The value of the monocyte-to-lymphocyte ratio and osteopontin (SPP1) in tuberculosis treatment response monitoring

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Abstract

There is an urgent need to rapidly diagnose tuberculosis (TB) disease and effectively monitor anti-TB treatment responses. Host-directed therapy (HDT) is a promising platform to mitigate challenges in TB diagnosis and anti-TB treatment response monitoring. Identifying changes in systemic proteins and immune cell distributions during the disease is an integral aspect of developing targeted therapies. Here, samples were collected from healthy individuals (CTRL) [n=32 plasma, n=9 bronchoalveolar lavage (BAL)] and newly diagnosed TB patients (TB treatment group) [n= 82 plasma, n= 28 BAL] to analyze full blood count, secreted levels of full-length osteopontin (OPN), and inflammatory markers. Peripheral blood and BAL samples were collected at a single time-point from CTRL, while in TB participants, they were collected at TB diagnosis (TBDx), week 1 (TBW1), month 2 (TBM2), and month 6 (TBM6). We observed a significantly increased monocyte-to-lymphocyte ratio (MLR) and plasma OPN in TB group at TBDx compared to the CTRL group. Inflammatory markers including IL-6, VEGF-A, and sFasL showed significant increase at TBDx when compared to CTRL, but these significantly declined by TBM6. Plasma OPN significantly declined at TBW1 and TBM2 when compared to TBDx but significantly increased at TBM6. BAL OPN showed no significant differences between CTRL and TB patients at TBDx, whereas a significant increase was observed in TB group between TBDx and TBM6. Given the study limitations, these findings should be considered preliminary and exploratory. Our results add to literature and identify MLR and plasma OPN as potential targets for early TB diagnosis and treatment monitoring.

Introduction

Mycobacterium tuberculosis (Mtb) infection is listed amongst the deadliest communicable diseases, with 10.7 million new cases and 1.23 million deaths reported in 2024 (1). Currently, diagnosis of infected individuals relies on the evaluation of patient history, clinical symptoms, radiographic examination, and detection of *Mtb* bacilli through acid-fast staining and Mycobacterium Growth Indicator Tube (MGIT) or alternatively through solid media such as Lowenstein–

Jensen and Middlebrook 7H10/7H11 (2,3). Delays in the diagnosis of *Mtb* infection enhances progression to active TB disease leading and a continued anthroponosis, which is contrary to the “End-TB-strategy” which is aimed at reducing TB incidences by 2035 as proposed by the World Health Organization (WHO) (1). Despite advances in TB diagnosis, sputum culture tests still represents the gold standard for TB diagnosis and treatment monitoring (4) even though it has been reported to have limitations such as the quality of sputum, contamination, and turnaround time (up to 6 weeks). Advances in TB diagnostics increasingly rely on molecular diagnostic approaches using nucleic acid amplification techniques, such as the Xpert MTB/RIF and Xpert Ultra assays. These tests rapidly detect Mtb DNA directly from clinical samples with high sensitivity (85-91%) and specificity (95-99%). However, their use is limited by factors such as high cost, limited accessibility, and the need for specialized technical expertise (5). This suggests the need to identify additional host targets which may lead to efficient and timeous diagnosis of *Mtb* infection and/ or anti-TB treatment outcomes. Although several biomarkers for tuberculosis have been identified and have contributed significantly to improving diagnosis and disease monitoring, their performance remains variable across different clinical contexts. Many currently available biomarkers show limited sensitivity or specificity in certain populations, such as individuals with HIV co-infection, children, or patients with extrapulmonary TB, and are often insufficient to distinguish active from latent infection or to monitor treatment response. Given the biological heterogeneity of TB, there remains a need to identify additional biomarkers or biomarker combinations that can complement existing markers and improve clinical applicability. The monocyte-to-lymphocyte ratio (MLR) has been suggested as a potential diagnostic target which can be indicative of individuals who are at a higher risk of TB disease (6). A study by La Manna *et al*. (2017) highlighted significant differences in MLR, with an increased MLR in active TB disease patients compared to healthy donors, latently infected, and cured-TB patients. The authors further reported MLR as a potential marker for discriminating active TB from healthy donors, with a sensitivity of 91% and a specificity of 93% at a cut-off value of 0.285 (7). This data was concurred by Estévez *et al*. (2020) and Grassi *et al*. (2021) who showed an increased MLR in active TB patients compared to healthy and latently infected individuals which further showed a positive correlation with polymorphonuclear-myeloid-

