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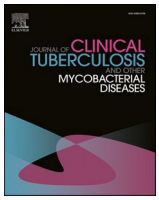

Neutrophils and lymphocytes in relation to MMP-8 and MMP-9 levels in pulmonary tuberculosis and HIV co-infection

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ABSTRACT

Background: Matrix metalloproteinase (MMP) activity has an important role in lung cavitary formation occurred in pulmonary tuberculosis (TB). Low number and viability of CD4 + T-lymphocytes in patients with TB/HIV co-infection leads to impaired neutrophils production, causing further impaired MMPs production.
Objective: To explore association of neutrophils and lymphocytes count to MMP-8 and MMP-9 among pulmonary TB patients with cavitary lesion and HIV co-infection.
Methods: We conducted a cross-sectional study using a purposive sampling technique among patients with non-cavitary TB (n = 50), cavitary TB (n = 50) and TB/HIV (n = 27). Complete blood count was examined, including neutrophils and lymphocytes count. MMP-8 and MMP-9 were measured from plasma samples using ELISA method. Statistical analysis was conducted to determine the relation between neutrophils, lymphocytes and MMPs.
Result: MMP-8 and MMP-9 were positively correlated with neutrophils, but not to lymphocytes in all groups. Neutrophils, lymphocytes, and MMP-9 were significantly lower in TB/HIV co-infection, whereas MMP-8 was higher compared to new pulmonary TB. Interestingly, in cavitary TB, low lymphocytes were significantly correlated with higher level of MMP-8 and larger extent of lung affected.
Conclusion: MMP-8 and MMP-9 are associated with neutrophil count, suggesting that neutrophils contribute significantly to their secretion. MMP-8 is significantly higher in TB/HIV co-infection and extent of lung damage in cavitary TB with lower lymphocyte count. This study suggests that lower lymphocyte level is related to higher neutrophil orchestrated inflammation, leading to tissue destruction.

1. Introduction

Tuberculosis (TB) has recently surpassed HIV as the primary infectious disease killer worldwide, but the two diseases continue to display lethal synergy.[1] The impact of these diseases on one another are bidirectional. HIV increases risk of TB disease progression while TB slows CD4 + T-lymphocyte recovery as well as accelerating progression to AIDS and death among the HIV infected.[1] It is estimated that 1.2 million people has died from TB globally, and among these, 17% is HIV infected. In Indonesia, there are 845 thousands new TB cases yearly, and 2.2% of them are HIV positive.[2].

Despite trying to understand virulence factor of *Mycobacterium tuberculosis* (Mtb), host immunological responses that contribute both protection and pathology remains a great challenge.[3] As outcome, the infection depends on host ability to mount effective protection and balances inflammatory responses.[3,4] Neutrophils are innate immune

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cells which are implicated in both processes.[4] The factors released by neutrophils, such as neutrophil collagenase (MMP-8)[5,6] and gelatinase B (MMP-9)[7,8] indiscriminately damage bacterial and host cells.[9] Thus, neutrophils constitute a potent population of effector cells that can mediate both antimycobacterial activity and immunopathology during Mtb infection.[9].

Members of the matrix metalloproteinases (MMP) subfamily of zinc-dependent metalloproteinases, such as MMP-8 and MMP-9, are released by degranulating polymorphonuclear neutrophils (PMNs)[10] and promote pericellular proteolysis by binding to PMN surfaces in catalytically-active.[6] MMP-9 cleaves a wide variety of extracellular matrix (ECM) proteins and various non-matrix proteins to alter their biologic activities.[6] Previous studies have explained the role of MMP-9 in pulmonary granuloma formation.[11] Furthermore, MMP-8 is also believed to degrade extracellular matrix (ECM).[11] ECM destruction promotes tissue necrosis and cavitary, creates an area where macrophages and granulocytes can poorly penetrate and form an immune-sheltered zone of bacterial growth.[11,12].

Neutrophils in Mtb infection also facilitate the activation of lymphocyte.[13] Neutrophils has a role in presenting Mtb to dendritic cells (DCs) and promoting development of lymphocytes.[3] Among HIV infection, DCs also present HIV antigens to lymphocyte and initiate an immune response.[14] Some studies explained that HIV infection is associated with dysregulation of innate and adaptive immunity, including decreased number of DCs.[15] Both neutrophils and lymphocytes play an important role in maintaining successful immune control of Mtb,[16] however, the immune parameters that are modified by HIV co-infection and subsequently contribute to loss of immune control of Mtb infection have not been well defined.[15].

