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Lysine and threonine plasma concentrations in Ivorian patients living with human immunodeficiency virus

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Côte d'Ivoire is one of the most affected countries in West Africa with HIV/AIDS, with a prevalence of 3.4%. Essential amino acids are needed by the organism as they play key roles in the immune system and they are supplied through diet. The objective of this study was to determine the plasma lysine and threonine status for better medical and nutritional management of patients living with HIV. This study involved 254 individuals: 127 HIV positive and 127 HIV negative (serving as controls) after confirmation of their HIV status through an HIV test (test DETERMINE® and GENIE II). Lysine and threonine were assayed using high performance liquid chromatography (HPLC) on plasma and CD4 lymphocyte count by the method of flow cytometry (FacsCalibur) from whole blood containing EDTA. This study showed that deficiency of lysine was more observed in male HIV infected individuals (66.7%) and threonine deficiency in female HIV infected individuals (17.1%) as compared to the controls subject. The amino acid concentrations as a measure of the degree of immunosuppression was significant for lysine (P = 0.0006) and not significant for threonine (P = 0.8640). The deficiency observed in HIV infected patients taking antiretrovirals is therefore probably due to viral infection and insufficient lysine intake in diet. The threonine concentration depends on the health condition of the subject.

Key words: Amino acids, essential, Côte d'Ivoire, HIV-infected patients, lysine, threonine.

INTRODUCTION

Human immunodeficiency virus (HIV) infection is a major public health problem (Alqudah et al., 2016). During HIV infection, activation of immune cells causes an increase in the body requirements of specific amino acid, and affected immune cells require an exogenous supply of certain amino acids (McGaha et al., 2012). In actual fact, HIV infection causes hypermetabolism of proteins and amino acids in the muscles, liver and adipose tissues in patients taking antiretroviral therapy (Zou and Berglund, 2007).

Lysine is an essential amino acid that helps build the body immune system by contributing to the development of antibodies; it has antiviral properties (Lukasheva and Berezov, 2002). A plasma lysine requirements increases in cases of acute infectious diseases which are common in many developing countries (Ghosh et al., 2010).

Furthermore, lysine deficiency leads to active HIV replication (Butorov, 2013).

Threonine is a key nutrient to the intestine. In the intestine, this amino acid plays a major role in the synthesis of mucin, a glycoprotein required for the protection of the intestinal epithelium (Ruth and Field, 2013). Like mucins, immunoglobulins are globular glycoproteins rich in threonine. Due to the high threonine concentration in immunoglobulins, the deficiency of threonine may affect the production of immunoglobulins (Richard and Galanaud, 1995). During HIV infection, nutritional care and support are very important to prevent the development of nutritional deficiencies and therefore, improve the quality of life of people living with HIV (WHO, 2002).

Few clinical data exist on plasma lysine and threonine profiles in HIV-infected patients (Butorov, 2013). In Côte d'Ivoire, the status of essential amino acids, particularly lysine and threonine in patients living with HIV, is not documented. For better medical and nutritional care for people living with HIV, it is therefore necessary to evaluate the levels of lysine and threonine, the essential nutrients for proper functioning of the living organism.

The objective of this study was to determine the plasma lysine and threonine concentrations and analyze their relationship with the degree of immunosuppression in people living with HIV/AIDS taking antiretrovirals in Côte d'Ivoire.

MATERIALS AND METHODS

Period and study design

The study was carried out in the Clinical and Fundamental Biochemistry Department of the Institut Pasteur of Côte d'Ivoire from December 2014 to November 2016. It was a cross-sectional descriptive study involving a cohort of individuals from whom blood samples were collected. The rapid tests Determine™ HIV-1/2 and *GENIE II* HIV-1/HIV-2 were used for selection of the individuals.

Blood sample collection

Blood samples from HIV positive and negative subjects were required for the various biochemical and serological analyses. Therefore, two blood tubes containing EDTA (Termo, Tokyo, Japan) were used to determine plasma concentrations of essential amino acids (Maeda et al., 2010) and CD4+ T cell count (blood total) in HIV positive subjects, respectively. A tube of blood without anticoagulant (dry tubes) was used to carry out HIV serological tests and biochemical parameters assay. Finally, blood glucose was determined on serum from a tube containing potassium oxalate and sodium fluoride. Blood samples from pregnant women and children, regardless of their serology status were not included in this study.

