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Deficiency in Fat-soluble Vitamins A, D, E in Patients with Pulmonary Multidrug-Resistant Tuberculosis

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ABSTRACT

Objective: The treatment management of Multidrug-Resistant Tuberculosis (MDR-TB) is a major global public health problem. The development of this form of tuberculosis increases immune deficiency and the production of free radicals in the body. Micronutrients, especially fat-soluble vitamins A, D, and E, play an essential role in the immune system by protecting and renewing cells. The objective of this study is to determine the profiles of vitamins A, D, and E in order to evaluate the performance of the immune defenses of MDR-TB under second-line anti-TB treatment.

Methods: The analysis of vitamins A, D and E was carried out using an HPLC chain, in isocratic mode by UV-Visible detection after prior extraction of the lipid fraction from the serum in the hexane protected away from light.

Results: MDR-TB showed a significant decrease in the concentration of vitamins A, D, and E ($p<0.05$) with high reduction levels of 80%, 40% and 50%, respectively.

Conclusion: The persistence of this deficit after six months of TB treatment highlights the need for corrective measures to be taken, such as the supplementation of vitamins A, D, and E. *J Microbiol Infect Dis 2020; 10(4): 199-207.*

Keywords: *Micronutrients, Multidrug-resistant, Tuberculosis, HPLC, Côte d'Ivoire*

INTRODUCTION

Multidrug-Resistant Tuberculosis (MDR-TB) is a life-threatening infectious disease caused by mycobacterium tuberculosis, which is resistant to at least the two major anti-tuberculosis drugs of the first-line treatment, Isoniazid and Rifampicin [1]. MDR-TB is a real threat to the eradication of tuberculosis [2]. About 480,000 people suffered from MDR-TB with 190,000 deaths worldwide [3]. The prevalence of MDR-TB in Africa and Côte d'Ivoire is 14% [4] and 2.5%, respectively [5]. Encountering new resistance and high rates of treatment failure when taking second-line anti-tuberculosis drugs make medical treatment management difficult. This situation severely threatens the Sustainable Development Goals (SDGs) in its efforts to eradicate TB in all its forms by 2030 [6]. In fact, in 2010, the rate of treatment failures was more than 50%; in 2012, about 9.6% of these MDR-TB evolved into a form of Extensive Drug Resistant Tuberculosis (XDR-TB) [3].

MDR-TB is characterized by a reduction in macrophage phagocytic activities, a decrease in lymphocytes proliferation and a reduction in erythropoiesis [7,8]. Additionally, the increased oxidative stress caused by mycobacterial infection during the inflammatory process contributes to the deterioration of the immune cells [9].

Another characteristic of mycobacterium tuberculosis infection, the causative agent of tuberculosis, is the differentiation of infected macrophages into foamy cells rich in lipids [10]. These cells accumulate lipid droplets, lipid storage that are necessary for intracellular bacillary growth [11] probably by their ability to provide mycobacteria with micronutrients (vitamins and trace elements) that are essential

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for the proper functioning of the human body [10].

The antioxidant and immunostimulant properties of vitamins A, D and E have been the subject of several studies in recent years. Vitamins A and D are essential for the normalization of lymphocytes B and T cell functions, antibody production and macrophage activation [12, 12]. Vitamin E increases lymphocyte proliferation and phagocytic activity of macrophage cells. It also protects the integrity of immune cells against free radical aggression [13]. Therefore, supplementation with these micronutrients in MDR-TB cases taking TB treatment may help to selectively strengthen important parts of their immune defenses [14]. Given the importance of these fat-soluble vitamins in the fight against tuberculosis, studying the relationship between micronutrients and MDR-TB is vital to improve the medical treatment management of the MDR-TB patients.

Therefore, this study therefore is a preliminary study with the aim of contributing to the better management of patients with MDR-TB. The overall goal was to evaluate the disorder of fat-soluble vitamins A, D and E in patients with MDR-TB.

METHODS

Study Design, Site and Study Population

This is an experimental study carried out on MDR-TB patients from the pneumology section of the University Teaching Hospital (UTH) in Cocody and in the five anti-tuberculosis centers (ATC) in the city of Abidjan (Abobo, Adjamé, Koumassi, Port-Bouet and Yopougon). Informed consent was obtained from patients for the use of their blood.