Mtb typically causes pulmonary disease with cavity formation, which further drives dissemination of the bacilly.[17] Among TB patient with HIV co-infection, pulmonary cavity occurred less often.[11] Previous study have also demonstrated that TB patients with HIV co-infection shows reduced MMP activity compared to TB without HIV co-infection.[18] Massive depletion of lymphocytes affect the effectiveness of neutrophil[19] which then impair the MMP production and decrease cavity formation in TB/HIV co-infection.[11] However since the comprehensive analysis of the relation between neutrophils, lymphocytes, and MMPs and their relationship to TB disease pathology has not been carried out, we report these results that have been conducted among cavitary TB, non-cavitary TB and TB/HIV co-infection.

2. Methods

2.1. Study design settings

We conducted a cross-sectional study by including new pulmonary TB patients and HIV negative with cavitary lesions and without cavitary lesion based on chest radiography. In parallel, we also included new pulmonary TB patients with HIV comorbidity. Patients were consecutively enrolled in sampling blocks based on sex and age categories to allow similar distribution between groups. New pulmonary TB patients were enrolled in community health centers and were further invited to follow research protocol in the TB research clinic of the Faculty of Medicine, Universitas Padjadjaran, Bandung. These TB patients were a subset of TANDEM study (http:www.tandem-fp7.eu/).[20] TB patients with HIV co-infection were enrolled in the HIV and TB research clinic of the Faculty of Medicine, Universitas Padjadjaran.

Upon identification of a highly suspected TB cases whose had at least TB symptoms and suggestive chest Xray, we assessed eligibility of enrollment based on sex and age. We then asked for patient's consent, followed by interview and examination, collecting information regarding clinical symptoms and body mass index. Sputum collection was conducted for microscopy examination and for Mtb culture. The diagnosis of active pulmonary TB was made based on comprehensive clinical symptoms, including chest radiography, sputum smear and culture. Active pulmonary TB was confirmed with at least one of the following criteria: (1) positive Acid-Fast Bacillus (AFB) smear derived from sputum; (2) having clinical TB with positive chest Xray, growth of Mtb in sputum culture, and responding to TB treatment in the first month. The TB patients were further categorized as newly pulmonary TB patient with and without cavity based on chest radiography result, and TB with HIV co-morbidity based on HIV result test.

Similar process was conducted in the HIV clinic. If HIV patient found to have TB, patients were enrolled, interviewed and then blood was collected at the baseline (<72 h before TB treatment). The TB patients were further categorized as newly pulmonary TB patients with and without HIV co-infection.

Chest X-ray results were read by two investigators, using a check list to determine grades, the extent of lung damage and presence of cavity.[21] This study was approved by Research Ethics Committee, Universitas Padjadjaran with ethical number 653/UN6.KEP/EC/2020.

2.2. Laboratory examinations

Blood was drawn in EDTA tube and sent to the hospital central laboratory. Complete blood count was tested (Sysmex XN-1000 hematology analyzer). Neutrophils and lymphocytes proportion and absolute count information was extracted from the laboratory report.

Using separated plasma from patient's whole blood collected in plain tube, matrix metalloproteinase (MMP) −8 and −9 were examined using ELISA method, following the instruction manual from the company (R&D System® Elisa Kit cat no. DY908 and DY911, respectively). To determine the optimal ELISA result, we conducted several testing in serial plasma concentration using PBS as dilution. MMP-8 were measured at concentration dilution of 8 times, whereas MMP-9 were measured at concentration dilution of 128 times.

2.3. Statistical analysis

The data was analyzed using SPSS program version 22 and Prism 8 (GraphPad) licensed to Universitas Padjadjaran. Characteristic and clinical symptoms among non cavitary TB, cavitary TB and TB/HIV co-infection were presented as frequencies and percentages. Differences between groups were analysed using Chi-square and Kruskal-Wallis test. Laboratory result were presented with median and IQR, and the difference between groups were compared using Mann-Whitney and t-independent test. Correlation between neutrophils, lymphocytes and MMP-8, MMP-9 were determined using Pearson and Spearman test.

