Association between severe anaemia and inflammation, risk of IRIS and death in persons with HIV: A multinational cohort study

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Summary

Background After initiating antiretroviral therapy (ART), approximately 25% of people with HIV (PWH) may develop Immune Reconstitution Inflammatory Syndrome (IRIS), which is associated with increased morbidity and mortality. Several reports have demonstrated that low haemoglobin (Hb) levels are a risk factor for IRIS. To what extent the severity of anaemia contributes to the risk of IRIS and/or death is still insufficiently explored.

Methods We investigated both the presence and severity of anaemia in PWH in a multinational cohort of ART-naïve patients. A large panel of plasma biomarkers was measured pre-ART and patients were followed up for 6 months. IRIS or deaths during this period were considered as outcomes. We performed multidimensional analyses, logistic regression, and survival curves to delineate associations.

Findings Patients with severe anaemia (SA) presented a distinct systemic inflammatory profile, characterized by higher TNF, IL-6, and IL-27 levels. SA was independently associated with IRIS, with a higher risk of both early IRIS onset and death. Among IRIS patients, those with SA had a higher risk of mycobacterial IRIS.

Interpretation PWH with SA display a more pronounced inflammatory profile, with an elevated risk of developing IRIS earlier and a statistically significant higher risk of death.

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Keywords: Tuberculosis; Systemic inflammation; IRIS; Death; HIV

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Research in context

Evidence before this study

Despite the success of antiretroviral therapy (ART) in improving health status of people with HIV (PWH), a portion of these patients may experience rapid clinical deterioration after ART, known as Immune Reconstitution Inflammatory Syndrome (IRIS), that can significantly increase morbidity and mortality. Anaemia is a common complication of HIV disease progression and can be a risk factor for unfavourable outcomes such as IRIS. Identifying risk factors for IRIS is important to provide insights on optimization of clinical management of these patients. We explored the relationship between anaemia severity and IRIS, examining the inflammatory profile and IRIS occurrence in PWH according to different degrees of anaemia.

Added value of this study

Previous studies have shown low haemoglobin level as a risk factor for unfavourable outcomes in PWH, however to what extent the severity of anaemia contributes to the risk of IRIS and/or death is still insufficiently explored. Our results show that PWH with moderate and severe anaemia display a more pronounced systemic inflammatory profile, with an elevated risk of developing IRIS mainly by mycobacteria and a statistically significant higher risk of death.

Implications of all the available evidence

By identifying moderate and severe anaemia as a risk factor for IRIS and death, we demonstrate that these patients should be carefully monitored before and after ART initiation and, if possible, anaemia should be thoroughly evaluated to assess possible undiagnosed underlying infections or malignancies.

Introduction

Despite the success of antiretroviral therapy (ART) in strengthening both the immunologic responses and health status of people with HIV (PWH), a portion of these patients may experience rapid clinical deterioration shortly after ART commencement.[1] This phenomenon, known as Immune Reconstitution Inflammatory Syndrome (IRIS), can affect from 5% to 50% of the severely immunosuppressed individuals and can significantly increase their morbidity and mortality.[2] Mechanistically, IRIS is characterized by a tissue-destructive inflammation that occurs concomitantly to the emergence of functionally active $CD4^+$ T-cells in the setting of opportunistic co-infections. These infections can be caused by several distinct pathogens, for example tuberculosis (TB),[3] parasites, fungi and viruses, such as herpes virus 3 (varicella zoster virus – VZV) and 8 (Kaposi sarcoma virus).[4,5] Risk factors for TB-IRIS development include severe $CD4^+$ T-cell lymphopenia, short interval between ART and antitubercular treatment initiation, previously diagnosed *Mycobacterium* spp. infection, and low levels of haemoglobin (Hb).[3,6,7]

Importantly, anaemia, characterized by low haemoglobin (Hb) levels (<12 g/dL in women and <13 g/dL in men), is a frequent comorbidity observed among PWH, affecting between 1.3 and 95% of patients, depending on clinical and social factors.[8] The cause of anaemia in PWH is multifactorial and can be associated with chronic disease, bone marrow suppression or infiltration by infections, iron status or nutritional deficiencies, concomitant medications such as Trimethoprim/sulfamethoxazole or others, haemolysis, and sustained inflammation.[9] Several studies have demonstrated a strong relationship between low Hb levels with the occurrence of IRIS and unfavourable treatment outcomes after initiating ART.[7,10,11] In addition, ART-naïve patients who have concomitant anaemia and systemic inflammation are at high risk for failing ART, and timely identification and appropriate management of these may help reduce adverse outcomes.[12]

The association between anaemia and HIV progression is known, but no studies have reported how the severity of anaemia affects the treatment outcomes of PWH. The present study aimed to investigate the relationship between anaemia severity and IRIS using several multidimensional statistical analyses and logistic regression models, examining the pre-ART inflammatory profile of PWH with different degrees of anaemia (mild, moderate, severe), who were followed for up to 6 months of treatment. A better understanding of the effect of anaemia severity on ART outcomes is of utmost importance to provide insights on optimization of clinical management to minimize the risk of IRIS and mortality.

Methods

Ethics statement

All patients provided written informed consent before inclusion in accordance with the Declaration of Helsinki. The study protocol was approved by the ethics committees of the participating sites and registered on National Institute of Health (NIH) Clinical Trials website (Identifier: NCT00286767).

Overall study design

The study was a retrospective analysis of data from a previously reported international observational cohort study conducted across three countries: United States, Kenya, and Thailand[7] and registered at NIH Clinical

Trials website (Identifier: NCT00286767). Each clinical research site enrolled persons ≥18 years of age with an HIV infection diagnosis, CD4 count ≤100 cells/μL, without previous ART, and residence within a 120-mile radius of the clinical sites, between 2006 and 2018. Exclusion criteria were pregnancy and substance use disorder. Further details are included in Supplementary Material 1 and in the study protocol, attached as Supplementary Material 2. In the original study design, the required sample size calculated to identify baseline variables capable of predicting IRIS was 400, with a power of around 90%.

