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Effect of anti-tuberculosis treatment on the systemic levels of tissue inhibitors of metalloproteinases in tuberculosis – Diabetes co-morbidity

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ABSTRACT

Objectives: To study the association of Tissue inhibitors of matrix metalloproteinases (TIMP) levels with tuberculosis-diabetes comorbidity (TB-DM) comorbidity at baseline and in response to anti-TB treatment (ATT).
Methods: We examined the levels of TIMP-1, -2, -3 and -4 in pulmonary tuberculosis alone (TB) or TB-DM at baseline and after ATT.
Results: TIMP-1, -3 and -4 were significantly increased in TB-DM compared to TB at baseline and after ATT. ATT resulted in a significant reduction in TIMP-2 and -3 levels and a significant increase in TIMP-1 in both TB and TB-DM. TIMP-1, -3 and -4 were also significantly increased in TB-DM individuals with bilateral, cavitary disease and also exhibited a positive relationship with bacterial burden in TB-DM and HbA1c in all TB individuals. Within the TB-DM group, those known to be diabetic before incident TB (KDM) exhibited higher levels of TIMP-1, -2, -3 and -4 at baseline and TIMP-2 at post-treatment compared to those newly diagnosed with DM (NDM). KDM individuals on metformin treatment exhibited lower levels of TIMP-1, -2 and -4 at baseline and of TIMP-4 at post-treatment.
Conclusions: TIMP levels were elevated in TB-DM, associated with disease severity and bacterial burden, correlated with HbA1c levels and modulated by duration of DM and metformin treatment.

1. Introduction

The TIMP family consists of four members (TIMP-1, -2, -3 and -4) with significant homology, that inhibit matrix metalloproteinases (MMPs) with some specificity. TIMPs are endogenous inhibitors of MMPs and regulate MMP response by forming 1:1 complexes with MMPs [1,2]. TIMP family is a biological inhibitor of several MMP enzymes, which are involved in the process of the tumor and cell invasion through the extracellular matrix and its expression is stimulated by several physiological triggers in various cell types [3,4]. TIMP-1 was previously determined to be critical in the immune response to TB [5]. TIMPs may also be crucial in the growth of fibrosis [6], which is characteristic of healing TB infection [7]. TIMPs have been advocated as potential biomarkers for TB with good sensitivity and specificity to discriminate TB from healthy individuals [8,9]. TIMPs (TIMP-1, -2 and -3) help in the remodeling and repair of tissue following destruction by matrix metalloproteinases (MMPs). Therefore, proteolytic balance between MMPs and TIMPs is vital in normal tissue remodeling and various pathological conditions [10].

Published studies have reported that TIMP levels were higher in serum and pleural fluid of TB patients compared to serum of healthy controls and non-TB pleural fluid [9]. We have also previously reported that TIMP-4 is a significant biomarker for the discrimination of TB-DM from TB [11]. However, a comprehensive analysis of the relationship of TIMPs with TB-DM and their association to disease pathology or bacterial burdens has not been performed. We have previously demonstrated that the clinical and biochemical characteristics of newly diagnosed DM individuals with TB are significantly different from those with TB and known DM [11]. Metformin is the most widely-used medication for type 2 diabetes and published studies have reported that it may be a

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candidate for host-directed therapy for TB [12,13]. Retrospective human studies indicate that metformin diminishes the risk of progression to active TB disease [14,15]. Similarly, the association of TIMPs with TB individuals with KDM or NDM or of TB-KDM individuals with or without metformin use has never been examined.

Therefore, the aim of this study was to examine the association of the systemic levels of TIMP-1, -2, -3 and -4 in TB-DM individuals and compare them with TB individuals without DM and healthy controls. We demonstrate elevated levels of TIMPs in association with TB-DM in comparison to TB and healthy controls. ATT resulted in a significant reduction in TIMP-2 and -3 levels and a significant increase in TIMP-1 in both TB and TB-DM.

2. Materials and methods

2.1. Ethics statement

The Ethics Committees of the Prof. M. Viswanathan Diabetes Research Center and NIRT approved this study. Informed written consent was obtained from all participants.

2.2. Study population

All the study participants were prospectively recruited from ten participating clinics (TB units) in and around Chennai. Study participants were identified on the basis of being smear positive for acid-fast bacilli and enrolled on being *Mycobacterium tuberculosis* culture positive on solid media. Study participants were 25–60 years of age and excluded if they had prior episode of TB disease, had received >7 days of treatment for TB disease, had taken more than seven doses of a fluoroquinolone within the past 30 days, were pregnant or nursing, were seropositive for HIV, or were receiving immunosuppressive therapy.

