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**INSTITUTO GONÇALO MONIZ**

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**Curso de Pós-Graduação em Patologia Humana e Experimental**

**TESE DE DOUTORADO**

# DETERMINANTES DA RELAÇÃO ENTRE ANEMIA E DESFECHOS CLÍNICOS DESFAVORÁVEIS EM PESSOAS VIVENDO COM HIV

**MARIANA ARAÚJO PEREIRA**

**Salvador - Bahia**

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**MARIANA ARAÚJO PEREIRA**

Tese apresentada ao Curso de Pós-Graduação em Patologia Experimental para a obtenção do grau de Doutora**.**

Orientador: Prof. Dr. Bruno Bezerril Andrade

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"DETERMINANTES DA RELAÇÃO ENTRE ANEMIA E DESFECHOS CLÍNICOS DESFAVORÁVEIS EM PESSOAS VIVENDO COM HIV ".

**Mariana Araújo Pereira**

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“Se vivemos como respiramos,
prendendo e soltando, não
poderemos errar.”

**(Clarissa Pinkola Estes)**

PEREIRA, Mariana Araújo. **Determinantes da relação entre anemia e desfechos clínicos desfavoráveis em pessoas vivendo com HIV.** 2022. 235 f. Tese (Doutorado em Patologia) – Universidade Federal da Bahia. Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2022.

## RESUMO

**INTRODUÇÃO:** Pessoas vivendo com HIV (PVHIV) frequentemente apresentam anemia como complicação. A anemia em pacientes com HIV pode ser associada a uma exacerbação do perfil inflamatório, com aumento de citocinas no sangue e progressão da doença mais acelerada. Entretanto pouco se tem relatado sobre o impacto da gravidade da anemia nos desfechos clínicos desfavoráveis durante tratamento desses pacientes com antirretrovirais, como tuberculose (TB) incidente, síndrome de imunorreconstituição inflamatória (SIRI) e morte. **OBJETIVO:** Caracterizar o perfil inflamatório de pacientes anêmicos com HIV, bem como identificar as associações entre a presença de anemia e sua gravidade com os desfechos desfavoráveis de tratamento desses pacientes. **METODOLOGIA**: Esta tese reúne um conjunto de seis manuscritos que visam caracterizar e associar a anemia aos desfechos clínicos de PVHIV. Ao todo, foram avaliados dados de 2403 participantes. Anemia e sua gravidade foram definidos de acordo com os critérios da Organização Mundial da Saúde utilizando hemoglobina (Hb) como marcador. Para cada manuscrito foi utilizada uma diferente coorte, e em cada uma delas foram analisados os níveis plasmáticos de marcadores inflamatórios, tal como dados clínicos, socioeconômicos e laboratoriais. Foram aplicados métodos de estatística descritiva e um conjunto de técnicas multidimensionais, incluindo análises de rede, análises integrativas, regressões logísticas e cálculo de grau de perturbação inflamatória (GPI). **RESULTADOS**: De forma geral, identificamos que PVHIV anêmicos pré-tratamento antiretroviral (TARV) apresentam um risco aumentado de desfechos desfavoráveis, e uma maior perturbação inflamatória sistêmica se comparados aos não anêmicos. Estratificando por manuscrito, (1) PVHIV anêmicos apresentaram um risco 2.3 vezes maior de TB incidente após o início do tratamento antirretroviral (TARV), (2) aqueles com anemia grave apresentaram 8 vezes maior risco de SIRI por micobacterias, (3) a anemia está associada a uma maior disseminação da TB e (4) maior perturbação inflamatória. Nestes manuscritos, a anemia moderada a grave está associada consistentemente ao desfecho de óbito, independente de outros fatores como contagem de CD4 e carga viral de HIV. Em relação aos desfechos de tratamento anti-TB (TAT), indivíduos (5) com anemia persistente ao longo do tratamento ou (6) com anemia moderada a grave pré-TAT possuem maior risco de óbito como desfecho. **CONCLUSÕES**: Em conjunto, os manuscritos desta tese adicionam importantes informações quanto à importância da anemia no contexto da coinfecção por HIV-TB. Demonstramos que a anemia pré-TARV ou pré-TAT pode ser um importante marcador de mau prognóstico para TB incidente, SIRI, TB disseminada, perturbação inflamatória e óbito. Tendo em vista que esse é um marcador cuja quantificação já está implementada na rotina clínica, as informações aqui contidas são úteis no direcionamento de novas abordagens para otimizar o manejo clínicos e melhorar a qualidade e expectativa de vida de PVHIV.

**Palavras-chave**: HIV. Anemia. Inflamação sistêmica. Desfecho. Tuberculose.

PEREIRA, Mariana Araújo. Mariana. **Determinants of the relationship between anemia and unfavorable clinical outcomes in people living with HIV.** 2022. 235 f. Thesis (Doctorate in Pathology) – Universidade Federal da Bahia. Instituto Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, 2022.

## ABSTRACT

**INTRODUCTION:** People living with HIV (PLHIV) often have anemia as a complication. Anemia in HIV patients may be associated with an exacerbation of the inflammatory profile, with an increase in blood cytokines and more accelerated disease progression. However, little has been reported on the impact of anemia severity on unfavorable clinical outcomes during treatment of these patients with antiretroviral drugs, such as incident tuberculosis (TB), immunoreconstitution inflammatory syndrome (IRIS) and death. **OBJECTIVE**: To characterize the inflammatory profile of anemic patients with HIV, as well as to identify the associations between the presence of anemia and its severity with the unfavorable treatment outcomes of these patients. **METHODS**: This thesis brings together a set of six manuscripts that aim to characterize and associate anemia with the clinical outcomes of PLHIV. Altogether, data from 1617 PLHIV were evaluated. Anemia and its severity were defined according to World Health Organization criteria. For each manuscript, a different PLHIV cohort was used, and in each of them the plasma levels of inflammatory markers were analyzed, as well as clinical, socioeconomic and laboratory data. Descriptive statistical methods and a set of multidimensional techniques were applied, including network analyses, integrative analyses, logistic regressions and calculation of the degree of inflammatory perturbation (DIP). **RESULTS**: In the first study, we concluded that anemic PLHIV are more likely to develop incident TB after starting antiretroviral treatment (ART) compared to non-anemic individuals. In the second, it was found that severe anemia increases the risk of IRIS, mainly from TB, during ART. In the third study, it was observed that anemia is associated with a greater dissemination of TB in PLHIV. In the three aforementioned studies, moderate/severe anemia are risk factors for death. The fourth study demonstrated that low hemoglobin levels were associated with higher DIP in HIV-TB patients. Next, we observed that HIV-TB patients with persistent anemia have a higher risk of dying than non-anemic patients during anti-TB treatment (ATT). This last result was reinforced by the sixth study, where moderate/severe anemia pre-ATT was a risk factor for death. **CONCLUSIONS**: Together, the manuscripts of this thesis add important information regarding the importance of anemia in the context of HIV-TB co-infection. By identifying moderate and severe anemia as a risk factor for incident TB, IRIS, disseminated TB, inflammatory disturbance, and death, we demonstrate that anemic PLHIV should be carefully monitored and, if possible, anemia should be carefully evaluated as a hallmarker of possible opportunistic infection and worse prognosis. In this context, this information is useful in directing new approaches to optimize clinical management and improve the quality and life expectancy of PLHIV.

**Keywords**: Tuberculosis. HIV. Anemia. Systemic inflammation. Outcome.

# LISTA DE FIGURAS

# LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| **AFP** | Anemia Ferropriva |
| **AIC** | Anemia por Inflamação Crônica |
| **CCR5** | Receptor de Quimiocina Tipo 5 |
| **CD4** | Cluster of Differentiation |
| **CMV** | Citomegalovírus |
| **CXCR4** | Receptor de Quimiocina 4 |
| **DNA** | Ácido Desoxiribonucleico |
| **GPI** | Grau de Perturbação Inflamatória |
| **Hb** | Hemoglobina |
| **HBV** | Vírus da Hepatite B |
| **HCV** | Vírus da Hepatite C |
| **HIV** | Human Immunodeficiency Virus |
| **IC** | Intervalo de Confiança |
| **IIQ** | Intervalos Interquartis |
| **IL** | Interleucina |
| **IO** | Infecção Oportunista |
| **ISP-HIV** | Inflamação Sistêmica Persistente Por HIV |
| **LT CD4+** | Linfócito T Auxiliar |
| **LT CD8+** | Linfócito T Citotóxico |
| **MAC** | Complexo *Mycobacterium Avium* |
| **Mtb** | Mycobacterium Tuberculosis |
| **OMS** | Organização Mundial da Saúde |
| **PCR** | Proteína C Reativa |
| **PTB** | Tuberculose Pulmonar |
| **PVHIV** | Pessoas Vivendo com HIV |
| **RNA** | Ácido Ribonucleico |
| **SIDA** | Síndrome da Imunodeficiência Adquirida |
| **SIRI** | Síndrome da Imunorreconstituição Inflamatória |
| **TARV** | Terapia Antiretroviral |
| **TAT** | Tratamento Antitubercular |
| **TB** | Tuberculose |

| | |
|---|---|
| **TNF** | Tumoral Necrosis Factor |
| **TR** | Transcriptase Reversa |
| **VHS** | Velocidade de Hemossedimentação |

## SUMÁRIO

**MANUSCRITOS**

ESTA TESE É BASEADA NOS SEGUINTES MANUSCRITOS

1. Impact of the relationship between anaemia and systemic inflammation on the risk of incident tuberculosis and death in persons with advanced HIV: A sub-analysis of the REMEMBER trial
2. Severe anemia in persons with HIV leads to a distinct inflammatory profile that increases the risk of immune reconstitution inflammatory syndrome during antiretroviral therapy.
3. Relationship between anemia and systemic inflammation in people living with HIV and tuberculosis: a sub-analysis of the CADIRIS clinical trial.
4. Severe anaemia as an indicator of tuberculosis dissemination, systemic inflammation and a predictor of mortality in persons with advanced HIV: a prospective cohort study.
5. Impact of Persistent Anemia on Systemic Inflammation and Tuberculosis Outcomes in Persons Living With HIV.
6. Effect of anemia on anti-tuberculosis treatment outcome in persons with pulmonary tuberculosis: a multi-center prospective cohort study.

# 1 **INTRODUÇÃO**

## 1.1 HIV: Um grande problema de saúde pública mundial

### 1.1.1 Descoberta do HIV e SIDA

A infecção pelo vírus da imunodeficiência humana (HIV, do inglês *human immunodeficiency virus)* é um grande problema de saúde pública mundial que se espalhou possivelmente no início do século XX (GAO et al., 1999; KEELE et al., 2006). Curiosamente, o conhecimento da existência desse vírus é relativamente novo, já que foi descoberto apenas na década de 1980 e descrito pela primeira vez por pesquisadores do Instituto Pasteur (França) como a Dra. Françoise Barré-Sinoussi e Dr. Luc Montagnier (BARRÉ-SINOUSSI et al., 1983), que anos depois foram laureados pelo prêmio Nobel por conta deste grande feito.

O interesse que levou à descoberta do HIV surgiu a partir de um súbito aumento de casos de imunodeficiência avançada de causa desconhecida, principalmente em homens, no início da década de 1980. Essa patologia foi descrita como uma síndrome de imunodeficiência adquirida (SIDA, ou AIDS do inglês *adquired immunodeficiency syndrome)* e dois anos após a sua descrição, o HIV foi descrito como seu agente causador (BARRÉ-SINOUSSI et al., 1983; GALLO et al., 1983).

Atualmente, estima-se que existam 37,7 milhões de pessoas vivendo com HIV (PVHIV) globalmente (**Figura 1**). A cada ano, aproximadamente 1,5 milhão de pessoas são infectadas e cerca de 690.000 morrem por complicações causadas pela infecção (UNAIDS, 2021). O número atual de PVHIV é 47% maior que no início do século XXI, tendo em vista que em 2000 foram estimados 25.5 milhões de PVHIV em todo o mundo. Isso reflete a contínua transmissão do HIV, apesar de reduções na incidência, e do acesso significativamente ampliado à terapia antirretroviral (TARV), que ajudou a reduzir o número de mortes relacionadas ao HIV desde então (UNAIDS, 2021). Dado que a infecção atinge principalmente adultos em idade economicamente ativa (25-49 anos), além dos danos à saúde do indivíduo infectado, o alastramento da infecção pode também impactar a economia global (DEEKS et al., 2015).

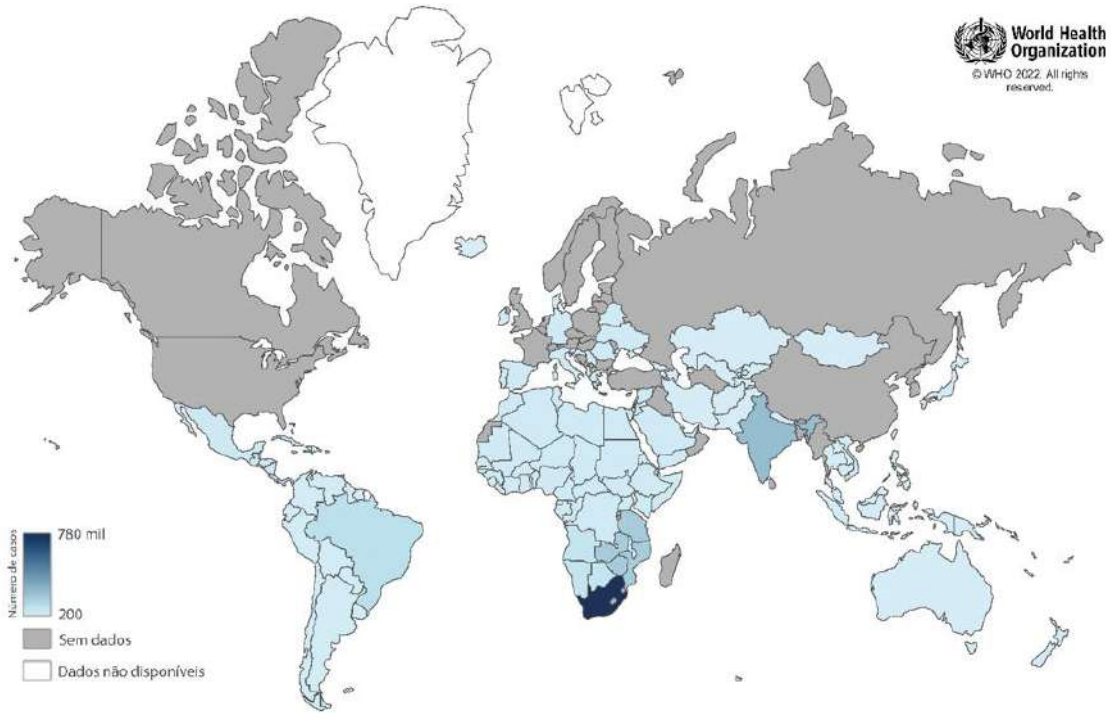


**Figura 1** - Distribuição global de casos estimados de HIV (todas as idades) em 2021. O mapa foi criado utilizando dados atualizados (acesso em maio de 2022) do Observatório Global de Saúde da Organização Mundial de Saúde.
**Fonte**: (WHO, 2022)

### 1.1.2 História natural da infecção por HIV

HIV é um retrovírus envelopado que pertence à família *Retroviridae*, gênero *Lentivirus* (BARRÉ-SINOUSSI et al., 1983). Pela sua natureza retroviral, esse vírus é capaz de integrar o seu material genético no genoma do hospedeiro, o que dificulta a sua eliminação (DEEKS et al., 2015). O HIV pode ser do tipo 1 (HIV-1) ou do HIV-2. O HIV-1 é mais prevalente e mais patogênico do que o HIV-2 e é responsável pela grande maioria da pandemia global (WORLD HEALTH ORGANIZATION, 2021).

O vírus infecta células que expressam a molécula CD4, como os linfócitos T auxiliares (LT $CD4^+$), monócitos, macrófagos e células dendríticas. A glicoproteína do envelope viral interage com CD4 presente na superfície celular e o receptor de quimiocina 4 (CXCR4) ou 5 (CCR5), o que possibilita a sua entrada na célula **(Figura 2)**. Depois de infectar a célula, o RNA viral de fita simples é transcrito reversamente em DNA, que é então integrado ao DNA do hospedeiro. Utilizando a maquinaria do hospedeiro, o HIV é transcrito, as proteínas são produzidas e clivadas e os vírions maduros são liberados (ENGELMAN; CHEREPANOV, 2012).

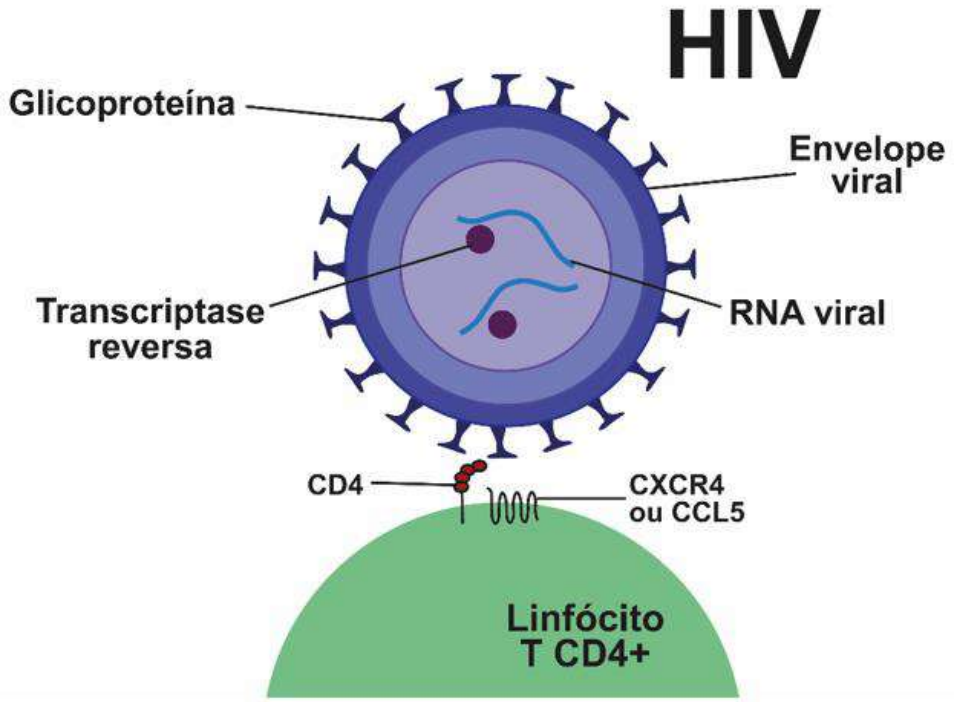


**Figura 2** - Estrutura do HIV e interação entre a glicoproteína do vírus e os receptores e correceptores de superfície dos linfócitos T CD4+.
**Fonte**: Elaboração da autora

Os principais alvos para a infecção são os LT $CD4^+$ ativados, que são mais permissivos à infecção do que as células em repouso. A infecção causada por HIV é caracterizada então pela perda progressiva de LT $CD4^+$, o que – dado o seu importante papel na resposta imune adaptativa - conduz a facilitação da ocorrência de infecções oportunistas. As células dendríticas são mais difíceis de ser infectadas se comparadas aos LT, mas apresentam um importante papel na disseminação do vírus ao "capturar" e transportá-lo para os linfonodos, onde irá infectar outros LT (WU; KEWALRAMANI, 2006).

O curso clínico de infecção por HIV inclui três estágios: infecção primária, latência clínica e SIDA. A infecção primária, como o nome sugere, ocorre a partir da transmissão do vírus para um hospedeiro ainda não infectado. Essa transmissão ocorre a partir do contato com fluídos corporais provenientes de um indivíduo infectado, como sangue, sêmen, fluído pré-seminal, retal, vaginal ou leite materno (UNAIDS, 2021).

Após um evento de transmissão, o HIV se instala nos tecidos da mucosa e rapidamente se espalha para os órgãos linfoides. Nas primeiras semanas após a infecção, surgem os sintomas agudos e inespecíficos, com extensiva viremia e elevado número de LT CD4+ infectados. O sistema imunológico então responde à infecção primária, principalmente com a ativação de LT citotóxicos (LT CD8+) até atingir o controle da infecção, onde um estado de latência clínica é

estabelecido. Esse estado é caracterizado pelo baixo nível de replicação do HIV e baixo número de células infectadas, que pode durar cerca de uma década. Durante esse período, mesmo em baixo número, a replicação continua ocorrendo, principalmente nos tecidos linfoides. Isso provoca a redução gradual de LT CD4+ e aumento da carga viral. Progressivamente surge uma imunodeficiência profunda e os indivíduos desenvolvem complicações infecciosas a partir de infecções oportunistas que caracterizarão o último estágio da infecção: SIDA (WORLD HEALTH ORGANIZATION, 2021).

A SIDA é definida por uma contagem de LT $CD4^+$ abaixo de 200 células/microlitro (µL) de sangue ou pela identificação de pelo menos uma doença definidora de AIDS, a exemplo da tuberculose (TB) (WORLD HEALTH ORGANIZATION, 2021). TB é uma das doenças definidoras de AIDS mais frequentemente diagnosticada, representando até 50% dos casos a depender da população estudada (BELLAMY; SANGEETHA; PATON, 2004; REINHARDT et al., 2017; TANAKA et al., 2021).

### 1.1.3 Tratamento antirretroviral e síndrome de imunoreconstituição inflamatória

A epidemia da SIDA se estabilizou nas últimas décadas, com redução na incidência e na mortalidade relacionada à síndrome. Mesmo com a estabilização, devido ao seu caráter crônico, a infecção por HIV segue sendo um grande problema de saúde pública, principalmente em países em desenvolvimento. Mais de 95% das infecções causadas por HIV concentram-se nesses países e 68% do total ocorrem na África subsaariana (UNAIDS, 2021).

O avanço mais crítico para a estabilização da epidemia foi o desenvolvimento e implementação da TARV, que levou a reduções significativas na morbimortalidade por meio da reconstituição imune e atenuação da ruptura homeostática, além da redução da incidência e da gravidade de infecções oportunistas em PVHIV. A OMS recomenda que a TARV seja aplicada a todos os pacientes infectados por HIV, independente da contagem de LT $CD4^+$. De acordo com o último boletim da OMS, cerca de 70% dos PVHIV estão em uso da TARV (WORLD HEALTH ORGANIZATION, 2021).

Atualmente existem 46 drogas ARV aprovadas pelo FDA que estão disponíveis para o tratamento da infecção por HIV (FOOD & DRUG ADMINISTRATION, 2020). Estas drogas pertencem a cinco diferentes classes, correspondentes ao tipo de atuação: (1) inibidores nucleosídeos e nucleotídeos da transcriptase reversa (TR); (2) Inibidores não-nucleosídeos da

TR; (3) Inibidores de protease; (4) Inibidor da integrase e (5) Inibidores de entrada (FOOD & DRUG ADMINISTRATION, 2020).

No entanto, apesar dos resultados significativos obtidos a partir da TARV, em um subconjunto de PVHIV o início do tratamento pode desencadear piora clínica com ativação imunológica contra as infecções oportunistas já existentes, caracterizada pela produção descontrolada de citocinas. Esse quadro é conhecido como síndrome de imunoreconstituição inflamatória (SIRI) (CEVAAL; BEKKER; HERMANS, 2019; SHARMA; SONEJA, 2011).

A redução da capacidade imune que ocorre em PVHIV sem tratamento permite a reativação de infecções por patógenos dormentes e aumenta a suscetibilidade do indivíduo a novas infecções oportunistas por vírus, bactérias, fungos e protozoários (BOULOUGOURA; SERETI, 2016). Combinando esse ambiente inflamatório desregulado com a reconstituição imune que ocorre após o início da TARV, pode haver o desenvolvimento da SIRI (SHARMA; SONEJA, 2011). A inflamação sistêmica persistente associada ao HIV (ISP-HIV) pode também levar à regulação inadequada da ativação inflamatória, contribuindo assim para rupturas homeostáticas sistêmicas (DEEKS et al., 2015).

SIRI é definida como uma condição que ocorre mais frequentemente em até 3 meses após o início da TARV, apesar de poder ocorrer após este período em pacientes que experimentam reconstituição imunológica tardia. Na sua forma mais frequente, esta síndrome é marcada por rápida deterioração clínica e com processos inflamatórios descontrolados, apesar da supressão da carga viral do HIV e aumento de LT CD4+. Podem ocorrer em dois cenários: (1) pelo desmascaramento de uma infecção oportunista clinicamente silenciosa (SIRI desmascarada); ou (2) pela deterioração do estado clínico após a introdução da TARV, apesar ter sido realizada uma terapia específica para o patógeno anteriormente identificado e do paciente ter apresentado uma melhora clínica inicial (SIRI paradoxal) (SHARMA; SONEJA, 2011).

Estudos anteriores indicam que, na ausência de tratamento, a ISP-HIV está associada a níveis sistêmicos aumentados de citocinas pró-inflamatórias, como interleucina (IL)-6, fator de necrose tecidual (TNF) e IL-1β (BORGES et al., 2015; NEUHAUS et al., 2010). Após o início da TARV, a maioria dos indivíduos apresenta declínio acentuado em algumas concentrações de citocinas circulantes, enquanto outros marcadores, como IL-6 e proteína-C reativa (PCR) permanecem elevados (HSU et al., 2016; NEUHAUS et al., 2010). Embora a reconstituição do sistema imunológico por meio de TARV seja fundamental para a redução da mortalidade em

PVHIV, a inflamação descontrolada através do desenvolvimento de SIRI pode levar rapidamente à deterioração clínica (**Figura 3**).

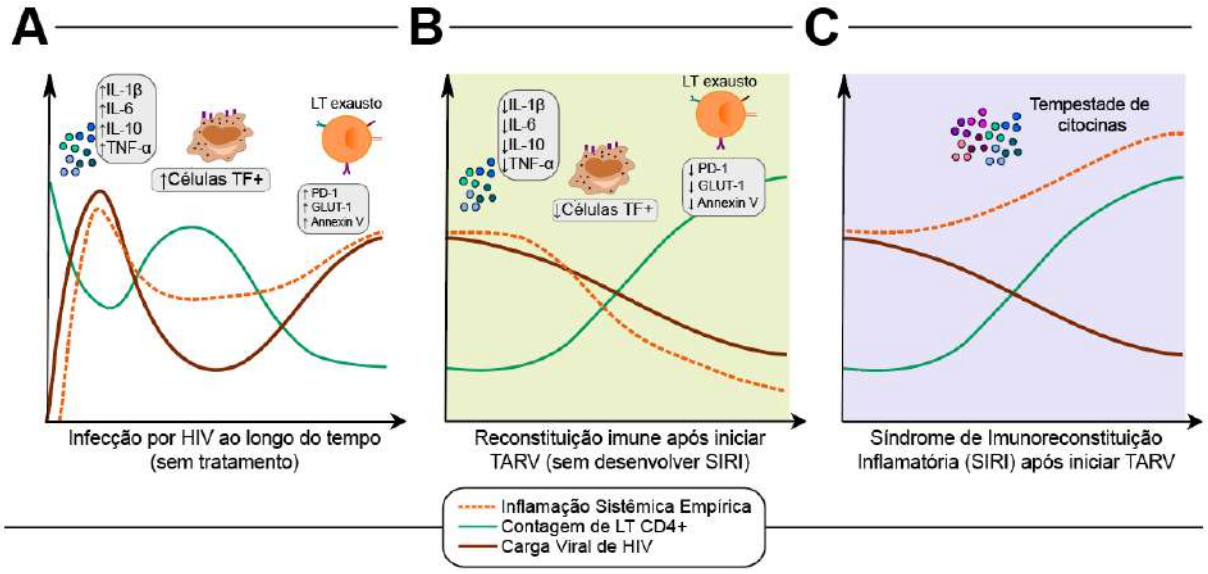


**Figura 3** - Influência da terapia antirretroviral (TARV) na inflamação sistêmica. (A) Após a infecção por HIV, há alta carga viral com concomitante diminuição da contagem de linfócitos T (LT) CD4+. Ao longo da infecção por HIV, há disfunção na ativação imunológica e exaustão de LT, resultando em inflamação sistêmica. (B) Após o início da TARV, ocorre uma restauração gradual da resposta imune, resultando em aumento de LT CD4+ e diminuição da carga viral. Este processo é seguido por inflamação sistêmica diminuída. (C) Com a síndrome inflamatória de reconstituição imune (SIRI), há melhora clínica inicial, seguida de deterioração marcada por aumento do nível de inflamação.
**Fonte**: adaptado de (VINHAES ET al., 2021).

## 1.2 Tuberculose: a infecção oportunista mais frequente em PVHIV

A infecção por bactérias do gênero *Mycobacterium* é a infecção oportunista mais comum associada à ocorrência de SIRI, principalmente quando se fala de *Mycobacterium tuberculosis (Mtb),* causadora da TB. Estima-se que 800.000 PVHIV estão coinfectadas por TB, e que cerca de 214.000 óbitos são registrados em decorrência da coinfecção HIV-TB, tornando essa a principal causa de morte nesta população (UNAIDS, 2021; WORLD HEALTH ORGANIZATION, 2021).

A TB é uma doença infectocontagiosa transmitida pelas vias aéreas e provocada principalmente por *Mtb.* A infecção por *Mtb* ocorre através do contato com partículas infectantes lançadas pela tosse de indivíduos com TB ativa (PAI et al., 2016). Ao entrar em contato com o bacilo, esses indivíduos inicialmente não-infectados podem resolver rapidamente a infecção, desenvolver a forma latente, a forma subclínica ou desenvolver a doença em sua

forma ativa, que pode ser restrita no pulmão ou apresentar sítios extrapulmonares (PAI et al., 2016).

PVHIV têm um risco aumentado em 20 vezes para o desenvolvimento de TB ativa (GETAHUN et al., 2010). Da mesma forma, foi relatado que a TB exacerba a infecção por HIV (MODJARRAD; VERMUND, 2010). PVHIV coinfectados por TB (HIV-TB) têm uma menor qualidade de vida e saúde (SAAG et al., 2004; VOLBERDING, 2002), além da aceleração do declínio das funções imunológicas e risco de morte substancialmente maior que aqueles monoinfectados (KWAN; ERNST, 2011; PAWLOWSKI et al., 2012).

A coinfecção HIV-TB pode levar ao aumento da inflamação sistêmica. A própria infecção por TB leva a alterações nas proteínas inflamatórias e nos mediadores lipídicos que persistem mesmo após o término da terapia anti-TB (TAT), culminando em intenso desequilíbrio inflamatório (OLIVEIRA-DE-SOUZA et al., 2019; VINHAES et al., 2019). Este processo promove a replicação do HIV através da modulação do metabolismo redox do hospedeiro. Em comparação a monoinfectados, aqueles HIV-TB expressam níveis mais elevados de citocinas inflamatórias como IL-6, TNF, IL-10 e IL-1β (TYAGI et al., 2020).

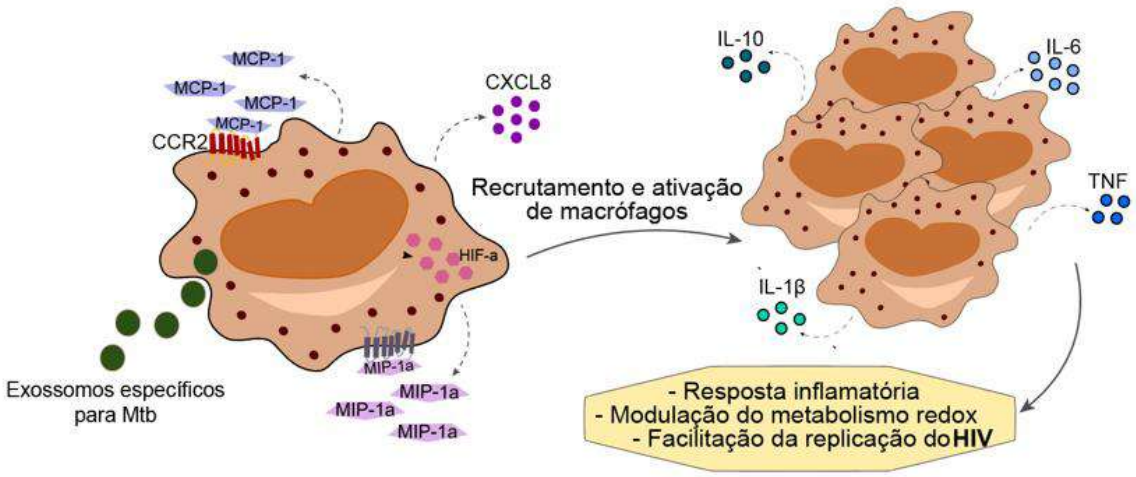


**Figura 4 -** A coinfecção HIV-TB facilita a replicação do HIV. A coinfecção HIV-TB aumenta os níveis de citocinas e quimiocinas produzidas por macrófagos ativados, levando ao recrutamento de macrófagos para o local da infecção. Esse mecanismo aumenta a produção de mediadores pró-inflamatórios, aumentando assim as respostas inflamatórias com modulação do metabolismo redox do hospedeiro que, em última análise, facilita a replicação do HIV.
**Fonte**: adaptado de (VINHAES et al., 2021).

## 1.3 **Anemia em pessoas com HIV: um agravo substancial**

A causa da anemia em PVHIV é multifatorial. O próprio vírus induz a ISP-HIV e sob essas condições inflamatórias, o ferro dietético é bloqueado da liberação de enterócitos, enquanto o ferro circulante é redistribuído em locais de armazenamento celular, como macrófagos. Além disso, a supressão de várias células hematopoiéticas e a ocorrência de infecções oportunistas contribuem ainda mais para a anemia durante a infecção por HIV (QUIROS-ROLDAN et al., 2017).

Como consequência da exacerbação da inflamação sistêmica, a anemia pode se desenvolver e afetar o sucesso do tratamento com terapia anti-TB e/ou antirretroviral, além de poder aumentar o risco de desenvolvimento de SIRI. Em indivíduos monoinfectados por HIV ou por TB, a progressão das doenças e morte são comumente associadas a um menor nível de concentração de Hb no sangue periférico (BELPERIO; RHEW, 2004; MOCROFT et al., 1999; WENGER et al., 1988).

De acordo com a Organização Mundial da Saúde (OMS), a anemia é uma condição caracterizada por baixos níveis de Hemoglobina (Hb) no sangue e pode ser classificada com base na gravidade de declínio da Hb em três graus: leve, moderada e grave. A anemia leve é definida para adultos como concentração de Hb entre 11,0-11,9 g/dL para mulheres não grávidas e entre 11,0-12,9 g/dL para homens. A anemia moderada, por sua vez é definida como o valor de Hb de 8,0–10,9 g/dL, enquanto a anemia grave é definida como o valor de Hb inferior a 8,0 g/dL para ambos os sexos (WORLD HEALTH ORGANIZATION, 2011).

A inflamação crônica contribui para o desenvolvimento de anemia e faz com que ela seja a anormalidade hematológica mais comum em PVHIV, podendo ser um fator preditivo de morbidade e mortalidade associadas ao HIV, independentemente da contagem de CD4 (BELPERIO; RHEW, 2004; MCDERMID et al., 2013; SHIVAKOTI et al., 2015). A anemia por inflamação crônica (AIC), é uma síndrome clínica caracterizada pelo desenvolvimento de anemia como consequência de uma doença crônica como infecção por HIV, doenças inflamatórias, auto imunes e neoplásicas (GANZ, 2019). Nestes casos, a patologia é caracterizada por hipocromia normocítica leve a moderada e está associada à diminuição dos níveis de ferro sérico e da capacidade total de ligação do ferro, bem como ao aumento dos níveis de ferritina (GANZ, 2019).

A anemia reduz a qualidade de vida e pode impactar diretamente na efetividade da resposta imunológica frente a infecções (JONKER; BOELE VAN HENSBROEK, 2014). Em indivíduos anêmicos, a resposta imune mediada por células e a capacidade bactericida dos leucócitos são significativamente suprimidas, aumentando a morbimortalidade de forma geral (VALDEZ et al., 2009). Em PVHIV sem outras coinfecções identificadas, a anemia pode ser um fator preditivo de desfecho (MOCROFT et al., 1999). Além disso, esses achados também foram encontrados em indivíduos apenas com TB, onde já foi demonstrado que a anemia está associada a níveis aumentados de biomarcadores inflamatórios (GIL-SANTANA et al., 2019; MUKHERJEE et al., 2019; OLIVEIRA et al., 2014),

A associação entre anemia e progressão do HIV conhecida, mas não há muitos estudos prospectivos relatando como a gravidade da anemia está associada a marcadores inflamatórios e como afeta os resultados da TARV e desenvolvimento da SIRI. Além disso, identificar como a anemia está associada à progressão das doenças e como pode impactar na ocorrência de desfechos desfavoráveis de tratamentos (TAT e TARV) em indivíduos coinfectados é de grande importância para a saúde pública. Uma melhor compreensão do efeito da gravidade da anemia em PVHIV pode fornecer *insights* sobre a otimização do manejo clínico para minimizar o risco de SIRI e mortalidade nessa população.

## 2 JUSTIFICATIVA

Apesar do avanço significativo no combate ao HIV a nível mundial, a exemplo da expansão do oferecimento e implementação de TARV e do surgimento de políticas públicas locais e globais a fim de melhorar a qualidade de vida e erradicar a infecção, anualmente cerca de 690 mil vidas ainda são ceifadas anualmente com causas de mortes relacionadas a essa infecção. No total, cerca de 33 milhões de pessoas já morreram em decorrência da infecção por HIV no mundo (UNAIDS, 2021). Assim, ainda são necessárias investigações que visem identificar e estudar fatores de risco associados à infecção que possam impactar no desfecho de tratamento, a fim de posteriormente elucidar como esses fatores podem ser controlados visando a melhora da qualidade de vida e diminuição da morbimortalidade dos PVHIV. Um desses fatores é a anemia.

Embora o exame de quantificação de Hb seja realizado na rotina clínica, não se compreende totalmente como a anemia afeta o perfil da inflamação sistêmica dos pacientes, como afeta o curso das doenças concomitantemente e como pode ser um fator determinante para o desfecho terapêutico em relação a TAT ou a TARV. Ainda que diagnóstico de anemia com mensuração de Hb seja de baixo custo e amplamente disponível em ambientes clínicos, não há no momento diretriz ou política estabelecida para considerar pacientes anêmicos como o grupo de risco para desenvolvimento de TB ativa ou para o aumento da ocorrência de desfechos desfavoráveis de tratamento como desenvolvimento de SIRI, falha terapêutica e morte em PVHIV. Portanto, a realização de estudos que investiguem o impacto da anemia e seus diferentes graus de gravidade na progressão do HIV e desenvolvimento de TB em PVHIV é de extrema importância.

Por fim, dentre os poucos estudos sobre anemia em PVHIV publicados, a avaliação dos pacientes ao longo do tratamento (estudos de coorte) é escassa (revisado por CAO et al., 2022). Além disso, para além dos marcadores clínicos comumente utilizados para anemia, como mensuração de Hb, essa tese se propôs a avaliar biomarcadores inflamatórios sistêmicos no sangue periférico e aspectos clínicos e socioepidemiológicos de diferentes coortes de PVHIV utilizando abordagens da biologia de sistemas, a fim de obter uma análise global que possibilite a predição de fatores determinantes de desfecho e associação da anemia com distintos perfis inflamatórios. Os resultados obtidos a partir desses estudos podem auxiliar na identificação de pacientes que necessitem de uma estratégia terapêutica distinta, impactando a pesquisa e a prática clínica em casos de HIV, principalmente em indivíduos co-infectados com TB.

## 2.1 Hipótese

PVHIV anêmicos possuem um perfil inflamatório sistêmico mais acentuado e, por consequência, um maior risco de desenvolver TB, assim como de apresentar desfechos desfavoráveis de tratamento como desenvolvimento de SIRI, maior progressão das doenças (HIV e/ou TB) e morte.

# 3 OBJETIVOS

### 3.2.1 Objetivo geral

Avaliar se a gravidade da anemia influencia no desenvolvimento de TB em PVHIV e como está relacionada aos desfechos desfavoráveis de tratamento antirretroviral e antituberculose.

### 3.2.2 Objetivos específicos

1. Examinar a influência da gravidade da anemia no desenvolvimento de TB e óbito em PVHIV;
2. Identificar se os graus de anemia estão associados desenvolvimento de SIRI e óbito após o início do TARV;
3. Caracterizar o perfil inflamatório sistêmico de pacientes HIV-TB anêmicos;
4. Avaliar se há uma associação da gravidade da anemia com a disseminação da TB em pacientes HIV-TB;
5. Testar a associação da anemia e da inflamação sistêmica com o risco de desfecho de tratamento desfavorável em pacientes HIV-TB;
6. Avaliar a associação da gravidade da anemia com o risco de desfecho de tratamento desfavorável em pacientes TB (com e sem HIV);

# 4 METODOLOGIA

Para o desenvolvimento dos trabalhos que compõem essa tese foram utilizados dados de cinco coortes prospectivas de PVHIV e uma coorte de pacientes TB (com e sem HIV), como descrito no tópico I desta seção. Para cada uma dessas coortes foi utilizada uma metodologia de análise quantitativa dos dados, com metodologias de estatística descritiva e preditiva, análises de redes, aprendizado de máquina e estratégias de avaliação quantitativa de marcadores como a adaptação de uma metodologia matemática para o desenvolvimento do Grau de Perturbação Inflamatória (GPI), como explicado no tópico II desta seção.

## 4.1 COORTES

Os dados das coortes foram previamente coletados e disponibilizados por colaboradores para a confecção dos manuscritos desta tese. Nestas coortes constam dados de PVHIV anêmicos e não anêmicos, atendidos em centros de referências em 13 diferentes países: África do Sul, Brasil, Estados Unidos, Haiti, Índia, Malawi, México, Peru, Quênia, Tailândia, Uganda, Zâmbia e Zimbábue (**Figura 5**).

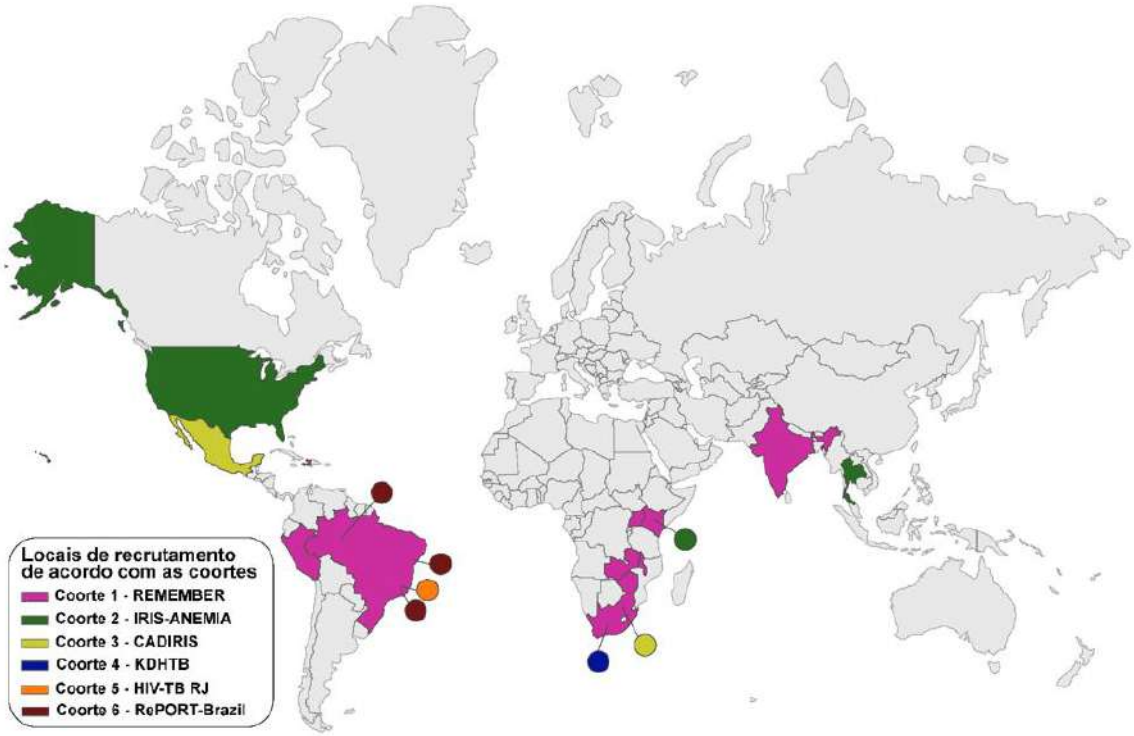


**Figura 5** - Países de recrutamento de PVHIV para os estudos, de acordo com cada uma das coortes estudadas. Em rosa estão os países referentes à coorte 1 (REMEMBER); em verde aqueles referentes a coorte 2 (IRIS-ANEMIA); em azul a África do Sul, local de recrutamento da coorte 3 (KDHTB); em amarelo os países

referentes à coorte 4 (CADIRIS), em laranja o local de recrutamento referente a coorte 5 (HIV-TB RJ), no Brasil; por fim, ainda no Brasil, estão em vermelho os locais de recrutamento da coorte 6 (RePORT-Brazil).
**Fonte**: Elaboração da autora

Os dados incluíram aspectos clínicos, laboratoriais e de biomarcadores desses pacientes, além de informações de desfecho como desenvolvimento de TB ativa, de SIRI, falha de tratamento, cura, abandono e óbito. As informações referentes ao número de pacientes, frequência de anêmicos, assim o critério de contagem de LT CD4+ e desfecho estudado estão sumarizados no **quadro 1** para cada coorte.

**Quadro 1** - Dados de anemia, critério de CD4 e desfechos para cada coorte.

| | | N | % de anêmicos | Critério de CD4 | Desfecho do estudo | Referência da coorte |
|---|---|---|---|---|---|---|
| | Coorte 1 – REMEMBER | 269 | 76.2% | <50 | TB ativa | (HOSSEINIPOUR et al., 2016) |
| | Coorte 2 – IRIS-ANEMIA | 502 | 83.7% | <100 | SIRI/Óbito | (SERETI et al., 2020) |
| | Coorte 3 – CADIRIS | 159 | 71.0% | <100 | Perturbação Inflamatória | (SIERRA-MADERO et al., 2014) |
| | Coorte 4 – KDHTB | 496 | 92.7% | <350 | Disseminação/ Óbito | (SCHUTZ et al., 2019) |
| | Coorte 5 – HIV-TB RJ | 191 | 85.6% | - | Pert. Inflamatória/ Desfecho de TAT | (DEMITTO et al., 2019) |
| | Coorte 6 – RePORT-Brasil | 786 | 56% | - | Desfecho de TAT | (ARRIAGA et al., 2021) |

Nota de quadro: O critério de contagem de CD4 considerou a unidade de medida células/$mm^3$.
Abreviaturas: TB – tuberculose; SIRI: síndrome de imunoreconstituição inflamatória; TAT: tratamento anti-TB.
**Fonte**: Elaboração da autora

Cada uma das seis coortes estudadas deu origem a um manuscrito dessa tese, que responde a cada um dos objetivos específicos listados na seção 3, e em conjunto respondem ao objetivo geral proposto. Em todas as coortes, os protocolos utilizados foram aprovados pelos comitês de ética dos centros de recrutamento, e todos os participantes assinaram o Termo de Consentimento Livre e Esclarecido (TCLE) correspondente a cada estudo.

### 4.1.1 Manuscrito I

A coorte utilizada nesse estudo foi obtida a partir do ensaio clínico randomizado aberto REMEMBER (Identificador: NCT0138008), que recrutou 269 PVHIV virgens de TARV e os acompanhou por 4 anos (48 meses). Os pacientes foram arrolados entre os anos de 2011 e 2014, e atendidos em 18 ambulatórios de pesquisa distribuídos em dez países: África do Sul, Brasil, Haiti, Índia, Malawi, Peru, Quênia, Uganda, Zâmbia e Zimbábue. Os critérios de inclusão para

esses estudos foram: pacientes com diagnóstico de HIV, virgens de TARV, com idade ≥13 anos, contagens de LT CD4+ <50 células/mm$^3$, que não tinham evidência de TB ativa e que eram elegíveis para tratamento preventivo de TB. Os critérios de exclusão foram o uso de dose única de nevirapina nos 2 anos anteriores, recebimento de tratamento de TB ou preventivo dentro de 96 e 48 semanas antes da entrada no estudo, respectivamente, e histórico de exposição domiciliar a TB-multidroga resistente. Para as análises do manuscrito contido nessa tese, também excluímos pacientes sem dados de citocinas e perda de seguimento em 48 meses. Neste estudo, usamos apenas mensurações basais (baseline, no momento do arrolamento no estudo) de biomarcadores e resultados de desfecho relatados ao final de 48 meses. A coorte final deste estudo apresentou um número igual a 269 PVHIV **(Figura 6)**.

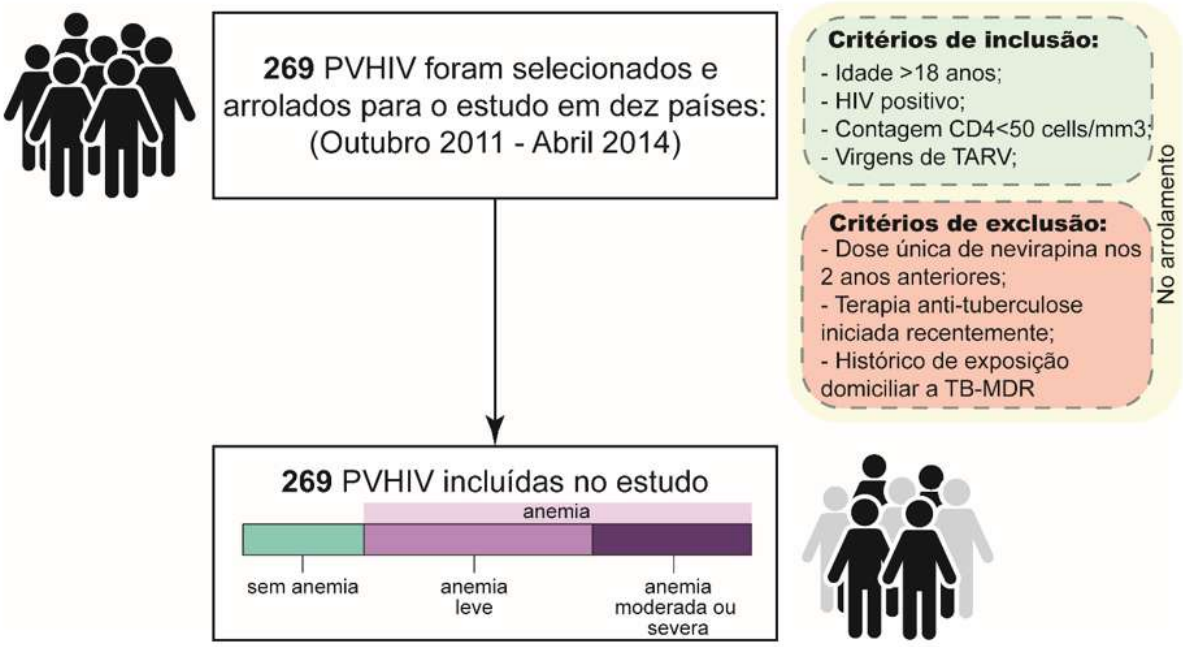


**Figura 6 - Fluxograma de estudo – Manuscrito I. 269 PVHIV foram arrolados para o estudo entre os** anos de 2011 e 2014 em um estudo multicêntrico realizado em dez países. Dentre os participantes, 76.2% eram anêmicos.
**Fonte**: Elaboração da autora.

### 4.1.2 Manuscrito II

Trata-se de uma coorte prospectiva, que foi criada e acompanhada durante um ensaio clínico realizado em três países: Estados Unidos, Quênia e Tailândia; e registrado no site de ensaios clínicos do NIH (Identificador: NCT00286767). Foram arrolados 506 pacientes, que foram acompanhados desde o início da TARV (semana 0) por até 6 meses (24 semanas), e durante esse período foram registrados desfechos como desenvolvimento de SIRI ou morte.

Cada centro de pesquisa clínica recrutou pessoas com idade maior ou igual 18 anos, com diagnóstico de infecção por HIV, contagem de LT CD4+ ≤100/μL e sem TARV anterior. Os critérios de exclusão foram gravidez e abuso de substâncias. O momento e o regime de TARV foram escolhidos de acordo com as diretrizes locais de tratamento. As equipes clínicas nos locais de estudo identificaram a ocorrência de SIRI e os apresentaram a um comitê de revisão de desfechos. Os critérios e procedimentos diagnósticos foram descritos na figura abaixo **(Figura 7)**.

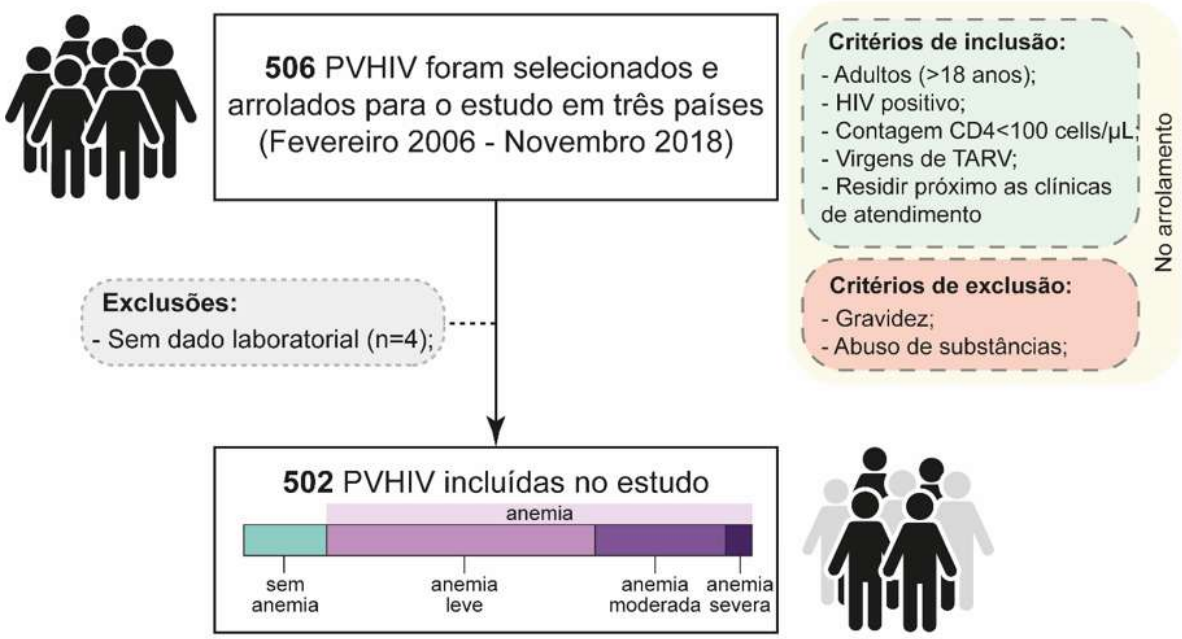


**Figura 7** - Fluxograma de estudo – Manuscrito II. 506 PVHIV foram arrolados para o estudo, entretanto 4 foram removidos das análises deste manuscrito por não cumprirem os critérios necessários, restando 502 PVHIV. Destes, 83.7% eram anêmicos.
**Fonte**: Elaboração da autora

### 4.1.3 Manuscrito III

A coorte prospectiva desse estudo foi proveniente de um estudo ensaio clínico randomizado duplo-cego (Identificador: NCT00988780) que recrutou PVHIV e os acompanhou por 1 ano (12 meses). Os pacientes foram arrolados entre os anos de 2009 e 2012 em cinco centros clínicos no México e da África do Sul para o ensaio denominado CADIRIS (CCR5 *Antagonism to Decrease the Incidence of IRIS in HIV*). Os pacientes elegíveis eram aqueles infectados por HIV, com idade mínima de 18 anos, virgens de TARV e com contagem de células LT CD4+ igual ou inferior a 100/μL. Pacientes com Hb <8g/dL foram excluídos. Para o manuscrito utilizamos apenas os dados de base (semana 0) e incluímos apenas pacientes com

doenças definidoras de AIDS ou síndrome debilitante associada ao HIV no momento da inscrição, resultando em um número total de 159 PVHIV **(Figura 8)**.

