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Modulation of iron status biomarkers in tuberculosis-diabetes co-morbidity

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Abstract

Tuberculosis (TB) and diabetes mellitus (DM) remain vital disease burdens in developing countries and the dual burden of DM and TB clearly signifies a growing global public health concern. While modulation of iron status biomarkers in TB is well described, very little is known about the association of these markers with TB-DM. To examine the association of circulating iron status biomarkers in TB disease, we examined the systemic levels of ferritin, hepcidin, soluble transferrin receptor (sTfR), transferrin, apotransferrin and hemopexin in pulmonary TB (PTB) individuals with DM (PTB-DM), without DM (PTB) and those with diabetes only (DM). Circulating levels of ferritin and hepcidin were significantly enhanced in PTB-DM and PTB compared to the DM group. On the other hand, the circulating levels of transferrin and apotransferrin were significantly diminished in PTB-DM and PTB compared to the DM group. The levels of ferritin and hepcidin exhibited a significant positive relationship with HbA1c, whereas apotransferrin exhibited negative relationship with HbA1c in PTB-DM and PTB. ROC analysis revealed that ferritin, hepcidin and transferrin are markers that can distinguish PTB-DM from DM individuals. Our results suggest that some of these circulating iron status markers could prove useful as biomarkers to monitor disease severity.

Introduction

The emerging epidemic of diabetes mellitus (DM) has substantially affected developing countries, where tuberculosis (TB) is also co-endemic [33]. DM is commonly associated with a higher susceptibility to pulmonary infections, with the most common pathogen being *Mycobacterium tuberculosis* (Mtb) [1]. The association of DM and TB has significant public health implications; patients with DM are approximately three times more likely to progress

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active TB than those without DM [2] and globally it is been reported that 15–25% of annual incident TB cases are attributable to DM [3, 4]. Fighting this double burden is challenging given that the mechanisms underlying the increased susceptibility of individuals with DM to TB remain unclear. The convergence of TB and DM diseases and increased morbidity and mortality raises meaningful issues regarding infection control and the reemergence of intracellular bacterial infections [5].

Iron is a fundamental cofactor for both mycobacterial growth during infection and for an effective protective immune response by the host [6]. Host iron status and iron trafficking among the host and pathogen may affect the serious host-pathogen battle for necessary iron, and thus influence the development of TB [7]. Excessive free iron is toxic to the cell and can also offer a source of iron for invading pathogens [34]. In contrast, very low iron prevents the host from mounting a successful host immune response during infection [8]. Thus, the regulation of iron by the host must be precisely balanced by providing adequate iron for successful host immune responses [9]. It has been also reported that anemia is more likely to occur among TB patients compared to healthy controls [10]. More notably, anemia is associated with more severe forms of TB [11]. However the role of the iron status biomarkers (ferritin, hepcidin, soluble transferrin receptor (sTfR), transferrin, apotransferrin and hemopexin), which are known to influence the development of anemia) in TB-DM has never been explored.

We, therefore, examined the systemic levels of iron status biomarkers in pulmonary TB patients with DM (PTB-DM), without diabetes (PTB) and diabetes (DM) group. Our data reveal that iron status biomarkers are significantly altered in PTB patients with and without DM. Our data also shows a significant association of plasma levels of ferritin, hepcidin and apotransferrin with HbA1c and significant ability of ferritin, hepcidin and transferrin to distinguish PTB-DM from DM. Our results also suggest that the factors mentioned above could serve as accurate biomarkers for monitoring disease severity.

Materials and Methods

Ethics statement

All individuals were examined as part of a natural history study protocol approved by the Institutional Review Board of the National Institute of Research in Tuberculosis, and informed written consent was obtained from all participants.