Plasma samples were collected from 64 individuals with TB-DM and 24 individuals with TB without DM and 24 healthy control individuals, recruited in Chennai, India. This was the same set of individuals previously used for studying the association of MMPs with TB-DM [16]. To define cavitary disease as well as unilateral versus bilateral lung involvement, chest X-rays were used. To define bacterial burdens smear grades were used and they are as classified as 1+, 2+ and 3+. Glycemic status (DM or normoglycemia) was diagnosed on the basis of oral glucose tolerance test and/or HbA1c levels (for known diabetics), according to the WHO criteria. Amongst the 64 TB-DM individuals, 32 were KDM and 32 were NDM. Amongst the KDM individuals, 16 were on metformin containing anti-diabetic medication and 16 were not. The study groups were matched with regard to age and gender and the baseline characteristics of the study participants are shown in Table 1. Standard ATT was administered to TB-DM individuals using the directly observed treatment, short course (DOTS) strategy. At 6 months following ATT initiation, fresh plasma samples were obtained. All TB-DM and TB individuals were culture negative at the end of ATT.

2.3. ELISA

Circulating levels of TIMP-1, -2, -3 and -4 were estimated using a multiplex luminex assay system (Bio-Rad Laboratories, Inc) in plasma samples. The lowest detection limits were as follows: TIMP-1, 0.02 ng/mL; TIMP-2, 0.067 ng/mL; TIMP-3, 0.059 ng/mL; TIMP-4, 0.0067 ng/mL.

2.4. Statistical analysis

Geometric means (GM) were used for measurements of central tendency. Statistically significant differences between the two groups were analysed using the Mann Whitney test with Holm's correction for multiple comparisons. Linear trend post-test was used to compare TIMPs concentrations with smear grades (reflecting bacterial burdens) and Spearman rank correlation was used to compare TIMPs concentrations with HbA1c levels. Analyses were performed using GraphPad PRISM Version 7.

Table 1
Demographics of the study groups and biochemical parameters in TB-DM and TB.

Study Demographics	TB-DM	TB	HC	p Value
No. of subjects recruited	64	24	24	–
KDM/NDM	32/32	–	–	–
Metformin Rx Yes/No	16/16	–	–	–
Gender (Male/Female)	44/20	17/7	14/10	NS
Median Age (Range)	52 (31–70)	43 (30–67)	35(27–62)	NS
Median Height, cm	159 (129–176)	164 (121–181)	162 (125–190)	NS
Median Weight, kg	49 (31–64)	44 (30–90)	55 (45–90)	NS
Smear Grade: 0/1+/2+/3+	0/22/24/18	0/9/9/6	NA	–
Fasting Blood Glucose, mg/dL	158 (109–427)	93 (73–103)	88 (75–105)	**p < 0.0001**
Post Prandial Glucose, mg/dL	220 (183–448)	112 (80–129)	110 (78–120)	**p < 0.0001**
Glycated hemoglobin level, %	10.3 (7.3–15.6)	5.6 (5.0–5.8)	5.5 (5.0–5.7)	**p < 0.0001**

The values represent the geometric mean (and the 95% confidence intervals) except for age where the median (and the range) are depicted.

3. Results

3.1. Study population characteristics

The baseline characteristics including demographic and biochemical features of the study population are shown in Table 1. As shown, TB-DM individuals had significantly higher levels of fasting and post-prandial glucose as well as HbA1c compared to TB. No significant differences were observed in age, sex, smear or culture grades at baseline between the TB-DM and TB groups (Table 1).

3.2. Heightened levels of circulating TIMPs in TB-DM and alterations following ATT

We examined the systemic levels of circulating TIMPs in TB-DM, TB and HC individuals by measuring the circulating levels of TIMP-1, -2, -3 and -4 (Fig. 1). As shown, Fig. 1A, systemic levels of TIMP-1 (GM of 36.5 ng/ml in TB-DM vs. 22.2 ng/ml in TB vs 16.97 ng/ml in HC), TIMP-2 (GM of 4.4 ng/ml in TB-DM vs 3.1 ng/ml in HC), TIMP-3 (GM of 2.2 ng/ml in TB-DM vs. 1.1 ng/ml in TB vs 0.58 ng/ml in HC) and TIMP-4 (GM of 2.7 ng/ml in TB-DM vs 1.6 ng/ml in HC) were significantly higher in TB-DM compared TB or HC individuals.

We also examined the effect of ATT on TIMP levels in TB-DM individuals. As shown in Fig. 1B, there were consistent and statistically significant trends for a reduction in TIMP-2 and -3 in TB-DM. In marked contrast to the other TIMPs measured, the levels of TIMP-1 were consistently higher at TB treatment completion than at baseline in TB-DM. Thus, treatment of TB results in alteration of circulating levels of TIMPs, albeit with TIMP-1 trending in the opposite direction as the other TIMPs measured.

