteopontin (OPN), a pro-inflammatory cytokine secreted by a wide variety of cells including T cells and macrophages and elevated in tuberculosis, is linked with CD4 T helper (Th1) cell lineage stimulation and was reported to regulate *Mycobacterium avium* in cattle (Karcher et al. 2008). It was also claimed that OPN expression correlates with effective immune and inflammatory responses in MAC-infected individuals (Nau et al. 2000). We also measured pentraxin-3 (PTX-3), which is associated with the acute-phase response and involved in innate immunity. PTX-3 is produced from mononuclear phagocytes, dendritic cells, and endothelial cells in response to inflammatory signals. PTX3 binds with high affinity to the complement and activates the classical pathway of complement and facilitates pathogen recognition by macrophages. (Garlanda et al. 2005; Inforzato et al. 2012). The plasma PTX-3 level, which was reported to reflect the degree of inflammation in MTB infection (Azzuri et al. 2005), can be highly expressed *in vitro* on human PBMCs and monocytes stimulated with lipoarabinomannan (LAM) (Vouret-Craviari et al. 1997).

The current study was designed to compare various serological markers that are associated with TB, including OPN, PTX-3, leptin and soluble IL-2 receptor (sIL-2R), in NTM patients with or without LTBI to characterize their status.

Materials and Methods

Study population

All the patients that participated in this study were receiving care at Tohoku University Hospital between January 2008 and July 2010. We enrolled 9 patients (N1-9) who met the 2007 American Thoracic Society (ATS) microbiological criteria for pulmonary NTM diseases (Griffith et al. 2007). Six patients diagnosed as active TB (T1-6) were served as disease controls (Table 1). Among them, 2 patients (N7, T3) were withdrawn themselves from the study. NTM and MTB were confirmed from the site of infection by culture. In addition to culture, we repeatedly tested the samples from patients by PCR (Roche amplicon), and MTB was never detected in the NTM group. All the patients were assessed for clinical features, medical history including prior tuberculosis disease, treatment history and chest CT scan finding. Individuals with HIV/AIDS infection or who were receiving immunosuppressive therapy were excluded from the study. Five healthy volunteers who were without any symptoms relevant to active tuberculosis were enrolled as negative controls. The study was approved by the Ethics Committee of Tohoku University Hospital (2007-136; 2007-257). We obtained written informed consent from all the participants. All work was conducted in accordance with the Helsinki declaration. Plasma was obtained from EDTA-containing blood by centrifugation and was aliquoted to cryotubes and stored at −80°C for future use. Simultaneously, PBMCs were isolated over Ficoll-Paque Plus gradient and suspended in RPMI 1,640 supplemented with 2 mM L-glutamine, penicillin (100 U/ml), gentamycin (5 μg/ml) and 10% heat-inactivated FCS (Sigma) for further assay.

All the laboratory data including blood cell counts, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) and sIL-2R were measured at Tohoku University Hospital.

RD1 PCR analysis

Mycobacterial isolates from 6 NTM patients were kindly provided by the central research laboratory of TUH. DNA was extracted from the clinical isolates as described previously (Nakajima et al.

Table 1. Patients profile and result of IFN-γ release assay (IGRA).

Patients ID	Gender	Age (yr)	Diagnosis	Mycobacteria	Duration of illness	IGRA
N1	M	79	NTM	*M. avium*	5 yrs	negative
N2	F	76	NTM	*M. avium*	5 yrs	positive
N3	F	62	NTM	*M. avium*, *M. intracellulare*, *M. fortuitum*	6 months	negative
N4	F	83	NTM	*M. avium*	7 years	negative
N5	F	68	NTM	*M. avium*	7 yrs	positive
N6	M	43	NTM	*M. abscesus*	6 months	positive
N8	M	61	NTM	*M. avium*	13 yrs	negative
N9	F	76	NTM	*M. intracellulare*	3 yrs	positive
T1	F	78	ETB	*M. tuberculosis*	2 months	positive
T2	F	71	ETB	*M. tuberculosis*	1 month	positive
T4	M	65	ETB	*M. tuberculosis*	1 month	positive
T5	F	32	ETB	*M. tuberculosis*	1 month	positive
T6	F	57	PTB	*M. tuberculosis*	1month	positive

NTM, non-tuberculosis mycobacterium; ETB, extra-pulmonary tuberculosis. PTB, pulmonary tuberculosis.

