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$CD4^+$ T-Lymphocyte counts among under-5 children with protein-energy malnutrition as seen in Usmanu Danfodiyo University Teaching Hospital , Sokoto, Nigeria.

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Abstract *Background:* Protein-energy malnutrition is a prevalent public health problem in the developing countries. It affects body systems including cell-mediated immunity.
Objectives: To determine the effect of PEM on $CD4^+$ T-lymphocyte counts among under - 5 children.
Methods: This was a prospective cross-sectional study conducted among HIV-negative children aged 6 - 59 months with PEM and the HIV-negative well-nourished children between November 1st, 2010 and July 31st, 2011. The socio-demographic characteristics, weight and some haematological indices of the both groups were documented. The $CD4^+$ T-lymphocyte count was determined using Partec cytoflow machine.
Result: One-hundred children were recruited for each group over a 9 month period. The two study groups were comparable in age (p= 0.53) and sex (p= 0.65). The mean $CD4^+$ T-lymphocyte count in children with PEM was 1705.5±605.6 cells/µL as compared to 2314.3±491.1 cells/µL among the controls (p= 0.0001). A statistically significant difference was observed in the mean $CD4^+$ T-lymphocyte count of the different types of PEM with the highest value observed among children with kwashiorkor (2097.7±712.9 cells/µL) and lowest value observed among those with marasmus (1449.3±368.2 cells/µL). There were significant differences in the mean $CD4^+$ T-lymphocyte count of the control (2314.3±491 cells/µL) when compared to those of marasmus (1449±368 cells/µL) (p= 0.001), marasmic-kwashiorkor (1888±762 cells/µL) (p= 0.002), underweight (1559±452 cells/µL) (p= 0.001) and underweight-kwashiorkor (1534±402 cells/µL) (p= 0.001), but it was comparable with that of kwashiorkor group (2098±713 cells/µL) (p= 0.21). A statistically significant difference was observed in the mean $CD4^+$ T-lymphocyte count of the different types of PEM with the highest value observed among children with kwashiorkor (2097.7±712.9 cells/µL) and lowest value observed among those with marasmus (1449.3±368.2 cells/µL).
Conclusion: The PEM has deleterious effects on the $CD4^+$ T-lymphocyte counts among under-5 children with PEM with the lowest count observed among those presenting with marasmus.

Key words: $CD4^+$ T-Lymphocyte, Count, PEM, Under-5.

Introduction

Protein-energy malnutrition (PEM) applies to a group of pathological conditions arising from absolute or relative lack of protein and/or calorie in varying proportions, occurring commonly in infants and young children[1]. It includes a wide range of clinical stages, the extreme forms being Marasmus and Kwashiorkor, while the mild and moderate forms express themselves as varying degrees of growth retardation. It is a complex situation as low intake of calories, protein and micronutrients; poverty, infectious diseases, poor breastfeeding and

weaning practices are implicated factors[1-3]. However, in addition to these predisposing factors, kwashiorkor has also been ascribed to aflatoxins[4]. Lack of food and clean water, poor sanitation, social unrest and political instability lead to PEM. These factors are prevalent especially in sub-Saharan Africa; hence the persistence of the problem in the region[5].

PEM is globally the most important risk factor for morbidity and mortality, contributing to more than half of deaths in children worldwide.[6] PEM is responsible, directly or indirectly, for 54% of the 10.8 million deaths per year in children under five years of age. It also contributes to every second death associated with infectious diseases among children under five years of age in developing countries[6,7]. In Nigeria, it is associated with about 30-40% of deaths in preschool children.[8] Children with severe PEM are at risk of several life-threatening problems like hypoglycaemia, hypothermia, serious infections and severe electrolyte disturbances.

Adequate nutrition is essential for the maintenance and integrity of the body systems and structures including body's immunity. It has been shown that there is strong association between PEM and infections. The interaction of PEM and infection results in increase morbidity and mortality among under-5 children in developing countries. In the presence of PEM, ordinary childhood diseases result in severe consequences. These and similar observations suggest a defective immune response in PEM. PEM is the single most common cause of immunosuppression or immunodeficiency in children[7].