This study was conducted from January 2014 to December 2015, at the Institut Pasteur of Côte d'Ivoire (IPCI) which is one of the biological monitoring centers for MDR-TB patients.

The samples included in this study were taken from 100 patients with MDR-TB after confirmation of resistance to both first-line anti-TB drugs (Isoniazid and Rifampicin) through microscopic and molecular analyses performed by the National TB Reference Laboratory, according to the WHO recommended tests (Gene Xpert) [15,16].

Samples from children, pregnant women and subjects with sensitive TB and HIV positive were excluded from this study.

In the same site, 100 non-tuberculosis samples were collected, which showed no clinico-biological signs of active tuberculosis and who voluntarily accepted to give consent for the use of their blood for research purpose in this study to serve as control subjects. Patients and controls were divided equally into 50 men and 50 women. The age range of all participants is between 18 and 50 years. However, samples from the controls who did not give their consent to participate in this study, samples from children, pregnant women and patients with sensitive tuberculosis were not included in the study.

Collection of samples and reagents

The biological material consisted of the whole blood of MDR-TB patients being treated and non-tuberculous voluntary controls taken in a dry tube without anticoagulant. Sampling was performed one time only in control subjects and at different stages (M0, M3 and M6) of treatment follow-up in MDR-TB patients.

A total of 300 MDR-TB samples and 100 control samples were collected and used for this study. The samples were centrifuged at 3000 rpm for five minutes using a Horizon 642 VES centrifuge manufactured by The Drucker Co., USA. The sera were collected in Eppendorf® tubes and stored at -20ºC until the performance of micronutrient analysis.

Reference standard solutions (1 g/L vitamin A, 1 g/L vitamin D and 10 g/L vitamin E) and an internal standard (retinyl acetate, 1 g/L) were used to prepare different diluted concentrations from which calibration curves were plotted.

Determination of vitamins A, D and E by HPLC

The analysis of vitamins A, D and E was carried out using a Waters® HPLC chain, USA, in isocratic mode by UV-Visible detection after prior extraction of the lipid fraction from the serum in the hexane protected away from light. The extraction and the determination of the vitamin concentrations were carried out as follows: In a hemolysis tube containing 300 µL of serum, 300 µL of retinyl acetate (internal standard) prepared at 1 mg / L [17] and 300 µL

of ethanol were added successively and the mixture was vortexed for 20 seconds. After the homogenization of the mixture, 1200 µL of hexane (extraction solvent) was added to the mixture and the whole was again vortexed twice for 30 seconds successively and then centrifuged at 3500 rpm for 15 minutes. Then, 900 µ L of the supernatant (hexane phase) was recovered and passed through nitrogen gas (pressure: 0.5 bar), to evaporate the solvent. Three hundred microliters (300 µL) of methanol (elution solvent) was added to the residue obtained after evaporation. The mixture, obtained by stirring gently, was the extract used for the quantitative analysis of vitamins. Twenty microliters (20 µL) of the mixture (residue methanol) was injected automatically into the injection loop of the C18 column in the chromatographic system. The peaks corresponding to the different vitamins A, D and E with retention times of 3.84 minutes, 7.29 minutes and 8.23 minutes are presented on the chromatogram displayed by the recording integrator. Concentrations of vitamins are determined from the peak areas. The chromatographic conditions are as follows: A reverse phase C18 column by Waters Spherisorb® ODS2 was used as a stationary phase. It is preceded by a precolumn adapted to columns C18. The mobile phase consisted of a mixture of methanol/water (97/3, v/v) at a flow rate of 1 mL/min with an injection volume of 20 µL. The simultaneous detection of the three vitamins (A, D and E) was carried out at UV-Visible at the wavelength of 180 nm, and the analysis of the chromatographic data was done using Breeze 2. Moreover, the detection limits for vitamins A, D and E are 0.005 mg/L, 0.006 mg/L and 0.087 mg/L respectively. Serum reference values are 0.35–1.75 µmol/L for vitamin A; 60–105 nmol/L for vitamin D and 18–29 µmol/L for vitamin E [18].

The reduction percentages of vitamins are calculated by this formula:

Reduction (%) = [(Mean of Controls - Mean MDR-TB) / Mean of Controls] x 100

Statistical analysis

The mean values with the standard error on mean (mean ± SEM) of the data were calculated using the Graph Pad Prism 5.0 software (Microsoft, USA). The statistical analysis of the results was performed using the ANOVA, followed by the Tukey Multiple Comparison Test. The difference is significant when p-value <0.05.