2010). Briefly, glass beads (0.1 mm), the mycobacteria sample (500 μl in TE buffer) and 500 μl Chloroform were put in a 2 ml microcentrifuge tube for and oscillated by a minibeadbeater, at 4,800 rpm for 2 min. The aqueous phase was collected immediately upon centrifugation. DNA was isolated by 80% ethanol precipitation, dissolved in 50-μl sterile distilled water and stored in −20°C until assay. DNA samples were amplified for the RD1 region (150 bp) by PCR analysis using the same primers described previously (Parsons et al. 2002). DNA from the H37RV strain of *M. tuberculosis* was used as a positive control. PCR was performed with 5 μl DNA samples in a total volume of 50 μl of PCR mix, 5 μl 10 × buffer, 4 μl of 2.5 nM dNTP, 1 μl of Taq DNA polymerase and 0.2 μl of each primer (50 pmol/μl). The mixture was denatured at 95°C for 5 min and cycled for 45 times at 94°C for 30 s than 62°C for 45 s and 68°C for 45 s followed by a final 10-min extension at 68°C. The PCR product was visualized by UV transilumination of ethidine bromide staining after separation by 2% gel electrophoresis.

IFN-γ release assay (IGRA)

The assay was performed as described previously (Guio et al. 2010). Freshly isolated peripheral blood mononuclear cells (2.5×10^5 per well) were cultured on plates precoated with antibody against IFN-γ (IGRA ELISPOT. Oxford Immunotech, Oxford UK). After 16 hours stimulation of the cells with Early Secreted Antigenic Target-6 (ESAT-6) and CFP-10, the spots were developed according to the manufacturer's instructions. Spot-forming units (SFUs) were counted with an automated ELISPOT reader (KS ELISPOT Carl Zeiss MicroImaging-Germany). Only when the negative control well contained 5 SFUs or less, we subtracted this value from the SFUs counted in the wells of cells stimulated with MTB-specific antigens. Wells containing 5 SFUs or more were taken as positive as previously described.

Inflammatory markers

Anti-TBGL antibody which recognizes glycolipid (maily trehalose 6,6'-dimycolate) of MTB and NTMs was meausered as described (Mizusawa et al. 2008). The plasma OPN concentrations were determined using Human OPN Elisa kit (Immuno-Biological Laboratories, Takasaki, Japan) as described (Chagan-Yasutan et al. 2009), and the plasma PTX-3 levels were measured in special reference laboratory (SRL, Hachiouji, Japan) (Peri et al. 2000). Values were expressed as ng/ml. Plasma levels of leptin were measured as described (Siddiqi et al. 2012).

Statistical analysis

Data were entered into a Microsoft Excel spreadsheet and then copied into Statcel2 software (OMS, Tokyo, Japan) for statistical analysis. Continuous data were compared between groups by Mann Whitney *U* test and significance was considered a p value < 0.05.

Results

Subjects

Profiles of the patients are listed in Table 1. The NTM patients (43 to 83 years old (y.o.)) were infected predominantly by *M. avium complex* (Table 1) and mostly had pulmonary involvement except N6, who had extra-pulmonary involvement (nasal granulomatosis). The duration of illness was variable, ranging from 2 to 148 months. None of them were infected with *M. marinum, M. szulgai* or *M. kansasi*, which are known to possess the RD1 region. All the TB patients included in this study (32 to 78 y.o.) had extra-pulmonary disease except T6 (pulmonary TB). All 5 healthy volunteers (25 to 60 y.o.) were apparently healthy without any TB-related symptoms.

Verification of absence of RD1 region and M. tuberculosis strains

Absence of the RD1 region in the DNA samples from the clinical strains of the 6 NTM patients (N1-N6) was confirmed by PCR analysis. No positive band was observed at the 150 bp position for the RD1 region in any of the clinical isolates (Fig. 1).

