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The role of nutrition as a determinant of clinical, immunological and pharmacological outcome in HIV-infected children in Uganda

A dissertation submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy
by

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Declaration

This thesis has not previously been submitted for the award of a degree at the University of Dublin, Trinity College, or any other university, and is entirely the product of my own work.

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Judy Tatwangire Orikiiriza

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Dedication

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"Success is not final neither is failure fatal but it is the courage to continue that counts…..Failure is not the opposite of success it's the pathway to success, embrace your failures and use them as a springboard to reaching the stars".

PhD Output

Original research articles:

1. **Judy Orikiiriza**, Jane Nakawesi, Ben Kikaire, Dorothy Turitwenka, Walter Schlech, Andrew Kambugu, Mohammed Lamorde, Johan Normark, Martina Hennessey, Victor Musiime, Joseph Rujumba, Grace Ndeezi, Nazarius Mbona Tumwesigye, Derek G. Doherty, Jane Achan. Unmet needs persist in Pediatric HIV programmes: Lessons from selected case studies in Uganda. AIDS 31: 1196-1199, 2017.
2. **Judy Orikiiriza**. Roll out of efavirenz- based regimens in option B+ in the PMTCT programs: challenges and lessons learned from a post-exposure prophylaxis experience. AIDS 30(15):1 DOI10.1097/QAD.0000000000001212, 2016.
3. **Judy Orikiiriza**, Dorothy Turitwenka, Jane Nakawesi, Kuteesa Bisaso, Victor Musiime, Grace Ndeezi, Jane Achan. Severe acute malnutrition in a 3-month-old HIV exposed infant admitted at Mildmay- Uganda: Implications for clinical care in low resource settings. Manuscript submitted.
4. Pádraic J. Dunne, Christina O. Maher, Michael Freeley, Andreea Petrasca, **Judy Orikiiriza**, Margaret R. Dunne, Derval Reidy, Siobhan O'Dea, Aisling Loy, Jim Woo, Thomas R. Rogers, Fiona Mulcahy and Derek G. Doherty. Vδ1 T cells with low or absent CD3ε expression are expanded in patients with HIV infection but display properties of clonal inactivation. Front. Immunol., 02 May 2018 | https://doi.org/10.3389/fimmu.2018.00940

Conference presentations:

1. Concept presentation at the Infectious Disease Institute College of Health Sciences Makerere University on "The influence of nutrition on immune function and drug metabolism in HIV infected children in Uganda" during the research forum on 4/11/2014.
2. Protocol presentation "The influence of nutrition on immune function and drug metabolism in HIV infected children in Uganda" presented at the Higher Degree Research Ethics Committee meeting held on 1/12/2014 at the School of Public Health Makerere College of Health Sciences.

3. Protocol presentation on "The influence of nutrition on drug metabolism in HIV infected children in Uganda" in the Department of Pharmacology and Therapeutics Trinity College Dublin on 1/3/2016.
4. Protocol presentation on "The influence of nutrition on immune function in HIV infected children in Uganda" on 16/3/2016 in the Clinical Immunology meetings at St James Hospital Department of Immunology Trinity College Dublin.
5. Transfer viva for my PhD on "The influence of nutrition on immune function in HIV infected children in Uganda" in Trinity College Dublin on 4/4/2016.
6. Preliminary results presentation on "The influence of nutrition on immune function and drug metabolism in HIV infected children in Uganda at the Global Health Department Trinity College on 21/4/2016.
7. Preliminary results presentation on "The influence of nutrition on immune function and drug metabolism in HIV infected children in Uganda in the Department of Immunology at Trinity College Dublin in the Immunology meeting on 28/4/2016.
8. Preliminary results presentation on "The influence of nutrition on immune function and drug metabolism in HIV infected children in Uganda in the Department of Immunology at Trinity College Dublin in the Immunology meeting on 12/5/2016.
9. Preliminary results presentation on "The influence of nutrition on immune function and drug metabolism in HIV infected children in Uganda" at the Infectious Diseases College of Health Sciences Makerere University on 4/8/2016.
10. Care taker knowledge, perceptions and experience of using Ready to use therapeutic food among HIV infected children: A case study of Uganda. 10th World Congress of the World Society for Pediatric Infectious Diseases 2nd-5th December 2017.
11. The role of nutrition as a determinant of clinical, immunological and pharmacological outcome in HIV-infected children in Uganda. Trinity Translational Medicine Institute annual scientific conference 23rd March 2018.

Conferences attended

1. Irish Society Flow Cytometry Conference, UCD, Dublin, 2014
2. Uganda Society of Health Sciences for medicine Kampala, Uganda 2014
3. Conference of Retroviruses and opportunistic infections (CROI), Seattle 2015
4. Uganda National Pediatric HIV conference, Kampala 2016
5. Uganda Pediatric Association annual conference, Kampala 2016
6. Uganda Medical Association annual scientific conference, Kampala 2017
7. 10th World Congress of the World Society for Pediatric Infectious Diseases 2nd-5th December 2017, Shenzhen, China
8. Trinity Translational Medicine Institute annual scientific conference 23rd March 2018.

Awards.

Uganda Society of Health Sciences annual conference for Medicine, August 2014**. [Awarded the bester Poster prize].** Immunologic and virologic response among ART experienced children in Uganda. **Orikiiriza. J,** Nakawesi. J, Kikaire. B, Turitwenka. D, Mubiru. F, Nabatanzi R, Mugasha C, Doherty DG.

Courses attended:

1. Graduate course in Immunology, Trinity College Dublin, -2014
2. Flow Cytometry Course (Flocyte), Department of Immunology MMI, 2014
3. FACSCanto II Training (Becton Dickinson), Dublin, June 15-16th 2014
4. Pharmacokinetics and pharmacodynamics modelling from 21st-25th June 2014 at Makerere College of Health Sciences at the department of Pharmacology and Therapeutics.
5. Introduction to FlowJo software at MMI trinity College Dublin 2015
6. Training on statistical analysis of flow cytometer data at Biomedical Center Trinity College Dublin 2015
7. Dietary analysis course for 5 days at the Department of Nutrition Trinity College Dublin February, 2015

8. STATA course for a week at the UCI-Fred Hutchison research collaboration with Makerere University, November 2015
9. Research Methodology part I 2015 by Makerere University School of graduate studies
10. Research Methodology part II 2015 by Makerere University School of graduate studies
11. Philosophy of Methods 2015 at Makerere University School of graduate studies
12. IT courses at Trinity college Dublin 2016
13. Acute malnutrition: Improving treatment through research at Mwanamujjimu Nutrition rehabilitation center-research collaboration with Makerere University and University of Copenhagen from 14th-18th November 2016
14. Access course at TMMI on the 22nd May 2018
15. Excel curse at TMMI on the 5th June 2018
16. Postgraduate Skills Development Summer School 13th & 14th June 2018

Abstracts accepted and presented at the 22nd International AIDS Conference (AIDS 2018), held in Amsterdam, Netherlands, 23-27 July 2018.

1. Antiretroviral therapy effect on lymphocyte numbers and phenotypes in HIV infected children
2. Changes in circulating gamma delta T cell subset numbers in immunological and virological responders and non-responders among HIV-infected children on antiretroviral therapy

Abstracts submitted to the 10th IAS Conference on HIV Science (IAS 2019) to be held in Mexico City, Mexico, 21-24 July 2019.

1. Effect of Ready-to-Use Therapeutic Food on NNRTI plasma levels in malnourished HIV- infected children in Uganda.
2. Clinical outcomes of HIV-infected children in Pediatric HIV programs in resource-constrained-settings: Implications of achieving HIV viral suppression.

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Acronyms

3TC-Lamivudine

ABC- Abacavir

ACD-Acid citrate dextrose

AE- Adverse events

AIDS-Acquired Immune Deficiency Syndrome.

AOR- Adjusted odds ratio

ART-Anti retroviral Therapy.

BMD- Bone mineral density

CD- Cluster of differentiation

CD4^{+}- Cluster of Differentiation 4

CD8^{+}- Cluster of Differentiation 8

CI- Confidence interval

COR- Crudes odds ratio

CRP-C reactive protein

CTX-Cotrimoxazole

d4T- Didanosine

DNA-Deoxyribonucleic acid

DNCB-2-4-di-nitro-chlorobenzene

DTHR- Delayed type hypersensitivity response

EDTA- Ethylenediaminetetraacetic acid

EFV- Efavirenz

EMTCT- Elimination of mother to child transmission

ESR-Erythrocyte sedimentation rate

FBC-Full blood count

FCT- Food composition tables

FCS- Fetal calf serum

GC-Gas chromatography

GFATM-Global Fund to fight AIDS, Tuberculosis and Malaria

HAART-Highly Active Antiretroviral Therapy

HAZ- Height for age z-score

HIV-Human Immunodeficiency Virus

HPLC- high-performance liquid chromatography

IFN-ɣ- Interferon gamma

Ig- Immunoglobin

IL- Interleukin

iNKT- Invariant natural killer T cells

INR-immunological none responders

IRIS-Immune reconstitution Inflammatory Syndrome

IRB- Institutional review board

IRD-Immune Restoration Disease

JCRC-Joint Clinical Research Centre

LFT's-Liver function Tests

LT- Leukotrienes

LRS-Low resource settings

MAM- Moderately acute malnutrition

MDG's- Millennium Development goals

NK- Natural killer cells

IMAM-Integrated management of acute malnutrition

IMCI- Integrated management of childhood illnesses

IQR- Interquartile range

MOH- Ministry of Health

MRC- Medical research council

MTCT-Mother to child transmission

NAIDS- Nutritional acquired immunodeficiency syndrome

NVP-Nevirapine

OI's-Opportunistic Infections

PBS- Phosphate buffered saline

PCR-Polymerase chain reaction

PEPFAR-The President's Emergency Plan For AIDS Relief

PHA- Phyto-hemaglutinin

PhD- Doctor of Philosophy

PMTCT-Prevention of Mother to child transmission

PPD- Purified protein derivative

RFTs-Renal function Tests

RFS- Re-feeding syndrome

RNA- Ribonucleic acid

RPMI- Roswell Park Memorial Institute medium

SAE- Serious/severe adverse events

SD- standard deviation

TB- Tuberculosis

TCR- T cell receptor

TLC- Total lymphocyte count

TNFα- Tumor necrosis factor alpha

TREAT- Timetable for Regional Expansion of Antiretroviral Therapy Program

UNAIDS-United Nations program against AIDS

UNAIS-Uganda Aids Indicator Survey

UNICEF-United Nations International Children's emergency fund

UV-Ultra violet

WAZ- Weight for age z-score

WBC-White blood cells

WHZ- Weight for height z-score

WHO-World Health Organization

Operation definitions

Malnutrition: This was defined as a condition resulting from the imbalance between nutrient requirements and intake that results in cumulative deficits of energy, protein or micronutrients that may negatively affect growth, development and other relevant outcomes [1] .

Acute malnutrition in children: "Weight-for-height z score < - 2SD expected for age or mid upper arm circumference < 12.5 cm or weight-for-age z score < -2 SD = wasting [2]".

Moderately acute malnourished (MAM) children: "Weight-for-height z score < - 2SD to -3SD expected for age or mid upper arm circumference >11.5 -12.5cm or weight-for-age z score > -3 to -2 SD or BMI > 16 - 18.5kg/m^2 [2] ".

Severely acute malnourished (SAM) children: "Weight-for-height z score < - 3SD expected for age or mid upper arm circumference < 11.5 cm or weight-for-age z score < -3 SD = wasting or BMI ≤16 kg/m^2 [2]".

Well-nourished HIV infected children (WN): "Weight-for-height z score > - 1SD expected for age or mid upper arm circumference >12.5 cm or weight-for-age z score >-1 SD or BMI between 18.5 to 24.9 [2]".

Ready to use Therapeutic food (RUTF): This is a product which can be consumed by a child that provides sufficient nutrient intake for complete recovery and it is based on peanut butter or paste mixed with dried skimmed milk with vitamins and minerals made according to a standard, energy-rich composition defined by the World Health Organization [2].

Immunological non responders: These were defined as having a CD4+ T cell counts of <200 cells/μL after being on treatment for at least 6 months in our study or having <20% increase from baselines after 3 months of ART initiation [3].

Virological suppression: This was defined as viral load <20 copies/ml from the baseline measurements according to Medical Research Center laboratory lower limit of detection.

Pharmacokinetics: Is defined as the study of the time course of drug absorption, distribution metabolism and excretion.

Pharmacological responses or outcomes: For purposes of this study this was limited to measurements of plasma drug concentrations with special emphasis on non-nucleoside reverse transcriptase inhibitors (NNRTIs) pharmacokinetics.

Immunological outcomes or responses: For purposes of this study this was defined as the changes in numbers of circulating selected immune cell populations.

Adverse events (AE) in the study were defined as any untoward medical occurrence in a patient which may or may not necessary have a causal relationship with treatment with RUTF/ART. Therefore it may be any unfavorable and unintended sign including an abnormal laboratory finding, symptom, or disease temporarily associated with the use of RUTF/ART, whether or not related to the use of RUTF/ART.

Serious or severe adverse event (SAE): This was defined as any untoward medical occurrence whether or not related to RUTF/ART that meets the following criteria:

a. Results in death
b. Is life threatening
c. Requires inpatient hospitalization or prolongs existing hospitalisation
d. Results in persistent or significant disability/incapacity
e. Is an important medical event requiring medical or surgical intervention to prevent serious outcome.

Abstract

Introduction: Perinatally acquired HIV infection and malnutrition remain major public health challenges for sub-Saharan Africa health systems. HIV and malnutrition result in 50% mortality. The introduction of antiretroviral therapy (ART) and ready to use therapeutic food (RUTF) have significantly reduced the morbidity and mortality rates, however, clinical, nutritional, immunological and pharmacological responses to treatment remains understudied.

Objectives: The studies included in this thesis were designed to document the effects of nutritional status and nutritional supplementation using RUTF. This was achieved by: 1) describing the clinical cohorts of HIV infected Ugandan children; 2) determining the nutritional status and outcomes including anthropometry, nutrition biomarkers and dietary analysis; 3) determining lymphocyte phenotypes and how they are affected by HIV, malnutrition, RUTF and ART, and in immunological and virological responders and non-responders; 4) determining pharmacological responses, specifically efavirenz (EFV) and nevirapine (NVP) drug levels, virological failure and 5) the knowledge, perceptions of the primary carers on use of ART and RUTF in their children.

Methods: This was a prospective cohort study involving a population of HIV infected children who were ART-experienced (ART-E) or ART-naïve (ART-N) initiating ART and primary carers. It was a multi-center study involving pediatric HIV health facilities in the central region of Uganda. Study participants were followed-up for 12 weeks while on RUTF for those who were malnourished. HIV negative healthy controls were enrolled and sampled once for immunological comparisons. We aimed to examine the role of nutrition and nutritional supplementation on clinical, immunological and pharmacological outcomes in HIV infected children aged between 6 months and 12 years. At each visit, children had a complete clinical and anthropometric assessment. Biological samples were taken to measure markers indicative of nutritional, immunological and pharmacological responses at baseline and 12 weeks. Primary carers were interviewed using focus group discussions and key informant guide to explore processes that influence adherence and uptake of RUTF and ART in children in routine HIV care. This study was approved by the relevant ethical boards.

Results: A total of 278 HIV infected children were screened, of whom 156 fulfilled the study criteria, between January 2015 and December 2017. These included 66 ART-N and 90 ART-E HIV infected children. The age range of enrolled children was 9 months to 12 years and majority were between 5-12 years and this age category had the highest malnutrition burden. Overall, 41 of 66 (62.1%) ART-N children were moderately acutely malnourished (MAM) or severely acutely malnourished (SAM) and 48.9% of the ART-E children were MAM or SAM. The median length of time on ART at baseline for the ART-E children was 42 months (IQR: 15-66 months). The majority (85.5%) of the ART-N children had severe immunological suppression prior to ART initiation, with median $CD4^+$ T cell percentage <30% and an absolute counts less than 1000 cells/ml and a median viral load of 240,753 viral copies/mL (IQR: 45,752-1,286,388). The majority of ART-E patients (54.1%) at baseline had a median $CD4^+$ T cell percentage >30% and absolute cell counts less than 1000 cells/ml and a median viral load of 114 viral copies/mL. Virologic failure occurred in 80% of SAM, 58.5% of well-nourished (WN) and 50% of MAM patients. After 12 weeks virologic failure occurred in 54.6% of SAM, 26.7% of WN and 20.7% of MAM patients. The mortality rate was 5/156 (3.2%) in the 1st month of recruitment.

Anthropometrically, the HIV infected children added 2.3 kg, (P<0.0001) and the mid-upper-arm circumference (MUAC) of 1 cm (P<0.0001) by 12 weeks. The malnourished children added 2.5 kg, (P<0.0001) and 1.3 cm MUAC, (P<0.0001) after RUTF supplementation while WN children added 2 kg, (P=0.002) and 0.3 cm MUAC with no RUTF. Good adherence was reported in 24/50 (48%), moderate adherence in 1/50 (2%) while 26/50 (52%) were poorly adherent to RUTF. Overall there was high macronutrient consumption, however the malnourished children had reduced nutrient intake. ART impacted more significantly on lipid profile than did nutritional supplementation. Serum lipid levels (total cholesterol, high density lipoprotein and low density lipoprotein) were all low among ART-N compared to ART-E children at baseline (P<0.0001, P<0.0001 and P=0.0011 respectively). Inflammatory markers were higher in the WN children compared with the malnourished children. After 12 weeks follow up, they drastically decreased in the malnourished children but did not attain the expected normal levels.

Compared to HIV-negative children, ART-N children had significantly lower frequencies of circulating $CD4^+$ T cells, natural killer (NK) cells, $CD56^+$ T cells (natural T or NT cells),

invariant natural killer T (iNK T) cells and higher frequencies of $CD8^+$ T cells, $CD4^-CD8^-$ (double negative or DN) T cells. The Vδ1 subset of γδ T cells was expanded in children with HIV whereas Vδ2 and Vδ3 subsets were unchanged. When ART-N children were compared according to nutritional status, Vδ1 T cells were expanded and NT cells were depleted in the SAM patients. At baseline, malnourished and WN ART-E patients had higher frequencies and numbers of $CD4^+$ T cells compared to ART-N patients and lower frequencies and numbers of DN and Vδ1 T cells. Supplementation of malnourished ART-E children with RUTF for 12 weeks resulted in expansions of NK cells, DN T cells and Vδ2 T cells, but not $CD4^+$ T cells, and depletions of $CD8^+$ T cells but many of these changes were also seen in WN patients who were not given RUTF. Vδ1 T cell numbers were significantly higher in immunological and virological non-responders to ART, whereas Vδ2 T cell numbers were lower in the immunological responders and higher in the virological non-responders.

Children with sub-therapeutic and supra-therapeutic levels of plasma EFV and NVP were more common than those who had therapeutic levels at baseline and 12 weeks. However, 81/84 (96.4%) had a self-reported adherence score to ART.

Most of the primary carers expressed limited knowledge on nutritional rehabilitation using RUTF. The presence of malnutrition in the family and utilisation of RUTF in the community was associated with being HIV positive and carried stigma, hindering pursuit of nutritional support. On the contrary, carers attributed the challenge of food insecurity to poor adherence to ART.

Conclusion: There is high unmet need for effective and timely initiation of ART in pediatric programs, as children continue to access care late, often with AIDS defining illness, and with high rates of loss-to-follow once in care. Immune reconstitution was marginal with high virologic failure and high incidence of suboptimal and undetectable drug levels despite reported high ART adherence. Prompt therapeutic drug monitoring and ART switching to potentiate benefits of ART in pediatric HIV programs needs to be prioritized irrespective of nutritional status. Though ART leads to sustained expansion of $CD4^+$ T cells in both malnourished and WN children with HIV infection, it may cause depletions of other cells and complete immune recovery is not achieved. ART may have

adverse effects on innate T cell populations and NK cells. Since Vδ1 T and NK cells are expanded in HIV infection especially in those with SAM and Vδ1 T cells are reduced among immunological and virological responders. Immunotherapies using innate T cells and NK cells may potentiate ART effect to attain complete immune recovery, however further studies are urgently needed with the high level of incomplete immune reconstitution in the era of ART in the Pediatric HIV programs with high co-infection burden.

CHAPTER ONE: Introduction and Literature review

This chapter presents background information on the study variables, general literature reviews of what is current in the field of HIV and malnutrition in the context of pediatrics, immunology, antiretroviral therapy and nutritional supplementation in children.

Introduction: The Global Burden of HIV and Malnutrition.

Human Immunodeficiency Virus/Acquired Immunodeficiency Disease Syndrome (HIV/AIDS) is a global pandemic that is a widely known cause of infant and childhood morbidity and mortality especially in the low resource settings (LRS) such as sub-Saharan Africa (SSA) [4]. Today, HIV/AIDs is especially responsible for the increasing number of deaths in malnourished children [5, 6].

According to the global United Nations program against AIDS (UNAIDS) 2010 report and World Health Organisation (WHO), at the end of 2011, it was estimated that 3.4 million children less than 15 years of age were living with HIV with 91% living in SSA [7-9]. In 2009, an estimated 370,000 children (220,000–520,000) contracted HIV during the perinatal and breastfeeding period, decreasing dramatically from 500 000 (320 000–670 000) in 2001 [8]. The current trend in the HIV epidemic demonstrates appreciable progress in treatment with 20.9 million people accessing antiretroviral therapy (ART) in June 2017. In 2016, 36.7 million [30.8 million–42.9 million] people were living with HIV, newly infected people were estimated to be 1.8 million [1.6 million–2.1 million] and 1 million [830 000–1.2 million] from AIDS-related illnesses [10]. In spite of the availability of high impact HIV prevention and interventions which reduce mother to child transmission (MTCT) of HIV to less than 2%, children born to HIV positive mothers still contract HIV [8, 9]. The high HIV infection rate in children in Africa results directly from the high HIV infection rate in women of child bearing age. In Uganda the infection rate in women of reproductive age (15-45 years) is still unacceptably so high at 7.3% [11, 12]. It is currently estimated that in LRS, 1,600 children are infected daily majorly through MTCT, which accounts for over 95% of HIV transmissions in children [4]. In the absence of ART, the transmission risk is 5-10% during pregnancy, 10-20% during labor and delivery, and 10-20% during breast-feeding [13, 14]. Worldwide HIV accounts for 7.7% mortality in the

under 5s while AIDS alone accounts for a rise of more than 19% and 36% in infant and under five mortality respectively.

Malnutrition arises when one's daily diet is unable to provide satisfactory calories and protein for growth and maintenance or if they are incapable to wholly harness the food consumed due to illness and this is usually coupled with micronutrient deficiencies [15]. Globally malnutrition rates remain alarming with a total of 49.48 million suffering from malnutrition especially the under 5s with the largest burden being shouldered in LRS. Stunting rates are declining very slowly while wasting still impacts the lives of far too many young children. https://www.who.int/nutgrowthdb/jme-2019-key-findings.pdf?ua=1. HIV and malnutrition are common in low and middle income countries, nonetheless the largest magnitude is found in Africa where a recent study demonstrated a prevalence of malnutrition in HIV to be 42% [16]. In LRS, malnutrition accounts for over 300,000 deaths per year in children under five years and globally contributes indirectly to over a half of all the deaths in children [17]. Approximately a third of Africa's population is faced with persistent hunger most especially in the Sahel region, the region west of the Horn of Africa and East Africa [18]. This region comprises 11 countries with a population of about 23 million people affected by acute food insecurity and at risk of developing malnutrition [19]. This is further complicated by the fact that African countries have made the least progress towards accomplishing the millennium development goals (MDGs) of reducing hunger by half by 2015. Some of the major contributors are high population growth rates, conflicts, endemic poverty, climate change [20] and lack of political will.

Global Initiation of anti-retroviral drugs and access to antiretroviral therapy

ART has significantly altered the face of HIV infection in countries where it has been auspiciously introduced [21]. Children and infants can now survive to adolescence and adulthood with an increase in life expectancy in adulthood [22]. For a long time WHO guidelines for ART use in children were considered within the context of adult guidelines and were first published in 2003 however this is changing guided by the increased efforts invested to carry out research relevant to pediatric settings [23]. The agenda to rollout ART in LRS took shape in September 2003, where leaders of the WHO, UNAIDS, global fund to fight AIDS, tuberculosis (TB), and malaria (GFATM) joined hands to declare the lack of

access to antiretroviral (ARV) drugs a global emergency. In response the '3 by 5 initiative' was launched [24]. The 3 by 5 initiative was a global target to provide three million people living with HIV/AIDS (PLWHIV/AIDS) in low and middle income countries with ART by 2005. In 2009 alone, for the first time 1.2 million people received ART, resulting in a 30% increase in the number of people receiving treatment annually [25] and this lead to UNAIDS setting another ambitious target of virtual elimination of mother to child transmission (EMTCT) of HIV/AIDs by 2015. Treatment targets were also revised to have 10 million of the PLWHIV/AIDS on ART by 2025 [25]. This HIV treatment expansion resulted in a 19% decline in mortality between 2004 and 2009. Early 2010, it was noted that 10 million PLWHIV/AIDs eligible for treatment had not initiated ART as yet though there were more than 5 million people receiving ART; a major public health achievement, though this represented only 35% of the people who needed HIV therapy [23]. Therefore there was a large gap of unmet needs in ART treatment most especially in pediatrics. Since then, HIV treatment guidelines were updated twice meaning that there were even more PLWHIV/AIDs needing treatment [22]. As of 2013, 37% of PLWHIV/AIDs needing treatment received ART with disproportionately only 24% of children needing ART receiving it [26]. Overall, 54% of adults and 43% of children living with HIV are currently receiving lifelong ART. Global ART coverage for pregnant and breastfeeding women living with HIV is high at 76% [10]. The global community of HIV/AIDs has set new treatment targets of 90-90-90 to end the AIDS epidemic by 2020 that state, 90% of all people living with HIV will know their HIV status, 90% of all people with diagnosed HIV infection will receive sustained ART and 90% of all people receiving ART will have viral suppression [26]. Whether this will be achieved, especially in pediatrics, time will tell as we are just 2 years away.

Malnutrition and HIV in Uganda.

HIV/AIDS is still an outstanding public health problem in Uganda [27]. HIV prevalence among women and men aged 15-49 years has heightened from 6.4% [27] in the 2004-05 Uganda AIDS indicator survey (UAIS) to 7.3% in 2011 [11]. The 2011 UAIS also tested nearly 10,000 children less than five years of age for HIV and found that 0.7% of children were HIV positive. The HIV prevalence amongst children whose mother was dead was 4.3% and those whose mother was widowed was found to be 3.3% [11]. According to the

Uganda Demographic Health survey (UDHS) in 2010 the prevalence of HIV was found to range from 30-50% in malnourished children [27]. This clearly illustrates that sub-standard nutrition undermines the health of HIV infected children and puts them at an increased risk of morbidity and mortality and in addition is associated with the impaired mental development especially among those aged less than 5 years [28]. Therefore the importance of providing nutritional supplementation or feeding in children cannot be underscored [29].

Antiretroviral (ARV) coverage in Uganda

In June 2005 about 90,000 people were estimated to be in need of ART according to Ministry of Health (MOH) AIDs control program (ACP) HIV 2005 report [27]. Uganda's target was to have 60,000 people on ART by the end of 2005 which was superseded and by December 2005 80,000 people had been started on ART [30, 31]. According to the Uganda AIDs Indicator survey (UAIS), 2011 one-third to one-half of Ugandan adults who were eligible for receiving ART were treated [11]. Regarding the children, it had been estimated that 130,000 children under 14 years were living with HIV with 42,000 children in HIV care and treatment with only 17,000 of these (41%) receiving ART [11]. Goals of ART in children are to prolong the survival of HIV-infected children, promote optimal growth and development, preserve, enhance, or reconstitute the immune system and therefore reduce opportunistic infections (OIs), suppress HIV replication resulting in preventing disease progression and minimizing the generation of drug resistant strains thus reduction of morbidity and mortality of children and in turn improved quality of life [30].

Current ART guidelines for Infants, children and adolescents

Currently the treatment regimens extensively utilized by the HIV programs in LRS and subsequently adopted by countries [22] like Uganda are the non-nucleoside reverse transcriptase inhibitor (NNRTI). These constitute efavirenz (EFV) or nevirapine (NVP) plus a nucleoside reverse transcriptase inhibitor (NRTI) as the backbone constituting tenofovir (TDF) + lamivudine (3TC) or zidovudine (AZT) + lamivudine (3TC)[32, 33]. The preferred ARV 1st line treatment for infants and children in Uganda are AZT based regimens in combination with 3TC and NVP or EFV. In the event that AZT is found to be

contraindicated like in patients with anemia, abacavir (ABC) or didanosine (d4T) based regimen can then be used as 1st and 2nd alternative respectively. However EFV is still not recommended in children under 3 years (or 15 kg) in spite of the Food and Drug Authority (FDA) licensing its use in children aged 3 months and above in the USA. In infants and children <24 months who have been exposed to NVP or other NNRTIs in PMTCT the protease inhibitor (PI) lopinavir boosted by ritonavir (LPV/r) is currently recommended as part of a first-line regimen combination [32].

Several manufacturers have developed Fixed Dose Combination tablets (FDCs) primarily tailored for pediatric use improving dosing accuracy in children in comparison to previously used adult FDCs [32]. The pediatric FDC tablets are easier to prescribe and administer than individual single drug formulations and syrups. The tablets are scored, crushable and dispersible in water and may be dosed in children up to 24.9 kg including infants as small as 3 kg. Therefore the currently available pediatric FDCs include: Triple FDC tablets such as AZT/3TC/NVP (60/30/50 mg), d4T/3TC/NVP(6/30/50 mg) baby, d4T/3TC/NVP (12/60/100 mg) junior and duo FDC: AZT/3TC(60/30 mg), d4T/3TC(6/30 mg) baby, d4T/3TC (12/60 mg) junior and ABC/3TC(60/30 mg). The triple FDC have a higher proportion of NVP which makes them better suited for dosing in children who are rapid metabolizers of NVP than adults [32].

The choice of ART regimen in HIV-TB co-infection in children is mainly guided by the need to minimize drug-drug interaction most especially when rifampicin is co-administered with NVP or PIs. Rifampicin is a cytochrome P450 enzyme inducer and when co-administered with NVP or PIs results in sub-therapeutic levels of NNRTIs or PIs being present because the drugs are metabolized by this family of enzymes. Alternative drugs like rifabutin, which exhibits minimal interaction with NNRTIs and PIs, are not readily available under the TB program because of their cost. In case of availability, rifabutin is substituted for rifampicin and the ART regimens need not be adjusted [32].

Children diagnosed with TB after initiation on first line ARV regimen should be immediately started on anti-TB treatment too. Nonetheless, the ARV regimen should be reviewed to ensure optimal treatment of both TB and HIV and to minimise potential drug toxicities and drug–drug interactions [32]. In the following situations when co-

administering anti-TB and ARVs drugs in pediatrics the Ugandan treatment guidelines advice that:

1. For children 3 years and older receiving a NVP based regimen, NVP has to be substituted with EFV.
2. For children under 3 years, an attempt to maximize dose of NVP to 200 mg/m^2 is made or they are switched to a triple NRTI regimen (AZT/3TC/ABC)
3. Similarly patients on a LPV/r containing regimen will have ritonavir:LPV dose ratio adjusted to 1:1.
4. Children diagnosed with TB but are ART naive must immediately begin TB treatment and thereafter start ART as soon as it can be tolerated in the first 2-8 weeks of TB therapy, irrespective of the $CD4^+$ T cell count and WHO clinical stage of HIV [32].

In adolescents the recommended 1st line ARV regimen contains two NRTIs plus one NNRTI. Thus, TDF/3TC plus NVP or EFV alternatively AZT/3TC or ABC/3TC plus NVP or EFV. However ABC/3TC is for patients with poor renal function and/or anemic [33]. WHO currently recommends a newly introduced integrase inhibitor called doultegravir (DTG) as an alternative first-line ART for adolescents with HIV instead of EFV [34]. Currently, DTG is dispensed in combination with TDF and 3TC combined in a single fixed-dose combination pill and considered to be among the best current treatments for HIV, but its availability in LRS has been limited by its high cost. It is not considered for use in children, however, as of June 2017, DTG has been approved by the FDA for use among children 6 years and older (weighing at least 30 kg), and by the European Medicines Agency for children weighing more than 15 kg. However with more findings from HIV services and research in Botswana, which has provided DTG to pregnant women with HIV for more than 1 year, demonstrate that birth outcomes like stillbirth, neonatal death, preterm birth and smallness for gestational age are undermining the potential of DTG though were not shown to differ between women receiving EFV-based therapy and those receiving DTG-based therapy [34-36]. In the upcoming months, this study and other investigations will provide more data on the safety of DTG for infants exposed in utero which may once again change the treatment guidelines.

The treatment recommendation in adolescents co-infected with TB/HIV is that ART must be initiated within the first 2 weeks of starting TB treatment with the pre-requisite of ensuring they are stabilized on their TB therapy and will subsequently continue on ART indefinitely irrespective of their initial $CD4^+$ T cell count. Whereas in the adolescents with a CD4 >350/mm^3 they should start ART only after completion of the intensive TB treatment phase, which usually lasts for 2 months. In cases where a person needs to initiate TB and HIV treatment concurrently, the recommended first line treatment options are TDF/3TC + EFV or the alternative AZT/3TC + EFV. In the exceptional circumstances where $CD4^+$ T cell counts cannot be obtained, ART should be initiated 2-8 weeks after the start of TB therapy when the patient has stabilized on TB treatment. In severely immunosuppressed patients (CD4 less than 50) co-infected with TB, ART should be started immediately (within two weeks of determining eligibility) and monitor for occurrence of immune reconstitution syndrome [33].

Malnutrition in HIV

Non-oedematous malnutrition is more prevalent than oedematous malnutrition in patients with HIV [37]. Malnutrition in HIV is typically linked with multiple concurrent micronutrient deficiencies such as vitamin A, iron, selenium and zinc [38]. Mortality is elevated four fold in HIV infected malnourished children compared with uninfected children with malnutrition regardless of nutritional rehabilitation [37]. Initiation of ART is required to prevent HIV disease progression [21, 39, 40]. With both global and national movements geared to increase access to ART for all eligible HIV infected patients and to step up EMTCT there is need to describe the immunological and pharmacological response to nutritional supplementation and ART initiation in LRS [41] so as to better understand the intricacies of HIV pathology. Furthermore, malnutrition and HIV disease states may affect pharmacokinetics (PK) of ARVs through several mechanisms that preclude disrupting drug absorption, protein binding or metabolism [42]. Malnutrition and HIV unusually may result in reduced drug absorption as a result of villous atrophy of the intestinal lining leading to reduction in the absorptive surface area, reduced gastric acidity. The duo may also cause low serum albumin and as a drug carrier in the blood may lead to reduced drug binding or carriage resulting in reduced binding of some drugs in the target cells. In addition lead to increased free-drug levels in circulation thus increased

toxicity levels. Diarrhea and micronutrient deficiency may further impair PK of ART by reducing the transient time of the drugs in the gut and thus in turn reduce the amount of drug reabsorbed affecting immunological recovery.

Definition of Malnutrition.

A pediatric malnutrition definitions working group in 2013 revised the definition of malnutrition "as an imbalance between nutrient requirements and intake that results in cumulative deficits of energy, protein or micronutrients that may negatively affect growth, development and other relevant outcomes" [1]. They took into account 5 major domains in revising the definition and these included anthropometric measurements, growth, chronicity of malnutrition, aetiology and pathogenesis, and developmental/functional outcomes based on available evidence". The classification of malnutrition is either by acute malnutrition occurring less than 3 months in duration or chronic occurring more than 3 months in duration. Based on the aetiology of malnutrition it may be disease related or caused by environmental/behavioral factors associated with reduced nutrient intake/availability or both (Table 1).

Table 1. Main Classification and Definitions/Characteristics of Pediatric Malnutrition

[1]

Class	Definition/Characterization
1. Illness-related malnutrition (severe or moderate)	*Definition*: Illness-related malnutrition (disease/trauma specified), is caused by a nutrient imbalance and may be associated with one or more negative outcomes. *Etiology*: The associated disease/illness/trauma should be specified. If more than one condition is thought to affect nutrition status, specify the primary and secondary conditions. *Severity*[a]: The severity of malnutrition is based on the degree of deterioration in key anthropometric markers and may be severe (usually with evidence of severe inflammation[b]) or moderate (inflammation not severe). *Mechanism*: Nutrient imbalance resulting from one or more of the following conditions: decreased intake, increased requirement, increased losses, and altered utilization of nutrients. *Chronicity*: May be acute (duration less than 3 months) or chronic (more than 3 months).
2. Non–illness-related malnutrition: caused by environmental/behavioral factors (severe or moderate)	*Definition*: Malnutrition from environmental (starvation/socioeconomic) or behavioral factors, resulting from decreased nutrient intake (lower than required), and may be associated with one or more adverse developmental or physiologic outcomes. *Severity*[a]: The severity of malnutrition is based on the degree of deterioration in key anthropometric markers and may be severe or moderate. *Mechanism*: Nutrient imbalance resulting from decreased intake. *Chronicity*: May be acute (duration less than 3 months) or chronic (more than 3 months).

[a]Severity of malnutrition is determined by anthropometric measurements and the relationship of these parameters with standard/reference charts.

[b]The presence or absence of inflammation influences disease-related malnutrition and must be indicated in the definition when improved markers of inflammation become available in the future.

Epidemiology and risk factors of malnutrition in HIV infection

Globally, the prevalence of HIV amongst malnourished children has been estimated to be 30-50% [43]. Malnutrition is responsible for 1 million deaths per year in children younger than 5 years of age most especially those infected by HIV [44]. A systematic review and meta-analysis amongst children who presented with severe acute malnutrition (SAM) in

SSA reported an overall HIV sero-prevalence of 29% with data from 17 studies [45]. It also demonstrated that HIV-infected African infants and children commonly present with marasmus, and wasting was recognized as an independent risk factor for mortality in spite of receiving ART [37, 42, 46]. Urban referral hospitals had the highest burden of disease, with HIV prevalence rates as high as 50% and mortality of 30% among HIV-infected children with SAM [45]. In a cross national analysis of demographic health surveys of SSA countries in HIV infected children, the author documented that young age, male sex, multiple/twin births, low birth weight, mothers low educational status, poverty were risk factors for developing malnutrition in HIV in SSA households [47].

Pathogenesis of Malnutrition in HIV

A combination of HIV and malnutrition can result into profound immune impairment and loss of lean-body mass due to increased metabolic drive and chronic immune activation. This can lead to further immune deficiency, and rapid HIV disease progression. This is further complicated by presence of anemia [48]. Infection with HIV eventually culminates into profound reduction of $CD4^+$ T cells, lymphocyte proliferative responses, and delayed type hypersensitivity (DHTR) responses, all of which are independently associated with an increased risk of disease progression and mortality [49-51].

ART results in suppression of HIV replication, increase in $CD4^+$ T cell numbers and declines in morbidity and mortality from HIV [52]. However, this does not come without hurdles such as drug toxicities, metabolic derangements, immune reconstitution inflammatory syndrome (IRIS) and death [53, 54]. In addition overzealous nutritional supplementation can cause the re-feeding syndrome and may compound the complications of malnutrition [55]. Good nutrition increases resistance to infection and disease, increases energy availability, and thus improves the quality of life. Wasting syndrome in HIV, defined as loss of more than 10% of the body weight with a lack of other detectable cause of wasting other than the HIV infection, needs to be prevented as it is a risk factor of poor outcome even on ART. The current WHO and National Nutrition and ART guidelines recommend that nutritional improvement measures must be initiated before a patient reaches this stage for them to have a beneficial outcome while receiving ART [33, 44, 56].

The pathophysiology of malnutrition in HIV is not yet totally elucidated [57]. Suppression of HIV replication by ART often restores protective pathogen–specific immune responses, but in some patients the restored response is immunopathological and causes IRIS [49, 58]. The leading causes of malnutrition in patients with HIV are reduced intake of food because of reduced appetite, difficulty in ingesting food as a result of oral pathology like oral thrush, *Kaposi sarcoma* or *Candida oesophagitis* or common systemic OIs, fever, side effects of medicines, or depression or vomiting [59]. This may be further compounded with impaired absorption of nutrients as a result of diarrhea and/or vomiting probably due to bacterial infections such as *Salmonella* or *Mycobacterium avium*; viral infections such as cytomegalovirus (CMV) or parasitic infections such as *Giardia, Cryptosporidium parvum, Strongyloides stercoralis,* and *Isospora beri.* Malnutrition may also be caused by nausea/vomiting as a side effect of medications used to treat HIV or OIs or any other systemic infections or malignancies [60, 61]. This compounds the malabsorptive syndromes in malnutrition [62].

The gastrointestinal tract is directly affected by HIV infection and may compound the diarrhea and malabsorptive syndromes leading to inadequate availability of nutrients [55]. HIV causes damage to the intestinal cells by causing villus flattening and decreased D-xylose absorption. This mainly impairs carbohydrate and fat absorption thereby affecting fat soluble vitamins like vitamins A, D, E and K which are important for proper functioning of immune system [57].

There is increased demand for nutrients during fever and infection, including HIV infection, however, nutrients are poorly utilized by the body due to high metabolic over drive [63]. This leads to loss of weight and lean muscle tissue, further causing damage to the immune system. In LRS there is frequently a lack of iron in the diet, which compounded by infections such as malaria and hookworm can lead to anemia [64]. Anemia causes lethargy, reduces food intake and nutrient absorption, and disruption of metabolism, immune defense and muscle wasting [55]. Caretakers with AIDS-related dementia or neuropsychiatric impairment may be unable to care for their children and themselves forget to eat, or unable to prepare balanced meals. Therefore at household level the breadwinner, if HIV infected, will contribute to the nutritional status of his or her

dependents. Dietary intake also varies inversely with level of virus, suggesting that viral replication directly or indirectly suppresses appetite [60].

At a pathogen-host interaction level, the ability to control HIV infection or delay disease progression is probably controlled by a balance between viral and host factors. Studies done in HIV discordant couples have demonstrated that currently there is no consensus regarding the factors that are responsible for protection in HIV-exposed sero-negative people. Nonetheless, factors are certainly multifactorial and include contributions from the host, such as immunological and behavioral characteristics and genetic environment as well as from the virus properties, such as viral tropism, load, and subtype [65]. Furthermore studies done among commercial sex workers that remained HIV-negative after more than 5 years of active prostitution demonstrated that such individuals mimic a model of natural immunity to HIV, thus HIV resistance may be associated with the host's capacity to preserve systemic integrity by constraining immune activity and controlling inflammatory conditions at the mucosal point of entry by the virus. This relies on probably delicate balanced innate and adaptive immune responses [66]. However, impact of host genetic variation on HIV-1 susceptibility was identified early in the pandemic, with a major role attributed to the genes encoding class I human leukocyte antigens (HLA) and the chemokine receptor CCR5. Studies using genome-wide data set showed the strength of these associations relative to variants located throughout the rest of the genome. However, the extents to which additional polymorphisms influence HIV-1 disease progression, and how much of the variability in outcome can be attributed to host genetics, remain largely unclear [67], and even more recently to ART response.

Clinical presentation of Malnutrition in HIV-infected patients

The clinical presentation of malnutrition includes severe visible wasting (marasmus), nutritional edema (kwashiorkor), or a mixed picture (marasmic–kwashiorkor) [45, 68]. In SSA, marasmus occurs more commonly than kwashiorkor in HIV infected children [37], and marasmic children have a higher risk of mortality [55]. HIV-infected children with SAM or moderate acute malnutrition (MAM) are more likely to present with other comorbidities and complications such as TB and acute respiratory infections, persistent diarrhea, and oral candidiasis, which contributes to increased morbidity and mortality

[60]. Concurrent micronutrient deficiencies are common in most children with malnutrition and may contribute to growth failure and disease progression [69].

Management of Malnutrition in HIV

There is no evidence from randomized prospective trials on which to base recommendations for the prevention or management of malnutrition in patients with HIV [41, 70]. For complicated acute malnutrition, micronutrients including zinc, magnesium, selenium, copper, iodine, and iron are supplemented within the standard high-energy milk formulas (F75 and F100) while in the uncomplicated acute malnutrition ready-to-use therapeutic foods fortified with a variety of micronutrients are used [56]. Currently it is the standard of care to provide nutritional supplementation as a prerequisite in the MAM and SAM PLWHIV/AIDS [43]. In the uncomplicated acutely malnourished children it is the standard of care to initiate ART alongside nutritional supplementation whereas in the severely acutely malnourished children, WHO recommends starting ART after nutritional stabilization. Ready to use therapeutic food (RUTF) in the form of Plumpynut® is given to children with moderate-severe wasting (weight-for-height Z-score (WHZ) < −2SD) or are underweight (weight-for-age Z-score (WAZ) < −3SD) according to WHO guidelines and Integrated management of acute malnutrition (IMAM) guidelines [44]. The recommended dosage of RUTF ranges from 150-200 kilocalories per kilogram per day until they reach the target weight, which should be in 6 to 10 weeks [56].

Re-feeding syndrome and Immune Reconstitution inflammatory syndrome in Malnutrition and HIV

The re-feeding syndrome (RFS) and immune reconstitution Inflammatory syndrome (IRIS) are results of treatment interventions influencing the immune system and are two important possible aspects of treatment outcomes in the HIV infected malnourished patients [55, 71]. The RFS involves abnormalities in fluid balance, glucose metabolism, vitamin levels, hypophosphatemia, hypomagnesemia, and hypokalemia in patients usually exposed to enteral or parenteral nutrition after a period of prolonged starvation and weight loss. Patients that have the greatest risk of RFS include those with kwashiorkor or marasmus, anorexia nervosa, chronic malnutrition, alcoholism in adults, and prolonged fasting in cultures or religions that commonly practice extended fasting. IRIS is still a poorly studied problem, though there are some reports showing that children developed

SAM or its complications days after initiating intensive feeding or months after ART initiation [71]. However there is a paucity of data on RFS in HIV infected malnourished children. It has mainly been described in malnourished patients with chronic renal disease, surgical conditions while on parental feeding [72, 73].

IRIS in HIV-infected patients is an adverse consequence of the restoration of pathogen-specific immune responses commonly occurring in the initial months of ART [58]. Previously subclinical infections are "unmasked" or pre-existing OIs clinically deteriorate as host immunopathological inflammatory responses are "switched on". IRIS is most frequently associated with mycobacterial infections [58]. IRIS and RFS result in substantial morbidity and mortality and it is important for clinicians to recognize these two entities in order to provide proper and timely treatment. OIs should be treated before the start of ART to avoid IRIS [74]. ART usually improves the function of the cellular immune system and the more rapidly this improvement occurs the more likely IRIS ensues [75]. Systemic or local inflammatory reactions frequently occur at the sites of the preexisting infection. Unfortunately IRIS and RFS are diagnoses of exclusion: exclusion of common electrolyte imbalances, supra infection, adverse drug reactions, noncompliance, drug malabsorption and drug resistance [75]. In the case of IRIS there may be a temporal association with initiation of ART with good virological and immunological response [76]. Recently, there have been reports of children initiating ART and subsequently developing severe edematous malnutrition in the Anti-Retroviral Research for Watoto clinical trial that demonstrated that 3.2% of 1207 African children initiated on ART were hospitalized for SAM within 12 weeks of ART initiation [71]. Of the 220 children with severe disease, 7% developed kwashiorkor and 3.6% developed marasmus. Their $CD4^+$ T cell % rise was similar in both groups and mortality rate was highest in those who developed marasmus. The mechanism for this severe malnutrition is not clear, but the authors postulated that this may be a form of IRIS in those children with severe malnutrition and severe immune suppression who initiate ART [71].

Nutrition assessment and nutritional recovery

A Ugandan cross-sectional population-based survey of children aged 0–12 years was carried out amongst inhabitants of 25 neighboring villages in rural southwest Uganda. Nutritional status was assessed using the height for age, weight for age and weight for

height and were correlated to the children's HIV sero-status, maternal HIV sero-status and maternal vital status. Out of 5951 children studied, 30% were found to be underweight, 42% stunted and 10% wasted. HIV sero-prevalence was noted to be 0.7%. There was significantly higher HIV positivity amongst the underweight and stunted [77]. Weight is a commonly used anthropometric parameter, however, it is better optimized in relation to other anthropometric measurements as shown in the Ugandan study and according to WHO growth monitoring guidelines [77, 78]. Weight gain is an indication of early response to treatment but not necessary treatment success. Therefore there is need to routinely perform comprehensive nutritional assessments of children on ART in order to monitor treatment response [43]. Nutritional assessment is the systematic evaluation of nutritional status, diet (including caregiving practices and family food security), nutrition-related symptoms and biochemical or radiological markers. It is essential for the early identification of malnutrition and growth faltering. Growth monitoring can also contribute to monitoring HIV disease progression and treatment efficacy of children on ART [55].

A Tanzanian study investigating RUTF supplementation in children receiving ART demonstrated that RUTF had the potential of improving under nutrition as the percentages of underweight and wasting among non-RUTF-receivers were 12.4% and 16.5%; whereas those of RUTF-receivers were 3.0% and 2.8%, respectively. Furthermore among RUTF receivers, children treated for at least four months were less likely to have under-weight, wasting and stunting [79].

Assessing dietary intake in an environment where it is routinely done may be a pain staking exercise that needs to be approached in a multipronged style. This is because children generally should not be consuming single nutrients, food or even food groups but rather a combination of foods. However in LRS due to food insecurity, the methods of food storage, preparation and seasonal availability, this might not occur. Thus, when assessing dietary intake there is need to capture the different foods or nutrients in order to assess the quality of dietary intake against a predetermined standard. The other challenge is that children greatly depend on a carer and thus carer characteristics like availability, literacy and preference may affect the nutrition status of children [80]. A systematic review on the quality of dietary intake methodology and reporting in child and adolescent obesity intervention trials found that the most common dietary method employed in assessing

children's dietary intake was use of a food diary, 24-h recall, food frequency questionnaire, dietary questionnaire and recommended to add check lists [81]. These methods have their merits and demerits. The quality of dietary intake reporting varies according to several factors such as age of the respondent, literacy level, skill of the person administering the tool, time needed to administer the tool, interest of the researcher and appropriateness of the tool in the cultural setting.

Biochemical markers of Nutrition

Biochemical markers greatly supplement clinical and anthopometric assessment. Presently there is no single objective biomarker with the sensitivity and specificity to be a reliable index of malnutrition, predictor of nutrition-related complications, or indicator of food intake. Therefore in the present study, we will use several known biomarkers of nutritional status in combination with anthropometry, and dietary assessment to holistically assess nutritional status. Although markers of protein status such as albumin may assist the clinician to establish progress, they may not correlate with clinical observations of nutritional status. Nonetheless laboratory values are more useful in helping to establish overall prognosis and severity of illness.

Serum Protein

Overall about only 3% of the body's total protein is found in the plasma and extravascular fluids whereas approximately 97% is visceral organ and cellular protein [82]. Since plasma protein measurements are cost effective to perform and because many plasma proteins are synthesized in the liver, plasma protein has historically been used to assess protein status in the body [48]. However several studies have demonstrated that neither serum albumin nor pre-albumin are accurate markers of protein or nutritional status.

Albumin

Albumin has a long half- life of about 20 days so the concentration in the blood changes slowly and cannot be used to measure the impact of nutritional supplementation. There is also a large extravascular pool of albumin that can be available to return to the circulation when needed, thus skewing the results of laboratory tests. There are also many factors, such as infection, acute stress, surgery or cortisone excess, which can decrease albumin

levels even when a person's protein intake is adequate. Dehydration on the other hand may falsely elevate albumin levels [48].

Pre-albumin (PAB)

PAB (transthyretin and thyroxine-binding albumin) has a half-life of 2-3 days, so practitioners have historically assumed it to be a better indicator of protein status. However, PAB is subject to the same factors that make albumin a poor nutritional indicator like inflammatory stress, metabolic stress and zinc deficiency decrease PAB levels. In addition, PAB levels may also be maintained during malnutrition [48].

Haemoglobin

Studies done in SSA have demonstrated that anemia in PLWHIV/AIDs is an important predictor of morbidity and mortality. The prevalence of anemia in PLWHIV/AIDs ranges from 10% in asymptomatic patients to 92% in patients with AIDS. In Uganda, anemia has been shown to affect 92% of young HIV-infected children in a hospital-based cohort with an overall cumulative incidence of 100% [83]. In another study, the prevalence of anemia in HIV-infected patients on ART was found to be 57.6% with 62.2% having mild anemia, 32.0% moderate anemia and 4.8% with severe anemia. The mean haemoglobin (hb) was lower among children with more advanced HIV disease. Microcytic-hypochromic anemia (44.9%) was the commonest type of anemia. Anemia was independently associated with young age, advanced HIV WHO disease stage and low $CD4^+$ T cell count percentage. The children with anemia were most likely to be unable to attain viral suppression (53.4%) while on ART compared to those who had no anemia (86.7%) [84]. Another Ugandan study in 6 months to 12 year old children demonstrated that the prevalence of moderate to severe anemia in children infected with HIV in Mulago Hospital was 50.7%. Moderate to severe anemia was most prevalent among children aged 6 to 24 months indicating that there is early onset of anemia. Several types of anemia were identified and included microcytic normochromic (48.4%), normocytic normochromic (34.9%), microcytic hypochromic (12%), and macrocytic anemia (2.8%) [85].

Glucose

Overall dysglycemia, which comprises hypoglycemia and hyperglycemia, are associated with a high risk of mortality amongst children admitted to general paediatric hospitals

according to a study done in Madagascar in children aged 1 month to 15 years [86]. Hypoglycemia is a common complication in malnourished children and has been associated with high mortality whereas hyperglycemia is also associated with increased infection rates and very poor treatment outcomes in malnutrition. Studies done in Tanzania, Bangladesh, Zambia and Senegal indicated that hypoglycemia is common in malnutrition caused by severe sepsis and failure of feeding. Consequently, in severely malnourished children, dehydration and hypoglycemia increase the risk of dying more than in mild and moderate malnutrition [44].

Transferrin

Iron (Fe) is a fundamental component of hemoglobin and myoglobin, which are important components of the oxygen transporting and storage molecules. It is also a major component of many enzymes that catalyze the redox reactions required for the generation of energy (eg, cytochromes), the production of various metabolic intermediates, and for host defense (eg, nicotinamide adenine dinucleotide phosphate [NADPH] oxidase) [87]. Therefore in malnutrition Fe deficiency is one of the important micronutrient deficiencies. Ferritin is the major Fe storage compound, as well as a readily available source of Fe for metabolic requirements. Though Fe is an essential component in the human body needed for all metabolic pathways it must be tightly controlled especially in the face of infections since invading bacteria and protozoa, such as *Plasmodia* also need iron to survive [88]. Bacteria and *Plasmodia* which are common in the malnourished patients [89] have evolved multiple sophisticated mechanisms for acquiring Fe in environments where very little free Fe is available [88]. This is true for malnutrition states thus the national guidelines advice with holding iron supplementation until the patient is infection free and this depends on the clinician's judgment [56].

The use of serum ferritin and transferrin levels as nutrition diagnostic markers are challenging in diagnosing iron deficiency as they are acute phase reactants which can be affected by inflammation, stress and various infections. However they are used in combination with full blood count and blood film report to extensively describe and define the type of anemia present.

Lipids

HIV infection and its treatments are associated with abnormalities in lipid metabolism [90]. This can contribute to the increased cardiovascular disease risk in PLWHIV/AIDs. Before the advent of ART, a number of studies found that AIDS patients had elevated plasma triglyceride and free fatty acid levels whereas HIV-infected patients without AIDS had decreased total cholesterol and high density lipoprotein (HDL)-cholesterol [91]. In the Multicenter AIDS Cohort Study, total cholesterol, HDL-cholesterol, and low density lipoprotein (LDL)-cholesterol levels declined in PLWHIV/AIDs [92]. On initiation of ART in this study group, total cholesterol and LDL-cholesterol levels rose but HDL-cholesterol remained decreased. In the Swiss HIV Cohort Study, HIV PIs use was found to be associated with increases in plasma total cholesterol and triglycerides [93]. Discontinuation of antiretroviral therapy in the Strategies for Management of Antiretroviral Therapy (SMART) study resulted in a decline in total cholesterol and LDL-cholesterol, but HDL-cholesterol declined as well, leading to an unfavorable increase in the total/HDL-cholesterol ratio [94]. Even in childhood, HIV-infected children receiving antiretroviral therapy were found to have higher total cholesterol and triglycerides, and increased carotid intima-media thickness; thus cardiovascular risk may be heightened among HIV patients even at a young age [95]. A Ugandan study found a high prevalence among HIV-infected children that experienced fat redistribution and hyperlipidemia (27.0% and 34.0%, respectively). Of the children with hyperlipidemia, 16.8% exhibited hypercholesterolemia, 83% hypertriglyceridemia and 29% of children with fat redistribution had hyperlipidemia [96]. The omega-3 fatty acids are the key precursors for the production of eicosanoids like prostaglandins, prostacyclins, thromboxanes, and leukotrines that are necessary in a variety of host defensive mechanisms therefore lipid deficiency in the diet can impair cytokine synthesis which are needed for proper immune response [97].