The protocol was approved by the ethics committees of the participating sites (NIH, US IRB approval no.: 06-I-0086.; Kenya Medical Research Institute, Kenya IRB approval no.: 14702; South East Asia Research Collaboration with Hawaii, Thailand: IRB approval no.: 264/53; Bamrasnaradura Infectious Disease Institute: IRB approval no.: P009h/53). All study participants signed informed consent and were followed prospectively from the initiation of ART (week 0) for up to 6 months (24 weeks) for the development of IRIS or death. At baseline, sociodemographic characteristics and comprehensive medical data were collected. Peripheral blood samples were collected and stored at –80 °C for later testing. The timing and regimen of ART were chosen according to local treatment guidelines. The clinical teams at study sites identified IRIS events and presented them to an endpoint review committee. Diagnostic criteria and procedures were detailed in Supplementary Material 2.

Anaemia definition

According to the World Health Organization (WHO) guideline criteria, anaemia was defined as levels of Hb below (<)13 g/dL for men or <12 g/dL for women.[13] 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 anaemia was defined as Hb > 8 g/dL and ≤ 10 g/dL for both sexes.[13] Severe anaemia (SA) was defined as Hb < 8 g/dL for both sexes.

Biomarker measurements

Biomarkers were batch-tested in the same laboratory, from cryopreserved plasma collected from participants pre-ART. To measure C Reactive Protein (CRP), C-X-C motif chemokine ligand 10 (CXCL10), interleukin-2 (IL-2), IL-6, IL-8, IL-10, IL-27, interferon-γ (IFN-γ), and tissue necrosis factor (TNF), we used electrochemiluminescence by Meso Scale Discovery (MSD, MD). D-dimer was measured by enzyme-linked fluorescent assay (ELFA) on a VIDAS instrument (bioMerieux). Finally, hyaluronic acid (HA), myeloperoxidase (MPO), soluble cluster differentiation 14 (sCD14), sCD163, and tissue factor (TF) were measured by enzyme-linked immunosorbent assay (ELISA) using the manufacturer's instructions. Thus, we measured biomarkers involved in inflammation (CRP, IL-6, IL-8, MPO, TNF), coagulation (D-dimer), myeloid activation (TF, CXCL10, sCD14, sCD163) and lymphoid function (IL-2, IL-27, IFN-γ). HIV viral load, CD4 and CD8 T cells counts, Hb, white blood cell count (WBC), platelets, and glucose were measured by each site's clinical laboratory with approved assays.

Data collection and statistical analysis

The data from the study participants were collected at each study site and maintained in an electronic data system (CRIMSON). Data from CRIMSON Data System were collected directly from subjects during study visits and abstracted from subjects' diaries, and medical records. Data managers from each site were in close monitoring together with a central coordinator in US to perform quality checks and store the database in main data repository at the NIH.

Prior to the evaluation of the study database to answer to accomplish the aims of this study, all analyses were pre-specified. The data analysis plan is found in the Supplementary Material 3. Approximately 0.4–1.78% of the data on measurements of inflammatory biomarkers were missing. Given the small number of missing values, and that there are continuous data, the predictive mean matching method was used for data imputation through the R package "mice". 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 (%). The Pearson 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 Spearman rank test was used to assess correlations between Hb and biomarkers in each group/condition, where correlations were considered statistically significant if $|rho| \geq 0.25$ and $p < 0.05$.

To evaluate the overall profile of inflammation, we $\log_{10}$ transformed the biomarker data and performed an unsupervised hierarchical cluster analysis (Ward's method), with dendrograms representing the Euclidean distances.

The Cox proportional hazards models were used to evaluate association between anaemia grade and 26-weeks IRIS development and mortality. Cox regression analysis was conducted using a multivariable model (adjusted). Age, sex, and country were incorporated to adjust for patient specific variance. We used a multivariable binomial logistic regression (backward stepwise regression) analysis including all parameters in univariate analysis (comparing patients who developed IRIS versus whose who did not) to test independent associations between inflammatory biomarker levels, anaemia severity, and IRIS. The results were presented

in the form of adjusted Odds Ratio (aOR) and 95% confidence intervals (CI), with calculation of C-statistics.

A classification test was performed using conditional inference tree (CTree), implemented through the "party" R package, to classify patients according to IRIS occurrence. The decision tree was constructed based on the clinical, inflammatory markers and Hb values. CTree bases splitting decisions on univariate regression models, and following the initial split, subsequent inference takes place within subgroups. CTree selects the input variable with the highest p-value with response variable. In this analysis, when continuous variables are used, the split cut-off is defined so that the residual sum of squared error (squared difference between the observed outcome values and the predicted ones) is minimized across the training samples that fall within the sub partition. The split point is defined so that the population in within each sub partition is as pure as possible.[14] More information about CTRee is described in Supplementary Methods and Supplementary Fig. S1). Receiver operating characteristic (ROC) curve was used to evaluate the predictive effect of the variable selected by conditional inference tree to identify persons who further developed IRIS.

Kaplan–Meier analysis was calculated according to the log-rank (Mantel–Cox) test and applied to estimate the probability of the participants developing IRIS stratified based on the anaemia severity (without anaemia, mild, moderate, and severe anaemia). We also utilized this approach to estimate death probability stratified based on the anaemia severity. In all analyses performed on the manuscript, differences with p-values below 0.05 after Benjamini-Hochberg adjustment for multiple comparisons were considered statistically significant. The statistical analyses were performed using IBM SPSS version 25, and 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 had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Results

Characteristics of the study population

A total of 506 ART-naive PWH were enrolled in the three sites of the study: United States, Kenya, and Thailand. Four were removed from analyses of this current investigation due to a lack of data on baseline Hb levels (Supplementary Fig. S2). The cohort was stratified according to the occurrence and severity of anaemia. We found that 16.3% (n = 82) of the patients had normal Hb levels according to WHO definitions, as described in Materials and Methods. The remaining 83.7% (n = 420) had low Hb levels and were considered anaemic. Clinical characteristics between subgroups of anaemic and non-anaemic study participants are detailed in Supplementary Table S2. Of note, the characteristics of the study participants by country are detailed in Supplementary Table S3 and the characteristics of the participants by anaemia grade for each country are detailed in Supplementary Tables S4–S6.

We subsequently stratified the anaemic group according to the severity of anaemia into the following categories: mild (n = 265, 63.1%), moderate (n = 129, 30.7%), and severe (n = 26, 6.2%) (Fig. 1a, Table 1). Clinical characteristics of patients according to anaemia severity are detailed in Table 1.

