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Klinikum der Ludwig-Maximilians-Universität München

Inflammatory and prognostic biomarkers associated with pulmonary tuberculosis long-term sequelae after TB treatment in relation to HIV status and lung impairment

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List of abbreviations

APC – Allophycocyanin

ART- Antiretroviral treatment

ATB – Active Tuberculosis

ATS/ ERS – American Thoracic Society/European Respiratory Society

AUC – Area Under Curve

BCG – Bacille Calmette-Guerin

BFA – Brefeldin A

CFP 10 – Culture Filtrate Protein 10

CI – Confidence interval

COPD – Chronic Obstructive Pulmonary Disease

CRP – C reactive protein

DNA – Deoxyribonucleic acid

ESAT-6 – Early Secretory Antigenic Target 6

FEV – Forced expiratory volume

FVC – Forced vital capacity

GM-CSF – Granulocyte- Monocyte Colony-Stimulating factor

HIV- Human Immunodeficiency Virus

HLA-DR – Human Leukocyte Antigen – DR isotype

iFCS – inactivated Fetal Calf Serum

IFNγ – Interferon gamma

IL – Interleukin

LI – Lung impairment

LTBI – Latent Tuberculosis infection

LAMP – Loop-mediated isothermal amplification

LF-LAM – Lateral flow- lipoarabinomannan

MMP – Matrix Metalloproteinase

MPO – Myeloperoxidase

MTB – Mycobacterium tuberculosis

NCAM – Neural Cell Adhesion Molecule

PBMC – Peripheral Blood Mononuclear Cells

PLHIV – People Living with HIV

PPD – Purified Protein Derivative

RIF – Rifampicin

RNA – Ribonuclease nucleic acid

SEB – Staphylococcal Enterotoxin B

TAM-TB – T cell activation and maturation - tuberculosis

TB – Tuberculosis

Th – T helper cells

TNFα – Tumor necrosis factor alpha

WHO – World Health Organization

List of publications

Paper A:

Tuberculosis Treatment Response Monitoring by the Phenotypic Characterization of *MTB*-Specific *CD4*+ T-Cells in Relation to HIV Infection Status. **Nádia Sitoe**, Mohamed I M Ahmed, Maria Enosse, Abhishek Bakuli, Raquel Matavele Chissumba, Kathrin Held , Michael Hoelscher, Pedroso Nhassengo, Celso Khosa, Andrea Rachow, Christof Geldmacher. (2022) *Pathogens*. **2022 Sep 12**;11(9):1034. doi: 10.3390/pathogens11091034.

Paper B:

Effect of TB Treatment on Neutrophil-Derived Soluble Inflammatory Mediators in TB Patients with and without HIV Coinfection. **Nádia Sitoe**, Imelda Chelene, Sofia Ligeiro, Mohamed I M Ahmed, Kathrin Held, Pedroso Nhassengo, Celso Khosa, Raquel Matavele Chissumba, Michael Hoelscher, Andrea Rachow, Christof Geldmacher. (2023) *Pathogens* **2023, 12**, 794. https:// doi.org/10.3390/pathogens12060794.

1. My contribution to the publications

1.1. Contribution to paper A

The aim of the paper was to study the expression dynamics of phenotypic characteristics on *MTB*-specific CD4+ T-cells in relation to TB treatment and the severity of lung impairment in microbiologically cured TB patients. I conceived the study, selected the patient samples for testing, designed and standardized the laboratory protocol as well as tested all samples. I analyzed the crude flow cytometry results using the FlowJo software and organized the data for statistical analysis using GraphPad prism. I performed statistical analyses, drafted the manuscript, and interacted with the supervisors and co-authors for review and support. I addressed their comments and submitted the paper to the scientific journal as a correspondent author. After receiving the reviewers' comments, I revised the manuscript accordingly.

1.2. Contribution to paper B

The aim of the paper was to describe the dynamics of selected plasma biomarkers in relation to HIV status and the severity of lung impairment in cured TB patients before and after receiving TB treatment. I conceived the study defining the objectives and methodology, selecting the patients' sample, and standardizing the laboratory protocol. I ran the laboratory experiments, validated the experiment results, and organized the database. Using GraphPad prism, I performed the statistical analyses and drafted the manuscript. I interacted with co-authors for review and addressed their comments. I also submitted the paper, as corresponding author to the scientific journal and revised it according to the reviewer's comments.

2. Introductory summary

2.1 Background

2.1.1 Epidemiology of Tuberculosis and HIV coinfection

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis* bacteria (*MTB*) [1]. Active TB (ATB) is among the most important HIV-associated opportunistic infections and particularly affecting HIV patients in Southern Africa [2]. It is estimated that 10.6 million people were diagnosed with TB in 2021, of which 23% occurred in Africa. In Africa, 8.0% of TB cases were HIV positive, while in Southern Africa more than 50% were co-infected with HIV [3]. In Mozambique, one of the 30 highest prevalence countries for TB, the incidence of disease in the general population and in those coinfected with HIV is higher, 361 and 90 per 100 000 habitants, respectively [3].

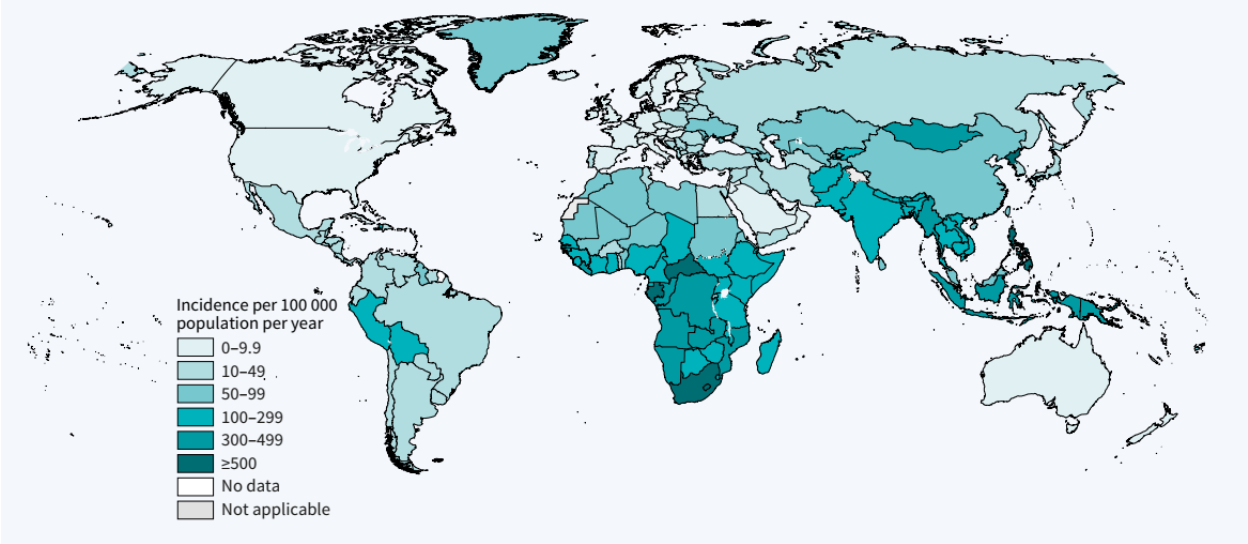

Figure 2.1:TB incidence rate in 2021 (Source: WHO, 2022 [3]).

One third of the global population is considered to have latent TB [1,4,5] of which approximately 5-10% will develop active disease during their lifetime [4–7]. The other 95% are still at risk of developing ATB during their lifetime, especially if they become immunocompromised due to an HIV infection or other illness [8,9]. PLHIV and immunocompromised individuals have a higher probability of developing active disease [6]. An individual with latent TB, who becomes HIV infected has a 26 times higher probability to progress to active TB when compared with HIV negative individuals [10]. If treated, active TB has a relatively high cure rate of 95% with relapse rates of 5%, but if untreated active TB has a probability 50% of death [9].

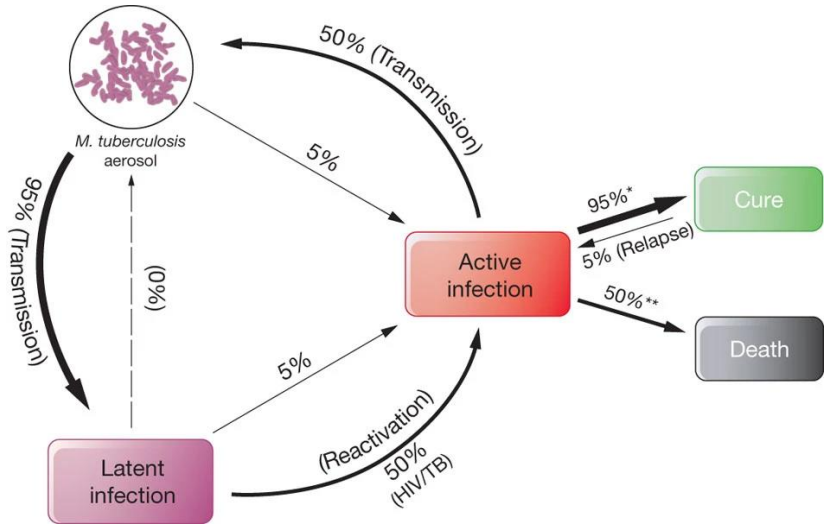

Figure 2.2: TB disease cycle during the lifetime after MTB exposure (Source: Koul et al. 2011 [9]).

2.1.2 Immune response to Tuberculosis and HIV coinfection

The immune response to TB starts in the lungs when the *MTB* is phagocytized by the alveolar macrophages that trigger a bactericidal mechanism, such as production of reactive nitrogen or oxygen intermediates [11,12] at the same time that induce the release of TNFα, IL12 and IL1, to activate the bacterial killing by macrophages; and IFNγ produced by CD4+ and CD8+ T-cells, Natural killer and dendritic cells [12]. Similar to macrophages, neutrophils releases reactive oxygen intermediates, peptides and enzymes that can lyse the *MTB* wall and its systemic reduction interfere the inhibition of bacteria growth [13]. The growth and proliferation of *MTB* can be controlled in the granuloma [12,14]. Granuloma is a cellular structure generated by the aggregation of infected macrophages, neutrophils, T and dendritic cells that inhibits the bacteria spreading, but also works as a reservoir [12,13,15,16]. Neutrophils mediators can influence the development of caseous necrotic granuloma that lead to ATB and *MTB* dissemination [13]. The chemokines and pro-inflammatory cytokines are crucial for the cellular recruitment, formation and stabilization of granuloma [12,17], but environmental factors such as malnutrition or co-infections with HIV or other diseases can reactivate the disease [12,18].

MTB-specific CD4+ T-cells plays an important role to control *MTB* growth and spread, and its early depletion from circulation or tissue-air interphase after HIV infection, contributes to the TB activation [2,19,20]. In early stages of HIV infection, the level of CD4 T-cells is stable, the granuloma is preserved, but in advanced stages, the number of CD4

T-cell counts drop, the granuloma disorganized and the bacteria are disseminated [17]. The granuloma disruption can be due to increasing of HIV viral load that leads to depletion on total number of CD4 T-cells, dysfunctionality of macrophages and changes on function of *MTB*-specific T-cells [21,22]. Furthermore, the depletion of CD4+ T cells is associated to the risk to develop TB in three hypotheses: (i) *MTB* creates an environment for HIV replication and increases the HIV replication in stimulated macrophages; (ii) HIV replicates at sites of *MTB* proliferation, reducing the bacteria containment; and (iii) HIV increases *MTB* replication in tissues and systemic cells migrate to the lungs in response to *MTB*, appearing as a reduction in systemic responses but not indicate a loss of specific responses [2,21–26].

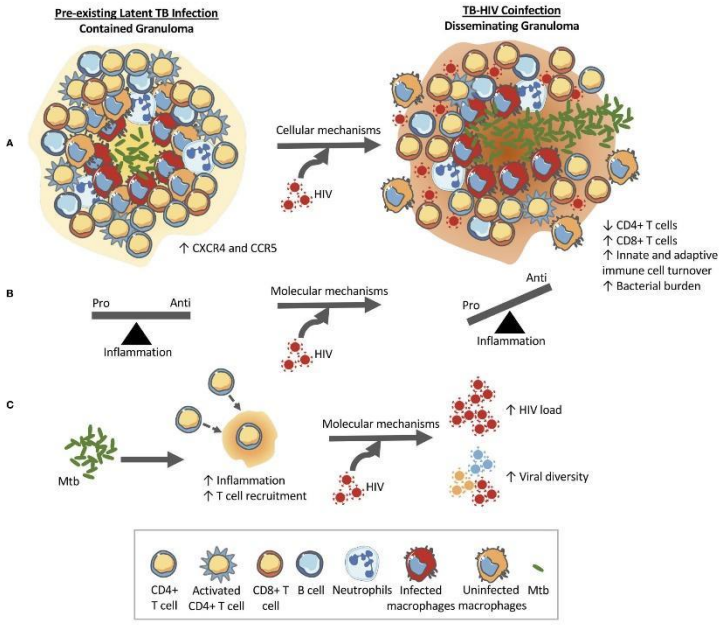

Figure 2.3: Influence of HIV infection on functional impairment of *MTB*-specific CD4+ T-cells (Source: Hoerter et al. 2022 [18]).

2.1.3 Laboratory diagnosis of Tuberculosis

2.1.3.1 Smear microscopy

The detection of *MTB* in sputum by smear microscopy for acid-fast bacilli is widely used at primary health care, is fast, simple and low cost, however has a very low sensitivity and is only positive in half of the patients with ATB with even lower sensitivity in children and PLHIV [27–29]. Furthermore, the efficacy of the test depends heavily on the operator

skills. The staining further is also not specific for *MTB*, but also detects other *Mycobacteria* [27,28]. Fluorescence light-emitting diodes (LED) microscopy, that use LED light sources was implemented to improve the sensitivity and efficacy of TB diagnosis [27], as is less expensive, require less power, can run on batteries and the bulbs do not release toxic products, but operator skills are still crucial [30]. Due the limitation for use of microscopy for TB diagnosis in children and PLHIV, as well as, low bacterial load 2-months post-TB treatment initiation, filtration and centrifugation of sputum samples is recommended to increase the sensitivity, although not usually conducted in limited resource settings [28,29,31].