3.3. Circulating TIMPs are markers of radiographic TB disease severity and bacterial burdens in TB-DM

Since the circulating TIMP levels were significantly enhanced in TB-DM individuals, we wanted to determine the association between the systemic levels of TIMPs and disease severity in TB-DM. To this end, we measured the circulating levels of TIMPs in TB-DM individuals with

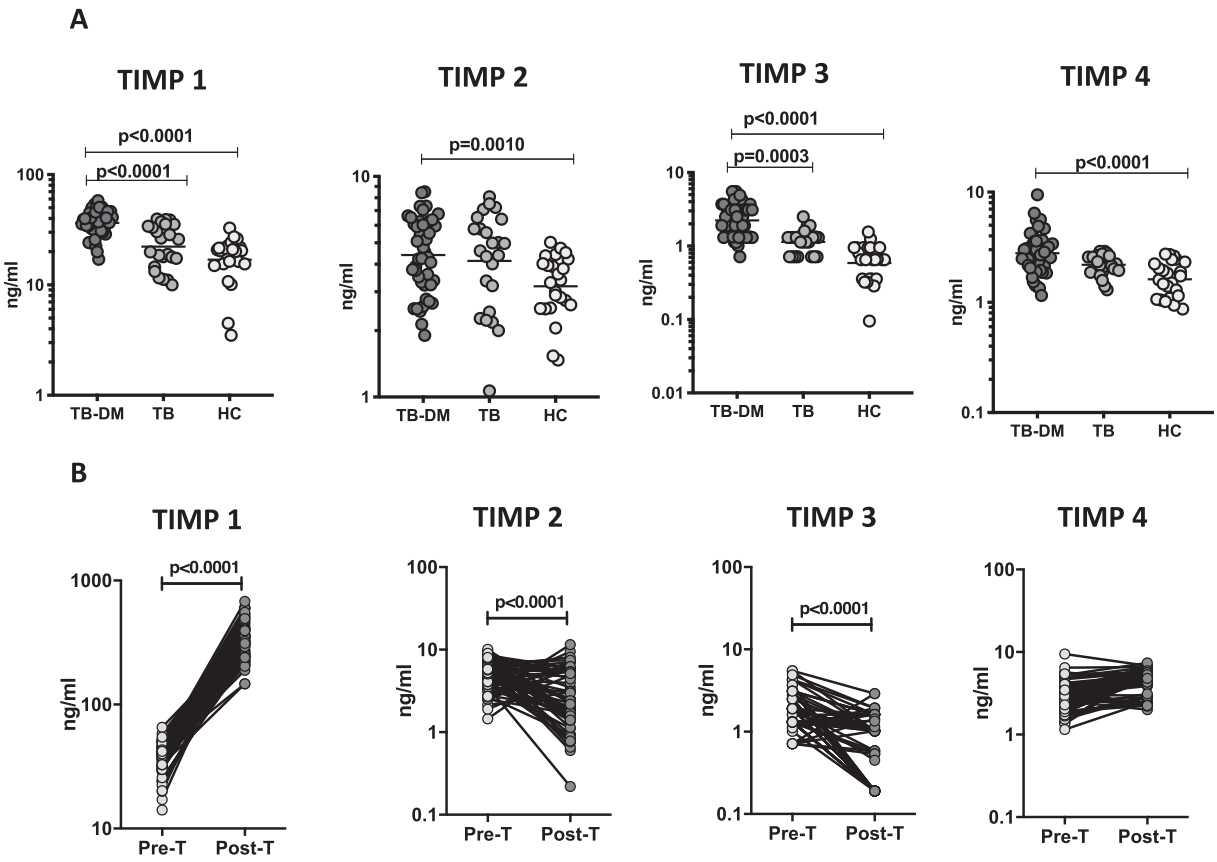

Fig. 1. Elevated circulating levels of TIMPs in TB-DM individuals. (A) The plasma levels of TIMP-1, TIMP-2, TIMP-3 and TIMP-4 were measured in TB-DM (n = 64), TB (n = 24) and HC (n = 24) individuals at baseline. The data are represented as scatter plots with each circle representing a single individual. P values were calculated using the Kruskal-Wallis test with Dunn's post-hoc for multiple comparisons. (B) The plasma levels of TIMP-1, TIMP-2, TIMP-3 and TIMP-4 were measured in TB-DM individuals at baseline (pre-T) and at 6 months of ATT (post-T). The data are presented as line graphs with each line representing a single individual. P values were calculated using the Wilcoxon signed rank test.

