Effect of the relationship between anaemia and systemic inflammation on the risk of incident tuberculosis and death in people with advanced HIV: a sub-analysis of the REMEMBER trial

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eClinicalMedicine 2023;60: 102030
Published Online xxx
https://doi.org/10.1016/j.eclinm.2023.102030

Summary

Background Tuberculosis (TB) is an infectious morbidity that commonly occurs in people living with HIV (PWH) and increases the progression of HIV disease, as well as the risk of death. Simple markers of progression are much needed to identify those at highest risk for poor outcome. This study aimed to assess how baseline severity of anaemia and associated inflammatory profiles impact death and the incidence of TB in a cohort of PWH who received TB preventive therapy (TPT).

Methods This study is a secondary posthoc analysis of the AIDS Clinical Trials Group A5274 REMEMBER clinical trial (NCT0138008), an open-label randomised clinical trial of antiretroviral-naïve PWH with CD4 <50 cells/μL, performed from October 31, 2011 to June 9, 2014, from 18 outpatient research clinics in 10 low- and middle-income countries (Malawi, South Africa, Haiti, Kenya, Zambia, India, Brazil, Zimbabwe, Peru, and Uganda) who initiated antiretroviral therapy and either isoniazid TPT or 4-drug empiric TB therapy. Plasma concentrations of several soluble inflammatory biomarkers were measured prior to the commencement of antiretroviral and anti-TB therapies, and participants were followed up for at least 48 weeks. Incident TB or death during this period were primary outcomes. We performed multidimensional analyses, logistic regression

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analyses, survival curves, and Bayesian network analyses to delineate associations between anaemia, laboratory parameters, and clinical outcomes.

Findings Of all 269 participants, 76.2% (n = 205) were anaemic, and 31.2% (n = 84) had severe anaemia. PWH with moderate/severe anaemia exhibited a pronounced systemic pro-inflammatory profile compared to those with mild or without anaemia, hallmarked by a substantial increase in IL-6 plasma concentrations. Moderate/severe anaemia was also associated with incident TB incidence (aOR: 3.59, 95% CI: 1.32–9.76, p = 0.012) and death (aOR: 3.63, 95% CI: 1.07–12.33, p = 0.039).

Interpretation Our findings suggest that PWH with moderate/severe anaemia display a distinct pro-inflammatory profile. The presence of moderate/severe anaemia pre-ART was independently associated with the development of TB and death. PWH with anaemia should be monitored closely to minimise the occurrence of unfavourable outcomes.

Funding National Institutes of Health.

Keywords: Tuberculosis; Haemoglobin; Systemic inflammation; HIV; Incident TB; Anaemia; Inflammation; Death

Research in context

Evidence before this study

We searched PubMed and Embase and databases on January 15, 2022, for articles published in English language using the terms: "anemia", "tuberculosis", "outcomes", "death", and "hiv" and retrieved 41 studies. Anaemia is a common complication in people living with HIV (PHW) and has been described as a risk factor for opportunistic infections, such as tuberculosis (TB), or for treatment outcomes, such as death. However, is still unknown if anaemia in this setting reflects a unique profile of immunopathological processes and the impact of this inflammatory disturbance on outcomes.

Added value of this study

Previous studies have linked low haemoglobin levels to unfavourable outcomes in PWH. Nevertheless, it is unclear whether the severity of anaemia contributes to incident TB and/or mortality. Our findings demonstrate a more pronounced systemic inflammatory profile, a raised risk of incident TB, and an increased risk of death in PWH with moderate and severe anaemia.

Implications of all the available evidence

The presence of moderate/severe anaemia pre-ART was independently associated with the development of TB and death. Given that, these patients should be carefully monitored before and after starting ART, and anaemia should be thoroughly evaluated as a hallmark of a distinct systemic inflammation profile, opportunistic infections and poor outcomes.

Introduction

In 2021, an estimated 700 thousand people with HIV (PWH) were living with active tuberculosis (TB).[1] The risk of TB increases by 2.5-fold in early HIV infection,[2] and individuals with TB-HIV co-infection exhibit a substantially higher risk of death.[3] The World Health Organization (WHO) advocates for the use of TB preventive therapy (TPT), including isoniazid preventive therapy (IPT), in adults and adolescents living with HIV who are unlikely to have active TB.[4] The use of TPT can reduce the incidence of TB by 36% in PWH.[5] However, other risk factors for TB in PWH also need to be investigated to reduce active TB occurrence in this population.

Low baseline CD4 count, low body mass index (BMI), and tobacco use are major risk factors for incident TB in PWH.[6,7] Another risk factor for incident TB is anaemia,[8] a condition characterised by low haemoglobin (Hb) levels (<12 g/dL in women and <13 g/dL in men) according to the WHO guidelines.[9] Anaemia is common among PWH, and its prevalence increases proportionally to the progression of HIV disease to the next stages until acquired immunodeficiency syndrome (AIDS).[10–12] The aetiology of anaemia in PWH is multifactorial and includes red blood cell destruction (haemolysis), blood loss and ineffective red blood cell production, associated with deficiencies of vitamin B12, folate, or iron.[13]

Anaemia is an independent prognostic indicator among PWH, associated with HIV disease progression.[11,14,15] In previous studies of our group, we have linked anaemia with higher and sustained inflammatory perturbation in TB-HIV initiating ART.[16] The increased inflammatory perturbation of TB-HIV anaemic

individuals includes increased levels of IL-6.[17] IL-6 is a multifunctional cytokine that regulates the immune response, inflammation, and haematopoiesis and appears to be the central mediator of anaemia of inflammation.[18] IL-6 also induces hepcidin production, that leads to anaemia by inhibiting iron absorption, blocking the release of iron from macrophages, and interfering with heme delivery to erythroid cells.[19]

In individuals with TB-HIV co-infection, anaemia has also been associated with a higher incidence of unfavourable adverse TB treatment outcomes, such as death, loss to follow-up, and treatment failure.[16] More recently, in a distinct cohort of PWH, anaemia has been associated with augmented systemic inflammatory disturbance, which was more pronounced in those with active TB.[17] However, little is known about the influence of anaemia severity on the development of active TB in PWH. In this study, we analysed data from the AIDS Clinical Trials Group A5274 REMEMBER trial (NCT0138008), an open-label randomised clinical trial of antiretroviral-naïve PWH with CD4 <50 cells/μL,[20,21] to investigate the influence of anaemia on the development of incident active TB or death following the initiation of ART and IPT vs. empiric TB treatment. Our primary aim was to investigate the association between anaemia, the inflammatory profile, incident TB or death after initiation of ART and TPT. Our hypothesis is that anaemia is a marker of high inflammatory dysregulation, which may be associated with a poor prognosis for PWH. To achieve these goals, we used multidimensional methods, including logistic regressions and Bayesian inference, to assess the severity of anaemia in PWH with an innovative approach.