Study population

We studied a group of 88 individuals with PTB, 44 of whom had DM (PTB-DM) and 44 of whom had no diabetes (PTB). We also studied another 44 individuals who had diabetes alone (DM) 44. The baseline characteristics including demographics and biochemical parameters of the study population are shown in Table 1. PTB diagnosis was based on culture positivity of sputum samples. Two sputum samples per subject were examined by fluorescence microscopy, processed by the modified Petroff's method and cultured on Lowenstein-Jensen medium. Presence of acid-fast bacilli (AFB) in sputum smears was also documented. LTb was diagnosed on the basis of being positive for the Mantoux skin test

(induration > 12 mm) using 2TU PPD (Serum Statens Institute) and also being Quantiferon-in-Tube Gold assay positive with no chest symptoms of TB and a normal chest radiograph. Type 2 DM was diagnosed on the basis of glycated hemoglobin (HbA1c) levels and random blood glucose, according to the American Diabetes Association criteria (all Type 2 DM individuals had HbA1c levels > 6.5% and random blood glucose > 200 mg/dL) [35]. All the individuals were HIV negative. All individuals were anti-tuberculosis treatment naïve. Biochemical parameters, including plasma glucose and HbA1c were obtained using standardized techniques.

ELISA

Plasma cytokines were measured using ELISA, according to the manufacturer's instructions. The parameters analyzed were ferritin (Orgentec Diagnostika); transferrin and hemopexin (Genway); hepcidin and apotransferrin (USCN Life Science Inc). Soluble transferrin receptor (sTfR) was measured using Quantikine ELISA kit (R&D Systems). The lowest detection limits were as follows: ferritin, 15 ng/mL; transferrin, 9.375 ng/mL; hemopexin, 6.25 ng/mL; hepcidin, 62.5 ng/mL; apotransferrin, 0.156 ng/mL; sTfR, 3 nmol/L. We have assigned the lowest standard value to the samples that were below the threshold of detection.

Statistical Analysis

Geometric means (GM) were used for measurements of central tendency. Statistically significant differences between the three groups were analyzed using the Kruskal-Wallis test with Dunn's multiple comparisons. Receiver Operator Characteristics (ROC) curves was designed to test the power of each candidate iron status biomarker to distinguish PTB from PTB-DM or DM. Analyses were performed using Graph-Pad PRISM Version 6.

Results

Circulating Iron Status Biomarkers Are Significantly Altered in PTB and PTB-DM

To determine the systemic levels of iron status biomarkers in PTB-DM and PTB, we measured the circulating levels of ferritin, hepcidin, sTfR, transferrin, apotransferrin and hemopexin in PTB-DM, PTB and DM individuals, (Fig. 1). As shown in Fig. 1, the systemic levels of ferritin (GM of 36.51 ng/ml in PTB-DM versus 25.68 ng/ml in PTB and 5.03 ng/ml in DM) and hepcidin (GM of 284.4 ng/ml in PTB-DM versus 207.3 ng/ml in PTB and 94.03 ng/ml in DM) were significantly enhanced in PTB-DM and PTB compared to DM individuals. In contrast, systemic levels of transferrin (GM of 26.97 ng/ml in PTB-DM versus 55.95 ng/ml in PTB and 96.63 ng/ml in DM) were diminished in PTB-DM and PTB compared to DM. The systemic levels of apotransferrin (GM of 1.561 ng/ml in PTB-DM versus 3.19 ng/ml in PTB versus 1.91 ng/ml in DM) was significantly diminished in PTB-DM and DM compared to PTB individuals. Thus, PTB-DM and PTB are associated with altered circulating levels of iron status biomarkers.