cavitary versus non-cavitary disease and unilateral versus bilateral disease at baseline. As shown in Fig. 2A, the circulating levels of TIMP-1 (GM of 42.962 ng/ml in cavitary vs. 33.993 ng/ml in non-cavitary disease), TIMP-3 (GM of 2.927 ng/ml in cavitary vs. 1.663 ng/ml in non-cavitary disease) and TIMP-4 (GM of 3.841 ng/ml in cavitary vs. 2.557 ng/ml in non-cavitary disease) were higher in TB-DM individuals with cavitary disease compared to those without. Similarly, as shown in Fig. 2B, the circulating levels of TIMP-1 (GM of 42.817 ng/ml in bilateral vs. 33.993 ng/ml in unilateral disease), TIMP-2 (GM of 62.412 ng/ml in bilateral vs. 1.692 ng/ml in unilateral disease) and TIMP-4 (GM of 3.489 ng/ml in bilateral vs. 2.522 ng/ml in unilateral disease) were higher in TB-DM individuals with bilateral disease compared to those with unilateral disease. To determine the association of circulating TIMPs and bacterial burdens, we performed a correlation of the circulating levels of TIMP family in TB-DM individuals with smear grades. As shown in Fig. 2C, TIMP-1, -3 and -4 exhibited a significant positive correlation with smear grades in TB-DM individuals, indicating a positive association of these factors with bacterial burdens. Thus, disease severity and bacterial burden in TB-DM are associated with elevated systemic levels of circulating TIMPs at baseline.

3.4. Circulating TIMPs exhibit a positive relationship with HbA1c in TB individuals and are increased in individuals with KDM

To elucidate the association between systemic levels of circulating TIMPs and glycemic control in TB patients with or without DM at baseline, we determined the relationship between the circulating levels of TIMPs in TB individuals with or without DM with HbA1c levels (Fig. 3A). As shown, the circulating levels of TIMP-3 and TIMP-4 exhibited a significant positive association with HbA1c levels in TB individuals, with or without DM at baseline showing a significant association of these factors with poor glycemic control. To determine whether TIMP levels differ based on the duration of diabetes in TB-DM, we estimated the systemic levels of TIMPs in KDM (Median HbA1c 10.5%) (n = 32) and NDM (Median HbA1c 6.8%) (n = 32) individuals. As shown in Fig. 3B, systemic levels of TIMP-1 (GM of 41.716 ng/ml in KDM vs. 31.369 ng/ml in NDM), TIMP-2 (GM of 6.359 ng/ml in KDM vs. 3.864 ng/ml in NDM), TIMP-3 (GM of 2.600 ng/ml in KDM vs. 1.437 ng/ml in NDM) and TIMP-4 (GM of 3.274 ng/ml in KDM vs. 2.479 ng/ml in NDM) were significantly higher in KDM compared to NDM individuals at baseline. As shown in Fig. 3C, systemic levels of TIMP-2 (GM of 3.552 ng/ml in KDM vs. 2.051 ng/ml in NDM) alone were significantly increased in KDM compared to NDM individuals upon completion of ATT. Thus, KDM is associated with elevated systemic levels of circulating TIMPs at baseline and TIMP-2 following standard ATT.

3.5. Metformin treatment is associated with diminished circulating TIMPs

Use of the anti-diabetic drug metformin has been associated with lower risk for TB infection, progression from TB infection to active TB disease and for mortality in TB-DM. To test whether this protective effect of metformin was reflected by differences in circulating TIMPs, we compared plasma TIMP levels in KDM individuals who reported use of metformin at baseline (n = 16) compared to those on non-metformin antidiabetic regimens (n = 16). Importantly, no significant differences were observed in HbA1c levels between KDM individuals on metformin (Median HbA1c 11.3%) compared to KDM individuals not on metformin (Median HbA1c 10.2%). As shown in Fig. 4A, systemic levels of TIMP-1

