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IP-10 differentiates between active and latent tuberculosis irrespective of HIV status and declines during therapy

I. Wergeland [a], N. Pullar [b,c], J. Assmus [d], T. Ueland [e,f], K. Tonby [e], S. Feruglio [e], D. Kvale [e,g], J.K. Damås [h,i], P. Aukrust [e,f,j], T.E. Mollnes [e,h,k], A.M. Dyrhol-Riise [a,e,g,*]

[a] *Department of Clinical Science, University of Bergen, N-5021 Bergen, Norway*
[b] *Department of Internal Medicine, Section for Infectious Diseases, University Hospital of Northern Norway, N-9038 Tromsø, Norway*
[c] *Department of Clinical Medicine, University of Tromsø, N-9037 Tromsø, Norway*
[d] *Center for Clinical Research, Haukeland University Hospital, N-5020 Bergen, Norway*
[e] *Institute of Clinical Medicine and K.G. Jebsen IRC, University of Oslo, N-0424 Oslo, Norway*
[f] *Research Institute of Internal Medicine, Oslo University Hospital, N-0424 Oslo, Norway*
[g] *Department of Infectious Diseases, Oslo University Hospital, N-0424 Oslo, Norway*
[h] *Centre of Molecular Inflammation Research, Norwegian University of Science and Technology, Trondheim, Norway*
[i] *Department of Infectious Diseases, St Olav's Hospital, Trondheim, Norway*
[j] *Section of Clinical Immunology and Infectious Diseases, Oslo University Hospital, N-0424 Oslo, Norway*
[k] *Research Laboratory, Nordland Hospital, Bodø, and Faculty of Health Sciences, K.G. Jebsen TREC, University of Tromsø, Tromsø, Norway*

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Summary *Objectives:* Biomarkers for diagnosis and therapy efficacy in tuberculosis (TB) are requested. We have studied biomarkers that may differentiate between active and latent TB infection (LTBI), the influence of HIV infection and changes during anti-TB chemotherapy.
Methods: Thirty-eight plasma cytokines, assessed by multiplex and enzyme immunoassays, were analyzed in patients with active TB before and during 24 weeks of anti-TB chemotherapy (n = 65), from individuals with LTBI (n = 34) and from QuantiFERON-TB (QFT) negative controls (n = 65). The study participants were grouped according to HIV status.

* Corresponding author. Department of Infectious Diseases, Oslo University Hospital, PO Box 4956, Nydalen, NO-0424 Oslo, Norway. Tel.: +47 92857261; fax: +47 22119181.
E-mail address: a.m.d.riise@medisin.uio.no (A.M. Dyrhol-Riise).

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Results: Plasma levels of the CXC chemokine IP-10 and soluble TNF receptor type 2 (sTNFr2) significantly differentiated active TB from the LTBI group, irrespective of HIV status. In the HIV-infected group the sensitivity and specificity was 100% for IP-10 with a cut-off of 2547 pg/mL. Plasma IP-10 declined gradually during anti-TB chemotherapy (12–24 weeks, $p = 0.002$) to a level comparable to LTBI and QFT negative control groups. sTNFr2 fluctuated throughout therapy, but was decreased after 12–24 weeks ($p = 0.006$).
Conclusions: IP-10 distinguished with high accuracy active TB from LTBI irrespective of HIV infection and declined during anti-TB chemotherapy. Plasma IP-10 may serve as a diagnostic biomarker to differentiate between the stages of TB infection and for monitoring therapy efficacy.

Introduction

Tuberculosis (TB) is a major global health problem, especially in the developing world. The World Health Organization estimates that one third of the world's population has latent TB infection (LTBI), and that there were 9.0 million incident active TB cases and 1.5 million deaths caused by TB in 2013.[1] Human immunodeficiency virus (HIV) infection is globally the major risk factor contributing to the resurgence of TB,[2] and the emergence of multi- and extensively drug resistant (MDR and XDR) strains of *Mycobacterium tuberculosis* (*Mtb*) have further aggravated the situation.[1]

Strategies for TB control aim to reduce transmission by identification and treatment of infectious cases as well as to reduce development of active disease by offering preventive therapy to individuals with LTBI. However, there is shortage of reliable and rapid diagnostic tools that can identify and discriminate between latent and active TB, and predict therapy responses.[3,4] The currently available tests for diagnosing LTBI are the century old tuberculin skin test (TST) and the more recent interferon gamma (IFN-γ)-release assays (IGRAs). The IGRAs offer better specificity compared to the TST, but both tests have suboptimal sensitivity in HIV co-infected individuals, and cannot distinguish between the various stages of TB infection or be used for evaluation of treatment efficacy.[3,5] Sputum culture conversion within the first 2 months of anti-TB chemotherapy could serve as a predictor of a favorable outcome,[6,7] but may also fail as predictive marker of cure in individual patients,[8,9] and are not applicable in extra-pulmonary and culture negative TB infection. Further, the need of alternative tools for monitoring treatment efficacy and for identifying patients at high risk of relapse is emphasized by the challenges of MDR- and XDR-TB cases, which require long lasting treatment with potentially inefficient and toxic drugs. Tools for monitoring treatment are also essential for accelerating drug development.

Alternative biomarkers have been sought to improve immune diagnosis of TB and monitoring of treatment efficacy. Various single or combinations of markers have been suggested to differentiate between latent and active TB infection.[10–15] However, a recent review concludes that there is no clear pattern of markers able to differentiate between the various TB infection stages.[16] Tests based on IFN-γ -inducible Protein 10 (IP-10)/CXCL10, one of the most studied surrogate biomarkers, perform comparably to the QuantiFERON-TB (QFT) in most patient groups,[17] but may increase the diagnostic accuracy of TB infection in both HIV-infected individuals[18–20] and children.[21–24] It has also been suggested that IP-10 can be used to monitor treatment efficacy,[14,25–29] but longitudinal studies performed on diverse patient populations are limited.

The aims of this study were to examine the potential of 38 selected cytokines, including interleukins, chemokines and growth factors to differentiate between the stages of TB infection as well examine the influence of HIV co-infection and the changes in these markers during anti-TB chemotherapy. Plasma IP-10 appeared to be the most consistent of the biomarkers studied, as it was the only marker that significantly differentiated active TB from both the LTBI and QFT negative control groups irrespective of HIV status and also gradually and significantly declined during anti-TB chemotherapy.

Materials and methods

Study participants and sample collection

The study population was included from clinical studies performed at three different hospitals in Norway in the period 2006–2012; Haukeland University Hospital,[30,31] University Hospital of Northern Norway[31] and Oslo University Hospital.[32] The study participants were recruited from patients diagnosed with active TB and from individuals referred to the hospitals for medical evaluation of LTBI based on a positive TST and/or exposure of TB.[30,32] HIV-infected individuals were recruited from outpatient infectious disease clinics.[31] QFT negative/HIV-uninfected individuals from age-matched employees with no known exposure to TB served as controls.

Thoracic X-ray and clinical examination were performed in all participants and an induced sputum sample and/or biopsy was obtained for acid fast staining and culture by BACTEC 960 MGIT liquid culture media (BD) or Lövenstein Jensen solid media. The patients diagnosed with active TB were given standard anti-TB chemotherapy according to national guidelines.[33] Subjects with a positive QFT, but no clinical signs or symptoms of active TB and no prior TB that could explain QFT positivity, were defined as LTBI.