derived suppressor cells (PMN-MDSC) (8,9). Immune cells, including monocytes, lymphocytes and granulocytes are recruited to the site of infection during TB disease through elevated secretion of chemo-attractants affecting MLR (10–13). Additionally, MLR has been reported as a reliable predictor of disease severity in other disease settings, including rheumatic and cancer studies (14,15). It is well documented that *Mtb* infection triggers both cellular and humoral immune responses leading to increased secretion of pro-inflammatory cytokines, anti-inflammatory cytokines, chemokines, growth factors and other biological proteins which are known to enhances cellular processes (16). Particularly, Osteopontin (OPN, also known as secreted phosphoprotein 1 (SPP1)) has been shown to play a crucial role in inflammatory responses through mediating immune cell functions, including cell adhesion (17), migration (18), differentiation (19), and phagocytosis (20,21). OPN is an integrin-binding glycophosphoprotein which is secreted in the bone matrix and contributes in bone resorption, formation (22,23), and tissue remodeling. It has been shown to be involved in defense against pathogens through enhancing T-helper 1 (Th1) activity and its capabilities as a chemo-attractant which recruits immune cells to the site of infection (24,25). Elevated levels of OPN has been reported in *Mtb* infection studies evaluating both transcription levels (25–27) and secreted proteins in plasma samples (28). OPN has been described as having different isoforms which may perform distinct functions due to their localization, these includes secreted OPN (sOPN) and intracellular OPN (iOPN) (21). In a study by Kanayama et al. (2017) iOPN was shown to reduce the frequency of both myeloid progenitor cells and myeloid cells while sOPN enhanced the expansion of lymphoid cells suggesting that these OPN isoforms may result in skewed leukocyte cell population (29). In addition, OPN may undergo proteolytic fragmentation where the cleaved fragments have been described to activate different signaling pathways resulting in different cytokine-like functions (18,30–32). In this study we evaluated secreted full-length OPN (sOPN) in plasma and Bronchoalveolar lavage (BAL) samples collected from healthy individuals and active TB patients at W1, M2 and M6 of anti-TB treatment. We aimed to investigate the secretion profile of OPN, cytokines (IL-6, VEGF-A, sFasL, and TRAIL) in BAL and blood samples. The analytes were selected based on their biological functions and evidence from TB studies. IL-6 is a key pro-inflammatory cytokine that is consistently elevated in active TB and correlates with disease severity, while VEGF

serves as a central regulator of angiogenesis and vascular permeability. TRAIL and sFasL are involved in apoptosis signaling, providing complementary insights into host cell death pathways. Additionally, we evaluated the MLR in blood, to determine the diagnostic value as potential targets for pulmonary tuberculosis (PTB) progression during anti-TB therapy. Furthermore, we postulated that OPN and MLR may play distinctive roles during active TB disease which may elucidate a better understanding of TB pathophysiology during anti-TB treatment and monitoring of treatment outcomes.

Results

Clinical and demographic characteristics: This study included 60 participants with mean age of 31.18 years. Study participants were stratified into two groups which included healthy controls (CTRL) (n=32) and TB treatment group (n=28) with mean age of 29.1 and 33.4, respectively. As shown in Table 1, 28 (46.6%) of study participants were female and all included study participants were HIV-negative.

Table 1: Study participant demographic information.

	All	**CTRL**	**TB GROUP**
Number of participants, n	60	32	28
Age in years, mean (SD)	31.18 (11.96)	29.10 (11.84)	33.41 (11.90)
Female, n (%)	28 (46.6)	14 (43.7)	14 (50.0)

Increased Plasma OPN concentrations in TB group at Dx and month 6 compared to CTRL group: This study compared plasma OPN levels between the CTRL group (n=32) and the TB treatment group (n=28). As highlighted in Figure 1A, OPN was evaluated at four distinct time points for TB treatment group, including TBDx (n=28), TBW1 (n=8), TBM2 (n=7) and TBM6 (n=39). Our results showed a significant upregulation in secreted OPN plasma level in the TB group at TBDx (p=0.006) and TBM6 (0.004) when compared to the CTRL group. However, comparison between TB treatment time points showed significant downregulation in OPN plasma levels at TBW1 (p=0.044) and TBM2 (p=0.043) when compared to the

TBDx time point. Moreover, significant upregulation was observed at TBM6 (p=0.018) when compared to TBM2.

Significant increased BAL OPN concentrations in the TB group at Month 6 compared to TB diagnosis: Figure 1B illustrates the data obtained for OPN levels in BAL samples between CTRL group (n=9) and the TB group at TBDx (n=8) and TBM6 (n=20) time points. There was no significant difference observed in the OPN levels between the CTRL group and the TB group at either TBDx or TBM6. Contrary to plasma OPN levels, a significant upregulation in BAL OPN levels was observed at TBM6 when compared to the TBDx time point (p=0.020).

Paired analysis for OPN plasma levels at TBDx and TBM6: Using the Wilcoxon signed rank test, there was no significant difference observed in paired plasma samples from the TB group (n=19) at TBDx (mean=23 669 pg/mL) and TBM6 (mean=21 102 pg/mL) as highlighted in Figure 1C.

