Evaluation of the T-SPOT.TB test, oxidation-related factors, and antimicrobial peptide LL-37 in the diagnosis of pulmonary tuberculosis with type 2 diabetes

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Abstract
Objective: To investigate the diagnostic value of the T cell spot (T-SPOT.TB) test, oxidation-related factors (ORF), and antimicrobial peptide LL-37 in patients with pulmonary tuberculosis (PTB) with type 2 diabetes.
Methods: A total of 560 patients with PTB admitted to our hospital from January 2019 to April 2021 were retrospectively included in this study. Of these, 232 patients with PTB and type 2 diabetes were assigned to the combined group, and 328 patients without complications were assigned to the PTB group.
Results: Areas under the curve (AUCs) for the number of spot-forming cells in CFP10 and ESAT-6 test panels detecting PTB with type 2 diabetes were 0.892 (95% confidence interval [CI] 0.831–0.921) and 0.893 (95% CI 0.841–0.935), respectively. CFP10 combined with ESAT-6 had the highest diagnostic value, with sensitivity and specificity levels and an AUC of 87.73%, 88.93%, and 0.942 (95% CI 0.907–0.967), respectively. The levels of total antioxidant capacity, superoxide dismutase, and catalase in the combined group were lower than in PTB and control groups.
Conclusion: The combination of T-SPOT.TB, ORF, and LL-37 in the diagnosis of pulmonary tuberculosis with type 2 diabetes mellitus has a high diagnostic value and clinical application value.
Introduction

According to the World Health Organization, the cumulative tuberculosis (TB) mortality rate in 2018 was the highest of all diseases worldwide, even exceeding that of HIV/AIDS; this led to 1.5 million deaths in that year alone.\(^1\) Diabetes mellitus is an important risk factor for the aggravation of TB,\(^2\) and, with increasing aging within China, the prevalence of diabetes combined with TB is rising annually. Studies have shown that the worldwide incidence of TB combined with diabetes is 19.3% to 24.1%.\(^3\) Compared with patients with TB alone, patients with TB and diabetes have a worse prognosis and a higher mortality rate during anti-TB treatment.\(^4\)

Therefore, a reliable diagnostic method is of great clinical importance for defining the treatment plan and improving the survival rate of patients with TB.

Acid-fast bacterial staining is the most convenient method of diagnosing TB, but its sensitivity is low.\(^5\) In recent years, a T cell-based interferon (IFN)-\(\gamma\) release test and Mycobacterium tuberculosis T cell spot test (T-SPOT.TB) have been developed, and are now some of the most common methods for detecting TB.\(^6\) The antimicrobial peptide LL-37 is the main cathelicidin member in humans and has a broad spectrum of antibacterial effects, including killing mycobacteria. Recent studies found that LL-37 plays an important role in regulating the inflammatory response, cell proliferation, and apoptosis.\(^7\) Additionally, the gene expression of antimicrobial peptides was shown to be decreased in patients with TB and diabetes, which may be a main reason for patients with diabetes being more prone to active TB.\(^8\)

Under normal circumstances, the generation of nitric oxide in the body and removal of the antioxidant capacity are maintained in a dynamic equilibrium. However, after infection with \(M.\) tuberculosis, lesions lead to the generation of oxygen-derived free radicals, which cause an oxidative system and antioxidant imbalance, resulting in oxidative stress.\(^9\) In recent years, type 2 diabetes was reported to aggravate TB pathology by increasing the occurrence of drug-resistant mutations, increasing sputum test positivity, decreasing the cure rate, and causing more pulmonary lesion cavities.\(^10\) However, few studies have explored whether this is related to oxidative stress. Moreover, little is known about whether the T-SPOT.TB test, oxidation–antioxidant balance, and LL-37 can be used as diagnostic criteria for TB complicated with type 2 diabetes. Herein, this study explored the application value of these diagnostics in the identification of patients with TB with type 2 diabetes.

Materials and methods

Study patients

Between January 2019 and April 2021, 560 patients with pulmonary TB (PTB) admitted to our hospital were
retrospectively included in the study. Of these, 232 patients with PTB combined with type 2 diabetes were assigned to the combined group, and 328 patients without complications were assigned to the PTB group; 359 individuals who underwent physical examination at our hospital during the same period were enrolled as the control group. This study was approved by the Ethics Committee of Beijing Geriatric Hospital, and written informed consent was obtained from all participants for study participation and publication of the results.