Micronutrients

Micronutrients include vitamins and minerals, many of which act as co-enzymes for numerous metabolic processes in the body [62]. They assist the body to optimize macronutrient metabolism to provide energy. For example, the B group of vitamins are required for energy metabolism, lipid and nucleic acid synthesis. Vitamin B6 is a co-

enzyme in metabolic pathways of amino acids and their transport from an amino group to a ketoacid [98]. Micronutrients are also involved in nucleic acid synthesis and formation of new cells, for example folate acid in conjunction with vitamin B_{12} is required in the synthesis of red blood cells and integrity of the bone marrow and gastrointestinal cells. Zinc and vitamin A are fundamental components of thymic hormones and are involved in the synthesis of nucleic acid. Zinc deficiency influences both lymphocyte and phagocyte cell functions and affects more than 100 metalloenzymes that are zinc dependent [98]. Thus micronutrient deficiencies, as seen in malnutrition, may result in metabolic derangements at all levels.

Metabolic complications associated with ART in children have been found to be increasing with increased access to ARVs and the length of ART treatment though were previously thought to be rare. The long term metabolic complications include lipodystrophy, dyslipidemia, insulin resistance, hyperlactatemia, osteopenia or decreased bone mineral density (BMD) [55]. It has been postulated that micronutrients play an important role in the pathogenesis of HIV disease. Numerous anecdotal reports, clinical observations, and newly published studies provide evidence of the growing interest and concern about evidence that HIV-infected adults and children have a range of micronutrient deficiencies with increasing severity in advanced disease and in the presence of malnutrition. It appears that micronutrient deficiencies contribute to immune dysfunction, infectious morbidity and disease progression [99].

The Immune system and HIV/Malnutrition

Immunology of Paediatric HIV

Perinatally acquired HIV infection occurs at a crucial time when the child's immune system is developing and most vulnerable [100]. There are striking age related differences in the progression of HIV disease and these may result from the perturbations of HIV related to the age specific differences of a developing immune system. In addition HIV infection gravely disrupts the maternal immune system upon which the infants depend upon for both protection and immune instruction in order to develop their own independent immune system [101].

Until recently, there was limited information on the distribution of HIV target cells in infants and thus the understanding of immunology of pediatric HIV was limited. It is now well established that the HIV virus mainly utilises the CCR5 co-receptor and activated $CD4^+$ T cells for invasion and replication [102]. However, the majority of infant immune cells are dormant and in infants have a remarkable absence of $CD4^+CCR5^+$ T cells in cord blood with a few in lymph nodes. Subsequently, levels of $CD4^+CCR5^+$ expression on peripheral T cells have been found to attain adult levels at about 5 years age. A group of researchers have recently demonstrated that fetal and neonatal intestines have a profound abundance of memory $CD4^+CCR5^+$ T cells with predominantly Th1 and Th17 phenotypes concentrated in gut epithelial layers and lymphoid tissues of the gut submucosa [103]. These memory $CD4^+CCR5^+$ T cells were found to be highly susceptible to HIV-infection *in vitro* without prior activation and are presumably the predominant site of HIV infection, replication, and destruction in infants and children. In adults it has been demonstrated that early HIV invasion and destruction occurs in the $CD4^+$ T cells residing in the gastrointestinal tract and this is a hallmark of the early events in HIV-1 immunopathogenesis in adults, and most of the depleted gut $CD4^+$ T cells belong to the Th17 subset. It is likely that the gut is an important site of viral replication and destruction in infants as well [100]. The distinctive event of HIV infection is depletion of $CD4^+$ T cells with increased viral load leading to an increased susceptibility to OIs. Progressively the large $CD4^+$ T-cell repertoire has been suggested as a mechanism of age-related differences in viral loads and disease progression in children. However, this takes place in a very complex homeostatic environment deranged by HIV infection [100]. In the majority of children a continual state of immune activation characterised by cascade of inflammatory processes stimulated by the presence of HIV infection and coupled with associated metabolic derangements will ensue during this critical window of development. Therefore in the absence of ART the immune dysregulation will profoundly progress to a state of AIDS and finally mortality.

Immune recovery in Malnutrition and HIV

A South African randomized clinical study in PLWHIV with self-reported weight loss, were randomized to receive ART plus FutureLife porridge® nutritional supplement (388 kcal/day) or ART alone for 6 months. This study showed that nutritional supplementation

in combination with ART resulted in significant increases in weight, body mass index (BMI), $CD4^+$ T cells, hemoglobin concentration (Hb), white blood cells and red blood cells. In addition the interventional arm demonstrated better physical activity in patients who had initial weight loss. [104]. A Zambian study carried out in HIV uninfected and infected children who were ART naive found out that there was a $CD4^+$ T cell decline despite nutritional recovery in HIV infected Zambian children. It also showed that, among HIV negative children with severe malnutrition there was no reduction in $CD4^+$ T cell counts thus malnutrition did not affect the $CD4^+$T cell counts. It also demonstrated that the raised $CD4^+$ T cell at baseline were due to probably a reaction to infection burden in this population but normalized after nutritional recovery [105]. In a Ugandan study of HIV uninfected and infected malnourished patients, Bachou noted that $CD4^+$ T cell frequencies in both HIV-positive and HIV-negative children with marasmus were significantly depleted lower than those in children with oedema, however those with HIV demonstrated a much lower $CD4^+$ T cell frequency [37]. This study demonstrated that irrespective of the HIV serostatus, malnutrition affected the $CD4^+$ Tcells most especially if one presented with marasmus.

Immunological and Virological responses to ART in HIV

After the HIV virus has invaded the body, the immune system initiates anti-HIV antibody and cytotoxic T cell production. This process may take one to six months to produce measurable quantities of antibody that are then detected on an HIV serology test. Subsequently the immune response is weakened as memory T cells ($CD4^+$ $CXCR4^+$/$CCR5^+$) are progressively destroyed. Upon ART initiation among HIV-infected patients, there is usually a dramatic change resulting in diminished viral replication, increased $CD4^+$ T cell counts, a reversal of most immunological disturbances, and a reduction in risk of morbidity and mortality. Nonetheless, about 20% of all HIV-infected patients have been found not to achieve optimal immune reconstitution despite viral load suppression. These patients are now recognized as immunological non-responders (INRs) however in some literature this phenomenon is referred to as discordant immune response [106]. Recent studies are now showing that INRs present with severely altered and deregulated immunological functions such as decreased cell production by the lymphopoetic tissue and malfunction. Subsequently there is an increased immune activation,

immunosenescence, and apoptosis [107]. Therefore INRs have demonstrated an increased risk of morbidity and mortality compared to HIV-infected patients with an optimal immune reconstitution. The reason for immunological nonresponse is not well understood.

Several possible mechanisms promoting INR have been suggested. These include: damage to $CD4^+$ T cells that begins long before ART initiation due to direct effects of HIV on thymic tissue, and depletion of progenitor cells [108]. Thymic output may be disproportionally affected in patients who start ART at lower $CD4^+$ T cell counts leading to under-reconstitution of naïve $CD4^+$ T [109]. Lymph node fibrosis is also major feature and correlates with duration of HIV infection prior to ART initiation [110, 111]. Untreated HIV infection leads to a significant activation of the immune system, resulting in a cycle of systemic inflammation, persistent T cell activation, exhaustion and death [112]. The magnitude of HIV replication is measured in plasma to provide plasma HIV RNA levels and is a sensitive and specific predictor of HIV disease progression.

Innate Immune system

Innate or natural immunity is so named because it is present at birth and does not have to be learned through exposure to an infectious agent [113]. It is responsible for providing an immediate response to pathogens; though it is not pathogen-specific [79]. It recognises a limited number of identifying substances called pathogen-associated molecular patterns (PAMPs) on pathogen surfaces which may be common to a wide variety of microorganisms. The commencement of any immune response requires specific receptors of the innate immune system or adaptive immune system to recognise ligands or patterns on organisms or particle. The sensory arm of innate defence system comprises of specific receptors on cells such as macrophages, neutrophils, dendritic cells and natural killer (NK) cells. Examples of such receptors are macrophage mannose receptor, scavenger receptors, toll-like receptors (TLRs), retinoic acid-inducible gene –I (RIG-1) like receptors, nucleotide oligomerization domain (NOD)-like receptors (NLRs) and soluble receptor molecules (mannose-binding lectin, MBL, and complement). These are called pattern recognition receptors (PRRs) and they have a central role in the proper functioning of the innate immune system. They detect pathogens such as bacteria, viruses and parasites. Deficiencies in PRRs are associated with increased susceptibility to infections. Deficiency

in MBL receptors lead to increased risk of cryptosporidiosis and malaria. Ligands of PRRs are molecules characterized by repeated motifs that are recognized in a class-specific manner, so-called PAMPs [114]. Thus, PRRs recognize prokaryotic and viral molecules that exhibit these repetitive patterns. Innate immunity, unlike acquired immunity, has no memory of the encounters, does not remember specific foreign antigens, and does not provide protection against future infection [113]. The white blood cells (WBCs) involved in innate immunity include monocytes (which develop into macrophages), neutrophils, eosinophils, basophils and natural killer (NK) cells. These cells communicate by their response to soluble factors in blood such as acute phase proteins and complement components [113, 115].

About 245 studies published from 1957 to 2014 describing immune characteristics in malnourished children aged 0-5 years were recently studied in a systematic review. They noted that the majority of studies were outdated and used archaic immunological methods. In addition these studies focused on children between 0-5 years, they were cross-sectional in design and were hospital based. [116]. This meant that the knowledge describing nutritional acquired immunodeficiency (NAIDs) in those with non-complicated malnutrition was limited and thus to-date there is still a paucity of information. Nonetheless, the current evidence from these studies demonstrates that both innate and adaptive immunity are impaired by under malnutrition. Signs and symptoms of innate immune dysfunction include impaired epithelial barrier function of the skin and gut, reduction in granulocyte function, circulating dendritic cells, complement components, but preserved white blood cell counts and acute phase response. While adaptive immune dysfunction was shown to include reduced levels of antibodies, such as, soluble IgA in saliva and tears, atrophy of the thymus, tonsils, and/or peyers patches, dampened delayed-type hypersensitivity responses (DTHR), depleted circulating B cells, and a shift from Th1-associated to Th2-associated cytokine production. Lymphocytes in peripheral blood of malnourished children exhibited reduced responsiveness to phytohemagglutinin, however, these children had preserved lymphocyte and immunoglobulin levels. In addition, most malnourished children appear to have a good vaccine response, needless to say, the timing, quality, and longevity of vaccine-specific responses may be impaired [117, 118]. Therefore there is an urgent need for current studies in malnutrition in

children to use up-to-date functional immunological methods in well-characterized longitudinal cohorts of children and to use appropriate well-nourished comparison groups, and evaluate associations between immune parameters and clinical outcomes. In addition there are no articles that have described the expression of pattern recognition molecules like Toll-like receptors in NAIDs in children yet this is an important area in the functioning of the innate immune system.

White Blood cells of the Innate Immune system

Several studies have described an increase in the numbers of circulating granulocytes in the HIV negative malnourished children compared to the malnourished HIV positive children. Generally granulocytes and lymphocytes counts are not decreased in peripheral blood of malnourished children, however in the HIV infected children with malnutrition the majority of children have these parameters depressed [57, 62]. Chemotaxis of granulocytes was reduced in NAIDs, together with diminished ability to adhere to foreign material, reduced ability to ingest particles or bacteria and increased evidence of apoptosis and DNA damage which has also been described in HIV associated malnutrition [119-121]. Three West African studies: two from Nigeria and one from Ghana on children with NAIDs found no difference in the percentages of NK cells compared to the normal children but noted that the malnourished children had lower numbers of NK cells which has not been described in HIV malnourished children [122, 123].

The acute phase response

Acute phase proteins are usually produced as a result of trauma and presence of infection [62]. Cytokines such as interleukin 6 (IL-6), IL-1β and tumor necrotic factor-α (TNFα) are the main mediators of acute phase response. This leads to secretion of positive acute phase proteins (APP), such as C-reactive protein (CRP) and serum amyloid–A (SAA), complement factors, ferritin and α-1-acid-glycoprotein with depression of other proteins, such as pre-albumin, albumin, α-feto-protein, α-2-HS-glycoprotein, otherwise known as the negative APPs [124]. In most studies in malnourished children it was found that positive APP levels were upregulated, especially CRP, α-1-acid-glycoprotein and haptoglobulin, and the negative APP, including transferrin and pre-albumin were abnormally low in the presence of infection [57]. However, some studies showed inconsistent results. Exposure to vaccines in malnourished children demonstrated normal or increased levels of positive

APPs with febrile response to measles vaccine and this was more marked on repeat exposure after nutritional rehabilitation [57].

The complement system

The complement system consists of plasma proteins which are synthesized in the liver and activated in response to the presence of pathogens, in particular bacteria. Complement activation involves the sequential proteolytic cleavage of inactive precursors to yield the production of a series of proteins that bind the pathogen surface and stimulate local inflammation. The result is to recruit immune cells to the site of infection, to opsonize pathogens for phagocytosis or cytolytic killing by other cells and to form a membrane attack complex, which directly kills bacterial cells [113]. The complement system is activated through three main pathways namely the classical pathway, alternative pathway and mannose binding lectin pathway with the complement protein C3 playing a pivotal role in all the pathways [125].

Acquired Immunity

The adaptive immune system is also known as the acquired immune system and is composed of highly specialized, systemic cells and processes that eliminate pathogens or prevent their growth. The adaptive immune system includes the B cells that have a B cell receptor also known as immunoglobin (Ig). They mature in the bone marrow where they are synthesised and produce one type of antibody in response to specific antigen [126]. Apart from producing antibodies, B cells are APCs, secrete cytokines, and set up the architecture of lymphoid organs. The T cells are part of the acquired immune system and have the T-cell receptor (TCR) for which the ligand is antigen presented by the human leucocyte antigen (HLA) molecules on APCs. The TCR molecule is a heterodimer made up of an α and a β polypeptide. The structure of TCRs is almost analogous to the antibody molecule, however in contrast to antibodies the TCR is able to recognise amino acids within a peptide of 8–11 amino acids bound to a specialised groove on the surface of an MHC Class I molecule and 10–30 amino acids for MHC Class II molecules. $CD4^+$ cells carry TCRs that recognize antigen presented by MHC Class II and $CD8^+$ T cells recognize antigens presented by MHC Class I molecules. TCRs are formed by rearrangements of genetic determinants expressed in germ line cells, rearrangements that occur during maturation of lymphocytes. The acquired immune system is called into action against pathogens that

are able to evade or overcome innate immune defenses. There are two main arms of adaptive immunity: humoral immunity, mediated by antibodies produced by B lymphocytes, and other soluble factors, and cell-mediated immunity, mediated by T lymphocytes. The acquired immune system also orchestrates tolerance to self and harmless material, such as the gut mictobiota [113, 115]. The acquired immune responses are characterised by the secretion of antibodies by the B cells or activation of sensitised T cells. The participation of the adaptive response when invaded entails several discrete steps and they appear to delay in response. These broadly include recognition of the intruder or antigen through the interaction of various lymphocyte subpopulations; activation and proliferation of the responding cells; transcription of genes; synthesis of proteins; and generation of the specific end products such as antibodies, cytokines, etc.

The thymus and lymphatic system

The thymus and lymphatic system is the main lymphatic organ in the adaptive/acquired immune system where the maturation of the T-lymphocytes occurs. The thymus is located in the mediastinum and it is largest at birth and gradually undergoes involution after childhood with progressive reduction of T-lymphocyte production with age [127]. Autopsy studies in malnourished children demonstrated severe thymus atrophy which they referred to as “nutritional thymectomy.” This involves the destruction of the thymus during malnutrition resulting in depletion of thymocytes which are replaced by connective tissue with decreased cortico-medullary differentiation [128]. In large vaccination studies in Guinea Bissau and Bangladesh it was demonstrated by serial ultrasound scans that nutritional thymectomy improved with nutritional rehabilitation however the thymus never achieved the optimal size for age. Breast fed infants often had larger thymuses compared to formula fed children and had a higher chance of survival [129]. Autopsy studies on histology reported reduction of germinal centers and depletion of lymphocytes in the para-cortical regions of the lymph nodes, spleen tonsils, appendix and Peyer’s patches in malnourished children [130, 131]. Studies in living children demonstrated smaller tonsillar sizes in the malnourished children in comparison to the well-nourished children [132].

Delayed type hypersensitivity response (DTHR)

The function of the cellular immune system is commonly assessed by DTHR using purified protein derivative (PPD) or commonly known as the Mantoux test named after Charles Mantoux who discovered it in 1912. PPD is manufactured from cultures of *M. tuberculosis* [133]. Other intradermal applications of substances used are *Candida,* phytohaemagglutinin (PHA) and local contact sensitizer called 2-4-di-nitro-chlorobenzene (DNCB) [57]. A standard dose of 5 tuberculin units which are 0.1 mls by volume of PPD is injected intradermally into the skin. It is commonly injected on the forearm in the cubital fossa or the posterior aspect of the thigh in the popliteal fossa and read 48 to 72 hours later. The skin reaction of interest is characterized by skin induration and or occasional vesiculation or necrosis whose diameter is then measured in mm or cm. The immunological reaction involves T cells previously exposed and sensitised to TB infection are recruited to the skin site where the PPD has been injected and through the release of cytokines, the skin appears inflamed and raised in most reactions. The cytokines induce the induration through local vasodilatation, edema, fibrin deposition, and recruitment of other inflammatory cells to the area [134]. The results of this test must be interpreted carefully. A positive PPD test is when the induration is ≥5mm in diameter in an HIV positive person and ≥10mm in diameter in an HIV negative person irrespective of the nutritional status. The majority of studies indicated that there is frequently no positive reaction that is an induration of ≤ 5mm in diameter in malnourished children most especially those infected with HIV and previously vaccinated with Bacillus Calmette-Guérin (BCG) in the attempt to screen for TB [132]. Some studies described more diminished reactivity in odematous malnutrition while others described it in the non-odematous type of malnutrition [132]. Zinc supplementation was found to improve the DTHR in malnourished children in general [135].

Lymphocytes in Blood

Lymphocytes are one of several different types of WBC. Each type of lymphocyte has a specific function, and they all work together to fight illness and disease. In earlier studies, T lymphocyte cells were identified as those that formed rosettes with sheep red blood cells while latter studies use monoclonal antibodies to CD3. The alpha-beta (αβ) heterodimeric T-cell antigen receptor binds antigen in association with major histocompatibility complex

(MHC) proteins on host cell surfaces. Two disulfide-linked glycoproteins, α and β chains are associated on T-cell surfaces with a complex of proteins called CD3. Human CD3 consists of at least 4 proteins: gamma (γ), delta (δ), epsilon (ε), and zeta (ʒ). The anti CD3 antibodies attach to the epsilon chain of the CD3 protein complex. In most of the earlier studies using the rosetting method, lower levels of T lymphocytes were found in malnourished children [136]. More recent studies have shown that T-lymphocytes and $CD4^+$ T cell counts appear normal in malnourished children when measured by flow cytometry. These levels may to be altered by infection rather than nutritional status and do not correlate with malnutrition related immunodeficiency [137].

In healthy normal children when T lymphocytes are incubated with PHA there was proliferation however in malnourished children there was a dampened response. In addition malnourished children supplemented with zinc showed improved proliferation [135].

With the advent of CD4 Pima™ machines for enumeration of T cells in the whole blood samples in the HIV programs under the President's Emergency Plan for AIDS Relief a number of studies have investigated the effect of malnutrition on $CD4^+$ T cell numbers. Malnourished children were found to have decreased $CD4^+$ and $CD8^+$ T cell numbers in whole blood samples compared to well-nourished children. The majority of studies have shown similar or higher levels of $CD4^+$ T cells in HIV negative malnourished children, however, the presence of bacterial infections caused depression of the $CD4^+$ T cells [137]. In the presence of HIV, $CD4^+$ T cells were reduced and despite nutritional rehabilitation the $CD4^+$ T cells further declined. Therefore changes in $CD4^+$ T cell numbers may not be attributed to malnutrition regardless of whether the child is HIV positive or not as malnourished children usually have a multitude of underlying infections and untreated HIV further affects the $CD4^+$ T cells [105].

In a Mexican study using flow cytometry to assess surface markers of T lymphocytes in malnourished children and healthy children with bacterial infections, it was found that malnourished children had lower numbers of circulating effector T lymphocytes that expressed CD62L and CD28, low numbers of activated T lymphocytes that expressed CD68 or CD25 and markedly reduced memory T lymphocytes that expressed CD45RO+.

However in contrast a Ghanaian study found similar numbers of activated T lymphocytes expressing HLA-DR in malnourished and well-nourished children [137].

On the contrary other studies have demonstrated that malnutrition leads to decreased adipocyte mass resulting in changes in adipocyte-secreted hormones and T-cell numbers and function as a result of decreased circulating leptin levels [138]. There is growing evidence on the relationship and role leptin, inflammation especially adipocytokines and malnutrition. Leptin has been found to be an important mediator for regulation of immunity and nutrition. Leptin has also been shown to modulate both innate and adaptive immune responses as its deficiency has been found to be associated with dysregulation of cytokine production, increased susceptibility toward infectious diseases, autoimmune disorders, malnutrition and inflammatory responses [139]. Probably immunodeficiency in malnutrition may be partly driven by leptin levels that are low which have also been demonstrated to predict mortality in children with SAM [140]. Leptin is an adipokine that has direct and indirect effects on T cell numbers and functions. It promotes Th1 and Th17 cell development and cytokine production while inhibiting Th2 cytokine production and Treg proliferation [141]. A Ugandan study in 2014 demonstrated that low levels of leptin were the single most important biomarker to predict mortality during inpatient treatment of malnourished children but did not report its effect on T lymphocyte population [142]. Furthermore there is emerging evidence on the effect of treatment on leptin levels in infectious diseases and this has been studied among TB infected children who usually will present with malnutrition. A study done in Indonesia showed that leptin is crucial in improving cellular immune response as the leptin levels increased this enhanced weight and fat gain [143] Immune responses need large amounts of energy to drive the process. Activated T cells have demonstrated high consumption of energy evidenced by the increase in glucose utilisation and aerobic glycolysis to drive the process in normal situations. In the presence of malnutrition as it is a state of deficiency of nutrients it has been found to be associated with profound immune deficiency and increased infection risk. Although it is clear that immunity is suppressed in times of nutrient stress, mechanisms that link systemic nutrition to T cell function are poorly understood. However, growing evidence showing that fasting may result into persistent defects in T cell activation and metabolism, as T cells from fasted animals had low glucose uptake and

decreased ability to produce inflammatory cytokines, even when stimulated in nutrient-rich media. Together, these data demonstrate that induction of T cell metabolism upon activation is dependent on systemic nutritional status, and leptin links adipocytes to metabolically license activated T cells in states of nutritional sufficiency [144]. Future studies are required to access the specificity of serum leptin levels as a marker of treatment response in children with malnutrition and HIV as they invariably present with wasting.

B-lymphocytes and antibody levels

B cells are derived from pluripotent haematopoietic stem cells and are produced in the human foetal liver at early stages of gestation, and in the bone marrow by 14-17 weeks of gestation. B cells are at the centre of the adaptive humoral immune system and are responsible for mediating the production of antigen-specific Ig called antibodies directed against invasive pathogens. Previous studies carried out prior to the 1990s found that B lymphocyte levels were unaffected or even higher in the malnourished children while the later studies found reduced B lymphocytes in the malnourished children [136]. B lymphocytes, macrophages and Kupffer cells are clearly decreased during nutritional deficiencies. In some studies, Ig levels in blood were frequently similar or higher in malnourished children in comparison to healthy controls. In particular, IgG, IgM and IgA levels were found significantly raised in odematous malnutrition with dermatosis while IgE and IgD showed no clear pattern [122].

Immense efforts in the field of neutralising antibodies specific to HIV have been demonstrated by the numerous studies done over the decades. Advances in technology regarding HIV specific antibodies using single-cell antibody cloning techniques have led to the isolation and characterisation of antibodies from people with HIV infection that can neutralize several HIV variants [145]. These antibodies are referred to as broadly neutralizing antibodies detected in about 25% of persons with untreated HIV infection. This may probably imply the presence of continuous host immune response towards ongoing viral replication, generation of large numbers of viral variants, and shifting antigen exposure. However studies have shown that though broadly neutralising antibodies may exert some selective pressure as they develop, they generally do not reduce viral burden, improve health, or slow the progression of disease [146].

Vaccine responses were mainly studied in children with mild-moderately malnourished children and this was assessed either by sero-conversion rates or antibody titre response. Studies looking at sero-conversion rates in malnourished children showed a mixed pattern of results; however, in the severely malnourished children they demonstrated a reduced sero-conversion rate or a slow conversion rate. In the severely malnourished children findings showed reduced antibody titers [147]. The majority of the studies indicated that malnourished children had similar adverse reactions to vaccines compared to the well-nourished children except for the measles vaccine where they reported higher incidences of diarrhea, pneumonia and fever post vaccination [148].

Cytokines

Cytokines are effector molecules that act locally between cells or systemically [57]. Cytokines that have been found to be low in serum in malnourished children include IL-1, IL-2, interferon gamma (IFN-γ) and granulocyte macrophage colony stimulating factor. Furthermore some studies have demonstrated no response after *in-vitro* stimulation of T cells with lipopolysaccharide (LPS) and normal response with leptin [149, 150]. Cytokines found to be consistently high in malnourished children include IL-10, TNF-α, IL-4 and IL-6. Therefore in malnutrition, though cytokine production is reduced it appears to shift the balance of pro-inflammatory Th1 versus anti-inflammatory Th2 cytokine by expressing an increased production of Th2 cytokines in comparison to Th1 cytokines [151-153].

Innate T cells and NK cells (gamma/delta T cells, iNK T cells, MAIT cells and NK cells).

HIV infection invariably leads to AIDS in the absence of ART through extensive destruction of $CD4^+$ T cells. AIDS characterized by immune dysregulation affecting several cells including $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages, NK cells, and ɣ/δ T cells [154]. In humans, γδ T cells comprise a minor subset (1 to 5% on average) of circulating T cells but may represent as much as 50% of the T cells present within the mucosa-associated lymphoid tissue [155]. γδ T cells are involved in recognition of microbial pathogens and as a result play an important role in the adaptive immune system in stimulation of Th1, Th2 and Th17 cytokine production [156]. There are two main γδ T-cell subsets, that express either the first variable region (V1) or the second variable region (V2) of the δ locus from the T-cell receptor (TCR) [157]. The $V\delta1^+$ γδ T-cells are found predominately

at mucosal sites and can respond to non-classical MHC complex molecules expressed on infected or tumour cells whereas Vδ2^{+} γδ T-cells are mainly found in the peripheral circulation and respond to non-peptide phosphoantigens [158]. In HIV infection Vδ2 T cells are depleted from the circulation and Vδ1 T cells are expanded. γδ T cells like CD4^{+} T cells can express CXCR4 and CCR5 co-receptors which act as receptors for the attachment and entry of the HIV virus [159]. With ART treatment there is gradual repopulation of Vδ2 and improved response to HIV and OIs. The impact of malnutrition in combination with HIV on γδ T cells has not been nor has been the impact of nutritional rehabilitation.

No studies to date investigated immune response in the presence of nutritional rehabilitation in malnourished children receiving ART despite the high burden of malnutrition and HIV and their combined contribution to high morbidity and mortality.

Pharmacokinetics of ART in Malnutrition and HIV

Numerous studies have shown that malnutrition is a disease spectrum with varying effects on drug pharmacokinetics (PK) [160]. Most studies on PK in malnutrition were done in the early seventies and eighties before the event of ART. PK studies have drastically decreased and therefore there are limited studies on ART PK among malnourished PLWHIV/AIDs. Despite the enormous global burden of malnutrition and its association with a wide spectrum of infections and severe complications, the PKs of drugs used for management of co-morbidities that occur in malnutrition states have not been extensively studied. Malnutrition significantly decreases total clearance and increases the half-life of many drugs primarily metabolised in the liver, which may indicate a need for modifications of the doses of these drugs in the acute phase management of infections in malnutrition. The current WHO guidelines state that all children infected with HIV below 15 years of age must initiate ART [22]. A study on ART outcomes in LRS for HIV-infected children aged less than 5 years showed that providing ART for this age group is feasible, with a mortality rate over 48 months of follow up being 6.5%, 55% of whom died in the first 3-6 months with a loss of follow up of 10.3%. Overall, 3.8% experienced severe drug toxicity necessitating switching from 1st line to 2nd line ART [40]. A retrospective analysis was performed on a Ugandan SAM HIV infected cohort to evaluate the mortality among malnourished children in relation to the timing of ART initiation and found that mortality

was higher in those children who initiated ART within 10 wk of diagnosis as opposed to those who initiated ART later (OR: 2.8; 95% CI: 1.33–5.90; P = 0.007) after adjustment for age, sex, $CD4^+$ T cell frequencies, and WHO clinical stage [71]. In this cohort, early initiation of ART was associated with a higher mortality and may have been related to concurrent infections and/or complications of SAM. The authors suggested that the optimum time to initiate ART in severely malnourished children needed further evaluation [71]. However other studies have shown delay of ART in SAM children after nutritional rehabilitation is also associated with further worsening of the immunosuppression [105]. Moderate wasting that is unresponsive to nutritional therapy is a WHO stage III condition and severe wasting is a stage IV condition, both of which qualify HIV-infected children with MAM or SAM for the initiation of lifelong ART. However, the optimal timing of ART initiation in children with SAM and optimal dosing in these children has not been established [55]. The initiation of ART in children with SAM is complicated by the complex interplay between their unpredictable and changing physiology or metabolic status related to both HIV and SAM, including numerous oxidative stressors, therapeutic feeding options, ART, HIV viral load and persistent immune activation, changes in lean body tissue and drug distribution, mitochondrial dysfunction and ARV toxicity, hepatic and renal dysfunction, altered intestinal absorption of oral medications, potential impact of nutritional supplementation like re-feeding syndrome, potential interactions with anti-TB, anti-fungal, anti-malaria medications, potential occurrences of OIs and the possibility of triggering IRIS [161].

The Cytochrome P450 system

Cytochrome P450 system (CYPs) is a family of proteins that are oxidative enzymes containing heam as a cofactor. They are localized on the cytosolic side of the endoplasmic reticulum with a few on the matrix of mitochondrial inner membrane. In humans, xenobiotic and drug metabolizing CYPs are mainly located in the liver and intestines but can be found in the kidney, skin, brain and lungs. Twelve CYP families have been studied to date and most drug metabolizing enzymes belong to the highly polymorphic CYP1, CYP2 and CYP3 subsets that are responsible for over 80% of all phase 1 metabolism of drugs in clinical use. Thus the human CYPs constitute several subfamilies with varying expression, activity and substrate specificity [162].

CYP2B6 and CYP3A enzymes

CYP2B6 is a member of the cytochrome P450 family that constitutes about 2-10% of the total hepatic CYP content [163]. It is also expressed in the brain and is thought to be an important factor in the metabolism of drugs acting at the central nervous system (CNS) level with their neurological effects [164, 165]. It is inducible by several drugs and xenobiotics and has a high inter-individual variation [163]. CYP3A is also a member of the cytochrome P450 and has a wide substrate spectrum, high propensity to induction or inhibition. CYP3A accounts for over 35% of all the CYPs expressed in the human liver and metabolises about 60% of all currently marketed drugs [166] including ARVs such as PIs and NNRTIs. Several diseases such as HIV, hepatitis B (HBV), hepatitis C (HCV) affect the liver function and the expression of CYPs and are further affected by inflammatory, nutritional and drug-drug interactions [167]. The CYP enzymes are generally inducible through a process involving de novo ribonucleic acid (RNA) and protein synthesis that has been demonstrated in studies using transcription and translation inhibitors. The commonest mechanism of induction is by ligand activation of key transcription factors including pregnane X receptor (PXR) and constitutive androstane receptor (CAR) resulting into increased transcription.

Other drugs known to increase CYP3A4 activity include NVP, benzodiazepines, fluconazole, ketoconazole, oral contraceptives, clarithromycin, calcium channel blockers, morphine, methadone, steroids like dexamethazone and rifampicin [168]. Considering our study population are children aged 6 months to 12 years in a LRS, the most likely drugs that they may receive alongside the ARVs are rifampicin and antifungals, because of the high TB and fungal infection burden in malnourished and HIV patients, and benzodiazepines in case of convulsive disorders. Therefore there is a high chance of having drug-drug interactions during the treatment of HIV malnourished children and the co-infections they sometimes develop.

Pharmacogenetics of CYP3A4 inducers

Nevirapine

NVP comprises the backbone of most of the first-line ART in Uganda in both adults and children and there is no exception for those who are malnourished [32, 33]. NVP is the still

the commonest NNRTI used in several ART regimens currently [169]. NVP is taken orally and has different formulations. It is well absorbed with absorption rate >90% and it is metabolized in the liver by the CYP450 system and has a low protein affinity therefore crosses the blood brain barrier with ease achieving high concentrations in the central nervous system (CNS). A Ugandan study in children aged between 1 year and 4 years demonstrated that the PK of ARVs varied with the formulation received and in an adult study on stavudine, NVP levels were also found to be low [170, 171]. Hepatic metabolism of NVP is mainly through induction of CYP enzymes 3A4 and 2B6, forming 2-, 3-, 8-, and 12-hydroxynevirapine. These products are then glucuronidated by uridine 5'-diphospho-glucuronosyltransferase (UDP)-glucuronosyltransferase (UGT) enzymes and excreted in the urine. Other CYP enzymes that metabolise NVP are CYP3A5, CYP2C9 and CYP2D6 however this is at a minimal level [172]. NVP has a property of auto-induction where it induces production of CYP3A4 resulting in increased metabolism of NVP. This also results in decreased plasma half-life, from approximately 45 hours using a single dose of NVP to approximately 25 - 30 hours (multiple dosing with 200 - 400 mg/day). Auto-induction is usually complete in 28 days resulting in a steady-state plasma NVP trough concentration of 4.7 mg/ml ranging from 3.6-6.4 mg/ml and this is stable for at least one year of therapy [173]. Several studies have shown that NVP therapy is associated with serious skin rash, categorized as Steven Johnson's syndrome, and fatal hepatotoxicity. It also exhibits drug-drug interactions with rifampicin leading to suboptimal NVP blood levels in children on anti-TB treatment [174] and with artemether lumenfantrine leading to suboptimal levels of this antimalarial drug. EFV was also been found to cause suboptimal levels of artemether-lumefantrine in patients receiving ART in Uganda [175].

Efavirenz and Rifampicin

The majority of HIV malnourished children will be treated for TB co-infection especially in the 1st 6 months after diagnosis of HIV. This implies that malnourished HIV infected children will have to be initiated or switched to an EFV based regimen. In addition, TB diagnosis also poses a diagnostic challenge in pediatrics, moreso in malnutrition in the face of HIV so it increases the probability of being treated with anti TB drugs. EFV exhibits pharmacokinetic variability causing varied clinical response with a narrow therapeutic range. Plasma concentration levels greater than 4 μg/ml is associated with more CNS

toxicity including sleep disorders, hallucinations, insomnia and dizziness [176]. EFV concentrations below 1 μg, which is suboptimal for treatment of HIV, are associated with increased rate of virological failure. Inter-patient variability results from variability in hepatic metabolism and the extent of P-glycoprotein (Pg-P, a multi-drug resistant transport protein) mediated movement across plasma membranes [177]. EFV absorption is increased by fat-containing meals, potentially increasing plasma concentrations of the drug by 50%, of which 99.75% is bound to albumin. Hypothetically, in the current study we expected higher concentrations of EFV in the children who were supplemented with RUTF due to the high fat content in RUTF. EFV metabolism primarily is via CYP3A4, and 2B6; undergoes autoinduction (20-40%) during first two weeks of therapy whose major metabolite is inactive [178].

The inactive metabolites of EFV are hydroxylated metabolites including 8-hydroxyefavirenz (8-hydroxy-EFV), produced predominately by the enzymatic action of CYP2B6 an isoenzyme of cytochrome P450 (CYP450) system. CYP3A4, CYP3A5, CYP1A2 and CYP2A6 may play a minor role in this xenometabolic step. EFV can also be hydroxylated to 7-hydroxy-EFV by CYP2A6, a minor pathway of EFV metabolism accounting for around 23% of overall EFV metabolism *in vitro*. Studies of human liver microsomes have shown that the formation rate of 8-hydroxy-EFV displays considerable variability in different samples. EFV metabolism has been shown to be majorly dependent on CYP2B6 activity; which exhibits variable levels of expression that is associated with genetic polymorphism. However, more recent studies have shown that EFV is extensively metabolized by intestinal and hepatic CYP3A4 enzyme and EFV is also known as to be a potent CYP3A4 modulator coupled with having drug-drug interactions. However there is paucity of data regarding CYP3A4 activity in HIV infected malnourished patients most especially children [178]. The lack of knowledge in addition to the inter-individual and intra-individual variability in metabolism of EFV poses a challenge on the extent of drug left available to clear the HIV infection.

A common polymorphism of CYP2B6 is a single nucleotide polymorphism, with a base change at position 516 from G to T (CYP2B6–G516T), which is associated with reduced EFV metabolism resulting in elevated plasma EFV concentrations. Homozygous CYP2B6–

516T/T carriers can have substantially higher EFV plasma values compared with CYP2B6–516G/G homozygotes [179]. The CYP2B6–516T allelic variant is more common in African populations with a reported prevalence of 36% to 60% in adult populations. It was recently found out that CYP2B6*6 accounts for about 50% of inter-individual variability of EFV PK as the main predictor of EFV plasma concentration in Ugandan HIV infected adults on an EFV based regimen [180]. This is not as extensively studied in children; however Saitoh et al reported the median oral clearance rate of EFV was significantly lower in children with the CYP2B6–516T/T genotype than in children with either the G/T or the G/G genotype [181] whereas expression of the CYP2B6*6-516GG genotype in children was associated with 50-70% of suboptimal EFV concentration of <1µg/ml [182]. Physiological changes in growth and development, immature enzyme systems and clearance mechanisms greatly affect drug pharmacogenetics in children. Recent studies done in children have found out that recommended dosing guidelines do not necessarily achieve optimal EFV concentrations [183]. EFV has been found to cause CNS toxicity, with 20% to 40% of adults reporting CNS symptoms or neuropsychiatric adverse events though similar findings in children were noted to occur at a lower frequency and these included dizziness, nightmares, insomnia, mood changes, and less frequently, more severe psychiatric symptoms such as epilepsy, depression, suicidal ideation, and psychosis [184].

The predominant mode of EFV excretion is as glucuronides in the urine, with 8-hydroxy-EFV-glucuronide being the major metabolite found. EFV can be directly glucuronidated to EFV-N-glucuronide (though this is a minor pathway after the first dose of EFV), which *in vitro* studies have shown is carried out by uridine 5'-diphospho-glucuronosyltransferase (UGT2B7). Multiple UGT isoforms can act upon the three hydroxylated EFV metabolites to produce glucuronide forms, including UGT1A1, 1A3, 1A7, 1A8, 1A9, 1A10, and 2B7 [178]. Rifampicin induces expression and activity of CYP2B6, which is the main metabolic enzyme for EFV. In human hepatocytes, the increase in CYP2B6 activity due to rifampicin varies widely from 2.5 fold to 13 fold [185]. *In vivo*, co-administration of rifampicin and EFV may lead to 22-26% reduction of EFV compared to administration of EFV alone although more recent studies in humans report no significant differences in EFV concentration with or without rifampicin co-treatment. Inter individual differences in the inducibility of EFV metabolizing enzymes by co-administered rifampicin have been

reported. The variability in EFV concentration was found to be greater with rifampicin administration than without. Many malnourished patients in Uganda are co-infected with HIV and TB and require simultaneous treatment for both diseases [186]. There is a paucity of data on the complex pharmacokinetic interactions between antiretroviral and anti-TB drugs in malnourished children and the available data are not representative of the African setting, hence it is important that these complex drug interactions be characterized fully.

In summary no studies have to date investigated the pharmacological responses in the presence of nutritional rehabilitation in malnourished children receiving ART despite the high burden of malnutrition and HIV and their combined contribution to high morbidity and mortality. Most evidence on the PK and PD studies in children are done in older children who were well nourished and adults. Most evidence is extrapolated from adult studies and western studies that are not so relevant to African children.

Knowledge, perceptions and experience towards RUTF and ART

There is paucity of information on knowledge, perceptions and attitudes in nutrition supplementation in HIV and most studies are around infant feeding [187]. A few studies done recommend medical workers to observe the patient take the RUTF in the clinic to ensure they appreciate the taste for the adults and the children are able to swallow the RUTF while for the children they assess the patients ability to swallow. However studies on adherence to RUTF are still few and mainly concentrate on the acceptability of using RUTF in programs [188, 189]. A study done in India on the nutrition-related knowledge, attitude, and practices among PLHIV was low and lacking in in-depth information [190]. Its findings are similar to the Ethiopian study which found out that despite health workers providing RUTF for treatment reasons in children, their caregivers use it also for meeting broader food and economic needs of the household endangering the benefits to the affected children and would explain the poor adherence rates seen at program level [191].

Problem statement, justification and objectives.

Problem Statement

Children remain at risk of HIV infection, mainly through vertical transmission (95%) [10]. Most of these infections are diagnosed late, often with advanced HIV disease. Globally, it is estimated that more than 2 million children are living with HIV infection and >90% of these reside in sub-Saharan Africa (SSA). Severe acute malnutrition (SAM) remains a major problem for HIV-infected children who live in LRS, and SAM has been documented to be an important risk factor for mortality. SAM in HIV-infected children is associated with life threatening conditions such as electrolyte disorders, micronutrient deficiencies, and severe infections, which contribute to the high mortality [56, 75]. In countries where ART has been successfully introduced, children and infants can now survive to adolescence and adulthood [33, 39]. WHO guidelines for ART in malnourished children recommend starting ART after nutritional stabilization is achieved, but there is a paucity of literature describing the response to ART in SAM [37]. This greatly affects the timing of introduction of ART in these children as nutrition stabilization may take a longtime especially in the very sick children and HIV disease therefore progresses leading to an increase of viral reservoir [145].

In general chronic illnesses affect PK of drugs through various mechanisms such as through interruption of absorption, protein binding and metabolism. In severe malnutrition there is markedly reduced drug absorption as a result of atrophy of the villi that form the small intestinal lining, reduced gastric acidity, reduced serum albumin leading to reduced binding and transportation of various drugs like EFV. This is further compounded by diarrhea and micronutrient deficiency that impacts on absorption.

SAM may present clinically with severe visible wasting (marasmus), nutritional edema (kwashiorkor), or a mixed picture (marasmic–kwashiorkor) with the most prevalent form of SAM in HIV being the non-oedematous malnutrition [30] which is associated with high risk of mortality with severe immunosuppression compared to the oedematous form of acute malnutrition. SAM HIV infected children are more susceptible to other comorbidities like pneumonia, oral candidiasis, persistent diarrhea, TB, malignancies that will further result in reduced food intake and absorption in spite of an increased metabolic or immune

activation [56, 75]. This is compounded by micronutrient deficiencies such as iron, zinc, selenium and vitamin A leading to rapid disease progression. Though vitamin A supplementation has shown to reduce all-cause mortality in HIV infected children, micronutrient supplementation alone does not adequately restore the depleted stores, improve weight gain and growth, thus there is need for both macronutrient and micronutrient supplementation in therapeutic doses. ART-naïve SAM patients with HIV have been documented to have a mortality rate of more than 4 times that of HIV uninfected SAM patients as a result of metabolic complications and disease burden. Bachou et al reported a mortality rate of 24% in Ugandan malnourished hospitalized patients with more than 70% of the deaths occurring in the first one week of admission [3]. Though, HIV infected ART naïve SAM patients respond as well as HIV uninfected SAM during nutritional rehabilitation they remain severely immunosuppressed needing ART. In addition studies in Uganda and Zambia have indicated SAM HIV ART naïve patients have longer hospitalization, poor weight gain and increased risk to relapse to severe malnutrition with high fatality [68].

Successful initiation of ART in children is followed by a rapid decline in viral load, a rebound in $CD4^+$ T cell count, a reduction in mortality, and a rapid gain in weight, especially in the first six to twelve months of ART. However, clinicians have noted that certain patients deteriorate after starting ART despite virological suppression and immunological improvement with a paradoxical emergence of certain OIs thus IRIS. A Ugandan study demonstrated the burden of IRIS in children to be 38% and 55% of IRIS events occurred in the first month of ART initiation [119]. Malnourished children have also been noted to deteriorate after initiating therapeutic feeding and the highest mortality has been noted to occur in the first 72 hours of admission [3, 57]. Therefore the combination of IRIS and re-feeding syndrome (RFS) in HIV malnourished children after ART initiation and nutritional supplementation may result from rapid immune reconstitution leading to severe morbidity and mortality. In addition, some studies have demonstrated that starting ART when severely immunosuppressed may result in a higher risk of having INR despite spending several years on ART [192]. Studies on ART timing in malnourished children are unknown as most studies were performed in adults. It is imperative therefore that the extent of nutritional supplementation is optimized for malnourished children on ART

initiation. Secondly there is need to document the effect of ART on nutritional, immunological recovery and pharmacological response during nutritional rehabilitation.

Justification of study

In the area of nutrition and HIV, children deserve special attention because of their additional needs to ensure growth and development and their dependency on adults for adequate care. Therefore it is important to devise means to ensure that this vulnerable population receives optimum nutrition and care so as to improve their survival. Adequate nutrition is important for optimal immune and metabolic function. Appropriate dietary support and ART timing will improve clinical, nutritional, immunological and pharmacological outcomes by reducing the incidence of drug toxicities, and attenuating progression of HIV disease. This study could go a long way towards documenting the appropriate management of moderately and severely malnourished HIV infected children in the context of the impact on immune response and drug metabolism. It will also document unwanted effects of ART and RUTF such as IRIS, re-feeding syndrome and drug toxicities with the aim of sensitizing the health care workers on its burden and plan for prompt management to reduce morbidity and mortality. Finding of absence of EFV drug levels in the blood will indicate that the child is not receiving the ART and this will assist in identifying children who are in this category and therefore advocate for their proper management. The study will contribute to the knowledge gap on immune reconstitution in malnourished HIV infected children with supplementation with the novel RUTF while on ART in HIV infected children attending malnutrition clinics in Uganda. This study will contribute towards policy formulation through provision of more information in regards to detailed management of the most at risk population doubly infected by HIV and malnutrition.

Research objectives

The overall study objective is to determine the influence of nutritional status and nutritional supplementation on the clinical, immunological and pharmacological responses to ART for HIV infection in children and to evaluate the utility of supplementing patients with RUTF during treatment.

Primary Objectives

Objective I

To compare the nutritional, clinical and immunological responses among well-nourished and undernourished (MAM and SAM) HIV infected children initiating ART and RUTF in Uganda.

Objective II

To compare the effect of ART and RUTF on pharmacological responses among HIV-infected well-nourished or moderately or severely malnourished children initiating ART in Uganda.

Objective III

To compare the effects of RUTF supplementation on the nutritional, immunological and pharmacological status of malnourished children who are ART experienced for more than 6 months with those in well-nourished ART experienced children.

Objective IV

To assess the knowledge, practices and attitudes of carers of HIV-infected children towards feeding malnourished children and the use of RUTF and ART.

Study hypotheses

1. Well-nourished HIV infected children newly initiating ART will have better nutritional, clinical and immune responses to ART than malnourished children receiving RUTF.
2. Well-nourished HIV infected children newly initiated on ART will have better pharmacological responses and ART tolerance than malnourished children receiving RUTF.
3. Well-nourished HIV infected children receiving ART for at least 6 months will have better nutritional, clinical, immunological and pharmacological responses to ART than the malnourished children receiving RUTF.

4. Carers of malnourished HIV-infected children who are knowledgeable about nutrition will display improved adherence to RUTF and ART and their children will have better treatment outcomes.

The conceptual framework in figure 1 illustrates the relationship of food intake and its impact on nutritional status, and the combined infect on immunological and pharmacological status HIV infected malnourished patients receiving ART. Other factors that may impact on immune responses are ART medication, patient adherence to the RUTF and ART, and duration on ART and RUTF.

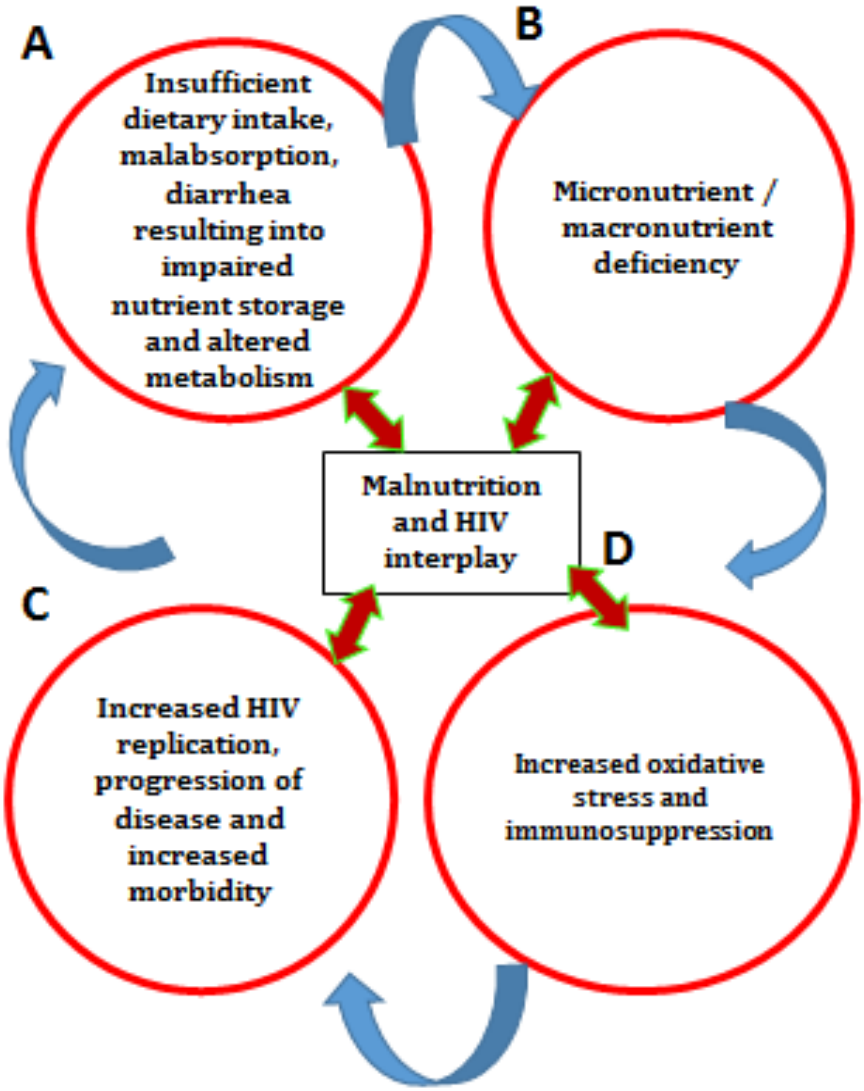


Figure 1: The conceptual framework of HIV/Malnutrition interaction or vicious cycle.

A, Factors affecting *the dietary intake and absorption of diet will gradually lead to* ***B,*** *nutrient deficiency and this will result into* ***C,*** *increased oxidative stress and immunosuppression that result in* ***D,*** *progression of HIV disease and risk of morbidity and mortality. The vicious cycle is usually worsened by the presence of an ongoing infection or a new infection resulting in rapid progression of HIV-malnutrition duo in the absence of treatment.*

CHAPTER TWO: Methodology

This chapter looks at methods used in the study under the sub headings of the clinical, nutritional, immunological and pharmacological aspects of the study. It focuses on the study design, sample size, instruments, study tools, reliability, validity, procedure, ethical issues, data management and analysis. The fulcrum of the study hinges on the clinical aspect of the study because it was the entry point of collecting all the data that we are using to answer the study objectives downstream.

Clinical methods

Study Design

This was a prospective cohort study involving a population of HIV infected children on ART or initiating ART and primary carers. The HIV infected children were followed-up over a period of 12 weeks while on RUTF for those who were malnourished. The well-nourished controls did not receive RUTF supplementations though they were also followed up for 12 weeks. For ease of implementation the study was divided into 3 cohorts.

Cohort I

We aimed to examine the role of nutrition and nutritional supplementation on clinical, immunological and pharmacological outcomes in ART-naïve, HIV-infected children aged between 6 months and 12 years. This comprised well-nourished (WN), moderately acute malnourished (MAM) and severely acute malnourished (SAM) patients (at least 26 in each group) initiating ART and RUTF when appropriate.

Cohort II

We aimed to examine the role of nutrition and nutritional supplementation on clinical, immunological and pharmacological outcomes in ART-experienced, HIV-infected children aged between 6 months and 12 years. These included WN and undernourished (MAM and SAM) with at least 36 in each group.

At each visit, patients had a complete anthropometric assessment. Biological samples were taken to measure specific markers indicative of nutritional, immunological and pharmacological responses at baseline and 12 weeks.

All children who did not exhibit acceptable changes in weight gain or BMI after 12 weeks of treatment with RUTF were maintained on supplementary feeding until they reached target weight gain or BMI by the end of the study. Loss to follow up was minimised by calling the study participants to remind them of their visit a week earlier and a day prior to the appointment, and those who did not come were traced through the clinic system.

Cohort III

This was a cohort study design adopting qualitative methodology projected to involve 160 primary carers whose children were either enrolled in cohorts I & II, or were attending the HIV clinics at the study sites. These were randomly selected to participate in 12 focus group discussions (FGDs) comprising 8-12 primary carers each, which took place at or around the time of treatment commencement and after 12 weeks. We also aimed to carry out 16 key informant interviews that involved carrying out an in-depth interview (IDIs) with primary carers such as the grandmothers, fathers or good Samaritans. Qualitative techniques were primarily used to explore processes that influence adherence and uptake of RUTF and ART in children in routine HIV care centres.

Study setting

This was a was a multicentre study carried out at the Infectious Diseases Institute affiliated Kampala City Council Authority (KCCA) Clinics namely (Kiswa, Kisenyi, Komamboga, Kawala, Kitebi and Kawempe), Mildmay Malnutrition ward; and the Mwanamugimu Nutritional Rehabilitation Center in Mulago Hospital (Figure 2).

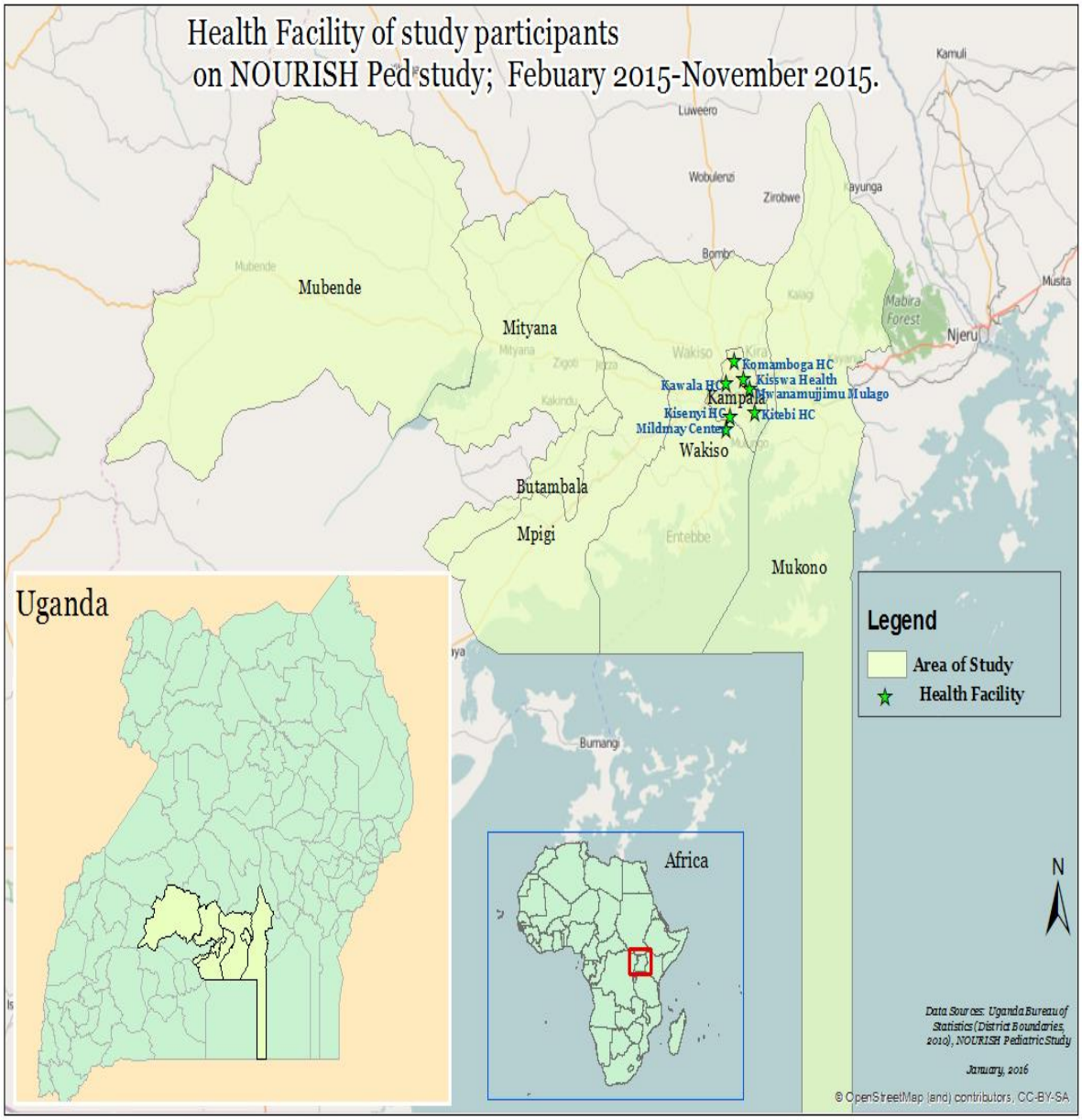


Figure 3: Map of location of the study sites-KCCA-IDI affiliated clinics, Mwanamujimu rehabilitation centre, Mildmay Centre.

