

Original Article

Cytokines related to nutritional status in patients with untreated pulmonary tuberculosis in Indonesia

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Although several studies have dealt with the patterns of cytokine production in tuberculosis, little is known about the association between nutrient deficiencies and cytokines in tuberculosis. The objective of this study was to assess the concentration of cytokines related to nutritional status during tuberculosis. In 41 untreated tuberculosis patients and matched healthy controls in an urban hospital in Indonesia, we measured: height and weight, parameters of iron, vitamin A and zinc; and cytokines concentrations in the circulation and production in whole blood cultures. Plasma interleukin-6 (IL-6) and interleukin-1 receptor antagonist (IL-1ra) were significantly higher in patients than in controls. Patients with cavities (n=26) had higher concentrations of IL-6 than patients without cavities (n=15). Body mass index <18.5 kg/m² was associated with high concentrations of tumor necrosis factor- α (TNF- α) and IL-6. Anaemia was associated with high concentrations of IL-6 and IL-1ra. Zinc deficiency was associated with high LPS-stimulated production of TNF- α and IL-1ra. Marginal plasma retinol concentrations were associated with high concentrations of IL-6 after LPS stimulation. In conclusion, low concentrations of micronutrients in tuberculosis were associated with increased cytokine production. An intervention study would allow causality to be examined.

Key Words: interleukin-6, tumor necrosis factor- α , interleukin-1 receptor antagonist, body mass index, micronutrient

Introduction

Malnutrition is frequently observed during tuberculosis¹, and is thought to influence host defence and thereby outcome of the infection. How malnutrition affects host defence is incompletely understood, but it may involve changes in the balance of pro- and antiinflammatory cytokines.

Manifestations of tuberculosis such as fever, weight loss, prolonged acute-phase response and granuloma formation, are mediated by proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6). Antiinflammatory cytokines such as IL-4, IL-10 and IL-1 receptor antagonist (IL-1ra) may also influence host defence against *Mycobacterium tuberculosis*. Deficiencies of micronutrients, as well as protein-energy malnutrition, diminish the non-specific host defence during infection²⁻⁴ including the production of cytokines.^{5,6} Vitamin A deficiency induces a regulatory T-helper (Th) cell imbalance.⁷ Zinc deficiency decreases phagocytosis⁸ and numbers of circulating T cells in animals⁹. Zinc defi-

ciency also leads to decreased production of IFN- γ , IL-1 and TNF- α .⁶ Conversely, supplementation of micronutrients such as vitamin A may increase production and/or release of IL-1 β and TNF, and inhibit production of interferon- γ (IFN- γ) and interleukin-2 (IL-2) in vitro.¹⁰⁻¹² Zinc supplementation can regulate the production of IL-1 α from alveolar macrophages in patients with pulmonary tuberculosis or bacterial pneumonia.¹³ In addition, vitamin A and zinc may improve the proliferation of T lymphocytes during the response to tuberculin, Purified Protein Derivative (PPD).¹⁴

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Although several studies have dealt with the patterns of cytokine production in tuberculosis, little is known about the association between nutrient deficiencies and cytokine production during active tuberculosis. The objective of this study was to assess concentrations of both circulating and ex-vivo produced cytokines, as well as the association between nutritional status and cytokine production in tuberculosis patients and controls. Since cavitary tuberculosis is considered a more serious form of tuberculosis, in which necrosis prevails¹⁵, we also investigated whether cytokine patterns were different in patients with and without cavitary lesions.

Materials and methods

Patients

The study was carried out as a case-control study at the Pulmonology Clinic of Cipto Mangunkusumo University Hospital associated with the Medical Faculty of the University of Indonesia in Central Jakarta, Indonesia. Sample size was calculated based on the ability to determine a difference with $\alpha=0.05$ and $1-\beta=0.95$ using one-tailed test for concentrations of cytokines, plasma retinol, zinc and of blood haemoglobin. It was calculated that with a sample size of 35 in each group, zinc concentrations as the parameter requiring the largest sample size could be detected with between-group difference of $0.46 \mu\text{mol/L}$.¹⁶ Assuming that 25% of patients may not fulfil the inclusion criteria, we recruited 45 subjects for each group. Newly diagnosed out-patients with pulmonary tuberculosis were included as cases if they fulfilled all of the following 4 criteria: 1) age 15-55 yrs, 2) at least two sputum specimens positive for acid-fast bacilli by direct microscopy or one sputum specimen positive for culture, 3) abnormalities on chest X-ray, and 4) clinical examination consistent with pulmonary tuberculosis.¹⁷