Blood samples were obtained before start of any anti-TB chemotherapy and drawn into EDTA tubes. Plasma was

harvested after centrifugation, snap-frozen and stored at −80 °C until analysis. Plasma samples from patients with active TB were also obtained at 2–4 weeks, 6–12 weeks and 12–24 weeks after the start of anti-TB chemotherapy.

Written informed consent was obtained from all participants. The study was approved by the respective Regional Committees for Ethics in Medical Research (REK-Vest, REK-Nord and REK-Sør-øst).

QuantiFERON-TB Gold In-tube assay

The QuantiFERON TB-Gold In-tube® assay was performed according to the manufacturer's instructions (Cellestis Ltd, Qiagen, Chadstone, VIC, Australia) at the respective hospitals. The cut-off value for positive test was $\geq$0.35 IU/ml.

Multiplex cytokine analysis

Plasma samples were analyzed using a multiplex cytokine assay (Bio-Plex Human Cytokine 27-Plex Panel; Bio-Rad Laboratories Inc., Hercules, CA) containing assays for interleukin (IL)-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12(p70), IL-13, IL-15, IL-17, basic fibroblast growth factor (bFGF), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), IFN-γ, eotaxin/CCL11, IP-10/CXCL10, macrophage chemotattractant protein 1 (MCP-1)/CCL2, macrophage inflammatory protein 1 alpha (MIP-1α)/CCL3, MIP-1β/CCL4, regulated on activation, normal T cell expressed and secreted (RANTES)/CCL5, tumor necrosis factor (TNF), platelet-derived growth factor -BB (PDGF-BB) and vascular endothelial growth factor (VEGF).

The samples were analyzed on a Multiplex Analyser using Bio-Plex Manager 6.0 (Bio-Rad Laboratories) according to the manufacturer's instructions. Intra- and inter-assay coefficients of variation were <12% for all analytes. Nine of the markers evaluated (IL-1β, IL-2, IL-4, IL-5, IL-9, IL-10, IL-13, IL-15 and GM-CSF) were below the lower detection level (LDL) or detected at very low levels in all samples (>80% below the LDL of the assay), and were therefore excluded from further statistical analysis. The remaining occasional values (<20%) below the LDL were replaced by a defined common value below the LDL (0.001), which allowed non-parametric statistical analysis.

Enzyme immunoassays (EIA)

Plasma levels of CXCL16, pentraxin 3 (PTX3), soluble TNF receptor 2 (sTNFr2), Fas Ligand (FasL), thymus and activation regulated chemokine (TARC)/CCL17), osteoprotegerin (OPG), activated leukocyte cell adhesion molecule (ALCAM), IL-23, secreted frizzled-related protein 3 (sFRP3) and C-reactive protein (CRP) were measured by EIAs (R&D Systems, Stillwater, MN). Intra-and inter-assay coefficients of variation were <10% for all except FasL (<15%). MD-2 was analyzed by an in-house ELISA as previously described.[34] Briefly, MD-2 was captured on immune plates coated with a TLR-4-Fc fusion protein and detected with digoxygenin labelled MD-2 mAb 5D7 or digoxigenin labelled IIC1 and anti-digoxygenin-HRP (Roche). The MD-2 standard was purchased from R&D Systems, Stillwater, MN.

Statistical analysis

All statistical analyses were performed using IBM SPSS statistics 21, Matlab 7.10. or GraphPad Prism 6. We initially analyzed the HIV-infected and HIV-uninfected TB groups separately using Mann–Whitney U-test to detect pairwise differences between the study subgroups at baseline. In addition, we applied a binary logistic regression model to evaluate whether each of the markers was able to differentiate between active, LTBI and QFT negative controls pairwise, when adjusted for age, sex and HIV-status.

Mann–Whitney U-test was used to compare HIV-uninfected MDR-*Mtb* vs. drug sensitive *Mtb*, and extrapulmonary vs. pulmonary active TB cases, at time points where the sample size in each of these groups were $n \geq 5$. Wilcoxon signed rank test was used to evaluate changes in the levels of markers in patients with active TB from baseline to each follow up time point during anti-TB chemotherapy. Spearman's correlations were used to detect relationships between the CD4 counts and selected markers in the HIV-infected group.

The general significance level was set to 0.05. Taking into account effects of multiple testing, the Bonferroni adjustment would be too conservative due to the dependence between the markers. Thus, we decided to use a marginal significance level of 0.005 (corresponding to Bonferroni adjustment for 10 tests), and to describe results with p-values equal to or above 0.005 as significant and less than 0.05 ($0.005 \leq p < 0.05$) as tendencies.

Receiver operator characteristic (ROC) curve analyses were performed for the most promising marker differentiating between active and LTBI based on the results of the aforementioned tests. The optimal cut-off levels were defined by the minimum Euclidian distance to maximal specificity and sensitivity.

Results

Study participant characteristics

A total of 164 study participants had plasma samples available for analysis and these were classified into three groups; 1) Active TB (n = 65), 2) QFT-positive LTBI (n = 34) and 3) QFT negative controls (n = 65). The patients were further grouped according to HIV status. The clinical characteristics of the study participants are summarized in Table 1. Six (9.2%) of the patients with active TB, 23 (67.6%) of the individuals with LTBI and 52 (80.0%) of the QFT negative controls were HIV-infected. A total of 51 (63%) of the HIV patients were treated with antiretroviral therapy at inclusion in the study. Two of the QFT negative HIV-infected controls had acquired immune deficiency syndrome (AIDS)-defining infections (*Herpes simplex virus*, *Mycobacterium avium complex*, *Varicella zoster virus*), with high HIV viral load and CD4 counts below 100, but with no clinical signs of TB. AIDS-defining infections were not seen in the LTBI group.

The active TB diagnosis was based on positive *Mtb* culture in 60 of the patients, whereas in five patients the

Table 1 Characteristics of the study participants.

	Active TB (n = 65)		LTBI (QFT-positive, n = 34)		QFT-negative controls (n = 65)	
	HIV-infected (n = 6)	HIV-uninfected (n = 59)	HIV-infected (n = 23)	HIV-uninfected (n = 11)	HIV-infected (n = 52)	HIV-uninfected (n = 13[a])
Median age (range)	46 (38–64)	30 (16–91)	38 (21–48)	44 (24–61)	37 (19–68)	36 (26–70)
Sex: males/females	4/2	29/30	13/10	4/7	24/28	5/8
Origin: TB high/low endemic country	5/1	52/7	23/0	8/3	34/18	1/12
Median CD4 count (cells/μL, range)	286 (50–425)	ND	475 (110–1870)	ND	394 (3–1270)	ND
HIV patients on antiretroviral therapy (%)	4 (67)	ND	9 (39)	ND	38 (73)	ND
TB localization: pulm./ extrapulm./disseminated	6/0/0	39/18/2				
MDR-TB/mono-resistant TB[b]	1/1	10/4				
Culture: positive/negative or not performed	5/1	55/4				

[a] 5 QFT-negative/HIV-uninfected controls were included in the Multiplex bead assay. 13 QFT-negative/HIV-uninfected controls were included in the ELISA assays. ND: Not determined/relevant.
[b] Monoresistance against isoniazid, streptomycin or pyrazinamide.

diagnosis was based on clinical evaluation and characteristic histopathological or radiological findings. There were 11 MDR-TB cases and two patients with disseminated TB disease.