RESULTS

The results showed a significant decrease in the concentrations of vitamins A, D and E in men and women with MDR-TB, both at baseline (M0) and during treatment follow-ups (M3, M6) compared to non-tuberculous controls (p< 0, 05) (Figure 1 and Table 1). This decrease is revealed by percentages reduction of vitamin concentrations above 80%, 40% and 50% respectively for the vitamins A, D and E in MDR-TB subjects. It should be noted that these reductions in vitamin concentrations are observed in all stages of MDR-TB treatment follow-up (Figure 2).

Therefore, these results showed no significant variation in the concentrations of vitamins A and E during second-line TB treatment (Table I).

The concentration of vitamin D has increased significantly (p< 0.05) compared to the baseline stage (Table 1).

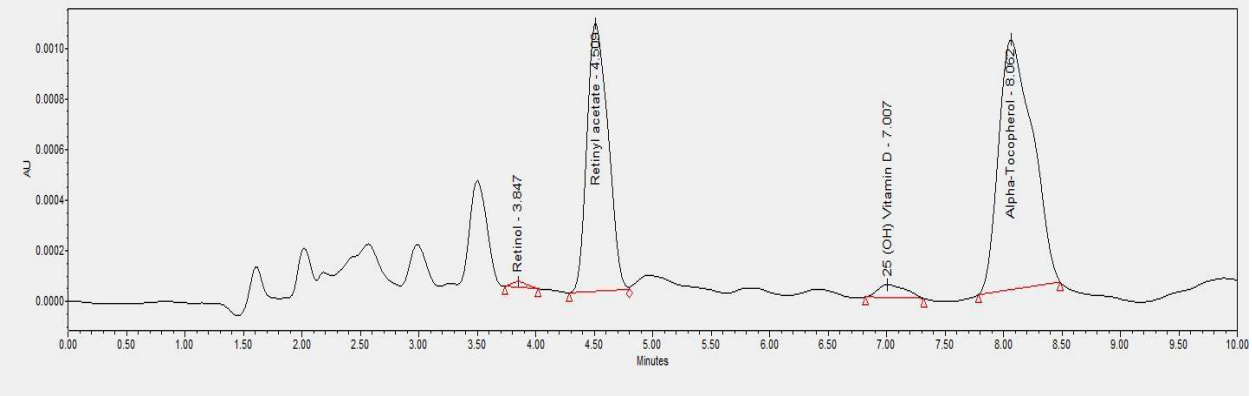
However, the concentration of vitamin A in non-tuberculosis controls subjects, although within the range of normal values, is significantly lower than the average of usual values (p <0.05).

A comparative analysis of the concentrations of vitamins A, D and E according to sex in the MDR-TB sample (Table II) shows significant decrease in women's concentrations of vitamins A, E at all stages M0, M3, M6, and vitamin D at the M0 stage.

DISCUSSION

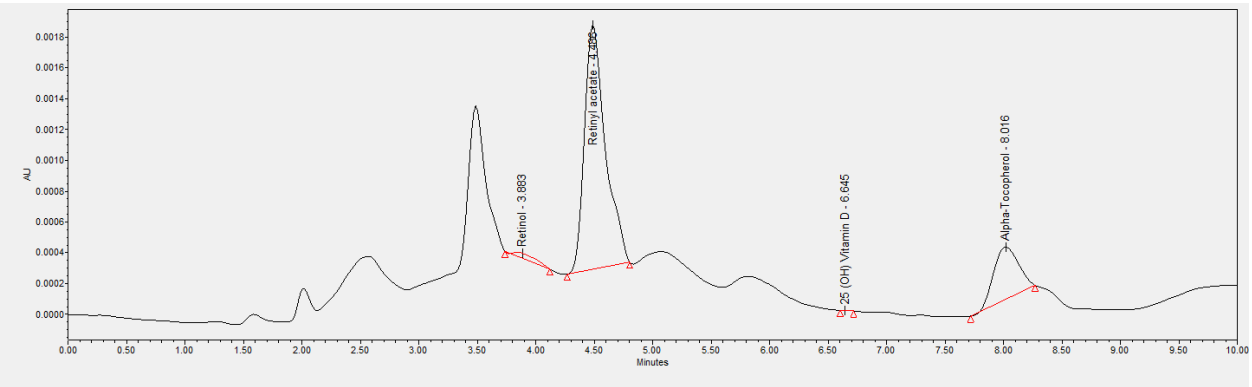
In this study, low concentrations of fat-soluble vitamins A, D and E are observed in MDR-TB sample compared to non-tuberculosis control. This could be explained due to malnutrition [19] and inflammation. In fact, acute and chronic inflammation leads to malabsorption of lipid fractions containing fat-soluble vitamins [20]. Similar vitamin concentration levels have been reported in previous studies in patients with active tuberculosis, particularly in India [21] and Nigeria [22]. Other more specific mechanisms related to the metabolism of each fat-soluble vitamin. Thus, the decrease in serum vitamin A concentrations in MDR-TB may be due to an increase in the urinary excretion of this vitamin