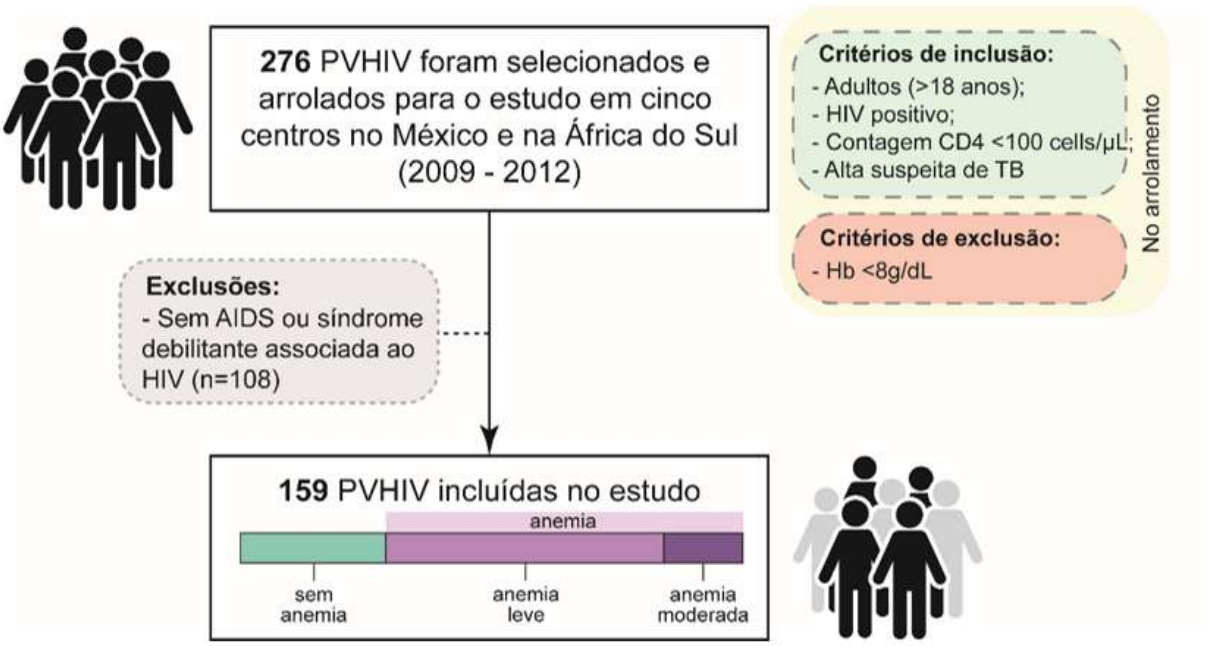


**Figura 8** - Fluxograma de estudo – Manuscrito III. 267 PVHIV foram arrolados para o estudo, entretanto 108 foram removidos das análises deste manuscrito por não cumprirem os critérios necessários, restando 159 PVHIV. Destes, 71% eram anêmicos.
**Fonte**: Elaboração da autora

### 4.1.4 Manuscrito IV

Para a confecção desse manuscrito foi utilizada dados de uma coorte prospectiva composta inicialmente por 659 PVHIV hospitalizados e com suspeita de TB, inscritos em uma mediana de 2 dias (IQR:1-3) após a apresentação em um hospital de referência da Cidade do Cabo, África do Sul. Os pacientes foram arrolados entre os anos de 2013 e 2016. Os critérios de inclusão foram: pacientes com diagnóstico de HIV, com idade ≥18 anos, contagens de CD4 <350 células/mm3, com alta suspeita de TB. Os critérios de exclusão foram: gravidez e TAT recente. Para o manuscrito, incluímos apenas casos de TB confirmados ou prováveis, excluindo possíveis diagnósticos e pacientes TB. Também excluímos pacientes sem dados de citocinas e perda de seguimento ao longo de 6 meses (84 semanas), resultando em 496 PVHIV **(Figura 9)**.

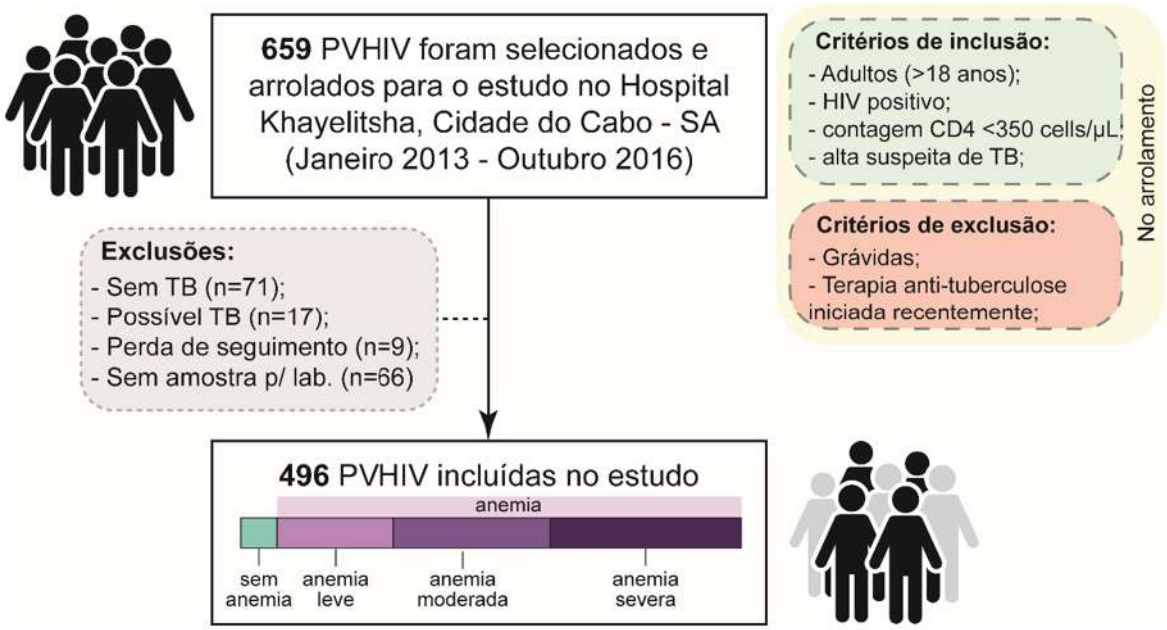


**Figura 9** - Fluxograma de estudo – Manuscrito IV. 659 PVHIV foram arrolados para o estudo, entretanto 193 foram removidos das análises deste manuscrito por não cumprirem os critérios necessários, restando 496 PVHIV. Destes, 92.7% eram anêmicos.
**Fonte**: Elaboração da autora

### 4.1.5 Manuscrito V

Uma coorte prospectiva vem sendo acompanhada no Laboratório de Pesquisa Clínica em Micobactérias (LAPCLIN-TB) do Instituto Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, Brasil, desde 2000 até o momento. O presente estudo é uma avaliação retrospectiva realizada com dados de 2008 e 2016 obtidos desta coorte. Os dados foram coletados de prontuários eletrônicos com base em informações padronizadas registradas em cada visita de cada paciente. Foram incluídas PVHIV com 18 anos ou mais, com sinais e sintomas clínicos de TB. O diagnóstico de TB foi feito quando a detecção de *Mtb* foi positiva em qualquer amostra coletada (esfregaço de bacilos álcool-ácido resistente, Gene Xpert ou cultura de espécimes clínicos). Nos casos sem confirmação bacteriológica, o diagnóstico foi estabelecido por exame de imagem sugestivo, exame histopatológico, juntamente com achados clínicos e epidemiológicos compatíveis com TB. Para aqueles que apresentaram cultura negativa, considerou-se teste terapêutico positivo com medicamentos para TB, após exclusão de outras doenças oportunistas para diagnóstico diferencial. Foram excluídos os pacientes que iniciaram o ATT e aqueles que foram diagnosticados posteriormente com micobactérias não tuberculosas,

bem como aqueles que apresentaram resistência à rifampicina e à isoniazida (multirresistência). Assim, a coorte final foi composta por 191 PVHIV **(Figura 10)**.

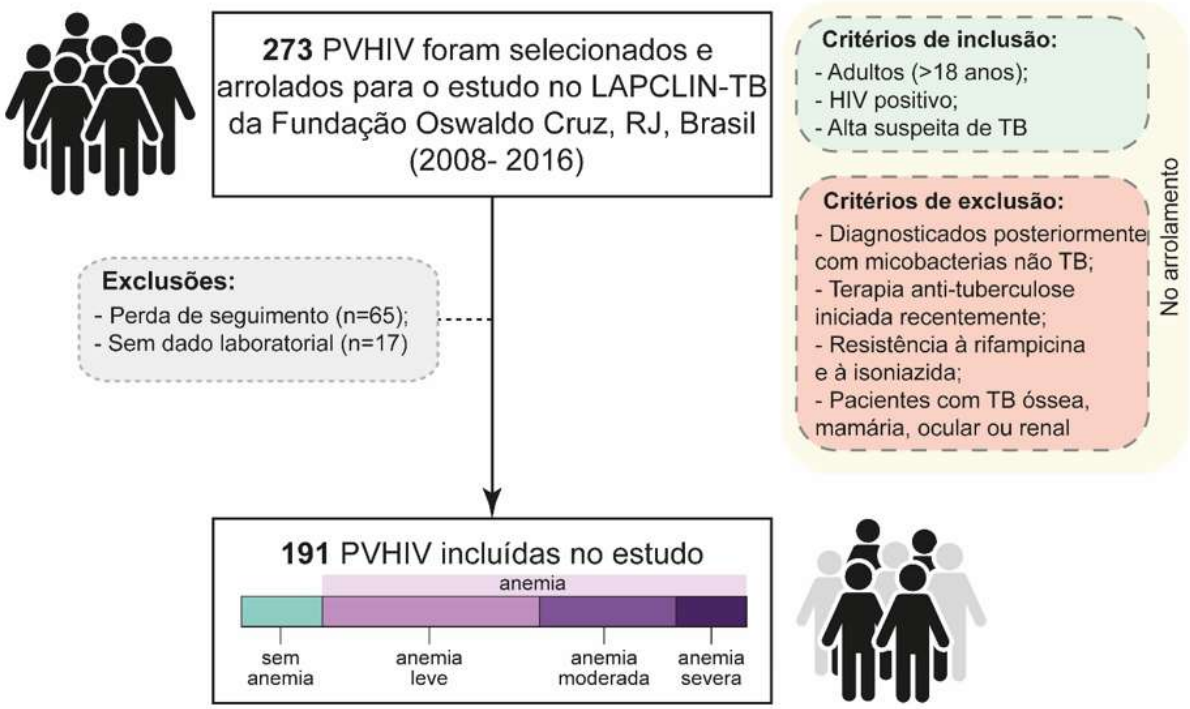


**Figura 10** - Fluxograma de estudo – Manuscrito V. 273 PVHIV foram arrolados para o estudo, entretanto 82 foram removidos das análises deste manuscrito por não cumprirem os critérios necessários, restando 191 PVHIV. Destes, 85.6% eram anêmicos.
**Fonte**: Elaboração da autora

### 4.1.6 Manuscrito VI

O Regional Prospective Observational Research in Tuberculosis (RePORT)-Brazil é um dos consórcios de pesquisa que compõem o consórcio RePORT-international, focado nos países do BRICS: RePORT-India, RePORT-África do Sul, RePORT-China, RePORT-Indonésia, RePORT-Filipinas. O objetivo primário do RePORT-Brazil é descrever os determinantes de desfechos clínicos do TAT no Brasil e a ocorrência de TB ativa e latente entre contatos próximos desses casos de TB. Os centros de recrutamento do RePORT estão localizados em três estados brasileiros (Amazonas, Bahia e Rio de Janeiro). A fase 1 do projeto arrolou 1187 pessoas com TB ativa entre 2015 e 2019. As informações epidemiológicas e amostras para exames laboratoriais foram coletadas durante as visitas do estudo. O banco de dados foi monitorado continuamente quanto à qualidade e integridade dos dados e atualizado conforme apropriado. No presente trabalho, utilizamos dados coletados pré-tratamento e o desfecho de tratamento em até 24 meses. Foram incluídos pacientes TB com 18 anos ou mais, com diagnóstico de TB através da cultura de escarro confirmada, HIV positivo ou negativo.

Foram excluídos os pacientes que não tinham dados de Hb, apresentaram resistência à rifampicina e/ou isoniazida, mulheres grávidas e pacientes sem informações de desfecho. Assim, a coorte final foi composta por 786 pacientes TB, com e sem coinfecção por HIV **(Figura 11)**.

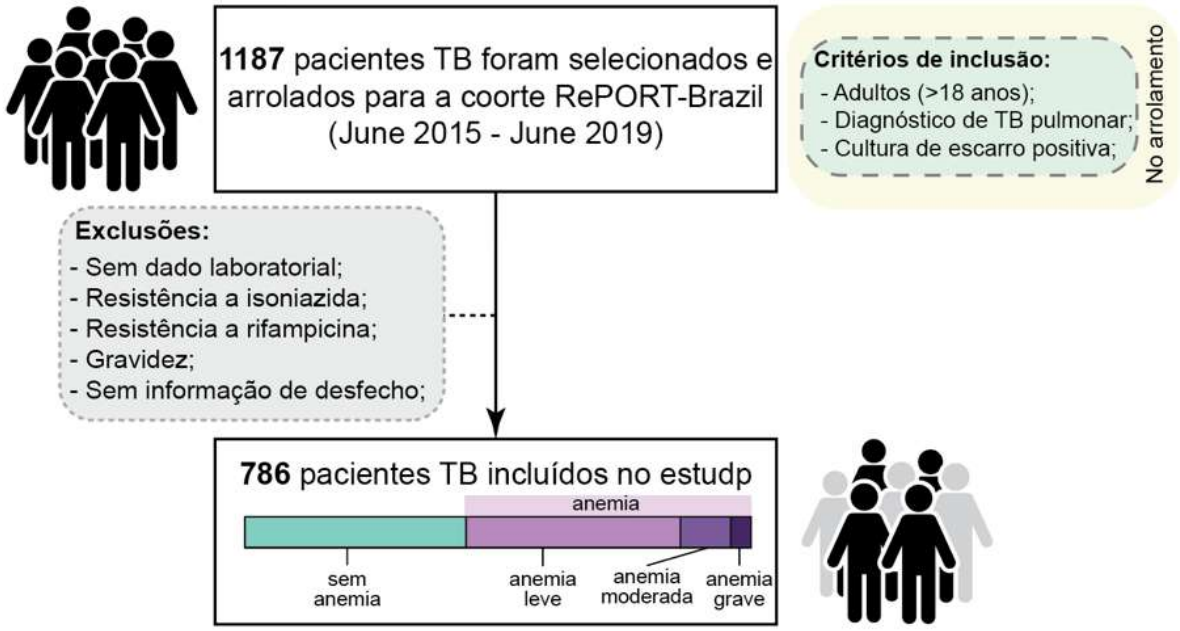


**Figura 11** - Fluxograma de estudo – Manuscrito VI. 1187 pacientes TB (com e sem HIV) foram arrolados para o estudo, entretanto 401 foram removidos das análises deste manuscrito por não cumprirem os critérios necessários, restando 786 pacientes TB. Destes, 56% eram anêmicos.
**Fonte**: Elaboração da autora

### 4.2 Grau de Perturbação Inflamatória

O grau de perturbação inflamatória (GPI) é baseado no grau de perturbação molecular, uma adaptação da distância molecular à saúde descrita anteriormente (GONÇALVES et al., 2019). Nos estudos realizados com as coortes 4 e 5, ao invés de usarmos os valores de expressão gênica, inserimos as concentrações de biomarcadores. Para o estudo 5, adicionalmente acrescentamos carga viral do HIV e contagem de células sanguíneas. Em ambos os casos, o cálculo de GPI foi utilizado seguindo a mesma lógica: a média e o desvio padrão de um grupo de referência foram calculados para cada parâmetro. O escore GPI de cada biomarcador individual foi definido pela normalização do escore Z, onde as diferenças nos níveis de concentração da média do grupo de referência foram divididas pelo desvio padrão de referência.

A pontuação GPI representa as diferenças por número de desvios padrão do grupo teste em relação ao grupo controle (**Figura 12**).

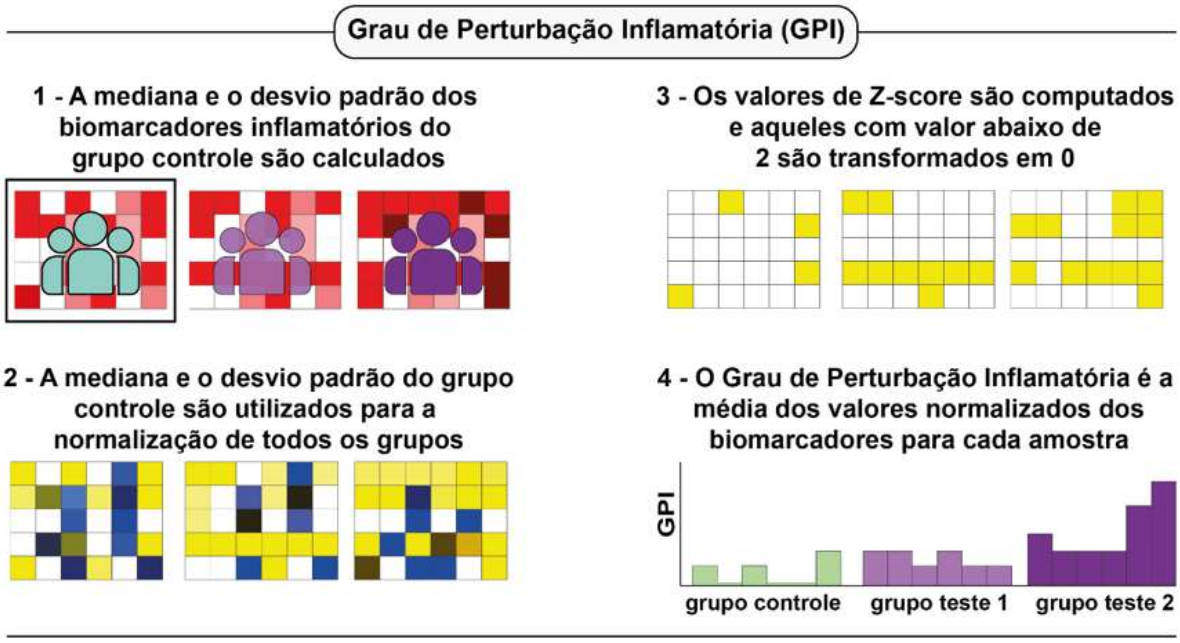


Figura 12 - Grau de perturbação inflamatória (GIP). GPI é baseado no Grau de Perturbação Molecular, mas ao invés de usar dados da expressão gênica, usamos mensurações de marcadores biomarcadores plasmáticos inflamatórios. O GPI foi calculado usando a mediana e o desvio padrão dos marcadores do grupo controle como ponto de partida. Em seguida, calculou-se o Z-score para todos os grupos, estabeleceu-se um ponto de corte e, por fim, realizou-se um cálculo de perturbação média para cada amostra.
**Fonte**: adaptada de (UNIVERSITY OF SÃO PAULO; UNIVERSITY OF CHILE, 2022)

### 4.3 Análise Estatística

Após a análise exploratória e etapas de limpeza e classificação (como classificação da anemia a partir dos níveis de Hb e transformação logarítmica de marcadores, quando necessário), foram realizados testes de normalidade e covariância dentro de cada um dos bancos. Em todas as coortes o resultado do teste de normalidade foi não normal (não paramétrico). Sendo assim, para a apresentação dos dados foi utilizada estatística descritiva, utilizando os valores da mediana e intervalos interquartis (IIQ) como medidas de tendência central e dispersão, respectivamente, para as variáveis contínuas. As variáveis categóricas foram descritas por meio de número absoluto e proporções (%).

Foram realizados diferentes testes estatísticos de acordo com o objetivo de cada análise e características de cada coorte. Para determinar a relação entre anemia e características clínicas e inflamatórias dos pacientes, foi utilizado o teste de Qui-quadrado para variáveis categóricas.

Além disso, para a comparação de variáveis contínuas em dois grupos distintos/não pareados (exemplo: anêmicos *vs* não-anêmicos), o teste de Mann–Whitney *U* foi utilizado. Em casos de estudos com mais de 2 grupos, como por exemplos naqueles onde os graus de anemia foram comparados (não-anêmicos, leve, moderada e/ou grave), foi utilizado o teste de Kruskall-Wallis. Adicionalmente, para realizar análises comparativas entre tempos de tratamento (exemplo: basal, durante e após o tratamento) foi utilizado o teste de Wilcoxon.

A análise de agrupamento hierárquico (método de Ward) usando valores de dados normalizados de escore z foi empregada para representar o perfil geral de expressão dos marcadores indicados nos subgrupos de casa um dos estudos. Nas análises que compõem essa tese, os dendrogramas representam a distância euclidiana (inferindo grau de similaridade). A determinação do GPI dos pacientes foi realizada conforme previamente descrito no tópico II dessa seção e a comparação entre os valores mensurados para cada grupo foi realizada pelo teste de Mann–Whitney *U*.

A fim de avaliar se biomarcadores de anemia estavam correlacionados a níveis de biomarcadores de inflamação, para cada grupo e tempo (quando existente) foi utilizado o teste de correlação de Spearman. Os dados de correlação foram utilizados para as análises de rede, onde parâmetros como densidade da rede e conectividade nos nodos também foram avaliados. Para avaliar a associação das características clínicas e dos biomarcadores de anemia e inflamação com o risco de desfechos desfavoráveis, foram realizados modelos de análise de regressão de Poisson, regressão logística binária e multivariada de acordo com os objetivos de cada manuscrito, para identificar determinantes independentemente associados aos desfechos de tratamento estudados. Os resultados foram apresentados na forma de Odds Ratio ajustado (aOR) e IC de 95%.

Além disso, uma análise por rede Bayesiana for utilizada para descrever e visualizar dependências condicionais entre múltiplas variáveis clínicas e inflamatórias. Variáveis contínuas foram discretizadas em inferior ou superior à média de toda a população do estudo. Logo, a relação avaliada na rede refere-se a medidas de maiores valores. O algoritmo de aprendizado usado para estabelecer a estrutura da rede foi baseada no método heurístico Hill-climb. As dependências foram representadas qualitativamente por um gráfico acíclico direcionado no qual cada nó está correspondendo a uma variável e um arco direto entre os nós representa influência direta. A robustez dos arcos foi avaliada usando um teste não-paramétrico *bootstrap* (100x replicado). Arcos com mais de 40% de suporte foram representados. As

associações mais fortes foram consideradas aquelas que permaneceram estatisticamente significativas em ≥50% dos *bootstraps*.

A análise Kaplan-Meier foi avaliada de acordo com o teste Breslow (Wilcoxon Generalizado) e aplicado para estimar a ocorrência de TB e probabilidade de morte em pacientes estratificados baseados no grau de anemia. Além disso, a análise Kaplan-Meier também foi utilizada para estimar a probabilidade de pacientes desenvolverem IRIS estratificados por grau de anemia e, nesse caso, calculada de acordo com o teste log-rank (Mantel-Cox).

Adicionalmente, o teste de permutação Jonckheere-Terpstra e teste assintótico (para séries temporais) foram usados para comparar variáveis contínuas ao longo do tempo, assim como análises de tendência. Para todos os testes, as diferenças com valores de p abaixo de 0,05 (em comparações bicaudais) foram consideradas estatisticamente significantes.

## 5 MANUSCRITOS

Os resultados encontrados durante o desenvolvimento do projeto resultaram em cinco manuscritos, de forma que cada um está associado a um dos objetivos específicos descritos na seção três (“3. Objetivos”) desta tese. Os principais resultados de cada um dos manuscritos estão sumarizados na **figura 13**, onde pode-se observar os seguintes pontos: (1) PVHIV apresentam uma alta prevalência de anemia, cuja prevalência de gravidade varia de acordo com a população estudada; (2) a anemia está associada ao desenvolvimento de TB e (3) de SIRI por micobactérias, (4) Pacientes HIV-TB anêmicos apresentam uma maior perturbação inflamatória, indicando uma resposta imune desordenada em comparação àqueles não-anêmicos ou apenas com HIV, (5) assim como foi encontrada uma correlação negativa entre os níveis de Hb e a disseminação do *Mtb*; (6) Pacientes anêmicos apresentam um maior risco de óbito se comparados aos não anêmicos, independente de outros fatores como contagem de CD4 e alteração de outros parâmetros bioquímicos. (7) Por fim, pacientes TB (com e sem HIV) com anemia apresentam não só um maior GPI como maior risco de desfecho desfavorável de TAT.

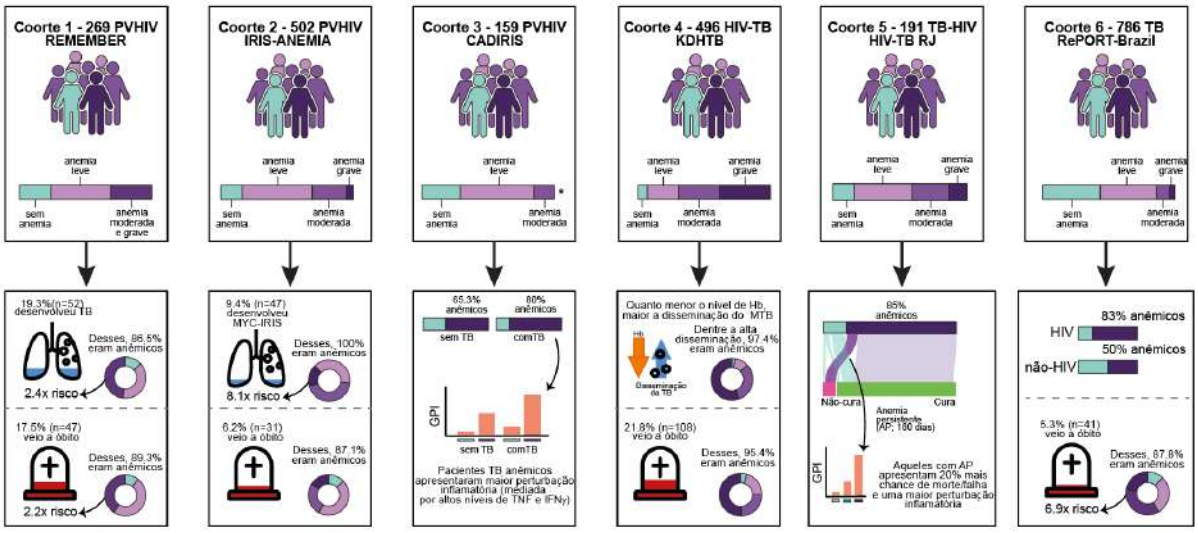


**Figura 13** - Representação gráfica dos principais resultados de cada uma das coortes estudadas. Os estudos das coortes 1 e 2 mostraram que PVHIV com anemia moderada e/ou grave apresentam mais risco de desenvolver TB, SIRI por micobacterias (MYC-IRIS) ou vir a óbito se comparadas àqueles sem anemia; A coorte 3 avaliou o perfil inflamatório de pacientes HIV-TB e mostraram que aqueles com anemia apresentam uma maior perturbação inflamatória sistêmica. A coorte 4 demonstrou que a anemia estava associada a uma maior disseminação da TB. A coorte 5 associou a anemia persistente com desfechos desfavoráveis de tratamento anti-TB, enquanto a coorte 6 demonstrou que pacientes HIV-TB apresentam maior frequência de anemia e que a anemia moderada/grave é um grande fator de risco para óbito nesses pacientes.
**Fonte**: Elaboração da autora

## 5.1 Manuscrito I

### Título

"Impact of the relationship between anaemia and systemic inflammation on the risk of incident tuberculosis and death in persons with advanced HIV: A sub-analysis of the REMEMBER trial"

### Objetivo

Esse trabalho teve como objetivo examinar a associação entre a ocorrência de anemia e de TB incidente em PVHIV.

### Resumo de resultados

A coorte utilizada nesse estudo foi proveniente do ensaio clínico REMEMBER, e composta por 269 PVHIV, dos quais 76,2% eram anêmicos. A partir de análises multidimensionais, curvas de sobrevida e rede bayesiana, delineamos associações entre anemia, parâmetros laboratoriais e desfechos clínicos. Nesse estudo foi observado que PVHIV com anemia moderada/grave exibiram um perfil inflamatório sistêmico mais acentuado que aqueles com anemia leve ou sem anemia, marcado por um aumento substancial nas concentrações plasmáticas de IL-6. A anemia moderada/grave também foi associada a TB incidente e óbito por TB. Além disso, os níveis de IL-6 foram maiores em pacientes que sofreram TB incidente e/ou morreram. A anemia moderada/grave é um fator de risco para TB incidente e aumenta significativamente o risco de morte. PVHIV com anemia devem ser monitoradas de perto para minimizar a ocorrência de desfechos desfavoráveis.

### Status do manuscrito

Este trabalho foi submetido ao periódico internacional ***The Lancet Microbe*** (Fator de Impacto JCR 2021 = 86,208).

# Impact of the relationship between anaemia and systemic inflammation on the risk of incident tuberculosis and death in persons with advanced HIV: A sub-analysis of the REMEMBER trial

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**Keywords**: tuberculosis; anaemia; death; haemoglobin; systemic inflammation;

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## ABSTRACT

**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 randomized clinical trial of antiretroviral-naïve PWH with CD4<50 cells/μL, from 18 outpatient research clinics in 10 low- and middle-income countries 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, survival curves, and Bayesian network analyses to delineate associations between anaemia, laboratory parameters, and clinical outcomes.

**Findings:** Of all participants, 76·2% were anaemic. 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 and death.

**Interpretation:** PWH with moderate/severe anaemia displays a distinct pro-inflammatory profile. Moderate/severe anaemia is a risk factor for incident TB and significantly increases the risk of death. PWH with anaemia should be monitored closely to minimize the occurrence of unfavourable outcomes.



**Funding:** National Institutes of Health and the Johns Hopkins Clinical Trial Unit.

**Keywords:** HIV, incident TB, anaemia, inflammation, death

**Research in context**

***Evidence before this study***

*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. It is still unknown if anaemia in these settings reflects a unique profile of immunopathological processes and the impact of this inflammatory disturbance on outcomes. Identifying risk factors for incident TB and death is important to provide insights into the optimization of the clinical management of these patients. We explored the relationship between haemoglobin levels, systemic inflammation, incident TB and death PWH according to different degrees of anaemia.*

***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***

*We demonstrate that moderate and severe anaemia as a risk factor for incident 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 2020, an estimated 1 million 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 treatment (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 characterized by low haemoglobin (Hb) levels (<12g/dL in women and <13g/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. 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 (10).

Anaemia is an independent prognostic indicator among PWH, associated with HIV disease progression (11–13). In previous studies of our group, we have linked anaemia with higher and sustained inflammatory perturbation in TB-HIV initiating ART (14). The increased inflammatory perturbation of TB-HIV anaemic individuals includes increased levels of IL-6 (15). 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 (16). 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 (17).

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 (14). 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 (15). 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 randomized clinical trial of antiretroviral-naïve PWH with CD4 <50 cells/μL (18,19), to investigate the influence of anaemia on the development of incident active TB or death following the initiation of ART and IPT versus empiric TB treatment. 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 at Johns Hopkins University and participating site institutions. The sponsors of the study had no role in study design, data collection, analysis, or interpretation, or in the writing of the paper.

## Study Design

We conducted a case-cohort study from participants enrolled in the REMEMBER clinical trial. This was an open-label randomized 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 study design and study procedures are available in previous papers using this cohort (18,19).

## 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. (19) and detailed in **Supplementary Methods**.

## Anaemia definition

Aligned with the WHO definition, anaemia was defined as Hb<13 g/dL for men or <12 g/dL for women (20). 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 (20).

## Laboratory Procedures

Plasma samples 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 minimize 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. (19).

## 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 (%). 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 visualize non-linear associations between the multiple clinical and inflammatory variables, as described in **Supplementary Methods.**

Kaplan-Meier analysis was evaluated according to the Breslow (Generalized 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 (stepwise regression) analysis including all parameters in Table 1 to test independent associations between clinical data, anaemia severity, and incident TB or death. The results were presented in the form of adjusted Odds Ratio (aOR) and 95% confidence intervals (CI). IN all analysis, 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 1**.

### 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.

## RESULTS

### Characteristics of the study population

Our cohort was composed of 269 PWH, 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 continuously, no significant correlations were observed either (CD4 p=-0·05; HIV VL p=-0·12) (**Supplementary Figure 1**).

### 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 (**Figure 1a, Table 2**). 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 Figure 2, Supplementary Table 2)**. The overall measurements of inflammatory markers at pre-ART according to anaemia severity are detailed in **Supplementary Table 3**.

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 (**Figure 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 Figure 1**). Of note, no difference between Hb or DIP values was observed when comparing the different arms of the original study(18) (**Supplementary Figure 3a-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) (**Figure 2a**). We next designed analyses to test whether the DIP score values are somehow related to incident TB (Figure 2b). The DIP values were higher in those who presented incident TB in contrast with patients who did not develop TB (p=0·009; **Figure 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; **Figure 2c**). Furthermore, mortality was substantially lower in non-anaemic participants (10·9%; p=0·05) than in participants from the other clinical groups. (**Figure 2d**). Indeed, patients who died displayed substantially higher DIP score values than those who survived (p=0·017; **Figure 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; **Figure 2f**).

Next, we evaluated the distribution of anaemia severity and systemic inflammatory profile according to the development of unfavourable outcomes (i.e., incidence 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 (**Figure 3a**). The frequency of anaemia was lower in participants who did not have TB and survived, and higher than in the other groups in 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 (**Figure 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 **Figure 3c**.

A binomial logistic regression analysis was performed to test independent associations between anaemia severity and clinical parameters with incident TB. We found that the presence of moderate/severe anaemia pre-ART was independently associated with development of TB (aOR: 3·25, 95%CI: 1·23-8·58, p=0·018) independent of other confounding factors (**Figure 4a**). Similar to the abovementioned results on occurrence of TB, another binomial logistic regression analysis was 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: 3·25, 95%CI: 1·05-10·1, p=0·041) independent of other confounding factors (**Figure 4b**).

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 Figure 3**). Te 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 Figure 4**).

## 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 increased risk of developing TB and/or death. Understanding the association of anaemia with systemic inflammation may help to optimize 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 (21). 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 (22). Although HIV infection is known to often reduce neutrophil counts (23), 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 (14), 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 (14,24–26). 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 (27). IFN-γ acts directly on macrophages and prompts blood cell uptake, leading to consumptive anaemia of inflammation (28). 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. Higher levels of IL-6 are commonly described in PWH (29–33) and are associated with anaemia in this population, providing evidence of activation of coagulation (12,29). 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 (17). Based on our results and prior studies, we showed that anaemia in PWH is associated with increased levels of IL-6 and hypothesize 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. Although patients were extensively screened during baseline, it is not impossible that baseline anaemia results from unidentified TB infections, given that those with anaemia have a shorter TB-free time. 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 (10). 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 (34). In the EuroSIDA study, the 12-month survival rate was 96·9% among non anemic PWH and was 59·2% among those with severe anaemia (11). 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 (30) as well with HIV complications and death in PWH on ART (35). In relation to TB in PWH, high levels of IL-6 were detected in TB-HIV co-infection (36) and a previous study of our group demonstrated that higher IL-6 levels are strongly associated with TB-IRIS (37). 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 responses that may contribute substantially to disease progression (38,39).

Finally, we performed multidimensional analyses to explore the relationship between Hb values, inflammation, incident TB and death. Albeit our study do not permit to identify whether anemia 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, 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 (13). It would be interesting to extend this study to a population with less advanced HIV infection. Furthermore, when evaluating death and incident TB together, we had only 13 participants. If the sample was larger, it would be interesting to assess the impact of different grades of anaemia within this population. Regardless of such limitations, our study provides strong basis to elucidate the influence of anaemia on 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 (14). Thus, we believe that PWH with moderate-to-severe anaemia should be carefully monitored before and after ART commencement in PWH.

## CONTRIBUTORS

Study Design and funding acquisition: M.A-P., S.K., M.H., G.B, B.B.A. and A.G.,; Conceptualization, M.A-P, S.K and B.B.A.; Data Collection: M.H., G.B, S.B-F., K.N., J.R.L., L.M, V.M.; Data curation and verification of the underlying data, M.A-P., S.K, B.B.A. and A.G.; Investigation, M.A-P., S.K., B.B.A. and A.G.; Formal analysis, M.A-P. and B.B.A.; Methodology, M.A-P. and B.B.A.; Software, M.A-P. and B.B.A.; Supervision, B.B.A. and A.G.; Writing—original draft, M.A-P., S.K., B.B.A. and A.G.; 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. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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## FIGURES AND TABLES

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

| | 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) |
| **Hemoglobin (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 (male), n (%):** | 36 (56·2) | 70 (57·9) | 29 (34·5) |
| **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) |
| | | | |
| **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, 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) |

**Table note:**

Data are shown as median and interquartile (IQR) range 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). Bold font and letters in "p value" column indicate statistical significance (p<0·05). [a]without anaemia x mild anaemia; [b]without anaemia x moderate/severe anaemia; [c]mild x moderate/severe. 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.

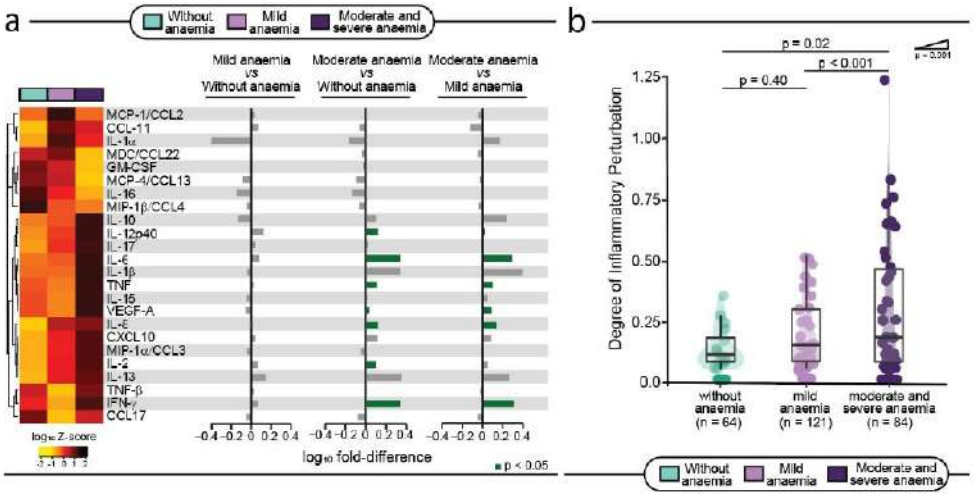



**Figure 1. Association between anaemia severity and the systemic inflammatory profile.** Among all people with HIV (PWH) (n=269), 76·2% had anaemia: 45% mild anaemia and 31·2% moderate/severe anaemia. (a) A heatmap was designed to depict the overall pattern of inflammatory markers. A one-way hierarchical 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 >13g/dL for man and >12g/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.

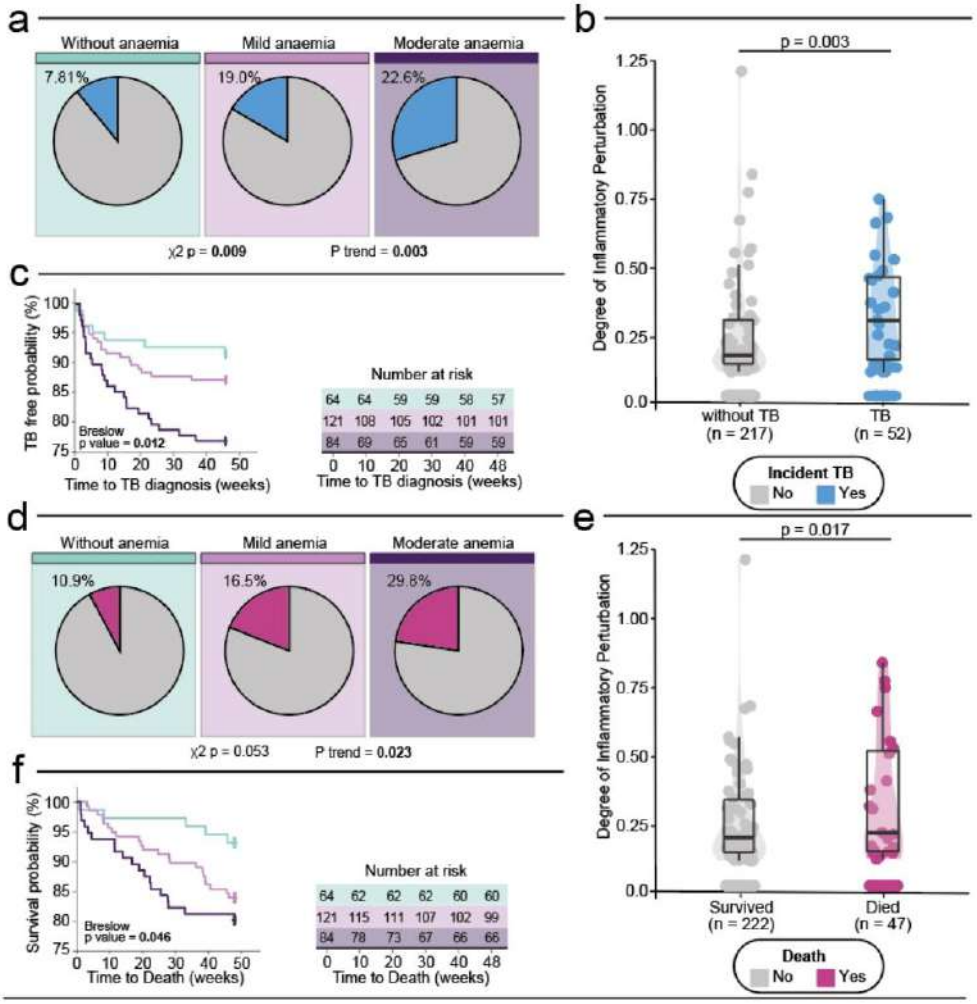



**Figure 2. The association between anaemia and incident TB and death.** (a) Distribution of TB occurrence according anaemia severity. (b) Scatter plots of the DIP value grouped according to incident TB. Lines in the scatter plots represent median values and data were compared using the Mann–Whitney 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 >13g/dL for man and >12g/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.

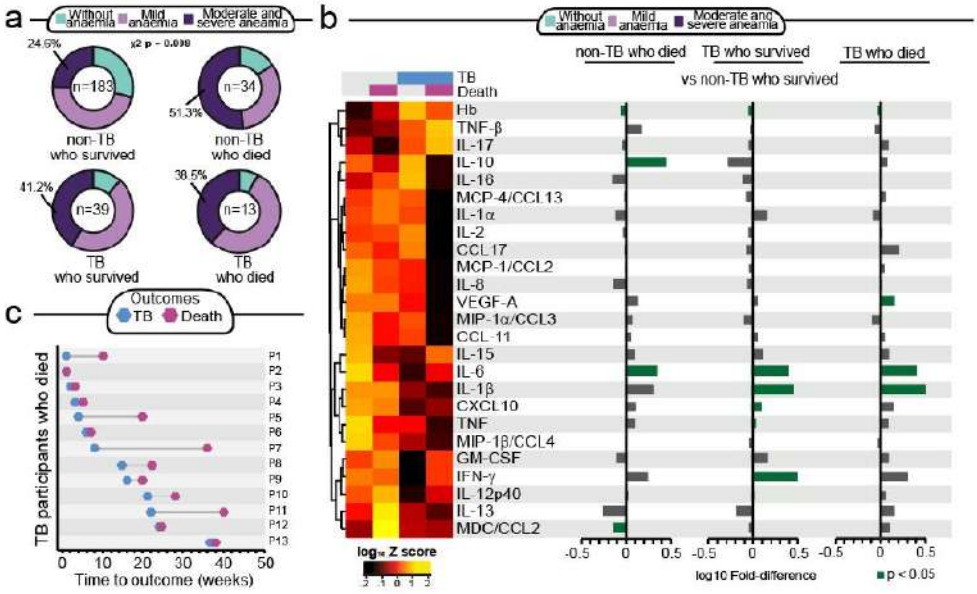



**Figure 3. Inflammatory profile of individuals according to TB development and death in people with HIV**. (A) Four groups were established: non-TB who survived (n=189); non-TB who died (n=34); TB who survived (n=39) and TB who died (n=13). (B) Right panel: A heatmap was designed to depict the overall 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 ($p < 0{\cdot}05$) 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 >13g/dL for man and >12g/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

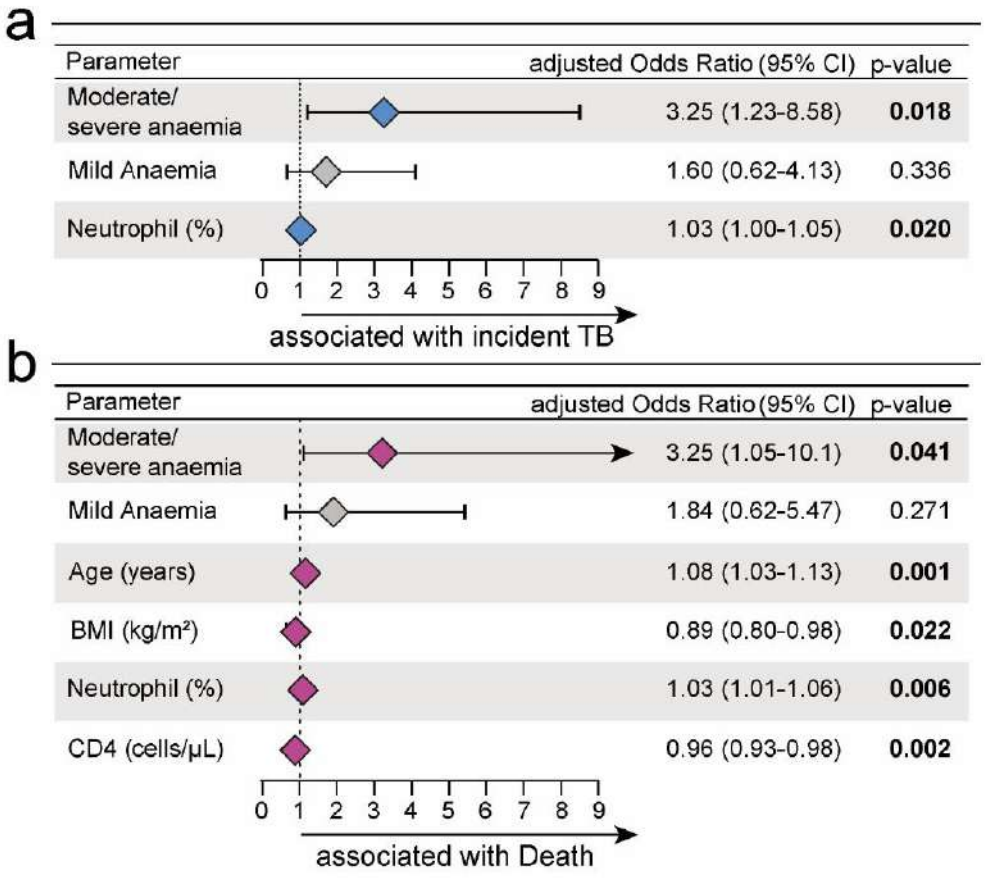



**Figure 4. Binomial logistic regression (stepwise method) to assess the independent risk factor for incident TB and death in PWH.** Binomial logistic regression with stepwise method was used to test independent associations between all clinical measurements (described in table 1) and (a) incident TB or (b) death. Odds are per increase in 1 unit of the continuous variables. 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 >13g/dL for man and >12g/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.

**Supplementary Material**

**Content:**

## Supplementary Methods

### Case-control design

The case-cohort design used in this study was previously designed by Manabe et al. (1). 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.

### Bayesian Network

Bayesian analysis is a more robust approach to finding the associations between these different types of parameters, compared to monovarietal approaches or using linear correlations. Continuous variables were discretized using median values of all participants for each measurement (lower or higher than average of the entire study population). Thus, the relationship evaluated on the network refers to higher values of measurements. The learning algorithm used to establish the network structure was based on the heuristic Hill climb method accessed by "bnlearn" package in R 4·4·1.

The dependencies are represented qualitatively by a directed acyclic graph where each node corresponds to a variable and a direct arc between nodes represents a direct influence. The robustness of the arcs was scored by a non-parametric bootstrap test (100 × replicates). Arcs with more than 40% support were depicted. The strongest associations were considered those remaining statistically significant in ≥60% bootstraps. All analyses were pre-specified.

### Degree of Inflammatory Perturbation

The degree of inflammatory perturbation (DIP) was calculated to identify the general inflammatory environment of the participants. DIP was adapted from the molecular degree of perturbation, which has been described previously(2). In this study, the DIP calculation included the concentrations of the plasma inflammatory markers instead of gene expression values in the original analysis model(2). Thus, herein, the average level and standard deviation of a baseline reference group (without anemia) were calculated for each biomarker. The DIP score of each biomarker was defined by z-score normalization, where the differences in concentration values from the average of the biomarker in reference group was divided by the reference standard deviation. Therefore, the DIP score represents the differences by number of standard deviations from the control group. Similar approaches resulting in DIP-like scores have been previously employed using biomarker measurements by our group(3,4).

**Supplementary Table 1. Packages used for the statistical analyses.**

| Objective | R package | Version | Reference |
|---|---|---|---|
| **Heatmap** | **ComplexHeatmap** | **2.12.0** | (5) |
| **Plot graphs** | **ggplot2** | **3.3.6** | (6) |
| **Plot graphs** | **ggpubr** | **0.4.0** | (7) |
| **Spearman correlations** | **Hmisc** | **4.7.0** | (8) |
| **Bayesian network** | **bnlearn** | **4.7.1** | (9) |
| **Logistic regression** | **MASS** | **7.3.58** | (10) |

**Supplementary Table 2. Spearman correlation of clinical and immunological markers at baseline.**

| Source | Target | Spearman correlation | P value |
|---|---|---|---|
| **IL-6** | Hb | -0.3 | **<0.001** |
| **IL-12p40** | Hb | -0.18 | **0.002** |
| **TNF** | Hb | -0.18 | **0.003** |
| **IL-2** | Hb | -0.18 | **0.004** |
| **IL-8** | Hb | -0.16 | **0.007** |
| **VEGF-A** | Hb | -0.15 | **0.011** |
| **IL-1β** | Hb | -0.15 | **0.013** |
| **IFN-γ** | Hb | -0.14 | **0.019** |
| **IL-13** | Hb | -0.12 | **0.04** |
| **CXCL10** | Hb | -0.12 | **0.04** |
| **IL-10** | Hb | -0.11 | 0.08 |
| **MIP-1α/CCL3** | Hb | -0.1 | 0.11 |
| **CD4** | Hb | -0.1 | 0.12 |
| **MDC/CCL22** | Hb | 0.08 | 0.20 |
| **IL-15** | Hb | -0.08 | 0.20 |
| **IL-16** | Hb | 0.08 | 0.21 |
| **IL-17** | Hb | -0.07 | 0.27 |
| **MIP-1β/CCL4** | Hb | 0.06 | 0.34 |
| **TNF-β** | Hb | 0.05 | 0.40 |
| **CCL11** | Hb | -0.04 | 0.49 |
| **MCP-4/CCL3** | Hb | 0.03 | 0.58 |
| **CCL17** | Hb | 0.03 | 0.63 |
| **MCP-1/CCL2** | Hb | 0.02 | 0.74 |
| **CD8** | Hb | 0.02 | 0.78 |
| **GM-CSF** | Hb | 0.02 | 0.8 |
| **IL-1α** | Hb | 0.01 | 0.99 |

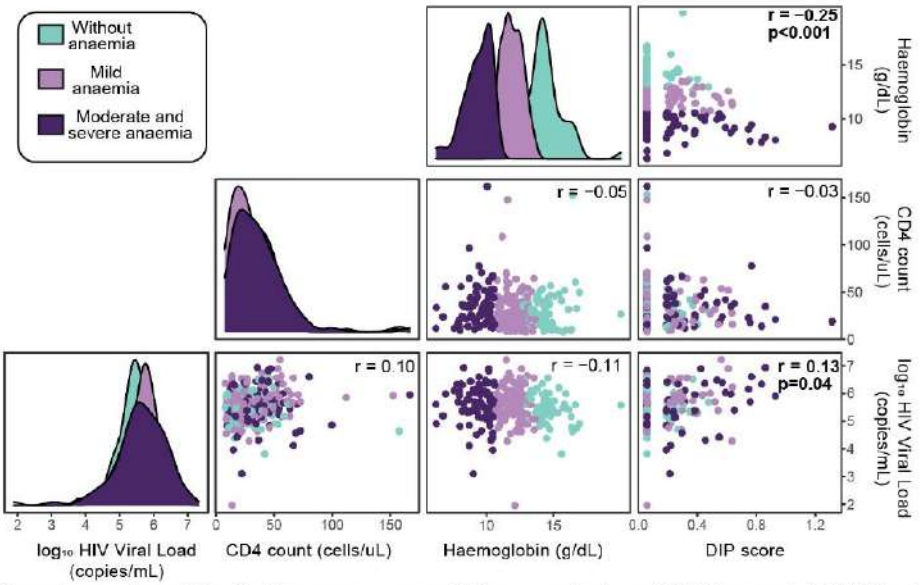
**Table note:** Abbreviations: Haemoglobin (Hb); C Reactive Protein (CRP), C-X-C motif chemokine ligand 10 (CXCL10), interleukin-2 (IL-2), IL-6, IL-8, IL-10, IL-27, interferon-γ (IFN-γ), tissue necrosis factor (TNF), C-C Motif Chemokine Ligand 2 (CCL2), CCL3, CCL4, CCL11, CCL17. Bold type font indicates statistical significance (p-value below 0.05).