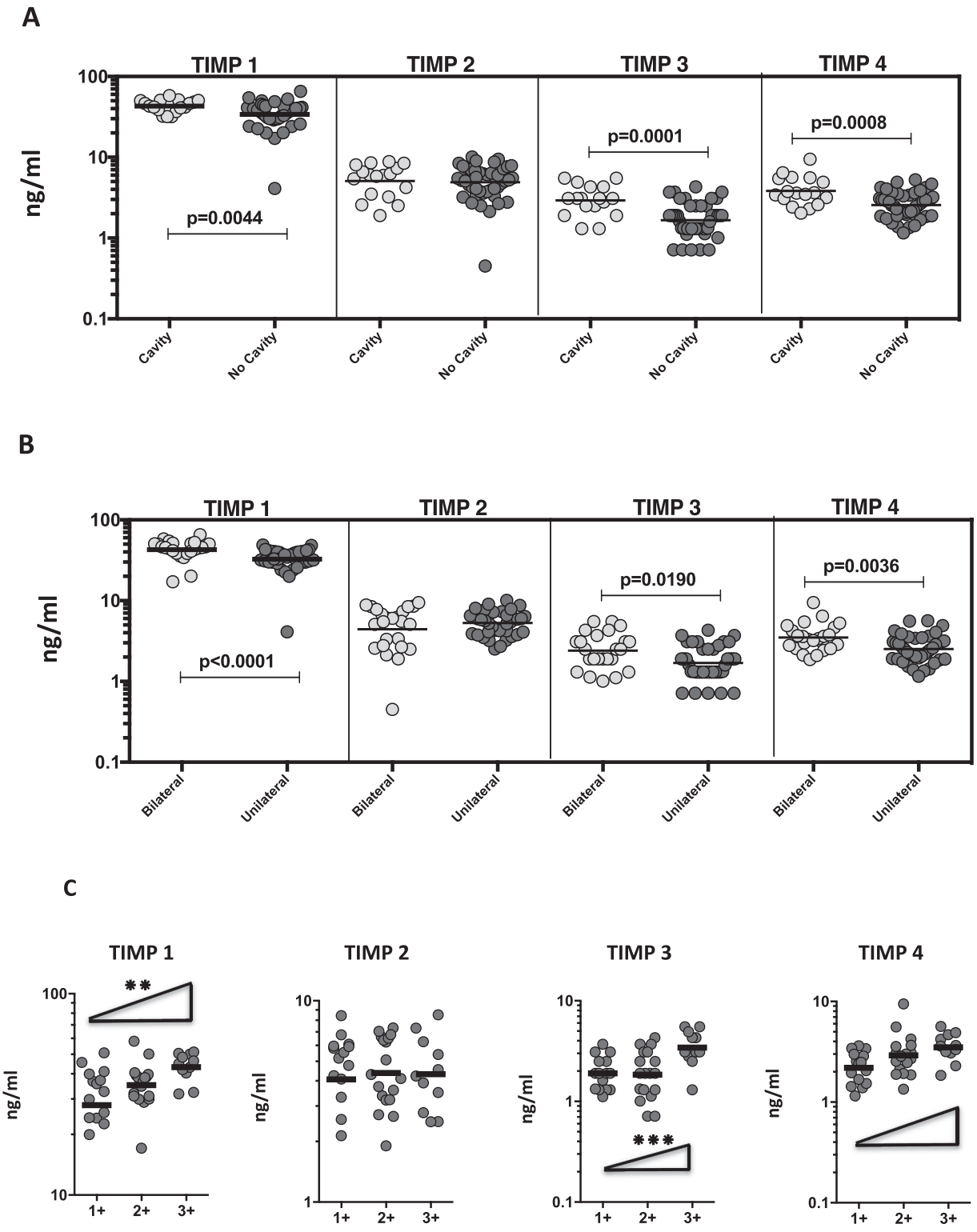

Fig. 2. Elevated circulating levels of TIMP 1, 3 and 4 in cavitary and bilateral disease in TB-DM individuals and relationship to bacterial burdens (A) The plasma levels of TIMP-1, TIMP-2, TIMP-3 and TIMP-4 were measured in TB-DM individuals with cavitary versus non-cavitary disease. (B) The plasma levels of TIMP-1, TIMP-2, TIMP-3 and TIMP-4 were measured in TB-DM individuals with bilateral versus unilateral disease. (C) The relationship between the plasma levels of TIMP-1, TIMP-2, TIMP-3 and TIMP-4 and smear grades as estimated by sputum smears was examined in TB-DM individuals. The data are represented as scatter plots with each circle representing a single individual. For bilateral and cavitary disease P values were calculated using the Mann-Whitney test with Holm's correction for multiple comparisons. For bacterial burden relationship P values were calculated using the Linear trend post – test.