IGRA and association with the clinical condition

All healthy controls except one were negative for IGRA. Four of 8 NTM patients and all TB patients were

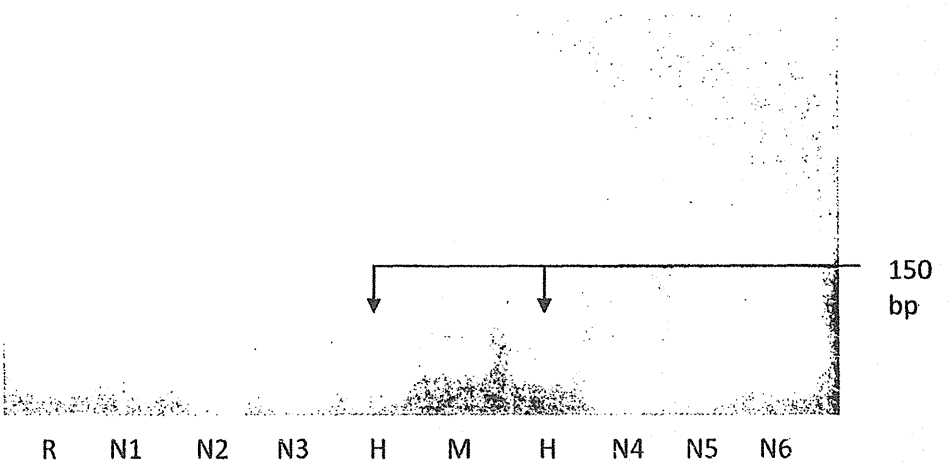

Fig. 1: PCR amplification of RD1 region using DNA obtained from mycobacteria isolated from NTM patients. Arrows indicate the positive bands at 150 bp. N1 to N6, (DNA from 6 NTM patients); H, DNA from *Mycobacterium tuberculosis* (H37RV); M, Marker; R, reagent only.

positive by IGRA (Table 1). Repeated examination by culture and PCR showed negative for MTB infection in all NTM patients. We further stratified these NTM patients into two subgroups, IGRA-positive as NTM with LTBI (NTM+) and IGRA-negative as NTM without LTBI (NTM−).

Patients in the NTM+ group had pulmonary pathology related to NTM disease based on clinical and radiographic features: consolidation and pulmonary infiltration in N2, both cavity and nodule in N5 and N9 had a combination of bronchiectasis, cavity, consolidation and nodules. Patients of the NTM− group also had typical radiographic features related to NTM infection. The patients of the NTM+ group had symptoms for longer periods (36 to 84 months), whereas the NTM patients had symptoms for variable durations (2 to 148 months), although the difference was not significant. No differences in age or clinical presentation were observed between the NTM+ and NTM− groups. Only patient N5 from the NTM+ group had a history of prior TB infection at the age of 18 that was cured by anti-TB therapy. Among all the NTM patients, only 2 NTM+ patients (N5, N9) had been receiving treatment for NTM infection because of clinical and radiological severities. Patient N5 was treated with rifampicine (RFP), clarithromycine (CAM), ciprofloxacin (CPFX) and ehtambutol (EB) for 6 months starting from July 2001. From May 2004, he was treated again with RFP and CAM, and treatment was continued until October 2005. Since then, he has been treated with different quinolone derivatives including gatifloxacin, CPFX, levofloxacin until June 2006. Finally, the patient was on combined therapy of RFP, CAM and a quinolone derivative until the time of the assay. Patient N9 was also treated with RFP, EB and CAM for 2 months before enrolling in the study. All the other patients hadn't received any drugs for NTM infection. Patients, N5 and N9 had died before the writing of this paper, though detailed information was not available.

Laboratory markers

Laboratory findings are listed in Table 2. There were no significant differences in the conventional markers between the NTM− and + groups. The levels of leptin were apparently lower in the NTM+ and TB groups than in the NTM− group, but no significant differences were found among the groups (Table 2, Fig. 2F).