3. Result

In total, 127 new pulmonary TB patients were included, consisting of 50 patients with non cavitary presentation, 50 with cavitary and 27 TB patients with HIV co-infection. Patient characteristics, clinical symptoms and X-ray findings were described in Table 1. Low BMI (<18.5 kg/m^2) and loss of weight >5 kg were significant prevalent in TB/HIV with $p = 0.01$ and $p = 0.027$, respectively. Cough duration >3 weeks, haemoptysis and night sweat were significantly higher in TB with cavity with $p = 0.00$, $p = 0.02$ and $p = 0.001$, respectively, whereas consolidation was more common in non-cavitary TB ($p = 0.024$). Age, gender, pleural effusion and percentage of visible lung affected were not significantly correlated with any patient categories. Bacteriological confirmation or culture positive of Mtb was achieved with higher proportion in TB patient with cavity (90%) and without cavity (96 %); however, in TB with HIV comorbidity culture positive of Mtb was only 48% (Table 1).

The average of neutrophils in TB with cavity compared to TB without was 8.27 ± 1.45 and 6.61 ± 1.40 respectively, and this was significantly higher ($p = 0.004$) (Table 2). The neutrophils and MMP-9 were also significantly higher in cavitary TB compared to non-cavitary TB and TB/HIV (neutrophils, $p = 0.003$; MMP-9, $p = 0.002$) (Table 2). However,

Table 1
Characteristic, clinical symptoms and radiographic findings among new pulmonary tuberculosis patients with no cavity, with cavity and with HIV co-infection.

	TB				TB/HIV (*N* = 27)		*p*
	Non Cavitary (*N* = 50)		Cavitary (*N* = 50)				
	N	%	*N*	%	*N*	%	
Gender							
Male	25	50.0	29	57.9	18	66.6	0.36
Female	25	50.0	21	42.0	9	33.3	
Age							
18–29	19	38.0	19	38.0	13	48.1	0.195
30–50	21	42.0	23	46.0	14	51.8	
>50	10	20.0	8	16.0	0	0.0	
Weight loss of weight *							
<5kg	36	72.0	31	62.0	11	40.7	**0.027***
>5kg	14	28.0	19	38.0	16	59.2	
BMI category (kg/m^2)							
<18.5	24	48.0	30	60.0	22	81.4	**0.01***
≥18.5	26	52.0	20	40.0	5	18.5	
Clinical symptoms							
Cough duration							
≤2 weeks	4	8.0	2	4.0	10	37.0	**0.00***
≥3 weeks *	46	92.0	48	96.0	17	62.9	
Haemoptysis							
No	30	60.0	29	57.9	22	81.4	**0.02***
Mild Haemoptysis	10	20.0	4	8.0	4	14.8	
Massive Haemoptysis	10	20.0	17	34.0	1	3.70	
Dyspnea							
Yes	29	57.9	34	68.0	18	66.6	0.55
No	21	42.0	16	32.0	9	33.3	
Night sweat *							
Yes	41	82.0	45	90.0	15	55.5	**0.001***
No	9	18.0	5	10.0	12	44.4	
Bacteriology confirmation							
Sputum AFB							
Positive	46	92.0	50.0	100	20	74.0	**0.001***
Negative	4	8.0	0	0	7	25.9	
Mtb Culture							
Positive	45	90.0	48	96.0	13	48	**0.000***
Negative	2	4	0	0	10	37	
Xpert Mtb pos	6	12.0	7	25.0	NA	NA	NA*
Chest X-ray reading							
Consolidation							
Any	23	18.1	19	15	4	3.1	**0.024***
None	27	21.3	31	24.4	23	18.1	
Pleural effusion							
Yes	5	3.9	5	3.9	2	1.6	0.99
No	45	35.4	45	35.4	16	12.6	
% of visible lung affected							
<30%	15	11.8	14	11	10	7.9	0.067$^{\beta}$
30–60%	21	16.5	20	15.7	13	10.2	
≥ 60%	14	11.0	16	12.6	4	3.1	

*Clinical symptom occurred in >75% of new pulmonary tuberculosis patients. p values were calculated using Chi-square test. ^{B}p values were calculated using Kruskal-Wallis test *was significant p-values. NA, Not available. NA*, Not Applicable; BMI, body mass index; HIV, human immunodeficiency virus; TB, tuberculosis.

there was no significant different among lymphocytes, MMP-8 and MMP-9 in cavitary TB compared to non-cavitary TB (Table 2).

