Immunological profiling of tuberculosis-associated immune reconstitution inflammatory syndrome and non-immune reconstitution inflammatory syndrome death in HIV-infected adults with pulmonary tuberculosis starting antiretroviral therapy: a prospective observational cohort study

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Summary

Background Patients co-infected with advanced HIV and tuberculosis are at risk of tuberculosis-associated immune reconstitution inflammatory syndrome (IRIS) and death soon after initiation of antiretroviral therapy (ART). Tuberculosis-associated IRIS has been associated with quicker recovery of cellular immune responses after ART initiation and early mortality with slower recovery of these responses. We aimed to assess whether patients who have these outcomes have distinct immunological profiles before and after ART initiation.

Methods We undertook this prospective cohort study at 22 public clinics and the main public hospital in Gaborone, Botswana, in ART-naive adults (aged ≥21 years) with advanced HIV (CD4 cell counts ≤125 cells per μL) and pulmonary tuberculosis. We obtained data for clinical variables and for levels of 29 plasma biomarkers, quantified by Luminex assay. We classified patients as having tuberculosis-associated IRIS, early mortality, or survival without a diagnosis of tuberculosis-associated IRIS (controls), on the basis of outcomes recorded in the 6 months after ART initiation. We used rank-sum or χ^2 tests, and logistic regression with odds ratios (OR) and 95% CIs, to assess the association between variables measured before and 4 weeks after ART initiation with death and tuberculosis-associated IRIS, compared with controls.

Findings Between Nov 12, 2009, and July 3, 2013, we enrolled 201 participants. 31 (15%) patients left the study before ART initiation, leaving 170 (85%) patients for analysis. Patients with tuberculosis-associated IRIS had reduced pre-ART concentrations of several pro-inflammatory biomarkers, including interleukin (IL)-6 (adjusted OR per 1 $\log_{10}$ increase 0·40 [95% CI 0·18–0·89]). However, patients with early death had increased pre-ART concentrations of inflammatory biomarkers, including monocyte chemoattractant protein-1 (adjusted OR 9·0 [95% CI 1·0–80·0]) and tumour necrosis factor (TNF)α (7·8 [1·1–55·2]). At week 4 after ART initation, tuberculosis-associated IRIS was independently associated with greater increases in several inflammatory biomarkers, including IL-6 (adjusted OR 1·7 [95% CI 1·2–2·5]) and TNFα (1·5 [1·0–2·2]), versus controls. Death was likewise associated with greater increases in systemic inflammatory markers, including granulocyte colony-stimulating factor (adjusted OR 2·8 [95% CI 1·3–6·1]), IL-12p40 (1·8 [1·0–3·4]), and IL-15 (2·0 [1·1–3·7]), versus controls. However, changes in CD4 cell count during ART, which were similar between controls and patients with tuberculosis-associated IRIS (p=0·45), were substantially lower in patients who died (p=0·006).

Interpretation Distinct immunological profiles before and after ART initiation characterise patients with advanced HIV and tuberculosis who have tuberculosis-associated IRIS and death. Interventions that decrease inflammation while promoting cellular immune recovery during ART should be considered in patients co-infected with HIV and tuberculosis.

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Introduction

In 2013, HIV-infected patients accounted for 13% of 9·0 million tuberculosis cases and 24% of 1·5 million tuberculosis-associated deaths worldwide.[1] Initiation of antiretroviral therapy (ART) during tuberculosis treatment can decrease mortality in HIV-infected individuals.[2] The need for ART is particularly urgent in patients with advanced HIV, because patients with the lowest CD4 T-cell counts have improved survival when ART is started within the first few weeks of anti-tubercular therapy.[3–5] Nonetheless, patients with advanced HIV and tuberculosis have a persistently high risk of death despite starting therapy for both diseases. For example, 120 (18%) of 661 patients and 55 (7%) of 783 patients in two trials of ART timing in patients with HIV and tuberculosis died within 48 weeks despite

starting both ART and anti-tubercular therapy (M Kendall, Harvard School of Public Health, personal communications).[3,5]

Early ART initiation after tuberculosis treatment is also associated with an increased risk of paradoxical tuberculosis-associated immune reconstitution inflammatory syndrome (IRIS),[3–7] characterised by pathological inflammation after ART initiation in patients concurrently treated for tuberculosis.[8–13] Although mortality from tuberculosis-associated IRIS is low,[14] morbidity can be substantial.[7,14,15] Studies of outcomes in patients with HIV and tuberculosis have frequently focused on tuberculosis-associated IRIS while excluding early deaths; as such, mechanisms of early mortality after ART in patients with HIV and tuberculosis are largely unknown. However, failure to recover CD4 cell counts despite virological suppression has been associated with early death after ART initiation in adults with advanced HIV and tuberculosis.[16] This finding is notable because the diminished cellular immune recovery reported in early mortality[16] contrasts with the more rapid cellular immune responses often noted in patients with tuberculosis-associated IRIS,[8,12,13,17] suggesting that the underlying immunopathogenesis for tuberculosis-associated IRIS and early mortality differ. Understanding of the risk factors for these outcomes is important, because interventions designed to prevent tuberculosis-associated IRIS could increase early mortality if the immunological processes, such as rapid immune recovery, driving that disorder are associated with survival. In view of these differences, we postulated that patients who go on to develop tuberculosis-associated IRIS would differ from those who die early after ART initiation in terms of immune activation and recovery before and early after ART initiation.

We investigated the relation of clinical variables and immunological characteristics before and 4 weeks after ART initiation to risk of non-traumatic death (early mortality) and paradoxical tuberculosis-associated IRIS in adults with advanced HIV and tuberculosis.

See Online for appendix

Methods

Study design

We did a prospective cohort study at 22 public clinics and Princess Marina Hospital (the main public hospital) in Gaborone, Botswana, with previously described methods.[16] Eligible patients (aged ≥21 years) were HIV-infected, ART-naive citizens of Botswana with pre-ART CD4 cell counts of 125 cells per μL or less, a new diagnosis of pulmonary tuberculosis, and plans to initiate ART within 2 months of starting standard anti-tuberculosis therapy.[18] Diagnosis of pulmonary tuberculosis required a sputum smear positive for acid-fast bacilli, a positive GeneXpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA), or meeting WHO criteria for smear-negative pulmonary tuberculosis.[18] We included patients with extrapulmonary involvement. We excluded patients who were pregnant, taking steroids or other immune modulators, or had drug-resistant tuberculosis or close contact with drug-resistant tuberculosis at screening. The study sample included all available participants with data and specimens for analysis. For the baseline analysis, we included all enrolled patients who started ART. For the analysis of early responses, we included patients who started ART and had baseline and week-4 plasma available for biomarker analysis.