Inflammatory markers

The data of inflammatory markers are shown (Fig. 2). The TBGL antibody levels are elevated in the NTM+ group and such elevations were unexpectedly not seen in the MTB group, probably because the most of the TB patients were the mild, extra-pulmonary type (Fig. 2A). Patients of the NTM+ group had increased levels of OPN (859 to 1,499 ng/ml) (normal value: < 820 ng/ml; according to Chagan-Yasutan et al. 2009) and the levels were significantly higher than those of the NTM− ($p < 0.05$) (Fig. 2B). The OPN titers in patients N5 and N9 were very high 1,150 ng/ml and 1,499 ng/ml, respectively (Fig. 2B). The CRP levels were

Table 2. Clinical characteristics of patients enrolled in the study.

Laboratory data	Ref. range	Median		
		NTM−	NTM+	TB
RBC (10^6/ul)	3.93-5.03	3.915	3.74	3.76
WBC (/ul)	3.2-9.6	5,250	5,650	4,100
Neutrophil %	31-73	62	58.5	69
Eosinophil %	0-7	1	2	1
Basophil %	0-3	0.5	0.5	0.005
Lymphocyte %	18-51	27	30.5	5
Monocyte %	1-12	8	7.5	5
Platelets (10^3/ul)	155-347	232.5	229.5	277
Hb (g/dl)	11.7-14.8	12.9	11.2	11.7
CRP (mg/dl)	0-0.2	1.2	2.45	0.5
Albumin (g/dl)	4.2-5.3	4.1	3.6	3.7
IgG (mg/dl)	748-1,694	1,320	1,542	1,564
IgM (mg/dl)	33-254	86.5	103	115.5
IgA (mg/dl)	91-391	281	336	295
ESR (30 min) (mm)	(−)	8	11	23
ESR (1 hr) (mm)	(−)	22.5	25	53
KL-6 (U/ml)	105-435	310	315.5	347.5
Leptin (pg/ml)	4,700-32,500	8,105	3,437	4,013

NTM−, IGRA negative non-tuberculosis mycobacterium patients; NTM+, IGRA positive non-tuberculosis mycobacterium patients; TB, tuberculosis patients.

