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**Tuberculosis after commencing antiretroviral therapy for HIV infection is associated with elevated CXCL9 and CXCL10 responses to *Mycobacterium tuberculosis* antigens**

Benjamin G. Oliver, BSc (Hons),<sup>1</sup> Julian H. Elliott, MB BS, PhD,<sup>2,3,4</sup> Patricia Price, MSc PhD,<sup>1</sup> Michael Phillips B.Sc.(Hons), M.Med.Sci.<sup>5</sup>, David A. Cooper, MB BS, MD,<sup>4</sup> Martyn A. French, MB ChB, MD,<sup>1</sup>.

1. School of Pathology and Laboratory Medicine, University of Western Australia, Perth, Australia.
2. Alfred Hospital, Melbourne, Australia.
3. Burnet Institute, Melbourne, Australia.
4. National Centre in HIV Epidemiology and Clinical Research, University of New South Wales, Sydney, Australia.
5. Western Australia Institute for Medical Research, University of Western Australia, Perth, Australia.

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**Corresponding author:**

Professor Martyn French

School of Pathology and Laboratory Medicine

Level 2 MRF Building

Rear 50 Murray St

Perth, 6000

Australia

Tel: +61 8 9224 0205

Fax: +61 8 9224 0204

Email: [martyn.french@uwa.edu.au](mailto:martyn.french@uwa.edu.au)

## **Abstract**

*Background:* Commencing antiretroviral therapy (ART) in HIV patients with treated or unrecognised *Mycobacterium tuberculosis* disease may trigger tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) or antiretroviral therapy-associated tuberculosis (ART-TB). We have shown that whole blood interferon-gamma (IFN- $\gamma$ ) release assays may aid in the prediction and diagnosis of ART-TB. Here, we investigate IFN- $\gamma$ -inducible chemokines CXCL9 and CXCL10.

*Methods:* CXCL9 and CXCL10 responses to region of difference 1 (RD1) antigens and purified protein derivative (PPD) were assayed in plasma from whole blood cultures collected before and after 4, 12 and 24 weeks of ART from 15 TB-IRIS cases, 11 ART-TB cases and matched controls.

*Results:* Relative to matched controls, ART-TB cases had elevated CXCL10 responses to RD1 antigens pre-ART ( $P=0.02$ ) and to PPD and RD1 antigens over 24 weeks of ART ( $P\leq 0.02$  and  $P\leq 0.03$ ). In contrast, TB-IRIS cases had higher CXCL10 responses to RD1 antigens before and after 4 weeks of ART only ( $P=0.04$  for both). CXCL9 responses to PPD and RD1 antigens were similar but less pronounced in ART-TB cases and did not differ between TB-IRIS cases and controls. CXCL10 responses to RD1 antigens performed as well as, or better than, IFN- $\gamma$  responses in the prediction and diagnosis of ART-TB.

*Conclusions:* Tuberculosis after commencing ART is associated with increased CXCL10 and, to a lesser extent, CXCL9 responses to *M. tuberculosis* antigens. Assessment of antigen-

induced CXCL10 responses to RD1 antigens may assist in the prediction and diagnosis of ART-TB.

**Key words:** human immunodeficiency virus, antiretroviral therapy, tuberculosis-associated immune reconstitution inflammatory syndrome, antiretroviral therapy-associated tuberculosis, CXCL10, CXCL9, IFN- $\gamma$

## INTRODUCTION

*Mycobacterium tuberculosis* disease is the leading cause of illness and death in patients with HIV infection <sup>1</sup>. *M. tuberculosis*-specific Th1 cells are depleted during HIV infection <sup>2,3</sup> but are partly restored by antiretroviral therapy (ART) <sup>4,5</sup>. In some HIV patients, the restored immune response to *M. tuberculosis* may cause immunopathology known as immune restoration disease (IRD) <sup>6</sup>. Paradoxical worsening of treated tuberculosis (TB) after ART is a form of IRD known as tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) <sup>7</sup>. Tuberculosis presenting during the first three months of ART might also be a form of IRD. Many cases present as ‘unmasking TB-IRIS’ whereby previously subclinical TB is ‘unmasked’ after starting ART. However, newly acquired TB may also present after ART initiation, so cases of TB which present after ART are referred to as antiretroviral therapy-associated tuberculosis (ART-TB) <sup>7</sup>. ART-TB has been associated with increased interferon-gamma (IFN- $\gamma$ ) responses to mycobacterial antigens compared to HIV patients with no history of TB <sup>8</sup>.