HIV test and CD4+ T lymphocytes count

For the detection of antibodies anti-HIV, two rapid tests were performed. They were the "Alere Determine™ HIV-1/2 kit" (Alere Medical Co., Japan) which is an immunochromatographic test

based on the principle of formation of an antigen-antibody complex revealed after staining (Tang et al., 2008) and the GENIE II HIV-1/HIV-2 kit (BIO-RAD, France) which is a confirmatory immunoenzymatic assay. The principle of this test is based on the detection of anti-HIV1 and anti-HIV2 antibodies directed specifically against antigens (Ouassa et al., 2007; Laforgerie et al., 2010).

The CD4 T lymphocyte count was performed using flow cytometry system on the automated FacsCalibur from the whole blood taken from EDTA tube. The principle is based on the quick analysis of moving particles (cells) in a single file in front of a laser beam (Ormerod and Imrie, 1990).

Indeed, the CD4+ T lymphocyte count was determined by pouring a volume of 20 µL of Tri TEST (CD3, CD4 and CD45) in each Trucount tubes. A volume of 50 µL of whole blood and a lysis solution diluted by 1:10 at a rate of 500 µL was added to each tube. The tubes were then homogenized and incubated for 15 min in the dark. This operation (homogenization and incubating the tubes in the dark) was repeated a second time; then the Trucount tubes were placed on the rack of FacsCalibur device after third homogenization. The mixture was drawn into the flow cytometric counter and the result is shown electronically.

The normal reference values for CD4 count according to WHO are: 600 to 1750 cells/mm^3 (31 to 60%). All HIV-1 infected patients were classified into four groups according to their CD4+ T cells count: no significant immunodeficiency (≥ 500 cells/mm^3), average immunodeficiency (350 to 499 cells/mm^3), advanced immuno-deficiency (200 to 349 cells/mm^3) and severe immunodeficiency (< 200 cells/mm^3) (Schneider et al., 2008).

Biochemical analysis

The serum concentrations of creatinine, urea, glycaemia, alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) were measured on the COBAS INTEGRA 400 plus controller. It is a spectrophotometer based on the reaction of TRINDER which is an enzyme, and colorimetric method which uses a chromogen whose colour intensity developed is directly proportional to the concentration of the measured substance, was used (Deyhimi et al., 2006).

Plasma lysine concentration and threonine were determined by high performance liquid chromatography (HPLC) according to the method of Teerlink et al. (1994).

Stock solutions of 1000 µmol/L of lysine and threonine as well as the internal standard (L-Norvaline) were prepared in acidified methanol (0.1% HCl). For each amino acid, successive dilutions were made from these solutions to obtain the following concentrations: 5, 10, 25, 50, 100, 200 and 400 µmol/L. These concentrations were selected according to the limits of detection (LOD) and quantification (LOQ), and the physiological concentrations of lysine and threonine in blood plasma. For this analysis, the detection limits of lysine and threonine are respectively 8 and 44 fmol/L. The quantification limits of lysine and threonine are 27 and 146 fmol/L, respectively. A calibration line is made using the entered values for the concentrations of amino acid in the standard. For each of the analysts, the concentrations in the "unknown" samples are calculated separately.

After thawing at room temperature, 200 µL of plasma was introduced into a tube containing an equal volume (200 µL) of acidified methanol (0.1% HCl). The mixture obtained was centrifuged at 3000 rpm for 15 min, and then the collected supernatant was filtered on millipore of 0.45 µm in diameter. 15 µL of internal standard L-Norvaline (400 µmol/L), an equal volume (15 µL) of sample filtrate (plasma, standards or mixed standards) and 60 µL of MilliQ water were added to 270 µL of derivatization reagent (OPA / 3-MPA, pH 10.0). The solution obtained after homogenization was incubated in the Autosampler for 3 min before the injection.