Study unit

This was a child aged 6 months to 12 years both initiating RUTF and ART, or initiating RUTF in those who were ART experienced while continuing ART during the study period and primary carers.

Sample size estimation

Objective I: To compare the nutritional, clinical and immune responses among well-nourished versus undernourished (MAM and SAM) HIV infected children initiating ART and RUTF in Uganda.

Sample size was calculated using the formulae of sample sizes for two independent samples with continuous outcome below.

$$n = \left(\frac{Z_{1-\alpha/2} + Z_{1-\beta}}{ES} \right)^2$$

Sample size was calculated to detect the change in mean of Vδ2 T cells between the MAM versus the WN and the SAM versus the WN.

where α is the selected level of significance and Z 1-α/2 is the value from the standard normal distribution holding 1- α/2 below it, 1- β is the selected power and Z 1-β is the value from the standard normal distribution holding 1- β below it and ES is the effect size, defined as:

$$ES = \frac{\mu_d}{\sigma_d} = \frac{10}{20} = 0.50$$

where μd is the mean difference expected under the alternative hypothesis, H1, and σd is the standard deviation of the difference in the outcome (e.g., the difference based on measurements over time or the difference between matched pairs)[193].

Sample size for objective 1was determined to be 77 with a power of 80% however due to challenges of few newly diagnosed ART-N children accessing the study sites who fulfilled the study criteria we were able to accrue 66 ART-N children.

Objective II: To compare the effect of ART and RUTF on pharmacological responses among HIV-infected well-nourished or moderately or severely malnourished children initiating ART in Uganda.

Pharmacokinetic studies only require small sample sizes (12-20 participants) to achieve significance. The Food and Drug Administration (FDA) recommend a minimum number of 12 subjects should be included in any replicated crossover bioequivalence studies, with sufficient number of subjects in the study to allow for dropouts. For this reason we chose to have above 20 study participants per group.

Objective III: To compare the effect of RUTF supplementation on the nutritional, immunological and pharmacological status of malnourished children who are ART experienced for more than 6 months with well-nourished ART experienced children. A total sample size of 72 was determined to be sufficient with 36 well-nourished children and 36 acutely malnourished who were ART-E for at least 6 months prior to recruitment in the study. There is no need of sample size calculation as this was a proof of concept.

Objective IV: To assess the knowledge, practices and attitudes of carers of HIV-infected children towards feeding malnourished children and the use of RUTF and ART using FGDs. We planned to carry out 12 FGDs and 16 key informant interviews all together.

Sampling procedure and selection criteria

Eligible patients were consecutively sampled as they were reviewed at the study sites during the study period. Six sets of study groups of HIV infected children were selected namely WN, MAM and SAM initiating ART or on ART for at least 6 months and primary carers were selected.

Selection criteria for cohorts 1 and 2

The study participants enrolled in cohort 1 and 2 were HIV-infected children aged 6 months to 12 years, including WN, MAM and SAM patients initiating on ART within 2 weeks or having received ART for 6 months, whose primary carer was aged ≥18 years and had provided informed consent. In addition assent was given by the children older than 7 years according to the Uganda National Council of Science and technology Research guide

lines 2014 [148]. The exclusion criteria utilised in the screening process of the patients are in table 2.

Table 2. Exclusion criteria for the study

1. Previous enrolment in a nutritional therapeutic program in the last 3 months
2. Children involved in an on-going nutrition study
3. Children who have previously received the tuberculin skin test (TST) or mantoux or purified protein derivative (PPD) in the last 12 months.
4. Children with clinically suspected or confirmed malignancy
5. Children exhibiting any specific food intolerance, acute or chronic diarrhoea *
6. Children who were vomiting profusely (over 3 times daily)*
7. Children living outside 100 km radius from the study site in Kampala.
8. Children whose carers did not disclose their home address for any reason.
9. Children whose cause of malnutrition was compounded by congenital malformations, chromosomal disorders, metabolic disorders, congenital immune disorders, cerebral palsy
10. Children with a severe disability limiting the possibility of investigations
11. Children who planned to leave the catchment area in the next 6 months

*In this study we excluded any child who had food intolerance, chronic diarrhea and vomiting in the last 3 days. This is because we were using an oral supplement and the commonest anticipated side effects were gastrointestinal related and we didn't want to have this masked or worsened because of the food supplement we were introducing. Thus we wanted to be able to quickly detect these changes in case they occurred since these were very ill children that would rapidly deteriorate if they developed GI symptoms. Therefore those with malabsorption and diarrhea were excluded.

Cohort 3 Inclusion and exclusion criteria

We enrolled carers of children receiving routine HIV care and treatment at the study sites. We excluded carers of children who were not living with the children consistently for the last 6 months before enrolment into the study.

Study Instruments

Interviews were conducted using the following tools outlined in Table 3.

Table 3. Study Tools used in conducting the interviews

Number	Name of study tool	Appendices number
1.	Patient screening log	4
2.	Standardised clinical questionnaire at baseline to assess the clinical signs, symptoms and nutritional assessments.	5
3.	Standardised follow up clinical questionnaire to assess the clinical signs, symptoms and nutritional assessments at 12 weeks follow up time point and adherence form	6
4.	Dietary assessment form	8
5.	In-depth interview guide for key informants and focus group discussions	9
6.	Household questionnaire	7

Ethical clearance

Approval was sought in a stepwise systematic fashion and received from the following institutes in the order below:

1. Trinity College Dublin Institutional Ethics Review Board in October 2014
2. Infectious Diseases Institute Scientific Review Committee (IDI- SRC) in December 2014
3. Higher Degrees - Research Ethical Committee (HD-REC) School of Public Health Makerere University College of Health Sciences in December 2014
4. Uganda National Council of Science and Technology (UNCST) on 7th January 2015
5. In addition site approvals were sought and received from Mildmay Centre on the 29th January 2015, KCCA headquarters on the 22nd February 2015 and Mwanamujimu Rehabilitation centre on the 6th May 2015.

Due to the slow accrual of patients we had to apply for extension of the study and this was approved on the 18th December 2015 for 1 more year. By 18th December 2016 we had to apply for another extension to be able to recruit HIV negative healthy controls and we completed the study field activities in October 2017.

Data collection

Clinical data collection procedures

Patients suspected to have malnutrition during the outpatient consultation at the study sites were assessed by a clinic nurse on duty using the screening log (Appendix 4) and then sent to the PhD student or Research Assistant (RA) for evaluation. The PhD student or RA explained the study in detail to the primary carer. They provided an information sheet (Appendix 1) in the language the carer understood. If willing to participate they then provided written consent (Appendix 2) and children 8-12 years of age assented to join the study according to the UNCST ethics guidelines (Appendix 3). Though we aimed at recruiting 5 patients daily until the sample size was achieved this was not possible and we recruited about 1-3 per week. For cohort I, the malnourished patients were initiated on RUTF and ART at baseline and subsequently followed up at 12 weeks while the WN were only initiated on ART and followed up for 12 weeks. We therefore would use this cohort to assess the combined impact of both RUTF and ART as it was unethical to deny food supplements to malnourished children. For cohort 2 only the malnourished children were initiated on RUTF and both malnourished and WN children were followed up for 12 weeks. This would enable us to assess the impact of RUTF on the study outcomes. At each of the two visits demographic, anthropometric and dietary intake data were obtained. Demographic characteristics captured included age, gender, ethnicity, and anthropometric measures including height, height-for-age, weight, weight–for-age, weight-for-height, head circumference, mid upper arm circumference and waist circumference. In addition, HIV/AIDS clinical staging (using WHO guidelines), and details of ART, medical history of OIs, current medications and vital signs were collected. Mother's demographics, HIV status, weight, height and BMI were taken. A 2 ml spot urine sample was collected at baseline and at 12 weeks for metabolomics analysis. Other laboratory diagnostics carried out on routine samples were full blood count, total protein, full lipid profile, renal function tests, liver function tests, inflammatory markers such as lactate

dehydrogenase (LDH), CRP and micronutrients which included potassium, phosphate, calcium, magnesium, chloride and other appropriate markers. A total of 5-10 mls of blood was collected from the patients for laboratory investigations. Viral loads were done using a Cobas AmpliPrep/Cobas Taqman 48 (CAPCTH) machine.

Dietary data collection and analysis procedures

In order to estimate the individual dietary intakes of the study participants, interactive 24 hour recall method was used. The 2 researchers were trained by an expert nutritionist on how to administer the 24 hour dietary recall tool. This entailed administering a 30 min structured interview to the primary carers for children below 7 years while for those above 7 years both the primary carer and child were interviewed. Participants were asked to recall all the foods they had consumed in the last 24 hours and the characteristics captured were the time of food consumption, type of food or drink, method of cooking, whether the meal consumed was homemade or bought and from what vendor they bought the food or drink, amount or portion measured in terms of spoons, cups, millilitres, weights or cost. It also captured whether the patient had an adequate appetite, and if otherwise the possible reasons to the inadequate appetite state. In addition occasions such as parties that potentially would affect the dietary intake were also captured to better interpret the dietary information. The researchers probed further to ensure that the description of food consumed was exhaustive in the following categories: specific food item, specific part of food item (e.g. chicken leg, with skin), fresh or dried state (e.g. fresh or dried peas, beans), processing state (e.g. refined verses whole grain, smoked, salted), colour (as relevant for nutrient content or identification like brown verses white rice), stage of maturity or ripeness (ripe mango or raw mango), raw or cooked; if cooked, the cooking method used (e.g., boiled, steamed, roasted, fried: shallow or deep fried). We performed one recall interview per participant at baseline and 12 week visit from Monday to Friday.

Estimation of portions and amounts of foods consumed were determined by the following methods during the interview:

a. Standardised utensils were used in the estimation of portions and amounts for majority of the foods using validated set of dietary spoons and cups (table 4).

b. Common foods like *samosa, chapatti, mandazi,* boiled eggs, bread slices, fruits such as bananas, mangoes, unmashed *matooke* (boiled/steamed fingers), and sugarcane portion sizes were estimated using the costs of the food type eaten, number of units of the food type eaten or the size i.e. small, medium or large.

The researchers then converted the estimated food portions reported by the participants into weight in grams using the following methods:

1. For food portions generated using the standard utensils, a volume to weight conversion factor of the food composition tables (FCT) conversion spreadsheet was used.
2. The researchers identified the commonly used cups and measured their quantities in terms of water, millet porridge, maize porridge and tea. This was done using a feeding graduated syringe and measuring jug. The volumes generated were converted into weight using the FCT conversion spreadsheet.
3. For the food portions reported in small, medium and large for foods such *as samosa, chapatti, mandazi,* boiled eggs, bread slices, fruits such as bananas, mangoes, unmashed *matooke* (boiled/steamed fingers) and sugarcane fruits the researcher, weighed the different portions in grams using a home kitchen scale and generated the average weights and these were the ones used in calculating the nutrient composition.

Then, the portion sizes were converted into gram equivalents since the food conversion tables contain nutrient composition per 100 g of the foods.

Regarding the mixed dishes/recipes like *"katogo"* the proportions of individual ingredients present as reported were taken into consideration guided by the Harvestplus recipe catalogue.

For foods reported in millilitres of liquid, such as milk, conversion factors were provided by Harvestplus Uganda and utilized to convert them into grams with the exception where the researcher had weighed the porridge. Care was taken to consider dilutions wherever necessary so that the nature and form of foods matched those contained in the food composition and nutrient conversion databases.

Table 4. Standardised utensils used in the estimation of portions and amounts for majority of the foods assessed in the 24 hour dietary recall interviews

Number	Utensil used	Equivalent	mls	Dry weight
1	½ Tea spoon	----------	2 mls	2.2 gm
2	1 Tea spoon	----------	4 mls	6.4 gm
3	½ Table spoon	----------	5 mls	7 gm
4	Table spoon	----------	10 mls	14 gm
5	Green spoon	1 cup	236.64 mls	230 gm
6	Blue spoon	½ a cup	118.32 mls	115gm
7	Pink spoon	¼ a cup	59.16 mls	60 gm
8	Purple spoon	1/8 of a cup	29.58 mls	28.8 gm
9	*Nice* cup	----------	250 mls	230 gm
10	*Tumpeco* cup	----------	500 mls	470 gm

We used food composition and conversion tables developed by a group of Ugandan and International researchers in Uganda [194, 195] for nutrient composition analysis for each patient. Harvestplus Uganda food FCT is a compilation of existing and assigned food composition data for foods commonly consumed in Central and Eastern Uganda. Therefore this FCT is representative of the foods that are commonly consumed in the area where we carried out our study, which is part of central Uganda. However the majority of the primary nutrient composition data in Harvestplus Uganda's is generated from the United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference.

For foods absent from the Harvestplus Uganda database, such as the artificial juices, therapeutic foods F100, F75, *plumpynut*, etc, nutritional information as recorded in the labels was used to obtain the nutrients. For therapeutic foods, in addition WHO guidelines

were followed to access the nutrition composition. There was a challenge obtaining full nutrient information, especially the particular micronutrients since these are not detailed in the labels. This was not included in the nutrient summations, i.e, they were taken to supply none (zero) of those particular nutrients not specified. However, energy and protein from these foods were added to the final sum.

Thereafter each food consumed was assigned a food code according to Harvestplus Uganda FCTs depending on the method used for food preparation (raw, steamed, boiled, fried, roasted etc), food state (ripe, raw, dry, fresh, smoked, salted etc) or by recipes.

Computing the nutrient composition of the recipes collected in the 24 hr dietary recalls, the weight of each cooked ingredients was recorded with the exception of the hybrid food items or ingredients where the raw weight amount was used. Thus for hybrid foods nutrient losses from that ingredient due to cooking would not be accounted for.

Calculating the individual participants total nutrients consumed in 24 hour period was done using the Harvestplus Uganda food conversion algorithms.

1. Individual food nutrient composition: Using the codes obtained from the FCT the corresponding food items consumed were identified on the food conversion algorithm spreadsheet, and the total amounts in grams were entered in the spreadsheet. The total nutrient intake was then generated using the inbuilt formulae in the spreadsheet.
2. Common recipe nutrient composition: The proportions of different food items making up a given recipe were identified from the FCT spreadsheets. To estimate the amounts of the individual items in the recipe consumed by an individual; the proportion of the food item was multiplied by the total amount the recipe consumed. To calculate the individual nutrient composition step 1 above was followed.

Immunology methods

Procedures for the phenotypic analysis of human peripheral blood mononuclear cells.

Materials: Materials used in this investigation and their sources are listed in Tables 5-8 and 10.

Table 5. General reagents

Reagent	Supplier	Catalogue Number
Phosphate buffered saline	Gibco Life Technologies	14190-094
RPMI glutamax medium	Gibco Life Technologies	61870-010
HEPES	Gibco Life Technologies	15630-056
Penstrep	Gibco Life Technologies	15140-122
Fungizone	Gibco Life Technologies	15290-026
Trypan Blue	Gibco Life Technologies	15250-061
Lymphoprep	Axis-Shield, Norway	2016-05
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich	472301
One Comp Beads	eBioscience	01-1111-42
Bovine serum albumin	Fisher Scientific	BP9703-100
Sodium azide	Sigma-Aldrich	S2002
4% paraformaldehyde	Santa Cruz Biotechnology	SC-281692
Foetal calf serum	HyClone or other company	
Antibodies – see Table 4		
FACSCanto II Reagents (CS&T Beads, FACS flow, FACS Clean etc)	BD Biosciences	

Table 6. Plasticware

Item	Supplier	Catalogue Number
3.5 ml transfer pipettes	Sarstedt	86.1171.010
Automatic pipette tips	local supplier	
EDTA blood tubes	local supplier	
Centrifuge tubes - 30 ml Universal tubes	Fisherbrand	FSH30APS
- or 15 ml Falcon tubes	Sarstedt	62.554.502
Cryovials	Thermo Scientific / Nalgene	5000-0020
Flow cytometry tubes (5 ml polystyrene)	BD Falcon	352008

Table 7. Equipment

Equipment type	Manufacturer/Supplier
Automatic pipettes – (2-20 µl, 20-200 µl and 200-1000 µl or equivalent)	Fisher scientific
Bench centrifuge	Eppendorph
Laminar air flow cabinet	Telstar, Laboratory Equipment Woerden, The Netherlands
Haemocytometer slide	
Vortex	IKA, Staufen im Breisgau, Germany
Microscope	Nikon, Tokyo, Japan
Centrifuge tube and flow cytometry tube racks-	Eppendorf, Hamburg, Germany
"Mr. Frosty" 1^{o}C freezing container – Nalgene 5100-0001	Fisher
+4^{o}C fridge	
-20^{o}C freezer	
-80^{o}C freezer	Mason Technology
Liquid nitrogen	BOC gases
FACSCanto II flow cytometer	Becton Dickinson High Wycombe, UK

Peripheral blood mononuclear cells (PBMC) were prepared from whole blood by standard density gradient centrifugation over Lymphoprep (Axis-Shield, Norway) and cryopreserved in the Makerere University College of Health Sciences Immunology Laboratory in Uganda. Plasma was aliquoted and stored at -80 ^{o}C. All the antibodies were titrated to determine the optimal amount to use to stain 0.1-0.2x10^{6} PBMC. We used the optimal amounts of antibodies, which are shown in Table 8.

The Vδ3 antibody was labelled with APC using the Lightning Link kit (Innova Biosciences) in our laboratory.

Table 8. Antibodies

Catalogue Number	Cell marker	Fluorochrome	Clone	Antibody source
558117	CD3	Pacific Blue	UCHT1	BD-Pharmingen
557852	CD4	PECy7	SK3	BD-Pharmingen
552825	Invariant NKT cell	PE	6B11	BD-Pharmingen
TCR2055	TCR Vδ1	FITC	TS8.2	Thermo Fisher
555369	CD8α	APC	RPA-T8	BD-Pharmingen
558117	CD3	Pacific Blue	UCHT1	BD-Pharmingen
555738	TCR Vδ2	FITC	B6	BD-Pharmingen
BULK1TCR	Vδ3	APC	P11.5B	Beckman Coulter
557835	CD19	PECy7	SJ25C1	BD-Pharmingen
555516	CD56	PE	B159	BD-Pharmingen
558117	CD3	Pacific Blue	UCHT1	BD-Pharmingen
557852	CD4	PECy7	SK3	BD-Pharmingen
555340	CD3	PE	HIT3a	BD-Pharmingen
555369	CD8α	APC	RPA-T8	BD-Pharmingen
555634	CD8α	FITC	HIT8a	BD-Pharmingen

Collection of blood samples from the field in Uganda

Patients who fulfilled the study inclusion criteria were enrolled into the study at the Ugandan sites. Samples were collected at enrollment and at 12 weeks after enrollment. Whole blood was collected in Acid Citrate Dextrose (ACD) vacutainer tubes for immunophenotyping and cell enumeration, ethylenediaminetetra-acetic acid (EDTA) vacutainer tubes for full blood count, plasma processing, immunological monitoring, viral load testing and serum separation vacutainer tubes (SST) for biochemistry studies and serum separation. After receiving consent to collect the blood sample, the venopuncture site was identified, swabbed with alcohol swabs to observe aseptic conditions, a tourniquet was used briefly to enhance the veins and samples were then collected from the cubital forsa of any arm for the majority of patients.

The samples for biochemistry analysis were separated in the field to avoid hemolysis. This was done by centrifuging the SST tubes for 5 mins at 400 x g. Thereafter transported to the Medical Research (MRC) Laboratory in Mengo where serum was aliquoted and

transported for biochemical analysis and storage in Entebbe, table 9. The remaining serum was stored in a -80 ^{0}C refrigerator in cryovials. The other investigations carried out at the MRC laboratory included FBC, blood smear for malaria parasites, renal function tests, liver function tests, lipid profiles, immunological profiling and viral load testing. Plasma was processed from the EDTA tube and stored for performing drug ARV pharmacokinetics (PK) in a -80^{0}C refrigerator.

Venous blood (5-8 ml) was collected into ACD tubes for preparation of PBMCs for immunophenotyping and cell enumeration. These were transported from the study site to the Department of Molecular Immunology at the College of Health Sciences at Makerere University. All the samples from the field were processed within 4 hours of collection.

Table 9. Tests done at the Medical Research Laboratory in Entebbe Uganda

Number	Name of test	Sample used for testing
	Baseline tests	
1	Full blood count	Whole blood
2	Blood film report	Whole blood
3	Serum electrolytes (K+, Na+, Cl-, Ph, Ca2+, Mg, Mn)	Serum
4	Renal function testes (blood urea & nitrogen - BUN and creatinine)	Serum
5	Liver function tests (ALT, AST, ALP, Bilirubin)	Serum
	Diagnostic tests	
6	Blood smear for malaria parasites	Whole blood
7	Tuberculin skin test	Not applicable
	Nutritional markers	
8	Total protein	Serum
9	Albumin	Serum
10	Globulin	Serum
11	Lipid profiles (Total Cholestrol, HDL, LDL, triglycerides)	Serum
12	Lactate dehydrogenase	Serum
13	C-reactive protein	Serum
	Monitoring tests	
16	Routine immunological profiling	Whole blood
17	Viral loads	Whole blood

Preparation of peripheral blood mononuclear cells

The preparation of PBMCs was carried out within a class II biosafety cabinet with appropriate sterile techniques. Personal protection gear such as gloves and a laboratory coat was always utilized. Blood wastes or any utensil used with blood were first decontaminated using diluted Virkon for at least 1 hour, followed by autoclaving of the remaining material. PBMC were prepared from whole blood by standard density gradient centrifugation over Lymphoprep (Axis-Shield, Norway) and cryopreserved in the Makerere University College of Health Sciences Immunology laboratory in Uganda.

The whole blood was diluted in a 1:1 with phosphate buffered saline (PBS) containing 1% foetal calf serum (FCS: heat-inactivated for 30 min at 56^{o}C). The diluted blood was then carefully layered onto Lymphoprep (~7.5 ml in a 30 ml Universal tube or ~4 ml in a 15 ml Falcon tube) as shown in Figure 3. With the brake off (minimum acceleration and deceleration) the diluted blood on Lymphoprep was centrifuged for 25 min at 400 x g (1,410 rpm in an Eppendorf 5810 centrifuge). The top plasma layer was removed using a plastic pipette, as shown in Figure 3, and discarded. The buffy coat (cloudy layer that sits on top of the Lymphoprep) plus the Lymphoprep layer (in order to obtain all the buffy coat) were carefully transferred to a clean tube ensuring that none of the red cell pellet was taken. The buffy coat layers were then topped up with PBS + 1% FCS, mixed and centrifuged for 8 min at 450 x g (1,500 rpm in an Eppendorf 5810 centrifuge) with the brake on. The supernatant was discarded by tipping off once and the cell pellet was resuspended in a small volume (<1 ml or the amount of liquid remaining) and vortexed. This was then topped up with PBS + 1% FCS, mixed and centrifuged for 8 min at 450 x g with the brake on. The supernatant was discarded by tipping off *once*. The remaining pellet was resuspeneded in a small amount of complete RPMI medium (RPMI 1640 containing 25 mM HEPES, 2 mM L-glutamine, 50 μg/ml streptomycin, 50 U/ml penicillin and 10% heat-inactivated FCS). (The complete RPMI was made up by adding 50 ml FCS, 12.5 ml 1 M Hepes, 4 ml of Penstrep and 4 ml of Fungizone to 500 ml RPMI 'Glutamax'). The samples were stored in Uganda in -80^{0}C freezers and thereafter were shipped using the dry shippers and dry ice from Uganda to Ireland following the International Air Transport Association guidelines of shipping biological samples.

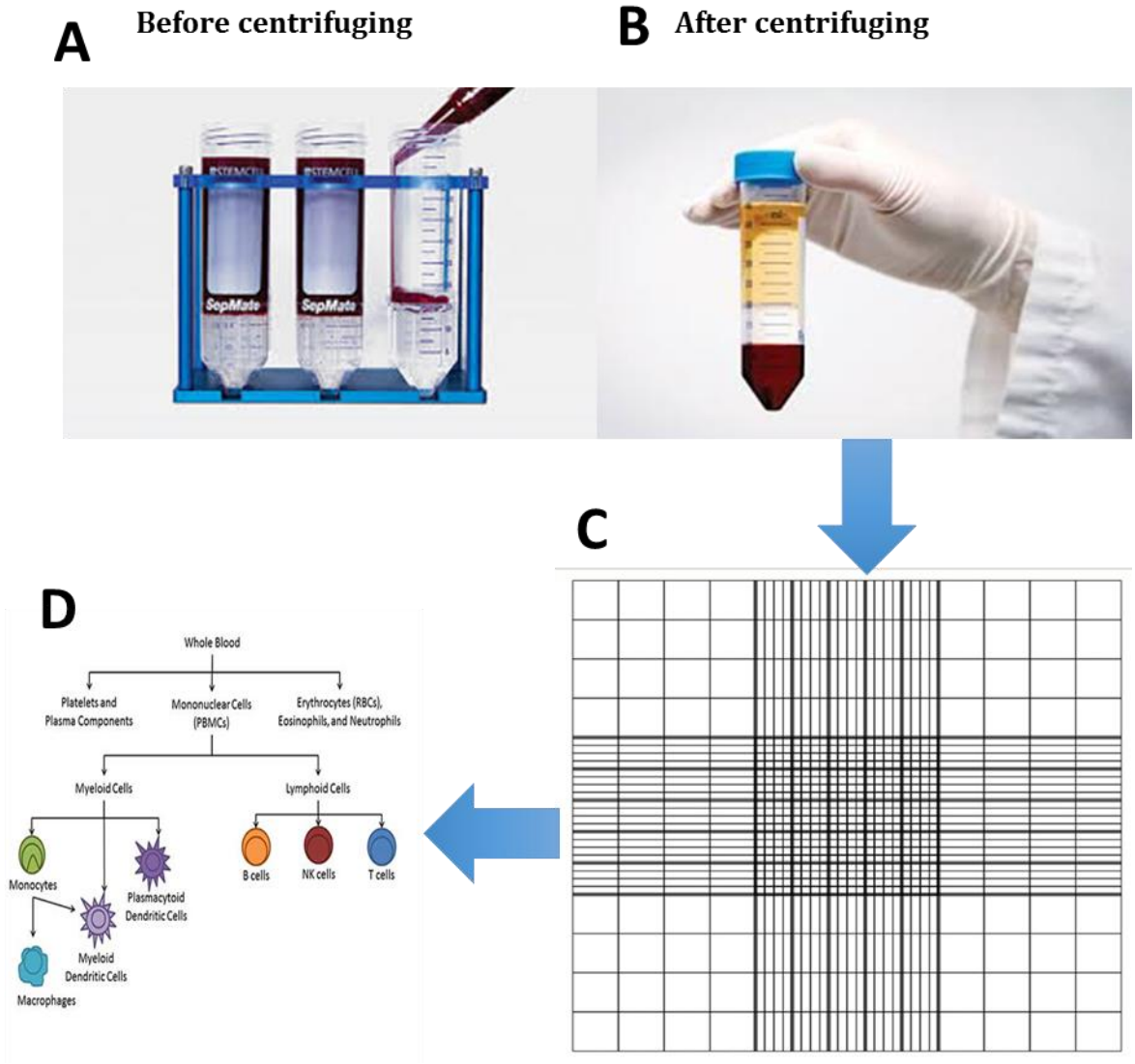


Figure 4: Isolation of peripheral blood mononuclear cells from whole blood using density gradient separation in Uganda.

A, *Whole blood collected in an Acid Citrate Dextrose (ACD) tube was slowly layered on Lymphoprep,* ***B,*** *thereafter centrifugation was carried out at 400xg for 25 mins at room temperature in order to achieve separation of blood components according to their density. Peripheral blood mononuclear cells (PBMCs) gather in a white-coloured ring, separated from granulocytes and erythrocytes by the Lymphoprep reagent. Upon separation the PBMC layer was removed using a plastic pipette and placed in 15 mls tubes for washing. A Haemocytometer was used to count the cells before freezing down.* ***C,*** *Neubauer chamber used for counting cells prior to freezing and calculating sample viability. Image courtesy of Hausser Scientific (www.hausserscientific.com).* ***D,*** *Flow chart illustrating the different blood components that are separated by the density gradient method.*

Determination of cell counts

A Neubauer chamber was used for counting cells prior to freezing and calculating sample viability. 10 μl cell of suspension was added to 190 μl ethidium bromide (EBO) and counted on a hemocytometer slide. The original number of cells (millions) after the 1:20 dilution per ml was taken to be equal to the number of cells counted in two groups of 16 small squares containing a total volume of 0.2 mm^3 divided by 10 in figure 3. For example, if you counted 56 cells in two groups of 16 small squares, you would have $5.6x10^6$ cells/ml. Thereafter this value was multiplied by the number of ml of cell suspension to get the total number of PBMC obtained.

Storage of PBMC in Uganda

In Uganda, the viability of PBMC before storage was determined using a Neubauer chamber prior to freezing. We only stored PBMC with viability above 80% before freezing down. To store the PBMC we used a cell freezing mixture that consisted of 90% FCS + 10% dimethyl sulphoxide (DMSO). This was freshly made each time the PBMCs were to be stored by adding 0.5 ml DMSO to 4.5 ml FCS and allowing it to cool for about 10 minutes, because FCS and DMSO produce heat when mixed. PBMC were pelleted by centrifugation for 7 min at 450 x g, the supernatant was discarded and resuspended the cell pellet in 2 ml of freezing mixture after cooling down. The PBMC with freezing mixture were immediately transferred to previously-labelled cryovials (with study identification code and date) and then placed in a -80°C freezer overnight in a "Mr. Frosty" container. This allows the cells to cool gradually at 1°C/minute. The cryovials were then transferred the next day to liquid nitrogen.

Recovery of PBMC from liquid nitrogen at Trinity College Dublin

To thaw the PBMC the laminar airflow cabinet was switched on and the RPMI medium warmed to 37°C and tubes labelled and placed in the laminar flow cabinet ready for action as we needed to work fast to avoid the PBMC sitting in DMSO unnecessarily. PBMC were thawed rapidly without allowing them to heat up beyond room temperature and then diluted rapidly in culture medium as DMSO is toxic to the cells. This was done by gently shaking continuously the PBMC cryovials under a hot tap or in a water bath. This ensured the transfer of heat throughout the vial. The vial was then removed from the hot water

before it was completely thawed (i.e. left a small lump of ice floating in the liquid to ensure that the liquid doesn't heat up too much, which could kill the cells). The suspension of thawed cells were then transferred into a universal or Falcon tube using a sterile plastic pipette and complete RPMI medium was added dropwise, while shaking the tube to mix continuously. The mixture was then centrifuged for 10 minutes at 450 x g and the supernatants discarded. The pellets were resuspended in ~2 ml complete RPMI medium. Cells were then counted as described above and illustrated in figure 3 above.

Phenotypic analysis of PBMC

We used flow cytometry to perform phenotypic analysis of the PBMC.

The principle of flow cytometry

Flow cytometry is a method widely used for differentiating immune cells since the 1980s. It involves quantitative single cell analysis based on its optical and fluorescence characteristics (e.g. cells, nuclei, chromosomes) during their passage within a fluid channel, figure 4. The current state-of-the art flow cytometers are capable of analyzing up to 20 parameters (forward scatter, side scatter, 18 colors of immunofluorescence) per cell at rates up to 100,000 cells per second.

Forward scatter (FSC) delineates the size of the cell while side scatter (SSC) is a correlate to the internal complexity of the cell allowing discrimination of mononuclear and polymorphonuclear cells.

Globally flow cytometry systems are comprised of three major parts: the fluidics, optics and electronics. The fluidics system is composed of a tubing apparatus carrying a solution called "sheath fluid" through the machine. The sheath fluid constitutes a vehicle for single cell suspensions, which are channeled in the centre of its stream through a nozzle and towards an "interrogation point".

The interrogation point is the key site of the optics system, which is composed of a set of lasers, filters, mirrors and photomultiplier tubes. The lasers used in flow cytometry machines are typically identified by the colour of visible light they are excited by, e.g. as violet (405 nm), blue (488 nm) and red (640 nm). Light from the lasers hits cells at the interrogation point, exciting the fluorochrome-conjugated antibodies they are stained with and causing them to emit fluorescence

Any fluorescent molecule present in or on the particle will absorb energy from the laser light and release the absorbed energy at longer wave length. The emitted light is collected by lenses and detectors and emitted fluorescence intensity is proportional to the amount of fluorescent compound on the particle. The scattered light from particles passing the laser light is converted to digital values that stored in the computer for analysis.

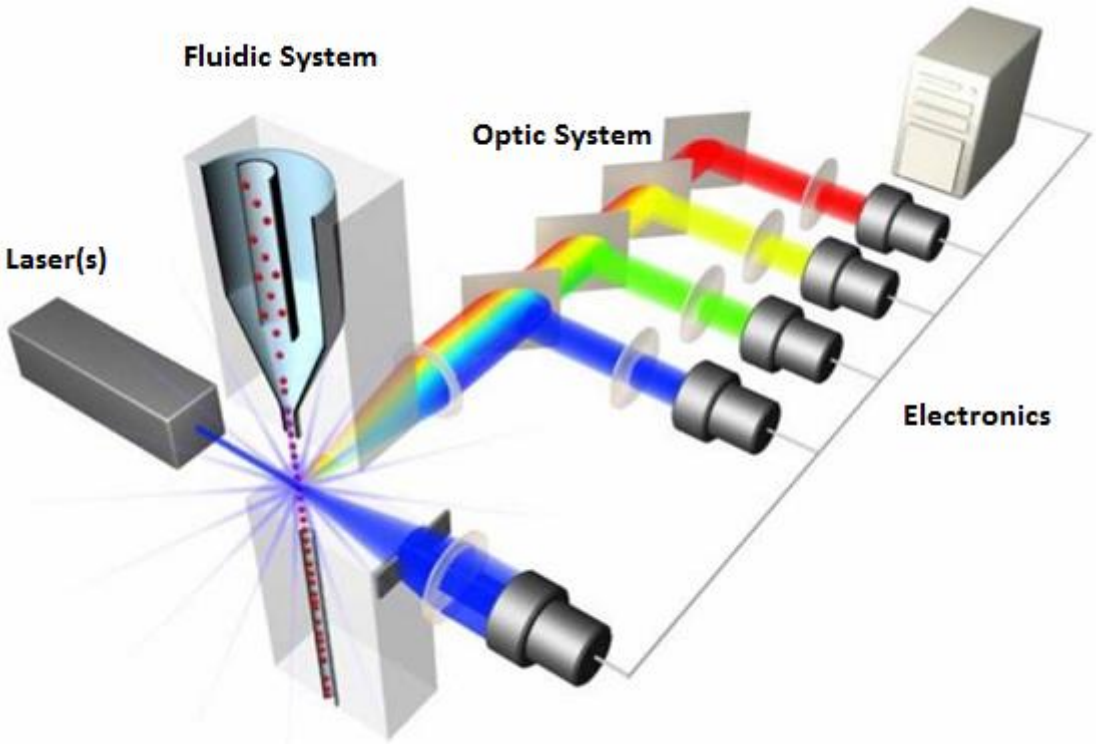


Figure 5: Flow cytometry principles and clinical application.
This is composed of a light source in form of a Laser system, flow cell, optical components to focus light of different colours on to the detectors, electronics to amplify and process the resulting signals and a computer.

Flow cytometry tubes were labelled as in Table 10. For every subject, PBMC were stained with mAbs specific for the cell surface markers, shown for Tubes 7 and 8. An unstained sample was also treated similarly. Additionally, on every day of analysis, single staining for each fluorochrome was carried out using One Comp Beads (Tubes 1-5).
Finally, fluorescence-minus-one (FMO) control stainings were carried out as shown for Tubes 9-11 in table 10. FMO control stains were done for each experiment for antibodies that stain few cells, i.e. iNKT, Vδ1, Vδ2 and Vδ3. For example, the FMO for Vδ1 would consist of all antibodies in tube 7 except Vδ1 (iNKT-PE, CD8α-APC, CD4-PECy7 and CD3-Pacific Blue but not Vδ1-FITC) and this should be used as a negative control to set the quadrants for Vδ1 expression in tube 7.

Cells were first stained with a dead cell stain (Fixable Viability Dye, diluted 1/1,000 in PBS for 15 minutes. They were then washed with PBS, centrifuged at 450 x g and the supernatants discarded. Each antibody was added to the correct tubes, as per Table 11. During this process antibodies were kept on ice in the dark to avoid being denatured. A new tip was used after dispensing each antibody and up to 5 antibodies were added per tube, we devised a system whereby every time an antibody was added the tube was moved to the next row, so as to remember which antibodies have been added. After adding all antibodies, PBA buffer was added to each tube to bring the total volumes to 50 µl per flow cytometry tube.
To make up PBA buffer we used PBS containing 1% bovine serum albumin (BSA) and 0.02% sodium azide. We added 20 ml of 30% BSA and 1.2 ml 10% sodium azide to 580 ml of PBS.

Approximately 0.1-0.2x10^6 PBMC were transferred to each flow cytometry tube in about 100 µl PBA buffer and the tubes were incubated for 15 min at room temperature in the dark (covered in tin foil). Thereafter, the cells were washed by adding ~2 ml PBA buffer, centrifuging for 7 min at 450 x g and discarding supernatants (once). Before acquiring the cells on the flow cytometer all the human samples were first fixed with paraformaldehyde, as a safety precaution to protect users of the flow cytometer against possible infection.
After PBMCs were stained with fluorochrome-labelled antibodies specific for CD3, CD4, CD8, CD19, CD56 and the Vα24Jα18, Vδ1, Vδ2 and Vδ3 T cell receptor (TCR) chains,

frequencies and absolute numbers of lymphocyte subsets were analysed by flow cytometry. To acquire cells on the flow cytometer we used tube 6 to set up the detectors and parameters and tubes 1-5 to set up the compensations. Thereafter we plotted forward scatter against side scatter and adjusted voltages so that the lymphogate was visible. A gate was drawn around the lymphocytes and used to examine fluorescence. The gating strategy used for all the cells is shown in Figures 5-9. Enumeration of lymphocyte subsets was carried out using the phenotypic markers as shown in Table 12. Absolute numbers of each cell type (per µl of blood were calculated from the full blood counts.

Table 10. Flow cytometry tubes used for each PBMC sample for phenotyping

Control samples – used 1 drop of One Comp Beads

Tube	FITC	PE	APC	PECy7	PB
1	CD8α	-	-	-	-
2	-	CD3	-	-	-
3	-	-	CD8α	-	-
4	-	-	-	CD4	-
5	-	-	-	-	CD3

Test samples – do for each sample

Tube	FITC	PE	APC	PECy7	PB
6	-	-	-	-	-
7	Vδ1	iNKT	CD8α	CD4	CD3
8	Vδ2	CD56	Vδ3	CD19	CD3

FMO controls – do for each sample

Tube	FITC	PE	APC	PECy7	PB
9	-	iNKT	CD8α	CD4	CD3
10	Vδ2	-	Vδ3	CD19	CD3
11	Vδ2	CD56	-	CD19	CD3

Table 11. Amount of each antibody used per tube

Cell marker	Fluorochrome	Clone	Dilution	Amount per tube
CD3	Pacific Blue	UCHT1	1/20	2 µl
CD4	PECy7	SK3	1/20	4 µl
iNKT cell	PE	6B11	neat	3 µl
TCR Vδ1	FITC	TS8.2	1/5	3 µl
CD8α	APC	RPA-T8	1/5	3 µl
CD3	Pacific Blue	UCHT1	1/20	2 µl
TCR Vδ2	FITC	B6	1/20	2 µl
TCR Vδ3	APC	P11.5B	neat	2 µl
CD19	PECy7	SJ25C1	1/20	3 µl
CD56	PE	B159	neat	1 µl
CD3	Pacific Blue	UCHT1	1/20	2 µl
CD4	PECy7	SK3	1/20	4 µl
CD3	PE	HIT3a	1/5	3 µl
CD8α	APC	RPA-T8	1/5	3 µl
CD8α	FITC	HIT8a	neat	1 µl

Table 12. Identification of lymphocyte subsets by flow cytometry

Cell type	Phenotype	Tube
T cells	CD3+	7
$CD4^+$ T cells	CD3+ CD4+	7
$CD8^+$ T cells	CD3+ CD8+	7
$CD4^+CD8^+$ T cells	CD3+ CD4+ CD8+	7
$CD4^-CD8^-$ T cells	CD3+ CD4- CD8-	7
Vδ1 T cells	CD3+ Vδ1+	7
iNKT cells	CD3+ Vα24Jα18+	7
B cells	CD3- CD19+	8
NK cells	CD3- CD56+	8
NT cells	CD3+ CD56+	8
Vδ2 T cells	CD3+ Vδ2+	8
Vδ3 T cells	CD3+ Vδ3+	8

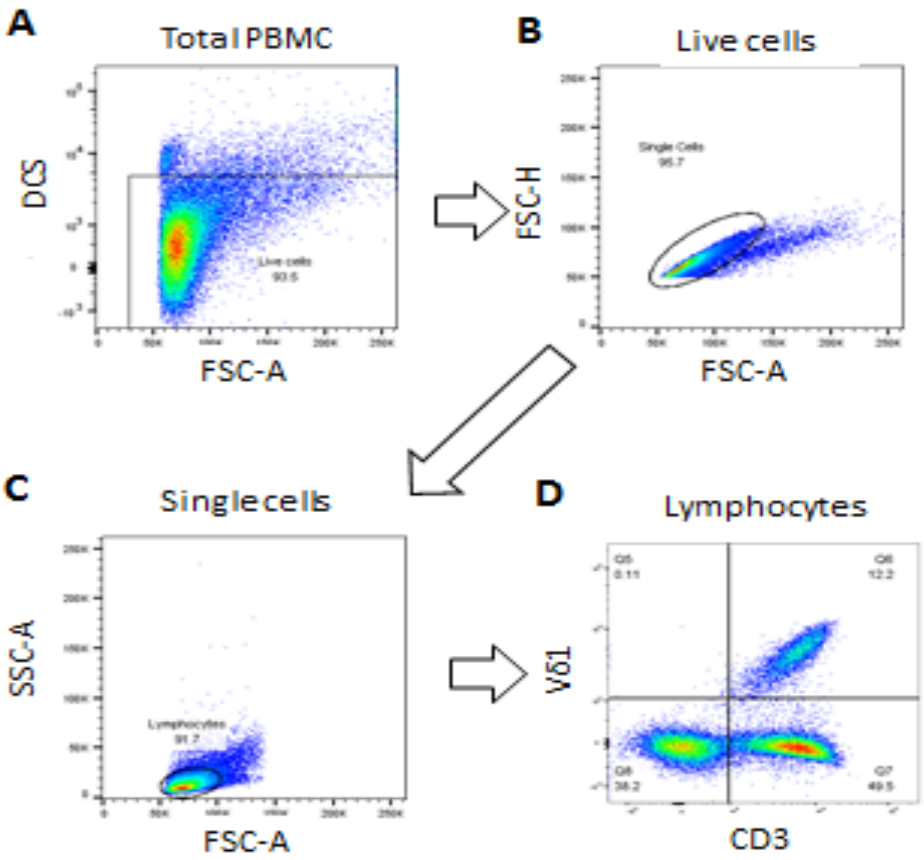


Figure 6: Gating strategy for phenotypic analysis of PBMC

*PBMC were stained with a dead cell stain (DCS) and mAbs specific for cell surface markers and acquired on a flow cytometer. A dot plot of FSC-A against DCS was generated (**A**) and a gate was drawn around the live cells. Live cells were used to generate a dot plot of FSC-A against FSC-H (**B**) and a gate was drawn around the singlet cells. Singlet live cells were used to generate a dot plot of FSC-A against SSC-A (**C**) and a gate was drawn around the lymphocytes. In the present example lymphocytes were then used to generate a dot plot of CD3 against Vδ1 (**D**) in order to determine the percentage frequency of Vδ1 T cells. Numbers in the quadrants show percentage frequencies of cell populations.*

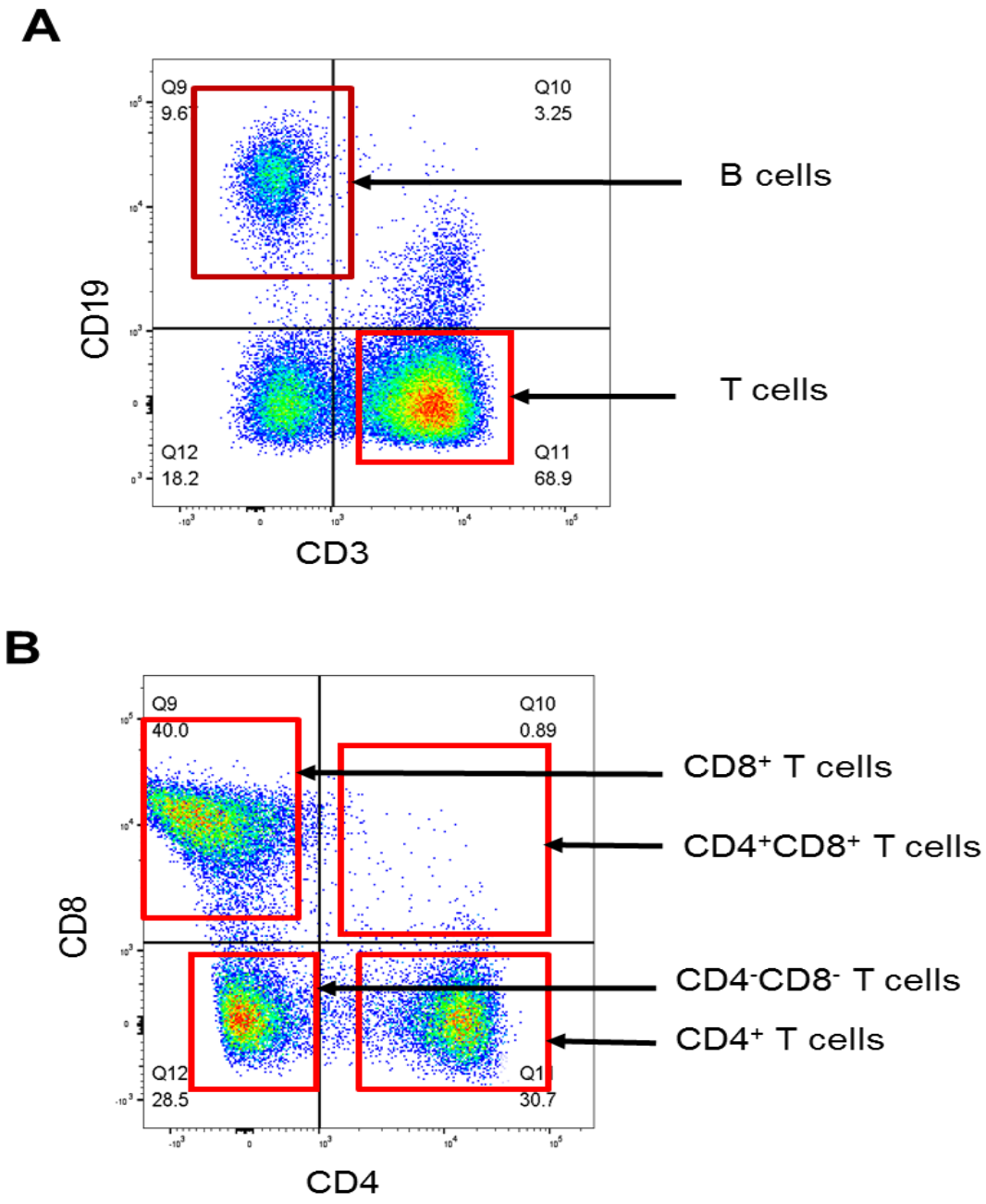


Figure 7: Flow cytometric enumeration of B cells and T cell subsets. ***A,*** *Representative flow cytometry dot plot showing CD3 and CD19 expression by gated lymphocytes after excluding dead cells and doublets, as shown in Figure 6.* ***B,*** *Representative flow cytometry dot plot showing CD4 and CD8 expression by gated CD3⁺ cells. The red boxes indicate how the different lymphocyte subsets were identified.*

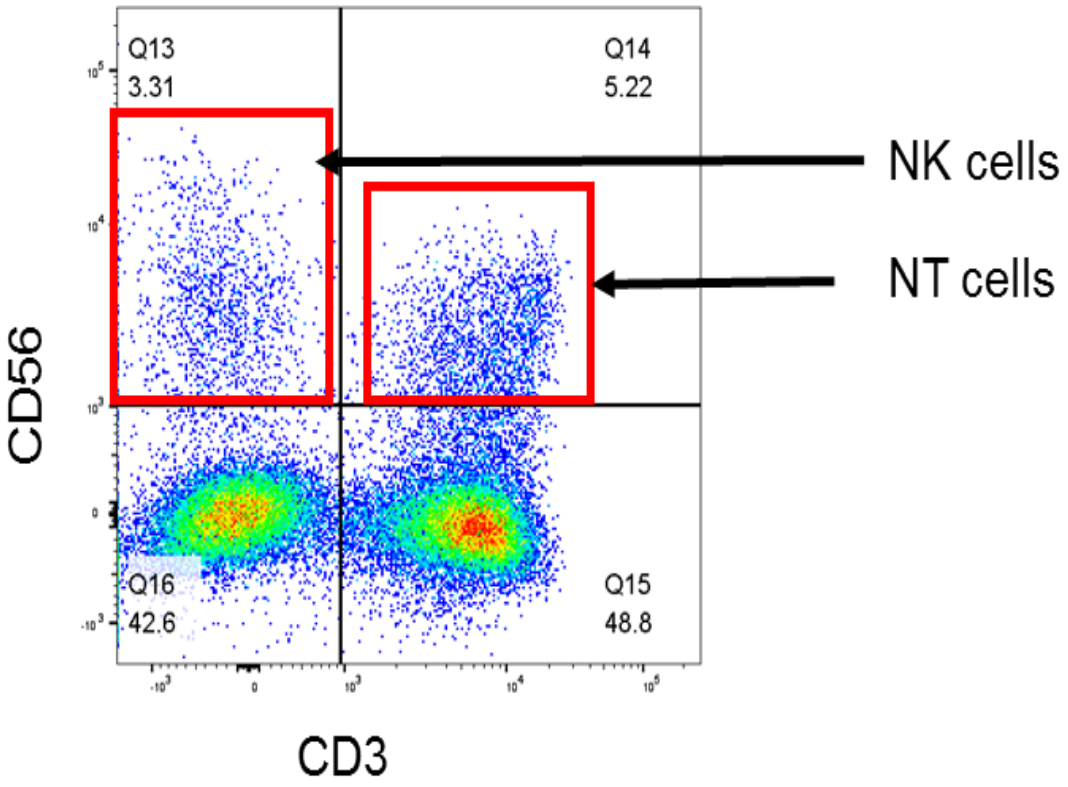


Figure 8: Flow cytometric enumeration of NK cells and NT cells.
Representative flow cytometry dot plot showing CD3 and CD56 expression by gated lymphocytes after excluding dead cells and doublets, as shown in Figure 5. The red boxes indicate how the different lymphocyte subsets were identified.

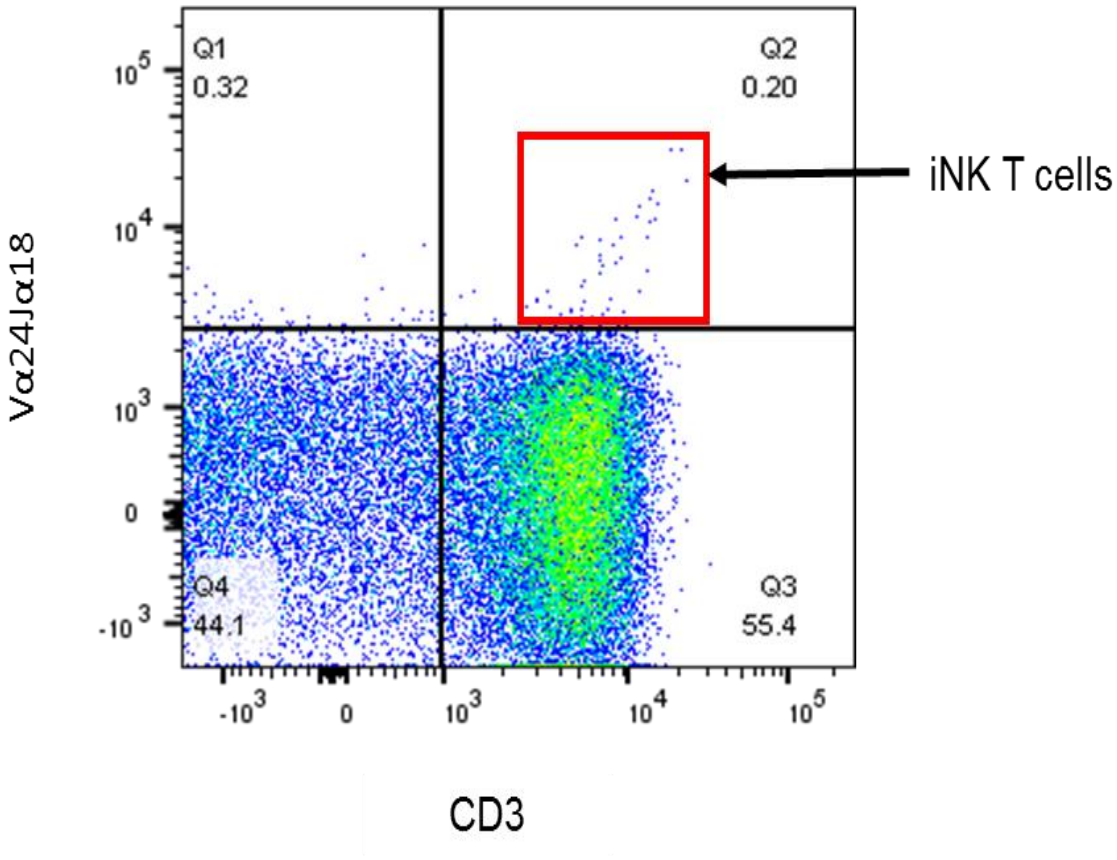


Figure 9: Flow cytometric enumeration of iNK T cells.
Representative flow cytometry dot plot showing CD3 and Vα24Jα18 expression by gated lymphocytes after excluding dead cells and doublets, as shown in Figure 5. The red box indicates how iNK T cells were identified.