The patients with active TB had plasma samples obtained at 2–4 weeks (n = 24), at 6–12 weeks (n = 24) and at 12–24 weeks (n = 20). Only two HIV-infected patients with active TB had available plasma samples during treatment, and these were therefore excluded from the statistical analyses. All the patients with plasma samples available during treatment were successfully treated, except one patient who developed concomitant serious disease with intestinal perforation and septicaemia leading to death after 8 weeks of therapy. Thus, this patient was excluded from the follow-up analyses.

Impact of HIV infection on cytokine levels in various stages of TB infection

We found significant higher levels of IP-10 and eotaxin ($p < 0.005$) and a tendency of higher levels of FasL and sTNFr2 ($0.005 \leq p < 0.05$) in HIV co-infected compared with HIV-uninfected active TB cases (Figs. 1 and 2). In contrast, there was no significant impact of HIV infection on the levels of any of the markers neither in subjects with LTBI nor in QFT negative controls, although a tendency of increased IP-10 and CXCL16 levels ($0.005 \leq p < 0.05$) were seen in the HIV-infected compared to the HIV-uninfected LTBI group. Among QFT negative controls a tendency of decreased level of MIP-1α, MIP-1β, sFRP3 ($0.005 \leq p < 0.05$) and increased level of OPG ($0.005 \leq p < 0.05$) were observed in HIV-infected compared with HIV-uninfected individuals.

We further analyzed whether there were any correlations between the CD4 count and the level of IP-10 and eotaxin, respectively, in the HIV-infected individuals. We found a significant negative correlation between the CD4 count and the level of IP-10 ($p < 0.005$, $r = -0.349$) (Fig. 3), but not with eotaxin ($p = 0.098$, $r = -0.185$).

Cytokines differentiating between the various stages of TB infection

Since we observed a significant impact of HIV co-infection on the pattern of biomarkers in patients with active TB we analyzed the HIV-infected and HIV-uninfected TB groups separately.

In HIV-infected individuals there were significantly higher levels of IP-10 and sTNFr2 in the active TB group compared with both the LTBI group and QFT negative controls ($p < 0.005$, Fig. 2), and there was a tendency of PTX3, eotaxin and MCP-1 differentiating between active and LTBI ($0.005 \leq p < 0.05$). In contrast, in HIV-uninfected individuals, PTX3 was the only marker that significantly differentiated between active and LTBI ($p < 0.005$, Fig. 2), but in addition IP-10, IL-8, VEGF, MD-2 and sFRP3 showed a tendency of differentiating ($0.005 \leq p < 0.05$). However, several markers significantly distinguished (IL-8, PDGF-BB, TARC and CRP, $p < 0.005$) or tended to distinguish (sFRP3, IL-7, eotaxin, OPG and CXCL16, $0.005 \leq p < 0.05$) active TB from QFT negative controls.

IP-10 and sTNFr2 also differentiated significantly ($p < 0.005$) between active TB and both LTBI and QFT negative controls when a binary logistic regression model adjusting for HIV status, age and gender was applied. In addition, in this model, PTX3 and CRP significantly differentiated between active TB and QFT negative controls ($p < 0.005$), and tended to differentiate between active TB and LTBI ($0.005 \leq p < 0.05$).

The diagnostic accuracy of IP-10 in differentiating between active TB, LTBI and the QFT negative control group was investigated by ROC curve analyses (Table 2). In HIV-infected individuals a cut-off level of 2547 pg/mL

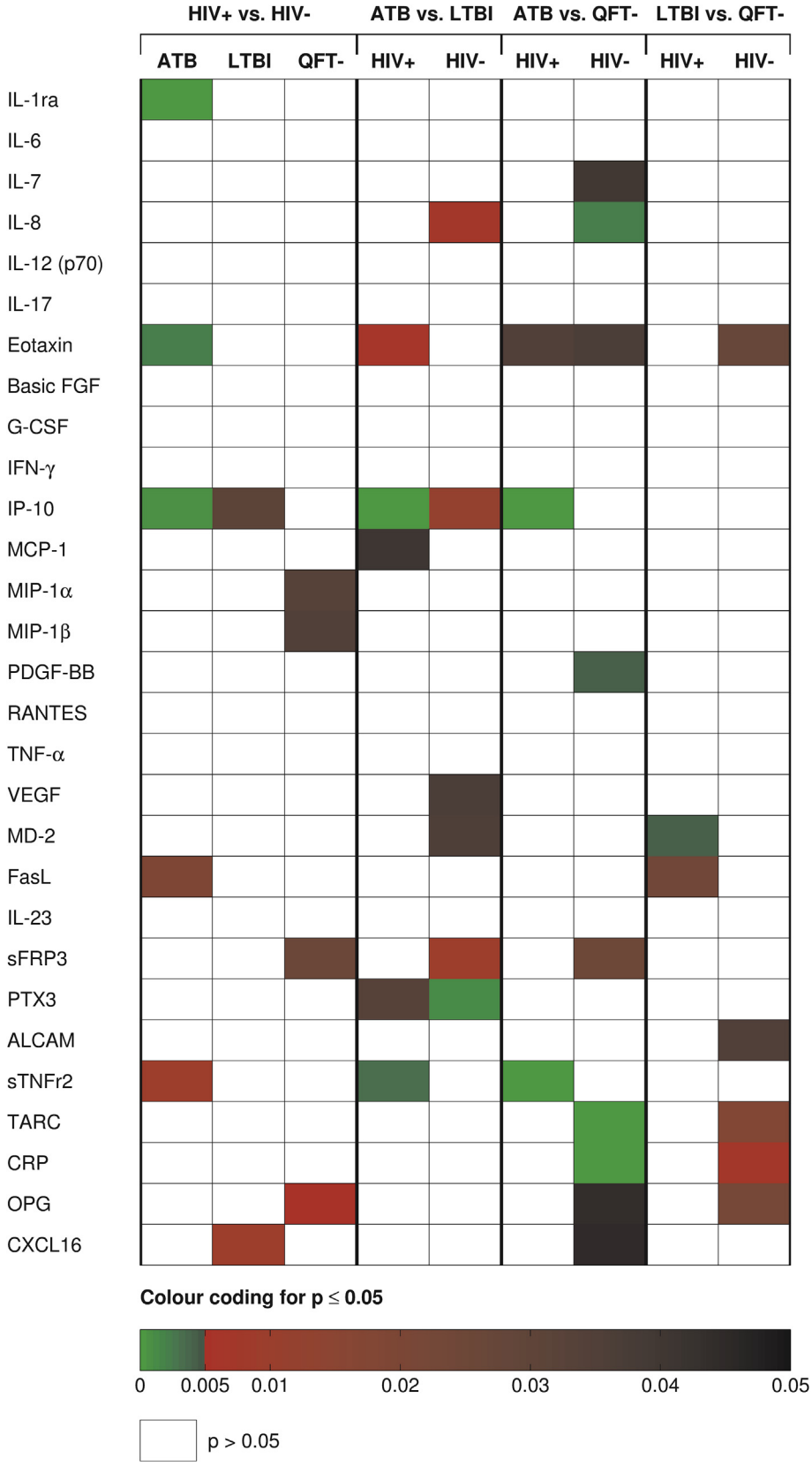

Figure 1 Comparisons of cytokine levels between patients with active TB, latent TB and QFT-negative controls and between HIV-infected and HIV-uninfected patients (Mann—Whitney U test). Significant p-values ($p < 0.005$) are shown as a colour scale in green, and p-values described as tendencies ($0.005 \leq p < 0.05$) are shown as a colour scale ranging from red to black. ATB: active TB infection. LTBI: Latent TB infection. QFT-: QuantiFERON-TB negative controls.