Methods

Ethics statement

This study was approved by ethics committees and institutional review boards from the following participating site institutions: Johns Hopkins University, Baltimore, MD, USA; Chennai Antiviral Research and Treatment (CART) CRS, Chennai, India; University of North Carolina, Lilongwe CRS, Malawi; Soweto ACTG CRS, Chris Hani Baragwanath Hospital, Johannesburg South Africa; Joint Clinical Research Centre (JCRC)/ Kampala CRS, Kampala, Uganda; Kenya Medical Research Institute/Walter Reed Project Clinical Research Centre (KEMRI/WRP) CRS, Nairobi, Kenya; Moi University Clinical Research Centre (MUCRC) CRS, Eldoret, Kenya; Les Centres GHESKIO Clinical Research Site (GHESKIO-INLR) CRS, Port-au-Prince, Haiti; Blantyre CRS, Malawi College of Medicine, Blantyre, Malawi; Milton Park CRS, University of Zimbabwe, Harare, Zimbabwe; CAPRISA eThekwini CRS, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa; Byramjee Jeejeebhoy Medical College (BJMC), Pune, India; GHESKIO Institute of Infectious Diseases and Reproductive Health (GHESKIO–IMIS), Port Au Prince, Haiti. Informed consent was provided by all participants of the study.

Study design

We conducted a case-cohort study from participants enrolled in the REMEMBER clinical trial. This was an open-label randomised clinical trial (NCT0138008) of PWH with CD4 T-cell counts <50 cells/μL who were antiretroviral-naïve. Individuals initiated ART and were randomized to receive either IPT or 4 drug empiric TB therapy and were followed longitudinally for incident TB or death. The complete study design and study procedures are available in previous papers using this cohort.[20,21]

In an open label randomised 1:1 trial design, we contrasted two different treatment modalities: ART + empiric TB therapy (Empiric arm) vs. ART + IPT (IPT arm). The ACTG Data Management Centre's computer developed the randomization sequence. Randomization was balanced by clinical trial unit and stratified by CD4+ T cell count (<25 vs. >25 cells/mm3) and the presence of any of the prognostic factors listed below, including anaemia (haemoglobin <8 g/dL), a hospitalization within the previous 30 days, a BMI of less than 18.5 kg/m^2, and reportable hospitalization. The assignment of groups was not concealed from the participants, site staff, or study statisticians. To mimic actual field situations where ART would be paired with either Empiric 4 medication therapy or IPT, the open-label pragmatic design was used.

The randomly selected sub-cohort comprised 193 participants all of whom had available archived baseline plasma specimens for the determination of biomarker levels prior to ART initiation and prior to the development of two outcomes of interest: incident TB disease or death. Additionally, all TB and death cases (n = 64), as well as patients who did not develop incident TB or death (n = 12) were included, outside of the randomly selected sub-cohort in accordance with case-cohort design principles, resulting in 269 participants.

Patient population and clinical procedures

From October 31, 2011, to June 9, 2014, 850 participants were recruited from 18 clinical research sites in 10 countries (Malawi, South Africa, Haiti, Kenya, Zambia, India, Brazil, Zimbabwe, Peru, and Uganda). Participants were ART naïve, aged 18 years and older, and had a CD4 T cell count <50 cells/μL with no evidence of active TB. Those with negative symptom screening for TB or positive symptom screening but no microbiological or presumptive diagnosis of TB were eligible for enrolment in the study. Individuals who were strongly suspected to have TB at screening were excluded. The case-cohort design used in this study was similar to the study previously designed by Manabe et al.[21] and detailed in Supplementary Methods.

Sample size calculation

We conducted a post hoc power calculation to evaluate the power to detect a significant difference in the incidence of TB between non-anaemic, mild anaemia, and moderate/severe anaemia groups. We classified participants into non-anaemic (n = 64), with mild anaemia (n = 121) and with moderate/severe anaemia (n = 84) and found that the incidence of TB was 7.8% in the non-anemic group, compared to 19.0% and 22.6% in the anaemic groups, respectively. We performed a chi-square test of independence with three groups, and considering the sample size of 269, an alpha level of 0.05, and effect size (odds ratio) of 1.38 we obtained a study power of 79%. Performing the same analysis for each comparison we observed that there is a reasonable chance of detecting a significant difference between non-anaemic and moderate/severe anaemia groups (effect size: 1.4; study power: 81%) but a lower chance of detecting a significant difference between non-anaemic and mild anaemia group (effect size: 1.11; study power: 46%).

We performed the same analysis for death and found that the mortality was 10.9% in the nonanemic group, compared to 16.5% and 29.8% in patients with mild and moderate/severe anaemia, respectively. For the comparison between non-anaemic and mild anaemia groups, the study was found to be underpowered with a post hoc power of 28.4%. On the other hand, for the comparison between non-anaemic and moderate/severe anaemia groups, the study had sufficient power to detect a significant difference with a post hoc power of 98.4%. Assuming a significance level of 0.05, a total sample size of 269, and an effect size of 0.266, the post hoc power for the comparison of mortality rates between the three groups was 70.6%.

Definitions

Aligned with the WHO definition, anaemia was defined as Hb <13 g/dL for men or <12 g/dL for women.[22] Mild anaemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate/severe anaemia was defined as Hb <10 g/dL for both sexes.[22] Patients of moderate/severe anaemia were grouped due to the low number of participants with severe anaemia (n = 13).

Patients who presented cough, headache, fever, weight loss, night sweats or palpable lymph nodes during the medical screening at baseline were considered as those who presented "any TB sign or symptom".

According to decisions made in REMEMBER protocol, we defined incident TB cases as those who developed TB within 48 weeks after randomization. We defined death as persons who died within 48 weeks after initiation of protocol.