The average neutrophils count was significantly higher in TB compared with TB/HIV, which were 7.4 ± 1.45 versus 5.14 ± 2.19, respectively ($p = 0.04$). As also with lymphocytes count which was significantly higher in TB compared to TB/HIV with $p < 0.01$ (1.57 ± 0.64 and 1.02 ± 0.57, respectively) (Table 2). MMP-9 (54,954.09 ± 2.00) in pulmonary TB was significantly higher compared to TB/HIV co-infection (30,902.95 ± 2.63) with $p = 0.008$ (Table 2). However, we unexpectedly found that MMP-8 among TB/HIV co-infection patient (4677.35 ± 3.89) was significantly increased compared to TB patient (2137.96 ± 2.88) with $p = 0.008$ (Table 2). Furthermore, MMP-8 was significantly higher among TB/HIV co-infection patient compared to TB patient with and no cavity ($p = 0.006$) (Table 2).

To investigate further, this study explored the correlation between neutrophils, and MMP-8, MMP-9 among cavitary TB, non-cavitary TB and TB/HIV co-infection. There was a significant positive correlation between neutrophil and MMP-8 among non cavitary TB, cavitary TB and TB/HIV co-infection with $p < 0.05$ ($r = 0.35$, $r = 0.48$, $r = 0.448$, respectively) (Fig. 1). Neutrophils and MMP-9 among non-cavitary TB, cavitary TB and TB/HIV co-infection also showed significant positive correlation with $p < 0.01$ ($r = 0.634$, $r = 0.669$ and $r = 0.560$, respectively) (Fig. 2). From these analyses, this study showed that there was consistent positive correlation between neutrophils and MMP-8, MMP-9. However, we identified that the correlation between MMP-9 and neutrophil was stronger than the correlation between MMP-8 and neutrophil (data not shown).

To determine whether lymphocyte affect neutrophil and MMP activity among TB patient with HIV co-infection, we also compared the relation of lymphocyte, MMP-8, MMP-9 among non-cavitary TB, cavitary TB and TB/HIV co-infection. Among non-cavitary TB and cavitary TB, there was an inverse correlation between lymphocyte and MMP-8 with $p < 0.01$ ($r = -0.35$, $r = -0.48$, respectively) (Fig. 3). Interestingly, we found no correlation between lymphocyte and MMP-8 among TB/HIV co-infection as well as between lymphocyte and MMP-9 (data not shown).

Since neutrophils and MMP were associated with lung destruction, we also examined the relation of neutrophils, MMP-8, MMP-9 with percentage of visible lung affected. In cavitary TB patients, neutrophil and MMP-8 were significantly increased among cavitary TB patient with >60% of visible lung affected compared with cavitary TB patient with ≤ 60% of visible lung affected with $p = 0.002$, $p = 0.04$, respectively, however, there was no significant difference of MMP-9 between cavitary TB with >60% vs ≤ 60% of visible lung affected (Fig. 4). We did not observe significant effect of neutrophils, lymphocytes, MMP-8 and MMP-9 on percentage of visible lung affected among non-cavitary TB patient and TB/HIV co-infection patient (data not shown).

4. Discussion

We have explored the relation of neutrophils, lymphocytes and MMP-8, MMP-9 among non-cavitary TB, cavitary TB and TB/HIV co-infection. This study found that neutrophils count among TB with cavity was significantly higher compared to TB without cavity and TB/HIV co-infection. There are several mechanisms whereby neutrophils may contribute to cavity formation. Specifically, neutrophils were suggested to play a role of "trojan horse" hiding Mtb from activated macrophages; together with their precursor, myeloid derived suppressor cells, neutrophils may suppress T-cells responses; finally, neutrophils may directly mediate lung tissue destruction and thereby creates the cavitary.[22]

Table 2
The level of neutrophils, lymphocytes, MMP-8 and MMP-9 among patients with non-cavitary TB, cavitary TB and TB/HIV co-infection.