2.1.3.2 *MTB* Culture

This method is the gold standard for TB diagnosis [27] and is approximately 50% [32] more sensitive than smear microscopy [28,33]. *MTB* culture is conducted in a high containment laboratory due the risk of *MTB* infection [34]. The bacterial growth is slow and can be in solid medium, taking up to 6 weeks, and in liquid medium taking up to 21 days [27,28,34]. *MTB* culture is useful for drug susceptibility tests specially for second line drugs in cases of multidrug resistance, and can allow TB treatment monitoring more efficiently [34,35]. Liquid culture is more sensitive, bacteria rapidly growth but easy for contamination. *MTB* culture contamination rate that can be a major issue, and the WHO recommends to use both growth media, where is possible [27,36].

The limitations for this method for TB treatment monitoring are the need for biosafety level S3 infrastructure, highly skilled personal, long time to diagnosis, and quality control [28,35].

2.1.3.3 Molecular tests

Molecular tests allow the rapid detection of *MTB* DNA and drug resistance. Line Probe assays (LPA) is a molecular method that detects *MTB* DNA after bacteria culture and determines the resistance for rifampicin, isoniazid fluoroquinolones, ethambutol and aminoglycosides [28,35]. This test is performed in sputum samples with known positive result, making it not useful for TB diagnosis [28].

GeneXpert (Cepheid) and Truenat MTB (Molbio Diagnostics) are other tests that detect bacterial DNA with a sensitivity and specificity are up to 85% and 90%, respectively [27,37,38]. Furthermore, these technologies are useful for paucibacillary TB cases in those smear negative or PLHIV [28,35].

The molecular tests are not recommended for TB treatment monitoring because these can detect both live and dead bacteria providing a positive result even if the patient is cured [39].

2.1.3.4 Whole and target genome sequencing

The importance of this assay has been in public health discussions and so far remains an impractical tool for TB diagnosis, yet is useful to formulate public health policies for TB contact tracing in outbreaks [27]. Whole genome sequencing is used for transmission dynamics, to investigate the structure of *MTB* complex, and emergence of drug resistance in a population [40]. Target genome sequencing that use direct sputum, has short turnaround time and is recommended to be used for monitoring of TB resistance [40]. A novel rifampicin gene mutation, not determined in the molecular assays described above, was discovered in Eswatini using this method [27,41]. This mutation was responsible for the MDR-TB outbreak between 2009 and 2010 in Eswatini, and leveraged the concern of an increase of false negatives for rifampicin resistance [27]. Limitation for use of TB genome sequencing is the need of highly skilled personnel, good infrastructure and the costs to maintain the equipment [40], as wells as is only useful for monitoring of TB drug-resistance.

2.1.3.5 Tuberculin Skin Test and Interferon Gamma release assays

Tuberculin skin test (TST) and interferon gamma release assay (IGRA) is common used for diagnosis of latent TB [42]. TST and IGRA are indirect markers of *MTB* exposure and for detect persistent mycobacteria-specific T-cell responses [35,43]. TST is in-vivo assay which purified protein derivate (PPD) is intradermally injected in arm of patient. Induration reaction with more than 15 mm after two to three days is related to past and present *MTB* infections [35,42]. IGRA is another in-vitro assay that use whole blood to detect IFNγ after stimulation with mycobacterial antigens, early secretory antigenic target 6 (ESAT-6) and

culture filtrate protein 10 (CFP-10) [35,42]. Only TST is influenced by BCG vaccination and exposure to environmental mycobacteria [42]. However, due to low sensitivity and inaccuracy to discriminate active and latent TB [43], these two assays are not useful for TB treatment monitoring.

2.1.4 Blood-based biomarkers for TB infection, disease, and treatment monitoring

The majority of assays for TB diagnosis and treatment monitoring are sputum-based, and there is a challenge to obtain good quality sample in patients without productive cough [44] or two months post-TB treatment initiation, as well as, in PLHIV [45] and children [46] who present paucibacillary TB [45,46]. Sputum production reduces with TB treatment and is a clinical signal of improvement [44]. Blood sample is an alternative, is easier to collect and accessible than sputum, especially in patients without a productive cough [47].

Blood biomarkers or profiles to monitor TB treatment are urgently sought after [48–59]. In PLHIV, the diagnosis of TB is a challenge in those with advanced disease because changes in the clinical presentation of disease are observed, pulmonary TB can be initially asymptomatic or the disease can be extrapulmonary [60]. Wallis et al (2009) and Doherty et al (2009) revising the literature, highlighted that there was a need to validate candidate biomarkers that should assess the TB disease status or the risk to progress to disease or relapse [54,58].

Activation, proliferation and maturation markers, expressed on *MTB*-specific T helper 1 and 2 cells, are widely identified. Adekambi et al (2015) identified host-blood biomarkers on *MTB*-specific CD4+ T cells that discriminated between active and latent TB, and could be used for monitoring of TB treatment response [51]. Ahmed et al (2018), in addition to the previous study, found that maturation markers were also useful to monitor TB treatment and showed that early decline in MTB-specific T cell activation correlates with the time to culture negativity [48]. Recent studies done in sub-Saharan Africa, endemic settings for TB/ HIV coinfection, showed that co-expression of activation markers on *MTB*-specific CD4+ T cells can be used for TB diagnosis, regardless of HIV status [56], and could distinguish recent QUANTIFERON-TB conversion and those with disease progression [55]. Most recently, Kroidl et al (2022) showed that *MTB*-specific CD4+ T-cell activation identifies 63% of incipient TB cases in PLHIV at 6 months before actual diagnosis based on GeneXpert and clinical symptoms [53].

Plasma and serum immunological proteins can also be an option to diagnose or monitor TB infection, mainly in pulmonary TB cases. Chemokines have been described as predictors of bacterial burden increase and delay in TB culture conversion [61]. In individuals living without HIV, plasma levels of vascular endothelial growth factor measured at the start of TB treatment could predict sputum culture conversion [62], while in those living with HIV, plasma biomarkers of acute inflammation and microbial translocation are identified as predictors of progression to and recurrence of TB disease [59]. These studies have been relevant as the results could guide future development of simple to use, point-of-care TB diagnostic tests based on immunological biomarkers [63].

2.1.5 Lung impairment in tuberculosis infection

Approximately 40% of cured TB patients are susceptible to develop lung impairment even after successful completion of their TB treatment [64,65]. Lung impairment is measured using spirometry test or X-ray radiography and is an essential clinical measurement [66] to evaluate the lung function or damage. Although both tests are crucial for clinical follow up of the TB cured patients, there are limitations for spirometry use at primary healthcare in constraint-resource settings because of: (i) duration of the test that can increase the time spent by the patient in the health facilities, (ii) difficulties to interpret the results of the test and needs of highly skilled professionals, and (iii) the needs of a dedicate consultation or task delegation for other healthcare workers *versus* the lack of the healthcare workers [67].

The TB-associated lung impairment is characterized by lung cavitation, fibrosis or functional impairment and is driven by the host genetic and immunological response [68]. The host genetic is responsible for regulating the immunological response that is characterized by intense activity of neutrophils, CD4+ T-cells, cytokines, chemokines, and matrix metalloproteinases [68]. An impaired *MTB*-specific Th1 response [69], described by *MTB*-specific CD4+ T-cell activation and maturation profiles pre-TB treatment, correlates with disease extent determined in a chest radiograph [70]. The phenotypic profile of neutrophils was also described as predictors of lung pathology as well as lung recovery before and after TB treatment [71]. Systemic levels of cytokines and chemokines were other biomarkers used for lung impairment. Serum levels of infection, inflammation, metabolism biomarkers, and the levels of hypo-albunemia are associated with radiographic measures of disease severity [57] or poor outcome of TB treatment [72]. Neutrophils-based matrix

metalloproteinases (MMP) and peroxidases (MPO) are proteins potentially contributing to lung impairment that can trigger lung damage and tissue injury after *MTB* infection. While the systemic levels of MMPs and MPO are reduced in patients with good outcome of TB treatment, the sputum levels increase among patients with lung damage at baseline visits [61,73,74], and this is correlated to the levels of circulating neutrophils. This is also observed in PLHIV under ART treatment with lung impairment post-TB cure [68,73]. Impairment of lung function is observed in other lung diseases such COPD which a combination of plasma biomarkers improve the predictive value of disease severity and mortality [75].

2.1.6 HIV and HIV Antiretroviral therapy on risk for TB

The use of antiretroviral therapy (ART) to control the HIV infection mitigate the risk for TB development by 54 to 90% [17] but even under ART, in general population, the risk to develop TB in HIV individuals is 2 to 5 times increased during early stages of HIV infection and more than 20 times increased in advanced stages of HIV infection [76]. In high TB burden countries, in PLHIV, even those with higher CD4+ T-cell counts, the risk for TB is 4.4 higher compared to those without HIV [17]. PLHIV has higher risk to develop TB probably due to the persistent functional impairment of the CD4+ T cell response or due to the fact that CD4+ T cells subsets do not recover quantitatively [17,77].

The standard TB treatment for all patients with drug sensitive TB above 12 years, regardless to the severity of TB or HIV status [78], consists of either a 6-month treatment regimen composed by two months of rifampicin, isoniazid, ethambutol and pyrazinamide, followed by four months of isoniazid and rifampicin, or a four months regimen composed by two months of rifampicin, rifapentine, moxifloxacin and pyrazinamide, followed by two months of rifapentine, isoniazid and moxifloxacin. The selection of the treatment depends on the availability of drugs, but in PLHIV receiving efavirenz ART is recommended to use rifampicin because this drug reduces the plasma concentration of nevirapine and proteases inhibitors [78].

2.2 Rationale

Characterizing the profile of cellular and soluble blood-based biomarkers is crucial for the understanding of the immune response to TB infection [2,71]. A better understanding of the host factors affecting the lung impairment and clinical outcome of pulmonary tuberculosis is important to design evidence-based strategies for improved and more personalized therapeutic intervention [79,80], as well as to predict pulmonary outcomes in patients initiating TB treatment [48,53,55,56]. Different studies described that host immune response-based biomarkers can be used to discriminate between ATB and latent TB infection (LTBI), for TB treatment monitoring [48,51,70,81], for early detection of progression to TB disease [53] and to measure the severity of pulmonary impairment [61,73,82]. The reduction of *MTB*-specific CD4+ T cells expressing activation and proliferation markers post-TB treatment initiation and the association of blood-based neutrophil inflammatory mediators with bacterial load, indicate that these biomarkers could serve as a TB treatment monitoring tool [50,56,77]. Studies to explore or validate these inflammatory biomarkers in higher burden countries for HIV and TB using blood samples are needed. These samples are a feasible option, easy to access and collect, and is not depended on the clinical status of patients.

2.3 Objectives

Based on the rationale, I hypothesized that HIV coinfection impacts the expression of activation and maturation markers by the *MTB*-specific CD4+ T-cells, and the plasma levels of neutrophils-based biomarkers after TB treatment initiation. In this PhD thesis, I aimed to describe and analyze the host response to *MTB* infection with a particular focus on HIV and lung impairment. The specific objectives were:

- To study phenotypic changes of *MTB*-specific CD4+ T-cells in relation to TB treatment outcome and severity of lung impairment in HIV and TB coinfected subjects.
- To investigate the dynamic changes in plasma concentrations of neutrophil-derived inflammatory mediators during TB treatment in context of HIV infection and severity of lung impairment.

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4. Publications

4.1 Paper A

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Article

Tuberculosis Treatment Response Monitoring by the Phenotypic Characterization of *MTB*-Specific *CD4*+ T-Cells in Relation to HIV Infection Status

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Abstract: HIV infection causes systemic immune activation, impacts TB disease progression and hence may influence the diagnostic usability of *Mycobacterium tuberculosis*-specific T cell profiling. We investigated changes of activation and maturation markers on *MTB*-specific CD4+ T-cells after anti-tuberculosis treatment initiation in relation to HIV status and the severity of lung impairment. Thawed peripheral blood mononuclear cells from TB patients with (n = 27) and without HIV (n = 17) were analyzed using an intracellular IFN-γ assay and flow cytometry 2 and 6 months post-TB treatment initiation. H37Rv antigen was superior to the profile *MTB*-specific CD4+ T-cells phenotype when compared to PPD and ESAT6/CFP10. Regardless of HIV status and the severity of lung impairment, activation markers (CD38, HLA-DR and Ki67) on *MTB*-specific CD4+ T-cells declined after TB treatment initiation ($p < 0.01$), but the expression of the maturation marker CD27 did not change over the course of TB treatment. The *MTB*-specific T cell phenotype before, during and after treatment completion was similar between people living with and without HIV, as well as between subjects with severe and mild lung impairment. These data suggest that the assessment of activation and maturation markers on *MTB*-specific CD4+ T-cells can be useful for TB treatment monitoring, regardless of HIV status and the severity of lung disease.

Keywords: tuberculosis; HIV; *Mycobacterium tuberculosis* (*MTB*)-specific; lung severity

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1. Introduction

Tuberculosis (TB) is a disease caused by *Mycobacterium tuberculosis* bacilli (*MTB*) [1,2]. In 2020, 10 million people were diagnosed with TB globally, resulting in 1.5 million deaths, 214,000 of which were recorded among people living with HIV. TB is an opportunistic infection in HIV patients, particularly in the countries of sub-Saharan Africa [1]. Mozambique is among the top 30 countries globally with the highest burden of TB disease [1]. In 2020, the TB incidence in the Mozambican population was 368 per 100,000, with HIV coinfection accounting for 27% of TB cases [1]. Particularly in the absence of antiretroviral therapy (ART), HIV-1 infection represents a major risk factor, increasing the risk to develop active TB (ATB) by 26-fold [3]. Studies have shown the importance of *MTB*-specific CD4+ T helper-1 cell responses in the control of *MTB* infection [4]; however, these cells are rapidly depleted from circulation during HIV infection [5,6]. Furthermore, the overall depletion of CD4+ T-cells caused by HIV-1 infection correlates with the risk of developing ATB infection [3,4,7,8]. ART initiation partially restores *MTB*-specific CD4+ T-cell responses and simultaneously reduces the risk of TB disease progression in people living with HIV [9].