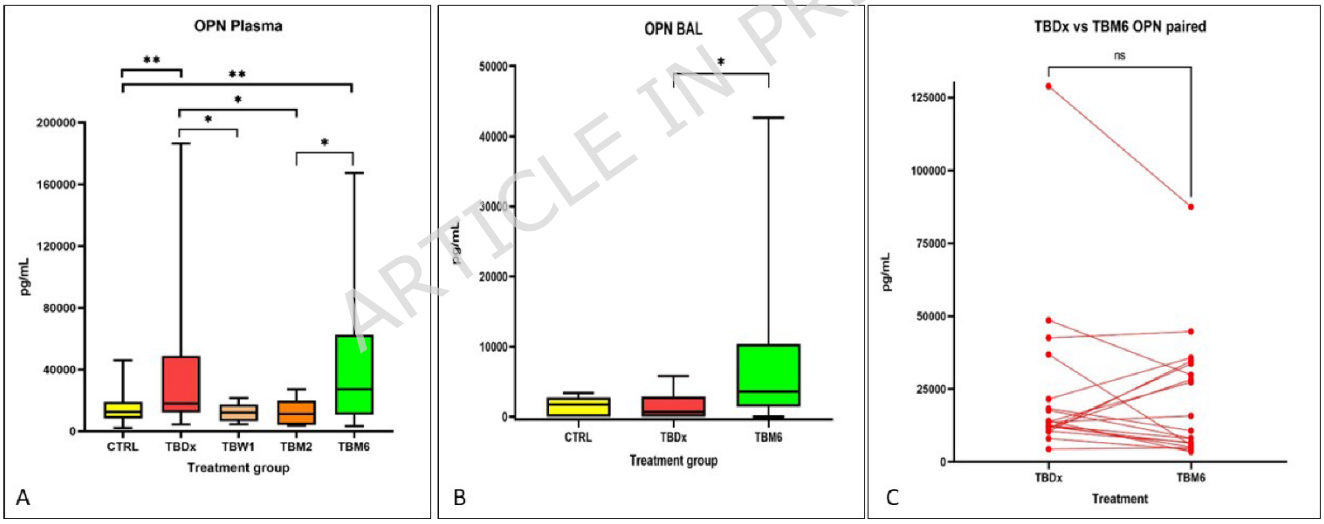

Figure 1. Osteopontin (OPN) secretion in plasma and BAL samples collected from healthy individuals and those with active TB disease. **A.** plasma OPN levels between CTRL (n=32), TBDx (n=28), TBW1 (n=8), TBM2 (n=7) and TBM6 (n=39); **B.** BAL OPN levels between CTRL (n=9), TBDx (n=8), and TBM6 (n=20); **C.** paired OPN plasma results, comparing TBDx against TBM6 (n=19). Data was analyzed using one-way analysis of variance (ANOVA) and *P* value symbols are indicated as $p < 0.0001$ (****), $p < 0.001$ (***), $p < 0.01$ (**) and $p < 0.5$ (*) in the figures, Ns is not significant. Total number (n), control (CTRL), TB diagnosis (TBDx), TB treatment at week 1 (TBW1), TB treatment at month 2 (TBM2), TB treatment at month 6 (TBM6).

Increased MLR in TB at diagnosis compared to CTRL group

To investigate the pathogenic role of the MLR in tuberculosis, we compared the MLR in blood samples between TB patients at diagnosis (n=22) and the control group (n=25). Using the Mann-Whitney test, a significant upregulation in MLR was observed in the TB group at TBDx when compared to the CRTL group with a p-value <0.010 as illustrated in (Figure 2A). Furthermore, the principal component analysis (PCA) showed that the MLR and other hematological factors including Mean Corpuscular Hemoglobin Concentration (MCHC), and platelet count distinguished the TB group from the CTRL group (Figure 2B). In the PCA plot, the control group was shown as blue circle while the red circle indicated the TB group at TBDx with PC1 (35%) and PC2 (12%).

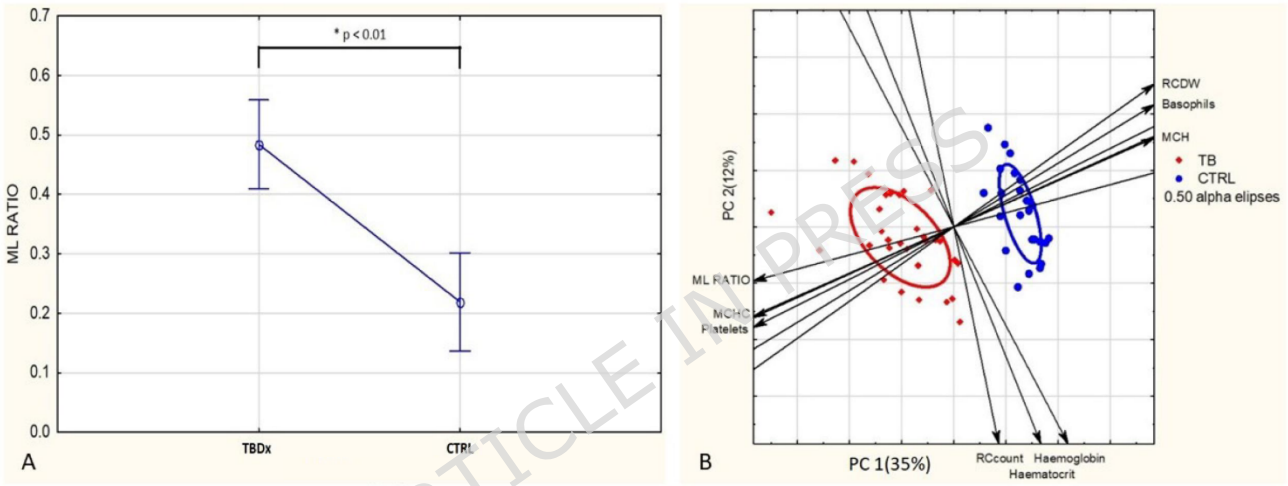

Figure 2. Discrimination between healthy individuals and active TB individuals. A. Monocyte-to-Lymphocyte ratio (MLR) between healthy individuals and those with TB disease at diagnosis. **B.** Principal Component Analysis showing distinction between the TB group at diagnosis and the CTRL group. The MLR statistical difference is assessed through Mann-Whitney test where *P* value symbol is indicated as $p < 0.05$ (*). Control (CTRL), TB diagnosis (TBDx).