**Supplementary Table 3. Baseline biomarker measurements according anaemia severity.**

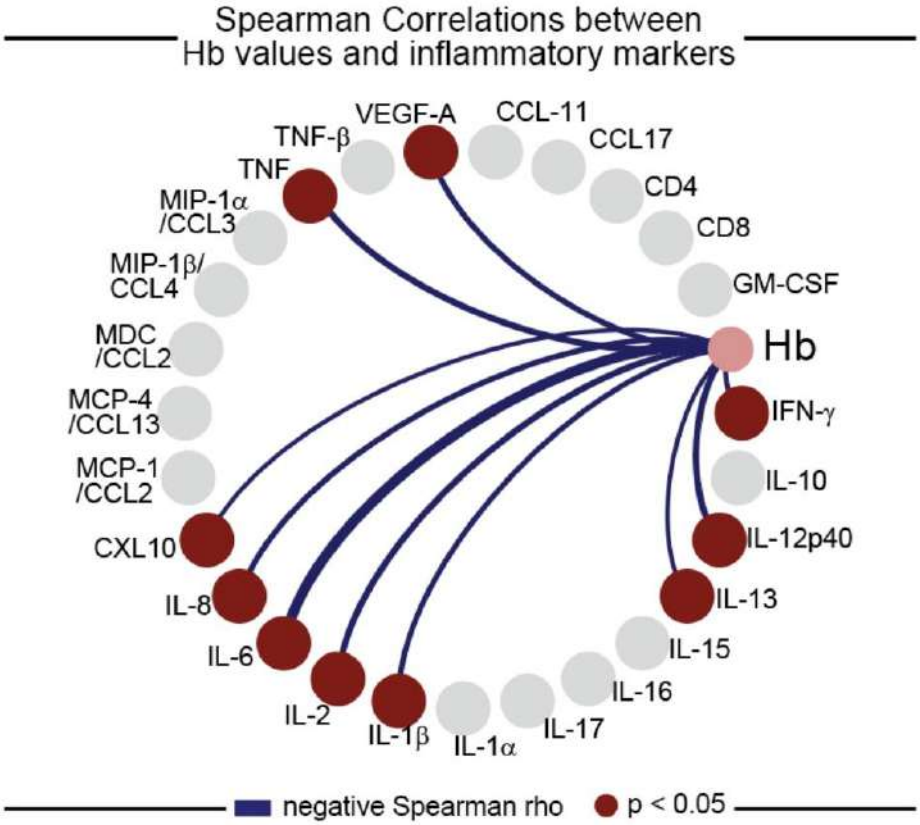
| | Without anaemia (n=64) | Mild anaemia (n=121) | Moderate/severe anaemia (n=84) | P value |
|---|---|---|---|---|
| **CCL-11 (pg/mL), median (IQR)** | 251 (182-431) | 276 (206-371) | 269 (204-372) | 0·924 |
| **CCL17 (pg/mL), median (IQR)** | 206 (122-310) | 183 (85·9-360) | 200 (117-304) | 0·672 |
| **GM-CSF (pg/mL), median (IQR)** | 0·17 (0·06-0·32) | 0·16 (0·04-0·32) | 0·15 (0·05-0·27) | 0·749 |
| **IFN-γ (pg/mL), median (IQR)** | 24·7 (11·8-49·5) | 19·7 (10·6-46·6) | 31·7 (14·8-107) | **0·016**[b,c] |
| **IL-16 (pg/mL), median (IQR)** | 210 (138-294) | 197 (126-297) | 183 (148-260) | 0·726 |
| **IL-1α (pg/mL), median (IQR)** | 0·14 (0·05-0·49) | 0·20 (0·07-0·40) | 0·17 (0·06-0·36) | 0·922 |
| **IL-1β (pg/mL), median (IQR)** | 0·01 (0·00-0·12) | 0·01 (0·00-0·19) | 0·03 (0·00-0·26) | 0·096 |
| **IL-10 (pg/mL), median (IQR)** | 1·08 (0·50-1·75) | 1·09 (0·61-1·86) | 1·16 (0·83-1·79) | 0·411 |
| **IL-12p40 (pg/mL), median (IQR)** | 227 (168-318) | 251 (166-379) | 326 (208-424) | **0·004**[b,c] |
| **IL-13 (pg/mL), median (IQR)** | 0·20 (0·00-0·64) | 0·38 (0·00-0·82) | 0·49 (0·07-0·76) | 0·100[b] |
| **IL-15 (pg/mL), median (IQR)** | 4·00 (2·82-4·63) | 3·88 (2·85-5·39) | 4·51 (2·94-5·88) | 0·356 |
| **IL-17 (pg/mL), median (IQR)** | 3·87 (2·67-6·68) | 4·39 (2·09-8·76) | 5·36 (3·50-7·90) | 0·183 |
| **IL-2 (pg/mL), median (IQR)** | 0·42 (0·25-0·78) | 0·54 (0·32-0·85) | 0·65 (0·44-0·82) | **0·022**[b] |
| **IL-6 (pg/mL), median (IQR)** | 1·38 (0·82-2·15) | 1·46 (0·94-3·07) | 2·61 (1·46-5·23) | **<0·001**[b,c] |
| **IL-8 (pg/mL), median (IQR)** | 6·42 (3·36-13·0) | 8·72 (5·36-16·9) | 9·22 (6·35-16·9) | **0·044**[a,b] |
| **CXCL10 (pg/mL), median (IQR)** | 3·16 (2·97-3·45) | 3·24 (2·99-3·46) | 3·32 (3·07-3·53) | 0·135 |
| **MCP-4/CCL3 (pg/mL), median (IQR)** | 103 (62·4-159) | 101 (65·3-140) | 89·4 (68·6-133) | 0·644 |
| **MCP-1/CCL2 (pg/mL), median (IQR)** | 197 (150-275) | 236 (160-299) | 197 (151-266) | 0·214 |
| **MDC/CCL22 (pg/mL), median (IQR)** | 1063 (760-1386) | 1094 (786-1424) | 954 (689-1352) | 0·296 |
| **MIP-1α/CCL3 (pg/mL), median (IQR)** | 44·4 (28·8-75·1) | 50·9 (33·1-89·1) | 57·7 (32·3-86·2) | 0·369 |
| **MIP-1β/CCL4 (pg/mL), median (IQR)** | 86·3 (59·4-129) | 77·4 (50·4-117) | 76·2 (61·1-104) | 0·332 |
| **TNF-β (pg/mL), median (IQR)** | 0·52 (0·32-0·74) | 0·47 (0·26-0·75) | 0·54 (0·21-0·72) | 0·715 |
| **TNF (pg/mL), median (IQR)** | 5·95 (4·05-8·05) | 5·71 (4·47-8·29) | 7·55 (5·49-9·91) | **0·007**[b,c] |
| **VEGF-A (pg/mL), median (IQR)** | 58·3 (29·3-94·5) | 53·1 (32·6-125) | 89·9 (42·2-176) | **0·009**[b,c] |

**Table note:** Bold font indicates statistical significance. Data are shown as median and interquartile (IQR) range 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). Bold font and letters

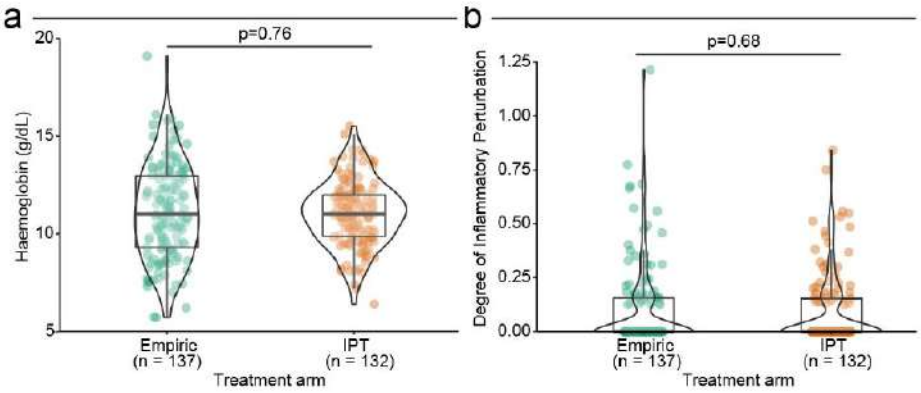
indicate statistical significance (p<0·05). [a]without anaemia x mild anaemia; [b]without anaemia x moderate/severe anaemia; [c]mild x moderate/severe. Without anaemia was defined as Hb value >13g/dL for man and >12g/dL for women. Mild anaemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate/severe anaemia was defined as Hb <=10 g/dL for both sexesAbbreviations: Interquartile Range (IQR); Haemoglobin (Hb); 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), tissue necrosis factor (TNF), hyaluronic acid (HA), myeloperoxidase (MPO), soluble cluster differentiation 14 (sCD14), sCD163, tissue factor (TF).



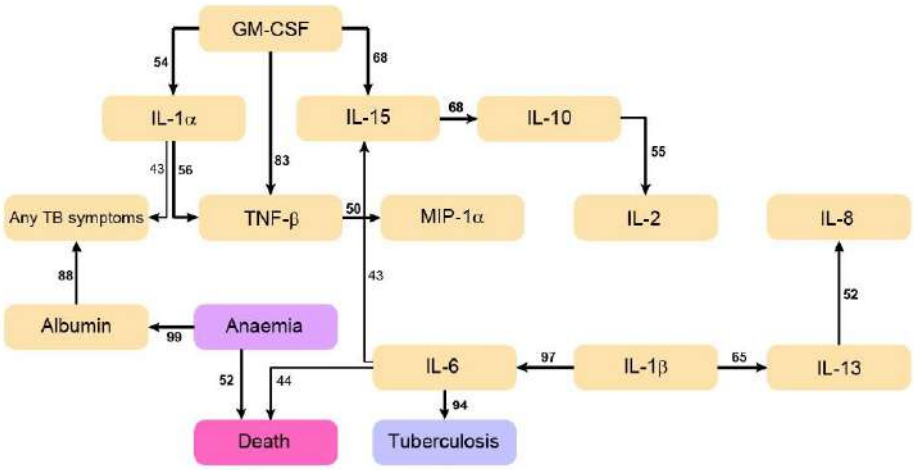

**Supplementary Fig 1. Spearman correlation analysis of CD4 count, HIV Viral Load, haemoglobin values and DIP score of people living with HIV at baseline**. Without anaemia was defined as Hb value >13g/dL for man and >12g/dL for women. Mild anaemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate/severe anaemia was defined as Hb <=10 g/dL for both sexes.




**Supplementary Fig 2. Spearman correlation network between haemoglobin levels and plama inflammatory markers.** Network displays statistically significant associations between plasma concentrations of the indicated biomarkers and haemoglobin levels in the entire study population. In this chart, all corelations were negative (blue lines). Only correlations which remained with p-value <0·05 are displayed (red circles).




**Supplementary Fig 3. Boxplots to compare haemoglobin and degree of inflammatory perturbation between two study treatment arms.** The original study treatment arms (empiric treatment, green; Isoniazid preventive treatment (IPT), orange) were compared in relation to haemoglobin levels (a) and degree of inflammatory perturbation score (b). Groups were compared using the Mann-Whitney test.




**Supplementary Fig 4. Bayesian network analysis to identify association chains between risk factors and incident TB or death.** The network was built with bootstrap (100x) and used to illustrate the statistically significant associations ($p<0.05$) between the parameters and the occurrence of TB and/or death in the study population. Lines represent direct associations. Associations that remained statistically significant on >40 times out of 100 bootstraps are plotted. Numbers of times each association persisted during bootstrap are shown. Bold lines highlight the strongest associations, which persisted more than 50 times in the bootstrap. Anaemia was defined as Hb value <13g/dL for man and <12g/dL for women.

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## 5.2 Manuscrito II

### Título

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

### Objetivo

Esse trabalho teve como objetivo entender como a gravidade da anemia contribui para o desenvolvimento da SIRI e morte em pacientes com HIV após o início do tratamento antirretroviral.

### Resumo de resultados

A coorte desse estudo foi composta por 502 pacientes com HIV, dos quais apenas 16,3% não eram anêmicos. O restante apresentava anemia leve, moderada ou grave de acordo com os critérios estabelecidos pela OMS. Após avaliar um amplo painel de marcadores plasmáticos e associá-los com os desfechos de tratamento ao longo de 6 meses, observamos que pacientes com anemia grave apresentam um perfil inflamatório distinto que os demais, com valores mais elevados de TNF, IL-6 e IL-27. Além disso, a anemia grave foi independentemente associada a ocorrência de SIRI e morte de forma mais significativa que os demais graus de anemia. É importante ressaltar que aqueles com anemia grave apresentaram maior risco de apresentar SIRI por micobactéria que os demais. Nesse estudo chamamos a atenção para a associação entre anemia grave e desfechos desfavoráveis de tratamento, evidenciando a importância de acompanhar de perto pacientes anêmicos que irão começar o tratamento antirretroviral a fim de minimizar a ocorrência desses desfechos.

### Status do manuscrito

Este trabalho foi publicado no periódico internacional ***eBiomedicine*** (Fator de Impacto JCR 2021 = 11,202) em 22 de outubro de 2022.

# 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.[11] 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.[11] 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

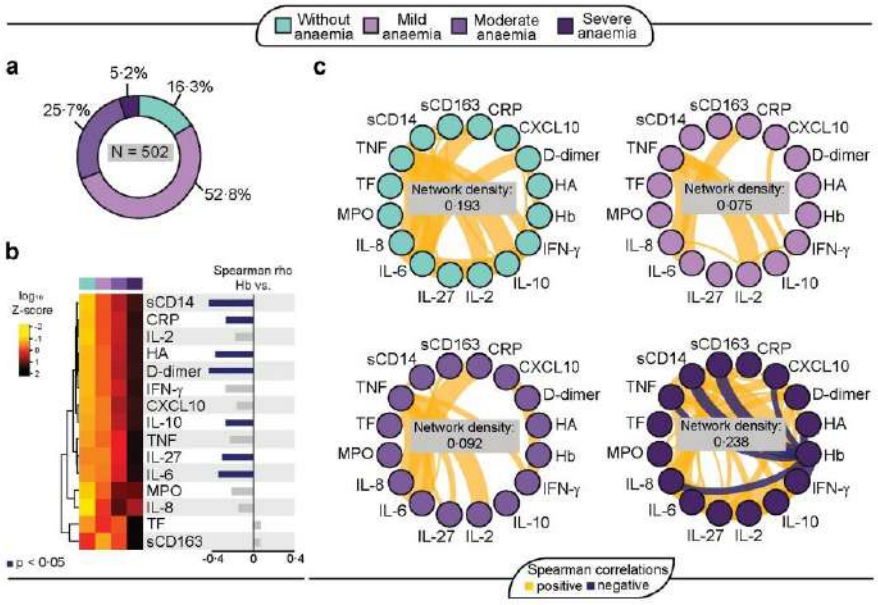


**Fig. 1:** Patients with severe anaemia present a distinct inflammatory profile. (a) Among all the PWH (n = 502), 83.7% had anaemia: 52.8% mild anaemia; 25.7% moderate anaemia; and 5.2% severe anaemia. (b) Right panel: A heatmap was designed to depict the overall pattern of inflammatory markers. A one-way 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.[13,16]

### 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

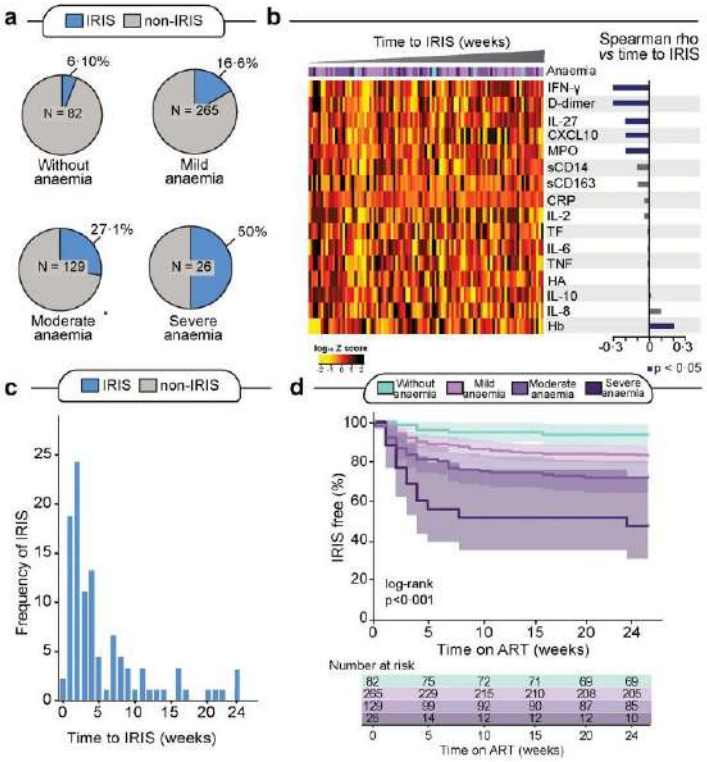


**Fig. 2:** Moderate and severe anaemia were associated with IRIS occurrence. (a) IRIS occurrence increased according to anaemia severity. (b) Left panel: data were log10 transformed and ranked and coloured in a heatmap from values detected for each inflammatory biomarker. Participants were ordered based 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, $r = -0.37$, $p = 0.04$)

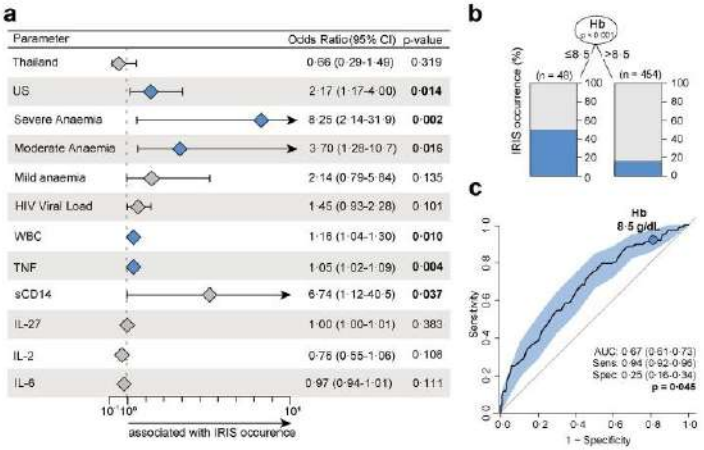


***Fig. 3:*** Moderate and severe anaemia are associated with IRIS occurrence. (a) Binomial logistic regression model (backward stepwise regression) to test independent associations between all the relevant measurements (Mann–Whitney *U* test p-value < 0.1 in Supplementary Table S7) and IRIS occurrence. 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.[3] 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,

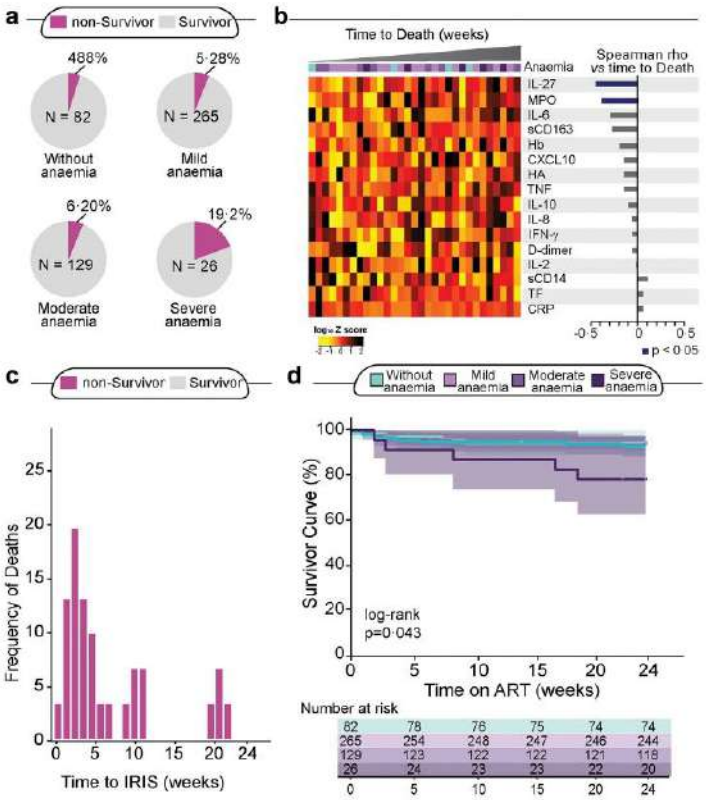


*Fig. 4:* Mortality was higher in patients with severe anaemia. (a) Mortality increased according to anaemia severity. Pink shaded areas represent the frequency of participants who died in each indicated clinical group. (b) Left panel: data were $log_{10}$ transformed, ranked and coloured in a heatmap 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.[23] 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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## Supplementary Material

**Content:**



### Supplementary Methods

#### Diagnosis of opportunistic infections and IRIS

Mycobacterial infections were diagnosed based on clinical and histologic and/or microbiologic evidence. Viral events were diagnosed as follows: cytomegalovirus events based on clinical and pathological evidence of end-organ disease, HHV-8-related events (Kaposi's sarcoma) based on clinical presentation and histologic confirmation, viral hepatitis flares (Hepatitis B virus and Hepatitis C virus) based on elevated liver-associated tests and viral molecular assays, and varicella zoster events based on clinical presentation, with molecular testing for confirmation when needed clinically. The committee adjudicated IRIS events using the ACTG IRIS definition criteria; evidence of ART initiation with a resultant increase in CD4 count (≥50 cells/μL or a ≥2- fold rise) and/or virologic suppression (>0.5 log10 decrease in plasma HIV viremia), clinical presentation consistent with an infectious or inflammatory condition, and the absence of an alternative aetiology such as the expected course of a previously recognized infection or side-effects of medications

**Conditional Inference Tree**

Conditional inference trees represent a machine-learning method that estimates a regression relationship by binary partitioning in a conditional inference framework. The algorithm works in three steps: (1) Test the global null hypothesis of independence between any of the input variables and the response and stop if this hypothesis cannot be rejected. Else, select the input variable with the strongest association to the response, measured by a p-value; (2) Implement a binary split in the selected input variable. (3) Recursively repeat steps 1) and 2).

The implementation utilizes a framework for conditional inference tests. The stop criterion in step 1 is either based on multiplicity-adjusted p-values (Bonferroni) or the univariate p-values. The selection of the input variable to split in is based on the univariate p-values avoiding a variable selection bias towards input variables with many possible cut-off points.

**Supplementary Table 1. Packages used for the statistical analyses.**

| Objective | R package | Version | Reference |
|---|---|---|---|
| **Heatmap** | **ComplexHeatmap** | **2.12.0** | (1) |
| **Plot graphs** | **ggplot2** | **3.3.6** | (2) |
| **Plot graphs** | **ggpubr** | **0.4.0** | (3) |
| **Spearman correlations** | **Hmisc** | **4.7.0** | (4) |
| **Decision Tree** | **Ctree** | **1.3.10** | (5) |

**Supplementary Table 2. Characteristics of the study participants according to anaemia status.**

| | Without anaemia (n=82) | With anaemia (n=420) |
|---|---|---|
| **Age, median (IQR)** | 35.0 (31.0-44.0) | 37.0 (31.0-45.0) |
| **Male, n (%)** | 54 (65.9) | 253 (60.2) |
| **Country, n (%)** | | |
| **Kenya** | 46 (56.1) | 152 (36.2) |
| **Thailand** | 16 (19.5) | 84 (20.0) |
| **USA** | 20 (24.4) | 184 (43.8) |
| **Glucose (mg/dL), median (IQR)** | 81.0 (76.3-88.0) | 85.5 (77.0-97.0) |
| **WBC ($10^6$/µL), median (IQR)** | 3.67 (2.70-4.82) | 3.20 (2.38-4.28) |
| **Platelets ($10^5$/µL), median (IQR)** | 198 (142-262) | 224 (169-291) |
| **Hb (g/dL), median (IQR)** | 13.8 (13.3-14.8) | 10.5 (9.40-11.6) |
| **$CD4^+$ count/µL, median (IQR)** | 40 (14-63) | 26 (10-53) |
| **$CD8^+$ count/µL, median (IQR)** | 618 (428-888) | 426 (258-703) |
| **BMI ($Kg/m^2$), median (IQR)** | 20.5 (18.2-23.0) | 20.2 (17.8-23.4) |
| **HIV Viral Load (Log10 copies/mL), median (IQR)** | 5.34 (4.93-5.60) | 5.30 (4.92-5.70) |

**Table note:**
Bold type font indicates statistical significance (p-value below 0.05). Data are shown as median and interquartile (IQR) range or frequency (percentage). To define anaemia according to haemoglobin, the cut-off point of 12 g/dL for women and 13 g/dL for men was used. Data were compared between the clinical groups using the Mann-Whitney *U* test (continuous variables).

**Supplementary Table 3. Characteristics of the study participants according to country.**

| | Kenya (n=198) | Thailand (n=100) | USA (n=204) | P value |
|---|---|---|---|---|
| **Age, median (IQR)** | 36.0 (32.0-42.0) | 36.5 (31.0-45.0) | 38.0 (31.0-46.0) | |
| **Male, n (%)** | 95 (48.0) | 62 (62.0) | 150 (73.5) | |
| **Anemia grade, n (%)** | | | | |
| **Without aneamia** | 46 (23.2) | 16 (16.0) | 20 (9.80) | |
| **Mild aneamia** | 99 (50.0) | 42 (42.0) | 124 (60.8) | |
| **Moderate anaemia** | 40 (20.2) | 32 (32.0) | 57 (27.9) | |
| **Severe anaemia** | 13 (6.57) | 10 (10.0) | 3 (1.47) | |
| **Glucose (mg/dL), median (IQR)** | 79.1 (74.0-85.7) | 81.0 (75.0-87.0) | 94.0 (85.0-108) | |
| **WBC ($10^6$/µL), median (IQR)** | 3.30 (2.50-4.30) | 3.79 (2.80-5.71) | 2.95 (2.24-3.92) | |
| **Platelets ($10^6$/µL), median (IQR)** | 232 (181-310) | 250 (196-321) | 191 (148-251) | |
| **Hb (g/dL), median (IQR)** | 11.2 (9.83-12.8) | 10.4 (8.97-12.5) | 10.9 (9.70-12.0) | |
| **$CD4^+$ count/µL, median (IQR)** | 35.5 (14.2-61.0) | 29.0 (12.8-54.8) | 19.0 (8.00-46.0) | |
| **$CD8^+$ count/µL, median (IQR)** | 531 (318-888) | 481 (273-710) | 412 (262-612) | |
| **BMI ($Kg/m^2$), median (IQR)** | 18.3 (16.9-20.8) | 19.2 (17.6-21.9) | 23.1 (20.6-25.8) | |
| **HIV Viral Load ($Log_{10}$), median (IQR)** | 5.46 (5.07-5.78) | 5.42 (5.06-5.98) | 5.13 (4.70-5.49) | |
| | | | | |
| **MPO (pg/mL)** | 4.16 (3.99-4.38) | 4.30 (4.15-4.47) | 4.17 (4.01-4.39) | **0.002** |
| **TNF (pg/mL)** | 16.0 (11.2-22.7) | 10.8 (7.81-16.5) | 9.79 (6.72-13.6) | **<0.001** |
| **CXCL10 (pg/mL)** | 2074 (1314-3381) | 2980 (1871-4159) | 2561 (1536-3876) | **0.005** |
| **sCD14 (pg/mL)** | 6.40 (6.27-6.52) | 6.36 (6.25-6.58) | 6.35 (6.27-6.44) | 0.073 |
| **HA (ng/mL)** | 77.0 (37.6-124) | 126 (83.0-176) | 111 (64.0-205) | **<0.001** |
| **IFN-γ (pg/mL)** | 3.80 (1.67-9.36) | 5.04 (2.52-19.6) | 2.87 (1.25-6.43) | **<0.001** |
| **IL-10 (pg/mL)** | 13.2 (6.12-23.2) | 7.72 (5.32-13.8) | 8.93 (5.58-14.9) | **0.001** |
| **IL-27 (pg/mL)** | 487 (309-762) | 727 (546-1078) | 390 (273-572) | **<0.001** |
| **IL-2 (pg/mL)** | 0.90 (0.00-1.48) | 0.00 (0.00-1.11) | 0.00 (0.00-0.94) | **<0.001** |
| **IL-6 (pg/mL)** | 3.98 (1.75-9.21) | 2.47 (1.53-5.33) | 1.75 (0.93-3.30) | **<0.001** |
| **IL-8 (pg/mL)** | 18.5 (10.5-35.9) | 8.39 (5.80-14.7) | 7.72 (4.04-12.9) | **<0.001** |
| **sTF (pg/mL)** | 72.1 (49.5-108) | 88.1 (64.3-112) | 78.5 (62.0-99.6) | 0.058 |
| **sCD163 (pg/mL)** | 466 (269-808) | 807 (471-1178) | 176 (5.46-473) | **<0.001** |
| **D dimer (pg/mL)** | 6.07 (5.84-6.33) | 5.98 (5.77-6.35) | 5.99 (5.75-6.29) | 0.124 |
| **CRP (pg/mL)** | 6.94 (6.23-7.57) | 6.56 (6.05-7.12) | 6.59 (5.98-7.01) | **<0.001** |

**Table note:**
Bold type font indicates statistical significance (p-value below 0.05). Anaemia was defined as levels of Hb <13 g/dL for men or <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 anaemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anaemia (SA) was defined as Hb<8g/dL for both sexes. Data are shown as median and interquartile (IQR) ranges. Data were compared between the clinical groups using the Kruskal-Wallis test and Mann-Whitney *U* test. [a]without anaemia x mild anaemia; [b]without anaemia x moderate anaemia; [c]without anaemia x severe anaemia; [d]mild x moderate; [e]mild x severe; [f]moderate x severe. MPO, sCD14, D-dimer and CRP were log10 transformed. Abbreviations: Interquartile Range (IQR); Haemoglobin (Hb); 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), tissue necrosis factor (TNF), hyaluronic acid (HA), myeloperoxidase (MPO), soluble cluster differentiation 14 (sCD14), sCD163, tissue factor (TF).

**Supplementary Table 4. Characteristics of the study participants from Kenya according to anaemia status.**

| | | Without anaemia (n=16) | Mild anaemia (n=42) | Moderate anaemia (n=32) | Severe anaemia (n=10) | P value |
|---|---|---|---|---|---|---|
| CLINICAL DATA | Age, median (IQR) | 37.5 (33.0-44.0) | 36.0 (32.0-40.5) | 35.0 (28.8-42.5) | 38.0 (35.0-41.0) | |
| | Male, n (%) | 23 (50.0) | 53 (53.5) | 14 (35.0) | 5 (38.5) | |
| | Glucose (mg/dL), median (IQR) | 78.4 (74.0-84.5) | 78.8 (73.4-85.3) | 84.9 (75.3-91.0) | 80.3 (75.6-85.0) | |
| | WBC ($10^6$/µL), median (IQR) | 3.15 (2.60-3.80) | 3.20 (2.45-4.25) | 3.60 (2.58-5.00) | 4.60 (3.90-5.30) | |
| | Platelets ($10^6$/µL), median (IQR) | 198 (136-254) | 238 (196-300) | 296 (223-372) | 243 (161-458) | |
| | Hb (g/dL), median (IQR) | 13.6 (12.8-14.1) | 11.3 (10.6-12.1) | 9.40 (8.80-9.62) | 7.30 (7.00-7.50) | |
| | $CD4^+$ count/µL, median (IQR) | 39.0 (20.2-58.0) | 33.0 (14.0-59.5) | 33.0 (9.75-71.0) | 41.0 (25.0-82.0) | |
| | $CD8^+$ count/µL, median (IQR) | 652 (443-972) | 492 (314-844) | 444 (238-736) | 864 (318-1060) | |
| | BMI ($Kg/m^2$), median (IQR) | 19.4 (17.9-21.3) | 18.2 (16.8-20.5) | 17.9 (15.3-19.7) | 18.9 (16.9-20.9) | |
| | HIV Viral Load ($Log_{10}$), median (IQR) | 5.47 (5.04-5.76) | 5.37 (5.03-5.71) | 5.58 (5.07-5.88) | 5.68 (5.64-5.82) | |
| BIOMARKER DATA | MPO (pg/mL) | 4.07 (3.88-4.24) | 4.15 (4.00-4.40) | 4.30 (4.02-4.49) | 4.32 (4.21-4.83) | **0.006** |
| | TNF (pg/mL) | 13.2 (8.40-17.8) | 16.2 (12.2-21.9) | 16.5 (12.8-23.8) | 26.3 (17.3-36.5) | **0.003** |
| | CXCL10 (pg/mL) | 2100 (1309-3283) | 2020 (1419-3224) | 1978 (1310-4627) | 2406 (1374-3140) | 0.923 |
| | sCD14 (pg/mL) | 6.31 (6.20-6.44) | 6.38 (6.28-6.52) | 6.49 (6.36-6.56) | 6.53 (6.42-6.59) | **<0.001** |
| | HA (ng/mL) | 53.3 (29.8-97.3) | 77.0 (39.0-117) | 86.1 (42.1-134) | 131 (92.7-289) | **0.009** |
| | IFN-γ (pg/mL) | 2.80 (1.11-4.85) | 3.71 (1.71-8.54) | 6.23 (3.20-23.7) | 7.77 (3.14-28.3) | **0.003** |
| | IL-10 (pg/mL) | 7.74 (4.66-14.4) | 12.6 (6.12-23.5) | 17.8 (12.9-26.2) | 19.5 (6.54-40.4) | **0.001** |
| | IL-27 (pg/mL) | 428 (221-578) | 463 (307-718) | 610 (451-834) | 801 (571-1100) | **0.001** |
| | IL-2 (pg/mL) | 0.86 (0.00-1.46) | 0.86 (0.00-1.35) | 0.97 (0.00-1.82) | 1.28 (0.72-1.57) | 0.316 |
| | IL-6 (pg/mL) | 2.13 (1.06-4.65) | 3.49 (1.78-8.43) | 7.05 (3.94-15.9) | 10.6 (4.06-18.3) | **<0.001** |
| | IL-8 (pg/mL) | 14.0 (8.79-20.9) | 21.2 (11.5-41.9) | 20.8 (12.7-37.7) | 17.4 (8.13-24.9) | **0.029** |
| | sTF (pg/mL) | 69.7 (49.9-102) | 72.7 (47.0-107) | 72.4 (49.6-104) | 83.2 (71.3-113) | 0.814 |
| | sCD163 (pg/mL) | 466 (231-879) | 485 (298-834) | 432 (266-617) | 532 (128-851) | 0.750 |
| | D dimer (pg/mL) | 5.89 (5.66-6.09) | 6.01 (5.83-6.26) | 6.33 (6.08-6.75) | 6.42 (6.19-6.62) | **<0.001** |
| | CRP (pg/mL) | 6.53 (5.96-7.02) | 6.92 (6.32-7.53) | 7.16 (6.74-7.88) | 7.61 (7.13-7.86) | **<0.001** |
| OUTCOME DATA | IRIS occurrence, n (%) | 2 (4.35) | 17 (17.2) | 8 (20.0) | 6 (46.2) | **<0.001** |
| | Time to IRIS* (weeks), median (IQR) | 26.0 (26.0-26.0) | 26.0 (25.5-26.0) | 26.0 (7.00-26.0) | 24.0 (4.00-26.0) | **0.016** |
| | Death, n (%) | 1 (6.25) | 2 (4.76) | 1 (3.12) | 3 (30.0) | 0.061 |
| | Time to Death* (weeks), median (IQR) | 26.0 (26.0-26.0) | 26.0 (26.0-26.0) | 26.0 (26.0-26.0) | 26.0 (26.0-26.0) | 0.776 |

**Table note:**
Bold type font indicates statistical significance (p-value below 0.05). Anaemia was defined as levels of Hb <13 g/dL for men or <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 anaemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anaemia (SA) was defined as Hb<8g/dL for both sexes. Data are shown as median and interquartile (IQR) ranges. Data were compared between the clinical groups using the Kruskal-Wallis test and Mann-Whitney *U* test. [a]without anaemia x mild anaemia; [b]without anaemia x moderate anaemia; [c]without anaemia x severe anaemia; [d]mild x moderate; [e]mild x severe; [f]moderate x severe. MPO, sCD14, D-dimer and CRP were log10 transformed. Abbreviations: Interquartile Range (IQR); Haemoglobin (Hb); 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), tissue necrosis factor (TNF), hyaluronic acid (HA), myeloperoxidase (MPO), soluble cluster differentiation 14 (sCD14), sCD163, tissue factor (TF). *Study participant that did not developed IRIS or survived throughout the entire study period had values of 26 weeks in the indicated parameters (time to IRIS and time to Death).

**Supplementary Table 5. Characteristics of the study participants from Thailand according to anaemia status.**

| | | Without anaemia (n=16) | Mild anaemia (n=42) | Moderate anaemia (n=32) | Severe anaemia (n=10) | P value |
|---|---|---|---|---|---|---|
| CLINICAL DATA | Age, median (IQR) | 34.5 (26.0-42.5) | 38.0 (32.0-44.8) | 36.0 (32.0-40.0) | 44.0 (33.5-49.5) | |
| | Male, n (%) | 14 (87.5) | 26 (61.9) | 17 (53.1) | 5 (50.0) | |
| | Glucose (mg/dL), median (IQR) | 85.5 (80.0-88.0) | 79.0 (72.0-88.0) | 80.0 (73.5-85.0) | 83.5 (80.8-90.0) | |
| | WBC ($10^6$/µL), median (IQR) | 5.81 (5.20-7.04) | 3.52 (2.73-4.69) | 3.54 (2.56-4.36) | 4.00 (2.97-7.77) | |
| | Platelets ($10^6$/µL), median (IQR) | 242 (196-278) | 254 (194-311) | 262 (210-350) | 214 (136-333) | |
| | Hb (g/dL), median (IQR) | 14.8 (13.8-15.3) | 11.5 (10.7-12.4) | 9.05 (8.57-9.50) | 7.15 (6.67-7.55) | |
| | $CD4^+$ count/µL, median (IQR) | 55.5 (36.5-73.8) | 25.5 (14.0-48.8) | 21.5 (10.0-50.0) | 16.5 (12.0-49.2) | |
| | $CD8^+$ count/µL, median (IQR) | 736 (545-786) | 480 (278-619) | 420 (260-610) | 298 (187-458) | |
| | BMI ($Kg/m^2$), median (IQR) | 22.6 (18.7-24.4) | 19.7 (18.2-21.7) | 18.0 (16.8-19.5) | 18.3 (16.4-20.4) | |
| | HIV Viral Load ($Log_{10}$), median (IQR) | 5.11 (4.89-5.48) | 5.45 (5.15-5.99) | 5.59 (4.95-5.99) | 5.37 (5.26-5.99) | |
| BIOMARKER DATA | MPO (pg/mL) | 4.32 (4.16-4.44) | 4.30 (4.17-4.46) | 4.29 (4.13-4.46) | 4.28 (4.23-4.66) | 0.841 |
| | TNF (pg/mL) | 8.87 (6.01-10.3) | 9.40 (7.67-13.3) | 16.1 (10.8-20.9) | 14.0 (8.81-38.8) | **0.001** |
| | CXCL10 (pg/mL) | 1488 (1098-2319) | 3002 (1857-3834) | 3609 (2356-4162) | 3827 (3163-4340) | **0.004** |
| | sCD14 (pg/mL) | 6.23 (6.10-6.26) | 6.35 (6.23-6.50) | 6.48 (6.30-6.63) | 6.68 (6.39-6.84) | **<0.001** |
| | HA (ng/mL) | 114 (83.6-136) | 97.0 (54.0-124) | 154 (125-243) | 285 (136-355) | **<0.001** |
| | IFN-γ (pg/mL) | 1.98 (1.38-3.37) | 6.97 (2.47-22.3) | 6.66 (3.45-22.5) | 7.96 (3.45-18.1) | **0.001** |
| | IL-10 (pg/mL) | 6.46 (5.50-8.61) | 6.50 (4.70-9.77) | 10.9 (6.00-17.7) | 19.1 (15.7-27.7) | **0.001** |
| | IL-27 (pg/mL) | 555 (396-714) | 674 (509-888) | 851 (706-1132) | 1107 (960-1349) | **<0.001** |
| | IL-2 (pg/mL) | 0.00 (0.00-0.76) | 0.00 (0.00-1.00) | 0.32 (0.00-1.55) | 1.17 (0.65-1.46) | 0.114 |
| | IL-6 (pg/mL) | 1.81 (1.18-2.23) | 1.97 (1.45-4.08) | 3.30 (1.81-6.63) | 6.75 (4.69-18.2) | **0.001** |
| | IL-8 (pg/mL) | 6.08 (5.46-8.40) | 7.97 (5.86-13.0) | 11.7 (7.16-16.5) | 11.3 (5.61-17.5) | 0.227 |
| | sTF (pg/mL) | 88.2 (61.5-118) | 88.6 (64.3-110) | 84.5 (65.2-109) | 105 (84.1-135) | 0.283 |
| | sCD163 (pg/mL) | 561 (296-1206) | 752 (501-1067) | 932 (504-1219) | 986 (638-1267) | 0.487 |
| | D dimer (pg/mL) | 5.71 (5.52-5.96) | 5.89 (5.72-6.21) | 6.21 (5.89-6.39) | 6.39 (6.23-6.63) | **<0.001** |
| | CRP (pg/mL) | 6.33 (5.80-6.50) | 6.58 (6.06-6.93) | 6.70 (6.17-7.39) | 7.15 (6.97-7.57) | **0.029** |
| OUTCOME DATA | IRIS occurrence, n (%) | 0 (0.0) | 3 (7.14) | 7 (21.9) | 6 (60.0) | **<0.001** |
| | Time to IRIS (weeks), median (IQR) | 26.0 (26.0-26.0) | 26.0 (26.0-26.0) | 26.0 (23.0-26.0) | 3.00 (1.25-20.0) | **<0.001** |
| | Death, n (%) | 1 (6.25) | 2 (4.76) | 1 (3.12) | 3 (30.0) | 0.061 |
| | Time to Death (weeks), median (IQR) | 26.0 (26.0-26.0) | 26.0 (26.0-26.0) | 26.0 (26.0-26.0) | 25.0 (21.0-26.0) | **0.026** |

**Table note:**
Bold type font indicates statistical significance (p-value below 0.05). Anaemia was defined as levels of Hb <13 g/dL for men or <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 anaemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anaemia (SA) was defined as Hb<8g/dL for both sexes. Data are shown as median and interquartile (IQR) ranges. Data were compared between the clinical groups using the Kruskal-Wallis test and Mann-Whitney *U* test. [a]without anaemia x mild anaemia; [b]without anaemia x moderate anaemia; [c]without anaemia x severe anaemia; [d]mild x moderate; [e]mild x severe; [f]moderate x severe. MPO, sCD14, D-dimer and CRP were log10 transformed. Abbreviations: Interquartile Range (IQR); Haemoglobin (Hb); 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), tissue necrosis factor (TNF), hyaluronic acid (HA), myeloperoxidase (MPO), soluble cluster differentiation 14 (sCD14), sCD163, tissue factor (TF).

**Supplementary Table 6. Characteristics of the study participants from the US according to anaemia status.**

| | | Without anaemia (n=16) | Mild anaemia (n=42) | Moderate anaemia (n=32) | Severe anaemia (n=10) | P value |
|---|---|---|---|---|---|---|
| CLINICAL DATA | Age, median (IQR) | 32.5 (27.8-40.0) | 38.0 (31.0-47.0) | 41.0 (31.0-46.0) | 48.0 (43.0-57.0) | |
| | Male, n (%) | 17 (85.0) | 97 (78.2) | 36 (63.2) | 0 (0.00) | |
| | Glucose (mg/dL), median (IQR) | 88.0 (81.0-95.0) | 96.0 (86.0-106) | 95.0 (85.0-114) | 123 (92.0-179) | |
| | WBC ($10^6$/µL), median (IQR) | 3.86 (3.12-4.38) | 2.91 (2.20-3.68) | 2.89 (2.21-3.93) | 4.95 (3.33-6.22) | |
| | Platelets ($10^6$/µL), median (IQR) | 160 (128-236) | 197 (156-248) | 200 (142-256) | 160 (130-174) | |
| | Hb (g/dL), median (IQR) | 14.1 (13.6-14.8) | 11.2 (10.6-12.0) | 9.30 (9.10-9.50) | 7.80 (7.65-7.80) | |
| | $CD4^+$ count/µL, median (IQR) | 18.0 (10.8-56.5) | 20.0 (10.0-45.2) | 19.0 (7.00-45.0) | 6.00 (6.00-8.00) | |
| | $CD8^+$ count/µL, median (IQR) | 466 (386-604) | 444 (274-672) | 338 (207-492) | 392 (300-402) | |
| | BMI ($Kg/m^2$), median (IQR) | 23.1 (21.1-24.5) | 23.5 (21.1-26.3) | 21.3 (19.4-24.7) | 31.9 (23.3-33.4) | |
| | HIV Viral Load ($Log_{10}$), median (IQR) | 5.09 (4.76-5.46) | 5.08 (4.66-5.46) | 5.21 (4.86-5.69) | 5.20 (5.19-5.28) | |
| BIOMARKER DATA | MPO (pg/mL) | 4.11 (3.99-4.35) | 4.17 (4.00-4.36) | 4.22 (4.06-4.52) | 4.26 (4.18-4.66) | 0.371 |
| | TNF (pg/mL) | 8.96 (5.51-11.3) | 9.90 (7.18-13.1) | 9.91 (6.74-15.0) | 11.4 (6.73-18.5) | 0.492 |
| | CXCL10 (pg/mL) | 2324 (1804-3716) | 2430 (1512-3688) | 2917 (1678-4303) | 3857 (2191-5266) | 0.482 |
| | sCD14 (pg/mL) | 6.29 (6.25-6.38) | 6.34 (6.27-6.44) | 6.41 (6.31-6.50) | 6.43 (6.27-6.44) | **0.004** |
| | HA (ng/mL) | 69.4 (30.8-116) | 93.7 (57.2-195) | 147 (104-283) | 230 (163-267) | **<0.001** |
| | IFN-γ (pg/mL) | 3.58 (1.31-6.10) | 2.66 (1.15-5.65) | 3.16 (1.68-7.82) | 6.26 (3.61-8.61) | 0.460 |
| | IL-10 (pg/mL) | 8.55 (4.97-13.7) | 7.30 (5.20-12.2) | 12.6 (9.05-18.3) | 28.5 (17.1-58.5) | **<0.001** |
| | IL-27 (pg/mL) | 408 (288-534) | 369 (261-534) | 447 (292-707) | 804 (761-903) | **0.017** |
| | IL-2 (pg/mL) | 0.00 (0.00-0.34) | 0.00 (0.00-0.94) | 0.00 (0.00-1.02) | 0.88 (0.44-1.10) | 0.327 |
| | IL-6 (pg/mL) | 0.99 (0.64-1.94) | 1.54 (0.86-2.90) | 2.89 (1.53-4.40) | 4.07 (2.04-10.4) | **<0.001** |
| | IL-8 (pg/mL) | 4.09 (3.04-9.18) | 7.44 (4.29-12.5) | 8.36 (5.36-13.7) | 2.55 (2.20-30.0) | 0.165 |
| | sTF (pg/mL) | 81.6 (70.8-104) | 82.1 (62.7-99.8) | 74.9 (56.6-92.8) | 73.1 (72.6-84.3) | 0.497 |
| | sCD163 (pg/mL) | 98.6 (0.00-583) | 176 (22.8-448) | 173 (1.87-473) | 381 (190-430) | 0.998 |
| | D dimer (pg/mL) | 5.83 (5.57-5.96) | 5.93 (5.70-6.23) | 6.20 (5.96-6.44) | 6.38 (6.31-6.47) | **<0.001** |
| | CRP (pg/mL) | 6.29 (5.78-6.67) | 6.43 (5.92-6.82) | 6.92 (6.39-7.26) | 7.59 (6.39-7.81) | **<0.001** |
| OUTCOME DATA | IRIS occurrence, n (%) | 0 (0.0) | 3 (7.14) | 7 (21.9) | 6 (60.0) | **<0.001** |
| | Time to IRIS (weeks), median (IQR) | 26.0 (26.0-26.0) | 26.0 (22.8-26.0) | 26.0 (6.00-26.0) | 25.0 (14.0-26.0) | 0.138 |
| | Death, n (%) | 1 (6.25) | 2 (4.76) | 1 (3.12) | 3 (30.0) | 0.061 |
| | Time to Death (weeks), median (IQR) | 26.0 (26.0-26.0) | 26.0 (26.0-26.0) | 26.0 (26.0-26.0) | 26.0 (26.0-26.0) | 0.978 |

**Table note:**
Bold type font indicates statistical significance (p-value below 0.05). Anaemia was defined as levels of Hb <13 g/dL for men or <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 anaemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anaemia (SA) was defined as Hb<8g/dL for both sexes. Data are shown as median and interquartile (IQR) ranges. Data were compared between the clinical groups using the Kruskal-Wallis test and Mann-Whitney *U* test. [a]without anaemia x mild anaemia; [b]without anaemia x moderate anaemia; [c]without anaemia x severe anaemia; [d]mild x moderate; [e]mild x severe; [f]moderate x severe. MPO, sCD14, D-dimer and CRP were log10 transformed. Abbreviations: Interquartile Range (IQR); Haemoglobin (Hb); 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), tissue necrosis factor (TNF), hyaluronic acid (HA), myeloperoxidase (MPO), soluble cluster differentiation 14 (sCD14), sCD163, tissue factor (TF).

**Supplementary Table 7. Concentrations of inflammatory markers in peripheral blood, according to anaemia severity.**

| | Without anaemia (n=82) | Mild anaemia (n=265) | Moderate anaemia (n=129) | Severe anaemia (n=26) | P value |
|---|---|---|---|---|---|
| **MPO (pg/mL)** | 4.13 (3.99-4.34) | 4.17 (4.01-4.38) | 4.28 (4.06-4.48) | 4.29 (4.22-4.74) | **0.002**[b,c,d,e] |
| **TNF (pg/mL)** | 10.6 (7.23-15.5) | 11.7 (8.19-16.2) | 14.2 (8.02-20.9) | 20.9 (10.9-36.0) | **0.001**[b,c,d,e,f] |
| **CXCL10 (pg/mL)** | 2098 (1308-3272) | 2229 (1473-3450) | 2857 (1670-4373) | 3204 (1627-4198) | **0.015**[b,d] |
| **sCD14 (pg/mL)** | 6.27 (6.20-6.40) | 6.35 (6.27-6.46) | 6.45 (6.32-6.55) | 6.51 (6.39-6.67) | **<0.001*** |
| **HA (ng/mL)** | 69.4 (32.7-120) | 88.3 (50.5-150) | 135 (82.2-227) | 188 (116-352) | **<0.001**[a,b,c,d,e] |
| **IFN-γ (pg/mL)** | 2.78 (1.25-4.72) | 3.51 (1.41-9.23) | 5.29 (2.54-12.2) | 7.59 (2.70-18.9) | **<0.001**[a,b,c,d,e] |
| **IL-10 (pg/mL)** | 7.33 (5.05-13.3) | 8.33 (5.33-15.5) | 13.9 (8.09-19.8) | 19.6 (6.94-37.2) | **<0.001**[b,c,d,e,f] |
| **IL-27 (pg/mL)** | 430 (283-602) | 433 (295-658) | 610 (411-879) | 970 (780-1212) | **<0.001**[b,c,d,e,f] |
| **IL-2 (pg/mL)** | 0.00 (0.00-1.19) | 0.30 (0.00-1.09) | 0.69 (0.00-1.43) | 1.17 (0.64-1.53) | **0.018**[b,e] |
| **IL-6 (pg/mL)** | 1.87 (0.99-3.16) | 2.08 (1.12-4.55) | 3.78 (1.81-7.84) | 6.75 (3.87-17.4) | **<0.001**[b,c,d,e,f] |
| **IL-8 (pg/mL)** | 9.32 (5.17-15.8) | 10.5 (5.96-21.7) | 11.9 (7.19-20.7) | 11.3 (5.12-21.5) | 0.500 |
| **sTF (pg/mL)** | 72.4 (56.2-106) | 79.0 (59.2-101) | 76.2 (56.5-100) | 94.5 (73.2-115) | 0.218 |
| **sCD163 (pg/mL)** | 441 (183-823) | 375 (99.8-769) | 423 (109-867) | 595 (288-1139) | 0.316 |
| **D-Dimer (pg/mL)** | 5.85 (5.59-6.03) | 5.98 (5.75-6.24) | 6.22 (5.97-6.50) | 6.40 (6.19-6.59) | **<0.001** |
| **CRP (pg/mL)** | 6.36 (5.92-6.87) | 6.64 (6.07-7.12) | 7.01 (6.38-7.47) | 7.43 (7.04-7.84) | **<0.001** |

**Table note:**
Bold type font indicates statistical significance (p-value below 0.05). Anaemia was defined as levels of Hb <13 g/dL for men or <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 anaemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anaemia (SA) was defined as Hb<8g/dL for both sexes. Data are shown as median and interquartile (IQR) ranges. Data were compared between the clinical groups using the Kruskal-Wallis test and Mann-Whitney *U* test. [a]without anaemia x mild anaemia; [b]without anaemia x moderate anaemia; without anaemia x severe anaemia; [d]mild x moderate; [e]mild x severe; [f]moderate x severe. MPO, sCD14, D-dimer and CRP were log10 transformed. Abbreviations: Interquartile Range (IQR); Haemoglobin (Hb); 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), tissue necrosis factor (TNF), hyaluronic acid (HA), myeloperoxidase (MPO), soluble cluster differentiation 14 (sCD14), sCD163, tissue factor (TF).

**Supplementary Table 8. Spearman correlation between Hb and immunological markers at baseline.**

| Source | Target | Spearman correlation | p-value |
|---|---|---|---|
| Hb | MPO | -0.19 | **<0.001** |
| Hb | TNF | -0.20 | **<0.001** |
| Hb | CXCL10 | -0.14 | **0.001** |
| Hb | sCD14 | -0.41 | **<0.001** |
| Hb | HA | -0.34 | **<0.001** |
| Hb | IFN-γ | -0.24 | **<0.001** |
| Hb | IL-10 | -0.25 | **<0.001** |
| Hb | IL-27 | -0.28 | **<0.001** |
| Hb | IL-2 | -0.15 | **<0.001** |
| Hb | IL-6 | -0.31 | **<0.001** |
| Hb | IL-8 | -0.12 | **0.005** |
| Hb | sTF | -0.06 | 0.188 |
| Hb | sCD163 | -0.05 | 0.226 |
| Hb | D-Dimer | -0.40 | **<0.001** |
| Hb | CRP | -0.25 | **<0.001** |

**Table note:** Abbreviations: Haemoglobin (Hb); 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), tissue necrosis factor (TNF), hyaluronic acid (HA), myeloperoxidase (MPO), soluble cluster differentiation 14 (sCD14), sCD163, soluble tissue factor (sTF). Bold type font indicates statistical significance (p-value below 0.05).

**Supplementary Table 9. Spearman correlation of clinical and immunological markers at baseline in patients without anaemia.**

| Source | Target | Spearman correlation | p-value |
|---|---|---|---|
| TNF | CXCL10 | 0.46 | **<0.001** |
| TNF | IFN-γ | 0.50 | **<0.001** |
| CXCL10 | IFN-γ | 0.46 | **<0.001** |
| TNF | IL-10 | 0.61 | **<0.001** |
| CXCL10 | IL-10 | 0.41 | **0.001** |
| IFN-γ | IL-10 | 0.46 | **<0.001** |
| TNF | IL-2 | 0.57 | **<0.001** |
| IFN-γ | IL-2 | 0.41 | **<0.001** |
| TNF | IL-6 | 0.53 | **<0.001** |
| sCD14 | IL-6 | 0.44 | **<0.001** |
| IFN-γ | IL-6 | 0.56 | **<0.001** |
| IL-10 | IL-6 | 0.42 | **<0.001** |
| TNF | IL-8 | 0.59 | **<0.001** |
| IFN-γ | IL-8 | 0.43 | **<0.001** |
| IL-10 | IL-8 | 0.49 | **<0.001** |
| IL-2 | IL-8 | 0.42 | **0,001** |
| IL-6 | IL-8 | 0.53 | **<0.001** |
| TNF | sCD163 | 0.44 | **<0.001** |
| IL-10 | sCD163 | 0.40 | **<0.001** |
| IL-27 | sCD163 | 0.40 | **<0.001** |
| IL-6 | D-Dimer | 0.55 | **<0.001** |
| IL-6 | CRP | 0.58 | **<0.001** |

**Table note:** Only correlations with |rho| > 0.4 and p value < 0.05. Abbreviations: Haemoglobin (Hb); 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), tissue necrosis factor (TNF), hyaluronic acid (HA), myeloperoxidase (MPO), soluble cluster differentiation 14 (sCD14), sCD163, soluble tissue factor (sTF), White Blood Cells (WBC). Bold type font indicates statistical significance (p-value below 0.05).

**Supplementary Table 10. Spearman correlation of clinical and immunological markers at baseline in patients with mild anaemia.**

| Source | Target | Spearman correlation | p-value |
|---|---|---|---|
| CXCL10 | IFN-γ | 0.43 | **<0.001** |
| TNF | IL-10 | 0.50 | **<0.001** |
| IFN-γ | IL-10 | 0.40 | **<0.001** |
| TNF | IL-2 | 0.59 | **<0.001** |
| IFN-γ | IL-2 | 0.41 | **<0.001** |
| TNF | IL-8 | 0.54 | **<0.001** |
| IL-2 | IL-8 | 0.40 | **<0.001** |
| IL-6 | IL-8 | 0.47 | **<0.001** |
| IL-6 | CRP | 0.53 | **<0.001** |

**Table note:** Only correlations with |rho| > 0.4 and p value < 0.05. Abbreviations: Haemoglobin (Hb); 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). Bold type font indicates statistical significance (p-value below 0.05).