Hematological features of the study population

To determine the hematological profile of PTB-DM, PTB and DM individuals, we measured hematological parameters like hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red

blood cells (RBC) and white blood cells (WBC) in our study population (Fig. 2). As shown in Fig. 2, PTB-DM and PTB exhibited significantly diminished levels of Hb (GM of 12.09 g/dL in PTB-DM versus 11.8 g/dL in PTB and 13.29 g/dL in DM) and MCV (GM of 77.62 fL in PTB-DM versus 76.39 fL in PTB and 83.63 fL in DM) in comparison with DM individuals. On the other hand, WBC levels (GM of $10.83 \times 10^3/\text{ml}$ in PTB-DM versus $10.15 \times 10^3/\text{ml}$ in PTB and $8.57 \times 10^3/\text{ml}$ in DM) were significantly enhanced in PTB-DM and PTB in comparison with DM individuals. No significant differences were seen in other hematological parameters like MCH, MCHC and RBC counts between the study groups.

Iron Status Biomarkers exhibit a borderline relationship with hematological parameters in PTB

To determine the relationship between iron status biomarkers and hematological indices in PTB-DM and PTB, we examined the correlation between iron status markers and hematology parameters in individuals with PTB with and without DM. As shown in Table 2, hepcidin exhibited a significant positive correlation with hemoglobin levels, whereas ferritin and sTfR exhibited a significant positive correlation with MCV and MCH. No significant correlations were seen in other iron status markers like transferrin, apotransferrin and hemopexin.

Iron Status Biomarkers exhibit a relationship with hyperglycemia in PTB

To determine the influence of hyperglycemia on iron status biomarkers in PTB-DM and PTB, we examined the correlation between iron status markers and HbA1c levels or random blood glucose in individuals with PTB with and without DM. As shown in Fig. 3A ferritin and hepcidin exhibited a significant positive correlation with HbA1c levels, while apotransferrin exhibited a significant negative correlation. On the other hand, as shown in Fig. 3B, iron status biomarkers exhibited no significant correlation with random blood glucose in individuals with PTB with and without DM. Overall, the results indicate an at least minimal association of these markers with hyperglycemia.

Iron Status Biomarkers don't exhibit a relationship with hyperglycemia in DM

To determine the influence of hyperglycemia on iron status biomarkers in pure diabetic population, we examined the correlation between iron status markers and HbA1c levels or random blood glucose level in individuals with DM. As shown in table 3 iron status biomarkers exhibited no significant correlation with HbA1c and random blood glucose in individuals with DM. Overall, the results indicate there is no association of these markers with hyperglycemia in diabetic population

Circulating Iron Status Biomarkers are markers of disease severity in PTB-DM and PTB

To determine the association between the systemic levels of iron status markers and disease severity in PTB-DM and PTB, we measured the circulating levels of ferritin, hepcidin, sTfR, transferrin, apotransferrin and hemopexin in PTB individuals with and without DM with unilateral versus bilateral disease and with cavitary versus non-cavitary disease (Figure 4). As shown in Figure 4A, the systemic levels of ferritin (GM of 47.62 ng/ml in bilateral vs. 26.95 ng/ml in unilateral disease) and hepcidin (GM of 362.3 ng/ml in bilateral vs. 211.6

ng/ml in unilateral disease) were significantly higher in PTB individuals with bilateral disease compared to unilateral disease, whereas transferrin levels (GM of 14.53 ng/ml in bilateral vs. 46.66 ng/ml in unilateral disease) were diminished in PTB individuals with bilateral disease compared to unilateral disease. Similarly, as shown in Figure 4B, the circulating levels of ferritin (GM of 43.33 ng/ml in cavitary vs. 24.33 ng/ml in non-cavitary disease) and hepcidin (GM of 360.1 ng/ml in bilateral vs. 197.9 ng/ml in unilateral disease) were significantly higher in PTB individuals with cavitary disease compared to those without cavitary disease. In contrast, transferrin levels (GM of 18.78 ng/ml in bilateral vs. 49.89 ng/ml in unilateral disease) were diminished in PTB individuals with cavitary disease compared to those without cavitary disease. Thus, disease severity in PTB is associated with altered systemic levels of circulating iron status markers.