Plasma cytokine (IL-6, VEGF-A, TRAIL, and sFasL) levels were monitored during anti-TB treatment period

The current study further examined the secretion profiles of IL-6, sFasL, TRAIL, and VEGF-A in plasma samples between the CTRL group (n=32) and the TB group at TBDx (n=28), TBW1 (n=8), TBM2 (n=7), TBM6 (n=39). Plasma cytokine levels are illustrated as box & whiskers plots in Figure 3A (IL -6), Figure 3B (sFasL), Figure 3C (TRAIL), and Figure 3D (VEGF-A) where statistical significance was determined

through one-way analysis of variance (ANOVA) with Dunn's multiple comparison. A significant upregulation in the secretion levels of plasma IL-6 (p<0.001), sFasL (p=0.005) and VEGF-A (p<0.001) were observed in the TB group at TBDx compared to the CTRL group. Non-significant results were obtained for TRAIL when comparing TBDx and the CTRL group. Additionally, the secretion levels of plasma cytokines between the TB group at TBM6 and the CTRL group showed significantly increased levels of IL-6 (p<0.001), sFasL (p<0.001), TRAIL (p<0.001) and VEGF-A (p<0.001). Interestingly, IL-6 (p=0.001), TRAIL (p=0.037), and VEGF-A (p=0.029) showed significant reduction in TB group at TBW1 while IL-6 (p<0.001) and TRAIL (p=0.020) further remained significantly reduced at TBM2 when compared to TBDx. At TBM6, the plasma level of IL-6 (p<0.001) showed significant reduction when compared to TBDx while non-significant change was observed for sFasL, TRAIL, or VEGF-A levels.

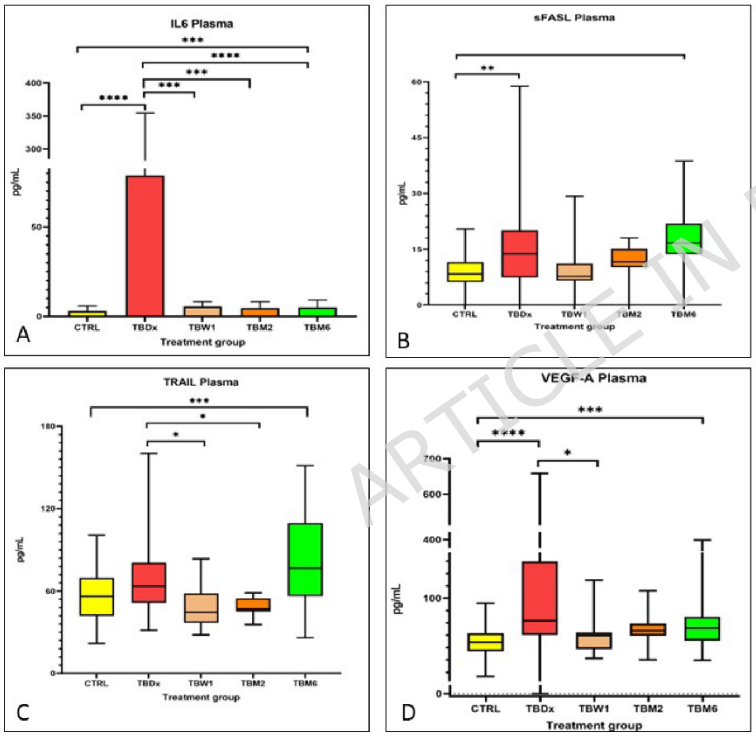

Figure 3. Plasma cytokine secretion over the course of anti-TB treatment and healthy controls. A. IL-6, **B.** sFASL, **C.** TRAIL, and **D.** VEGF-A. The number of plasma samples included in this experiment are as follows; TBDx (n=28), TBW1 (n=8), TBM2 (n=7), TBM6 (n=39) and CTRL (n=32). Statistical difference is assessed through a one-way non-parametric analysis of variance (ANOVA), P value symbols are indicated as $p < 0.0001$ (****), $p < 0.001$ (***), $p < 0.01$ (**) and $p < 0.5$ (*) in the figures. Control (CTRL), TB diagnosis (TBDx), TB treatment at week 1 (TBW1), TB treatment at month 2 (TBM2), TB treatment at month 6 (TBM6).