Exclusion criteria were pregnancy, lactation, previous anti-tuberculosis medication, moderate to severe injury or surgery during the previous month, use of corticosteroids or supplements containing vitamin A, zinc, or iron during the previous month and presence of liver disease (elevated serum levels of Aspartate Amino Transferase (ASAT) and Alanine Amino Transferase (ALAT)), diabetes mellitus (elevated fasting serum levels of blood glucose), neoplasm detected by clinical examination, chronic renal failure (elevated serum levels of urea or creatinine), or clinical signs of congestive heart failure.

Controls were selected from neighbours of the patients (non-family members) in the smallest administrative unit ('rukun tetangga') comprising about 20 houses, and were matched with cases for sex and age (within ± 2 yrs). For the selection of controls, a list of healthy subjects was requested from the head of the administrative unit (rukun tetangga) by the field workers and one control subject was selected randomly from the list of 3-7 persons. Controls had no history of tuberculosis and no evidence of disease by clinical examination and laboratory assessment (total white blood cell count, and concentrations of C-reactive protein and fasting blood glucose). Informed consent was obtained from all subjects. The study was approved by the Committee on Health Research Ethics, Faculty of Medicine, University of Indonesia, Jakarta.

Clinical examination

Patients and controls were screened by using a structured questionnaire to collect information related to inclusion and exclusion criteria. Patients who were eligible were examined both clinically including Karnofsky score and body temperature, and by chest X-ray by one of authors (ZA) who is a pulmonologist at the Cipto Mangunkusumo University Hospital.

Sputum examination

Three specimens of early morning sputum from patients with suspected tuberculosis were examined for acid-fast bacilli by direct microscopy after Kinyoun Gabett staining (a modification of the Ziehl-Nielsen method)¹⁸, and one sputum was cultured in Kudoh medium.¹⁸

Tuberculin skin test (Mantoux test)

Skin test responses were stimulated by injecting PPD 0.1 mL, 5 tuberculin units (TU) (Perum Biofarma, Pasteur Institute, Bandung, Indonesia) into the skin of the volar area of the forearm. Responses were assessed during 48-72 h after injection by measuring the mean diameter of induration to the nearest mm, while induration ≥ 10 mm was considered as positive.

Anthropometric measurements

Subjects were weighed without shoes using an electronic platform model weighing scale (770 alpha; SECA, Hamburg, Germany). The weight was recorded to the nearest 0.1 kg. Height was recorded to the nearest 0.1 cm using a microtoise. Body mass index (BMI) was calculated as body weight divided by height squared (kg/m^2). Subjects with BMI $< 18.5 \text{ kg/m}^2$ were regarded as malnourished.¹⁹

Blood sampling

Fasting blood samples (15 mL) were collected via venipuncture to determine haematocrit, white blood cells count, erythrocyte sedimentation rate, and concentrations of haemoglobin in blood, zinc-protoporphyrin in washed erythrocytes, and C-reactive protein, albumin, retinol, zinc and α -tocopherol in plasma. The blood was centrifuged at $750 \times g$ for 10 min at room temperature and the plasma collected and stored at -80°C until analysed. Determination of zinc-protoporphyrin, haemoglobin, haematocrit, white blood cells count, and albumin was carried out on the day of sampling.

Biochemical tests

Haemoglobin, haematocrit, white blood cell counts, ASAT and ALAT were measured directly using an automatic analyzer (Sysmex Microdilutor F-800, Kobe, Japan). The cut-off points for normal haemoglobin were $>120 \text{ g/L}$ and $>130 \text{ g/L}$ and for haematocrit >0.37 and >0.40 , for females and males respectively. The normal value for white blood cells was $5,000\text{-}10,000 \text{ cells/mm}^3$. Erythrocyte sedimentation rate was determined according to Westergreen²⁰ with normal values $<20 \text{ mm/h}$. Albumin was determined by the bromocresol green method²¹ with normal values of $35\text{-}50 \text{ g/L}$. C-reactive protein was measured using an immunoturbidimetric assay²² (Behringwerke, Marburg, West Germany) with normal values $<5 \text{ mg/L}$. Zinc-protoporphyrin as a measure of free