The analysis was carried out with an elution rate of 1 mL/min, a wavelength of 340 nm (excitation) and 455 nm (emission), an analysis time of 25 min, an analysis temperature of 37°C and a gain of 2. The injection volume was fixed at 10 µL of the sample, the standard or the mixed standards from the HPLC injection loop (Waters®, France). The NUCLEOSIL® 100 Å column (150 mm × 4.6 µm x 5 µm particle size) was cleaned with 100% of the mobile phase B (acetonitrile/water v/v) and rebalanced with 100% of the mobile phase A (potassium dihydrogen phosphate buffer, 30 mM, pH 7.0) between two injections. Normal reference values for lysine and threonine are 107 to 244 µmol/L and 74 to 175 µmol/L, respectively (Teerlink et al., 1994).

Statistical analysis

Statistical analyses were performed using Student's t-test for comparison of averages. The correlation between essential amino acid concentrations and CD4$^+$ T lymphocytes count was determined by the Pearson test. A P < 0.05 value was considered to be statistically significant.

Ethical considerations

The study was conducted in accordance with the Helsinki Declaration 2000 on HIV and AIDS research conducted in poor countries and in accordance with the local legislation regarding the national program on treatment management for People Living with HIV/AIDS (Decree No. 411 of December 23, 2001). The blood samples were collected from HIV-positive patients monitored at the Institut Pasteur of Côte d'Ivoire (IPCI), a reference center for public health programs in Côte d'Ivoire supported by the global fund for HIV/AIDS/malaria/tuberculosis. However, for research purposes, written consent was obtained from patients for the use of their blood samples taken during biological monitoring.

RESULTS

Background characteristics

This study involved 254 blood samples from 127 HIV-negative and 127 positive individuals. Their ages varied between 26 and 49 years and the average age of infected subjects was 37 ± 0.52 years against 32 ± 0.50 years for the controls. In addition, the HIV serological subtype found was 100% HIV1. Among these 127 HIV positive individuals, there were 70 women and 57 men on antiretroviral therapy (zidovudine + lamivudine + nevirapine).

Determination of biochemical parameters and analysis of amino acid concentrations

Biochemical parameters

The mean values of the biochemical parameters in the HIV-infected and the control samples analyzed were within the normal standard reference values [blood glucose: 4.16 to 6.11 mmol/L; creatinine: 53 to 106 µmol/L; urea: 1.66 to 5.83 mmol/L and transaminases

(ASAT): 8 to 49 IU/L; ALAT: 7 to 48 IU/L] and no significant difference was observed in the control samples.

Analysis of the plasma lysine and threonine profile

This study showed a significant increase (P = 0.0040) in lysine levels (148 ± 16.32 vs. 92 ± 6.14 µmol/L) and a non-significant decrease (P = 0.6124) in the threonine levels (324 ± 42.00 vs. 357 ± 48.06 µmol/L) in HIV positive subjects as compared to the controls subject, respectively. However, threonine levels were higher in the two groups of subjects as compared to normal standard values (control versus infected subject: 357 vs. 324 µmol/L).

Relating to sex: Regarding deficiency, 66.7% (38/57) male HIV-positive had deficiency of lysine as compared to threonine; 0.0% (0/57 HIV-positive) (Figure 1a). On the other hand, in female PLHIV, 37.2% (26/70) had lysine deficiency as compared to threonine; 17.1% (12/70) of PLHIV (Figure 1b).

