SCIENTIFIC Publishing Limited

Trends in Immunotherapy

https://ojs.ukscip.com/index.php/ti

Article

Pilot Randomized Trial of Vitamin D and Zinc Supplementation on Total Adenosine Deaminase in Pediatric Tuberculosis

Agustin Iskandar [1,2], Arnaz Adisaputra [1,2], Ery Olivianto [2,3,*] and Kusworini Handono [1]

[1] Department of Clinical Pathology, Faculty of Medicine, Brawijaya University, Malang 65111, Indonesia; agustin_almi@ub.ac.id (A.I.); arnazsaputra@student.ub.ac.id (A.A.); rinihandono.fk@ub.ac.id (K.H.)

[2] Saiful Anwar General Hospital, Malang 65111, Indonesia

[3] Department of Child Health, Faculty of Medicine, Brawijaya University, Malang 65111, Indonesia

[*] Correspondence: ery15.fk@ub.ac.id

Received: 22 June 2026; **Revised:** 24 July 2026; **Accepted:** 30 July 2026; **Published:** 1 September 2026

Abstract: Pediatric tuberculosis remains difficult to monitor using objective biomarkers. Total serum adenosine deaminase (ADA) reflects cellular immune activation, whereas vitamin D and zinc may modify antimycobacterial immunity. This pilot randomized trial evaluated whether adjunctive vitamin D or zinc altered serum ADA during the intensive phase of pediatric tuberculosis treatment. Thirty-three treatment-naive children with tuberculosis were randomized equally to standard anti-tuberculosis treatment (ATT) alone, ATT plus zinc, or ATT plus vitamin D. Total serum ADA was measured at baseline and after two months. The prespecified completer analysis compared change scores, while the primary adjusted analysis used analysis of covariance (ANCOVA) with post-treatment ADA as the outcome and baseline ADA as a covariate. A multiple-imputation intention-to-treat sensitivity analysis included all randomized participants. Twenty-two participants completed follow-up (ATT, n = 8; zinc, n = 8; vitamin D, n = 6). Unadjusted ADA decreased in all groups and most markedly in the vitamin D arm, but the between-group difference in change was not significant (p = 0.364). Baseline-adjusted ANCOVA also showed no treatment-group difference ($F_{2,18}$ = 0.79; p = 0.470). Compared with ATT alone, the adjusted effects were −4.35 U/L for vitamin D (95% CI −15.70 to 7.00) and +2.26 U/L for zinc (95% CI −8.07 to 12.59). The intention-to-treat sensitivity analysis produced the same overall interpretation. In this underpowered pilot trial, adjunctive vitamin D or zinc did not significantly reduce total serum ADA beyond standard therapy. The large crude decline in the vitamin D arm was attenuated after adjustment for baseline imbalance and regression to the mean. Larger multicentre trials should confirm micronutrient repletion, measure ADA2, and prespecify methods for missing data.

Keywords: Adenosine Deaminase; Pediatric Tuberculosis; Vitamin D; Zinc; Intensive Phase

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains a major infectious disease burden worldwide. Children carry a particular clinical risk: those younger than five years progress from infection to active disease more often than adults, and they are more prone to severe manifestations [1]. Loss to follow-up during pediatric tuberculosis treatment is likewise common, in part because a child's care depends on an adult caregiver, and it complicates both clinical management and research in this population [2].

Micronutrient deficiency is endemic among Indonesian children. Zinc, vitamin D, and iron deficiency each occur at high rates in school-aged populations and each impairs immune responses during active infection. Low

vitamin D status in particular has been associated with TB disease in children. Because vitamin D status has shown a particular association with TB disease in children, micronutrient supplementation warrants evaluation as an add-on to standard anti-tuberculosis therapy, not a substitute for it, to determine whether it improves treatment outcomes [3,4].

Vitamin D modulates antimycobacterial immunity through several mechanisms, including expression of the vitamin D receptor on innate and adaptive immune cells, macrophage activation, and the induction of antimicrobial peptides [5]. Randomized trials of adjunctive vitamin D in tuberculosis have yielded mixed results. In some populations, improved sputum conversion or more rapid symptom resolution has been reported [6,7], whereas others have demonstrated no benefit. In children specifically, the available evidence remains limited, and the role of vitamin D in prevention as opposed to treatment is still debated [8].

Zinc contributes to immune function through the maintenance of epithelial barrier integrity, T-cell activation, lymphocyte proliferation, and cytokine production, and its deficiency impairs cell-mediated immunity [9]. In adults with pulmonary tuberculosis, randomized trials and meta-analyses have reported changes in nutritional markers and biochemical outcomes following zinc supplementation, although effects on clinical endpoints remain inconsistent [10,11].

ADA is a purine-metabolizing enzyme that rises in serum during active cellular immune responses, driven by lymphocyte proliferation and monocyte-macrophage activation. Its isoform ADA2, secreted by activated monocytes and macrophages, differentiates active tuberculosis from latent infection. Serum ADA concentration also reflects mycobacterial burden and disease severity in pulmonary TB [12,13].

The enzyme's role does not stop at diagnosis. Systematic reviews show that serum ADA falls measurably during effective TB treatment, a pattern confirmed by recent data from Indonesian adults with pulmonary TB who had lower ADA values after completing two months of intensive-phase therapy [14,15], in extrapulmonary TB where ADA declines over six months of treatment [16], and in studies using ADA alongside ferritin and c-reactive protein (CRP) to monitor treatment response [17].

Evidence in the pediatric population remains sparse. Whether vitamin D or zinc supplementation alters serum ADA during pediatric TB treatment has not been established. We hypothesized that adjunctive vitamin D or zinc during the intensive phase would produce a greater reduction in serum ADA than standard therapy alone, and measured the effect of each supplement on total serum ADA levels in children with TB over the course of intensive-phase treatment.