Severe PEM, particularly kwashiorkor, during childhood results in extensive atrophy of the thymus, spleen and other lymphoid tissues.[9] It has been related to changes in cellular immunity, changes in peripheral lymphocyte subsets (mainly cluster cells of differentiation (CD): $CD3^+$, $CD4^+$, and $CD8^+$) and cytokines elaborated by these cells. This immunodeficiency represents a key factor in susceptibility to infections and has therefore been termed nutritionally acquired immunodeficiency syndrome (NAIDS).[7]

In patients with severe PEM, both acquired immunity i.e., lymphocyte functions as well as innate host defense mechanisms i.e., macrophages and granulocytes are affected. Diminished immune functions render undernourished patients more susceptible to infections, which further worsen the nutritional status of the child, energy loss, reduce productivity on the community level, and perpetuate the alarming spiral of PEM, infection, disease and poverty. This can only be interrupted by prompt and adequate nutritional rehabilitation.[7,9]

With the advent of the human immunodeficiency virus (HIV) pandemic, there has been a tendency to overlook the role of malnutrition in immunodeficiency, and indeed, only a handful of studies have investigated the $CD4^+$ T-lymphocyte subsets in children with PEM, especially in the developing countries despite the magnitude of the problem of malnutrition and infection especially in these countries. The results of these studies are inconsistent. In some studies an increase of lymphocyte proportion has been observed; while other studies show a decrease in lymphocyte proportions.[10-14] These inconsistent results may be probably due to difference in methodology applied, types or severity of malnutrition studied, the variables compared or perhaps there were other unknown factors that were not taken into consideration. Hence, this study was carried out to determine the pattern of $CD4^+$ T-lymphocyte count among the under-5 children with PEM.

Subjects and methods

The study was a prospective cross-sectional in which subjects were children with protein-energy malnutrition aged 6 months to 59 months and the controls were age and gender matched well-nourished apparently healthy children. The study was conducted between August, 1st, 2010 and July, 31st, 2011 at the General Paediatric Outpatient Clinic (GPOC), Immunization clinic, Emergency Paediatric Unit (EPU), and Paediatric Medical Ward (PMW) of Usmanu Danfodiyo University Teaching Hospital (UDUTH), Sokoto, the Sokoto State capital.

The hospital is a tertiary health facility that serves as a referral centre for people of Sokoto, Zamfara, and Kebbi states; and the neighbouring Niger and Benin Republics in the West African sub-region. Sokoto state is located at the extreme part of North-western Nigeria between longitude 3° and 7° East and between latitude 10° and 14° North of the Equator. It shares borders with Niger Republic to the north, Kebbi State to southwest and Zamfara State to the east[15].

Approval was obtained from the Ethics Committee of UDUTH, Sokoto, and written consent was also obtained from the parents/guardians of the patients. The age, sex, weight of the subjects and the controls; and the presence of oedema in them were documented. The nutritional status was classified using Modified Wellcome Classification of PEM.[16] Those with any form of allergic disorder, haematological disorder and malignancies were excluded from the study.

Laboratory Methods

The $CD4^+$ T-lymphocyte count was determined using Partec cytoflow machine and HIV infection was confirmed with ELISA for children >18 months and HIV-DNA PCR for those aged ≤18 months.
A total of 5mls of whole blood was collected from each of patients into two ethylenediamine tetraacetate (EDTA) vacuette containers, 2 mls in an EDTA vacuette container for complete blood count and HIV tests at the Haematology laboratory and 3mls in the other container for $CD4^+$ count using partec cytoflow machine which was done by the Investigator under the close supervision of the Laboratory Scientist in charge of the investigation. The sample collection and assay of $CD4^+$ T-

lymphocyte count was done between 8am and 12mid-day at the Immunology laboratory. The reagents and protocol for $CD4^+$ T-lymphocyte count were obtained from Partec (Munster, Germany). A total of 20 µL of well-mixed whole blood in EDTA was placed in the test tube provided, and 20 µL of CD4-PE monoclonal antibody was added. The contents of the tube was mixed gently and incubated in the dark at room temperature for 15 min. Following incubation, 800 µL of non-lyse buffer was added to the tube. The tube was mixed gently for 5 seconds to re-suspend the cells immediately before counting. Calibration of the cytoflow instrument was done with standard stained beads of known concentration to obtain the best peak and resolution for counting $CD4^+$ T cells.