Data from large prospective studies indicate that mortality associated with TB-IRIS is low but morbidity is substantial in HIV/TB patients who have pre-ART CD4<sup>+</sup> T cell counts <50 cells/ $\mu$ l and initiate ART within four weeks of starting TB treatment <sup>9,10</sup>. Morbidity and mortality from ART-TB are also significant, as HIV patients who present with TB during the first three months of ART are 3.25 times more likely to die than HIV patients presenting with TB at other times <sup>11</sup>.

Prediction and diagnosis of TB-IRIS and ART-TB might be improved by a better understanding of their immunopathogenesis. TB-IRIS has been associated with increased pro-inflammatory and Th1 cytokine responses to mycobacterial antigens <sup>8,12-14</sup>, but these are

also observed in co-infected patients who do not develop TB-IRIS after commencing ART<sup>8,15,16</sup>, so other immune responses may also contribute to the immunopathology<sup>17,18</sup>. ART-TB has also been associated with a prominent Th1 response to mycobacterial antigens, particularly those encoded by the region of difference 1 (RD1) domain of the *M. tuberculosis* genome<sup>8</sup>.

We have used a whole blood IFN- $\gamma$  release assay to examine IFN- $\gamma$  responses to mycobacterial antigens [purified protein derivative (PPD) and RD1 antigens] in a cohort of Cambodian HIV patients with or without treated TB<sup>8</sup>. To identify novel biomarkers of TB-IRIS and ART-TB, we examined antigen-induced production of CXCL9 (monokine induced by gamma-interferon, MIG) and CXCL10 (interferon- $\gamma$ -inducible protein-10, IP-10) in plasma from these assays. These chemokines are induced by IFN- $\gamma$  and are important in several inflammatory conditions<sup>19-25</sup>.

## **METHODS**

### **Cases and controls.**

Patients were recruited from the National Centre for HIV/AIDS, Dermatology and Sexually Transmitted Diseases Social Health Clinic in Phnom Penh, Cambodia, and were a subgroup of those reported previously<sup>8</sup>. Seventy five patients were being treated for active TB upon starting ART and 15 (20%) developed TB-IRIS after a median time of 10 (range 7-89) days on ART. Eleven out of 231 patients (4.8%) with no prior history of TB developed ART-TB at after a median time of 10 (range 1-28) days on ART. We used case definitions of TB-IRIS and ART-TB proposed by the International Network for the Study of HIV-associated IRIS<sup>7</sup>. This subgroup differed from those reported previously<sup>8</sup> as each TB-IRIS and ART-TB case was matched with two controls from the cohort by sex, pre-ART CD4<sup>+</sup> T cell count and TB

history. The study was approved by the Cambodian National Ethics Committee and the human research ethics committees of the University of New South Wales and Royal Perth Hospital. All patients provided written and informed consent.

#### **Assay of plasma levels of CXCL10 and CXCL9 from whole blood cultures.**

Plasma samples from unstimulated and PPD and RD1-stimulated tubes of Quantiferon-TB Gold™ in-tube (QFTGIT) assays (Cellestis, Carnegie, Australia) were collected pre-ART, after 4, 12 and 24 weeks of ART and at the suspected time of TB-IRIS or ART-TB and cryopreserved at -80°C. Levels of CXCL9 and CXCL10 were assayed using BD Cytometric Bead Array Flex Sets (BD Biosciences, San Jose, CA). 300 events were collected per analyte using a BD FACSArray machine and BD FACSArray System Software v1.0.3 (BD Immunocytometry Systems, San Jose, CA). Analysis was performed using FCAP Array Software v1.0.1 (BD Biosciences). All samples were diluted at least 1/5 and the lowest limit of detection was 5pg/ml. To analyse the effect of PPD and RD1 antigens on CXCL10 and CXCL9 production, levels of CXCL10 and CXCL9 in unstimulated tubes were subtracted from levels measured in PPD- and RD1-stimulated tubes.