**Supplementary Table 11. Spearman correlation of clinical and immunological markers at baseline in patients with moderate anaemia.**

| Source | Target | Spearman correlation | p-value |
|---|---|---|---|
| TNF | sCD14 | 0.42 | **<0.001** |
| TNF | IFN-γ | 0.44 | **<0.001** |
| CXCL10 | IFN-γ | 0.49 | **<0.001** |
| TNF | IL-27 | 0.44 | **<0.001** |
| TNF | IL-2 | 0.55 | **<0.001** |
| TNF | IL-6 | 0.49 | **<0.001** |
| sCD14 | IL-6 | 0.40 | **<0.001** |
| TNF | IL-8 | 0.55 | **<0.001** |
| IL-6 | IL-8 | 0.47 | **<0.001** |
| IL-27 | sCD163 | 0.42 | **<0.001** |
| IL-6 | CRP | 0.57 | **<0.001** |

**Table note:** Only correlations with |rho| > 0.4 and p value < 0.05. Abbreviations: Haemoglobin (Hb); 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), tissue necrosis factor (TNF), hyaluronic acid (HA), myeloperoxidase (MPO), soluble cluster differentiation 14 (sCD14), sCD163. Bold type font indicates statistical significance (p-value below 0.05).

**Supplementary Table 12. Spearman correlation of clinical and immunological markers at baseline in patients with severe anaemia.**

| Source | Target | Spearman correlation | p-value |
|---|---|---|---|
| TNF | sCD14 | 0.65 | **<0.001** |
| CXCL10 | sCD14 | 0.55 | **0.005** |
| MPO | IFN-γ | 0.42 | **0.043** |
| TNF | IFN-γ | 0.70 | **<0.001** |
| CXCL10 | IFN-γ | 0.51 | **0.011** |
| sCD14 | IFN-γ | 0.81 | **<0.001** |
| TNF | IL-10 | 0.52 | **0.009** |
| CXCL10 | IL-10 | 0.46 | **0.023** |
| TNF | IL-27 | 0.41 | **0.048** |
| CXCL10 | IL-27 | 0.51 | **0.010** |
| sCD14 | IL-27 | 0.45 | **0.028** |
| IFN-γ | IL-27 | 0.59 | **0.002** |
| IL-10 | IL-27 | 0.47 | **0.022** |
| TNF | IL-2 | 0.49 | **0.016** |
| IFN-γ | IL-2 | 0.52 | **0.011** |
| IL-10 | IL-2 | 0.44 | **0.032** |
| TNF | IL-6 | 0.41 | **0.049** |
| IFN-γ | IL-6 | 0.53 | **0.008** |
| IL-27 | IL-6 | 0.44 | **0.030** |
| MPO | IL-8 | 0.52 | **0.009** |
| TNF | IL-8 | 0.71 | **<0.001** |
| sCD14 | IL-8 | 0.56 | **0.004** |
| IFN-γ | IL-8 | 0.82 | **<0.001** |
| IL-2 | IL-8 | 0.56 | **0,004** |
| IL-6 | IL-8 | 0.52 | **0,009** |
| CXCL10 | sCD163 | 0.43 | **0.038** |
| IL-27 | sCD163 | 0.42 | **0.043** |
| MPO | D-dimer | 0.43 | **0.038** |
| sCD14 | D-dimer | 0.43 | **0.040** |
| IFN-γ | D-dimer | 0.57 | **0.004** |
| IL-8 | D-dimer | 0.52 | **0.009** |
| IL-6 | CRP | 0.55 | **0.006** |
| Hb | TNF | -0.51 | **0.012** |
| Hb | CXCL10 | -0.49 | **0.014** |
| Hb | sCD14 | -0.61 | **0.002** |
| Hb | HA | -0.54 | **0.006** |
| Hb | IFN-γ | -0.64 | **<0.001** |
| Hb | IL-8 | -0.51 | **0.010** |
| Hb | sCD163 | -0.62 | **0.001** |

**Table note:** Only correlations with |rho| > 0.4 and p value < 0.05. Abbreviations: Haemoglobin (Hb); 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), tissue necrosis factor (TNF), hyaluronic acid (HA), myeloperoxidase (MPO), soluble cluster differentiation 14 (sCD14), sCD163, tissue factor (TF), White Blood Cells (WBC). Bold type font indicates statistical significance (p-value below 0.05).

**Supplementary Table 13. Hazard Ratio for IRIS occurrence according anaemia grade**

| | Hazard Ratio (HR, Cox Regression) | | |
|---|---|---|---|
| | **HR** | **95% CI** | **p value** |
| **Without anaemia** | - | - | - |
| **Mild anaemia** | 2.56 | 1.01-6.49 | 0.048 |
| **Moderate anaemia** | 4.91 | 1.90-12.66 | 0.001 |
| **Severe anaemia** | 14.03 | 4.91-40.09 | 0.001 |

**Table note:**

Anaemia was defined as levels of Hb <13 g/dL for men or <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 anaemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anaemia (SA) was defined as Hb<8g/dL for both sexes. Variables included in the model: anaemia grade, age, sex and country. Abbreviations: Hazard Ratio (HR); Confidence Interval (CI).

**Supplementary Table 14. Clinical data and concentrations of inflammatory markers in peripheral blood, according to IRIS development.**

| | Without IRIS (n=405) | With IRIS (n=97) | P value |
|---|---|---|---|
| **Age, median (IQR)** | 37.0 (32.0-45.0) | 37.0 (30.0-44.0) | |
| **Country, n (%)** | | | |
| **Kenya** | 165 (40.7) | 33 (34.0) | |
| **Thailand** | 84 (20.7) | 16 (16.5) | |
| **USA** | 156 (38.5) | 48 (49.5) | |
| **Male, n (%)** | 247 (61.0) | 60 (61.9) | |
| **Anaemia grade, n (%)** | | | |
| **Without anaemia** | 77 (19.0%) | 5 (5.15%) | |
| **Mild anaemia** | 221 (54.6%) | 44 (45.4%) | |
| **Moderate anaemia** | 94 (23.2%) | 35 (36.1%) | |
| **Severe anaemia** | 13 (3.21%) | 13 (13.4%) | |
| **Glucose (mg/dL), median (IQR)** | 85.0 (77.0-94.3) | 85.0 (76.4-96.1) | |
| **WBC ($10^6$/µL), median (IQR)** | 3.25 (2.47-4.28) | 3.20 (2.38-4.96) | |
| **Platelets ($10^6$/µL), median (IQR)** | 221 (161-285) | 219 (174-302) | |
| **Hb (g/dL), median (IQR)** | 11.2 (9.80-12.7) | 10.0 (8.80-11.2) | |
| **CD4$^+$ count/µL, median (IQR)** | 29 (12-58) | 22 (7-49) | |
| **CD8$^+$ count/µL, median (IQR)** | 478 (281-747) | 384 (237-656) | |
| **BMI (Kg/m$^2$), median (IQR)** | 20.5 (17.9-23.4) | 19.7 (17.9-23.0) | |
| **HIV Viral Load ($Log_{10}$ copies/mL), median (IQR)** | 5.28 (4.87-5.70) | 5.37 (5.07-5.70) | |
| **MPO (pg/mL)** | 4.20 (4.00-4.38) | 4.24 (4.11-4.53) | **0.029** |
| **TNF (pg/mL)** | 11.6 (7.87-16.6) | 13.9 (9.05-21.7) | **0.009** |
| **CXCL10 (pg/mL)** | 2274 (1469-3668) | 2955 (1663-4429) | **0.027** |
| **sCD14 (pg/mL)** | 6.34 (6.25-6.48) | 6.43 (6.34-6.53) | **<0.001** |
| **HA (ng/mL)** | 96.0 (57.2-160) | 146 (92.7-231) | **<0.001** |
| **IFN-γ (pg/mL)** | 3.78 (2.02-9.09) | 6.62 (3.25-15.0) | **<0.001** |
| **IL-10 (pg/mL)** | 9.32 (5.46-16.8) | 13.5 (6.38-24.0) | **0.001** |
| **IL-27 (pg/mL)** | 468 (293-718) | 576 (407-980) | **<0.001** |
| **IL-2 (pg/mL)** | 1.11 (0.85-1.55) | 1.31 (0.90-1.77) | 0.181 |
| **IL-6 (pg/mL)** | 2.36 (1.45-6.07) | 3.62 (2.10-7.26) | **0.002** |
| **IL-8 (pg/mL)** | 10.3 (5.82-18.6) | 14.1 (7.24-30.6) | **0.024** |
| **sTF (pg/mL)** | 76.6 (58.7-103) | 80.9 (59.0-111) | 0.491 |
| **sCD163 (pg/mL)** | 494 (235-885) | 555 (338-957) | 0.225 |
| **D-Dimer (pg/mL)** | 5.99 (5.75-6.26) | 6.20 (5.95-6.52) | **<0.001** |
| **CRP (pg/mL)** | 6.62 (6.09-7.17) | 6.98 (6.65-7.50) | **<0.001** |

**Table note:**
Anaemia was defined as levels of Hb <13 g/dL for men or <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 anaemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anaemia (SA) was defined as Hb<8g/dL for both sexes. Data are shown as median and interquartile (IQR) range. Data were compared between the clinical groups using the Mann-Whitney *U* test. Abbreviations: CRP: C Reactive Protein; HA: Hyaluronic Acid; Hb: Haemoglobin; IL: interleukin; IQR: Interquartile Range; MPO: myeloperoxidase; TF: Tissue Factor; TNF: tumour Necrosis Factor. WBC: white blood cells. Bold type font indicates statistical significance (p-value below 0.05).

**Supplementary Table 15. Clinical data and concentrations of inflammatory markers in peripheral blood, according to mycobacterial-IRIS development.**

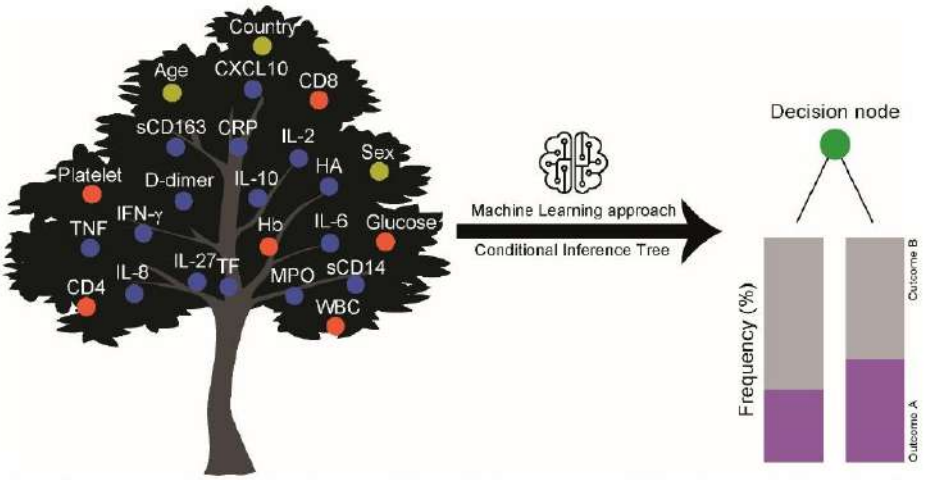
| | Other IRIS (n=50) | MYC-IRIS (n=47) | P value |
|---|---|---|---|
| **Age, median (IQR)** | 39.0 (30.8-43.2) | 36.0 (29.0-44.0) | |
| **Country, n (%)** | | | |
| **Kenya** | 15 (31.2) | 18 (37.5) | |
| **Thailand** | 8 (16.7) | 7 (14.6) | |
| **USA** | 25 (52.1) | 23 (47.9) | |
| **Male, n (%)** | 31 (64.6) | 28 (58.3) | |
| **Anaemia grade, n (%)** | | | |
| **Without anaemia** | 5 (10.4) | 0 (0.00) | |
| **Mild anaemia** | 25 (52.1) | 19 (39.6) | |
| **Moderate anaemia** | 13 (27.1) | 21 (43.8) | |
| **Severe anaemia** | 5 (10.4) | 8 (16.7) | |
| **Glucose (mg/dL), median (IQR)** | 83.5 (76.9-89.5) | 86.0 (76.1-107) | |
| **WBC ($10^6$/µL), median (IQR)** | 3.48 (2.58-5.09) | 3.10 (2.30-4.88) | |
| **Platelets ($10^6$/µL), median (IQR)** | 219 (171-275) | 216 (175-320) | |
| **Hb (g/dL), median (IQR)** | 10.6 (9.17-11.9) | 9.65 (8.40-10.5) | |
| **$CD4^+$ count/µL, median (IQR)** | 18 (7-50) | 27 (8-47) | |
| **$CD8^+$ count/µL, median (IQR)** | 388 (247-635) | 350 (161-790) | |
| **BMI (Kg/$m^2$), median (IQR)** | 20.7 (18.1-24.3) | 18.8 (16.6-21.2) | |
| **HIV Viral Load ($Log_{10}$ copies/mL), median (IQR)** | 5.28 (4.96-5.59) | 5.64 (5.21-5.88) | |
| **MPO (pg/mL)** | 4.18 (4.09-4.35) | 4.33 (4.13-4.61) | 0.069 |
| **TNF (pg/mL)** | 11.6 (8.69-17.2) | 14.8 (10.9-28.4) | **0.022** |
| **CXCL10 (pg/mL)** | 2188 (1537-3911) | 3391 (2051-4742) | **0.036** |
| **sCD14 (pg/mL)** | 6.39 (6.30-6.49) | 6.47 (6.40-6.60) | **0.013** |
| **HA (ng/mL)** | 111 (59.2-170) | 184 (120-287) | **0.003** |
| **IFN-γ (pg/mL)** | 4.77 (2.72-10.4) | 8.53 (4.94-20.3) | **0.007** |
| **IL-10 (pg/mL)** | 8.77 (5.96-19.8) | 18.0 (12.1-27.0) | **0.003** |
| **IL-27 (pg/mL)** | 471 (379-733) | 738 (490-1125) | **0.005** |
| **IL-2 (pg/mL)** | 1.43 (1.00-1.78) | 1.21 (0.80-1.72) | 0.446 |
| **IL-6 (pg/mL)** | 3.29 (1.97-5.44) | 4.05 (2.36-10.6) | 0.086 |
| **IL-8 (pg/mL)** | 13.4 (6.47-20.8) | 16.5 (8.22-36.1) | 0.157 |
| **sTF (pg/mL)** | 81.2 (58.3-111) | 77.6 (63.6-108) | 0.979 |
| **sCD163 (pg/mL)** | 448 (324-758) | 673 (385-1195) | 0.079 |
| **D-Dimer (pg/mL)** | 6.15 (5.85-6.39) | 6.30 (6.01-6.58) | **0.019** |
| **CRP (pg/mL)** | 6.95 (6.64-7.36) | 7.09 (6.66-7.63) | 0.171 |

**Table note:**
Anaemia was defined as levels of Hb <13 g/dL for men or <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 anaemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anaemia (SA) was defined as Hb<8g/dL for both sexes. Data are shown as median and interquartile (IQR) range. Data were compared between the clinical groups using the Mann-Whitney *U* test. Abbreviations: CRP: C Reactive Protein; HA: Hyaluronic Acid; Hb: Haemoglobin; IL: interleukin; IQR: Interquartile Range; MPO: myeloperoxidase; MYC: mycobacterial; TF: Tissue Factor; TNF: Tumour Necrosis Factor. WBC: white blood cells. Bold type font indicates statistical significance (p-value below 0.05).

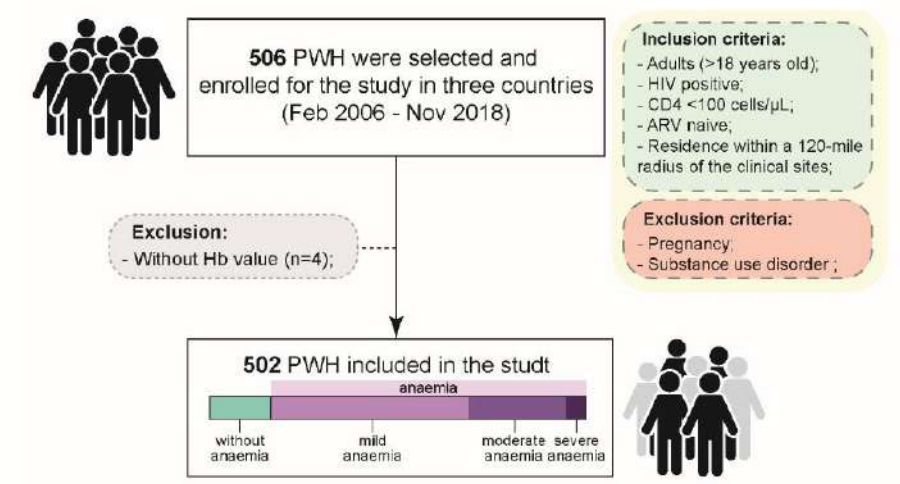
**Supplementary Table 16. Hazard Ratio for Death according to anaemia grade.**

| | Hazard Ratio (HR, cox Regression) | | |
|---|---|---|---|
| | **HR** | **95% CI** | **p value** |
| **Without anaemia** | - | - | - |
| **Mild anaemia** | 1.44 | 0.47-4.39 | 0.524 |
| **Moderate anaemia** | 1.66 | 0.49-5.63 | 0.415 |
| **Severe anaemia** | 3.52 | 0.92-13.4 | 0.065 |

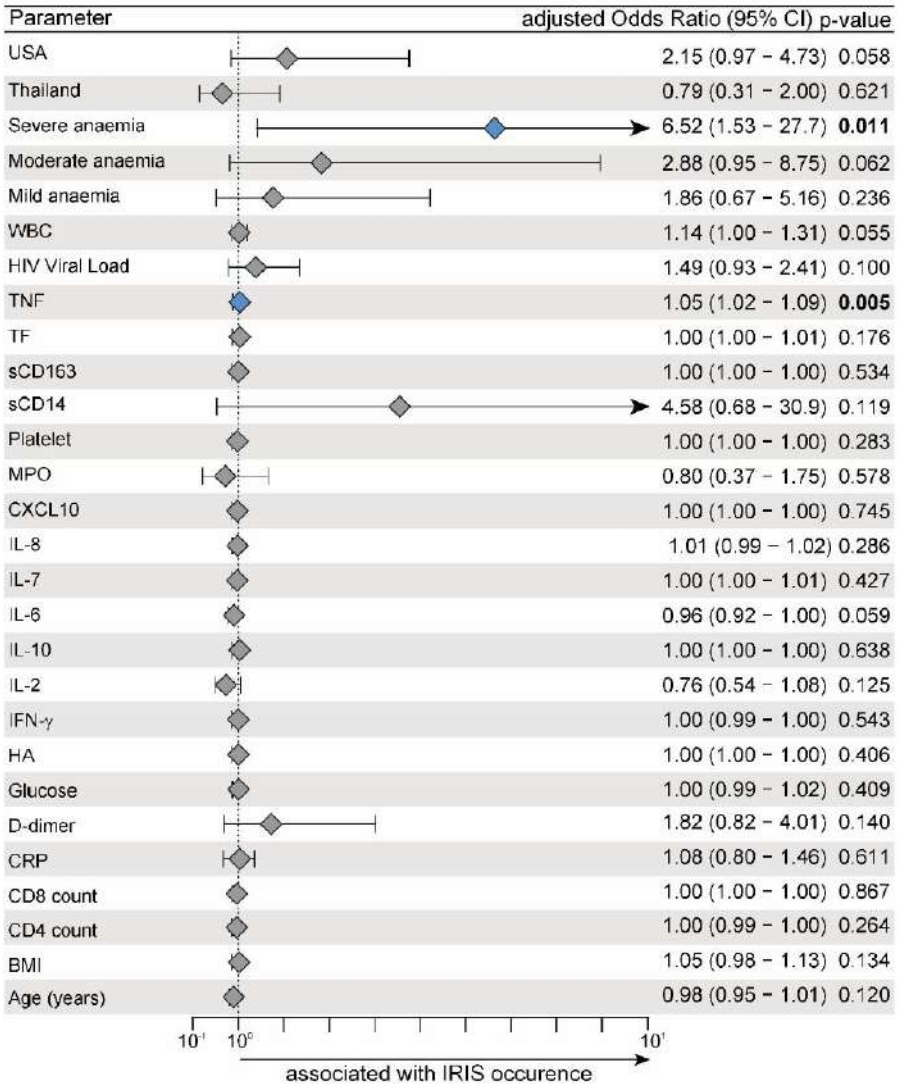
**Table note:**
Anaemia was defined as levels of Hb <13 g/dL for men or <12 g/dL for women. Mild anaemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate anaemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anaemia (SA) was defined as Hb<8g/dL for both sexes. Variables included in the model: anaemia grade, age, sex and country. Abbreviations: Hazard Ratio (HR); Confidence Interval (CI).



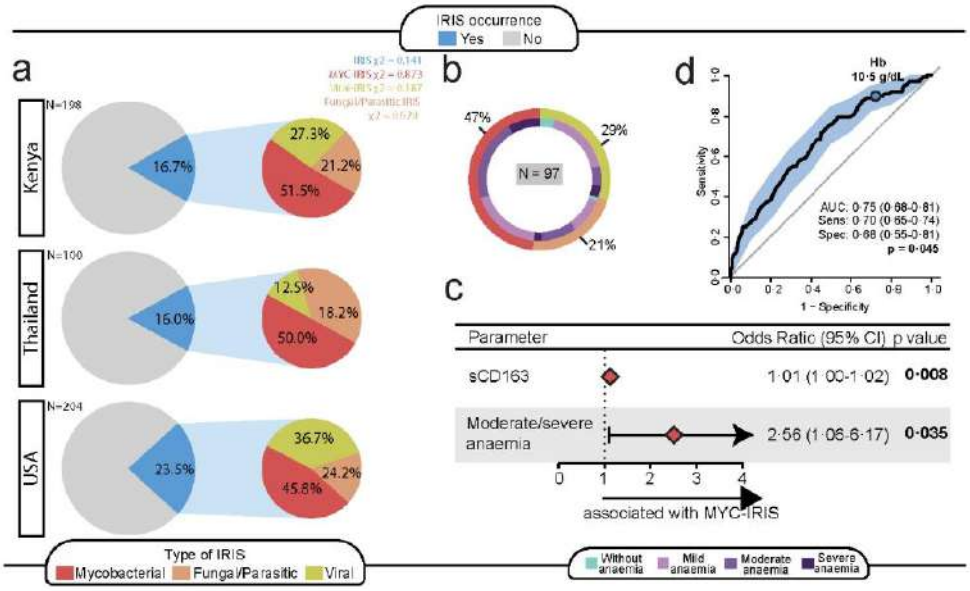

**Supplementary Figure 1.** The conditional inference tree (CTree) was built using the clinical, inflammatory markers and Hb values. This approach bases splitting decisions on univariate regression models, and following the initial split, subsequent inference takes place within subgroups. CTree select the input variable with the highest p-value with response variable.



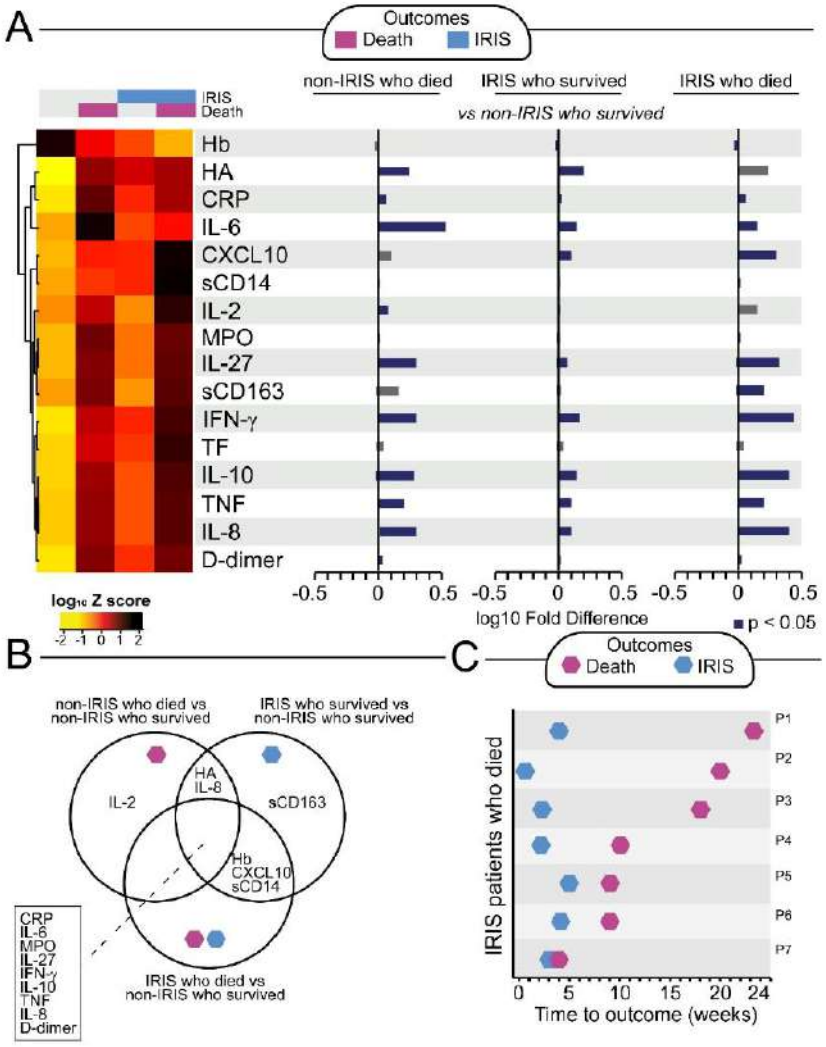

Supplementary Figure 2. Study Flow Chart. 506 people with HIV (PWH) were firstly included in the cohort. However, four patients were removed due to lack in haemoglobin data.



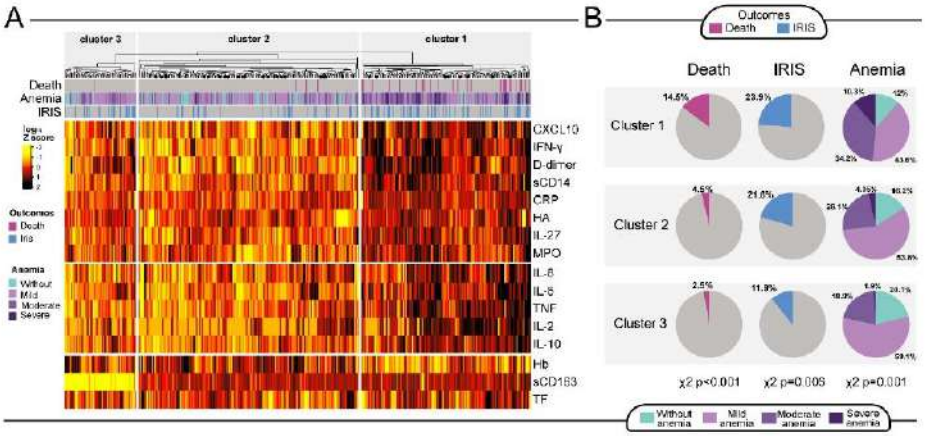

Supplementary Figure 3. Binomial logistic regression model (backward stepwise regression) to test independent associations between all clinical and laboratory parameters (Supplementary Table 11) and IRIS occurrence.




Supplementary Figure 4. Moderate and severe anaemia were associated with mycobacterial-IRIS occurrence. (a) IRIS occurrence according to country of origin. Each country has your number of patients reported, as well frequency of IRIS occurrence and the frequency according to types of IRIS. The chi-square test between countries for each variable (IRIS and type of IRIS) were calculated and have demonstrated that there are not statistically significant differences of occurrence of IRIS and type of IRIS between countries of origin. (b) IRIS occurrence according to types and anaemia severity. (c) Binomial logistic regression model (backward stepwise regression) tests independent associations between all the relevant parameters (p value < 0.1 in Supplementary Table 10) and mycobacterial-IRIS occurrence in IRIS population. Only measurements that remained in the last step are shown. Measurements entered on step 1: anaemia grade (dichotomized), BMI, HIV Viral Load, TNF, CXCL10, HA, IFN-γ, IL-10, IL-27, IL-2, IL-6, sCD163, MPO, sCD14. (d) ROC curve analysis to evaluate the discrimination power of Hb.




Supplementary Figure 5. Profile of patients according to IRIS development and death. (A) Right panel: A heatmap was designed to depict the overall pattern of inflammatory markers in patients according to IRIS and death occurrence. Four groups were established: non-IRIS who survived (n=391); non-IRIS who died (n=24); IRIS who survived (n=90) and IRIS who died (n=7). 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-IRIS who survived). Significant differences ($p < 0.05$) are highlighted in dark blue bars. (B) Venn diagram of inflammatory markers with statistical difference between comparisons. (C) Panel shows the time of IRIS development (in blue) and death (in purple) during the study for the IRIS patients who died.




Supplementary Figure 6. A more pronounced inflammatory profile is associated with the occurrence of IRIS and death. (a): An unsupervised two-way hierarchical cluster (Ward's method) was performed with all 502 participants. Data were log10 transformed and ranked and coloured in a heatmap from values detected for each inflammatory biomarker. Dendrograms represent Euclidean distance. Three main clusters were defined. (b) For each cluster, shaded areas represent the frequency of death (pink), IRIS (blue), and anaemia grade (light blue to dark purple). A chi-squared test was performed for each variable comparing clusters (Death: Pearson chi square p<0.001; IRIS: Pearson chi square p<0.001; Anaemia: Pearson chi square p<0.001).

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## 5.3 Manuscrito III

### Título

"Relationship Between Anemia and Systemic Inflammation in People Living With HIV and Tuberculosis: A Sub-Analysis of the CADIRIS Clinical Trial"

### Objetivo

Esse trabalho teve como objetivo demonstrar que a ocorrência da anemia em pacientes HIV-TB está associada a um perfil inflamatório sistêmico mais acentuado em comparação a pacientes HIV-TB não anêmicos.

### Resumo de resultados

Esse estudo utilizou uma coorte de 159 PVHIV, dos quais 61 eram coinfectados por TB. Através da análise de marcadores clínicos, laboratoriais de rotina e da dosagem de um amplo perfil de marcadores inflamatórios, aplicamos uma estratégia matemática aqui chamada de "Grau de Perturbação Inflamatória (GPI)" para avaliar o perfil sistêmico inflamatório de pacientes com e sem anemia nos grupos com e sem TB. Observamos inicialmente que pacientes com TB apresentavam anemia em maior frequência e em maior gravidade que aqueles sem TB. Foi observado também que apenas no grupo HIV-TB havia uma diferença de perfil inflamatório entre pacientes com e sem anemia. Nesse mesmo grupo baixos níveis de anemia foram associados a maiores níveis de TNF, sCD14 e sCD163. Pacientes HIV-TB apresentaram um GPI mais alto que os não TB. Por fim, aqueles com HIV-TB e anemia apresentaram o maior GPI em comparação a todos os outros grupos. O alto GPI foi relacionado a níveis mais altos de TNF e IFN-γ, assim como níveis mais baixos de Hb. Assim, o alto GPI em PVHIV foi associado à coinfecção por TB e ocorrência de anemia antes do tratamento antirretroviral.

### Status do manuscrito

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# Relationship Between Anemia and Systemic Inflammation in People Living With HIV and Tuberculosis: A Sub-Analysis of the CADIRIS Clinical Trial

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People with HIV (PWH) are at increased risk of developing active tuberculosis (TB), and anemia is a common complication in both conditions. Anemia in TB patients has been linked to immune activation, levels of inflammatory biomarkers in blood, and risk for HIV disease progression and death. In this study we show that anemia was associated with a more pronounced inflammatory profile in HIV-TB coinfected persons in a cohort of 159 individuals with advanced HIV disease (CD4 count < 100 cells/μL) recruited as part of a randomized clinical trial (NCT00988780). A panel of plasma biomarkers was assessed on plasma obtained prior to combination antiretroviral therapy (cART) initiation. We performed a series of multidimensional analyses including clinical variables and concentrations of inflammatory biomarkers to profile systemic inflammation of PWH with and without anemia. We observed that TB participants presented with moderately lower levels of hemoglobin than non-TB participants. These participants also presented a higher Degree of Inflammatory Perturbation (DIP) score, related to increased levels of IFN-γ and TNF. The DIP was associated with TB coinfection and anemia before cART initiation. Future mechanistic studies are warranted to assess the determinants of such associations and the implications on treatment outcomes.

**Keywords: HIV, Tuberculosis, inflammation, degree of inflammatory perturbation, anemia**

## INTRODUCTION

By the end of 2020 it was estimated that there were 38 million people with HIV (PWH) globally. Every year approximately 1.7 million people are newly infected and about 690,000 people die from complications caused by HIV infection (1). When not treated, PWH typically progress through stages, the last being the acquired immunodeficiency syndrome (AIDS) (2).

AIDS is defined by a CD4+ T-cell count below 200 cells/μL or by the identification of at least one AIDS-defining illness. Tuberculosis (TB) is among the most frequently diagnosed AIDS-defining illnesses (2). TB is an infectious disease caused by Mycobacterium tuberculosis (*Mtb*), and in 2019, approximately 10 million people were living with active TB (PLTB) and 1.3 million TB-associated deaths were estimated. Also according to these statistics, 8% of PLTB are thought to be co-infected with HIV, and 16% of TB-associated deaths occurred in this population of HIV-TB co-infected persons (1).

PWH coinfected with TB have a substantially higher death risk (3), a lower quality of life and health (4), and lower hemoglobin (Hb) concentrations in peripheral blood (5), compared to mono-infected patients (i.e. either TB or HIV alone). Low levels of Hb are associated with increased levels of inflammatory biomarkers in blood of TB patients (6) and with the acceleration of HIV disease progression (7, 8). A previous study of our group had demonstrated, using routine clinical laboratory parameters, that anemia is a risk factor for unfavorable anti-TB treatment outcomes, and that Hb levels are associated with a heightened degree of inflammatory perturbation in TB-HIV patients (9). In the present study, we expand the investigation of inflammatory disturbance associated with anemia in the context of TB-HIV by examining levels of inflammatory cytokines using plasma samples of PWH with advanced disease participating in a randomized clinical trial (10). Our aim was to evaluate the relationship of low Hb levels and the inflammatory profile of PWH and AIDS-TB.

## METHODS

### Study Outline

The CADIRIS study (CCR5 Antagonism to Decrease the Incidence of immune reconstitution inflammatory syndrome (IRIS) in HIV infected participants, NCT00988780) enrolled 276 participants from 2009 to 2012 at five clinical sites in Mexico and South Africa in a double-blind, randomized, placebo-controlled study that followed participants for 1 year after cART initiation (10). Participants were adults (age at least 18 years) with HIV-infection, cART-naïve, and with CD4 cell count equal to or lower than 100 cells/μL; individuals with Hb <8g/dL were excluded. For this sub-study, we included CADIRIS participants with AIDS-defining illnesses or HIV-associated wasting syndrome at enrollment, and with available blood samples for analysis (n=159). We evaluated data of nationality, sex, TB status (active TB or no TB), anemia severity (healthy, mild and moderate), and levels of biomarkers measured in cryopreserved plasma specimens from study participants collected at baseline (pre-cART).

### Ethical Statement

CADIRIS study was approved by the Ethics Review Committee at all participating institutions. All participants provided written informed consent before any study procedures.

### Definitions

Anemia was defined according to World Health Organization (WHO) guideline criteria as levels of Hb below (<)13 g/dL for men or <12 g/dL for women (11). Mild anemia was defined as Hb >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate anemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anemia (Hb<8g/dL) was an exclusionary criterion as previously stated. TB diagnoses were made with mycobacterial blood culture, tuberculin skin tests, and chest radiographs.

### Biomarker Measurement

Collection and cryopreservation of blood derived specimens have been described previously (12). We measured biomarkers using commercial kits to evaluate C-Reactive Protein (CRP), Interferon-γ (IFN-γ), interleukin (IL)-1β, IL-6, IL-8, IL-10, IL-12p70, IL-17, interferon-inducible protein (CXCL) 10, P-selectin, serum amyloid A (SAA), tumor necrosis factor-α (TNF), Leukotriene B4 (LTB4), soluble (s)CD14, sCD40 ligand, sCD163, Von Willebrand Factor (vWF), fibrinogen (FIB), proteins C and S, Hyaluronic Acid (HA), D dimer and 25-hydroxyvitamin-D levels, using electrochemiluminescence (MESO scale discovery), enzyme-linked immunosorbent assays (R&D Systems, AdipoBioscience, Zymutest, ALPCO), enzyme-linked fluorescent assay (Biomeriuex) and enzyme coagulation kit (Corgenix) methods.

### Statistical Analysis

Descriptive statistics were used to present data, using median values with interquartile ranges (IQR) as measures of centrality and dispersion for continuous variables. Categorical variables were described using frequency (no.) and proportions (%). The Pearson's chi-square test was used to compare categorical variables between study groups (i.e. TB and non-TB). The Mann–Whitney U test (for two unmatched groups; i.e. TB and non-TB) was used to compare continuous variables. The Spearman rank test was used to assess correlations between Hb and biomarkers.

The degree of inflammatory perturbation (DIP) was calculated to identify the general inflammatory environment of the participants. DIP was based on the molecular degree of perturbation (MDP) (13) and calculated as previously described (9). Details about this method are described in **Supplementary Figure 1**.

We used backward stepwise linear regression to examine the association between biomarkers and Hb levels. All biomarkers were included in this analysis and all values were log 2 transformed. Next, we used multi-level Poisson regression models to estimate the association between units of biomarkers and Hb with DIP score levels. The results were presented in the form of adjusted Odds Ratio (aOR) and 95% confidence intervals (CI).

Differences with p-values below 0.05 after adjustment for multiple comparisons (Holm-Bonferroni) were considered statistically significant. The statistical analyses were performed using IBM SPSS version 25; and R (version 4.4.1), using mdp (version 1.8.0), rstatix (version 0.4.0), stats (version 3.6.2), metaphor (version 2.4.0) and questionr (version 0.7.1) R packages.

## RESULTS

### Characteristics of the Study Participants

We included 159 PWH from Mexico and South Africa, mostly men (74.8%) in both countries. All participants were diagnosed with an AIDS-defining illness detailed in **Supplementary Table 1**. We stratified these participants into two main groups: non-TB (n=98) and TB (n=61). We found that 65.6% of participants with TB were from South Africa, while 78.6% of the non-TB group were from Mexico (p<0.001). TB participants were more frequently self-reported Blacks, whereas "Mixed" was the race most often self-declared in non-TB participants (p<0.001) (**Table 1**).

TB participants presented with lower levels of Hb with a median of 11.3 g/dL (9.80-13.0) in comparison to non-TB participants, with 12.2 g/dL (11.3-13.6) (p=0.002). Of note, this difference was observed comparing all participants according to TB status, as well as comparing TB status according to sex (non-TB male: 12.2(11.1-13.8) *vs* TB male: 11.8(10.3-13.2), p = 0.081; non-TB female: 12.4(11.5-12.7) *vs* TB female: 10.8 (9.57-11.2), p=0.005). Interestingly, TB participants had lower CD8 T cell counts (p = 0.037) and higher CD4/CD8 ratio values when compared to the non-TB group (p=0.004). Baseline plasma HIV levels and CD4 T-cell counts were similar in the groups (**Table 1**).

We decided to examine how participants were distributed in terms of anemia status and we characterized their inflammatory profile. About 65% (n=64) of the non-TB group presented with anemia, whereas in the TB group this frequency was higher, at 80% (n=49). Noteworthy, we found no difference in occurrence of anemia when comparing patients from Mexico and South Africa. Most anemic participants in both groups, had mild anemia (non-TB: 86%; TB: 67.3%). Participants in the TB group exhibited a higher proportion of moderate anemia (33%) than participants in the non-TB group (14%, p=0.007) (**Figure 1A**).

### Anemia Is Linked to the Inflammatory Profile in TB

After classifying the participants according to anemia status, the biomarker levels of the anemic and non-anemic participants were compared within each clinical group, to identify the relationship of anemia to systemic inflammation. A hierarchical heatmap was built using log2-transformed and Z-score normalized data and clustered according to anemia status to analyze the differences of inflammatory profile between groups and conditions (**Figure 1B**). In the non-TB group, anemia appeared not to be significantly linked to levels of inflammatory cytokines, except for IL-6 levels which were increased in anemic participants, with a median of 2.45 pg/mL (IQR:1.68-3.64) in comparison to levels in non-anemic participants (2.14 [IQR:1.29-2.63], p = 0.046) (**Figure 1B**; **Supplementary Table 2**). In contrast, anemic participants in the TB group presented an inflammatory profile distinct from that of non-anemic participants, with significant increases in FIB, IL-10, sCD40L, IL-8, D Dimer, TNF, HA, CRP, IFN-γ and sCD14 (**Figure 1B**) levels. Medians and p-values of these comparisons are detailed in **Supplementary Table 2**.

Next, a Spearman correlation network analysis between Hb concentrations and inflammatory marker levels was performed for each group (non-TB and TB). CD4 and CD8 T-cell counts were also included in the analysis. Hemoglobin values displayed negative correlations with inflammatory markers in both groups, being more frequently identified in the TB group (**Figure 2**).

**TABLE 1** | Characteristics of the study population.

| | ALL (n=159) | non-TB (n=98) | TB (n=61) | P-value |
|---|---|---|---|---|
| **Country, no. (%):** | | | | **<0.001** |
| **Mexico** | 98 (61.6) | 77 (78.6) | 21 (34.4) | |
| **South Africa** | 61 (38.4) | 21 (21.4) | 40 (65.6) | |
| **Male, no. (%):** | 119 (74.8) | 76 (77.6) | 43 (70.5) | 0.418 |
| **Race, no. (%):** | | | | **<0.001** |
| **Asian** | 1 (0.63) | 1 (1.02) | 0 (0.00) | |
| **Black** | 58 (36.5) | 18 (18.4) | 40 (65.6) | |
| **Mixed** | 88 (55.3) | 70 (71.4) | 18 (29.5) | |
| **White** | 12 (7.55) | 9 (9.18) | 3 (4.92) | |
| **Hemoglobin (g/dL), median (IQR):** | 12.0 (10.8-13.2) | 12.2 (11.3-13.6) | 11.3 (9.80-13.0) | **0.002** |
| **HIV Viral Load log10, median (IQR):** | 5.27 (4.93-5.62) | 5.40 (5.00-5.76) | 5.51 (5.15-5.82) | 0.181 |
| **CD4 (cells/μL), median (IQR):** | 31 (16-58) | 31 (14-53) | 32 (21-62) | 0.210 |
| **CD8 (cells/μL), median (IQR):** | 475 (342-760) | 524 (385-800) | 423 (296-671) | **0.037** |
| **CD4/CD8, median (IQR):** | 0.06 (0.03-0.10) | 0.05 (0.02-0.10) | 0.07 (0.05-0.13) | **0.004** |

*Bold font indicates statistical significance at p<0.05. Data are shown as number and frequency (percentage). Data were compared between groups using the Pearson chi-square test for categorical variables and the Wilcoxon test for continuous variables.*
*IQR, interquartile range.*

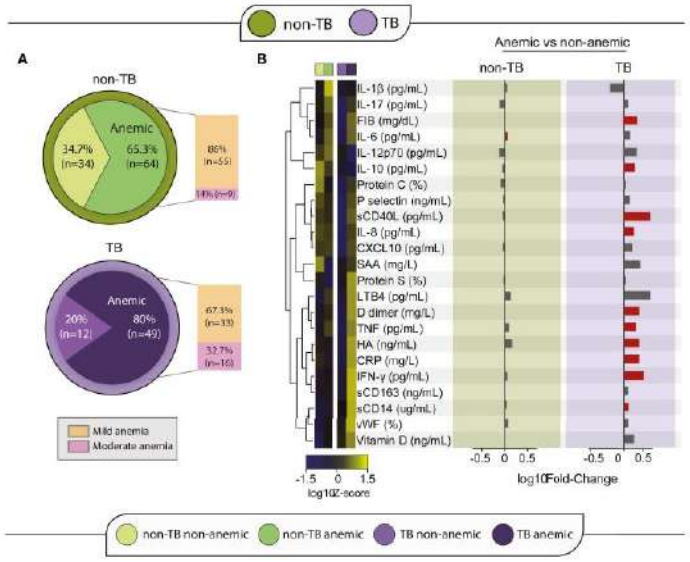


**FIGURE 1** | Anemia is linked to a differential inflammatory profile in TB-HIV participants. **(A)** Participants of each group were divided according to anemia status and grade. To define anemia grade, the cut-off point for hemoglobin of 12 g/dL for women and 13 g/dL for men was used to define normal levels, the cut-off of 10 g/dL and lower than normal levels was used for mild anemia and hemoglobin between 8 and 10 g/dL was classified as moderate anemia. **(B)** 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. Right panel: A log10 of fold-change was calculated and statistical analyses were performed using the Mann–Whitney U adjusted test. Significant differences ($p < 0.05$) between groups are highlighted in red bars.

In the non-TB group, significant albeit weak correlations were noted between Hb and two markers: TNF (r=-0.21, p=0.03) and IL-6 (r=-0.22, p=0.03). In the TB group correlations were found between Hb and IL-1β (r=-0.28, p=0.04), IL-6 (r=-0.29, p=0.04), sCD163 (r=-0.3, p=0.04), sCD40L (r=-0.32, p=0.02), FIB (r=-0.36, p=0.007), HA (r=-0.38, p=0.005), D Dimer (r=-0.39, p=0.005), IL-8 (r=-0.44, p=0.001), sCD14 (r=-0.47, p<0.001) and TNF (r=-0.51, p<0.001). Of note, in the TB group the negative correlation of Hb was also seen with the CD4 count (r=-0.5, p<0.001). Moreover, the correlation between Hb values and CD8 count was not statistically significant (r=0.068; p=0.06) (**Figure 2A**).

A network analysis between biomarkers in groups stratified according to TB and anemia was also performed. In this analysis, it was observed that CRP was the most interconnected marker with other markers among all groups, but especially in anemic TB participants. In this group, there was also a high amount of cytokine connectivity with IL-6. This greater interconnectivity in HIV-TB participants with anemia reflects a greater network density compared to the other groups and may be associated with a coordinated immune response (**Supplementary Figure 2**).

When we performed a backward stepwise linear regression to identify the independent associations between cytokine measurements and Hb values in the two main study groups, we found that higher values of pro-inflammatory cytokines were associated with low Hb values in both groups (non-TB and TB) (**Figure 2B**). In the non-TB group, decreasing levels of IL-6 (β coefficient: -0.09; 95%CI: -0.12 to -0.05; p<0.001), LTB4 (β coefficient: -0.03; 95%CI: -0.06 to -0.01; p=0.003) and sCD14 (β coefficient: -0.08; 95%CI: -0.14 to -0.01; p=0.031) as well as increasing levels of IL-10 (β coefficient: 0.03; 95% CI: 0.01 to 0.06; p=0.034) and IL-1β (β coefficient: 0.04; 95% CI: 0.01 to 0.06; p=0.009) were independently associated with an increase of one Hb (log2) unit. In the TB group, decreasing levels of TNF (β coefficient: -0.12; 95%CI: -0.21 to 0.04, p=0.003), sCD14 (β coefficient: -0.18; 95%CI: -0.29 to -0.07, p=0.002) and sCD163 (β coefficient: -0.05 95%CI: -0.09 to -0.01, p=0.01) were associated with an increase of one Hb (log2) unit. These associations indicate that, in PWH, low Hb levels are associated with greater systemic inflammation.

## PWH With Anemia Have Higher Degrees of Inflammatory Perturbation Even in the Absence of TB

With the individuals classified according to TB infection status in two main groups, as well as subdivided accordingly to their anemia status in four groups (non-TB non-anemic, non-TB anemic, TB non-anemic and TB anemic), we employed DIP approach, to estimate the overall degree of inflammation and/or immune activation in TB participants. In the first comparison,

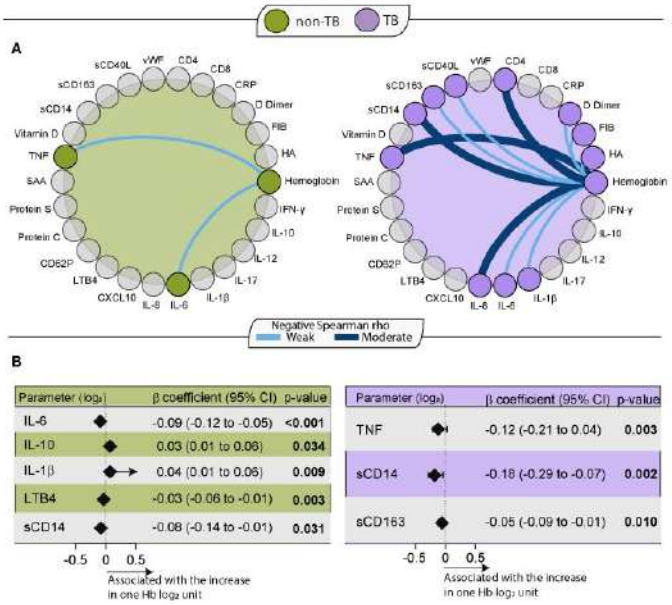


**FIGURE 2** | Hemoglobin levels are related to the inflammatory profile of HIV-TB participants. **(A)** Spearman correlation test between Hb levels and laboratory measurements for each group (Green: non-TB; Purple: TB). Light blue lines indicate a weakly negative (rho <0.40) correlation, and dark blue lines indicate a moderately strong negative (0.4≤ rho <0.6) correlation between the linked parameters. All correlations in this chart had p value less than 0.05. **(B)** Backward stepwise linear regression to identify biomarker measurements independently associated with Hb levels. All parameters were log2 transformed. Only significant parameters in the last step are shown. Left: non-TB (green). Right: TB (purple).

non-TB participants were used as controls (**Figure 3A**). Thus, it was possible to observe that TB coinfection is linked to an increased DIP (p<0.001) in PWH (**Figure 3A**).

We next assessed whether the groups with anemia had a different DIP than those without anemia, using the non-TB non-anemic group as control (**Figure 3B**). First, it was observed that TB non-anemic participants had the same low profile of perturbation as did the control group (p=0.810). DIP values were elevated in anemic participants without (p=0.004) and with TB (p=0.004) when compared to their respective non-anemic peers. The anemic TB group had a higher median DIP value than the non-anemic TB group (p=0.001), arguing that anemia in HIV-TB context is associated with inflammatory disturbance/activation (**Figure 3B**).

The top 10 biomarkers that contributed to inflammatory perturbation were: sCD14, IFN-γ, Protein S, HA < IL-8, TFN, sCD163, Hb, CD62P and vitamin D (**Figure 3C**). By performing Poisson regression in order to identify factors associated with higher DIP, we found that an increase in one unit of each DIP score was associated with 1 log increases in plasma IFN-γ (Exp (β): 1.68; 95%CI: 1.25-2.26, p=0.001) and TNF (Exp(β): 3.97; 95%CI: 1.96-8.06, p=0.001), as well as decreases in Hb levels (Exp (β): 0.01; 95%CI:0.001-0.16, p=0.001) in TB participants (**Figure 3D**), enabling us to identify the main markers involved in inflammatory disturbance in TB participants and, also associating low hemoglobin levels with this perturbation.

## DISCUSSION

In this retrospective analysis of a multicenter prospective study, we evaluated the relationship between low levels of hemoglobin on the inflammatory profile in PWH and TB. We performed a comprehensive investigation, measuring numerous host soluble inflammatory mediators collected prior to antiretroviral therapy initiation, in persons with advanced HIV infection.

We found that participants with TB coinfection presented with lower Hb levels and were more often anemic than non-TB participants. This finding corroborates those of an Ethiopian cohort of 230 participants, where HIV-TB participants also had lower levels of Hb than did non-TB participants (14). In our cohort, the frequency of anemia in TB participants was 80%. This frequency was similar to the findings of an earlier study by our group, where in a Brazilian cohort with HIV-TB, 84% of participants were anemic at baseline (9).

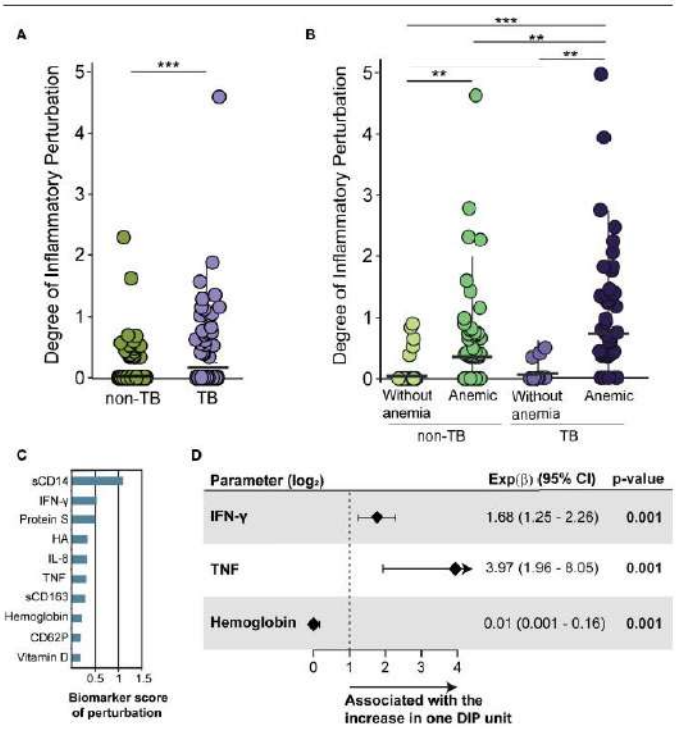


**FIGURE 3** | The degree of inflammatory perturbation increases in anemic participants. **(A)** Scatter plots of the degree of inflammatory perturbation (DIP) by sample in the main groups: non-TB (green) and TB (purple); **(B)** Scatter plots of DIP by sample in each group: non-TB non-anemic, used as control (light green); non-TB anemic (dark green); TB non-anemic (light purple); and TB anemic (dark purple). DIP score values were compared between groups according to TB and anemia status. Lines in the scatter plots represent median values and data were compared using the Mann–Whitney U test. $^{**}p < 0.01$; $^{***}p < 0.001$. **(C)** Top 10 biomarker score of perturbation in the comparison non-TB vs TB. The score was obtained using DIP approach. **(D)** Poisson regression to identify biomarker measurements independently associated with increases of DIP score in HIV-TB participants. All parameters are log2 transformed. Only significant parameters are shown.

Stratifying each main group according to anemia status, we observed that in TB, anemic participants had a distinct inflammatory profile in comparison to non-anemic participants. Other investigations have reported a higher inflammatory profile in TB participants in comparison to non-TB participants, showing that the increased systemic inflammation can be associated with a higher risk of unfavorable outcomes in HIV-TB (9, 14).

In TB participants, Hb measurements were strongly and negatively related to CD4 T-cell counts. The negative correlation with CD4 counts, interestingly, is contrary to what is reported in the literature, where a positive correlation between Hb and CD4 levels is described (15, 16). We hypothesize that in people with AIDS (or PWH and advanced disease), there may be opportunistic infections that affect the white blood cell count, which in turn can affect the CD4 T-cell count value. It has already been described that chronic diseases, infections and drug use can cause this effect among PWH (17).

Measurements of most of these inflammatory markers, especially TNF, sCD14, IL-8 and IL-6 also were strongly and negatively correlated with Hb levels. All these factors have already been described associated with a pro-inflammatory condition in PWH and reported as risk factors for the progression of HIV infection and increased morbidity and mortality (18–21). Of note, these markers identified in our data, in addition to Hb, were used to develop a composite score to predict mycobacterial IRIS and death in PWH in a separate observational cohort study (22).

In the linear regression performed here, higher levels of TNF and sCD14 appeared again associated with low Hb levels, with the addition of sCD163, which also presents this pattern in HIV-TB. TNF also was associated with increases in DIP in HIV-TB participants. TNF is potent inflammatory molecules primarily secreted by macrophages and required for control of growth of *Mtb* (23), however, this same cytokine is known to activate HIV replication in macrophages. Thus, TNF inhibits *Mtb* growth while

enhancing HIV replication (24). Linking this information to our results in anemic participants, we can infer that in PWH with anemia, there is an exacerbation of TNF production. Whether anemia itself is directly causing a boost in TNF production or if augmented inflammation is leading to the establishment and/or persistence of anemia cannot be determined with our data and warrants further mechanistic investigations.

The increased concentrations of sCD14 and sCD163 likely indicate an activation of monocyte/macrophages. sCD14 is a marker of monocyte response, described as an independent predictor of mortality (21) in PWH, whereas CD163 is an important surface marker expressed on monocyte-macrophage lineage cells and shed in soluble form during inflammation (25). In PWH, Hb levels have been associated with monocyte activation, reflecting in an increased risk of inflammatory events such as atherosclerosis (26).