2. Materials and Methods

2.1. Study Design and Setting

This was a single-centre, pilot randomized, single-blind, controlled trial using a pretest-posttest control-group design with three parallel arms. The trial was conducted at the Department of Child Health, Dr. Saiful Anwar General Hospital, Malang, with laboratory analysis performed at the Clinical Pathology Laboratory, Faculty of Medicine, Universitas Brawijaya. Recruitment ran from August 2023 to January 2024, following approval by the institutional ethics committee. Reporting follows the Consolidated Standards of Reporting Trials (CONSORT) 2010 statement, and participant flow is shown in **Figure 1**.

2.2. Study Participants

The target population was children with TB. The accessible population comprised pediatric TB patients receiving outpatient or inpatient treatment at the Pediatric Respirology Clinic of the study hospital. Children were eligible if they were aged 18 years or younger, newly diagnosed with TB according to the 2023 national technical guideline, had received no prior anti-TB treatment, and had written informed consent from a parent or legal guardian.

Children were excluded if they had another condition producing symptoms or signs that overlapped with the parameters of the pediatric TB scoring system, or if they had an immunosuppressive disorder or were receiving immunosuppressant therapy. Further exclusion criteria were chronic liver disease, non-tuberculous infectious disease, renal failure, nephrotic syndrome, malabsorption syndrome, diabetes mellitus, HIV infection, heart failure, and neoplasm. Children were also excluded if they had a history of corticosteroid use or of taking supplements containing zinc or vitamin D, or if they had already received more than one week of anti-tuberculosis treatment.

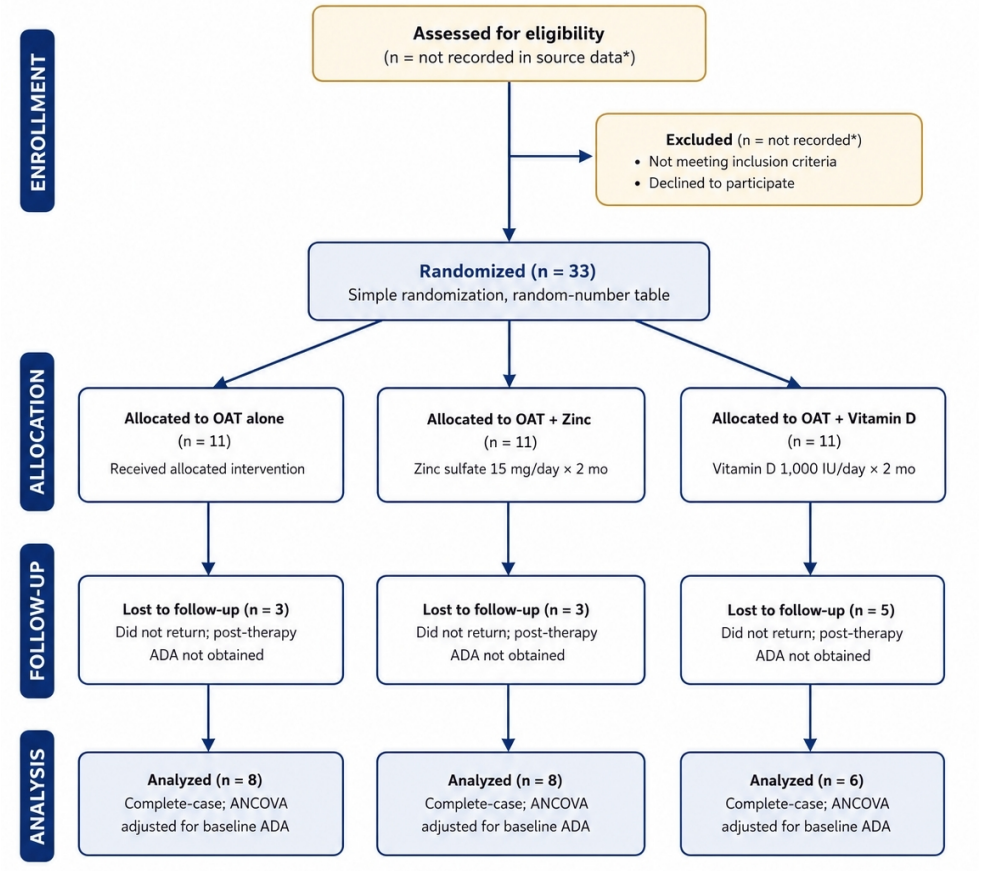

Figure 1. Participant flow diagram.

Diagnosis used the Indonesian pediatric TB scoring system referenced in the 2023 technical guideline and aligned with world health organization (WHO) guidance on TB in children [18]. It is a weighted point-based tool combining contact history, tuberculin skin-test result, nutritional status, clinical features (persistent fever, chronic cough, weight or growth faltering), peripheral lymph node findings, and chest radiography, with a total score of six or more supporting a TB diagnosis. Consistent with the guideline, children with a score below six were also eligible if they had at least one specific feature: cough for three weeks or more, fever for two weeks or more, cervical lymphadenopathy not improving after two weeks of non-specific treatment, a chest radiograph suggestive of TB, or close contact with a confirmed adult TB case verified by tuberculin testing.

2.3. Sample Size

The original trial was formally powered a priori for the between-arm difference in change in serum ADA. Using a minimally important mean difference of 6.52 U/L and a standard deviation of 5.97 U/L from the available literature, with a two-sided alpha of 0.05 and 90% power, the calculation yielded nine completers per arm (27 in total). Eleven participants were therefore randomized per arm to allow for attrition. Only 22 participants completed follow-up, including six in the vitamin D arm; consequently, the achieved sample was below the planned analysable sample. The study is therefore presented as a pilot trial, and effect estimates and confidence intervals are emphasized rather than definitive efficacy claims.