#### **Statistical analysis.**

Demographic characteristics of TB-IRIS and ART-TB cases and controls were assessed using the Mann-Whitney *U* test and Fisher's exact test. Data was assessed for normality using the Shapiro-Wilk *W* test. As the data were not normally distributed, median levels of CXCL9 and CXCL10 were compared between TB-IRIS or ART-TB cases and matched controls pre-ART and after 4, 12 and 24 weeks of ART using the Mann-Whitney *U* test. Receiver operating characteristic (ROC) curves were used to determine if levels of CXCL10 and CXCL9 pre-ART and at the time of TB-IRIS or ART-TB (or an equivalent time post-ART for controls)



alone or in combination may aid in prediction or diagnosis of TB-IRIS or ART-TB. Comparisons were also made with data for IFN- $\gamma$ . Correlations were performed using the Spearman rank test. Analyses were performed using STATA version 11 (Chicago, IL, USA) and Prism v5.02 (San Diego, CA, USA). Statistical significance was defined as  $P < 0.05$ .

## RESULTS

### *Demographic characteristics.*

Demographic characteristics of the TB-IRIS and ART-TB cases and controls are summarized in Table 1 and are similar to those of the entire cohort<sup>8</sup>. Clinical information about patients with TB-IRIS and ART-TB have been reported previously<sup>8</sup>. Patients who developed TB-IRIS had a shorter interval between starting anti-tubercular therapy (ATT) and ART compared to those who did not ( $P=0.04$ ). The use of corticosteroid therapy for TB-IRIS was very infrequent.

### *CXCL10 responses were most clearly elevated in ART-TB cases.*

Prior to commencing ART, CXCL10 responses to RD1 antigens alone were higher in both ART-TB cases ( $P=0.02$ ) and TB-IRIS cases ( $P=0.04$ ) compared with their respective controls (Figure 1C and 1D). In contrast, CXCL10 responses to both PPD and RD1 antigens were higher during 24 weeks of ART in HIV patients who developed ART-TB compared to controls (PPD: for week 4:  $P=0.0009$ , for week 12:  $P=0.002$ , for week 24:  $P=0.02$ ; RD1: for week 4:  $P=0.001$ , for week 12:  $P=0.008$ , for week 24:  $P=0.03$ ) (Figure 1A and 1C) whereas TB-IRIS cases demonstrated a significantly higher CXCL10 response to RD1 antigens at week 4 only ( $P=0.04$ ) (Figure 1D).

IFN- $\gamma$  responses to PPD and RD1 antigens have been reported in these patients<sup>8</sup>. In general, CXCL10 responses to PPD and RD1 antigens correlated with IFN- $\gamma$  responses in TB-IRIS and ART-TB cases and their controls over 24 weeks of ART (data not shown). A strong correlation between IFN- $\gamma$  and CXCL10 responses to RD1 antigens was evident in patients who developed ART-TB over 24 weeks of ART (For week 0, 4, 12 and 24 of ART;  $r \geq 0.87$ ,  $P < 0.05$ ) (data not shown).

**CXCL9 responses to RD1 antigens were higher in ART-TB cases compared to controls.**

Prior to ART, CXCL9 responses to PPD and RD1 antigens were not significantly different between ART-TB or TB-IRIS cases and their controls (Figure 2). In ART-TB cases, CXCL9 responses to RD1 antigens were significantly higher compared to controls after 4, 12 and 24 weeks of ART (for week 4:  $P = 0.02$ , for week 12:  $P = 0.048$ , for week 24:  $P = 0.002$ ) (Figure 2C) whereas CXCL9 responses to PPD were higher only after 4 weeks of ART compared to controls ( $P = 0.02$ ; Mann-Whitney) (Figure 2A). CXCL9 responses to PPD and RD1 antigens were similar in TB-IRIS cases and controls (Figure 2B and 2D).