The institutional review boards of the University of Pennsylvania, Botswana Ministry of Health, and the Princess Marina Hospital approved this study. All patients provided written informed consent.

Data collection

We obtained clinical data, including medical history and non-tuberculosis opportunistic illnesses, at baseline and every month thereafter.[16] We obtained baseline CD4 cell counts from medical records or measured counts independently at an accredited laboratory in Gaborone. Blood was collected at baseline and week 4 of ART for HIV viral load (NucliSENS Easy Q HIV-1, BioMérieux, France), plasma, and peripheral blood mononuclear cells. Patients were actively traced by phone or by home visit if they did not return for follow-up, and were deemed lost to follow-up if their vital status was unknown at the 6-month follow-up visit. We defined paradoxical tuberculosis-associated IRIS with a modified version of the International Network for Study of HIV-associated IRIS (INSHI) case definition, by which we adjudicated patients as probable if they met INSHI criteria, or as suspected if criteria for probable disease developed after 3 months of ART or patients developed otherwise unexplained new or worsening respiratory symptoms anytime during the 6 months of follow-up.[19] Furthermore, to assess the strength of our diagnoses of tuberculosis-associated IRIS, we retrospectively assessed all INSHI-defined cases with the AIDS Clinical Trials Group IRIS definition used by Grant and colleagues (appendix).[20] We used medical records or information from patients' families to assess possible cause of death.

Procedures

We used a 29-cytokine, chemokine, and growth-factor magnetic-bead Luminex panel (EMD Millipore, Billerica, MA, USA) to measure epidermal growth factor (EGF); vascular endothelial growth factor (VEGF); granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF); interferon (IFN)α and IFNγ; interleukin (IL)-1RA, IL-1α, IL-1β, IL-2, IL-3, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-15, and IL-17a; IFNγ-induced protein-10; monocyte chemoattractant protein (MCP)-1; macrophage inflammatory protein (MIP)-1α and MIP-1β; eotaxin; and tumour necrosis factor (TNF)α in plasma before, and 4 weeks after, ART initiation. Concentrations of IL-13 and IL-4, and TNFβ, were lower than the limit of detection

and therefore excluded. Undiluted plasma, which was previously stored at –80°C, was tested in duplicate, as per the manufacturer's protocol for the Bio-Plex2000 Luminex platform (Bio-Rad, Hercules, CA, USA). We used a 5-point log–log standard curve to analyse data with Bio-Plex Manager software (version 5.0).

We undertook enzyme-linked immunosorbent spot assays of freshly isolated peripheral blood mononuclear cells, as per the manufacturer's protocol[16] (BD Bioscience, San Jose, CA, USA). IFNγ spots in response to 5 μg/mL purified protein derivative (Statens Serum Institute, Copenhagen, Denmark), ionomycin (500 ng/mL) and phorbol 12-myristate 13-acetate (50 ng/mL; Sigma-Aldrich, St Louis, MO, USA), or RPMI 1640 medium containing 10% fetal bovine serum (Lonza, Basel, Switzerland), 1% L-glutamine, and penicillin–streptomycin (Gibco, Life Technologies, Grand Island NY, USA) as a negative control, were enumerated with an ImmunoSpot plate reader (Cellular Technology, Shaker Heights, OH, USA).

Statistical analysis

We compared patients who had tuberculosis-associated IRIS or early mortality with those who survived without an IRIS diagnosis (controls) in the 6 months after ART initiation. If a patient diagnosed with tuberculosis-associated IRIS died, they were counted as a case of IRIS in the primary analysis because that was the proximal event that might have led to death; such patients were later reclassified as deaths in sensitivity analyses. We summarised continuous variables with medians and IQRs or with means and SDs, dependent on distributions. In unadjusted analyses, we assessed continuous and categorical variables with the Wilcoxon rank-sum test or the χ^2 test. We assessed the distribution of the strength and direction of associations between specific biomarker concentrations and outcomes with odds ratios (ORs) and 95% CIs. Specifically, pre-ART plasma biomarker concentrations were $\log_{10}$ transformed and analysed with logistic regression to produce ORs relating values increased by 1 $\log_{10}$ with each outcome. For early response analyses, in which both increases and decreases were possible, we calculated ORs with quartiles of change. Additionally, we present raw biomarker values to aid interpretation. In primary analyses, p values are unadjusted for multiple comparisons, but univariate biomarker comparisons include p values adjusted with the Benjamini-Hochberg method.[21] Tests were two-sided.

In adjusted analyses, we regarded clinical variables associated with either outcome at p values of 0·20 or less to be potential confounders. Factors that changed the unadjusted association by 10% or more were regarded as actual confounders and included in multivariate logistic regression models. In this setting, use of nevirapine-based ART was reserved mainly for women. In view of this colinearity, and that our cohort included more women than nevirapine users, when both sex and nevirapine were associated with outcome, we included only sex in the models. We did unadjusted sensitivity analyses after inclusion of all participants without tuberculosis-associated IRIS (including those who died) as controls in the analysis of risk factors for IRIS, and