erythrocyte protoporphyrin was estimated using the portable front-face hematofluorometer (AVIV Biomedical Co, Lakewood, NJ 08701). To determine the influence of components in plasma, we measured zinc-protoporphyrin in washed erythrocytes. For the washing procedure, we centrifuged the blood for 4 min at 1000xg just before the measurement, then removed the plasma and replaced it with isotonic saline to restore the original volume. After repeating the washing procedure, zinc-protoporphyrin was measured in 20 μ L of the washed erythrocyte suspension.²³ Values <40 μ mol/mol heme were regarded as normal. Plasma retinol concentration was measured by HPLC (Bondapak C-18 column, Waters, Milford, MA; model 6PD-6AV, Shimadzu, Tokyo; standards from Sigma, St Louis, MO) with methanol:water (95:5, vol:vol) as described previously and values <0.70 μ mol/L were regarded as indicating marginally low vitamin A status.²⁴ Plasma zinc was measured by atomic absorption spectrometry with values <10.7 μ mol/L regarded as zinc deficiency.²⁵

Cytokine measurement

For measurement of circulating cytokines, venous blood was sampled in sterile 4-mL EDTA tubes, and platelet-poor plasma was obtained by centrifugation. Ex-vivo production of cytokines was performed as previously described.²⁶ Briefly, for unstimulated cytokine production, a 4-mL EDTA tube was incubated for 24 h at 37°C. For LPS-stimulated production, a similar tube was incubated with LPS (*Escherichia coli* serotype 055:b5; Sigma, St. Louis, final concentration 10 mg/L). After incubation for 24 h, both LPS-stimulated and unstimulated samples were centrifuged and handled thereafter like the first tube. IL-1 β , TNF- α , and IL-1ra were measured in duplicate by nonequilibrium RIA²⁶. Recombinant human IL-1 β , TNF- α , and IL-1ra were calibrated against standards provided by the National Institute of Biological Standards and Control (Potters Bar, UK) with the sensitivity of the assay with 100 μ L sample of 40 pg/mL, 40 pg/mL, and 80 pg/mL, respectively. IL-6 was measured with an ELISA (CLB, Amsterdam, The Netherlands).²⁷ The detection limit was 4 pg/mL.

Statistical analysis

One sample Kolmogorov-Smirnov test was used to check whether the variables were normally distributed. Data are expressed as mean \pm standard deviation (SD) for normally distributed parameters while median and 25th-75th percentiles are used for non-normally distributed parameters. The independent sample *t* test was used for variables with normal distribution and when the frequency distribution was not normal, the Mann-Whitney *U* test was used. Pair-wise analysis using Wilcoxon rank sum test for unmatched samples was done to compare differences between patients with cavities and non-cavities, and controls. The SPSS software package (Windows version 7.5.2, SPSS Inc., Chicago, IL) was used for all statistical analyses. Results were considered statistically significant at *p*<0.05.

Results

Forty-one tuberculosis patients and 41 healthy control subjects (25 males and 16 females in both groups) were included in the study. Four patients had severe hemoptysis and were excluded from the study. Patients exhibited poorer nutritional status than controls (Table 1). Twenty-six patients (63%) had 3 positive sputum smears for acid fast bacilli, and 15 (37%) had 2 positive sputum smears, while 24 (59%) patients had a positive sputum culture. The main symptoms and signs exhibited by the patients were productive or unproductive cough (95%), hemoptysis (51%) and weight loss (83%). Five patients also demonstrated extra-pulmonary involvement as evidenced by lymph node enlargement (neck and axilla). Tuberculin testing of patients was positive (\geq 10 mm) in 34 patients (83%) and in 2 controls (5%).

As reported earlier²⁸, haematocrit and the concentrations of haemoglobin, albumin, plasma zinc and plasma retinol in active pulmonary tuberculosis patients were significantly lower, whereas zinc-protoporphyrin concentration was higher than in healthy controls. A significant correlation between C-reactive protein and the concentration of micronutrients (plasma retinol, zinc and zinc-protoporphyrin) was not found²⁸.