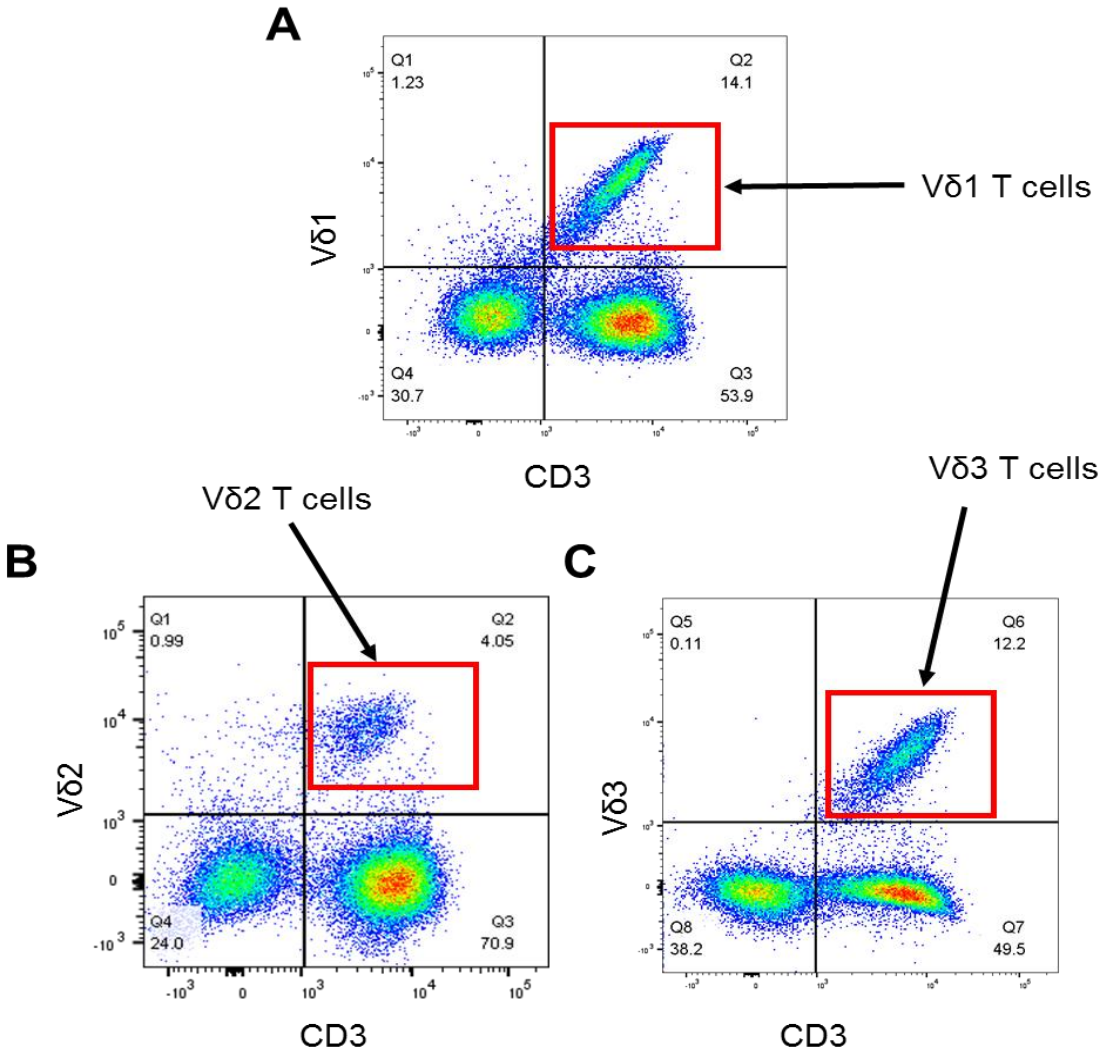


Figure 10: Flow cytometric enumeration of γδ T cell subsets.
*Representative flow cytometry dot plot showing CD3 and Vδ1 (**A**), Vδ2 (**B**) and Vδ3 (**C**) T cell receptor expression by gated lymphocytes after excluding dead cells and doublets, as shown in Figure 5. The red boxes indicate how the different γδ T cell subsets were identified.*

Analysis of flow cytometry findings was done using Flo-Jo version 10 and data generated were further analysed using graph prism version and results are presented in chapter 5.

Pharmacology methods and procedures

A blood sample was removed from study participants at the 2 study visits after administration of ART. EFV or NVP at the 2 study visits were given the previous evening to the ART-E and for the ART-N at 12 weeks study visit only. Pharmacological outcome measures included pharmacokinetics (PK) of mid dose EFV and trough levels (C min) for NVP at baseline and after nutritional supplementation. These were measured in the Pharmacology Laboratory at Makerere University using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection by a trained RA. Regarding the ART-E cohort if their viral loads at baseline were found to be above 1000 viral copies per ml, we called them back to receive intense adherence counselling and sent a sample for resistance testing at Joint Clinical Research Centre in Uganda (JCRC). JCRC has laboratories certified for viral resistance testing and is the centre for HIV viral resistance testing for several Eastern and Southern African countries. Assessment and management of adverse effects of ARVs and RUTF was done at 6 weeks and 12 weeks. Primary carers were asked to call in or report using a telephone call or they could choose to come in outside their appointment days. This would be captured or transferred to the questionnaire for monitoring adverse reactions to drugs and RUTF on the appointment day. Side effects due to RUTF supplementation in this study that were routinely assessed included diarrhoea, abdominal discomfort and skin hives. In the event that there were adverse events to RUTF or ART we categorised them according to severity and admitted all the severe cases if they consented. In case of mild-moderate adverse events category we went ahead and admitted the patient if the carer consented for 24 hours to properly assess the category of side effects in case it was an adverse effect that would warrant the child to stop the RUTF or ART or both. If the carer declined we provided information on how to manage the child and in case the symptoms worsened they would call or return to the health facility for management. We also emphasized the need for the carer to report any new symptoms occurring after enrolment of their child into the study. Considering the children initiating ARVs we worked together with the clinic doctor in the ART clinic to monitor and evaluate any possible side effects and adverse effects of the ART regimen the children were initiated

on during the study period and followed the management protocol and reporting systems in place. We used the standard reporting card issued by Ministry of Health.

High-performance liquid chromatography (HPLC) with ultraviolet (UV) detection.

As shown in the schematic diagram in Figure 10, HPLC machine is made up of a pump, injector, column, detector and integrator or acquisition and display system. The core of the system is the column where separation occurs. The HPLC machine has several applications which include resolution, identification and quantification of a compound. In addition it may be used in separation and purification of chemicals. This is done through a series of processes handled by the different parts. In the solvent reservoir is where the mobile phase or HPLC solvent are stored. This is composed of a mixture of polar and non-polar solvents (e.g. water, acetonitrile and/or methanol). Their concentrations are varied depending on the composition of the sample for analysis. A pump aspirates the mobile phase from the solvent reservoir and forces it through the system's column and detector. Generating very high pressures that are dependent on a number of factors like column dimensions, particle size of the stationary phase, the flow rate and composition of the mobile phase. The sample injector of an HPLC system may be a single injection or an automated system. An injector for an HPLC system should provide injection of the liquid sample within the range of 0.1-100 mL of volume with high reproducibility and under high pressure (up to 4000 psi) through a column which is the heart of an HPLC system. Polished stainless steel columns are usually used and are between 50 and 300 mm long with an internal diameter between 2 and 5 mm and are filled with a stationary phase with a particle size of 3–10 μm. The temperature of the mobile phase and the column should be kept constant during an analysis. The HPLC detector is located at the end of the column and detects the analytes as they elute from the chromatographic column. Commonly used detectors are UV-spectroscopy, fluorescence, mass-spectrometric and electrochemical detectors. Data collection is done by special devices attached in the form of chart recorders or electronic integrators that vary in complexity and in their ability to process, store and reprocess chromatographic data. The computer integrates the response of the detector to each component and places it into a chromatograph that is easy to read and interpret [196].

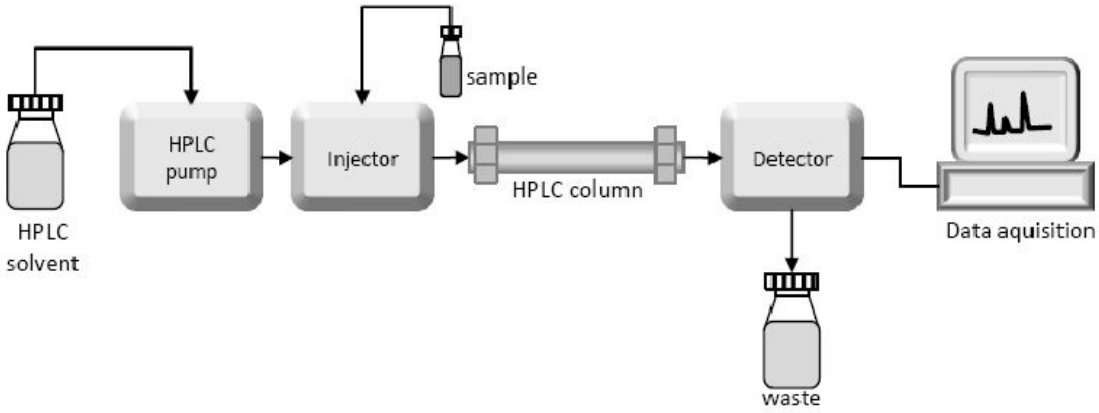


Figure 11: High-performance liquid chromatography (HPLC).
HPLC instrumentation includes a pump, injector, column, detector and integrator or acquisition and display system. The heart of the system is the column where separation occurs.
https://www.shimadzu.eu/sites/default/files/LC_World_Talk_Special_Issue_Volume1.pdf

Table 13. Materials and reagents used for EFV PK levels in the study

Reagents used for the EFV analysis	Equipment used for the EFV analysis
30% acetonitrile	HPLC machine of UV detection A4 (Shimadzu)
30% methanol	Column was Ace C18 3µm 50X3mm
10 molL^{-1} acetic acid	100 µL pipettes, autosampler tubes
4 molL^{-1} potassium hydroxide	Vortexer machine
The above reagents made up the mobile phase and were mixed at a pH of 4.3	Centrifuge 5424 eppendorf

Preparation of samples for Efavirenz analysis on an HPLC machine

Materials and reagents used for determining the EFV PK of the samples are in table 11. Plasma samples were thawed at room temperature. The thawed sample was then vortexed and 100 µL of plasma was pipetted into an eppendorf tube. To the plasma 200 µL of acetonitrile were added to precipitate the plasma proteins and a cap was placed on the tube. Thereafter the tube was vortexed for 10 seconds to mix the contents. The mixture

was then centrifuged at 3000 rpms for 5 mins. 6 µL of the supernatant was then transferred into an insert of an autosampler vial to mount onto the HPLC for analysis.

Determination of plasma concentration of Efavirenz

Plasma EFV was determined by reverse-phase HPLC with UV detection as previously described [197]. The HPLC machine, used consisted of a system controller (model SCL-10AVP), a solvent deliverer pump (model LC-10ATVP), auto injector (model SIL-10ADVP), column oven (model STO-10ASVP) and a spectrophotometric UV-vis detector (model SPD-10AVP) all supplied by Shimadzu co-operation Kyoto Japan. The column used was Ace3C18, 3 mm 50 X 30mm (Advanced Chromatography Technologies, Aberdeen, UK). The mobile phase consisted of 30% acetonitrile, 30% methanol, 4 mmol l-1 potassium hydroxide and 10 mmol l-1 acetic acid (pH 4.3). Plasma proteins were precipitated with acetonitrile before centrifuging. Supernatant (6 µL from the precipitation of plasma and acetonitrile as described above) were injected and eluted at 0.80 ml min-1 for 3.5 min. The retention time for EFV was 2.42 min as detected at UV-VIS 1, 210 nm, UV-VIS 2,220 nm. This method was linear with a within-day coefficient of variation of 3.2, 3.3 and 5.1% at concentrations of 2.0 mM (n = 17), 8.0 mM (n = 17), and 20 mM (n = 16), respectively, and a between-day coefficient of variation of 4.1% (n = 50). The limit of quantification for the method was set at 0.35 mM. The limit of quantification is the smallest concentration of a drug concentration and that can be reliably measured by an analytical procedure and may be referred to as the limit of detection too.

Preparation of plasma blood samples before analysis for Nevirapine PK levels

Materials and reagents used for determining the NVP PK of the samples are in table 12. Plasma samples were thawed at room temperature. The thawed sample was then vortexed and 100 µL of plasma was pipetted into an eppendorf tube. To the plasma 200 µL of acetonitrile were added to the plasma to precipitate the plasma proteins and a cap was placed on the tube. Thereafter the tube was vortexed for 10 seconds to mix the contents. The mixture was then centrifuged at 5000 rpms for 5 mins. 6 µL of the supernatant was then transferred into an insert of an autosampler vial to mount onto the HPLC for analysis.

Determination of plasma concentration of Nevirapine

Plasma NVP was determined by reverse-phase HPLC with UV detection as previously described by Minzi et al in 2010 [198]. The HPLC machine, used consisted of a system controller (model SCL-10AVP), a solvent deliverer pump (model LC-10ATVP), auto injector (model SIL-10ADVP), column oven (model STO-10ASVP) and a spectrophotometric UV-vis detector (model SPD-10AVP) all supplied by Shimadzu co-operation Kyoto Japan. The column used was zorbax eclipse XBD-phenyl 5 µm C18, 4.6 mm X 150 mm (Agilent Technologies, USA). The mobile phase consisted of 75% phosphate buffer and 25% acetonitrile. Plasma proteins were precipitated with acetonitrile before centrifuging. Supernatant (6 µL) was injected and eluted at 50 µl min-1 for 15 min. The retention time for nevirapine was 7.5 min as detected at UV-VIS 280 nm. This method was linear, with a within-day coefficient of variation of 3.0, 2.3 and 4.2% at concentrations of 1.0 µg/ml (n = 17), 5.0 µg/ml (n = 17), and 16.0 µg/ml (n = 16), respectively, and a between-day coefficient of variation of4.1% (n = 50). The limit of quantification for the method was set at 0.4 µg/ml, (table 14).

Table 14. Materials and reagents used for NVP PK levels in the study

Reagents used for the NVP analysis	Equipment used for the NVP analysis
25% acetronile	HPLC machine of UV detection A4 (Shimadzu)
4.29g of potassium hypochlorate	Column was zorbax eclipse XBD-phenyl 5µm C18, 4.6mm X 150mm
1.914g of sodium hypochlorate	100 µL pipettes, autosampler tubes
The above reagents made up the mobile phase	Vortexer machine, Centrifuge 5424 eppendorf centrifuge

Qualitative study methods and procedures

The general objective of this qualitative study was to explore and understand the knowledge, practices and attitudes of carers of HIV-infected children towards feeding malnourished children and the use of RUTF and ART. The specific objectives were:

1. To assess knowledge and perception of health and malnutrition.

2. To examine the malnutrition burden and attitudes in the community.

3. To describe the interventions/programmes in place for management of malnutrition.

4. Establish RUTF and ART perceptions and behaviour.

5. Obtain suggestions for the improvement of nutrition service delivery.

This was part of the cohort study design that involved mixed methods of data collection. Data were collected between January 2015 and March 2016. The study participants included carers whose children were accessing HIV care at the study sites. Following establishment of the study team led by the principle investigator other team members involved were two research assistants. The study team piloted the study tool and adjusted it accordingly.

Data collection methods

The data were collected using in-depth interview (IDI) method through audio recorded interviews, using IDI guides. IDIs enabled researchers to explore individual experiences of the women and men in the management of malnutrition in HIV. The study tools were translated into "*Luganda*", the main local language spoken in Central Uganda. The study tools were then pre-tested on two men and four women. This led to adjustment of the tools particularly, rearranging the questions to maintain a good flow of the interviews. Participants were asked for language preference for *Luganda* or English and they chose *Luganda*. Key informant interviews were carried out with carers who were opinion leaders among the carers and were not able to join FGD due to lack of time and busy schedules, Otherwise carers were invited to participate in FGDs. FGD and key informant data were collected by a doctor as a RA trained in qualitative methods and a counsellor

trained in anthropology. These staff received Good Clinical Practice (GCP) training as well as training in conducting FGDs and administering individual Key informant interviews. The English transcripts were reviewed by a third person.

Informed written consent was sought from the carers before enrolment in the study. Consenting to participate in the qualitative study was done in two stages. We started off by sensitising the primary carers of HIV infected children receiving HIV care services at the study sites about the study. Primary carers were provided with detailed information of the study using the information sheet in the language they could read. After reading the information sheet and asking questions of the study they were asked if they wanted to participate in FGDs. Those who verbally accepted to participate were randomly selected to get 8-12 participants per FGD group. Two FGDs per site were to be held, each lasting 30-45 mins involving a male group and female group. Participants were given an appointment according to the most convenient day they identified. The FGD were all to be carried out mid-morning and the seating arrangement was in a circle. The team of researchers conducting the FGDs consisted of a moderator and a recording person who also doubled as a time keeper. The moderator introduced the team of researchers, provided details of the research and guidelines of carrying out the research once again. Audio recording to capture the data was done if the group agreed to be recorded otherwise the main stay of data capture was by the minute taker.

Regarding IDIs, these were carried out with key informants from among the primary carers who couldn't make a complete FGD group or were identified as the key opinion leaders by the study sites or fellow carers. These included but were not limited to grandmothers, grandfathers, expert patients/clients who were informed about management of children with HIV and were having children under their carer. We planned to carry out 2 IDIs per site. Participants were identified through the health facility administration and inquiring from the carers during their visits to help in identification of their leaders. The IDIs were to be taken for 30-45 mins after the participant provided informed written consent. No audio recordings were done.

At week 0 the main issues were to assess their global knowledge, practices and attitude in feeding malnourished children and their perceptions of RUTF. At week 12 weeks we

aimed to assess their experience in managing their children with regard to using the RUTF and ART adherence.

Study outcome measures

The primary outcome of this study was the unspecified change in T, B and NK cells and their subsets and drug levels at 12 weeks.

Data management and analysis

Primary data sets were cleaned and edited by the Principal Investigator and are protected by a series of passwords.

Cleaned versions of the clinical, nutrition, immunology, pharmacology data sets were transferred to Stata version 12 (Stata Corp., College Station, TX, USA) for overall analysis. However sub-analysis for the nutrition, immunology and pharmacology data sets was done using PRISM version 7 in order to plot the standard curves and then calculate the drug concentrations. Regarding the immunological outcomes, flow cytometry data was analysed using FloJo software and transferred to PRISM version 7. Thereafter the frequencies and absolute counts generated into EXCEL then exported to Stata for pooled analysis.

Categorical variables were summarized as proportions, continuous variables as means or median and standard deviation depending on their distribution. Associations between variables of interest and outcomes were analysed using the Chi square test if outcomes are reported as proportions (for example; association between gender of carer versus adherence) and using the t test for normally distributed outcomes expressed as mean values, and the Mann-Whitney test if not normally distributed. Confounders were controlled for at the bivariate level utilizing stratification. We used the ANOVA (Analysis of Variance) test to compare means of continuous variable that were normally distributed for more than 2 groups eg comparing the average $CD4^+$ T cells with nutrition status. To adjust for confounder's we used multiple linear regression, and logistic regression techniques for continuous and binary outcomes respectively.

In analysing the qualitative study, the audio interviews were transcribed verbatim and typed into Microsoft Word version 7. The research team read through typed transcripts

and filled in gaps by listening back and forth to the audio recordings for the FGDs. Content analysis was used to analyse the qualitative data to mainly assess adherence to ART and RUTF. Data analysis was guided by Graneheim and Lundman 2004 framework to capture latent and manifest content in the interview script [199]. Multiple readings of scripts were done to make sense of the material, identify study themes and sub-themes relating to adherence of RUTF and ART by study participants [200].

Quality assurance

Quality control was assured by using pretested, pre-coded and standardized questionnaire to collect the clinical data and this was done by trained research assistant or PhD student both certified in Good Clinical Practice. Instruments used for measuring temperature, weight etc were checked and calibrated daily. Aseptic conditions were observed in sample collection and reputable accredited laboratories were used in Uganda and Ireland.

Study Limitations/challenges

We were unable to carry out a randomised clinical trial (RCT) as previously planned among the MAM to assess the impact of RUTF on our study outcomes. This was because Uganda as a country updated the policy on management of malnutrition to have all MAMs and SAM in outpatient therapeutic centers supplemented with RUTF making an RCT design unethical. Secondly it was difficult to randomly select patients who were ART naïve while enrolling them in the study as they were so few therefore we enrolled them consecutively. We were not able to collect samples on every follow up visit therefore subtle immunological changes may have been missed. Lastly, regarding the sample size calculation, we used the Vδ2 T cells to calculate the sample size was because the primary outcomes were based on answering the immunology objectives not the clinical outcomes since we could no longer carryout an RCT. The clinical information provided the picture of what cohorts or background we have based on in the thesis. In addition the study was well powered for the immunology and pharmacology outcomes however for the clinical outcomes we would need very large sample sizes to address the clinical outcomes statistically and this would have a large cost implication.

Dissemination of results from the study

The results of this study will be disseminated to the department of Immunology School of Postgraduate studies at Trinity College Dublin, Research department of IDI and the IRBs that reviewed this protocol. Ministry of Health and Uganda National Council for Science and Technology will also be provided the findings of this study. Results will be presented at scientific gatherings such as conferences, seminars etc and also be sent to a reputable journal for publication.

CHAPTER THREE: Clinical outcome results

This chapter presents the clinical results of this study that were collected during the recruitment period of the study participants. All the enrolled study participants acquired the HIV infection perinatally.

Introduction

Perinatally acquired HIV infection is the commonest mode of MTCT of HIV infection and occurs at a crucial time of the immune system development of a child. In the absence of ART, 50% of these children do not survive to celebrate their 2nd birthday and 75% will have died by their 5th birthday. In 2003 before the rollout of ART in India a pediatric HIV study, where the mean age of study participants was 4 years demonstrated the common clinical manifestations of HIV. The constellation of clinical signs of pediatric HIV were oral candidiasis (43%), pulmonary tuberculosis (PTB) (35%), recurrent respiratory infections (26%), bacterial skin infection (21%), pruritic-papular dermatitis (19%), hepatosplenomegaly and lymphadenopathy (14%) and chronic or persistent diarrhea (7%) [201]. While another study undertaken to determine mortality rates and clinical predictors of mortality during the period prior to ART initiation in a Zambia documented the mortality rate was 8% and factors associated with mortality included younger age, anemia, and malnutrition [202]. Another study to determine the impact of HIV on child mortality and risk factors for mortality among HIV-infected and HIV-exposed uninfected children in a longitudinal cohort in rural Uganda showed mortality rate was six times higher in ART-naive HIV infected children than in HIV-exposed uninfected children (HR = 6.4, 95% CI: 2.4–16.6). Among HIV-infected children, mortality was highest in those who were less than 2 years of age. Malnutrition and young age were the commonest risk factors of mortality [203]. With the event of ART, children are now able to survive to adulthood with better quality of life. However, in the setting of malnutrition, their survival is threatened and the gains of ART are reversed. In addition, understanding that children still present with advanced AIDs in the era of ART will alert physicians to actively look out for the children who have missed opportunities to access ART timely so that they are prioritised to access ART so as to achieve the 90-90-90 global target and improve child survival. Therefore we aimed at studying an understudied group of children that currently

has the highest pediatric burden of HIV and these are children presenting with malnutrition. We provided RUTF to the malnourished HIV infected children and clinically investigated the impact of nutritional status and supplementation on the clinical outcomes and in order to achieve the study outcomes the methodology is summarized in figure 10. The specific outcomes of interest were the rate of OIs the children had during the study period, type of OI occurrence and hospitalisation rate which we described as the average number of times study participants were hospitalised during the study period. We investigated the IRIS rate in this cohort and the type of IRIS event the study participants presented with and the presence of the re-feeding syndrome. Finally we investigated the rate of mortality in this cohort.

Since adults were not included in the study, it is difficult to explore if there is a difference in immune responses regarding the horizontal and vertically transmitted effect. Generally, the majority of studies done have shown that infant immune responses are distinct from those of adults [204]. Due to the abrupt transition of a child at birth from the mother's sterile environment in the intrauterine period, through the birth canal with its microbe environment and finally to the environment they are to live in they are exposed to a barrage of repeated immune stimuli. Substantial evidence demonstrates that the neonatal immune system is highly adapted to this abrupt transition and early life. In contrast, immunologically mature adults have acclimated to persistent antigen exposure, including a host of commensal bacteria and viruses that reside in the gut and skin, and as a result, orchestrate immune responses differently than infants. In the context of HIV, although ordinarily infants and adults respond differently to antigenic stimulation, HIV invariably causes immune suppression in the absence of ART with disease progressing faster in the young child than adult. Infants exposed to HIV intrauterine have also shown to have immunological impairment as demonstrated by lower vaccine response and have increased risk of infections and mortality compared to HIV un-exposed children but the cause remains unknown.

Phenotypic and functional characteristics of immune cells between infants and adults have substantial differences. Neonatal neutrophils have lower chemotactic responses and reduced phagocytic capacities compared to adult neutrophils [205]. Adults demonstrate a higher number of dendritic cells than neonates do while neonate dendritic cells secrete

higher levels of interleukins, demonstrating that they are not deficient in cytokine production [206]. Cord blood also contains higher proportions of NK cells than adult blood, but they have distinct expression levels of activating and inhibitory markers. NK cells in cord blood have a reduced capacity to respond to stimuli and lower cytotoxic capacity than adult NK cells though this can be enhanced in vitro [207].

For a long time, it was thought that infants have deficient $CD4^+$ T cell responses. On the contrary, recent studies show that the apparent infant deficiency is largely modulated by T regulatory cells. Children generally have much higher $CD4^+$ T cell counts than adults do. Babies have higher $CD4^+$ T cell counts than children do. Because of this difference for a longtime WHO HIV guidelines recommend children are monitored using $CD4^+$ percentage rather than the counts. However, within the CD4+ repertoire, infants have lower numbers of circulating $CD4^+$ CCR5+ T cells, the main target for HIV; although a high, proportion was recently reported in the infant's gut. Infants also exhibit a bias toward Th2 responses and this is likely due to the high levels of Th2-promoting cytokines, such as IL-10, and prostaglandin E2 in early life. In addition, infants have impaired humoral responses than adults, however infant immunization studies have provided a proof that infants can mount comparable or higher immune responses than adults following vaccination thus have provided information that has guided immunization schedules [204]

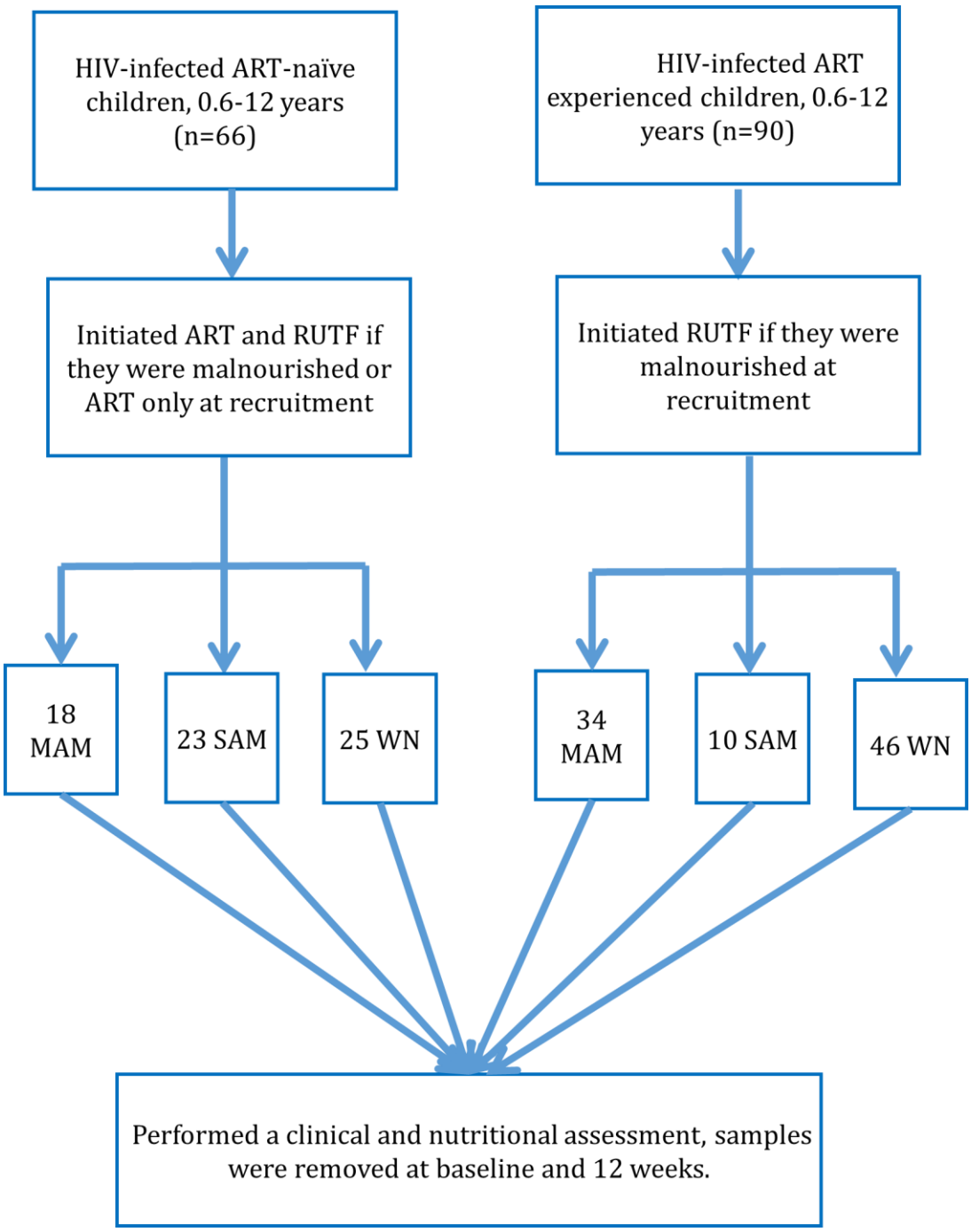


Figure 12: Study profile.

The ART naïve children were consecutively enrolled, while the ART experienced children were randomly enrolled. Malnourished ART naïve children received both ART and RUTF while the ART experienced malnourished children received only RUTF. All the well-nourished children received ART. Patient samples were collected at baseline and 12 weeks follow up. Data analysis was done using the appropriate soft-ware and triangulated with the qualitative study.

Results

Screening of study participants

In order to enroll the appropriate study population, we screened all HIV positive children attending the pediatric HIV services at each study site, during the study period. We used the screening log (appendix 4) and WHO growth charts to identify the MAM, SAM and WN guided by the WAZ, WHZ, BMI and MUAC z score [208]. A total of 278 HIV infected children at the study sites were screened, 156 fulfilled the study criteria and were enrolled into the study between January 2015 to December 2017 (Figure 11). One hundred and twenty-two children were excluded from the study. As shown in table 15 the commonest reason for exclusion from the study was having mild malnutrition and being HIV exposed but negative. The study patients came from 7 main districts in central Uganda. Figure 12 shows the map of the different districts that the patients were residing in during the period of study. The majority were from Wakiso district.

Table 15. The commonest reason for exclusion from the study

Reasons for not being recruited	Numbers	Percentages
Suspected or confirmed malignancy	2	1.64
Vomiting	6	4.92
Congenital disorders	4	3.28
Cerebral palsy	4	3.28
ART naïve critically ill children	10	8.20
Odematous malnutrition	3	2.46
Recent RUTF use in the last 3 months	1	0.80
Stunted	9	7.38
Controversial HIV tests	3	2.46
Mild malnutrition	34	27.87
Minor with minor**	3	2.46
HIV exposed negative	33	27.05
Doctor busy	6	4.92
Inappropriate age range	4	3.28
Total	**122**	**100**

** Minor with a minor refers to a child who was brought by a carer who was < 18 years old but otherwise fulfilled the anthropometry assessment.

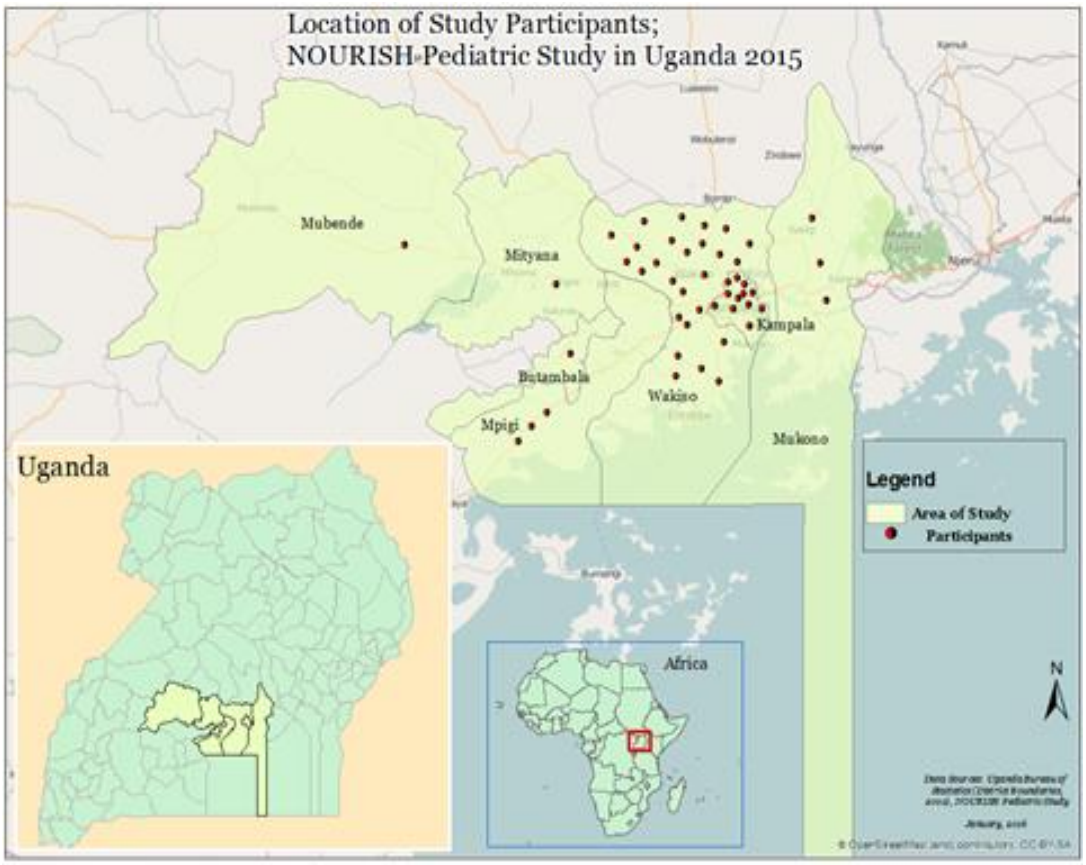


Figure 13: Distribution of the enrolled study participants according to the district of residence during the study period.

The map of Africa showing the location of Uganda in the mid-bottom of the figure in a red box. In an anticlockwise direction is the Ugandan map showing the districts where the participants came from. In the upper half of the figure is the map if central Buganda districts with a legend showing the red dots representing the physical addresses of study participants. The study participants were form 7 districts from Central Uganda namely: Mubende, Mityana, Butambala, Mpigi, Wakiso, Kampala and Mukono.

The age range of the children accrued in the study was 9 months to 12 years though the proposed study age was 6 months to 12 years. The majority of patients recruited in the study were between 5-12 years (table 16) and this age category had the highest malnutrition burden.

Table 16. Age distribution by nutrition status of the children recruited on the study

Nutrition status	Age in categories in years ≤1	1-5	≥5-12	Total
MAM	2	19	30	51
	3.92	37.25	58.82	100.00
	18.18	28.36	38.46	32.69
SAM	6	19	9	34
	17.65	55.88	26.47	100.00
	54.55	28.36	11.54	21.79
WN	3	29	39	71
	4.23	40.85	54.93	100.00
	27.27	43.28	50.00	45.51
Total	11	67	78	156
	7.05	42.95	50.00	100.00
	100.00	100.00	100.00	100.00

MAM-moderate acute malnutrition, SAM-severe acute malnutrition, WN-well-nourished

Immunisation status as reported by the primary carer of the enrolled study participants.

Child survival in LRS depends on the synergy of several known child survival strategies addressing the challenge/threats to child survival. The threats to child survival change with age and many times overlap in childhood. One intervention that has transformed the lives of children is ensuring a continuum of high vaccination coverage as the burden of childhood diseases that threaten child survival in their infancy are highly preventable by vaccination. According to the Ugandan National Expanded Programme on Immunisation, a child is fully vaccinated if they have received one dose of BCG injection at birth, four doses of oral polio vaccine that are given at birth (denoted as polio 0), 6 weeks (polio 1), 10 weeks (polio 2) and 14 weeks (polio 3), three doses of diphtheria, pertussis and tetanus (DPT), hepatitis B (Hep B), H. influenza type b (Hib) as a penta-vaccine that is given at 6 weeks, 10 weeks and 14 weeks and one dose of the measles vaccine at 9 months [209]. Additionally, the timeliness of vaccinations is crucial and is defined for each vaccine according to WHO recommended time ranges: BCG (birth–8 weeks), polio 0 (birth–4 weeks), three polio and three pentavalent vaccines (4 weeks–2 months; 8 weeks–4 months; 12 weeks–6 months) and measles vaccine (38 weeks–12 months) [210]. We assessed the timeliness of receiving the vaccines by the study participants by interviewing the primary carers. Overall, 127/156 (81.4%) received BCG and polio 0 vaccines at birth,

125/156 (80.1%) received polio 1 and DPT, Hib, Hep B vaccines at 6 weeks, 125/156 (80.1%) received polio 2 and DPT, Hib, Hep B at 10 weeks, 123/156 (78.9%) received polio 3 and DPT, Hib, Hep B at 14 weeks and 115/156 (73.7%) received measles at 9 months.

Clinical signs and symptoms reported by the primary carer of the enrolled children

We assessed the occurrence of fever by interviewing the primary carer if the child had fever at enrolment and by taking the axillary temperature using a mercury thermometer. Overall, 41/156 (26.3%) reported having fever at study enrolment and 15/156 (9.6%) had documented fever (temperature above 37.5^0C). The mean axillary temperature was 38.7^0C indicative of the presence of fever.

Cough was present in 91/156 (58.3%) with difficulty in breathing being reported in 16/156 (10.3%) at recruitment and 21/156 (13.5%) with rapid respiratory rate and physical signs of respiratory distress. Convulsions were present in 3/156 children who got admitted.

On clinical examination 30/156 (19.2%) had mild pallor, 10/156 (6.4%) moderate pallor and 7/156 (4.5%) severe pallor while 111/156 (71.2%) had no pallor of the mucus membranes and 3 had no documented assessment. Overall, 5/156 (3.2%) had jaundice and 59/156 (37.8%) had significant lymphadenopathy.

After a detailed clinical assessment, WHO HIV clinical staging of HIV disease was done. The majority of the study participants were WHO stage 1, 76/156 (48.7%), while stage 2 disease was 9/156 (5.8%), stage 3 was 33/156 (21.2%) and stage 4 disease was 38/156 (24.4%). Considering that the study design involved malnourished and WN HIV infected children, the two groups are generally well represented in this study.

Effect of nutrition status on baseline clinical outcomes among HIV-infected children

The effect of nutritional status on baseline characteristics of interest among the ART naïve and ART experienced cohorts was assessed and will be reported in succession as these are distinctively different cohorts at baseline according to the ART status. HIV is an

independent and non-modifiable risk factor for poor nutritional outcomes in those who are already infected, but an improvement in their nutritional status may significantly decrease their morbidity, thus we used RUTF as the choice of supplement (Figure 18 in chapter 4 for the description of RUTF).

ART naïve HIV infected children cohort results

The ART naïve cohort accounted for 66/156 (43.3%) of the study participants (Figure 11). The median age in years among the ART naïve children was 1.95 years (IQR=0.5-5.3). Their age distribution is shown in Figure 12. Table 17 illustrates the socio-demographic characteristic of the ART naïve children. The male: female ratio was 1:1.1, with 41/66 (62.1%) having malnutrition (MAM and SAM). The proportion of ART naïve children recruited on the study with MAM was 25.8%, SAM was 36.4% and WN was 37.9%. Forty-five and a half percent were enrolled into the study from Mildmay Center and the majority (63.6%) was from the Ganda tribe. The study participants whose mothers were their next of kin or primary carer comprised 72.7% and 88.3% of the study participants had their mother alive. Only 69.4% of the mothers reported to be on ART and 57.9% knew the HIV sero-status of their spouses. Overall 72.9% of the ART naïve children were fully immunised.

The past medical history was assessed by conducting interviews with the primary carers among the ART naive cohort. The parameters assessd included previous TB exposure, treatment, persistent diarrheal episodes, recent hospitalisation, blood trasfusion and malnutrition episodes as this would provide vital information on the HIV disease progression. Generally, 10.6% were previously treated for TB. Overall TB contact was reported at 9.1% while previous diarrheal episodes were experienced in 30.3%. In addition, 1.5% of the patients were previously admitted in the last 3 months prior to enrolment in the study and 25.8% had experienced a malnutrition episode in the last 2 years (Table 18).

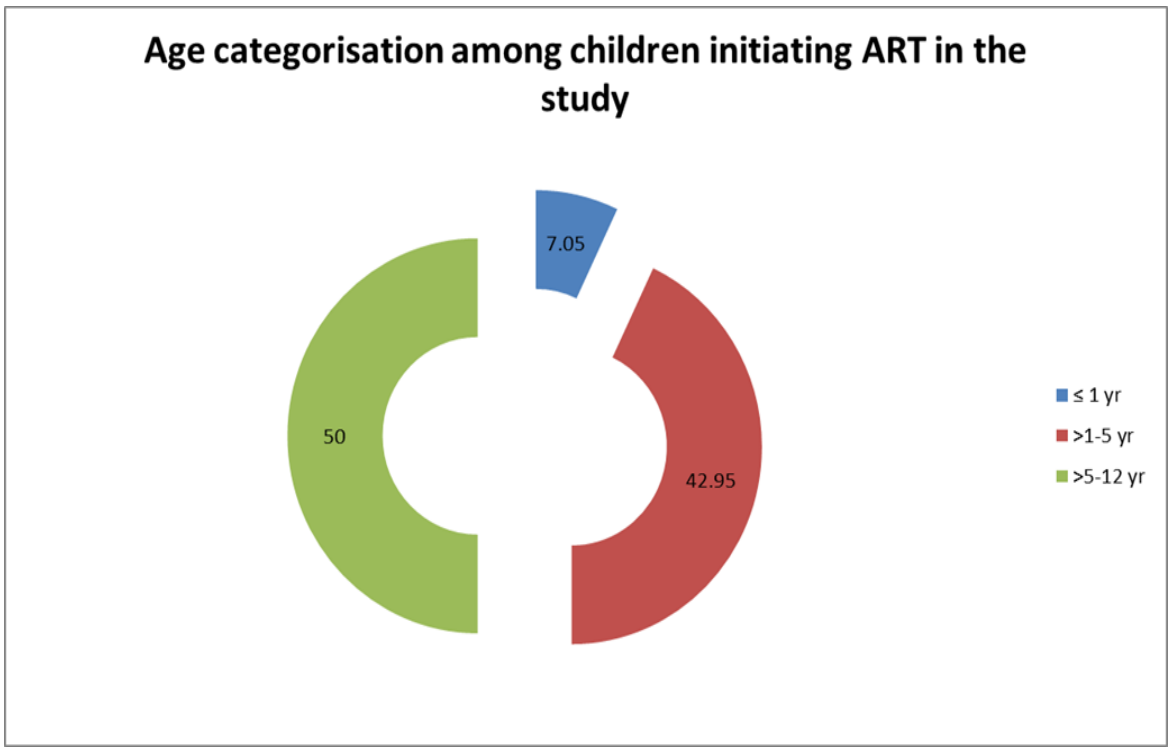


Figure 14: Age distribution of ART naïve HIV infected children enrolled on the study.
This figure shows a pie chart of the age distribution of ART naïve HIV infected children enrolled on the study. They are divided into 3 age categories where those ≤ 1 years of age are represented in blue, >1- 5 years of age are in maroon and in green are those >5-12 years of age.

The commonest clinical presentation among the ART-N cohort was having a cough for more than 2 weeks 43/66 (65.2%) and fever 26/66 (39.4%). Other clinical presentations included convulsions 2/66 (3%) and oral sores 8/66 (12.1%). On clinical examination significant lymphadenopathy was 21/66 (32.3%) with pallor of the mucus membranes in 30/66 (45.5%).

The haematological indices (Table 19) of the ART naïve patients on the study were assessed using automated cell counting and analysis machines at the MRC Laboratory in Uganda. The median haemoglobin (Hb) concentration was 10.0 g/dl (IQR: 8.6-11), Mean corpuscular haemoglobin (MCH) of 23.6 pg (IQR: 21.5-23.6), Mean corpuscular volume (MCV) of 76 fL (IQR: 71.7-80) and red blood cell distribution width (RDW) % of 17.25 (IQR: 15.2-19.1) indicating anemia, at ART initiation. All the other indices were within the normal ranges.

Table 17. The social-demographic characteristics of the ART naïve children at baseline

Demographic characteristic	N/66 (Percentage)
Sex : Male	31 (46.97)
Female	35 (53.03)
Nutrition status	
Malnourished	41 (62.12)
Well nourished	25 (37.88)
Study site	
Mildmay center	30 (45.45)
KCCA clinics	28 (42.42)
Mwanamugimu Unit	8 (12.12)
Tribe as Ganda	42 (63.64)
Next of Kin as mother	48 (72.73)
Mother on ART	34 (69.39)
Mother alive	53 (88.33)
Mother aware of HIV sero-status of father to patient	22 (57.89)
Immunisation status	43 (72.88)

Table 18. Past Medical history of the ART naïve children enrolled on the study

Item	Yes	No
Previous TB treatment	7 (10.6%)	59 (89.4%)
TB contact	6 (9.1%)	60 (90.9%)
Previous persistent diarrhea episodes in the last 6 months	20 (30.3%)	40 (69.7%)
Previous admissions in the last 3 months	1 (1.5%)	65 (98.5%)
Previous blood transfusion in the last 3 months	0 (0%)	66 (100%)
Previous malnutrition episode in the last 2 years	17 (25.8%)	49 (74.2%)

Serum electrolytes of the ART naïve children on the study were analysed from MRC Laboratory in Uganda. These were prepared from 66 ART naïve HIV-infected children sampled at baseline. The median, inter-quartile (IQR) and normal ranges are provided for each electrolyte analysed as shown in table 20. All the electrolytes analysed had a median that was within the normal range except for serum level of phosphates that was high.

Table 21 and 22 are showing the renal and liver function test results of the ART naïve HIV positive children on the study respectively. Serum samples were prepared from 66 HIV-infected ART naïve children sampled at baseline. The median, IQR and normal range for each tested variable has been given. In table 21 median serum creatinine levels are lower than expected while in table 22 the median serum alkaline phosphatase (ALP) levels are high.

The majority of the ART naïve children 53/66 (85.5%) at ART initiation had a median $CD4^+$ T cell percentage <30% and median $CD4^+$ T cell absolute count of ≤1000 cells/ml using fresh whole blood. The median viral load of the 66 patients initiating ART was 240,753 cps/mL (IQR: 45,752-1,286,399).

Table 19. Haematological indices of the ART naïve children enrolled on the study at baseline

Hematological indices	Median	IQR	Normal range
WBC absc ($x10^3/\mu L$)	10.4	7.09-13.40	$4.0\text{-}14x10^3$
RBC absc ($x10^3/\mu L$)	4.31	3.95-4.57	$4.5\text{-}6.5x10^6$
Hb (g/dl)	**10.0***	8.6-11	11.5-15.5
HCT (%)	32.7	27-35.7	35-45
MCV(fL)	**76***	71.7-80	77-95
MCH (pg)	23.6	21.5-23.6	23-31
MCHC (g/dl)	30.8	29.3-31.9	28-33
RDW (%)	17.25	15.2-19.1	11-16
Platelets ($x10^3/\mu L$)	382	289-493	$150\text{-}400x10^3$
MPV (fL)	9	7.9-9.8	6-10

***denotes parameter that is outside the normal range, RBC: red blood cells, Hb: haemoglobin, HCT: Haematocrit concentration, MCV: mean corpuscular volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, RDW: red blood cell distribution width, MPV: mean platelet volume.**

At ART initiation 62/66 (93.94%) received an NNRTI based regimen, 4.55% a triple nucleoside based regimen and 1.52% a PI based regimen. Overall 3 children died in this group in the first 2 weeks of recruitment and they were all severely malnourished with multiple complications. One WN control was found to have cryptococcal meningitis at presentation; he progressively became severely malnourished as he was in coma for over 2 weeks, later developed cerebral palsy like condition.

Table 20. The serum electrolytes of the ART naïve children on the study at baseline

Serum electrolytes	Median	IQR	Normal range
Calcium (mmol/L)	2.23	2.07- 2.32	2.15-2.55
Chloride (mmol/L)	102.2	99.7 -103.8	98-107
Magnesium (mmol/L)	0.83	0.79-0.90	0.7-1.05
Phosphates (mmol/L)	**1.64***	1.5-1.81	0.87-1.45
Potassium (mmol/L)	4.5	4.1- 5.5	4.0-5.0
Sodium (mmol/L)	134.5	132-136	136-145

***denotes parameter that is outside the normal range**

Table 21. The Renal function test results of the ART naïve children on the study at baseline

Renal function tests	Median	IQR	Normal range
Creatinine (µmol/L)	**21***	18-27	47-209
Blood urea nitrogen (mmol/L)	2.05	1.3-2.7	2.0-11.9

***denotes parameter that is outside the normal range**

Table 22. The Liver function test results of the ART naïve children on the study at baseline

Liver function tests	Median	IQR	Normal range
ALT (U/L)	20	13-31	8-61
AST (U/L)	41	32-56	14-60
ALP (U/L)	**190.5***	140-253	48-164
Tot bilirubin (µmol/L)	2.4	1.4-4.3	3.9-37

***denotes parameter that is outside the normal range**

ART experienced HIV infected children cohort results

A total number of 90 ART experienced children were enrolled in this cohort, 48.9% were malnourished and 51.1% were well-nourished. Two children died after enrolment in the study. These died from home and attempts to get a detailed verbal autopsy were futile.

The average age of the ART experienced cohort was 6.8 years (IQR: 4.5-10 years). Figure 13 shows the distribution of the malnourished and well-nourished child in the 3 age categorisations. There were no malnourished patients in the age group ≤ 1 years and the majority of malnourished patients were ≥ 5 years-12 years (figure 14). Among the ART experienced cohort about half were females and half were malnourished. The median length of time on ART in months was 42 (IQR: 15-66 months). About 73% had their mothers as the next of kin and 93% were on ART. However about 67% knew the HIV status of the father to the study patient. Overall the immunisation coverage was 83.2% among the enrolled study participants, table 23. The enrolled children were on 8 types of ART regimen combination, figure 15. Of the 8 combinations 5 were NNRTI based, 2 were PI based and 1 was a triple nucleoside based regimens. Thirty-three were receiving AZT+3TC+NVP, 16 were on ABC+3TC+LPV/r, and 15 were on ABC + 3TC + EFV and 10 on ABC + 3TC + NVP.

In this cohort of ART experienced HIV infected children, 28.1% were previously treated for TB as shown in table 24. Overall TB contact was reported at 23.6%. Previous diarrheal episodes were experienced in 6.7%. Though 4.5% were admitted in the last 3 months prior to enrolment in the study, 24.7% had experienced a malnutrition episode in the last 2 years.

Table 25 shows the clinical signs and symptoms exhibited by the ART experienced children at baseline. The 1st column shows the clinical sign or symptom ie clinical characteristics studied, 2nd column shows the symptom/sign were present on clinical examination, 3rd column is illustrating the numbers for each response for each clinical variable and the 4th column the proportions/percentages of each response for each variable. The commonest clinical manifestations were cough for more than 2 weeks (52.81%), lymphadenopathy (42.57%) and skin rashes or dermatosis (29.21%).

The vital clinical signs exhibited by the ART experienced children at baseline included moderate-severe anemia in 4/89 (4.5%), 3.4% had jaundice of the mucus membranes, 10.11% had some dehydration and none were severely malnourished. Overall, 4.5% had recorded fever. The median respiratory rate was 15 breaths per minute (IQR: 12-16) and median pulse rate was 88 beats per minute (IQR: 76-105).

The hematological indices were all within normal ranges, Table 26.

Table 23. The social-demographic characteristics of the ART experienced children at baseline enrolled on the study

Demographic characteristic	N/90 (Percentage)
Sex	
Male	44 (48.9)
Female	46 (51.1)
Nutrition status	
Malnourished	44 (48.9)
Well nourished	46 (51.1)
Study site	
Mildmay center	75 (84.27)
KCCA clinics	13 (14.61)
Tribe as Ganda	65 (73.03)
Next of Kin as mother	65 (73.03)
Mother on ART	70 (93.33)
Mother alive	79 (88.76)
Mother aware of HIV sero-status of father to patient	56 (66.67)
Immunisation status	74 (83.15)

Table 24. The past medical history of the ART experienced patients enrolled on the study at baseline

Item	Yes	No
Previous TB treatment	25 (28.09%)	64 (71.91%)
TB contact	21 (23.60%)	68 (76.40%)
Previous persistent diarrhea episodes in the last 6 months	6 (6.74%)	83 (93.26%)
Previous admissions in the last 3 months	4 (4.49%)	85 (95.51%)
Previous blood transfusion in the last 3 months	0 (0%)	89 (100%)
Previous malnutrition episode in the last 2 years	22 (24.72%)	67 (75.28%)

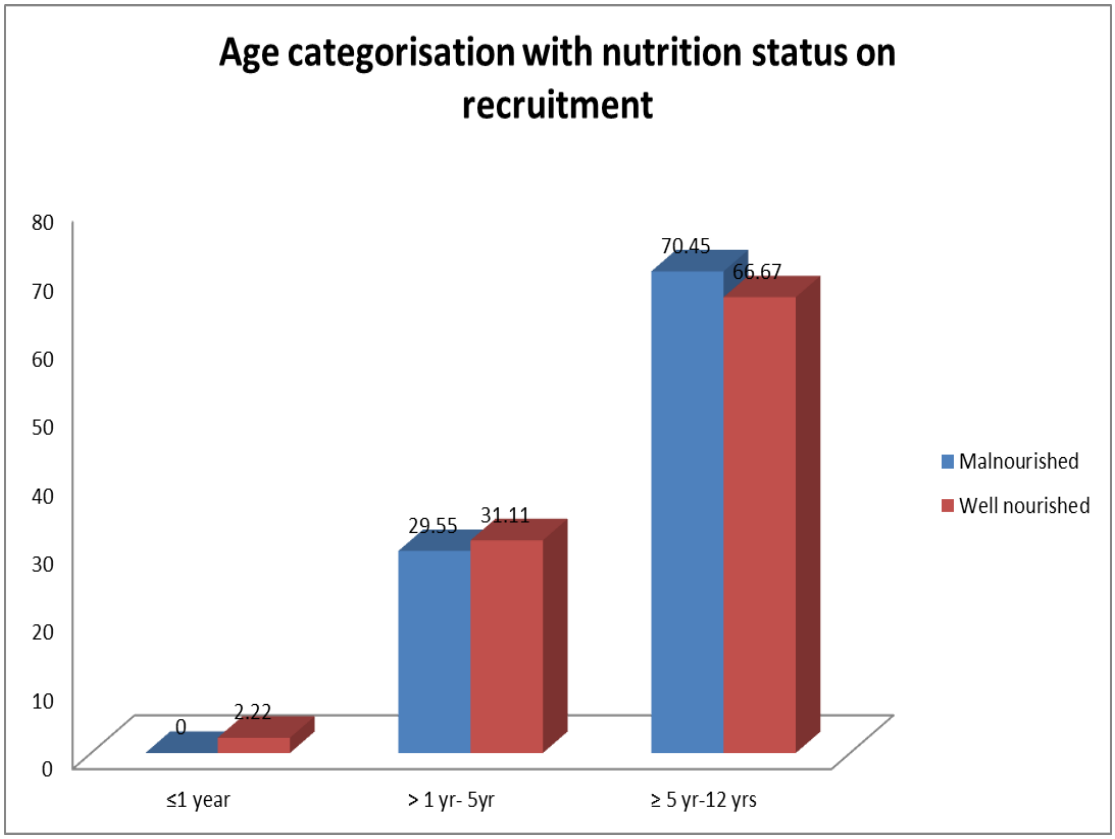


Figure 15: Age distribution of study patients among the ART experienced cohort in relation to nutrition status at baseline.

There were no malnourished patients in the age group ≤ 1 years and the majority of malnourished patients were ≥ 5 years-12 years. The well-nourished are in the maroon bars while the malnourished are shown in the blue bars in the graph.

