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MECHANISMS OF PATHOGENESIS

Iron homeostasis and progression to pulmonary tuberculosis disease among household contacts

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SUMMARY

Early identification of individuals at risk for progressing to active tuberculosis (TB) disease may limit new transmission and improve clinical outcomes. Evidence indicates altered iron homeostasis may identify those at greater risk of disease progression in HIV co-infection. We aimed to investigate iron homeostasis biomarkers as risk factors for progression to TB. Archived plasma samples were analyzed from household contacts of pulmonary TB index cases in The Gambia. Contacts were classified as asymptomatic non-progressors (n = 17) or TB-progressors (n = 10), which included two HIV-infected participants. Iron homeostasis (hemoglobin, ferritin, hepcidin, soluble transferrin receptor, transferrin) was assessed in all contacts at study recruitment. Plasma was collected a median of 910 days prior to TB diagnosis. Low transferrin around the time of known exposure to infectious TB was a disease progression risk factor among all TB-progressors (Poisson incidence rate ratio: 0.55; 95% CI: 0.35–0.89). Iron homeostasis also differed between early and delayed TB-progressors, with higher ferritin and hepcidin concentrations observed among early TB-progressors (mean ferritin 50.2 vs. 26.2 ng/ml; $P = 0.027$; mean hepcidin 37.7 vs. 5.6 ng/ml; $P = 0.036$). Iron homeostasis is associated with progression to TB among household contacts. Further studies are needed to elucidate mechanisms and determine the clinical utility of monitoring iron homeostasis biomarkers.

1. Introduction

Progression from uninfected or latent tuberculosis infection to clinical tuberculosis (TB) disease is frequently asymptomatic or associated with only mild symptoms during the first few months of disease [1]. This can significantly delay diagnosis and treatment, resulting in higher TB mortality [2–4] and ongoing transmission [5,6]. Indeed, treatment delays are considered an important contributor to the approximately 1.5 million deaths and 9.0 million new TB cases reported each year [7,8]. Current approaches to reduce treatment delays include improved TB diagnostics [9] and active case finding strategies [10]. While these approaches are helpful [11–13], identification of individuals at risk of disease progression could lead to further improvements.

Altered host iron status has been previously identified as a risk factor for progression to TB among HIV-infected individuals [14,15], and a number of studies have indicated that *Mycobacterium tuberculosis* (*Mtb*) iron acquisition plays an important role in TB pathogenesis [16–20]. A complex and intricate host-pathogen iron competition begins early in most infections. In TB, immune recognition of *Mtb* by the human host induces a pro-inflammatory reaction that restricts *Mtb* iron access [16]. In response, *Mtb* manufactures siderophores, molecules capable of binding iron more strongly than host iron-storage proteins [reviewed in [21]]. Siderophore biosynthesis has been shown to be essential for *Mtb* growth and virulence [17,19], suggesting that the success of *Mtb* in iron-scarce environments is due in part to its ability to acquire host iron. Despite the toxicity associated with iron excess, *Mtb* appears to also thrive when iron availability is increased [16]. High macrophage iron stores have been linked to an increased likelihood of contracting *Mycobacterium spp.* infections [22], and dietary iron

Abbreviations: BMI, body mass index; CI, confidence interval; Hb, hemoglobin; IQR, interquartile range; IRR, incidence rate ratio; MCV, mean corpuscular volume; Mtb, *Mycobacterium tuberculosis*; PTB, pulmonary tuberculosis; sTfR, soluble transferrin receptor; TB, tuberculosis; TBCC, TB case-contact; TST, tuberculin skin test.

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overload has been associated with an increased risk of developing pulmonary TB (PTB) [23] or dying from TB [24].

Only a minority of exposed or infected people ever progress to clinical TB disease during their lifetimes. Among those that do progress, it typically occurs after a long clinical latency period. This presents considerable challenges to studying TB susceptibility risk factors since biomarkers of TB susceptibility need to be collected prior to the initiation of disease pathogenesis. As a result, biomarker studies require the prospective follow-up of large numbers of contacts of known infectious TB cases for a period of several years. To overcome this obstacle, this case-cohort study was designed to investigate iron homeostasis biomarkers as risk factors for progression to TB using archived plasma samples that were obtained as part of a larger TB case-contact study.

2. Methods

2.1. Study participants and definitions

Cryopreserved plasma and data from household contacts of PTB index cases recruited to the ongoing TB case-contact (TBCC) platform at the Medical Research Council Unit in The Gambia were used. The TBCC is a unique research platform designed to identify TB disease susceptibility risk factors in a large cohort of household contacts and has been described in detail elsewhere [25]. Household contacts of index PTB cases were eligible to participate in the current case-cohort study if ≥15 years of age, maintained a shared residence with an index PTB case for ≥3 months prior to index case disease diagnosis and had a sufficient plasma sample archive for iron homeostasis analysis. All eligible TB-progressors cases identified within the larger cohort were included, as well as a random selection of non-progressors. In addition, TB-progressors and non-progressors were compared with a third group of culture-confirmed active PTB cases at TB diagnosis (Supplemental Figure 1). Ethical approval for the TBCC platform was granted by The Gambian Government/Medical Research Council Unit joint ethics committees, and additionally, for this case-cohort study by Cornell University.