***CXCL10 responses to RD1 antigens may aid in the prediction and diagnosis of ART-TB.***

We have previously shown that pre-ART levels of IL-18, CXCL10 and CCL2 in combination with IFN- $\gamma$  responses to PPD and RD1 antigens in plasma from QFTGIT assays are predictive of TB-IRIS, while IFN- $\gamma$  responses to PPD and RD1 antigens are predictive of ART-TB<sup>8,17</sup>. Here, ROC analyses demonstrated that CXCL10 responses to RD1 antigens were moderately predictive of ART-TB [area under the curve (AUC), 0.75] and were a better predictor than IFN- $\gamma$  responses (AUC, 0.61) (Table 2). In contrast, CXCL9 and CXCL10 responses to PPD and RD1 antigens were poorly predictive of TB-IRIS (Table 2).

We have also reported that IFN- $\gamma$  responses to PPD and RD1 antigens in plasma from QFTGIT assays may aid in the diagnosis of ART-TB<sup>8</sup>. Here, CXCL10 responses to PPD and RD1 antigens were assessed at the time of disease in TB-IRIS and ART-TB cases and at an equivalent time post-ART in matched controls. CXCL10 responses to PPD and RD1 antigens were strongly diagnostic of ART-TB (AUC, 0.86; 0.85) but not TB-IRIS (AUC, 0.53; 0.56). However, the diagnostic ability of CXCL10 was not better than IFN- $\gamma$  alone or when combined with CXCL9 and IFN- $\gamma$  (Table 3).

## **DISCUSSION**

CXCL9 and CXCL10 responses to PPD and RD1 antigens of *M. tuberculosis* were assessed in plasma from the QFTGIT assays undertaken in HIV patients who developed ART-TB or TB-IRIS and matched controls, to elucidate mechanisms of immunopathogenesis and seek biomarkers of IRD associated with *M. tuberculosis* infection after commencing ART. Patients who developed ART-TB had higher CXCL10 responses to RD1 antigens pre-ART and higher CXCL10 responses to both RD1 antigens and PPD during 24 weeks of ART compared to controls. In addition, patients who developed ART-TB had higher CXCL9 responses to RD1 antigens during 24 weeks of ART and higher responses to PPD at week 4 compared to controls. In contrast, TB IRIS cases displayed higher CXCL10 responses to RD1 antigens than controls pre-ART and at week 4 only. Neither CXCL9 nor CXCL10 responses to PPD differed between TB-IRIS cases and controls.

As RD1 antigens are secreted by *M. tuberculosis*<sup>26</sup>, the increase in CXCL9 and CXCL10 responses to RD1 antigens in ART-TB cases may reflect an elevated antigen-specific T cell response against active *M. tuberculosis* infection. These findings therefore provide further evidence that ART-TB is associated with increased T cell responses to mycobacterial

antigens, as observed by the measurement of IFN- $\gamma$ <sup>8</sup>, suggesting that most, if not all, cases are likely to be a form of IRD. Differences in antigen-induced CXCL9 or CXCL10 responses were less pronounced in patients who developed TB-IRIS compared to controls. This could be interpreted as evidence that T-cells mediate ART-TB but not TB-IRIS. However, two other possible explanations should be considered. Firstly, differences in T-cell responses between ART-TB cases and controls may be clearer because ART-TB controls were not infected by *M. tuberculosis* so their T cell responses to PPD and RD1 antigens are low. Secondly, assays of CXCL9 and CXCL10 in antigen-stimulated whole blood cultures may not be sensitive enough to distinguish T cell responses of TB-IRIS cases from controls.