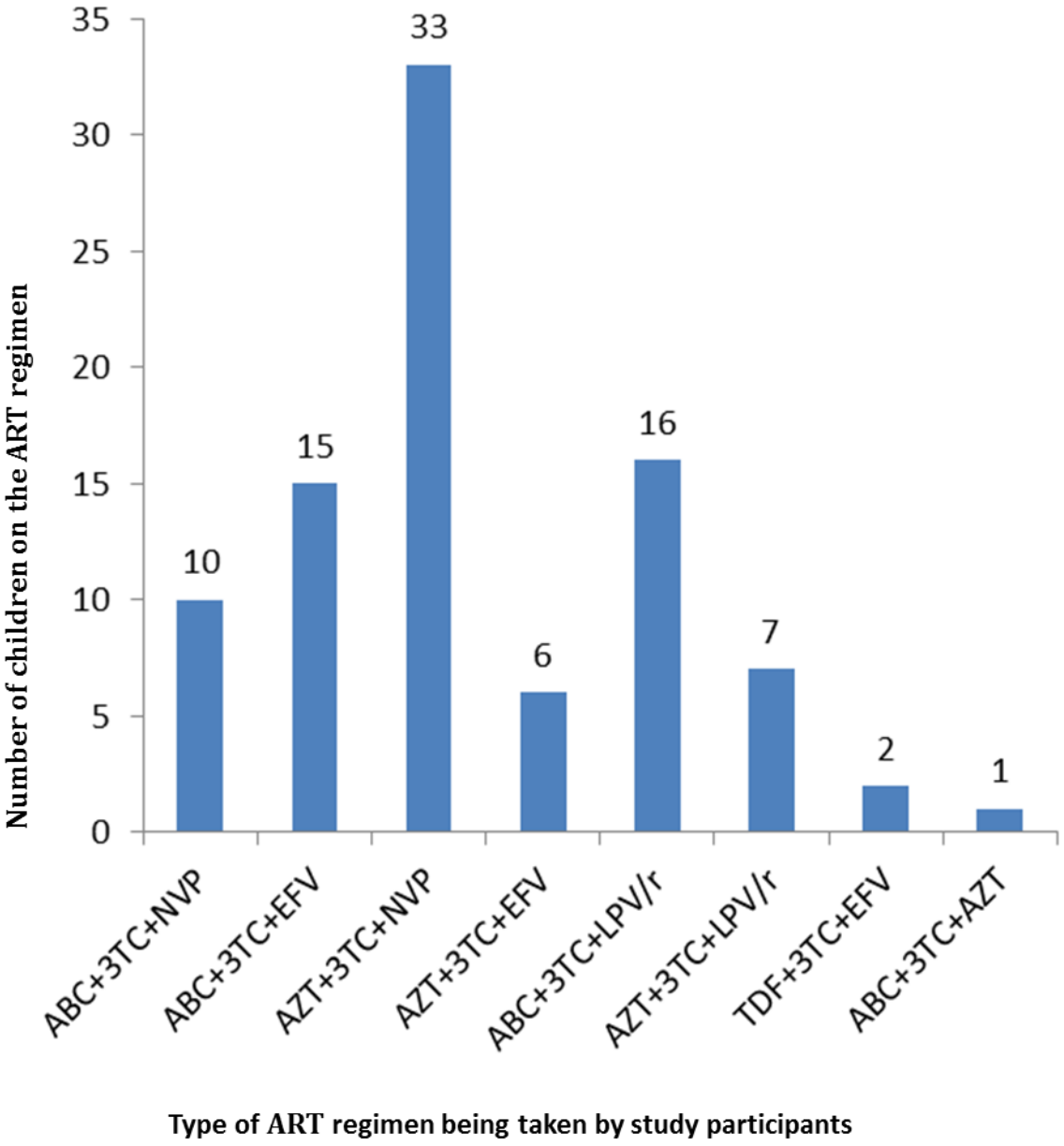


Figure 16: The type of ART regimens the ART experienced children were receiving on the study at baseline.

The enrolled children were on 8 types of ART regimen combination as shown on the horizontal axis. The vertical axis shows the number of the enrolled children receiving that type of regime. Of the 8 combinations 5 were NNRTI based, 2 were PI based and 1 was a triple nucleoside based regimen. Thirty-three were receiving AZT+3TC+NVP, 16 were on ABC+3TC+LPV/r, and 15 were on ABC + 3TC + EFV and 10 on ABC + 3TC + NVP.

Table 25. The clinical signs and symptoms exhibited by the ART experienced children enrolled on the study at baseline

Clinical characteristic	Presence	N=89	Percentage
Presence of cough for > 2 weeks	Yes	47	52.81
	No	42	47.19
Presence of fever	Yes	15	16.85
	No	74	83.15
Digital clubbing	Yes	25	28.09
	No	64	71.91
Dental carries	Yes	22	24.72
	No	67	75.28
Lymphadenopathy	Yes	37	41.57
	No	52	58.43
Oral lesions	Yes	5	5.62
	No	84	94.38
Presence of rashes	Yes	26	29.21
	No	63	70.79
Presence of organomegaly	Yes	7	7.87
	No	82	92.13

Table 26. The haematological indices of the ART experienced children enrolled on the study at baseline.

Hematological indices	Median	IQR	Normal range
WBC absc ($x10^3/\mu L$)	6.8	5.5-8.4	4.0-14
RBC absc ($x10^3/\mu L$)	4.07	3.69-4.38	4.5-6.5
Hb (g/dl)	12.1	11.1-12.9	11.5-15.5
HCT (%)	36.8	33.3-39.2	35-45
MCV(fL)	89	82-96	77-95
MCH (pg)	29.7	26.6-31.9	23-31
MCHC (g/dl)	33	32.2-34	28-33
RDW (%)	13.4	12.4-14.4	11-16
Platelets ($x10^3/\mu L$)	379	321-488	150-400
MPV (fL)	8.1	±0.74	6-10

RBC: red blood cells, Hb: haemoglobin, HCT: Haematocrit concentration, MCV: mean corpuscular volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, RDW: red blood cell distribution width, MPV: mean platelet volume.

Table 27 and 28 are showing the renal and liver function test results of the ART experienced HIV positive children on the study respectively. Serum samples were prepared from 90 HIV-infected children sampled at baseline. The median, IQR and normal range for each tested variable has been given. In table 27, creatinine serum levels are lower than expected while in table 28, the ALP serum levels are high and serum bilirubin levels are low.

Table 27. The Renal function test results of the ART experienced children enrolled on the study at baseline

Renal function tests	Median	IQR	Normal range
Creatinine (µmol/L)	**30***	22-34	47-209
Blood urea nitrogen (mmol/L)	2.4	1.9-2.8	2.0-11.9

***denotes parameter that is outside the normal range**

Table 28. The Liver function test results of the ART experienced children enrolled on the study at baseline

Liver function tests	Median	IQR	Normal range
ALT (U/L)	18	14-25	8-61
AST (U/L)	29	25-34	14-60
ALP (U/L)	**278***	229-344	48-164
Tot bilirubin (µmol/L)	**1.8***	0.9-2.7	3.9-37

***denotes parameter that is outside the normal range, ALT: Alanine transaminase, AST: aspartate transaminase, ALP: alkaline phosphatase**

Serum samples were prepared from 90 ART experienced HIV-infected children at baseline, table 29. The median, IQR and normal range for each tested variable has been given. The serum phosphate levels were the only electrolyte that was out of the normal range and was high.

Table 29. The baseline serum electrolyte levels of the ART experienced children enrolled on the study.

Serum electrolytes	Median	IQR	Normal range
Calcium (mmol/L)	2.29	2.22-2.39	2.15-2.55
Chloride (mmol/L)	102	100.5-103.6	98-107
Magnesium (mmol/L)	0.86	0.82-0.92	0.7-1.05
Phosphates (mmol/L)	**1.56***	1.42-1.75	0.87-1.45
Potassium (mmol/L)	4.5	4.1-5.4	4.0-5.0
Sodium (mmol/L)	136	134-138	136-145

***denotes parameter that is outside the normal range**

The majority of the ART-E children 54.1% at baseline had a median $CD4^+$ T cell percentage >30% and median absolute count >1000 cells/ml using fresh whole blood. The median $CD4^+$ T cell percentage for the ART-E HIV infected children at baseline was 32% (IQR: 25-39) and $CD4^+$ T cell absolute count was1064 (IQR: 686-1379) cells/ml. Comparing the immunological status using $CD4^+$ T cell percentage by nutritional status, figure 16, children with SAM had the lowest $CD4^+$ T cell percentage and when compared to the WN group the difference was statistically significant. The children with MAM had the highest $CD4^+$ T cell percentage.

The average viral load at baseline was 114 viral copies/mL (IQR: 19-17136). Though there was a near significant trend in association with nutrition status (p value=0.065), there was no statistical difference of viral load profiling in relation to nutrition status at baseline (figure 17). Among the ART-E children the highest virological failure was among SAM (80%), followed by the WN (58.5%) and then MAM (50%), figure 17.

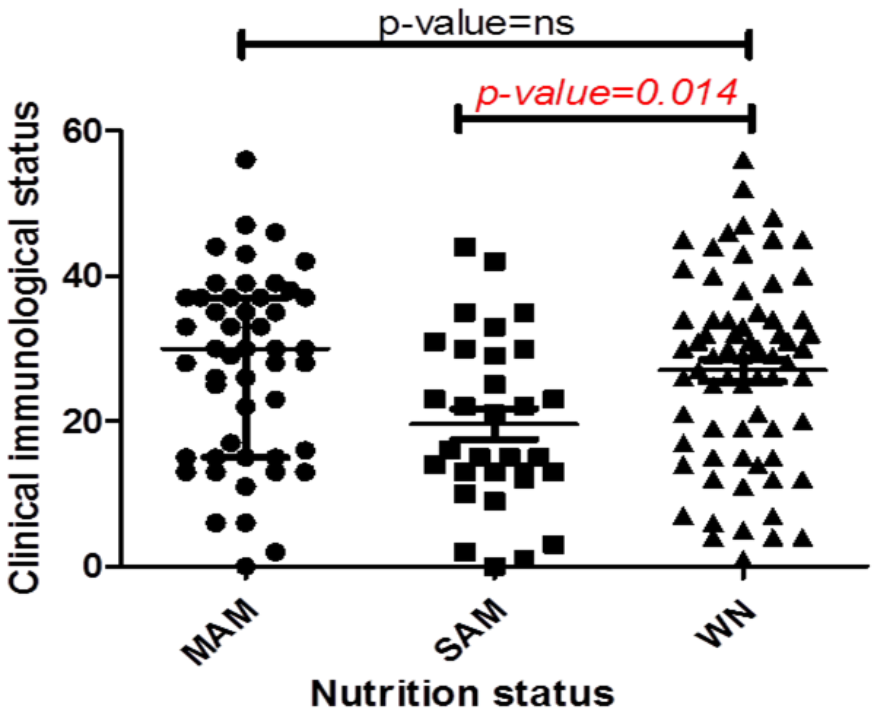


Figure 17: The immunological status among the ART-N and ART-E HIV infected children in comparison to the nutritional status at baseline.
Out of the 156 children enrolled on the study who had CD4⁺ T cell percentages done; 51 were moderately acutely malnourished (MAM), 34 severely acutely malnourished (SAM) and 71 well-nourished (WN). Data were compared using the Kruskall Wallis statistical test ie "one way ANOVA on ranks". Numbers in the graph indicate p- values and those in red and are all statistically significant. For the p-value that is not statistically significant it is denoted by ns.

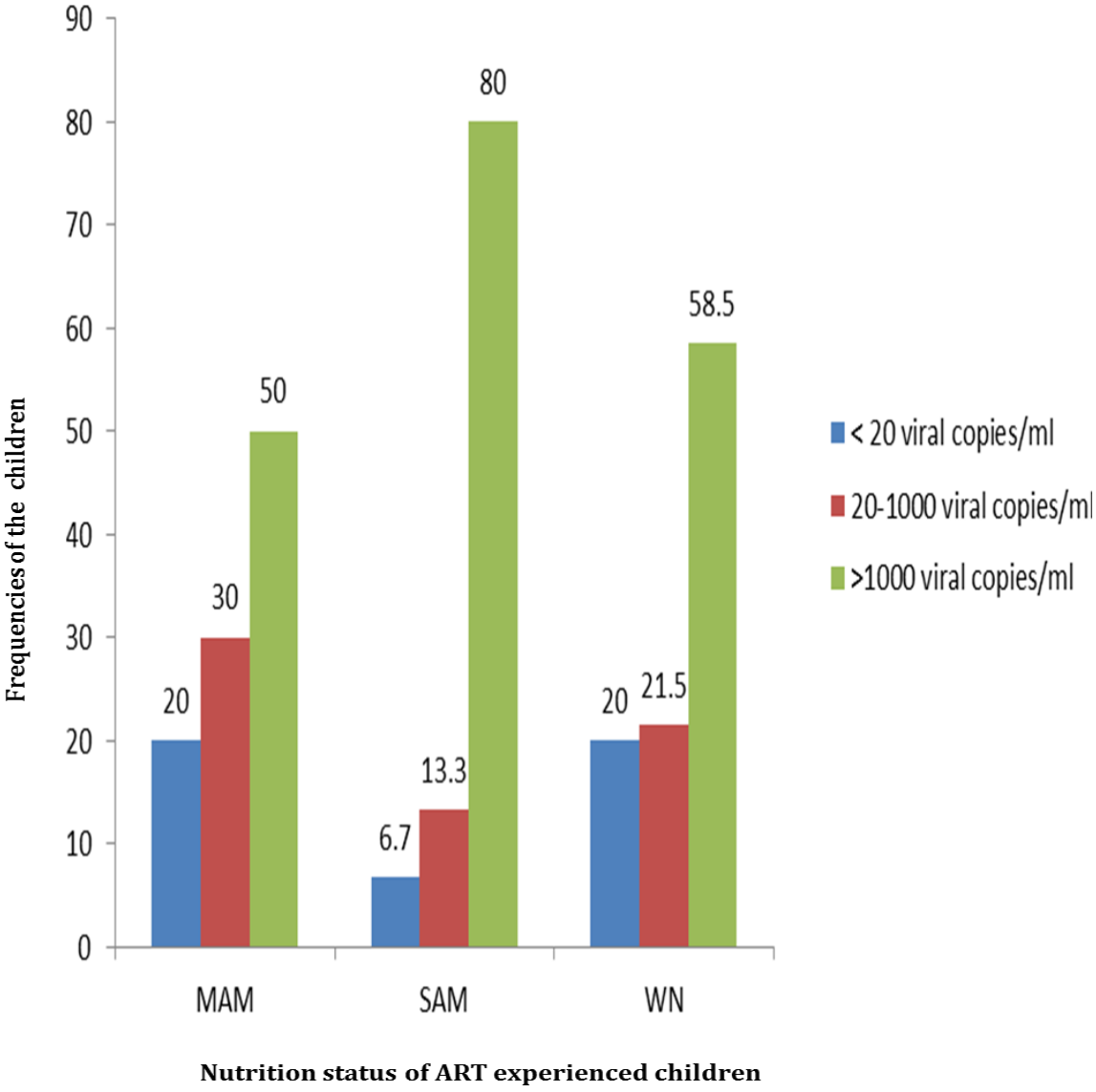


Figure 18: Baseline nutritional status and viral loads of ART experienced HIV infected children.

The blue bars designate children who are well controlled according to the lowest detectable level of the machine used in measuring viral load. The maroon bars show children who are not responding adequately to ART while the green bars show the children who have virological failure according WHO guidelines.

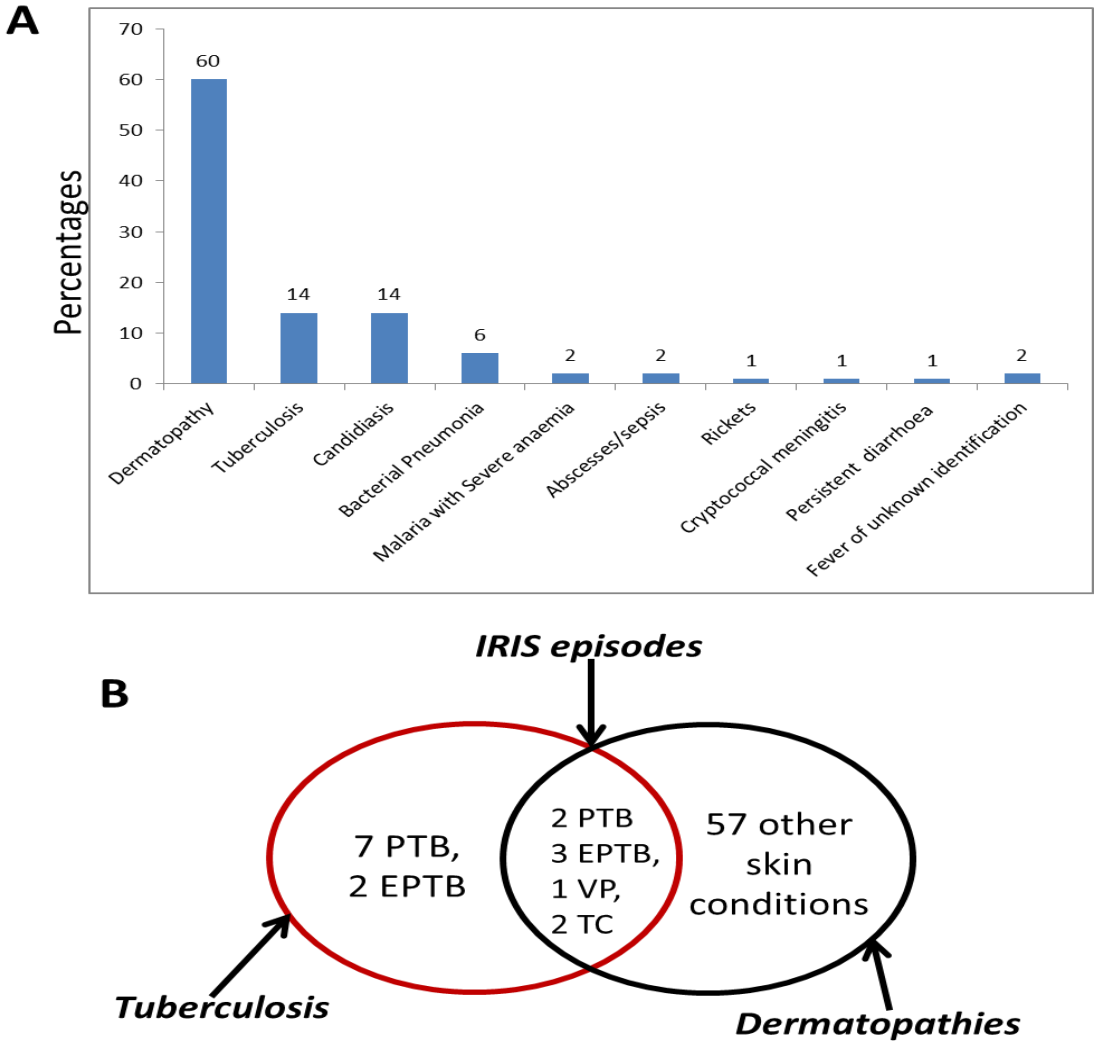


Figure 19: Co-morbidities among HIV infected children enrolled on the study at baseline.

Of the 156 HIV infected children at baseline were examined for co-morbidities. ***A,*** *the commonest comorbidities were dermatopathies and OIs. OIs included oral candidiasis, tuberculosis and cryptococcal meningitis and persistent diarrhoea. The other co-infections were malaria with severe anaemia, bacterial pneumonia, sepsis with abscess formation. Other comorbidities included rickets and fever whose cause could not be identified.* ***B,*** *The two circles show the total tuberculosis cases and dermatopathies. The intersection is the episodes of Immune Reconstitution Inflammatory Syndrome (IRIS) presentation which are 2 pulmonary TB (PTB), 3 extra-pulmonary TB (EPTB), 1 verruca planus (VP) and 2 Taenia capitis (TC).*

All the children who were hospitalised were ART-N except for the child who died in a private clinic. The overall hospitalisation rate in the study was 35/156 (35.3%) and was highest at baseline at 25/156 (16%) in comparison to no hospitalisation at 12 weeks. During the study period 10/156 (6.4%) were hospitalised. The average times an individual was hospitalised during the 12 week study period were 2 times (range: 1-4). However 2 children were hospitalised beyond 12 weeks of follow up.

Mortality rate was 5/156 (3.2%) and occurred in the 1st month of recruitment. Three children who were ART-N died in the first 2 weeks of admission: a 7 year old female, a 5 year old and a 3 year old male. These children all had SAM with severe bacterial pneumonia, metabolic derangements and anemia. The other 2 children that died were 4 year old and 2 year old males that were WN. One developed acute diarrhoea and was managed from a private health facility outside the study site and had a poor outcome according to the mother's report and the other we failed to ascertain the possible circumstances leading to his death due to change of multiple carers.

There were 5 children aged between 5-11 years with the mean age of 8.8 years who developed 8 IRIS events and all had TB IRIS. Three patients with TB-IRIS also had dermatopathy. They were 3 males and 2 females and the earliest presentation of IRIS was after 5 days of ART initiation and the latest presentation was at 2.5 months. Three children had EPTB with extensive worsening of dermatopathy (1 verruca planus, 2 extensive teania capitis) and the other 2 had PTB (1 with miliary type and the other extensive right lobar pneumonia with cervical adenitis), figure 18. No re-feeding syndrome was observed during the study.

Overall, there were 103/156 (66%) comorbidities among the HIV infected children at baseline. The majority were dermatopathies (60/156), candidiasis (14/156), TB (14/156). The OI rate was 30/156 (19.2%). All the OI episodes were diagnosed at baseline figure 18, except for all the IRIS episodes that were diagnosed during the course of the study. However the OIs and co-morbidities were resolving at the 12 week visit except for the 2 children with EPTB admitted beyond 12 week follow up time point, the 5 children that died and the one who had cryptoccocal meningitis. He progressed to become

severely malnourished with severe neurological squealae characterised by spastic quadriplegia, loss of speech, hearing, vision and profoundly impaired cognition.

Effect of nutrition supplementation on clinical outcomes among HIV-infected children

After 12 weeks of follow up all children had received ART and the malnourished received RUTF. We therefore report the ART-N and ART-E cohorts together. Out of the 84 children who came back for follow up the median age was 7 years (IQR: 3.5-10 years). The male: female ratio was 1:1. In table 30, all haematological indices assessed were within normal range.

Serum ALP was increased almost doubles the upper limit. The other liver function tests and renal function tests were within the normal range, (Table 31).

Serum phosphorous levels were increased at 12 weeks after nutritional supplementation however the other serum electrolytes were in the normal range, Table 32.

Table 30. Haematological indices of the HIV infected children after nutritional supplementation.

Hematological indices	Median	IQR	Normal range
WBC absc ($x10^3/\mu L$)	6.99	5.75-10.34	4.0-14
RBC absc ($x10^3/\mu L$)	4.23	3.85-4.59	4.5-6.5
Hb (g/dl)	11.75	10.5-12.35	11.5-15.5
HCT (%)	37.6	34.1-38.7	35-45
MCV(fL)	85.4	78.8-95	77-95
MCH (pg)	27.4	24.5-30.2	23-31
MCHC (g/dl)	31.8	30.4-32.4	28-33
RDW (%)	14.1	12.5-16.1	11-16
Platelets ($x10^3/\mu L$)	378	310-450	150-400
MPV (fL)	9	8.1-9.9	6-10

RBC: red blood cells, Hb: haemoglobin, HCT: Haematocrit concentration, MCV: mean corpuscular volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration, RDW: red blood cell distribution width, MPV: mean platelet volume.

Table 31. Renal and liver function tests of the HIV infected children after nutritional supplementation

Serum levels	Median	IQR	Normal range
Renal function tests			
Creatinine (µmol/L)	25	19-32	47-209
BUN (mml/L)	2.3	1.7-2.7	2.1-11.9
Liver function tests (U/L)			
ALT	20	15-38	8-61
AST	31	25-38	14-60
ALP	**294.5***	240-361	48-164
Total-bilirubin (µmol/l)	1.7	1.3-2.6	3.9-37

***denotes parameter that is outside the normal range, BUN: blood urea nitrogen, ALT: Alanine transaminase, AST: aspartate transaminase, ALP: alkaline phosphatase**

Table 32. The serum electrolytes of the study children after nutritional supplementation

Serum electrolytes	Median	IQR	Normal range
Calcium (mmol/l)	2.33	2.26-2.42	2.15-2.55
Chloride (mmol/l)	102.1	100.5-104	98-107
Magnesium (mmol/l)	0.87	0.83-0.92	0.7-1.05
Phosphorous (mmol/l)	**1.74***	1.57-1.85	0.87-1.45
Potassium (mmol/l)	4.5	4.3-4.8	4.0-5.0
Sodium (mmol/l)	136	135-137	136-145

***** indicates the electrolyte levels that are out the normal range.**

Table 33. CD4+ T cell change after nutritional supplementation among the HIV infected children.

CD4+T cell profile	Baseline (IQR)	12 weeks (IQR)	P-value
CD4+ T cell %	15 (6-22)	29 (20-38)	**0.07**
CD4+ T cell abc	555 (270-1895)	1088(653-1564)	0.13

At baseline the CD4+ T cell profiling indicated severe immune-depression, however by 12 week follow up using the CD4+ T cell percentage there was more than 50% increment. The absolute counts also doubled by 12 weeks (Table 33). However the change was not statistically significant.

The change in viral loads were not statistically significant after 12 weeks follow up (p-value=0.250). Adequate suppression (<20 viral copies/ml) was achieved in the WN (43.3%), MAM (37.9%) and SAM (18.2%) at 12 weeks of follow up. Using the WHO definition of virologic failure (>1000 viral copies/ml), the SAM patients had the highest proportion of children found to have virologic failure (54.6%), WN (26.7%) and MAM with the lowest at 20.7%. In comparison to the WHO cut offs for viral suppression at 12 weeks of follow up which is far from the 90-90-90 targets set for viral suppression. Moderate suppression was highest among the MAM (41.4%), WN (30%) and lowest among the SAM children at 27.3%), Figure 19.

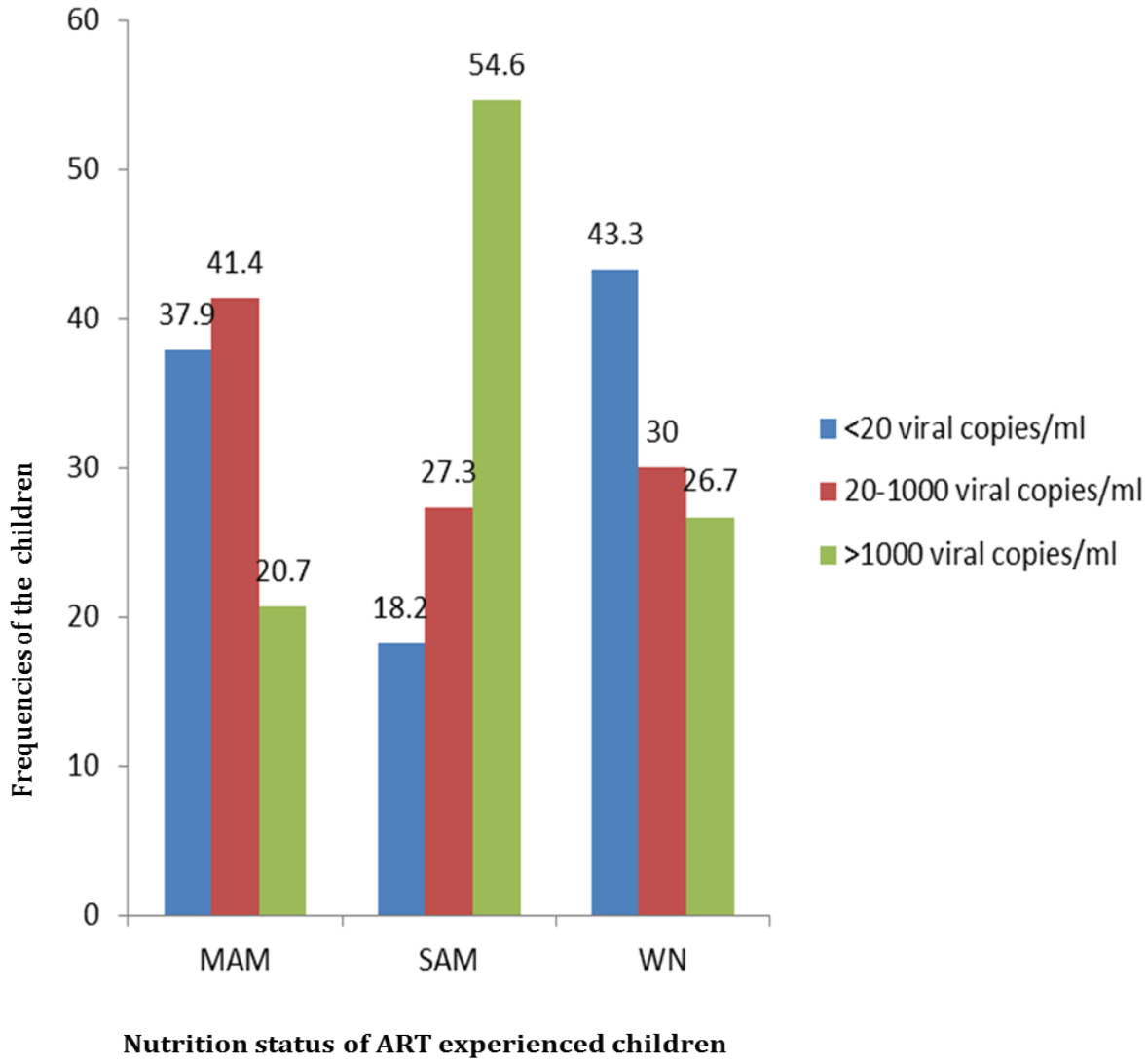


Figure 20: Viral loads of HIV infected children at 12 weeks on the study.
The blue bars designate children who are well controlled according to the lowest detectable level of the machine used in measuring viral load. The maroon bars show children who are not responding adequately to ART while the green bars show the children who have virological failure according WHO guidelines.

Discussion

Introduction

The introduction of ART for the management and prevention of HIV has greatly reduced HIV incidence on the global platform [211]. Furthermore the quality of lives of those infected with HIV has immensely improved with reduction of OIs [212]. Nutrition on the other hand has been neglected for long, however, in the context of HIV has recently gained the interest on the international health platform due to the introduction of RUTF. This is mainly because of the model being applied, where RUTF is being used in the management of stable malnourished children in a home environment. This mode of intervention has been reported to reduce the burden of hospitalisation both on patients' families and hospitals [213]. The HIV programs in pediatrics have a multi-prong approach in LRS and are aiming at elimination of pediatric HIV, early initiation of all HIV infected children on ART in order to achieve the 90-90-90 targets by 2020 with sustained continuum of treatment for those on ART. However there are still children who are missed by these programs and we reported these recent findings in this study [214]. I will limit the present discussion to the clinical outcomes noted in the prospective pediatric cohorts set up in this study in Uganda.

A total of 278 HIV infected children were screened at all the study in order to accrue 66 ART-N and 90 ART-E HIV infected children. ART-N HIV infected children were enrolled consecutively while ART-E HIV infected children were randomly enrolled. During the enrollment period we found out that incident cases of perinatally transmitted HIV had markedly decreased due to the tremendous effort invested in the last decade to eliminate pediatric HIV [215]. Several studies have begun reporting on the positive strides achieved in the management and prevention of pediatric HIV/AIDs [216]. Therefore we were faced with the challenge in accruing the numbers of ART-N HIV infected children in the study and opted to consecutively recruit them. We also noted that the critically ill children were mainly ART-N HIV infected children indicating that though progress has been made, there are still children who present late with AIDS defining illnesses. These findings illustrate a missed opportunity in identifying and treating these children timely [214]. The ART-E HIV infected cohort was accrued by random selection and this was possible as more and more children in the pediatric HIV programs are now on ART treatment as the Ugandan

guidelines had begun to treat all children 15 years and below as early as 2013 ahead of all countries in LRS.

The study was a multicentre study carried out at the Infectious Diseases Institute affiliated KCCA clinics namely (Kiswa, Kisenyi, Komamboga, Kawala, Kitebi and Kawempe), Mildmay Malnutrition ward; and the Mwanamugimu Nutritional Rehabilitation Center in Mulago Hospital. This explains why the majority of the children were from the Ganda tribe as it is the predominant tribe found in central Uganda. Of the 156 HIV infected children recruited on the study, the majority (50%) were aged ≥ 5-12 years and they also had the highest burden of malnutrition (41.2%). This showed that the older children in the HIV programs also are facing malnutrition management challenges, as a result of possibly delayed HIV diagnosis with programs designed to focus on managing malnutrition in those less than 5 years of age. Children who miss early HIV diagnosis and treatment and survive tend to present later with AIDs defining illness. However, for those who are ART-E, malnutrition within a food insecure homestead has become an issue [217]. This is probably because management of malnutrition within national programs have mainly focused on the under-fives; hence poor nutrition in older age group has not been addressed.

Demographic characteristics of study cohorts

The ART-N children accounted for 66/156 (43.3%) of the study participants, the median age in years was 1.95 years (IQR=0.5-5.3). The findings are consistent with late diagnosis of children in LRS where children continue being missed in the EID programs. Presentation with malnutrition in itself is a late diagnosis of HIV as malnutrition is an AIDS defining condition according to the WHO clinical staging of HIV. The proportion of ART-N children recruited on the study with MAM was 25.8%, SAM was 36.4% and WN was 37.9%. Our results demonstrate that children are still presenting late to access HIV services for the first visit despite the multipronged interventions since the late 90s in PMTCT [218]. A study demonstrated that though ART in paediatric programs is effective in increasing survival among HIV infected children and early ART initiation was associated with a high survival probability, active TB, malnutrition, and delayed ART initiation remain predictors of poor survival among children living with HIV [219]. Previous studies have shown the direct effect of HIV infection on child survival through MTCT and indirectly by maternal

HIV status, as maternal mortality has a devastating impact on the child survival [220]. Estimates of child mortality from community-based cohorts demonstrate that children of HIV infected mothers have higher mortality rates than the children of uninfected mothers, and that child mortality is closely linked with maternal health status [221], therefore irrespective of HIV infection the importance of having a mother as next of kin cannot be overstated. In the current study, participants whose mothers were their next of kin or primary carer comprised 72.7% and 88.3% of the study participants had their mother alive. Only 69.4% of the mothers were reported to be on ART and 57.9% knew the HIV sero-status of their spouses. The figures for mothers reported to be on ART are still unsatisfactory and below the 90-90-90 target for treatment on ART. To further improve prediction of maternal survival we recommend assessment of maternal viral suppression however, we were unable to do this as part of our study. The Uganda Population-Based HIV Impact Assessment (UPHIA), a household-based national survey, conducted from August 2016 to March 2017 to assess the progress of Uganda's national HIV response noted that although the HIV epidemic in Uganda had declined from a prevalence of 7.3% in 2011 to 6.2% in 2016 it remained concentrated in women of child bearing age at 7.6%. These are at risk of transmitting HIV to their children perinatally against a background of 5% MTCT of HIV of all new HIV infections [222]. Therefore the HIV programs need to optimise targeting the mother to ensure the provision of adequate HIV care for the survival of her child as she remains the commonest carer in paediatric HIV programs. Overall 72.9% of the ART-N children were fully immunised.

The annual Health Sector Performance report 2017/18 has indicated immunisation coverage of >95%. The findings suggest Uganda had achieved a target of 99.2% for the DPT3HibHep3 coverage however, our study reports a lower coverage among the ART-N cohort [223].

Regarding the ART-E HIV infected cohort, the median age was 6.8 years (IQR: 4.5-10 years). As previously emphasised in LRS children on ART programs are usually in the older age categories. There are various reasons which may explain why, despite the evidence that providing early ART before 5 months of age is a predictor of long time survival with minimal immune activation, as the HIV reservoir is still underdeveloped. HIV programs are still young and paediatric HIV services were previously accessed by older children.

These patients presented late but had survived to their 2nd and 5th birthday, being elite controllers of HIV or "slow progressors". This may imply that the majority did not survive long or infants are still diagnosed late thus less of the younger children on ART in the HIV programs [224]. The distribution of the malnourished children in the ART-N cohort in this study according to the 3 age categorisations demonstrates an absence of malnourished patients in the age group ≤ 1 year while the majority of malnourished patients were found in the age category of ≥ 5 years-12 years. Among the ART-E, cohort about half were malnourished and the older age group was most affected. The reason for the higher levels of malnutrition in the older age groups may be due to the combined effects of HIV disease progression, poor intake or muscle wasting as the result of high catabolism. It should be noted that from a programming perspective, until recently there were few nutrition programs targeting those older than 5 years, with the majority targeting children less than 5 years of age. This picture is characteristic of the paediatric HIV programs in SSA where HIV-infected African children commonly present with MAM or SAM, and wasting is recognized as an independent and predictive risk factor for mortality even when receiving ART [225-227].

The median length of time on ART in months was 42 (IQR: 15-66 months). Among the ART-E cohort, about 73% had their mothers as the next of kin and 93% were on ART. Similar to other paediatric HIV programs, the commonest carers are usually mothers and this has been reported in multiple studies in Africa [224]. This study demonstrates greater ART coverage of mothers whose children are ART-E compared with mothers of the ART-N cohort. In addition a larger proportion (67%) of them knew the HIV status of the father to the study patient. This implies in order to achieve the 90-90-90 treatment targets for HIV, increased disclosure to children and partners and strengthening male participation are recommended [228]. The present study is further evidence of this. Overall, immunisation coverage was 83.2% among the enrolled study participants, higher than that for the ART-N children. Since the ART-E children in this study had been on ART for at least 42 months their health seeking behaviour are expected to have improved and leading to better access and utilisation of the immunisation services that are integrated in the programs [223]. The immunisation rate may have been higher, however, at the beginning of the study we used immunisation cards to confirm the immunisation status,

and unfortunately most of the cards we assessed were not properly completed regarding vital immunisation and growth parameters. This may be better assessed by performing targeted antibody levels in serum in response to specific vaccines. Therefore we considered it pointless for the research team to continue referring carers to return with poorly completed cards so we used self-reporting instead. There is need for the Ministry of Health in Uganda to ensure the health care workers complete the growth cards of the children.

The enrolled children were on 8 types of ART regimen combination. Of the 8 combinations 5 were NNRTI based, 2 a PI based and 1 a triple nucleoside based regimen. Thirty-three were receiving AZT + 3TC + NVP, 16 were on ABC + 3TC + LPV/r, and 15 were on ABC + 3TC + EFV and 10 on ABC + 3TC + NVP. The type of regimen dispensed in the programs are guided by the national treatment guidelines of Uganda where the NNRTI based regimen forms the back bone of 1st line choice and PI the backbone of second line by the time of the study [212]. This partly explains why majority of the ART-E children on this study were on first line treatment despite being on ART for 42 months. The process of changing of an ART regimen in the paediatric study sites was not only guided by the WHO guidelines, but also by site-specific guidelines where an ART switch meeting to change treatment of a patient suspected of having clinical failure requires to be discussed prior to consensus being reached. This may contribute to a delay whereby children remain on a less than optimal regimen for a longer period.

Clinical findings among the study cohorts

In the current study, ART-N HIV infected children had lower proportions of TB treatment levels (10.6%) in comparison to the ART-E HIV infected children (28.1%). This is probably due to fact that the ART-E cohort had better health seeking behaviour dictated by the paediatric HIV programs that provide treatment and care. The previous admission rates were higher among the ART-E cohort, 4.5% compared to 1.5% among the ART-N cohort. This may still be due to the improved health seeking pattern by the ART-E cohort as they are reviewed every 3 months and ailments necessitating admission are identified and managed. [212, 229]. In addition, reporting of TB contacts was lower among the ART-N cohort (9.1%) in comparison to 23.6% among the ART-E cohort. This further demonstrates that being on the HIV program for some time improves knowledge and

understanding of TB management, thus may explain the higher rates of reporting as they probably appreciate the health information provided. There is usually aggressive routine TB screening and other OIs enshrined in routine clinical assessments in the HIV management package every 3 months. In comparison to some studies, a Ugandan study in 2011 on TB in HIV infected children initiating ART revealed that the proportion of new TB cases diagnosed after starting ART decreased by 70%, [230]. A systematic review on TB risk in children established that children are at high risk of progression to TB disease following infection with *Mycobacterium tuberculosis,* most especially those who were HIV co-infected and children in the first 2 years of life, who often develop non-pulmonary forms of TB. It is not surprising therefore that there are high treatment rates in ART-E especially in the older age group [211].

There was a higher proportion of reporting of persistent diarrheal episodes among the ART-N cohort (30.3%) in comparison to ART-E cohort (6.7%). This is not surprising given that, in severely immunosuppressed people one of the main presentations of OIs is persistent diarrhoea caused by aetiologies such as giardiasis, *Strongyloid stercoralis, Isospora beri, Cryptosporidium parvum, Cryptosporidium hominis, Enterocytozoon bieneusi* etc. The high presentation among the ART-N cohort may be due to some of the above organisms in this cohort as a result of being immunosuppressed in the absence of cotrimoxazole prophylaxis. Routine cotrimoxazole prophylaxis is routinely provided to HIV infected children and dramatically reduces the incidence of diarrhoea. In the presence of ART and routine cotrimoxazole prophylaxis these episodes reduce dramatically as demonstrated among the ART-E HIV infected cohort in this study who had lower proportions of persistent diarrhoea similar to other studies [231].

The only abnormal haematological indices among the ART-N HIV infected children were low Hb concentration levels in blood and low MCV while among the ART-E HIV infected children all the haematological indices were normal. In a Ugandan study where the authors sought to study the anaemia burden among HIV infected children admitted at Mulago Hospital, substantiated that anaemia was a common haematological complication of HIV infection most especially among those who had not initiated ART. They went ahead to show that anaemia had a significant impact on the quality of life and clinical outcome [232]. After RUTF supplementation all haematological indices were normal even among

those who were previously ART-N at baseline implying that ART improves or restores haematological indices though the mechanism is not well elucidated.

Serum creatinine levels were low in the ART-N and ART-E cohorts at baseline and also after 12 weeks of supplementation with RUTF among the malnourished children. Though HIV infection has been found to cause renal derangement as a result of the HIV virus affecting kidney cells the presentation of renal derangements is variable. Proteinuria, elevated serum creatinine or both have been shown in several studies to frequently occur and are the commonest manifestations of renal disease in HIV infected children pre-ART era [233]. In our study we did not assess for proteinuria or do a renal biopsy to properly describe the renal derangements resulting in low creatinine that persisted beyond the supplementation.

The only liver function tests noted to be deranged at baseline among the ART-N and ART-E cohorts were ALP serum levels and these increased with RUTF supplementation. Serum bilirubin was low in the ART-E at baseline but normalised after RUTF supplementation. HIV-infected children have been shown to have compromised bone health compared to their healthy uninfected counterparts. The bone changes observed in HIV-infected children include alterations in the rate of bone metabolism and this may explain the persistently high ALP and phosphatase serum level in all the study cohorts even after RUTF supplementation [234].

Immunological and virological characteristics of study participants

ART-N HIV infected children in this study demonstrated severe immunological suppression prior to ART initiation. The majority of ART-N (85.5%) had a median $CD4^+$ T cell percentage <30% and an absolute count ≤1000cells/ml. The median viral load was 240,753 viral copies/mL (IQR: 45,752-1,286,388). This is comparable to other studies that have found that in the absence of ART there is worsening of immunosuppression and increase in viral replication as evidenced by high plasma viral loads [203], as reported in the ART-N cohort. In addition since, malnutrition has for long been known to be an indicator of HIV disease progression and this cohort had a high proportion of malnourished children, it is therefore not surprising to find that the children with SAM

had the highest viral loads [235], in addition malnutrition is used as one of the AIDs defining clinical presentations [4].

It is now well established that ART leads to the improvement of $CD4^+$ T cells even when initiated in the presence of severe immunosuppression [236]. This was evident in our study where among the ART-N cohort at baseline the $CD4^+$ T cell profiling indicated severe immune-depression in the majority. Upon ART initiation for 12 weeks there was more than 50% increase in $CD4^+$ T cell frequency and doubling of absolute counts. The current policy is that anyone found to be HIV positive at the earliest opportunity is initiated on ART thus the several initiatives such as test and treat follow this principle in order to achieve the ambitious targets of the 90-90-90 goal and EMTCT by 2020 [228]. In those who are found to have poor immunological response after ART initiation, adherence to ART is strengthened and sometimes $CD4^+$ T cell levels increase towards attaining normal ranges in most patients however there are those who do not improve despite achieving viral suppression [237].

The majority of ART-E (54.1%) at baseline had a median CD4+ T cell percentage >30% and absolute cell counts >1000 cells/ml. The median viral load at baseline was 114 viral copies/mL with the highest virologic failure among the SAM (80%), WN (58.5%) and MAM (50%). According to the WHO definition of virologic failure (>1000 viral copies/ml), adequate viral suppression was least achieved in those who had SAM (18.2%) in comparison to 43.3% in the WN and 37.9% in MAM at 12 weeks in this study. Consequently, SAM had the highest proportion of children found to have virologic failure (54.6%), WN (26.7%) and MAM with the lowest at 20.7%. In comparison to the WHO cut offs for viral suppression at 12 weeks of follow up the outcomes are far from the 90-90-90 targets set for viral suppression. Despite the enormous progress made in in LRS in rolling out ART and increased paediatric treatment, many challenges remain; some of them include the early identification and management of patients who are failing on the first-line regimen [238]. Presently less than 3% of patients are receiving second-line treatment, whereas 15-25% in current studies shows detectable viral loads 12 months or more on treatment, of whom a substantial proportion could be due to virological failure [239]. In addition the process to switch children who are failing on a particular regimen undergo

stringent scrutiny and this may delay the process and undermine the gains of prompt ART switching.

Opportunistic infections (OI)

The plethora of co-infections among the study participants included dermatopathies, OIs such as oral candidiasis, TB, cryptococcal meningitis and persistent diarrhoea. The other co-infections were bacterial pneumonias, malaria with severe anemia, sepsis with abscess formation and dermatitis such as verucca planus and taenia capitis. The probable reason of low OIs in this study population may be explained by the stringent exclusion criteria which were geared to ensure we enrolled children with uncomplicated malnutrition that could be managed by RUTF in a home setting. In the absence of ART or poor adherence to ART, HIV disease progresses thus increased susceptibility to OIs [94]. We mainly used clinical methods to screen for OIs this could explain the lower rate of OIs identified as compared to utilising molecular techniques like disease specific ELISA tests.

Hospitalisation

The children hospitalised in the present study were those enrolled as ART-N HIV infected children. The overall hospitalisation rate in the study was 35/156 (22.4%), the rate of hospitalization was highest at baseline at 25/156 (16%) in comparison to no hospitalisation at 12 weeks. The average times an individual was hospitalised during the 12 week study period were 2 times (range: 1-4). However 2 children were hospitalised beyond 12 weeks of follow up. The trend in this study is similar to the pattern seen in the pre-ART era where morbidity and mortality rates were high in comparison to the ART era [203], and demonstrates that the impact of ART on patient outcomes improves their survival resulting in fewer OI episodes and severity in turn reduction of hospitalisation [226].

Immune reconstitution inflammatory syndrome and re-feeding syndrome

It is difficult to define as a single clinical entity however the clinical pattern of IRIS manifestation in this study is not similar to previous reports and are fewer cases noted. There were 8 events of IRIS, 5 children developed IRIS events and all were TB IRIS. IRIS can manifest with a wide variety of clinical symptoms, depending on the target of the

inflammatory response and the prevailing infections in the locality. This study was done in Uganda, which has a high prevalence of TB; hence, TB IRIS was the commonest clinical pattern of IRIS. Similar findings were demonstrated by some studies where TB-IRIS was the commonest form of presentation. The commonest symptoms were fever, cough and lymphadenopathy as shown in this study. They presented in the first 2 months of ART initiation and they were higher in those who were severely immunosuppressed and older age group. The prevalence of TB-IRIS in this study was lower than previous studies. Bakeera et al in Uganda, reported a prevalence of 17% [240], Thai study 43.7% [241] and Ugandan study 29% [242].

No re-feeding syndrome was observed during the study however re-feeding syndrome is usually referred to as a constellation of metabolic derangements characterised by hypophosphatemia, hypokalemia, hypocalcemia, and hypoalbuminemia. In the current study the serum electrolytes examined were calcium, chloride, magnesium, phosphate, potassium and sodium at baseline and 12 week time point. All the serum electrolytes were normal except for serum phosphate which was raised above normal in ART-N, ART-E at baseline and after RUTF supplementation. The only study where an oral therapeutic diet was introduced in SAM children and where cases of re-feeding syndrome occurred was in children with coeliac disease and the presentation of re-feeding syndrome overlapped the presentation of celiac crisis. This was a retrospective study which may not be comparable to our study by design [243]. In our study we were not able to perform Vitamin D levels due to the high related costs. None-the-less bone metabolism in HIV is dysregulated. Though the high level of serum phosphate in the HIV-infected children in this study is the reverse of what was expected, a study in malnourished adults in urban cities of Tanzania and Zambia noted similar findings. This phenomenon characterised by high incidence of above-normal serum phosphate suggests that malnourished, HIV-infected children like the adults have much lower metabolic capacity to handle this electrolyte than do healthy individuals thus the high levels. We measured the serum levels at 2 time points while this adult study measured over 6-7 times over a 12 week period. The pertinent questions are whether these changes in serum phosphate levels resulted in any adverse effects to the patients and whether relatively high intakes of electrolytes through the supplementation affected the occurrence of adverse events that the study may have missed. Could they have

been more strongly associated with mortality than the absolute electrolyte levels and treatment may have accentuated the effects, but the numbers of the mortality cases were small we observed. Labile serum electrolyte levels support the idea of poor metabolic control among this malnourished population with advanced HIV, and high supplementary electrolytes has been reported in some incidences to probably further impair this control [244].

Mortality rate

The high mortality rate during the first months of ART among HIV infected children in LRS has been reported in several HIV studies the world over. Mortality in children and adolescents vertically infected by HIV receiving care at a referral hospital in Brazil showed a high mortality prior to initiation of ART of 14.7% with majority (61.5%) in children <3 years of age. Mortality rate decreased over time and the distribution of deaths was homogenous over the years of evaluation. Early initiation of ART before 6 months of age was associated with improved child survival. The main causes of mortality were severe bacterial infections (57%) and other OIs (33.3%) [245]. According to a study on hospitalisation and mortality among HIV infected children after receiving ART in Thailand, mortality rate decreased from 5.7% in the first 24 weeks to 0%–0.6% in the subsequent 24-week intervals after ART initiation [246]. It was also documented in a Ugandan study comparing mortality among HIV-infected children and uninfected in an 8 year longitudinal cohort in rural south-west Uganda demonstrated a mortality rate that was six times higher in ART-naive HIV infected children than in HIV-exposed and uninfected children and was highest among those aged <2 years. In this current study mortality rate was 5/156 (3.2%) and occurred in the 1st month of recruitment. It was much lower than what is described in most studies and this was probably because one of the cohorts had experience in ART use. Three children who were ART-N died in the first 2 weeks of admission: 7 year old female, 5 year old and 3 year old males. These children all had SAM with severe bacterial pneumonia, metabolic derangements and anemia. The other 2 were 4 year old and 2 year old males that were WN. One developed acute diarrhoea and was managed from a private health facility outside the study site and had a poor outcome according to the mother's report and the other one we failed to ascertain the possible circumstances leading to his death due to change of multiple carers.

Conclusion

The present study has demonstrated that the use of ART in HIV infected children significantly reduces hospitalisation, OIs and mortality rates. We have also demonstrated that children attending the HIV programs are still being initiated on ART very late with severe immunosuppression. Nonetheless, this study has demonstrated the presence of significant immune reconstitution with inadequate virologic suppression. We also show that majority of the children were on a first line ART regimen for a protracted period and nutrition status was not associated with immunologic or virologic suppression.

CHAPTER FOUR: Nutrition outcome results

Introduction

Malnutrition and HIV impact negatively on the health of those directly and indirectly affected by both epidemics. Multiple studies over the last 30 decades have documented the devastating impact of HIV/AIDS epidemic on health, nutrition, food security and overall socioeconomic development in countries that have been greatly affected by the disease. Therefore the hallmark of currently managing HIV/AIDS epidemic is enshrined in ensuring that timely nutritional support is available to potentiate the gains made by universal access to ART. Malnutrition poses a challenge across SSA in particular, where the majority of the population is faced with food insecurity and the world's largest HIV/AIDs burden [10]. Studies have shown that malnutrition is an independent risk factor of poor outcome in HIV and the younger age group is at even greater risk of death [202, 203]. Therefore efforts in combating HIV/AIDs and malnutrition need to be an integral element of the health systems of the countries affected. A cross-sectional study on the prevalence of malnutrition among HIV-infected children in Central and West-African HIV-care program in 2011 revealed that the burden of malnutrition remained high even when on ART. Anthropometric measurements and appropriate nutritional care of malnourished HIV-infected children remained profoundly insufficient [16].

The introduction of RUTF has revolutionalised the management of malnutrition most especially in LRS. It has turned the management of stable severe cases of malnutrition from hospital to home settings thus reducing the cost of admission on weak health systems [215]. Nutrition status is a strong risk factor for the disease progression of HIV, survival and quality of life of a person who is infected with the HIV virus. Lack of essential macronutrients and micronutrients may not only contribute to the decrease of $CD4^+$ T cells but may have a suboptimal response to ART. In our study, we investigated the effects of nutrition status at baseline and nutritional supplementation at 12 weeks on anthropometry, dietary assessment and nutrition biochemical markers among HIV-infected children. The therapeutic nutrition supplement used in the study was RUTF (Plumpy'nut®) which was provided to the MAM and SAM at 150 kcal / kg / day during the 12 weeks of the study [247]. After the 12 weeks of treatment, if any study participant was found to have become malnourished or had poor response in WAZ or MUAC they were

either initiated on RUTF or continued on RUTF until they achieved a WAZ or MUAC z-score above -2 z-score. However, study parameters are only reported in this thesis until the 12 week time point.

Nutritional requirements in HIV/AIDs and Malnutrition

The management of malnutrition targets both prevention and treatment options in an outpatient or community and inpatient level through provision of nutrition counselling, therapeutic supplementation and treatment in context of other requirements the patients need to survive. Therefore a nutritional management package for PLWHIV/AIDS comprises of nutrition counselling, care and support interventions and these vary according to nutritional status ie whether one has MAM/SAM/mild malnutrition or adequate nutrition status and the extent of disease progression [248]. This may be accessed at an OPD or inpatient level depending on the stability of the patient. In addition, HIV-related infections, such as TB and persistent diarrhea need to be considered as they confound the benefits of nutritional management.

The macronutrients that are consumed are grouped as energy, protein and fats. In patients with HIV/AIDs, energy requirements are likely to increase by 10% in order to maintain body weight and physical activity in asymptomatic HIV-infected adults [249], and growth in asymptomatic children. During symptomatic disease, and subsequently AIDS, energy requirements are estimated to increase by approximately 20% to 30% to maintain adult body weight as evidenced from increased energy requirements in those who were receiving ART [250]. Thus energy intake need to be increased by 50% to 100% over normal requirements in children experiencing weight loss though there is low evidence for it. Regarding proteins and fats there is still insufficient data to support an increase in protein and fat requirements due to HIV infection.

In LRS, micronutrient deficiencies are common where HIV infection is prevalent. Though some studies yielded conflicting findings [83], others show that different micronutrient supplements provide a variable range of beneficial outcomes [251]. The positive role that micronutrients play in immune function and infectious disease is well established. In observational studies, low blood levels and decreased dietary intakes of some micronutrients are associated with faster HIV disease progression and mortality, and

increased risk of HIV transmission. However, the specific role of individual and multiple micronutrients in the prevention care and treatment of HIV infection and related conditions merits further attention or research. In children, periodic zinc and vitamin A supplementation has been shown to reduce all-cause mortality [251, 252], pneumonia and diarrhea morbidity in vitamin A-deficient children, including HIV-infected children. HIV-infected adults and children are encouraged to consume healthy diets [252, 253]. Nonetheless, dietary intake of micronutrients at recommended daily allowances (RDA) levels may not be sufficient to correct nutritional deficiencies in HIV-infected individuals. Adequate micronutrient intake is best achieved through an adequate diet [254].

Ready to use therapeutic food (RUTF)

Ready-to-use therapeutic food called Plumpy'nut® is a lipid-based groundnut paste now widely used in the treatment of acute malnutrition in SSA. It is packaged in 92 g individual sachet each containing 500 kcal (Figure 20 A). It is composed of vegetable fat, peanut butter, skimmed milk powder, lactoserum, maltodextrin, sugar, mineral and vitamin complex (Figure 20 B). It was initially developed for management of childhood malnutrition but its use has been extended to the adults with malnutrition.

The advantages of RUTF arise from being a high nutrient density nutrient mix with low water content; thus, it does not support bacterial growth even without refrigeration. There is no need for cooking or other preparation and can be fed directly from the packaging with no need for training. The introduction of RUTFs in the management of malnutrition has provided the opportunity to extend this treatment beyond hospital settings to the comfort and convenience of one's home. Consequently case fatality rates due to malnutrition have reduced and cost and the burden on in-patient health care facilities have also reduced allowing for increased coverage [2]. However due to its low water content one still needs to drink water as it is sticky and thick so that swallowing is eased. In addition a malnourished child needs increased fluid intake and this has not been addressed sufficiently after the introduction of RUTF.