	Control (n=120)	TB-associated IRIS (n=33)*	p value†	Deaths (n=17)	p value‡
Sex					
Male	71 (59%)	19 (58%)	0·87	6 (35%)	0·06
Female	49 (41%)	14 (42%)	0·87	11 (65)	0·06
Age (years)	36 (8)	37 (7)	0·98	39 (10)	0·21
Time between anti-tubercular therapy and ART initiation (days)	27 (18–47)	30 (21–44)	0·54	29 (22–49)	0·59
Nevirapine-based ART	12 (10%)	7 (21%)	0·08	5 (29%)	0·02
Presence of extrapulmonary TB at baseline	6 (5%)	2 (6%)	0·81	1 (6%)	0·88
Presence of non-TB opportunistic infections at baseline	10 (8%)	5 (15%)	0·24	5 (29%)	0·01
CD4 T-cell count (cells per μL)	64 (32–94)	61 (36–86)	0·79	34 (14–66)	0·02
HIV viral load ($\log_{10}$ copies per mL)§	5·5 (4·9–6·0; 116)	5·5 (5·0–5·9; 32)	0·77	5·7 (5·5–5·9; 17)	0·24
Body-mass index (kg/m^2)					
Median (IQR; N)§	19·0 (16·4–21·8; 119)	20·4 (18·3–22·9; 33)	0·08	19·3 (17·7–24·5; 15)	0·66
<19	61 (51%)	10 (30%)	0·03	7 (47%)	0·74
Smear-negative for acid-fast bacilli	30 (25%)	6 (18%)	0·41	6 (35%)	0·37
Purified protein derivative response (SFU per 10^6 PBMC)§	266 (12–860; 98)	318 (64–1508; 20)	0·19	68 (35–370; 12)	0·54

Data are n (%), mean (SD), median (IQR), or median (IQR; N), unless otherwise indicated. TB=tuberculosis. IRIS=immune reconstitution inflammatory syndrome. ART=antiretroviral therapy. SFU=spot-forming units. PBMC=peripheral blood mononuclear cells. *Includes one patient who died and had TB-associated IRIS. †For controls (non-TB-associated IRIS survivors) versus patients with TB-associated IRIS. ‡For control patients versus deaths. §Numbers of patients are provided alongside the index to indicate missing data.

Table 1: **Baseline characteristics**

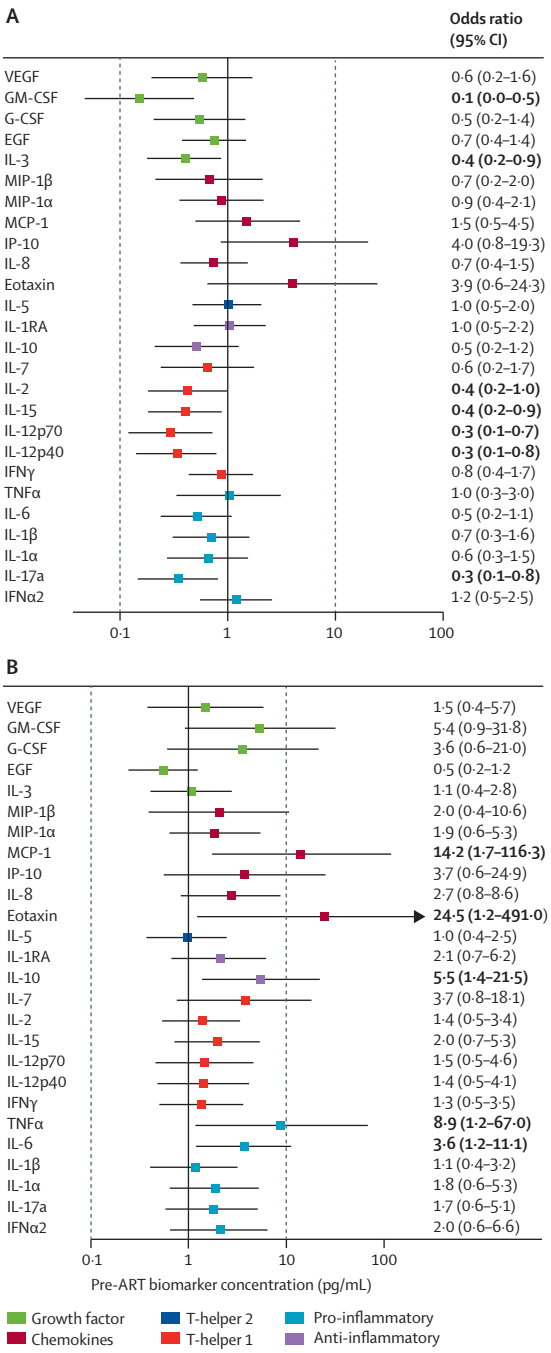

***Figure 1*: Unadjusted ORs relating 1 log_{10} increases in baseline biomarker concentration to risk of outcomes after initiation of antiretroviral therapy in patients with advanced HIV and tuberculosis who had tuberculosis-associated immune reconstitution inflammatory syndrome (A) or early death (B), compared with controls**

Data are OR (95% CI). Data in bold show markers that were significantly associated with outcome. OR=odds ratio. VEGF=vascular endothelial growth factor. GM-CSF=granulocyte-macrophage colony-stimulating factor. EGF=epidermal growth factor. MIP=macrophage inflammatory protein. MCP=monocyte chemoattractant protein. IP= IFNγ induced protein. IL=interleukin. IFN=interferon. TNF=tumour necrosis factor. G-CSF=granulocyte colony-stimulating factor.

after inclusion of all those who survived (including those with IRIS) as controls in the analysis of risk factors for death. Because biomarker concentrations during ART were obtained at the week-4 visit, we assessed IL-6 values in patients with early (diagnosed at week 4) versus late (diagnosed after week 4) tuberculosis-associated IRIS to establish whether pooling of these patients had affected the results. We did analyses with Stata (version 11.0).

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report manuscript. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between Nov 12, 2009, and July 3, 2013, we enrolled 201 participants. Before ART initiation, 31 (15%) patients left the study: three withdrew, ten transferred out, five died, and 13 were discontinued from the study because of failure to follow study procedures, leaving 170 (85%) patients for analysis. The median CD4 cell count of included participants was 60 cells per μL (IQR 31–90), nearly three-quarters of patients had smears positive for acid-fast bacilli or GeneXpert assays positive for pulmonary tuberculosis, and the median time to ART initiation after tuberculosis