There are limitations to our study. One of the inclusion criteria for CADIRIS was Hb >8g/dL, effectively excluding severe anemia which might have provided better power to related Hb with other markers, as well as having an impact on regression analyses. Baseline biomarker measurement allowed for the investigation of inflammatory profile in the context of TB and anemia but did not allow us to evaluate temporal changes during treatment. Moreover, we did not evaluate latent TB infection or co-infection with helminths in these patients, even is know that this can impact in immune response. It is also important to note that TB was more frequent in participants in South Africa than among those in Mexico. Of note, in a previous study, our group demonstrated that the different inflammatory profiles of the participants were associated with their country of origin (12). This could be explained by a higher incidence of HIV-TB among participants as reported by WHO (577 vs. <1 per 100 000 cases in 2009), which is consistent with distribution of TB-cases in this study (27). Although the TB prevalence observed here was higher in South Africa compared to that in Mexico, we found no difference in occurrence of anemia between the study participants stratified by country. Africa is known for being an epicenter of a number of hemoglobinopathies while Mexico is not, and thus one could infer that such difference could underlie discrepancies in anemia and its related information. Our study did not explore the impact of conditions affecting Hb metabolism and consequently further studies are warranted to test this hypothesis.

The present study demonstrates that there is an association between lower Hb concentration and augmented inflammatory disturbance in PWH and advanced disease regardless of TB. The inflammatory activation, characterized in our paper by increased levels of TNF and IFN-γ, and low Hb levels are described in the literature as a risk factor for adverse treatment outcomes of HIV and TB separately, and our study demonstrates that both factors are often present in TB coinfected participants. Such associations described here between soluble markers in peripheral blood and anemia may underlie the pathogenesis of advanced HIV which may drive unfavorable disease outcomes.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

CADIRIS study was approved by the Ethics Review Committee at all participating institutions. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

MA-P, PB-Z, ML, ISe, and BA designed the study. LM, AR, ISe, and JS-M collected the data. MA-P, BB-D, MA and BA analyzed and interpreted the data. MA-P, BB-D and BA drafted the manuscript. PB-Z, LJM, ML, ISe, and BA critically revised the manuscript. ML and JS-M obtained the funding. JS-M provided administrative, technical, and material support and supervised the study. All authors contributed to manuscript revision, read, and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.916216/full#supplementary-material

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The remaining 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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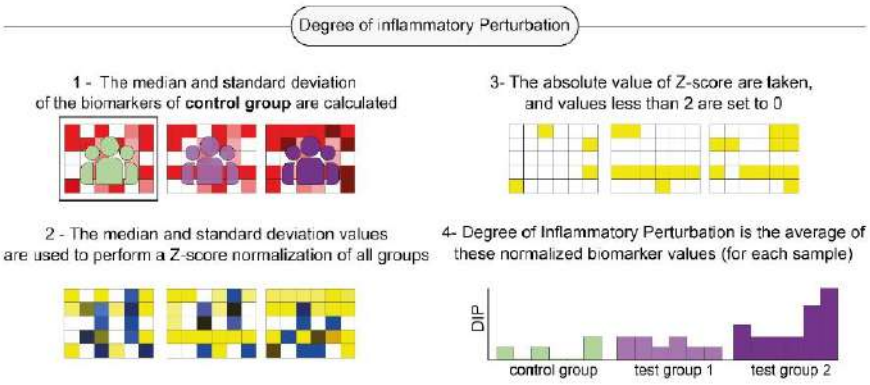
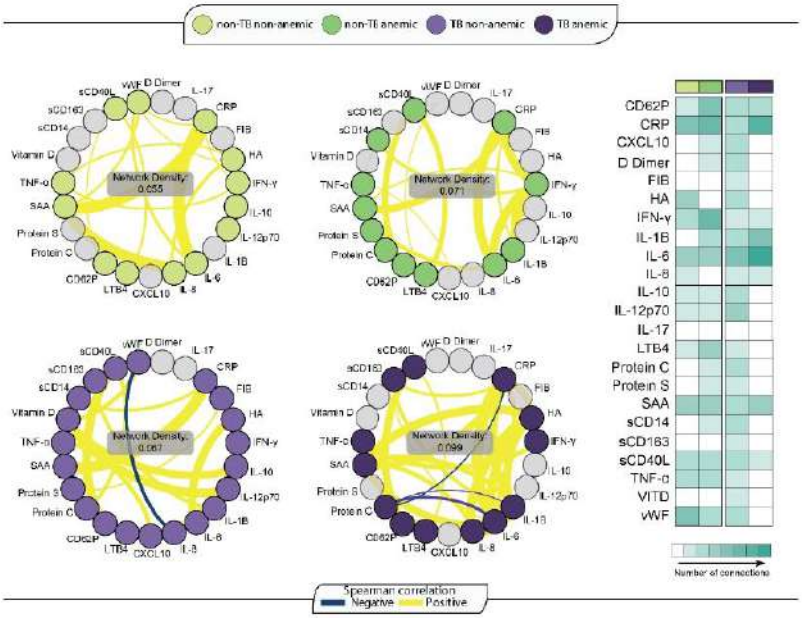
## *Supplementary Material*




**Supplementary Figure 1.** Degree of inflammatory perturbation (DIP) is based on Molecular Degree of Perturbation, but instead of using gene expression, we used biochemical and cellular markers. DIP was calculated using the median and standard deviation of the control group as a starting point. Then, the Z-score was calculated for all groups, a cut-off point was established and, finally, an average disturbance calculation was performed for each sample. This figure was adapted https://mdp.sysbio.tools/about.




**Supplementary Figure 2.** Anemic TB patients have greater interconnectivity between biomarkers. Left: Spearman correlation test between laboratory measurements for each group according anemia and TB status. Blue lines indicate negative correlation, and yellow lines indicate a positive correlation between the linked parameters. All correlations in this chart had p value less than 0.05. Right: Number of connections by biomarker and group.

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**Supplementary Table 1. AIDS defining illness**

| AIDS-defining illness | n |
|---|---|
| ***Candidiasis* of esophagus** | 10 |
| ***Cryptococcosis*** | 5 |
| ***Cryptosporidiosis*** | 5 |
| ***Cytomegalovirus*** | 4 |
| ***Histoplasmosis*** | 3 |
| ***Isosporiasis*** | 1 |
| ***Pneumocystis jirovecii pneumonia*** | 22 |
| ***Kaposi Sarcoma*** | 4 |
| ***Toxoplasmosis*** | 4 |
| ***Mycobacterium tuberculosis*** | 58 |
| **Wasting syndrome attributed to HIV** | 40 |

**Supplementary Table 2. Biomarker levels according to clinical groups.**

| | non-TB | | | TB | | |
|---|---|---|---|---|---|---|
| | Non-anemic at baseline (n=34) | Anemic at baseline (n=64) | P value | Non-anemic at baseline (n=12) | Anemic at baseline (n=49) | P value |
| D Dimer (mg/L) | 0.74 (0.52-1.31) | 1.17 (0.62-1.77) | 0.062 | 0.45 (0.31-1.11) | 1.54 (1.17-2.20) | **0.003** |
| CRP (mg/L) | 2.77 (1.22-6.62) | 4.44 (1.37-9.88) | 0.256 | 4.07 (1.01-6.99) | 9.30 (2.82-26.2) | **0.043** |
| SAA (mg/L) | 3.85 (1.27-7.29) | 7.28 (2.76-15.6) | 0.09 | 5.00 (1.49-10.1) | 12.5 (2.60-32.7) | 0.218 |
| CD62P (ng/mL) | 56.3 (44.2-69.8) | 51.5 (40.1-71.1) | 0.613 | 40.6 (29.1-43.2) | 55.0 (35.4-80.7) | 0.095 |
| IFN-γ (pg/mL) | 3.47 (2.43-5.60) | 3.93 (1.98-7.31) | 0.852 | 3.15 (1.84-4.06) | 8.62 (3.79-14.5) | **0.009** |
| IL-10 (pg/mL) | 13.9 (9.60-20.3) | 12.3 (8.74-19.4) | 0.351 | 6.91 (6.42-9.87) | 12.4 (8.82-15.9) | **0.037** |
| IL-12p70 (pg/mL) | 1.31 (0.77-2.93) | 0.99 (0.40-2.62) | 0.382 | 0.59 (0.36-0.98) | 1.31 (0.89-2.92) | 0.071 |
| IL-1β (pg/mL) | 0.27 (0.10-0.51) | 0.30 (0.00-0.80) | 0.981 | 0.43 (0.09-0.91) | 0.20 (0.05-0.47) | 0.415 |
| IL-6 (pg/mL) | 2.14 (1.29-2.63) | 2.45 (1.68-3.64) | **0.046** | 1.60 (1.14-1.88) | 2.44 (1.50-4.16) | 0.095 |
| IL-8 (pg/mL) | 11.3 (6.53-20.5) | 11.6 (7.35-17.8) | 0.932 | 5.79 (2.35-8.62) | 9.42 (6.54-14.6) | **0.043** |
| TNF- α (pg/mL) | 16.1 (14.0-20.2) | 20.1 (13.7-27.5) | 0.071 | 12.2 (9.68-14.9) | 22.5 (16.4-29.1) | **0.001** |
| IL-17 (pg/mL) | 0.41 (0.16-0.58) | 0.32 (0.17-0.63) | 0.539 | 0.24 (0.15-0.39) | 0.32 (0.17-0.50) | 0.255 |
| CXCL10 (pg/mL) | 2810 (1588-4240) | 2531 (1821-3837) | 0.963 | 1766 (1148-3014) | 2695 (1867-3972) | 0.109 |
| sCD14 (ug/mL) | 1997 (1634-2306) | 2199 (1796-2461) | 0.247 | 2502 (2088-2770) | 3148 (2665-3755) | **0.015** |
| sCD40L (pg/mL) | 930 (411-1320) | 833 (265-1316) | 0.56 | 272 (93.4-494) | 1027 (514-1536) | **0.001** |
| HA (ng/mL) | 51.3 (37.7-77.7) | 74.5 (45.0-98.9) | 0.179 | 42.8 (15.2-69.9) | 92.1 (49.2-176) | **0.033** |
| sCD163 (ng/mL) | 654 (419-991) | 639 (458-828) | 0.963 | 649 (450-948) | 805 (444-955) | 0.593 |
| LTB4 (pg/mL) | 10.2 (10.2-27.7) | 13.7 (10.2-50.9) | 0.061 | 12.6 (10.2-17.9) | 46.9 (10.2-62.8) | 0.106 |
| FIB (mg/dL) | 761 (505-1403) | 758 (513-1368) | 0.96 | 640 (449-1028) | 1423 (576-2203) | **0.043** |
| vWF (%) | 8510 (6978-11725) | 10059 (8006-12456) | 0.231 | 8935 (8553-11762) | 11120 (9608-13806) | 0.086 |
| Protein S (%) | 3824 (3425-4408) | 3560 (3116-4134) | 0.204 | 3817 (3009-4386) | 4112 (3177-5273) | 0.407 |
| Protein C (%) | 4242 (3351-4770) | 3519 (3220-4125) | 0.054 | 3414 (3071-4072) | 3761 (3163-4385) | 0.522 |
| Vit. D (ng/mL) | 9.35 (6.39-16.6) | 9.65 (4.99-15.0) | 0.913 | 6.32 (4.41-11.9) | 10.4 (5.99-17.0) | 0.134 |

**Table Note:**

Bold font indicates statistical significance, at $p<0.05$. Data are shown as median and (IQR). Data were compared between groups using the Wilcoxon test for continuous variables. IQR: Interquartile range;

## 5.4 Manuscrito IV

### Título

"Severe anaemia as indicator of tuberculosis dissemination, systemic inflammation and a predictor of mortality in persons with advanced HIV: a prospective cohort study"

### Objetivo

Esse trabalho teve como objetivo investigar como a ocorrência da anemia e suas gravidades estão associadas a disseminação de *Mtb* em pacientes HIV-TB e a sua influência no perfil inflamatório e risco de morte desses pacientes.

### Resumo de resultados

Essa foi uma coorte composta por 496 PVHIV hospitalizados e com suspeita de TB, das quais verificamos que 460 (92,7%) apresentavam anemia no momento de entrada no estudo. Através das análises de diversos parâmetros clínicos e laboratoriais, nossos resultados mostram que pessoas hospitalizadas com HIV-TB com anemia moderada e grave apresentam um perfil inflamatório sistêmico mais acentuado, com elevada disseminação de *Mtb* e risco substancialmente maior de morte. As descobertas também revelam que os baixos níveis de Hb estão estritamente correlacionados a um perfil de ativação imune no sangue periférico que precede a morte dentro de 7 dias após a admissão no hospital. Finalmente, descobrimos que os níveis de Hb podem ser usados de forma confiável como um preditor de morte precoce. A identificação desses pacientes por meio da medição dos níveis de Hb pode levar a um monitoramento mais próximo para reduzir a mortalidade. Investigações futuras são necessárias para testar se as intervenções precoces impactam a sobrevivência dessa população vulnerável.

### Status do manuscrito

Este trabalho foi submetido ao periódico internacional ***The Lancet Microbe*** (Fator de Impacto JCR 2021 = 86,202) e está em etapa de avaliação editorial.

# Severe anaemia as an indicator of tuberculosis dissemination, systemic inflammation and a predictor of mortality in persons with advanced HIV: a prospective cohort study

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**Keywords:** anaemia, HIV, tuberculosis, mortality, systemic inflammation

## Abstract

**Background:** Anaemia frequently affects people living with HIV (PLHIV). Nevertheless, the impact of anaemia on treatment outcomes of patients with HIV-associated tuberculosis (TB) and the underlying molecular profiles are not fully characterized. The aim of this study was to investigate the impact of anaemia on the inflammatory profile, dissemination of *Mycobacterium tuberculosis* (Mtb) and the link between anaemia and death in patients with HIV-associated TB.

**Methods:** Hospitalized PLHIV ≥18 years old, with CD4 count <350 cells/μL and high clinical suspicion of new TB infection were enrolled in Cape Town between 2014 and 2016. Patients were classified according to anaemia severity in non-anaemic, mild, moderate, or severe anaemia. Clinical, microbiologic, and immunologic data (plasma cytokines and chemokines) were collected at baseline. The primary outcome was vital status at week 12. Hierarchical cluster analysis, degree of inflammatory perturbation, survival curves and C-statistics analyses were performed to assess the impact of anaemia severity on Mtb dissemination, systemic inflammation and mortality.

**Findings:** Through the analysis of several clinical and laboratory parameters, we observed that those with severe anaemia exhibited greater systemic inflammation, characterized by high concentrations of IL-8, IL-1RA and IL-6. Furthermore, severe anaemia was associated with a higher Mtb dissemination score and a higher risk of death, particularly within 7 days of admission. Cluster analysis revealed that most of the patients who died had severe anaemia and had a more pronounced systemic inflammatory profile in comparison with the cluster composed of participants who more frequently survived.

**Interpretation:** Severe anaemia is associated with greater Mtb dissemination of Mtb and increased risk of death in PLHIV. Early identification of such patients through measurement of Hb levels may drive closer monitoring to reduce mortality. Future investigations are warranted to test whether early interventions impact survival of this vulnerable population.


**Funding:** Wellcome Trust (098316, 203135, and 211360).

**Keywords**: tuberculosis, systemic inflammation, IRIS, death, HIV;

**Research in context**

***Evidence before this study***

*It is estimated that approximately 1/4 of the world population is affected by some type of anaemia, such prevalence is substantially higher in immunocompromised patients. Indeed, anaemia is a common complication in people living with HIV (PLHIV) and/or tuberculosis (TB). In these populations, anaemia has been described as a risk factor for unfavourable outcomes such as death. Whether in such settings anaemia hallmarks a specific profile of individuals undergoing underlying microbiologic and immunologic pathological processes is still unknown.*

***Added value of this study***

*Our results show that hospitalized persons with HIV-associated TB patients presenting with moderate and severe anaemia display a more pronounced systemic inflammatory profile, with an elevated mycobacteria dissemination and a substantially higher risk of death. The findings also uncover that haemoglobin levels strictly correlate a unique profile of immune activation in peripheral blood precedes death within 7 days of hospital admission. Finally, we found that haemoglobin levels can be reliably used as an accurate predictor of death.*

***Implications of all the available evidence***

*By identifying moderate and severe anaemia as a risk factor for TB dissemination and death, we demonstrate that these hospitalized patients should be carefully monitored and, if possible, anaemia should be thoroughly evaluated to assess possible undiagnosed underlying infections or malignancies. Moreover, rather than just representing more advanced disease, haemoglobin levels hallmark a unique profile of systemic inflammation that may be further tested as targets for host-directed interventions.*

## Introduction

It is estimated that about 24·8% of the world population is affected by some type of anaemia(1), being especially prevalent in immunocompromised patients(2). Anaemia is defined by a decrease in haemoglobin (Hb) values below well-established cut-offs (<13 g/dL for men; and <12 g/dL for women)(3) and is closely associated with malnutrition, micronutrient deficiencies, inflammation, and infectious diseases(3,4).

Importantly, anaemia is a very frequent comorbidity in people living with HIV (PLHIV), with prevalence ranging from 21 to 71%(5), and is associated with greater all-cause mortality(6), higher HIV viral load, lower CD4 count, and HIV disease progression(7,8). The aetiology of anaemia in PLHIV is multifactorial, being commonly attributed to chronic inflammation/disease(9).

Anaemia is also a prevalent finding in tuberculosis (TB) patients, ranging from 44 to 89%(10), and is related to higher rates of treatment failure. Of note, HIV is well described as a risk factor for the development of active TB. HIV-associated TB (hereafter mentioned as HIV-TB) was responsible for more than 200,000 deaths in 2020(11). Therefore, deciphering whether anaemia in HIV-TB patients undermines prognosis during hospitalization through association with unfettered systemic inflammation and TB dissemination is critical to improve at least two aspects of clinical management. First, early identification of anaemia could help identifying patients at higher risk of death. Lastly, the identification of the relationships between mycobacterial dissemination, systemic inflammation and anaemia severity could uncover immune mechanisms underlying increased mortality.

The present study investigated the impact of anaemia on the inflammatory profile in a cohort of hospitalized persons with HIV-TB from South Africa. We also linked the

severity of the anaemia to important clinical features on presentation and outcomes, such as dissemination of *Mycobacterium tuberculosis* (*Mtb*) and death, respectively, in context of HIV-TB infection.

## METHODS

### Ethical Review of the Study & Informed Consent of Study Participants

The study was approved by the University of Cape Town Human Research Ethics Committee (UCT HREC), reference number 057/2013. Participants provided written informed consent when possible. Details about informed consent are in **Supplementary appendix.**

### Study population and procedures

This study is a sub-analysis of a prospective observational cohort which recruited participants in Khayelitsha Hospital, Cape Town. Thus, this is a convenience sample. The study was conducted from January 2013 to October 2016 as previously described(12). PLHIV ≥18 years old, with CD4 count <350 cells/μL and a high clinical suspicion of new TB were eligible for enrolment. Pregnant women, history of anti-TB therapy within the last month, or those who were recently initiated and received three or more doses of anti-TB therapy were not eligible for enrolment(12). Detailed description of the cohort is found in a previous publication(12).

### Laboratory assays

Sputum TB cultures, sputum Xpert *Mtb*/RIF assay (Cepheid), urine Xpert *Mtb*/RIF assay, *Mycobacterial* blood culture and the GenoType *Mtb*DRplus assay (Hain Lifesciences) were performed at the National Health Laboratory Services (NHLS) and used to provide TB diagnosis. CD4 count, HIV viral load, full blood count, differential count, renal

function, liver function, C-reactive protein (CRP), procalcitonin, venous lactate, and cytomegalovirus (CMV) viral load tests were performed on all participants by the NHLS, as previously reported(12). Details on measurements of plasma mediators of inflammation are depicted in **Supplementary appendix**.

**Data collection and definitions**

Clinical data were obtained from the patient's hospital folder, and clinical review at enrolment and captured on standard case record forms. The primary outcome was vital status at week 12. Participants with a health system record entry performed beyond week 12 were assumed to be alive at week 12.

Urine lipoarabinomannan (LAM) more than or equal to grade 1 by two independent readers was regarded as positive. "Microbiologically confirmed TB" was defined as participants with *Mtb* on at least 1 culture or Xpert *Mtb*/RIF test from any clinical sample. Early deaths were deaths that occurred within 7 days of enrolment, and late deaths are all deaths that occurred after 7 days and within 12 weeks of enrolment.

**Anaemia definitions**

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(3). The **Supplementary Fig 5** describes further details on stratification of anaemia severity (3).

**Degree of Mycobacterial Dissemination**

The degree of *Mtb* dissemination was defined with a three-point dissemination score, as previously described by our group(13). Participants were allocated 1 point for the following: urine LAM test positive, mycobacterial blood culture positive and identified

153 as *Mtb* and urine Xpert *Mtb*/RIF assay positive for *Mtb*, yielding a score ranging from 0-
154 3(14).

155 **Inflammatory Profile and Degree of Inflammatory Perturbation**

156 To evaluate the overall profile of systemic inflammation and how it related to degree of
157 anaemia, we $\log_{10}$ transformed the biomarker values and performed an unsupervised
158 hierarchical cluster analysis (Ward's method), with dendrograms representing the
159 Euclidean distances. Description of the calculations of the degree of inflammatory
160 perturbation (DIP) is shown in the **Supplementary appendix**.

161 **Statistical analysis**

162 Descriptive statistics were used to present data, and median values with interquartile
163 ranges (IQR) were used as measures of central tendency and dispersion, for continuous
164 variables. Only complete cases were evaluated. Categorical variables were described
165 using frequency (no.) and proportions (%). The Pearson's chi-square test was used to
166 compare categorical variables between study groups. The Mann-Whitney *U* test (2
167 groups) or the Kruskal–Wallis test (>2 groups) were used to compare continuous
168 variables. The Cochran–Armitage test for trend was used to assess for the presence of an
169 association between the DIP levels and clinical characteristics with the severity of
170 anaemia.

171 Kaplan-Meier analysis was performed using the log-rank (Mantel-Cox) test and applied
172 to estimate death probability of the participants stratified based on the hierarchical cluster.
173 Differences with p-values below 0.05 after adjustment for multiple comparisons (Holm-
174 Bonferroni) were considered statistically significant. The statistical analyses were
175 performed using and R language (version 4·4·1).

176 **Data Availability**

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

## RESULTS

### Characteristics of the cohort

This prospective cohort was composed of 659 hospitalized PLHIV and with suspected TB, enrolled a median of 2 days (IQR:1-3) after hospital admission. We excluded patients without cytokines data and those lost to follow-up, resulting in 496 patients in the analysis **(Supplementary Fig 1a)**.

The cohort was further stratified according to the occurrence and severity of anaemia. We found that 7·3% (n=36) of the patients had normal Hb levels according to WHO definitions. The remaining 92·7% (n=460) had low Hb levels and were considered anaemic. Comparisons of these two main groups are detailed in **Supplementary Table 1**.

Next, we stratified the anaemic patients according to the severity into mild (23·4%, n=116), moderate (31·2%, n=155), and severe (38·1%, n=189) **(Table 1)**. We found a predominance of female sex among participants with severe anaemia (without anaemia: 41·7%; mild: 42·2%; moderate: 51·6%; severe: 62·4%; p=0·003). Importantly, a decrease of CD4 T-cell counts was detected according to anaemia severity (without anaemia: 110 cells/µL [50-162]; mild: 69 cells/µL [23·8-140]; moderate: 57 cells/µL [25·5-120]; severe: 42 cells/µL [17-101]; p<0·001) **(Supplementary Fig 1b)**. Furthermore, a higher proportion of positive *Mtb* blood cultures was observed as anaemia severity worsened (without anaemia: 22.6%; mild: 33·3%; moderate: 36·4%; severe: 48·4%; p=0·014). The same phenomenon was observed for positive Urine *Mtb* Xpert tests (without anaemia:

18·8%; mild: 32·0%; moderate: 48·1%; severe: 59·6%; p<0·001). **(Supplementary Fig 1b, Table 1)**.

We further investigated in more details the relationships with Hb values and key clinical laboratory parameters. In this cohort, Hb values were weakly positively associated with CD4 counts (rho: 0·16; p<0·001) and not related with HIV viral loads (rho: -0·02; p=0·71) **(Supplementary Fig 2a-b)**. As expected, CD4 counts were inversely correlated with HIV viral loads **(Supplementary Fig 2c)**.

**Association between anaemia severity, TB dissemination and death**

Next, we assessed the *Mtb* dissemination score and mortality according to the severity of anaemia. Patients without anaemia displayed a lower frequency of positive *Mtb* dissemination score (score 1, 2 and 3) than the other groups(p<0·001). The frequency of participants with a positive *Mtb* dissemination score increased proportional to augmented anaemia severity (p-value for trend <0·001) **(Figure 1a)**. Mortality, including that occurring within the first 7 days of hospitalization (early death) tended to increase according to the severity of anaemia (overall mortality: p-value for trend=0·009; early death: p-value for trend=0·018) **(Figure 1b)**. When Hb values were examined as a continuous variable, we observed that its levels gradually decreased following increases in the *Mtb* dissemination score (p-value for trend <0·001) **(Figure 1c)**. A correlation analysis confirmed those findings that the *Mtb* dissemination score and Hb values are inversely correlated (rho: -0·33; p<0·001). The opposite association was observed when comparing Hb levels with time of death, where those who had an early death presented the lowest Hb values compared with those who experienced late death (after 7 days of hospital admission) or survived (p-value for trend <0·001) **(Figure 1d)**. Furthermore, mortality elevated following increases in the *Mtb* dissemination score (chi-square for trend p-value: <0·001; **Supplementary Fig 3**).

A receiver operator characteristic (ROC) curve analysis was used to evaluate the accuracy of Hb levels for identifying persons with a high *Mtb* dissemination score (**Figure 1e**) or those who died early (**Figure 1f**). The results demonstrated that a Hb cut-off of 8·05g/dL was associated with an accuracy of 65% (AUC: 0·65; 95%CI: 0·58-0·72), with a sensitivity of 58% (95%CI: 47-68) and specificity of 63% (95%CI: 58-68) (p<0.001) for identification of patients with high *Mtb* dissemination score (**Figure 1e**). Moreover, using a Hb cut-off of 7.95g/dL resulted in an accuracy of 61% (AUC: 0·61, 95%CI: 0·51-0·72), with sensitivity of 66% (95%CI: 61-70) and specificity 60% (95%CI: 43-77, p=0·014) for identification of patients who died (**Figure 1f**).

**Severity of anaemia is associated with the degree of inflammatory perturbation in HIV-associated TB**

Lymphocyte (p<0·001) and monocyte (p<0·001) counts, as well as the concentrations of ALT (p=0·02) and albumin (p<0·001), decreased whereas values of CRP (p<0·001), procalcitonin (p<0·001), D-dimer (p<0·001), urea (p<0·001) and creatinine (p=0·018) showed a tendency to be higher according to anaemia severity (**Supplementary Table 2-3**). Other comparisons between subgroups of individuals with different degrees of anaemia are shown in **Supplementary Table 2-3**.

The study groups were also compared according to the plasma concentrations of a variety of inflammatory markers to delineate the immunologic profile associated with anaemia (**Figure 2a**). Trend analysis of the circulating levels of IL-1β, IL-8, CXCL10, IL-6, CCL4, IL-1RA and CCL2 uncovered that rising levels of these markers are proportional to increases in anaemia severity **(Figure 2a, right panel; Supplementary Table 4).**

The abovementioned observations suggested that there is an intriguing disturbance of the immune activation systemically, which characterizes HIV-TB persons with severe

anaemia. To quantify such disturbance, we calculated the DIP scores in all the clinical groups, considering the non-anaemic group as the reference group (**Figure 2b**). The resulting DIP scores were shown to inversely correlate with Hb values (rho: -0·22; p=0·007), and with CD4 cell counts (rho: -0·28; p=0·007) but were not related to HIV viral loads (**Supplementary Fig4a-c**).

It was observed that the DIP score values increased following the severity of anaemia (p-value for trend=0·005), reinforcing the idea that severe anaemia in hospitalized patients with HIV-TB is associated with substantial inflammatory disturbance in the peripheral blood (**Figure 2b**). The top 10 biomarkers most contributing to this inflammatory perturbation (assessed through the DIP score) were IL-8, IL-1RA, IL-6, CCL4, CCL2, IL-10, IL-12p70, IL-1β, GM-CSF and Eotaxin (**Figure 2c**).

We next designed analyses to test whether the DIP score values are somehow related to the degree of *Mtb* dissemination (**Figure 3a**). The DIP values gradually elevated proportional to increases of the *Mtb* dissemination score (p-value <0·001 for all the ad hoc comparisons). A correlation analysis confirmed that DIP values and *Mtb* dissemination score levels are directly related (rho: 0·31; p<0·001). This argues that systemic inflammatory profile is dramatically altered in patients that experience *Mtb* dissemination and that this association may be linked to anaemia severity (**Figure 3a**). We next observed that DIP values were inversely correlated to the time to death (rho: -0·32; p=0·002). Indeed, patients who died within 7 days of hospital admission displayed substantially higher DIP score values than those who died at later timepoints or those who survived (p<0·001; **Figure 3b**).

Next, we used C-statistics analysis to evaluate the accuracy of the DIP score values identification of persons with a high *Mtb* dissemination score (score = 3) or of those who experienced early death. The accuracy for *Mtb* dissemination score using the DIP cut-off

274 of 0·14 was 65% (AUC: 0·65; 95%CI: 58-72), with a sensitivity of 55% (95%CI: 0·44-
275 0·66) and specificity of 72% (95%CI: 67-77) (p<0·001) (**Figure 3c**). The result of the
276 ROC curve for early death was similar, with an accuracy of 76% (AUC:0·76; 95%CI:
277 0·66-0·86), with sensitivity of 81% (95%CI: 77-86) and specificity of 68% (95%CI: 52-
278 84) (p<0·001) using a DIP cut-off point of 0·53 (**Figure 3d**). Importantly, altogether, the
279 results presented so far revealed that anaemia (Hb values), systemic inflammation (DIP
280 score values) and *Mtb* dissemination are all interrelated and impact overall mortality and
281 time to death.

282 Finally, in the unsupervised hierarchical cluster analysis, three main clusters of patients
283 were defined (**Figure 4a**). The cluster #1 displayed a higher frequency of participants
284 who died during the follow up and had the uppermost occurrence of patients with high
285 *Mtb* dissemination scores and severe anaemia (p<0·001 in both comparisons) (**Figure**
286 **4b**). The cluster #2 exhibited a lower frequency of anaemic participants, lower frequency
287 of people with any *Mtb* dissemination and lower mortality than the other clusters/sub-
288 groups and was considered as the reference for fold difference comparisons (**Figure 4a,**
289 **right panel**). The cluster #3 included participants with an intermediate phenotype,
290 without any characteristics that specifically defined this group (**Figure 4b**). When we
291 compared those three clusters, the individuals within the cluster #1 presented relatively
292 higher values of cytokines and chemokines than those in the other clusters
293 (**Supplementary Table 5**). This finding shows once again that these inflammatory
294 mediators are involved with the inflammatory exacerbation in patients with severe
295 anaemia and that this setting is related with *Mtb* dissemination and death in the study
296 population. Finally, the survival analysis demonstrated that patients from cluster #1 had
297 higher mortality than those from the other clusters (p=0·008) (**Figure 4c**).

298 **DISCUSSION**

299 Anaemia affects one third of the world's population, and mainly in PLHIV and in those
300 with TB(15,16). In a previous study from our group examining persons with HIV-TB co-
301 infection, anaemia was reported in 84% of the participants(17). In addition, in many
302 reports, the majority of the participants examined present with mild anaemia(2,18). The
303 present cohort study revealed that 92.7% of study participants had anaemia, with a high
304 proportion of severe anaemia (38%). This higher frequency may be explained by the fact
305 that we enrolled only hospitalized patients. Yet, regardless of its imposing pervasiveness
306 in such setting, anaemia is frequently overlooked in the clinical practice when patients
307 with HIV-TB are managed. No consensus on how anaemia in HIV-TB patients should be
308 addressed has been documented. The findings presented here demonstrate that Hb levels
309 not only infer TB dissemination but also indicate degree of inflammatory disturbance.
310 More importantly, Hb levels are predictive of early mortality in hospitalized persons with
311 HIV-TB. Whether anaemia is a cause or a consequence of the HIV-TB-driven chronic
312 inflammation and/or disease progression is less important than its utility as a biomarker
313 that can identify persons at higher risk of death. Early identification of such patients
314 through a simple measurement of Hb levels must alarm the healthcare professionals to
315 take a closer look and optimize management to reduce the odds of mortality. Future
316 investigations are warranted to test whether early interventions, such as use of adjunct
317 therapies, fostered by Hb measurement at hospital admission impact survival of this
318 vulnerable population.

319 In this cohort, anaemic patients were shown to have a lower weight and lower CD4 count,
320 while having higher frequency of positive urine *Mtb* Xpert test and detectable CMV in
321 blood. Other studies have demonstrated that anaemic patients frequently have weight loss
322 and lower CD4 counts(5).The association between low Hb values with low CD4 T
323 lymphocyte count and high frequency of CMV co-infection suggests that anaemia can be

caused by advanced HIV with opportunistic infections such as TB and/or CMV. Decreased levels of Hb have been described as predictive markers for HIV disease progression to AIDS(19).

Our multidimensional analyses exploring the relationships between Hb values and TB progression confirmed the previously established hypothesis that anaemia hallmarks advanced TB disease. The data on *Mtb* dissemination score reported here highlight that persons with severe anaemia are those presenting with more frequent detection of *Mtb* in extrapulmonary compartments such as urine and blood. *Mtb* dissemination is reported to occur when growth of mycobacteria is unfettered, which is observed when the infected host is unable to adequately respond with a robust and efficient immune response (reviewed in (20)). Such incapacity to defeat *Mtb* is frequently observed in immunocompromised persons, that include PLHIV. When we combine our results on relationships between Hb values and surrogates of HIV (CD4 count and HIV viral load) or of TB (dissemination score), we can argue that anaemia in this study population is likely more noticeably related to progression of TB progression than that driven by HIV. Corroborating with this idea, a retrospective cohort study of PLHIV (CD4 <100 cells/μL) reported that those with TB diagnosis more frequently had anaemia and exhibited more pronounced inflammatory profile than those without this comorbidity(17).

An important criticism commonly emerges during discussion of results such as those reported here. That is related with the difficulty to establish whether anaemia is an underlying factor driving the inflammatory abnormalities or is a consequence of sustained immunopathology. In fact, our analysis demonstrated that there is a strong relationship between the severity of anaemia and the degree of systemic inflammatory perturbation. The study design does not allow us to determine causality. Instead, it sanctions the use of Hb levels as a proxy of inflammatory disturbance and of a unique immune activation

profile that relates with *Mtb* dissemination and mortality. Thus, Hb is a simple, low-cost parameter that deserves more attention, especially in limited-resource regions.

The unique inflammatory profile observed in patients with severe anaemia who experienced TB dissemination includes high concentrations of IL-1RA, IL-8 and IL-6, all of which have been previously described to be involved in mycobacteria-associated immunopathology in both clinical and experimental settings(21–26). Moreover, these heightened levels of these cytokines have been previously reported as risk factors of TB progression and infer increased morbidity and mortality(27–29). Curiously, these cytokines are closely associated with innate immune responses, and cells described to rapidly respond to its induced signals are macrophages and neutrophils, which have been placed as critical cells driving both immunity against TB(30,31) and immune-driven tissue damage(32,33). The predominance of signals deriving from activation of innate immune responses over the molecules fostering T cell activity favours the hypothesis that innate cells may play a more substantial role in induction of the systemic inflammatory perturbation reported here. Whether these cytokines are insufficiently attempting to control TB dissemination or are solely promoting immunopathology that is leading to worse outcomes is still a matter of debate and deserves additional investigations.

The worse possible outcome that can occur in patients severely afflicted by TB and HIV infections is death. In our cohort, 22% of the participants died, and 7% died within a week of admission. An omnipresent feature of the patients who died seemed to be the presence of anaemia, given that only 5 (5%) participants who died at any timepoint were not anaemic at baseline. Thus, Hb levels are a strong predictor of mortality in persons with HIV-TB. This important observation is supported by a systematic review which demonstrated that anaemia, regardless of its type, is associated with an increased risk of all-cause mortality in PLHIV(10). Discriminant analysis using C-statistics demonstrated

that a Hb value <8 g/dL can identify patients with high *Mtb* dissemination score (score=3). Similarly, baseline Hb values can reliably predict early mortality (AUC: 0·63, p=0·008), which is higher than that of other parsimonious biomarkers previously described, such as CRP (AUC: 0·31, p=1·0) and D-dimer (AUC: 0·30, p=0·06). If validated in other studies, assessment of Hb should be not ignored as an important predictor which may drive change/optimization of clinical management.

This study has certain limitations. Samples were obtained only at baseline, not allowing the evaluation of changes during time of treatment. Additionally, we did not have autopsy information on causes of death. Regardless, our study identifies previously underexplored nuances of the key relationships between anaemia, inflammation, and control of pathogen loads/dissemination in highly susceptible patients with HIV-associated TB. More importantly, the findings propose the systematic implementation of Hb measurement as a mandatory policy that may reduce the extremely high mortality in this population.

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404 **DECLARATION OF INTERESTS**

405 The authors declare that the research was conducted in the absence of any commercial or
406 financial relationships that could be construed as a potential conflict of interest. All other
407 authors declare no competing interests.

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409 The funders had no role in study design, data collection and interpretation, or the decision
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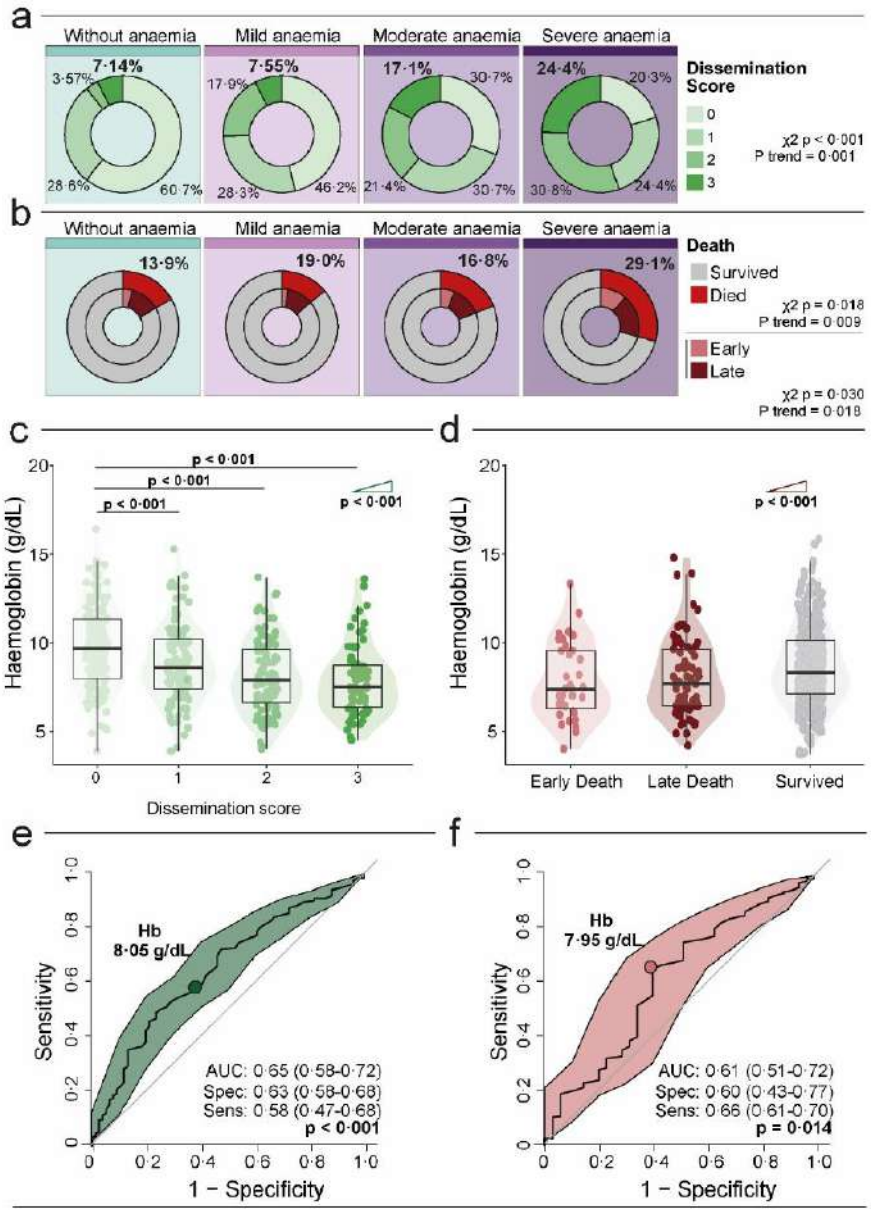
**Table 1. Clinical characteristics of HIV-TB patients according to anaemia**

| | Without anaemia (n=36) | Mild anaemia (n=116) | Moderate anaemia (n=155) | Severe anaemia (n=189) | p value |
|---|---|---|---|---|---|
| Sex (female), (%): | 15 (41.7) | 49 (42.2) | 80 (51.6) | 118 (62.4) | **0.003*** |
| Age (years), median (IQR): | 37.6 (31.8-50.2) | 37.8 (32.5-43.9) | 35.8 (31.1-42.6) | 35.1 (30.0-41.3) | **0.046*** |
| Weight (kg), median (IQR): | 57.0 (49.2-75.8) | 54.0 (49.0-60.8) | 53.0 (46.4-61.0) | 54.0 (47.0-62.0) | 0.063 |
| ART naive, n (%): | 19 (52.8) | 50 (43.5) | 65 (41.9) | 64 (33.9) | 0.181 |
| CD4 (count), median (IQR): | 110 (50.0-162) | 69.0 (23.8-140) | 57.0 (23.5-120) | 42.0 (17.0-101) | **<0.001*** |
| HIV ($log_{10}$ copies/mL) VL, median (IQR) | 5.25 (4.35-5.70) | 5.28 (3.77-5.68) | 5.18 (3.64-5.75) | 5.15 (3.84-5.77) | 0.953 |
| Blood detected CMV, n (%): | 6 (16.7) | 44 (38.3) | 61 (40.1) | 81 (43.3) | **0.028*** |
| *Mtb* blood culture, n (%): | 7 (22.6) | 37 (33.3) | 55 (36.4) | 89 (48.4) | **0.014*** |
| Urine Xpert *Mtb*/RIF assay positive, n (%): | 6 (18.8) | 33 (32.0) | 64 (48.1) | 96 (59.6) | **<0.001*** |

**Table note:**

Bold font indicates statistical significance. 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. Severe anaemia was defined as Hb<8g/dL for both sexes. The severity of anaemia according to the Hb levels is defined in methods. Data are shown as median and interquartile (IQR) range 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 Kruskall-Wallis (for more than two groups) or Mann-Whitney *U* test (for two unmatched groups). *Represent p <0.05 in Cochran–Armitage test for trend.

543 **Figure Legends**




544

545 Figure 1. Association of anaemia severity with *Mtb* dissemination and death. (a)
546 Distribution of dissemination score according to anaemia severity. (b) Distribution of
547 deaths and time to death according to anaemia severity. Early deaths were defined as
548 deaths occurring within 7 days of enrolment, and late deaths were all deaths that occurred
549 after 7 days and within 12 weeks of enrolment. (c) Hb levels according to dissemination

score. (d) Hb levels according to time of death. Groups 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. (e, f) ROC curve analysis was used to evaluate the accuracy of Hb values to discriminate high dissemination score (*Mtb* dissemination score 3) (e) and early death (f). Coloured dots indicate the cut-off values of Hb extracted from the ROC curve analyses that resulted in the optimal ratio between sensitivity and specificity; these values are described in the indicated panels. 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. Severe anaemia was defined as Hb<8g/dL for both sexes.

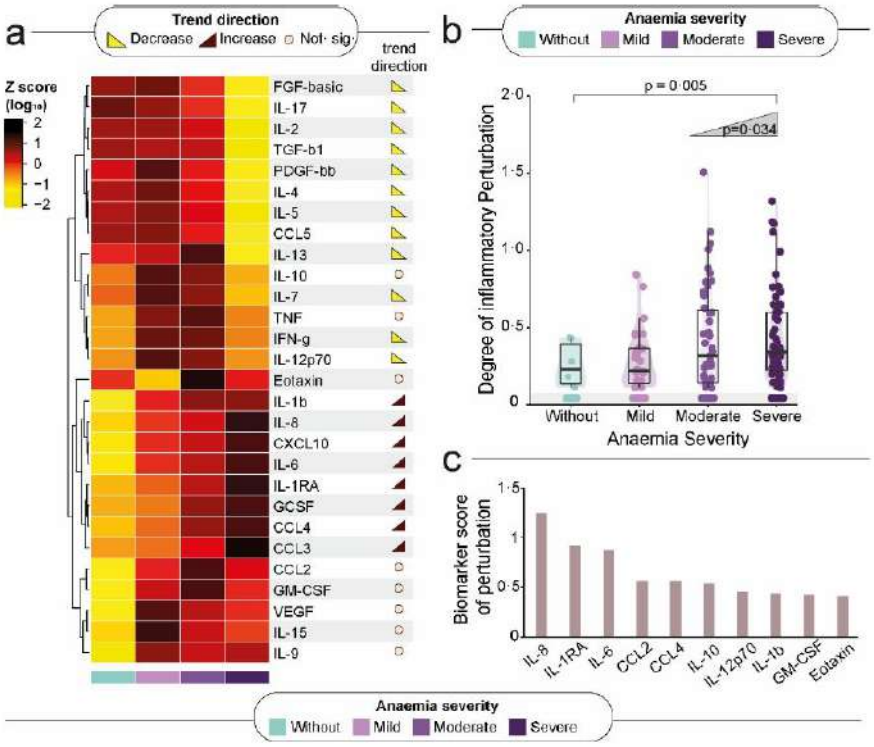



Figure 2. Association of anaemia severity with inflammatory profile. (a) Left panel: A heatmap was designed to depict the overall pattern of inflammatory markers. A one-way hierarchical cluster analysis (Ward's method) was performed. Dendrograms represent Euclidean distance. Right panel: Several analyses were performed to identify trends of increasing or decreasing of biomarker levels across anaemia severity. Significant differences ($p < 0.05$) are highlighted in red-brown trend symbol when the trend is decreasing and in yellow when the trend is increasing. For those of no significance, there is a beige circle. (b) Scatter plots of the degree of inflammatory perturbation (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. (c) We identified the Top 10 biomarker scores contributing to overall perturbation. The score was obtained using DIP approach. 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. Severe anaemia was defined as Hb<8g/dL for both sexes.

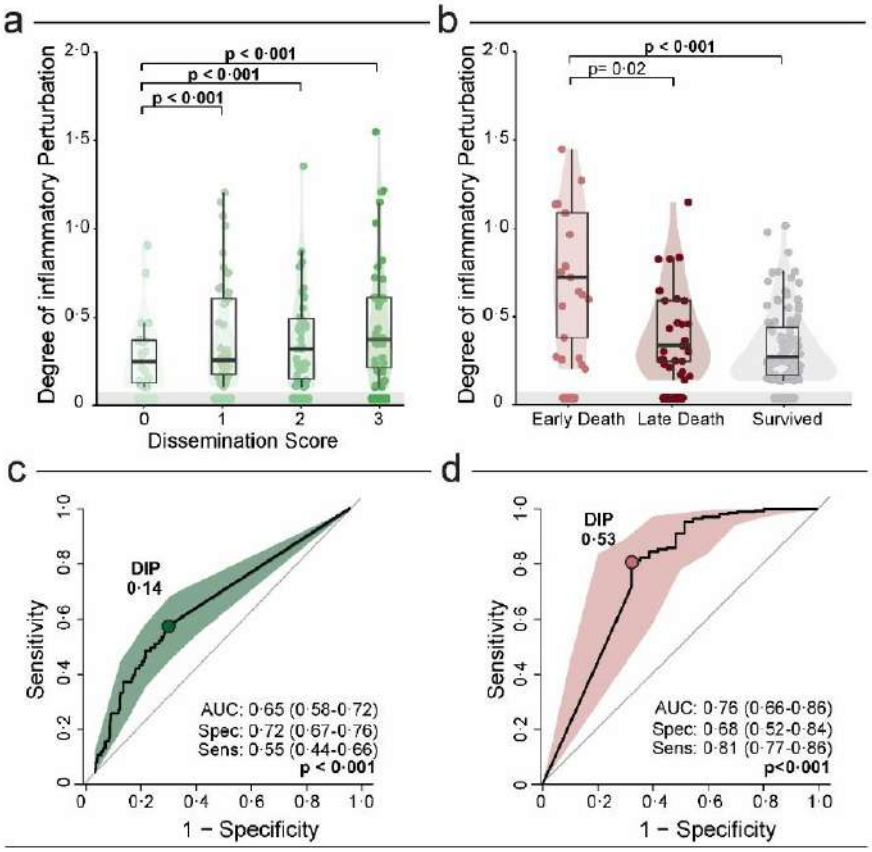



Figure 3. Degree of Inflammatory Perturbation (DIP) according to dissemination score and death. (a) Scatter plots of the DIP value grouped according to anaemia severity. Lines in the scatter plots represent median values and data were compared using the Mann–Whitney *U* test. The Cochran–Armitage test for trend was used to assess the tendency of increased levels or frequencies among groups. (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. (c,d) ROC curve analysis was used to evaluate the accuracy of DIP values to discriminate high dissemination score (*Mtb* dissemination score 3) (c) and early death (d). Coloured dots indicate the cut-off values of DIP extracted from the ROC curve analyses that resulted in the optimal ratio between sensitivity and specificity; these values are described in the indicated panels. 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. Severe anaemia was defined as Hb<8g/dL for both sexes.

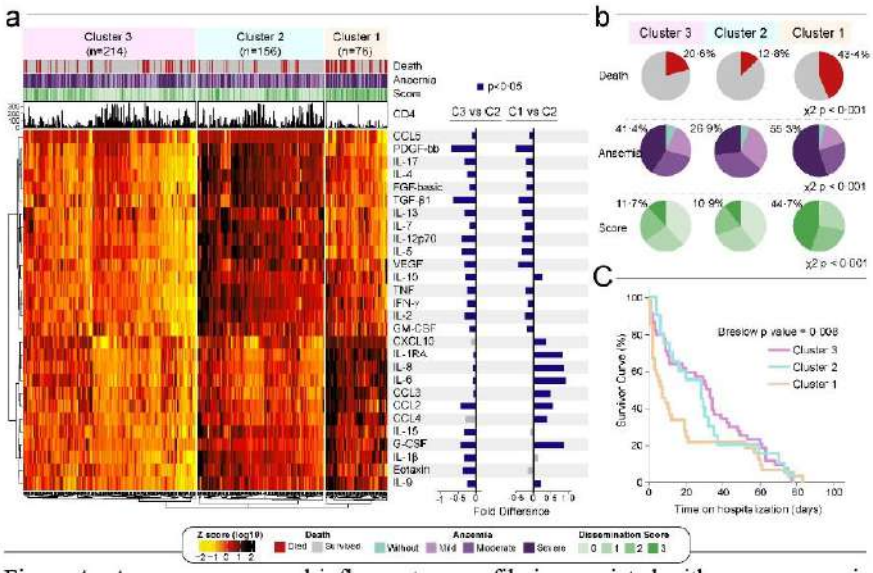



Figure 4. A more pronounced inflammatory profile is associated with severe anaemia and death. (a) Left panel: An unsupervised two-way hierarchical cluster (Ward's method) was performed with all 496 participants. Data were log10 transformed and ranked and coloured in a heatmap from values detected for each inflammatory biomarker. Dendrograms represent Euclidean distance. Three main clusters were defined. Right panel: A log10 fold change was performed comparing each cluster with reference cluster (C3, due to lowest frequency of dissemination and death). Significant differences ($p < 0.05$) are highlighted in blue bars. (b) For each cluster, shaded areas represent frequency of death (red), anaemia severity grade (light blue to dark purple) and *Mtb* dissemination score (light green to dark green) and. Chi squared test was performed to each variable comparing clusters. (c) Survivor curves show the probability of survival over 12 weeks for each cluster. 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. Severe anaemia was defined as Hb<8g/dL for both sexes.

**SUPPLEMENTARY APPENDIX**

## SUPPLEMENTARY METHODS

### Informed Consent of Study Participants

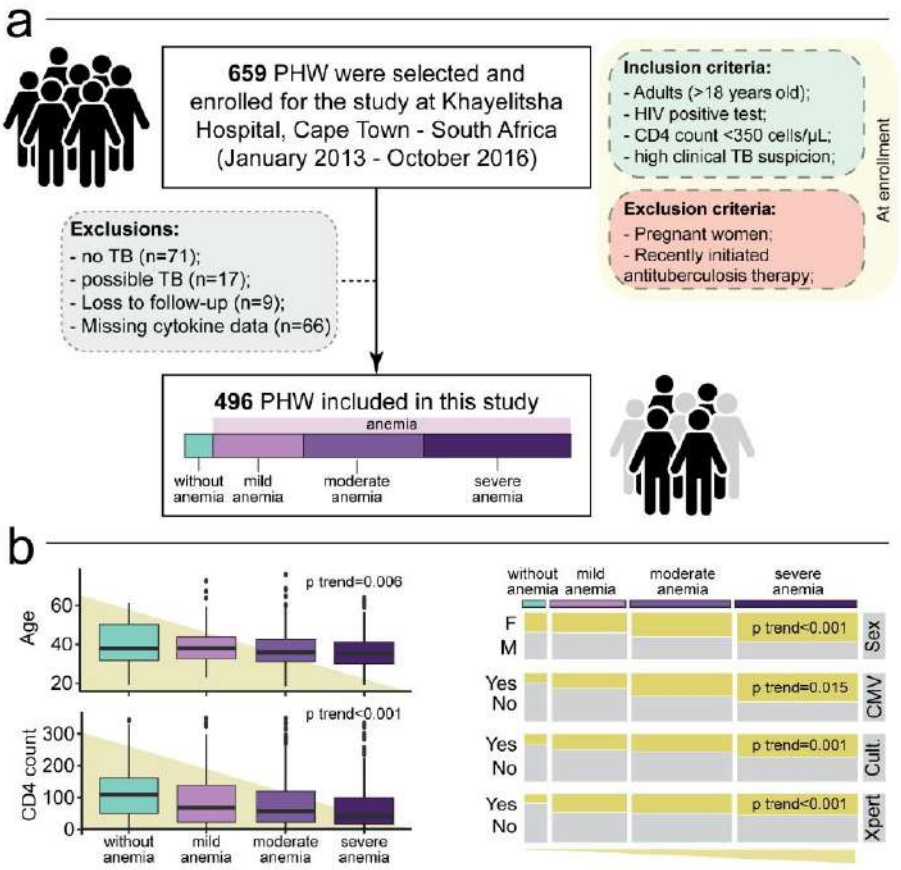
Eligible patients with a decreased level of consciousness were enrolled and followed up daily until they regained capacity to participate in the informed consent process, and if not agreeable to participate, were withdrawn from the study. The UCT HREC approved the use of information from participants who died prior to providing informed consent by the end of study follow-up.