2.4. Randomization and Blinding

Allocation used simple randomization from a random-number table. Each participant received a sequential enrolment number and was assigned to one of the three arms according to a pre-generated allocation list, giving 11 participants per arm. The randomization sequence was generated before participant enrollment. Allocation concealment was maintained by an independent study coordinator who was not involved in participant recruitment,

clinical management, outcome assessment, or statistical analysis. The coordinator assigned participants according to the predetermined randomization sequence after eligibility had been confirmed. The trial was single-blind. Because the ATT-only arm received no matching placebo, participants and their caregivers could not be masked to the presence or absence of a supplement; masking therefore applied to outcome assessment. Laboratory personnel who measured serum ADA and vitamin D analysed coded samples and were blinded to arm assignment, so that the primary biochemical outcome was determined without knowledge of allocation.

2.5. Interventions

Standard pediatric ATT was given at body-weight-adjusted doses across the two-month intensive phase, comprising at least rifampicin, isoniazid and pyrazinamide. Zinc sulfate syrup was given at 15 mg daily for two months in the zinc arm. Vitamin D was given at 1,000 IU daily for two months in the vitamin D arm. Supplements were added to, and did not replace, standard therapy.

2.6. Laboratory Methods

Serum ADA was measured at baseline and at two months, coinciding with the end of intensive-phase therapy. Blood samples were allowed to clot for 10 to 20 min at room temperature, then centrifuged at 2,000 to 3,000 rpm for 20 min, and serum was stored under standard laboratory conditions pending analysis. Total ADA was measured on a Mindray BS-200E chemical analyzer using Mindray reagent via enzymatic colorimetric assay, with a reference interval of 4 to 24 U/L, linearity of 1 to 200 U/L, and a detection limit of 1 U/L. Two levels of Mindray internal quality control material were run daily before sample measurement.

Serum vitamin D (25-hydroxyvitamin D2 and D3) was measured on a Cobas c801 analyzer using Elecsys Vitamin D total III reagent and electrochemiluminescence binding assay. The measuring range was 6.00 to 120 ng/mL. PreciControl total III (two-level material) was used daily for internal quality control, after reagent replacement, and following instrument calibration.

2.7. Statistical Analysis

Data were analyzed using IBM SPSS Statistics version 23. Normality was assessed with the Shapiro-Wilk test, chosen because all group sizes were below 50. Levene's test assessed homogeneity of variance. For normally distributed and homogeneous data, within-group comparisons used the paired *t*-test and between-group comparisons of ADA change used one-way ANOVA. For non-normally distributed data, the Wilcoxon test was used within-groups and the Kruskal-Wallis test between groups. Spearman correlation examined the relationship between change in vitamin D and change in ADA. The significance threshold was set at $p < 0.05$.

Because the arms were imbalanced at baseline and a pretest–posttest randomized design is more efficiently and less biasedly analysed by covariate adjustment, we additionally fitted an ANCOVA with post-treatment ADA as the outcome, treatment arm as a fixed factor, and baseline ADA as a covariate; adjusted (estimated marginal) means and between-arm contrasts with 95% confidence intervals were derived from this model. A secondary ANCOVA additionally adjusted for baseline vitamin D and nutritional status, interpreted cautiously given the limited residual degrees of freedom. Regression to the mean was examined directly by correlating baseline ADA with within-subject change. Robustness was checked with a rank-based Kruskal–Wallis test on change and an ANCOVA on change scores. Spearman correlation examined the relationship between change in vitamin D and change in ADA. The significance threshold was $p < 0.05$; post-hoc pairwise comparisons used Bonferroni correction [19].

2.8. Research Ethics

The procedure for this research has obtained ethical approval from Dr. Saiful Anwar General Hospital, Malang through the ethical approval number 400/179/K.3/102.7/2022.

3. Results

3.1. Participant Enrollment and Baseline Characteristics

Thirty-three children were randomized, 11 per arm. During the two-month follow-up, 11 (33.3%) did not provide complete post-treatment data, mainly because they did not return for the follow-up visit at which post-

treatment ADA was drawn. Complete data were therefore available for 22 children: eight in the ATT arm, eight in the ATT + zinc arm and six in the ATT + vitamin D arm (**Figure 1**). Attrition was thus both high and differential, being greatest in the vitamin D arm (5/11, 45%). Baseline characteristics are shown in **Table 1**.

Table 1. Baseline characteristics by treatment group.

Characteristic	ATT (n = 8)	ATT + Zinc (n = 8)	ATT + Vitamin D (n = 6)
Age (years), median (min-max)	10 (1–17)	13.5 (1.75–17)	8 (4–16)
TB score, median (min-max)	7 (3–8)	7 (6–8)	7 (6–9)
ADA pre-treatment (U/L), median (min-max)	16 (8–20)	22.5 (3–28)	16.5 (5–47)
ADA post-treatment (U/L), median (min-max)	9.5 (7–32)	11.5 (7–41)	10 (2–22)
Vitamin D pre-treatment (ng/mL), median (min-max)	15.9 (3–27.6)	9.9 (3–20)	3 (1–9.2)
Vitamin D post-treatment (ng/mL), median (min-max)	15 (3–27)	12.6 (3–40)	16.5 (9–25.5)
Male, n (%)	3 (37.5)	4 (50.0)	2 (33.3)
Female, n (%)	5 (62.5)	4 (50.0)	4 (66.7)
Poor nutrition, n (%)	1 (12.5)	5 (62.5)	3 (50.0)
Under nutrition, n (%)	5 (62.5)	3 (37.5)	2 (33.3)
Good nutrition, n (%)	2 (25.0)	0 (0.0)	1 (16.7)
Pulmonary TB, n (%)	5 (62.5)	6 (75.0)	4 (66.7)
Extrapulmonary TB, n (%)	3 (37.5)	2 (25.0)	2 (33.3)
Body weight pre-treatment (kg), median (min-max)	27 (9–49)	35.5 (7–40)	21.15 (9–46)
Body weight post-treatment (kg), median (min-max)	27.5 (11–49)	37 (8–48)	25 (11–50)

Note: ADA, adenosine deaminase; ATT, anti-tuberculosis treatment; TB, tuberculosis.