BAL cytokine (IL-6, VEGF-A, TRAIL, and sFasL) levels were monitored during anti-TB treatment

The secretion profiles of IL-6, sFasL, TRAIL, and VEGF-A were evaluated in BAL samples obtained from the CTRL group (n=9) and the TB group at TBDx (n=8) and TBM6 (n=20). Contrary to plasma analysis, BAL samples were not collected at TBW1 and TBM2 during the anti-TB treatment. The BAL cytokine levels are highlighted in Figure 4A (IL-6), Figure 4B (sFasL), Figure 4C (TRAIL) and Figure 4D (VEGF-A). Our results showed a significant upregulation in IL-6 (p=0.011) and TRAIL (p=0.014) levels in the TB group at TBDx when compared to CTRL group. Furthermore, IL-6 (p=0.047) and TRAIL (p=0.002) remained significantly elevated in the TB group at TBM6 when compared to CTRL group. There were no significant changes observed in the secretion profiles of sFasL and VEGF-A between the CTRL group and the TB group at both TBDx and TBM6. Furthermore, there were no significant changes that were observed in the TB group between anti-treatment time points for IL-6, sFasL, and TRAIL while VEGF-A (p=0.039) showed a significant upregulation between TBDx and TBM6 time points.

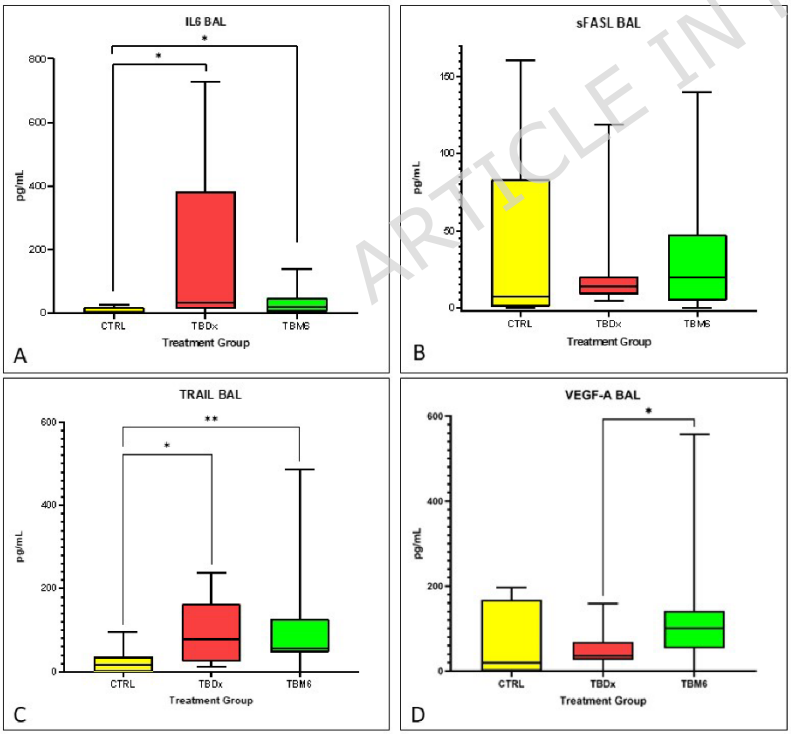

Figure 4. Level of BAL cytokines between the TB group and healthy controls. A. IL-6, **B.** sFASL, **C.** TRAIL, and **D.** VEGF-A. The number of BAL samples included in this study are as follows; TBDx (n=8), TBM6 (n=20) and CTRL (n=9). Statistical difference is assessed through a one-way non-parametric analysis of variance (ANOVA), *P* value symbols are indicated as $p < 0.0001$ (****), $p < 0.001$ (***), $p <$

0.01 (**) and $p < 0.5$ (*) in the figures. Control (CTRL), TB diagnosis (TBDx), TB treatment at week 1 (TBW1), TB treatment at month 2 (TBM2), TB treatment at month 6 (TBM6).

Haematological analysis between healthy controls and TB treatment group

Evaluation of full blood count showed significant upregulation of WCcount, MCHC, platelets, neutrophils, and eosinophils in the TB group at TBDx when compared to healthy controls (CTRL). Conversely, Haemoglobulin, MCH, RCDW, and basophils showed significant downregulation in the TB group at TBDx compared to the control group (Table 2).

Table 2: Haematological analysis between healthy controls and the TB treatment group.

Haematogical Parameters	**CTRL (Mean ± SD)**	**TBDx (Mean ± SD)**	**p-value**
Whit Cell count	8.33 ± 3.58	10.75 ± 3.49	0.0089
Red cell count	4.92 ± 0.69	4.72 ± 0.71	0.3866
Haemoglobin	14.42 ± 1.76	12.84 ± 2.20	0.0110
Haematocrit	0.43 ± 0.05	0.40 ± 0.06	0.692
MCV	89.14 ± 6.52	85.43 ± 8.00	0.1000
MCH	33.00 ± 0.85	27.20 ± 3.21	<0.0001
MCHC	3.53 ± 6.21	31.80 ± 1.24	<0.0001
RCDW	285.3 ± 77.99	6.06 ± 7.65	<0.0001
Platelets	5.44 ± 17.01	505.70 ±168.60	<0.0001
Monocytes	2.54 ± 8.13	0.54 ± 0.39	0.0979
Lymphocytes	0.30 ± 1.12	1.23 ± 0.90	<0.0001
Neutrophils	0.37 ± 1.11	31.92 ± 35.26	<0.0001
Eosinophils	0.07 ± 0.23	1.17 ± 2.67	<0.0001
Basophils	29.42 ± 2.35	0.13 ± 0.15	<0.0001

Notes: MCV = mean corpuscular volume, MCH = mean corpuscular haemoglobin, MCHC = mean corpuscular haemoglobin concentration, RCDW = red cell distribution width, SD = standard Deviation, CTRL = Healthy Controls, TBDx = TB diagnosis.