	TB-associated IRIS*	Death†
GM-CSF	0·15 (0·05–0·48)‡	3·0 (0·49–18·9)
IL-2	0·41 (0·18–0·96)‡	1·4 (0·54–3·4)
IL-3	0·39 (0·17–0·86)‡	1·1 (0·41–2·8)
IL-12p40	0·32 (0·14–0·75)‡	1·4 (0·48–4·2)
IL-12p70	0·28 (0·12–0·69)‡	1·4 (0·46–4·6)
IL-15	0·39 (0·18–0·86)‡	2·0 (0·72–5·3)
IL-17a	0·33 (0·14–0·78)‡	1·7 (0·58–5·1)
IL-6	0·40 (0·18–0·89)‡	2·8 (0·93–8·4)
IL-10	0·5 (0·21–1·2)	3·5 (0·89–13·5)
MCP-1	1·5 (0·49–4·5)	9·0 (1·0–80·0)‡
TNFα	0·99 (0·32–3·0)	7·8 (1·1–55·2)‡
Eotaxin	5·4 (0·78–36·5)	3·6 (0·58–22·5)
G-CSF	0·53 (0·20–1·4)	3·6 (0·61–21·0)
IP-10	4·0 (0·84–19·3)	2·9 (0·50–16·8)

Data are adjusted odds ratio (95% CI). Log_{10} transformed baseline values of biomarkers that were associated with TB-associated IRIS or death at p<0·10 in unadjusted analyses (see table 3) were used to establish association with paradoxical TB-associated IRIS and death in a logistic regression model. TB=tuberculosis. IRIS=immune reconstitution inflammatory syndrome. GM-CSF=granulocyte-macrophage colony-stimulating factor. IL=interleukin. MCP=monocyte chemoattractant protein. TNF=tumour necrosis factor. G-CSF=granulocyte colony-stimulating factor. IP=IFNγ induced protein. *TB-associated IRIS associations are adjusted for body-mass index and nevirapine use. †Models included CD4 cell count before antiretroviral therapy, female sex, and presence of baseline opportunistic infections. ‡Independent association between biomarker and outcome.

***Table 2*: Relation of baseline biomarker concentrations to TB-associated IRIS and early mortality in patients with advanced HIV and tuberculosis starting antiretroviral therapy**

diagnosis was roughly 28 days (IQR 20–47; appendix). 33 (19%) of 170 patients had paradoxical tuberculosis-associated IRIS after ART initiation; eight (24%) of these cases were probable and 25 (76%) cases were suspected (appendix). All probable and suspected cases also met the ACTG case definition.[20] 18 (11%) of 170 patients died, leaving 120 controls. The median time to tuberculosis-associated IRIS onset was about 28 days (IQR 28–56) and to death was about 81 days (43–93). One (1%) patient was lost to follow-up at week 16. One (6%) patient who died had dyspnoea and paradoxical tuberculosis-associated IRIS 4 weeks after ART initiation, but the IRIS symptoms resolved before death, which happened 2 weeks later. This patient was categorised as an IRIS case and not a death in the primary analysis. The appendix provides clinical details of cases of tuberculosis-associated IRIS and deaths. One (3%) patient with tuberculosis-associated IRIS and two (2%) control patients did not have plasma available for analysis, leaving 32 patients with tuberculosis-associated IRIS and 118 controls for baseline analyses.

At baseline, patients with tuberculosis-associated IRIS were similar to controls in terms of clinical characteristics, including CD4 cell count and time to ART initiation; however, they had a significantly higher body-mass index (BMI; table 1).

Figure 1 shows the unadjusted ORs relating tuberculosis-associated IRIS to $\log_{10}$ increases in pre-ART biomarker concentrations compared with controls. Of the 26 biomarkers assessed, 19 (73%) had point estimates for the unadjusted ORs that were lower than 1·0, and seven (27%) also had upper limits of the 95% CI that did not cross 1·0 (figure 1), suggesting that lower concentrations of these seven biomarkers are associated with an increased risk of tuberculosis-associated IRIS. These biomarkers included plasma concentrations of factors secreted mainly by monocytes or macrophages and endothelial cells such as GM-CSF; soluble markers that are produced by or promote T-helper-1 responses, including IL-2, IL-15, IL-12p40, and IL-12p70; growth factors such as IL-3; and the pro-inflammatory cytokine

	Control (n=118)	TB-associated IRIS (n=32)*	p value†	p_{corr}†	Death (n=17)	p value‡	p_{corr}‡
EGF	146·9 (55·6–235·3)	92·7 (46·3–201·5)	0·17	0·28	78·6 (38·7–156·5)	0·10	0·36
VEGF	123 (76·1–181·7)	106·2 (65·4–160·2)	0·21	0·30	173·3 (97·4– 219·6)	0·28	0·50
G-CSF	124·1 (86·6–174·1)	88·4 (65·3–132·8)	0·01§	0·04§	138·4 (97·7–243·1)	0·29	0·50
GM-CSF	34·5 (20·0–51·4)	18·3 (12·2–31·9)	0·0008§	0·02§	46·9 (35·5–66·1)	0·03§	0·20
IL-1RA	78·7 (39·8–153·7)	90·4 (34·2–200·0)	0·74	0·74	115·5 (50·8–160·2)	0·25	0·50
IL-1α	20·1 (9·4–57·3)	9·4 (9·4–44·7)	0·20	0·30	44·2 (9·4–109·5)	0·32	0·52
IL-1β	1·2 (0·8–3·9)	0·8 (0·8–3·2)	0·16	0·28	1·6 (0·8–1·9)	0·77	0·80
IL-2	2·4 (1·0–7·3)	1·0 (1·0–3·0)	0·02§	0·07	3·7 (1·0–11·4)	0·40	0·58
IL-3	3·7 (1·3–9·1)	1·5 (0·7–5·9)	0·007§	0·04§	2·8 (1·5–13·2)	0·69	0·75
IL-5	2·8 (1·5–6·5)	3·0 (1·1–4·8)	0·62	0·70	3·1 (0·5–7·7)	0·97	0·97
IL-6	14·7 (7·5–28·1)	10·3 (5·0–21·3)	0·04§	0·12	19·8 (11·8–33·2)	0·11	0·36
IL-7	21·0 (14·1–29·4)	15·8 (12·2–22·7)	0·14	0·28	23·0 (19·0–47·5)	0·15	0·36
IL-8	16·8 (9·6–28·6)	14·6 (6·8–20·8)	0·15	0·28	22·2 (11·9–38·8)	0·14	0·36
IL-10	18·8 (11·0–32·5)	14·5 (8·3–21·3)	0·07	0·17	39·8 (13·1–98·2)	0·03§	0·20
IL-12p40	15·0 (7·4–36·0)	7·4 (7·4–12·0)	0·002§	0·03§	20·7 (7·4–27·9)	0·65	0·74
IL-12p70	9·8 (5·8–18·7)	6·3 (3·4–12·0)	0·01§	0·04§	11·4 (8·1–19·0)	0·49	0·66
IL-15	4·3 (1·2–10·0)	1·7 (1·2–4·8)	0·02§	0·07	5·8 (2·5–13·3)	0·27	0·50
IL-17a	3·3 (1·6–5·9)	1·4 (0·7–3·8)	0·005§	0·04§	4·2 (2·4–6·2)	0·35	0·54
IFNα	53·3 (29·8–98·6)	71·8 (31·1–110·0)	0·36	0·49	57·4 (35·4–91·4)	0·55	0·68
IFNγ	18·8 (9·2–35·9)	16·3 (5·0–34·9)	0·56	0·66	20·2 (9·3–43·4)	0·64	0·74
IP-10	3390 (2298–4267)	4185 (2827–5931)	0·06	0·16	3935 (2964–5203)	0·12	0·36
MCP-1	548·7 (388·4–779·4)	649·3 (378·2–841·6)	0·71	0·74	832·2 (652·3–1331·2)	0·003§	0·08
MIP-1α	13·3 (5·7–22·9)	10·4 (6·8–15·4)	0·41	0·53	19·7 (8·2–25·0)	0·14	0·36
MIP-1β	66·9 (47·1–112·2)	63·7 (39·1–106·9)	0·45	0·56	99·2 (49·3–114·3)	0·51	0·66
Eotaxin	146·5 (119·6–191·8)	180·0 (132·9–220·0)	0·08	0·17	189·8 (140·6–258·7)	0·06	0·31
TNFα	41·4 (28·9–63·0)	38·2 (27·0–60·5)	0·65	0·71	56·6 (44·9–74·8)	0·02§	0·20