Inflammatory profile of patients according to anaemia grade

We next evaluated the systemic inflammatory profile in our study population by assessing the plasma levels of biomarkers associated with innate immune activation, adaptive immune responses, and Hb levels. We observed distinct profiles according to anaemia severity, in which patients with moderate and severe anaemia presented higher levels of all biomarkers in comparison to mild anaemia and non-anaemic participants (Fig. 1b). The overall measurements of inflammatory markers pre-ART according to anaemia severity is described in Supplementary Table S7.

We observed weak negative correlations between Hb values and concentrations of Hyaluronic Acid (HA) (Spearman correlation test, $r = -0.34$, $p < 0.001$), IL-6 (Spearman correlation test, $r = -0.31$, $p < 0.001$), CRP (Spearman correlation test, $r = -0.25$, $p < 0.001$), IL-10 (Spearman correlation test, $r = -0.25$, $p < 0.001$), IL-27 (Spearman correlation test, $r = -0.28$, $p < 0.001$), and a moderate negative correlation between Hb levels and D-dimer (Spearman correlation test, $r = -0.40$, $p < 0.001$) and sCD14 (Spearman correlation test, $r = -0.41$, $p < 0.001$) levels (Fig. 1b, Supplementary Table S8). A network analysis of Spearman correlations was built to visualize only moderate correlations (set arbitrarily as rho value $r \geq \pm 0.4$) in subgroups of study participants stratified according to anaemia severity. This analysis demonstrated a higher number of strong correlations between concentrations of biomarkers in the group of persons with SA, resulting in a higher network density compared to the other clinical groups (Fig. 1c). The correlations displayed in Fig. 1c are detailed in Supplementary Tables S9–S12. In the SA group, Hb values negatively correlated to a moderate or strong degree with concentrations of HA (Spearman correlation test, $r = -0.54$, $p = 0.006$), CXCL10 (Spearman correlation test, $r = -0.49$, $p = 0.014$), sCD163 (Spearman correlation test, $r = -0.62$, $p = 0.001$), sCD14 (Spearman correlation test, $r = -0.61$, $p = 0.002$), TNF (Spearman correlation test, $r = -0.51$, $p = 0.012$), IL-8 (Spearman correlation test, $r = -0.51$, $p = 0.010$) and

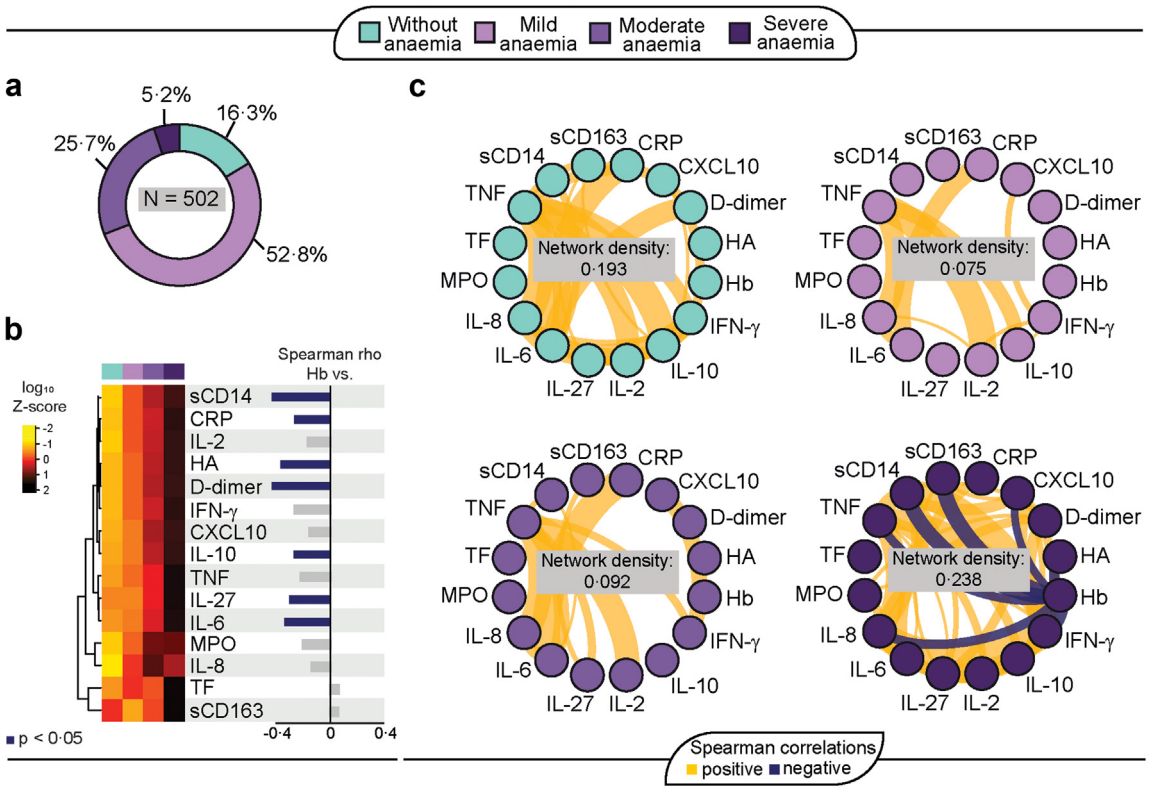

Fig. 1: Patients with severe anaemia present a distinct inflammatory profile. (a) Among all the PWH (n = 502), 83.7% had anaemia: 52.8% mild anaemia; 25.7% moderate anaemia; and 5.2% severe anaemia. (b) Right panel: A heatmap was designed to depict the overall pattern of inflammatory markers. A one-way hierarchical cluster analysis (Ward's method) was performed. Dendrograms represent Euclidean distance. Left panel: A Spearman correlation test was performed between Hb and biomarker levels. Statistically significant correlations ($p < 0.05$) are highlighted in dark blue bars. (c) Spearman correlation test between Hb and biomarkers for each group (Green: Without anaemia; Light Purple: mild anaemia; Purple: moderate anaemia; Dark Purple: severe anaemia). Dark blue lines indicate moderate/strong negative Spearman correlation (rho < −0.40) and orange lines indicate moderate/strong positive Spearman correlation (rho >0.40). All correlations in this chart had p values less than 0.05.