Lipid profiles in HIV

Though HIV infected ART naïve and experienced adults have demonstrated some form of dyslipidemia, most studies on lipid levels have been performed with the aim of assessing

the ART impact and therefore have mainly been done on those receiving ART [255]. A retrospective case-control study in HIV positive and negative adults assessed the lipid profiling before patients commenced on ART and they found that the mean serum LDL, HDL and total serum cholesterol levels was higher among the HIV positive cases compared with the controls. There was no statistically significant difference in the mean TGL serum levels [256]. However, on the contrary, an Indian study observed low cholesterol, HDL, LDL and an increase in TGL, as the $CD4^+$ T cell count decreased in HIV infected ART naïve when compared against HIV negative controls. Males had higher cholesterol, TGL, LDL and lower HDL cholesterol than females when the values between sexes compared in HIV infected ART naïve patients. The ART-E HIV-infected patients had a significant increase in serum total cholesterol; HDL, LDL and decrease in TGL compared to HIV infected ART naïve patients [255]. In a study on ART experienced children in Uganda dramatically raised incidences of hypercholesterolemia and hypertriglyceridemia were observed [96]. Another Ugandan study done at Mbarara Regional referral hospital documented a prevalence of dyslipidemia of 74% in HIV infected ART naïve children. Of the children with dyslipidemia, 56.6% had low HDL, 22% had hypertriglyceridemia, 15.6% had high LDL and 11% had hypercholesterolemia. The dyslipidemia was significantly associated with WHO clinical stage at ART initiation [257].

In summary, malnutrition is still a major cause of death in young children in LRS, but the precise benefits of nutritional supplementation for HIV-infected children are still under appreciated. Several reviews have mainly focused on specific micronutrient supplementation for HIV-infected children such as serum vitamin levels as a primary outcome. Generalisable research regarding macronutrients is still limited.

This chapter will present the nutrition outcome results that include the rate of change of anthropometric measurements, dietary intake, nutrition biomarkers, RUTF adherence and reported adverse effects to RUTF.

A

B

Ready to use therapeutic food -Plumpy'Nut® formula: elements for 92 g* used on the study					
Energy	500 kcal	Copper	1.5 mg	Vitamin B1	0.46 mg
Proteins	12.8 g	Iron	10.3 mg	Vitamin B2	1.5 mg
Lipids	30.3 g	Iodine	98 µg	Vitamin B6	0.55 mg
Carbohydrates	45 g	Selenium	28 µg	Vitamin B12	1.5 µg
Calcium	302 mg	Sodium	165 mg	Vitamin K	14.4 µg
Phosphorus	343 mg	Vitamin A	0.79 mg	Biotin	56 µg
Potassium	1 171 mg	Vitamin D	14 µg	Folic acid	184 µg
Magnesium	80 mg	Vitamin E	18.4 mg	Pantothenic acid	2.8 mg
Zinc	11.8 mg	Vitamin C	46 mg	Niacin	4.6 mg

Figure 21: Ready to use therapeutic food (RUTF) supplement used on this study.
RUTF is the therapeutic food supplementation given to those who were malnourished at 150kcal/kg per day for 12 weeks. ***A,*** *92 gm RUTF sachet containing 500 kcal.* ***B,*** *Table showing the nutrient composition of RUTF in the form of plumpy'nut ®. *The values given in this table are based on Nutriset's knowledge of the intrinsic nutrient content of the raw materials and their variability, as well as the variability of the process.* https://www.nutriset.fr/products/en/plumpy-nut

Results

Effect of nutrition status at baseline and nutritional supplementation at 12 weeks on anthropometry, dietary assessment, lipid profile, protein profile and inflammatory markers among HIV-infected children

Assessing the nutrition status of a child is a complex exercise that needs to be approached from multiple perspectives. In our study we used a combination of parameters for nutritional assessment and how they change after nutritional supplementation. We performed anthropometry, 24 hour dietary recalls and biochemical markers to assess nutritional status. We used the WAZ, WHZ, BMI or MUAC to screen the potential study participants. They were then catogorised into 3 groups MAM or SAM or WN according to the z score. The children with MAM and SAM were provided with RUTF from the study for 12 weeks. ART-N children were initiated on ART according to Ugandan ART treatment guidelines.

Overall the HIV infected children gained 2.3 kg by 12 weeks (*p-value<0.0001*) and the MUAC increased by 1 cm from baseline (*p-value<0.0001*). The malnourished children added 2.5 kg by 12 weeks after RUTF supplementation (*p-value<0.0001*) and a 1.3 cm MUAC increase from baseline, *p–value<0.0001* (Table 34). The WN children added 2 kg by 12 weeks with no RUTF supplementation (p-value=0.002) and the MUAC increased by 0.3 cm at 12 weeks, Table 34. The largest magnitude of increase in anthropometry was observed among the malnourished children.

The expected or target weight gain after receiving RUTF at 150-200 kcal/kg/day for 6-8 weeks in treatment of malnutrition is 10-15 g/kg/day [247]. Out of the 45 malnourished children who came at 12 weeks for follow-up only 5/45 (11.1%) had achieved a rate of weight gain of 5 g/kg/day and 1 (2.2%) achieved a weight gain of 10 g/kg/day (Table 35) despite being prescribed and provided with RUTF for 12 weeks. This is beyond the recommended 8 weeks period usually prescribed for therapeutic supplementation.

Adherence to RUTF was assessed using an adherence form (Appendix 6). Primary carers were interviewed at 12 weeks and asked if the child consumed RUTF as prescribed and how many sachets were consumed. There after adherence was categorised in 3 groups:

>80-100%, 50-80% and <50% and denoted as good, fair and poor adherence respectively. Overall malnourished children were provided RUTF at 150-200kcal/day. Good adherence was reported in 24/50 (48%), moderate adherence in 1/50 (2%) while 26/50 (52%) were poorly adherent to taking RUTF (Figure 21). These results showed the poor adherence to RUTF was high in this study population.

Baseline dietary assessment was done using a 24-h dietary recall tool shown in Appendix 8. Using the household questionnaire (Appendix 7) we were able to assess the most frequently eaten foods and the family monthly income while with the 24-h recall we were able to assess the dietary intake by administering an interview to collect quantitative data consumed by the child in the last 24 hours. We mainly asked the primary carer, and for the older children who could recall foods and drinks, they consumed in the 24 hours prior to the interview provided supplemental information. We then performed nutrient analysis to determine the nutritional content of foods and drinks the 151 study participants consumed as discussed in detail in chapter 2.

The overall nutrient consumption of the study participants as assessed by the 24 hour dietary recalls at baseline among the HIV infected children was high with energy intake of 1,745 Kcal, 46.7g of protein, 39.8g of lipids, 297g of carbohydrates and 16g of fibre per 100g of food daily, Table 36. Baseline analysis of the 24-h dietary intake grouped by nutrition status among the HIV positive children on the study in Table 36 demonstrates that the malnourished HIV infected children had reduced nutrient intake which is about half of that taken by WN HIV infected children. The differences in nutrient intake between the malnourished and WN groups are statistically different, Figure 23. The ART-N children in Table 37 had a lower nutrient intake compared to the ART-E HIV infected children. Figure 24 shows that the difference in nutrient analysis at baseline of the foods and fluid consumed by ART status was significant statistically. The low intake was most marked in the ART-N malnourished children.

Table 34. Effect of nutrition status and nutritional supplementation at 12 weeks on anthropometry

Anthropometric measurement	Baseline Median (IQR)	12 weeks Median (IQR)	P-value
Overall weight (kg)	14.7 (10-21)	17 (12-24)	***<0.0001****
Overall MUAC (cm)	14.5 (13.5-16)	15.5 (14.4-16.7)	***<0.0001****
Weight for malnourished children (kg)	14 (9-17)	16.5 (10.3-21.8)	***<0.0001****
MUAC for malnourished children (cm)	13.7 (12-15)	15 (14-16)	***<0.0001****
Weight for WN (kg)**	18 (12.5-26.3)	20 (13-27)	***0.002****
MUAC for WN (cm)**	15.7 (14.8-18)	16 (15-17.8)	0.26

***denotes statistically significant p-values, **denotes absence of nutritional supplementation for the WN HIV infected children. The carer only received nutritional counselling while the children received ART or continued on ART.**

Table 35. Comparison of rate of weight gain among the malnourished children who received RUTF

Rate of weight gain (5g/Kg/day	Numbers (freq)	Rate of weight gain (10g/Kg/day)	Numbers (freq)
< 5g/Kg/day	40 (88.9)	<10g/Kg/day (inadequate nutrition response)	44 (97.8)
≥ 5g/Kg/day	5 (11.1)	≥10g/Kg/day (adequate nutrition response)	1 (2.2)

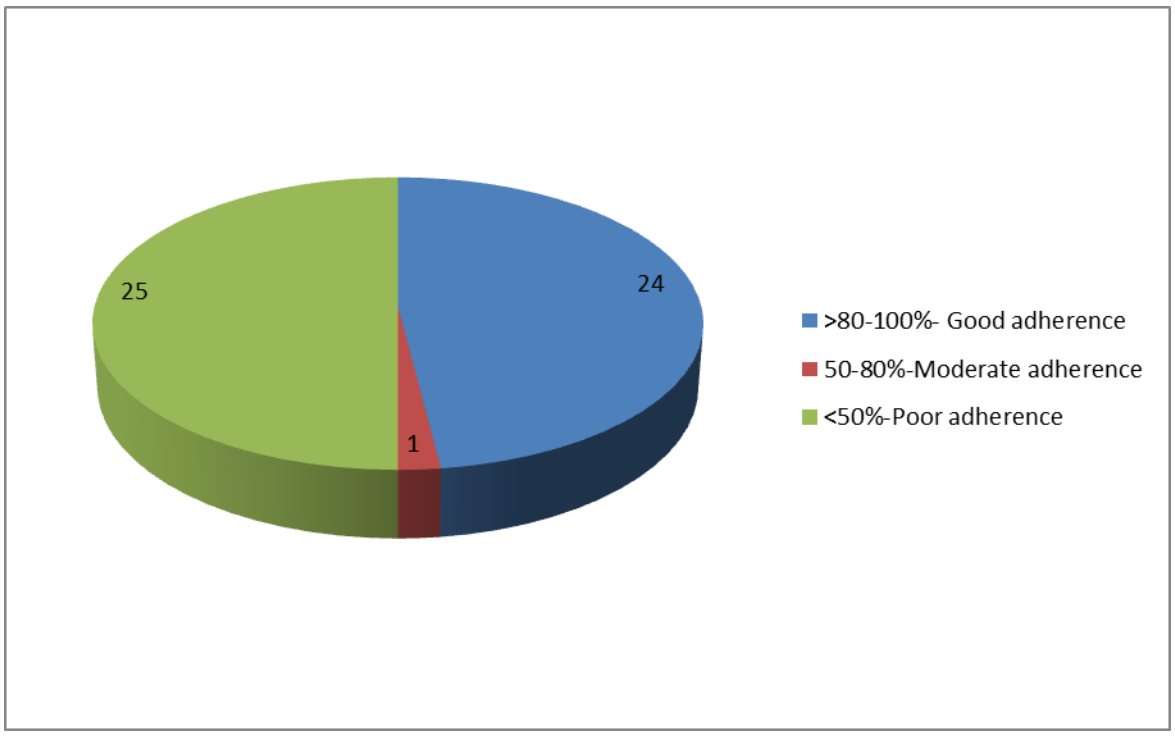


Figure 22: Ready to use therapeutic food (RUTF) adherence status of the malnourished HIV infected children on the study at 12 weeks.

Adherence to RUTF was assessed by the researcher through interviewing the primary carers of the 50 HIV infected children who returned at 12 weeks follow up. Carers were asked whether the malnourished child consumed the RUTF as prescribed. Thereafter the adherence category was calculated by the number of RUTF sachets consumed and completed at 12 weeks/the total number of RUTF sachets prescribed for 12 weeks X 100. They were then categorized in 3 groups namely good adherence (>80-100% adherence rate), moderate adherence (50-80% adherence rate) and poor adherence (< 50% adherence rate).

When comparing the nutrient intake by females to males (Figure 25), females had lower nutrient consumption in comparison to the males on this study. HIV infected children whose carers were mothers had the lowest nutrient intake in comparison to where the father or grandparents were the carers. In addition the family income where the mother was the carer was found to be higher. The family income was assessed by asking the primary carer to total up all monies they received from whichever source that was used in financing the family. The average monthly income for a family was Uganda Shillings (UGS) 206,369/= ($54.3) at an exchange rate of 1 $: 3,800 UGS at the time of the study, Table 38.

Table 36. Baseline nutrient analysis of the 24-h dietary intake grouped by nutrition status among the HIV positive children on the study

Nutrient	Malnourished group	WN group	Overall	P-value
Energy (kcal)	1,301	2,266.1	1,745	***<0.0001****
Protein (g)	35	60.5	46.7	***0.0003****
Total lipids (g)	31	50.2	39.8	***0.015****
Carbohydrate (g)	212.6	397.2	297	***<0.0001****
Fibre (g)	11.5	21.3	16	***0.0009****

***denotes statistically significant p-values. Malnourished group includes MAM and SAM**

Table 37. Baseline nutrient analysis of the 24-h dietary intake grouped by ART status among the HIV positive children on the study

Nutrient	**ART-N**	**ART-E**	**P-value**
Energy (kcal)	1,321.3	2,043.5	***<0.0001****
Protein (g)	35.7	54.5	***0.0006****
Total Lipids (g)	26.6	49.2	***0.0002****
Carbohydrates (g)	223.8	349.4	***0.0002****
Fibre (g)	10.6	19.8	***<0.0001****

***denotes statistically significant p-values.**

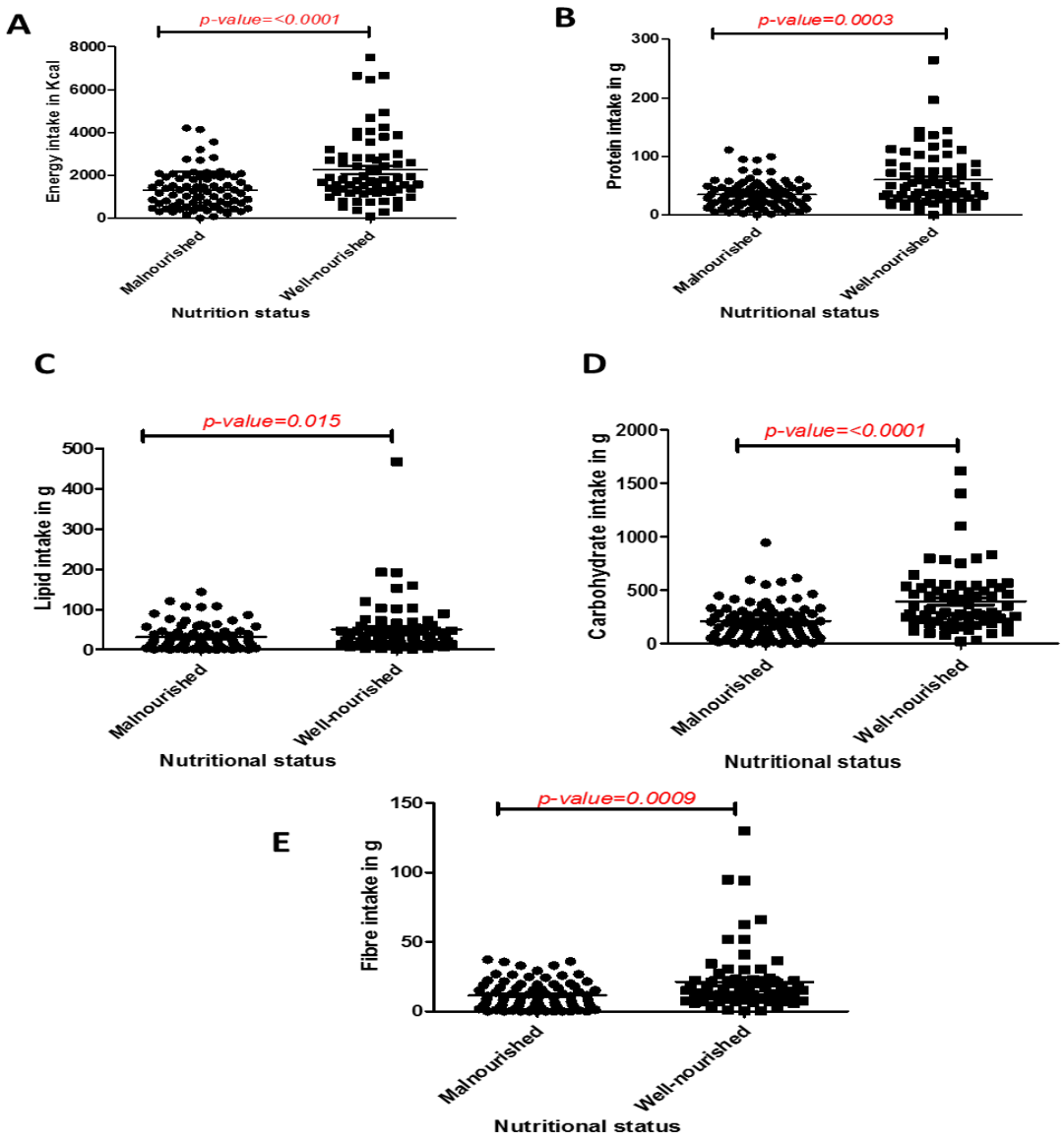


Figure 23: Nutrient intake in HIV infected children compared by nutrition status at baseline.

*Out of the 156 HIV children enrolled on the study at baseline we were able to perform 155, 24 hour dietary interviews. One child died before we could administer the tool, 4 were reported to be breastfeeding exclusively and one had not consumed any food in the last 72 hours. We report 150 patients whose nutrient analysis was done, 81 were malnourished with moderate or severe acute malnutrition and 69 were well-nourished. Scatter plots show the amounts of each nutrient intake analysed (**A**) energy in Kcal, (**B**) protein in g, (**C**) total lipids in g, (**D**) carbohydrates in g and (**E**) fibre in g. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values in red and are all statistically significant.*

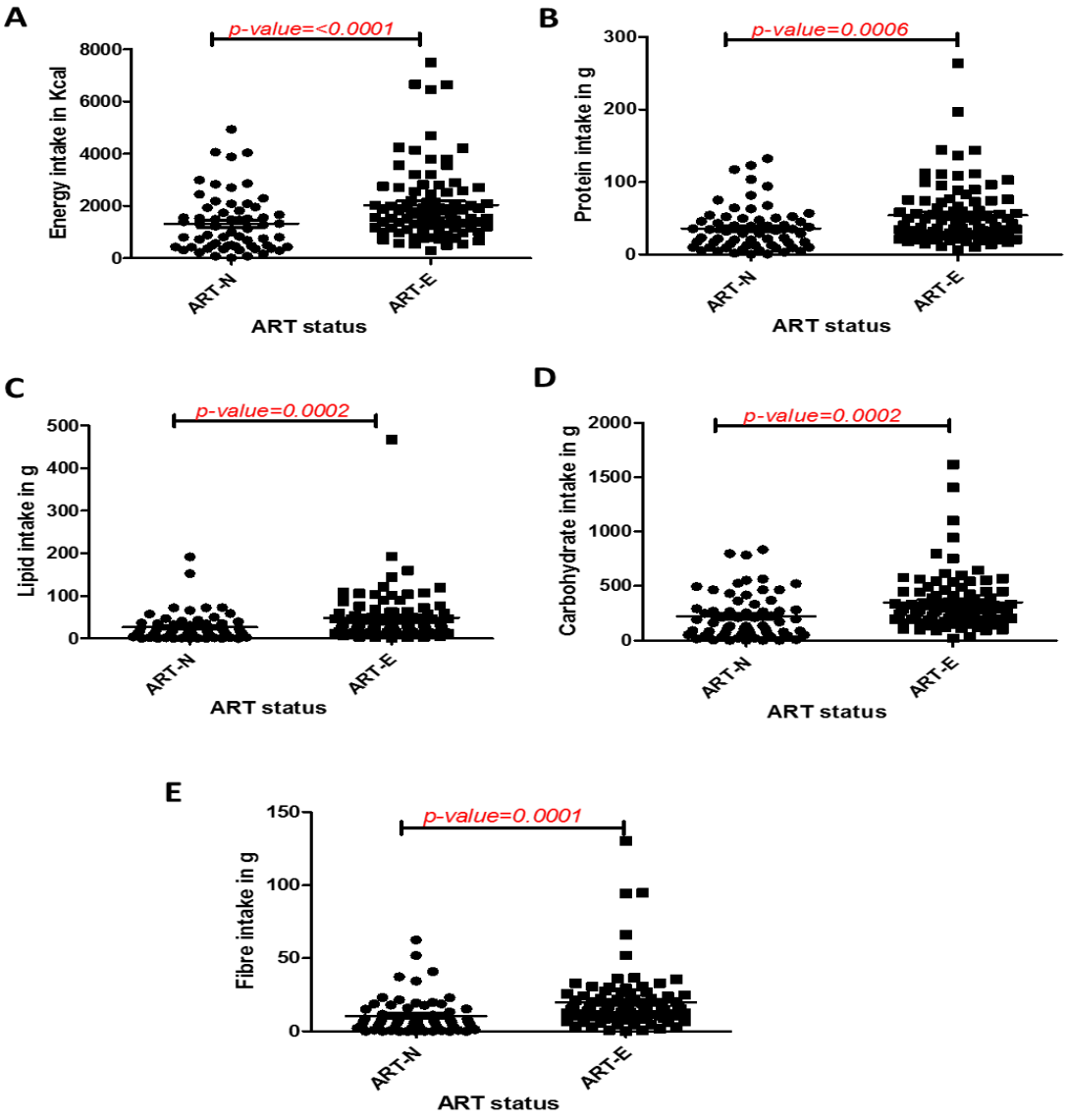


Figure 24: Nutrient intake in HIV infected children compared by ART status at baseline.

*Out of the 156 HIV children enrolled on the study at baseline we were able to perform 155, 24 hour dietary interviews. One child died before we could administer the tool, 4 were reported to be breastfeeding exclusively and one had not consumed any food in the last 72 hours. We report 150 patients whose nutrient analysis was done, 62 were ART-naïve (ART-N) and 88 were ART-experienced (ART-E). Scatter plots show the amounts of each nutrient intake analysed (**A**) energy in Kcal, (**B**) protein in g, (**C**) total lipids in g, (**D**) carbohydrates in g and (**E**) fibre in g. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values in red and are statistically significant.*

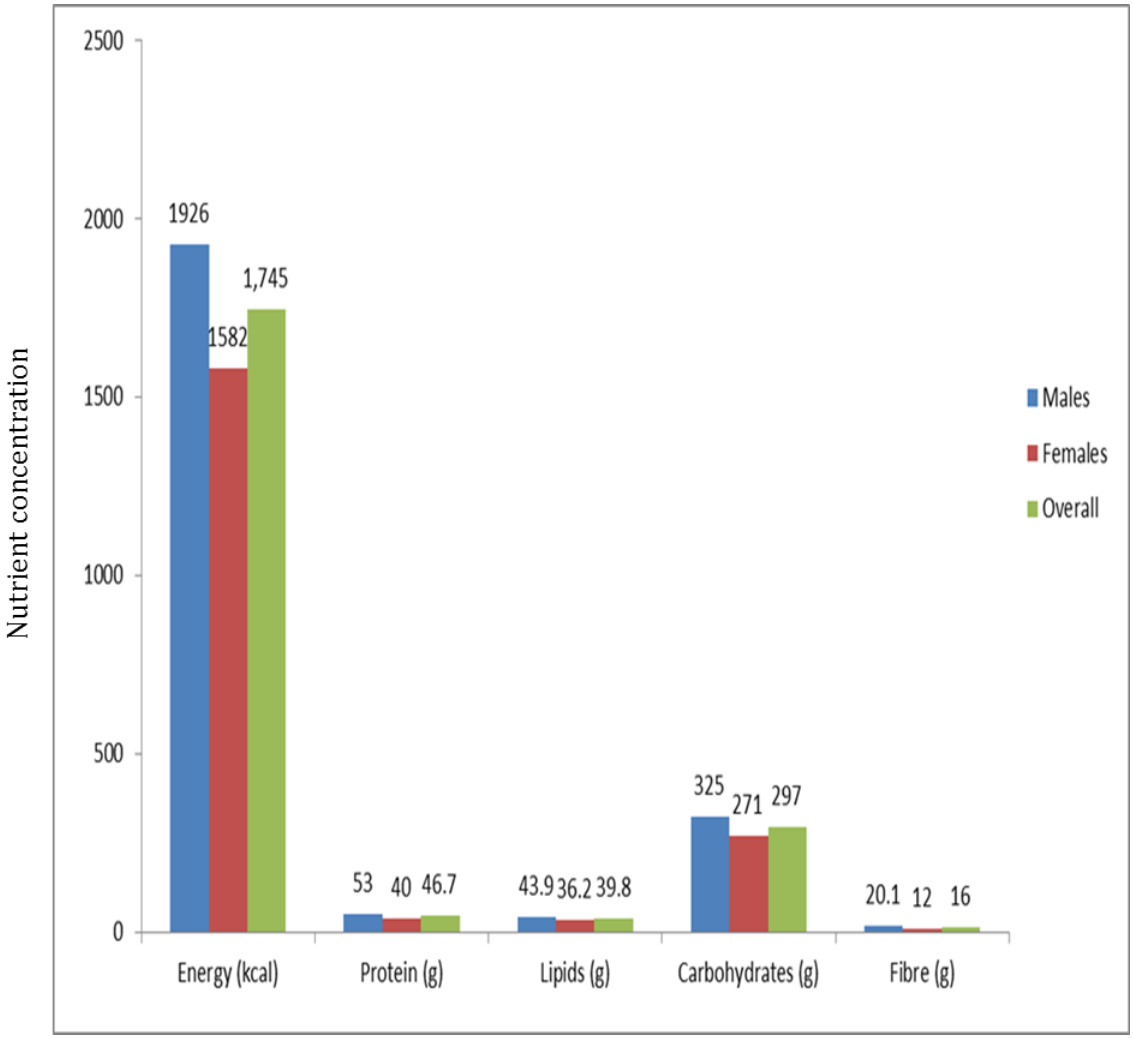


Figure 25: The 24-h nutrient intake at baseline compared by sex on this study.
The dietary intake (food and drinks) of the HIV infected children at baseline were assessed in the last 24 hours by interviewing their primary carers. We assessed 152 children's dietary intake by taking care to record the type of food consumed, when, amount and how it was prepared. We also assed the diet intake from Sunday to Thursday only but also ensured to assess if there was any special occasion. We also ensured to assess if the where the food was prepared from for example at home, restaurant, school, prepackaged foods and drinks etc. The males generally had the highest intake of each nutrient consumed.

Table 38. Comparison of 24 h nutrient intake of study participants and monthly income of the family as reported by the primary carer

Nutrient	Mother	Father	Grandparents	Others*
Energy (kcal)	1,520.7	2,935	2,184.3	2,139.5
Protein (g)	42.5	62	51.3	58.2
Lipids (g)	38.2	49.9	54.6	39.1
Carbohydrates (g)	250	55.9	384.5	379.2
Fibre (g)	14.3	19.3	20.3	21.3
Average monthly income in UGS (USDs)	208,898 (55)	175,500 (46.2)	98,750 (26)	259,556 (68.3)

***Others comprise of carers who were aunties, uncles, brother, sister, and good Samaritan**

In Figure 26, we categorised the Hb concentration into 3 groups and these were severe anaemia: Hb ≤6 g/dl, mild-moderate anaemia: Hb >6-10.9 g/dl and normal Hb concentration Hb≥11 g/dl). We compared the nutrient intake by the Hb categories and the results show that nutrient intake was lowest among those who had severe anaemia, followed by mild-moderate anaemia and those with normal Hb concentration had the highest nutrient intake for all the nutrients assessed, Figure 28.

Immunological status was classified in 2 groups, presence of immunosuppression (ISS) with $CD4^+$ T cell percentage of < 30% and "No ISS" with $CD4^+$ T cell percentage of ≥ 30%. We compared the nutrient intake between the 2 categories; the entire nutrient intake assessed was similar with the exception of the daily fibre intake that was found to be statistically significantly lower in those with ISS, Figure 27.

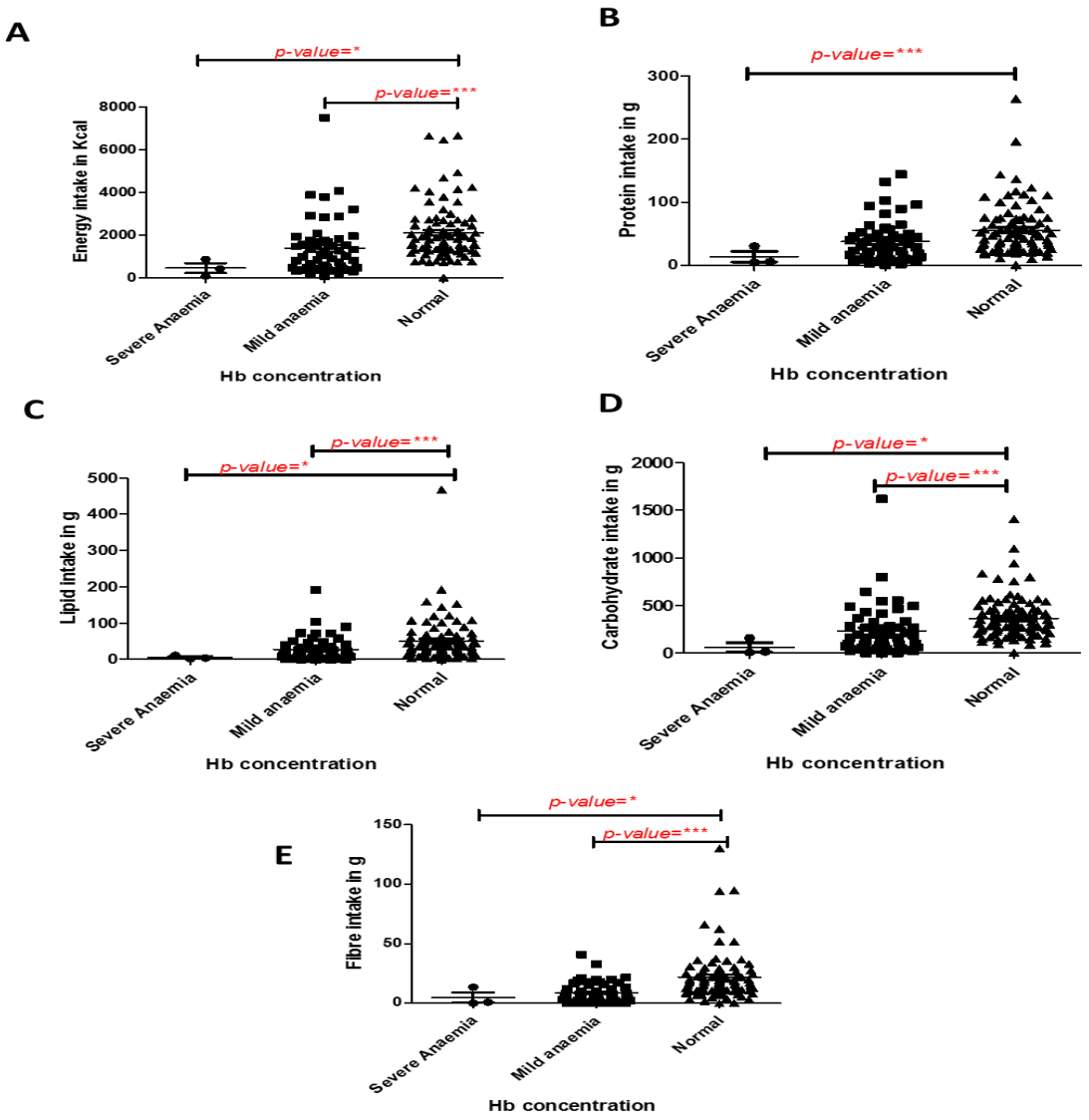


Figure 26: Nutrient analysis assessed by 24 hour dietary intake in HIV infected children compared by hemoglobin concentration at baseline.

*Out of the 156 HIV children enrolled on the study at baseline we were able to perform 155, 24 hour dietary recall interviews. One child died before we could administer the tool, 4 were reported to be breastfeeding exclusively and one had not consumed any food in the last 72 hours. We report 150 patients whose hemoglobin concentration (Hb) was classified in 3 groups (severe anaemia: Hb ≤6 g/dl, mild-moderate anaemia: Hb >6-10.9 g/dl and normal Hb concentration Hb≥11 g/dl). Nutrient analysis was done for 5 children with severe anaemia, 58 with mild anaemia, 86 with normal Hb concentration and 7 had missing values. Hb concentration was assessed by automated Coulter machine. Scatter plot show the amounts of each nutrient intake analysed (**A**) energy in Kcal, (**B**) protein in g, (**C**) total lipids in g, (**D**) carbohydrates in g and (**E**) fibre in g. Data were compared using the Kruskall Wallis statistical test ie "one way ANOVA on ranks". * in the graphs indicate P values in red and are all statistically significant.*

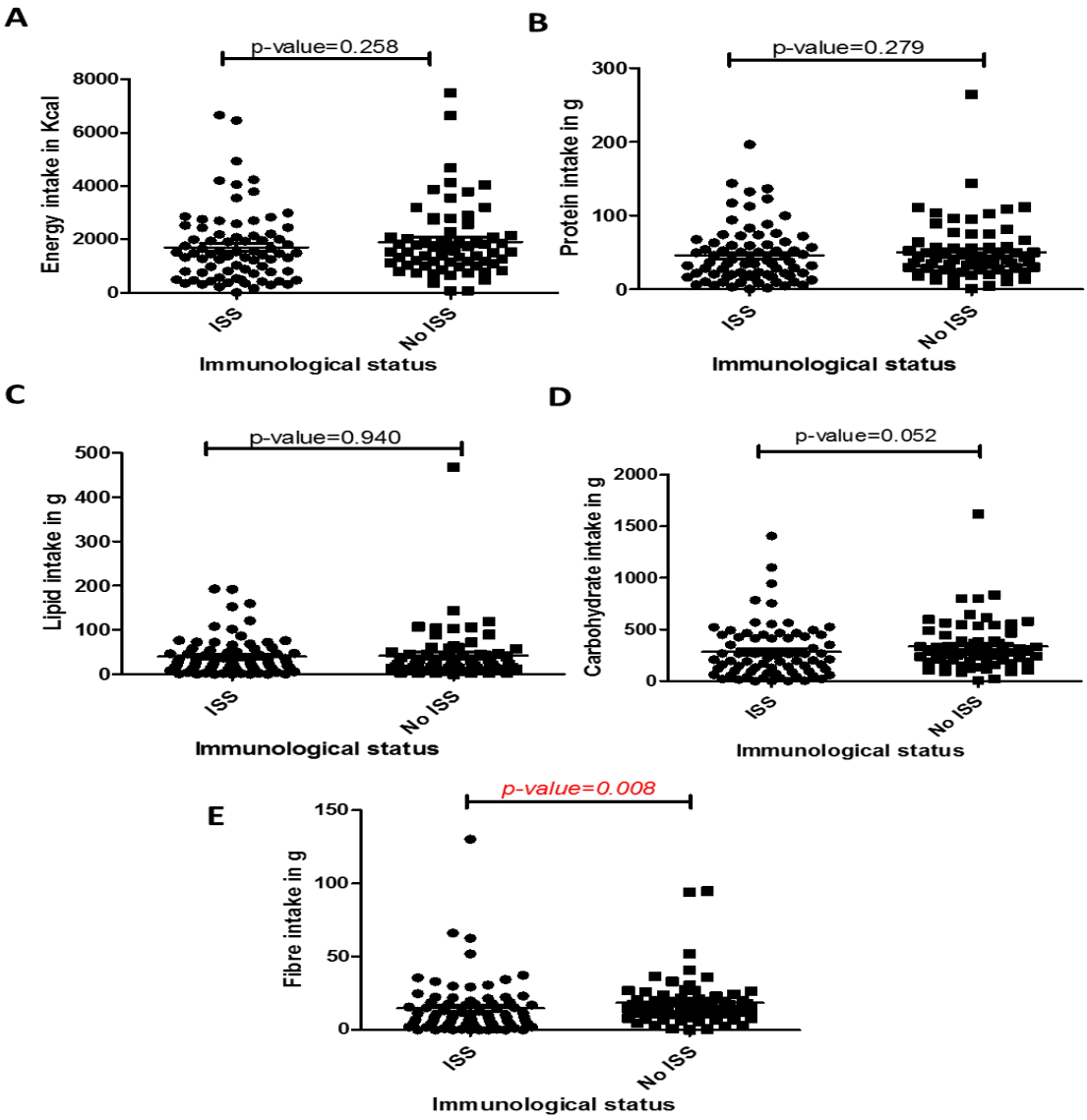


Figure 27: Nutrient analysis assessed by 24 hour dietary intake in HIV infected children compared by Immunosuppression (ISS) at baseline.

*Out of the 156 HIV children enrolled on the study at baseline we were able to perform 155, 24 hour dietary recall interviews. One child died before we could administer the tool, 4 were reported to be breastfeeding exclusively and one had not consumed any food in the last 72 hours. We report 150 patients whose immunological status was classified in 2 groups (Immunosuppression (ISS) with CD4⁺ T cell percentage of < 30% and "No ISS" with CD4⁺ T cell percentage of ≥ 30%. Nutrient analysis was done for 66 children with no ISS, 83 with ISS and 6 had missing values. CD4⁺ T cell percentage was assessed by automated flow cytometer machine. Scatter plot show the amounts of each nutrient intake analysed (**A**) energy in Kcal, (**B**) protein in g, (**C**) total lipids in g, (**D**) carbohydrates in g and (**E**) fibre in g. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values and the ones in red are statistically significant.*

Serum samples were prepared from whole blood collected from eligible study participants before they took any food in the morning. In Figure 28, the changes in lipid profiling over the study period, the total cholesterol, HDL, LDL were reduced at baseline among the malnourished HIV infected children in comparison to the WN except for TGL which was higher in the malnourished HIV infected children. Upon nutritional supplementation there was a marked increase in total cholesterol, HDL and LDL with a decline of TGL to acquire similar levels demonstrated in the WN HIV infected children.

In Table 39, while examining for the impact of ART on lipid profiles at baseline, the total cholesterol, HDL and LDL serum levels were all lower among the ART-N compared to the ART-E children and the TGL was higher among the ART-N in comparison to ART-E children. The difference was statistically significant for total cholesterol, HDL and LDL (*p-value≤0.0001, p-value≤0.0001* and 0.0011 respectively). After receiving ART for a period of 12 weeks among those who were enrolled as ART-N their lipid profile changed towards the trend among the ART-E.

Serum levels of inflammatory markers and protein profiles were measured at baseline and 12 week follow up time points. At baseline, we found an overall increase of CRP that was dramatic, about 5 times increase above the normal level, 19.5 mg/dl (normal level is <4 mg/dl). CRP levels were higher among the WN in comparison to the malnourished children, (Figure 29.A). At 12 week follow up there was a dramatic fall in CRP serum levels that was most pronounced in the malnourished who received nutritional supplementation compared to the decrease among the WN children who did not receive any supplementation. Overall, LDH was raised above the normal level (<250 IU/L) at baseline. It was higher in the WN children compared with the malnourished children. After 12 weeks follow up, LDH sharply decreased among the malnourished children compared to the WN children. Overall there was a sharp decrease but did not attain the expected normal levels (Figure 29.B).

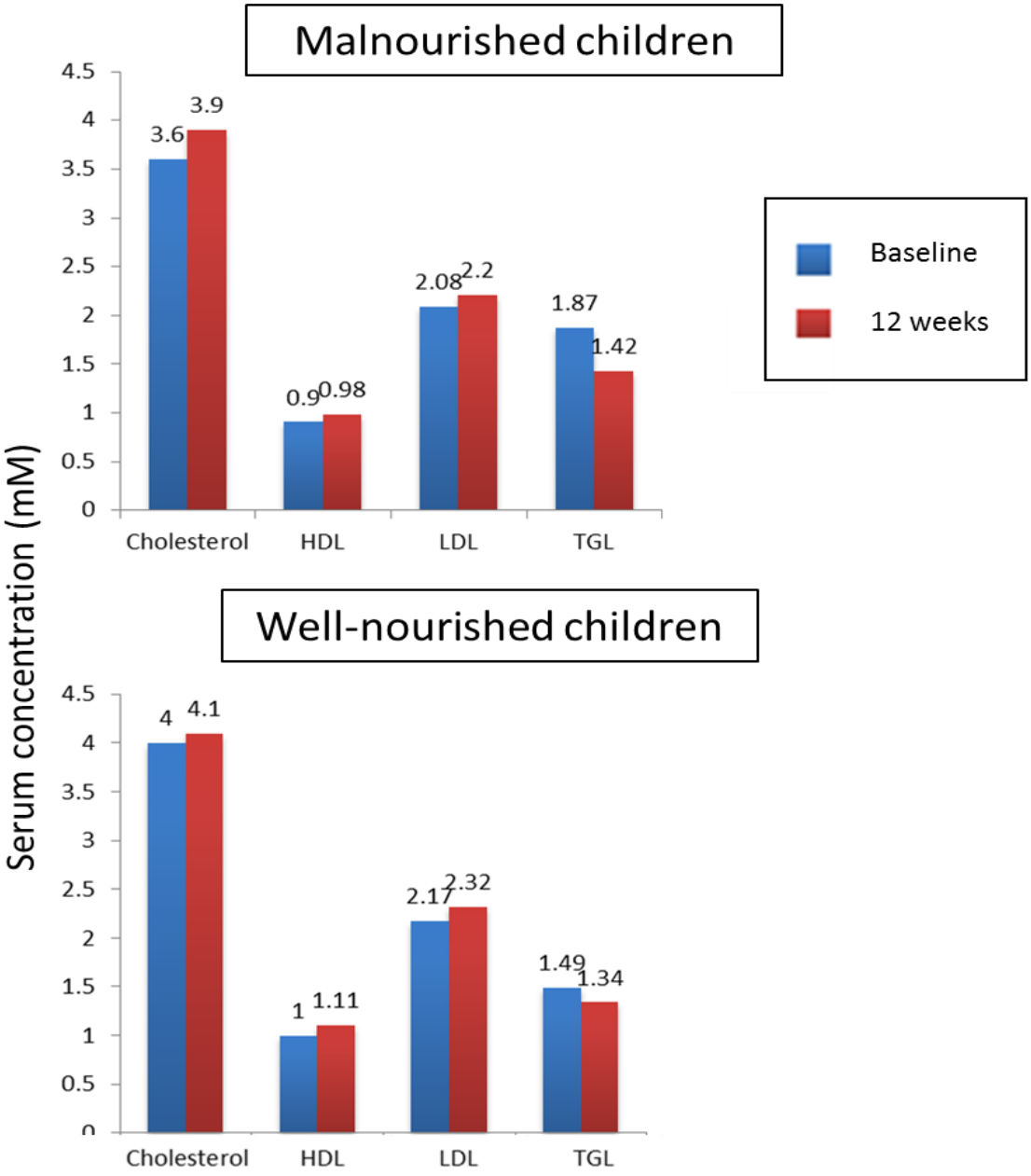


Figure 28: Comparison of lipid profiles at baseline and 12 weeks by nutritional status.

Both ART naïve (ART-N) and ART experienced (ART-E) HIV infected children were sampled at baseline and 12 week time points to determine the serum lipid levels. Malnourished children were all supplemented with ready to use therapeutic food (RUTF). The ART-N children initiated ART while the ART-E continued with ART. Well-nourished children only received ART and nutritional counselling.

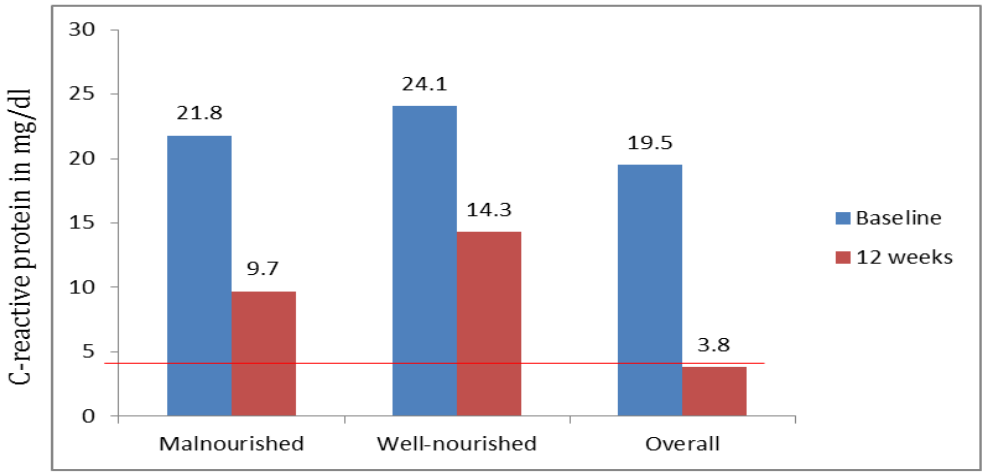
Table 39. Comparison of lipid profiles at baseline and 12 weeks by ART status in HIV infected children

Characters	Baseline			12 weeks		
ART status	**ART –N**	**ART-E**	**P-value**	**ART-N**	**ART-E**	**P-value**
Cholesterol	3.1 (2.3-4.1)	4 (3.4-4.45)	***≤0.0001****	4.1 (3-4.6)	4 (3.4-4.5)	0.833
HDL	0.59 (0.26-0.79)	1.1 (0.89-1.4)	***≤0.0001****	0.95 (0.71-1.14)	1.15 (0.83-1.33)	0.076
LDL	1.52 (0.8-2.3)	2.2 (1.65-2.7)	***0.0011****	2.26 (1.45-2.82)	2.21 (1.84-2.64)	0.892
TGL	2 (1.2-2.57)	1.59 (1.29-2.41)	0.273	1.33 (1.02-2.51)	1.45 (1-2.24)	0.9

***denoted statistically significant p-values.**

Total serum protein levels were higher at baseline among the malnourished children in comparison to the WN children. However both levels were within the normal expected range of serum protein levels. At 12 weeks serum protein levels were the same and within the normal range (6.4-8.3 mg/dl). Serum albumin levels showed an increased trend from baseline to 12 week time point in both malnourished and WN children, however, at baseline the malnourished children had lower levels than the WN children. Overall the albumin levels were the same at baseline and 12 week follow up time points. Generally the levels were within normal expected serum levels, 3.5-5.2 mg/dl, (Figure 30).

A Serum levels of C-reactive protein (mg/dl) in the study



B Serum levels of Lactate dehydrogenase (LDH in IU/L) in the study

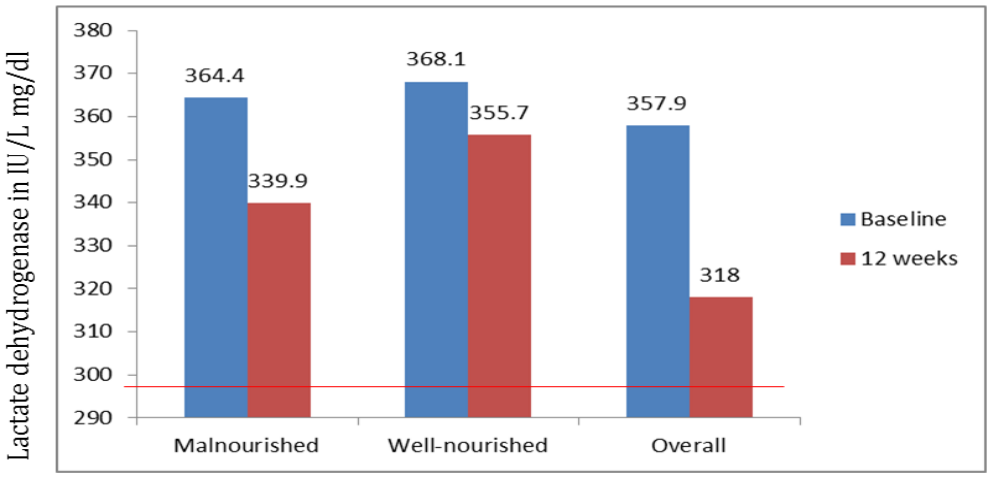
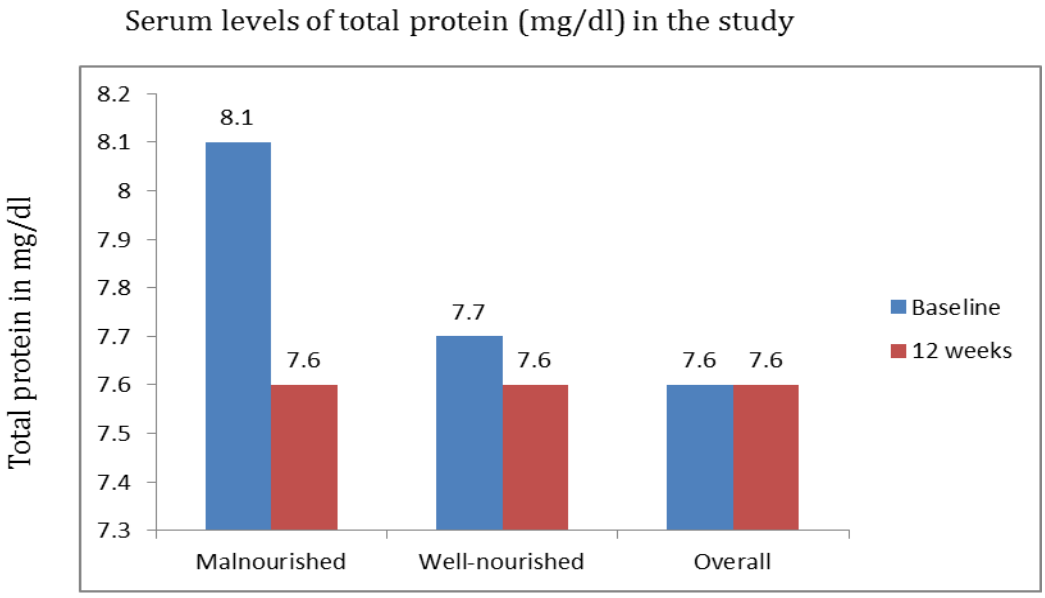


Figure 29: Effect of nutritional supplementation on inflammatory markers among HIV positive children on the study.

Serum samples were prepared from 151 HIV-infected children sampled at baseline and 81 at 12 weeks. Medians were determined for CRP and Lactate dehydrogenase (LDH). ***A,*** *CRP serum levels of both ART naïve and experienced children.* ***B,*** *LDH serum levels among ART naïve and experienced children. The red lines represent the upper limit of the normal range for the inflammatory markers.*

A



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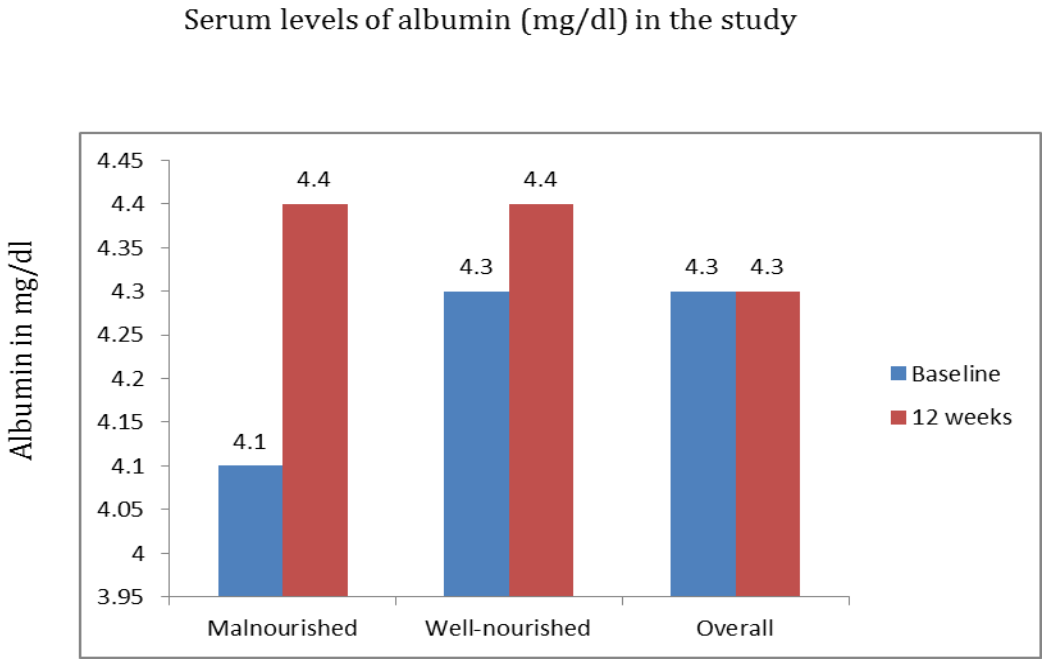


Figure 30: Effect of nutritional supplementation on serum protein levels among HIV positive children on the study.

Serum samples were prepared from 151 HIV-infected children sampled at baseline and 81 at 12 weeks. Medians were determined for total protein and albumin serum levels. ***A,*** *Represents total serum protein of both ART naïve and experienced children.* ***B,*** *Represents serum albumin levels among ART naïve and experienced children.*

Discussion

In Africa, HIV/AIDs was first described as the 'Slim disease.' This was the result of progressive wasting away and subsequently became the hallmark of the nutritional consequence of HIV progression. Finally patients developed AIDS in the absence of ART. Later in the epidemic, having MAM or SAM then become one of the recognised AIDS defining illnesses using WHO clinical staging of HIV disease [258]. Globally, association between HIV infection and growth faltering in children has become topical and crucial in order to address HIV/AIDs mortality and to improve quality of life in a more relevant approach. Moreover the dual tragedy has particular relevance in SSA where HIV/AIDs and malnutrition have significant geographical overlap as food insecurity and moderate and severe malnutrition are also common in the high HIV prevalent countries [18]. A study carried out in Swaziland on predictors of survival among HIV positive children on ART demonstrated that ART paediatric services are effective in increasing survival and children initiated timely on ART have high survival probability. However, malnutrition was also cited as a major predictor of mortality. Active TB and delayed ART initiation also remained predictors of poor survival among HIV infected children [219]. Since 2007, WHO recommended the use of RUTF for the outpatient nutritional rehabilitation of children with uncomplicated MAM and SAM, and most SSA countries have adopted national guidelines for RUTF-based nutritional rehabilitation of children <5 years, and Uganda has extended it to those above 5 years and included adults who are HIV positive or co-infected with TB [247]. In our study we aimed at describing the effect of nutrition status at baseline and nutritional supplementation at 12 weeks on anthropometry, dietary assessment, Hb concentration, lipid profile, protein profile and inflammatory markers among HIV infected children. We used RUTF as the therapeutic food supplement.

Our study demonstrated an overall increase of weight (2.3 kg) and MUAC (1 cm) by 12 weeks in the HIV infected children. After RUTF supplementation the malnourished children showed increases in weight and MUAC of 2.5 kg and 1.3 cm respectively. In addition, in the absence of RUTF supplementation the WN children too added 2 kg and the MUAC increased by 0.3 cm at 12 weeks. Basically, anthropometric measurements improved probably due to the effect of both ART and RUTF in the malnourished and ART in those who were WN, however, the largest increase was observed among the

malnourished indicating the effect of RUTF supplementation on anthropometric change was appreciated. The improvement on anthropometrics is further demonstrated by the rate of change for each index daily. The target weight gain after receiving RUTF at 150-200 kcal/kg/day for 6-8 weeks in treatment of malnutrition is 10-15 g/kg/day [247]. Though there was improved anthropometry in this study the majority did not attain the recommended targets despite providing it for a longer period. An Ethiopian study on anthropometric improvement among HIV infected pre-school children following initiation of first line ART demonstrated similar findings to those of our study where there was a significant improvement in all anthropometric indices at any follow-up after initiation of first-line ART among under-five children [259]. This study revealed the effects of ART as they did not receive RUTF supplementation. In addition emergence of progressive lipodystrophy syndrome may affect the achievement of anthropometric targets reported in this study as this syndrome underscores the use of anthropometry however we did not assess for it. A systematic review undertaken to evaluate the impact of nutritional supplementation on clinical outcomes for HIV infected children in LRS in 2015 revealed a paucity of supporting evidence in the use of specific elements of supplementation, especially macronutrient supplementation, using common therapeutic food supplements. RUTF in the form of plumpy-nut has just been initiated in programs so the studies are still limited. Compiled evidence mainly showed that macronutrient supplementation improved anthropometrics for HIV infected children. RUTF in the presence of ART increased the average weight gain in HIV infected children [213]. There are no studies which look at the effect of RUTF alone in HIV as it is unethical to withhold ART or RUTF in children who need the RUTF or ART.

Our study used a 24 hour dietary recall method to assess the nutrient intake among the HIV infected children. The use of this method was validated in Ugandan children and was found to satisfactorily correlate with weighted food records [260]. On the contrary, other studies have shown that using this method usually over estimates the children's daily intake. We present similar findings in our study where the 24 hour recalls demonstrated that children were receiving over twice the recommended macronutrient intake. This study also reveals that no one method of measuring nutritional status alone is sufficient and combining methods offers a more complete picture. Alternatively, since it is well

understood that malnutrition causes atrophy of intestinal villi reducing the absorptive surface area, [261], this may be further affected by the combination of both HIV and malnutrition resulting in inadequate reabsorption of food across the study cohort. The implication of this is that in the presence of HIV and malnutrition, there may be need for a longer period of supplementation to give ample time for the repair of the intestinal villi for it to then absorb nutrients as required. A study in HIV infected malnourished children in high resource settings showed that providing enteral feeding in comparison to parental feeding for the period of malnutrition improved the $CD4^+$ T cells in those who received enteral feeding and there was no impact in those who received parenteral nutrition. Probably, nutritional supplementation in HIV with malnutrition may necessitate a treatment approach where exposure to therapeutic foods needs to first restore the microbiota and prime the repair of the absorptive surface if one is to gain from nutritional supplementation. The adherence to RUTF was also suboptimal among the study participants due to several factors such as sharing of the RUTF with other household members. This has been shown to occur but is under-reported [262], resulting in the suboptimal anthropometric increase observed in this study.