Data are median pg/mL (IQR). TB=tuberculosis. IRIS=immune reconstitution inflammatory syndrome. p_{corr}=Benjamini-Hochberg-corrected p value. EGF=epidermal growth factor. VEGF=vascular endothelial growth factor. G-CSF=granulocyte colony-stimulating factor. GM-CSF=granulocyte-macrophage colony-stimulating factor. IL=interleukin. IFN=interferon. IP=IFNγ induced protein. MCP=monocyte chemoattractant protein. MIP=macrophage inflammatory protein. TNF=tumour necrosis factor. *Includes one patient who had TB-associated IRIS and died. †p and p_{corr} values comparing controls with patients with TB-associated IRIS. ‡p and p_{corr} values comparing controls with deaths. §Significant associations.

***Table* 3: Baseline biomarker concentrations associated with TB-associated IRIS and early mortality in patients with advanced HIV and TB starting antiretroviral therapy**

IL-17a (figure 1). Associations between these seven biomarkers and tuberculosis-associated IRIS were mostly unchanged in models adjusted for baseline BMI and nevirapine (table 2). Pre-ART concentrations of IL-6 were not associated with tuberculosis-associated IRIS in univariate analysis (figure 1), but became significantly associated in adjusted analysis (table 2). After adjustment for multiple comparisons, circulating concentrations of GM-CSF, IL-3, IL-12p40, IL-12p70, and IL-17a were all significantly reduced in patients with tuberculosis-associated IRIS (table 3). Secondary analysis comparing patients with tuberculosis-associated IRIS to all those without IRIS produced similar results (appendix). Pre-ART biomarker concentrations were similar in early and late tuberculosis-associated IRIS cases, and pooling of these patients did not change these associations (data not shown).

Before ART, patients who died were more likely than controls to be female, to initiate nevirapine-based ART, to have lower CD4 cell counts, and to have a non-tuberculosis opportunistic infection. The two groups were similar in terms of time to ART initiation (table 1).

Figure 1 also shows the unadjusted ORs relating death to $\log_{10}$ increases in pre-ART biomarker concentrations compared with controls. Of the 26 biomarkers assessed, 25 (96%) had point estimates for the unadjusted ORs that were higher than 1·0, and five (19%) also had lower limits of the 95% CI that did not cross 1·0, suggesting that higher pre-ART concentrations of these five biomarkers are associated with an increased risk of death (figure 1). These biomarkers included plasma concentrations of IL-10, MCP-1, TNFα, IL-6, and eotaxin (figure 1). Increased pre-ART concentrations of MCP-1 and TNFα were significantly associated with death after assessment of female sex, non-tuberculosis opportunistic infections, and CD4 cell count as possible confounders (table 2). Adjustment for multiple comparisons resulted in non-significant associations between death and MCP-1 and TNFα (table 3). Secondary analyses comparing deaths to survivors gave similar results (appendix).

Between baseline and week 4 of ART, seven (4%) of 170 patients died and four (2%) patients did not have plasma collected at week 4 for biomarker analysis. Pre-ART clinical characteristics of the 159 remaining patients were similar to those of the 170 patients included overall (data not shown).

Compared with controls, patients with tuberculosis-associated IRIS had similar increases in CD4 cell count and purified protein derivative-specific immune responses, whereas those who died had minimum immune recovery despite virological control during ART (appendix). Each quartile increase in CD4 cell count was associated with an approximate 60% reduction in the odds for death (table 4). Tuberculosis-associated IRIS was associated with significantly greater increases in G-CSF, IL-6, IL-8, IL-17a, and TNFα compared with controls at week 4 of ART (figure 2, appendix). Although GM-CSF decreased both in patients with tuberculosis-associated IRIS and in controls, the decrease in controls was greater (figure 2, appendix). P values for these biomarkers were somewhat increased after adjustment for multiple comparisons (appendix). In adjusted analyses, increases in IL-6, TNFα, IL-8, and G-CSF remained independently associated with tuberculosis-associated IRIS (table 4). Additionally, increases in concentrations of IL-6 in the 17 (53%) of 32 patients with early tuberculosis-associated IRIS (median 14·5 pg/mL [IQR –0·40 to 25·1]) were greater than the increases in the 15 (47%) patients with late IRIS (–2·5 pg/mL [–5·0 to 5·6]; p=0·01). The increase in IL-6 concentration, from baseline to week 4 after ART initiation, in patients with early IRIS was also significantly greater than in controls (median –3·0 pg/mL [IQR –10·8 to 8·3]; p=0·0006, Benjamini-Hochberg corrected p=0·02; adjusted OR 2·7 [95% CI 1·5–4·9]).