The utility of activation and maturation markers expressed by *MTB*-specific CD4+ T-cells for TB diagnosis and treatment monitoring in peripheral blood has been well described [10–14]. Assessing the expression of the activation and proliferation markers CD38, HLA-DR and Ki-67 can assist in discriminating between ATB and latent TB infection (LTBI) and can be used for monitoring TB treatment response [10–15] and, potentially, for the early detection of TB disease progression [16]. TB treatment also induces the reduction of activation markers on *MTB*-specific CD4+ T-cells in HIV/TB patients, indicating that these markers could serve as a TB diagnosis tool regardless of HIV status [17–19]. Together, these studies suggest that HIV infection does not per se have a major impact on the phenotypic profiles of *MTB*-specific T cells utilized for the diagnostic discrimination of ATB versus LTBI or cured TB.

The lungs are among the major organs affected by TB disease; more than 40% of treated pulmonary TB patients develop chronic lung impairment [20,21], as shown by chest radiography (CXR) abnormalities [22] and the reduced capacity in lung functional tests or physical exercises [23]. The risk factors associated with pulmonary function deterioration include smear-positive disease, extensive pulmonary involvement prior to anti-tuberculosis treatment, prolonged anti-tuberculosis treatment and limited radiographic improvement after treatment [24]. Apart from this, *MTB*-specific T cell functional profiles have been linked to pulmonary cavities in several studies [13,25,26]. TNF-α and IFN-γ producing *MTB*-specific T cells within both the CD4+ and CD8+ T-cell compartments were significantly reduced in TB patients with cavities as compared to those with mere lung infiltrates [26]. Furthermore, Fan et al. (2015) has shown that *M. tuberculosis* antigen-specific Th1 response decreases when pulmonary TB lesions develop to severe cavities [25].

Systemic immune activation—assessed on circulating CD8+ or CD4+ T-cells by the co-expression of HLA-DR and CD38—is a hallmark of HIV [26–29] and TB [13,19] infections and correlates with more rapid HIV disease progression [27]. Indeed, systemic immune activation correlates with CD4+ T-cells depletion [27], is a major determinant of survival in advanced HIV-1 disease [29], and may have utility in the clinical management of HIV-infected persons [30]. To what degree HIV influences the activation profile of *MTB*-specific CD4+ T-cells as bystander activation or because of the higher pathogen activity associated with HIV infection at, before or after ending TB treatment is currently unclear. This is of particular interest for the expression of the activation marker CD38, which differentiated best in previous studies between active TB and LTBI in HIV negative TB patients [11,12]. Furthermore, there are gaps concerning the diagnostic usefulness of *MTB*-specific T cell phenotypic assessments in HIV/TB coinfection in relation to lung damage and the severity of lung impairment before and after TB treatment. Based upon observations that treatment-induced reductions in *MTB*-specific T cell activation correlate with the time to sputum-culture conversion in HIV-negative TB patients [11], we hypothesized that reduction in the activation marker expression of *MTB*-specific CD4+ T-cells is primarily driven by TB treatment, reflecting reductions in the in vivo bacterial load regardless of HIV status and the severity of lung impairment.

Here, we therefore studied the dynamics of *MTB*-specific CD4+ T-cells' phenotypic characteristics in relation to TB treatment, the severity of lung impairment and clinical outcomes in well characterized TB patients in a Mozambican cohort in the context of HIV coinfection and lung function impairment.

2. Results

2.1. Characteristics of the Study Participants

We had valid results from 44 participants and stratified these based on HIV status into TB monoinfected (n = 17) and HIV/TB coinfected (n = 27) groups. The median age was 37 years [IQR: 30.6–48.1] and 39 years [IQR: 27.4–43.9] for the HIV/TB coinfected and TB monoinfected groups, respectively (Table 1). Most of the TB patients were male (31 out of 44) and had converted to TB culture negativity at month 2 (M2) (38/44), reflecting the pattern also observed in the main TB sequel cohort. The remaining TB culture-

positive patients were negative by the end of TB treatment (month 6). The median ratio monocyte/lymphocyte was similar among the HIV/TB coinfected and TB monoinfected groups—0.47 [IQR: 0.37–0.88] and 0.41 [IQR: 0.38–0.63], $p = 0.99$, respectively. The median levels of AST and ALT were also similar between the two groups, $p = 0.9041$ and $p = 0.6041$, respectively. The median Ralph score (RS) at baseline (BL) was 15.0 [IQR: 10.0–45.0] for the HIV/TB coinfected group and for the TB monoinfected group [IQR: 10.0–35.0]. At month 6 (M6), the median Ralph score was higher in the TB monoinfected group (RS = 10.0) compared to the HIV/TB coinfected group (RS = 6.0), but the difference was not statistically significant ($p = 0.1$). Twenty-nine of the TB patients analyzed had lung function impairment at baseline according to spirometry, and the impairment was not completely resolved at the end of TB treatment (Table 1).

Table 1. Characteristics of the study participants.

	HIV$^+$/TB$^+$	**TB$^+$**	***p*-Value**
N	**27**	**17**	
Median of age (range), years	37.01 (20.66–61.73)	38.57 (23.78–52.70)	0.7117
Gender (Male/Female)	19/8	12/5	
BMI [1] at BL (95% CI)	18.40 (17.54–20.34)	18.85 (17.94–20.35)	0.6127
Number of HIV-positive patients ART naïve	8		
Median CD4+ T-cells counts at BL (Min-Max), cells/mm^3	279 (1–812)	____ [6]	
Median CD4+ T-cells counts at BL on HIV ART naïve (range), cells/mm^3	156 (66–365)	____ [6]	
Median ratio monocytes/lymphocytes at BL (95% CI)	0.47 (0.37–0.88)	0.41 (0.38–0.63)	0.9901
Median AST [2] level at BL (95% CI), in U/L	22.0 (21.96–37.24)	20.0 (16.81–58.52)	0.9041
Median ALT [3] level at BL (95% CI), in U/L	31.0 (29.13–46.50)	26.0 (24.43–45.46)	0.6041
TB culture positivity at month 2, N	3	3	
Median ralph score at BL (Min-Max)	15.0 (5–85)	15.0 (7–55)	0.9557
Median ralph score at M6 (Min-Max)	6.0 (2–60)	10.0 (3–58)	0.1038
Presence of lung cavities at M0/M6, N	8/1	4/3	
Any lung impaired spirometry at Month 0, in %	69.57% (16/23 [4])	81.25% (13/16 [5])	
Any lung impaired spirometry at Month 6, in %	65.22% (15/23 [1])	76.47% (13/17)	

[1] BMI—Body Mass Index. [2] AST—Aspartate Aminotransferase enzyme. [3] ALT—Alanine Aminotransferase enzyme. [4] Four subjects had spirometry data missing. [5] One subject had spirometry data missing. [6] Not applicable.

2.2. TB Treatment Reduces the Expression of Activation Markers on MTB-Specific CD4+ T-Cells but Not on Total CD4+ T-Cells, Regardless of HIV Status

As described above, we considered samples that had at least 20 CD4 IFN-γ + T-cell counts for the analyses of phenotypic characteristics. Overall, the TB monoinfected subjects showed a higher probability of responding to *MTB*-specific peptides as compared to the HIV/TB coinfected subjects. The average frequencies of responders with valid results after stimulation with H37Rv, Purified Protein Derivative (PPD) and Early Secretory Antigen Target Protein 6/Culture Filtrate Protein 10 (ESAT-6/CFP-10) were 92.2%, 82.4% and 49.0% for the TB monoinfected group and 78.8%, 62.9% and 55.6% for the HIV/TB coinfected group, respectively (Supplementary Table S1). Hence, the stimulation with antigen H37Rv

resulted in the most robust detection of *MTB*-specific T cell responses, and we therefore focused our analyses of *MTB*-specific T-cell phenotypes on this antigen.

We then compared the frequencies of activation (CD38, HLA-DR and Ki67) and maturation (CD27) markers on *MTB*-specific CD4+ T-cells (representative dot plots presented in Supplementary Figure S1) at baseline, month 2 and month 6 between the HIV/TB coinfected and TB monoinfected subjects and within each group. Overall, combining both the TB monoinfected and HIV/TB coinfected groups, we observed that the expression of each of the activation markers was significantly reduced as early as 2 months into treatment, while the expression of the maturation marker $CD27^{pos}$ did not change much over time (Figure 1A–D). We also compared the HIV/TB coinfected and TB monoinfected groups at each timepoint (Figure 1E–H). At baseline, we observed that the frequencies of *MTB*-specific CD4+ T-cells expressing CD38 (median: 37.80% versus 27.0%, $p = 0.4$), HLA-DR (median: 17.40% versus 18.70, $p = 0.9$) and Ki67 (median: 11.84% versus 6.5%, $p = 0.1$) were largely comparable between HIV/TB coinfected and TB monoinfected patients. Additionally, at the same time point, the frequencies of *MTB*-specific T cells with a $CD27^{pos}$ phenotype in the HIV/TB coinfected group (median: 14.25%) were slightly lower than those in the TB monoinfected group (median: 22.90%); however, the difference was not statistically significant ($p = 0.08$).

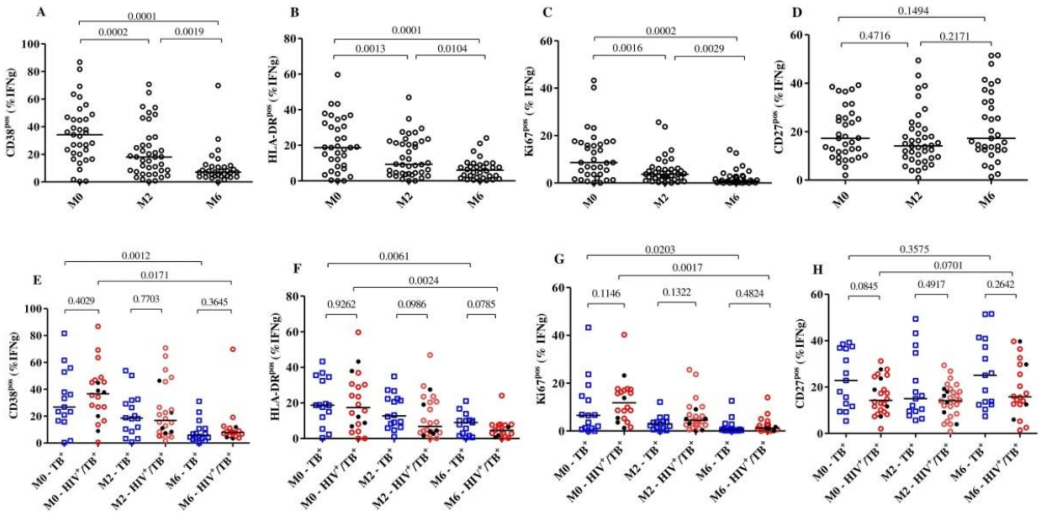

Figure 1. Detection of dynamic changes in the expression of the activation and maturation markers on *MTB*-specific CD4+ T-cells upon TB treatment initiation. The frequency of T cells expressing the activation markers CD38, HLA-DR, Ki67 and CD27 in all subjects ($n = 44$, (**A**–**D**)) and per group (**E**–**H**): HIV/TB coinfected ($n = 27$) and TB monoinfected ($n = 17$) at baseline ($n = 35$), 2 months ($n = 40$) and 6 ($n = 33$) months after TB treatment. The red circles and blue squares represent HIV/TB coinfected and TB monoinfected subjects, respectively. The black dots in the HIV/TB coinfected plots represent the subjects ART naïve at baseline. *MTB*-specific CD4+ T-cells were characterized after H37Rv stimulation. Bars represent medians. Statistical analyses were performed using the Mann–Whitney test for unmatched samples and with the Wilcoxon signed rank test for paired samples. *p*-values are indicated.

The frequencies of the *MTB*-specific CD4+ T-cells expressing CD38 were significantly reduced from baseline (median: 37.8%) to month 2 (median: 15.20%) ($p = 0.0079$) among HIV/TB coinfected patients, while reductions in the TB monoinfected group were relatively minor but statistically significant (median: 26.8% to 18.5%, $p = 0.0052$). At month 6, significant reductions were observed in both the HIV/TB coinfected (median: 37.80% to 7.45%, $p = 0.0171$) and TB monoinfected (median: 26.80% to 3.60%, $p = 0.001$) groups

compared to baseline. These changes in the frequencies of *MTB*-specific CD4+ T-cells were not observed in the total CD4+ T-cells expressing CD38 or HLA-DR. The profile was similar over 6 months of TB treatment in both the HIV/TB coinfected and TB monoinfected subjects (Supplementary Figure S2). We also observed that five of the six TB patients who were still sputum-culture *MTB*-positive at month 2 had substantially decreased frequencies of *MTB*-specific CD4+ T-cells that expressed CD38.