	TB		All TB (N = 100)	TB/HIV (N = 27)	P^a	P^b	P^c
	Non Cavitary (N = 50)	Cavitary (N = 50)					
Neutrophil (x10^3/μL)	6.61 ± 1.40	8.27 ± 1.45	7.4 ± 1.45	5.14 ± 2.19	**0.004***	**0.04***	**0.003***
lymphocyte (x10^3/μL)	1.63 ± 0.66	1.51 ± 0.61	1.57 ± 0.64	1.02 ± 0.57	0.344	**0.00***	**0.001***
MMP-8 (pg/mL)	1178.28 ± 2.69	2630.27 ± 2.95	2137.96 ± 2.88	4677.35 ± 3.89	0.065	**0.008***	**0.006***
MMP-9 (pg/mL)	48977.88 ± 1.95	63095.73 ± 2.04	54954.09 ± 2.00	30902.95 ± 2.63	0.052	**0.002***	**0.002***

Note. The value presented as geometric mean ± SD. *significant p-values.
P^a = TB with and without cavity; P^b = all TB and TB/HIV; P^c = TB with and without cavity and TB/HIV. MMP, Matrix Metalloproteinase; HIV, human immunodeficiency virus; TB, tuberculosis.

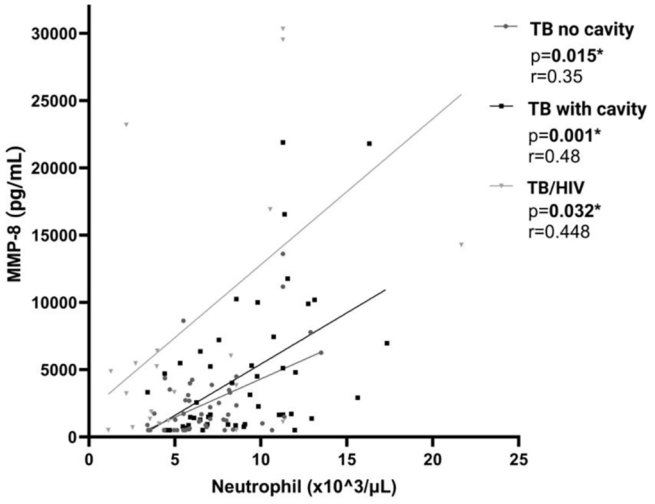

Fig. 1. Significant positive correlation between neutrophils and MMP-8 among TB patients with no cavity, with cavity and TB/HIV co-infection. MMP, Matrix Metalloproteinase; HIV, human immunodeficiency virus; TB, tuberculosis.

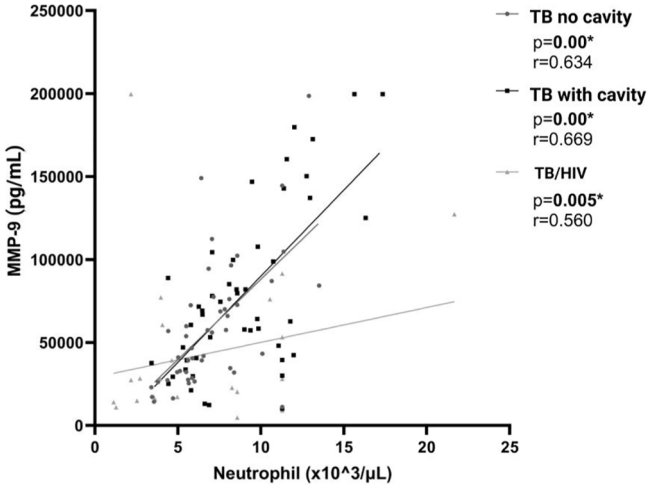

Fig. 2. Significant positive relation between neutrophils and MMP-9 among TB patients with no cavity, with cavity and TB/HIV co-infection MMP, Matrix Metalloproteinase; HIV, human immunodeficiency virus; TB, tuberculosis.

High oxygen concentration within the cavity also provides a rich environment with high rates of bacterial replication, leading to a large bacillary burden at the inner edge of the cavity (10^7 –10^9 bacilli).[12] Virulent strains of Mtb induce high levels of type I IFN by triggering multiple cell surface expression and promote overactivation of neutrophil.[23].

