Severely reduced antioxidant and impaired mitochondrial biomarkers could be linked to post-tuberculosis lung disease in a cohort from South Africa

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Background. Post-tuberculosis lung disease (PTLD) refers to unresolved lung damage and impaired lung function after successful treatment of tuberculosis (TB). Its pathogenesis is not fully understood, and we hypothesised that antioxidant-oxidant and mitochondrial factors may be instrumental.

Objectives. To investigate the involvement of mitochondrial and antioxidant-oxidant biomarkers in TB patients who had had more than one previous TB episode and were in the post-TB stage of disease.

Methods. Enzyme-linked immunosorbent assays were conducted on patient serum.

Results. Lipid peroxidation (measured with the thiobarbituric acid reactive substances assay) was within the normal range. In contrast, the mitochondrial regulator metallothionein-1 was 240 times lower, catalase activity 7.5 times lower, and superoxide dismutase activity 273 times lower than the normal ranges for these markers. Hypoxia-inducible factor-1-alpha (HIF-1α) was below the limit of detection. The mitochondrial markers were similar across the stratified groups after stratifying the patients based on the number of previous TB episodes. Age positively correlated with the ratio of early diastolic mitral inflow velocity to early diastolic mitral annulus velocity (E/e′), a marker of left ventricular filling pressure, and it marginally correlated with pulmonary artery systolic pressure, while there were no other notable correlations.

Conclusion. Our data demonstrate that antioxidant enzyme activities are extremely low in post-TB patients. A key mitochondrial protein, HIF-1α, showed no role in this context, which could suggest that these patients are not hypoxic. A novel contributor to PTLD could therefore be a limitation of antioxidant capacity and mitochondrial pathways, which is not linked to the number of previous TB episodes but may highlight the need to consider antioxidant therapy during the post-TB stage. Further research is warranted.

Keywords. Post-tuberculosis lung disease, underlying mechanisms, pulmonary hypertension, mitochondrial dysfunction, antioxidant capacity.

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Study synopsis

What the study adds. This is one of the first studies to link reduced antioxidant and impaired mitochondrial biomarkers to the pathogenesis of post-tuberculosis (TB) lung disease.

Implications of the findings. This study will allow for a better follow-up management plan of patients after resolution of TB symptoms, in order to prevent post-TB lung impairment.

Pulmonary tuberculosis (TB) is caused by a single infectious agent, *Mycobacterium tuberculosis*, and spreads between individuals via aerosol droplets.[1] TB is a leading cause of death globally, with a global prevalence of 14 million cases in 2022, with a further six million cases that are believed to remain undiagnosed.[2] Accumulating evidence demonstrates that TB has several long-term consequences, also termed post-TB lung disease (PTLD),[3] that can manifest as moderate or severe impairment of pulmonary function, obstructive pulmonary ventilation defects, and opportunistic infections.[4] The development of PTLD is not well understood, and several pathways have been hypothesised to contribute to it, including inflammatory[5] and endothelial function.[6] Another pathway that

may contribute is mitochondrial, and previous work has highlighted the key roles of mitochondria in TB and PTLD.[7,8] In brief, mitochondrial free radicals and mitochondrial antioxidant systems play a role in the development of mitochondrial dysfunction,[7,8] which in turn contributes to cellular death and the pathogenesis of forms of PTLD such as pulmonary hypertension (PH).[9] However, the field is poorly understood, and further research is needed to better comprehend the involvement of mitochondrial pathways in the post-TB context.

Methods

This article investigates mitochondrial and antioxidant-oxidant biomarkers in patients with ≥1 previous TB episodes, who were successfully treated for TB at least 1 year before enrolment. This substudy is nested within the Pulmonary Artery Pressures in Pulmonary Tuberculosis Study II (PuPPeT II) trial, which was a cross-sectional study that evaluated the prevalence of PH in TB patients.[10] For the parent study, and therefore this substudy, all patients completed a questionnaire on age, sex, heart disease, HIV status, smoking status, and sociodemographic status. We also included data on pulmonary artery systolic pressure (PASP) and estimated left ventricular diastolic function and filling pressure, determined using the ratio of early diastolic mitral inflow velocity to early diastolic mitral annulus velocity (E/e′), after transthoracic echocardiography.[10] The inclusion criteria of the parent study required that participants be aged ≥18 years and that they met the criteria of having ≥1 previous TB episodes and being successfully treated for TB at least 1 year before enrolment. Exclusion criteria were failure to provide written and informed consent, multidrug-resistant or extensively drug-resistant TB, active malignant disease, the presence of dementia or delirium if the patient was deemed medically unstable by the study team, or if any condition was present that may have made for an unreliable echocardiography measurement of pulmonary pressure, such as pericardial tamponade. Ethical approval was obtained from the Stellenbosch University Human Research Ethics Committee (ref. no. N18/08/091).