Discussion

Although several biomarkers associated with TB have previously been identified, their diagnostic and prognostic performance has not been consistently validated across different populations, disease stages, and biological compartments. Many reported biomarkers have primarily been evaluated in peripheral blood, while limited information exists regarding their expression at the site of disease. In this study, we evaluated these biomarkers in both blood and BAL samples, providing additional evidence of their potential utility and contributing to the growing body of knowledge on host immune responses during TB disease. OPN has been described as a multifunctional matrix protein that is associated with the regulation of Th1/Th2 immune responses (28,33). This protein has been reported to be elevated during *Mtb* infection and is described as a potential marker of acute infection, which may be linked to disease severity (28,34). Furthermore, OPN was suggested as a potential molecule which can be used to monitor TB disease progression (35). Our study reports a significantly increased plasma OPN levels in individuals who have active TB disease and undergoing anti-TB treatment compared to the healthy control group. This data concurs with other studies which have previously reported an increased OPN levels in TB patients compared to both unexposed and latently infected individuals (34,36–38). We further demonstrated that at month 6 of anti-TB treatment, plasma OPN levels remain significantly upregulated in the TB group when compared with the control group. Additionally, we observed a significant increase in OPN levels which were detected in BAL samples of individuals with active TB disease when comparing TB diagnosis with the end of anti-TB treatment at month 6. This is an interesting finding since there was no significant change observed when comparing TB diagnosis and month 6 for plasma OPN, indicating that OPN effects on immune responses may be local rather than systemic due to seroconversion and reduced bacterial load by month 6. Previously, OPN was shown to decrease following sputum conversion during TB disease (28) while other studies reports that OPN levels may decrease after 2 months of anti-TB treatment (39). Interestingly, our study

showed that plasma OPN levels significantly decreased in TB patients at week 1 and month 2 of anti-TB treatment. This further indicates that OPN may be regarded as a reliable indicator of improved disease outcome from the early stage of anti-TB treatment period. However, in the current study, we observed a significant increase in plasma OPN levels between month 2 and month 6 of anti-TB treatment (p=0.018). The factors associated with the observed upregulation at month 6 in this study population remains elusive but may be linked with anti-TB regimens change as per the South African standard of care guidelines for TB or *Mtb* bacilli re-activation. Further investigations aimed at elucidating the mechanisms underlying these results will be valuable and will enhance our understanding of the role of OPN in pulmonary tuberculosis, especially in endemic settings. Previous studies reported a dual role of OPN during inflammatory state, where it was shown to induce (40,41) or suppress immune responses (40,42). However, from this study, we conclude that OPN has immunomodulatory roles, and it is significantly elevated during the critical phase in individuals who are infected with *Mtb* as compared to healthy individuals. Secretion of cytokines, chemokines and growth factors are well described during early *Mtb* infection, but their secretion may also be regulated by presence of matrix proteins such as OPN. In this study four pro-inflammatory cytokines including IL-6, sFasL, TRAIL, and VEGF-A were evaluated from the study participants. Particularly, pro-inflammatory cytokines, including IL-6 are known to mediate early immunopathogenesis during *Mtb* infection, contribute to the disease severity, and promote treatment failure (43). In this study, the increased plasma IL-6 and VEGF-A levels at TB diagnosis were significantly downregulated towards the end of TB treatment as previously demonstrated in a study by Siawaya *et. al* (2009) (44). Furthermore, Shete *et al*. (2020) documented the severity of TB disease correlating to higher IL-6 and OPN levels in individuals with active TB (34).

There is an increasing effort directed at identifying host biomarkers which may aid in better disease monitoring and TB disease outcomes. Recently, the MLR has been seen as a promising route in TB studies for discriminating infected against non-infected individuals and monitoring responders against non-responders during the treatment phase (7,45,46). The blood MLR can be used for TB disease progression in both children and adults determination and it is regarded as cost effective and may be routinely achievable in limited-resource setting (6,47). This could be useful,

especially in patients where it is difficult to collect sputum samples, for example in young children. Furthermore, MLR has been widely reported to be increased in pathological conditions characterized by systemic inflammation, including chronic kidney disease (CKD), cardiovascular diseases (CVD), autoimmune disorders, malignancies, and infectious diseases (48–50). Previous studies have suggested that inflammatory cytokines, particularly IL-6, play a key role in modulating MLR by stimulating myelopoiesis and monocyte release from the bone marrow, while simultaneously regulating lymphocyte survival and function (51,52). This inflammatory milieu leads to high MLR due to increased monocyte expansion and activation, accompanied by a relative decrease in lymphocyte counts, partly due to stress-induced lymphopenia (52). In our study, the MLR distinguished TB patients from the control group at the time of TB diagnosis. This data agrees with the study by La Manna et al. (2017) who showed that at a cut-off of 0.285 the MLR displayed 91% sensitivity and 94% specificity in identifying HIV negative patients with culture-confirmed TB (7). Even though MLR was evaluated only at TB diagnosis in this study, other reports have shown that MLR may also be used for monitoring treatment responses (46). Furthermore, full blood count analysis revealed that TB patients at diagnosis had elevated WCC, neutrophils, eosinophils, MCHC, and platelets, alongside reduced haemoglobin, MCH, RCDW, and basophils, which maybe be reflective of systemic inflammation. These changes corresponded with an increased monocyte-to-lymphocyte ratio (MLR), indicative of monocyte expansion and lymphopenia driven by inflammatory cytokines such as IL-6 (52). Together, these haematological and biomarker alterations highlight the interplay of inflammation, immune cell redistribution, and cell death during active tuberculosis. This approach may represent an impactful diagnostic approach for TB disease which may further lead to the identification of other host directed targets for efficient TB diagnosis, particularly in areas with limited resources.