Laboratory procedures

Plasma samples from baseline (pre-ART) were thawed from storage at –80 °C. Thawed samples were then filtered, aliquoted, and frozen again for storage at –80 °C until ready for use to minimise subsequent freeze–thaw cycles during analysis. Using Meso Scale Discovery (MSD) multiplexed immunoassay kits as per the manufacturer's recommendations (www.mesoscale.com) we quantified: V-PLEX Proinflammatory Panel 1 Human Kit (K15049D; interferon γ [IFN-γ], interleukin [IL]-1β, IL-2, IL-6, IL-8, IL-10, IL-13, tumour necrosis factor α [TNF-α]), V-PLEX Cytokine Panel 1 Human Kit (K15050D; GM-CSF, IL-1α, IL-12/IL-23p40, IL-15, IL-16, IL-17A, TNF-β, VEGF-A), and V-PLEX Chemokine Panel 1 Human Kit (K15047D; eotaxin (CCL-11), macrophage inflammatory protein [MIP]-1β/CCL4, TARC (CCL-17), CXCL-10, MIP-1α/CCL3, IL-8, monocyte chemoattractant protein [MCP]-1/CCL2, myeloid dendritic cell [MDC/CCL22], MCP-4/CCL3). For soluble CD14 (sCD14) and IL-1 R1 analysis, R&D Systems Human CD14 DuoSet enzyme-linked immunosorbent assay (ELISA) (DY383) and Human IL-1 RI DuoSet ELISA (DY269) were used, respectively, as per manufacturer's recommendations (www.rndsystems.com). SoftMax Pro 5.3 was used to acquire and compute concentration values. Laboratory procedures were described in detail by Manabe et al.[21]

Inflammatory profile analysis

To evaluate the overall profile of inflammation, we log10 transformed the biomarker data and performed an unsupervised hierarchical cluster analysis (Ward's method), with dendrograms representing the Euclidean distances. Log10 fold-change also were calculated. In addition, we performed a Degree of Inflammatory Perturbation (DIP) approach, as detailed in Supplementary Methods.

Data analysis

Descriptive statistics were used to present data, and median values with interquartile ranges (IQR) were used as measures of central tendency and dispersion, for continuous variables. Categorical variables were described using frequency (no.) and proportions (%). Only complete cases considering the baseline and outcome data, were evaluated. The chi-square test was used to compare categorical variables between study groups. The Mann–Whitney *U* test (for two unmatched groups) and Kruskal–Wallis test (for more than 2 unmatched groups) were used to compare continuous variables. The Cochran–Armitage test for trend was to assess for the presence of an association between the measurements and the severity of anaemia. The Spearman rank test was used to assess correlations between Hb and biomarkers in each group/condition, where correlations were considered significant if p-value <0.05. Bayesian network learning was used to describe and visualise non-linear associations between the multiple clinical and inflammatory variables, as described in Supplementary Methods.

Kaplan–Meier analysis was evaluated according to the Breslow (Generalised Wilcoxon) test and applied to estimate incident TB and death probability of the participants stratified based on the anaemia severity.

We used a multivariable binomial logistic regression analysis including all parameters in Table 1 to test independent associations between clinical data, anaemia severity, and incident TB or death. Once we obtained the global model, we implemented a stepwise backward selection process to reduce the variables in order to transform it into a more practically useful prediction model. The alpha-to-enter and alpha-to-remove values were equal to 0.15. The results were presented in the form of adjusted Odds Ratio (aOR) and 95% confidence intervals (CI). To determine the model's discriminatory capacity, the area under the curve (AUC) was calculated. Additionally, internal validation was conducted using the bootstrap method with 1000 resamples.

In all analyses, differences with p-values below 0.05 after adjustment for multiple comparisons (Benjamini-Hochberg) were considered statistically significant. The statistical analyses were performed using R (version 4.4.1). The R packages used to perform the analysis in this paper were described in Supplementary Table S1.

Role of the funding source

The funders participated in study design of the parent clinical trial but not in the design, data collection or interpretation, nor the decision to submit this work for publication. All authors have reviewed and had full access to the data in the study and agreed to take responsibility for the decision to submit the work for publication.

Results

Characteristics of the study population

Our cohort was composed of 269 PWH (50.2% male and 49.8% female), who were grouped according to anaemia severity. Of the total number of participants, 23.8% (n = 64) did not have anaemia, 45% (n = 121) had mild anaemia, and 31.2% (n = 84) had moderate or severe anaemia. At baseline, those with moderate/severe anaemia were younger compared to PWH without anaemia and with mild anaemia. Those with moderate/severe anaemia had the highest neutrophil percentage and lowest albumin levels (Table 1). Interestingly, the levels of CD4 count and HIV viral load (VL) did not differ according to the severity of anaemia. When evaluating the correlation of these HIV markers with Hb

	Without anaemia (n = 64)	Mild anaemia (n = 121)	Moderate/severe anaemia (n = 84)
Continent of origin, n (%)			
Africa	53 (82.8)	83 (68.6)	54 (64.3)
America	9 (14.1)	31 (25.6)	23 (27.4)
Asia	2 (3.12)	7 (5.79)	7 (8.33)
Haemoglobin (g/dL), median (IQR):	13.5 (13.0–14.2)	11.2 (10.7–11.8)	8.90 (8.10–9.52)
Age (years), median (IQR):	36.0 (31.8–40.0)	38.0 (32.0–45.0)	34.0 (30.0–40.2)
Sex, n (%):			
Male	36 (56.2)	70 (57.9)	29 (34.5)
Female	28 (43.8)	51 (42.1)	55 (65.5)
Race (black), n (%):	58 (90.0)	106 (87.6)	72 (85.7)
BMI, median (IQR):	20.9 (19.1–23.4)	20.0 (18.3–22.2)	20.5 (18.2–22.2)
Hospitalization, n (%):	5 (8.33)	4 (3.51)	6 (7.89)
CD4 count (cells/μL), median (IQR):	19.5 (8.75–33.0)	18.0 (8.00–32.0)	25.5 (12.5–37.2)
Log10 HIV Viral Load (copies/mL), median (IQR):	5.21 (4.94–5.52)	5.41 (5.05–5.70)	5.36 (4.96–5.80)
CD8 count (cells/μL), median (IQR):	467 (321–612)	460 (279–611)	492 (294–704)
WBC x 10^9/L, median (IQR):	3.71 (2.70–2150)	3.50 (2.60–6.60)	4.70 (3.03–2545)
Neutrophil percentage, median (IQR):	52.6 (39.5–66.8)	54.9 (44.0–65.7)	60.4 (49.8–71.9)
Albumin (g/dL), median (IQR):	36.0 (4.15–40.6)	4.20 (3.50–36.0)	3.60 (3.00–34.0)
Creatinine (mg/dL), median (IQR):	0.72 (0.60–0.90)	0.70 (0.60–0.82)	0.70 (0.57–0.84)
Any TB sign or symptom, n (%):	36 (56.2)	79 (65.3)	63 (75.0)