In patients, both circulating concentrations and unstimulated ex-vivo production of IL-6 and IL-1ra were

Table 1. Characteristics of patients with active pulmonary tuberculosis and healthy controls *

	Tuberculosis patients	Controls	<i>p</i> [‡]
No. of persons	41	41	-
Female/male ratio	18/23	18/23	-
Age (yrs)	28 \pm 9	28 \pm 9	-
Body weight (kg)	46.1 \pm 9.5	54.8 \pm 9.3	<0.001
Body mass index (kg/m ²)	18.1 \pm 3.1	21.9 \pm 3.0	<0.001
Body temperature (°C)	37.5 (37.0 - 38.2)	36.9 (36.0 - 37.0)	<0.01
Karnofsky score	87.5 \pm 4.5	100	<0.001
ESR (mm/h)	52 (25-82)	22 (12-31)	<0.001
C-reactive protein (mg/L)	35 (24-60) †	0	<0.001
White blood cells (cells/mm ³)	8626 \pm 3207	6434 \pm 1712	<0.001

* Data are mean \pm SD or median (25th-75th percentiles). † C-reactive protein was not detectable in 14 patients and in none of the controls. ‡ Independent sample *t* test for normally distributed data; Mann-Whitney *U* test for data not-normally distributed.

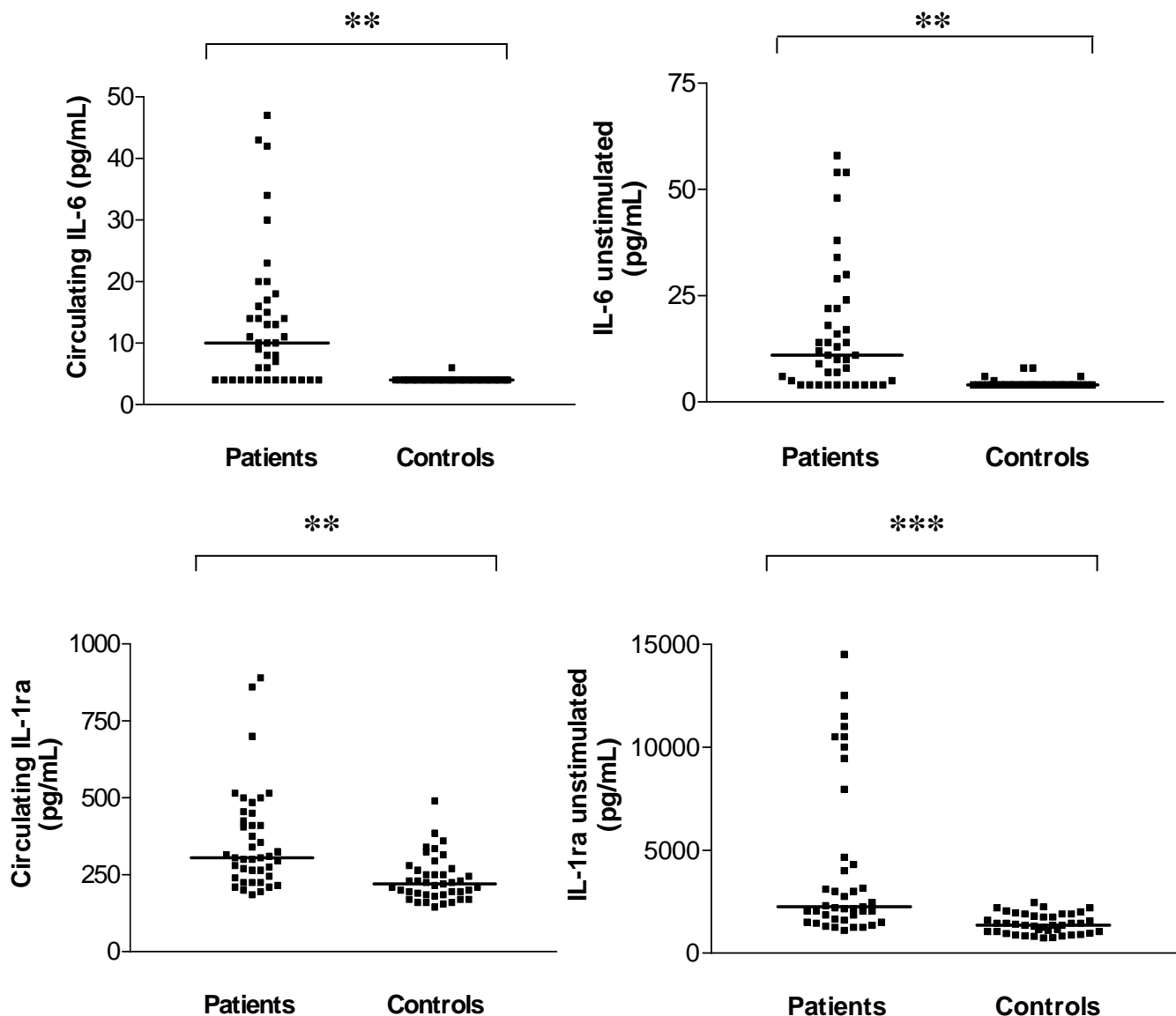


Figure 1. Distribution of circulating concentrations and unstimulated ex-vivo production of IL-6 and IL-1ra in patients with tuberculosis (n=41) and healthy controls (n=41). Each point represents one person. The median value for each group is indicated by a horizontal line. Significant differences were found between patients and controls (Mann-Whitney *U* test, ***: $p < 0.001$).