Neutrophils are important source of MMP-8[5] and MMP-9, whereas

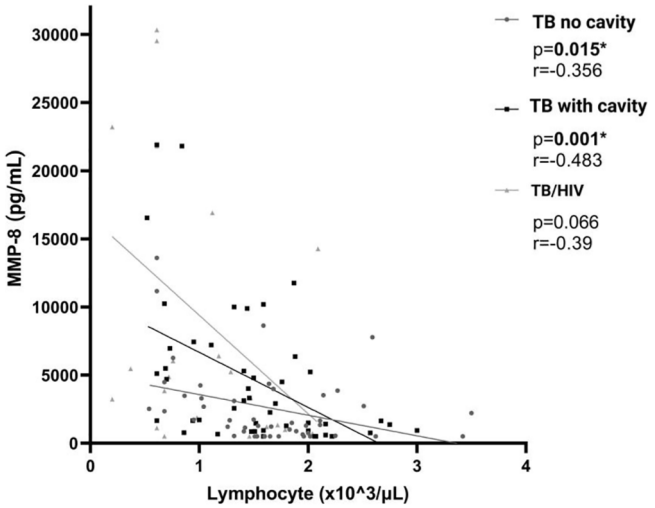

Fig. 3. Significant negative correlation between lymphocyte and MMP-8 among TB with cavity and non-cavitary TB, but no in TB/HIV co-infection. MMP, Matrix Metalloproteinase; HIV, human immunodeficiency virus; TB, tuberculosis.

increased neutrophil level is associated with increased MMP-8 and MMP-9.[7,8] This study has shown that neutrophil and MMP-8 have significant association to percentage of visible lung affected. This result supports other study that demonstrates elevated concentrations of neutrophil derived MMP-8 in TB patient with high radiological and clinical TB severities and the ability of MMP-8 to cause tissue matrix destruction.[5].

In contrast to all TB and especially TB with cavity, TB/HIV co-infection has showed significantly lower neutrophil count. Previous study explains that among TB/HIV co-infection, depending on the timing of infection, HIV infection in turn inhibit type 1 IFN which then result decreased activation of neutrophil.[24] Low levels of neutrophils and neutrophil-derived peptides have been associated with increased risk of TB.[22] However, neutrophils can mediate both anti-mycobacterial activity and immunopathology during Mtb infection.[9] Neutrophils have a role in granuloma formation and Mtb killing, thus the lower concentration could also drive lung pathology with less specific pattern in TB/HIV coinfection.[22].

However, we unexpectedly found that MMP-8 was significantly increased among TB/HIV co-infection compared to TB patient without HIV co-infection. Furthermore, we also found that linear correlation between neutrophil and MMP-8 among TB/HIV co-infection was also higher than TB patient without HIV co-infection. These result supports the previous study that describes the effectiveness of neutrophils may be encumbered as a consequence of diminished numbers of CD4 + T-lymphocyte cells among TB/HIV patient with immunocompromised state [19].

To investigate further, we also analyze the correlation between neutrophils, MMP-8 and lymphocytes. Interestingly, we found that