Conclusion

Despite the effectiveness of anti-TB treatment in clearing the infection, the treatment regimen has been reported to result in other medical complications such as septic shock (53), pneumothorax (54), bronchiectasis (55), malignancies (56), and an increased risk of osteoporosis (57), thereby suggesting the need to identify host-

directed diagnostic and anti-TB treatment monitoring targets. Our study demonstrated that OPN, MLR, and IL-6 may be associated with tuberculosis and the relationship between these markers may serve as a potential prognostic indicator for anti-TB treatment outcomes and help identify patients who might require follow-up after the anti-TB treatment period. Evaluation of these biomarkers in both peripheral blood and BAL samples contributes to a better understanding of host immune responses at both systemic and site-of-disease levels. Undoubtedly, additional studies with larger population sizes, and including other pulmonary diseases, are required to elucidate the exact roles which are mediated by these potential markers during TB progression.

Study Limitations

We acknowledge that this study had some limitations, including a relatively small sample size, a low number of active TB cases for both blood and BAL samples, and a limited number of participants with complete follow-up time points. Furthermore, latent tuberculosis infected (LTBI) individuals were not included in this study to account for subclinical *Mtb* infection. Bacterial counts were not performed for TB participant to correlate with analyzed biomarkers or disease severity. Despite these limitations, this study provides preliminary data for future evaluation.

Materials and Methods

Ethical Considerations

The study was conducted in accordance with the Declaration of Helsinki and International Conference of Harmonizing guidelines. Ethical clearance was obtained from the Health Research Ethics Committee of Stellenbosch University (N05/11/187) and the City of Cape Town City Health. Written informed consent was obtained from all participants before their enrolment into the study.

Participant recruitment, and clinical findings

The study participants were recruited around Cape Town, Western Cape, South Africa. These were recruited from two community areas, including Kuilsriver and Ravensmead. Study participants were above 18 years old, and they were enrolled regardless of gender. Newly diagnosed individuals with the first episodes of TB

disease were enrolled into the study before the commencement of anti-TB treatment and these were stratified into TB treatment group (Figure 5). These patients presented with a positive sputum culture, positive TB X-ray, and clinical symptoms suggestive of TB disease. Healthy controls (CTRL) were recruited from the same communities where they were defined by a negative QuantiFERON (QFT) and negative *Mtb* culture test with no clinical symptoms of disease. The TB cases were followed over the 6-months of anti-TB treatment and biological samples (bronchoalveolar lavage and blood) were collected at different time points (Figure 5). This study excluded HIV-positive patients, individuals with diabetes, those with other lung diseases, and individuals with a history of previous TB disease.