Phlebotomy and serum collection

After informed consent was obtained, blood was collected in vacutainer blood tubes (PLAS-GD100EK2 and PLAS-VAC-Y5; Gongdong Medical Technology Co., Ltd, USA). Plasma blood collection tubes contained ethylenediaminetetra-acetic acid, which blocks the coagulation cascade by binding to calcium ions, while serum collection tubes were used to isolate serum samples. The serum collection tubes were coated with a clot activator and gel, which allows for serum separation and therefore high-quality serum specimens for laboratory analysis. Specimens were taken to the laboratory, where all blood tubes were centrifuged at 3 000 rpm for 10 minutes. The centrifuge (Centrifuge 5810; Eppendorf North America, USA) was solely used for TB work by the Division of Medical Physiology at Stellenbosch University, and all processes were performed in a class II biosafety cabinet (centrifugal fan H14 HEPA filter; Biobase, China). Plasma and serum were stored as 50 µL aliquots at –80°C in a freezer designated for potentially biohazardous specimens, according to Centers for Disease Control and Prevention guidelines.

Enzyme-linked immunosorbent assays

The thiobarbituric acid reactive substances (TBARS) assay measures lipid peroxidation, a proxy for oxidative stress. It measures malondialdehyde, a reactive compound formed during lipid peroxidation, which is caused by reactive oxygen species (ROS). The method described by Jentzsch *et al.*[11] was used. Samples were read at an absorbance of 532 nm on the FLUOStar Omega plate reader (BMG LABTECH, Germany) with Omega software. The final concentration of malondialdehyde in the samples was calculated using the Beer-Lambert law and the extinction coefficient of 1.54 × 105 M-1/cm-1. Final values were expressed as µmol malondialdehyde/mL serum.[11] Metallothionein-1 (MT-1) was measured to assess serum levels of this mitochondrial regulator. The 96-well plate was placed into the plate reader and immediately read at 450 nm. The final MT-1 data were expressed as pg/mL. The catalase (CAT) activity assay measured the final CAT activity in the samples, specifically the CAT activity of the patient serum. The assay is based on the principle of CAT, an antioxidant enzyme that catalyses the reduction of hydrogen peroxide to water and oxygen.[12] The assay was performed in a 96-well UV F-bottom plate (Greiner Bio-One, Austria), which was inserted into the plate reader and read at 240 nm. CAT activity was expressed as IU/mg protein/min. The superoxide dismutase (SOD) activity in patient serum was measured using a method adapted from Maarman *et al.*[13] The assay is based on xanthine oxidase catalysing the oxidation of xanthine to uric acid, producing ROS. Cytochrome c is reduced during this reaction, and the reduction rate is measured at a wavelength of 550 nm. The final SOD activity was determined and expressed in IU/mg protein/min.[13] A hypoxia-inducible factor-1-alpha (HIF-1α) subunit enzyme-linked immunosorbent assay kit (Invitrogen, USA) was used to measure HIF-1α protein levels in patient serum. The absorbance was measured using a plate reader at 450 nm, and the final data were expressed as ng/mL.

Statistical analyses

Statistical analyses were performed using Stata 16 (StataCorp, USA). Statistical significance was considered at $p<0.05$. Data underwent normality testing using the Shapiro-Wilk test. For normal data, an independent *t*-test or one-way analysis of variance was performed. For non-normal data, non-parametric tests were used, including Mann-Whitney and Kruskal-Wallis tests. Correlations were performed using multiple regression analysis and the Spearman's rank correlation coefficient test.

Results

Patient demographics

The majority of the patients were between the ages of 18 and 39 years, while 15.3% were in the 40 - 49-year age bracket, 19.4% in the 50 - 59-year age bracket, and 4.2% aged >60 (Table 1). There was a relatively equal split between females (41.7%) and males (58.3%), and the majority of patients were current smokers (51.4%). In addition, 63.9% of the patients were HIV negative, whereas 34.7% were HIV positive and treatment adherent; one person did not know their status. The majority (76.4%) of patients did not have hypertension, 20.8% had diagnosed and treated hypertension, and 2.8% did not know if they had the disease. Lastly, 88.9% did not have known heart disease, 9.7% had heart disease, and one person did not know if they had heart disease (Table 1).