Biomarkers also increased relative to controls in patients who died (figure 2, appendix). P values for associations between increases in biomarkers and death and tuberculosis-associated IRIS increased slightly after adjustment for multiple comparisons (appendix). In a logistic regression model, increases from baseline in G-CSF, IL-3, IL-12p40, IL-15, and IL-1RA were significantly and independently associated with death (table 4).

	TB-associated IRIS*	Death†
IL-6	1·7 (1·2–2·5)‡	1·8 (0·94–3·3)
TNFα	1·5 (1·0–2·2)‡	1·2 (0·67–2·1)
IL-8	1·4 (1·0–2·0)‡	1·7 (0·91–3·1)
G-CSF	1·5 (1·0–2·1)‡	2·8 (1·3–6·1)‡
IL-3	1·1 (0·80–1·6)	2·3 (1·1–4·6)‡
IL-12p40	1·2 (0·89–1·8)	1·8 (1·0–3·4)‡
IL-15	1·3 (0·92–1·9)	2·0 (1·1–3·7)‡
IL-1RA	1·1 (0·76–1·6)	2·2 (1·1–4·4)‡
GM-CSF	1·3 (0·93–1·9)	1·3 (0·71–2·2)
IL-10	1·3 (0·93 1·9)	1·4 (0·77–2·4)
IFNγ	1·4 (0·98–2·0)	1·7 (0·91–3·2)
IL-5	1·0 (0·71–1·4)	1·7 (0·91–3·2)
IL-12p70	1·2 (0·81–1·6)	1·8 (0·93–3·3)
CD4	1·2 (0·85–1·8)	0·38 (0·17–0·85)‡
PPD	1·2 (0·78–1·9)	0·75 (0·37–1·5)

Data show adjusted OR (95% CI). ORs and 95% CIs indicated are per quartile change in each biomarker. Changes in biomarker concentrations from baseline to week 4 after ART that were associated with TB-associated IRIS or death at p<0·10 in unadjusted analyses (appendix) were stratified into quartiles and assessed for association with either outcome in logistic regression models. TB=tuberculosis. IRIS=immune reconstitution inflammatory syndrome. IL=interleukin. TNF=tumour necrosis factor. G-CSF=granulocyte colony-stimulating factor. GM-CSF=granulocyte-macrophage colony-stimulating factor. IFN=interferon. PPD=purified protein derivative. *TB-associated IRIS associations are adjusted for body-mass index, nevirapine use, and pre-ART concentrations of respective biomarker. †Model included pre-ART CD4 cell count, female sex, presence of baseline opportunistic infections, and pre-ART concentrations of respective biomarker. ‡Independent association between biomarker and outcome.

***Table 4*: Association between changes in biomarker concentrations from baseline to week 4 after ART initiation and TB-associated IRIS and early mortality in patients with advanced HIV and TB**

Sensitivity analyses reclassifying the patient who died after diagnosis of IRIS did not meaningfully change the results (appendix). Reclassification of the patient lost to follow-up as having died also had minimum effect (data not shown).

Discussion

Our findings show that individuals who had early mortality or paradoxical tuberculosis-associated IRIS had substantially different pre-ART biomarker concentrations compared with those with uncomplicated immune recovery. Furthermore, patients with IRIS and early mortality had rapid increases in immune activation and inflammation early during ART, but differed substantially in terms of the magnitude of early cellular immune recovery after ART initiation.

Although several studies[8-12,22-24] have assessed biomarker concentrations in patients with tuberculosis-associated IRIS, to our knowledge, no previous reports have compared cellular immune responses and biomarker profiles between patients who died or had paradoxical tuberculosis-associated IRIS and internal controls. As such, a major strength of our study is a novel design that places risk factors for tuberculosis-associated IRIS in the context of risk factors for death. Other strengths include a large number of paradoxical tuberculosis-associated IRIS cases and non-IRIS controls relative to published immunological reports,[8–11,20] comprehensive assessment of relevant biomarkers before and soon after ART initiation, and the study setting, which has a high burden of HIV and tuberculosis.

Limitations include possible misclassification of tuberculosis-associated IRIS, the few deaths and scarcity of data for precise causes of death, and assessment of circulating biomarkers rather than those at the site of infection. Major bias from IRIS misclassification is unlikely because our event adjudication was masked to biomarker data and was consistent with definitions used by other groups.[7,20] Since many IRIS cases were mild, some incident respiratory symptoms could have been due to the heterogeneous course of tuberculosis rather than to overt inflammation. However, mixing of true IRIS cases with these patients would probably lead to bias towards the null by comparisons with controls, such that we might have underestimated true differences.