All participants had a Tuberculin Skin Test (TST) (2 tuberculin units of Purified Protein Derivative RT23, Staten Serum Institute, Denmark) and underwent a clinical evaluation that included a co-morbidity assessment with TB-specific clinical questions. Household contacts with symptoms consistent with TB were classified as "TB-progressors" if they developed active TB ≥90 days after study enrollment or were classified as "non-progressors" if they did not have evidence of TB throughout the follow-up period (Supplemental Figure 1). TB-progressors were further categorized as "early TB-progressors" if they progressed to TB within two years of study entry or "delayed TB-progressors" if they developed TB after two years of follow-up.

2.2. Clinical and laboratory data

Enrollment characteristics included data on age, gender, body mass index (BMI), HIV-seropositivity, hemoglobin (Hb), white blood cell count and mean corpuscular volume (MCV). Plasma ferritin (Immuno-biological Laboratories, Germany), soluble transferrin receptor (sTfR) (R&D Systems, UK) and transferrin (Cygnus Technologies, USA) were measured by ELISA. Hepcidin was measured using a competitive enzyme immunoassay (Bachem, USA). All assays were optimized for use with plasma, and test samples, standards and controls were assayed in duplicate and concentrations interpolated from 4-parameter logistic standard curves (log/log **curves for sTfR). All standard curves were generated using SoftmaxPro 6 (Molecular Devices,** USA). Samples with an intra-assay coefficient of variation >15%, were re-assayed. Lower limits of detection for all assays, with the exception of hepcidin, were defined by the manufacturer. For hepcidin, the limit of detection (0.02 ng/ml) was interpolated at three standard deviations from the all plate mean (e.g. wells that contained diluent in lieu of hepcidin standard or sample).

2.3. Statistical analysis

Median values of biomarkers and clinical measurements with non-normal distributions were compared using the Mann–Whitney *U*-test. Unpaired Student's *t* tests were similarly used for normally distributed biomarkers and measurements as well as comparisons of small samples. Chi-square tests were used to compare frequency distributions. Since median and/or mean concentrations of ferritin, sTfR and hemoglobin fell within normal clinical reference ranges, and a reference range for hepcidin has not yet been defined, iron homeostasis biomarker concentrations were standardized and modeled continuously. Univariate associations between standardized iron biomarkers and the binary outcome of "TB progression" or "no TB progression" were assessed using Poisson regression with robust variance estimates. Poisson regression is considered an alternative to log-binomial regression when outcomes are common and odds ratios cannot be interpreted as relative risks [26]. Given the mixed HIV-status of the TB-progressor group, all analyses were run in parallel with two groups of TB-progressors: one inclusive of the two HIV-seropositive individuals, the other excluding them. Examination of iron homeostasis biomarker concentrations (hemoglobin, ferritin, hepcidin, soluble transferrin receptor, transferrin) and clinical characteristics (BMI, MCV, white blood cell) suggested that HIV-infected TB-progressors were not statistically or clinically different from HIV-negative TB-progressors in measured risk factors. However, since the absolute CD4 cell counts were unknown and because HIV infection is known to be a strong risk factor for TB progression, the results of both the HIV-inclusive and HIV-exclusive analyses are presented. Statistical analyses were performed using STATA 13.0 (College Station, TX USA) and Prism 6.0 (La Jolla, CA USA).

3. Results

Demographic and clinical characteristics of the TBCC platform, the case-cohort subsample drawn from the TBCC platform and the active TB cases are summarized in Table 1. Overall, the case-cohort subsample was representative of the larger TBCC platform as participants were not significantly different based on age, gender, BMI, white blood cell and MCV. The median time to progression for all case-cohort TB-progressors was 910 days [interquartile range (IQR): 470–1337 days, n = 10], and slightly longer at 1047 days (IQR: 611 to 1426, n = 8) among TB-progressors who were HIV-negative. Early case-cohort TB progressors were diagnosed at approximately one year following study entry, and this did not differ according to HIV-seropositivity, while delayed progressors were diagnosed at a median of 3.2 years. Known TB risk factors like age, gender and HIV status were not statistically different between TB-progressors, non-progressors or active TB cases. While BMI did not differ between TB-progressors and non-progressors, TB cases had significantly lower BMI and MCV and higher white blood cells. Additionally, significant differences were not detected in the comparison between TB-progressors when grouped with or without the two HIV-seropositive TB-progressors.

A dose-response relationship for concentrations of iron homeostasis biomarkers in non-progressors, early or delayed TB-progressors and active TB is shown in Figure 1. The lowest