and a reduction in the hepatic production of its transport to serum, particularly the retinol-binding protein (RBP). This protein is necessary for the plasma mobilization of vitamin A (Retinol) stored in the liver [23]. Furthermore, zinc deficiency and high levels of oxidative stress lead to a high production of oxygen and nitrogen reactive species during mycobacterial infection [21,24].



	Molecules	Retention Time	Area	% Area	Height	Amount	Units
1	Retinol	3.847	195	0.57	22	0.600	mg/L
2	Retinyl acetate	4.509	13237	38.68	1064	9.000	mg/L
3	25 (OH) Vitamin D	7.007	855	2.50	52	1.000	mg/L
4	Alpha-Tocopherol	8.062	19937	58.25	988	20.000	mg/L

Figure 1a. Non-tuberculous controls: Normal profile of vitamins A, D and E in non-tuberculous controls.



	Molecules	Retention Time	Area	% Area	Height	Amount	Units
1	Retinol	3.883	455	1.81	33	0.468	mg/L
2	Retinyl acetate	4.486	19704	78.24	1589	10.471	mg/L
3	25 (OH) Vitamin D	6.645	4	0.01	1	0.000	mg/L
4	Alpha-Tocopherol	8.016	5021	19.94	340	3.789	mg/L

Figure 1b. MDR-TB patients: Abnormal profile of vitamins A, D and E in MDR-TB patients.

Table 1. Levels of vitamins A, D and E in MDR-TB patients and non-tuberculous controls.

Variables		Control	MDR-TB M_0	MDR-TB M_3	MDR-TB M_6
Male	Vitamin A (Ref.: 0.35–1.75 µmol/L)	0.50 ± 0.21	0.10 ± 0.02*	0.14 ± 0.02*	0.15 ± 0.14*
	Vitamin D (Ref.: 60–105 nmol/L)	108 ± 4.8	13 ± 2.60*º	26 ± 5.20*	57 ± 2.60*
	Vitamin E (Ref.: 18–29 µmol/L)	24 ± 5.38	3 ± 0.90*	6 ± 1.47*	7 ± 3.68*
Female	Vitamin A (Ref.:0.35–1.75 µmol/L)	0.44 ± 0.19	0.08 ± 0.04*	0.12 ± 0.08*	0.14 ± 0.17*
	Vitamin D (Ref.: 60–105 nmol/L)	105 ± 3.40	5 ± 2.60*º	26 ± 5.80*	54 ± 2.60*
	Vitamin E (Ref.: 18–29 µmol/L)	30 ± 2.87	5 ± 1.71*	5 ± 1.35*	10 ± 3.48*

M_0: baseline value; M_3: Value at three months of treatment follow-up; M_6: Value at six months of treatment follow-up
*: Significant difference between MDR-TB and non-tuberculous controls, P <0.05
º: Significant difference between different stages of follow-up, P <0.05