The gold standard for the diagnosis of PTB was a \textit{M. tuberculosis}-positive smear or positive culture, or a positive clinical diagnosis. For PTB patients with type 2 diabetes, the severity of the lesion was graded according to chest imaging examination findings. Mild refers to the involvement of one lung lobe without significant voids; moderate refers to the involvement of two or more lobes in a single lung with a cavity <4 cm in diameter; and severe refers to extensive involvement of both lungs with multiple cavities found on imaging.

Inclusion criteria for the combined group were: (1) diagnosed with TB according to guidelines for the diagnosis and treatment of TB published by the Chinese Medical Association,\textsuperscript{11} including pathological tissue biopsy and etiological examination; (2) diagnosed with type 2 diabetes according to guidelines for the prevention and treatment of type 2 diabetes in China;\textsuperscript{12} (3) positive acid-fast staining of a sputum smear; (4) complete clinical data; and (5) written informed consent. Exclusion criteria were: (1) type I diabetes or diabetic complications such as a diabetic foot or diabetic nephropathy; (2) extrapulmonary TB; (3) malignant tumors, familial hereditary disease, or other inflammatory infectious disease; (4) long-term use of hormones and/or addictive drugs; (5) autoimmune disease; (6) complicated with pneumonia, allergic disease, heart, brain, liver, or kidney lesions, and immune dysfunction; and (7) false-positive acid-fast staining of a sputum smear or T-SPOT. TB test.

\textbf{Laboratory procedures}

\textit{T-SPOT.TB assay}. A total of 8 mL of fasting peripheral venous blood collected in the morning was placed in a heparin sodium anticoagulant test tube. One-third of this was added to lymphocyte separation medium (Absin Bioscience Inc., Shanghai, China), then centrifuged three times at 1600 × g for 28 minutes at 18°C to separate lymphocytes from whole blood. Then, 10 μL sample was added to the counting plate to count the number of living cells. Culture filtrate protein 10 (CFP10) antigen, early secretory antigenic target 6-kD protein (ESAT-6), and negative control liquid were added to the culture plate to 50 μL. Next, 100 μL of 2.5 × 10^5 lymphocytes was added to each well and incubated at 37°C with 5% CO₂ for 16 hours. Then, 50 μL enzyme-labeled antibody fluid was added to the culture plate, incubated at 4°C for 1 hour, and 50 μL substrate solution was added for a further incubation at room temperature for 7 minutes in the dark. Distilled water was used to stop the reaction, and the number of blotch-forming cells was counted automatically after drying.

If the number of spots in the negative control wells was 0 to 5, the number of spots in any ESAT-6 or CFP10 well was subtracted from the number in the negative control wells, and a result ≥6 was deemed positive. If the number of spots in the negative control wells was 6 to 10, and the number of spots in any ESAT-6 or CFP10 well was more than twice that of the negative control, the results were judged to be positive. If the above criteria were not met, and the positive control wells were normal, the test results were determined as negative.
Oxidation-related factors (ORF) assay. One-third of the peripheral venous blood was centrifuged at 600 × g for 7 minutes at 18°C, and the separated plasma was stored at −50°C. The total antioxidant capacity (T-AOC), reactive oxygen species (ROS), malondialdehyde (MDA), superoxide dismutase SOD (SOD), and catalase (CAT) levels were detected by radioimmunoprecipitation using kits purchased from Beijing Furui Runze Biotechnology Co., Ltd. (Beijing, China), according to the manufacturer’s instructions.

LL-37 assay. The remaining third of peripheral venous blood was centrifuged, and the serum extracted into a polypropylene tube and stored at −60°C. LL-37 levels were measured by solid-phase enzyme-linked immunosorbent assay (Hycult Biotech, Wayne, PA, USA).