Additionally, we correlated the proportion of the *MTB*-specific CD4+ T-cells expressing the three activation markers, CD38, HLA-DR and Ki67, and the maturation marker, CD27, confirming the previous results reported by Ahmed and colleagues that a positive correlation between the activation markers is present [11]. The strongest correlation was observed between CD38 and HLA-DR expression on *MTB*-specific CD4+ T-cells (r = 0.78 and $p < 0.0001$ in TB monoinfection) (Supplementary Figures S3 and S4). HIV infection slightly decreased the strength of correlation of HLA-DR^{pos} with CD38^{pos} and Ki67^{pos} T cells (r = 0.34, $p = 0.04$ and r = 0.33, $p = 0.0427$, respectively) and increased the strength of correlation of CD38^{pos} with Ki67^{pos} T cells (from r = 0.49 to r = 0.66). No correlation was observed between CD38 and CD27 expression on *MTB*-specific CD4+ T-cells, regardless of HIV status (r = −0.0002, $p = 0.99$ for TB monoinfected and r = −0.26, $p = 0.12$ for HIV/TB coinfected). Together, these results mostly confirm our previous results in HIV negative TB patients and showed that active TB patients, in our setting, typically have activated *MTB*-specific T cells, which decrease rapidly after TB treatment initiation, regardless of HIV infection.

We then intended to address whether the level of reduction in T-cell activation between baseline and month 2 was specific to the *MTB*-specific CD4+ T-cell compartment in both HIV-positive and HIV-negative TB patients. Overall, there were no differences observed in CD38, HLA-DR and Ki67 expression within the total CD4+ T-cells between baseline and month 2 in either group (Figure 2). The expression dynamics for these three markers were significantly different between the total and *MTB*-specific CD4+ T-cells (CD38^{pos}, Ki67^{pos} and HLA-DR^{pos}, $p = 0.003$, $p = 0.02$ and $p = 0.03$, respectively) for the HIV/TB coinfected. Only the slope of the *MTB*-specific T cells expressing HLA-DR was significantly lower than the total CD4+ T-cells in the TB monoinfected subjects ($p = 0.02$) (Figure 2). Hence, the reduction in cellular activation between baseline and month 2 upon TB treatment initiation was specific for the *MTB*-specific T cell compartment and was more pronounced in the HIV/TB coinfected patients.

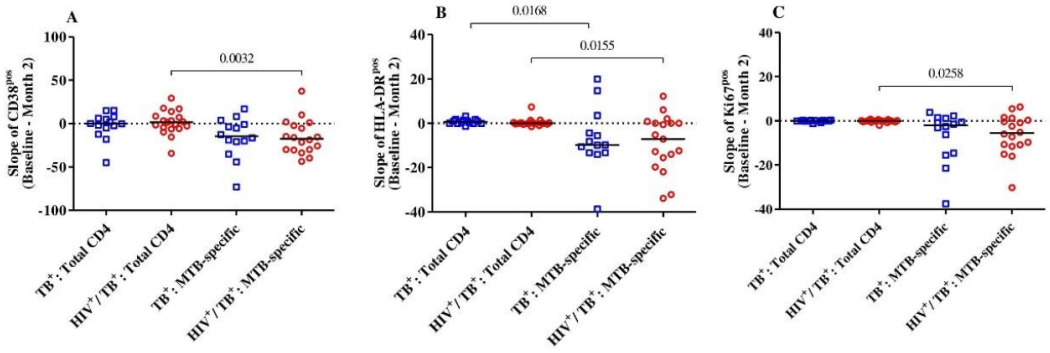

Figure 2. The slopes (difference from baseline to month 2) of the expression of activation markers CD38 (**A**), HLA-DR (**B**) and Ki67 (**C**) on *MTB*-specific and total CD4+ T-cells were compared between and among the HIV/TB coinfected ($n = 19$) and TB monoinfected ($n = 14$) groups. The red circles and blue squares represent HIV/TB coinfected and TB monoinfected subjects, respectively. Bars represent medians. Statistical analyses were performed using the Mann–Whitney test. *p*-values are indicated.

2.3. Frequency of MTB-Specific CD4+ T-Cells Expressing the Activation Markers Reduces over 6 Months of TB Treatment Regardless of the Severity of Lung Impairment

Lung impairment can be a consequence of pulmonary TB infection. Using spirometry, we measured the lung impairment at the end of treatment (month 6). We categorized subjects according to their end-of-treatment lung impairment into "severe" (n = 13), "moderate" (n = 4) and "mild" (n = 11) and compared the phenotypic CD4+ T-cells profile at each time point (baseline, month 2 and month 6).

Overall, we observed that the expression of the activation markers CD38 and HLA-DR on *MTB*-specific CD4+ T-cells was significantly reduced after 6 months of TB treatment (p = 0.003 for CD38, p = 0.0027 for HLA-DR and p = 0.0020 for Ki67), and all these markers were already significantly reduced at month 2. The expression of the maturation marker CD27 on *MTB*-specific CD4+ T-cells did not change much over the observed time (p = 0.31).

Subjects with severe lung impairment after treatment completion had slightly higher frequencies of MTB-specific CD4+ T-cells expressing CD38, HLA-DR and Ki67 compared to subjects with mild impairment, but these differences were not statistically significant (Figure 3A–C). Additionally, for the expression of activation and maturation markers among the severe or mild lung impairment groups, we observed that only the frequency of MTB-specific CD4+ T-cells expressing HLA-DR among subjects with mild lung impairment did not significantly change over 6 months of TB treatment (median: 13.85% [IQR: 3.08–28.53%] at baseline to 4.43% [IQR: 1.25%–7.72%] at month 6, p = 0.10). The median frequencies of *MTB*-specific CD4+ T-cells expressing CD27 did not alter over the duration of TB treatment for subjects with severe (p = 0.92) and mild lung impairment (p = 0.054) (Figure 3D).

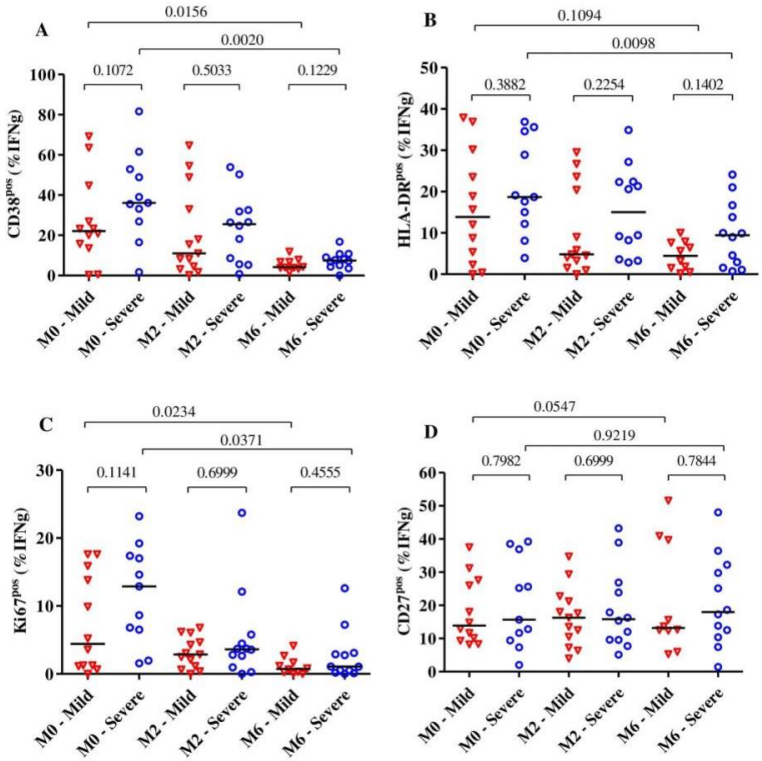

Figure 3. The dynamic of the expression of the activation markers CD38 (**A**), HLA-DR (**B**) and Ki67 (**C**) as well as the maturation marker CD27 (**D**) on *MTB*-specific CD4+ T-cells from the beginning to

the end of TB treatment (n = 28). The expression of these markers was analyzed among subjects with severe (n = 13) and mild (n = 15) lung function impairment (**A**–**D**) at baseline (n = 23), month 2 (n= 25) and month 6 (n = 22). The blue circles and red triangles represent subjects with severe and mild lung impairment, respectively. *MTB*-specific CD4+ T-cells were characterized after H37Rv stimulation. The mild group includes subjects with mild and moderate lung impairments. Bars represent medians. Statistical analyses were performed using the Mann–Whitney test for unmatched samples and the Wilcoxon signed rank test for paired samples. p-values are indicated.

3. Discussion

Indeed, some data indicate that TB patients living with HIV may be affected by more continuous *MTB*-specific T cell activation, even at the end of treatment, and hence have a disease that is probably not completely resolved. Our present study addresses the dynamics of the expression of activation and maturation markers on *MTB*-specific CD4+ T-cells in well characterized pulmonary TB patients upon TB treatment initiation in a high TB and HIV endemic setting. We focused on the potential impact of HIV coinfection and correlations with lung function impairment.

Our study tested PPD, an ESAT-6/CFP-10 peptide pool and the H37Rv antigen; the H37Rv antigen stimulation enabled the detection of a maximum of IFN-γ *MTB*-specific T-cell events for most subjects and time points tested, consistent with a previous study [17]. H37Rv is known to detect mycobacterial responses induced by *MTB*, BCG vaccination or exposure to environmental mycobacteria [9]. In contrast, stimulation with ESAT6/CFP10 antigens often did not result in the detection of sufficient IFN-γ *MTB*-specific cell events to allow for an accurate phenotypic analysis, and this was particularly noteworthy in HIV+ patients. It has been reported that the response to ESAT6/CFP10-specific T-cell immune response in *MTB* subjects involves only a few specific T cells, which may contribute to the low detection rates of *MTB*-specific T cells using this antigen [31]. Additionally, HIV+ patients with low CD4+ T-cell counts and percentages and an advanced disease stage [32] can have false-negative results in the interferon-gamma release assay (IGRA) [32–34] due to reduction in the production of *MTB*-specific IFN-γ [35]. The choice of "whole" *MTB* protein antigens, such as H37Rv or PPD, that increase the number of responders compared to ESAT6/CFP10 alone is therefore important to obtain a maximal number of valid assay results, particularly in people living with HIV. Riou et al. (2020) have also successfully tested an MTB300 peptide pool in people living with HIV with good results; furthermore, similar peptide pools with a small number of *MTB*-derived peptides have been developed [13,16,36]. The inclusion and combination of additional immunodominant *MTB* antigens into so called megapools is an approach that should therefore be considered to optimize the detection and phenotypic characterization of *MTB*-specific T cells, particularly in people living with HIV for diagnostic purposes [36,37].

Our results show that *MTB*-specific CD4+ T-cells activation and proliferation were significantly elevated in TB patients before treatment and decreased within months 2 and 6, with no significant difference between HIV+ and HIV- patients. It is noteworthy that even though the H37Rv antigen may not be specific only for *MTB*-infection [17], a TB treatment-induced decline of the activation profile on *MTB*-specific CD4+ T-cells was observed, suggesting that potential exposure to non-*MTB* mycobacteria did not interfere with the observed TB treatment-induced activation decline. Overall, these results confirm previous studies involving HIV-negative patients [10–12,37,38], and recent studies involving HIV/TB patients [17,19,37,39] and therefore add to the growing evidence that the TAM-TB assay can be utilized for TB diagnosis and treatment monitoring regardless of HIV infection.

The progressive depletion of systemic CD4+ T-cells is the hallmark of HIV infection [14–18]. ART partially reverses the loss of CD4+ T-cells and also reduces HIV-associated systemic T cell activation, as defined by single or co-expressed CD38 [16] and HLA-DR [13,17]. Consistent with Mupfumi et al. (2020), we found a significant reduction in activation marker expression within *MTB*-specific T cells [13,16,17] but not within total CD4+ T-cells at 2 months of TB treatment, which were more pronounced in the HIV coinfected patients compared to the HIV-negative TB patients. This reinforces the notion that these markers can be used to discriminate active TB and

LTBI as well as to monitor declines in mycobacterial burden after two months of ART [16,17]. However, the lack of stratified analysis by ART status at baseline limited the extrapolation of our findings to HIV/TB ART naïve patients. Interestingly, although at baseline our study had the frequencies of *MTB*-specific CD4+ T-cells expressing CD38, HLA-DR and Ki67 in HIV/TB coinfected patients similar to other studies [16,17], we observed that our cohort had lower frequencies of *MTB*-specific CD4+ T-cells expressing HLA-DR compared to the study by Riou et al. (2020) [13]. Differences in the gating strategy, equipment, cellular staining procedure and type of specimen used for antigen stimulation can justify the results. Moreover, our study had 70.4% of HIV patients previously exposed to ART, of whom 30% had CD4+ T-cell counts below 100 cells/mm^3. Riou et al. (2020) had only 38.9% of patients living with HIV on ART [13]. The HIV ART reduces the level of HIV-specific CD4+ T-cells responses [40], and this may be extrapolated to *MTB*-specific responses.

The dynamics of *MTB*-specific CD4+ T-cells activation over 6 months of TB treatment in ATB subjects have not yet been studied in relation to lung function impairment. We observed that regardless of lung impairment severity, there was a significant reduction in activation markers on *MTB*-specific T cells, suggesting that the dynamic changes within these markers are primarily linked to TB treatment-induced reductions in *MTB* bacterial load and not with lung function outcomes. We have previously shown that the slope of *MTB*-specific CD4+ T-cells activation reductions during early treatment inversely correlates with the time to *MTB* culture negativity [11]. Subjects with severe lung impairment had higher frequencies of activated *MTB*-specific CD4+ T-cells compared to those with mild lung impairment, but the difference was not statistically significant. Ravimohan et al. (2018) described that *MTB*-specific T cells secreting IFN-γ perhaps trigger the activation of effectors that result in excessive inflammation and subsequent lung disability [41]. Nevertheless, we did not measure these activation effectors in supernatants after stimulation with *MTB* antigens.