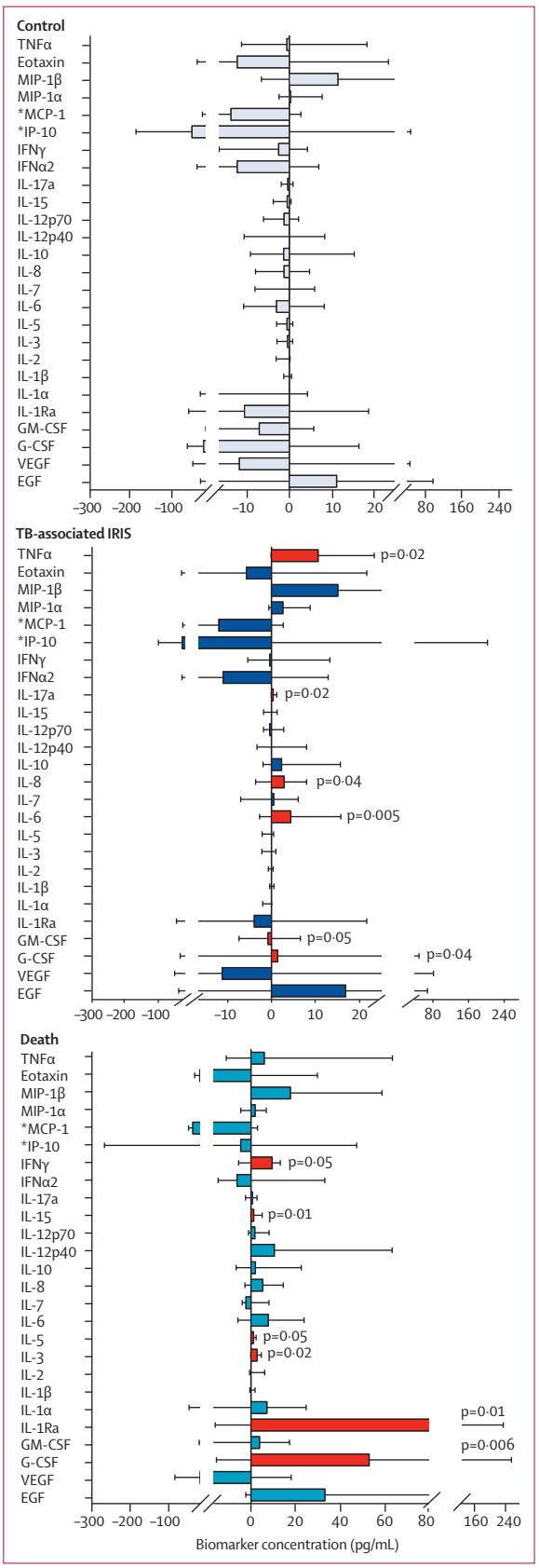

***Figure 2*: Early changes in biomarker concentration after initiation of antiretroviral therapy in controls, patients with TB-associated IRIS, and patients who died**

Error bars show median (IQR) change in plasma biomarkers from baseline to week 4 after ART initiation (appendix). Red bars show biomarkers that were significantly associated with TB-associated IRIS or death compared with controls by Wilcoxon rank-sum tests. TB=tuberculosis. IRIS=immune reconstitution inflammatory syndrome. TNF=tumour necrosis factor. MIP=macrophage inflammatory protein. MCP=monocyte chemoattractant protein. IP=IFNγ induced protein. IFN=interferon. IL=interleukin. GM-CSF=granulocyte-macrophage colony-stimulating factor. G-CSF=granulocyte colony-stimulating factor. VEGF=vascular endothelial growth factor. EGF=epidermal growth factor. *We transformed MCP-1 and IP-10 values by a factor of 1/10 to enable plotting with other biomarkers.

Furthermore, we did not investigate in detail whether patients had IRIS associated with non-tuberculosis opportunistic infections. Study of circulating biomarkers is unlikely to have substantially biased our results, because responses at the site of infection are generally positively correlated with those taking place in the periphery.[25] Additionally, although the incidences of early mortality and paradoxical tuberculosis-associated IRIS in our study suggest generalisability to similar settings,[14,26] the findings might be less generalisable to patients with CNS infections, in whom IRIS is more often fatal.[27] Multiple testing is another limitation, and further studies in other settings are needed. Nonetheless, a focus on ORs enabled an assessment of the strength and direction of the associations shown in figure 1, which strongly suggest systematic differences between these patient groups.

Our analysis of early and late tuberculosis-associated IRIS cases, and strong associations between cytokine increases at the time of IRIS onset reported by others,[8,9,11,12,22,23] suggest that we might have underestimated changes during ART by obtaining samples at week 4 and not at the time of event. These data show that, in this superficially homogeneous group, patients who have early mortality have profound immunological dysfunction at the time of ART initiation. Other studies have shown that high pre-ART concentrations of inflammatory cytokine are associated with mortality in patients with HIV,[28] and concentrations of TNFα and other cytokines generally decrease during tuberculosis treatment.[29] Although we do not have data for biomarker concentrations at the time of tuberculosis diagnosis, findings from autopsy studies showing overwhelming *Mycobacterium tuberculosis* infection in advanced HIV patients being treated for tuberculosis,[30] and studies linking increases in MCP-1 with disseminated tuberculosis,[31] suggest that tuberculosis might have been more advanced at presentation in patients who died than in survivors. By contrast, in adjusted analyses in our study concentrations of several circulating inflammatory markers, including IL-6 and the T-helper 1, 2, and 17 cytokines IL-2, IL-3, IL-12, IL15, and IL-17a, were lower in patients with tuberculosis-associated IRIS than in non-IRIS controls. These findings are consistent with a study by Goovaerts and colleagues,[22] who reported that lower pre-ART concentrations of IL-6 and G-CSF were associated with an increased risk of paradoxical tuberculosis-associated IRIS.

Although our data superficially conflict with results linking raised pre-ART pro-inflammatory cytokine concentrations to tuberculosis-associated IRIS in a report by Narendran and colleagues,[10] this finding might be related to a shorter time to ART initiation and more advanced HIV-disease stage in the IRIS cases compared with controls in that study.Andrade and colleagues[32] showed that circulating biomarkers and *M tuberculosis* sputum load decrease with time spent on anti-tubercular therapy before ART initiation. Because time to ART initiation and antigen burden are also associated with risk of tuberculosis-associated IRIS,[23,32,33] findings showing higher pre-ART biomarker concentrations in patients with tuberculosis-associated IRIS who started ART weeks earlier than those who did not develop the syndrome[10,23] might not inform this association when examined in populations in whom time to ART is relatively uniform.

Panel: Research in context

Systematic review

We searched PubMed for studies published before Dec 19, 2014, with no restrictions, for studies describing tuberculosis-associated immune reconstitution inflammatory syndrome (IRIS) or early mortality in patients with HIV and tuberculosis starting antiretroviral therapy (ART) with the search terms "HIV" AND "Tuberculosis" AND "immune reconstitution inflammatory syndrome" AND "ART", and then by addition of the phrase AND "death". Our search identified several papers.[2–14,16,20,22–24,28,32,37] Relevant studies,[8–13,16,17,22–24,28,32] investigated circulating biomarkers or cellular immune responses before and after ART initiation and assessed the association with tuberculosis-associated IRIS or death. We identified no study that compared immunological profiles of patients with paradoxical tuberculosis-associated IRIS or death to non-IRIS survivors within a single cohort at risk for both outcomes. Furthermore, although many studies, including key studies,[8,12,13,17,24] assessed tuberculosis (purified protein derivative)-specific immune response and its association with tuberculosis-associated IRIS, no studies other than our previously published report[16] assessed immune response (CD4 cell count or purified protein derivative-specific responses) during ART and its association with early mortality in patients co-infected with HIV and tuberculosis.