Adherence to RUTF in this study was categorised in 3 groups: >80-100%, 50-80% and <50% were denoted as good, fair and poor adherence respectively. Overall all malnourished were provided RUTF at 150-200kcal/day, 24/50 (48%) reported good adherence while 26/50 (52%) poorly adhered to taking RUTF. This result showed that poor adherence to RUTF was high in this study population. Originally RUTF was developed for paediatric use for children below 5 years; however its use has extended to involve PLWHIV through food-by-prescription programs in LRS. A study on acceptability of outpatient RUTF based protocols in HIV infected Senegalese children and adolescents showed that greater than half the children had successful weight recovery, although adherence to RUTF prescription was suboptimal. They recommended the need for further research in therapeutic foods with improved palatability, alternative and simpler intervention design, and procedures for continuous and tailored psychosocial support to improve adherence in children [263].

A study on the nutritional assessment and lipid profile in HIV infected Latin American children and adolescents treated with ART showed that food intake was similar between

the groups and no changes were observed after twelve months follow-up, except for a decrease in energy, protein and carbohydrate intake in the control group. This study demonstrated that decreased nutrient intake is not encountered in children with HIV infection which was similar to the findings described in our study where nutrient intake especially for macronutrients were found to be over double the recommended intakes [264]. Another study confirmed that dietary intake among children with HIV infection is reported to be equal to or greater than those in non-infected children [265], and additional variables, such as metabolic abnormalities, could be the reason for this escalated intake such as progressive lipodystrophy syndrome, gastrointestinal intolerance leading to higher intakes as we observed [266]. The current supplementation of RUTF and nutritional habits in LRS as shown by the nutrient distribution in this study may contribute to the observed excessive caloric intake and a shift in dietary composition toward lipids suggesting the need to monitor dietary intake in HIV infected children to avoid increased risk of non-communicable diseases such as cardiovascular disease. Despite the fact that, a higher proportion of energy and carbohydrate intake than lipids or proteins were verified in all the comparison groups in the current study, females had a lower nutrient intake in comparison to the males. Reasons for this observation are not well understood, however, in Africa the boy child is usually given preferential treatment in comparison to females and this may explain the difference in nutrient intake [267]. In addition, though the family income was noted to be highest in families where the mother was the primary carer of the HIV infected child, these children surprisingly had the lowest nutrient intake in comparison to where the father or grandparents were the primary carers. It is thought that when women are economically empowered, this indirectly may result to better health choices for the children under their care, however, the mothers in this study may have had several competing priorities such as other children needing support thus though they seemed to have a higher income at a household level the demand was high. However, we may not be able to substantiate this since we did not assess for the other dependents in the household. A study in two developing countries on the effect of women's empowerment on child health revealed that the empowerment of women was a direct indicator of child survival and its relationship to child health outcomes were a strong and positive influence of the active participation of women in making decisions in the

household on their children's health status after investigating a sample of 22,462 under five years' children in Nigeria and 45,516 India [268]. This study is a useful addition.

Daily food intake is affected by several factors beyond food insecurity. Some of the individual factors may be due to the presence of painful sores in the mouth, pharynx and oesophagus that will affect the patients' ability to swallow which has been well demonstrated in several studies in children with advanced HIV disease or severe malnutrition [269]. Some data have shown that fatigue and depression, including changes in mental state, may also play a significant role by affecting appetite and interest in food [270, 271]. Therefore reductions in food intake are believed to be an important cause of slow and progressive weight loss despite providing adequate food supplementation and may be a result of systemic conditions [270, 272], such as anaemia, increased nutritional demands, malabsorption, and altered metabolism [273]. It is well known that anaemia affects a significant proportion of children with HIV in LRS, [274], resulting in a range of impaired mental or cognitive capacity to life threatening conditions like congestive cardiac failure and respiratory failure [275]. However, most studies performed have focused on certain age groups, such as children younger than 5 years of age. As a contributor to reduced dietary intake a few studies have alluded to it as a risk of developing poor food intake among HIV negative children. In our study we report that nutrient intake was lowest among those who had any form of anaemia in comparison to those who had a normal Hb concentration for all the nutrients assessed. Anaemia causes loss of appetite, lethargy, oral sores due to poor wound healing and in severe causes altered mentation and respiratory distress resulting inability to feed thus reduced intake [276].

It is generally accepted that nutrition is an important determinant of immune responses and epidemiological and clinical studies have for long suggested that nutritional deficiencies alter immuno-competence and increase the risk of infection. It is agreed that poor dietary intake contribute to susceptibility to infection which worsens the malnutrition. Previous research findings have confirmed that impaired immunity is a critical adjunct factor for a good appetite and indirectly causes reduced food intake in patients with malabsorption, resulting from different diseases or systemic infections due to the release of specific factors that inhibit appetite at the central nervous system level [277]. Several studies in the past have documented that AIDs and infection exacerbate

problems malnutrition in children [271]. From birth HIV infected children have challenges of adequate and optimal infant feeding practices which have affected their health in terms of enhancing HIV progression especially if the mother is malnourished as well [278]. This current study revealed that having immunosuppression in comparison to the absence of immunosuppression did not affect the dietary intake with the exception of the daily fibre intake that was found to be profoundly lower in those who had immunosuppression. Deficiency of high fibre diet is not well studied in HIV infected children. However, fibre is known to be a key component of a balanced diet. The main benefit of fibre in diet is to aid the digestive system by ensuring adequate bowel movements and has also been found to reduce the risk of developing inflammatory bowel conditions [279]. A study in HIV infected men showed that consumption of a high-fiber diet may be beneficial in preventing the development of fat deposition in persons infected with HIV [280]. This suggests that fibre supplementation trial studies may be indicated in malnourished children though high fibre diet may bind micronutrients and wastes space in the diet for more energy dense foods in the malnourished children.

A review article showed that prior to the introduction of ART, HIV infection itself caused dyslipidaemia. This was characterised by declines in total cholesterol, LDL and HDL particularly in those with more advanced disease than HIV negative controls and with lower HDL concentrations associated with higher circulating HIV viral loads and longer duration of HIV infection. This pattern or trend is well demonstrated in the current study [281]. The pattern of lipid profile in this current study was characterised by the reduction in serum levels of total cholesterol, HDL, LDL at baseline among the malnourished HIV infected children in comparison to the WN except for TGL which was higher in the malnourished HIV infected children compared to the WN. After nutritional supplementation, there was a marked increase of the total cholesterol, HDL and LDL with a decline of TGL to acquire the similar levels demonstrated in the WN HIV infected children. Examining for the impact of ART on lipid profiles at baseline, the total cholesterol, HDL and LDL serum levels were all lower among the ART-N compared to the ART-E children and the TGL was higher among the ART-N in comparison to ART-E children. The difference was statistically significant for total cholesterol, HDL and LDL. After receiving ART for a period of 12 weeks among those who were ART-N, their lipid

profile changed towards the trend observed among the ART-E. The lipid changes in this study were mainly due to the ART effect other than nutritional supplementation. Other studies have shown similar findings and a study in the United Kingdom seeking to describe the effect of specific ART drugs on lipid changes and therefore the need for lipid management in children with HIV, demonstrated that on average, ART-N children had normal lipids except for low HDL levels [282]. These findings were similar to the current study showing HDL levels were lower than the normal expected in children. A recent Ugandan study to determine the prevalence of lipid abnormalities and correlation to $CD4^+$ T cell count and HIV clinical stage among HIV infected children found a high prevalence of dyslipidaemia of 74%. Among those with dyslipidaemia, 56.6% had low HDL, 22% had hypertriglyceridemia, 15.6% had high LDL and 11% had hypercholesterolemia [257]. In comparison to the current study we compared cohorts and nutrition groups and our study design was a cohort while this study was a cross-sectional study however the effect of ART on lipid was profoundly similar in both studies. An Indian study done to assess the prevalence of dyslipidaemia and lipodystrophy in children receiving NNRTI based ART and the associated risk factors, revealed high prevalence of dyslipidaemia of 38.3% and of these 25% were ART-N [283]. Dyslipidaemia in a cohort of HIV infected Latin American children receiving ART showed 20.5% had hypercholesterolemia and 29.4% had hypertriglyceridemia [284] and our study demonstrated a trend towards developing dyslipidaemia due the rapid change of lipids after a short duration of starting ART. Therefore our findings are in favour of deducing that ART had more profound effects on altering the lipid profiles in our study cohorts compared to nutritional supplementation. Therefore there is need to incorporate routine services to identify non-communicable diseases with chronic use of ART in LRS.

At baseline we found a dramatic increase of CRP, about 5 times above the normal level. CRP levels were surprisingly higher among the WN in comparison to the malnourished children. At 12 week follow up there was a dramatic fall in CRP serum levels that was most pronounced in the malnourished who received nutritional supplementation compared to the decrease among the WN children who did not receive any supplementation. There is limited information on CRP and HIV more so in combination with malnutrition and therapeutic supplementation. Naturally, CRP being an acute phase reactant should

increase in patients with HIV disease progression and further be compounded by the high microbial translocation and immune activation as HIV progresses in the absence of ART. However, a Ugandan study in adults reported that despite the absence of microbial translocation there was significant immune activation as measured by high levels of CRP correlating with HIV disease progression. However, there was no association of CRP levels with markers of microbial translocation namely LPS, endotoxin antibody, LPS-binding protein, and soluble CD14 [235]. In the current study, LDH levels in serum were also raised above the normal level (<250 IU/L) at baseline. It was higher in the WN children compared with the malnourished children. At 12 weeks follow up LDH sharply decreased among the malnourished children compared to the WN children. Overall there was a sharp decrease but did not attain the expected normal levels. Our study is the first to document the differences of LDH levels by nutritional status and nutritional supplementation in HIV infected children. A study done in the USA to document the association of serum LDH level with selected OIs and HIV progression in adults found that patients with a serum LDH level ≤225 IU/L had a higher mean $CD4^+$ T cell count compared to patients with a serum LDH level >225 IU/L [285]. A case series report of febrile HIV infected immigrants in the Netherlands demonstrated high LDH in these patients and were found to have histoplasmosis [286]. Our findings show persistently high levels of LDH and this may indicate the presence of ongoing antigenimia of OIs that we did not test for or may be indicative of immune active. LDH is an enzyme present in plants, animals and microorganisms such as bacteria and yeast. It has a pivotal role in glucose metabolism. An Indian study on elevated activities of serum LDH in HIV positive adults on ART showed high levels of LDH activities compared to HIV negative after 3-6 months of ART [287]. LDH provides an alternative pathway of oxygen generation from lactate in the absence of hypoxic conditions and later converts lactate to pyruvate when oxygen is available. In tissue injury, disturbed cell metabolism due to anoxia, cancers, and many other infectious and non-infectious conditions may trigger release of high activities of LDH thus resulting into high levels. ART by itself has been shown to be responsible for toxic effects on various organs and their role in inflammatory response may contribute to increased activities of LDH in HIV infected individuals while in ART-N microorganism translocation may contribute to the increased levels. The increased levels in this study may also be due to the body's response to presence of RUTF metabolites. However further studies need to be

done to assess the persistent high levels in people who are on ART and on RUTF supplementation.

In routine management of malnutrition we estimate serum total protein and serum albumin level in order to assess the nutritional status of the patient and monitor the patient or probably prognostic them. Malnutrition is an important complication of HIV infection and nutrition biomarkers have not been found to have added value in treatment monitoring. In children malnutrition, age, microbial translocation, monocyte, and $CD8^+$ T cell activation have been shown to be associated with decreased rates of $CD4^+$ T cell % immune recovery after 48 weeks of ART. SAM is associated with increased microbial translocation, immune activation, and immune exhaustion and with worse prognosis and impaired immune recovery in HIV infected children on ART [288]. Malnutrition and OIs not only cause altered body metabolism but are also associated with reduced oral intake, which seems to be the most important determinant of weight loss. The present study attempted to assess the serum profile of protein levels before and after nutritional supplementation in HIV infected children. Total serum protein levels were higher at baseline among the malnourished children in comparison to the WN children. However both levels were within the normal expected range of serum protein levels. At 12 weeks serum protein levels were the same and within the normal range (6.4-8.3 mg/dl). Serum albumin levels showed an increased trend from baseline to 12 week time point in both malnourished and WN children, however, at baseline the malnourished children had lower levels than the WN children. Overall the albumin levels were the same at baseline and 12 week follow up time points. Generally the protein levels were within normal expected serum levels, 3.5-5.2 mg/dl. Since plasma protein measurements are cost effective to perform and because many plasma proteins are synthesized in the liver, plasma protein has historically been used to assess protein status in the body. However several studies have demonstrated limitations of using serum albumin as a reliable marker of protein or nutritional status. Albumin has a long half- life of about 20 days so the concentration in the blood changes slowly and may not be a precise measure of nutritional supplementation. There is also a large extravascular pool of albumin that can be available to return to the circulation when needed, thus skewing the results of laboratory tests. In our study population the children had high levels of inflammatory markers at both study

time points and could result in a continuous mobilisation of protein and albumin levels in the intravascular space thus rendering the normal levels observed in this study [82].

Conclusion

Malnutrition is still a burden in children infected with HIV even when receiving ART. Nutritional supplementation improves anthropometry as observed in several studies and current study; however, the majority did not attain the recommended targets. Adherence to RUTF in this study was either poor or good and may explain the failure to meet the recommended anthropometric targets. There was an increase of serum levels of cholesterol, HDL and LDL with a decrease of TGL, however, ART demonstrated a similar effect and may need monitoring of lipid profiles to prevent or timely identify possible emergency of non-communicable diseases in the chronic use of ART. Inflammatory markers declined towards normal levels after 12 weeks of nutritional supplementation with ART, however, did not attain normal levels. This is probably due to the continued immune activation, occult infection or response to RUTF metabolites or a combination of all these options. Total protein and albumin serum levels were similar after nutritional supplementation as previously documented in studies in malnourished children thus confirming the notion that they are not sensitive nutrition bio-markers.

CHAPTER FIVE: Immunological outcome results

Introduction

HIV and malnutrition negatively impact each other. The magnitude of this interaction and its consequences on the entire immune system is still poorly understood, most especially in children infected with HIV/AIDs. It is however well established that good nutrition with exercise; supports a properly balanced immune system [289]. HIV gradually causes appetite loss, reduced food/nutrient intake and absorption in the absence of ART. Malabsorptive syndromes occur, resulting in altered cellular metabolism and weight loss which can lead to severe emaciation. To reverse the malnutrition in the HIV context necessitates a multipronged approach comprising ART initiation, OI treatment, food security, and well balanced diet, mitigation of drug side effects and psychosocial support with a strong component of continual monitoring and adherence support. However as HIV progresses it becomes harder to correct the nutrition status.

By the end of 2016, 36.7 million were PLWHIV/AIDs and of these, 2.1 million were children [10]. The incidence of HIV has stabilized worldwide due to the impact of evidence based HIV programs. The introduction of ART globally, has revolutionalised the management of HIV/AIDs and infected people can now survive longer and lead productive life styles. However, nutrition in HIV and beyond HIV is still a challenge. Malnutrition poses a threat to the positive gains made in management of HIV most especially in children. Malnutrition constitutes a spectrum of two extremes: deprivation can result in mild malnutrition, stunting, marasmus, kwashakori in/or marasimic-kwashakori while over nutrition can lead to is obesity [225]. This thesis is focused on the malnutrition resulting from deprivation or inability to utilize the nutrients specifically the wasting type: marasmus.

In the current HIV programs $CD4^+$ T cell percentages or counts are used for immunological monitoring of HIV associated disease and treatment response. Recently the use of viral suppression indices has been thought to be a better indicator of treatment response and this feeds directly into the 90-90-90 target. With the strides made since the introduction of ART and the ability to restore $CD4^+$ T cell counts, it has become more and more apparent that other components of the immune system may be directly or indirectly affected by HIV

infection, resulting in ongoing susceptibility to infection. There is a need to study other types of lymphocyte behavior in pediatric HIV considering the immune system of a child is still developing in order to appreciate the immunopathogenesis of perinatally acquired HIV. There is also need to find out the impact of nutritional supplementation and ART on the lymphocyte cell profile as malnutrition is an eminent threat to gains made in the management of HIV. In this study, the cells of interest included: T cells subsets ($CD8^+$, $CD4^+$, $CD4^-CD8^-$, $CD4^+CD8^+$, Vδ1, Vδ2 and Vδ3), B cells, NK, iNK T and NT cells.

T Cells

Cells of the lymphoid lineage are derived from the lymphoid progenitor cells that differentiate from bone marrow hematopoietic stem cells. The precursors of T cell migrate to the thymus, undergo a developmental process and thereafter the thymus eliminates auto-reactive cells by a process known as thymic selection. Progenitor cells migrating into the thymus do not express the dominant CD4 and CD8 markers and are termed "double-negative" thymocytes. T cells complete their development in the thymus and the rate of their production in the thymus is highest in children [126]. During adulthood the thymus shrinks since the complete T cell repertoire is primarily thought to be established in childhood before the pubertal stage occurs. Once the process of development in the thymus is complete they enter the blood circulation where they pass through the peripheral lymphoid tissues. Here they encounter specific antigens and differentiate into effector T cells that mediate adaptive immune responses. Mature T cells that have not encountered a specific antigen are called naïve T cells and after encountering an antigen they proliferate and differentiate into effector T cells. These cells rapidly respond to the specific antigen they were originally exposed to. In order for naïve T cells to differentiate into effector T cells they need to be presented a specific antigen by specialized cells called antigen presenting cells (APCs). The commonest APC is the dendritic cell whose currently only recognised function is to internalise the antigen and present it to the naïve T cell [126], bound to an MHC molecule. A tissue dendritic cell may internalise at a site of infection. It then migrates via the lymphatic system to a local lymph node where it matures into a highly effective APC to T cell. The mature APC expresses co-stimulatory molecules that are required for activation of naïve T cells. In addition to dendritic cells, macrophages and B cells are also capable of presenting antigens to T cells resulting in their

differentiation into helper cells that reciprocally activate the macrophages and B cells. Thus macrophages, B and dendritic cells are all referred to as the professional APCs [126].

Effector T cells are classified into 5 functional classes that detect and target peptide antigens derived from different compartments within cells. Peptides derived from intracellular pathogens are carried to the cell surface by MHC class I molecules and presented to $CD8^+$ T cells. $CD8^+$ T cells differentiate into cytotoxic T cells which target and kill infected target cells, such as killing HIV infected $CD4^+$ T cells. Extracellular bacteria and toxins internalised by APCs are presented on the cell surface by MHC class II molecules and presented to $CD4^+$ T cells. These can differentiate into several effector T cells including Th1, Th2, Th17 and regulatory T (T regs) cells. The activation of T cell in response to an antigen and their subsequent proliferation and differentiation is called a primary immune response. The stimulation of effector T cells generates immunological memory that provides protection from subsequent infection of same pathogen [126].

T cells play an important role in immunoregulation and immunostimulation. Th1 cells produce IL-2 and IFN γ and are involved in cellular immunity, and Th2 cells, produce IL-4, IL-5 and IL-13 and are involved in humoral immunity. The Th1/Th2 is regulated by regulatory Treg cells. Their capacity to produce cytokines is suppressed by immunoregulatory cytokines such as transforming growth factor (TGF)-β and IL-10 or by cell-to-cell interactions. Th17 cells, which produce the pro-inflammatory cytokine, IL-17, play important roles for the induction of inflammation. They have been proposed as a pathogenetic mechanism in autoimmune diseases and acute transplant rejection. On the other hand, Treg cells play important roles in immunoregulation and tolerance induction. Treg cells are now known to inhibit proliferation and cytokine production in both $CD4^+$ and $CD8^+$ T cells, immunoglobulin production by B cells, cytotoxic activity of NK cells, and maturation of dendritic cells, resulting in induction of tolerance. Several lines of evidence demonstrate that Treg and Th17 cells exhibit some key shared differentiation pathways, thus, both cell types require TGF-β and IL-2 for their differentiation and are predominantly present in the gut to maintain homeostasis. Both Treg and Th17 cells exhibit specificity toward commensal-derived antigens or self-antigens and their speciation transcriptional program shows direct interaction. Of the two major classes of APCs in the gut, dendritic cells are recognised to promote Th17 cell responses while

macrophages promote Treg responses. Treg and Th17 cells were shown to predominantly maintain gut homeostasis but their interplay in other diseases that include those caused by infections is beginning to be appreciated.

B cells

B cells are form one of the lymphocyte subtypes and is a white blood cell. They are important cells of the adaptive immune system and their main function is in the humoral immunity component when stimulated secreting antibodies. Additionally, B cells are one of the professional APCs and secrete cytokines. B cells, express B cell receptors on their cell membrane that allow binding to a specific antigen, against which it will initiate an antibody response [126]. Most studies assessing the impact of HIV on B cells have mainly been carried out in adults and this information has been extrapolated to the pediatric population despite the different modes of HIV transmission. There are, of course, important differences in the immunological characteristics of perinatally acquired HIV, and adult infection and caution must be taken when extrapolating from adult data to paediatric populations: perinatally acquired HIV results in an insult to a naive and still-developing immune system, which is reflected in differences in the dynamics of viral replication, immunosuppression, clinical progression and response to ART where as in an adult the infectious insult is on a mature immune system. Children with perinatally acquired HIV have been found to have significantly lower memory B and $CD4^+$ T cell percentages. Detectable viraemia in HIV infected children in developed countries was associated with higher activated or exhausted/tissue-like memory B cells. However children who were ART experienced have higher proportions of memory B cells. These results suggest that early and sustained suppressive ART may preserve B and T cell phenotypes in perinatally acquired HIV and limit deficits in humoral immunity [290].

When the B cell compartment is affected during HIV infection, other outcomes have been found to lead to reduced vaccine-induced B cell responses, polyclonal B cell activation, and the development of functionally impaired and exhausted virus-specific atypical B cells [291-293]. The hallmark of progressive of HIV disease is a result of depletion of $CD4^+$ T cells, but this may not be fully reversed by successful ART [294]. The depletion of $CD4^+$ T cells in mucosal tissues, such as the gut, during acute HIV infection may lead to weakened adaptive barriers [295, 296], as indicated by the observed reduction of mucosal IgA during

HIV infection [297]. HIV may also disrupt switching of mucosal B cells to IgA [297], as indicated by the observed reduction of mucosal IgA during HIV infection. The effect of these changes in the B cell compartment has been demonstrated by findings from several studies showing that in the absence of ART, PLWHIV/AIDS are at a higher risk of developing bacterial infections, particularly invasive pneumococcal disease, resulting in high levels of mortality and morbidity [298]. Although the reasons for this are multifactorial, impaired humoral immune responses are thought to be a major contributing factor. A few pediatric studies and several adults studies show that untreated HIV infection results in impaired serum responses to pneumococcal vaccines and reduced numbers of circulating memory B cells, including a reduction in the number of $CD27^{+}IgD^{+}IgM^{+}$ memory B cells, reside in the marginal zone of the murine spleen and provide first-line defense against invasive disease with encapsulated bacteria. Successful treatment with ART resulting in HIV viral suppression can restore antibody responses to pneumococcal vaccine; however, the reduction in the total number of memory B cells appears to be irreversible, despite control of the HIV viral load and recovery of the $CD4^{+}$ T cell count. The loss of $CD27^{+}IgD^{+}$ memory B cells correlates with reduced titers of naturally acquired pneumococcal polysaccharide antibodies [299].

Natural Killer (NK) cells

NK cells are a component of the innate immune system that defends the body by eliminating malignant and virally infected cells through cytolytic killing and cytokine production and secretion. The receptors that regulate NK cell function may be categorized on the basis of their ligand specificity for MHC-I and related molecules [300]. In humans, one of the most important groups of receptors responsible for NK cell function are killer cell Ig-like receptors (KIRs). KIRs are expressed at the surface of NK cells and recognize HLA class I molecules [301]. Expression of the KIR ligands by target cells, or lack thereof, determines the response of NK cells, resulting in either tolerance or cytolytic killing of the target cell.

ART has notably improved the life expectancy of PLWHIV/AIDs. Before the availability of ART, immune suppression related complications represented the predominant cause of mortality among HIV-infected individuals. NK cells were discovered 40 years ago and since then, a plethora of research has uncovered their phenotypic and functional capacity

against virally infected cells most especially in HIV. NK cells are CD3 negative multifunctional effector lymphocytes and are defined based on levels of CD56 and CD16 expression. The majorities of NK cells in the peripheral blood are $CD56^{dim}$ and mainly become cytotoxic upon activation. Upon activation they release pro-apoptotic cytoplasmic granules composed of granzymes and perforins [302]. $CD56^{dim}$ NK cells can also induce cytolysis via induction of Fas and TRAIL-dependent apoptotic signals. The minority of NK cells express CD16 receptors that bind to IgG antibodies that can bind to viral antigens expressed on the surface of infected cells. This antibody conjugation of NK cell and antibody-coated target cell, leads to NK cell activation and target cell lysis, known as antibody-dependent cell-mediated cytotoxicity (ADCC). A distinct subset of $CD56^{bright}$ cytokine-producing NK cells with a limited cytotoxic capacity is more abundantly present in lymph nodes. By producing IFN-γ, TNF-α, IL-10, and chemokines, this NK subset predominantly modulates other subsets of lymphocytes, thereby regulating dendritic cell maturation, differentiation of helper T cells, and B- and T-cell-specific immune responses [302].

HIV infection leads to a downregulation of surface MHC-I expression (HL-A and B) as a mechanism to avoid T cell recognition but in turn renders target cells more susceptible to NK-cell-mediated cytolysis. However, HIV has developed immune evasion mechanisms by maintaining the expression of HLA-C and HLA-E which transduce inhibitory signals through the KIR and CD94 on the NK cells. Besides interfering with self-recognition HIV leads to persistent activation and consequently T cell and NK cell immune exhaustion. Despite viral suppression and normal $CD4^+$ T cell counts in the majority of HIV infected persons on ART, NK cell phenotype and functionality are not fully restored, suggesting that these individuals may be more susceptible to long-term comorbidities associated with immune dysfunction, such as HIV-related malignancies [303].

$CD8^+$ T cells

$CD8^+$ T cells are commonly referred to as cytotoxic T cells and they contribute to the body's adaptive immune response and are characterised by a CD8 protein on the cell surface that recognises, bind and or kill cells infected by intracellular pathogens and cancer cells. They are developed in the bone marrow and mature in the thymus, and are regulated by transcription factors and signaling components [126]. Naive $CD8^+$ T cells are primed by

APCs in secondary lymphoid organs such as lymph nodes and spleen. Naive $CD8^+$ T cells first contact the antigen-bearing dendritic cell in the subcapsular sinus region or the interfollicular region of the draining lymph nodes. Particulate antigen and pathogens arrive via the lymphatics at the subcapsular sinus of the draining lymphnode. The first and major cell population infected by pathogen is CD169+ macrophages lining the subcapsular sinus. However, instead of these antigen-rich macrophages, naive $CD8^+$ T cells favor the dendritic cell population to stimulate them in order to initiate their differentiation to effector cells [304].

$CD8^+$ T cells are a critical component of the cellular immune system and they play a crucial role in the control of viral infection. During HIV infection, $CD8^+$ T cells recognize infected cells through an MHC class I dependent process and kill infected cells through the process of perforin and granzymes secretion. These cytotoxic T lymphocytes (CTL) can also eliminate virally infected cells through the engagement of death-inducing ligands expressed by $CD8^+$ T cells with death receptors on the surface of the infected cell. In addition, $CD8^+$ CTL secretes soluble factors such as beta-chemokines and the $CD8^+$ antiviral factor that suppress viral binding and transcription, respectively. In order for HIV to evade the immune system, the virus has adopted numerous strategies to escape the $CD8^+$ T cell response. The high rate of mutation by the HIV virus has enabled the virus to elude $CD8^+$ T cell recognition in addition to its propensity to suppress the expression of surface MHC-I by infected cells. Also, by altering the pattern of cytokine production and engagement of cellular receptors, HIV disrupts $CD8^+$ TCR signaling resulting in anergic state in these cells. By affecting the function of $CD4^+$ T cells, HIV is also able to decrease the circulating pool of effector and memory $CD8^+$ T cells that are able to combat viral infection. As a result there is derangement of $CD8^+$ T cell function [305].

$CD4^+$ T cells

MTCT of HIV occurs in utero, intrapartum, or through breast milk and the highest risk is from an HIV infected mother with high viral load [14]. The main type of cell that has been extensively studied is the $CD4^+$ T cell in the context of HIV. Loss of $CD4^+$ T cells is the hallmark of the HIV pathology resulting in the immune system dysfunction characterised by depletion of helper T cells to fight off OIs and cancers. The other factor that has been established as a driver of HIV disease progression to AIDS is the increased levels of

immune activation and inflammation that arise during HIV infection. $CD4^+$ Tregs have been established to have a pivotal role in suppressing elevated levels of immune activation but these are also depleted during HIV infection. CD4 is a membrane glycoprotein that is a member of the immunoglobulin (Ig) supergene family and a co-receptor in MHC class II-restricted T cell activation. It is central to the differentiation of T cells and the regulation of T and B cells. CD4 is the primary receptor for HIV virus entry and is expressed in a large proportion of thymocytes (80–90%) and over 50% of the peripheral blood T cells. Most thymocytes co-express CD4 and CD8 while monocytes, macrophages, and Langerhans cells can express CD4. ART is effective in inducing $CD4^+$ T cell recovery most especially if it is initiated before the age of 5 months in children who are infected with HIV perinatally. Studies are beginning to show that low age at initiation of ART correlates to better and sustainable $CD4^+$ T cell count. In a Zambian study on $CD4^+$ T cell recovery in the country wide HIV program, they noted that mortality was high just after ART initiation as most children initiated treatment when they were severely immunosuppressed. Mortality in the children less than 5 years of age was associated with decreasing WAZ score, while among older than 5 years of age mortality was associated with anemia [237]. However the surviving children had very good $CD4^+$ T cell responses. The average child in the cohort experienced a more than doubling of $CD4^+$ T cell percentage in the first year of ART. Improvement in weight was more pronounced in those less than 5 years, with the average child gaining approximately 1 SD in the WAZ score compared with the older children. $CD4^+$ T cell percentages and counts are widely used to monitor response to ART in LRS. However, the utility of such monitoring in terms of predicting virologic response to therapy is not well studied. $CD4^+$ T cell count monitoring does not accurately identify PLWHIV/AIDs on ART with virologic failure neither does virological monitoring alone identify inadequate immune responders [229]. Thus there is need to study other virologic or immune markers that can predict better immune recovery.

$CD4^-CD8^-$ T cells

Double-negative (DN) T cells express a TCR but are negative for CD4 and CD8. They exist in the peripheral circulation and lymphoid organs as a small cell population of lymphocytes normally accounting for 1%-5% [306]. DN T cells have been previously described as having regulatory roles in autoimmunity and transplantation in animal

models, and recent findings have shown that they have $CD4^+$ T cell-like functions during HIV infections. It could be possible therefore that DN T cells continue to drive the HIV infection as the $CD4^+$ T cells get depleted [307]. There is a paucity of work done on DN T cells in the field of perinatally acquired HIV. A recent study has shown that DN T cells that express HIV protein may be part of the HIV reservoir. The HIV reservoir is postulated to comprise HIV infected cells that evade treatment in HIV-infected patients on ART that leads to rebound of virus if treatment is stopped [308]. Understanding the regulation of viral protein expression during ART will be fundamental to designing effective strategies to eradicate HIV reservoirs.

$CD4^+CD8^+$ T cells

$CD4^+CD8^+$ double positive (DP) T cells comprise a small peripheral blood lymphocyte population. After cellular activation, CD4 expression has been found to occur on $CD8^+$ T cells and in patients with chronic HIV infection, DP T cells have been linked to generalized immune activation and is a crucial area evolving in order to understand the immunopathology of HIV. DP T cells in PLWHIV/AIDS frequently express CD38, HLA-DR, and programmed death (PD)-1. A significantly higher DP T cell population was observed in the patients with advanced HIV disease ($CD4^+$ T cell counts below 200 cells/μl), as compared to patients with $CD4^+$ T cell counts above 500 cells/μl. DP T cells from patients with AIDS had a significantly increased activation and exhaustion levels, compared to asymptomatic HIV patients and to single positive T cells from the same study patients. The proportions of CD38 and PD-1 expressing total and HIV specific DP T cells correlated positively with HIV plasma viremia and negatively with $CD4^+$ T cell counts. HIV infection results in a marked increase of DP T cell population with higher immune activation and exhaustion in adults but this phenomenon has not been studied in perinatally acquired HIV [309].

Invariant Natural Killer T (iNK T) cells

Natural killer T cells (NKT cells) are a subset of T lymphocytes that express natural killer (NK) cell surface markers. A subset of NKT cells, termed invariant NK T cells (iNK T), express a highly restricted TCR and responds to CD1d-restricted lipid ligands. iNK T cells are now appreciated for their role they play in linking innate and adaptive immune responses. In chronic HIV-infected patients iNK T cells have been found to express activity

in controlling immune activation. In addition researchers are carrying out work to harness iNK T ligands as vaccine adjuvants capable of improving vaccination-induced cellular immune responses [310]. iNK T cells have been shown to be depleted early in the adults affected with HIV however are under studied in the pediatric context of HIV.

Natural T cells (NT)

This is a subset of human T lymphocytes that expresses the NK cell-associated receptor CD56 and is capable of MHC unrestricted cytotoxicity against a variety of autologous and allogeneic tumor cells. When they become activated they express CD56 and are then easier to re-activate. They end up having functions of NK cells and produce cytokines whose activities make them potential targets for immunotherapy for infectious and immune-mediated disease [311]. They also accumulate in chronic infections. They make up < 5% of the lymphocytes in the peripheral circulation and 50% of T cells in the liver and intestines. They are capable of mounting potent perforin/granzyme-mediated killing of several tumor cells *in vivo* and *in vitro*. They are not well studied in HIV [312, 313].

γδ T Cells in HIV Disease

Human γδ T cells comprise 3 predominant cell populations (Vδ1, Vδ2 and Vδ3) based upon differences in the δ chain of the TCR. In addition to the TCR, γδ T cells express several other stimulatory receptors, including NKG2C, NKG2D, NKp30, toll-like receptors and the β-glucan receptor, dectin 1 [314]. γδ T cells account for 0.5-5% of total T lymphocytes in circulation however are mostly found in higher proportions in visceral tissues such as gut and liver [314]. They are known to identify unconventional antigens such as phosphorylated microbial metabolites and lipid antigens in an MHC unrestricted manner [315, 316]. They respond rapidly by killing target cells, releasing cytokines and providing ligands that mediate the activation and differentiation of other cells of the immune system [317-319]. They also respond early in infections compared to αβ T cells that seem to appear later on in infection. In some of the tissues γδ T cells are 5 times as numerous as αβ as T cells while in the circulation αβ T cells outnumber γδ T cells over 50 times [320]. Dysregulation of γδ T cells occurs early in HIV infection as part of an immune evasion mechanism. Patients with HIV infection have numerical and functional defects in their γδ T cell repertoires. With ART γδ T cells would be expected to return to normal levels,

however, this has been shown not to be the case even with patients with adequate immunological response, normal γδ T cell levels and function are not recovered [321].

Vδ1 T cells are predominantly resident in the tissues such as gut epithelium, lymph nodes, skin [322-324] and they represent over 50% of foetal blood γδ T cells at birth [325]. Vδ1 T cells recognise stress-inducible molecules, MICA and MICB that are related to MHC class I and lipid antigens presented by CD1 molecules [326, 327]. Vδ1 T cells are expanded in HIV patients [328], cytomegalovirus [329] and *Candida* infection and B cell chronic lymphocytic leukaemia [330]. They are found at higher frequencies in the blood, intestinal mucosa and bronchoalveolar fluid of patients with HIV compared with healthy subjects perhaps to compensate for the loss of $CD4^+$ T cells [331-336]. Studies have shown the Vδ1 T cells are potent producers of IL-17 and IL-22 [333, 337]. They can be activated through the TCR, NKp30 or NKG2C and they proliferate, release cytokines such as IFN-γ, tumor necrosis factor-α and interleukin-17 (IL-17), chemokines such as CCL3, CCL4 and CCL5, and they can kill $CD4^+$ T cells in vitro [338-342]. In the presence of ART they have been reported to reduce but do not regain their normal levels, however this phenomenon has not been studied in children with HIV [336].

Vɣ9Vδ2+ γδ T cells (hereafter referred to as Vδ2 T cells) represent the largest γδ T cell population in the peripheral blood circulation and account for 50-95% of γδ T cells in healthy individuals. In cord blood they are present in very small numbers and exposure to environmental microorganisms stimulates their expansion. They also tend to change with and differ between sex [343]. Their TCRs display specificity for pyrophosphate antigens produced by some bacteria and protists, such as 4-hydroxy-3-methyl-but-2-enyl pyrophosphate, an intermediate of the non-mevalonate pathway of isoprenoid synthesis [344]. Vδ2 T cell numbers have been found to decrease in the presence of HIV and repopulate in patients on ART but do not get restored to normal levels [342]. Vδ2 T cells are depleted from the blood, intestine and cervix of patients with HIV [333, 345], and exhibit impaired proliferation, cytotoxicity and interferon-γ (IFN-γ) production compared to Vδ2 T cells from healthy donors [346].

Vδ3 T cells represent the third most common subset in peripheral circulation of the γδ T cells and little is known about this subset. The antigens recognised by the Vδ3 TCR remain to be identified, but Vδ3 T cells that recognize HLA-A2 [347], and CD1d have been reported. Vδ3 T cells have been reported to be expanded in chronic viral infections, leukemia, renal and stem cell transplant patients [348]. The frequencies and functions of Vδ3 T cells in patients with HIV have not been reported [349].

Immunological responders and Virological responders

MTCT of HIV occurs at three distinct time points during pregnancy and after pregnancy: in utero, intrapartum, or through breast milk. The transmission risk is highest among HIV infected ART naïve mothers with transmission rates of approximately 5–10%, 10–20%, and 5–15%, in utero, intrapartum and breastfeeding periods respectively [14]. Nonetheless, 80% children exposed to HIV do not become infected even when primary HIV infection occurs during pregnancy and levels of viremia are extremely high [350]. Perinatally HIV infected children tend to exhibit higher plasma viral loads and experience a more rapid disease progression in comparison to infected adults in the absence of ART. The evolution of HIV infection in adults, in the absence of ART, is slow with a 10 year median progression to AIDS or death. In the weeks following acute infection, plasma viremia decreases and is relatively stable attaining a 'viral load set-point' for many years. The plasma HIV RNA level and the absolute $CD4^+$ T cell count at this set-point are independent predictors of disease progression. In contrast, during the first few months post-infection, infant plasma viremia generally increases to levels that are much higher than those in adults. Most untreated infants have plasma HIV RNA levels of >100,000 viral copies per ml, and levels of a million or greater per ml are not uncommon. In children, HIV RNA levels slowly decline and do not reach a set-point until around 5 years of age in the absence of ART. Upon ART initiation, there is usually a dramatic change resulting in diminished viral replication, increased $CD4^+$ T cell counts, a reversal of most immunological disturbances, and a reduction in risk of morbidity and mortality. Nonetheless, about 20% of all HIV-infected patients have been found not to achieve optimal immune reconstitution despite viral load suppression. These patients are now recognized as immunological non-responders (INRs) however in some literature this phenomenon is referred to as discordant immune response [106]. The mean time required

to reach viral levels below detection thresholds was noted to be longer in children compared with that in adults. Probably this difference in viral response suggests that HIV dynamics may be different in children, and that these differences may necessitate different treatment strategies or modalities.

Aims of the present study

This chapter will present the immunological results of the study. The main immunological objectives of interest were:

1. Effects of HIV infection on lymphocyte subsets.
2. Effects of nutritional status on lymphocyte subset frequencies and numbers among the ART naïve at baseline.
3. Effects of ART on lymphocyte subset frequencies and numbers in malnourished children at baseline.
4. Effects of ART on lymphocyte subset frequencies and numbers in well-nourished children at baseline.
5. Effects of RUTF supplementation on lymphocyte subset frequencies and numbers in ART-experienced malnourished children.
6. Change in lymphocyte subset frequencies and numbers in ART-experienced well-nourished children.
7. Comparison of Vδ1, Vδ2 and Vδ3 T cell frequencies and numbers in immunological and virological responders and non-responders.

Results

Objective 1: Effects of HIV infection on lymphocyte subset frequencies and numbers

To study the effects of HIV infection on circulating lymphocyte subset frequencies and numbers, we isolated PBMC at baseline from 61 ART-N patients with HIV infection, of whom 27 were well-nourished (WN), 15 were moderately acutely malnourished (MAM) and 19 were severely acutely malnourished (SAM). We also recruited 21 HIV negative healthy children (HIV-NC). Cells were stained with monoclonal antibodies specific for CD3, CD4, CD8, CD19, CD56 and the Vα24Jα18, Vδ1, Vδ2 and Vδ3 T cell receptor (TCR) chains

(Table 12). Frequencies and absolute numbers of lymphocyte subsets were analysed by flow cytometry. We were unable to calculate the absolute numbers for the HIV-NC as we did not perform full blood counts.

The frequencies of T cells, as percentages of total lymphocytes, were significantly lower in the ART-N children compared to the HIV-NC, (p-value=0.004). When the ART-N HIV-infected children were divided according to nutritional status, T cell frequencies were lower only in the malnourished ART-N HIV infected children (p-value=0.003, Figure 31). In contrast, B cell frequencies were similar across the groups (Figure 32). NK cell frequencies were significantly lower in both patient groups irrespective of nutritional status, compared to healthy control subjects (Figure 33).

As expected, the percentages of T cells that expressed CD4 were highly significantly lower in both groups of children with HIV compared to the HIV-negative healthy children ($P<0.0001$; Figure 35) and this was associated with a concomitant increase in $CD8^+$ T cells in the total ($P<0.004$) and malnourished ($P<0.001$) HIV-positive ART-N children (Figure 34).

$CD4^+CD8^+$ T cells were significantly expanded in both WN and malnourished HIV positive ART-N groups compared to the HIV-NC, ($P<0.0001$; Figure 36), whereas the frequencies of $CD4^-CD8^-$ T cells were similar in all the groups (Figure 37). T cells expressing CD56, denoted NT cells, were also less frequent in both groups of HIV-infected ART-N patients, in particular the WN group ($P=0.0002$; Figure 38).

The frequencies of innate T cell populations were also altered in the HIV-infected ART-N children compared to the HIV negative children. A striking finding was a highly significant depletion of iNK T cells in both groups of HIV-infected ART-N patients compared to HIV-NC ($P=0.002$ and 0.008 comparing the WN and malnourished patients, respectively; Figure 39). Of the 3 subsets of γδ T cells measured, only Vδ1 T cell frequencies were significantly altered in the patients, being expanded in the total ART-N children (p-value=<0.0001), and the malnourished children (p-value=0.0009), but not the WN children (Figure 40). Vδ2 T cell frequencies were raised in all patient groups compared to the HIV-NC, but these differences were not significant, (Figure 41). Vδ3 T cell frequencies were higher among all the groups of ART-N children in comparison with HIV-NC, however, only the malnourished ART-N children had a significant expansion, (p-value=0.013; Figure 42).

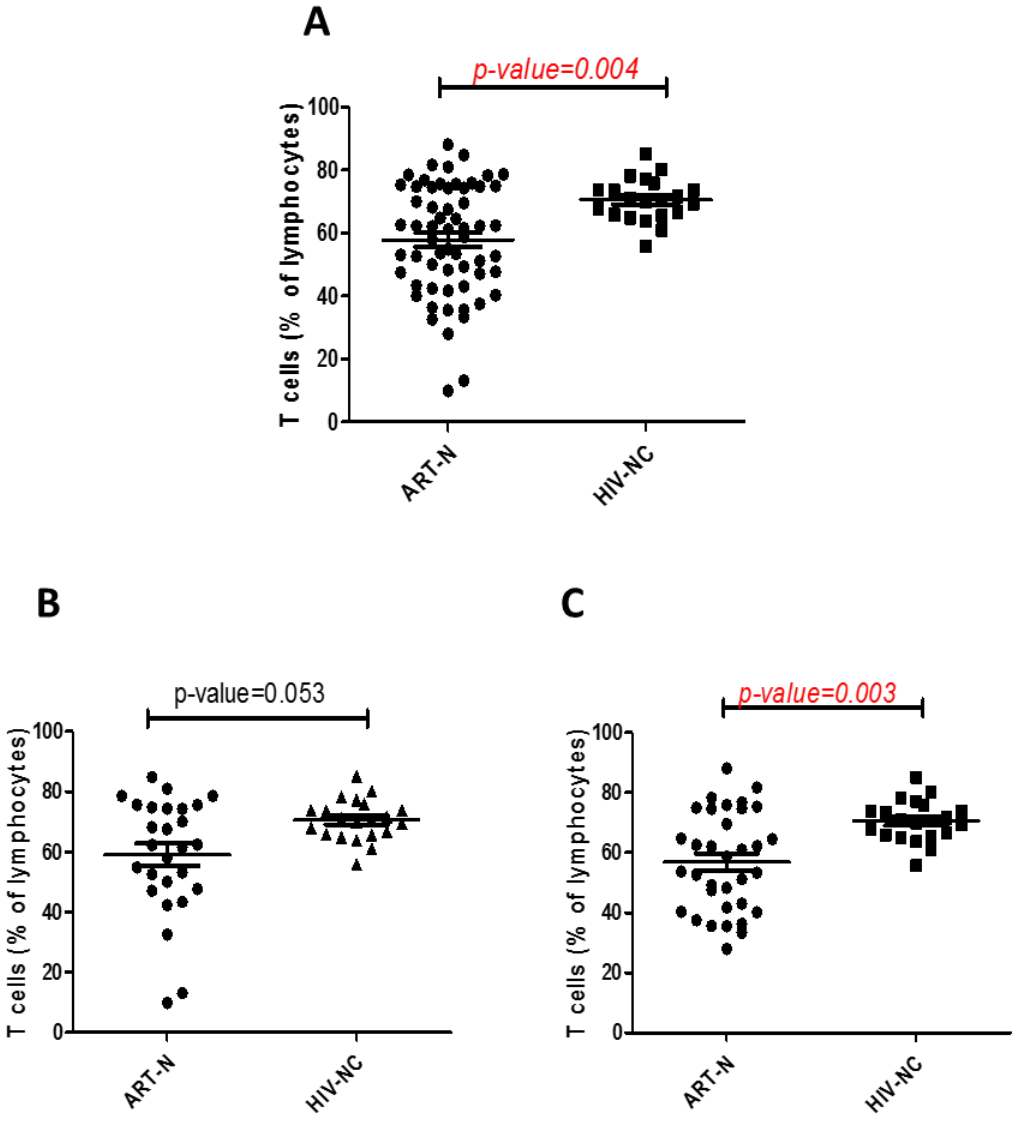


Figure 31: Comparison of T cell frequencies in ART naïve (ART-N) HIV infected children and HIV negative healthy children (HIV-NC).

*PBMC were isolated from 21 HIV-NC and 61 ART-N patients with HIV infection, of whom 34 malnourished and 27 were well-nourished (WN). Cells were stained with monoclonal antibodies specific for CD3 and analysed by flow cytometry. T cells were defined as lymphocytes that were positive for CD3. Scatter plot show comparisons of T cell frequencies between (**A**) all ART-N and HIV-NC, (**B**) ART-N WN and HIV-NC and **(C)** ART-N malnourished children and HIV-NC. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

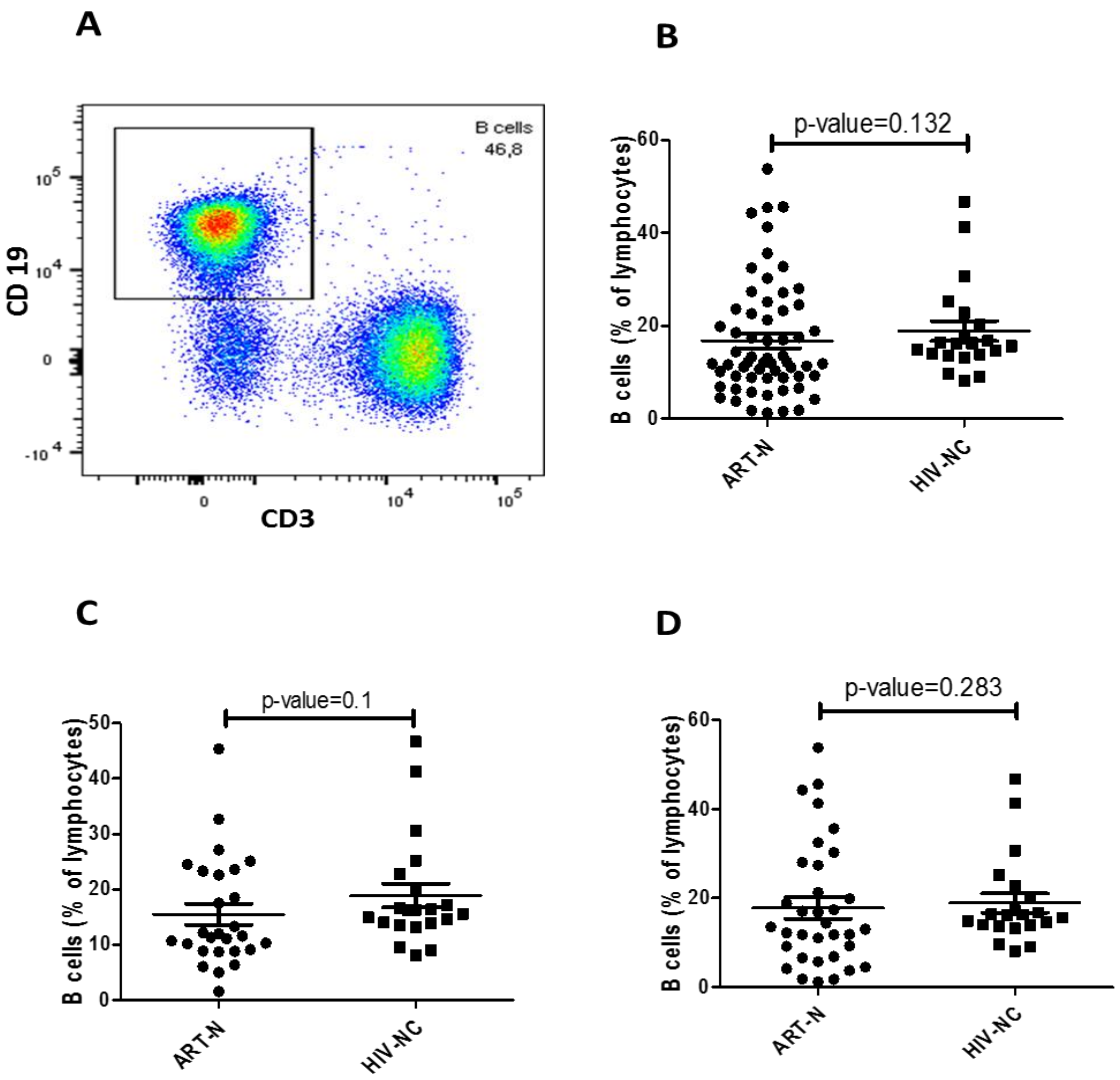


Figure 32: Comparison of B cell frequencies in ART naïve (ART-N) HIV infected children and HIV negative healthy children (HIV-NC).

*PBMC were isolated from 21 HIV-NC and 61 ART-N patients with HIV infection, of whom 34 malnourished and 27 were well-nourished (WN). Cells were stained with monoclonal antibodies specific for CD3 and CD19 and analysed by flow cytometry. B cells were defined as lymphocytes that were positive for CD19 and negative for CD3. (**A**) Shows dot plot of B cells. Scatter plot show comparisons of B cell frequencies between (**B**) all ART-N and HIV-NC, (**C**) ART-N WN and HIV-NC and **(D)** ART-N malnourished children and HIV-NC. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

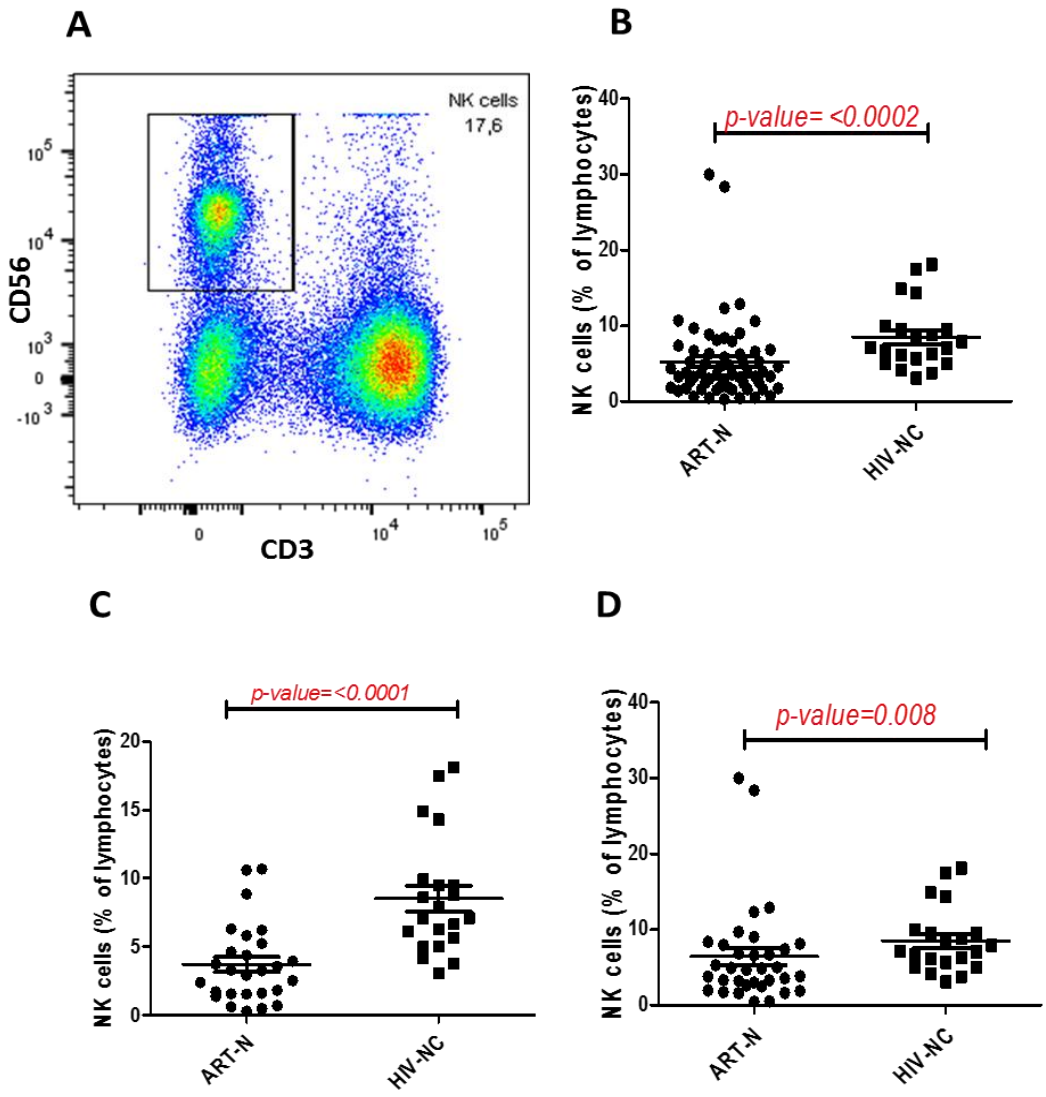


Figure 33: Comparison of NK cell frequencies in ART naïve (ART-N) HIV infected children and HIV negative healthy children (HIV-NC).