The frequency of *MTB*-specific T cells expressing CD27 did not alter over 6 months of TB treatment in the studied subjects, consistent with our previous observations [11]. CD27 expression on *MTB*-specific T cells was also independent of lung impairment. CD27 has been assessed as a surrogate of TB treatment outcome [11,14,19,42,43] and disease extent [13]. Differently to our study, Riou et al. (2020) observed that 4 weeks after TB treatment, the frequencies of *Mtb*300-specific IFN-γ CD4+ T-cells expressing CD27 increased irrespective of HIV status [13]. In the HIV-negative subjects, these frequencies are similar to those of the latent TB subjects [13]. Hence, the dynamics of CD27 expression on *MTB*-specific T cells upon treatment initiation may be influenced by the nature of the stimulation antigen used for in vitro restimulation. One study found that, at baseline, CD27 expression was strongly correlated with TB disease severity, which includes the presence of pulmonary cavities, showing a co-dependent association between these two factors [13]. Other studies found that, 12 months after treatment initiation, CD27 expression had increased in most subjects [42,44], suggesting slower dynamics of CD27 re-expression as compared to the downregulation of activation marker expression on *MTB*-specific T cells upon treatment initiation [44]. Nikitina et al. (2012) found that the accumulation of CD27 negative *MTB*-specific CD4+ T-cells in the blood is associated with lung destruction [45]. Differences in the cohort characteristics, the criteria to define lung severity, the ICS methodologies and the small sample sizes may explain the differences in the results between these studies and ours. Additionally, our study analyzed CD27-positive *MTB*-specific CD4+ T-cells, which can also drive this difference.

The limitations of this study were the relatively small sample size of subjects that were HIV/TB coinfected, ART naïve and lung function-impaired. Further, no data on the ART response, such as virus suppression or CD4+ T-cell counts after ART initiation, were available for this study. Our study did not classify *MTB*-specific CD4+ T-cells based on TNF-α production. A previous study suggested that the identification of *MTB*-specific CD4$^+$ T cells defined by the production of TNF-α could be more robust than that of IFN-g and IL-2 [46]. However, TNF-α often has an unspecific background in unstimulated CD4+ T-cells

during intracellular cytokine staining experiments, which complicates the differentiation of activation marker expression on truly *MTB*-specific CD4+ T-cells versus none-specific "background staining". Another study limitation was the addition of brefeldin A without a pre-stimulation of PBMCs. Kaven et al. (2012) showed that pre-stimulation varying up to 6 h, before the addition of Golgi protein inhibitors, significantly increased the frequency of multifunctional responses CD4+ T-cells antigenic specific, rising from 0.07% to 1.31% [47].

Our study was the first to investigate TAM-TB assay phenotypic profiles in the context of pulmonary lung impairment. However, future studies with a higher number of TB patients with post-TB treatment lung impairment and HIV infection should therefore further shed light on whether or how HIV infection and lung impairment may be correlated with activation phenotypes of *MTB*-specific CD4+ T-cells.

4. Materials and Methods

4.1. Study Populations

Xpert MTB/RIF (Rifampicin)-positive study participants were recruited in Machava and Mavalane TB Research Centers in Mozambique as part of the TB sequel study [48]. Consenting TB patients with positive Xpert MTB/RIF (Cepheid, Sunnyvale, CA, USA) results were tested for HIV following the national algorithm and were followed from baseline until 12 months after TB therapy initiation during seven study visits (baseline, 14 days and 2, 4, 6, 9 and 12 months). All study participants were TB drug-sensitive. Forty-one had 6 months of a TB regimen, an intensive phase for two months with isoniazid, rifampicin, ethambutol and pyrazinamide and a continuous phase for four months with isoniazid and rifampicin. Three participants were relapsed TB cases when they were recruited into our study and received 8 months of TB treatment. The HIV-positive participants received an antiretroviral treatment composed of Tenofovir/Lamivudine/Efavirenz. During each study visit under TB treatment, liquid and solid TB culture (Lowenstein–Jensen) was performed. In those who were positive for HIV, CD4+ T-cell levels were determined at baseline. Whole blood samples were collected for Peripheral Blood Mononuclear Cells (PBMCs) processing and cryopreservation at baseline, month 2 and month 6 for all participants. Forty-six subjects were selected for our analyses based on the availability of PBMC samples and available data on HIV status, CD4+ T-cell counts, pulmonary function and spirometry at baseline, month 2 and month 6. Samples with poor cellular quality or viability were excluded (Figure 4).

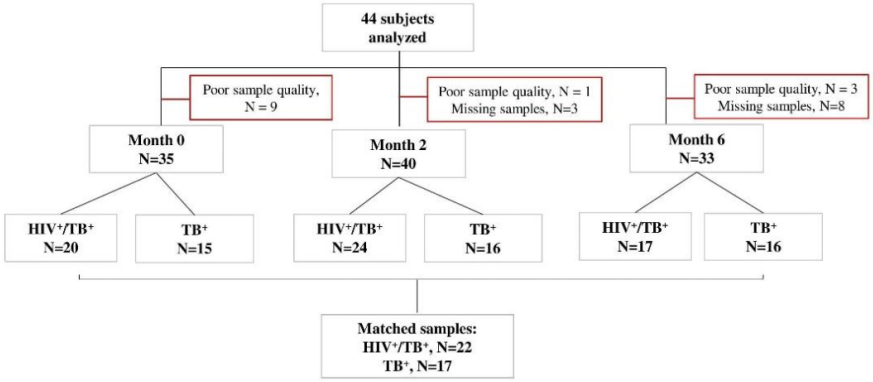

Figure 4. STARD flow diagram of study subjects. Peripheral blood mononuclear cell samples from TB patients with or without HIV (n = 44) were selected at baseline, month 2 and month 6 after TB treatment initiation, in vitro stimulated with *MTB* antigens and analyzed by flow cytometry. The number of missing visits (n = 11) and those excluded due to CD4 IFN-γ count <20 (poor quality samples, n = 13) is indicated for each time point. All samples responded to positive control antigen, SEB.

4.2. Assessment of Lung Function and Damage

Pulmonary function impairment was categorized based on spirometry results and X-ray analysis. X-ray results were read according to Ralph score [49] by two independent readers. Spirometry was performed according to the American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines. The values for Forced Ventilatory Capacity (FVC) and Forced Expiratory Volume in one second (FEV1) as well as the FEV1/FVC ratio were standardized for age, sex and height using Global Lung Function Initiative (GLI) reference equations. Pulmonary function impairment was classified as mild for an FVC and FEV1/FVC ratio >85% of predicted, moderate for an FVC or FEV1/FVC ratio 55–85% of predicted and severe for an FVC or FEV1/FVC ratio <55% predicted [23].

4.3. Assessment of MTB-Specific T-Cell Activation and Maturation

PBMCs were isolated using standard FICOLL centrifugation and LeucoSep tubes (Greiner Bio-One, Kremsmünster, Austria). After the last wash, the PBMCs were re-suspended at a concentration of 10 million PBMC/mL/vial using inactivated Fetal Calf Serum (iFCS) supplemented with 10% Dimethylsulfoxide (DMSO) and then controlled-rate overnight cryopreserved to −80 °C using Mr. Frosty Isopropanol containers. The samples were then transferred to liquid nitrogen the next day. PBMC samples from one patient were thawed simultaneously for all three time points and were later thawed and immediately processed simultaneously using freshly prepared thawing media prewarmed to 37 °C before usage. RPMI-1640 medium with glutamax (Gibco, Invitrogen, Göteborg, Sweden), 0.2% of benzonase endonuclease (Merk, Darmstadt, Germany), 10% of heat inactivated FCS (Sigma-Aldrich, St.Louis, DE, USA) and concentration penicillin–streptomycin (Gibco, Invitrogen, Göteborg, Sweden) were added. Intracellular cytokine staining was performed using an adapted protocol from Ahmed et al., 2018 [11]. In brief, PBMCs were stimulated overnight at 37 °C and 5% CO_2 for 20 h with Early Secretory Antigen Target Protein 6/Culture Filtrate Protein 10 (ESAT6/CFP10, 2µg/peptide/mL, Peptides & Elephants, Hennigsdorf, Germany), Purified Protein Derivative (PPD, 10 µg/mL, Serum Staten Institute, København, Denmark), *Mycobacterium tuberculosis*, strain H37Rv, whole cell lysate (the following reagent was obtained in the BEI Resources, NIAID, NIH: *Mycobacterium tuberculosis*, Strain H37Rv, Whole Cell Lysate, NR-14822) and Staphylococcal Enterotoxin B (SEB, 1 µg, Sigma-Aldrich, St.Louis, DE, USA) as a positive control and no added peptide as a negative control. A total of 50 µL of a co-stimulation cocktail composed of anti-CD49d (1 µg/mL, L25, BD, San Diego, CA, USA), anti-CD28 (1 µg/mL, L293, BD, San Diego, CA, USA) and Brefeldin A (BFA, 5 µg/mL, Sigma-Aldrich, St. Louis, DE, USA) was also added. Cells were stained with anti-CD4 APC (clone 13B8.2, Beckman Coulter, Brea, CA, USA), anti-CD27 ECD (clone 1A4CD27, Beckman Coulter, Brea, CA, USA), anti-CD38 APC fire (clone LS198-4-3, Biolegend, San Diego, CA, USA) and anti-HLA-DR PECy5 (clone Immu-357, Biolegend, San Diego, CA, USA), followed by fixation and permeabilization using FoxP3 Perm/Fix buffer and diluent (eBioscience, San Diego, CA, USA). Intracellular staining was performed using anti-IFN−γ FITC (clone B27, BD Pharmingen, San Diego, CA, USA), anti-Ki67 BV421 (clone B56, BD Pharmingen, San Diego, CA, USA) and anti-CD3 APC-A700 (clone UCHT1, Beckman Coulter, Brea, CA, USA).

Cells were acquired on a CytoFlex Flow cytometer (Beckman Coulter, Brea, CA, USA). All samples with a response in the positive control and no response in the negative control were analyzed using FlowJo_V10 (BD, San Diego, CA, USA) in a gating strategy represented in Supplementary Figure S5. A subject with a positive *MTB*-specific CD4+ T-cell response was considered when: (1) the frequency of the IFN−γ+ CD4+ T-cells after H37Rv, PPD, ESAT6/CFP10 and SEB stimulation was above ≥0.03%; (2) there was a ≥2-fold increase over the negative control and (3) there were at least 20 IFN−γ+ CD4 T cell events recorded.

4.4. Statistical Analysis

We classified the study groups as HIV/TB coinfected or TB monoinfected based on HIV status and mild or severe pulmonary impaired based on the spirometry test.

The frequencies of *MTB*-specific CD4+ T-cells expressing activation and maturation (TAM-TB) markers for each time point were compared regarding the HIV status and the severity of lung impairment using the non-parametric Mann–Whitney and Wilcoxon matched pair tests for unmatched and matched samples, respectively. Additionally, the age and Ralph scores at baseline and month 6 were compared between the HIV/TB coinfected and TB monoinfected groups using the non-parametric Mann–Whitney test. A non-parametric Spearman correlation was used to measure the correlation within the TAM-TB markers. All data were analyzed using GraphPad Prism V5 (GraphPad Software, La Jolla, CA, USA). A p-value < 0.05 was considered statistically significant.

5. Conclusions

In summary, our study supports the concept that dynamic changes in the expression of the biomarkers CD38, HLA-DR and Ki67 on *MTB*-specific CD4+ T-cells over 6 months of TB treatment can be used to monitor TB treatment outcomes among HIV-positive patients. Differences in lung function had no dramatic effects on *MTB*-specific T cell activation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/pathogens11091034/s1, Figure S1: Representative dot plots for the phenotypic characterization of *MTB*-specific CD4+ T-cells, Figure S2: Detection of dynamic changes in the expression of the CD38 and HLA-DR markers on *MTB*-specific CD4+ T-cells and total CD4+T-cells after TB treatment, Figure S3: Correlation analysis of activation and maturation markers expression on *MTB*-specific CD4+ T-cells before and after TB treatment, Figure S4: Correlation analysis of activation and maturation markers expression on *MTB*-specific CD4+ T-cells before and after TB treatment, separately, Figure S5: Gating strategy, Table S1: Percentage of responders per antigen in each time point.

Author Contributions: Conceptualization, N.S. and C.G.; Data curation, A.B.; Formal analysis, N.S., M.I.M.A., M.E. and K.H.; Investigation, N.S.; Methodology, N.S. and C.G.; Project administration, P.N., C.K. and A.R.; Resources, C.G.; Supervision, C.G.; Validation, C.G.; Writing—original draft, N.S.; Writing—review & editing, M.I.M.A., R.M.C., K.H., M.H., P.N., C.K., A.R. and C.G. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the National Bioethical Committee of Mozambican Health of Ministry of Health (protocol code 292/CNBS/21 form 31.05.2021) and the Ethical Committee of Ludwig-Maximillians University of Munich (protocol code 786-16 from 12.10.2018). Informed consent was obtained from all subjects involved in the study.

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Conflicts of Interest: The authors declare no conflict of interest.

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4.2 Paper B

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Article

Effect of TB Treatment on Neutrophil-Derived Soluble Inflammatory Mediators in TB Patients with and without HIV Coinfection

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Abstract: The mycobacteriological analysis of sputum samples is the gold standard for tuberculosis diagnosis and treatment monitoring. However, sputum production can be challenging after the initiation of TB treatment. As a possible alternative, we therefore investigated the dynamics of neutrophil-derived soluble inflammatory mediators during TB treatment in relation to HIV ART status and the severity of lung impairment. Plasma samples of TB patients with (N = 47) and without HIV (N = 21) were analyzed at baseline, month 2, month 6 (end of TB treatment) and month 12. Plasma levels of MMP-1, MMP-8, MPO and S100A8 markedly decreased over the course of TB treatment and remained at similar levels thereafter. Post-TB treatment initiation, significantly elevated plasma levels of MMP-8 were detected in TB patients living with HIV, especially if they were not receiving ART treatment at baseline. Our data confirm that the plasma levels of neutrophil-based biomarkers can be used as candidate surrogate markers for TB treatment outcome and HIV-infection influenced MMP-8 and S100A8 levels. Future studies to validate our results and to understand the dynamics of neutrophils-based biomarkers post-TB treatment are needed.