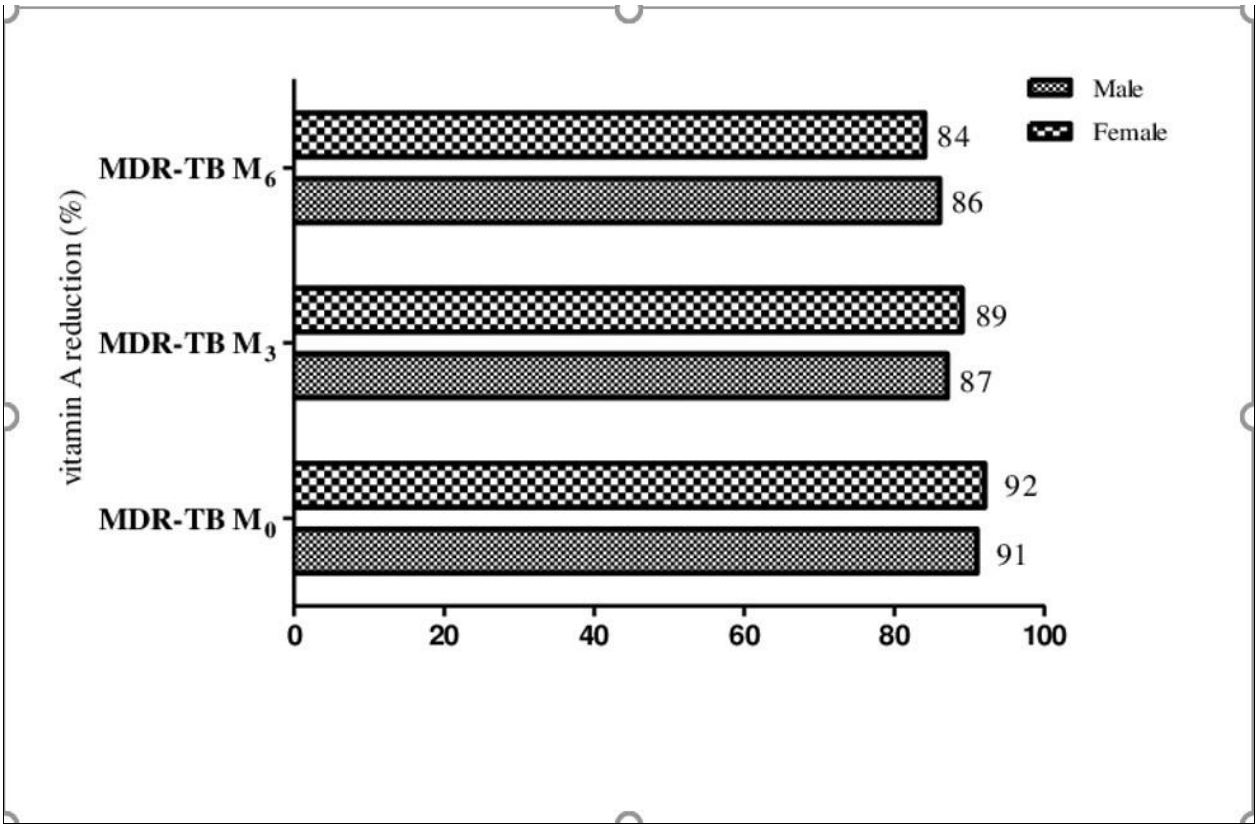


Figure 2a. Vitamin A.