Statistical analysis
Statistical analysis was performed using SPSS software version 23.0 (IBM Corp., Armonk, NY, USA). Normally distributed data were expressed as means ± standard deviation (SD), and compared using the Student’s t-test. Receiver operating characteristic analysis was performed to determine the best cut-off value for all test indicators. Areas under the curve (AUCs) and cut-off values with the highest sensitivity and specificity values were identified. Cohen’s kappa coefficient was calculated to evaluate the consistency of the diagnostic test compared with the gold standard. The test level α was 0.05 on both sides. P < 0.05 was considered statistically significant.

Results

Demographic data
A total of 560 patients with PTB and 359 controls were included in the study. The combined group included 111 men and 121 women, with an average age of 53.29 ± 10.28 years (range, 28–72 years). The PTB group included 172 men and 156 women, with an average age of 52.65 ± 9.99 years (range, 28–71 years). The control group included 186 men and 173 women, with an average age of 53.01 ± 10.56 years (range, 29–70 years). There was no significant difference in demographic data among the three groups.

Diagnostic efficacy of T-SPOT.TB
In the combined group, 191 cases had a positive T-SPOT.TB test, giving an accuracy rate of 82.33% (191/232); 285 cases had a positive T-SPOT.TB test in the PTB group, giving an accuracy rate of 86.91% (285/359); the T-SPOT.TB test was negative in the control group. Of the 560 patients with PTB, 476 had positive T-SPOT.TB tests. The total sensitivity, specificity, positive predictive value, negative predictive value, and kappa coefficient of the T-SPOT.TB test in the diagnosis of TB were 86.05%, 20.21%, 84.24%, 22.62%, and 0.515, respectively, which represented moderate consistency (Table 1).

Diagnostic efficacy of the spot-forming cell (SFC) number in CFP10 and ESAT-6 test panels
A comparison of the mean number of SFCs/2.5 × 10⁵ peripheral blood mononuclear cells

| Table 1. Comparison of T-spot test findings for Mycobacterium tuberculosis infection with pathological results. |
|-------------------------------------------------------------|
| Result of T-SPOT test (n) | Pathologic result (n) |
| Positive | Negative | Total (n) |
|----------|----------|----------|
| Positive | 401      | 75       | 476      |
| Negative | 65       | 19       | 84       |
| Total    | 466      | 94       | 560      |
(PBMCs) in ESAT-6 and CFP10 test panels revealed a significant difference among groups, with the highest number seen in the combined group (P < 0.001; Table 2).

AUCs for CFP10 and ESAT-6 in the diagnosis of PTB with type 2 diabetes were 0.892 (95% confidence interval [CI] 0.831–0.921) and 0.893 (95% CI 0.841–0.935), respectively. The cut-off value for CFP10 was 16.13 SFCs/2.5 × 10⁵ PBMCs, with a sensitivity and specificity of 85.73% and 74.87%, respectively. The cut-off value for ESAT-6 was 14.97 SFCs/2.5 × 10⁵ PBMCs, with a sensitivity and specificity of 85.98% and 75.87%, respectively. The AUC of CFP10 combined with ESAT-6 was 0.942 (95% CI 0.907–0.967), with a sensitivity and specificity of 87.73% and 88.93%, respectively (Figure 1).

**Comparison of ORF and LL-37**

The levels of T-AOC, SOD, and CAT in the combined group were significantly lower than in PTB and control groups, and were also significantly lower in the PTB group than in the control group (all P < 0.001). Moreover, the levels of ROS, MDA, and LL-37 in the combined group were significantly higher than in PTB and control groups, and were also significantly higher in the PTB group than in the control group (all P < 0.001) (Table 3).

**Table 2.** Comparison of the mean number of SFCs/2.5 × 10⁵ PBMCs in ESAT-6 and CFP10 test panels among groups.

| Group                  | n   | CFP10     | ESAT-6    |
|------------------------|-----|-----------|-----------|
| Combined group         | 232 | 23.63 ± 1.82 | 23.01 ± 1.67 |
| Pulmonary tuberculosis group | 328 | 16.23 ± 1.56 | 15.03 ± 1.25 |
| Control group          | 359 | 4.97 ± 0.23   | 4.87 ± 0.21  |
| F-measure              |     | 26099.060    | 18900.112  |
| P                      |     | <0.001      | <0.001     |

SFCs, spot-forming cells; PBMCs, peripheral blood mononuclear cells; CFP, culture filtrate protein; ESAT, early secretory antigenic target.
ROS, MDA, and LL-37 in the mild group were significantly lower than in moderate and severe groups, and were also significantly lower in the moderate group than in the severe group (all \( P < 0.001 \)) (Table 4).