### Laboratory assays

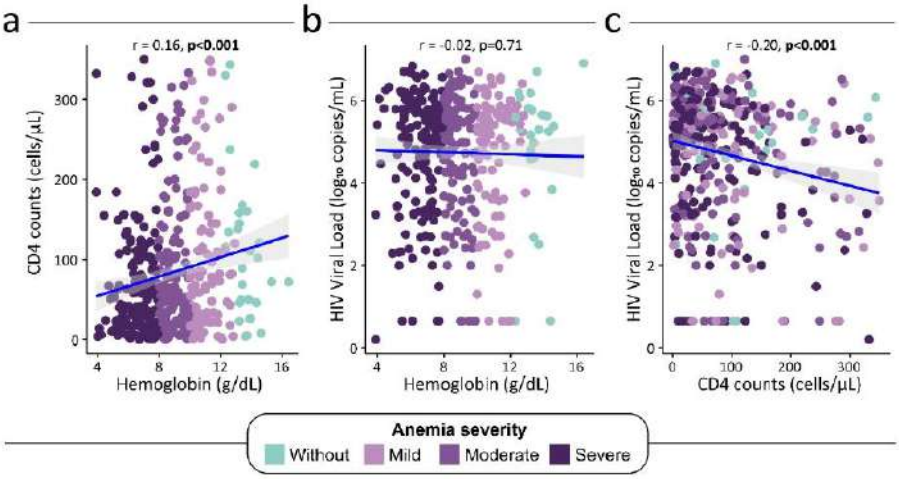
Plasma was stored at −80°C for immunology assays. Soluble inflammatory mediators were tested on stored plasma (1:2 dilution) using Luminex technology (Bio-Plex Pro Human Cytokine Standard 27-Plex kit). The following analytes were measured: interleukin (IL)-1β, IL-1 receptor antagonist (IL-1Ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17A, eotaxin, basic fibroblast growth factor (FGF), granulocyte colony stimulating factor (G-CSF)/colony stimulating factor 3 (CSF3), granulocyte-macrophage colony stimulating factor (GM-CSF/CSF2), interferon gamma (IFN-γ), interferon gamma-induced protein (IP-10)/ C-X-C motif chemokine ligand 10 (CXCL10), monocyte chemoattractant protein-1 (MCP-1)/C-C motif chemokine ligand 2 (CCL2), macrophage inflammatory protein-1 alpha (MIP-1α/CCL3), MIP-1 beta (MIP-1β/CCL4), platelet-derived growth factor-BB (PDGF), regulated on activation, normal T cell expressed and secreted (RANTES/CCL5), tumor necrosis factor-alpha (TNF), and vascular endothelial growth factor (VEGF). For statistical analyses, mean fluorescence intensity (MFI) values of the plasma markers were used. Such approach allows for analysis of analytes of low abundance and does not require censoring or correction for background(38–40).

**Degree of Inflammatory Perturbation**

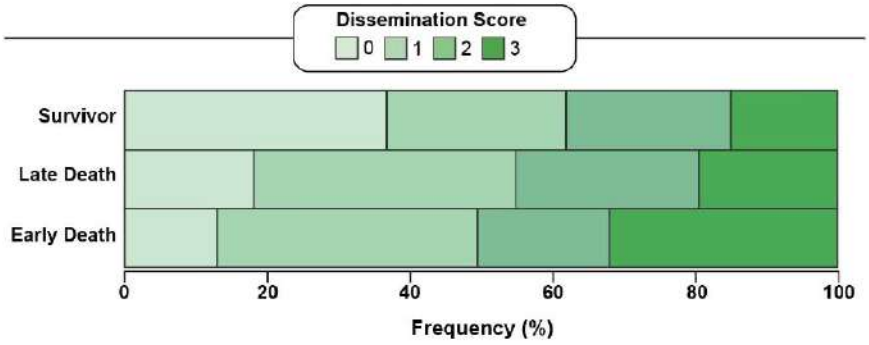
The degree of inflammatory perturbation (DIP) was calculated to identify the general inflammatory environment of the participants. DIP was adapted from the molecular degree of perturbation, which has been described previously(42). For this study, the DIP calculation included the concentrations of the plasma inflammatory markers instead of gene expression values in the original analysis model(42). Thus, herein, the average level and standard deviation of a baseline reference group (without anaemia) were calculated for each biomarker. The DIP score of each biomarker was defined by z-score normalization, where the differences in concentration values from the average of the biomarker in reference group was divided by the reference standard deviation. Therefore, the DIP score represents the differences by number of standard deviations from the control group. Similar approaches resulting in DIP-like scores have been previously employed using biomarker measurements by our group(16,43). We ranked the top 10 markers which contributed the most for the DIP score values, to identify the most informative soluble mediators contributing to the overall inflammatory disturbance.




Supplementary Fig 1. Study Flow chart. (a) 659 People with HIV (PWH) were selected and enrolled for the study, however, 193 were removed for this analysis, leaving 496 patients. Of these, the majority (92.7%) were anaemic with different severities. (b) Clinical characteristics of patients compared according to anaemia severity. Left panel: Decreasing trends in age and CD4 counts were observed with increasing severity of anaemia. The trend direction is represented by the coloured shaded triangle. On the other hand, there were also trends towards an increase in frequency (shown as %) of the female sex, CMV viremia, positive *Mtb* blood culture, and positive Urine Xpert as the severity of anaemia increased (right panel). The Cochran–Armitage test for trend was used to assess the tendency of increased levels or frequencies among groups. Mild anemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate anemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anaemia was defined as Hb<8g/dL for both sexes.

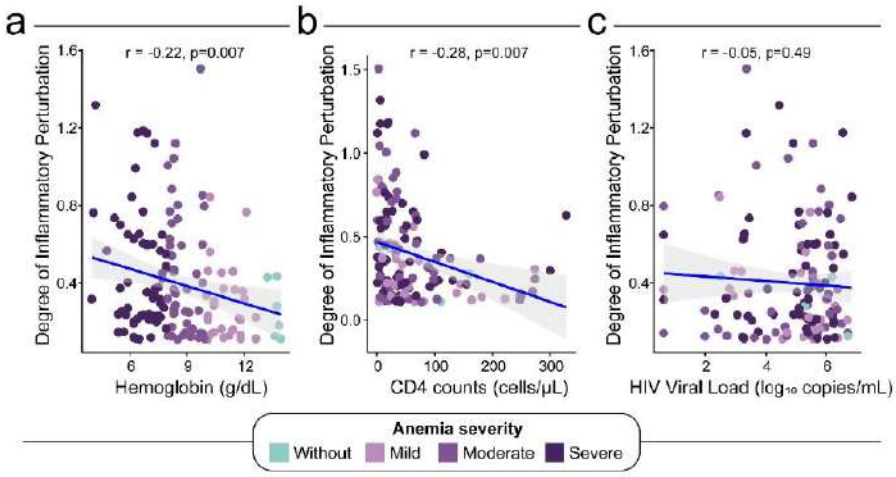



Supplementary Fig 2. Spearman correlation analysis between haemoglobin values, CD4 counts, and log10 HIV viral load. (a) Spearman correlation between CD4 counts vs haemoglobin. (b) Spearman correlation between log10 HIV viral load vshaemoglobinn. (C) Spearman correlation between log10 HIV viral load vs CD4 count. (Mild anemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate anemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anaemia was defined as Hb<8g/dL for both sexes.




| | Dissemination Score 0 | Dissemination Score 1 | Dissemination Score 2 | Dissemination Score 3 |
|---|---|---|---|---|
| Survivor | 36.7% | 25.2% | 22.9% | 15.2% |
| Late Death | 18.2% | 36.4% | 25.8% | 19.7% |
| Early Death | 12.9% | 35.5% | 19.4% | 32.3% |

Supplementary Fig 3. Frequency of *Mtb* dissemination score according to time of death. Upper panel: The frequency (%) of *Mtb* dissemination score is shown in a bar graph, with groups stratified by time of death (early, late, and survivor). Down panel: The frequency (%) of *Mtb* dissemination score is shown in a table format, with groups stratified by time of death (early, late, and survivor). Early death was defined as death that occurred in the first seven days after hospitalization. Groups were compared using the chi-square test and chi-square for trend.




Supplementary Fig 4. Spearman correlation analysis between Degree of Inflammatory Perturbation (DIP), haemoglobin values, CD4 counts, and log10 HIV viral load. (a) Spearman correlation between DIP vs haemoglobin. (b) Spearman correlation between DIP vs CD4 counts. (c) Spearman correlation between DIP vs log10 HIV viral load. Mild anemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate anemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anaemia was defined as Hb<8g/dL for both sexes.

| Definition | Hb Level (g/dL) |
|---|---|
| Anemia | <13 for men<br><12 for women |
| Mild Anemia | 10 ≤ <13 for men<br>10 ≤ <12 for women |
| Moderate anemia | 8 ≤ <10 for men<br>8 ≤ <10 for women |
| Severe Anemia | <8 for both sexes |

Supplementary Fig 5. Anaemia definition according to the WHO Criteria, using haemoglobin levels (World Health Organization, 2011).

**Supplementary Table 1. Clinical characteristics according to the presence of anemia**

| | All (n=496) | Without anemia (n=36) | With anemia (n=460) | p value |
|---|---|---|---|---|
| Sex (female), n (%): | 262 (52.8) | 15 (41.7) | 247 (53.7) | 0.223 |
| Age (years), median (IQR): | 35.9 (30.9-43.2) | 37.6 (31.8-50.2) | 35.9 (30.9-42.8) | 0.306 |
| Weight (kg), median (IQR): | 54.0 (47.0-62.0) | 57.0 (49.2-75.8) | 54.0 (47.0-61.0) | **0.025** |
| ART naive, n (%): | 198 (40.0) | 19 (52.8) | 179 (39.0) | 0.256 |
| CD4 (count), median (IQR): | 57.0 (21.0-117) | 110 (50.0-162) | 55.0 (20.0-111) | **0.001** |
| HIV VL ($log_{10}$ copies/mL), median (IQR) | 5.22 (3.83-5.75) | 5.25 (4.35-5.70) | 5.21 (3.75-5.75) | 0.809 |
| CMV detected, n (%): | 192 (39.2) | 6 (16.7) | 186 (41.0) | **0.007** |
| MTB blood culture, n (%): | 188 (39.4) | 7 (22.6) | 181 (40.6) | 0.139 |
| Urine Xpert positive, n (%): | 199 (46.4) | 6 (18.8) | 193 (48.6) | **0.002** |

**Table note:**

Bold font indicates statistical significance. Data are shown as median and interquartile (IQR) range 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). Mild anemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate anemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anemia was defined as Hb<8g/dL for both sexes. Abbreviations: IQR: Interquartile range; ART: Antiretroviral treatment; VL: viral load; CMV: cytomegalovirus; MTB: *Mycobacterium tuberculosis;*

**Supplementary Table 2. Cellular and biochemical profile according anemia severity**

| | Without anaemia (n=36) | Mild anaemia (n=116) | Moderate anaemia (n=155) | Severe anaemia (n=189) | p value | p trend |
|---|---|---|---|---|---|---|
| Hemoglobin (g/dL), median (IQR): | 13.4 (12.9-14.2) | 10.8 (10.4-11.4) | 8.80 (8.30-9.35) | 6.80 (6.00-7.30) | **<0.001** | **<0.001** |
| Mean Corpuscular Volume (fl) | 85.2 (81.4-90.3) | 85.1 (81.2-89.1) | 81.2 (77.5-86.0) | 79.1 (74.0-84.9) | **<0.001** | **<0.001** |
| White cell count ($\times 10^9$/L), median (IQR) | 6.42 (4.76-9.40) | 6.96 (4.86-9.88) | 7.15 (4.41-9.80) | 7.25 (4.07-11.7) | 0.824 | 0.896 |
| Abs. lymphocyte count ($\times 10^9$/L), median (IQR) | 1.02 (0.58-1.22) | 0.63 (0.38-1.15) | 0.56 (0.33-0.85) | 0.50 (0.29-0.82) | **<0.001** | **<0.001** |
| Abs. monocyte count ($\times 10^9$/L), median (IQR) | 0.45 (0.19-0.66) | 0.42 (0.24-0.64) | 0.31 (0.15-0.51) | 0.28 (0.13-0.56) | **0.001** | **0.001** |
| Abs. neutrophil count ($\times 10^9$/L), median (IQR) | 5.11 (3.42-7.26) | 5.40 (3.34-8.12) | 5.84 (3.14-8.63) | 5.72 (3.21-8.88) | 0.831 | 0.414 |
| Platelet count ($\times 10^9$/L), median (IQR) | 258 (190-297) | 279 (187-362) | 272 (174-362) | 252 (163-332) | 0.148 | 0.124 |

**Table note:**
Bold font indicates statistical significance. Mild anemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate anemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anaemia was defined as Hb<8g/dL for both sexes. Data are shown as median and interquartile (IQR) range 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 Kruskall-Wallis test. [a]without anaemia x mild anaemia; [b]without anaemia x moderate anaemia; [c]without anaemia x severe anaemia; [d]mild x moderate; [e]mild x severe; [f]moderate x severe. Abbreviations: IQR: Interquartile range.

**Supplementary Table 3. Cellular and biochemical profile according anemia severity**

| | Without anaemia (n=36) | Mild anaemia (n=116) | Moderate anaemia (n=155) | Severe anaemia (n=189) | p value | p trend |
|---|---|---|---|---|---|---|
| Random glucose (mmol/L), median (IQR) | 5.30 (4.68-5.93) | 5.20 (4.60-6.00) | 5.20 (4.80-6.00) | 5.30 (4.70-6.20) | 0.746 | 0.553 |
| Venous lactate (mmol/L), median (IQR) | 1.45 (1.20-2.10) | 1.70 (1.28-2.10) | 1.80 (1.30-2.75) | 2.00 (1.40-2.70) | 0.06 | **0.008** |
| C-reactive protein (mg/L), median (IQR) | 77.8 (44.8-155) | 148 (75.1-217) | 146 (96.4-228) | 175 (113-234) | **<0.001** | **<0.001** |
| Procalcitonin (µg/L), median (IQR) | 0.30 (0.08-1.97) | 0.72 (0.21-4.67) | 2.04 (0.34-6.13) | 4.74 (1.52-19.0) | **<0.001** | **<0.001** |
| D-dimer (mg/L), median (IQR) | 0.66 (0.40-1.39) | 1.19 (0.84-2.70) | 1.27 (0.97-3.63) | 2.35 (1.10-4.12) | **<0.001** | **<0.001** |
| AST (U/L), median (IQR) | 45.5 (29.5-85.2) | 52.0 (31.0-92.5) | 57.0 (34.0-118) | 57.0 (36.0-92.0) | 0.43 | 0.26 |
| ALT (U/L), median (IQR) | 33.0 (22.5-57.0) | 29.0 (17.0-50.0) | 30.5 (16.0-54.0) | 23.0 (15.0-38.0) | **0.002** | **0.002** |
| GGT (U/L), median (IQR) | 74.0 (41.0-122) | 75.0 (37.5-182) | 85.5 (49.2-161) | 73.0 (42.0-138) | 0.454 | 0.867 |
| Alkaline phosphatase (U/L), median (IQR) | 101 (63.2-172) | 106 (79.2-148) | 122 (83.0-192) | 118 (73.0-179) | 0.141 | 0.299 |
| Total bilirubin (µmol/L), median (IQR) | 7.50 (5.00-13.0) | 8.00 (5.00-11.8) | 8.00 (6.00-12.0) | 7.00 (5.00-12.0) | 0.776 | 0.787 |
| Conjugated bilirubin (µmol/L), median (IQR) | 4.00 (2.00-7.75) | 4.00 (2.00-6.00) | 4.00 (3.00-7.50) | 4.00 (3.00-8.00) | 0.502 | 0.242 |
| Total protein (g/L), median (IQR) | 78.5 (73.0-87.0) | 76.0 (70.0-84.0) | 76.0 (67.0-85.0) | 72.0 (65.2-81.0) | **0.002** | **<0.001** |
| Albumin (g/L), median (IQR) | 33.5 (28.0-39.0) | 27.0 (23.0-30.0) | 25.0 (21.0-28.0) | 23.0 (19.0-26.0) | **<0.001** | **<0.001** |
| Sodium (mEq/L), median (IQR) | 130 (126-133) | 129 (125-132) | 128 (124-131) | 128 (125-131) | 0.357 | 0.15 |
| Potassium (mmol/L), median (IQR) | 4.20 (3.85-4.65) | 4.10 (3.60-4.55) | 3.90 (3.50-4.60) | 3.90 (3.40-4.43) | 0.149 | 0.022 |
| Urea (mg/dL), median (IQR) | 4.10 (2.88-7.53) | 4.95 | 5.00 (3.45-8.60) | 6.50 (4.00-11.4) | **0.003** | **<0.001** |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | (3.42-8.97) | | | | |
| Creatinine (µmol/L), median (IQR) | 75.0 (62.2-97.0) | 79.5 (61.0-111) | 77.0 (58.0-116) | 89.0 (63.0-158) | 0.06 | **0.018** |

**Table note:**

Bold font indicates statistical significance. Mild anemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate anemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anemia was defined as Hb<8g/dL for both sexes. Data are shown as median and interquartile (IQR) range 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 Kruskall-Wallis test. [a]without anemia x mild anemia; [b]without anemia x moderate anemia; [c]without anemia x severe anemia; [d]mild x moderate; [e]mild x severe; [f]moderate x severe. Abbreviations: IQR: Interquartile range.

**Supplementary Table 4. Inflammatory profile according to anemia severity**

| | Without anaemia (n=36) | Mild anaemia (n=116) | Moderate anaemia (n=155) | Severe anaemia (n=189) | p-value | p trend |
|---|---|---|---|---|---|---|
| **IL-1β** | 1.72 (1.57-1.86) | 1.79 (1.63-1.89) | 1.83 (1.71-1.94) | 1.83 (1.72-1.97) | **0.001** | **<0.001** |
| **IL-1RA** | 1.99 (1.85-2.31) | 2.10 (1.94-2.47) | 2.31 (1.99-2.71) | 2.46 (2.14-2.88) | **<0.001** | **<0.001** |
| **IL-2** | 1.83 (1.72-1.92) | 1.83 (1.75-1.91) | 1.83 (1.75-1.92) | 1.79 (1.71-1.88) | 0.057 | **0.02** |
| **IL-4** | 1.69 (1.53-1.80) | 1.71 (1.59-1.84) | 1.68 (1.57-1.80) | 1.61 (1.46-1.75) | **<0.001** | **<0.001** |
| **IL-5** | 1.50 (1.36-1.66) | 1.52 (1.36-1.69) | 1.48 (1.34-1.65) | 1.38 (1.23-1.53) | **<0.001** | **<0.001** |
| **IL-6** | 2.04 (1.77-2.28) | 2.25 (2.04-2.51) | 2.35 (2.10-2.67) | 2.45 (2.25-2.72) | **<0.001** | **<0.001** |
| **IL-7** | 1.51 (1.45-1.62) | 1.57 (1.45-1.68) | 1.56 (1.44-1.67) | 1.49 (1.38-1.59) | **0.002** | **0.002** |
| **IL-8** | 1.87 (1.79-2.09) | 1.99 (1.84-2.19) | 2.06 (1.92-2.28) | 2.18 (2.00-2.46) | **<0.001** | **<0.001** |
| **IL-9** | 2.18 (2.02-2.32) | 2.20 (2.08-2.31) | 2.19 (2.08-2.33) | 2.19 (2.08-2.32) | 0.713 | 0.498 |
| **IL-10** | 1.82 (1.72-1.89) | 1.86 (1.73-1.97) | 1.85 (1.76-1.95) | 1.82 (1.73-1.91) | 0.061 | 0.078 |
| **IL-12p70** | 1.69 (1.61-1.87) | 1.77 (1.64-1.89) | 1.76 (1.62-1.91) | 1.69 (1.59-1.82) | **0.007** | **0.009** |
| **IL-13** | 1.57 (1.46-1.72) | 1.59 (1.48-1.76) | 1.61 (1.47-1.85) | 1.51 (1.34-1.69) | **<0.001** | **<0.001** |
| **IL-15** | 1.93 (1.86-2.06) | 1.97 (1.87-2.07) | 1.96 (1.88-2.06) | 1.95 (1.86-2.05) | 0.544 | 0.528 |
| **IL-17** | 1.87 (1.64-1.98) | 1.86 (1.71-2.02) | 1.81 (1.71-1.97) | 1.73 (1.62-1.87) | **<0.001** | **<0.001** |
| **Eotaxin** | 1.81 (1.66-1.89) | 1.80 (1.70-1.90) | 1.83 (1.71-1.97) | 1.81 (1.70-1.96) | 0.227 | 0.159 |
| **FGF** | 1.75 (1.62-1.86) | 1.76 (1.65-1.87) | 1.72 (1.64-1.83) | 1.68 (1.59-1.77) | **<0.001** | **<0.001** |
| **GCSF** | 1.79 (1.66-1.88) | 1.80 (1.69-1.91) | 1.85 (1.74-1.99) | 1.86 (1.73-2.04) | **0.012** | **0.002** |
| **GMCSF** | 1.90 (1.86-2.01) | 1.94 (1.85-2.07) | 1.96 (1.87-2.06) | 1.93 (1.82-2.04) | 0.148 | 0.706 |
| **IFN-γ** | 1.66 (1.51-1.93) | 1.75 (1.59-1.88) | 1.74 (1.61-1.88) | 1.67 (1.54-1.83) | **0.006** | **0.017** |
| **CXCL10** | 3.48 (3.08-3.78) | 3.76 (3.42-4.07) | 3.85 (3.56-4.10) | 3.99 (3.76-4.16) | **<0.001** | **<0.001** |
| **CCL4** | 1.95 (1.79-2.05) | 1.97 (1.87-2.12) | 2.00 (1.89-2.15) | 2.01 (1.88-2.20) | **0.019** | **0.003** |
| **CCL2** | 1.86 (1.73-2.14) | 1.96 (1.80-2.17) | 2.03 (1.84-2.25) | 1.97 (1.83-2.28) | **0.049** | 0.106 |
| **PDGF-bb** | 2.30 (1.97-2.67) | 2.40 (1.98-2.72) | 2.27 (1.91-2.56) | 2.06 (1.80-2.41) | **<0.001** | **<0.001** |

| | | | | | | |
|---|---|---|---|---|---|---|
| **CCL3** | 2.74 (2.55-3.02) | 2.76 (2.61-2.99) | 2.83 (2.63-3.04) | 2.91 (2.68-3.17) | **0.001** | **<0.001** |
| **CCL5** | 4.19 (4.14-4.23) | 4.20 (4.12-4.22) | 4.18 (4.11-4.22) | 4.14 (3.98-4.20) | **<0.001** | **<0.001** |
| **TNF** | 1.60 (1.52-1.69) | 1.65 (1.57-1.73) | 1.65 (1.57-1.75) | 1.61 (1.51-1.72) | **0.028** | **0.14** |
| **VEGF** | 1.96 (1.81-2.14) | 2.05 (1.87-2.23) | 2.03 (1.91-2.21) | 2.01 (1.88-2.16) | 0.341 | 0.513 |
| **TGF-β1** | 1.49 (1.19-1.71) | 1.49 (1.19-1.78) | 1.47 (1.19-1.75) | 1.25 (1.10-1.56) | **<0.001** | **<0.001** |

**Table note:**

Bold font indicates statistical significance. Mild anemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate anemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anemia was defined as Hb<8g/dL for both sexes. Data are shown as median and interquartile range (IQR). The Kruskall-Wallis test was used to assess the statistical differences between all groups. The Cochran–Armitage test for trend was used to assess for the presence of an association between the measurements and the severity of anemia. Abbreviations: IQR: Interquartile range.

**Supplementary Table 5. Clinical and Inflammatory profile according to cluster**

| | Cluster 3 (n=214) | Cluster 2 (n=156) | Cluster 1 (n=76) | P value |
|---|---|---|---|---|
| **Anaemia severity, n (%)** | | | | **<0.001** |
| **Without** | 16 (7.48) | 9 (5.77) | 3 (3.95) | |
| **Mild** | 46 (21.5) | 48 (30.8) | 12 (15.8) | |
| **Moderate** | 64 (29.9) | 57 (36.5) | 19 (25.0) | |
| **Severe** | 88 (41.1) | 42 (26.9) | 42 (55.3) | |
| **Dissemination score, n (%)** | | | | **<0.001** |
| **0** | 81 (37.9) | 62 (39.7) | 1 (1.32) | |
| **1** | 57 (26.6) | 46 (29.5) | 20 (26.3) | |
| **2** | 51 (23.8) | 31 (19.9) | 21 (27.6) | |
| **3** | 25 (11.7) | 17 (10.9) | 34 (44.7) | |
| **Deaths, n (%)** | 44 (20.6) | 20 (12.8) | 33 (43.4) | **<0.001** |
| **Time to death, median (IQR)** | 32.0 (9.00-50.0) | 28.0 (10.2-35.5) | 7.00 (2.00-20.0) | **0.014** |
| | | | | |
| **CD4 (count), median (IQR)** | 68.0 (24.2-122) | 66.5 (33.5-147) | 17.5 (6.00-44.0) | **<0.001** |
| **Hb (g/dL), median (IQR)** | 8.30 (7.03-10.5) | 9.15 (7.88-10.4) | 7.60 (6.20-9.80) | **<0.001** |
| | | | | |
| **IL-1β** | 1.7 (1.61 - 1.78) | 1.9 (1.84 – 2.00) | 1.94 (1.83 - 2.09) | **<0.001** |
| **IL-1RA** | 2.17 (1.91 - 2.52) | 2.23 (2.04 - 2.58) | 3.15 (2.86 - 3.45) | **<0.001** |
| **IL-2** | 1.74 (1.68 - 1.79) | 1.91 (1.86 – 2.00) | 1.83 (1.75 - 1.9) | **<0.001** |
| **IL-4** | 1.55 (1.43 - 1.64) | 1.83 (1.76 - 1.91) | 1.63 (1.54 - 1.69) | **<0.001** |
| **IL-5** | 1.36 (1.25 - 1.46) | 1.67 (1.57 - 1.76) | 1.34 (1.23 - 1.44) | **<0.001** |
| **IL-6** | 2.28 (1.98 - 2.51) | 2.33 (2.12 - 2.5) | 2.82 (2.65 - 3.15) | **<0.001** |
| **IL-7** | 1.46 (1.38 - 1.51) | 1.66 (1.59 - 1.74) | 1.46 (1.39 - 1.56) | **<0.001** |
| **IL-8** | 1.96 (1.83 - 2.15) | 2.08 (1.97 - 2.21) | 2.58 (2.44 - 2.99) | **<0.001** |
| **IL-9** | 2.08 (2 - 2.17) | 2.28 (2.19 - 2.37) | 2.36 (2.25 - 2.48) | **<0.001** |
| **IL-10** | 1.74 (1.68 - 1.81) | 1.94 (1.88 - 2.02) | 1.88 (1.8 - 2.01) | **<0.001** |
| **IL-12p70** | 1.62 (1.55 - 1.71) | 1.91 (1.81 - 2.02) | 1.65 (1.59 - 1.75) | **<0.001** |
| **IL-13** | 1.48 (1.34 - 1.63) | 1.72 (1.61 - 1.91) | 1.43 (1.34 - 1.53) | **<0.001** |
| **IL-15** | 1.88 (1.8 - 1.93) | 2.02 (1.98 - 2.11) | 2.06 (1.95 - 2.16) | **<0.001** |
| **IL-17** | 1.69 (1.59 - 1.78) | 2.00 (1.91 - 2.08) | 1.77 (1.7 - 1.88) | **<0.001** |
| **Eotaxin** | 1.72 (1.63 - 1.8) | 1.91 (1.83 - 2.03) | 1.88 (1.76 - 2.02) | **<0.001** |

| | | | | |
|---|---|---|---|---|
| **FGF** | 1.65 (1.57 - 1.71) | 1.87 (1.79 - 1.94) | 1.68 (1.61 - 1.73) | **<0.001** |
| **GCSF** | 1.72 (1.63 - 1.81) | 1.90 (1.82 - 1.99) | 2.08 (1.91 - 2.4) | **<0.001** |
| **GMCSF** | 1.87 (1.77 - 1.96) | 2.02 (1.95 - 2.12) | 1.97 (1.88 - 2.06) | **<0.001** |
| **IFN-γ** | 1.59 (1.49 - 1.68) | 1.88 (1.8 - 1.95) | 1.76 (1.64 - 1.9) | **<0.001** |
| **CXCL10** | 3.77 (3.51 - 3.99) | 3.84 (3.54 - 4.06) | 4.23 (4.12 - 4.35) | **<0.001** |
| **CCL4** | 1.89 (1.8 - 1.99) | 2.03 (1.94 - 2.15) | 2.3 (2.14 - 2.62) | **<0.001** |
| **CCL2** | 1.85 (1.71 - 2.08) | 2.03 (1.91 - 2.21) | 2.38 (2.13 - 2.6) | **<0.001** |
| **PDGF-bb** | 1.97 (1.8 - 2.31) | 2.65 (2.43 - 2.86) | 1.91 (1.77 - 2.13) | **<0.001** |
| **CCL3** | 2.77 (2.59 - 2.95) | 2.81 (2.61 - 3.02) | 3.4 (3.11 - 3.54) | **<0.001** |
| **CCL5** | 4.15 (4.03 - 4.2) | 4.2 (4.17 - 4.23) | 4.01 (3.73 - 4.14) | **<0.001** |
| **TNF** | 1.54 (1.47 - 1.61) | 1.75 (1.69 - 1.84) | 1.64 (1.57 - 1.72) | **<0.001** |
| **VEGF** | 1.92 (1.8 - 2.02) | 2.2 (2.09 - 2.34) | 2.04 (1.91 - 2.18) | **<0.001** |
| **TGF-β1** | 1.21 (1.07 - 1.46) | 1.74 (1.53 - 1.9) | 1.19 (1.13 - 1.4) | **<0.001** |

**Table note:**

Bold font indicates statistical significance. Mild anemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate anemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anemia was defined as Hb<8g/dL for both sexes. Data are shown as median and interquartile range (IQR). The Kruskall-Wallis test was used to assess the statistical differences between all groups. The Cochran–Armitage test for trend was used to assess for the presence of an association between the measurements and the severity of anemia. Abbreviations: IQR: Interquartile range.

## 5.5 Manuscrito V

### Título

"Impact of Persistent Anemia on Systemic Inflammation and Tuberculosis Outcomes in Persons Living With HIV"

### Objetivo

Esse trabalho teve como objetivo caracterizar a dinâmica dos níveis de Hb em pacientes TB/HIV, examinar as relações entre anemia e distúrbios inflamatórios sistêmicos, bem como a associação entre anemia persistente e desfechos de tratamento desfavoráveis em uma coorte de 256 pacientes do Brasil durante 180 dias de ATT.

### Resumo de resultados

Através das análises de diversos parâmetros bioquímicos, verificamos que 101 (63,63%) dos pacientes com anemia pré-ATT persistiram com tal condição até o dia 180. Tais indivíduos apresentaram grau elevado de perturbação inflamatória, que por sua vez foi inversamente correlacionado aos níveis de Hb. A recuperação da anemia foi associada ao aumento dos níveis de albumina pré-ATT, enquanto a anemia persistente foi relacionada a níveis mais elevados de proteína total no soro. A análise de regressão multivariada revelou que apresentar um baixo valor de Hb pré-ATT é o principal determinante dos desfechos desfavoráveis. Nossos achados demonstraram que a anemia persistente em pacientes TB/HIV durante o curso do ATT está intimamente relacionada com a perturbação inflamatória crônica.

### Status do manuscrito

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# Impact of Persistent Anemia on Systemic Inflammation and Tuberculosis Outcomes in Persons Living With HIV

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Tuberculosis (TB) is associated with systemic inflammation and anemia, which are aggravated in persons living with HIV (PLWH). Here, we characterized the dynamics of hemoglobin levels in PLWH coinfected with TB undergoing antitubercular therapy (ATT). We also examined the relationships between anemia and systemic inflammatory disturbance as well as the association between persistent anemia and unfavorable clinical outcomes. Data on several blood biochemical parameters and on blood cell counts were retrospectively analyzed in a cohort of 256 TB/HIV patients from Brazil during 180 days of ATT. Multidimensional statistical analyses were employed to profile systemic inflammation of patients stratified by anemia status (hemoglobin levels <12 g/dL for female and <13.5 g/dL for male individuals) prior to treatment and to perform prediction of unfavorable outcomes, such as treatment failure, loss to follow up and death. We found that 101 (63.63%) of patients with anemia at pre-ATT persisted with such condition until day 180. Such individuals exhibited heightened degree of inflammatory perturbation (DIP), which in turn was inversely correlated with hemoglobin levels. Recovery from anemia was associated with increased pre-ATT albumin levels whereas persistent anemia was related to higher total protein levels in serum. Multivariable regression analysis revealed that lower baseline hemoglobin levels was the major determinant of the unfavorable outcomes. Our findings demonstrate that persistent anemia in PLWH during the course of ATT is closely related with chronic inflammatory perturbation. Early intervention to promote recovery from anemia may improve ATT outcomes.

**Keywords: HIV, tuberculosis, anemia, inflammation, treatment outcome**

## INTRODUCTION

Tuberculosis (TB) remains as a leading cause of death from infection by a single pathogen and also among people living with human immunodeficiency virus (HIV) (1). Persons living with HIV (PLWH) exhibit up to 19 times higher risk of developing active TB (2). In addition, TB is one of the most common opportunistic infections in PLWH. In fact, a total of 1.5 million people died from TB in 2018, including 251,000 PLWH (1). Understanding the determinants of clinical outcomes of PLWH coinfected with TB is critical to improve patient care.

Anemia is also a global public health problem and is diagnosed based on concentration of hemoglobin (Hb), specifically when it falls below established cut-off values; 12.0 g/dL for women and 13.5 g/dL for men (3). Low concentrations of Hb are a frequent complication of both TB and HIV infections, and its occurrence is associated with increased morbidity and mortality (4). Several causes of anemia are described, including iron deficiency and chronic inflammation (5–7). Prevalence of anemia in TB patients is reported to range between 32 and 96% (8), whereas in PLWH, this estimate varies from 1.3 to 95% (4). The extreme discrepancies in frequency of anemia associated with either TB and/or HIV infections published by several studies are thought to be influenced by factors that include study design, geographic location as well as clinical and epidemiological characteristics of patients.

Many studies have associated anemia with poor prognosis and increased mortality after TB diagnosis (6, 7, 9). In patients with TB, anemia has been attributed to be caused by chronic inflammation (10). It has also been shown that anemia is related to accelerated HIV/AIDS disease progression in PLWH (11). This latter study concluded that Hb levels is a robust biomarker to predict death independent of $CD4^+$ T-cell count and HIV viral load values (11). More recently, a prospective investigation of antiretroviral therapy (HAART)-naïve PLWH reported that concurrent anemia and systemic inflammation were associated with higher risk of HAART failure (12). A potential explanation for the association between anemia and poor outcomes in HIV/AIDS and/or TB is that low Hb concentrations reflect more advanced disease staging. It is still to be defined the relationship between anemia and systemic inflammation in the context of antitubercular treatment (ATT) in PLWH and whether recovery from anemia during ATT in PLWH is related to improved prognosis.

In a study from Brazil, we have recently described that risk factors for mortality were distinct between HAART-naïve and HAART-experienced PLWH patients coinfected with TB. Indeed, in HAART-naïve patients, but not in those who were already undertaking antiretrovirals, the odds of death were substantially higher in patients who developed immune reconstitution inflammatory syndrome (IRIS) during the study follow up (13). This finding suggests that inflammation during the course of ATT in PLWH is related to unfavorable outcomes. In the present study, we expanded our analyses to investigate the relationship between the presence and severity of anemia and the cellular and biochemical profile of systemic inflammation in PLWH and TB in Brazil. We also tested whether low levels of Hb measured at pre-ATT could be used to predict unfavorable outcomes.

## MATERIALS AND METHODS

### Ethics Statement

The study was approved by the Institutional Review Board of the Instituto Nacional de Infectologia Evandro Chagas (INI) (CAAE: 71191417.8.0000.5262). Written informed consent was obtained from all participants, and all clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

### Population and Design

A prospective cohort has been followed at the Clinical Research Laboratory on Mycobacteria (LAPCLIN-TB) of the INI Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil, since 2000. The present study is a retrospective assessment performed between 2008 and 2016, with data obtained from this cohort. Data were collected from electronic medical records based on standardized information of a defined template used in each patient's visit for the whole cohort. PLWH 18 years and older, with clinical signs and symptoms of TB were included. The diagnosis of TB was made when *Mycobacterium tuberculosis* (Mtb) detection was positive in any sample collected (acid fast bacilli smear, Gene Xpert or culture from clinical specimens). In cases without bacteriological confirmation, the diagnosis was established by suggestive imaging analysis, histopathological examination, together with clinical and epidemiological findings consistent with TB. For those who had a negative culture, a positive therapeutic test with TB drugs was considered, after excluding other opportunistic diseases for differential diagnosis. Patients that initiated TB treatment and were diagnosed later with non-tuberculous mycobacteria as well as those who showed rifampicin and isoniazid resistance (multidrug resistance) were excluded. Patients with bone, mammary, renal or ocular TB were excluded, since these clinical forms can have very subtle, asymptomatic presentations, making it difficult to be compared to the other forms.

### Definitions

Anemia was defined according to World Health Organization (WHO) guideline criteria: Hb value $< 13.5$ g/dL for men and $<12$ g/dL for women.

Tuberculosis was classified as pleuropulmonary (when restricted to the lungs and/or pleura), extra-pulmonary (when just one extra-pulmonary site was identified) or disseminated (involving spleen, liver, bone marrow, or at least 2 non-contiguous sites).

Discharge due to cure, with or without etiologic confirmation of the diagnosis of TB, was considered a favorable outcome. Patients were defined as cured through clinical and/or radiologic improvement. Unfavorable outcome was defined as death, loss to follow up and treatment failure following the WHO guidelines. The cause of death was determined after thorough review of

relevant clinical, microbiological and pathological data of each deceased patient.

### Antiretroviral and Antitubercular Therapies

Highly active antiretroviral therapy was offered according to contemporary Brazilian National Guidelines that were periodically updated (14). The first line ATT regimen was the combination of rifampicin, isoniazid and pyrazinamide during the two initial months, followed by rifampicin and isoniazid for 4 months, except when the continuation phase needed to be extended to 7 months such as in cases with central nervous system TB. From July 2009 on, ethambutol was added to the intensive phase regimen following a new recommendation of the National TB program of the Brazilian Ministry of Health (15). TB treatment scheme was adjusted in cases of severe adverse reactions, drug resistance and HAART regimens that precluded the use of rifampicin.

### Follow Up Visits

Visits included in this study were done at baseline, 60 and 180 days after TB therapy initiation. HAART were initiated after TB treatment according to decision from each physician and following the Brazilian TB treatment Guidelines (14). Information collected at the baseline visit included socio demographic data as well as previous TB and HAART, clinical presentation of TB, comorbidities like diabetes, hypertension, hepatitis (B and C), opportunistic diseases as well as $CD4^+$ T-cell count and HIV VL among other variables. At baseline and in the follow up timepoints, patients underwent blood tests according to the INI's clinical laboratory routine, with complete blood count and biochemical tests (creatinine, urea, total and direct bilirubin, albumin, alkaline phosphatase, uric acid, AST, GGT, ALT and total proteins).

Some patients ($n$ = 06) who abandoned TB treatment (ATT loss to follow up) had recorded data on Complete Blood Count (CBC) and biochemical assessments in blood after the date of the outcome established by the present study (non-compliance), because those patients had been following up at INI by other specialties outside the TB outpatient clinic.

### Statistical Data Analysis

Three timepoints were considered: baseline, day 60 (D60) and day 180 (D180) of ATT. To perform baseline analysis, were used data from 256 patients. Due to lack of data in the subsequent timepoints (6.6% were missing data at D60 and 25.4% at D180), only 191 (74.6%) patients with complete laboratory data at all timepoints were considered for longitudinal analysis. Descriptive statistics was used to present data, use the median values with interquartile ranges (IQR) as measures of central tendency and dispersion, respectively, 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), the Wilcoxon matched pairs test (for two matched groups), the Kruskal–Wallis test (for more than 2 unmatched groups) or the Jonckheere-Terpstra permutation and asymptotic test (for time series) were used to compare continuous variables. The Spearman rank test was used to assess correlations between indicated markers, conditions and timepoints. A multivariable logistic regression analysis model was used to identify independent determinants of persistent anemia and unfavorable treatment outcomes. The results were presented in the form of adjusted odds ratio (aOR) and 95% confidence intervals (CI).

The degree of inflammatory perturbation (DIP) is based molecular degree of perturbation (MDP) (16), an adaptation of the molecular distance to health previously described (17). In the present study, instead of using gene expression values, we inputted biochemical markers concentrations, HIV viral load and blood cells counts. Thus, herein, the average level and standard deviation of a baseline reference group (non-anemic at baseline) were calculated for each biomarker. The DIP score of each individual biomarker was defined by z-score normalization, where the differences in concentration levels from the average of the biomarker in reference group was divided by the reference standard deviation. The DIP score represents the differences by number of standard deviations from the control group.

Hierarchical cluster analysis (Ward's method) using values of z-score normalized data was employed to depict the overall expression profile of indicated markers in the study subgroups. In this analysis, the dendrograms represent the Euclidean distance (inferring degree of similarity).

All analyses were pre-specified. Differences with $p$-values below 0.05 after adjustment for multiple comparisons (Holm-Bonferroni) were considered statistically significant. The statistical analyses. were performed using mdp (version 1.8.0), rstatix (version 0.4.0), stats (version 3.6.2), and caret (version. 6.0.86) R packages.

## RESULTS

### Characteristics of the Study Participants

During the period from 2008 to 2016, 273 patients were screened, but 17 were excluded from all the analyses because of lack of data at baseline. Thus, the initial analysis included 256 patients, out of whom 219 (85.6%) were anemic and 37 (14.4%) were not anemic at baseline. The vast majority of study participants were male (71%), and the median age was 37 years old (IQR: 31–46). Individuals with anemia at baseline were similar to non-anemic participants with regard to, age, sex, overall frequency of comorbidities and life-habits (**Table 1**). Anemic patients more frequently self-reported weight loss (>10% of body weight) before initiating treatment and displayed lower $CD4^+$ T-cell counts and higher HIV viral loads than those non-anemic at the study baseline (**Table 1**). Frequency of HAART use before TB diagnosis was higher in non-anemic study participants (65% in non-anemic vs. 43% in anemic, $p = 0.021$; **Table 1**).

**TABLE 1** | Characteristics of the study population.

| Characteristic | All ($n$ = 256) | Anemic at baseline ($n$ = 219) | Non-anemic at baseline ($n$ = 37) | $p$-value |
|---|---|---|---|---|
| Age (years), median (IQR) | 37 (31–46) | 37 (30.7–46) | 37 (33–46) | 0.504 |
| Sex, no. (% male) | 182 (71) | 157 (71.7) | 25 (67.6) | 0.996 |
| Weight loss (> 10%), no. (%) | 190 (74.2) | 174 (79.5) | 16 (43.2) | <0.01 |
| Smoking, no. (%) | 131 (51.2) | 114 (52) | 17 (45.9) | 0.948 |
| Use of illicit drugs, no. (%) | 74 (28.9) | 60 (27.3) | 14 (37.8) | 0.185 |
| Alcohol abuse[1], no. (%) | 88 (34.3) | 80 (36.5) | 8 (21.6) | 0.133 |
| Baseline CD4 count (cells/mm$^3$), median (IQR) | 170.5 (52–321.2) | 153 (42.5–304.5) | 294 (158–560) | <0.01 |
| Baseline Viral Load log10 (copies/mL), median (IQR) ($n$ = 185) | 4.3 (1.69–5.23) | 4.41 (2.5–5.31) | 1.79 (1.69–4.22) | <0.01 |
| D180 CD4 count (cells/mm$^3$), median (IQR) | 292.5 (165–432) | 258 (157.5–403) | 423 (266–603.5) | 0.018 |
| D180 Detectable Viral Load log10 (copies/mL), median (IQR) ($n$ = 76) | 3.21 (1.80–4.67) | 3.17 (1.79–4.68) | 3.42 (2.03–4.03) | 0.766 |
| D180 Undetectable VL, no. (%) | 133 (52) | 105 (65.2) | 25 (38.5) | 0.320 |
| Days until outcome[2], median (IQR) | 189 (178.7–259.7) | 189 (180–265) | 189 (168–247.5) | 0.564 |
| Viral Hepatitis (B and/or C), no. (%) | 25 (9.7) | 21 (9.48) | 4 (10.8) | 0.945 |
| Hypertension, no. (%) | 21 (8.2) | 16 (7.3) | 5 (13.5) | 0.270 |
| Diabetes, no. (%) | 32 (12.5) | 28 (12.7) | 4 (10.8) | 0.946 |
| Previous tuberculosis (%) | 64 (25) | 52 (23.7) | 12 (32.4) | 0.355 |
| Complete TB treatment previous, no. (% of previous TB) | 44 (68.8) | 35 (67.3) | 9 (75) | 0.742 |
| HAART use before TB, no. (%) | 118 (46) | 94 (42.9) | 24 (64.8) | 0.021 |
| HAART during TB treatment, no. (%) | 235 (91.7) | 202 (92.3) | 33 (89.2) | 0.763 |
| IRIS upon HAART initiation, no. (%) | 12 (4.68) | 12 (5.47) | 0 (0) | – |

*To define anemia according to baseline (D0) hemoglobin, the cut-off point of 12 g/dL for women and 13.5 g/dL for men was used. Data are shown as median and interquartile (IQR) range or frequency (percentage). Data were compared between the clinical groups using the Mann–Whitney U test (continuous variables) or the Pearson's $\chi$ 2 test (for data on frequency). Complete data at baseline: 256 patients; Complete data at day 60: 239 (93.4%) patients, Complete data at day 180: 191 (74.6%) patients. [1]The physicians also collected information about current use of illicit drugs and alcohol (Y/N to each) during the baseline interview. Potential problematic alcohol use was assessed with the CAGE questionnaire, with scores of 2 or greater indicating clinically significant alcohol problems. [2]Outcomes: Favorable (cure) and Unfavorable (failure, loss follow-up or death). IQR, Interquartile Range; IRIS, Immune reconstitution Inflammatory Syndrome; TB, Tuberculosis; HAART, Highly Active Antiretroviral Therapy.*

To perform the longitudinal analysis, 82 of these patients were excluded because due to lack of data at some time point of the TB treatment (as described in "Materials and Methods"). Thus, 191 patients were further considered, out of whom 161 (84.3%) were anemic and 30 (15.7%) were not anemic at baseline. The median TB treatment period was 189 days for both groups. At day 180 of treatment, $CD4^+$ T-cell counts increased in both study groups, but values in the group of participants who were anemic at the study baseline persisted substantially lower than those measured in non-anemic patients ($p$ = 0.018; **Table 1**). Nevertheless, both frequency of individuals with undetectable HIV viral loads and median values with detectable viral loads were indistinguishable between study participants stratified based on anemia at baseline. There was no difference in the type of antitubercular treatment regimen between the study groups.

## Presence of Anemia Is Associated With Specific Cellular and Biochemical Profiles in Peripheral Blood of PWH Coinfected With TB

The overall differences in cell counts and values of biochemical parameters measured at pre-ATT for anemic and non-anemic TB patients are described in **Supplementary Table 1**. As expected, erythrocyte counts, and values of hematocrit and hemoglobin were lower in anemic compared to non-anemic study participants. In addition, anemic patients exhibited lower counts of several leukocytes including lymphocytes and eosinophils at the study baseline (**Supplementary Table 1**). Additional analyses of the CBC parameters using hierarchical clustering of z-score normalized data and computation of fold change were performed to evaluate the dynamicity of the values over time in each group (**Figure 1A**). We observed a distinct profile between the groups, with three clusters defined in the heatmap, where the latter cluster (hemoglobin, hematocrit and erythrocyte) was the most consistent in both groups, with few changes mainly in the group of patients without anemia before treatment (baseline). Furthermore, it was possible to observe that, in the anemic participants, there was a significant difference in all parameters over time, mainly when comparing the baseline with the end of TB treatment (D180).

In regard to biochemical parameters, statistically significant differences were found in levels of ALT, AST and GGT, which were all higher in anemic patients at baseline, whereas the levels of albumin were lower

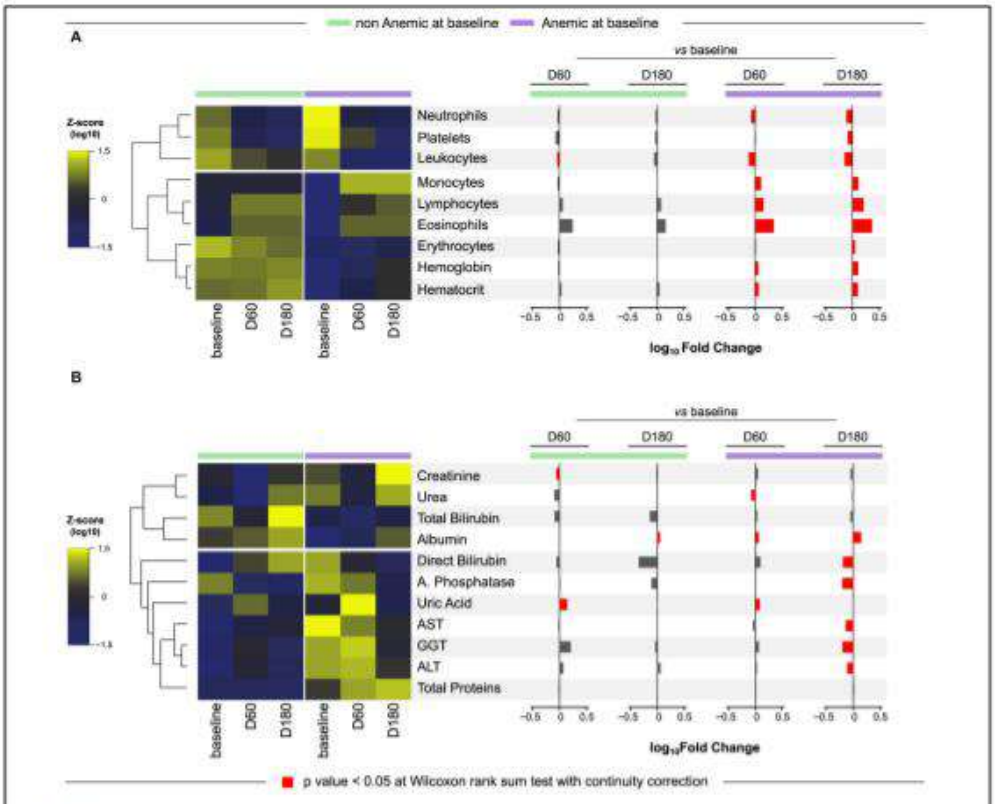


**FIGURE 1 |** Differential change in biomarkers between anemic and non-anemic patients. A Heatmap was designed to depict the overall pattern of complete blood counts (CBC) **(A)** and biochemical markers **(B)** at all timepoints in anemic and non-anemic at different study timepoints of anti-tubercular treatment. A two-way hierarchical cluster analysis (Ward's method) was performed. Dendrograms represent Euclidean distance. Expression scale represents Z-score normalization from the median at each timepoint and group. To define anemia according to baseline hemoglobin, the cut-off point of 12 g/dL for women and 13.5 g/dL for men was used. A $\log_{10}$ of fold-change was calculated and statistical analyses were performed using the Mann–Whitney *U* adjusted test. Significative differences ($p < 0.05$) between anemic and non-anemic patients for each time point are highlighted in red bars. Data are from 191 patients who had complete information on cell counts and biochemical measurements at all study timepoints.

(**Supplementary Table 1** and **Figure 1B**). Additional hierarchical cluster and fold change analysis performed with biochemical parameters revealed a distinct profile between the groups (**Figure 1B**). Again, small changes over time in the group without anemia at baseline were observed, with increased levels of uric acid and decreased levels of creatinine at D60 and with increase in albumin levels at D180, comparing with baseline. In the group that presented anemia at baseline, the differences in levels of biomarkers were more pronounced. We found that, at D60, a decrease in urea levels and increase in uric acid and albumin levels were detected compared to baseline. At D180, there were significantly higher values of albumin and lower values of direct bilirubin, alkaline phosphatase, AST, GGT and ALT, than those measured at the study baseline.

## Correlation Between Cells and Biochemical Parameters With Hemoglobin

The results presented above indicate that anemia is associated with a distinct profile of cell counts and biochemical parameters in peripheral blood of patients with HIV-TB coinfection prior to initiation of ATT. We next examined the correlations between Hb levels and cell counts or

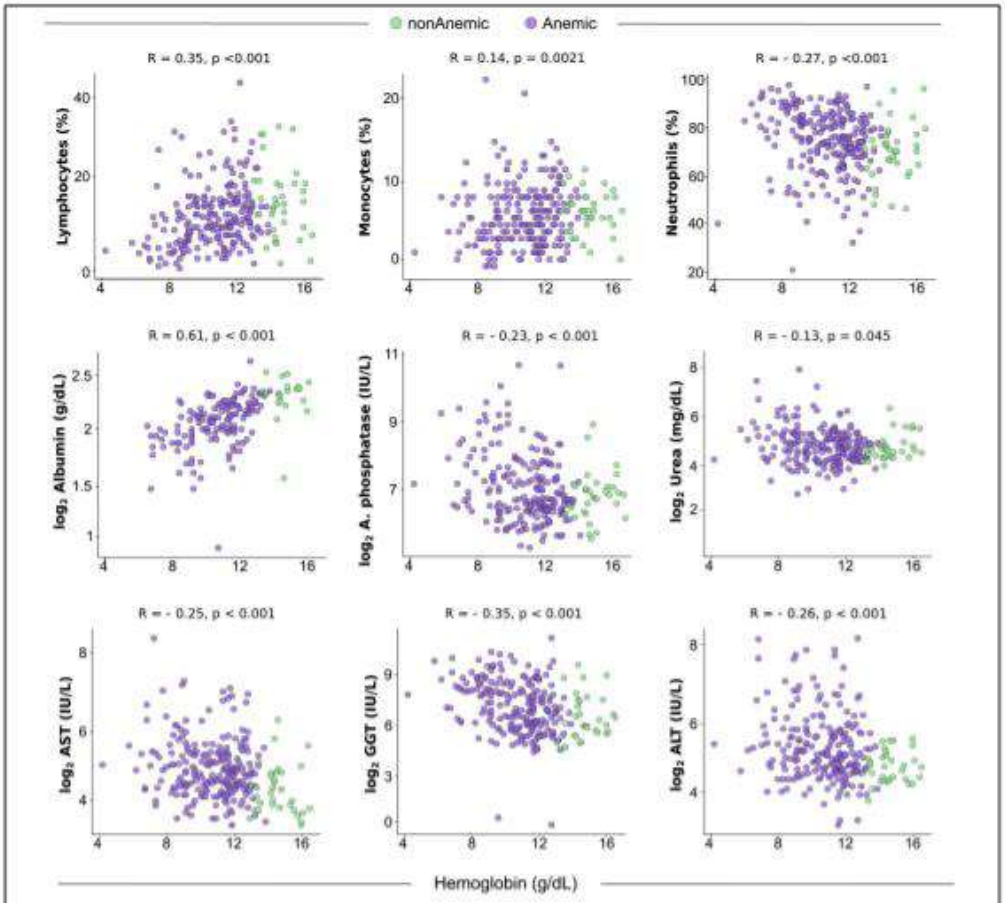


**FIGURE 2 |** Spearman correlation analysis of cells and biochemical parameters versus hemoglobin in blood of TB-HIV prior to antitubercular treatment initiation. Plots from statistically significant Spearman correlations between biochemical parameters and hemoglobin levels at study baseline (pre-ATT) are shown (*n* = 256). To define anemia according to baseline hemoglobin, the cut-off point of 12 g/dL for women and 13.5 g/dL for men was used. ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase.

values of the biochemical parameters (**Figure 2**). We observed that gradual increases in Hb values were related with decreases in percentage of neutrophils ($r = -0.27$; $p < 0.001$) and levels of ALT ($r = -0.26$; $p < 0.001$), AST ($r = -0.25$; $p < 0.001$), GGT ($r = -0.35$; $p < 0.001$), Alkaline Phosphatase ($r = -0.23$; $p < 0.001$), and Urea ($r = -0.13$; $p = 0.045$). Furthermore, frequency of lymphocytes ($r = 0.35$; $p < 0.001$) and monocytes ($r = 0.14$; $p = 0.021$), as well as levels of albumin ($r = 0.61$; $p < 0.001$) were increased proportionally to elevations in Hb levels (**Figure 2**). These findings reinforce the idea that degree of anemia is associated with changes in cellular and biochemical disturbances in peripheral blood.