Data are shown as median and interquartile range (IQR) or frequency (percentage). Categorical data were compared between the clinical groups using the Chi-squared tests. Continuous data were compared between the clinical groups using the Mann–Whitney *U* test (for two unmatched groups) or Kruskal–Wallis (for all groups). The countries considered for each continent were: Brazil, Haiti and Peru (America); Kenya, Malawi, South Africa, Uganda and Zambia (Africa); and India (Asia). Mild anaemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate/severe anaemia was defined as Hb ≤10 g/dL for both sexes.
Abbreviations: IQR: Interquartile range; BMI: body mass index, WBC: white blood cells. Any sign or symptom considered cough, headache, fever, weight loss, night sweats or palpable lymph nodes.

Table 1: **Clinical characteristics according anaemia severity.**

continuously, no significant correlations were observed either (CD4 p = –0.05; HIV VL p = –0.12) (Supplementary Fig. S1).

Inflammatory profile pre-ART of PWH according to anaemia severity

We observed distinct biomarker profiles according to anaemia severity, in which participants with moderate/severe anaemia presented higher levels of IFN-γ, IL-6, IL-12p40, TNF and VEGF-A in comparison to study participants with mild anaemia or non-anaemia (Fig. 1a). Those with moderate/severe anaemia exhibited higher levels of IL-2 (p = 0.007), IL-8 (p = 0.014) and IL-13 (p = 0.039) in contrast with those without anaemia. Plasma levels of these eight cytokines/growth factors were negatively correlated with Hb levels: IL-6 (p < 0.001), IL-12p40 (p = 0.003), TNF (p = 0.004), IL-2 (p = 0.004), IL-8 (p = 0.007), VEGF-A (p = 0.01), IL-1β (p = 0.02), IFN-γ (p = 0.03), IL-13 (p = 0.04) and CXCL10 (p = 0.04) (Supplementary Fig. S2, Supplementary Table S2). We also performed a supplementary analysis to assess the logarithmic relationship between Hb levels and the biomarkers, described in Supplementary Table S3. The overall measurements of inflammatory markers at pre-ART according to anaemia severity are detailed in Supplementary Table S4.

These observations suggested a disturbance of the immune activation in PWH with moderate/severe anaemia. To quantify this disturbance, we calculated the DIP score in all the clinical groups, considering the non-anaemic group as the reference group. Thus, we observed that the resulting DIP scores increased according to anaemia severity (Fig. 1b). In addition, DIP scores were shown to inversely correlate with Hb values (rho: –0.25; p < 0.001), and with HIV Viral Loads (rho: 0.13; p = 0.04) but were not related to CD4 counts (Supplementary Fig. S1). Of note, no difference between Hb or DIP values was observed when comparing the different arms of the original study[20] (Supplementary Fig. S3a and b).

Direct association between anaemia, TB and death

In our cohort, individuals without anaemia less frequently experienced incident TB than those from the other clinical groups; 7.8% of nonanemic participants developed TB in contrast with 19.0% and 22.6% detected in the mild and moderate/severe anaemia groups respectively (p = 0.009). Incident TB occurrence increased according to the severity of anaemia, with the highest frequency being detected in the group of severe anaemia (p = 0.003) (Fig. 2a). We next designed analyses to test whether the DIP score values are somehow