TB episodes and spirometry

Of the patients, 47 (65.3%) had had 1 previous TB episode, 16 (22.2%) 2 episodes, 3 (4.2%) 3 episodes, and 6 (8%) >3 episodes (Table 2). In terms of lung function, mean lung function for the whole study population was poor for the spirometry parameters forced vital capacity (% predicted) and forced expiratory volume in 1 second (% predicted) (Table 2).

Mitochondrial markers

The TBARS level was within the normal range. At the same time, MT-1 was 240 times lower, CAT activity 7.5 times lower, and SOD activity 273 times lower than the normal ranges for these mitochondrial markers (Table 3). In contrast, HIF-1α was undetectable in all samples. After stratifying patients by the number of previous TB episodes, mitochondrial markers were similar across the groups (1, 2, 3 and 4) (Fig. 1).

Serum markers correlated with clinical endpoints

The Spearman test showed that age was positively correlated with E/e′ (r=0.28; p=0.02) and marginally correlated with PASP (r=0.23; p=0.07) (Table 4). There were no other notable correlations.

Discussion

We measured TBARS, MT-1, SOD, CAT, and HIF-1α serum markers in a group of patients with ≥1 previous TB episodes, and who had been successfully treated for TB at least 1 year before enrolment. The main findings of this study were that TBARS levels were within the normal range, whereas MT-1 was 240 times lower, CAT activity 7.5 times lower, and SOD activity 273 times lower than the normal ranges for these markers. HIF-1α was below the limit of detection in all the samples. At the same time, the mitochondrial markers were similar across the stratified groups after stratifying the patients based on the number of previous TB episodes. Age positively correlated with E/e′ and marginally correlated with PASP, while there were no other notable correlations.

TBARS, a proxy marker for oxidative stress,[18] was within the normal range. Generally, TBARS levels are higher in TB patients compared with healthy individuals.[19-21] Our data may corroborate previous work showing that TBARS levels in TB patients were within the normal range.[21] The data could suggest that there was no oxidative stress in these patients, which is highly unlikely, as previous studies demonstrated a clear link between TB and elevated TBARS.[19-21] Our findings could also mean that, because the patients were successfully treated, the treatment was able to reduce TBARS, as observed in previous work.[22] Alternatively, another plausible reason for our finding is that oxidative stress could manifest in a different form in these patients, other than lipid peroxidation, for example, protein

Table 1. Descriptive data on the study population of post-tuberculosis patients (N=72)

Variable	*n* (%)
Age (years)	
18 - 29	21 (29.2)
30 - 39	23 (31.9)
40 - 49	11 (15.3)
50 - 59	14 (19.4)
≥60	3 (4.2)
Sex	
Female	30 (41.7)
Male	42 (58.3)
Smoking status	
Current	37 (51.4)
Ex-smoker	23 (31.9)
Never	12 (16.7)
HIV status	
Positive	25 (34.7)
Negative	46 (63.9)
Unknown	1 (1.4)
Hypertension	
Yes	15 (20.8)
No	55 (76.4)
Unknown	2 (2.8)
Known heart disease	
Yes	7 (9.7)
No	64 (88.9)
Unknown	1 (1.4)

Table 2. Data pertaining to the number of previous TB episodes and lung function in the study population of post-TB patients (N=72)

Variable	*n* (%)*
Previous TB episodes	
1	47 (65.3)
2	16 (22.2)
3	3 (4.2)
>3	6 (8.3)
Spirometry, mean (SD)	
FVC (L)	2.6 (0.86)
FVC (% predicted)	64 (17)
FEV1 (L)	1.8 (0.78)
FEV1 (% predicted)	56 (22)
FEV1/FVC	0.72 (0.19)
FEF 25 - 75%	1.7 (1.2)

TB = tuberculosis; SD = standard deviation; FVC = forced vital capacity; FEV1 = forced expiratory volume in 1 second; FEF 25 - 75% = forced expiratory volume at 25 - 75% of FVC.
*Except where otherwise indicated.