The complete blood count was analyzed at the haematology laboratory using the Automated (Coulter) method using Swelab Alfa 3-part haematology analyzer (Boule Medical, Stockholm, Sweden, 2006). The 2ml of whole blood collected earlier was mixed well using a mixer before analysis using the afore-mentioned machine following the manufacturer's instruction.

Data Analysis

The data entry and analysis were done using SPSS statistical package version 17.0. The comparison of means was done using Student's t test. The comparison of proportions of gender and socio-economic class of the malnourished and well-nourished groups were done using Chi-square test. A p value of 0.05 or less at 95% confidence interval was regarded as statistically significant.

Results

One-hundred children were recruited each as subject group and control group over a year period. The two study groups were comparable in age (p= 0.53), sex (p= 0.65) as depicted in Table 1.

Table 1: Some demographic characteristics of the subjects and the control

Variable	PEM n=100	Control n=100	χ2	t	p
Age (month)					
Range	6.0 - 59.9	6.0 - 59.9			
Mean±S.D.	18.7±9.4	19.1±9.7	-	0.63	0.53
Gender					
Female	36 (36%)	37(37%)			
Male	64 (64%)	63(63%)	0.20	-	0.65

The mean haematocrit value was significantly lower in children with PEM (29.4±3.4%) compared to the controls (34.0±1.9%) (t= -12.0, p= 0.0001). There were significant differences in the haematocrit (F= 39.9, p= 0.001), total leucocyte count (F= 8.5, p= 0.0001), absolute neutrophil count (F= 4.3, p = 0.002) and absolute lymphocyte count (F=7.8, p= 0.0001) of the controls when compared with those of various clinical types of PEM as depicted in Table 2. The mean TLC and absolute lymphocyte count were observed to be higher among children with kwashiorkor.

Table 2: Some haematological parameters of the children with PEM according to clinical type of PEM and the control

Parameters	Control	Mean±SD kwashiorkor	values of Underweight-kwashiorkor	Complete Underweight	blood count Marasmic-kwashiorkor	Marasmus
#Haematocrit	34.0±1.8	28.6±2.4	28.9±1.7	31.8±3.7	29.2±4.3	28.3±3.3
ϕTLC*	7.2±1.6	9.2±1.4	7.1±1.2	8.7±2.2	8.6±1.4	8.5±1.9
±ANC*	2.3±0.8	2.7±0.8	2.5±0.7	3.0±1.5	2.9±0.9	2.9±1.0
†ALC*	4.2±1.1	5.6±1.5	3.9±0.7	4.9±1.3	4.9±0.9	5.1±1.2
‡APC*	285±72	253±73	249±70	295±81	270±87	200±61

Key: ANC= Absolute Neutrophil Count; ALC= Absolute Lymphocyte Count; APC= Absolute Platelet Count; TLC= Total Leucocyte count; Kwash= Kwashiorkor; *figures are in 10^9 cells/L.
= (F=39.9, p= 0.0001); **Φ** = (F=8.5, p= 0.0001); **±** = (F=4.3, p= 0.002);
† = (F=7.8; p= 0.0001); **‡** = (5.7, p= 0.0001) (DELETE as this can go for short report moreso it not part of your objective for this paper).

The mean absolute $CD4^+$ T-lymphocyte count in the subjects was 1705.5 ± 605.6 cells/µL and the absolute count ranges between 444 cells/µL and 3220 cells/µL in children with PEM as compared to 2,314.3±491.1 cells/µL (range 1,434 cells/µL to 3,775 cells/µL) among the control group respectively. The difference in mean $CD4^+$ T-lymphocyte count was statistically significant (t= -7.8, p= 0.0001). This significant difference was evident among children aged 36 months and below when segregated by age group as shown in Table 3.