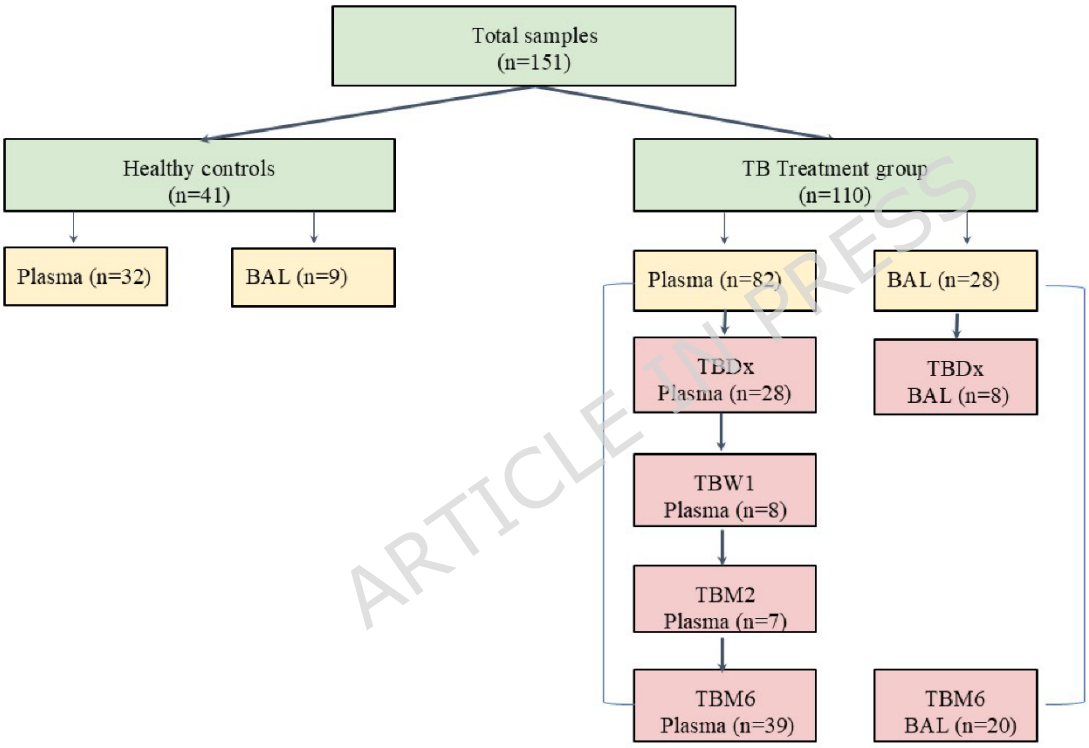

Figure 5. Plasma and BAL sample collection from the control (CTRL) group and the TB group at specific time points. Total number (n), control (CTRL), tuberculosis (TB), TB diagnosis (TBDx) and Bronchoalveolar Lavage (BAL).

Sample collection

Blood and BAL samples (n=151) were collected from recruited study participants as illustrated in Figure 5. These were stratified into two study groups: (1). Healthy community controls (CTRL) (n=41) and (2). TB treatment group (n=110). Healthy control samples were collected at a single time point where 32 blood samples and 9 BAL samples were collected. The TB treatment group samples were collected at four

distinct time points where a total of 82 blood and 28 BAL samples were collected. The plasma samples from the TB group were collected at all four time points and distributed across TB diagnosis (TBDx) (n=28), week 1 of anti-TB treatment (TBW1) (n=8), month 2 of anti-TB treatment (TBM2) (n=7), and month 6 of anti-TB treatment (TBM6) (n=39). However, BAL samples were only collected at TBDx (n=8) and TBM6 (n=20). All samples were transported to the laboratory under controlled conditions. Sodium heparin tubes were used to collect peripheral blood, samples were centrifuged, plasma aliquoted, labeled, and cryopreserved in temperature-controlled freezers at -80℃ until testing. The BAL samples were centrifuged, supernatants were aliquoted, labeled, and preserved in temperature-controlled freezers at -80℃ until testing. Once all the samples were received for this study, one aliquot per patient was thawed and spun down before batch processing.

Determination of cytokine and inflammatory mediators

The analysis kit was selected based on the literature search highlighting biomarkers which are positively associated with TB infection (44,58–64), we considered the following markers for this study: OPN, IL-6, VEGF-A, sFasL, and TRAIL. Plasma and BAL samples were analyzed using the Milliplex Map Human circulating cancer Biomarker panel I (sFasL, IL-6, OPN, TRAIL, and VEGF-A) assay kit (Millipore, Burlington, MA, USA, cat#HCCBP1MAG-58K-05), according to the manufacturer's instructions. This panel had no cross reactivity between analyte antibodies, and the minimum detection limits for the analytes were as follows: sFasL = 8.4 pg/ml, IL-6 = 0.2 pg/ml, OPN = 285.3 pg/ml, TRAIL = 0.5 pg/ml, and VEGF = 6.4 pg/ml. Data was acquired using MAGPix and BioPlex (BioRad Laboratories, Irvine, CA), instruments and analyzed using Bio-Plex manager software (Bio-Rad). The cytokine concentrations were extrapolated using 5- Parameter Logistic (PL) standard curves and reported in pg/mL.

Full Blood Counts Analysis

Quantification of white cell counts (WCC), red cell counts (RCC) Hemoglobin (Hb), Hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RCDW), Platelets, Neutrophils, Monocytes, Lymphocytes, Eosinophils, Basophils

were performed at the National Health Laboratory Service (NHLS) Tygerberg Hospital, Hematology laboratory on the Beckman Coulter Counter (Beckman Coulter, Brea, California, USA) instrument range.

Statistical Analysis

The statistical analyses were performed using GraphPad Prism 9.0.2 software (Graphpad, San Diego, USA). This study was exploratory by design; therefore, the sample size and statistical power were not predetermined. Data were analyzed using one-way analysis of variance (ANOVA) with Dunn's multiple comparison, and the Mann-Whitney *t*-tests. The Wilcoxon sign rank paired t-test was used for paired samples i.e., TB participants with treatment follow-up data. Principal component analysis (PCA) was performed using Statistica 12.0 software (StatSoft, Southern African Analytics Pty Ltd, SA). Statistical differences with $p \leq 0.05$ were considered significant where *p*-value is shown as $p \leq 0.0001$ (****), $p \leq 0.001$ (***), $p \leq 0.01$ (**) and $p \leq 0.5$ (*) in the figures.

Data Availability Statement

Data are contained within the article.

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Author Contributions

Conceptualization, A.G.L.; methodology, B.M., A.H., C.I.S., and I.R.; formal analysis, K.S.; writing—original draft preparation, B.M. and A.H.; writing—review and editing, K.S., I.R., C.I.S; supervision, A.G.L.; funding acquisition, A.G.L. All authors have read and agreed to the published version of the manuscript.

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Declaration of Interest

The authors have nothing to declare

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