Regarding excess amino acids, 74.1% (43/57) HIV-positive men had excess threonine and 0.0% (0/57) had excess lysine (Figure 1a). On the other hand, 50.0% (35/70) female HIV-positive have excess threonine and 12.8% have excess lysine (9/70) (Figure 1b). However, excess threonine was 100% in the male control subjects and 50.0% in women against total absence of excess in lysine in both sexes.
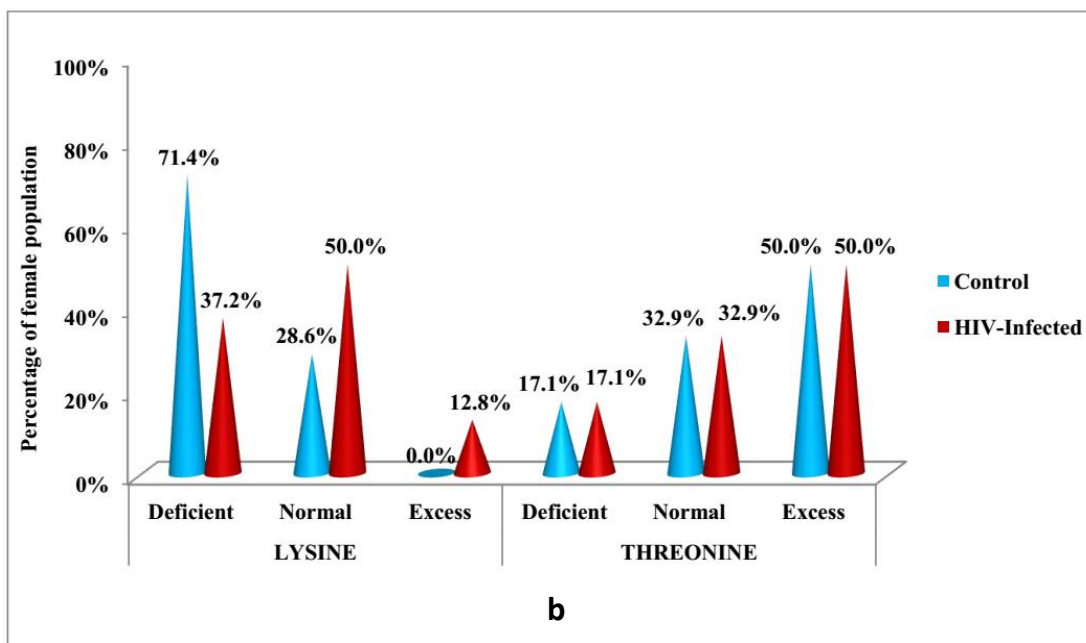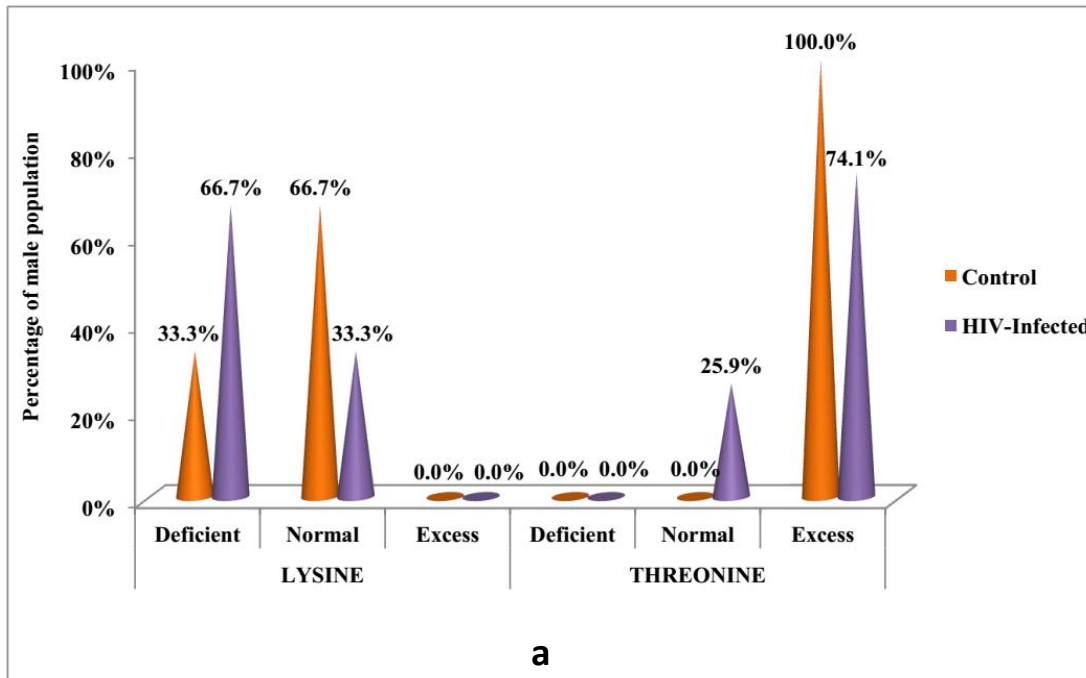
In HIV infected male, mean concentrations of lysine and threonine were significantly lower, 97 ± 3.00 and 329 ± 27.36 µmol/L, respectively, as compared to the control (Table 1). However, in HIV infected females, average concentrations of lysine and threonine were respectively higher (173 ± 19.61 and 320 ± 49.05 µmol/L) as compared to the controls (83 ± 5.27 and 243 ± 36.32 µmol/L) with a significant difference for lysine (P < 0.0001) and non-significance for threonine (P = 0.2101) (Table 1).