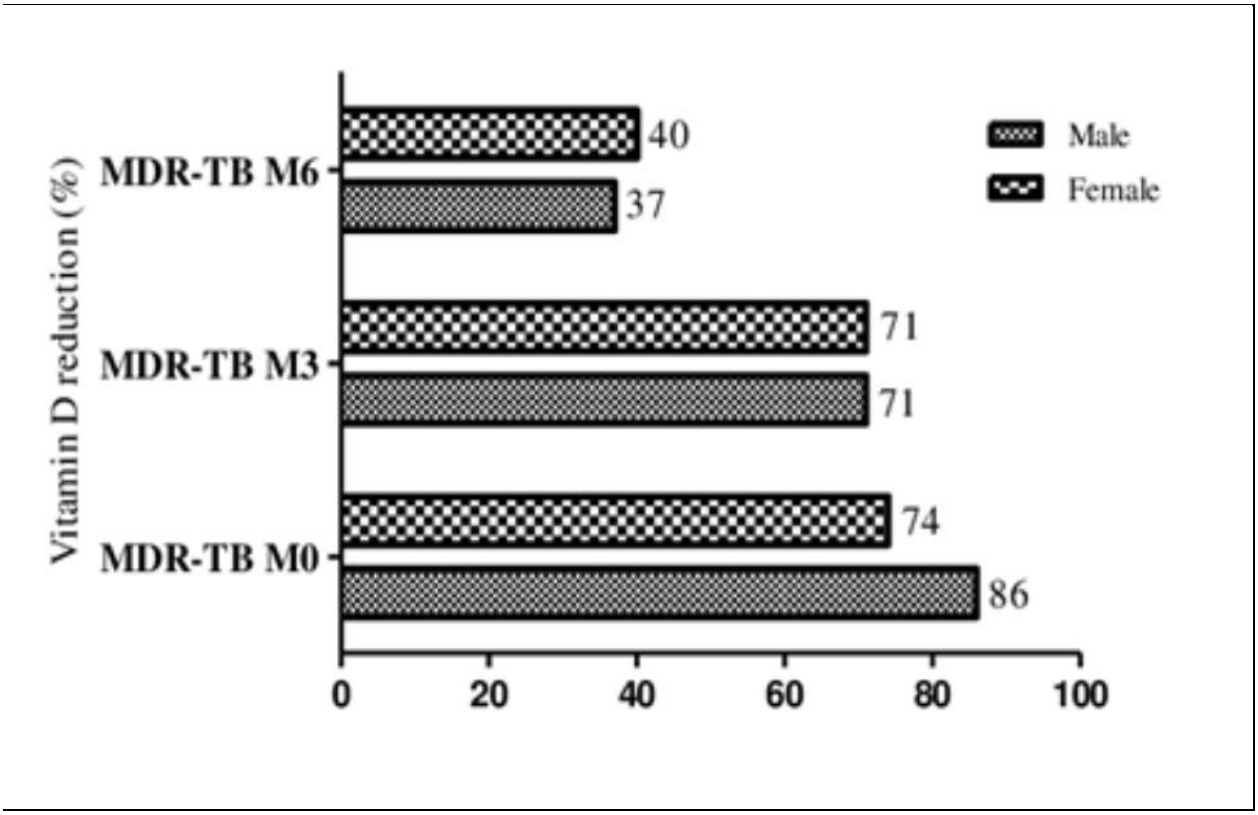


Figure 2b. Vitamin D.