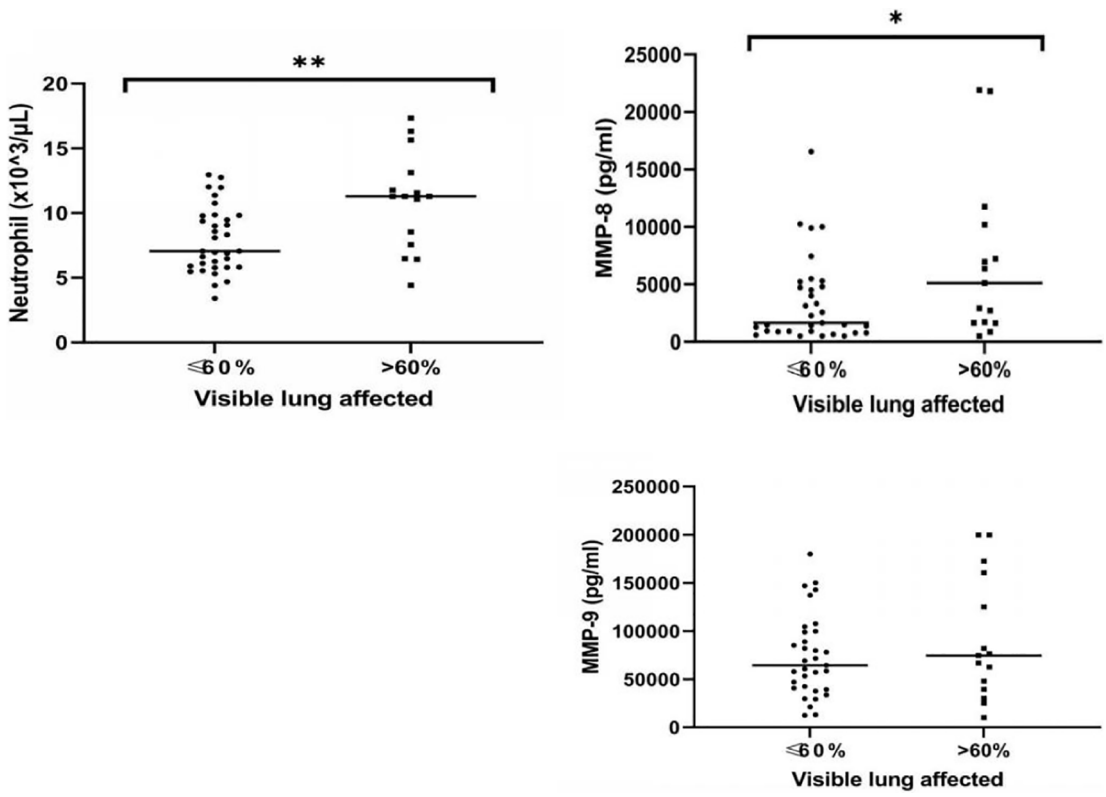

Fig. 4. In pulmonary TB with cavity, neutrophil (**$p < 0.01$) and MMP-8 (*$p < 0.05$) were significantly increased among patient with >60% of visible lung affected, however, MMP-9 was not significant. MMP, Matrix Metalloproteinase; TB, tuberculosis.

lymphocytes had substantial effect to MMP-8 among TB patient without HIV co-infection. Among TB patient, low lymphocyte level was significantly correlated with high level of MMP-8. Although mechanisms underlying this association are not fully clear, this result proves that patients with low lymphocyte level had highly inflammatory clinical patterns.[25] Previous study also explained that patients with low lymphocyte level had more severe TB manifestations and were less likely to achieve a cure.[25] Other study explained that lymphocytopenia cause decrease production of IFN-γ,[26] and decrease IFN-γ also cause prostaglandin E2 (PGE2) decrease which then results increase MMP-8 secretion.[8,27].

Furthermore, in this study, the relation between lymphocyte and neutrophil didn't show significant correlation. Some study has been shown the evidence for the influential role of neutrophils on lymphocyte during TB through neutrophil extracellular traps (NETs).[3] However the exact role of NETs during TB infection still controversial. NETs are able to trap mycobacteria but not to kill them[28], which could increase local concentrations of antimicrobial agents and make Mtb susceptible to the available antimicrobial immune responses,[3] then decrease the influential role of NETs on lymphocyte.

This study has several limitations, mainly concerning the low number of TB/HIV cases we can include in this study. Therefore, additional studies with larger populations might be helpful to a better understanding of the importance and role of neutrophils, lymphocytes, MMP-8 and MMP-9 in the context of TB with HIV co-infection. We also did not consider other opportunistic infections that might have affected lymphocyte counts, such as HBV co-infection[29] and plasmodium co-infection[30], and also affected neutrophil counts and MMP level, such as HCV co-infection.[31] There is high possibility that some patients are co-infected with other infectious diseases. Furthermore, this study did not include the number of CD4 + count among TB/HIV co-infection patient.

5. Conclusion

MMP-8 and MMP-9 are associated with neutrophil count, suggesting that neutrophils contribute significantly to their secretion. Compared to TB patient without HIV co-infection, neutrophils, lymphocytes and MMP-9 among TB/HIV co-infection patients are significantly lower. However, MMP-8 among TB/HIV co-infection is significantly higher compared to others and among cavitary TB patient with lower lymphocyte count, suggesting substantial effect of lymphocytopenia to neutrophil secretion of MMP-8. The result of this study has shown that low lymphocyte level is related to higher tissue damage caused by unregulated inflammation led by neutrophils.

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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