Relating to HIV infection: Concerning lysine, 50.4% (64/127) of HIV-infected patients and 54.3% (69/127) of the control subjects had lysine deficiency. On the other hand, 9 (7.10%) samples from HIV patients had excess of lysine, as opposed to the control subjects that showed no excess in lysine concentration (Table 2).

Concerning threonine, 9.5% of both HIV patients and controls (12/127) had threonine deficiency; however, 61.4% (78/127) of the HIV-infected patients and 72.4% (92/127) of the controls had excess threonine (Table 2).

Finally, the number of HIV infected patients having lysine deficiency (64/127) was higher than those having threonine deficiency (12/127) (Table 2).

Relating to the WHO classification of CD4 lymphocytes: In HIV-infected female who had CD4

Figure 1. a. Lysine and threonine status of male HIV infected and control population. **b.** Lysine and threonine status of female HIV infected and control population, in the study according to sex.

Table 1. Concentrations of L-lysine and L-threonine in HIV patients and control population according to gender.

Amino acids	Males			Females		
	HIV infected	Control	P-values*	HIV infected	Control	P-values*
Lysine (107-244 µmol/L)	97 ± 3.00	115 ± 4.27	0.0012	173 ± 19.61	83 ± 5.27	< 0.0001
Threonine (74-175 µmol/L)	329 ± 27.36	697 ± 23.31	< 0.0001	320 ± 49.05	243 ± 36.32	0.2101

*The difference is significance at $P < 0.05$.

Table 2. Mean concentrations of lysine and threonine in HIV infected patients and controls.

Amino acids	HIV infected patients		Control		P value
Reference values (µmol/L)	Mean concentrations (µmol/L)	Population (%)	Mean concentrations (µmol/L)	Population (%)	
Lysine					
< 107	82 ± 4.92	64 (50.4%)	71 ± 10.34	69 (54.3%)	0.3301
107 - 244	172 ± 16.76	54 (42.5%)	124 ± 8.13	58 (45.7%)	0.0522
> 244	427 ± 3.08	9 (7.1%)		0 (0%)	
Threonine					
< 74	61 ± 0.74	12 (9.5%)	27 ± 0.58	12 (9.5%)	< 0.0001
74 -175	124 ± 8.13	37 (29.1%)	75 ± 0.26	23 (18.1%)	< 0.0001
> 175	447 ± 80.51	78 (61.4%)	518 ± 75.23	92 (72.4%)	0.5369

Table 3a. Average concentrations of lysine and threonine in HIV-infected women according to CD4$^+$ count.

CD4$^+$ Range	HIV infected female (n = 70)	Lysine		Threonine	
		Mean concentration (µmol/L)	Chi^2 P*	Mean concentration (µmol/L)	Chi^2 P*
> 500	35	243 ± 27.86	0.00	296 ± 27.86	0.87
499 – 350	17	143 ± 8.09	0.03	443 ± 67.76	0.70
349 – 200	07	104 ± 1.09	0.03	350 ± 3.57	0. 03
< 200	11	87 ± 0.49	0.64	107 ± 0.81	0.18

*Difference is significance at P < 0.05.

Table 3b. Average concentrations of lysine and threonine in HIV-infected men according to CD4$^+$ count.