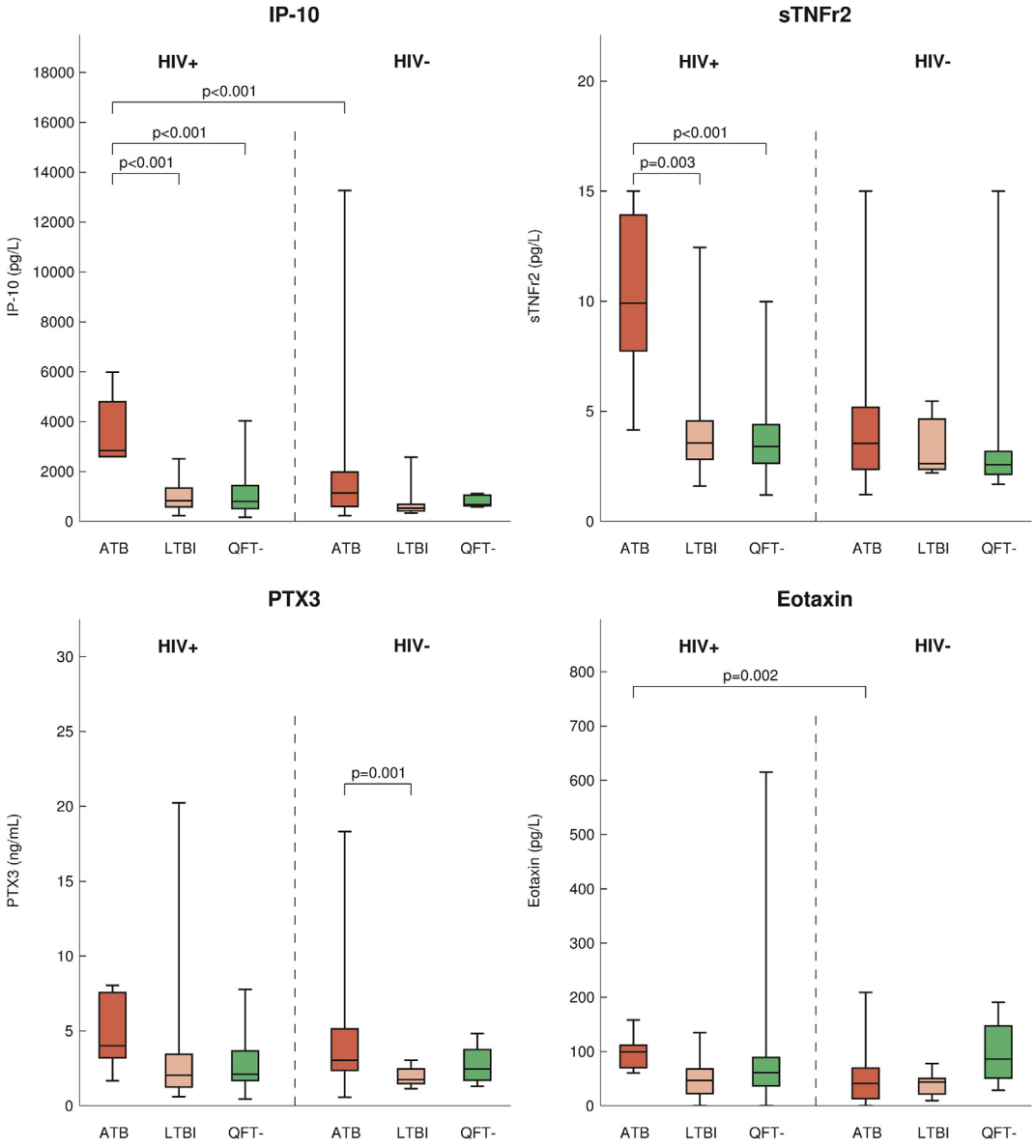

Figure 2 Plasma levels of IP-10, sTNFr2, PTX3 and eotaxin in HIV-infected and HIV- uninfected patients with active TB, latent TB and in QFT negative controls. The boxes show the median and interquartile range, and the whiskers show minimum and maximum values. Statistical analysis was performed using the Mann—Whitney U test. Brackets represent statistically significant differences ($p < 0.005$).

discriminated active TB from LTBI with 100% sensitivity and specificity, whereas in HIV-uninfected individuals the optimal cut-off level was 689 pg/mL, and the sensitivities and specificities were 71% and 82%, respectively.

Changes in cytokine levels during anti-TB chemotherapy

In HIV-uninfected patients with active TB, we analyzed the levels of the various markers at different time-points throughout therapy. The level of IP-10 decreased gradually during treatment. There was a tendency of decrease observed already after 6—12 weeks ($p = 0.022$, Fig. 4). However, the decrease was significant first after 12—24 weeks ($p = 0.002$), and the level was then comparable to that seen both among the LTBI and in the QFT negative control group. By using the optimal cut-off level of 674 pg/mL for differentiation between active TB and non-TB in HIV-uninfected patients, 43/59 (73%) of the active TB patients scored positive for plasma IP-10 at baseline, whereas 9/20 (45%) were positive at week 12—24. Only one patient with extrapulmonary TB scored negative at baseline and became positive during treatment. However, this patient had IP-10 levels close to cut-off both at baseline and during therapy. sTNFr2 tended to decrease to the level of LTBI cases and QFT negative controls after 12—24 weeks of treatment ($p = 0.006$), but in contrast to IP-10, which showed a uniform decrease over time, the level of sTNFr2 fluctuated (Fig. 4).

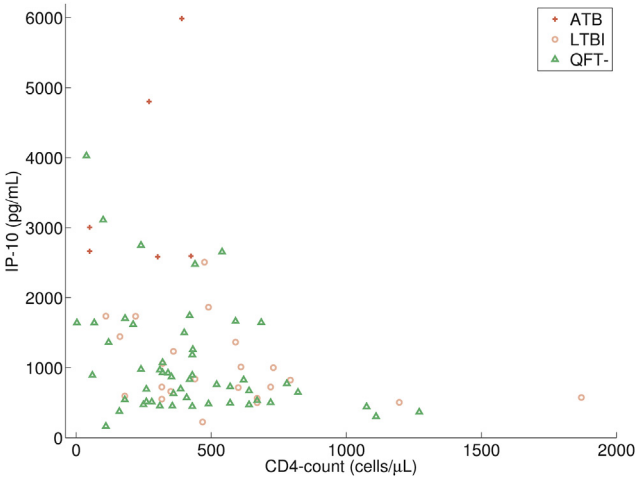

Figure 3 Negative correlation between CD4 counts and plasma IP-10 levels in HIV-infection. Spearman's correlations were used to detect relationships between the CD4 counts and selected markers in the HIV-infected group. There was a significant negative correlation between the CD4 count (cells/μL) and the plasma level of IP-10 (pg/mL) in HIV infected individuals ($p < 0.005$, $r = -0.349$). ATB: active TB infection. LTBI: Latent TB infection. QFT-: QuantiFERON-TB negative controls.