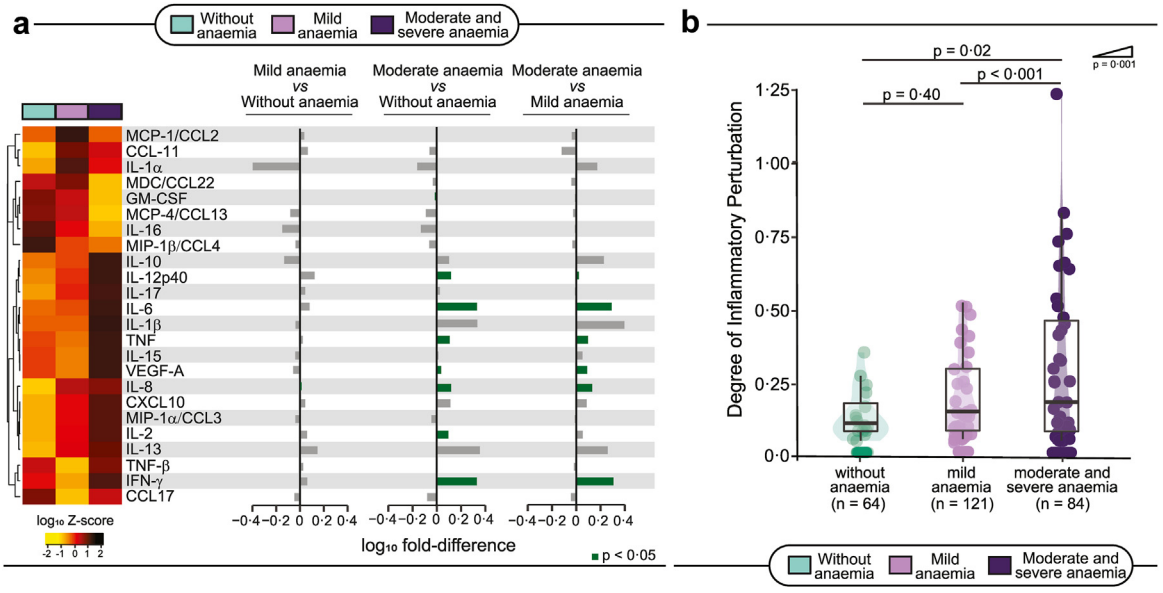

Fig. 1: **Association between anaemia severity and the systemic inflammatory profile.** Among all people with HIV (PWH) (n = 269), 76.2% had anaemia: 45% mild anaemia and 31.2% moderate/severe anaemia. (a) A heatmap was designed to depict the overall pattern of inflammatory markers. A one-way hierarchical cluster analysis (Ward's method) was performed. Dendrograms represent Euclidean distance. A log10 fold change was performed comparing groups. Significant differences (p < 0.05) are highlighted in green bars. (b) Scatter plots of the DIP value grouped according to anaemia severity. Lines in the scatter plots represent median values and data were compared using the Mann–Whitney *U* test. The Cochran–Armitage test for trend was used to assess the tendency of increased levels or frequencies among groups. Without anaemia was defined as Hb value >13 g/dL for man and >12 g/dL for women. Mild anaemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate/severe anaemia was defined as Hb ≤10 g/dL for both sexes.

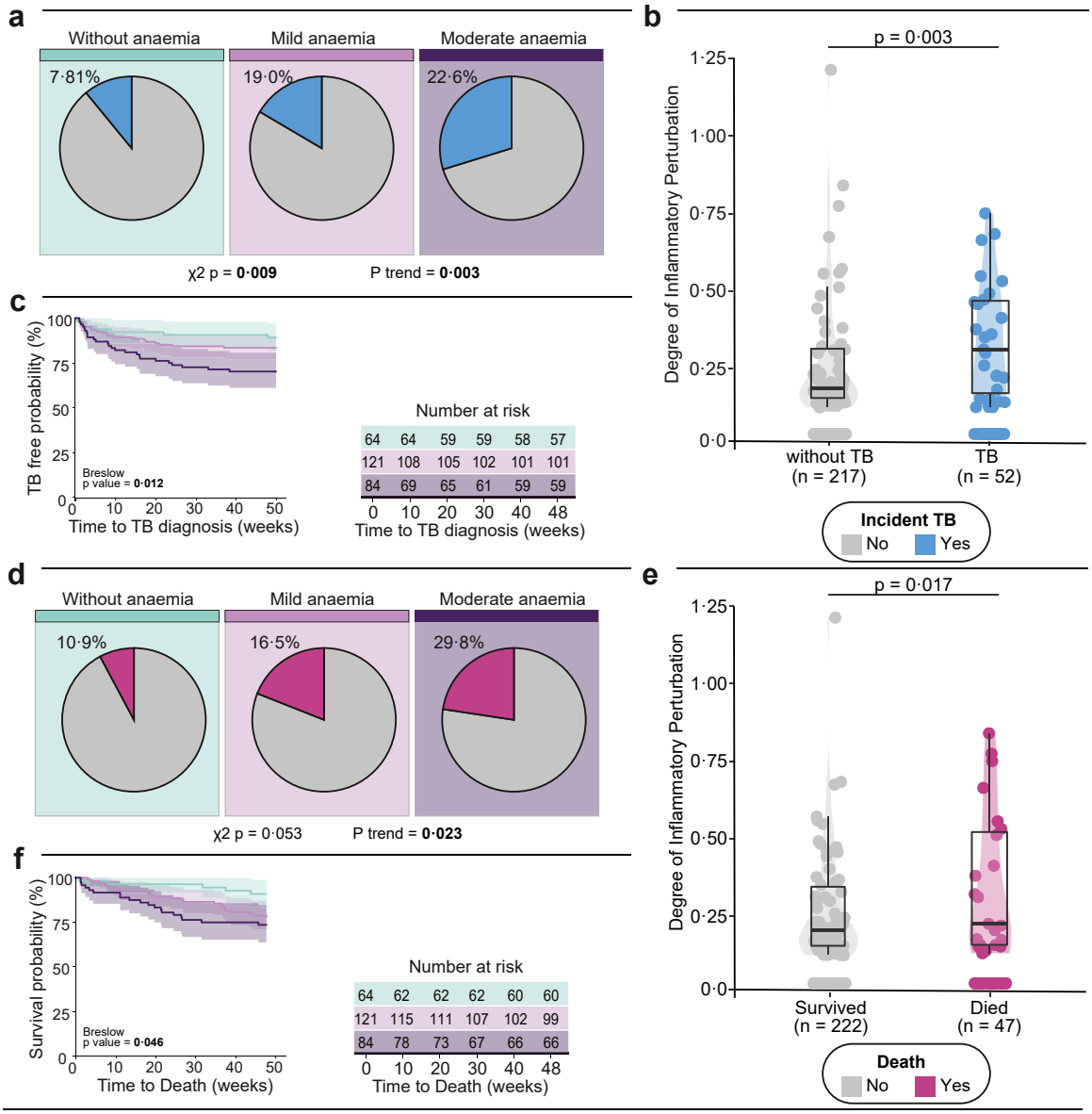

Fig. 2: **The association between anaemia and incident TB and death.** (a) Distribution of TB occurrence according anaemia severity. (b) Scatter plots of the DIP value grouped according to incident TB. Lines in the scatter plots represent median values and data were compared using the Mann–Whitney U test. (c) Kaplan–Meier curves show percentage of TB occurrence over 48 weeks. (d) Distribution of deaths according anaemia severity. (e) Scatter plots of the DIP value grouped according to mortality. Lines in the scatter plots represent median values and data were compared using the Mann–Whitney U test. (f) Kaplan–Meier curves show percentage of death occurrence over 48 weeks. Definitions: Without anaemia was defined as Hb value >13 g/dL for man and >12 g/dL for women. Mild anaemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate/severe anaemia was defined as Hb ≤10 g/dL for both sexes.

related to incident TB (Fig. 2b). The DIP values were higher in those who presented incident TB in contrast with patients who did not develop TB (p = 0.009; Fig. 2b). Individuals with moderate/severe anaemia presented a shorter TB-free survivor (37.1 weeks, standard deviation [SD] = 17.7) compared with that observed in the other groups (without anaemia = 44.1 [14.6]; mild anaemia = 41.8 [12.3], p = 0.012; Fig. 2c). Furthermore, mortality was substantially lower in non-anaemic participants (10.9%; p = 0.05) than in participants from the other clinical groups. (Fig. 2d). Indeed, patients who died displayed substantially higher DIP score values than those who survived (p = 0.017; Fig. 2e). Participants with moderate/severe anaemia presented a lower mean

overall survival time in weeks (40.9 [14.5] compared with that observed in the other groups (without anaemia = 46.2 [7.8]; mild anaemia = 43.6 [11.2]; p = 0.046; Fig. 2f).