In this patient cohort pre-ART IFN- $\gamma$  responses to PPD and RD1 antigens, when corrected for pre-ART CD4<sup>+</sup> T cell count, are predictive of ART-TB<sup>8</sup>. Here, pre-ART CXCL10 responses to RD1 antigens were higher in patients who subsequently developed TB-IRIS or ART-TB compared to matched controls. This would be expected given that IFN- $\gamma$  is a potent inducer of CXCL10 and CXCL9. However, analysis of the data using ROC analyses demonstrated that CXCL10 responses to RD1 antigens were more predictive of ART-TB than IFN- $\gamma$  responses (Table 2). It must be stressed, however, that the number of patients analysed in our study was small and further studies on larger numbers of patients are required to determine if assessment of CXCL10 responses to RD1 antigens has clinical utility in predicting ART-TB.

These findings indicate that T cell responses against *M. tuberculosis* are present before ART is commenced in patients who develop TB-IRIS or ART-TB and that assay of CXCL10 in whole blood cultures stimulated with RD1 antigens may be more informative than assays of IFN- $\gamma$ . They also raise the possibility that demonstration of these responses might be used to

detect 'subclinical' *M. tuberculosis* and predict the unmasking of infection by restoration of an immune response against mycobacterial antigens. Recent data suggests that HIV patients residing in areas endemic for TB should be screened for TB by sputum culture even in the absence of TB symptoms, as smear-positive TB cultures are indicative of subclinical disease that progresses to symptomatic disease in the majority of cases<sup>27,28</sup>. Assessment of CXCL10 responses to RD1 antigens in plasma from the QFTGIT assay may be a more effective screening tool for the detection of asymptomatic TB in HIV patients, as many HIV/TB patients have smear-negative pulmonary disease<sup>29</sup>. CXCL10 may be superior to IFN- $\gamma$  in diagnosing *M. tuberculosis* infection in HIV patients as CXCL10 detects a greater number of HIV patients with TB compared to IFN- $\gamma$ <sup>30,31</sup>.

We have previously shown that IFN- $\gamma$  responses to PPD and RD1 antigens in the QFTGIT assay may aid in the diagnosis of ART-TB<sup>8</sup>. Analysis of the data presented here demonstrated that CXCL10 responses to PPD and RD1 antigens were strongly diagnostic of ART-TB using ROC analyses (AUC=0.86 and 0.85) and performed as well as or better than IFN- $\gamma$  responses. This finding should be investigated further in a larger cohort of patients.

In patients with ART-TB, CXCL9 responses to PPD and/or RD1 antigens were higher than controls after ART. These findings are similar to those for CXCL10 and further suggest that ART-TB is associated with increased Th1 responses against mycobacterial antigens<sup>8</sup>. In contrast, CXCL9 responses to PPD and RD1 antigens did not differ between TB-IRIS cases and controls over 24 weeks of ART. These findings are similar to patterns seen with CXCL10.

This study has some limitations which should be considered. The number of patients studied was small so larger cohorts of patients are required to validate the clinical utility of assaying CXCL10 in antigen-stimulated QFTGIT assays. Levels of CXCL9 and CXCL10 measured in TB-IRIS cases may have also been affected by treatment with corticosteroids, but very few patients received corticosteroids before sample collection. Mycobacterial load pre-ART was not examined and might have influenced pre-ART immune responses against mycobacteria<sup>32</sup>, especially as patients who developed TB-IRIS had a shorter interval between starting ATT and ART compared to patients who did not. Due to the difficulties in defining latent *M. tuberculosis* infection, some ART-TB control group may have not been exposed to *M. tuberculosis*, some may have resolved previous infection and some may harbour latent infection. Future studies should include careful analysis of clinical histories.

In summary, patients who developed TB-IRIS and ART-TB exhibited higher CXCL10 responses to RD1 antigens of *M. tuberculosis* than controls before the initiation of ART. In patients who developed ART-TB, CXCL10 responses to RD1 antigens rose further on ART while CXCL10 responses to PPD and CXCL9 responses to both RD1 antigens and PPD also increased and remained higher than controls for 24 weeks. In contrast, TB-IRIS was characterised by a higher CXCL10 response to RD1 antigens pre- and after 4 weeks of ART only. Assessment of T cell responses to RD1 antigens by assaying CXCL10 in whole blood assays performs as well as, or better than, IFN- $\gamma$  and may have clinical utility in the detection of subclinical *M. tuberculosis* infection and the prediction and diagnosis of ART-TB.