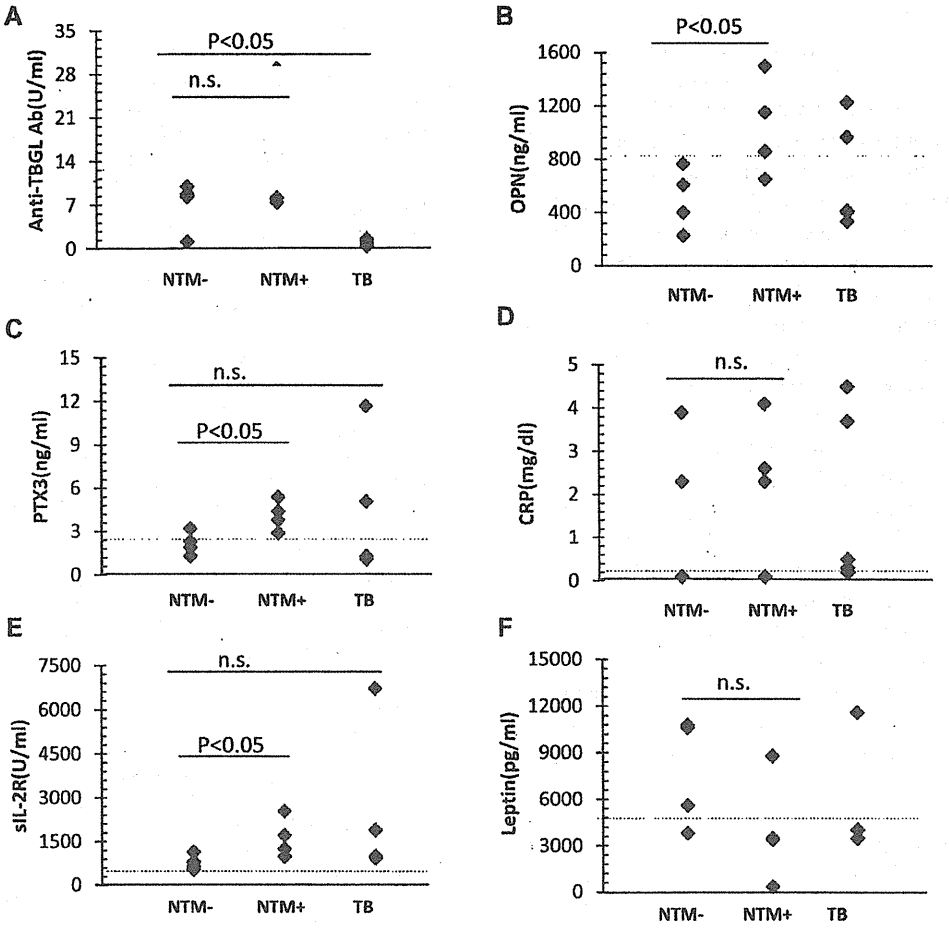

Fig. 2. Comparison of plasma inflammatory molecules.
NTM−, NTM patients without LTBI ($n = 4$); NTM+, NTM patients with LTBI ($n = 4$); TB, active TB patients ($n = 5$).

not different between the NTM+ and NTM− groups, but the PTX-3 levels (normal value: < 2.3 U/ml) and sIL-2R (normal value: 122-496 U/ml) were elevated in all NTM+ patients and the difference from the NTM− group was significant ($P < 0.05$) (Fig. 2C, D and E).

Discussion

In the current study, 4 of 8 NTM patients (50%) were found to be LTBI, and their average age was 74.5 y.o. In addition, PCR analysis showed that all of our NTM isolates lacked the RD1 region. An age-dependent increase of LTBI has already been described in Japan, where 9.8% for those aged 60-69 were IGRA ELISA positive (Mori et al. 2007). The excellence of IGRA ELISA to differentiate NTM and TB infection in children in non endemic countries was reported (Detjen et al. 2007). In Japan, only 1-8% of IGRA ELISA positive rate in Japanese patients with MAC disease was reported (Kobashi et al. 2006; Kobashi et al. 2009). However 34-49% IGRA ELISA positive cases were reported in NTM in endemic countries (Ra et al. 2011). The reasons of high rates of IGRA-positive rates in this study could be explained by IGRA ELISPOT assay employed here. It is known that IGRA ELISA tends to show false negative results among thin elderly people, presumably due to decreased immune levels, whereas IGRA ELISPOT assay might have detected LTBI more sensitively in the elderly patients with NTM disease. It was reported that the long-lasting positive IFN-γ response to antigenic challenge continues for 5 to 10 years following anti-TB therapy (Adams et al. 2008).

NTM+ patients had significantly higher titers of inflammatory markers such as OPN, PTX-3 and sIL-2R, though CRP did not show significant differences (Fig. 2). We also observed sustained high levels of OPN after treatment in NTM+ patients. It is possible that anti-NTM was ineffective because two patients died. Alternatively, persistent elevations of OPN after chemotherapy were already reported by us in AIDS patients treated by anti retroviral therapy (Chagan-Yasutan et al. 2009). Additionally our recent study supports the idea of immune-modulator effect of quinolone which enhance the production of OPN in human lung epitheliai cell line A549 *in vitro* (Shiratori et al. in press). We assume that the quinolone treatment may be one of the factors of persistent OPN elevation. The

increased plasma OPN in TB patients contributed to the disease pathology by activating the IL-12 mediated Th1 immunity (Koguchi et al. 2003). It was also found that OPN expression correlates with an effective inflammatory response and contributes to human resistance against MTB (Nau et al. 2000). In cattle, it was proposed that OPN is a key regulator against *M. avium* (Karcher et al. 2008). Immune responses by *M. avium complex* preferentially depend on the phase of infection in human. Early acute infection causes increased IFN-γ secretion, while the chronic phase has been reported to be associated with copious IL-10 production (Azouaou et al. 1997) with an inclination toward Th2 cytokines (Vouret-Craviari et al. 1997) that may provide protection against chronic diseases. We have already reported that the plasma levels of IFN-γ, OPN and leptins did not show any significant changes between LTBI and non-LTBI health care workers (HCW). Though only LTBI HCWs showed the association of TBGL-IgA antibody titer and serum IFN-γ (Siddiqi et al. in press). Our finding may imply that NTM co-infection with LTBI can synergistically induce large amounts of OPN. The synergistic effect could be explained by the natural resistance associated macrophage protein 1 (NRAMP1) because it was reported as host genetic factor for development of both tuberculosis and NTM, however the involvement of NRAMP1 in OPN production was not studied (Li et al. 2011; Sapkota et al. 2012).