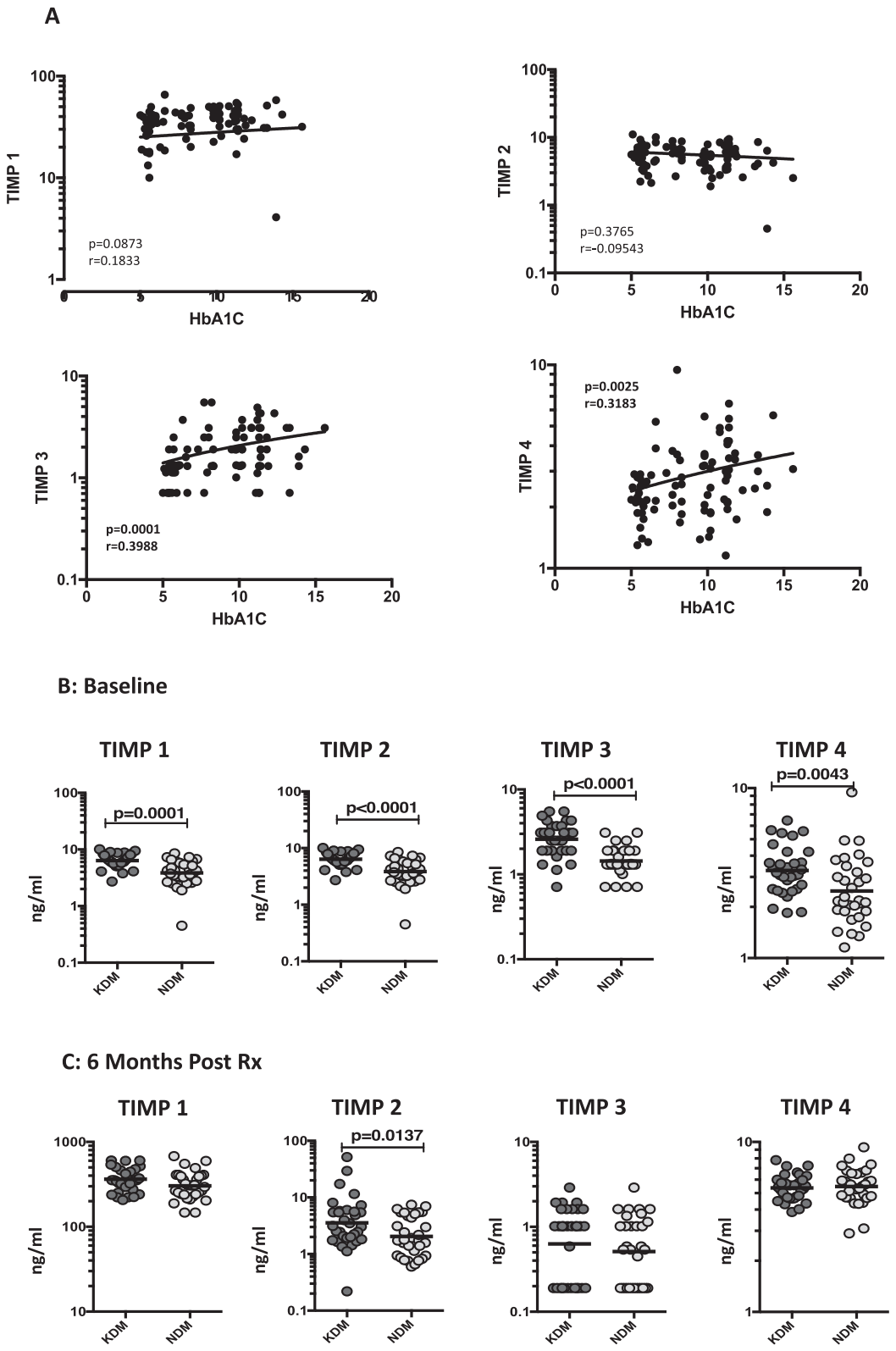

Fig. 3. Elevated TIMPs exhibit a positive relationship with HbA1c and are elevated in individuals with KDM (A) The relationship between the plasma levels of TIMP-1, TIMP-2, TIMP-3 and TIMP-4 and HbA1c levels was examined in all TB individuals with and without DM. The data are represented as scatter plots with each circle representing a single individual. (B) The plasma levels of TIMP-1, TIMP-2, TIMP-3 and TIMP-4 were measured in TB-DM individuals with known diabetes. (KDM) versus newly diagnosed diabetes (NDM) at baseline. (C) The plasma levels of TIMP-1, TIMP-2, TIMP-3 and TIMP-4 were measured in TB-DM individuals with known diabetes (KDM) versus newly diagnosed diabetes (NDM) at 6 months of ATT. The data are represented as scatter plots with each circle representing a single individual. For HbA1c P values were calculated using the Spearman Rank Correlation. For KDM, P values were calculated using the Mann-Whitney test with Holm's correction for multiple comparisons.