There was an inverse relationship between the age and the mean $CD4^+$ T-lymphocyte count (r= - 0.2, p= 0.04) in the children with PEM. A similar inverse relationship between the age and the mean $CD4^+$ T-lymphocyte count (r= - 0.52, p = 0.0001) was also observed in the controls as shown in figure 1. The mean value of $CD4^+$ T-lymphocyte count was comparable in both sexes (female= 1692.2±605.9 cells/µL; male= 1712.4±609.9 cells/µL) (t=- 0.16, p=0.87) as shown in Table 4. In both groups, the mean $CD4^+$ T-lymphocyte percentage ranges between 49.1% and 51.4% across the age groups; and it was 50.3% in both genders. There were no significant differences in the mean $CD4^+$ T-cell percentage across the age group (F= 0.28, p= 0.89) and between the gender (t=0.03, p=0.98) among the malnourished. There were no significant differences in the mean $CD4^+$ T-cell percentage across the age group (F= 0.38, p= 0.92) and between the gender (t=0.31, p=0.88) among the controls.

Table 3: Comparison of mean of $CD4^+$T-Lymphocyte counts of the Subjects and the Controls according to age group.

Age Group (month)		Mean±SD $CD4^+$T-Lymphocyte Count (cells/µL) Subjects (n=100)	Control (n=100)	t	*p*
6.0-11.9		909.9±672	2675.7±464.5	-4.7	0.0001
	n	32	32		
12.0-23.9		1657.8±594.0	2204.7±414.7	-3.8	0.0001
	n	53	56		
24.0-35.9		1484.3±351.8	1881.4±238.4	-4.6	0.003
	n	11	8		
36.0-47.9		1374.0±132.9	1877±60.8	-	-
	n	2	2		
48.0-59.9		1247.5±238.3	1770.0±70.7	-	-
	n	2	2		
Total		1705.5±605.6	2314.3±491.1	-7.8	0.0001

Fig 1: A graph showing mean absolute $CD4^+$T-lymphocyte count of the subjects and the controls according to age group

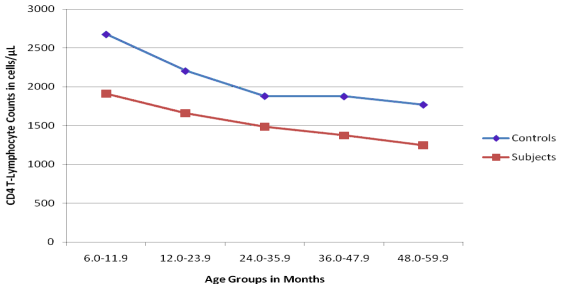

Table 4: The levels of $CD4^+$T-Lymphocyte Count in children with PEM according to age and sex.

Age (months)		Gender Male n=64	Female n=36	t	*p*
6.0-11.9	Mean±SD	1829.1±663.8	2087.8±695.2	1.04	0.33
	Range	950-3118	1492-3220		
	n	22	10		
12.0-23.9	Mean±SD	1702.3±618.5	1571.4±543.4	-0.74	0.47
	Range	978 - 3080	444 – 2900		
	n	35	18		
24.0-35.9	Mean±SD	1643.5±646.5	1705.8±755.2	0.14	0.89
	Range	1068-2150	1027-1774		
	n	5	4		
36.0-47.9	Mean±SD	1280.0±0.0	1468.0	-	-
	n	1	1		
48.0-59.9	Mean±SD	1416±0.0	1079±0.0	-	-
	n	1	1		
Total	Mean±SD	1712.4±609.9	1692.2±605.9	- 0.16	0.87

A statistically significant difference was observed in the mean $CD4^+$ T-lymphocyte count of the different types of PEM with the highest value observed among children with kwashiorkor (2097.7±712.9 cells/µL) and lowest value observed among those with marasmus (1449.3±368.2 cells/µL). There were significant differences in the mean $CD4^+$ T-lymphocyte count of the control (2314.3±491 cells/µL) when compared to those of marasmus (1449±368 cells/µL) (t= 7.46, *p*= 0.001), marasmic-kwashiorkor (1888±762 cells/µL) (t= 3.20, *p* = 0.002), underweight (1559±452 cells/µL) (t= 6.36, *p*= 0.001) and underweight-kwashiorkor (1534±402 cells/µL) (t= 6.67, *p*= 0.001), but it was comparable with that of kwashiorkor group (2098±713 cells/µL) (t= 7.46, *p*= 0.21) as shown in Table 5.