Keywords: patients living with HIV; TB; multiplex; TB treatment

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1. Introduction

Tuberculosis (TB) and HIV coinfection is a public health problem in TB-endemic countries such as Mozambique [1]. HIV infection increases the risk of developing active TB disease upon infection and of reactivating latent TB [2,3]. The diagnosis of active TB is commonly based on the use of sputum samples [4,5] via the detection of acid fast bacilli using smear microscopy, the culture detection of *Mycobacterium tuberculosis*, or by molecular diagnosis [6–9]. HIV infection reduces the sensitivity of both the GeneXpert molecular test [10,11] and smear microscopy [6,12] as it is often associated with paucibacillary tuberculosis. Both techniques, sputum smear microscopy and mycobacterial culture, have low sensitivity and modest specificity for predicting TB treatment failure [13]. This can be partly explained by the fact that treatment reduces the ability of patients to produce sputum. Therefore, treatment monitoring using sputum-independent host response biomarkers in blood would be a potential and feasible alternative to the laborious and time-intensive microbiological culturing of sputum.

Various blood-based biomarker signatures aiding in the diagnosis of active TB disease and in monitoring TB treatment [4,5,14–17] and the severity of pulmonary impairment [18–20] have been proposed. The systemic host response before and after TB treatment initiation can probably be used to predict pulmonary and treatment outcomes; however, it is still unknown to what extent this is possible [21]. Macrophage and neutrophil levels and activation markers, inflammatory mediators, matrix metalloproteinase (MMP) levels and other factors involved in the host inflammatory response to TB are linked to the inflammatory response that influences the clinical outcome of TB [21]. Rambaran et al. (2020) identified plasma signatures associated with TB culture conversion 8 weeks after the initiation of TB treatment and with lung cavitation in active TB cases [15]. Muefong and Sutherland (2020) described that levels of pro-inflammatory neutrophil-derived mediators in blood, such as MMP-1, -2, -3, -8 and -9, are linked to lung damage at baseline and lung recovery [18,22]. Consistent with these results, another study also showed that levels of certain neutrophil mediators decreased upon TB treatment initiation in a cohort of TB patients living with HIV; hence, such MMPs can be potential biomarkers for monitoring TB treatment regardless of HIV coinfection [23].

Nonetheless, little is known about the profile of blood-based neutrophil mediators at the end of TB treatment and whether they may have potential to predict the outcome of TB in the context of the severity of lung impairment in high HIV and TB burden settings. Our study therefore aimed to study the concentrations of neutrophil-derived proinflammatory mediators during TB treatment in the context of HIV infection and post-TB lung impairment.

2. Material and Methods

2.1. Study Population

TB patients with a positive result on culture methods or Xpert MTB/RIF or Ultra (Cepheid, Sunnyvale, CA, USA) were recruited at the Machava General Hospital and Mavalane Health Center, in Maputo, Mozambique, as part of the TB sequel study [24]. This study aims to understand the clinical, microbiological, immunological and socioeconomic risk factors affecting the long-term outcomes of pulmonary TB in four African countries. All participants who consented to participate in the study were tested for HIV following the national testing algorithm, and those who knew their HIV status were asked about their ART status. Participants were followed for 24 months after the initiation of TB therapy over nine visits: at baseline (BL), after 14 days, and after 2 (M2), 4, 6 (M6), 9, 12 (M12), 18 and 24 months. Sixty-eight subjects were selected for our analyses based on the availability of plasma samples at BL, M2, M6 and M12, HIV and ART status information, and TB culture results at months 2 and 6. Sixty-five participants received standard TB treatment, an intensive phase for two months and a continuous phase for four months accordingly to the WHO TB treatment guideline [25]. Three study participants were TB-drug-resistant and had a different regimen of TB treatment [26].

For all participants living with HIV (PLHIV), the CD4+ T-cells counts at baseline were determined. During each study visit, liquid and solid TB cultures (Lowenstein-Jensen) were performed. The study protocol was approved by the Ludwig-Maximillians University (approval number 786-16, from 10 December 2018) and Mozambican Ethical Committees (approval number 292/CNBS/21, from 31 May 2021).

2.2. Assessment of Lung Function and Damage

Spirometry was carried out at each study visit to assess lung function over the time of TB treatment and thereafter to assess pulmonary function impairment. The American Thoracic Society/European Respiratory Society (ATS/ERS) guidelines were used to interpret the spirometry results. The values for forced ventilatory capacity (FVC) and forced expiratory volume in one second (FEV1), as well as the FEV1/FVC ratio, were standardized for age, sex and height using the Global Lung Function Initiative (GLI) reference equations. The pulmonary function impairment was categorized as mild for an FVC value and FEV1/FVC

ratio > 85% of the predicted value, moderate for an FVC value or FEV1/FVC-ratio 55–85% of the predicted value, and severe for an FVC value or FEV1/FVC-ratio < 55% of the predicted value [27]. As severe TB disease was experienced at baseline by some participants, not all baseline spirometry data were conclusive; therefore, spirometry data from the first study visit, day 0 and day 14 were used herein as baseline lung function determinants.

Pulmonary damage was determined by independent readers of thoracic X-ray results and graded using a Ralph scoring system [28].

2.3. Luminex Assay

Whole blood was collected in heparin tubes, centrifuged at 2500 rpm for 10 min for plasma separation and stored at −80 °C. Plasma samples tested in different batches were diluted 1:2, and the levels of plasma biomarkers were measured using commercial kits, Human Magnetic Luminex assays (Lot number L129636 R&D, Minneapolis, MN, USA), as previously published [29]. The kit detected 15 soluble proteins, namely, IFN-gamma, NCAM, TNF-alpha, IL-8, IL-10, IL1b, GM-CSF, IL-13, IL-12, CD40-ligand, MMP-1, MMP-2, MMP-8, S100A8 and MPO. The sample plates were read on the same day using a MAGPIX system, xMAP instrument (Luminex, Austin, TX, USA). Finally, xPONENT software, version 4.3 (Luminex, Austin, TX, USA), was used for bead acquisition and analysis.

2.4. Statistical Analysis

The continuous clinical and demographic characteristics of people living with and without HIV were compared using the Mann–Whitney non-parametric test.

Protein plasma levels were compared between groups at different time points (baseline and at months 2, 6 and 12) using Kruskal–Wallis and Mann–Whitney non-parametric tests. The Wilcoxon signed rank test was used for comparisons among the study groups at different time points. Spearman's coefficient was used to assess the correlation of the plasmatic biomarkers with the neutrophil counts and the Ralph score at baseline and months 6 and 12. All statistical analyses were performed in GraphPad Prism software, version 5 (GraphPad software, San Diego, CA, USA). p values < 0.05 were considered statistically significant.

3. Results

3.1. Characteristics of Study Population

Table 1 provides an overview of patient characteristics stratified by HIV status. Sixty-eight TB sequel study participants with a median age of 38.2 years [min–max: 19.01–60.60] were selected for the analyses of plasma protein markers based on HIV and ART status and on the completeness of the relevant data on lung damage and function after the end of TB treatment. The majority of the participants were male 57.35% (39/68), and 69.12% (47/68) were living with HIV. Among the active TB (aTB) patients living with HIV, 48.94% were ART-naïve at enrollment, and the median CD4 T-cell count was 134.0 cells/mm^3 [95% CI: 66.50–343.0]. The majority of the subjects then received standard TB treatment of 6 months upon study inclusion (65/68) except for three participants, who had TB caused by rifampicin-drug-resistant MTB and were treated for 8 months instead of 6.

Six months of TB treatment reduced lung damage, as assessed via X-ray, with declining median Ralph scores from 15 at baseline before treatment to 5.00 after 6 months of TB treatment ($p < 0.0001$). TB treatment also reduced the proportion of patients with lung impairment; 75.76% of the patients had a lung impairment at baseline (BL) compared to 61.22% at month 6 and 60.78% at month 12 post TB treatment initiation. Moreover, TB treatment reduced peripheral blood neutrophil levels (median: BL = 3.85 cells/mm^3 and M6 = 1.61 cells/mm^3; $p = 0.01$), consistent with a reduction in TB-associated systemic inflammation after treatment completion.

Table 1. Characteristics of participants included in analyses.

	Total	PLHIV	HIV Negatives
N	68	47	21
Males, % (n/N)	57.35 (39/68)	53.19 (25/47)	66.67 (14/21)
Median age, years (min-max)	38.20 (19.0–60.6)	38.43 (23.8–60.6)	35.23 (19.0–59.2)
HIV- and ART [a] -naïve at BL [b], % (n/N)	48.94 (23/68)	48.94 (23/47)	NA
TB treatment, % (n/N)			
Standard	95.59 (65/68)	93.62 (44/47)	100 (21/21)
TB-DR [c]	4.41 (3/68)	6.38 (3/47)	0 (0/21)
Smear result at BL, % (n/N)			
Negative	11.74 (8/68)	14.89 (7/47)	4.76 (1/21)
1+	8.82 (6/68)	4.26 (2/47)	19.05 (4/21)
2+	20.59 (14/68)	23.40 (11/47)	14.29 (3/21)
3+	36.76 (25/68)	36.17 (17/47)	38.09 (8/21)
Scanty	22.06 (15/68)	21.28 (10/47)	23.81 (5/21)
Ralph score, in median (IQR [d])			
At BL (n = 64)	15.00 (8.00–42.50)	12.0 (8.00–20.00)	20.00 (9.00–48.00)
At month 6 (n = 57)	5.00 (3.00–10.00)	5.00 (3.00–8.00)	7.00 (3.00–10.00)
Spirometry, % (n/N)			
Any lung impairment at BL	75.76 (25/33)	71.43 (15/21)	76.92 (10/13)
Any lung impairment at month 6	61.22 (30/49)	69.69 (23/33)	50.0 (9/18)
Any lung impairment at month 12	60.78 (31/51)	67.57 (25/37)	50.0 (8/16)
CD4 T-cell count, median in cells/mm^3 (IQR) (n = 45)	NA	134.0 (66.50–343.00)	NA
Neutrophils, median in cells/mm^3 (IQR)			
At BL (n = 67)	3.85 (2.78–5.33)	3.77 (2.67–5.24	4.15 (3.5–4.48)
At month 6 (n = 61)	1.61 (1.24–2.24)	1.73 (1.26–2.30)	1.53 (1.21–1.93)
At month 12 (n = 52)	1.79 (1.35–2.56)	1.63 (1.33–2.57)	2.14 (1.51–2.42)

[a] ART: antiretroviral treatment; [b] BL: baseline; [c] TB-DR: TB-drug resistant; [d] IQR: interquartile range.

3.2. MMP-2 Plasma Levels Strongly Correlate with Neutrophils and Ralph Score, and Increased after TB Treatment Initiation

First, we analyzed the plasma concentrations of the inflammatory biomarkers (MMP-1, -2,-8, S100A8 and MPO) over the course of TB treatment within the whole cohort, regardless of HIV status or clinical outcomes. The concentration of MMP-2 moderately increased during the 6 months of TB treatment (median: 38,290.0 pg/mL at BL versus 40,080.0 pg/mL at M2 ($p < 0.0001$) and 41,450.0 at M6 ($p = 0.0032$)) but did not change between months 6 and 12 (median: 41,450.0 pg/mL versus 42,030.0 pg/mL, $p = 0.4417$) (Figure 1B). In contrast, consistent with the results reported by Muefong et al. (2021) [29], the overall plasma concentrations of MMP-1, MMP-8, S100A8 and MPO were significantly reduced from BL to months 6 and 12. These reductions were most pronounced at 2 months after TB treatment initiation, while the concentrations of these analytes did not change between months 6 and 12 (Figure 1A,C–E).

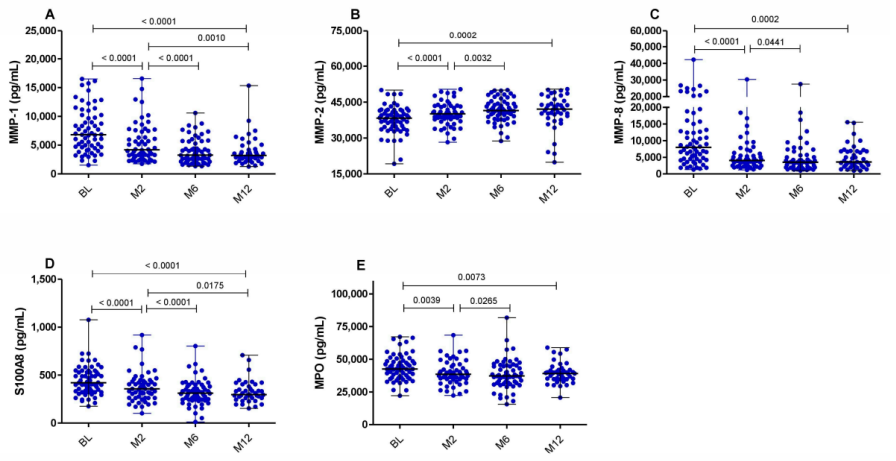

Figure 1. Dynamic changes in the concentrations of neutrophil-related inflammatory biomarkers upon TB treatment initiation. The concentrations of the biomarkers MMP-1 (**A**), MMP-2 (**B**), MMP-8 (**C**), S100A8 (**D**) and MPO (**E**) in all subjects (n = 68) at baseline (n = 62), 2 months (n = 56), 6 months (n = 59) and 12 months (n = 42) after TB treatment initiation are shown. Bars represent medians and interquartile range. Statistical analyses were performed using the Kruskal–Wallis and the Wilcoxon signed rank test for paired samples. A p-value of < 0.05 was considered significant.