Next, we evaluated the distribution of anaemia severity and systemic inflammatory profile according to the development of unfavourable outcomes (i.e., incident TB or death) during the follow-up. Of the 269 participants in our cohort, 68.0% did not develop TB and survived after 48 weeks (control group, n = 183), 14.6% developed TB and survived (n = 39), 12.6% did not develop TB but died (n = 34), and 4.8% developed TB and died (n = 13) during follow-up (Fig. 3a). The causes of death of these patients are described in Supplementary Table S5. The frequency of anaemia was lower in participants who did not have TB and survived, and higher than in the other groups of participants who had TB and/or died (p = 0.008). Hb concentrations were significantly lower whereas IL-6 levels were consistently higher in all groups that developed any unfavourable outcome (Fig. 3b). In the group of individuals who had TB and died, 1 (7.7%) was non-anaemic, 7 (53.8%) had mild anaemia, and 5 (38.5%) had moderate or severe anaemia. The time in weeks between diagnosis of incident TB and death did not vary according to anaemia severity, with an overall median of 4.7 weeks (IQR: 1.3–8.7) between outcomes. The time between the development of TB and death in individuals is illustrated in Fig. 3c.

A stepwise binomial logistic regression analysis was performed to test independent associations between anaemia severity and clinical parameters with incident TB. In such model, all the variables are imputed and only the once highly associated with outcome remaining in the last step of model. We found that the presence of moderate/severe anaemia pre-ART was independently associated with development of TB (aOR: 3.59, 95% CI: 1.32–9.76, p = 0.012) independent of other confounding factors, such as neutrophil frequency (Fig. 4a). The AUC of the model was 0.74 (with a 95% CI: 0.73–0.74). Using the bootstrap method with 1000 resamples, the AUC for internal validation was 0.70 (95% CI: 0.65–0.74).

Similar to the abovementioned results on occurrence of TB, another binomial logistic regression analysis was

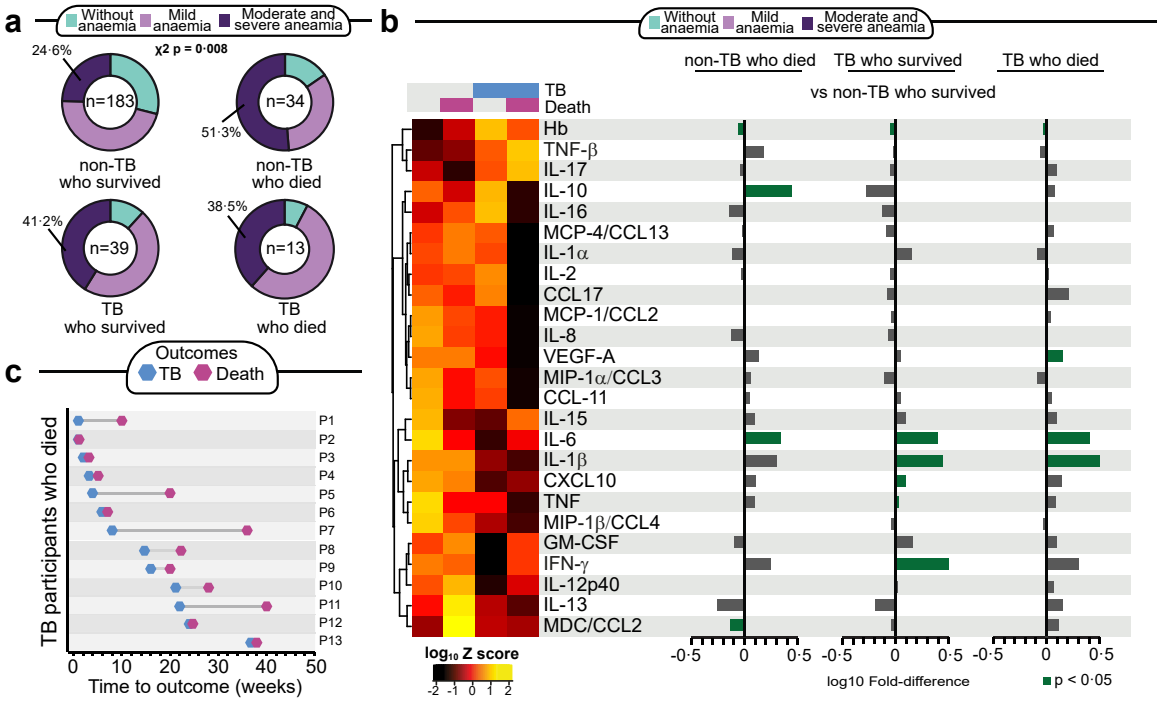

Fig. 3: **Inflammatory profile of individuals according to TB development and death in people with HIV**. (A) Four groups were established: non-TB who survived (n = 189); non-TB who died (n = 34); TB who survived (n = 39) and TB who died (n = 13) (B) Right panel: A heatmap was designed to depict the overall pattern of inflammatory markers in participants according to TB and death occurrence. A one-way hierarchical cluster analysis (Ward's method) was performed. Dendrograms represent Euclidean distance. Left panel: A log10 fold change was performed comparing each group with control (non-TB who survived). Significant differences (p < 0.05) are highlighted in green bars (C) Panel shows the time of TB development (in blue) and death (in purple) during the study for the TB participants who died. Without anaemia was defined as Hb value >13 g/dL for man and >12 g/dL for women. Mild anaemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate/severe anaemia was defined as Hb ≤10 g/dL for both sexes.