CD4$^+$ range	HIV infected male (n = 57)	Lysine		Threonine	
		Mean concentration (µmol/L)	Chi^2 P*	Mean concentration (µmol/L)	Chi^2 P*
> 500	20	126 ± 0.83	0.29	473 ± 18.84	0.05
499 – 350	11	96 ± 0.17	0.46	273 ± 6.69	0.38
349 – 200	13	92 ± 0.74	0.85	249 ± 20.42	0.72
< 200	13	83 ± 0.18	0.84	322 ± 1.13	0.47

*Difference is significance at P < 0.05.

lymphocytes >500 and between 350 and 499 cells/mm^3, the amino acid concentrations were normal for lysine (243 and 143 µmol/L, respectively) with a significant difference (P < 0.05) and higher for threonine (296 and 443 µmol/L) with no significant difference (P > 0.05) as compared to the normal reference values (lysine: 107 to 244 µmol/L; threonine: 74 to 175 µmol/L). However, when CD4 lymphocytes count was <200 cells/mm^3 and between 200 and 349 cells/mm^3, these values were lower for lysine (87 and 104 µmol/L, respectively). In the case of threonine, these values were higher (350 µmol/L) with a CD4 range of 200 to 349 cells/mm^3 (P < 0.05) and lower (107 µmol/L) with a CD4 range < 200 cells/mm^3 (P > 0.05) (Table 3a).

In HIV-infected male, mean concentrations of lysine were normal (126 µmol/L) with CD4 range > 500 cell/mm^3 and reduced with CD4 < 500 cells/mm^3 (P > 0.05). The mean concentrations of threonine was high in any CD4 range with no significant difference in all cases (P > or = 0.05) (Table 3b).

Finally, according to CD4 lymphocytes count, the correlation is significant for lysine (P = 0.0006) and not significant for threonine (P = 0.8640) (Table 4).

DISCUSSION

Lysine deficiency was observed in 66.7% male and 37.2% female HIV patients which showed the significant use of lysine by the body in the fight against HIV

Table 4. Correlation between CD4^{+}T lymphocytes cells and plasma amino acids in HIV infected patients.

Amino acids	CD4^{+} T lymphocytes count	
	Correlation	P value
Lysine	r = 0.840	0.0006*
Threonine	r = 0.056	0.8640

r Value denotes degree of positive or negative correlation; *P denotes statistically significant value; the difference is significant at P < 0.05.

infection. This amino acid, like other essential amino acids, cannot be synthesized by the body itself and therefore must rely on adequate dietary intake to function properly. During viral infection (herpes virus, shingles, HIV, etc.), the virus uses the amino acid, arginine in its viral replication process. However, lysine competes with arginine in this process. This competition is expected to slow down viral replication (Walsh et al., 1983) and therefore reduce the synthesis of nitric oxide (NO) which contributes to the establishment of cardiovascular disease (Liaudet et al., 1997). The higher lysine deficiency observed in men may be due to increased uses of this amino acid in the production of various enzymes, hormones and anti-infectious antibodies (Weinert et al., 2013). In general, the deficiencies observed in people living with HIV taking ARV may be therefore due to viral infection and insufficient amount of lysine intake in their diet. Lysine deficiency varies depending on the CD4 count (Butorov, 2013).

In the control population, the opposite was observed: lysine deficiency was more common in women (71%) than in men (33%). Lysine deficiency may occur in situations of intense stress or malnutrition (Thorne-Lyman et al., 2010) which can lead to a decrease in the immune system and disorders such as stunted growth, slow healing of wounds, anemia, reproductive problems, osteoporosis, lipid disorders, diarrhea, anxiety and stress (Smriga et al., 2002; Thorne-Lyman et al., 2010). Generally, deficiency in lysine may be due to several phenomena. It could be either an insufficient intake of lysine-rich diet, which is mostly the case in many developing countries, poor absorption, liver disease, and/or increased lysine utilization or loss in urine during acute and chronic infection (Flodin, 1997), or a lack of signal transduction in vision color. This defect is related to the level of vitamin A (Bhagavan and Ha, 2015). Unfortunately, in Côte d'Ivoire, Boyvin et al. (2013) showed dyslipidemia, phosphocalcic disruption, a link between very high reduction in vitamin A and HIV infection, which is so disastrous for HIV patient.