Neutrophils contribute to the development of lung cavities [18,30], and we therefore assessed the correlation of the neutrophils and the concentrations of the plasma biomarkers MMP-1, MMP-2, MMP-8, MP0 and S100A8 at BL, month 6 (end of TB treatment) and month 12 (6 months post TB treatment). Baseline neutrophil counts were inversely correlated to the levels of MMP-2 at baseline (r = −0.42 and p = 0.0096) and month 6 (r = −0.46 and p = 0.014), and positively correlated with MMP-1 at month 12 (r = 0.46 and p = 0.04) (Table 2). We also found that the neutrophil count at month 12 inversely correlated with the levels of MMP-1 (r = −0.35 and p = 0.046) and S100A8 (r = −0.46 and p = 0.48 and p = 00009) at month 6 (Table 2).

Table 2. Correlation of the plasmatic biomarkers and neutrophil counts at baseline and months 6 and 12.

Time Point	MMP-1	Neutrophils at Baseline	Neutrophils at Month 6	Neutrophils at Month 12
Baseline	MMP-1	0.18 (p = 0.27)	−0.063 (p = 0.72)	−0.32 (p = 0.06)
	MMP-2	−0.42 (p = 0.0096)	−0.03 (p = 0.86)	0.07 (p = 0.69)
	MMP-8	−0.04 (p = 0.83)	0.12 (p = 0.48)	0.14 (p = 0.42)
	S100A8	0.19 (p = 0.23)	0.12 (p = 0.48)	−0.05 (p = 0.75)
	MPO	0.12 (p = 0.46)	0.09 (p = 0.46)	0.10 (p = 0.55)
Month 6	MMP-1	0.26 (p = 0.14)	−0.071 (p = 0.7)	−0.35 (p = 0.046)
	MMP-2	−0.42 (p = 0.014)	−0.22 (p = 0.22)	−0.05 (p = 0.79)
	MMP-8	0.22 (p = 0.22)	−0.02 (p = 0.89)	−0.01 (p = 0.95)
	S100A8	0.03 (p = 0.85)	−0.06 (p = 0.75)	−0.46 (p = 0.009)
	MPO	−0.0005 (p = 0.99)	−0.02 (p = 0.91)	−0.18 (p = 0.34)
Month 12	MMP-1	0.46 (p = 0.04)	−0.14 (p = 0.54)	−0.21 (p = 0.36)
	MMP-2	−0.28 (p = 0.22)	0.06 (p = 0.81)	−0.18 (p = 0.43)
	MMP-8	−0.22 (p = 0.16)	−0.36 (p = 0.11)	−0.08 (p = 0.71)
	S100A8	0.19 (p = 0.39)	−0.11 (p = 0.65)	−0.13 (p = 0.58)
	MPO	0.04 (p = 0.85)	−0.13 (p = 0.59)	−0.25 (p = 0.28)

Then, we investigated the correlation of these plasma biomarkers with lung damage (Table 3). The baseline Ralph score did not show any association with BL plasma biomarkers. However, the baseline Ralph score positively correlated with month 6 plasma levels of MMP-8 (r = 0.36, p = 0.04) and MPO (r = 0.39, p = 0.02) and negatively correlated with

MMP-2 (r = −0.37 and $p = 0.03$); the Ralph score at month 6 correlated with the level of MMP-8 at month 12 (r = 0.52 and $p = 0.02$).

Table 3. Correlation of the plasmatic biomarkers and Ralph score at baseline and months 6 and 12.

Time Point	Biomarker	RS at Baseline	RS at Month 6
Baseline	MMP-1	−0.036 ($p = 0.83$)	0.31 ($p = 0.08$)
	MMP-2	−0.24 ($p = 0.15$)	−0.1 ($p = 0.58$)
	MMP-8	0.06 ($p = 0.73$)	0.18 ($p = 0.32$)
	S100A8	0.05 ($p = 0.79$)	0.22 ($p = 0.22$)
	MPO	0.07 ($p = 0.68$)	0.12 ($p = 0.50$)
Month 6	MMP-1	−0.13 ($p = 0.46$)	−0.01 ($p = 0.96$)
	MMP-2	−0.37 ($p = 0.03$)	−0.07 ($p = 071$)
	MMP-8	0.36 ($p = 0.04$)	0.08 ($p = 0.67$)
	S100A8	0.03 ($p = 0.89$)	−0.01 ($p = 0.95$)
	MPO	0.39 ($p = 0.02$)	0.10 ($p = 0.58$)
Month 12	MMP-1	−0.32 ($p = 0.15$)	0.10 ($p = 0.67$)
	MMP-2	−0.062 ($p = 0.79$)	0.23 ($p = 0.33$)
	MMP-8	0.07 ($p = 0.77$)	0.52 ($p = 0.02$)
	S100A8	0.08 ($p = 0.72$)	0.28 ($p = 0.24$)
	MPO	0.09 ($p = 0.68$)	0.35 ($p = 0.13$)

We also tested for correlations between plasma biomarkers amongst each other and found that S100A8 correlated to MMP-1 and MMP-8 at baseline and at months 6 and 12. S100A8 and MPO, however, correlated at months 6 and 12 (M6: r = 0.38 ($p = 0.02$) and M12: r = 0.69 ($p < 0.0001$)), respectively. MPO correlated with MMP-2 at baseline and month 12, and it also correlated with MMP-8 at months 6 and 12 (Table 4).

Table 4. Spearman correlation among the plasmatic biomarkers at baseline and months 6 and 12.

Time Point	Biomarker	MMP-1	MMP-2	MMP-8	MPO
Baseline (n = 38)	MMP-1	NA	−0.24 ($p = 0.15$)		
	MMP-8	0.14 ($p = 0.41$)	0.09 ($p = 0.57$)		0.27 ($p = 0.09$)
	S100A8	0.63 ($p < 0.0001$)	0.01 ($p = 0.097$)	0.33 ($p = 0.04$)	0.06 ($p = 0.74$)
	MPO	0.01 ($p = 0.94$)	0.39 ($p = 0.02$)		NA
Month 6 (n = 37)	MMP-1	NA	−0.14 ($p = 0.39$)		
	MMP-8	0.07 ($p = 0.66$)	−0.04 ($p = 0.82$)		0.71 ($p < 0.0001$)
	S100A8	0.74 ($p < 0.0001$)	−0.06 ($p = 0.73$)	0.36 ($p = 0.03$)	0.38 ($p = 0.02$)
	MPO	0.11 ($p = 0.51$)	0.23 ($p = 0.17$)		NA
Month 12 (n = 42)	MMP-1	NA	−0.24 ($p = 0.13$)		
	MMP-8	0.14 ($p = 0.37$)	0.23 ($p = 0.15$)		0.54 ($p = 0.0003$)
	S100A8	0.42 ($p = 0.0057$)	0.28 ($p = 0.07$)	0.61 ($p < 0.0001$)	0.69 ($p < 0.0001$)
	MPO	0.19 ($p = 0.22$)	0.31 ($p = 0.04$)		NA

3.3. ART-Naïve Patients Living with HIV Coinfected with TB Exhibit Markedly Elevated Levels of MMP-8 and S100A8

Next, we studied the concentrations of MMP-1, 2, 8, S100A8 and MPO analytes in relation to HIV and ART status. HIV infection status did not alter MMP-1 levels over the entire period study (Figure 2A). PLHIV on ART with active TB had significantly elevated MMP-2 plasma concentrations at baseline and at months 6 and 12 compared to TB

mono-infected patients (Figure 2B). PLHIV who were ART-naïve had 2.1-fold increased concentration levels of MMP-8 at baseline compared to TB mono-infected patients (median: 10,050.0 pg/mL versus 4,805.0 pg/mL, $p = 0.03$). Similar differences, albeit at a lower level, were observed at months 2 (median: 5,097.0 pg/mL versus 3,893.0 pg/mL, $p = 0.031$) and 6 (median: 3,980.0 pg/mL versus 2,215.0 pg/mL, $p = 0.018$). Furthermore, plasma levels of MMP-8 were also higher in ART-naïve PLHIV compared to individuals on ART at months 2 ($p = 0.048$) and 6 ($p = 0.045$) (Figure 2C). At the end of treatment, we had similar findings in the concentration levels of S100A8 in ART-naïve PLHIV compared to individuals on ART (median: 357.2 pg/mL versus 311.9 pg/mL, $p = 0.043$) (Figure 2D).

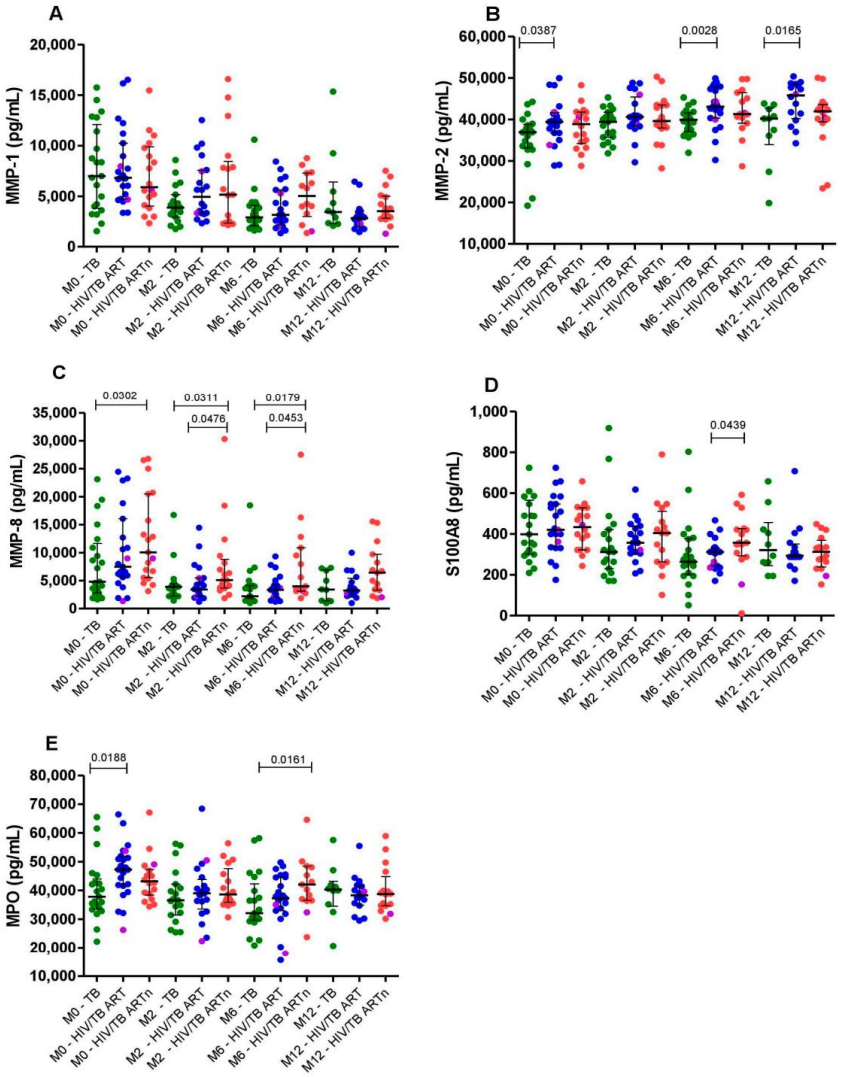

Figure 2. Comparisons of neutrophil-related inflammatory biomarkers MMP-1 (**A**), MMP-2 (**B**), MMP-8 (**C**), S100A8 (**D**) and MPO (**E**) between TB mono-infected (n = 21, green dots), HIV/TB coinfected patients on ART (n = 23, blue dots) and HIV/TB coinfected ART-naïve patients (n = 19, red dots) at baseline, month 2, month 6 and month 12 study visits. The magenta dots are the subjects with drug-resistant TB. Bars represent the median and interquartile range. The Mann–Whitney test was used for comparisons between the groups at different time points. $p < 0.05$ was considered significant.

Regarding plasma levels of MPO, PLHIV on ART and ART-naïve PLHIV had higher concentrations compared to TB mono-infected patients at baseline (median: 47,230.0 pg/mL versus 37,790.0 pg/mL, $p = 0.019$) and month 6 (median:42,050.0 pg/mL versus 32,060.0 pg/mL, $p = 0.016$), respectively (Figure 2E).

There were three PLHIV with TB-drug resistance at baseline in our study; one of them was ART-naïve at BL. At month 6 of TB treatment, the PLHIV ART-naïve patient developed cavities in the lungs (RS = 15.0 at BL and RS = 60.0 at month 6). In summary, these data show that MMP-2, MMP-8, S100A8 and MPO neutrophile-derived inflammatory markers are elevated in PLHIV before and after the initiation of TB treatment.

3.4. Higher Levels of MMP-8 and MPO before Treatment Initiation Are Linked to More Severe Lung Impairment at the End of TB Treatment Initiation

Based on spirometry results at baseline and month 6, we grouped TB sequel participants with normal and mild lung impairment as the "less severe" lung impairment group and those with moderate and severe lung impaired as the "more severe" lung impairment group. Of note, participants not able to undergo a spirometry test at baseline mostly did so on day 14 of the study.

Considering the spirometry results from BL/D14 visits and month 6, the levels of MMP-1 and MMP-2 were similar between the patients with more severe and less severe lung impairment at all study points (Figure 3A,B). However, using the BL/D14 spirometry, patients with less severe lung impairment had higher plasma levels of MMP-8 and S100A8 at the month 12 visit compared to those with more severe lung impairment, $p = 0.0007$ and $p = 0.018$, respectively (Figure 3C,D). Moreover, using the BL/D14 spirometry results, we observed that 6 months after the initiation of TB treatment, plasma levels of MPO in the more severe patients were higher than the less severe patients (median: 40,430.0 pg/mL versus 30,460.0 pg/mL, $p= 0.0134$) (Figure 3E).