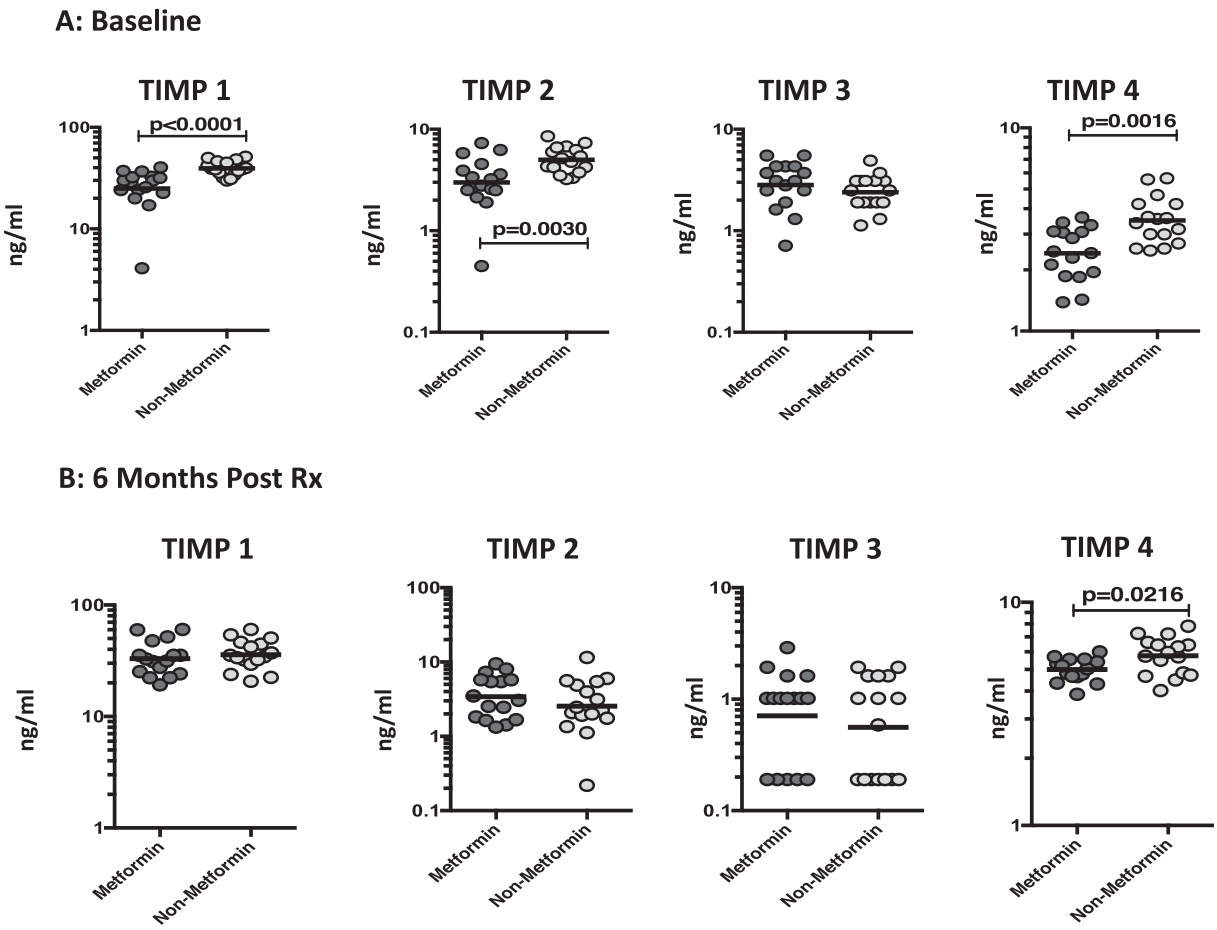

Fig. 4. Diminished circulating levels of TIMPs in KDM individuals on metformin treatment (A) The plasma levels of TIMP-1, TIMP-2, TIMP-3 and TIMP-4 were measured in KDM individuals on metformin treatment versus no metformin treatment at baseline. (B) The plasma levels of TIMP-1, TIMP-2, TIMP-3 and TIMP-4 were measured in KDM individuals on metformin treatment versus no metformin treatment at 6 months of ATT. The data are represented as scatter plots with each circle representing a single individual. P values were calculated using the Mann-Whitney test with Holm's correction for multiple comparisons.

(GM of 24.97 ng/ml in Metformin vs. 39.408 ng/ml in Non-Metformin), TIMP-2 (GM of 2.988 ng/ml in Metformin vs. 4.997 ng/ml in Non-Metformin) and TIMP-4 (GM of 2.414 ng/ml in Metformin vs. 3.506 ng/ml in Non-Metformin) were significantly diminished in KDM individuals on metformin compared to KDM individuals not on metformin. As shown in Fig. 4B, TIMP-4 (GM of 4.993 ng/ml in Metformin vs. 5.756 ng/ml in Non-Metformin) alone was significantly diminished in KDM individuals on metformin compared to KDM individuals not on metformin upon completion of ATT. Thus, metformin therapy in KDM individuals is associated with diminished systemic levels of circulating TIMPs.

4. Discussion

Many epidemiological and clinical studies have revealed that DM is one of the major risk factors for TB infection and DM is allied with a two to four-fold increased risk of active TB. Evidence from the published studies also reports that DM patients with uncontrolled blood glucose are at advanced risk to active TB than individual with controlled DM [17,18]. The interfaces amongst DM and TB are multidimensional and poorly known, though changes have been observed in innate and adaptive immune responses [19]. The detrimental effects of DM on TB incidence and consequences are now broadly accepted. More than a few studies have shown higher susceptibility to TB in animal models of TB-DM co-morbidity [20,21]. The actual mechanisms causing this susceptibility to TB are still vague and are in need of comprehensive evaluation. In addition to the heightened risk for TB, persons with TB-diabetes comorbidity have poorer ATT outcomes with longer times to sputum culture conversion, which in in turn leads to higher risk of death or treatment failure, and increased risk of relapse after successful completion of anti-TB treatment [22,23].