Circulating Iron Status Biomarkers can distinguish PTB-DM from DM individuals

To determine the discriminatory power of circulating iron status biomarkers in distinguishing PTB-DM from PTB and PTB-DM from DM, we performed ROC analysis of ferritin, hepcidin, sTfR, transferrin, apotransferrin and hemopexin in PTB-DM versus PTB (Fig. 5A) and in PTB-DM versus DM (Fig. 5B) individuals. As shown in Fig. 5A iron status markers did not exhibit a significant AUC and sensitivity and specificity in discriminating PTB-DM from PTB individuals. In contrast, as shown in Fig. 5B, ferritin, hepcidin and transferrin exhibited a significant AUC and sensitivity and specificity in discriminating PTB-DM from DM individuals. Thus, ferritin, hepcidin and transferrin exhibit the potential to serve as accurate biomarkers of active TB to distinguish active PTB-DM from DM.

Discussion

Diabetes mellitus is related to compromised immunity and greater susceptibility to pulmonary infections, with the most common pathogen being *Mycobacterium tuberculosis* [1]. DM is associated with an augmented risk of infectious diseases and their complications, including an average two fold higher risk of mortality compared with non diabetic individuals [12]. The biological basis for the increased susceptibility to TB in DM patients is likely due to their dysfunctional immunity [13, 14]. Previously published data reported that there is an altered host iron status and this iron status acts as a risk factor for progression to TB among HIV-infected individuals [7, 15], and other studies have also indicated that *Mtb* iron acquisition plays an important role in TB pathogenesis. [16, 17]. However, the role of circulating iron status biomarkers in PTB-DM, PTB and DM has not been explored in detail.

Ferritin plays an important role in iron homeostasis by binding and separating intracellular iron. It acts as an acute-phase reactant, and elevated levels ferritin are typically connected with both acute and chronic inflammatory conditions [18]. Previously published data reports that serum ferritin is significantly elevated in patients with PTB compared to tuberculin skin test positive or negative individuals [19–21]. Our data clearly expand on these reports and confirm that ferritin exhibits enhanced levels in PTB-DM and PTB compared to DM and in addition, our data also report that ferritin levels were also positively correlated with hyperglycemia and hemoglobin associated markers like MCV and MCH among patients with tuberculosis with and without DM.

Hepcidin has an important regulatory role in host iron homeostasis during infection, and pathogen iron homeostasis including both iron acquisition and storage influences TB pathogenesis [22]. Inflammation or excess iron stores lead to enhanced levels of hepcidin expression and synthesis [23]. Hepcidin has antimicrobial properties and appears to have an important role in the innate immune response against *Mtb* infection [24, 25]. Limited number of clinical studies have demonstrated an association between elevated hepcidin concentrations and TB [15, 26]. Previously published data reports that increased plasma hepcidin levels, may indicate anemia of inflammation, which indicates harmful effects of iron supplementation often observed during infections [27]. Hepcidin also displays a similar profile to ferritin in our study. Hepcidin also exhibited enhanced levels in PTB-DM and PTB compared to DM. Similar to our finding, studies from only tuberculosis or HIV associated tuberculosis patients have shown enhanced hepcidin levels compared patients without tuberculosis [26, 28]. In addition, our results also report that hepcidin concentrations were correlated with hyperglycemia and hemoglobin levels among PTB patients with and without DM. Our results indicate that hepcidin concentrations are related to tuberculosis-diabetes co-morbidity.

Transferrin is an iron-binding glycoprotein that controls the level of free iron, which binds to the iron transport protein, transferrin, for delivery to tissues and erythroblasts [36]. Like transferrin, apotransferrin also has a physiological role in the transportation and distribution of iron among the body organs. Serum soluble transferrin receptor (sTfR) levels reflect the degree of availability of iron for cells [29]. Previously published data reports that transferrin is diminished, whereas sTfR is increased in patients with tuberculosis compared to tuberculin skin test positive or negative individuals [26]. In our study, transferrin and apotransferrin were significantly diminished in PTB-DM and PTB compared to DM. On the other hand, sTfR displayed no significant differences between the groups. Other immunological studies from HIV infected patients also report that altered host iron status characterized by low transferrin has been identified as a risk factor for progression to TB among HIV-infected individuals [30]. These results indicate that altered levels of iron status markers are associated with iron homeostasis and inflammation in PTB patients with and without DM.