Table 3. Mitochondrial biomarkers across the entire patient group, along with their normal ranges as reported in the literature

Mitochondrial biomarker	Patient data	Normal range
TBARS (μmol/mL)	0.6	0 - 2.5[14]
MT-1 (pg/mL)	0.2	48[15]
CAT (IU/mg/min)	31	235[12]
SOD (IU/mg/min)	0.6	164[16]
HIF-1α (ng/mL)	< limit of detection	0 - 1[17]

TBARS = thiobarbituric acid reactive substances; MT-1 = metallothionein-1; CAT = catalase activity; SOD = superoxide dismutase activity; HIF-1α = hypoxia-inducible factor-1-alpha.

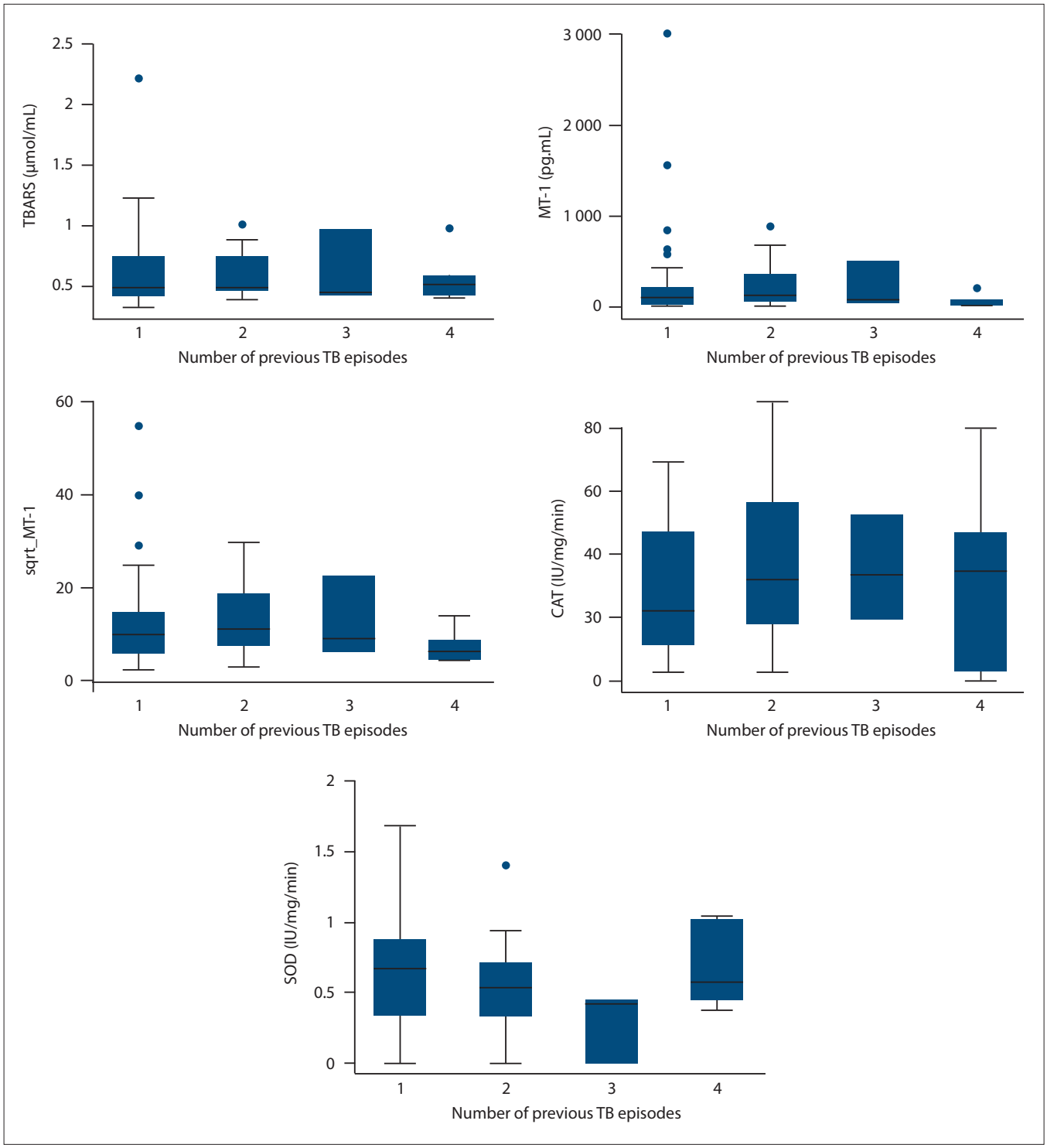

Fig. 1. Mitochondrial marker comparisons were performed when patients were stratified based on the number of previous episodes of TB. (TB = tuberculosis; TBARS = thiobarbituric acid reactive substances; MT-1 = metallothionein-1; sqrt_mt1 = square root of MT-1; CAT = catalase activity, SOD = superoxide dismutase activity.)