IFN-γ (Spearman correlation test, r = –0.64, p < 0.001) (Fig. 1c). Collectively, these results demonstrate that extremely low levels of Hb (<8 g/dL) are associated with greater activation of immune responses and may reflect a monocyte/macrophage activation, indicative of augmented systemic inflammation.[15,16]

Moderate and severe anaemia associates with a higher risk of IRIS occurrence

Over the 24-week follow-up period, 97 (19.3%) patients of our cohort developed IRIS. Of note, IRIS was more frequently diagnosed in anaemic patients, and the risk of IRIS development was associated with anaemia severity (Fig. 2a). An heatmap plot was generated with biomarker concentrations, and participants were ordered based on time to event (Fig. 2b) and the biomarkers were ordered according to Spearman correlation rho values. Baseline Hb values were positively correlated with time to IRIS (Spearman correlation test, r = 0.24, p = 0.01), meaning that the lower the levels of Hb, the faster a study participant developed IRIS (Fig. 2b). Importantly, the analysis also uncovered that higher pre-ART concentrations of D-dimer (Spearman correlation test, r = –0.27, p = 0.007), IL-27 (Spearman correlation test, r = –0.21, p = 0.04), MPO (Spearman correlation test, r = –0.20, p = 0.04), CXCL10 (Spearman correlation test, r = –0.20, p = 0.04) and IFN-γ (Spearman correlation test, r = –0.31, p = 0.002) were all negatively, albeit weakly, correlated with time to IRIS (Fig. 2b). Thus, the lower the Hb levels, the higher the pre-ART concentrations of key inflammatory markers which were related to earlier onset of IRIS events.

The distribution of study participants based on time to IRIS development is shown in Fig. 2c. Patients with severe anaemia presented with IRIS at an average of 12.7 weeks after ART commencement, compared with 17, 19 and 21.1 weeks observed in those without, mild and moderate anaemia respectively (log-rank p < 0.001;

	Without anaemia (n = 82)	Mild anaemia (n = 265)	Moderate anaemia (n = 129)	Severe anaemia (n = 26)
Age, median (IQR)	35.0 (31.0–44.0)	37.0 (32.0–45.0)	36.0 (30.0–45.0)	41.0 (35.5–47.2)
Male, n (%)	54 (65.9)	176 (66.4)	67 (51.9)	10 (38.5)
Country, n (%)				
Kenya	46 (56.1)	99 (37.4)	40 (31.0)	13 (50.0)
Thailand	16 (19.5)	42 (15.8)	32 (24.8)	10 (38.5)
USA	20 (24.4)	124 (46.8)	57 (44.2)	3 (11.5)
Glucose (mg/dL), median (IQR)	81.0 (76.3–88.0)	85.5 (77.0–97.0)	85.6 (79.2–100)	83.0 (75.8–90.0)
WBC (10^6/μL), median (IQR)	3.67 (2.70–4.82)	3.17 (2.33–4.00)	3.10 (2.38–4.45)	4.60 (3.20–6.93)
Platelets (10^6/μL), median (IQR)	198 (142–262)	221 (169–277)	237 (172–329)	208 (136–346)
Hb (g/dL), median (IQR)	13.8 (13.3–14.8)	11.3 (10.6–12.1)	9.30 (8.80–9.60)	7.35 (6.93–7.68)
$CD4^+$ count/μL, median (IQR)	39 (14–63)	26 (12–53)	22 (9–51)	25 (12–65)
$CD8^+$ count/μL, median (IQR)	618 (428–888)	467 (279–745)	375 (215–602)	400 (226–878)
BMI (kg/m^2), median (IQR)	20.5 (18.2–23.0)	20.7 (18.0–24.2)	19.4 (17.7–22.4)	18.9 (16.4–21.2)
HIV VL (log10 copies/mL), median (IQR)	5.34 (4.93–5.60)	5.25 (4.84–5.64)	5.33 (4.95–5.79)	5.64 (5.24–5.88)

Note: Continuous data are shown as median and IQR, and categorical data are shown as number and frequency (percentage).
Abbreviations: BMI: Body Mass Index; Hb: haemoglobin; IQR: interquartile range; HIV: Human Immunodeficiency Virus; USA: United States of America; WBC: white blood cells.

Table 1: **Characteristics of study participants at baseline according to anaemia severity.**

Fig. 2d). Using a Cox regression, the Hazard Ratio (HR) for these patients was HR: 14.03 (95%CI: 4.91–40.09; p = 0.001) (Supplementary Table S13).

A binomial logistic regression analysis was performed inputting all the variables exhibited in univariate analysis (age, country, anaemia grade, BMI, CD4 count, CD8 count WBC, HIV VL, TNF, TF, sCD163, sCD14, platelet, MPO, CXCL10, IL-10, IL-8, IL-7, IL-6, IL-2, IFN-γ, HA, glucose, D-dimer and CRP) (Supplementary Table S14) to test independent associations between inflammatory biomarker levels, anaemia severity, and IRIS. Our data demonstrated that increases of 1 unit (pg/mL) in TNF levels (adjusted Odds Ratio [aOR]: 1.04, 95%CI: 1.01–1.06, p = 0.003), of 1 unit (10^6/μL) in WBC levels (aOR: 1.16, 95%CI: 1.04–1.30, p = 0.010) and of 1 unit (pg/mL) in sCD14 levels (aOR: 6.74, 95%CI: 1.12–40.5, p = 0.037) were independently associated with increased odds of IRIS occurrence. In addition, US country (aOR: 2.17, 95%CI: 1.17–4.00, p = 0.014) and moderate (aOR: 4.48, 95%CI: 1.59–12.5, p = 0.004) or severe anaemia (aOR: 9.1, 95%CI: 2.5–33.7, p = 0.001) were also independently associated with IRIS occurrence. Of note, C-statistics of the model was equal to 0.75 (Fig. 3a). Additionally, we performed another binomial logistic regression analysis with an ENTER method, using all clinical and laboratory variables (age, country, anaemia grade, BMI, CD4 count, CD8 count WBC, HIV VL, TNF, TF, sCD163, sCD14, platelet, MPO, CXCL10, IL-10, IL-8, IL-7, IL-6, IL-2, IFN-γ, HA, glucose, D-dimer and CRP). In this analysis, we have found that severe anaemia was independently associated with IRIS occurrence (aOR: 6.52, 95%CI: 1.53–27.7, p = 0.011) (Supplementary Fig. S3). Next, we performed a machine-learning based decision tree analysis with clinical variables (age, country, sex, BMI, CD4 count, CD8 count, Glucose, WBC, Platelets), Hb and cytokine values, to identify persons at study baseline who further developed IRIS. The results from the decision tree contained just one decision node: Hb, with a cut-off point equal to 8.5 g/dL, which corresponds to moderate and severe anaemia (Fig. 3b). The discriminative power of this classifier was then evaluated by ROC curve. The area under the curve (AUC) was 0.671 (95%CI: 0.61–0.73), with a calculated high sensitivity (94%, 95%CI:0.92–0.96) but low specificity (25%, 95%CI:0.16–0.34) (Fig. 3c). The k-fold cross-validation of this model had an accuracy of 0.999 (95% CI 0.993–1.000), a non-information rate equal to 0.807 and a p-value <0.001.