The arms were not well balanced at baseline, despite randomization, a pattern that is not unusual in small trials and that motivated the ANCOVA reported below. Median baseline vitamin D was lowest in the vitamin D arm (3.0 ng/mL) and higher in the ATT and zinc arms (15.9 and 9.9 ng/mL), and mean baseline ADA was highest in the vitamin D arm (21.0 U/L) versus 14.9 and 18.4 U/L. The cohort spanned ages 1 to 17 years and included both pulmonary and extrapulmonary disease.

3.2. Within-Arm Change in Serum ADA

Serum ADA decreased numerically in all three groups after two months of intensive-phase therapy (**Table 2**). The mean decrease was 1.38 U/L in the ATT group ($p = 0.752$), 1.75 U/L in the ATT plus zinc group ($p = 0.670$), and 10.33 U/L in the ATT plus vitamin D group ($p = 0.154$). None reached statistical significance within-groups.

Table 2. Changes in serum ADA after two months of intensive-phase therapy.

Group	Pre-Treatment ADA, Mean ± SD (U/L)	Post-Treatment ADA, Mean ± SD (U/L)	Mean Change (U/L)	Within-Group *p*-Value
ATT (n = 8)	14.88 ± 4.67	13.50 ± 8.90	1.38	0.752
ATT + Zinc (n = 8)	18.38 ± 10.49	16.63 ± 12.39	1.75	0.670
ATT + Vitamin D (n = 6)	21.00 ± 16.43	10.67 ± 6.56	10.33	0.154
Between-group comparison of ADA change	-	-	-	One-Way ANOVA $p = 0.364$

Note: ADA, adenosine deaminase; ATT, anti-tuberculosis treatment; SD, standard deviation.

ADA change met assumptions for one-way ANOVA. Shapiro-Wilk testing confirmed normal distribution in the ATT group ($p = 0.081$), ATT plus zinc group ($p = 0.639$), and ATT plus vitamin D group ($p = 0.157$). Levene's test showed homogeneous variance ($p = 0.969$). One-way ANOVA found no statistically significant between-group difference in ADA change ($p = 0.364$).

3.3. ANCOVA Adjusted for Baseline

Because baseline ADA differed between treatment groups, post-treatment ADA was compared using ANCOVA with baseline ADA included as a covariate. After adjustment, no statistically significant difference was observed between groups ($F_{2,18} = 0.79$, $p = 0.470$), and baseline ADA was not independently associated with post-treatment ADA ($\beta = 0.25$, $p = 0.242$). The adjusted mean post-treatment ADA at the grand mean baseline value of 17.82 U/L was 14.23 U/L in the ATT group, 16.49 U/L in the zinc group, and 9.88 U/L in the vitamin D group. Compared with ATT alone, the adjusted treatment effect was −4.35 U/L for vitamin D (95% CI −15.70 to 7.00; $p = 0.431$) and +2.26 U/L for zinc (95% CI −8.07 to 12.59; $p = 0.651$).

Adjustment for baseline ADA materially changed the estimated treatment effect. Before adjustment, the crude difference in ADA reduction between the vitamin D and ATT groups was 8.96 U/L. After adjustment, this difference decreased to 4.35 U/L, indicating that approximately half of the apparent treatment effect was explained by baseline imbalance rather than by vitamin D supplementation. To examine the robustness of this finding, a secondary ANCOVA additionally adjusted for baseline vitamin D status and nutritional status. This analysis likewise showed no statistically significant difference between treatment groups (p = 0.480) and further attenuated the estimated vitamin D effect toward the null. Because this expanded model retained only 15 residual degrees of freedom, the results should be interpreted cautiously owing to the increased risk of overfitting.

3.4. Regression to the Mean

The observed pattern is consistent with regression to the mean. Across the full cohort, higher baseline ADA values were associated with larger within-subject declines during follow-up (r = −0.67, p = 0.001). This inverse correlation indicates that participants entering the study with the highest ADA concentrations tended to show the greatest reductions, regardless of treatment allocation. The association was strongest in the vitamin D group (r = −0.92, p = 0.010), which also had the highest baseline ADA values, and remained significant in the ATT group (r = −0.75, p = 0.032). By contrast, the relationship was weaker and not statistically significant in the zinc group (r = −0.35, p = 0.402). Taken together, these findings suggest that part of the larger decline observed in the vitamin D arm can be explained by its higher starting values. The data therefore support regression to the mean as a plausible contributor to the apparent treatment effect, alongside any biological effect that vitamin D supplementation may or may not have produced.

3.5. Intention-to-Treat Analysis

To evaluate the potential impact of the high and differential attrition, an intention-to-treat analysis was performed including all 33 randomized participants using multiple imputation by chained equations with predictive mean matching. The results were consistent with those of the complete-case analysis. No statistically significant difference was detected between treatment groups (p = 0.66), and the adjusted treatment effect for vitamin D was further attenuated, decreasing from −4.35 U/L in the complete-case ANCOVA to −2.11 U/L in the intention-to-treat analysis (95% CI −11.86 to 7.65; p = 0.66; fraction of missing information = 0.28). The corresponding adjusted estimate for the zinc group was +1.99 U/L relative to ATT alone (95% CI −7.28 to 11.25; p = 0.66). Mean reductions in serum ADA after multiple imputation were 2.2 U/L in the ATT group, 2.1 U/L in the zinc group, and 8.8 U/L in the vitamin D group. The close agreement between the complete-case and intention-to-treat analyses indicates that missing outcome data had little influence on the overall interpretation of the trial. A comparison of the pooled treatment estimates from both analytical approaches is presented in **Table 3**.