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## Legends to figures

### Figure 1

A comparison of levels of CXCL10 in response to purified protein derivative (PPD) and region of difference 1 (RD1) antigens within antiretroviral therapy-associated tuberculosis (ART-TB) cases and controls (Figure A and C) and tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) cases and controls (Figure B and D) over 24 weeks of antiretroviral therapy. Cases are denoted by (●) and controls are denoted by (○). Differences in median levels of CXCL10 between cases and controls were calculated using the Mann-Whitney *U* test.

### Figure 2

A comparison of levels of CXCL9 in response to purified protein derivative (PPD) and region of difference 1 (RD1) antigens within antiretroviral therapy-associated tuberculosis (ART-TB) cases and controls (Figure A and C) and tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) cases and controls (Figure B and D) over 24 weeks of antiretroviral therapy. Cases are denoted by (●) and controls are denoted by (○). Differences in median levels of CXCL10 between cases and controls were calculated using the Mann-Whitney *U* test.

Figure 1  
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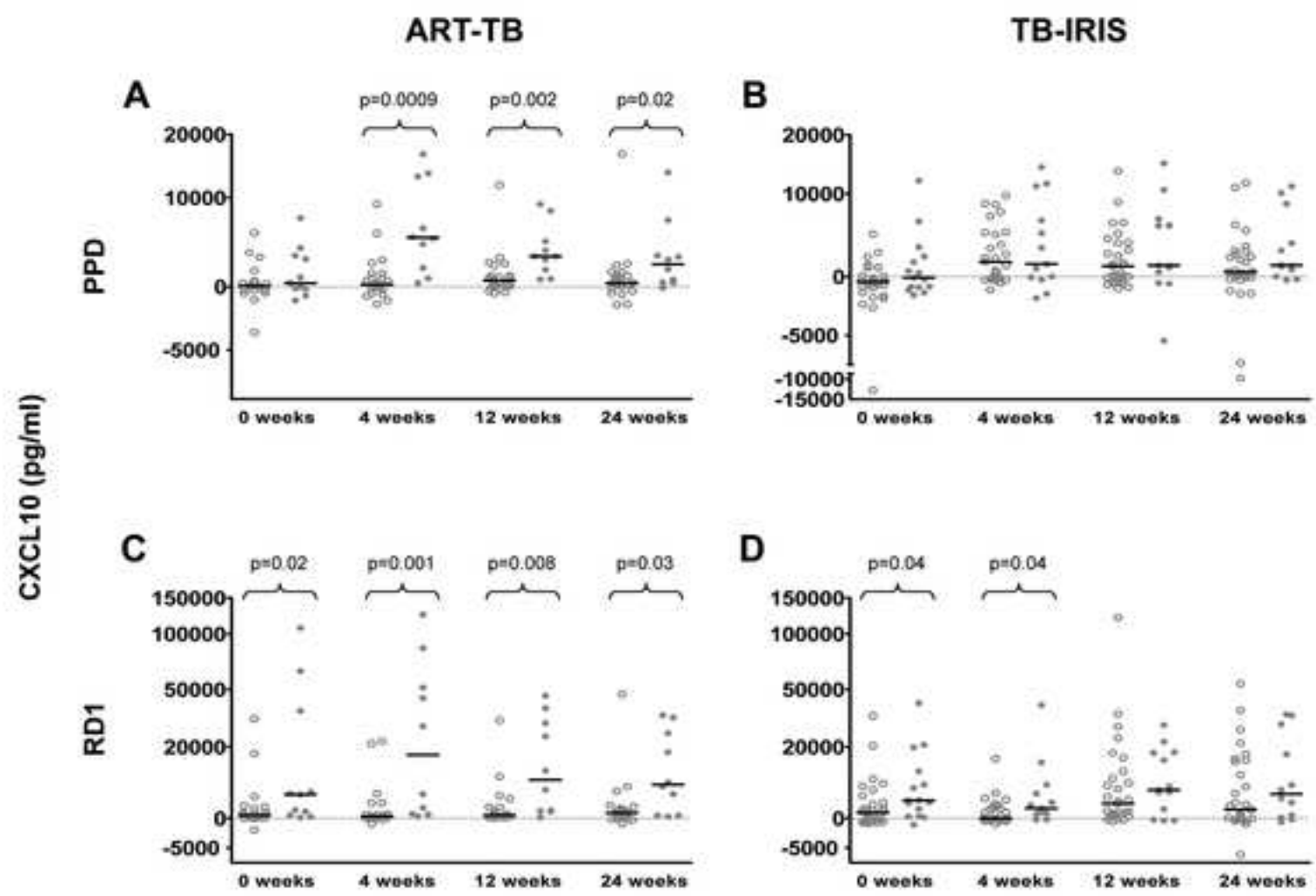
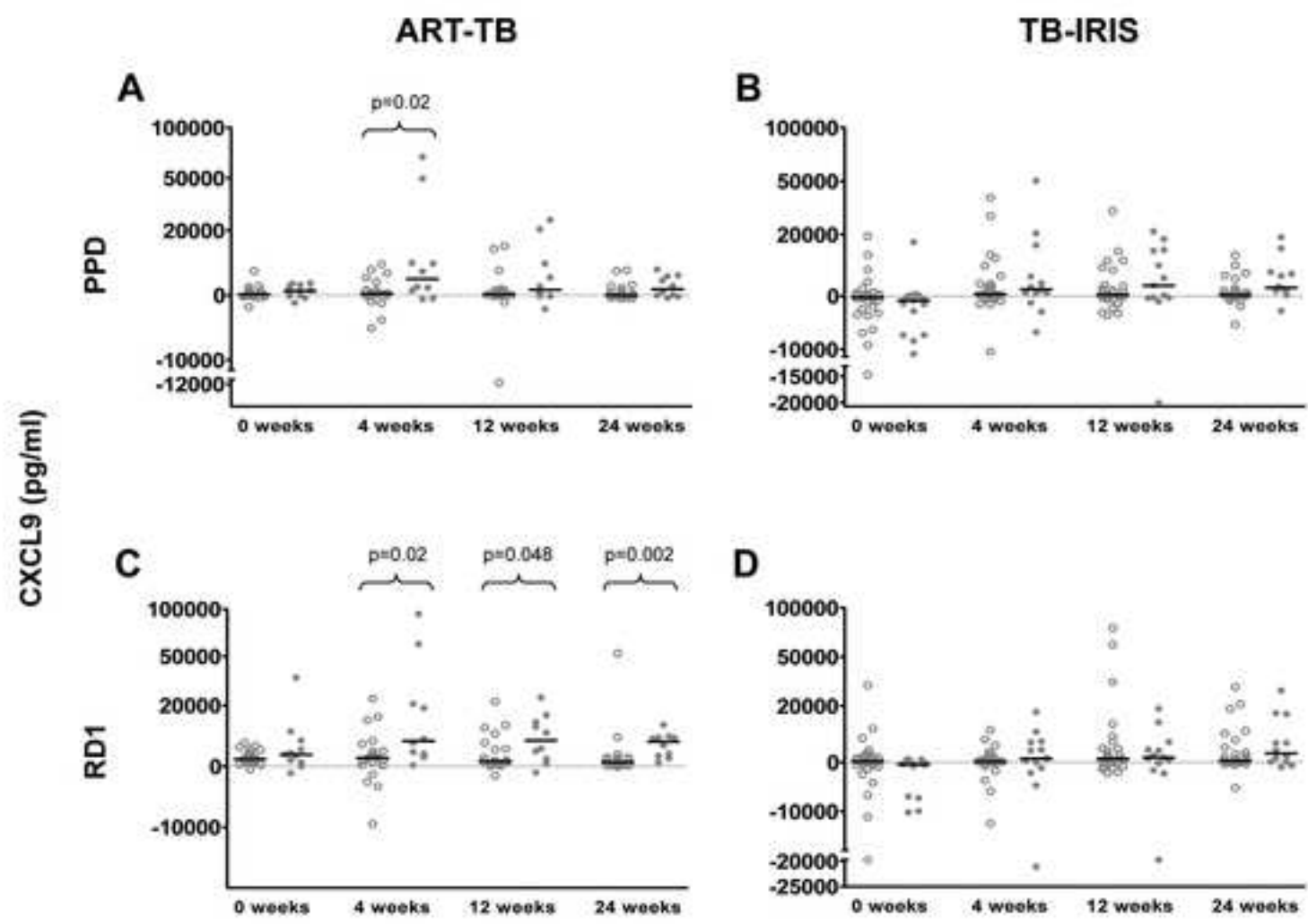