Association between anaemia severity and mycobacterial-IRIS

The occurrence of IRIS was similar in relation with country of origin (Pearson chi square test, p = 0.141), as well with the type of IRIS (Supplementary Fig. S4a). Among IRIS patients, 48.5% (n = 47) developed mycobacterial-IRIS and 51.5% (n = 50) developed viral or fungal/parasitic IRIS (Supplementary Fig. S4b). Stratifying according to anaemia, only 5.2% (n = 5) were not anaemic, 45.4% (n = 44) had mild anaemia, 36% (n = 35) moderate anaemia, and 13.4% (n = 13) severe anaemia (Supplementary Fig. S4b). Due to the low number of participants and the observed association between both moderate and severe anaemia with IRIS in the logistic regression model reported above, moderate and severe anaemia cases were concatenated in a single category. Another binomial logistic regression analysis was performed considering only IRIS patients, to test independent associations between anaemia severity and biomarker levels with occurrence of

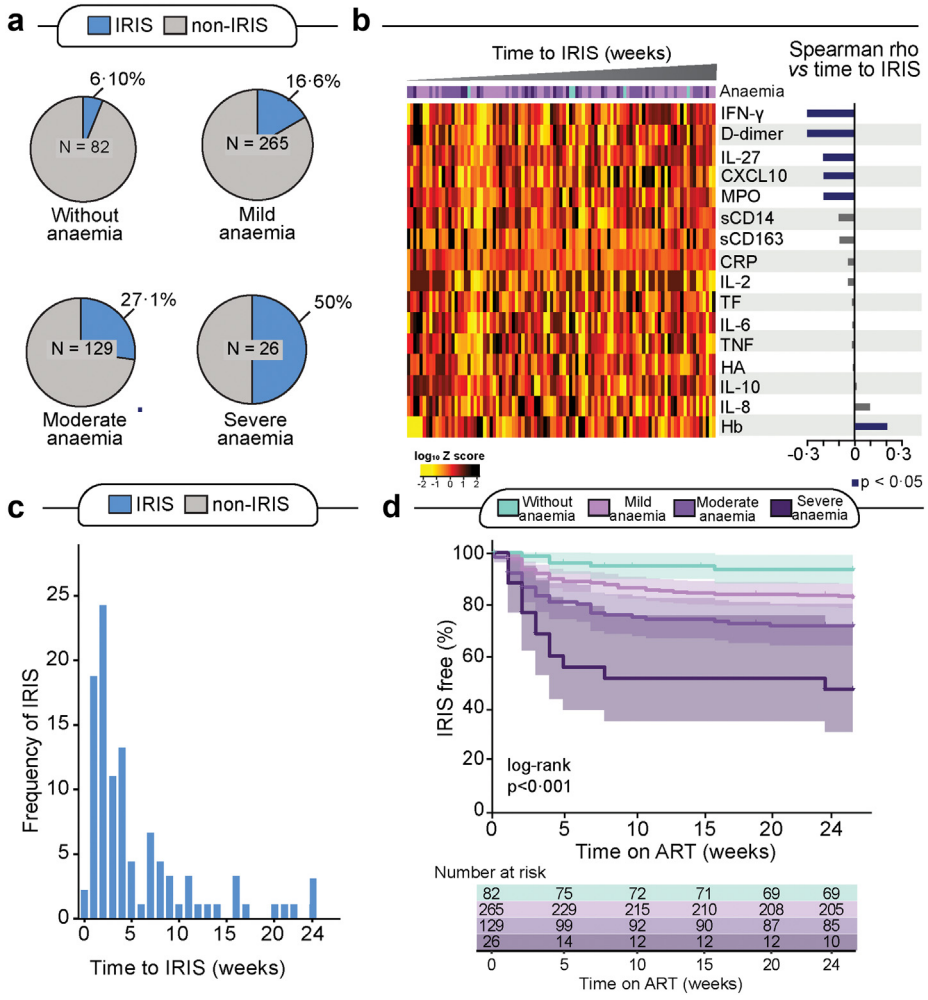

Fig. 2: Moderate and severe anaemia were associated with IRIS occurrence. (a) IRIS occurrence increased according to anaemia severity. (b) Left panel: data were log10 transformed and ranked and coloured in a heatmap from values detected for each inflammatory biomarker. Participants were ordered based on time to IRIS (in weeks), and plasma inflammatory biomarkers were clustered (Ward method) according to the distribution profile in the study population. Dendrograms represent Euclidean distance. Right panel: Spearman correlations for each mediator and time to IRIS. Green bars indicate statistically significant correlations ($p < 0.05$). (c) Histogram shows the frequency of participants who developed IRIS over time. (d) Kaplan–Meier curves show percentage IRIS free over 6 months and number at risk for each timepoint. The comparison between curves resulted in a log-rank p value < 0.001.

mycobacterial-IRIS relative to other types of IRIS. All variables with $p < 0.05$ in the univariate analysis (Supplementary Table S15) were included. We found that the presence of moderate/severe anaemia pre-ART was independently associated with development of mycobacterial-IRIS (aOR: 2.6, 95%CI: 1.1–3.2, $p = 0.035$) independent of other confounding factors (Supplementary Fig. S3c). Additionally, we performed a ROC curve to classify those who develop mycobacterial-IRIS from those who did not. With an optimal cut-off point of 10.55 g/dL of Hb, the AUC was 0.746 (95% CI: 0.68–0.81), with a calculated moderate sensitivity (79%, 95%CI:57–65) and specificity (61%, 95% CI:68–89) (Supplementary Fig. S4d).