Table 3. Complete-case versus multiple-imputation intention-to-treat estimates from the baseline-adjusted ANCOVA.

Analysis	Analysable (n)	Adjusted Vitamin D – ATT, U/L (95% CI)	Adjusted zinc – ATT, U/L (95% CI)	Omnibus between-Arm p
Complete-case ANCOVA	22	−4.35 (−15.70 to 7.00)	+2.26 (−8.07 to 12.59)	0.470
Multiple-imputation intention-to-treat	33	−2.11 (−11.86 to 7.65)	+1.99 (−7.28 to 11.25)	0.665

3.6. Correlation between Change in Vitamin D and Change in ADA

Spearman correlation between change in vitamin D and change in ADA was non-significant in every arm: r = −0.42 (p = 0.299) in the ATT arm, r = −0.69 (p = 0.060) in the zinc arm, and r = +0.70 (p = 0.125) in the vitamin D arm. With six to eight subjects per arm these coefficients are unstable, and the sign reversal in the vitamin D arm is more plausibly the effect of one or two extreme values on a small sample than a genuine biological divergence [20].

3.7. Change in Body Weight

Change in body weight differed across arms by Kruskal–Wallis test (p = 0.031). Post-hoc Mann–Whitney comparisons with Bonferroni correction (per-comparison α = 0.017) gave p = 0.357 (ATT vs. vitamin D), 0.025 (ATT vs. zinc) and 0.026 (vitamin D vs. zinc); no pair reached the corrected threshold.

4. Discussion

The numerical decline in serum ADA observed in all three groups after the two months intensive phase indicates that cellular immune activation decreased during anti-tuberculosis treatment. Although the vitamin D group showed the largest unadjusted reduction, neither the within-group nor the between group comparisons reached statistical significance. This pattern remained unchanged after adjustment for baseline ADA using ANCOVA. Taken together, these findings indicate that the present study did not detect an additional effect of vitamin D or zinc supplementation beyond standard anti-tuberculosis therapy. The larger crude decline observed in the vitamin D group appears to be explained, at least in part, by baseline imbalance and regression to the mean rather than by the intervention itself.

The decline in ADA across all treatment groups is biologically plausible. Adenosine deaminase reflects activation of T lymphocytes and the monocyte macrophage system during the host immune response to *Mycobacterium tuberculosis*. As bacterial burden decreases during effective treatment, immune activation also subsides, leading to lower circulating ADA concentrations. Previous studies have consistently shown that serum ADA parallels disease activity and declines during successful anti-tuberculosis therapy [13,21]. The reduction observed in the ATT only group therefore represents the expected response to standard treatment. Its relatively modest magnitude may reflect two characteristics of the present cohort. First, childhood tuberculosis is typically paucibacillary, particularly in children younger than five years, resulting in weaker and more variable inflammatory biomarker responses than those reported in adult pulmonary tuberculosis [18]. Second, the limited sample size and substantial variability in post treatment ADA values reduced the precision of the estimated treatment effect.

The apparent benefit observed in the vitamin D group requires careful interpretation because this group entered the study with both the highest baseline ADA concentration and the lowest serum vitamin D level. Groups with more extreme baseline values tend to show greater movement toward the population average on repeat measurement even in the absence of a true treatment effect. The present data support this explanation. Baseline ADA was inversely correlated with the magnitude of ADA change, and this relationship was strongest in the vitamin D group. More importantly, adjustment for baseline ADA reduced the crude difference between the vitamin D and ATT groups by approximately one half. This finding indicates that a substantial proportion of the observed decline can be attributed to baseline imbalance rather than supplementation. The issue extends beyond the instability expected in small correlation analyses and directly affects the primary comparison of treatment groups. For this reason, ANCOVA provides a more appropriate analytical framework than simple change score analysis in a pretest-posttest randomized trial. The adjusted analysis does not support the conclusion that vitamin D supplementation produced a measurable reduction in serum ADA.

Another consideration is whether the vitamin D intervention was sufficient to test the biological hypothesis. Participants assigned to this group had a median baseline 25 hydroxyvitamin D concentration of only 3.0 ng/mL, indicating severe deficiency. After two months of supplementation with 1,000 IU daily, the median concentration increased to 16.5 ng/mL. Although this represented biochemical improvement, most participants remained below the conventional deficiency threshold of 20 ng/mL and far below the commonly accepted sufficiency target of 30 ng/mL. The intervention therefore failed to achieve vitamin D repletion in the majority of children. This incomplete correction provides a plausible explanation for the absence of an observable effect on ADA. In contrast, Chandra and colleagues administered four oral doses of 100,000 IU vitamin D to deficient adults with pulmonary tuberculosis and reported significant reductions in ADA after four, six, and eight weeks of treatment [22]. Their cumulative dose of approximately 400,000 IU was substantially higher than the approximately 60,000 IU delivered in the present study. The discrepancy between the findings of the two studies is therefore more readily explained by differences in achieved vitamin D exposure than by differences between adult and pediatric populations alone. Future pediatric trials should employ dosing strategies capable of achieving confirmed vitamin D repletion and should incorporate serial monitoring of serum 25 hydroxyvitamin D concentrations throughout the intervention period before concluding whether vitamin D influences ADA responses during tuberculosis treatment.