Interpretation

To date, research attention has been mainly focused on the immunological correlates of paradoxical tuberculosis-associated IRIS in patients with advanced HIV and tuberculosis.[8–13,22,23] Early mortality is an adverse outcome for which these patients are at similar risk.[2,3,5] However, previous studies have excluded patients who die soon after ART initiaiton. Thus, in this prospective cohort study, we compared immunological risk factors before and early after ART initiation between patients who had either tuberculosis-associated IRIS or early mortality and surviving, non-tuberculosis-associated IRIS controls. Although patients with tuberculosis-associated IRIS had significantly lower pre-ART concentrations of several inflammatory biomarkers than controls, in line with one other study,[22] pre-ART concentrations of the biomarkers in patients who died were remarkably higher, similar to findings from Boulware and colleagues' study.[28] Soon after ART initiation, inflammatory markers of both the tuberculosis-associated IRIS and early mortality groups increased rapidly, but the two groups were distinguished by sharply divergent recovery of the adaptive immune system.

This study is the first to show that patients with tuberculosis-associated IRIS have linked recovery of markers of innate and adaptive immune function, whereas those who died have increases in inflammation without recovery of cellular immune responses that are known to be crucial to the control of *Mycobacterium tuberculosis*. Findings from this study have several implications for future research and clinical care. First, immunomodulatory therapies that inhibit inflammation without suppressing adaptive immune recovery should be prioritised as interventions in patients co-infected with HIV and tuberculosis. Second, future studies should assess the ability of immune markers to risk-stratify patients for early outcomes during ART. If accurate, such strategies could help direct different prevention interventions for tuberculosis-associated IRIS and early mortality to individuals at greatest risk. Third, triaging of care of patients with advanced HIV and tuberculosis with assessment of CD4 cell count in the initial weeks of ART initiation could have benefits in resource-limited settings and needs further assessment.

The findings of reduced pre-ART biomarkers in the patients with tuberculosis-associated IRIS in this study and in the study by Goovaerts and colleagues[22] are notable in this regard, because time to ART initiation in both cohorts was very similar between patients with tuberculosis-associated IRIS and non-IRIS controls. Inclusion of patients with unmasking IRIS due to an undiagnosed, untreated opportunistic infection at the time of ART initiation could also explain the increases in pre-ART cytokine concentrations in patients with IRIS in other reports.[20,28]

Cytokines such as IL-6, GM-CSF, IL-15, and IL-12, mainly produced by monocytes or macrophages and dendritic cells, mediate immunological control of *M tuberculosis* by stimulating T-cell and natural-killer-cell responses that activate macrophages.[34–36] Low pre-ART concentrations of these cytokines in patients with tuberculosis-associated IRIS patients might therefore be indicative of abnormal innate immune responses, which could lead to impaired pathogen clearance and high antigen loads at ART initiation, as postulated.[32,37] However, cytokine concentrations in patients with tuberculosis-associated IRIS in this study, in which timing of ART initiation was similar across groups, could also have been reduced because of reduced severity disease at the time of ART initiation. Irrespective of the mechanism, these data, and data for the generally favorable prognosis of pulmonary tuberculosis-associated IRIS reported by others,[6,14,23,38] suggest that an innate immune defect, if present, does not result in abnormally low protective cellular immune responses before ART or decreased patient survival thereafter. In view of these data, we would argue that the innate and cellular immune systems might actually be particularly functional in these patients, when all those who initiate ART are considered.

The divergent immunological profiles of patients with early mortality and tuberculosis-associated IRIS are further emphasised by the analysis of early changes after ART initiation. In adjusted analyses, we, and other investigators,[9–11,22,23] have noted a vigorous upsurge in pro-inflammatory cytokines, such as IL-6 and TNFα, in patients with tuberculosis-associated IRIS. The finding that these patients developed incident symptoms despite starting out with low IL-6 concentrations suggests that the clinical worsening after ART initiation characteristic of tuberculosis-associated IRIS is more reflective of the change in, than the absolute concentrations of, circulating cytokines. Patients who died also had increases in IL-6 but, by striking contrast, these increases were not accompanied by early recovery of CD4 cells.

Taken together, these findings have several implications for future research and clinical care. Several randomised trials are presently assessing or have recently investigated immunomodulatory therapies at the time of ART initiation in patients with advanced HIV and tuberculosis as a way to prevent tuberculosis-associated IRIS. Our data suggest that immunomodulatory therapies that inhibit inflammation without suppressing adaptive immune recovery should be prioritised. Indeed, the increases in inflammation noted in both patients who died and those who had tuberculosis-associated IRIS raise the possibility that, if inflammation hinders immune recovery, as one large study from Uganda has suggested,[39] selective anti-inflammatory therapies could decrease risk of both outcomes, moving patients towards the middle of the immune recovery range. Although corticosteroids might decrease inflammation without affecting pathogen-specific immune function in patients with HIV and tuberculosis-associated IRIS,[24] they might also impair T-cell recovery and proliferation, in addition to inducing T-cell death.[38,40] Another implication is that measurement of immunological profiles before and early after ART initiation could be useful as a means of stratification of risk of severe tuberculosis-associated IRIS and death and should be further investigated (panel).

In conclusion, our findings urge caution in treatment of patients with advanced HIV and tuberculosis as a homogeneous group. The somewhat inverse association between pre-ART cytokine concentrations in patients with tuberculosis-associated IRIS and early mortality, and the contrasting degrees of CD4 cell recovery, suggests that interventions that seek to prevent tuberculosis-associated IRIS could inadvertently increase risk of death. Further study is needed to identify ways to predict and improve outcomes in these individuals.

Contributors

GPB, DW, SR, and RG conceived and designed the study and experiments. SR and KN undertook the experiments. SR, GPB, and SLB analysed the data. GPB, DW, and SLB contributed reagents, materials, and methods of analysis. SR and GPB wrote the first draft of the manuscript. GPB, SR, DW, RG, RRM, SLB, NT, RL, and APS contributed to the writing of the manuscript. GPB, SR, DW, RG, RRM, SLB, NT, RL, APS, and KN agree with manuscript results and conclusions. NT and RL enrolled patients. NT and APS aided undertaking of the study in Botswana.

Declaration of interests

We declare no competing interests.

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