Mortality increases according to anaemia severity

Similar to the abovementioned results on occurrence of IRIS, overall mortality also increased according to the severity of anaemia. Thus, the frequency of deaths was 4.88% (n = 4), 5.28% (n = 14), 6.20% (n = 8), and 19.2% (n = 5) in the groups of patients without anaemia, with mild, moderate, or severe anaemia, respectively (Fig. 4a). We also plotted an heatmap with concentrations of inflammatory markers and Hb values where participants were ordered based on time to death. In this plot, biomarkers were ordered according to Spearman correlation rho values. In this analysis, pre-ART IL-27 (Spearman correlation test, $r = -0.46$, $p = 0.009$) and MPO (Spearman correlation test, $r = -0.37$, $p = 0.04$)

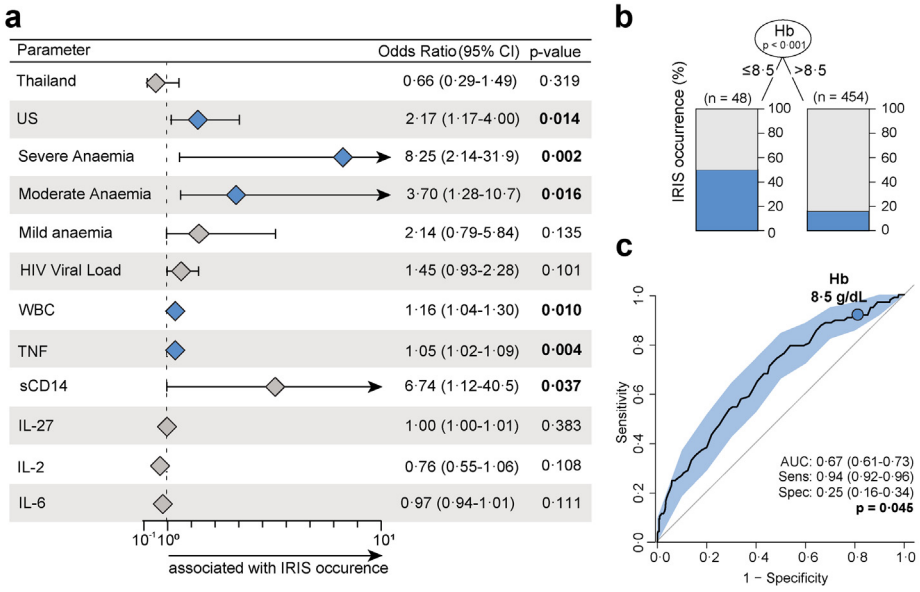

Fig. 3: Moderate and severe anaemia are associated with IRIS occurrence. (a) Binomial logistic regression model (backward stepwise regression) to test independent associations between all the relevant measurements (Mann–Whitney U test p-value < 0.1 in Supplementary Table S7) and IRIS occurrence. The c-statistic of the model was equal to 0.74. Only measurements that remained in the last step are shown: severe anaemia (adjusted Odds Ratio [aOR], p = 0.001), moderate anaemia (aOR p = 0.004), mild anaemia (aOR p = 0.055), D-dimer (aOR p = 0.056), IL-6 (aOR p = 0.090) and TNF (aOR p = 0.003). Measurements entered on step 1: anaemia grade, CD4, CD8, MPO, TNF, CXCL10, sCD14, HA, IFN-γ, IL-10, IL-27, IL-6, IL-8, D-dimer, and CRP. (b) Decision tree to identify individuals at baseline who further developed IRIS using biomarker values. Hb cut-off point was defined as 8.5 g/dL (p < 0.001). (c) ROC curve analysis to evaluate the discrimination power of Hb (p = 0.045).

levels exhibited statistically significant negative Spearman correlations with time to death (Fig. 4b). The distribution of time to death revealed that similar to what we observed in IRIS, deaths also occurred more frequently during the first 3 weeks of ART (Fig. 4c). The survival-curve analysis demonstrated a statistically significant difference between the clinical groups, so that those with severe anaemia died earlier and in greater numbers than the others (log-rank p = 0.043) (Fig. 4d). Using a Cox regression analysis, the Hazard Ratio (HR) for these patients was HR: 3.52 (95%CI: 0.92–13.4), p = 0.065 (Supplementary Table S16).

Additionally, we performed analysis to compare the profile of patients according to IRIS development and death during the follow-up. 381 (75.9%) patients were non-IRIS who survived, 24 (4.8%) were non-IRIS who died, 90 (17.9%) were IRIS who survived and 7 (1.4%) were IRIS who died. While analysing the inflammatory markers, we observed distinct biomarker profiles among these groups, in which patients with one (or both) unfavourable outcomes (IRIS or death) exhibited higher levels of inflammatory markers than non-IRIS survivors. Of note, patients who developed IRIS and survived or died presented with lower levels of Hb in comparison to non-IRIS who survived (Supplementary Figs. S5a, S5b). The time to outcome of participants who developed IRIS and died during the follow-up based on time of study is shown in Supplementary Fig. S5c.

Finally, a new unsupervised hierarchical cluster analysis was performed with all the study participants where three main clusters were defined. Cluster 1 exhibited in general higher levels of inflammatory cytokines than the other clusters (Supplementary Fig. S6a). In addition, comparing clusters, Cluster 1 also included a higher frequency of participants who: (I) died during the follow up (14.5%, p < 0.001), (ii) developed IRIS (23.9%, Pearson chi square test p = 0.006) and (iii) presented with moderate or severe anaemia at study baseline (44.5%, Pearson chi square test p = 0.001) (Supplementary Fig. S6b). Of note, Cluster 2 included 4.5% of all deaths, 21.6% of all IRIS cases, and 30.15% of the participants with moderate or severe anaemia. The frequencies of participants who died, experienced IRIS, and had moderate or severe anaemia in Cluster 3 were respectively 2.5%, 11.9%, and 20.8% (Supplementary Fig. S6b). This analysis reinforced the idea that augmented systemic inflammation and more severe anaemia are linked to higher odds of IRIS and death in persons with advanced HIV at early ART.