The zinc supplementation group likewise demonstrated a modest reduction in serum ADA, although the change was not statistically significant. Interpretation of this finding is constrained by the absence of serum zinc measurements before and after supplementation. Without these data, it is impossible to determine whether participants were zinc deficient at baseline, whether supplementation corrected that deficiency, or whether the administered

dose achieved biologically meaningful concentrations. As a result, the present study cannot distinguish between a true lack of biological effect and inadequate zinc exposure. Future trials should combine zinc supplementation with serial assessment of serum zinc concentrations to verify that the intervention produces the intended biochemical response before evaluating clinical or immunological outcomes.

The choice of biomarker may also have reduced the ability to detect treatment-related changes. This study measured total serum ADA, whereas the ADA2 isoenzyme is more closely associated with active tuberculosis because it is released predominantly by activated monocytes and macrophages during the antimycobacterial immune response [12,23]. Total ADA represents the combined activity of ADA1 and ADA2. Since ADA1 is widely distributed in many tissues and is less specific to tuberculosis associated inflammation, measurement of total ADA may dilute changes that occur primarily within the ADA2 fraction. A genuine treatment effect mediated through ADA2 could therefore remain undetected when only total ADA is measured. Future investigations should consider measuring ADA2 directly or reporting the ADA2 to total ADA ratio to improve the specificity of biomarker assessment during anti-tuberculosis therapy.

Previous exposure to anti-tuberculosis treatment represents another potential source of variability in baseline ADA concentrations. Eligibility criteria limited enrollment to treatment-naïve children or those who had received no more than one week of therapy, thereby increasing the likelihood that observed changes reflected the intensive treatment phase rather than prior drug exposure. Even so, complete exclusion of undocumented treatment before referral cannot be guaranteed. Dr. Saiful Anwar Hospital functions as a tertiary referral centre, and some children may have received incomplete treatment or unrecorded medications before referral. Such exposure could contribute to baseline heterogeneity in ADA concentrations despite careful participant screening. Future multicentre studies should document referral pathways and any previous anti-tuberculosis medication in greater detail to minimise this potential source of bias.

The absence of statistically significant differences between treatment groups should not be interpreted as evidence that vitamin D or zinc has no biological effect on tuberculosis related immune responses. Rather, it indicates that the present trial did not detect a difference within the limits imposed by its sample size and variability. The biological effects of micronutrient supplementation depend on multiple factors, including baseline nutritional status, host genetic background, inflammatory activity, adherence to supplementation, and the degree of deficiency before treatment. Small clinical trials may therefore fail to detect genuine treatment effects despite consistent directional changes. This limitation has been recognised previously. A Cochrane review that included 35 randomized controlled trials involving 8,285 participants concluded that most micronutrient supplementation trials in tuberculosis lacked sufficient statistical power to exclude clinically meaningful effects and that non-significant findings from small studies should not be interpreted as evidence of no effect [24]. The consistent decline in ADA observed across all study groups is compatible with the expected biological response to anti-tuberculosis therapy. The broad confidence intervals simply indicate that the present estimates lack precision.

Clinical heterogeneity within the study population probably contributed further to the observed variability. Participants ranged in age from one to seventeen years and included both pulmonary and extrapulmonary tuberculosis. These clinical entities differ in bacillary burden, immune activation, and inflammatory kinetics, all of which may influence circulating ADA concentrations. Most participants were also undernourished, a condition known to alter cellular immune function and inflammatory biomarkers [25,26]. In addition, susceptibility to tuberculosis and immune responses may vary according to sex during adolescence [27]. With only six to eight participants per treatment group, these sources of biological variation likely increased within-group variance and reduced statistical power. Age distribution and disease classification by treatment group are presented in **Table 1**. Future studies would benefit from narrower eligibility criteria or stratified randomization according to disease site and age category to reduce clinical heterogeneity and improve precision of treatment effect estimates.

Evidence from pediatric populations remains limited, and the closest comparison with the present study provides useful context. Tamara and colleagues conducted a double-blind randomized trial in Indonesian children with pulmonary tuberculosis using the same daily vitamin D dose of 1,000 IU and reported earlier resolution of fever and cough together with greater improvement in nutritional status compared with placebo [7]. These findings are not inconsistent with the present results. Their study evaluated clinical recovery, whereas the current trial examined a single biochemical marker with lower disease specificity in a smaller and more heterogeneous cohort. In addition, the baseline imbalance observed in the vitamin D group further complicated interpretation of treatment-related

changes. The available pediatric literature on ADA also focuses largely on its diagnostic performance, particularly in pleural fluid, rather than its role as a marker of treatment response in serum [23]. The present study therefore contributes new evidence regarding serum ADA during the intensive phase of childhood tuberculosis treatment, although the findings should be viewed as exploratory rather than definitive.

Several methodological improvements should be considered in future studies. Recruitment across multiple centres or a longer enrolment period would increase the likelihood of achieving the planned sample size and provide adequate statistical power. Intention-to-treat analysis with appropriate methods for handling missing data, such as multiple imputation, should be specified before recruitment begins. Micronutrient interventions should also be verified biochemically rather than assumed. Vitamin D supplementation should continue until serum 25 hydroxyvitamin D concentrations reach predefined target levels, while zinc supplementation should be accompanied by serial measurement of serum zinc concentrations. Biomarker selection could also be refined through measurement of the ADA2 isoenzyme or the ADA2 to total ADA ratio, both of which are expected to better reflect tuberculosis specific immune activity. For pretest-posttest randomized trials, covariate adjusted analyses such as ANCOVA should remain the primary analytical approach, accompanied by reporting of baseline to change correlations to allow readers to evaluate the potential influence of regression to the mean. Restricting enrolment to narrower age groups or specific forms of tuberculosis would further reduce biological heterogeneity and improve the precision of treatment effect estimates.