Several of the other markers that significantly differentiated between active TB and the LTBI and/or the QFT negative control groups at baseline also declined throughout therapy. PDGF-BB and TARC were significantly decreased already after 2–4 weeks ($p = 0.002$ and $p = 0.004$ respectively, Fig. 4), and there was a tendency of decrease in CRP and IL-8, which reached the significance level at week 12–24 ($p = 0.005$, Fig. 4). PTX3, that significantly differentiated between active and LTBI at baseline, did not decline during treatment. In contrast, a tendency of increasing concentration of PTX3 was found after 6–12 weeks of treatment ($p = 0.042$). However, when changes in biomarker levels were analyzed in the *Mtb* sensitive group separately, we found that the level of PTX3 tended to decrease at week 6–12 ($p = 0.013$), whereas there was an increase in PTX3 for the majority of the MDR TB patients in the same period. For the other markers analyzed we found no distinct differences in response patterns when studying the drug sensitive *Mtb* cases separately versus all active TB cases taken together.

There were also several markers which did not discriminate significantly between the various study groups at baseline, but where a significant decrease in concentrations were observed in active TB cases during treatment; RANTES (2–4 weeks), IL-1ra, IL-6, IL-12(p70), bFGF, IFN-γ, MIP-1α, TNF and VEGF (6–12 weeks), and G-CSF, MD-2 and IL-7 (12–24 weeks), $p < 0.005$, Fig. 4).

Discussion

In this study we have examined the potential of plasma cytokines to differentiate between active and LTBI in HIV-infected and HIV-uninfected individuals and longitudinal changes in these markers during anti-TB chemotherapy. For the first time we document that IP-10 and sTNFr2 can differentiate active TB cases from both individuals with LTBI and QFT negative controls irrespective of HIV status, age and gender. Moreover, we found that the level of IP-10 in HIV-uninfected active TB cases decreased gradually to the same level as LTBI and QFT negative controls after 12–24 weeks of anti-TB chemotherapy.

IP-10 is a chemokine produced by antigen presenting cells mainly in response to IFN-γ and TNF. It has been shown that plasma levels of IP-10 are increased in bacteremia[35] and infections with hepatitis C virus,[36] HIV[37,38] and TB,[14,25] and appear to correlate with the extent of inflammation. In our study, the level of IP-10 was significantly higher in HIV co-infected active TB cases compared with HIV-uninfected cases. This is in concordance with a study by Juffermans et al.,[39] but in contrast, Mihret et al.[14,27] and Riou et al.[28] found no significant differences in the level of IP-10 with respect to HIV-infection in patients with active TB before anti-TB chemotherapy. We found a significant negative correlation between the CD4 count and the level of IP-10 in HIV-infected individuals supporting studies of HIV-infected individuals without TB co-infection.[37,40] The discordant results could therefore be due to differences in CD4 counts between the HIV-infected groups in the various studies.

IP-10 circulates at much higher levels compared to IFN-γ and has been extensively studied as an alternative to IFN-γ for immunodiagnosis of TB.[16,17] In agreement with our results, it has been shown that plasma levels of IP-10 are elevated in active TB cases compared with household contacts.[14,25,41] Our study is a valuable contribution to the literature since HIV-infected subgroups are included both in the active TB, the LTBI and the QFT negative groups,

Table 2 ROC analyses with sensitivity and specificity of plasma IP-10 for tuberculosis in HIV-infected and HIV-uninfected patients.

		Cut-off (pg/mL)	Sensitivity (95% CI)	Specificity (95% CI)	AUC (95% CI)	p-value
HIV infected	ATB vs. LTBI	2547	100 (54.1–100)	100 (85.2–100)	1.00 (1.00–1.00)	<0.001
	ATB vs. QFT negative	2532	100 (54.1–100)	92.3 (81.5–97.9)	0.96 (0.91–1.00)	<0.001
HIV uninfected	ATB vs. LTBI	689	71.2 (57.9–82.2)	81.8 (48.2–97.7)	0.74 (0.59–0.89)	0.012
	ATB vs. QFT negative	674	72.9 (59.7–83.6)	60.0 (14.7–94.7)	0.66 (0.52–0.80)	0.235

ATB: Active TB infection. LTBI: Latent TB infection. QFT: QuantiFERON-TB. AUC: Area under the ROC curve. 95% CI: 95% confidence interval.

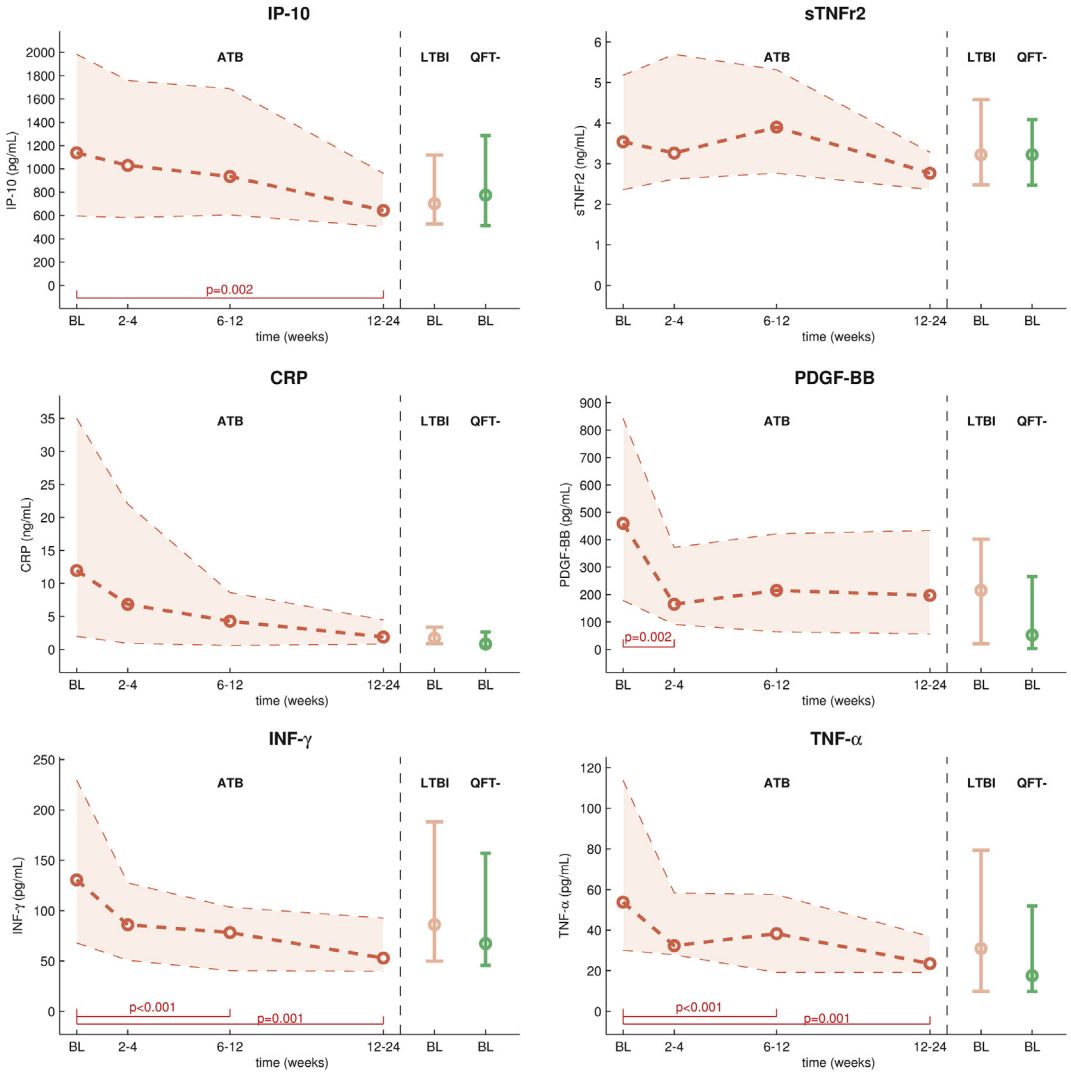

Figure 4 Plasma levels of selected soluble markers during anti-TB chemotherapy; IP-10, sTNFr2, CRP, PDGF-BB, IFN-γ and TNF-α in HIV-uninfected patients with active TB before and after 2—4, 6—12 and 12—24 weeks compared to the HIV-uninfected LTBI and QFT-negative groups. Changes in the plasma level of markers from baseline to each follow up time point during treatment were analyzed using Wilcoxon matched pairs signed rank test. Brackets represent statistically significant differences ($p < 0.005$). ATB: active TB infection. LTBI: Latent TB infection. QFT-: QuantiFERON-TB negative controls. BL: baseline.