Discussion

Anaemia is a common complication in PWH.[8] In this multinational cohort study, 83.7% of study participants were anaemic. Due to this high prevalence, we stratified patients according to anaemia severity (non-anaemic, mild,

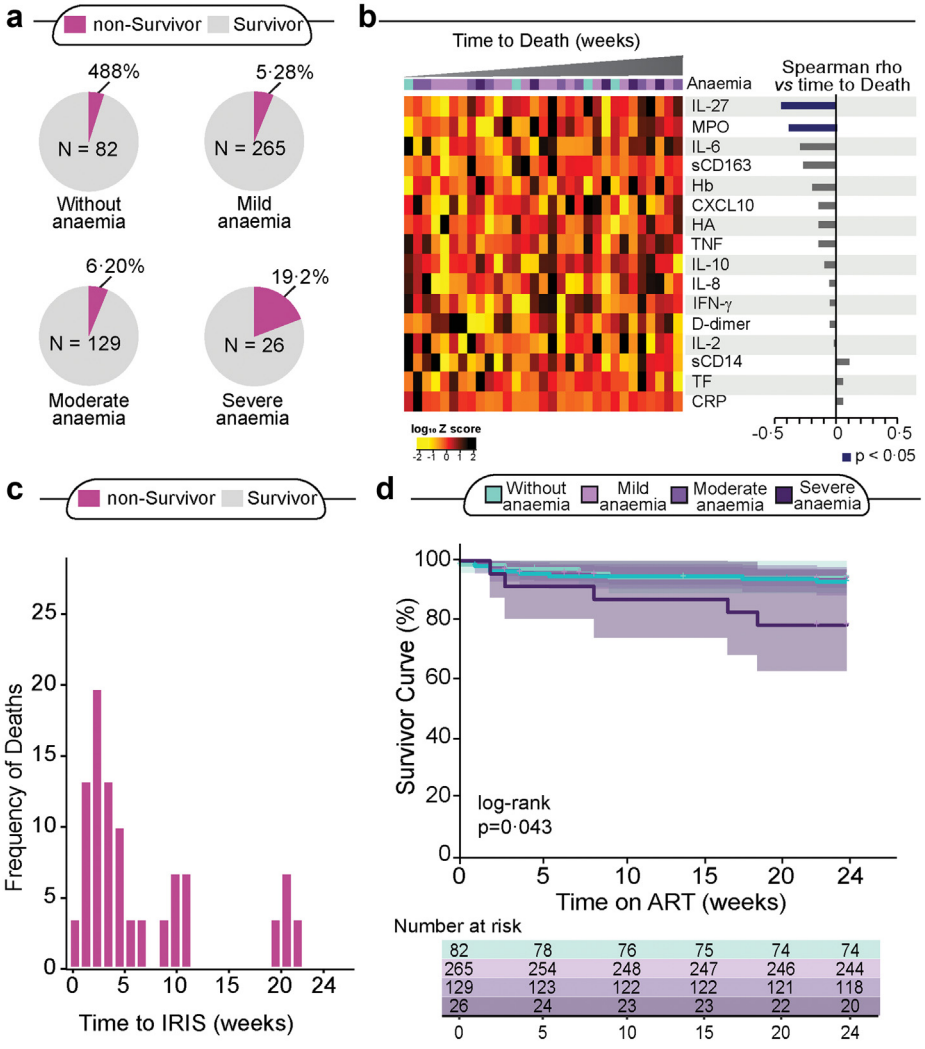

Fig. 4: Mortality was higher in patients with severe anaemia. (a) Mortality increased according to anaemia severity. Pink shaded areas represent the frequency of participants who died in each indicated clinical group. (b) Left panel: data were $\log_{10}$ transformed, ranked and coloured in a heatmap from values detected for each inflammatory biomarker. Participants (n = 502) were ordered based on time to death (in weeks), and plasma inflammatory biomarkers were clustered (Ward method) according to the distribution profile in the study population. Dendrograms represent Euclidean distance. Right panel: Spearman correlations for each mediator and time to death. Green bars indicate statistically significant correlations ($p < 0.05$). (c) Histogram shows the frequency of participants who died over time. (d) Kaplan–Meier curves show the probability of survival over 6 months in each group and number at risk for each timepoint (log-rank p = 0.043).

moderate, or severe) and evaluated whether the severity was associated with the systemic inflammatory profile, IRIS occurrence, and death during ART, in a multi-centre cohort including participants with severe immunosuppression at diagnosis. The results reported here favour the hypothesis that anaemia is associated with unfavourable outcomes, namely IRIS and death, in PWH.

Among anaemic patients in our study, 63.1% had mild and 30.7% had moderate anaemia, corroborating literature findings, where the most common severity of anaemia in PWH is mild to moderate.[8,17,18] Therefore, around 6.2% of our cohort had severe anaemia and presented higher values of HIV viral load, showing that patients with this severity of anaemia have higher viral replication with a possible direct impact on bone marrow function.

Analysis of the systemic inflammatory profile showed that, as the severity of anaemia increased, the levels and connectivity of inflammatory markers also increased. Previous studies of anaemia in PWH have described these associations as a consequence of immunologic dysregulation that may predispose to IRIS. In agreement with others, we found an augmented inflammation tracking the severity of the anaemia.[10,19–21]

Interestingly, we found an inverse correlation between IFN-γ, CXCL10, and Hb levels, similarly to reports in aplastic anaemia, that correlate the higher level of these markers with lymphocyte activation,[22] boosted Th1 responses, and inflammatory exacerbation.[22] Also, IFN-γ and IL-8 have been previously described as important mediators of anaemia, directly inhibiting the production of red blood cells in the progenitor cell level.[22–24] In addition, higher levels of pro-inflammatory markers of macrophage activation (sCD163 and sCD14) and tissue fibrosis (HA) were found in patients with severe anaemia.[25–27] This important finding highlights the link between anaemia, inflammation, and risk of IRIS in these groups.