oxidation, carbohydrate oxidation and nucleic acid oxidation.[23] It is perhaps noteworthy that future studies consider measuring several proxy markers of oxidative stress to better delineate its involvement in the pathogenesis of PTLD.

MT-1 is a protein that plays a role in cellular protection against oxidative damage induced by intracellular heavy metals and is considered a key regulator of mitochondrial function.[24] Considering that mitochondria are also responsible for antioxidant actions and ROS, MT-1 is generally elevated by increased free radical production[25] and has been correlated with PH, a recognised form of PTLD.[26] In patients with PH, MT-1 is elevated, suggesting its usefulness as a biomarker[7] for PTLD. MT-1 also regulates cell

Table 4. The estimates utilised and generated by the Spearman test, correlating the mitochondrial biomarkers to the clinical endpoints (E/e', PASP) and age*

	Age	E/e'	PASP	TBARS	MT-1	CAT	SOD
Age	1.00						
	72						
E/e'	0.2756	1.00					
	68	68					
	0.023	.					
PASP	0.2299	–0.0412	1.00				
	65	64	65				
	0.065	0.75	.				
TBARS	0.0011	0.0083	–0.2325	1.00			
	72	68	65	72			
	0.99	0.95	0.06	.			
MT-1	0.1441	0.238	0.2133	0.0489	1.00		
	72	68	65	72	72		
	0.23	0.05	0.08	0.68	.		
CAT	0.0598	–0.002	0.1225	0.0738	–0.0139	1.00	
	72	68	65	72	72	72	
	0.62	0.98	0.33	0.53	0.90	.	
SOD	–0.077	0.018	–0.1670	0.1298	–0.1650	–0.0126	1.00
	72	68	65	72	72	72	72
	0.51	0.88	0.18	0.28	0.17	0.9162	.

E/e' = ratio of early diastolic mitral inflow velocity to early diastolic mitral annulus velocity; PASP = pulmonary artery systolic pressure; TBARS = thiobarbituric acid reactive substances; MT-1 = metallothionein-1; CAT = catalase activity; SOD = superoxide dismutase activity.

*For this table, the number of observations included is a minimum of 64, an average of 69 and a maximum of 72. For each variable (age, E/e', PASP, TBARS, MT-1, CAT and SOD), the number of observations and p-value are provided on the three lines.

proliferation and vasoconstriction, which are also instrumental in the pathophysiology of PH.[7] It has not been linked with the post-TB state *per se*, and, given recent evidence that TB may predispose to PH,[10] we investigated its role in these post-TB patients. In our study, MT-1 levels were 240 times lower than the normal range. Considering that MT-1 plays a role in metal homeostasis, detoxification and antioxidant defence, extremely low levels, as reported here, could increase the susceptibility of lung cells to heavy metal toxicity, elevated DNA damage, and apoptosis.[27] Our findings could also suggest that MT-1 is not involved in the post-TB stage of disease; however, in conjunction with our other biomarkers, the extremely low MT-1 levels indicate that the post-TB stage is associated with a depletion of crucial mitochondrial players. Low MT-1 levels can put lung cells at a disadvantage, as MT-1 (at higher levels) could have assisted in protecting cells against TB infection and the subsequent toxic side reactions.