## Dynamic Change of Hemoglobin Levels Upon Initiation of Anti-TB Treatment

In order to better understand the impact of ATT commencement in the anemia, we prospectively investigated Hb levels at different time points of therapy (**Figure 3**). This approach revealed a differential dynamic of changes in Hb levels depending on the anemia status at the study baseline (**Figure 3A**). Indeed, a gradual

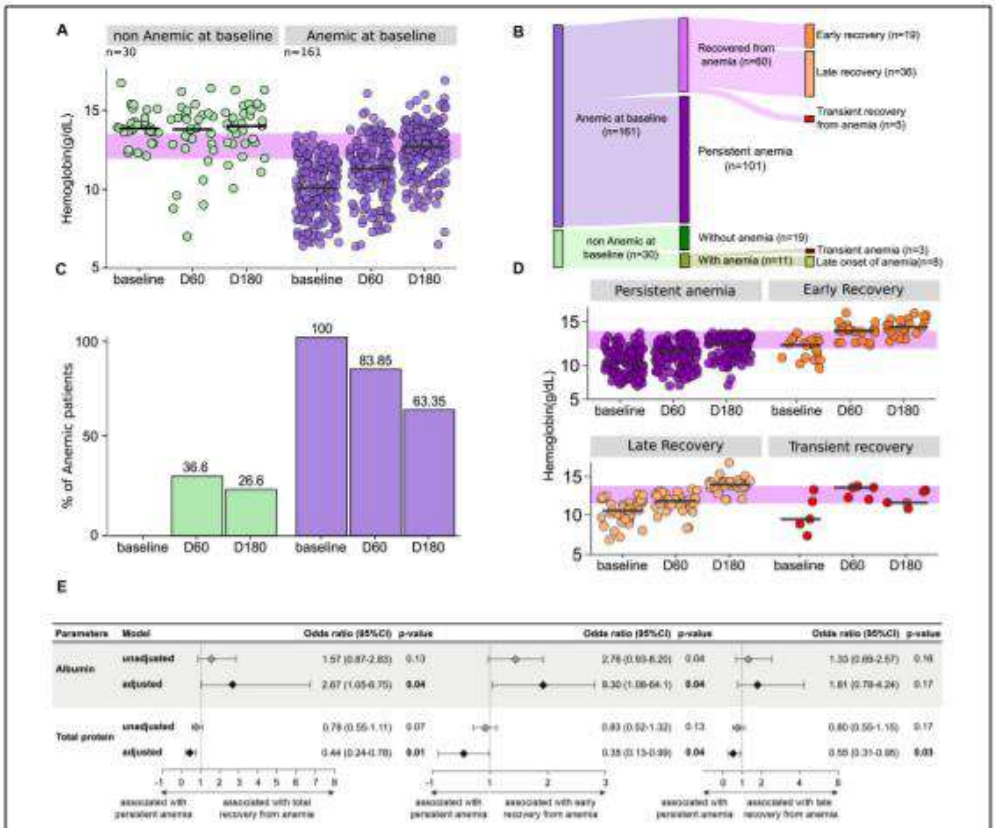


**FIGURE 3 |** The majority of the anemic patients at baseline persist with low levels of hemoglobin after initiation of anti-tubercular treatment. To define anemia according to baseline hemoglobin, the cut-off point of 12 g/dL for women and 13.5 g/dL for men was used. **(A)** Hemoglobin levels at different time points of antitubercular therapy in the longitudinal population ($n = 191$) as well as in the groups of patients with or without anemia at baseline of treatment are shown. Anemic group presented statistically significant difference with $p < 0.001$ between baseline and timepoints after 2 months (D60 and D180) using Wilcoxon rank sum test with corrections. On Jonckheere-Terpstra permutation test, where an increase in one variable results in an increase or decrease in another variable, both groups presented $p = 0.001$ to "increase" hypothesis, with number of permutation equal to 1000. Using Jonckheere-Terpstra asymptotic test, $p$-value of non-anemic group was 0.851 and $p$-value of anemic group was <2.2e-16. Green dots represent non-anemic TB patients at baseline and purple dots represent anemic TB patients at baseline. **(B)** To define a patient as recovered from anemia, were considered normal levels (above the cut-off) of hemoglobin in any time point after D0. Chi-square test comparing D0 and D180 in both groups returned $p < 0.00001$. Green bars represent non-anemic TB patients at baseline and purple bars represent anemic TB patients at baseline. **(C)** Of the 161 patients who had anemia before starting treatment, 37.3% ($n = 60$) increased the values to normal hemoglobin levels at some time point. Of these, 95% were completely recovered ($n = 55$), so that 35% ($n = 19$) were recovered early (D60), and 60% ($n = 36$) were recovered late (D180). Finally, 5% ($n = 5$) of the patients who were anemic at study baseline presented a transient recovery (recovered at day 60 but were once again anemic at D180). 36.6% ($n = 11$) of the 30 patients without anemia at baseline developed anemia in D60, but three of them recovered normal hemoglobin values at D180. **(D)** Hemoglobin levels at different time points of antitubercular therapy in the in the population of anemic patients at baseline, divided according to the time of recovery. Using Jonckheere-Terpstra asymptotic test and Wilcoxon rank sum test with corrections, only the transient recovery group (that showed higher levels of hemoglobin at time 60 but had anemia at 180) did not exhibit a significant $p$-value between the timepoints. **(E)** Logistic binomial regression model was used to test independent associations between biochemical and clinical parameters and total recovery from anemia status at baseline, early recovery (recovery from anemia in ≤60 days from baseline) or late recovery (recovery from anemia in >60 days from baseline). The condition persistent anemia (anemia from baseline to day 180) was used as reference to test associations. Only parameters which remained with $p \leq 0.2$ in univariate analysis (**Supplementary Table 2** for details) model were inputted in the adjusted model. (95%CI, 95% confidence interval). Associations reported in **(E)** are for increases in 1 unit in plasma concentrations of the indicated markers. Data are from 191 patients who had complete information on cell counts and biochemical measurements at all study timepoints.

increase in Hb levels over time on treatment was observed in the group of anemic participants (linear trend $p$-value: <0.001), whereas such levels did not substantially change in those who were not anemic at baseline. Curiously, 11 (36.6%) patients who were non-anemic at baseline developed anemia at day 60, from whom 8 (26.6% of the non-anemic group) were also anemic at day 180 of ATT (**Figure 3B**). Among the initially anemic patients, 83.85% were still anemic at day 60 and 63.35% persisted with anemia at day 180 of therapy (**Figure 3B**). A Sankey diagram was used to illustrate the dynamic change of anemia status over time on ATT (**Figure 3C**).

Hence, we observed that the vast majority of the participants who were anemic at the study baseline persisted with anemia until at least day 180 of therapy, whereas 19 (11.8%) individuals recovered from anemia at day 60 (early recovery), 36 (22.36%) recovered only by day 180 (late recovery), and 5 (3.1%) recovered at day 60 but were once again anemic at day 180 (transient recovery). The characteristics of these subpopulations are shown in the **Supplementary Table 2**. The dynamicity of hemoglobin levels in the different subgroups of anemic patients identified in the Sankey diagram is described in **Figure 3D**. Among the patients who had anemia at the baseline, with the exception of the transient recovery group, all exhibited a significant increase in hemoglobin levels over time of ATT ($p$-values < 0.05) (**Figure 3D**).

## Persistent Anemia Is Associated With Augmented Degree of Inflammatory Perturbation

Given that the majority of anemic patients persisted with anemia during the time of ATT regardless of the gradual increase in hemoglobin levels, we tested whether such condition was related to a chronic and unresolved inflammatory disturbance. To do so, we employed a mathematical maneuver named Molecular Degree of Perturbation (MDP), which has been used by our group and others to estimate the overall degree of inflammation and/or immune activation (18–20). In the present study, we included cells (from CBC), viral load, CD4 counts and biochemical parameters (creatinine, urea, total and direct bilirubin, albumin, alkaline phosphatase, uric acid, AST, GGT, ALT and total proteins) to create a score henceforth named Degree of Inflammatory Perturbation (DIP) (**Figure 4A**). We found that in general, anemia was associated with increased DIP values measured at both baseline (**Figure 4B**) and at day 180 of ATT (**Figure 4C**), with the highest levels being detected in the group of persistent anemia. Strikingly, the DIP score values exhibited strong inverse correlations with hemoglobin levels both at baseline ($r = -0.74$; $p < 0.001$) and at day 180 ($r = -61$; $p < 0.001$), highlighting that the degree of anemia and activation of inflammation are concurrent processes.

Additional analyses demonstrated that, as expected, patients who had an early recovery from anemia exhibited significantly higher baseline values for erythrocytes, Hb, hematocrit, neutrophils (**Supplementary Figure 1**) and albumin (**Supplementary Figure 2**) than those who did not recover. Patients who had a late recovery displayed significantly higher baseline values of Hb and hematocrit compared to those who persisted anemic (**Supplementary Figures 1**, **2**). The prospective comparisons have also identified discrepancies in cell counts and concentrations of biochemical parameters between the subgroups of patients based on recovery from anemia, which are summarized in **Supplementary Figures 1**, **2**.

The findings described above led us to hypothesize that the distinct profile of cell counts, and levels of biochemical parameters, measured at pre-ATT, is associated with persistent anemia. Thus, a stepwise binary multivariate logistic regression analysis was performed to test if biochemical parameters measured at pre-ATT (baseline) are able to predict recovery from anemia. Results demonstrated that increases in concentrations of albumin were directly associated with recovery from anemia (aOR: 2.67, 95% CI:1.05–6.75, $p = 0.04$) whereas increases in total proteins were directly associated with persistent anemia (aOR: 0.44, 95% CI: 0.24–0.78, $p = 0.01$) (**Figure 3E**). Similar trends in associations were observed when the major group of participants who recovered from anemia were further stratified in early and late recovery.

## Lower Concentrations of Hemoglobin at Pre-ATT Are Associated With Increased Risk of Unfavorable Treatment Outcome

In the longitudinal study cohort, 18 patients (9.4%) developed unfavorable outcomes (death attributed to TB: $n = 3$; death attributed to HIV: $n = 2$; ATT failure: $n = 1$; ATT loss to follow up abandonment: $n = 12$). The majority of the cases of unfavorable outcomes was composed by individuals who experienced persistent anemia (14 out of 18 participants, 77.8%) (**Figure 5A**). In fact, the median values of Hb levels gradually increased upon initiation of ATT in patients who were successfully treated (linear trend $p < 0.001$) but did substantially change in those who has unfavorable outcomes (**Figure 5B**). A hierarchical cluster analysis inputting average values of CBC (**Figure 5C**) and biochemical parameters (**Figure 5D**) demonstrated that there were differential trends in values between the study timepoints and the subgroups of favorable vs. unfavorable outcomes.

At study baseline, individuals who further developed unfavorable outcomes exhibited lower levels of Hb ($p = 0.052$), albumin ($p = 0.035$), uric acid ($p = 0.001$), urea ($p = 0.006$), and creatinine ($p = 0.008$) than those who were further successfully treated (**Supplementary Table 3**). A binomial logistic regression analysis was performed to test independent associations between the parameters analyzed and treatment outcome (**Figure 5E**). We found that increases in hemoglobin at pre-ATT were protective against unfavorable outcomes (aOR: 0.80, 95% CI: 0.64–0.99, $p = 0.04$) independent of the other factors (**Figure 5E**). These results highlight the importance of Hb as a prognostic marker in PLWH coinfected with TB.

## DISCUSSION

Anemia is a common complication associated with both TB and HIV, and it has been reported to occur in between 16 and 94% of TB patients (21–24); whereas in PLWH the prevalence ranges

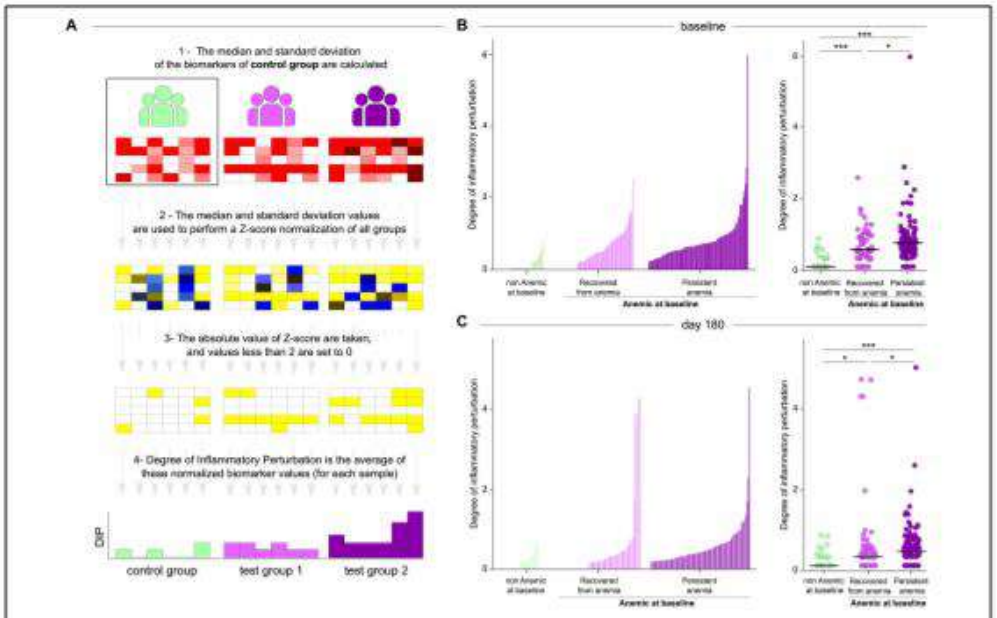


**FIGURE 4 |** Patients who persists with anemia exhibit substantially higher degree of inflammatory perturbation. **(A)** Degree of inflammatory perturbation (DIP) is based on Molecular Degree of Perturbation (16), but instead of using gene expression, we use biochemical and cellular markers. DIP was calculated was calculated using the median and standard deviation of the control group as a starting point. Then, the Z-score was calculated for all groups, a cut-off point was established and, finally, an average disturbance calculation was performed for each sample. This figure is adapted https://mdp.sysbio.tools/about. **(B,C) Left panels:** Histograms show the single sample degree of inflammatory perturbation (DIP) score values relative to the non-anemic at baseline group between patients recovered from anemia and with persistent anemia at baseline **(B)** and day 180 **(C)**. **Right panels:** Scatter plots of the summary data for each group are shown. DIP score values were compared between non-anemic at baseline (control group), recovered from anemia and persistent anemia at baseline **(B)** and day 180 **(C)**. Lines in the scatter plots represent median values. Data were compared using the Mann–Whitney *U* test. $^{*}P < 0.05$; $^{***}P < 0.0001$. Data are from 191 patients who had complete information on cell counts and biochemical measurements at all study timepoints.

from 39 to 71% (25–27). These observations were validated by the present study, which was focused on TB-HIV coinfection, and reported that 84.3% of the study participants were anemic at pre-ATT. In addition, our findings demonstrated that anemic patients exhibit higher inflammatory perturbation in the peripheral blood, which is sustained over the course of ATT in those who persisted with low Hb levels. Such condition is shown here to be closely associated with unfavorable outcomes. Early intervention focused on recovery from anemia could be a strategy to optimize the clinical management of PLWH with TB during ATT treatment.

In our cohort, anemic patients more frequently exhibited weight loss, lower $CD4^+$ T-cell counts and higher HIV viral loads than those who were not anemic. These observations reinforce the idea that anemia infers more advanced stage of disease progression. Our results are in agreement with other previously published findings which demonstrated that lower body mass index (27–29), higher HIV viral loads (28), and lower $CD4^+$ T-cell counts are all associated with higher prevalence of anemia (25, 26). As previously reported by us in a different cohort of TB patients, most of the anemia cases are attributed to chronic inflammation rather than to iron deficiency (10). A recent systematic review demonstrated that anemia is related to an increased risk of all-cause mortality and incident TB among PLWH, regardless of the anemia type (30). The magnitude of such effect is thought to be proportional to severity of anemia. Finally, iron supplementation in such cases is still a matter of debate, with inconsistent results reported by clinical trials. The probable determinants of anemia in the context of HIV/AIDS and TB are likely multifactorial and involve several factors including nutritional status (31), chronic inflammation and antibody-mediated erythrophagocytosis (32). Our results demonstrated that anemic patients also exhibit lower counts of other cell types, suggesting that a global effect on the bone marrow may be occurring. Additional mechanistic studies as well as large randomized clinical trials testing different approaches to reduce anemia are necessary to improve our knowledge regarding the molecular targets and to help delineate the best therapeutic schemes.

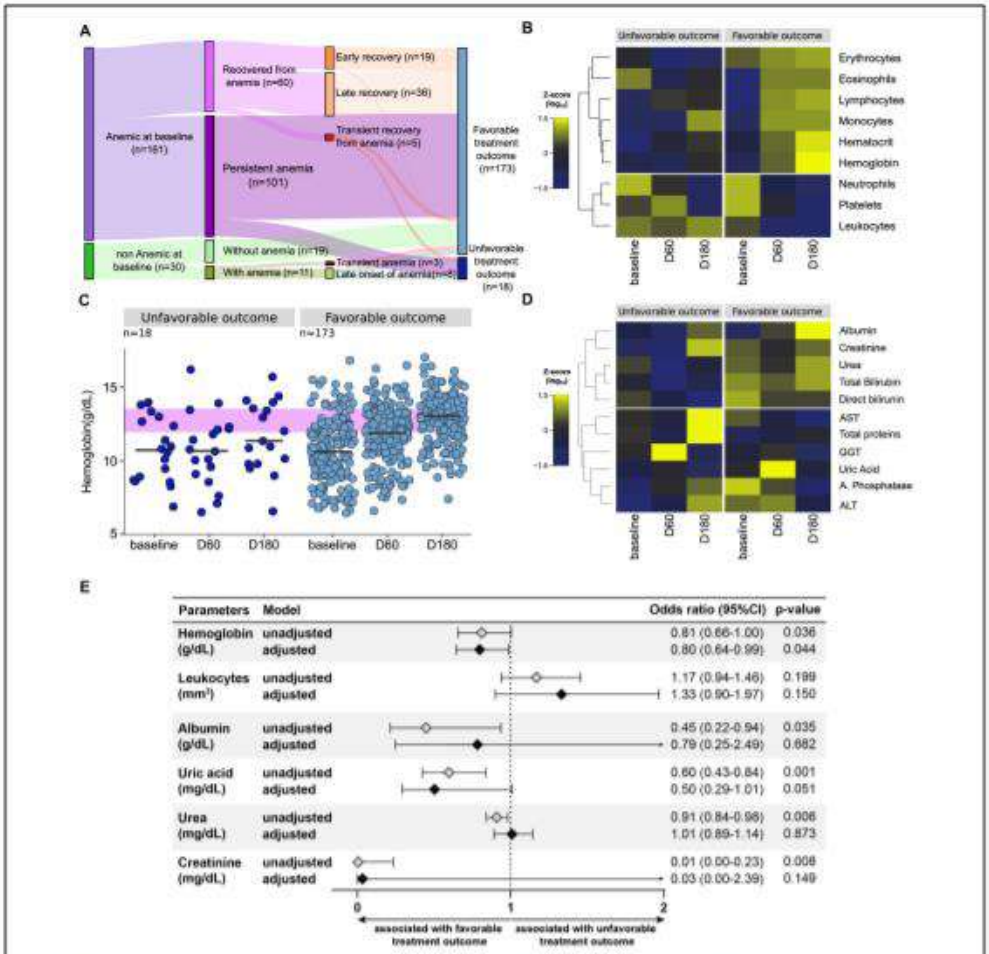


**FIGURE 5 |** Unfavorable outcome occurs mainly in patients with anemia. **(A)** Among all 191 patients who had complete laboratory data from all the study timepoints, only 18 had unfavorable treatment outcomes (death, failure or loss to follow up), whereas 173 were successfully treated (cure). **(B)** 14 of the 18 patients had anemia at baseline and 14 also had anemia at the end of the treatment. **(C,D)** A Heatmap was designed to depict the overall pattern of cells and biochemical markers at all timepoints in favorable and unfavorable outcome of anti-tubercular treatment. A two-way hierarchical cluster analysis (Ward's method) was performed. Dendrograms represent Euclidean distance. Expression scale represents Z-score normalization from the median at each timepoint and group. It is possible to observe that patients who had an unfavorable outcome have a profile opposite to that of patients with a favorable outcome. In **(C)** we can identify that patients with a favorable outcome had an increase in most of the blood count parameters over time, and also a decrease in neutrophils, platelets and leukocytes, returning to normal levels of these components. In patients with an unfavorable outcome, the parameters remain more similar to the baseline, with the exception of neutrophils and platelets, which also decrease. In **(D)** the biochemical markers of both groups do not seem to change much over time, but they visibly present different profiles when comparing favorable and unfavorable. Patients with a favorable outcome have higher levels of albumin, creatinine, urea and bilirubin throughout the treatment. **(E)** Logistic binomial regression model to significant biochemical parameters was used to test independent associations between biochemical and clinical parameters at baseline and treatment outcome after 180 days. The treatment outcome unfavorable (failure, death or loss to follow up) was used as reference to test associations. Only parameters which remained with $p \leq 0.2$ in univariate analysis model were inputted in the adjusted model. (95%CI, 95% confidence interval). Associations reported in **(E)** are for increases in 1 unit in plasma concentrations of indicated markers.

With regard to the biochemical parameters, our results indicate that low Hb levels accompanied higher values of ALT, AST and GGT, and lower concentrations of albumin. Such findings are similar to those previously published by our group in another cohort of TB patients and reinforce the idea that anemia is related to a distinct biochemical profile and linked to inflammation (10). In our study, the prevalence of hepatitis B or C in anemic patients (9.48%) was very similar to non-anemic patients (10.8%), suggesting that although this comorbidity is present, it is probably not the main factor driving the differences in the levels of liver transaminases. At the end of ATT, none of these biochemical markers demonstrated association with the clinical outcomes. Moreover, out of the 25 patients who had viral hepatitis, 20 (80%) had a favorable outcome, highlighting the low influence of this coinfection on the effectiveness of the treatment.

The results reported here demonstrated that among the study participants with anemia at the baseline, the vast majority persisted with low Hb levels until day 180 of ATT. In addition, within the group of patients who recovered from anemia under the course of ATT, most exhibited a late recovery, occurring between day 60 and day 180 of therapy. Other investigations have reported that anemia frequently has a benign course in TB patients without HIV coinfection, with complete recovery in 64.5% of patients undertaking ATT (5). The discrepancies between the findings presented here and this previous study can be likely explained but the fact that our cohort was composed by PLWH, which may have an additional detrimental effect on inflammation and its related anemia compared to the setting of TB in the absence of HIV. In our study, patients who recovered from anemia presented with relatively higher values of Hb and hematocrit at baseline compared to those who persisted anemic. Individuals who had early recovery from anemia also exhibited higher neutrophil counts and albumin levels. The multivariable logistic regression analysis performed here revealed that albumin was independently associated with recovery from anemia. This observation again reinforces the strong association of albumin levels with recovery from anemia. These findings suggest that the degree of anemia is associated with changes in concentrations of cells and biochemical markers and that more severe anemia before ATT indicates higher odds of persistent anemia for up to 6 months on therapy.

To describe the overall biochemical and cellular disturbances related to anemia in the study population, we used an adaption of the molecular degree of perturbation (18) to estimate the degree of inflammatory perturbation in PLWH and with TB according to anemia status. Our findings indicate that there are important discrepancies in the DIP values between patients with persistent anemia compared to those who recovered during ATT. Individuals who persisted with anemia in the course of ATT exhibited higher DIP values already at pre-ATT, and such profile was sustained ay day 180 of therapy. These findings argue that persistent anemia directly associates with increased disturbances in the biochemical and cellular profiles, which were sustained over the course of ATT. The inverse correlations between DIP values and Hb levels both at pre-ATT and at day 180 indicate that the degree of inflammatory perturbation is proportional to the severity of anemia. Whether anemia sets the stage for persistent inflammation or is just a hallmark of chronic, unfettered, dysregulation of inflammatory responses warrants further investigation. This association between low Hb levels and risk of inflammatory disturbance has been described in PLHW who experience IRIS (33, 34) and also in patients with HIV/TB coinfection (35).

Another important contribution of our study was to test whether lower concentrations of Hb at pre-ATT could be used to predict risk of unfavorable outcomes. We found that the majority of patients who had unfavorable outcomes experienced persistent anemia during the course of ATT. A previous study described that anemia is associated with a 2–3 times increase in the risk of death, recurrence of TB or ATT failure in PLWH/TB (7). Corroborating with these findings, the results from a logistic regression analysis presented here demonstrated that increases in Hb concentrations at pre-ATT play a protective role against unfavorable outcomes independent of other confounding factors.

Our study has some limitations, such as relatively small number of non-anemic participants and of unfavorable outcomes, although the latter is within the expected range in the outpatient clinic from our institution. The small sample size favors a potential bias, as well as the fact that we do not have data on these same patients prior to TB and/or HIV infection, so that we cannot determine whether the anemia was pre-existing or in fact is a consequence of the co-infection. The study population also included few IRIS cases, which precluded additional exploratory analyses. Regardless of such limitations, our study adds to the current knowledge in the field by demonstrating the relevance of persistent anemia in driving inflammatory disturbances related to worse prognosis of PLWH coinfected with TB. The fact that most patients with an unfavorable outcome persisted with anemia and with a high degree of inflammatory perturbation suggests that early intervention focused on recovery from anemia could be a strategy to optimize the clinical management of PLWH with TB during ATT treatment.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Instituto Nacional de Infectologia Evandro Chagas (INI). The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

FD, CS, FS'A, and VR contributed to conception and design of the study. FD also collected the data and organized the database. MA-P and MA performed the statistical analysis

and data visualization. FD, MA-P, and BA wrote the first draft of the manuscript. VR and BA supervised the project execution. All authors contributed to the article and approved the submitted version.

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## ACKNOWLEDGMENTS

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.588405/full#supplementary-material

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**Conflict of Interest:** 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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## *Supplementary Material*

**Supplementary Table 1. Concentrations of cells and biomarkers in peripheral blood, according to anemia status at study baseline (n=256).**

| Count Blood Cells and Hemoglobin | | | | Biochemical markers | | | |
|---|---|---|---|---|---|---|---|
| | Median (IQR1- IQR3) | | | | Median (IQR1- IQR3) | | |
| | **Anemic (n= 219)** | **non-Anemic (n= 37)** | **p-value** | | **Anemic (n= 219)** | **non-Anemic (n= 37)** | **p-value** |
| Basophils (%) | 0(0-0) | 0(0-0) | - | Albumin (g/dL) | 2.6(2.1-3.1) | 3.6(3.3-3.8) | **< 0.01** |
| Eosinophils (%) | 1(0-2) | 2(1-4) | **0.014** | A. Phosphatase (IU/L) | 125(97.5-236) | 130(97-156) | 0.306 |
| Erythrocytes (million/mm$^3$) | 3.6(3.1-4.1) | 4.6(4.1-4.8) | **< 0.01** | ALT (IU/L) | 39(28-58.3) | 30.4(24-38.5) | **< 0.01** |
| Hematocrit (%) | 30.4(26.8-34.5) | 39.8(35.8-42.4) | **< 0.01** | AST (IU/L) | 40.5(29-61) | 26(18.8-38.3) | **< 0.01** |
| Hemoglobin (g/dL) | 10.6(8.9-11.5) | 13.9(13.3-14.8) | **< 0.01** | Creatinine (mg/dL) | 0.87(0.72-1.1) | 0.83(0.74-0.94) | 0.42 |
| Leukocytes(mm$^3$) | 6550(4400-8770) | 6630(5170-8570) | 0.41 | Dir. Bilirubin (mg/dL) | 0.15(0.1-0.26) | 0.12(0.07-0.21) | **0.09** |
| Lymphocytes (%) | 18(12-26) | 25(16-33) | **< 0.01** | GGT (U/L) | 132(64.5-323) | 64(44-173) | **< 0.01** |
| Monocytes (%) | 7(4-10) | 8(6-10) | 0.20 | Tot. bilirubin | 0.36(0.25-0.59) | 0.43(0.29-0.62) | 0.52 |

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|---|---|---|---|---|---|---|---|
| | | | | (mg/dL) | | | |
| Neutrophils (%) | 71(60.5-80) | 64(56-72) | **0.016** | Tot. proteins (mg/dL) | 8.2(7.7-9.1) | 8(7.5-8.6) | 0.44 |
| Platelets (mil/mm$^3$) | 294(216-379) | 292(232-326) | 0.75 | Urea (mg/dL) | 25(17-34) | 22(18.5-25.6) | 0.54 |
| | | | | Uric acid (mg/dL) | 5.1(3.4-9.1) | 4.4(3.5-5.75) | 0.52 |

**Table note:**

Bold font indicates statistical significance.

[1]To define anemia according to baseline (D0) hemoglobin, the cut-off point of 12g/dL for women and 13.5g/dL for men was used. Abbreviations: IQR: Interquartile range; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; GGT: Gamma-Glutamyl Transferase;

Data are shown as median and interquartile (IQR) range or frequency (percentage). Data were compared between the clinical groups using the Mann-Whitney *U* test (continuous variables).

Clinical laboratory assessment was measured by cell blood count and specific biochemical tests as described in Methods.

**Supplementary Table 2. Characteristics of the study population and concentrations of cells and biomarkers in peripheral blood, according recovered from anemia.**

| Characteristics | Total recovery (n=55) | Early recovery (n=19) | Late recovery (n=36) | Persistent anemia (n=99) | p-value < 0.05[2] |
|---|---|---|---|---|---|
| Sex. no. (% male) | 44 (78.6) | 15 (71.4) | 29 (82.9) | 70 (70.7) | ns |
| Age (years). no. (IQR) | 37 (28-44) | 34 (26-41) | 39 (29-46) | 38 (31-47) | ns |
| Smoking. no. (%) | 30 (53.6) | 10 (47.6) | 20 (57.1) | 47 (48) | ns |
| Use of illicit drugs. no. (%) | 11 (19.6) | 2 (9.5) | 9 (25.7) | 27 (27.3) | ns |
| Alcohol abuse[1], no. (%) | 20 (35.7) | 7 (33.3) | 13 (37.1) | 37 (37.8) | ns |
| Weight loss (>10%) no. (%) | 47 (83.9) | 18 (85.7) | 29 (82.9) | 79 (79.8) | ns |
| Hypertension. no. (%) | 4 (7.4) | 1 (5) | 3 (8.8) | 9 (9.1) | ns |
| Diabetes. no. (%) | 5 (8.9) | 2 (9.5) | 3 (8.6) | 14 (14.1) | ns |
| Previous tuberculosis, no. (%) | 10 (17.9) | 5 (23.8) | 5 (14.3) | 19 (19.2) | ns |
| Complete TB treatment previous, no. (% of prior TB) | 7 (70) | 4 (80) | 3 (60) | 14 (73.7) | ns |
| HAART use before TB, no. (%) | 20 (35.7) | 8 (38.1) | 12 (34.3) | 31 (31.3) | ns |
| HAART during TB treatment, no. (%) | 55 (98.2) | 20 (95.2) | 35 (100) | 92 (92.9) | ns |
| IRIS upon HAART initiation, no. (%) | 3 (5.4) | 1 (4.8) | 2 (5.7) | 5 (5.1) | ns |
| **Count Blood Cells** | | | | | |

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|---|---|---|---|---|---|
| Erythrocytes (million/mm$^3$) | 3.96 (3.48-4.23) | 4.05 (3.65-4.28) | 3.82 (3.48-4.22) | 3.57 (3.01-4.12) | **a, b, c** |
| Hemoglobin (g/dL) | 10.8 (9.6-11.95) | 11.8 (9.8-12.3) | 10.6 (9.4-11.7) | 9.4 (8.1-11.1) | **a, b, c** |
| Hematocrit (%) | 32.6 (28.9-35.9) | 34.1 (30.3-36.3) | 31.9 (28.9-35.4) | 28.9 (24.7-33.6) | **a, b, c** |
| Leukocytes(mm$^3$) | 6.91 (4.65-8.89) | 7.22 (6.27-8.88) | 6.03 (4.64-9.53) | 6.1 (4.23-8.52) | **b** |
| Eosinophils (%) | 0 (0-2) | 0 (0-1) | 1 (0-3) | 1 (0-2) | **b** |
| Basophils (%) | 0 (0-0) | 0 (0-0) | 0 (0-0) | 0 (0-0) | ns |
| Neutrophils (%) | 74.5 (61.5-81) | 76 (73-80) | 64 (58-82) | 70 (61-80) | **b** |
| Lymphocytes (%) | 17.5 (12-26.5) | 15 (12-18) | 24 (11-27) | 18 (12-26) | ns |
| Monocytes (%) | 8 (3.5-11) | 7 (4-8) | 8 (3-12) | 6 (4-10) | ns |
| Platelets (mil/mm$^3$) | 3.01 (2.42-3.69) | 3.07 (2.4-3.54) | 3.01 (2.47-4.02) | 2.9 (2.08-3.78) | ns |
| **Biochemical markers** | | | | | |
| Tot. proteins (mg/dL) | 8.1 (7.4-8.7) | 7.9 (7.35-8.25) | 8.1 (7.4-8.8) | 8.4 (7.6-9.25) | **a** |
| Albumin (g/dL) | 2.8 (2.2-3.2) | 3.1 (2.65-3.2) | 2.69 (2.03-3.2) | 2.6 (2-3) | **b** |
| ALT (IU/L) | 41 (29-65) | 44 (27.5-65.5) | 40 (29-65) | 40 (30.5-61.5) | ns |
| AST (IU/L) | 42 (32-63) | 49.25 (38.5-62.09) | 39.3 (31-78) | 39 (29.32-61) | ns |
| A.Phosphatase (IU/L) | 132 (91-256.34) | 171.26 (87-242.64) | 130 (98.3-315) | 148 (96.5-244) | ns |
| GGT (U/L) | 117 (70-377) | 114 (70-253) | 117 (87-420) | 170 (74-369) | ns |

| | | | | | |
|---|---|---|---|---|---|
| Tot. bilirubin (mg/dL) | 0.39 (0.31-0.58) | 0.45 (0.27-0.58) | 0.4 (0.3-0.6) | 0.37 (0.25-0.6) | ns |
| Dir. Bilirubin (mg/dL) | 0.16 (0.11-0.27) | 0.19 (0.11-0.26) | 0.2 (0.1-0.3) | 0.18 (0.12-0.29) | ns |
| Uric acid (mg/dL) | 4.2 (3.7-8.3) | 4.2 (3.7-5.8) | 4.4 (3.7-9.2) | 5.7 (3.6-9.6) | ns |
| Urea (mg/dL) | 24.07 (18.09-30.88) | 20 (17-29) | 25 (20-33) | 27 (18-35) | ns |
| Creatinine (mg/dL) | 0.86 (0.74-1.11) | 0.86 (0.78-1.06) | 0.9 (0.7-1.2) | 0.85 (0.71-1.09) | ns |

**Table note:**

Bold font indicates statistical significance. To define anemia according to baseline (D0) hemoglobin, the cut-off point of 12 g/dL for women and 13.5 g/dL for men was used. Data are shown as median and interquartile (IQR) range or frequency (percentage). Data were compared between the clinical groups using the Mann-Whitney *U* test (continuous variables) or the Pearson's $\chi$ 2 test (for data on frequency).

[1]The physicians also collected information about current use of illicit drugs and alcohol (Y/ N to each) during the baseline interview. Potential problematic alcohol use was assessed with the CAGE questionnaire, with scores of 2 or greater indicating clinically significant alcohol problems.

[2] Significance: ns: not significant in all comparisons; a (significant in total recovery versus persistent anemia), b (significant in early recovery versus persistent anemia), c (significant in late recovery versus persistent anemia).

Abbreviations: IQR: Interquartile Range; IRIS: Immune reconstitution Inflammatory Syndrome; TB: Tuberculosis; HAART: Highly Active Antiretroviral Therapy;

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**Supplementary Table 3. Characteristics of the study population and concentrations of cells and biomarkers in peripheral blood, according to treatment outcome.**

| Characteristics | Unfavorable treatment outcome (n=18) | Favorable treatment outcome (n=172) | p-value |
|---|---|---|---|
| Sex. no. (% male) | 10 (55.6) | 129 (75.0) | 0.094 |
| Age (years). no. (IQR) | 37 (26-44) | 38 (31-46) | 0.546 |
| Smoking. no. (%) | 9 (50.0) | 83 (49.4) | 1.000 |
| Use of illicit drugs. no. (%) | 7 (38.9) | 41 (24.1) | 0.253 |
| Alcohol abuse[1], no. (%) | 6 (33.3) | 57 (33.5) | 1.000 |
| Weight loss (>10%) no. (%) | 13 (72.2) | 127 (74.3) | 0.785 |
| Hypertension. no. (%) | 0 (0.0) | 16 (9.5) | 0.369 |
| Diabetes. no. (%) | 1 (5.6) | 21 (12.2) | 0.700 |
| Previous tuberculosis, no. (%) | 4 (22.2) | 35 (20.3) | 0.776 |
| Complete TB treatment previous, no. (% of prior TB) | 1 (25.0) | 29 (82.9) | 0.032 |
| HAART use before TB, no. (%) | 8 (44.4) | 63 (36.6) | 0.610 |
| HAART during TB treatment, no. (%) | 13 (72.2) | 164 (95.3) | **0.003** |

| | | | |
|---|---|---|---|
| IRIS upon HAART initiation, no. (%) | 1 (5.6) | 8 (4.7) | 0.600 |
| **Count Blood Cells** | | | |
| Erythrocytes (million/mm$^3$) | 3.74 (3.17-4.32) | 3.87 (3.16-4.32) | 0.898 |
| Hemoglobin (g/dL) | 10.6 (8.9-12.15) | 10.8 (8.6-12.9) | **0.052** |
| Hematocrit (%) | 28.75 (26-36.2) | 32.1 (27.1-35.9) | 0.316 |
| Leukocytes(mm$^3$) | 6.46 (4.31-8.27) | 6.56 (4.65-8.8) | 0.199 |
| Eosinophils (%) | 3 (1-6) | 1 (0-2) | **0.013** |
| Basophils (%) | 0 (0-0) | 0 (0-0) | 0.573 |
| Neutrophils (%) | 69 (65-74) | 70 (60-80) | 0.986 |
| Lymphocytes (%) | 21 (10-28) | 19 (12-27) | 0.914 |
| Monocytes (%) | 7 (5-9) | 7 (4-10) | 0.883 |
| Platelets (mil/mm$^3$) | 3 (2-4) | 3 (2-4) | 0.901 |
| **Biochemical markers** | | | |
| Tot. proteins (mg/dL) | 8 (6-9) | 8 (8-9) | 0.521 |
| Albumin (g/dL) | 3 (2-4) | 3 (2-3) | **0.035** |
| ALT (IU/L) | 35 (30-61) | 39 (28-56) | 0.697 |

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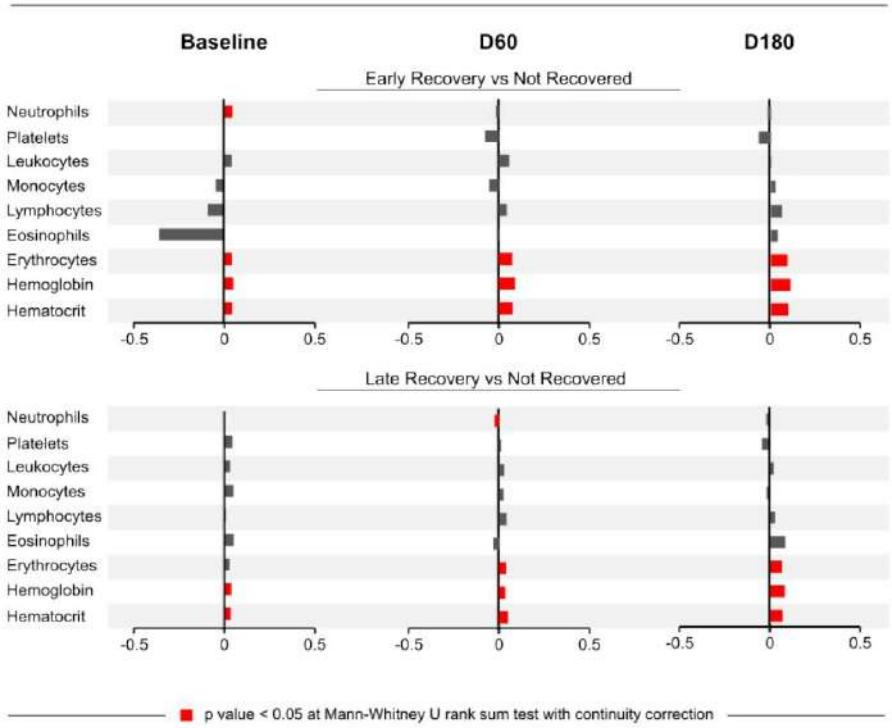
|---|---|---|---|
| AST (IU/L) | 39 (31-55) | 39 (28-60) | 0.908 |
| A.Phosphatase (IU/L) | 121 (90-173) | 135 (97-240) | 0.387 |
| GGT (U/L) | 161 (81-363) | 132 (63-341) | 0.536 |
| Tot. bilirubin (mg/dL) | 0 (0-1) | 0 (0-1) | 0.275 |
| Dir. Bilirubin (mg/dL) | 0 (0-0) | 0 (0-0) | 0.384 |
| Uric acid (mg/dL) | 5 (4-9) | 5 (3-8) | **0.001** |
| Urea (mg/dL) | 24 (18-26) | 25 (18-35) | **0.006** |
| Creatinine (mg/dL) | 1 (1-1) | 1 (1-1) | **0.008** |

**Table note:**

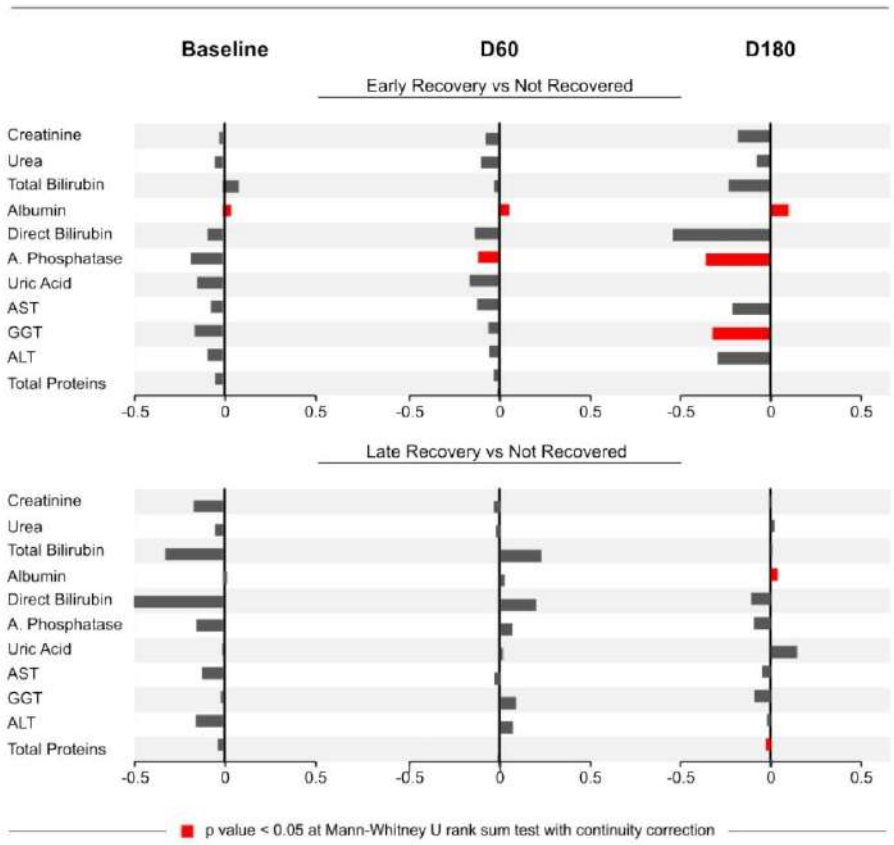
Favorable outcome: Cure; Unfavorable outcome: Death, loss to follow up and treatment failure

Abbreviations: IQR: Interquartile Range; TB: Tuberculosis; HAART: Highly Active Antiretroviral Therapy; ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; GGT: Gamma-Glutamyl Transferase; IRIS: Immune Reconstitution Inflammatory Syndrome Data are shown as median and interquartile (IQR) range or frequency (percentage). Data were compared between the clinical groups using the Mann-Whitney *U* test (continuous variables) or the Pearson's $\chi$ 2 test (for data on frequency). Data are from 191 patients who had complete information on cell counts and biochemical measurements at all study timepoints.




**Supplementary Figure 1. Differential change in complete cell blood counts between anemic and non-anemic patients.** Patients that were anemic at baseline (n=161) were divided according to recovery in Not Recovered, Early Recovery (D60), Late Recovery (D180) and Anemic at Baseline (were not anemic only at D60). A $\log_{10}$ of fold-change was calculated and statistical analyses were performed using the Mann-Whitney *U* adjusted test. Significative differences ($p < 0.05$) between groups for each time point are highlighted in red bars. Data are from 191 patients who had complete information on cell counts and biochemical measurements at all study timepoints.

Supplementary Material




**Supplementary Figure 2. Differential change in biochemical markers between subgroups of recovery in patients that were anemic at baseline.** Patients that were anemic at baseline (n=161) were divided according to recovery in Not Recovered, Early Recovery (D60), Late Recovery (D180) and Anemic at Baseline (were not anemic only at D60). A $\log_{10}$ of fold-change was calculated and statistical analyses were performed using the Mann-Whitney *U* adjusted test. Significative differences ($p < 0.05$) between groups for each time point are highlighted in red bars. Data are from 191 patients who had complete information on cell counts and biochemical measurements at all study timepoints.

## 5.6 Manuscrito VI

### Título

"Effect of anemia on anti-tuberculosis treatment outcome in persons with pulmonary tuberculosis: a multi-center prospective cohort study"

### Objetivo

Esse trabalho teve como objetivo avaliar o efeito da anemia dos desfechos desfavoráveis de TAT em uma coorte de 786 pacientes TB (com e sem HIV) acompanhados por 24 meses.

### Resumo de resultados

Através das análises de Hb, verificamos que 56% dos pacientes estavam anêmicos pré-ATT. A frequência de sintomas e de desfecho desfavorável aumentou de acordo com o aumento da gravidade da anemia, demonstrando uma possível associação entre os níveis de Hb e a apresentação clínica, assim como entre a Hb e os desfechos desfavoráveis de tratamento. A análise de regressão logística multivariada demonstrou que a anemia moderada/grave estava associada a um maior risco de óbito em pacientes TB de fatores como sexo, idade, gênero, IMC e disglicemia. Esse trabalho foi importante para reforçar que, embora mais evidente em PVHIV, a associação entre anemia e piores prognósticos parece estar intrinsecamente associada a co-infecção com TB.

### Status do manuscrito

Este trabalho foi submetido ao periódico internacional ***Clinical Infectious Diseases*** (Fator de Impacto JCR 2021 = 20,999).

# Effect of anemia on anti-tuberculosis treatment outcome in persons with pulmonary tuberculosis: a multi-center prospective cohort study

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**40-word summary of the article's main point**: TB patients with moderate/severe anemia have a high risk of death during anti-TB treatment independent of age, sex, BMI, HIV and glycemic status. Such condition must be closely monitored to minimize the occurrence of unfavorable anti-TB treatment outcomes.

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## ABSTRACT

**Background:** Tuberculosis (TB) remains a major plague of humanity. Persons with TB (PWTB) commonly present with anemia, which has been linked to immune activation and disease progression. Here, we tested whether the severity of anemia in PWTB prior to anti-TB treatment (ATT) is a risk factor of unfavorable outcomes.

**Methods:** Patients ≥ 18 years old with culture-confirmed drug-susceptible pulmonary TB enrolled between 2015 and 2019 in a multi-center Brazilian cohort were followed for up to 24 months and classified according to anemia severity (mild, moderate and severe), based on hemoglobin levels and according to the World Health Organization guidelines. A multinomial logistic regression model was employed to argue whether anemia was associated with death, failure, loss to follow-up, regimen modification or relapse (in contrast with cure or complete treatment).

**Results:** Among 786 participants who met inclusion criteria, 441 (56%) were anemic at baseline. Use of tobacco and diabetes or prediabetes status were more frequently observed in participants who had anemia. HIV exposure increased in accordance with increasing severity of anemia. Patients with moderate or severe anemia were more symptomatic and had higher frequencies of unfavorable outcomes compared to the other groups. The logistic regression demonstrated that moderate/severe anemia (adjusted OR [aOR]: 7.80, 95%CI:1.34-45.4, p=0.022) was associated with death independent of sex, age, BMI, HIV status and glycemic status.

**Conclusion:** Severity of anemia prior to ATT commencement is a risk factor of death. Such condition must be closely monitored to minimize the occurrence of ATT unfavorable outcomes.

## INTRODUCTION

Tuberculosis (TB) remains a major global health problem, with 1.3 million TB-associated deaths yearly, and around 10 million cases worldwide in 2020 (1). The clinical management of persons with TB (PWTB) depends on understanding the risk factors that may be associated with disease progression and poor treatment outcomes, such as HIV coinfection, consumption habits, diabetes mellitus (DM), and anemia (2–4).

According to the World Health Organization (WHO), anemia is defined as a decrease in hemoglobin (Hb) values below well-established cut-offs (<13 g/dL for men; and <12 g/dL for women) and is commonly associated with inflammatory and/or infectious conditions (5). This disorder is ordinarily identified in PWTB, in about 61.53% of the cases, and is frequently described as a marker of greater disease severity and/or more advanced disease (6).

The risk of developing active TB among anemic patients is described to be 3.56 times greater than in non-anemic patients; such risk seems to increase even more according to the severity of anemia (7). Anemic patients also more often develop severe clinical forms of TB, such as meningeal and disseminated TB (8). Understanding the effects of anemia in the context of anti-TB therapy (ATT) can provide insight for more focused, optimized, clinical management to improve outcomes.

In previous studies, we have demonstrated that persons affected by TB-HIV co-infection exhibit an increased dysregulation of immune activation (9). Furthermore, TB-HIV individuals who persist with low levels of Hb during the course of ATT have augmented risk of experiencing unfavorable outcomes, such as treatment failure, loss-to-follow-up, and death (3). Whether the severity of anemia assessed at pre-ATT differentially impacts the treatment outcomes regardless of HIV co-infection is not fully understood. The present study was aimed at answering this question in a multi-center prospective cohort that has been shown to be representative of the PWTB from the Brazilian National Tuberculosis Program registry (10). The findings provide the basis to support implementation of decision-making strategies to systematically screen for anemia in all the newly diagnosed TB cases and to close monitor those with severe anemia to minimize risk of unfavorable ATT outcomes.

## METHODS

### Ethics Statement

The study was conducted according to the principles of the Declaration of Helsinki. We used the cohort provided by the Regional Prospective Observational Research in Tuberculosis (RePORT-Brazil). The study was approved by the Institutional Review Boards at all enrollment sites (CAAE: 25102412.3.1001.5262) and at Vanderbilt; all participants provided written informed consent before inclusion. Participation in RePORT-Brazil was voluntary, and written informed consent was obtained from all participants.

### Study Design

This was a multi-center prospective observational study with data from RePORT-Brazil cohort (10). Study data were collected between June 2015 and June 2019. All participants in RePORT-Brazil cohort were at least 18 years old, with new or recurrent pulmonary TB, and had culture-positive sputum. For this study, only confirmed drug-susceptible patients were included. Epidemiological information was collected using standardized and validated clinical research forms during study visits at baseline, during and at the end of treatment, and up to 24 months after enrollment. Some of the collected variables are sex, age, self-reported race, weight, height, education level, use of alcohol, illicit drugs or tobacco, presence of comorbidities, and HIV status. Additionally, Hb values, radiographic evaluation of chest X-rays, drug susceptibility testing for anti-TB drugs, and CD4 counts (if HIV positive) were performed. In RePORT-Brazil, the treatment outcome was recorded at the last study visit (24 months after the initiation of treatment). Outcome definitions are described below.

Dysglycemia was defined according to baseline HbA1c, following American Diabetes Association (ADA) guidelines (11). Individuals were classified as having DM (HbA1c≥6.5%), prediabetes (HbA1c=5.7-6.4%) or normoglycemia (HbA1c<5.7%). In this study, participants with HbA1c≥5.7% were classified as having dysglycemia.

### Anemia definition

To define anemia, we used the WHO guideline criteria (5). Anemia was defined as levels of Hb below 13 g/dL for men or <12 g/dL for women. Mild anemia was defined

as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate anemia was defined as Hb>8 g/dL and ≤10 g/dL for both sexes. Severe anemia was defined as Hb<8g/dL for both sexes (5).

**Outcome definition**

A favorable treatment outcome was defined as cure or completed treatment. An unfavorable outcome was defined as treatment failure, lost to follow-up, recurrence, or death during treatment. The definitions for clinical and bacteriological cure, failure, lost to follow-up, recurrence, and death corresponded with the recently updated WHO guideline (12). The definitions in details of treatment outcomes for RePORT-Brazil cohort were previously described by our group (13) and reported in **Supplementary Table 1**.

**Statistical Analysis**

The median and interquartile range (IQR) were reported as measures of central tendency and dispersion respectively. Continuous variables were compared between groups according to TB and anemia grade using the Mann-Whitney *U* (between 2 groups) or Kruskal Wallis test (>2 groups). Categorical variables were reported as absolute values or relative frequencies (%) and compared using the Fisher's exact test (between 2 groups) or Pearson's chi-square test (more than 2 groups), when appropriate as indicates in the Results.

A multinomial logistic regression model (stepwise method) using all variables was performed to assess the odds ratio (OR) and 95% confidence intervals (CIs) of the associations with clinical and epidemiological characteristics and unfavorable outcomes. Only the results on anemia severity were reported, when adjusted in the logistic regression model. A p-value < 0.05 was considered statistically significant. Statistical analyses were performed using R language (version 4.5.1), with the following packages: compareGroups, nnet, stepAIC and ggplot2.

## RESULTS

### Characteristics of the study participants

A total of 1187 PWTB were enrolled into the RePORT-Brazil cohort. For this study, 401 individuals were removed from the analyses due to first line drug resistance, lack in Hb levels or outcome information, remaining 786 TB cases (details depicted in **Supplementary Figure 1)**. Of those, 441 (56%) had anemia. Anemic patients were more frequently of non-white race (anemic: 83%; non-anemic: 71.3%, p<0.001) and had higher frequency of HIV infection (anemic: 23%; non-anemic: 7.19%, p=0.003) and of dysglycemia (anemic: 65.2%; non-anemic: 54.7%, p=0.030). Anemia was also associated with increased proportion of tobacco use (anemic: 49.7%; non-anemic: 42.3%, p=0.048) compared to non-anemic patients (**Supplementary Table 2**).

Following stratification of the study participants according to anemia severity, we observed that 42% individuals had mild (n=333), 10% had moderate (n=80), and 4% had severe (n=28) anemia (**Figure 1a).** Anemia severity was shown to be independent of consumption habits (**Figure 1b**). In contrast, increased anemia severity was associated with a lower BMI value (p<0.001) (**Figure1c**) and a higher frequency of HIV infection (p<0.001) (**Figure 1d**). Furthermore, such subgroups of anemic patients also more regularly presented with TB-related symptoms, such as fever (p<0.001), weight loss (p=0.003) and fatigue (p=0.002). Nevertheless, the frequency of cough decreased according to anemia severity (p=0.015) (**Figure 1e**, **Table 1**).

### Determinants of TB Treatment Outcomes

Over the follow-up period, 592 (75.3%) patients had a favorable outcome whereas 194 (24.7%) experienced an unfavorable outcome. The frequency of unfavorable outcomes increased proportional to augmenting severity of anemia (p <0.001) (**Figure 2a**). Such trend was apparently driven by death, which substantially increased following the severity of anemia (p <0.001) (**Figure 2b**).

Patients with unfavorable outcomes more frequently were anemic (favorable: 51.9%; unfavorable: 69.1%, p<0.001), reported tobacco (favorable: 44.3%; unfavorable: 53.1%, p=0.040), alcohol (favorable: 79.1%; unfavorable: 89.2%, p=0.002), and illicit drug use (favorable: 23.5%; unfavorable: 35.2%, p=0.006) (**Table 2**). Patients who developed unfavorable outcomes also had higher frequency of HIV infection

(favorable:12.9%; unfavorable: 27.1%, p<0.001) and lower frequency of cavities on chest X-ray (favorable: 52.7%; unfavorable: 44.0%, p=0.003) **(Table 2)**.