and we demonstrate that the plasma level of IP-10 distinguish active TB from LTBI and QFT negative controls irrespective of HIV co-infection. The ROC curve analyses show that plasma IP-10 with a cut of 2547 pg/mL clearly differentiates between active TB and LTBI in HIV-infected individuals with 100% sensitivity and specificity. In HIV-uninfected individuals the diagnostic accuracy was less optimal and the cut-off level was 689 pg/mL which is notably higher than reported by Hong et al. (119.5 pg/mL).[41] This discrepancy is most likely explained by differences in methods of detection and variations in study populations, and emphasizes the importance of determining validated cut-offs before IP-10 can be used in clinical practice.

Our results support previous studies demonstrating a decrease in plasma level of IP-10 upon anti-TB chemotherapy of HIV-uninfected active TB cases,[14,25—28] whereas there are conflicting results in HIV co-infected cases. Riou et al. reported that IP-10 decreased irrespective of HIV infection,[28] but Mihret et al. documented a decline only for HIV-uninfected cases.[27] In a study by Azzuri et al., IP-10 increased in household contacts during progression to active TB and during relapse of TB in patients who previously had completed anti-TB chemotherapy.[25] Patients with active TB with moderate to high risk of relapse decline less in IP-10 during treatment compared with low risk patients.[26] It has also been suggested that serial determinations of serum IP-10 can be used to identify individuals with rheumatoid arthritis at high risk of developing active TB during TNF inhibitor treatment.[42] Further, plasma level of IP-10 correlates with the sputum smear acid fast bacilli grade[26,43] and the degree of lung involvement.[25] Although there was a tendency of decline already at 6—12 weeks, we found defined by our definition of significance level, a decrease in the level of IP-10 first after 12—24 weeks of treatment. We report a reduction in the fraction of active

TB patients with positive plasma IP-10 from 73% at baseline to 45% during anti-TB chemotherapy in line with a previous report.[26] Other markers, including PDGF-BB, TARC and IFN-γ showed an earlier significant decrease in response to therapy compared with IP-10, but these markers were not able to differentiate between the various stages of TB infection.

IP-10 measured directly in plasma is unspecific for TB, but IP-10 has also been studied as a potential readout biomarker in *in vitro Mtb* specific immunoassays. A review by Ruhwald et al. concludes that IP-10 release assays perform comparably to the QFT-test in most patient groups, but may improve the diagnostic accuracy in children and in HIV-infected individuals with low CD4 counts.[17] However, the *Mtb* specific immunoassays does not seem to be able to differentiate between active and LTBI,[3,17] and IGRAs cannot be used for monitoring treatment.[5] Hong et al. found that IP-10 secretion in response to QFT antigens decreased during anti-TB chemotherapy,[26] whereas Kabeer et al. could not confirm this, but rather detected decrease in response to RD1-selected peptides.[29]

The relatively high level of plasma IP-10 compared with other biomarkers, allows for simplification of analyzes. Methods for quantification of IP-10 in dried blood and plasma spots (DBS/DPS) have been developed, facilitating point-of-care tests and transport of samples at ambient temperatures.[44–46] DPS/DBS-based IP-10 release assays offer comparable diagnostic accuracy to the QFT.[44,47] IP-10, in contrast to IFN-γ and TNF-α, could also be detected in urine from TB patients and pulmonary TB patients have significantly higher urine IP-10 levels than healthy controls.[48] Moreover, the urine IP-10 level in cured TB patients were comparable to that found in healthy controls.[48] Urine samples have several advantaged including non-invasive collection, low cost and no need of special equipment or personnel. Still, care most be taken when detecting IP-10 in urine, as high urine IP-10 levels are also associated with chronic hepatitis C virus infection.[49] Thus, further studies are needed to determine whether urine IP-10 is a reliable alternative to plasma IP-10.

sTNFr2 can act as a TNF antagonist by competing with the cell membrane receptors for cytokine binding. It is the biologically active component of the TNF inhibitor etanercept, which, among other TNF inhibitors, has been associated with increased risk of reactivation of latent TB infection.[50] Elevated levels of TNF have been found in patients with active TB compared with household contacts and healthy controls.[51] Further, Jufferman et al. found that both sTNFr1 and sTNFr2 are increased in active TB cases and decline during treatment.[52] In our study, although finally declined, the level fluctuated during treatment. Riou et al. show that the level of TNF also fluctuates during treatment.[28] We found a gradual decrease in the level of TNF, which was significant after 6–12 weeks of therapy. However, since there were no significant differences in TNF level between any of our study groups at baseline this questions the usefulness as biomarker for therapy efficacy. Finally, if sTNFr2 may serve as a reliable marker of TNF activity is also unknown.

The pentraxin family, including CRP and PTX3, is involved in the acute phase reaction to inflammation.[53] PTX3 was the only marker that significantly differentiated between active and LTBI when HIV-uninfected individuals were studied separately, and also showed a tendency of differentiating between these groups in HIV-infected individuals. Azzuri et al. found that the plasma level of PTX3 was higher in patients with active TB compared with healthy household controls, decreased with successful treatment and increased in patients with treatment failure.[25] When patients with MDR-TB and drug sensitive *Mtb* were analyzed as one group we found a tendency of increase in the level of PTX3 during treatment. However, when analyzed separately, PTX3 tended to decrease in the drug sensitive *Mtb* group whereas there was an initial increase, followed by a decline after 24–48 months in MDR TB patients (data not shown). This may indicate that there is a delayed response to treatment in MDR compared with drug sensitive *Mtb*. CRP, which is extensively used as a marker of inflammation, declined steadily, but only near significantly, during treatment.

The main limitations of our study are the low sample size of HIV-infected patients with active TB and the lack of longitudinal follow up plasma samples from this group during treatment. This was included in the design of the original study,[31] but there was a low prevalence of active TB in the HIV-infected population, and only two HIV-infected patients had plasma samples obtained during treatment. Also, the changes in markers during treatment of active TB were assessed together regardless of drug resistant or sensitive *Mtb*, although the treatment differed in composition of antibiotics and length. We found indications that the kinetics of some of the biomarkers may differ between MDR and drug sensitive *Mtb*. Still, the majority of patients had *Mtb* sensitive strains, received standard anti-TB chemotherapy and all patients responded to treatment. Finally, since plasma IP-10 is unspecific for TB, our study should also have included patients with diagnoses other than TB, including AIDS-defining infections, to optimally validate the accuracy of the method. The two HIV patients in the QFT negative control group with other opportunistic infections clearly demonstrate this since they had plasma IP-10 levels above the median level of the HIV-infected active TB group. Still, our study was not designed to fully validate IP-10, but we recommend awareness of infections other than TB in the interpretation of plasma levels of IP-10, particularly in patients with HIV-confection.