In line with the above, we also found that SOD activity was 273 times lower and CAT activity 7.5 times lower than the normal ranges for these mitochondrial markers in all our samples. The SOD enzyme converts superoxide anion to hydrogen peroxide, and its role has been linked to several lung diseases, including TB.[28] The literature also reports a significant reduction in SOD activity in TB patients.[21] In turn, the CAT enzyme counteracts high concentrations of hydrogen peroxide by catalysing its decomposition into water and oxygen.[22] Studies comparing TB patients with healthy individuals found that patients infected with TB had significantly lower overall antioxidant status,[28,29] including significantly reduced CAT activity.[20] In addition, when investigating CAT activity before and after TB treatment, it was found that CAT activity was lower after treatment.[22] These findings corroborate ours and confirm that not only are mitochondrial role-players such as MT-1 extremely important, but also the antioxidant enzyme activities, which are closely linked to the optimal functioning of mitochondria.[30-32] Where previous studies suggest that increased CAT activity has beneficial effects, reduced CAT activity predisposes cells to impaired mitochondrial function and increased cellular damage,[33] which must be detrimental to organs like the lungs in the post-TB stage. Considering that SOD and CAT counteract toxic radicals (superoxide and hydrogen peroxide), our findings suggest that the radicals might remain high because these enzymes, which are meant to reduce them, have excessively reduced activities. This hypothesis is supported by literature showing that oxidative pathways (i.e. increased free radical production and reduced antioxidant capacity) may remain elevated despite successful TB treatment and may contribute to PTLD.[34]

Another key mitochondrial protein, HIF-1α, regulates oxygen homeostasis and plays a role in hypoxia, with studies showing elevated levels of HIF-1α in TB.[35] In our study, HIF-1α was below the limit of detection (≤30 pg/mL), with only three samples that had measurable levels. This finding could mean that it does not play a role in the post-TB context. Considering the role of HIF-1α in hypoxia,[36] our findings may further suggest that these patients were not hypoxic. This observation is in line with previous work.[10,37,38] Coincidentally, literature has already drawn a link between HIF-1α and mitochondrial dysfunction,[39] as low HIF-1α can lead to

increased free radicals, culminating in impaired mitochondrial function that could further contribute to lung diseases such as TB.[40] Alternatively, it suggests that future studies may have to find other hypoxia biomarkers (BNIP3, PDK1 and GLUT1, as well as CAIX, LDH-5, MCT1 and MCT4).[41]

The mitochondrial markers were similar across the stratified groups after stratifying the patients based on the number of previous TB episodes. This analysis was based on a suspicion that several previous TB episodes may worsen mitochondrial pathways and predispose to the development of PTLD. However, our data suggest that mitochondrial markers and antioxidant capacity are severely reduced, regardless of the number of previous TB episodes. A plausible reason is that the TB infection/antigens may elicit this phenomenon,[40] rather than the number of previous TB episodes. When correlations were drawn between mitochondrial markers and endpoints such as age, E/e′ and PASP, age positively correlated with E/e′ and marginally correlated with PASP, while there were no other notable correlations. Interestingly, PASP did not correlate with mitochondrial markers, as one would expect at least a strong negative correlation, where, for example, a higher PASP would be associated with reduced mitochondrial markers. However, we did not find any association, and although intriguing, it does not take away from the fact that the mitochondrial pathways were severely reduced in these patients either way. Our study reiterates the importance of mitochondrial hormesis, where a balance in mitochondrial pathways is beneficial, but tipping this balance (as we observed) could contribute to PTLD. Low mitochondrial involvement and antioxidant capacity have been shown to elevate oxidative stress, induce mitochondrial dysfunction, and predispose to impaired energy production, damaged mitochondrial structures, and the excessive activation of apoptotic pathways.[42] Ultimately, the reduced antioxidant activity can exacerbate conditions such as TB[43] and PTLD, and may highlight the therapeutic potential of potent antioxidant agents (*N*-acetylcysteine, liposomal glutathione, theophylline, melatonin) in the prevention or treatment of PTLD.[44-46] However, more research is needed to determine the effects of antioxidant therapy in patients with PTLD.

Conclusion

Our data suggest that mitochondrial pathways, especially antioxidant enzyme activities, are severely reduced in patients in the post-TB stage with moderately restricted lung function. Surprisingly, a key mitochondrial protein, HIF-1α, showed no role in this context, suggesting that these patients are not hypoxic. Our data indicate that a novel contributor to post-TB lung disease could be a limitation of antioxidant capacity and mitochondrial pathways, which is not linked to the number of previous TB episodes and may highlight the need to consider antioxidant therapy during the post-TB stage.

Data availability. The datasets generated and analysed during the present study are available from the corresponding author (GJM) on reasonable request. Additionally, any restrictions or additional information regarding data access can be discussed with the corresponding author.

Declaration. The research for this study was done in partial fulfilment of the requirements for CP's MSc degree at Stellenbosch University.

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