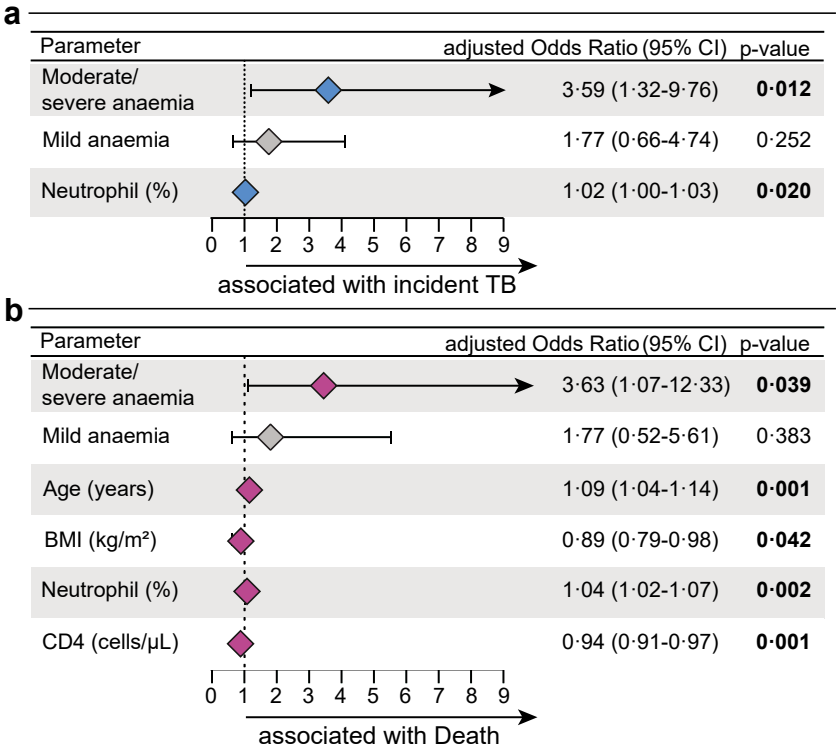

***Fig. 4:* Binomial logistic regression (stepwise method) to assess the independent risk factor for incident TB and death in PWH.** Binomial logistic regression with stepwise method was used to test independent associations between all clinical measurements (described in Table 1) and (a) incident TB or (b) death. Odds are per increase in 1 unit of the continuous variables. The alpha-to-enter and alpha-to-remove were equal to 0.15. Only variables remained in the last step were plotted. Abbreviations: CI: confidence interval; BMI: body mass index; TB: tuberculosis. Without anaemia was defined as Hb value >13 g/dL for man and >12 g/dL for women. Mild anaemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate/severe anaemia was defined as Hb ≤10 g/dL for both sexes.

performed to test independent associations between anaemia severity and other clinical parameters with death. We found that the presence of moderate/severe anaemia pre-ART was independently associated with death (aOR: 3.63, 95% CI: 1.07–12.33, p = 0.039) independent of other confounding factors, such as age, BMI value, neutrophil frequency and CD4 count (Fig. 4b). The AUC was 0.55 (with a 95% CI: 0.54–0.55). Using the bootstrap method with 1000 resamples, the AUC for internal validation was 0.61 (95% CI: 0.50–0.76). The general models (using ENTER method), with all clinical variables described in Table 1 is detailed in Supplementary Table S5 and stepwise models using Hb as a continuous variable in Supplementary Fig. S4. In these models, the decrease of 1 unit of haemoglobin (as a continuous variable) resulted in an aOR of 1.21 (95% CI: 1.02–1.44, p = 0.028) for incident TB and 1.37 (95% CI: 1.11–1.69, p = 0.004) for death.

Additionally, we applied Bayesian Network modelling to infer causal relations between anaemia, the occurrence of TB, and death in PWH and clinical laboratory parameters (Supplementary Fig. S5). The Bayesian network analysis confirmed the expected associations of anaemia with TB and death and indicated that higher inflammation was associated with higher values of IL-15. IL-6 formed an association chain with TB and death. Altogether, these data indicate that anaemia and higher IL-6 values are likely associated with incident TB disease and death in PHW (Supplementary Fig. S5).

Discussion

Our study of PWH with advanced immunosuppression who reside in diverse LMICs found a high prevalence of anaemia. Notably, we found that moderate to severe anaemia was associated with high markers of inflammation, including IL-6, and that these markers were significantly associated with an increased risk of developing TB and/or death. Understanding the association of anaemia with systemic inflammation may help to optimise clinical management and improve outcomes in PWH.

In our study, 76.2% of PWH were anaemic, specifically 45% of participants had mild anaemia, while 31.2% had moderate or severe anaemia, consistent with

literature that mild anaemia is the most prevalent degree of this condition in PWH.[12] Our participants with moderate/severe anaemia presented with a higher percentage of neutrophils and lower albumin levels. A prior analysis of risk factors for death in 5274 PWH accompanied during 48 weeks after ART initiation, documented an association between elevated neutrophil percent, lower albumin and lower haemoglobin levels and death.[23] Although HIV infection is known to often reduce neutrophil counts,[24] it has also been documented that there is a negative correlation between Hb and neutrophils in PWH, as well as a positive correlation between Hb and albumin,[16] which we also observed.

By analysing the systemic inflammatory profile of our cohort, we uncovered, in agreement with other previously reported investigations, that the severity of anaemia was linked to increased inflammation.[16,25–27] Innate inflammatory (IL-6, IL-8, and TNF), Th1 (IFN-γ, IL-2, IL-12p40), and Th2 (IL-13 and VEGF-A) cytokine concentrations increased following the degree of anaemia severity. Individuals with severe inflammation commonly present with cytopenia, and in some cases develop severe syndromes with an overproduction of IFN-γ, IL-2, IL-12, and TNF, by activated Th1 cells and macrophages.[28] IFN-γ acts directly on macrophages and prompts blood cell uptake, leading to consumptive anaemia of inflammation.[29] Together, the characteristics observed in PWH with moderate/severe anaemia demonstrate that those with this severity of anaemia may have greater inflammation and worse clinical presentation. Whether anaemia is a cause or consequence of the augmented systemic inflammation that leads to increased odds of unfavourable outcomes is yet to be determined. Rather, it permits the use of Hb levels, a straightforward and inexpensive parameter as an indicator of inflammatory disturbance and a distinct immune activation profile that is associated with TB and mortality. Higher levels of IL-6 are commonly described in PWH[30–34] and are associated with anaemia in this population, providing evidence of activation of coagulation.[11,30] In conjunction with IL-1 and TNF, IL-6 can induce apoptosis of red cell precursors and decrease the bone marrow's ability to respond to erythropoietin signalling.[19] Based on our results and prior studies, we showed that anaemia in PWH is associated with increased levels of IL-6 and hypothesise that this may occur due to increased inflammation or exacerbation of immune activation (i.e., anaemia of chronic and imbalanced inflammation). While immune activation is required to control the infection, its exacerbation can contribute to unfavourable outcomes in PWH, such as OIs and death.