Figure 2  
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**Table 1: Demographic characteristics of tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) and antiretroviral therapy-associated tuberculosis (ART-TB) cases and controls.**

	TB-IRIS			ART-TB		
	Cases	Controls	<i>P</i>	Cases	Controls	<i>P</i>
<b>Total</b>	15	30		11	22	
<b>Male/Female</b>	11/4	22/8		8/3	16/6	
<b>Age (years)</b>	35	36	0.66	35	38	0.53
<b>Pre-ART CD4<sup>+</sup> count</b>	45	44	0.83	37	38	0.91
<b>Change in CD4<sup>+</sup> count<sup>a,b</sup></b>						
<100 cells/ $\mu$ l	8	18	1.00	4	10	1.00
$\geq$ 100 cells/ $\mu$ l	4	11	1.00	6	11	1.00
<b>Interval between the start of ATT and ART (days)<sup>c</sup></b>	24	59	0.034			

**NOTE.** <sup>a</sup> Change in CD4<sup>+</sup> T cell count from pre-ART to week 24; <sup>b</sup> CD4<sup>+</sup> T cell count at week 24 was unavailable for 3 TB-IRIS cases, 1 TB-IRIS control, 1 ART-TB case and 1 ART-TB control;

<sup>c</sup> The interval between the start of ATT and ART could not be calculated for 1 TB-IRIS case and 4 TB-IRIS controls. Fisher's exact test was used to compare changes in CD4<sup>+</sup> count during 24 weeks of ART and the Mann Whitney *U* test was used for all other comparisons.

**Table 2:** Area under the curve values for receiver operator curve analyses of pre-ART antigen-induced CXCL9, CXCL10 and IFN- $\gamma$  levels in the prediction of tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) and antiretroviral therapy-associated tuberculosis (ART-TB).

	Responses to PPD		Responses to RD1 antigens	
	TB-IRIS (95% CI)	ART-TB (95% CI)	TB-IRIS (95% CI)	ART-TB (95% CI)
<b>IFN-<math>\gamma</math></b>	0.56 (0.38-0.75)	0.62 (0.39-0.85)	0.69 (0.52-0.87)	0.61 (0.37-0.84)
<b>CXCL10</b>	0.61 (0.42-0.80)	0.58 (0.35-0.81)	0.69 (0.52-0.86)	0.75 (0.57-0.94)
<b>CXCL9</b>	0.38 (0.20-0.55)	0.63 (0.40-0.86)	0.67 (0.51-0.83)	0.61 (0.37-0.84)
<b>IFN-<math>\gamma</math> + CXCL10</b>	0.61 (0.42-0.80)	0.66 (0.44-0.87)	0.70 (0.51-0.89)	0.75 (0.58-0.93)
<b>IFN-<math>\gamma</math> + CXCL9</b>	0.56 (0.37-0.75)	0.67 (0.47-0.87)	0.71 (0.54-0.88)	0.61 (0.38-0.84)
<b>IFN-<math>\gamma</math> + CXCL9 + CXCL10</b>	0.61 (0.43-0.80)	0.71 (0.52-0.90)	0.71 (0.53-0.89)	0.75 (0.58-0.93)

PPD: purified protein derivative; RD1: region of difference 1. ROC analyses were performed on pre-ART levels of IFN- $\gamma$ , CXCL10 and CXCL9 within TB-IRIS and ART-TB cases and matched controls.