Next, we performed analyses to assess the association between occurrence of anemia (or its different severity statuses) and each type of unfavorable outcome (treatment failure, death, LTFU and recurrence), using cure as the reference outcome. Due to the low number of participants in severe anemia group, moderate and severe anemia cases were concatenated in a single category. The multinomial logistic regression analysis demonstrated that moderate/severe anemia (adjusted OR [aOR]: 7.80, 95%CI:1.34-45.4, p=0.022) was associated with death independent on other confounding factors used in the final adjusted model, which included sex, age, BMI, HIV status and dysglycemia status **(Figure 3)**. However, no associations between anemia and other types of unfavorable outcomes were observed, arguing that the impact of anemia severity is more specifically focused on mortality.

## DISCUSSION

Our study of persons with pulmonary TB from a Brazilian multi-center prospective cohort revealed a high prevalence of anemia. Indeed, this condition is frequently diagnosed in PWTB, with reported frequencies between 32% and 86% (14). In our study, 56% of patients were anemic and 14% had moderate or severe anemia. Importantly, most of those participants with moderate/severe anemia were living with HIV. Previous studies have reported diminished Hb values in persons with TB-HIV co-infection compared to persons living with HIV and not with TB (9) or to PWTB who were unexposed to HIV (15). Therefore, the results reported in the present investigation on prevalence of anemia and its relationship with HIV infection in persons with TB are not particularly novel. Nevertheless, the findings described here, in a cohort which is representative of the Brazilian population of TB patients, add to the body of evidence that moderate to severe anemia is a robust risk factor for death in PWTB undergoing ATT. Noteworthy, such feature was shown to be independent on the effect of other important and well-known risk factors such as BMI values (16), HIV infection (17) and pre-DM or DM (4). These observations concur with a report from South India demonstrating that severe anemia prior to ATT commencement was associated with increased risk of death during the course of treatment (18). In this South Indian study, only 0.4% (n=5) of the study population were living with HIV. In addition, a prospective case-control study which enrolled pulmonary TB patients admitted to the emergency department of a referral

TB hospital also found that severe anemia composed a prediction score to infer early mortality, which as defined as death within one week of hospital admission (19). Altogether, the results strongly advocate that Hb values could be used as a predictor of mortality. Identification of anemic TB patients prior to ATT and the consequent estimation of prognosis may lead to optimize clinical management and improve treatment outcomes.

In PWTB with moderate and severe anemia, we found an increased frequency of disseminated TB and DM than in those without anemia. The relationship between anemia and severe clinical forms of TB has been aforementioned by Kerkhoff et al. (20). The authors demonstrated that the coincidence of anemia, low hepcidin levels and disseminated TB disease led to poor prognosis in TB-HIV patients. Yet, such relationships in the absence of HIV infection were not explored. In persons with DM and without TB, frequency of anemia was of 30% in a distinct study (21). In that scenario, anemia was shown to be an independent indicator of increased risk for DM-related macrovascular and microvascular complications. Even though both HIV co-infection and DM comorbidity have already been described to influence occurrence and severity of anemia in persons with TB, our results argue that anemia directly links to increased mortality independent of these conditions.

The present study has some limitations. In this prospective cohort, measurements of Hb were not performed at other study timepoints, precluding the exploration of whether transient vs. persistent anemia over the course of ATT could impact the outcomes. The study design also limits the capacity to decipher whether anemia itself causes deterioration of prognosis or it is just a hallmark of disease progression or even of an underlying process such as a unique profile of inflammatory disturbance. Moreover, it was not possible to dissect if anemia was caused by nutritional factors or use of medication that may have effect on erythrocyte lifespan. Regardless, our study established that moderate to severe anemia at pre-ATT directly affects the risk of death in PWTB, unrelatedly to HIV exposure. Consequently, new policy is required to implement systematic assessment of Hb values in all pulmonary TB patients prior to ATT initiation to estimate risk of mortality. This simple maneuver could lead to early interventions that might dramatically minimize such outrageous treatment outcome, especially in limited-resource settings.

## NOTES

**Potential conflicts of interest and disclaimer:** All authors: No reported conflicts of interest. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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**Authors and Contributors:** Conceptualization, M.A.P, T.R.S., M.C.F., M.C.S., V.C.R., and B.B.A.; Data curation, M.M.T., M.A-P., and B.B.A.; Investigation, M.A-P. M.C.F., A.L.K., V.C.R., T.R.S., M.C.S., and B.B.A.; Formal analysis, M.A-P., and B.B.A.; Funding acquisition, A.L.K., V.C.R., T.R.S., M.C.S., M.C.F., and B.B.A.; Methodology, M.A-P., and B.B.A.; Project administration, M.C.F., T.R.S., and B.B.A.; Resources, M.A-P., T.R.S., and B.B.A.; Software, M.A-P., M.C.F., and B.B.A.; Supervision, T.R.S., and B.B.A.; Writing—original draft, M.A-P., and B.B.A.; Writing—review and editing, all authors.

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## FIGURES AND LEGENDS

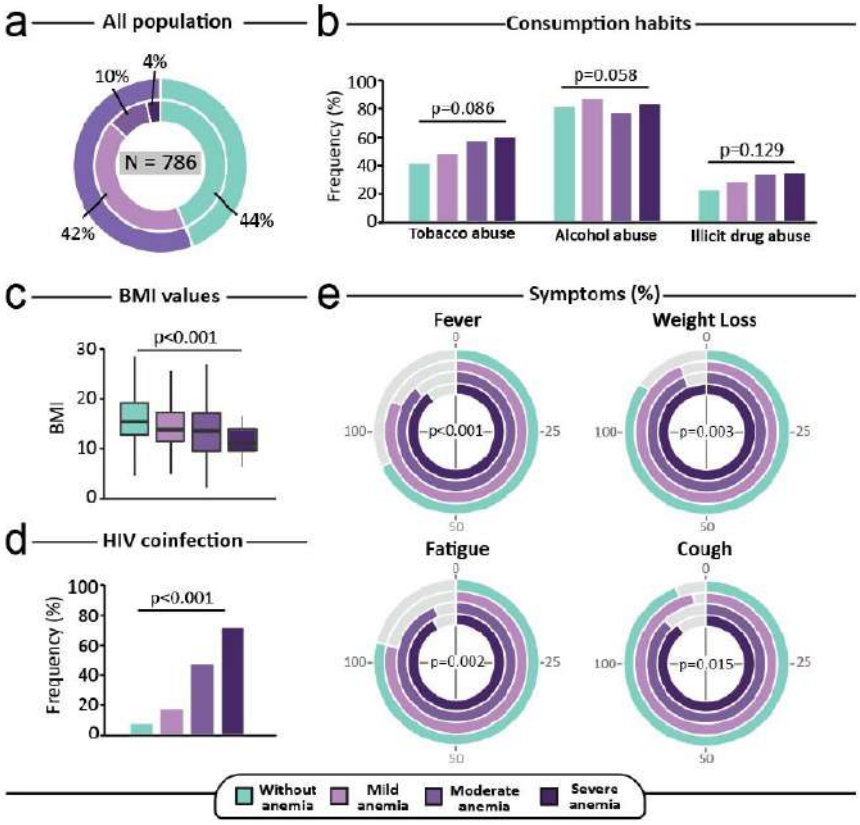



Figure 1. Increased in anemia severity is associated with poor consumption habits, higher frequency of comorbidities, and of TB-related symptoms. (a) Among all the study participants (n=786), 56% had anemia: 42% mild, 10% moderate, and 4% severe. (b) Frequency of consumption habits in patients stratified according to anemia severity. (c) BMI according to anemia severity. (d) Frequency of HIV according to anemia severity. (e) Frequency of TB clinical symptoms following the anemia severity. Groups were compared using the Pearson's chi-square test or the Kruskall-Wallis test.

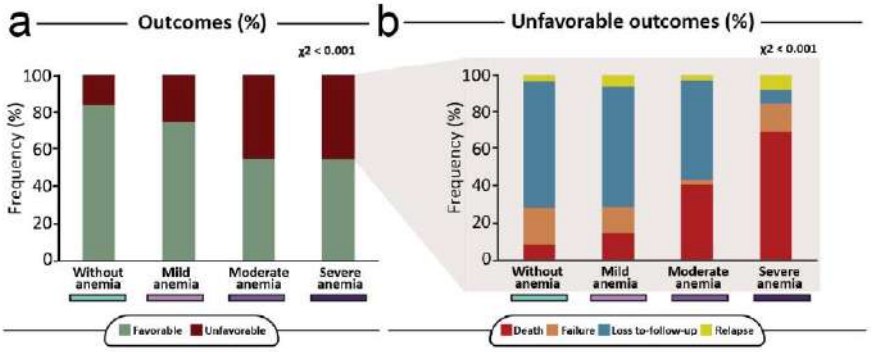



Figure 2. Augmented anemia severity is related with increased occurrence of unfavorable outcomes. (a) Frequency TB treatment outcomes according anemia severity. (b) Frequency of each type of TB unfavorable outcome according to anemia severity. Groups were compared using the Pearson's chi-square test.

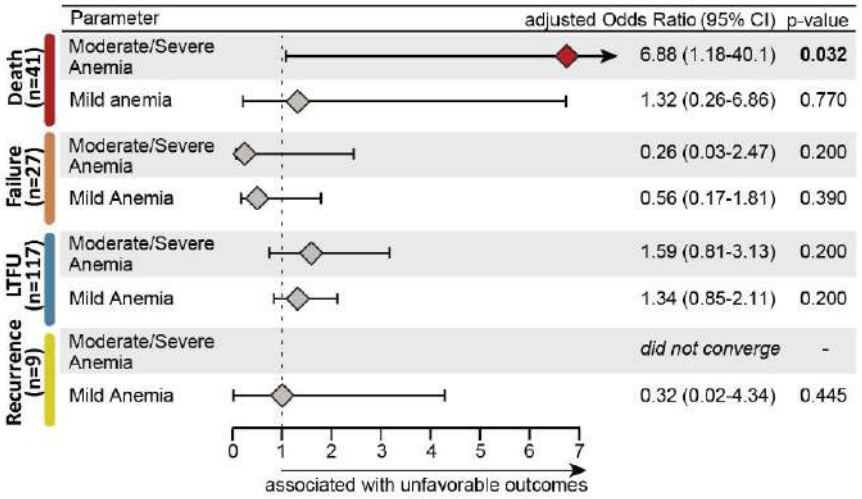



Figure 3. Moderate and severe anemia are associated with increased mortality in patients with TB undergoing treatment. A multinomial logistic regression model (backward stepwise regression) was designed to test independent associations between clinical characteristics and the indicated TB treatment outcomes. Cure or treatment complete was considered as reference outcome. The variables included in the adjusted model were anemia severity, sex, age, BMI, HIV status and dysglycemia status.

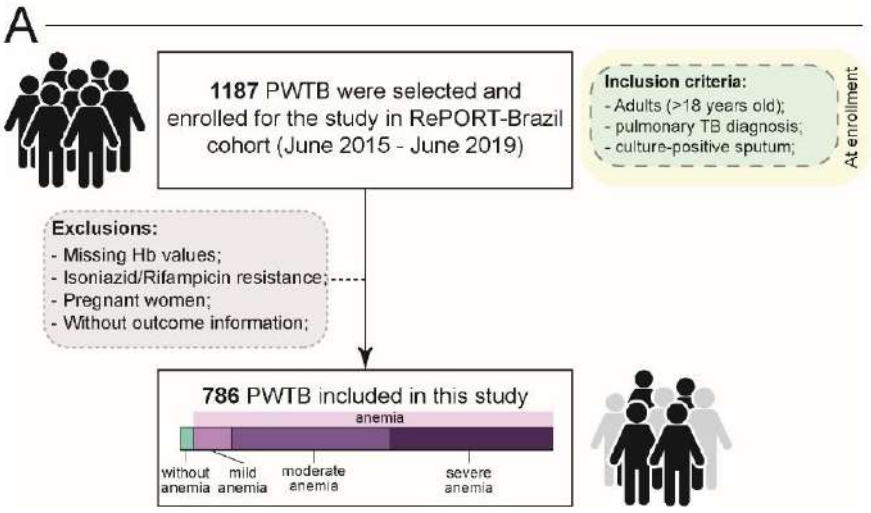
**Table 1. Characteristics of the study participants at baseline according anemia severity**

| | | Without anemia (n=345) | Mild anemia (n=333) | Moderate anemia (n=80) | Severe anemia (n=28) | P value |
|---|---|---|---|---|---|---|
| BASELINE CHARACTERISTICS AND CONSUMPTION HABITS | **Hemoglobin (g/dL), median (IQR)** | 13.6 (13.0-14.4) | 11.4 (10.8-12.0) | 9.25 (8.79-9.70) | 7.58 (7.30-7.73) | **<0.001** |
| | **Sex (male), n (%)** | 212 (61.4) | 224 (67.3) | 43 (53.8) | 16 (57.1) | 0.098 |
| | **Age, median (IQR)** | 37.0 (27.0-49.0) | 36.0 (25.0-49.0) | 37.5 (25.8-49.0) | 39.0 (30.5-48.5) | 0.640 |
| | **BMI, median (IQR)** | 21.0 (19.3-23.4) | 19.9 (18.4-22.2) | 19.7 (17.2-22.1) | 18.1 (17.2-20.0) | **<0.001** |
| | **Race (non-white), n (%)** | 246 (71.3) | 271 (81.4) | 71 (88.8) | 25 (89.3) | **<0.001** |
| | **HIV, n (%)** | 24 (7.19) | 50 (15.0) | 29 (36.2) | 11 (39.3) | **<0.001** |
| | | | | | | |
| | **Tobacco use, n (%)** | 146 (42.2) | 159 (47.7) | 43 (53.8) | 17 (60.7) | 0.086 |
| | **Alcohol use, n (%)** | 271 (78.6) | 282 (84.7) | 62 (77.5) | 37 (92.5) | 0.058 |
| | **Illicit drug use, n (%)** | 78 (23.2) | 80 (26.0) | 24 (25.8) | 6 (35.3) | 0.129 |
| | | | | | | |
| TB DATA AND COMORBIDITY | **Prior TB, n (%)** | 49 (14.2) | 48 (14.4) | 14 (17.5) | 1 (3.57) | 0.411 |
| | **Abnormal X-ray, n (%)** | 337 (98.3) | 317 (96.9) | 74 (92.5) | 26 (96.3) | 0.052 |
| | **Dysglycemia, n (%)** | | | | | **<0.001** |
| | **Diabetes** | 82 (23.8) | 65 (19.6) | 20 (25.0) | 11 (39.3) | |
| | **Prediabetes** | 116 (33.6) | 164 (49.4) | 20 (25.0) | 7 (25.0) | |
| | **Normoglicemia** | 147 (42.6) | 103 (31.0) | 40 (50.0) | 10 (35.7) | |
| | | | | | | |
| TB SYMPTOMS AT BASELINE | **Fever, n (%)** | 238 (69.0) | 276 (82.9) | 71 (88.8) | 25 (89.3) | **<0.001** |
| | **Weight loss, n(%)** | 295 (86.0) | 311 (93.4) | 74 (92.5) | 28 (100) | **0.003** |
| | **Fatigue, n (%)** | 270 (78.3) | 258 (77.5) | 75 (93.8) | 26 (92.9) | **0.002** |
| | **Night sweat, n (%)** | 230 (66.7) | 242 (72.9) | 51 (63.7) | 21 (75.0) | 0.193 |
| | **Chest pain, n (%)** | 232 (67.2) | 205 (61.7) | 49 (61.3) | 20 (71.4) | 0.363 |
| | **Cough, n (%)** | 320 (95.2) | 298 (96.8) | 59 (88.1) | 15 (88.2) | **0.015** |

**Table note:** Continuous variables are displayed as median and interquartile ranges (IQR) whereas categorical variables are shown as absolute number and frequency (%). Data were compared between the clinical groups using the Kruskal-Wallis (continuous) or the Pearson's chi square (categorical) tests. Mild anemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate anemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anemia was defined as Hb<8g/dL for both sexes. *Definition of alcohol use:* Past or current any consumption of alcohol. *Definition of tobacco use*: Past or current smoking of tobacco. *Definition of illicit drug use:* Past or current illicit drug use (marijuana, cocaine, heroin or crack). *Definition of non-white:* The following self-reported races: Asian, Black, Pardo and Indigenous. *Definition of type of TB*: Clinical form of TB regarding the disease's location. *Definition of prior-TB:* Previous TB history. **Abbreviations:** TB: tuberculosis, PTB: Pulmonary Tuberculosis, EPTB: Extrapulmonary Tuberculosis, HIV: Human Immunodeficiency Virus, DM: Diabetes Mellitus.

## Supplementary Material

**Supplementary Figure 1**
**Supplementary Table 1**
**Supplementary Table 2**




**Supplementary Figure 1. Flow chart of the study.**

**Supplementary Table 1. Definition of tuberculosis treatment outcomes**

| Type of outcome treatment | Outcome TB treatment | Definition |
|---|---|---|
| **Favorable** | Cure | Resolution of symptoms consistent with TB by the end of therapy. Patients without symptoms consistent with TB at the beginning of TB treatment cannot have their clinical response evaluated. |
| | Completed treatment | When the patient does not have treatment failure or loss to followup, and has received at least 90% of the total number of doses of the standard recommended anti-TB therapy by the National TB Program in a period up to one year for drug susceptible cases, and up to two years for MDR cases. For drug-susceptible TB, the drug regimen consists of isoniazid, rifampicin, pyrazinamide, and ethambutol for 2 months, followed by isoniazid and rifampin for 4 months. For drug-resistant TB, treatment is according to the presence of resistance. |
| **Unfavorable** | Death | A participant who dies for any reason after consenting to participate and prior to the end of the study. |
| | Failure | Sputum smear- or culture-positive at month 5 or later during treatment. |
| | Relapse | Patient who was declared cured or treatment completed by a physician, but who reports back to the health service and is now found to be sputum smear positive |
| | Loss to follow-up | A participant who no longer participates in study visit follow-up or when an outcome cannot be assigned due to insufficient information. |

**Supplementary Table 2. Clinical characteristics according to anemia status.**

| | | Non-anemic (n=345) | Anemic (n=441) | P value |
|---|---|---|---|---|
| BASELINE CHARACTERISTICS AND CONSUMPTION HABITS | **Hemoglobin (g/dL), median (IQR)** | 13.6 (13.0-14.4) | 11.1 (10.0-11.8) | **<0.001** |
| | **Sex (male), n (%)** | 212 (61.4) | 283 (64.2) | 0.478 |
| | **Age, median (IQR)** | 37.0 (27.0-49.0) | 37.0 (26.0-49.0) | 0.999 |
| | **BMI, median (IQR)** | 21.0 (19.3-23.4) | 19.8 (18.1-21.9) | **<0.001** |
| | **Race (non-white), n (%)** | 246 (71.3) | 367 (83.2) | **<0.001** |
| | **HIV, n (%)** | 24 (7.19) | 90 (23.1) | **<0.001** |
| | | | | |
| | **Tobacco use, n (%)** | 146 (42.3) | 219 (49.7) | **0.048** |
| | **Alcohol use, n (%)** | 271 (78.6) | 370 (83.9) | 0.068 |
| | **Illicit drug use, n (%)** | 78 (23.2) | 110 (28.1) | 0.160 |
| | | | | |
| TB DATA AND COMORBIDITY | **Prior TB, n (%)** | 49 (14.2) | 63 (14.3) | 0.999 |
| | **Abnormal X-ray, n (%)** | 337 (98.3) | 417 (96.1) | 0.119 |
| | **Dysglycemia, n (%)** | | | **0.017** |
| | **Diabetes** | 82 (23.8) | 96 (21.8) | |
| | **Prediabetes** | 116 (33.6) | 191 (43.4) | |
| | **Normoglicemia** | 147 (42.6) | 153 (34.8) | |
| | | | | |
| TB SYMPTOMS AT BASELINE | **Fever, n (%)** | 238 (69.0) | 372 (84.4) | **<0.001** |
| | **Weight loss, n(%)** | 295 (86.0) | 413 (93.7) | **0.001** |
| | **Fatigue, n (%)** | 270 (78.3) | 359 (81.4) | 0.315 |
| | **Night sweat, n (%)** | 230 (66.7) | 314 (71.4) | 0.181 |
| | **Chest pain, n (%)** | 232 (67.2) | 274 (62.3) | 0.171 |
| | **Cough, n (%)** | 320 (95.2) | 372 (94.9) | 0.968 |

**Table note:** Continuous data are shown as median and interquartile (IQR) ranges and categorical data as number and frequency (%). Data were compared between the clinical groups using the Mann-Whitney U test (continuous) and Pearson chi square test (categorical). Anemia was defined as levels of Hb <13 g/dL for men or <12 g/dL for women. Mild anemia was defined as Hb value >10 g/dL and <13 g/dL for men; and >10 and <12 g/dL for women, whereas moderate anemia was defined as Hb>8 g/dL and <=10 g/dL for both sexes. Severe anemia was defined as Hb<8g/dL for both sexes. *Definition of alcohol consumption:* Past or current any consumption of alcohol. *Definition of tobacco use*: Past or current smoking of tobacco. *Definition of illicit drug use:* Past or current illicit drug use (marijuana, cocaine, heroin or crack) *Definition of non-white:* The following self-reported races: Asian, Black, Pardo and Indignos. *Definition of type of TB*: Clinical form of TB regarding the disease's location. *Definition of prior-TB:* Previous TB history. **Abbreviations**: TB: tuberculosis, PTB: Pulmonary Tuberculosis, EPTB: Extrapulmonary Tuberculosis, HIV: Human Immunodeficiency Virus, DM: Diabetes Mellitus,

## 6 DISCUSSÃO

Essa tese foi construída a partir da análise de um conjunto de cinco coortes distintas de PVHIV virgens de TARV, recrutados em centros clínicos de 14 países em quatro continentes, com valores de Hb disponíveis no primeiro *timepoint* dos estudos. Em conjunto, essas coortes resultam em um número total de 1617 PVHIV, com e sem TB. Adicionalmente, uma coorte de pacientes TB, com e sem HIV, foi incluída a fim de expandir as análises acerca da anemia em TB-HIV. Juntos, os nossos resultados sugerem uma forte associação entre diferentes graus de anemia com a perturbação inflamatória sistêmica, co-infecção por *Mtb* e óbito durante os períodos de acompanhamento. Apesar dos desenhos de estudo não nos permite avaliar se a anemia é causa ou consequência do processo inflamatório dos participantes, em conjunto esses achados demonstram um eficiente uso de níveis de Hb como um *proxy* de distúrbio inflamatório, que se relaciona com TB e mortalidade em PVHIV. A Hb, um marcador de baixo custo e fácil acesso, merece mais atenção durante o acompanhamento de indivíduos vivendo com HIV e/ou TB, principalmente em regiões de recursos limitados.

Nos manuscritos que constituem essa tese, pacientes foram considerados anêmicos de acordo com os valores de referência da OMS (WORLD HEALTH ORGANIZATION, 2011), como descrito nos métodos. Entre as coortes de PHVIV aqui exploradas, a prevalência de anemia variou entre 71% e 92.7%, sendo que a maior parte apresentava anemia leve, seguida de anemia moderada ou severa. A frequência global de anemia relatada nas nossas coortes de PVHIV condiz com o descrito por outros estudos, como descrito por Akilimali e colaboradores (2015), ao investigar o status de anemia em 756 PVHIV atendidos em dois grandes hospitais de Goma, Congo, dos quais 69% eram anêmicos seguindo critérios similares ao dessa tese (AKILIMALI et al., 2015). Além disso, Kerkhoff e colaboradores (2015) também observaram frequência semelhante, de 72% ao investigar a anemia em uma coorte de PVHIV atendidos em um centro de referência na Cidade do Cabo, África do Sul (KERKHOFF et al., 2015).

Diferentes estudos avaliaram a associação da anemia com desfechos de óbito em PVHIV, demonstrando que quanto menor o nível de Hb, maior o risco de óbito em diferentes populações (BELPERIO; RHEW, 2004; KERKHOFF et al., 2015; MOCROFT et al., 1999). Entretanto, poucos estudos de coorte foram realizados para investigar como a anemia pode influenciar em outros desfechos, como desenvolvimento de TB, mesmo com a alta prevalência dessa condição em PVHIV. A OMS recomenda, desde 2011, que PVHIV sejam rotineiramente investigadas quanto à TB (WORLD HEALTH ORGANIZATION, 2019). Isso porque TB é a principal causa de hospitalização e morte em PVHIV (FORD et al., 2015, 2016; UNAIDS,

2021), demonstrando a importância de se investigar fatores associados ao desenvolvimento dessa coinfecção nessa população.

No **primeiro manuscrito (M1)** aqui apresentado, dos 269 PVHIV inseridos no estudo 45% apresentavam anemia leve e 31.2% anemia moderada ou grave. Aqueles com anemia moderada/grave apresentaram uma maior frequência de neutrófilos, maior número de sintomas e maior inflamação sistêmica, caracterizada pelo aumento de citocinas inflamatórias da resposta inata como IL-6, IL-8 e TNF, assim como da resposta adaptativa, como IFN-γ, associado ao perfil Th1. Além disso, observamos que a frequência de TB incidente e mortalidade aumentaram de acordo com a gravidade da anemia. Em seguida, demonstramos que PVHIV com anemia moderada/severa antes do início do TARV apresentavam um risco 2.4 vezes maior de TB incidente se comparado aos não-anêmicos após o início da TARV, além de um risco 2.2 vezes maior de vir a óbito. Por fim, a partir de uma análise bayesiana, identificamos uma associação direta entre altos níveis de IL-6 com TB incidente e morte.

Em uma coorte de PVHIV que foram acompanhados por até 8 anos após o início da TARV, também foi observado que a taxa de TB incidente aumentou de acordo ao aumento da gravidade da anemia. Anemia moderada e grave persistentes durante o tratamento foram independentemente associadas a TB incidente e morte nessa população (KERKHOFF et al., 2015**).** A contribuição do nosso estudo para essa observação é que no nosso trabalho, essas associações foram identificadas a partir do status de anemia do paciente pré-tratamento, demonstrando que PVHIV com anemia precisam ser melhor acompanhados desde o início da TARV independente de se recuperarem ou não durante o processo.

No **segundo manuscrito (M2)**, com 502 PVHIV, foi constatado que 83.7% dos indivíduos eram anêmicos, dos quais 36.9% tinham anemia moderada ou grave. Nesse estudo observamos que aqueles com anemia grave apresentavam maior carga viral de HIV, demonstrando uma possível associação dessa condição com o aumento da replicação viral. Além disso, encontramos correlações inversas dos níveis de Hb com citocinas como IFN-γ, CXCL10 e IL-6, assim como níveis significativamente aumentados de marcadores de inflamação macrofágica como sCD163 e sCD14 em pacientes com anemia grave. Por fim, a anemia grave foi independentemente associada a um aumento de 8.12 vezes de risco de desenvolvimento de SIRI, principalmente por micobactérias. Adicionalmente, na regressão logística o aumento de TFN também foi significativamente associado a SIRI de todos os tipos, assim como aumento de sCD163 foi especificamente associado a SIRI por micobactérias e o aumento de IL-27 foi associado a morte na coorte estudada.

As infecções por micobactérias (*Mtb* e do complexo *Mycobacterium avium (MAC)* são as infecções oportunistas mais comuns em PVHIV. A coinfecção por micobactérias em PVHIV pode levar à uma manifestação de SIRI multifocal, de acordo com os sítios de disseminação bacteriana, após o início da TARV (NARENDRAN et al., 2019). O envolvimento de marcadores de atividade macrofágica como sCD14, sCD163 e IL-27 (ABDALLA et al., 2015; ANDRADE et al., 2014; MUSSELWHITE et al., 2016) no desenvolvimento de SIRI e morte nos indivíduos da coorte investigada chamam a atenção para o envolvimento de macrófagos no processo fisiopatológico da SIRI.

Baixos níveis de Hb, assim como altos níveis de IFN-γ, sCD14 e sCD163 já foram descritos como fatores independentemente associados ao desenvolvimento de SIRI por *Mtb* e MAC (ANDRADE et al., 2014; BREGLIO et al., 2021; MUSSELWHITE et al., 2016; NARENDRAN et al., 2013, 2020; VINHAES et al., 2021), entretanto não foram encontrados estudos sobre a anemia estratificada por gravidade e a sua associação com SIRI. No nosso estudo evidenciamos que pacientes com anemia grave apresentam um alto risco de SIRI por micobactéria se comparados aos pacientes com anemia em grau moderado, leve ou sem anemia. Além disso, demonstramos que há uma modulação da atividade de macrófagos nos pacientes que desenvolvem SIRI ou morrem nessa população.

No **terceiro manuscrito (M3)**, com uma coorte composta por 159 PVHIV, encontramos que aqueles com HIV-TB apresentam menores níveis de Hb se comparados a PVHIV sem TB. Ao utilizar os valores de Hb para estratificar cada grupo de acordo com o status de anemia, observamos que uma maior frequência de anêmicos foi encontrada naqueles HIV-TB. Adicionalmente, avaliamos a correlação dos valores de Hb com mediadores solúveis inflamatórios, onde identificamos uma correlação negativa entre Hb e as citocinas TNF e IL-6 independente do status de TB. Em seguida, agrupamos os indivíduos utilizando o status de TB e anemia (TB anêmicos, TB não-anêmicos, não-TB anêmicos e não-TB não anêmicos) e os comparamos quanto ao perfil inflamatório sistêmico. Nessa comparação observamos que o GPI dos grupos anêmicos é maior que dos grupos não anêmicos, e que indivíduos com HIV-TB e anemia apresentam uma maior perturbação inflamatória caracterizada por altos níveis de IFN-γ e TNF.

As citocinas encontradas nesse manuscrito como TNF, sCD14, IL-8 e IL-6 estão envolvidas na condição pró-inflamatória associada a infecção por HIV, sendo inclusive fatores de risco para a progressão da infecção e aumento da morbimortalidade (BOULWARE et al., 2011; GRAHAM et al., 2019; SANDLER et al., 2011; TENFORDE et al., 2015). De maneira

interessante, no nosso trabalho essas citocinas foram fortemente e negativamente correlacionadas aos níveis de Hb, demonstrando que indivíduos anêmicos podem ter um pior prognóstico a partir da AIC.

Nesse trabalho novamente observamos a presença de marcadores de atividade de macrófagos, como sCD14 que é um marcador de resposta monocítica (SANDLER et al., 2011)**,** e TNF, uma potente molécula secretada por macrófagos que exerce um papel dúbio no controle da coinfecção HIV-TB. Essa dualidade no controle da infecção ocorre porque ao mesmo tempo que TNF é requerida para controlar o crescimento do *Mtb*, também é conhecida por ativar a replicação de HIV em macrófagos (KEDZIERSKA et al., 2003). Assim, ao identificar a correlação negativa entre os níveis de Hb e TNF como demonstrado no nosso trabalho, podemos inferir que em PVHIV anêmicos há uma exacerbação de produção de TNF, que poderia por sua vez levar a uma progressão mais acentuada da infecção por HIV.

No **quarto manuscrito (M4)**, com 496 PVHIV hospitalizados e com coinfecção por TB, observamos que 92.7% da coorte estava anêmica. Diferentemente das outras, aqui foi observada uma alta frequência de anemia grave, que correspondeu a 38,1% dos indivíduos. Identificamos que aqueles com anemia grave tinham menor contagem de LT CD4+ além de uma maior frequência de testes positivos para TB, assim como para a coinfecção por Citomegalovírus (CMV). Ao avaliar a inflamação sistêmica desses pacientes, observamos que o aumento da gravidade da anemia estava associado ao aumento da perturbação inflamatória, a partir da metodologia do GPI descrita nos métodos. Essa perturbação foi caracterizada principalmente por altos níveis de IL-8, IL-1RA e IL-6 em PVHIV com anemia grave.

Além disso, o aumento da gravidade da anemia foi associado ao aumento da disseminação da TB e morte precoce, nessa coorte. Ao avaliar os pacientes com maior disseminação da TB, foi observado que a maioria apresentava anemia grave e que o GPI daqueles com escore de disseminação 3 era significativamente aumentado em relação aos demais escores (sem disseminação, disseminação 1 e disseminação 2). Esse GPI foi caracterizado principalmente pelos altos níveis de IL-8. Por fim, o agrupamento hierárquico dos indivíduos a partir do perfil inflamatório demonstrou que aqueles com anemia grave e escore de disseminação mais elevado apresentaram um perfil de inflamação sistêmica similar, associado a alta taxa de morte na população estudada. As análises de acurácia demonstraram que a Hb é um bom marcador para predição de disseminação de TB e mortalidade precoce em PVHIV.

A associação entre baixos valores de Hb com baixa contagem de LT CD4+ e alta

coinfecção por CMV demonstram que possivelmente indivíduos com anemia grave estão em um estágio mais avançado da infecção por HIV, sendo identificada aqui pelos valores reduzidos de linfócitos e a maior frequência de infecções oportunistas. Níveis reduzidos de Hb já foram descritos como fatores de risco para a progressão do HIV (MOCROFT et al., 1999). A inflamação sistêmica, com participação de níveis elevados de IL-6, de PVHIV com anemia grave nessa população foi associada à disseminação do Mtb.

IL-6 é uma citocina multifuncional que induz a produção de hepcidina, que inibe a absorção de ferro, bloqueando a liberação de ferro dos macrófagos e a entrega de heme às células eritróides (ARMITAGE et al., 2014). Em indivíduos com TB, o aumento da hepcidina (diretamente associado ao aumento do IL-6) pode ser um reflexo da tentativa de limitar o crescimento bacilar extracelular, o que pode custar o quadro de anemia grave (KERKHOFF et al., 2016). A hepcidina já foi descrita como um preditor de morte, assim como de TB incidente e aumento de disseminação em TB associada ao HIV (KERKHOFF et al., 2016; MINCHELLA et al., 2014).

Assim, a coinfecção HIV-TB pode levar a uma resposta inata inicialmente protetora contra *Mtb*, com o aumento de IL-6 e consequente aumento de hepcidina. Entretanto, a longo prazo essa resposta potencialmente se torna prejudicial ao hospedeiro, com o desenvolvimento de uma AIC grave, que vai levar ao aumento da infecção e inflamação, com consequente risco aumentado de desfechos desfavoráveis como morte.

Em seguida, na coorte de 191 pacientes HIV-TB acompanhados durante o TAT por 6 meses, que deu origem ao **quinto manuscrito (M5)** desta tese, observamos uma frequência de 84,3% de indivíduos anêmicos. Em seguida, foi constatado que participantes HIV-TB anêmicos apresentaram contagens mais baixas de outros tipos de células sanguíneas, sugerindo que pode estar ocorrendo um efeito de depleção global na medula óssea. Além disso, identificamos também que baixos níveis de Hb acompanharam maiores valores de transaminases hepáticas, reforçando a ideia de que a anemia está relacionada a um perfil bioquímico distinto e ligada à inflamação. Ao utilizar os dados bioquímicos e celulares para calcular o GPI, foi identificado que indivíduos anêmicos apresentaram maior perturbação inflamatória, que se manteve ao longo da TAT. De acordo com os dados de acompanhamento da nossa coorte, observamos que a anemia persistente (180 dias) estava associada a óbito e falha de TAT nessa população. Além disso, não ter anemia pré-TAT é um fator protetor contra desfechos desfavoráveis independentemente de outros fatores.

Por fim, avaliamos uma coorte de 786 pacientes TB, com e sem HIV, que foram

acompanhados desde o início da TAT até 24 meses a fim de investigar se a gravidade da anemia era um determinante de desfecho desfavorável independente de outros fatores. Essa investigação deu origem ao **sexto manuscrito (M6).** Neste trabalho, observamos uma frequência de 56% de indivíduos anêmicos. Observamos também que a frequência de coinfecção por HIV aumentava em concordância com o aumento da gravidade da anemia. Na população geral, TB com e sem HIV, a anemia moderada/grave foi associada a uma maior frequência de sintomas, assim como de desfechos desfavoráveis. A análise de regressão multinominal demonstrou que essa gravidade (moderada/severa) é um fator de risco para óbito nessa população, mas não para os outros desfechos, independente de idade, sexo, raça e HIV. A importância desse estudo adicional, de pacientes TB com e sem HIV, é demonstrar que, embora mais acentuada no cenário de PVHIV, a associação entre anemia e desfechos desfavoráveis de tratamento antirretroviral e anti-TB parece estar intimamente associada a coinfecção por TB.

Estudos anteriores demonstraram que a anemia está associada a um aumento de 2 a 3 vezes no risco de morte, recorrência de TB ou falha de TAT (ISANAKA et al., 2012). Em um estudo realizado na Tanzânia com 1245 indivíduos com TB não-HIV, a anemia pré-TAT foi associada a um risco aumentado de um resultado positivo persistente no exame de cultura para Mtb até dois meses após o tratamento (NAGU et al., 2014). Os nossos estudos (M5-M6) demonstraram que em pacientes TB e/ou HIV, a anemia também desempenha um papel importante no curso do TAT, de forma que indivíduos anêmicos pré-TAT têm maior risco de apresentar um desfecho desfavorável de tratamento se comparados àqueles não-anêmicos. Esses estudos se complementam e adicionam um conhecimento atual da área ao demonstrar a relevância da anemia na condução de distúrbios inflamatórios relacionados ao desfecho de tratamento anti-TB em indivíduos vivendo com e sem HIV.

Em **todos os estudos** com a mensuração de mediadores solúveis provenientes de kits comercialmente disponíveis (M1-M4), os pacientes com anemia, principalmente anemia grave, também apresentaram associação com altos níveis de marcadores inflamatórios como IL-6, IFN-γ e TNF em comparação aos não-anêmicos. Os altos níveis de IL-6 podem estar associados a inflamação causada pela própria infecção por HIV, cujos processos metabólicos levam a anemia (LIPSHULTZ et al., 2015; RAJ, 2009; WEISS; GOODNOUGH, 2005). Já foi descrito que a AIC está associada à ativação de monócitos, e a IL-6 é essencial para esse processo (LIPSHULTZ et al., 2015). Em conjunto com TNF, IL-6 pode induzir a apoptose de precursores de glóbulos vermelhos e diminuir a capacidade da medula óssea de responder à sinalização da

eritropoietina (WEISS; GOODNOUGH, 2005).

As outras citocinas consistentemente identificadas (IFN-γ e TNF) também apresentam associação com o desenvolvimento da AIC, tendo em vista que o aumento IFN-γ diminui a expressão da ferroportina, levando à retenção de ferro em monócitos/macrófagos, e que o aumento de TNF promove a eritrofagocitose, agravando o acúmulo de ferro por macrófagos ativados (MOLDAWER et al., 1989; MOURA et al., 1998; YANG et al., 2002). Os altos níveis dessas citocinas encontrados em indivíduos anêmicos das coortes avaliadas, nos permitem compreender que a inflamação sistêmica exacerbada carreada pelo aumento desses três marcadores está contribuindo para o estabelecimento e aumento da gravidade da anemia. Por consequência, essa exacerbação de resposta imune encontrada principalmente em PVHIV com anemia grave pode estar associada aos desfechos estudados nessa tese.

PVHIV com anemia grave apresentam um maior risco de TB incidente (M1), SIRI – principalmente por micobactérias (M2), e maior perturbação inflamatória (M3) se comparados a indivíduos com anemia leve ou sem anemia. Além disso, participantes HIV-TB apresentam maior disseminação de Mtb (M4), um perfil inflamatório sistêmico mais exacerbado, tanto no que diz respeito aos mediadores solúveis inflamatórios (M4) quanto ao perfil bioquímico e celular (M5) desses pacientes. Pacientes com HIV-TB apresentam maor risco de morte (M1, M2, M4) se comparados a indivíduos com anemia leve ou sem anemia. Por fim, também foi observado que aqueles HIV-TB com anemia persistente durante o TAT apresentam maior risco de desfecho desfavorável de tratamento (M5), e que anemia moderada a grave pre-TAT é um fator de risco para óbito (M6) **(Figura 14)**.

Esses resultados podem implicar na necessidade de melhoria e criação de diretrizes, protocolos e escores de avaliação para o acompanhamento e tratamento de PVHIV com anemia. Pode-se propor, por exemplo, a investigação obrigatória da anemia antes do início da TARV (P1) com a classificação da gravidade (leve, moderada ou grave) a fim de identificar fatores de risco para os desfechos de tratamento. Em caso de identificação de anemia pré-TARV, pode ser interessante a criação de diretrizes específicas para o acompanhamento desses pacientes (P2), com investigação da possível causa da anemia, resolução quando possível e acompanhamento contínuo dos níveis de Hb (P3) até que volte aos níveis preconizados pela OMS. Além disso, a partir dos resultados encontrados, pode ser também interessante sugerir esse acompanhamento contínuo de Hb em indivíduos HIV-TB submetidos ao TAT (P4). Com todas essas informações, por fim, seria útil a criação de um escore e/ou esquema de direcionamento de abordagem terapêutica para PVHIV, onde poderia ser calculado o benefício de tratar a anemia pré-TARV

frente ao risco dos possíveis desfechos desfavoráveis (TB, SIRI ou morte) desses pacientes (P5) (**Figura 14**).

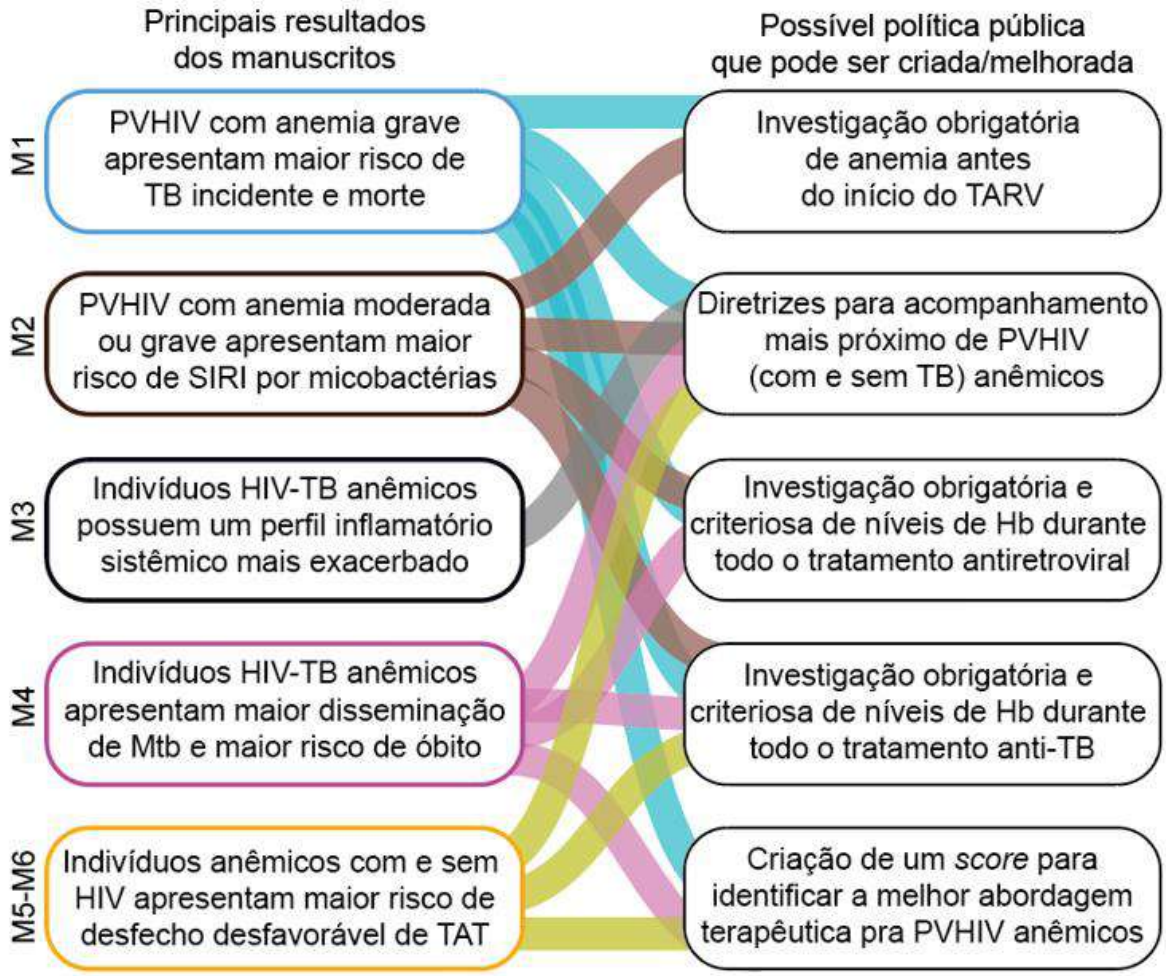



**Figura 14** - Associação entre os principais resultados de cada manuscrito e possíveis implicações nas políticas públicas para o acompanhamento e tratamento de PVHIV. M1: REMEMBER; M2: IRIS-Anemia; M3: CADIRIS; M4: KDHTB; M5: HIV-TB RJ; M6: RePORT-Brazil.
**Fonte**: elaboração da autora

## 7 CONCLUSÕES

Em conjunto os manuscritos apresentados nesta tese acrescentam importantes informações acerca da influência da anemia nos desfechos de tratamento antirretroviral e nos desfechos relacionados a coinfecção por TB em indivíduos vivendo com HIV. Observamos que os distintos níveis de gravidade da anemia exercem diferentes influências na inflamação e desfechos de tratamento desses pacientes. Enquanto não foram observadas associações estatisticamente significativas da anemia leve com nenhuma das implicações estudadas, ficou evidente que a anemia moderada e grave tem uma clara associação com TB incidente, desenvolvimento de SIRI, maior disseminação de TB, e morte. Isso evidencia que a investigação da anemia deve ser mais bem realizada no acompanhamento de PVHIV antes e durante a TARV, e quando possível deve-se trabalhar para a recuperação desses níveis de Hb e investigar a possível causa da anemia, a fim de identificar precocemente infecções oportunistas, melhorar a qualidade de vida e reduzir os riscos de desfecho desfavoráveis de tratamento antirretroviral e anti-TB nessa população.

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**Apêndice A - Desempenho da estudante quanto à produção científica**

A discente, graduada em Biotecnologia pela Universidade Federal da Bahia (UFBA), iniciou sua carreira científica em 2014 no Laboratório de Imunologia e Biologia Molecular (LABIMUNO-UFBA) como estudante de iniciação científica. No LABIMUNO, permaneceu por 3 anos sendo bolsista do CNPq e FAPEX-UFBA sendo orientada pela Dra. Songelí M. Freire. Publicou 19 resumos de trabalho e 4 artigos científicos referentes aos projetos desempenhados no local. Em seguida, entre 2017-2019, a estudante cursou o mestrado em Bioinformática no Instituto de Matemática e Estatística da Universidade de São Paulo (IME-USP), sob orientação do Dr. Helder Nakaya, onde publicou 2 artigos e apresentou 2 trabalhos em congresso referentes à sua dissertação e colaborações. Em 2020 iniciou o doutorado no Laboratório de Inflamação e Biomarcadores do Instituto Gonçalo Moniz (LIB-IGM) sob orientação do Dr. Bruno Andrade, onde publicou 34 artigos, incluindo 10 como primeira/co-primeira autora até o momento. Além disso, apresentou 2 trabalhos em congressos nacionais e internacionais e participou de outros 8 resumos apresentados em simpósios e congressos internacionais. A linha do tempo de produtividade de artigos científicos e as áreas correspondentes a eles podem ser conferidas abaixo (**Figura 1**). As colaborações que ocorreram com pesquisadores nacionais e internacionais durante o desenvolvimento desses artigos são demonstradas na **figura 2**.

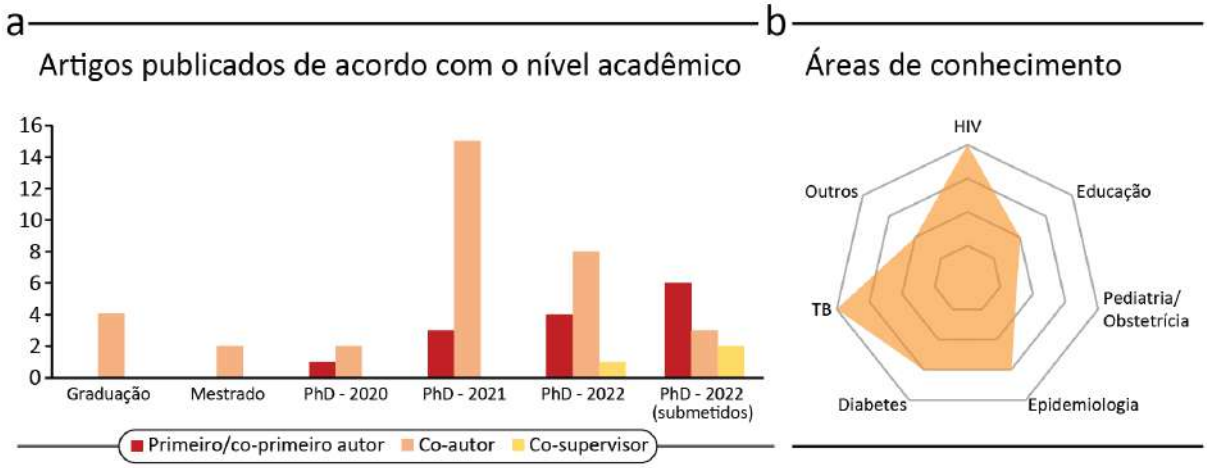


Figura 1. Número de artigos publicados de acordo com o nível acadêmico (a) e áreas de conhecimento de abrangência das publicações (b).

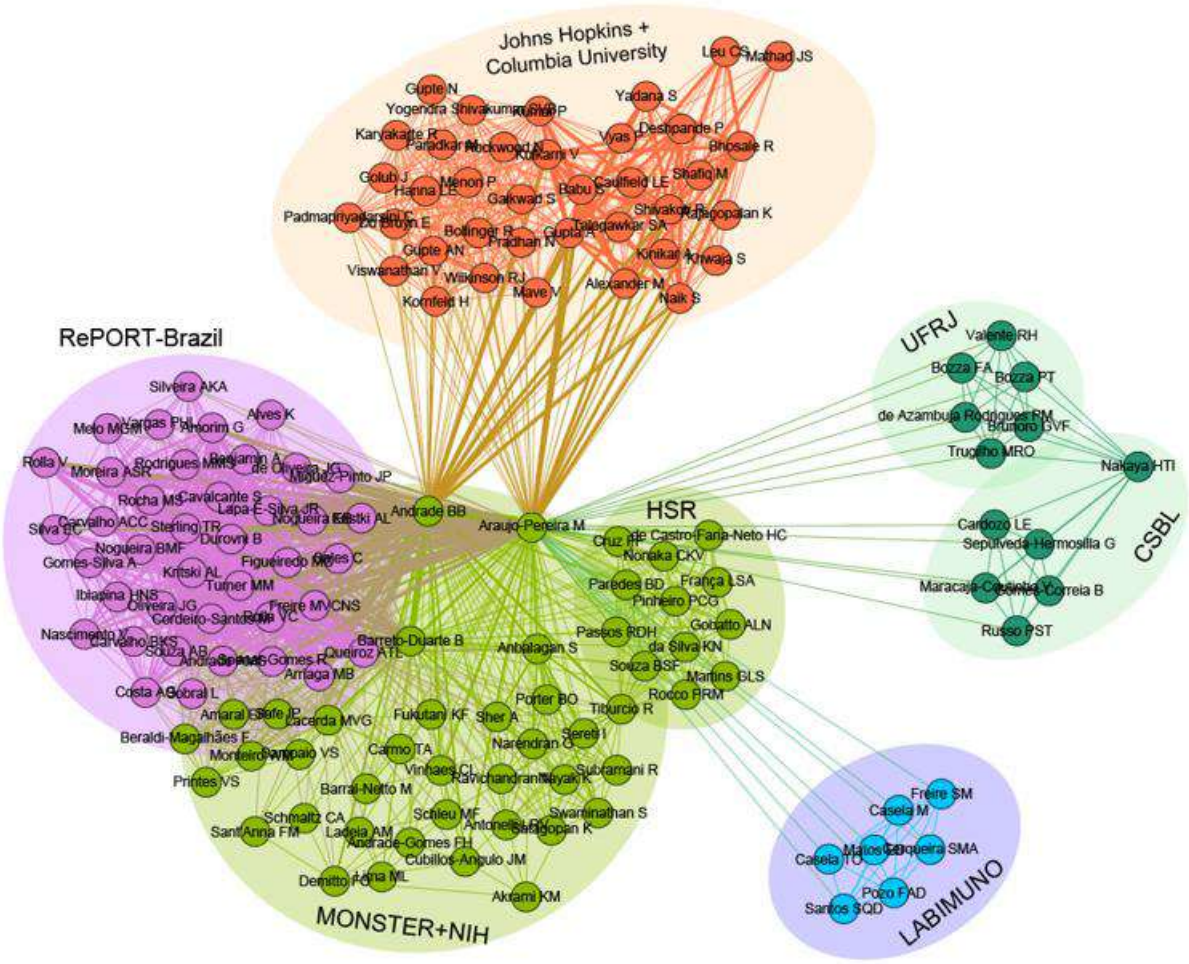


Figura 2. Rede de colaboradores de acordo com os artigos publicados até o momento. São identificados sete diferentes hubs, onde estão contidos colaboradores principalmente das seguintes instituições: (1) RePORT-Brazil; (2) Universidade Johns Hopkins + Universidade de Columbia; (3) Universidade Federal do Rio de Janeiro (UFRJ); (4) Computational and Systems Biology Laboratory (CSBL-USP); (5) Multinational Organization Network Sponsoring Translational and Epidemiological Research (MONSTER) + National Institute of Health (NIH); (6) Laboratório de Imunologia e Biologia Molecular (LABIMUNO-UFBA); e (7) Hospital São Rafael (HSR, Salvador-BA).

**Apêndice B - Lista de artigos publicados durante o doutorado 2020**

1. Impact of Persistent Anemia on Systemic Inflammation and Tuberculosis Outcomes in Persons Living With HIV. Demitto FO, **Araújo-Pereira M**, Schmaltz CA, Sant'Anna FM, Arriaga MB, Andrade BB, Rolla VC. Front Immunol. 2020 Sep 24; 11:588405. doi: 10.3389/fimmu.2020.588405. eCollection 2020.
2. Novel stepwise approach to assess representativeness of a large multicenter observational cohort of tuberculosis patients: The example of RePORT Brazil. Arriaga MB, Amorim G, Queiroz ATL, Rodrigues MMS, **Araújo-Pereira M**, Nogueira BMF, Souza AB, Rocha MS, Benjamin A, Moreira ASR, de Oliveira JG, Figueiredo MC, Turner MM, Alves K, Durovni B, Lapa-E-Silva JR, Kritski AL, Cavalcante S, Rolla VC, Cordeiro-Santos M, Sterling TR, Andrade BB; RePORT Brazil consortium.Int J Infect Dis. 2021 Feb;103:110-118. doi: 10.1016/j.ijid.2020.11.140. Epub 2020 Nov 14.
3. Association of Vegetable and Animal Flesh Intake with Inflammation in Pregnant Women from India. Yadana S, Talegawkar SA, Mathad JS, Alexander M, Rajagopalan K, Kumar P, Naik S, Leu CS, Kulkarni V, Deshpande P, **Araujo-Pereira M**, Bhosale R, Babu S, Andrade BB, Caulfield LE, Gupta A, Shivakoti R.Nutrients. 2020 Dec 8;12(12):3767. doi: 10.3390/nu12123767. 2021
4. Association between neurofibromatosis type 1 and cerebrovascular diseases in children: A systematic review. Barreto-Duarte B, Andrade-Gomes FH, Arriaga MB, **Araújo-Pereira M**, Cubillos-Angulo JM, Andrade BB.PLoS One. 2021 Jan 4;16(1):e0241096. doi: 10.1371/journal.pone.0241096. eCollection 2021.
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