significantly higher than in controls ($p < 0.001$) (Fig 1), while LPS-stimulated cytokine production of IL-6 and IL-1ra were not significantly different from controls (data not shown). Circulating concentrations and ex-vivo production of IL-1 β and TNF- α were not significantly different between patients and controls (data not shown). As shown in Fig. 2, circulating concentrations of IL-6, as well as unstimulated production of IL-6 were higher in patients with pulmonary cavities (n=26), than in patients without cavities (n=15) ($p < 0.05$) and control subjects (n=41) ($p < 0.001$). A similar trend was seen for IL-6 in LPS stimulated blood samples (data not shown). Such differences were not found for IL-1 β , TNF- α and IL-1ra (data not shown).

In healthy control subjects, there was no association between nutritional status and concentration of any of the cytokines. There was no significant association between IL-1 β and nutritional status (data not shown). Concentrations of circulating IL-6 were associated with low BMI, anaemia, and zinc deficiency, and marginal plasma retinol

concentration was associated with ex-vivo LPS stimulated IL-6 production in patients (Fig 3). Low BMI was also associated with higher concentrations of TNF- α in patients (Table 2). Patients with anaemia had significantly higher circulating IL-1ra concentrations than non-anaemic patients. Also, in patients with zinc deficiency, LPS-stimulated production of TNF- α and IL-1ra was higher than in patients with normal plasma zinc concentrations (Table 2).

Discussion

In this carefully controlled study in Indonesia, we found elevated levels of IL-6 and IL-1ra in the circulation and in unstimulated whole blood cultures from tuberculosis patients. Increased cytokine production was associated with a low BMI and deficiencies of zinc, iron and retinol. In addition, the presence of pulmonary cavities was related to increased production of IL-6.

The problems of assessing micronutrient status during infection have made it difficult to determine whether

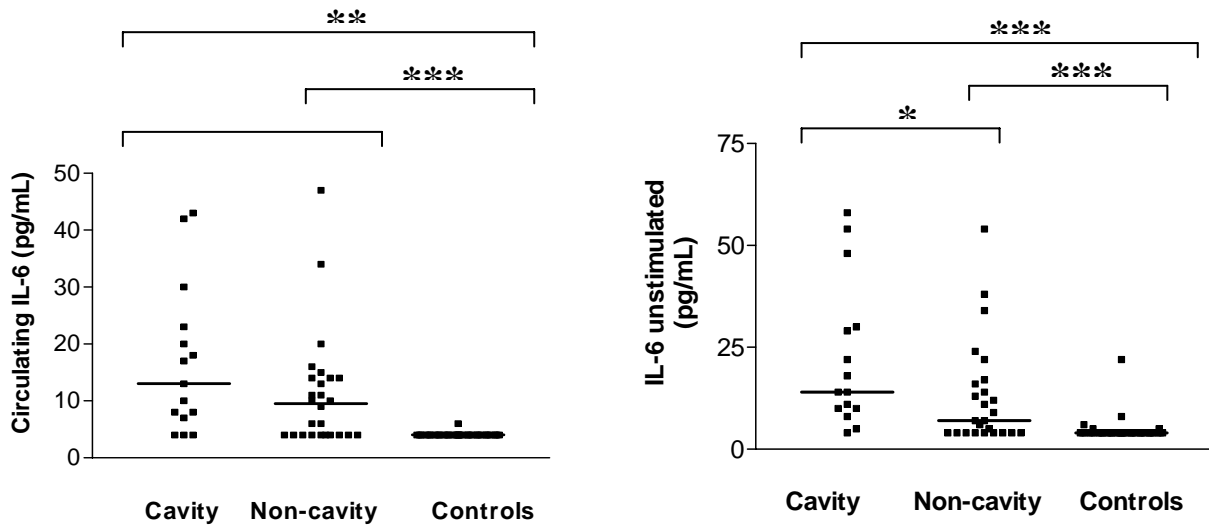


Figure 2. Distribution of circulating concentrations and unstimulated ex-vivo production of IL-6 in patients with cavitory (n=15) and non-cavitory (n=26), and control subjects (n=41). Each point represents one person. The median value for each group is indicated by a horizontal line. Significant differences were found between groups (Wilcoxon rank sum test, *: $p < 0.05$; ***: $p < 0.001$). f. female; m. male.