The study also has several strengths. Random allocation was performed prospectively, sample size estimation was conducted before recruitment, and only treatment-naïve children were enrolled, reducing confounding from previous anti-tuberculosis therapy. Laboratory analyses were performed under standardized quality-controlled conditions, and the results are presented transparently by distinguishing numerical trends from statistically supported findings. At the same time, several limitations should be acknowledged. The final sample size was smaller than originally planned, leaving the study underpowered and limiting the precision of between group comparisons. Serum zinc concentrations, dietary intake, treatment adherence, and biochemical confirmation of vitamin D repletion were not available, preventing direct assessment of treatment exposure. Attrition reached 33.3% and was greatest in the vitamin D group. Consequently, complete case analysis may have introduced bias if withdrawal was associated with disease severity, supplement related gastrointestinal symptoms, or other factors related to outcome [2,28]. The direction of this potential bias cannot be determined from the available data. If participants with poorer biochemical responses were more likely to discontinue vitamin D supplementation, the observed decline among completers would overestimate the true treatment effect. Conversely, if participants who responded well were disproportionately lost for reasons unrelated to treatment, the observed effect would underestimate the true response. Although the primary adjusted analysis did not demonstrate a statistically significant difference between groups, future adequately powered trials incorporating intention-to-treat analyses and formal methods for handling missing data are needed before firm conclusions regarding the immunomodulatory effects of vitamin D or zinc supplementation in childhood tuberculosis can be drawn.

5. Conclusions

During the two-month intensive phase of anti-tuberculosis treatment, serum ADA declined numerically in all study groups, with the largest unadjusted reduction observed among children receiving vitamin D supplementation. After adjustment for baseline ADA, however, neither vitamin D nor zinc supplementation produced a statistically significant reduction in total serum ADA compared with standard anti-tuberculosis therapy alone. The attenuation of the vitamin D effect after covariate adjustment indicates that much of the apparent benefit was attributable to baseline imbalance and regression to the mean rather than to the intervention itself. Interpretation of these findings should also consider the limited statistical power, incomplete vitamin D repletion, absence of serum zinc measurements, and reliance on total ADA rather than the more tuberculosis-specific ADA2 isoenzyme. Consequently, the present findings do not exclude a potential biological effect of micronutrient supplementation. Larger multicentre randomized trials with adequate statistical power, confirmation of micronutrient repletion, serial assessment of vitamin D and zinc status, measurement of ADA2, and intention-to-treat analyses are needed to determine whether adjunctive vitamin D or zinc supplementation modifies ADA responses during the treatment of childhood tuberculosis.

Author Contributions

Conceptualization, A.I.; methodology, A.I.; software, A.A.; validation, A.I. and A.A.; formal analysis, A.A.; investigation, E.O.; resources, A.I. and E.O.; data curation, E.O. and A.A.; writing—original draft preparation, A.A.; writing—review and editing, A.I., E.O. and K.H.; visualization, A.A.; supervision, A.I., E.O. and K.H.; project administration, A.A. and E.O.; funding acquisition, A.I. All authors have read and agreed to the published version of the manuscript.

Funding

This work was partially supported by Universitas Brawijaya (No: 20210929102527).

Institutional Review Board Statement

The study was approved by the Ethics Committee of Dr. Saiful Anwar General Hospital, Malang (approval number 400/179/K.3/102.7/2022).

Informed Consent Statement

Written informed consent was obtained from the parent or legal guardian of each participant.

Data Availability Statement

The datasets analyzed in this study are not publicly available due to institutional regulations but may be obtained from the corresponding author upon reasonable request.

Acknowledgments

We would like to thank PT Mindray Medical Indonesia for providing the chemical analyzer and reagents used in the serum ADA measurement for this study.

Conflicts of Interest

The authors declare no conflict of interest.

AI Use Statement

During the preparation of this manuscript, the authors used Anthropic (Claude) and OpenAI (ChatGPT) solely for language refinement. No AI tools were used for data analysis, interpretation, or generation of scientific content. All outputs were critically reviewed and edited by the authors. The authors take full responsibility for the integrity and accuracy of the work.

References

1. Maphalle, L.N.F.; Michniak-Kohn, B.B.; Ogunrombi, M.O.; et al. Pediatric Tuberculosis Management: A Global Challenge or Breakthrough? *Children* **2022**, *9*, 1120. [CrossRef]
2. Brooks, M.B.; van de Water, B.J.; Lecca, L.; et al. Tuberculosis treatment loss to follow-up in children exposed at home: A prospective cohort study. *J. Glob. Health* **2024**, *14*, 04194. [CrossRef]
3. Ernawati, F.; Efriwati; Nurjanah, N.; et al. Micronutrients and Nutrition Status of School-Aged Children in Indonesia. *J. Nutr. Metab.* **2023**, *2023*, 4610038. [CrossRef]
4. McArdle, A.J.; Keane, D.; Seddon, J.A.; et al. Vitamin D deficiency is associated with tuberculosis disease in British children. *Int. J. Tuberc. Lung Dis.* **2020**, *24*, 782–788. [CrossRef]
5. Papagni, R.; Pellegrino, C.; Di Gennaro, F.; et al. Impact of Vitamin D in Prophylaxis and Treatment in Tuberculosis Patients. *Int. J. Mol. Sci.* **2022**, *23*, 3860. [CrossRef]
6. Afzal, A.; Rathore, R.; Butt, N.F.; et al. Efficacy of Vitamin D supplementation in achieving an early sputum conversion in smear positive Pulmonary Tuberculosis. *Pak. J. Med. Sci.* **2018**, *34*. [CrossRef]
7. Tamara, L.; Kartasasmita, C.B.; Alam, A.; et al. Effects of Vitamin D supplementation on resolution of fever and cough in children with pulmonary tuberculosis: A randomized double-blind controlled trial in Indonesia. *J. Glob. Health* **2022**, *12*, 04015. [CrossRef]
8. Buonsenso, D.; Pata, D.; Colonna, A.T.; et al. Vitamin D and tuberculosis in children: A role in the prevention