It is also interesting that the PTX-3 levels were significantly higher in the NTM+ group while the CRP levels did not differ. It was documented that 5 of 220 TB contacts who developed active TB within 5 to 12 months of follow-up had elevated levels of PTX-3 (Azzuri et al. 2005). The PTX3 haplotype frequencies significantly differed in TB cases compared to controls, and a protective effect against MTB was found in association with a specific haplotype (Olesen et al. 2007). Hence, Th1 mediated PTX-3 production in mycobacterial infection also warrants further investigation.

In conclusion, frequent LTBI was detected in aged NTM patients, and these patients expressed higher levels of inflammatory markers than NTM without LTBI patients. The low number of patients is the main limitation of this study, but careful observation and extensive therapeutic intervention appear to be necessary in NTM with LTBI patients.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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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

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Anti-tubercular-glycolipid-IgG (TBGL-IgG) and -IgA (TBGL-IgA) antibodies, and the QuantiFERON-TB Gold test (QFT-G) 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 yrs) 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).

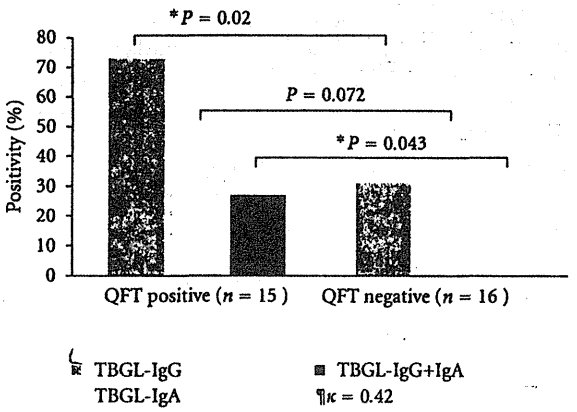

FIGURE 1: Positivity percentage of TBGL-IgG/IgA assay in QFT-positive/negative healthcare workers. The level of agreement between QFT and the TBGL-Ab assay was measured by Cohen's kappa (κ). ¶κ = 0.42; overall agreement 71%; 95% confidence interval: 0.1~0.73.* Significant difference ($P < 0.05$).

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 ($P < 0.05$).