Table 5: The mean value of CD4+ T-Lymphocytes counts of the various types of PEM and the controls

Types of PEM		Mean±SD $CD4^+$ T-Lymphocytes Counts (cells/µL) 6.0-11.9 mo	12.0-23.9 mo	24.0-35.9 mo	36.0-47.9 mo	48.0-59.9 mo	Total
Control							
	n	32	56	8	2	2	100
		2675.7±464.5	2204.7±414.7	1881.4±238.4	1877.0±60.8	1770.0±70.7	2314.3±491.1
[*]Kwashiorkor							
	n	4	14	2	-	-	20
		2334±1064	2044±675	1995±219	-	-	2098±713
[†]M-kwash.							
	n	9	10	1	-	-	20
		2192±696	1649±792	1540	-	-	1888±762
[‡]U-Kwashiorkor							
	n	1	18	1	-	-	20
		2440±0	1489±359	1428±0	-	-	1534±402
[ϕ]Underweight							
	n	4	6	6	2	2	20
		2112±448	1583±424	1332±319	1374±133	1247±238	1559±452
[±] Marasmus							
	n	14	5	1	-	-	20
		1512±396	1288±296	1380±0	-	-	1449±368

Keys: M-kwash= Marasmic-kwashiorkor, U-Kwashiorkor= Underweight-kwashiorkor
* = (t= 7.46, *p*= 0.21);† = (t= 3.20, *p*= 0.002); ‡= (t= 6.67, *p*= 0.001); ϕ= (t= 6.36, *p*= 0.001); ±= (t= 7.46, *p*= 0.001).

Discussion

Protein-energy malnutrition (PEM), especially severe forms had been demonstrated to affect almost all body systems as adequate nutrition is essential for the maintenance and integrity of the body systems and structures including body's immunity.[3]

The mean absolute $CD4^+$ T-lymphocyte counts were shown to be significantly lower among children with PEM in this series, and the difference was more obvious among children less than 3years of age. The comparable mean $CD4^+$ T-lymphocyte count between the two groups above 3 years of age was due to the small number of children recruited for the groups in these age groups. This finding is comparable with the findings of Yusuf *et al*[17], Najera *et al*[12] in Mexico, Fakhir and colleagues[13] in India and Bachou[14] and coworkers in Uganda but in contrast to the findings of Rikimaru and his colleagues[18] and Najera *et al*[19] who found no significant difference in the levels of $CD4^+$ T-lymphocyte subset among the malnourished and well-nourished Ghanaian and Mexican children respectively. This finding of lowered $CD4^+$ T-lymphocyte count in children with PEM in this study may suggest depressed cellular mediated immunity, hence, poor immune response among children with PEM as $CD4^+$ T-lymphocytes play a central role in regulating the body immune system and response to antigen challenge such purified protein derivative and vaccination[20,21]. Hence, these children may be susceptible to various forms of infections such as bacterial, viral and fungal infections; and may have poor response to vaccination and antigen challenge tests such as Mantoux test.

In this study the mean $CD4^+$ T-lymphocyte count was shown to be decreasing with increasing age in both the malnourished and well-nourished children with significant negative correlation between the age and $CD4^+$ T-lymphocyte count. This finding is similar to the pattern observed among the malnourished and well-nourished healthy young children as reported in previous studies

both in and outside Nigeria.[17,20-23] The absolute $CD4^+$ T-lymphocyte counts for age in this series was within normal reference values reported among American[21] and Saudi Arabian children[22] but were higher compared to the values reported by Emmanuel *et al*[20] among healthy Nigerian children in 2009 in Lagos. The difference may be related to the machine (FACScount machine) used in enumerating $CD4^+$ T-cells count in their study. The machine could only determine count ≤2000 cells/μL (Partec cytoflow machine used in this study can detect up to 4,000 cells/μL) and perhaps the larger sample size in Lagos series. The higher value of $CD4^+$ T-lymphocyte counts among infants compared to young children could be related to the higher absolute lymphocyte count among this age group as a positive correlation has been established between absolute lymphocyte count and the $CD4^+$ T-lymphocyte counts[24,25]. This observation implies that interpretation of the $CD4^+$ T-lymphocyte counts in under-5 children has to be age-adjusted and indirectly not reliable for monitoring of disease conditions such as HIV infection.