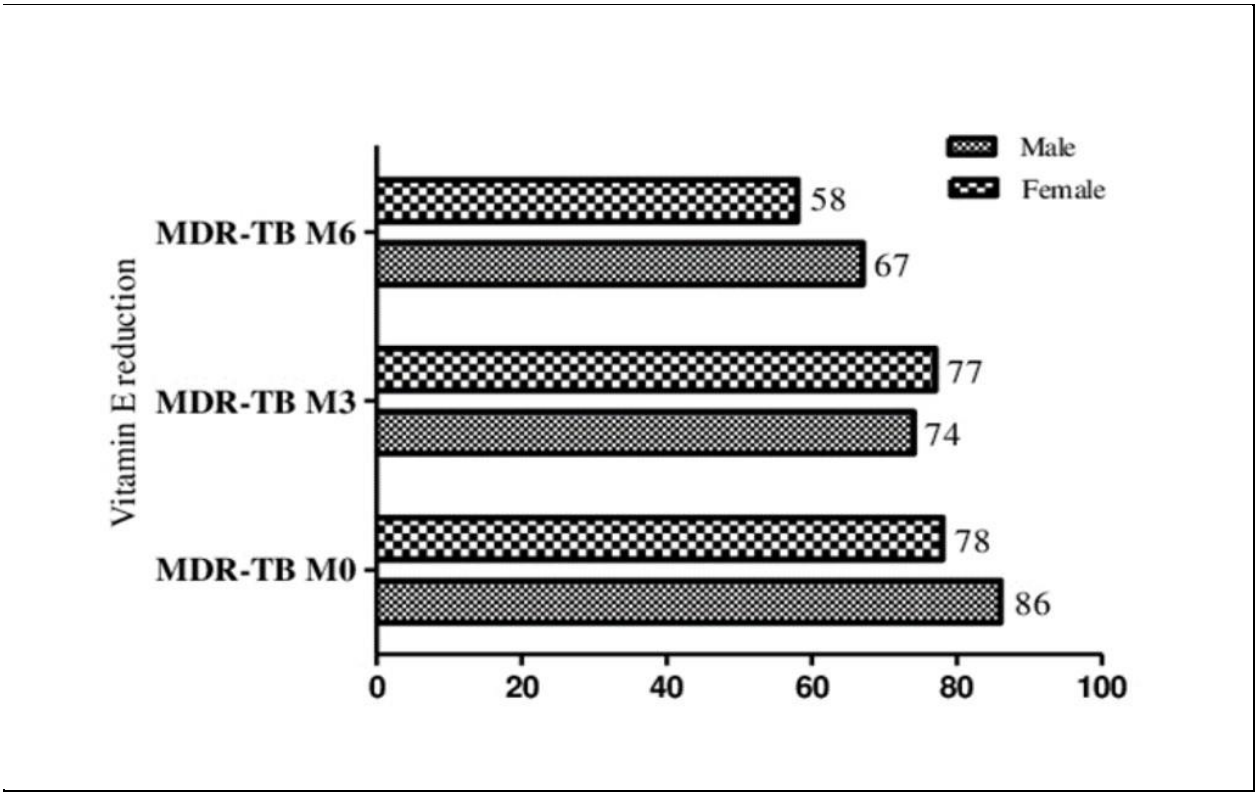


Figure 2c. Vitamin E

Figure 2: Percentages of vitamins A, D and E reduction in MDR-TB patients

Table 2. Comparative analysis of vitamin concentrations in MDR-TB according to sex.

Vitamins	Gender	MDR-TB M0	MDR-TB M3	MDR-TB M_6
Vit A (µmol/L)	Male	0.10 ± 0.017	0.14 ± 0.024	0.15 ± 0.140
	Female	0.08 ± 0.038	0.12 ± 0.084*	0.14 ± 0.174
	p-Value	P = 0.002	P = 0.001	P = 0.003
Vit D (nmol/L)	Male	13 ± 2.599	26 ± 5.198	57 ± 2.599
	Female	5 ± 2.599	26 ± 5.797	56 ± 2.599
	p-Value	P = 0.009	P < 0.927	P = 0.125
Vit E (µmol/L)	Male	3 ± 0.901	6 ± 1.467	8 ± 3.676
	Female	5 ± 1.711	6 ± 1.346	19 ± 3.483
	p-Value	p = 0.030	p = 0.002	p = 0.008

M0= initial baseline stage; M3= at three months of treatment follow-up; M6= at six months of treatment follow-up

The lower vitamin D concentrations could lead to insufficient nutrient intake, due to a lack of cutaneous synthesis of vitamin D3 and a reduction in the synthesis of its plasma transporter [25, 26]. The most possible explanation would be an abnormality of the vitamin D receptor (VDR). Variations in the polymorphism of the gene encoding vitamin D Binding Protein (VDBP) would result in a reduction of this protein in the plasma transporting vitamin D to the liver [27]. The increase in phosphatemia associated with a reduction in serum calcium and normal creatinemia would exclude the theory of renal failure and would be corroborated with that of hypoparathyroidism secondary to hypovitaminosis D observed in MDR-TB [28].

Vitamin E deficiency is mainly due to the high levels of oxidative stress caused by the overproduction of free radicals of oxygen and nitrogen during bacterial infection [29].

The deficiency of fat-soluble vitamins in MDR-TB patients is more pronounced at the initial baseline stage (M0) before commencement of treatment compared to treatment follow-up stages (M3 and M6). These results are in an agreement with those of Edem et al. [2] who showed an improvement in micro-nutritional status in MDR-TB at four and six months of second-line anti-tuberculosis treatment respectively. Low plasma levels of these fat-soluble vitamins may be one of the main causes of immune deficiency leading to therapeutic failure in MDR-TB patients [13,20, 23].

CONCLUSION

The persistence of these low concentrations of fat-soluble vitamins A, D and E after six months of the intensive phase of second-line TB treatment requires corrective measures during antibiotic therapy. Supplementation with certain essential micronutrients, especially vitamins A, D and E, would stimulate the immune system and restore the balance between the production of free radicals and the antioxidant capacity of these MDR-TB patients. However, additional studies would be needed to better understand the micro-nutritional status of these MDR-TB subjects. More specifically, the serum concentrations of RBP and albumin, which are important in the plasma transportation of vitamin

A and correlate with serum retinol concentrations, need to be determined. It would also be necessary to evaluate the concentrations and genetic profile of VDBP with the susceptibility of developing active tuberculosis in African populations.

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Ethics approval: This study was approved by the National Committee on Ethics and Research of Côte d'Ivoire (NCER). The approval and informed consent were obtained from MDR-TB patients and control participants for the use of their blood for research purpose.

Declaration of Conflicting Interests: The authors declare that they have no conflict of interest.

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