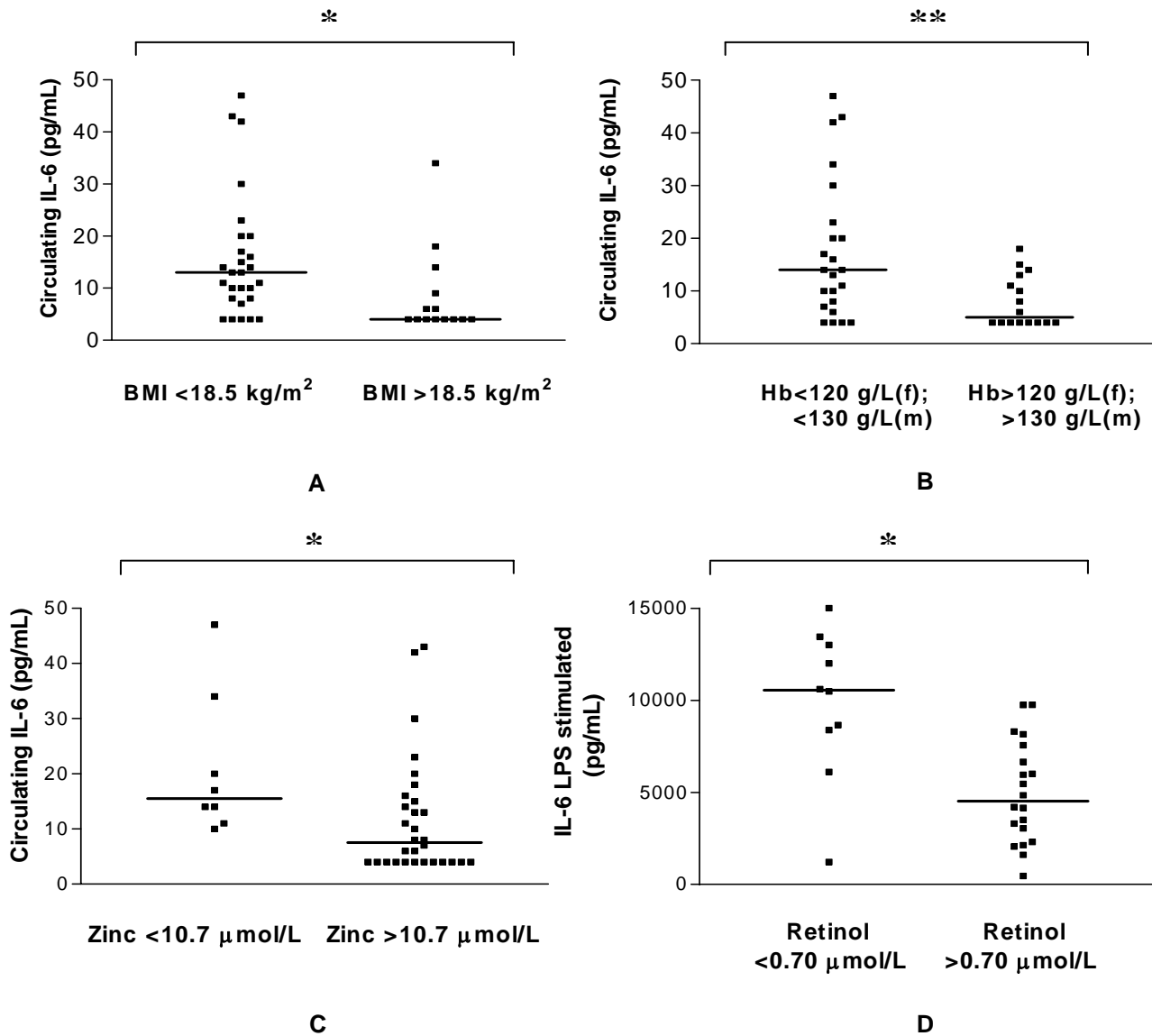


Figure 3. Distribution of circulating concentrations of IL-6 in patients with tuberculosis in relation to the nutritional parameters: (A) body mass index (BMI); (B) haemoglobin (Hb); (C) plasma zinc, and of concentrations of IL-6 after 24 h incubation with LPS in relation to the plasma retinol concentration (D). Each point represents one person. The median value for each group is indicated by a horizontal line. Significant differences were found between groups (Mann-Whitney U test, *: $p < 0.05$). f. female; m. male.