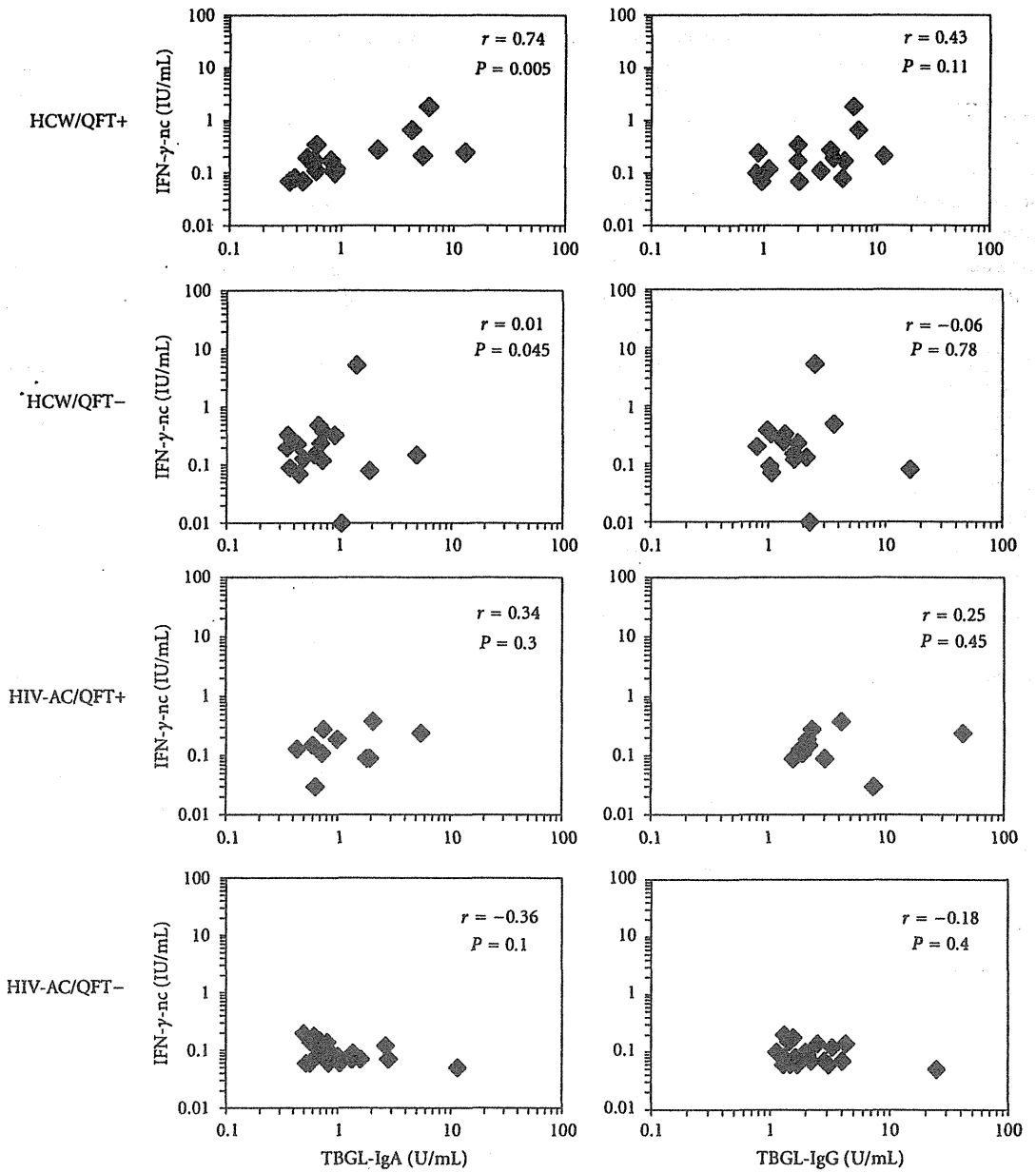

FIGURE 2: Correlations between TBGL-IgA or TBGL-IgG titers and IFN-γ concentrations measured in nonstimulated QFT-plasma samples (IFN-γ-nc) in QFT-positive/QTF-negative healthcare workers (HCWs) and asymptomatic HIV carriers (HIV-AC). The only significant positive correlation was observed between the 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 ≥ 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 the serum IgG and IgA in HCW and HIV-AC except for the

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 ($P < 0.05$).

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 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,

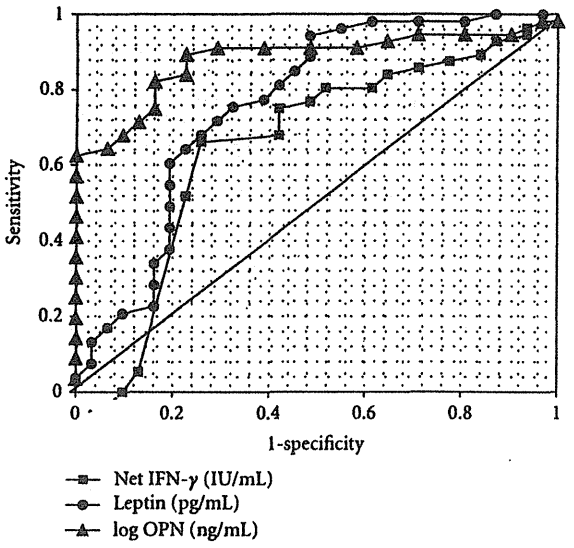

FIGURE 3: Receiver operating characteristic analysis for comparison of biomarkers between healthcare workers and asymptomatic HIV carriers. The result showed that the OPN plasma levels of OPN (log) exhibited the greatest ability to discriminate HIV from HCW based on the AUC (0.883), followed by leptin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

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

Both authors contributed equally to this work.

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