In conclusion, IP-10 appears to be the most consistent of the biomarkers studied as it was the only marker that significantly differentiated active TB from both LTBI and QFT negative controls irrespective of HIV status, and also significantly declined during anti-TB chemotherapy of HIV-uninfected active TB cases. Although not specific for TB, plasma level of IP-10 may give information about the stage of infection, and may also be used to monitor the effect of treatment, but further studies are needed to validate and standardize IP-10 assays.

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References

1. World Health Organization. *Global tuberculosis report*. 2014. Available: http://www.who.int/tb/publications/global_report/en/ [accessed 12.12.14].
2. Selwyn PA, Hartel D, Lewis VA, Schoenbaum EE, Vermund SH, Klein RS, et al. A prospective study of the risk of tuberculosis among intravenous drug users with human immunodeficiency virus infection. *N Engl J Med* 1989 Mar 2;**320**(9):545—50.
3. Pai M, Denkinger CM, Kik SV, Rangaka MX, Zwerling A, Oxlade O, et al. Gamma interferon release assays for detection of Mycobacterium tuberculosis infection. *Clin Microbiol Rev* 2014 Jan;**27**(1):3—20.
4. Parida SK, Kaufmann SH. The quest for biomarkers in tuberculosis. *Drug Discov Today* 2010 Feb;**15**(3—4):148—57.
5. Chiappini E, Fossi F, Bonsignori F, Sollai S, Galli L, de MM. Utility of interferon-gamma release assay results to monitor antitubercular treatment in adults and children. *Clin Ther* 2012 May;**34**(5):1041—8.
6. Holtz TH, Sternberg M, Kammerer S, Laserson KF, Riekstina V, Zarovska E, et al. Time to sputum culture conversion in multidrug-resistant tuberculosis: predictors and relationship to treatment outcome. *Ann Intern Med* 2006 May 2;**144**(9):650—9.
7. Wallis RS, Wang C, Doherty TM, Onyebujoh P, Vahedi M, Laang H, et al. Biomarkers for tuberculosis disease activity, cure, and relapse. *Lancet Infect Dis* 2010 Feb;**10**(2):68—9.
8. Benator D, Bhattacharya M, Bozeman L, Burman W, Cantazaro A, Chaisson R, et al. Rifapentine and isoniazid once a week versus rifampicin and isoniazid twice a week for treatment of drug-susceptible pulmonary tuberculosis in HIV-negative patients: a randomised clinical trial. *Lancet* 2002 Aug 17;**360**(9332):528—34.
9. Johnson JL, Hadad DJ, Dietze R, Maciel EL, Sewali B, Gitta P, et al. Shortening treatment in adults with noncavitary tuberculosis and 2-month culture conversion. *Am J Respir Crit Care Med* 2009 Sep 15;**180**(6):558—63.
10. Biselli R, Mariotti S, Sargentini V, Sauzullo I, Lastilla M, Mengoni F, et al. Detection of interleukin-2 in addition to interferon-gamma discriminates active tuberculosis patients, latently infected individuals, and controls. *Clin Microbiol Infect* 2010 Aug;**16**(8):1282—4.
11. Chegou NN, Black GF, Kidd M, van Helden PD, Walzl G. Host markers in QuantiFERON supernatants differentiate active TB from latent TB infection: preliminary report. *BMC Pulm Med* 2009;**9**:21.
12. Chegou NN, Detjen AK, Thiart L, Walters E, Mandalakas AM, Hesseling AC, et al. Utility of host markers detected in Quantiferon supernatants for the diagnosis of tuberculosis in children in a high-burden setting. *PLoS One* 2013;**8**(5):e64226.
13. Frahm M, Goswami ND, Owzar K, Hecker E, Mosher A, Cadogan E, et al. Discriminating between latent and active tuberculosis with multiple biomarker responses. *Tuberculosis (Edinb)* 2011 May;**91**(3):250—6.
14. Mihret A, Bekele Y, Bobosha K, Kidd M, Aseffa A, Howe R, et al. Plasma cytokines and chemokines differentiate between active disease and non-active tuberculosis infection. *J Infect* 2013 Apr;**66**(4):357—65.
15. Sutherland JS, de Jong BC, Jeffries DJ, Adetifa IM, Ota MO. Production of TNF-alpha, IL-12(p40) and IL-17 can discriminate between active TB disease and latent infection in a West African cohort. *PLoS One* 2010;**5**(8):e12365.
16. Chegou NN, Heyckendorf J, Walzl G, Lange C, Ruhwald M. Beyond the IFN-gamma horizon: biomarkers for immunodiagnosis of infection with M. tuberculosis. *Eur Respir J* 2014 May;**43**(5):1472—86.
17. Ruhwald M, Aabye MG, Ravn P. IP-10 release assays in the diagnosis of tuberculosis infection: current status and future directions. *Expert Rev Mol Diagn* 2012 Mar;**12**(2):175—87.
18. Goletti D, Raja A, Syed Ahamed KB, Rodrigues C, Sodha A, Carrara S, et al. Is IP-10 an accurate marker for detecting M. tuberculosis-specific response in HIV-infected persons? *PLoS One* 2010;**5**(9):e12577.
19. Kabeer BS, Sikhamani R, Raja A. Comparison of interferon gamma and interferon gamma-inducible protein-10 secretion in HIV-tuberculosis patients. *AIDS* 2010 Jan 16;**24**(2):323—5.
20. Vanini V, Petruccioli E, Gioia C, Cuzzi G, Orchi N, Rianda A, et al. IP-10 is an additional marker for tuberculosis (TB) detection in HIV-infected persons in a low-TB endemic country. *J Infect* 2012 Jul;**65**(1):49—59.
21. Alsleben N, Ruhwald M, Russmann H, Marx FM, Wahn U, Magdorf K. Interferon-gamma inducible protein 10 as a biomarker for active tuberculosis and latent tuberculosis infection in children: a case-control study. *Scand J Infect Dis* 2012 Apr;**44**(4):256—62.
22. Latorre I, Diaz J, Mialdea I, Serra-Vidal M, Altet N, Prat C, et al. IP-10 is an accurate biomarker for the diagnosis of tuberculosis in children. *J Infect* 2014 Dec;**69**(6):590—9.
23. Lighter J, Rigaud M, Huie M, Peng CH, Pollack H. Chemokine IP-10: an adjunct marker for latent tuberculosis infection in children. *Int J Tuberc Lung Dis* 2009 Jun;**13**(6):731—6.
24. Ruhwald M, Petersen J, Kofoed K, Nakaoka H, Cuevas LE, Lawson L, et al. Improving T-cell assays for the diagnosis of latent TB infection: potential of a diagnostic test based on IP-10. *PLoS One* 2008;**3**(8):e2858.
25. Azzurri A, Sow OY, Amedei A, Bah B, Diallo S, Peri G, et al. IFN-gamma-inducible protein 10 and pentraxin 3 plasma levels are tools for monitoring inflammation and disease activity in Mycobacterium tuberculosis infection. *Microbes Infect* 2005 Jan;**7**(1):1—8.
26. Hong JY, Lee HJ, Kim SY, Chung KS, Kim EY, Jung JY, et al. Efficacy of IP-10 as a biomarker for monitoring tuberculosis treatment. *J Infect* 2014 Mar;**68**(3):252—8.
27. Mihret A, Abebe M, Bekele Y, Aseffa A, Walzl G, Howe R. Impact of HIV co-infection on plasma level of cytokines and chemokines of pulmonary tuberculosis patients. *BMC Infect Dis* 2014;**14**:125.
28. Riou C, Perez PB, Roberts L, Ronacher K, Walzl G, Manca C, et al. Effect of standard tuberculosis treatment on plasma cytokine levels in patients with active pulmonary tuberculosis. *PLoS One* 2012;**7**(5):e36886.
29. Kabeer BS, Raja A, Raman B, Thangaraj S, Leportier M, Ippolito G, et al. IP-10 response to RD1 antigens might be a useful biomarker for monitoring tuberculosis therapy. *BMC Infect Dis* 2011;**11**:135.
30. Dyrhol-Riise AM, Gran G, Wentzel-Larsen T, Blomberg B, Haanshuus CG, Morkve O. Diagnosis and follow-up of treatment of latent tuberculosis; the utility of the QuantiFERON-TB Gold In-tube assay in outpatients from a tuberculosis low-endemic country. *BMC Infect Dis* 2010;**10**:57.
31. Pullar ND, Steinum H, Tonby K, Heggelund L, Leiva RA, Ofstad R, et al. Low prevalence of positive interferon-gamma tests in HIV-positive long-term immigrants in Norway. *Int J Tuberc Lung Dis* 2014 Feb;**18**(2):180—7.
32. Feruglio SL, Tonby K, Kvale D, Dyrhol-Riise AM. Early dynamics of T helper cell cytokines and T regulatory cells in response to