Table 2. Circulating concentrations and concentrations after 24 h incubation without LPS (unstimulated) and with LPS (LPS) of IL-6, TNF- α and IL-1ra related to nutritional status in pulmonary tuberculosis patients[†]

	n	IL-6 (pg/mL) [§]			TNF- α (pg/mL) [¶]			IL-1ra (pg/mL) [§]		
		Circulating	Unstimulated	LPS [‡]	Circulating	Unstimulated	LPS [‡]	Circulating	Unstimulated	LPS [‡]
BMI: <18.5 kg/m ²	27	13 * (8-20)	13 * (7-25)	6,960 (3,870-10,950)	124±20 *	328±150 *	4,310±2,990	340 (270-455)	2,150 (1,500-3,000)	12,000 (6,400-15,500)
≥18.5 kg/m ²	14	4 (4-10)	5 (4-15)	5,175 (3,060-9,590)	109±14	242±87	4,600±2,790	267 (221-346)	1,370 (1,250-2,050)	11,250 (3,900-13,250)
Haemoglobin: <120 ^f / ^m <130 ^m g/L	24	13 ** (7-22)	14 (8-30)	6,230 (3,110-11,650)	120±20	298±150	4,630±2,875	357 * (266-500)	2,100 (1,500-3,070)	12,000 (5,500-17,000)
>120 ^f / ^m >130 ^m g/L	17	4 (4-12)	6 (4-17)	6,150 (3,850-10,025)	117±17	280±115	4,130±2,995	280 (225-340)	1,500 (1,250-2,100)	10,500 (4,075-13,250)
Plasma retinol: <0.70 μ mol/L	10	14 (8-33)	13 (5-34)	10,540 * (7,810-13,110)	117±24	284±140	4,500±3,140	400 (258-538)	2,050 (1,185-3,000)	9,575 (5,625-16,625)
>0.70 μ mol/L	20	8 (4-15)	11 (5-24)	4,525 (2,485-8,000)	123±17	290±127	4,135±2,880	317 (251-443)	2,000 (1,460-3,140)	12,250 (3,940-15,125)
Plasma zinc: <10.7 μ mol/L	8	15 * (12-30)	14 (11-34)	9,450 (5,040-14,250)	123±22	294±131	6,595±2,215 *	285 (250-412)	1,650 (1,225-1,950)	16,500 * (12,250-19,500)
>10.7 μ mol/L	30	7 (4-15)	10 (4-23)	5,650 (2,670-10,110)	117±19	289±139	3,915±2,930	310 (225-451)	2,050 (1,290-2,820)	10,250 (3,710-13,125)

[†] Data are mean \pm SD or median (25th-75th percentiles).Significant differences between groups: *: $p < 0.05$; **: $p < 0.01$ (§: Mann-Whitney U test, ¶: Independent sample t test); f: female; m: male.[‡] LPS: lipopolysaccharide (LPS, *E. Coli* serotype 055:b5)

infections decrease micronutrient status itself in the long run. Many nutrients such as plasma retinol, zinc and iron are acute phase reactants, therefore, micronutrients show altered values during infections.²⁹ This may therefore hamper the interpretation of the differences which were found between patients and controls.

With regard to the assessment of cytokine production in patients, all established methods have limitations. In this study, we used a whole blood culture system for ex-vivo production of cytokines. Although this method is well established and attractive for clinical studies, it does suffer from variation.³⁰

The elevation of IL-6 in tuberculosis patients is moderate, especially when compared with the concentrations found in acute bacterial infections.³¹ Still, it could well be that the elevation of body temperature, the wasting and the elevation of C-reactive protein in tuberculosis are due to chronic elevation of this cytokine. An interesting finding in our study is that concentrations of IL-6, were higher in patients with pulmonary cavities, signifying more severe disease, than in patients without cavities. This is in agreement with the observation that tuberculosis patients who did not survive had higher circulating IL-6 levels than those who did.³² IL-6 is generally regarded as a cytokine that does not evoke tissue damage itself, in contrast to TNF- α for example. Production of IL-6 is, to a large extent, under the control of TNF- α and IL-1 and thus its concentration in blood may reflect local production of these cytokines in the lungs.