In this series, the mean absolute $CD4^+$ T-lymphocyte was similar in both males and females. A similar finding was reported by Yusuf T *et al*[17] in Sokoto, Nigeria and Foca M and colleagues[26] in USA, but in contrast to that reported by Mandala and coworkers[27] who reported significantly higher $CD4^+$ T-lymphocyte count among females compared to the male Malawian children. This shows that gender has no significant effect on the $CD4^+$ T-lymphocyte count in children in our community. Therefore, there is no need for different reference values for different gender in interpreting the $CD4^+$ T-lymphocyte count in both genders.
The $CD4^+$ T-cell percentage was comparable in all age groups below 5 years and between the genders as shown in this study. This is similar to earlier findings among under-5 children both in and outside Nigeria.[20-23] This implies that the $CD4^+$ T-cell percent is relatively stable with no significant change in children below 5years; hence, it is very useful and reliable as a guide in treatment decisions and monitoring of under-5 children with HIV infection.

A significant relationship between the levels of $CD4^+$ T-lymphocyte count and the clinical types of PEM has been demonstrated in this study. The $CD4^+$ T-lymphocyte count is much lowered among children with marasmus and highest among those with kwashiorkor which appeared normal. A similar pattern was earlier reported by Yusuf *et al*,[17] Bachou and his colleagues[14] and Stephen and his colleagues.[28] However, Rikimaru and his colleagues[18] found no relationship between the level of $CD4^+$ cells and the type of PEM. The observed higher absolute $CD4^+$ T-lymphocytes among children with kwashiorkor may be related to the high absolute lymphocyte count observed among children with kwashiorkor in this study as there is positive correlation between the total lymphocyte count and $CD4^+$ T-lymphocyte count.[24,25] It could also be related to the blood level of cortisol in children with kwashiorkor, as it was postulated in dysadaptation theory, that failure of the plasma cortisol to be maintained at sufficiently high level is responsible for the biochemical events in kwashiorkor. High levels of steroid in the body have been associated with low $CD4^+$ T-lymphocyte count, which may also explain the lower $CD4^+$ T-lymphocyte counts among children with marasmus in this study.[3,29] This observation may suggest that a certain degree of immunocompetence is required for the development of oedema in kwashiorkor.

This implies that there is likelihood of immunosuppression in children with PEM especially those presenting with marasmus. Severe PEM alters the immunological competence of the body via a number of mechanisms which include apoptosis of thymus gland, macro and micronutrients deficiencies[1,3]. The depletion of $CD4^+$ T-lymphocytes count is associated with impaired cellular immunity. As a result of this, children with PEM and especially marasmus are likely to develop severe form of infection with resultant high morbidity and mortality rates among children with severe PEM. The higher $CD4^+$ T-lymphocyte count observed among children with kwashiorkor may not suggest immunocompetence in this group of children as there may be other immunological derangements (which were not studied in this work) which make them susceptible to various forms of infections and higher morbidity and mortality rates. Further studies need to be conducted to further corroborate these findings.

Conclusion

In conclusion, the PEM has deleterious effects on the $CD4^+$ T-lymphocyte counts among under-5 children with PEM in our community, with the lowest count observed among those presenting with marasmus. These findings might reflect the effect(s) of PEM on the immune system. There is need for a large multi-centered study to be conducted to further elucidate immunological derangements in children with PEM. Furthermore, The $CD4^+$ T-lymphocyte percentage is relatively similar among under-5 irrespective of the age or gender, and therefore, can be useful in treatment decisions and monitoring under-5 children with HIV infection. A large cohort multicentre study would be needed to establish normal reference values for $CD4^+$ T-lymphocyte subsets in our community.

Authors Contribution
Yusuf T.: Source and analyzed the data, write up.
Jiya NM: Review of manuscript write up.
Ahmed H.: Conceptualized the research, review of the manuscript and write up

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