### Table 3. Comparison of ORF and LL-37 levels in each group (\( \bar{x} \pm s \)).

| Group                        | n    | T-AOC (U/L) | ROS (U/L) | MDA (\( \mu \text{mol/L} \)) | SOD (U/L) | CAT (U/L) | LL-37 (ng/mL) |
|------------------------------|------|-------------|-----------|-------------------------------|-----------|-----------|---------------|
| Combined group               | 232  | 2.31 ± 0.29 | 15.08 ± 0.27 | 13.23 ± 0.86                  | 44.86 ± 6.69 | 5.42 ± 0.23 | 53.56 ± 2.72  |
| Pulmonary tuberculosis group | 328  | 2.82 ± 0.32 | 8.87 ± 0.24 | 5.17 ± 0.76                   | 61.26 ± 7.32 | 22.46 ± 3.12 | 43.76 ± 2.54  |
| Control group                | 359  | 4.71 ± 0.52 | 2.54 ± 0.19 | 3.49 ± 0.82                   | 96.65 ± 7.65 | 58.92 ± 3.86 | 22.56 ± 2.01  |
| F-measure                    | 3050.815 | 212784.669 | 10937.052 | 3995.638                     | 24366.035 | 13277.399 |
| \( P \)                      | <0.001 | <0.001      | <0.001    | <0.001                       | <0.001    | <0.001    |

ORF, oxidation-related factors; T-AOC, total antioxidant capacity; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; LL-37, antimicrobial peptide LL-37.

### Table 4. Comparison of ORF and LL-37 levels in different severities of pulmonary tuberculosis in the combined group (\( \bar{x} \pm s \)).

| Group                | n    | T-AOC (U/L) | ROS (U/L) | MDA (\( \mu \text{mol/L} \)) | SOD (U/L) | CAT (U/L) | LL-37 (ng/mL) |
|----------------------|------|-------------|-----------|-------------------------------|-----------|-----------|---------------|
| Mild group           | 118  | 3.17 ± 0.23 | 7.37 ± 0.48 | 8.52 ± 0.56                  | 53.29 ± 2.39 | 13.69 ± 1.29 | 32.98 ± 2.13  |
| Moderate group       | 78   | 2.41 ± 0.31 | 13.21 ± 0.42 | 12.37 ± 0.63                 | 42.16 ± 2.01 | 6.01 ± 1.01 | 49.87 ± 2.69  |
| Severe group         | 36   | 1.78 ± 0.16 | 21.72 ± 0.57 | 15.87 ± 0.61                 | 36.26 ± 2.06 | 2.31 ± 0.89 | 58.92 ± 2.86  |
| F-measure            | 498.757 | 13234.376 | 2461.106 | 1080.732                     | 1855.361 | 2052.033 |
| \( P \)              | <0.001 | <0.001      | <0.001    | <0.001                       | <0.001    | <0.001    |

ORF, oxidation-related factors; T-AOC, total antioxidant capacity; ROS, reactive oxygen species; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; LL-37, antimicrobial peptide LL-37.

ROS, MDA, and LL-37 in the mild group were significantly lower than in moderate and severe groups, and were also significantly lower in the moderate group than in the severe group (all \( P < 0.001 \)) (Table 4).

### Diagnostic efficacy of ORF and LL-37 for the diagnosis of PTB with type 2 diabetes

In the diagnosis of PTB with type 2 diabetes, LL-37 had the highest AUC (0.656, 95% CI 0.571–0.729), while SOD with a cut-off value of 36.26 U/L had the highest sensitivity at 90.09% (Table 5, Figure 2).

### Performance of the T-SPOT.TB test combined with ORF and LL-37 in detecting PTB with type 2 diabetes

The AUC of the T-SPOT.TB test combined with ORF and LL-37 was the highest (0.938) of all diagnostics in detecting PTB with type 2 diabetes, as was the specificity; however, the sensitivity was lower than that of the T-SPOT.TB test combined with either ORF or LL-37. ORF combined with LL-37 had the lowest efficiency (Table 6).