treatment of active Mycobacterium tuberculosis infection. *Clin Exp Immunol* 2014 Oct 14.
33. Nasjonalt folkehelseinstitutt. *Tuberkuloseveilederen (Norwegian, Guidelines for tuberculosis)*. 2014. Available: http://www.fhi.no/publikasjoner-og-haandboker/tuberkuloseveilederen [accessed 23.05.14].
34. Sandanger O, Ryan L, Bohnhorst J, Iversen AC, Husebye H, Halaas O, et al. IL-10 enhances MD-2 and CD14 expression in monocytes and the proteins are increased and correlated in HIV-infected patients. *J Immunol* 2009 Jan 1;**182**(1):588—95.
35. Punyadeera C, Schneider EM, Schaffer D, Hsu HY, Joos TO, Kriebel F, et al. A biomarker panel to discriminate between systemic inflammatory response syndrome and sepsis and sepsis severity. *J Emerg Trauma Shock* 2010 Jan;**3**(1):26—35.
36. Askarieh G, Alsio A, Pugnale P, Negro F, Ferrari C, Neumann AU, et al. Systemic and intrahepatic interferon-gamma-inducible protein 10 kDa predicts the first-phase decline in hepatitis C virus RNA and overall viral response to therapy in chronic hepatitis C. *Hepatology* 2010 May;**51**(5):1523—30.
37. Keating SM, Golub ET, Nowicki M, Young M, Anastos K, Crystal H, et al. The effect of HIV infection and HAART on inflammatory biomarkers in a population-based cohort of women. *AIDS* 2011 Sep 24;**25**(15):1823—32.
38. Stylianou E, Aukrust P, Bendtzen K, Muller F, Froland SS. Interferons and interferon (IFN)-inducible protein 10 during highly active anti-retroviral therapy (HAART)-possible immunosuppressive role of IFN-alpha in HIV infection. *Clin Exp Immunol* 2000 Mar;**119**(3):479—85.
39. Juffermans NP, Verbon A, van Deventer SJ, van DH, Belisle JT, Ellis ME, et al. Elevated chemokine concentrations in sera of human immunodeficiency virus (HIV)-seropositive and HIV-seronegative patients with tuberculosis: a possible role for mycobacterial lipoarabinomannan. *Infect Immun* 1999 Aug;**67**(8):4295—7.
40. Liovat AS, Rey-Cuille MA, Lecuroux C, Jacquelin B, Girault I, Petitjean G, et al. Acute plasma biomarkers of T cell activation set-point levels and of disease progression in HIV-1 infection. *PLoS One* 2012;**7**(10):e46143.
41. Hong JY, Jung GS, Kim H, Kim YM, Lee HJ, Cho SN, et al. Efficacy of inducible protein 10 as a biomarker for the diagnosis of tuberculosis. *Int J Infect Dis* 2012 Dec;**16**(12):e855—9.
42. Chen DY, Shen GH, Chen YM, Chen HH, Lin CC, Hsieh CW, et al. Interferon-inducible protein-10 as a marker to detect latent and active tuberculosis in rheumatoid arthritis. *Int J Tuberc Lung Dis* 2011 Feb;**15**(2):192—200.
43. Chen YC, Chin CH, Liu SF, Wu CC, Tsen CC, Wang YH, et al. Prognostic values of serum IP-10 and IL-17 in patients with pulmonary tuberculosis. *Dis Markers* 2011;**31**(2):101—10.
44. Blauenfeldt T, Heyckendorf J, Graff JS, Lange C, Drabe C, Hermansen TS, et al. Development of a one-step probe based molecular assay for rapid immunodiagnosis of infection with M. tuberculosis using dried blood spots. *PLoS One* 2014;**9**(9):e105628.
45. Drabe CH, Blauenfeldt T, Ruhwald M. ELISA-based assay for IP-10 detection from filter paper samples. *Methods Mol Biol* 2014;**1172**:27—37.
46. Aabye MG, Eugen-Olsen J, Werlinrud AM, Holm LL, Tuuminen T, Ravn P, et al. A simple method to quantitate IP-10 in dried blood and plasma spots. *PLoS One* 2012;**7**(6):e39228.
47. Aabye MG, Latorre I, Diaz J, Maldonado J, Mialdea I, Eugen-Olsen J, et al. Dried plasma spots in the diagnosis of tuberculosis: IP-10 release assay on filter paper. *Eur Respir J* 2013 Aug;**42**(2):495—503.
48. Cannas A, Calvo L, Chiacchio T, Cuzzi G, Vanini V, Lauria FN, et al. IP-10 detection in urine is associated with lung diseases. *BMC Infect Dis* 2010;**10**:333.
49. Petrone L, Chiacchio T, Vanini V, Petruccioli E, Cuzzi G, Di GC, et al. High urine IP-10 levels associate with chronic HCV infection. *J Infect* 2014 Jun;**68**(6):591—600.
50. Wallis RS. Tumour necrosis factor antagonists: structure, function, and tuberculosis risks. *Lancet Infect Dis* 2008 Oct;**8**(10):601—11.
51. Wang S, Diao N, Lu C, Wu J, Gao Y, Chen J, et al. Evaluation of the diagnostic potential of IP-10 and IL-2 as biomarkers for the diagnosis of active and latent tuberculosis in a BCG-vaccinated population. *PLoS One* 2012;**7**(12):e51338.
52. Juffermans NP, Verbon A, van Deventer SJ, van DH, Speelman P, van der Poll T. Tumor necrosis factor and interleukin-1 inhibitors as markers of disease activity of tuberculosis. *Am J Respir Crit Care Med* 1998 Apr;**157**(4 Pt 1):1328—31.
53. Mantovani A, Garlanda C, Bottazzi B. Pentraxin 3, a non-redundant soluble pattern recognition receptor involved in innate immunity. *Vaccine* 2003 Jun 1;**21**(Suppl. 2):S43—7.