TIMPs are known to be important inhibitors of MMPs, and they are also gradually recognized to have impending roles in inflammatory response [24]. Published studies clearly report that presence of metalloproteinases and their inhibitors play an key role in integrity and remodeling of extra cellular matrix components in inflammatory conditions [25]. The imbalance of TIMP and MMP activities are linked to TB severity but this has not previously been explored in the context of TB-DM comorbidity. We, therefore hypothesized that alterations in TIMP levels would reflect disease pathogenesis, extent and severity of disease and response to treatment. Our existing analysis revealed that TB-DM patients exhibit significantly enhanced systemic levels of TIMP-1, -3 and -4 compared to TB individuals without DM and healthy controls. Other published studies have also reported that TIMP-1 concentrations were significantly elevated in TB patients in comparison to controls and also associated with disease severity [8]. In addition, a recently published study reported that TIMP-1 is a key biomarker for the diagnosis of TB [5]. It is also been well described and reported that TIMP-1 has been significantly elevated in the active TB disease in comparison to other pulmonary disorders like pneumonia [5]. Although the role of TIMPs in TB remain unclear, *M.tb* has been implicated to aggressively dysregulate the balance between MMPs and TIMPs [26]. Our study is one of the first to report on the systemic levels of TIMP expression following ATT. Our data suggest that while TIMP-1 levels are elevated, other TIMP levels

decrease significantly or do not change. A previous report on TIMP-1 levels in sputum showed decrease in TIMP-1 at 2 months post-treatment, which is different from our data on circulating levels albeit at a later time point [8]. Notably, Hwang et al [27] reported higher pleural fluid TIMP-1 levels in in TB pleuritis patients who went on to have residual pleural thickening. Our data also disclosed a significant relationship of TIMP levels with the severity of TB disease (as estimated by the bilateral and cavitary disease) and increasing bacterial burdens, showing that comorbid DM increases this response, which could reflect elevated bacterial load and/or a specific perturbation of immune function. Of further interest are the findings that TIMP levels are positively correlated with HbA1c, showing a relationship with poor glycemic control, which drives diabetic complications in all tissues [28,29].

Previously, we have reported that there was a bimodal distribution of baseline HbA1c between KDM and NDM individuals in our cohort, with significantly greater baseline A1c in the KDM group [30]. Our current study adds to this clear heterogeneity in the appearance of TB-DM co-morbidity. We determined that systemic TIMPs were significantly heightened in KDM in comparison to NDM at baseline and after completion anti-TB treatment, indicating the increased severity of TB disease in KDM individuals. Metformin is an approved antidiabetic drug in routine clinical use and has drawn attention as an impending adjunctive, host-directed therapy (HDT) for TB independent of its glucose-lowering activity [12,15,31]. Studies in mice reported that upon metformin treatment there is a lowering of bacterial burdens [31]. Enhanced immune control of TB in metformin-treated mice was associated with reduced systemic inflammation, which matches our finding that TIMP levels are lower in KDM individuals treated with metformin. Use of metformin has been linked to reduced risk for TB infection, for progression from TB infection to TB disease and for mortality during TB treatment in diabetic individuals [32]. Our results deliver new confirmation for a host-directed role for metformin in that individuals on metformin treatment revealed decreased systemic TIMP levels, recommending a host-protective effect of metformin in TB-DM with potential implications for its use in TB without DM.

Our results on TIMPs largely propose that heightened systemic levels of TIMPs is a typical characteristic of TB-DM co-morbidity. Our results suggest that specific TIMP-1, -3 and -4 may be a useful component of a biomarker panel for active TB-DM comorbidity with other clinical and immunological parameters. However, our study suffers from the limitation of a small sample size. Therefore, further validation of these biomarkers in different endemic populations and different geographical regions could serve as the basis to develop a biomarker test for active tuberculosis. At the end of successful anti-TB treatment, TIMP levels were found to remain increased in TB-DM compared to TB, which may be key in tissue remodelling and scarring that lead to long-term fibrosis. Our data reveal two non-mutually exclusive mechanisms for the elevation of TIMPs in TB and TB-DM. The first possibility is that TIMPs are directly driving pathological fibrosis and are therefore increased in these conditions [27,33]. The second possibility is that the elevations in the levels of TIMPs are merely a reflection of elevated levels of MMPs in these individuals. We plan on determining these different mechanistic possibilities in future studies. In addition, these markers could be beneficial as targets of host adjuncts to reduce the duration of chemotherapy as well as agents reducing the incidence of relapse or recurrence. Finally, our study reveals the complex network interlinking the pathogenesis of TB-DM to inflammatory pathology and possibly poor outcomes in TB-DM co-morbidity.

5. Conclusions

Our data reveal that heightened systemic levels of TIMPs are a typical characteristic of TB-DM co-morbidity. TIMP levels are correlated with the severity of pulmonary TB disease and with glycemic control.

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CRediT authorship contribution statement

NPK: Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft. **HK:** Conceptualization, Data curation, Funding acquisition, Resources, Writing - review & editing. **SB:** Conceptualization, Funding acquisition, Resources, Writing - original draft, Writing - review & editing. **VV:** Data curation, Project administration, Resources. **SH:** Data curation, Project administration. **SS:** Formal analysis, Investigation. **KM:** Investigation, Methodology.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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