It is hypothesized that the innate immune system hyperactivation that characterizes IRIS, when antigen-specific $CD4^+$ T cell numbers are restored after ART, is due to the uncoupling of innate and adaptive immune responses during an infection in the absence of $CD4^+$ T cells leading to an abrupt reversal of immune suppression.[2] Higher levels of inflammatory biomarkers such as IFN-γ and sCD14 in PWH at baseline have been found to be independently associated with risk of IRIS.[26] Furthermore, in this same cohort, CRP and low Hb levels were shown to be independently associated with TB-IRIS development.[26] In our study, severe anaemia was mainly independently associated with IRIS occurrence, and moderate/severe anaemia together were associated with mycobacterial IRIS specifically, highlighting the distinct pathophysiology of different degrees of severity of anaemia. Whether anaemia underlies specific immunopathological mechanisms that preferentially drive increased susceptibility to mycobacterial IRIS is still unknown and deserves experimental investigation.

The only plasma biomarker that showed statistically significant association with IRIS in the logistic regression analyses was TNF, and in agreement with this observation, anti-TNF drugs have been used in patients with IRIS refractory to the use of corticosteroids.[28–30] Furthermore, it was observed that higher levels of IL-27, MPO, IFN-γ, D-dimer, and CXCL10 as well as lower levels of Hb were correlated with earlier development of IRIS. This shows once again that lower Hb levels and a more exacerbated inflammatory profile are associated with the development of IRIS in PWH.

Moreover, using the classification analysis the use of only Hb values, with a cut-off of 8.5 g/dL, was enough to predict patients who developed IRIS with a high sensitivity, although with low specificity. This cut-off point is similar to the cut-off that defines severe anaemia (8 g/dL), showing that patients with this anaemia grade are indeed at higher risk to develop IRIS. Importantly, this described performance may be sufficient to adopt Hb values in the clinical practice to evaluate risk of IRIS at the time of ART commencement. In such setting, a test with high sensitivity is likely more useful for initial screening to identify those with a higher risk of IRIS. Furthermore, the results also suggested that the prediction performance of Hb levels is substantially higher to predict mycobacterial-IRIS. Additional investigations could take place together with a more careful monitoring to minimize the risk of IRIS.

Higher sCD163 concentrations were independently associated with mycobacterial IRIS in our study. As sCD14 was previously associated with IRIS,[26] higher sCD163 plasma levels have been detected in TB-IRIS patients in some studies.[31,32] In a previous study from our group, sCD163 concentrations remained increased in patients with TB-IRIS for 24 weeks of ART and the production of inflammatory cytokines by monocytes was higher compared to patients without IRIS.[32]

Low Hb levels are extensively related to increased mortality in PWH.[33] In our study, we also found that higher levels of IL-27 and MPO were associated with early death after starting ART. IL-27 is known to modulate macrophage activity during Mtb infection, favouring the pathogen by inhibiting phagosome acidification and the production of pro-inflammatory cytokines.[34] Additional studies are necessary to describe the exact mechanism by which IL-27 may contribute to early death in anaemic PWH.

We hypothesize that in PWH, anaemia is triggered by decreased red blood cell (RBC) production, increased RBC destruction and can be due to HIV viremia, other infections, and medications with inflammation contributing to its pathogenesis.[35] In addition, anaemia at baseline tends to be more severe in patients who developed IRIS, as reflected on the survival curves when we stratified patients according to the degree of anaemia. This is also consistent with the original study that found Hb < 8.5 as predictive of IRIS in decision tree analysis.[7] Therefore, further studies are needed to define the aetiology and improve the management of anaemia in patients with IRIS.

The study limitations include the relatively small number of participants who developed IRIS or died, preventing a more detailed analysis of variables. In addition, transfusions are more common in the US which may have underestimated the proportion of people with severe anaemia. We did not investigate IRIS caused by other pathogens, except for mycobacterial-IRIS, due to small number of participants in each subcategory. Additionally, other factors such as gastrointestinal bleeding, chronic inflammation and social-economic determinants could also contribute with the low Hb levels. It is important to emphasize that PWH may have opportunistic diseases or be using medications that can cause anaemia. Finally, in this study we only evaluated individuals with a CD4 count <100 cells/mcL, from three different countries and with an unknown cause of anaemia. However, to minimize the effects of low CD4 count or genetic variation, we used these variables as adjustments in logistic regression analyses. Regardless, our study was able to suggest that pre-ART moderate and severe anaemia were associated

with a higher risk of developing IRIS and dying compared to patients without anaemia or with mild anaemia.

Overall, our study demonstrates that severe anaemia before ART is closely associated with the development of IRIS and death. Severe anaemia may be a marker of progression of HIV infection, suggesting that PWH in our cohort with this condition have been infected for a long time and not diagnosed, given that they are ART-naïve. Thus, it is important to emphasize that efforts are needed to identify and treat patients earlier, in order to avoid unfavourable outcomes. In a previous article by our group evaluating clinical laboratory markers (such as CRP, bilirubin, albumin, total protein and liver transaminases), we observed that TB-HIV patients who recovered from anaemia during anti-TB treatment experienced a decreased systemic inflammatory disturbance compared to those who remained with persistent anaemia. Additionally, we identified that in our cohort, patients with moderate/mild anaemia who develop IRIS frequently have mycobacterial IRIS. This study was designed to explore the possibility of determining whether severe pre-ART anaemia is a risk factor for the development of IRIS and death in PWH. Whilst interesting, the findings reported here should be further validated in other cohorts of PWH from different epidemiological settings. We believe that PWH with severe anaemia should be carefully monitored before and after starting ART and, if possible, anaemia should be thoroughly evaluated to assess possible undiagnosed underlying infections or malignancies.

Contributors

Study Design and funding acquisition: I.S., D.S., J. A., and B.B.A.; Conceptualization, M.A-P, and B.B.A.; Data Collection: I.S., V.S., D.S., N.P., G.R; Laboratory Assays: A.R.; Data curation and verification of the underlying data, M.A-P., M.B.A. C.L.V., and B.B.A.; Investigation, M.A-P., M.B.A., B.B-D., C.L.V., R.T., M.P-A. and B.B.A.; Formal analysis, M.A-P., M.B.A., and B.B.A.; Methodology, M.A-P, M.B.A. and B.B.A.; Software, M.A-P., M.B.A. and B.B.A.; Supervision, I.S., and B.B.A.; Writing—original draft, M.A-P., B.B-D., R.T., M.P-A., and B.B.A.; Writing—review and editing, all authors. All authors have read 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2022.104309.

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