Evidence links iron with TB pathogenesis both from the side of the pathogen and the host. Upon infection, host immune recognition of *Mtb* induces a pro-inflammatory reaction that limits iron access, since host-acquired iron is a co-factor in essential *Mtb* cellular processes [16]. Published data clearly authenticate that decreasing iron availability reduces *Mtb* growth, and addition of iron to *Mtb* almost always enhances growth [16]. Characterizing the role of iron in PTB-DM and PTB from the perspective of the host immunity is important. Our study is the first to examine the circulating levels of iron status biomarkers in TB-DM co-morbidity and we report that ferritin, hepcidin and transferrin are important and accurate biomarkers for differentiating PTB-DM from DM and also that elevated circulating levels of ferritin and hepcidin reflect disease severity. In contrast, transferrin appears to be elevated only in DM individuals, although its biological significance is yet to be determined. These observations are consistent with animal and in vitro studies linking addition of iron to increased *Mtb* growth [16], and also existing evidence indicating that elevated ferritin and hepcidin along with decreased transferrin and hemoglobin are risk factors for incident TB in

a Gambian HIV-seropositive cohort [7, 15]. Since iron deficiency is recognized as an important contributor to TB-associated anemia, MCV reflects the mean RBC volume and has been the most broadly used index for the assessment of nutritional iron deficiency [37]. Similar to our finding of low MCV in PTB, low MCV values were also reported in other cohorts of TB patients [31, 32]. In addition, our finding also reports that ferritin and sTfR concentrations were correlated with MCV and MCH, while hepcidin concentrations were correlated with hemoglobin levels indicating that iron deficiency may be related with impaired immune function and reduced capacity to control infection.

Our study is the first to systematically explore the association of circulating iron status markers in human pulmonary TB patients with coexisting DM. While our study is purely descriptive in nature and does not prove any cause and effect, it however provides important evidence of association and therefore the need to explore the role of these factors in pathogenesis further. In addition, examination of iron homeostasis following completion of anti-TB treatment should provide an additional input on the pathogenesis of TB-DM co-morbidity. Moreover, monitoring iron homeostasis biomarkers in LTb individuals with or without DM may be a useful indicator, in combination with other early-onset biomarkers, to identify individuals at greater risk for progression to TB. Since some of the iron status biomarkers were strongly associated with disease severity, this implies that anemia may be a common and unfavorable consequence of this response. In summary, this study provides valuable insights on iron status markers that are potential use as diagnostic and prognostic biomarkers in TB-DM co-morbidity.

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Figure 1. Altered iron status biomarkers in PTB-DM and PTB

The plasma levels of iron status biomarkers (ferritin, hepcidin, sTfR, transferrin, apotransferrin and hemopexin) were measured in PTB-DM (n=44), PTB (n=44) and DM (n=44) individuals. The data are represented as scatter plots with each circle representing a single individual. P values were calculated using the Kruskal-Wallis test with Dunn's post hoc comparison.

Figure 2. Altered hematological profile in PTB-DM and PTB

Hematological parameters (Hb, MCV, MCH, MCHC, RBC, WBC) measured in PTB-DM (n=44), PTB (n=44) and DM (n=44) individuals. The data are represented as scatter plots with each circle representing a single individual. P values were calculated using the Kruskal-Wallis test with Dunn's post hoc comparison. .