### Discussion

Patients with TB and type 2 diabetes lack specific clinical symptoms in the early course of their disease, while patients with type 2 diabetes have metabolic disorders and decreased immunity. The interaction of the two diseases not only increases the complexity of clinical symptoms, but can also cause atypical symptoms leading to misdiagnosis, missed diagnosis, and delayed treatment. Additionally, type 2 diabetes
directly impinges on innate and adaptive immune responses, increasing the risk of TB infection and hypertension. Complicating diabetes can further increase the interaction between TB and hypertension, augment the risk of cardiovascular disease, and adversely affect the development, prognosis, and treatment of TB. Therefore, obtaining an accurate diagnosis of TB with type 2 diabetes is clinically important.

Current diagnostic methods for PTB combined with type 2 diabetes mainly include lung biopsy, bronchoscopy, tuberculin tests, sputum tests, and imaging examinations. The *M. tuberculosis* T cell spot assay is a new method for the diagnosis of TB that detects the number of IFN-γ T cells produced after the stimulation of a TB-specific antigen. Compared with conventional sputum smears and sputum culture, the T-SPOT.TB test has higher sensitivity and specificity, and is non-invasive. However, the IFN-γ content and antigen number are affected by disease activity, individual immunity, and other factors. Indeed, patients with TB combined with type 2 diabetes in particular have a weakened immune response, and reduced number of IFN-γ-producing T cells resulting from antigen activation, so appear non-reactive, leading to missed diagnosis and misdiagnosis. Shi et al. reported that the T-SPOT.TB test had a high sensitivity and low specificity for the diagnosis of active TB in areas with a high prevalence of infection, so failed to achieve an ideal diagnostic efficacy. Therefore, the choice of diagnostic test should be based on clinical manifestations.

A previous study reported sensitivity and specificity values for the T-SPOT.TB test in the diagnosis of *M. tuberculosis* of 95.1% and 91.3%, respectively. This is higher...
than our current findings of 76.60% and 64.61%, respectively, which might reflect differences in the study participants with respect to symptom severity and imaging characteristics. Moreover, we showed that combining multiple indicators improved the diagnostic efficiency by increasing the sensitivity to 87.73%, and the specificity to 88.93% in the combined diagnosis of CFP10 and ESAT-6 antigens.

Few clinical studies have been conducted on ORF and LL-37 in patients with PTB complicated with type 2 diabetes. Under normal physiological conditions, the production of oxides and the removal of antioxidants are in a state of dynamic balance, and the oxides produced in the body are quickly eliminated by the antioxidant system. Previous studies have suggested that patients with TB, like those with most other lung diseases, have a significant decrease in antioxidant capacity and increased oxidative metabolites. Commonly used indexes of oxidative lipid damage to detect oxidative stress in the blood include ROS and MDA, and protective indexes such as T-AOC, SOD, and CAT. SOD clears stress products and converts superoxide into hydrogen peroxide and oxygen through disproportionation, playing an important role in protecting against lipid, protein, and DNA damage. ROS is a type of free radical involved in the activation process of the oxidative stress reaction, which oxidizes local lipid components of tissues and generates large quantities of MDA. Therefore, the ROS content reflects the extent of lipid peroxidation during free radical attack. T-AOC reflects the overall ability to resist oxidation, while CAT scavenges hydrogen peroxide and plays an important role in the antioxidant process, and LL-37 is an important innate immune effector molecule that regulates immunity, and promotes apoptosis and cytotoxicity. Majewski et al. reported that LL-37 inhibited the growth of M. tuberculosis, with higher LL-37 levels having stronger inhibitory effects.