or treatment of the disease? *Monaldi Arch. Chest Dis.* **2022**, *92*. [CrossRef]
9. Costa, M.I.; Sarmento-Ribeiro, A.B.; Gonçalves, A.C. Zinc: From Biological Functions to Therapeutic Potential. *Int. J. Mol. Sci.* **2023**, *24*, 4822. [CrossRef]
10. Zolfaghari, B.; Ghanbari, M.; Musavi, H.; et al. Investigation of Zinc Supplement Impact on the Serum Biochemical Parameters in Pulmonary Tuberculosis: A Double Blinded Placebo Control Trial. *Rep. Biochem. Mol. Biol.* **2021**, *10*, 173–182.
11. Wagnew, F.; Alene, K.A.; Eshetie, S.; et al. Effects of zinc and vitamin A supplementation on prognostic markers and treatment outcomes of adults with pulmonary tuberculosis: a systematic review and meta-analysis. *BMJ Glob. Health* **2022**, *7*, e008625.
12. Delemarre, E.M.; van Hoorn, L.; Bossink, A.W.J.; et al. Serum Biomarker Profile Including CCL1, CXCL10, VEGF, and Adenosine Deaminase Activity Distinguishes Active From Remotely Acquired Latent Tuberculosis. *Front. Immunol.* **2021**, *12*, 725447. [CrossRef]
13. Sarkar, K.; Kashyap, B.; Jhamb, R.; et al. Correlation of serum Adenosine Deaminase levels with microbiological parameters in Pulmonary Tuberculosis. *Indian J. Clin. Biochem.* **2024**, *39*, 380–386. [CrossRef]
14. Zimmer, A.J.; Lainati, F.; Aguilera Vasquez, N.; et al. Biomarkers That Correlate with Active Pulmonary Tuberculosis Treatment Response: A Systematic Review and Meta-analysis. *J. Clin. Microbiol.* **2022**, *60*, e01859-21. [CrossRef]
15. Soedarsono, S.; Prinasetyo, K.A.I.; Tanzilia, M.; et al. Changes of serum adenosine deaminase level in new cases of pulmonary tuberculosis before and after intensive phase treatment. *Lung India* **2020**, *37*, 126–129. [CrossRef]
16. Muljono, C.G.; Soedarsono, S. Change in Adenosine Deaminase Serum Levels from Before and Six Months after Treatment in Patients with Tuberculous Lymphadenopathy. *J. Respir.* **2026**, *12*, 1–5. [CrossRef]
17. Nisa, Z.U.; Zeshan, B.; Ambreen, A.; et al. Plasma ferritin, C-reactive protein, and adenosine deaminase levels in tuberculous lymphadenitis and pleuritis and their role in monitoring treatment response. *BMC Infect. Dis.* **2024**, *24*, 1375. [CrossRef]
18. Direktorat Jenderal Pencegahan dan Pengendalian Penyakit. *Technical Guidelines for the Management of Tuberculosis in Children and Adolescents 2023*; Kementerian Kesehatan Republik Indonesia: Jakarta, Indonesia, 2023. (in Indonesian)
19. Kim, H.-Y. Statistical notes for clinical researchers: Post-hoc multiple comparisons. *Restor. Dent. Endod.* **2015**, *40*, 176. [CrossRef]
20. Cao, Y.; Chen, R.C.; Katz, A.J. Why is a small sample size not enough? *Oncologist* **2024**, *29*, 761–763. [CrossRef]
21. Arghir, I.A.; Arghir, O.C.; Otelea, M.R.; et al. Adenosine Deaminase and Systemic Immune Inflammatory Index—A Biomarker Duet Signature of Pulmonary Tuberculosis Severity. *Medicina* **2025**, *61*, 1096. [CrossRef]
22. Chandra, H.; Rahman, A.; Yadav, P.; et al. Effect of adjunct Vitamin D treatment in vitamin D deficient pulmonary tuberculosis patients: A randomized, double blind, active controlled clinical trial. *Indian J. Tuberc.* **2024**, *71*, 170–178. [CrossRef]
23. Na, F.; Wang, Y.; Yang, H.; et al. Performance of adenosine deaminase in detecting paediatric pleural tuberculosis: A systematic review and meta-analysis. *Ann. Med.* **2022**, *54*, 3128–3134. [CrossRef]
24. Grobler, L.; Nagpal, S.; Sudarsanam, T.D.; et al. Nutritional Supplements for People Being Treated for Active Tuberculosis. *Cochrane Database Syst. Rev.* **2016**, *2016*. [CrossRef]
25. Ockenga, J.; Fuhse, K.; Chatterjee, S.; et al. Tuberculosis and malnutrition: The European perspective. *Clin. Nutr.* **2023**, *42*, 486–492. [CrossRef]
26. Tellez-Navarrete, N.A.; Ramon-Luing, L.A.; Muñoz-Torrico, M.; et al. Malnutrition and tuberculosis: The gap between basic research and clinical trials. *J. Infect. Dev. Ctries.* **2021**, *15*, 310–319. [CrossRef]
27. Thakur, S.; Chauhan, V.; Kumar, R.; et al. Adolescent Females are More Susceptible than Males for Tuberculosis. *J. Glob. Infect. Dis.* **2021**, *13*, 3–6. [CrossRef]
28. Ceballos-Rasgado, M.; Lowe, N.M.; Mallard, S.; et al. Adverse Effects of Excessive Zinc Intake in Infants and Children Aged 0–3 Years: A Systematic Review and Meta-Analysis. *Adv. Nutr.* **2022**, *13*, 2488–2519. [CrossRef]