A

Figure 3. Relationship between iron status biomarkers and hyperglycemia in PTB-DM and PTB
 (A) The relationship between the plasma levels of iron status markers and HbA1c in all PTB individuals with or without DM (B) The relationship between the plasma levels of iron status markers and random blood glucose (RBG) in all PTB individuals with or without DM. The data are represented as scatter plots with each circle representing a single individual. P values were calculated using the Spearman Rank Correlation.

Figure 4. Altered circulating levels of iron status biomarkers in bilateral and cavitary disease in PTB individuals with or without DM

(A) The plasma levels of ferritin, hepcidin, sTfR, transferrin, apotransferrin and hemopexin were measured in PTB-DM and PTB individuals with bilateral unilateral disease. (B) The plasma levels of ferritin, hepcidin, sTfR, transferrin, apotransferrin and hemopexin were measured in PTB-DM and PTB individuals with cavitary versus non-cavitary disease. The data are represented as scatter plots with each circle representing a single individual. P values were calculated using the Mann-Whitney test

A : PTB DM vs PTB

B : PTB DM vs DM

Figure 5. ROC analysis to estimate the discriminatory power of systemic iron status biomarkers in PTB-DM and DM

(A) ROC analysis to estimate the sensitivity, specificity and area under the curve was performed using ferritin, hepcidin, sTfR, transferrin, apotransferrin and hemopexin to estimate the capacity of these factors to distinguish PTB-DM versus PTB individuals. (B) ROC analysis to estimate the sensitivity, specificity and area under the curve was performed using ferritin, hepcidin, sTfR, transferrin, apotransferrin and hemopexin to estimate the capacity of these factors to distinguish PTB-DM versus DM individuals.

Table 1

Demographics of study groups

	PTB-DM	PTB	DM	pValue
No. of subjects recruited	44	44	44	NA
Gender (M/F)	32/12	33/11	24/20	p=0.0806
Median Age (Range)	47 (18–63)	42 (18–67)	43 (18–65)	p=0.0912
Smear Grade: 0/1+/2+/3+	0/15/15/14	0/20/14/10	–	NA
Tuberculin Skin Test (TST)	–	–	>12mm	NA
Interferon gamma release assay	–	–	Positive	NA
Random Blood Glucose, mg/dl	252 (204 – 653)	98 (78 – 113)	174 (158 – 441)	p<0.0001
Glycated hemoglobin level, %	10.3 (7.01 – 16.16)	5.8 (5.01 – 6.05)	7.55 (6.8 – 11.72)	p<0.0001

Table 2

Relationship of Iron Status Biomarkers with Hematology Parameters

	Hb		MCV		MCH		MCHC	
	p Value	r Value	p Value	r Value	p Value	r Value	p Value	r Value
Ferritin	0.9786	-0.002978	0.0186	0.2611	0.0227	0.2529	0.3552	0.1041
Hepcidin	0.0318	-0.2344	0.5646	0.06495	0.2869	0.1198	0.4356	0.08783
sTfR	0.6293	-0.05343	0.0051	-0.3084	0.0064	-0.3004	0.8256	-0.02486
Transferrin	0.3626	0.1006	0.5519	0.06707	0.7557	0.0351	0.6414	-0.05252
Apotransferrin	0.8979	-0.01421	0.8563	-0.02044	0.3932	0.09615	0.3966	-0.09547
Hemopexin	0.3278	-0.1081	0.6645	0.04893	0.8735	0.01797	0.3966	-0.09547

Table 3

Relationship of Iron Status Biomarkers with hyperglycemia in diabetic population

	HbA1c		RBG	
	p Value	r Value	p Value	r Value
Ferritin	0.8052	−0.03828	0.8216	0.03542
Hepcidin	0.5407	0.09474	0.9831	0.003323
sTfR	0.9909	−0.001762	0.3955	−0.1329
Transferrin	0.2798	−0.1666	0.8658	0.02655
Apotransferrin	0.8776	−0.02389	0.4906	−0.108
Hemopexin	0.3024	−0.1591	0.6329	−0.07494