IL-1ra is a purely antiinflammatory cytokine, blocking IL-1 action at the receptor level, and excess of IL-1ra in the circulation is a common finding in disease.³³ Because IL-1ra production is under the control of IL-6, among other cytokines, the increased concentrations of IL-6 may have been responsible for those of IL-1ra. We did not find significant differences between patients and controls in concentration of the proinflammatory cytokines IL-1 β and TNF- α , but this is not remarkable, given the subacute nature of tuberculosis. However this finding does not preclude production of proinflammatory cytokines at the site of disease in the lungs, as has been established by others.^{34,35}

The primary aim of this study was to investigate the association between cytokine production and nutritional status. It seems that increased production of both pro- and antiinflammatory cytokines may contribute to the deficiencies of micronutrients in patients with tuberculosis. A low BMI was associated with higher concentrations of TNF- α and IL-6, which could fit in with the notion that both IL-6 and TNF- α induce anorexia and have catabolic properties at the level of muscle and fat tissue.³⁶

Micronutrient deficiencies were also associated with cytokine production. Tuberculosis patients with marginal plasma retinol concentrations exhibited higher production of IL-6. This would suggest that IL-6 production has an inhibitory effect on the hepatic synthesis of retinol-binding protein, thus suppressing the levels of plasma retinol-binding protein and plasma retinol. Alternatively, low concentrations of retinol and retinol-binding protein complex may be due to increased urinary excretion, as has been shown in shigellosis patients.³⁷ Anaemia was associated with higher concentrations of IL-6 and IL-1ra.

Anaemia of chronic disease seems to be a non-specific consequence of activation of the inflammatory cytokine network with simultaneous suppression of both erythropoietin production and the ability of erythroid progenitor cells to proliferate in response to the hormone.³⁸ Patients with low zinc concentrations showed increased concentrations of IL-6, both in plasma and whole blood cultures, and increased concentrations of TNF- α and IL-1ra in LPS-stimulated whole blood cultures. Proinflammatory cytokines may induce the production of metallothionein, a zinc-binding protein. Although metallothionein spills over into the plasma, the largest bulk of it remains in the liver, and this may lead to redistribution of zinc to the liver with a transient depression of plasma zinc levels.^{39,40} Alternatively, low plasma zinc concentration can also result from increased urinary zinc excretion as previously reported in patients with bladder cancer.⁴¹

So far, we have only been able to demonstrate associations between cytokines, nutrition and tuberculosis. Therefore, the biological significance of our findings remains to be established. To further elucidate pathogenic relationships, an intervention study is needed to investigate the effect of micronutrient supplementation on cytokine production and its effect on the clinical response to treatment of active tuberculosis.

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Original Article

Cytokines related to nutritional status in patients with untreated pulmonary tuberculosis in Indonesia

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印尼未治療肺結核病人細胞激素與營養狀況有關

雖然幾個研究已經探討過結核的細胞激素產生型態，但是結核病人的營養素缺乏與細胞激素間的關係所知有限。本研究目的為評估在結核病期間，細胞激素的濃度與營養狀況的相關。在印尼城市的醫院，41名未治療的結核病人與其配對的健康對照組，我們測量其身高與體重、鐵、維生素 A 及鋅；以及在循環中和全血培養產生的細胞激素濃度。病人的血漿中介白質-6(IL-6)及介白質-1接受體拮抗物(IL-1ra)顯著的較對照組高。有肺部空洞的病人(n=26)比起沒有空洞的病人(n=15)有較高的 IL-6 濃度。身體質量指數小於 18.5kg/m² 與較高濃度的腫瘤壞死因子- α (TNF- α)及 IL-6 有關。貧血者有較高的 IL-6 及 IL-1ra。鋅缺乏與高的 LPS-刺激產生 TNF- α 及 IL-1ra 有關。血漿視網醇濃度臨界缺乏與經 LPS 刺激後高濃度的 IL-6 相關。綜合以上，肺結核病人低微量營養素濃度與細胞激素產生增加有關。介入性研究將可評估其因果關係。

關鍵字：介白質-6、腫瘤壞死因子- α 、介白質-1 接受體拮抗物、身體質量指數、微量營養素。