The present study detected significantly lower levels of T-AOC, SOD, and CAT in the combined group than in PTB and control groups, and in the PTB group compared with the control group. Moreover, ROS, MDA, and LL-37 levels were significantly higher in the combined group than in PTB and control groups, and in the PTB group than in the control group. This suggested that patients with TB are in a state of imbalance between oxidation and antioxidants, with a reduced total antioxidant capacity and increased lipid oxidative damage. Additionally, LL-37 levels were positively correlated with the presence of TB. Zhang et al. reported that PTB combined with diabetes increased oxidative stress and DNA oxidative damage, and decreased lymphocyte proliferation activity in patients, while Li et al. showed decreased antioxidant capacity and lipid oxidative damage after infection with M. tuberculosis. Moreover, Wu et al. observed significantly higher LL-37 levels

### Table 6. Performance of the T-spot test combined with ORF and LL-37 for the diagnosis of pulmonary tuberculosis with type 2 diabetes.

| Diagnostic method | AUC (95% CI) | Sensitivity (%) | Specificity (%) |
|-------------------|--------------|----------------|-----------------|
| T-spot + ORF + LL-37 | 0.936 (0.901–0.963) | 79.87 | 93.29 |
| T-spot + LL-37 | 0.923 (0.889–0.949) | 82.98 | 89.42 |
| ORF + LL-37 | 0.704 (0.629–0.771) | 58.76 | 75.92 |
| T-spot + ORF | 0.929 (0.900–0.956) | 82.28 | 88.39 |

T-spot, T-spot test of Mycobacterium tuberculosis; ORF, oxidation-related factors; LL-37, antimicrobial peptide LL-37; AUC, area under the curve; CI, confidence interval.
in patients with TB compared with healthy controls, and varied LL-37 expression among patients with different types of active TB. Together, these findings indicate that complications of TB, such as type 2 diabetes, aggravate the state of oxidative stress in patients.

The present study also showed significantly higher levels of T-AOC, SOD, and CAT in patients with mild TB than in moderate and severe groups, and significantly higher levels in the moderate group compared with the severe group. Compared with mild and moderate groups, the severe group had significantly higher levels of ROS, MDA, and LL-37. This suggests that oxidation-related factors are involved in oxidative and lipid peroxidation damage in patients with PTB combined with type 2 diabetes, and that related indexes of the oxidation–antioxidant balance correlate with PTB severity. Likely specific mechanisms for this are as follows:27,28 (1) most patients with type 2 diabetes suffer from insulin resistance and a relative lack of insulin, so insulin is unable to regulate the interaction between enzymes and key transcription factors through relevant signaling pathways, thus damaging the antioxidant capacity; (2) diabetic patients are in a state of hyperglycemia, which generates a large amount of ROS through mitochondrial uncoupling and β-oxidation, resulting in oxidative stress. Excessive polysaccharide metabolites further aggravate the imbalance between oxides and the antioxidant system, while oxidative stress induced by hyperglycemia activates the stress sensitivity signaling pathway, exacerbates the functional defects of pancreatic β cells, and even causes β cell apoptosis and insulin resistance. Therefore, patients with TB in an oxidative stress state are more likely to be complicated with diabetes, which further promotes the oxidative stress response and the dynamic imbalance of oxidation and antioxidants.

LL-37 was previously shown to be highly expressed in patients with diabetes complicated with TB, and to have diagnostic value.29 Moreover, the T-SPOT.TB test combined with LL-37 with or without serum 25-hydroxyvitamin D3 was reported to effectively diagnose TB complicated with diabetes.30 The present study showed that ORF and LL-37 were less effective at diagnosing PTB with type 2 diabetes, while the T-SPOT.TB test combined with ORF and LL-37 had the largest AUC value in diagnosing PTB with type 2 diabetes. This suggests that combining multiple test indexes improves the diagnostic efficiency.

This study had some limitations. The first is its small sample size, which was limited to hospital inpatients. A good diagnostic study should calculate a matching sample size by referring to the sensitivity and specificity of previous studies or pretrials. Second, the cases derived from a single center, so selection bias may have occurred. Therefore, prospective studies with a larger sample size and more comprehensive design are needed to improve the quality of research results.

**Conclusion**

The T-SPOT.TB test alone had limited efficacy in diagnosing PTB with type 2 diabetes, but efficacy was increased by combining this with ORF and LL-37. Levels of ORF and LL-37 were positively correlated with the disease severity of PTB.

**Availability of data and material**

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

**Authors’ contributions**

LM contributed to the conception and design of the study; XC and SW performed the experiments, and collected and analyzed data; LM
wrote the manuscript. All authors reviewed and approved the final version of the manuscript.

**Declaration of conflicting interest**
The authors declare that there is no conflict of interest.

**Funding**
This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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