We next assessed incident TB and population survival and observed that incident TB, as well as mortality, increased according to the severity of anaemia. In addition, those with moderate/severe anaemia presented with a lower average of TB-free time and a lower average of survivor time in weeks. This short time for incident TB raises an important question about the cause of anaemia in these patients. Although patients were extensively screened during baseline, it is not impossible that baseline anaemia results from unidentified TB infections. Increasing severity of anaemia has been associated with exceptionally high rates of both incident TB and mortality during 8 years of follow-up after ART initiation in a South African cohort.[13] In a meta-analysis of cross-sectional and case–control studies, the pooled odds ratio for the association between anaemia and TB was 3.56, increasing with the severity of anaemia.[35] In the EuroSIDA study, the 12-month survival rate was 96.9% among non-anaemic PWH and 59.2% among those with severe anaemia.[14] Hb levels were significantly decreased in all groups of participants with unfavourable outcomes, and IL-6 was significantly increased in these comparisons. The Bayesian Network model confirmed the associations of anaemia with TB and death, as well association of higher IL-6 levels with TB and death. Increased levels of IL-6 have been associated with higher HIV RNA levels[31] as well with HIV complications and death in PWH on ART.[36] In relation to TB in PWH, high levels of IL-6 were detected in TB-HIV co-infection[37] and a previous study of our group demonstrated that higher IL-6 levels are strongly associated with TB-IRIS.[38] Moreover, higher pre-ART levels of IL-6 and greater increases in IL-6 on ART have been previously associated with death and TB-IRIS, respectively, in similarly advanced HIV/TB patients. TB-IRIS may be associated with an exaggerated cytokine response that may contribute substantially to disease progression.[39,40]

Finally, we performed multidimensional analyses to explore the relationship between Hb values, inflammation, incident TB and death. Albeit our study does not permit us to identify whether anaemia is a cause or a consequence of the inflammatory process, our analysis demonstrated that there is a strong relationship between systemic inflammation and anaemia severity. In addition, this systemic inflammation associated with low Hb levels was more prominent in those patients who had incident TB and/or died. After all, it endorses the use of Hb levels as a proxy for inflammatory perturbation that is associated with incident TB and mortality.

Our study has some limitations. We assayed only 26 biomarkers only at baseline, and a more comprehensive assessment including more targets could provide more details that could help delineate and/or answer the hypotheses discussed here. All participants were PWH with very low CD4 cell counts <50 cells/μL, which affects the generalizability of the results to the entire population living with HIV. However, we already described anaemia as a risk factor for incident TB in a cohort of participants with higher levels of CD4 cells/μL.[15] In addition, we could not evaluate individual countries due to limited number of participants. Rather,

we examined participants by continent, which resulted in more substantial sample size. Moreover, it would be interesting to extend this study to a population with less advanced HIV infection, and to expand the number of participants by country, to evaluate these populations separately. In addition, it was not possible to determine the cause of the anaemia or to measure other markers such as hepcidin or transferrin. Moreover, it was not possible to determine whether anaemia could be associated with other infections or socioeconomic determinants in our population. Our study design also did not provide conclusive evidence of causality between anaemia and incident TB. Furthermore, when evaluating death and incident TB together, we had only 13 participants. It is also important to mention that among patients with incident TB, there may be the possibility of previously subclinical TB, although we used strict TB assessment criteria. If the sample was larger, it would be interesting to assess the impact of different grades of anaemia within this population. It would also be important to validate the findings in other settings and populations. Regardless of such limitations, our study provides a strong basis to elucidate the associate of anaemia with the risk of incident TB and death in PWH with advanced immunosuppression, the very group with the highest incidence of TB and death.

Altogether, our findings indicate that moderate-to-severe anaemia and higher IL-6 values are associated with incident TB disease and death in PHW. Currently, it is unknown if resolving anaemia may mitigate the risk of TB and death in PHW. However, we have shown in a previous article that TB-HIV individuals who recovered from anaemia during anti-TB treatment experienced a decreased systemic inflammatory perturbation in comparison to those who remained with persistent anaemia.[16] Thus, we believe that PWH with moderate-to-severe anaemia should be carefully monitored before and after ART commencement in PWH.

Contributors

M.A-P., S.K., and B.B.A. were responsible for study design and funding acquisition, conceptualization, data curation, investigation, formal analysis, methodology, software and writing the original draft; M.H., G.B and A.G were responsible for funding acquisition; M.H., G.B, S.B-F., K.N., J.R.L., L.M. and V.M. were responsible for data collection; P.S.,Y.M., D.P.S., V.R., F.S., A.S., C.K., S.D., J.V., U.G.L., C.M.K., B.B.A. and A.G. were responsible for investigation; B.B.A. and A.G. were responsible for supervision and writing the original draft; All authors reviewed and edited the manuscript. All authors have read, had full access to the data and agreed to the submitted version of the manuscript.

Data sharing statement

The data that support the findings of this study will be available upon reasonable request to the corresponding author of the study.

Declaration of interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Acknowledgements

The authors thank the participants and the ACTG trial sites that participated in A5274 REMEMBER.

Research reported in this publication was supported by the AIDS Clinical Trials Group, which is supported by the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) under award numbers UM1 AI068634, UM1 AI068636, and UM1 AI106701. The work was also supported by The Johns Hopkins Baltimore-Washington-India Clinical Trials Unit (BWI CTU) and Johns Hopkins Baltimore India CTU (NIH/NIAID UM1AI069465 to A.G and S.K.)). M.A-P. received a fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Finance code: 001). The work of B. B. A. was supported by the intramural research program from FIOCRUZ and from the NIH (U01AI115940). S.K. was also supported by the National Institutes of Health T32 AI007291-27 and the Johns Hopkins University Clinician Scientist Award.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.eclinm.2023.102030.

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