Interestingly, before TB treatment initiation, patients with more severe lung impairment after TB treatment at month 6 had higher levels of MMP-8 and MPO compared to those with less severe lung impairment, $p = 0.016$ and $p = 0.001$, respectively. After TB treatment initiation, the levels of these two biomarkers were comparable between the two groups, although at months 2 and 6, MPO tended to be higher in the more severe compared to the less severe lung-impaired patients (Figure 4C,E). The levels of S100A8 were also comparable between subjects with more and less severe lung impairment (Figure 4D).

The Ralph score at baseline was not significantly associated with the Ralph score at the end of TB treatment (data not shown; r= 0.23 and $p = 0.087$), and the patterns of the plasma levels of MMP-8 and MPO in subjects with more severe lung impairment at enrollment were similar to those with any lung impairment at month 6 (Figure 4F).

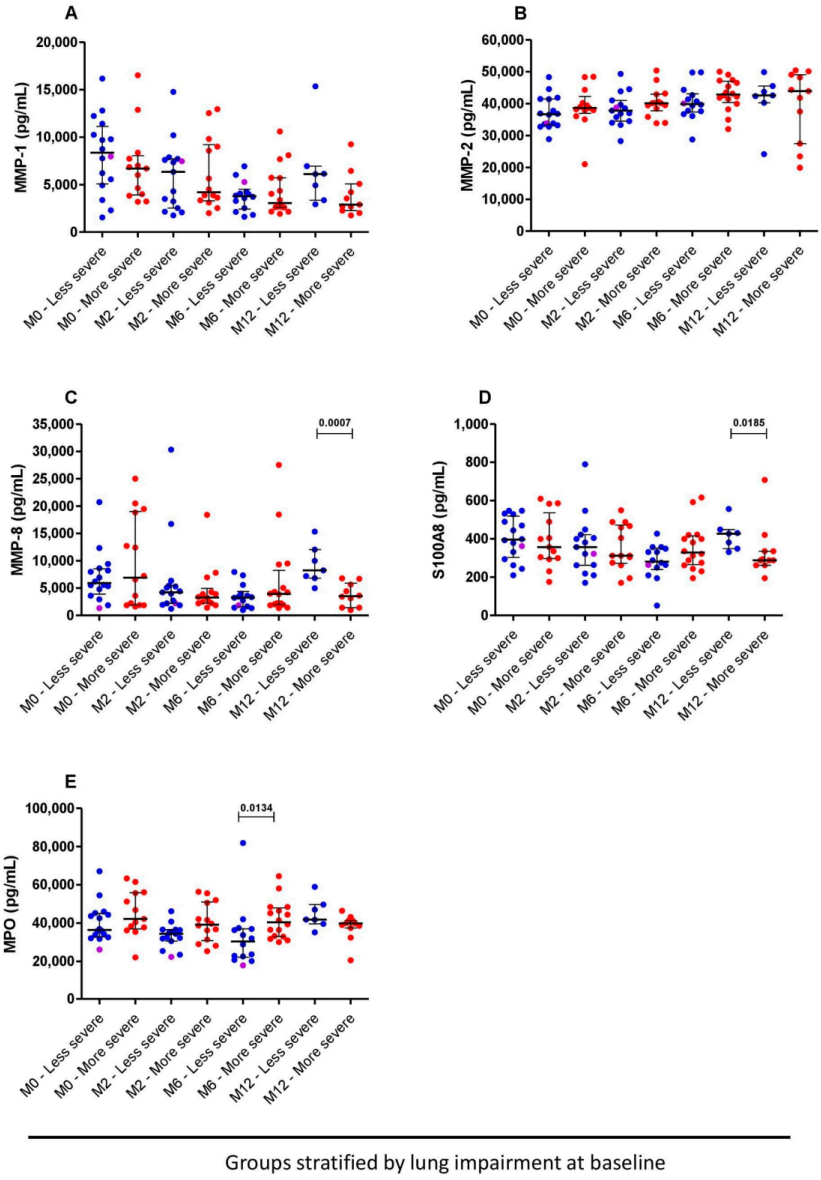

Figure 3. Comparisons of plasmatic biomarkers based on spirometry results from before TB treatment or on day 14: MMP-1 (**A**), MMP-2 (**B**), MMP-8 (**C**), S100A8 (**D**) and MPO (**E**) at baseline (n = 29), month 2 (n = 29), month 6 (n = 30) and month 12 (n = 17) between less (n = 16, blue dots) and more severe (n = 18, red dots) lung impairment. The more and less severe lung-impaired patients are represented by red and blue circles, respectively. The magenta dots represent the subjects with drug-resistant TB. Bars represent the median and interquartile range. Mann–Whitney tests were used for comparisons between the groups at different time points. $p < 0.05$ was considered significant.

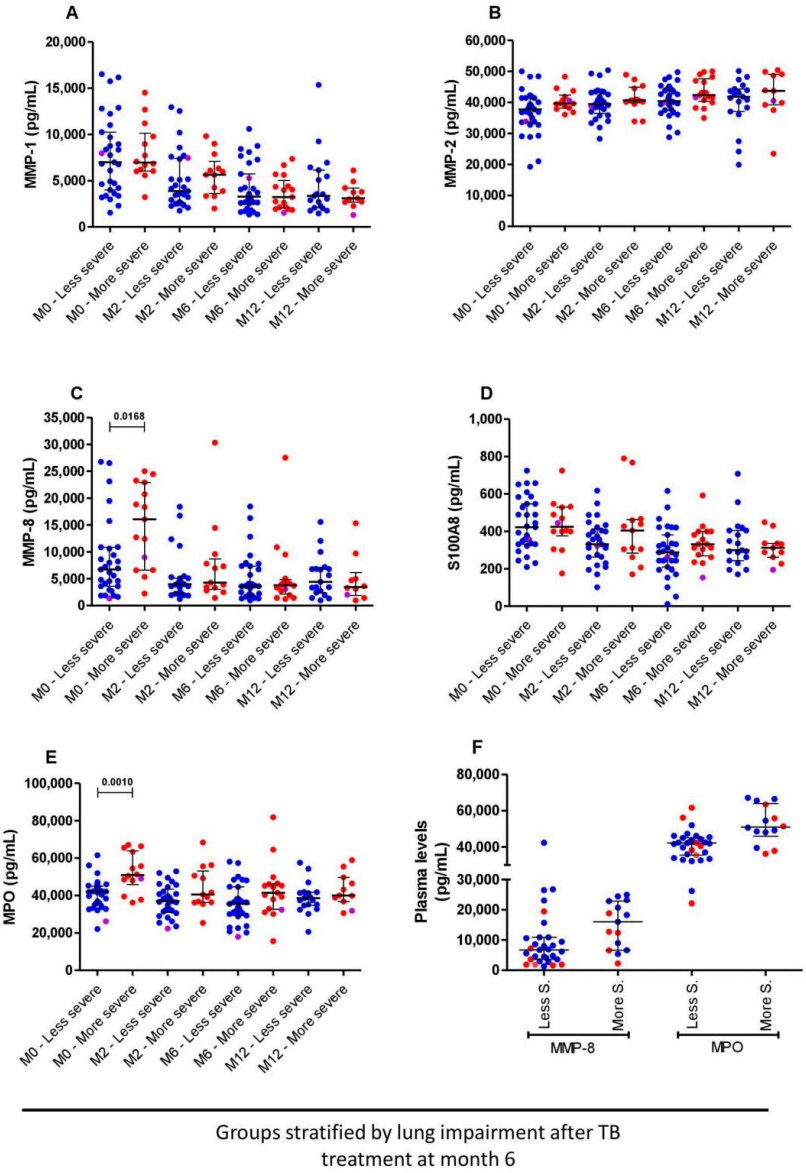

Figure 4. Comparisons of plasmatic biomarkers based on spirometry results of month 6: MMP-1 (**A**), MMP-2 (**B**), MMP-8 (**C**), S100A8 (**D**) and MPO (**E**) at baseline (n = 45), month 2 (n = 42), month 6 (n = 48) and month 12 (n = 31) between patients with more severe (n = 18) and less severe (n = 33) lung impairment. The more and less severe lung-impaired patients are represented by red and blue circles, respectively. The magenta dots represent the subjects with drug-resistant TB. (**F**) MMP-8 and MPO plasma levels at baseline of subjects with less and more severe lung impairment based on spirometry results at month 6, with red dots coding for subjects with more severe lung impairment at baseline. Bars represent the median and interquartile range. Mann–Whitney tests were used for comparisons between the groups at different time points. $p < 0.05$ was considered significant.

4. Discussion

Our study investigated several neutrophil-derived inflammatory mediators in relation to TB disease severity, HIV status and treatment outcome. Plasma levels of MMP-1, MMP-8, S100A8 and MPO were markedly reduced after TB treatment initiation regardless of HIV

infection status, with the most noteworthy decline occurring by month 2. Nonetheless, HIV coinfection influenced the plasma levels of some of these mediators; MMP-8, S100A8 and MPO were elevated at baseline and also at the end of TB treatment, albeit to a lower degree. The notable reduction in these inflammatory mediators at month 2 aligns with sputum conversion; all participants were sputum negative by month 2 post TB treatment initiation. This indicates that changes in the levels of these inflammatory mediators are linked with the early bactericidal effect during the first two months of intensive TB treatment. Similar results were observed in previous studies [18,19,23,29,31,32]. Neutrophil levels in blood correlated negatively with MMP-2 at baseline and month 6, and they also declined during TB treatment, with the opposite effect observed for MMP-2 levels. The other neutrophil-derived mediators did not correlate with neutrophil levels at baseline. One possibility is that it is particularly the activated neutrophils in the infected lung tissue that release these mediators. An in vitro study has shown that neutrophils in direct contact with MTB bacilli express high levels of MMP-8 [33]. Muefong and colleagues reported positive correlations of baseline Ralph scores and neutrophil counts with several neutrophil-derived inflammatory markers, such as MMP-8 and S100A8 [29]. These correlations were often not detected in our study. The abundance of low-density neutrophils in aTB patients is another possibility. These neutrophils are characterized by the inability to phagocyte the MTB and to produce reactive oxygen species [34], both of which are important for release of the neutrophils-derived inflammatory markers. HIV infection was associated with MMP-8 baseline levels in our study, and it is often associated with paucibacillary disease [35,36] and fewer cavities [37,38]. The high proportion of PLHIV and the low numbers of subjects with Ralph scores above 40 (indicative of cavities) and participants with CD4 counts below 200 in our study could therefore be responsible for these discrepancies. Moreover, our study had a smaller statistical power compared to the Muefong study.

It has been reported that HIV infection modulates the expression of matrix metalloproteinases (MMPs) [39]. In our cohort, the plasma levels of MMP-8 were higher in PLHIV, particularly those who were ART-naïve at baseline, even at the end of TB treatment and after ART initiation. Hence, other factors related to HIV infection, such the impact of HIV ART initiation, higher levels of systemic inflammation and/or microbial translocation probably contribute to this observation [40–44]. Our results are consistent with Alisjahbana et al. (2022), who found that a low lymphocyte level was significantly correlated to higher levels of MMP-8 [40]. Furthermore, TB-immune reconstitution inflammatory syndrome (TB-IRIS) often occurs in highly immunosuppressed patients after the initiation of ART [45]. Ravimohan et al. (2016) found an increase in the plasma levels of MMPs, including MMP-8, associated to TB-IRIS and a decrease in lung function post TB cure [41]; however, we did not specifically assess the proportion of PLHIV who developed TB-IRIS in our study.

Plasma levels of MMP-1, MMP-8, S100A8 and MPO before and after TB treatment initiation or 6 months after TB treatment were strongly correlated. This was also observed in animal and human models [46,47]. Gonzalez-Lopez et al. (2012) found that serum and plasma levels of MMP-8 modulate the levels of S100A8/9 [46], which is a contribution of the genetic polymorphisms of S100A8/9 genes [47]. In our study, participants with less severe levels of lung impairment had higher plasma levels of MMP-8 and S100A8 at month 12 post TB treatment initiation. Neutrophil-related inflammatory mediators correlate with pulmonary injury in humans [18,29,48] and animals [30,49,50]. S100A8, MMP-8 and MPO are involved in lung pathology [5,18,29,30,51] as consequences of lung tissue degradation in tuberculosis [18,29,30], emphysema [52], COVID-19 [53] or COPD [54]. Hence, while speculative, subjects starting TB treatment with less severe lung impairment may potentially develop a more severe lung condition after TB treatment as MMP-8 and S100A8 biomarkers are involved in the pulmonary damage. Indeed, at the end of TB treatment or 6 months after TB treatment, the plasma levels of the inflammatory mediators MMP-8, MPO and S100A8 were associated with the Ralph score, consistent with the notion that these biomarkers may contribute to lung damage and the impairment of lung function

after the end of TB treatment [55]. However, these findings must be confirmed in future studies with larger sample sizes and long-term follow ups.

The limitations of this study include the relatively small sample size of patients at 6 months post TB treatment and of those with mild and severe lung impairments. In addition, our study was of an exploratory nature, and we did not seek to confirm these results with an independent experiment and samples. Other limitations include the lack of availability of plasma HIV viral load data because it has been reported that HIV drives the dysfunction of neutrophils [56] that could probably influence the production and release of its inflammatory mediators.

In summary, our study confirmed that the plasma levels of neutrophil-based biomarkers markedly change with TB treatment regardless of HIV ART status and the severity of lung impairment. HIV infection status and ART negativity at baseline influenced MMP-8 and S100A8 levels. Future studies to validate our results and to explore the post-TB treatment dynamics of neutrophil-based biomarkers are needed.

Author Contributions: Conceptualization, N.S. and C.G.; data curation, N.S.; formal analysis, N.S.; investigation, N.S., I.C., S.L. and C.C.; methodology, N.S. and C.G.; project administration, P.N., C.K. and A.R.; resources, C.G.; supervision, C.G.; validation, C.G.; writing—original draft, N.S.; writing—review and editing, M.I.M.A., R.M.-C., K.H., M.H., P.N., C.K., A.R. and C.G. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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Conflicts of Interest: The authors declare no conflict of interest.

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