Concerning excess in lysine level, the absence of excess lysine observed in male PLVIH and the controls population, implied a normal catabolism of lysine by its two main pathways which are: through saccharopine (mainly mitochondrial) and pipecolate (essentially cytosolic

and peroxisomal) (Bender, 2012; Hallen et al., 2013). In contrast, the excess lysine found in 13% of female HIV individuals could be due either to hyperlysinaemia, a rare hereditary genetic disease caused by an enzyme deficiency that prevents breaking of lysine (Zhu et al., 2002), and associated with liver and kidney metabolic disorder which is associated with lack of signaling on both catabolism pathways of lysine (saccharopine and pipecolate). With regards to the metabolic disorder, no biochemical disruption of blood glucose, creatinine, urea and transaminases were observed in this study. In the study of Galindo et al. (2016), this same observation was reported.

Concerning threonine, both study populations (controls and HIV infected patients) generally had higher mean concentrations of threonine. Indeed, threonine is a key nutrient to the intestine. An important part of the ingested threonine is absorbed in the distal part of the intestine- the ileum. The remaining part (indigestible threonine) is found at the end of the ileum. Only 40% of the threonine intestinal light reaches the portal vein that collects nutrients from the digestion process. Enterocytes (cells in the wall of digestive tract) use 60% of the ingested threonine, which is two times more than that of lysine (Mao et al., 2011) for the synthesis of endogenous secretions, particularly mucus.

The plasma threonine deficiency observed in 17% of HIV positive patients and the female control population could be due to activation of threonine catabolism and/or synthesis of threonine-rich proteins (Laurichesse et al., 1998). Due to the high threonine concentration in immunoglobulins, threonine deficiency may affect the production of immunoglobulins (Richard and Galanaud, 1995). In actual fact, an infection or inflammation of the small intestine increases the synthesis of intestinal mucin and the use of threonine from arterial but non-luminous supply. This leads to the mobilization of endogenous proteins to meet the increased threonine demand associated with acute intestinal inflammation (Remond et al., 2009).

The absence of threonine deficiency observed in male in both groups (controls and PLHIV) showed the integrity of the intestinal barrier because mucus is an important component of the intestinal barrier that protects the intestine against digestive enzymes, physical damage and infections (Ruth and Field, 2013).

The excess threonine observed in HIV positive patients and the control population was not as a result of the high amount of threonine ingested which could disrupt liver function, causing the formation of excess urea, and consequently the toxicity of ammonia, in actual fact, the biochemical assessment of urea, creatinine and transaminases in this study revealed normal level which is similar to the study of Galindo et al. (2016) on the treatment of naive HIV infected patients.

This excess in threonine reflects the increased and maintained daily use of threonine for the synthesis of proteins in the intestinal wall and plasma proteins in

fighting against infections in HIV and against situations of intense stress and infections in the environment where malnutrition is often encountered, especially in developing countries. Threonine-rich diet intake in these two population groups and particularly in controls population is therefore essential for effective early nutritional management (Faure et al., 2007). Apart from its role as protein depositor, threonine is involved in maintenance processes, such as intestinal mucus renewal and immune protein synthesis (Bishop et al., 2013; Ruth and Field, 2013). In view of the importance of these digestive secretions to the intestines and to the digestive processes, an adequate amount of threonine must be provided by food to allow proper functioning of the digestive tract (Mao et al., 2011). Like mucins, immuno-globulins are threonine-rich globular glycoproteins. A poor and degraded health condition leads to threonine deficiency and must be filled (Nichols et al., 2008). Based on the degree of immunosuppression, no relationship was found between threonine deficiency and lower CD4 count. This confirms that the threonine level depends on the degradation of the health condition of the subject (Faure et al., 2007).

Conclusion

The deficiencies observed in HIV positive patients on antiretroviral therapy are therefore due to viral infection and insufficient nutritional intake of lysine. The threonine level depends on the degradation of the health condition of the subject. Therefore, effective early nutritional management of lysine and threonine is very essential to slow down viral replication in order to achieve a better quality of life for patients living with HIV.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

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