*PBMC were isolated from 21 HIV-NC and 61 ART-N patients with HIV infection, of whom 34 malnourished and 27 were well-nourished (WN). Cells were stained with monoclonal antibodies specific for CD3 and CD19 and analysed by flow cytometry. B cells were defined as lymphocytes that were positive for CD56 and negative for CD3. (**A**) Shows dot plot of B cells. Scatter plot show comparisons of B cell frequencies between (**B**) all ART-N and HIV-NC, (**C**) ART-N WN and HIV-NC and **(D)** ART-N malnourished children and HIV-NC. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

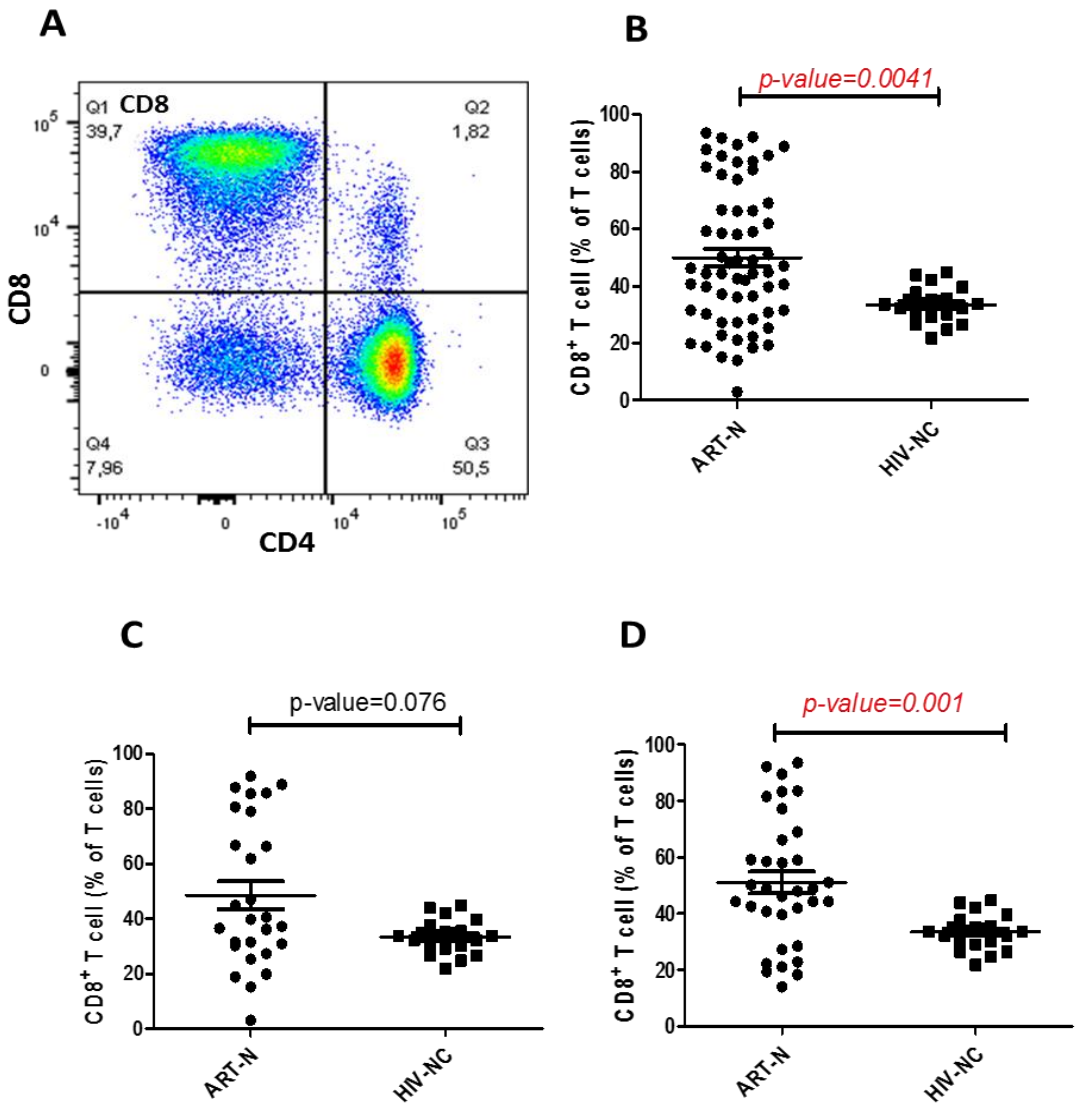


Figure 34: Comparison of CD8⁺ T cell frequencies in ART naïve (ART-N) HIV infected children and HIV negative healthy children (HIV-NC).

*PBMC were isolated from 21 HIV-NC and 61 ART-N patients with HIV infection, of whom 34 malnourished and 27 were well-nourished (WN). Cells were stained with monoclonal antibodies specific for CD3, CD4 and CD8 and analysed by flow cytometry. CD8 T cells were defined as lymphocytes that were positive for CD8 and CD3 and negative for CD4. (**A**) Shows dot plot of CD8⁺ T cells. Scatter plot show comparisons of CD8⁺ T cell frequencies between (**B**) all ART-N and HIV-NC, (**C**) ART-N WN and HIV-NC and **(D)** ART-N malnourished children and HIV-NC. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

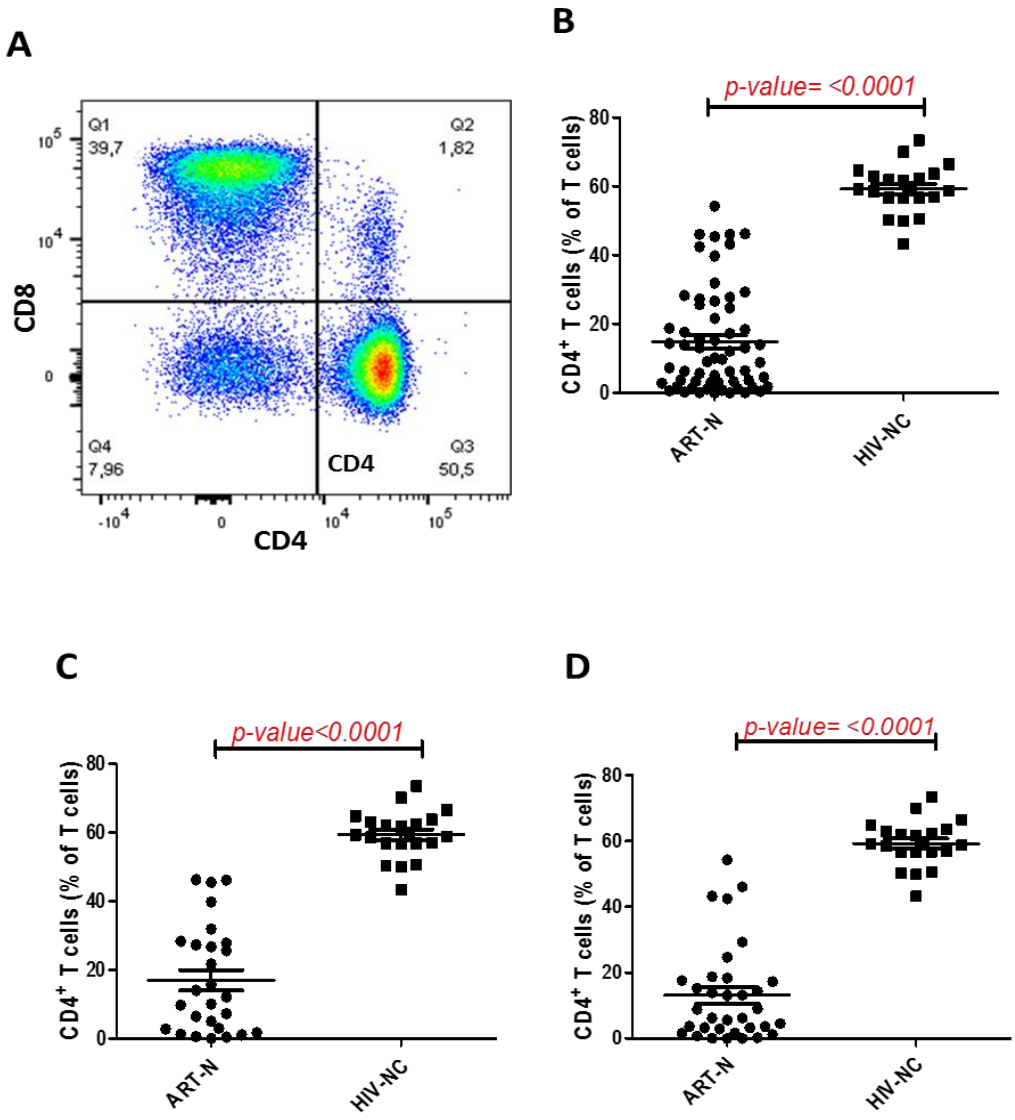


Figure 35: Comparison of CD4⁺ T cell frequencies in ART naïve (ART-N) HIV infected children and HIV negative healthy children (HIV-NC).

*PBMC were isolated from 21 HIV-NC and 61 ART-N patients with HIV infection, of whom 34 malnourished and 27 were well-nourished (WN). Cells were stained with monoclonal antibodies specific for CD3, CD4 and CD8 and analysed by flow cytometry. CD4 T cells were defined as lymphocytes that were positive for CD4 and CD3 and negative for CD8. (**A**) Shows dot plot of CD4⁺ T cells. Scatter plot show comparisons of CD4⁺ T cell frequencies between (**B**) all ART-N and HIV-NC, (**C**) ART-N WN and HIV-NC and **(D)** ART-N malnourished children and HIV-NC. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

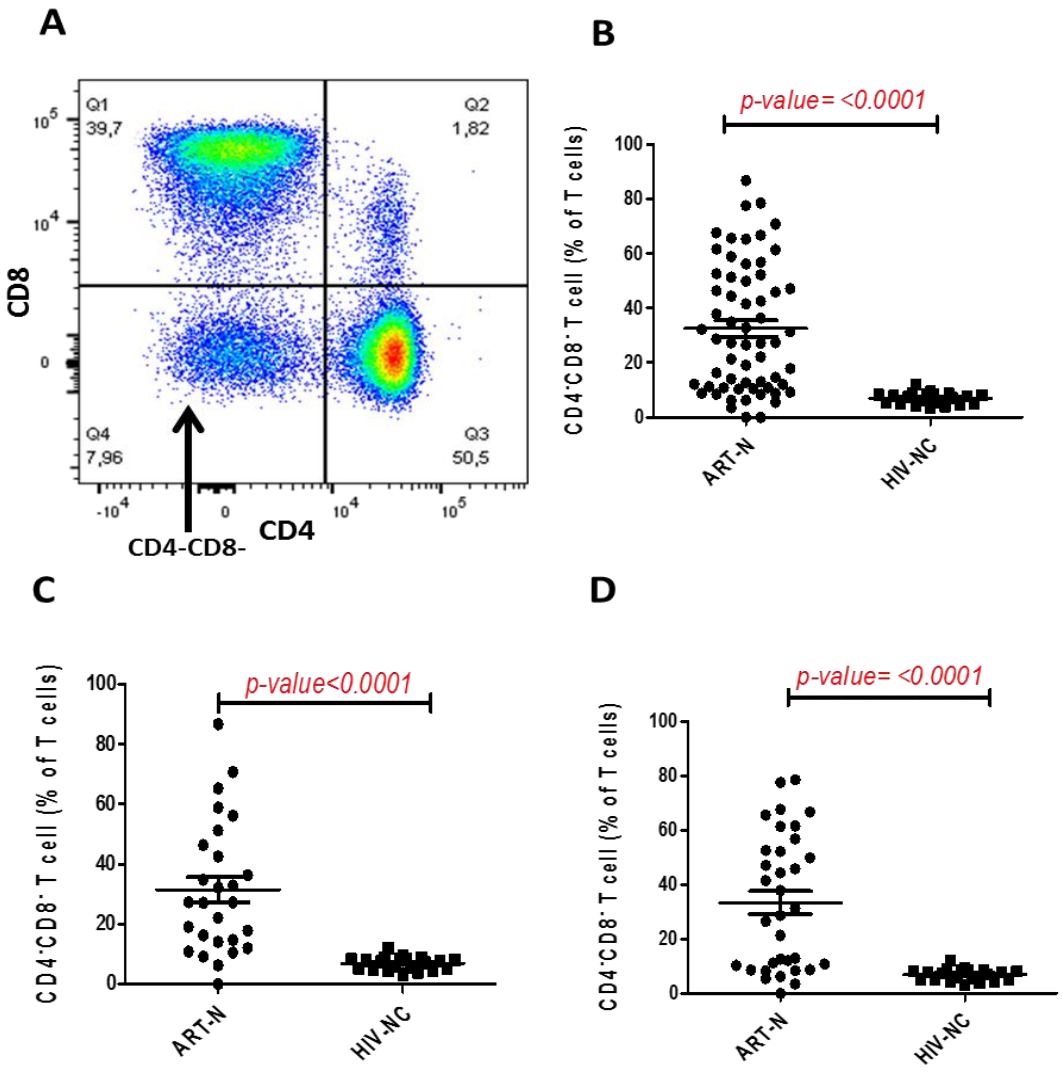


Figure 36: Comparison of CD4$^-$CD8$^-$ T cell frequencies in ART naïve (ART-N) HIV infected children and HIV negative healthy children (HIV-NC).

*PBMC were isolated from 21 HIV-NC and 61 ART-N patients with HIV infection, of whom 34 malnourished and 27 were well-nourished (WN). Cells were stained with monoclonal antibodies specific for CD3, CD4 and CD8 and analysed by flow cytometry. CD4$^-$CD8$^-$ T cells were defined as lymphocytes that were positive for CD3 and negative for CD4 and CD8. (**A**) Shows dot plot of CD4$^-$CD8$^-$ T cells. Scatter plot shows comparisons of CD4$^-$CD8$^-$ T cell frequencies between (**B**) all ART-N and HIV-NC, (**C**) ART-N WN and HIV-NC and **(D)** ART-N malnourished children and HIV-NC. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

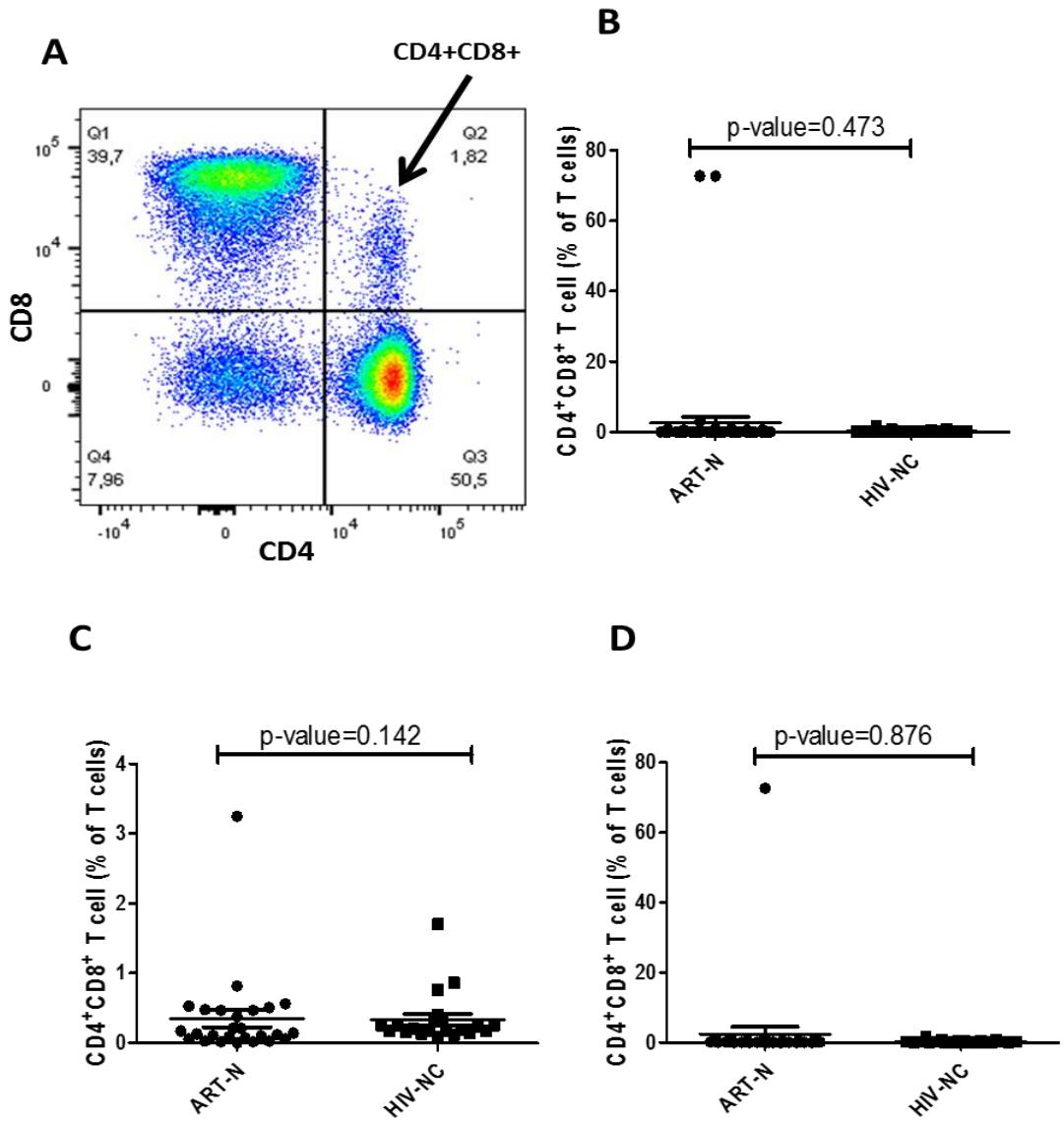


Figure 37: Comparison of CD4⁺CD8⁺ T cell frequencies in ART naïve (ART-N) HIV infected children and HIV negative healthy children (HIV-NC).

*PBMC were isolated from 21 HIV-NC and 61 ART-N patients with HIV infection, of whom 34 malnourished and 27 were well-nourished (WN). Cells were stained with monoclonal antibodies specific for CD3, CD4 and CD8 and analysed by flow cytometry. CD4⁺CD8⁺ T cells were defined as lymphocytes that were positive for CD3 and positive for CD4 and CD8. (**A**) Shows dot plot of CD4⁺CD8⁺ T cells. Scatter plot shows comparisons of CD⁺CD8⁺ T cell frequencies between (**B**) all ART-N and HIV-NC, (**C**) ART-N WN and HIV-NC and **(D)** ART-N malnourished children and HIV-NC. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

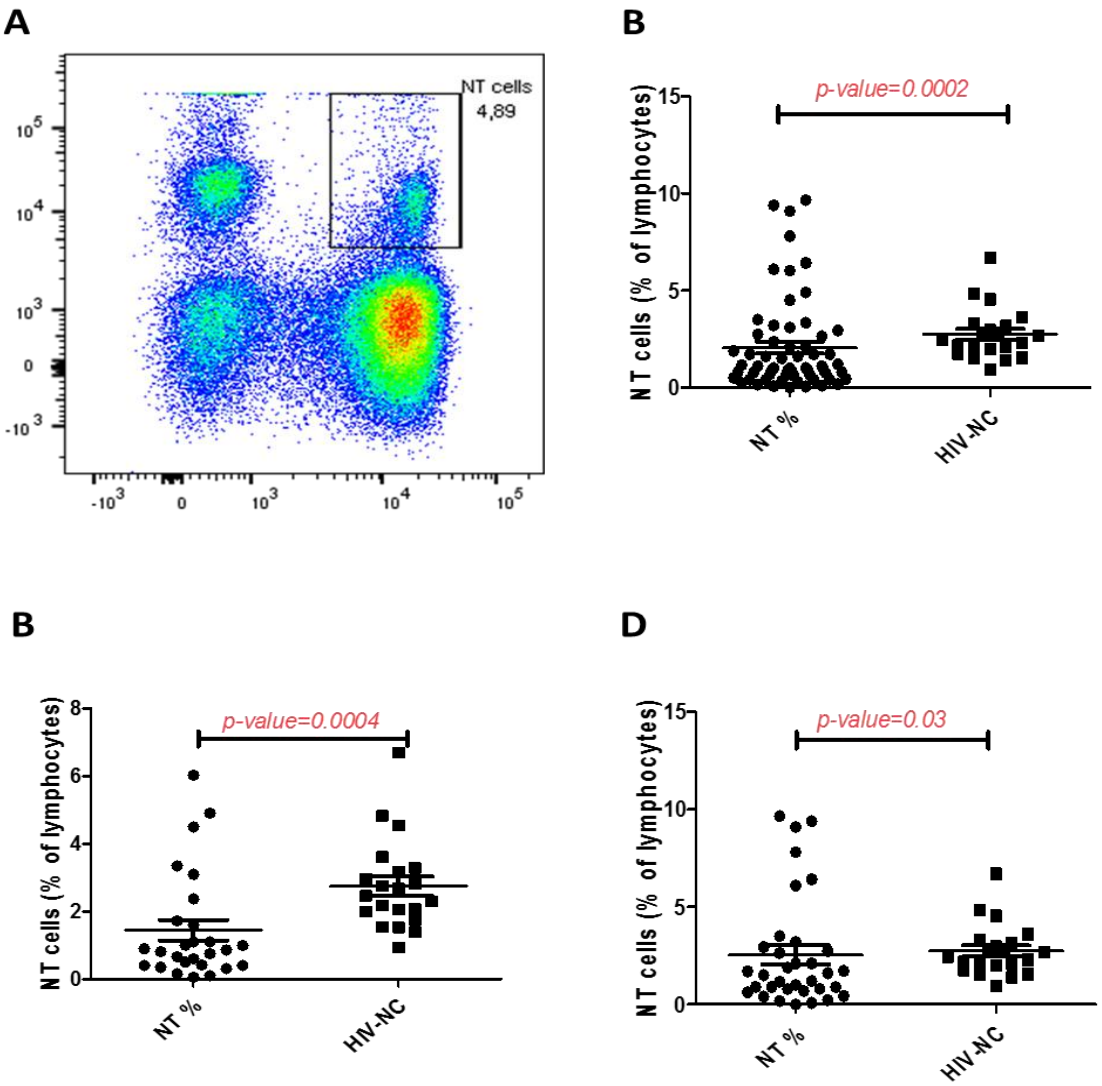
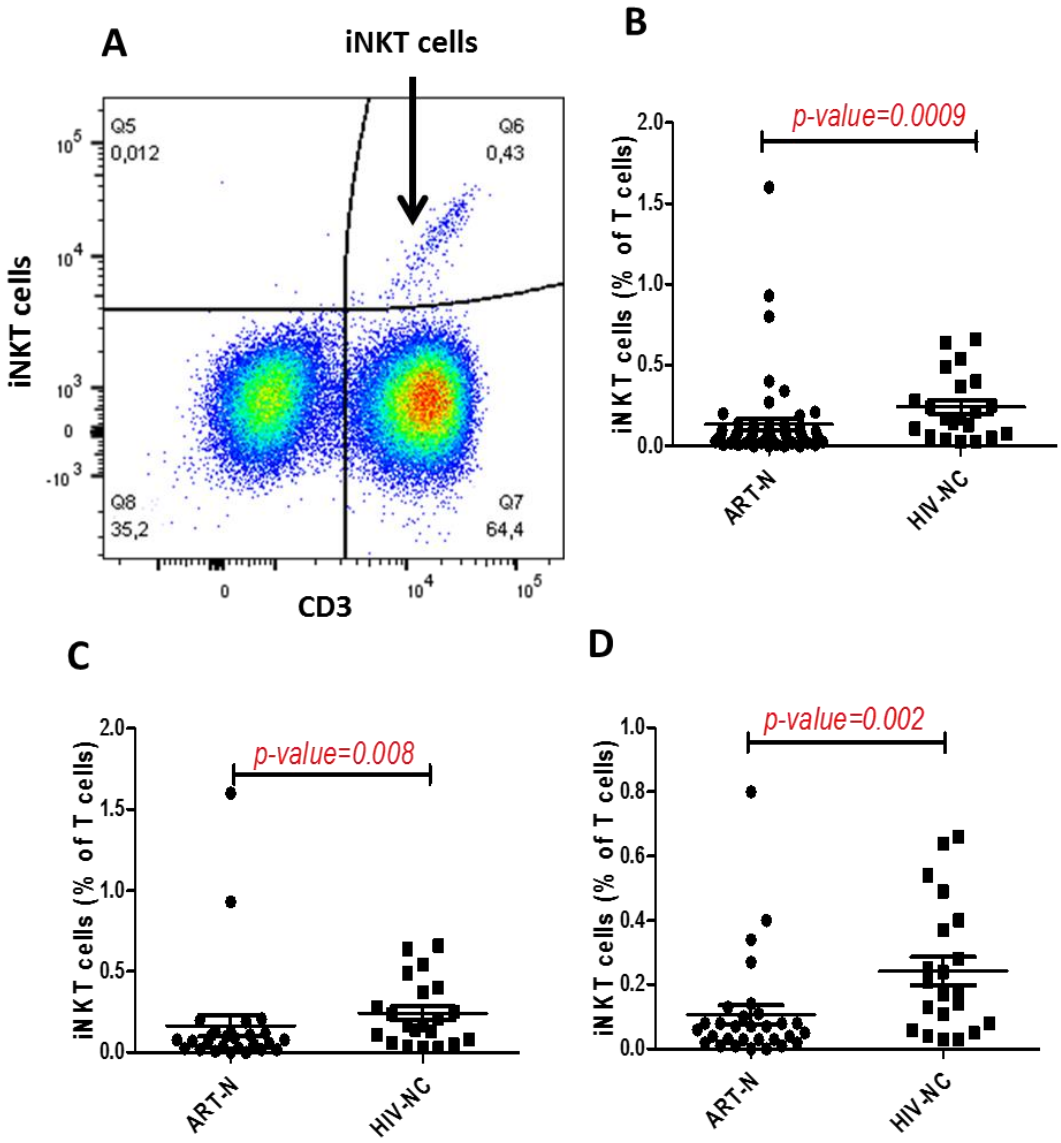


Figure 38: Comparison of NT cell frequencies in ART naïve (ART-N) HIV infected children and HIV negative healthy children (HIV-NC).

*PBMC were isolated from 21 HIV-NC and 61 ART-N patients with HIV infection, of whom 34 malnourished and 27 were well-nourished (WN). Cells were stained with monoclonal antibodies specific for CD3 and CD56 and analysed by flow cytometry. NT cells were defined as lymphocytes that were positive for CD56 and CD3. (**A**) Shows dot plot of NT cells. Scatter plot show comparisons of NT cell frequencies between (**B**) all ART-N and HIV-NC, (**C**) ART-N WN and HIV-NC and **(D)** ART-N malnourished children and HIV-NC. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*



F*igure 39: Comparison of iNK T cell frequencies in ART naïve (ART-N) HIV infected children and HIV negative healthy children (HIV-NC).*

*PBMC were isolated from 21 HIV-NC and 61 ART-N patients with HIV infection, of whom 34 malnourished and 27 were well-nourished (WN). Cells were stained with monoclonal antibodies specific for CD3 and Vα24Jα18 and analysed by flow cytometry. iNK T cells were defined as lymphocytes that were positive for Vα24Jα18 and CD3. (**A**) Shows dot plot of iNK T cells. Scatter plot show comparisons of iNK T cell frequencies between (**B**) all ART-N and HIV-NC, (**C**) ART-N WN and HIV-NC and **(D)** ART-N malnourished children and HIV-NC. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

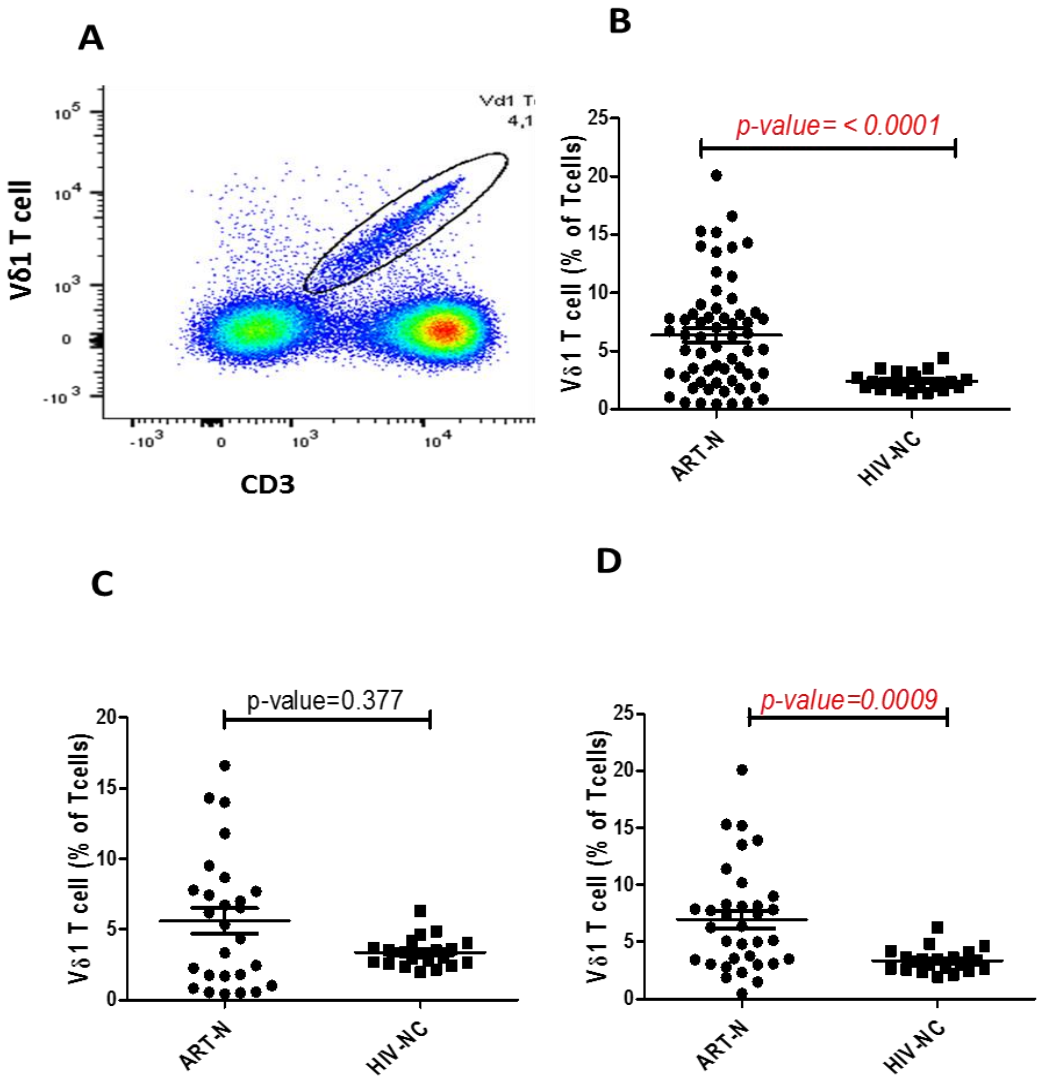


Figure 40: Comparison of Vδ1 T cell frequencies in ART naïve (ART-N) HIV infected children and HIV negative healthy children (HIV-NC).

*PBMC were isolated from 21 HIV-NC and 61 ART-N patients with HIV infection, of whom 34 malnourished and 27 were well-nourished (WN). Cells were stained with monoclonal antibodies specific for CD3 and Vδ1 and analysed by flow cytometry. Vδ1 T cells were defined as lymphocytes that were positive for Vδ1 and CD3. (**A**) Shows dot plot of Vδ1 T cells. Scatter plot show comparisons of Vδ1 T cell frequencies between (**B**) all ART-N and HIV-NC, (**C**) ART-N WN and HIV-NC and **(D)** ART-N malnourished children and HIV-NC. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

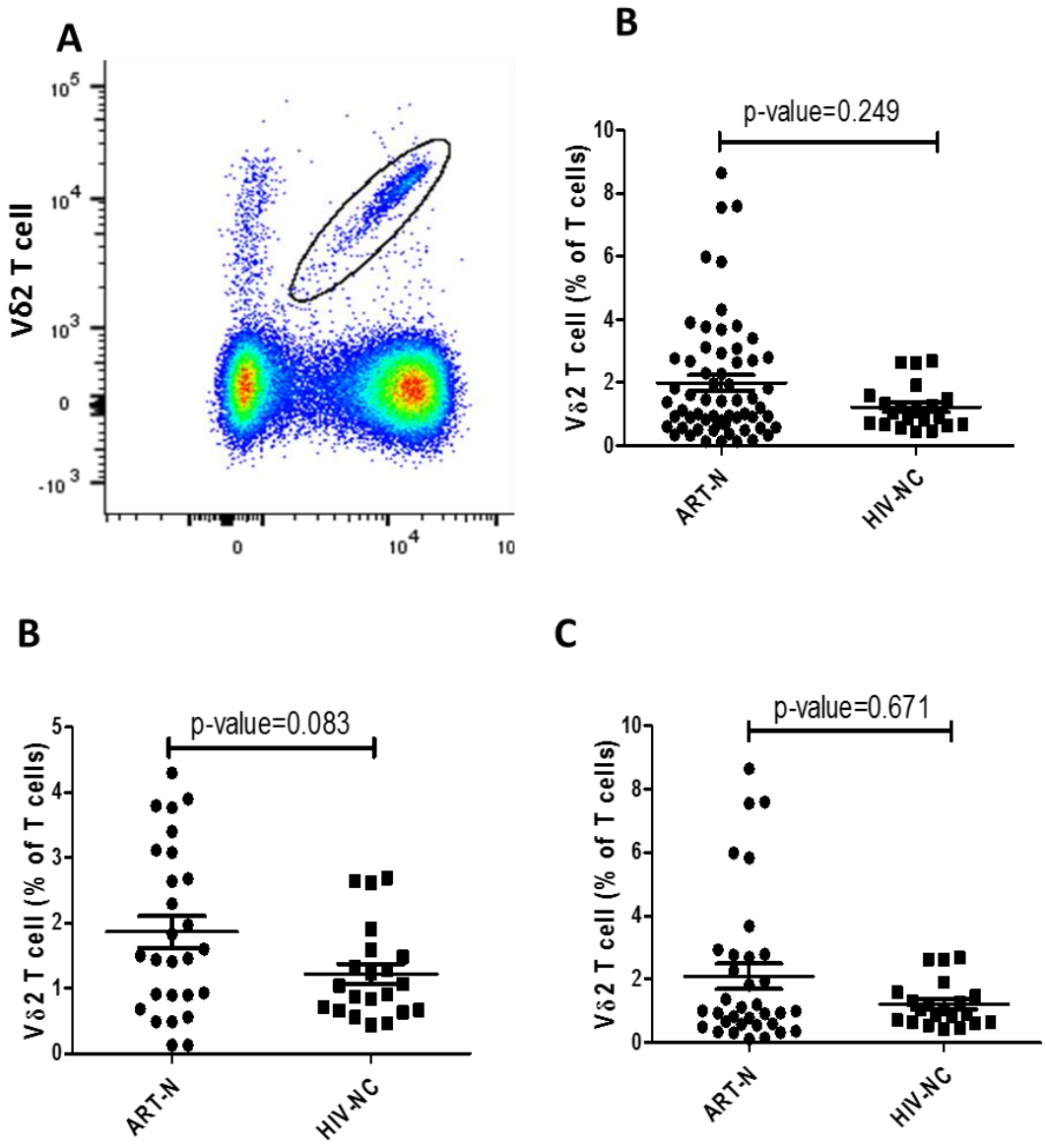


Figure 41: Comparison of Vδ2 T cell frequencies in ART naïve (ART-N) HIV infected children and HIV negative healthy children (HIV-NC).

*PBMC were isolated from 21 HIV-NC and 61 ART-N patients with HIV infection, of whom 34 malnourished and 27 were well-nourished (WN). Cells were stained with monoclonal antibodies specific for CD3 and Vδ2 and analysed by flow cytometry. Vδ2 T cells were defined as lymphocytes that were positive for Vδ2 and CD3. (**A**) Shows dot plot of Vδ2 T cells. Scatter plot show comparisons of Vδ2 T cell frequencies between (**B**) all ART-N and HIV-NC, (**C**) ART-N WN and HIV-NC and **(D)** ART-N malnourished children and HIV-NC. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

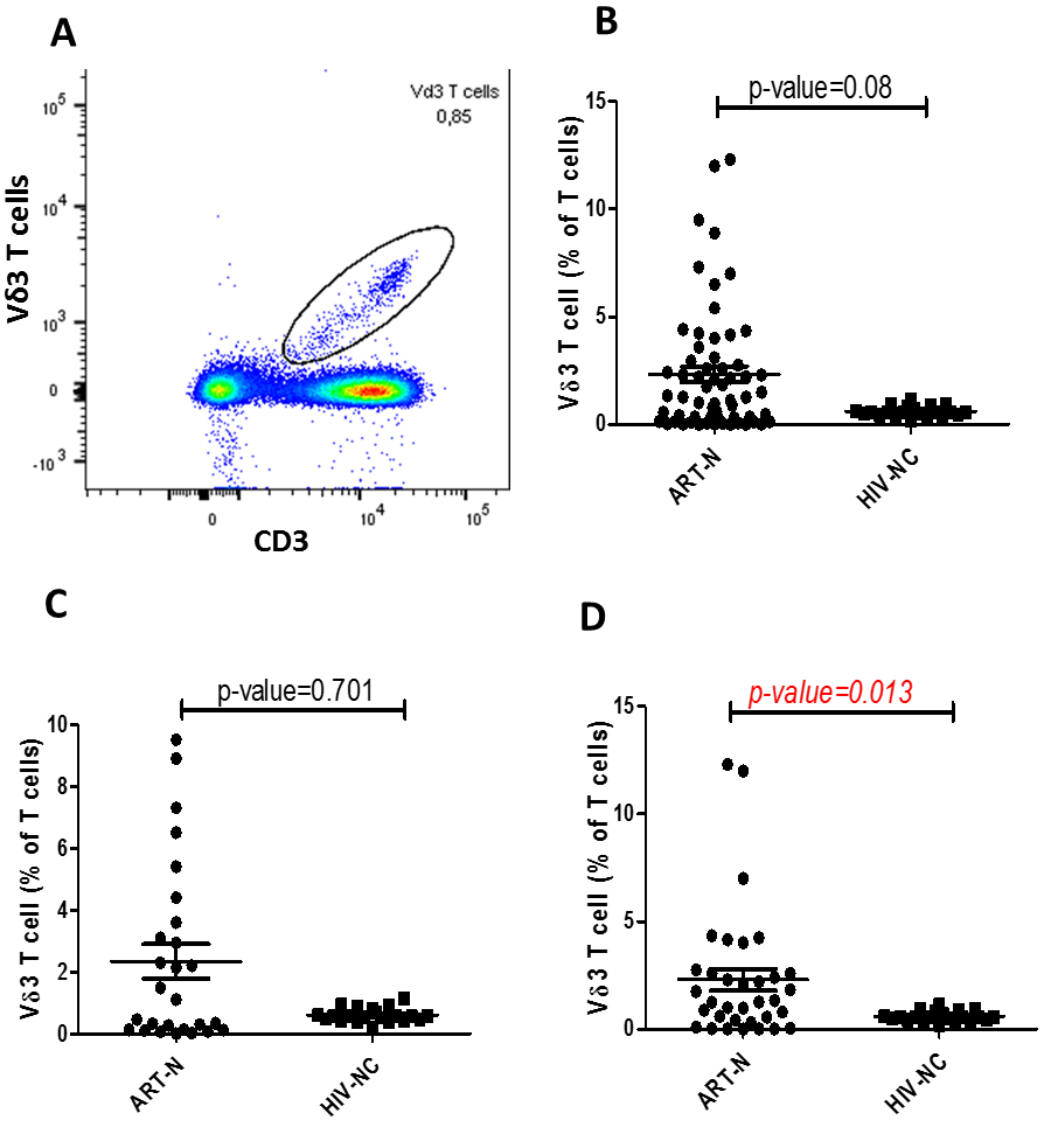


Figure 42: Comparison of Vδ3 T cell frequencies in ART naïve (ART-N) HIV infected children and HIV negative healthy children (HIV-NC).

*PBMC were isolated from 21 HIV-NC and 61 ART-N patients with HIV infection, of whom 34 malnourished and 27 were well-nourished (WN). Cells were stained with monoclonal antibodies specific for CD3 and Vδ3 and analysed by flow cytometry. Vδ3 T cells were defined as lymphocytes that were positive for Vδ3 and CD3. (**A**) Shows dot plot of Vδ3 T cells. Scatter plot show comparisons of Vδ3 T cell frequencies between (**B**) all ART-N and HIV-NC, (**C**) ART-N WN and HIV-NC and **(D)** ART-N malnourished children and HIV-NC. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

Table 40. Comparison lymphocyte subsets among ART-naïve (ART-N) and HIV healthy negative children (HIV-NC)

Cells	Median frequencies of ART-N	95% Confidence interval	Median frequencies of HIV-NC	95% Confidence interval	P-value
T cells	61.1	53.3-62.4	70.6	67.5-73.6	**0.004***
B cells	12.2	13.6-19.9	16.2	14.4-23.4	**0.132**
NK cells	3.7	3.8-6.6	7.1	6.6-10.5	**0.0002***
$CD8^+$ T cell	44.9	43.7-56.2	33.7	30.7-36.2	**0.004***
$CD4^+$ T cell	9.8	11-18.7	59.2	56.1-62.5	**<0.0001***
$CD4^-CD8^-$ T cell	27.4	26.6-38.5	7.6	5.9-7.9	**<0.0001***
$CD4^+CD8^+$ T cell	0.19	-0.6-6	0.2	0.17-0.5	**0.473**
iNK T cell	0.07	0.066-0.2	0.17	0.15-0.33	**0.0009***
NT cell	1.0	1.4-2.7	2.5	2.1-3.3	**0.002***
Vδ1 T cell	6.2	5.2-7.5	2.2	2.0-2.8	**<0.0001***
Vδ2 T cell	1.99	1.5-2.5	1.0	0.9-1.5	**0.249**
Vδ3 T cell	1.3	1.6-3.1	0.6	0.5-0.7	**0.08**

***Denotes statistical significance**

Table 41. Comparison lymphocyte subsets among ART-N well-nourished and HIV healthy negative children (HIV-NC)

Cells	Median frequencies of ART-N	95% Confidence intervals	Median frequencies of HIV-NC	95% Confidence intervals	P-value
T cells	62.3	51.4-66.7	70.6	67.5-73.6	**0.053**
B cells	12	11.6-19.3	16.2	14.4-23.4	**0.1**
NK cells	3.3	2.6-4.9	7.1	6.6-10.5	**<0.0001***
$CD8^+$ T cell	39.8	38.1-59	33.7	30.7-36.2	**0.076**
$CD4^+$ T cell	12.1	10.8-23.1	59.2	56.1-62.5	**<0.0001***
$CD4^-CD8^-$ T cell	27.3	22.8-40.2	7.6	5.9-7.9	**<0.0001***
$CD4^+CD8^+$ T cell	0.14	0.09-0.6	0.2	0.17-0.5	**0.142**
iNK T cell	0.08	0.03-0.3	0.2	0.15-0.3	**0.008***
NT cell	0.86	0.82-2.1	2.5	2.1-3.3	**0.0004***
Vδ1 T cell	5.4	3.8-7.4	3.3	2.9-3.8	**0.377**
Vδ2 T cell	1.5	1.4-2.4	1.0	0.9-1.5	**0.083**
Vδ3 T cell	1.1	1.2-3.5	0.6	0.5-0.7	**0.701**

***Denotes statistical significance**

Table 42. Comparison lymphocyte subsets among malnourished ART-N and HIV healthy negative children (HIV-NC)

Cells	Median frequencies of ART-N	95% Confidence intervals	Median frequencies of HIV-NC	95% Confidence intervals	P-value
T cells	56.3	51.2-62.6	70.6	67.5-73.6	**0.003***
B cells	13.3	12.9-22.6	16.2	14.4-23.4	**0.283**
NK cells	4.8	4.1-8.7	7.1	6.6-10.5	**0.008***
$CD8^+$ T cell	48.5	43.1-59	33.7	30.7-36.2	**0.001***
$CD4^+$ T cell	7.5	8.0-18.2	59.2	56.1-62.5	**<0.0001***
$CD4^-CD8^-$ T cell	30.1	24.8-42.0	7.6	5.9-7.9	**<0.0001***
$CD4^+CD8^+$ T cell	0.2	-1.9-6.8	0.2	0.17-0.5	**0.876**
iNKT cell	0.07	0.05-0.2	0.17	0.15-0.33	**0.002***
NT cell	1.6	1.56-3.53	2.5	2.1-3.3	**0.03***
Vδ1 T cell	6.3	5.4-8.6	3.3	2.9-3.8	**0.0009***
Vδ2 T cell	1.0	1.3-2.9	1.0	0.9-1.5	**0.671**
Vδ3 T cell	1.3	1.26-3.3	0.56	0.5-0.7	**0.013***

***Denotes statistical significance**

Objective 2: Effects of nutritional status on lymphocyte subset frequencies and numbers among the ART naïve at baseline.

To study the effects of nutritional status on lymphocyte subset frequencies and numbers, we isolated PBMC at baseline from 61 ART-N patients with HIV infection, of whom 27 were WN, 15 were MAM and 19 were SAM. Cells were stained with monoclonal antibodies specific for CD3, CD4, CD8, CD19, CD56 and the Vα24Jα18, Vδ1, Vδ2 and Vδ3 T cell receptor (TCR) chains (Table 12). Frequencies and absolute numbers of lymphocyte subsets were analysed by flow cytometry. Absolute numbers were calculated from full blood counts taken at the time of blood collection (Tables 19).

The frequencies and absolute counts of total T cells, B cells and NK cells were found to be similar in the WN, MAM and SAM groups (Figures 43-45), although a non-significant decrease was observed in T cell frequencies in MAM children, a non-significant increase in B cell frequencies and counts in MAM children, and a non-significant increase in NK frequencies in SAM children were observed.

Frequencies and absolute numbers of $CD4^+$, $CD8^+$, double negatives (DN) and double positives (DP) T cells were similar in the 3 groups (Figure 46-49), although a non-significant decrease in $CD4^+$ T cells was observed with a reciprocal increase of $CD8^+$ T cells in the children who had SAM.

The iNK T cell frequencies and absolute numbers were not altered by nutritional status (Figure 50) whereas absolute numbers of NT cells were significantly higher in SAM compared to WN children (p-value=0.028, Figure 51). Of the 3 subsets of γδT cells measured, only Vδ1 T cell frequencies were significantly altered in patient groups, being increased in the SAM group (p-value=0.032, Figures 52-54). These results show that nutritional status affects lymphocyte numbers in children with HIV. These results are summarised in Table 43 and 44.

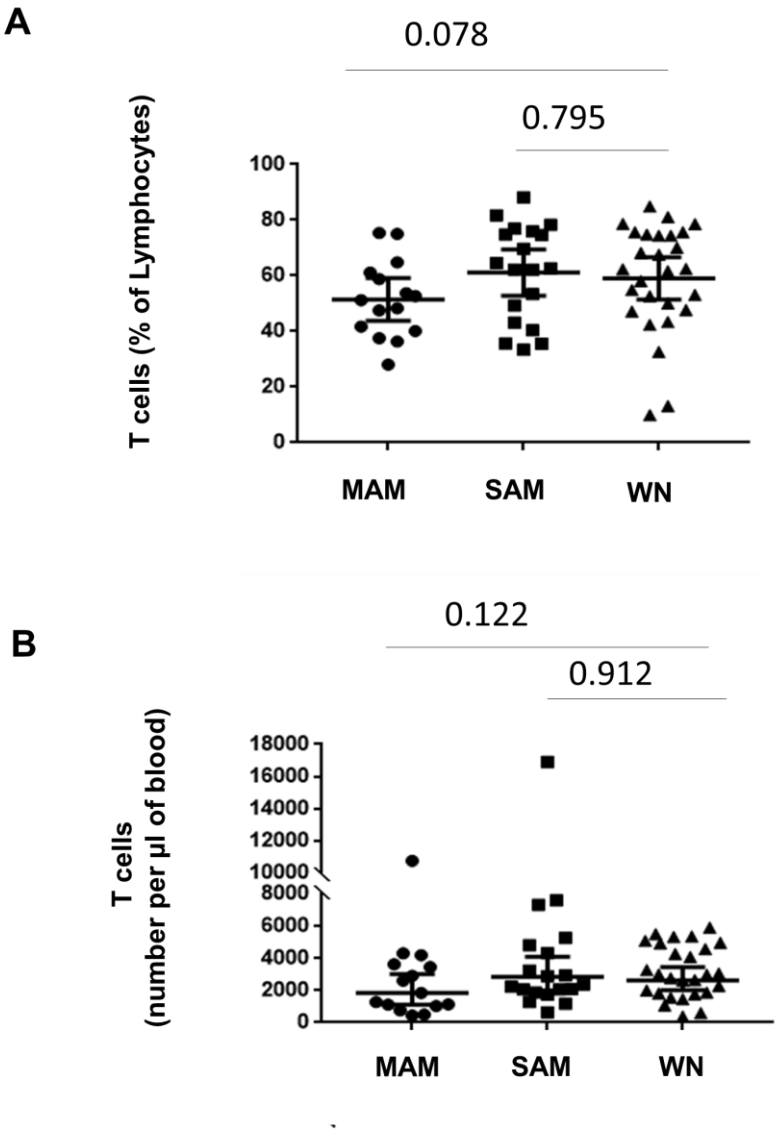


Figure 43: Effect of nutritional status on circulating T cell frequencies and numbers in ART-naïve children with HIV-infection.

*PBMC were isolated from 61 ART-naïve patients with HIV infection, of whom 27 were well-nourished (WN), 15 were moderately acutely malnourished (MAM) and 19 were severely acutely malnourished (SAM). Cells were stained with monoclonal antibodies specific for CD3 and analysed by flow cytometry. T cells were defined as lymphocytes that were positive for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values*

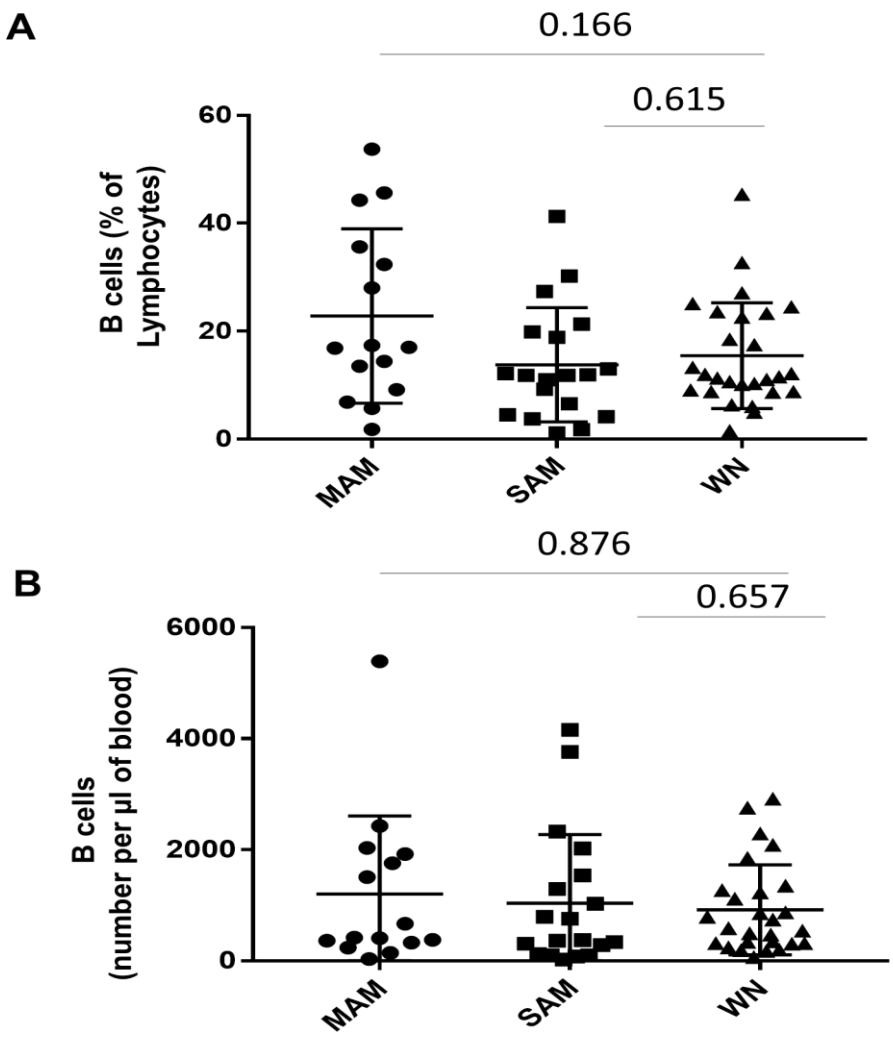


Figure 44: Effect of nutritional status on circulating B cell frequencies and numbers in ART-naïve children with HIV-infection.

*PBMC were isolated from 61 ART-naïve patients with HIV infection, of whom 27 were well-nourished (WN), 15 were moderately acutely malnourished (MAM) and 19 were severely acutely malnourished (SAM). Cells were stained with monoclonal antibodies specific for CD3 and CD19 and analysed by flow cytometry. B cells were defined as lymphocytes that were positive for CD19 and negative for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of B cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

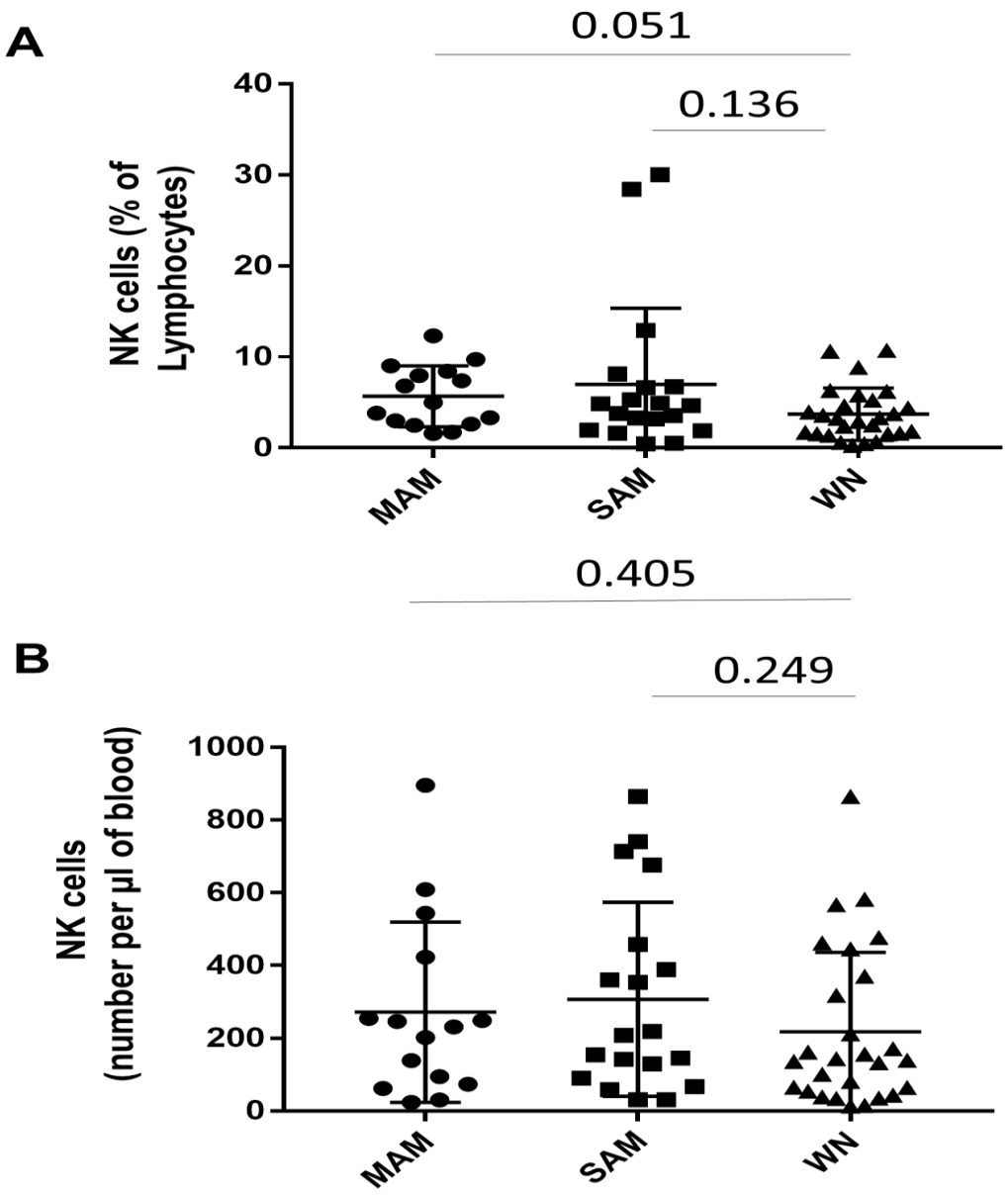


Figure 45: Effect of nutritional status on circulating NK cell frequencies and numbers in ART-naïve children with HIV-infection.

*PBMC were isolated from 61 ART-naïve patients with HIV infection, of whom 27 were well-nourished (WN), 15 were moderately acutely malnourished (MAM) and 19 were severely acutely malnourished (SAM). Cells were stained with monoclonal antibodies specific for CD3 and CD56 and analysed by flow cytometry. NK cells were defined as lymphocytes that were positive for CD56 and negative for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of NK cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

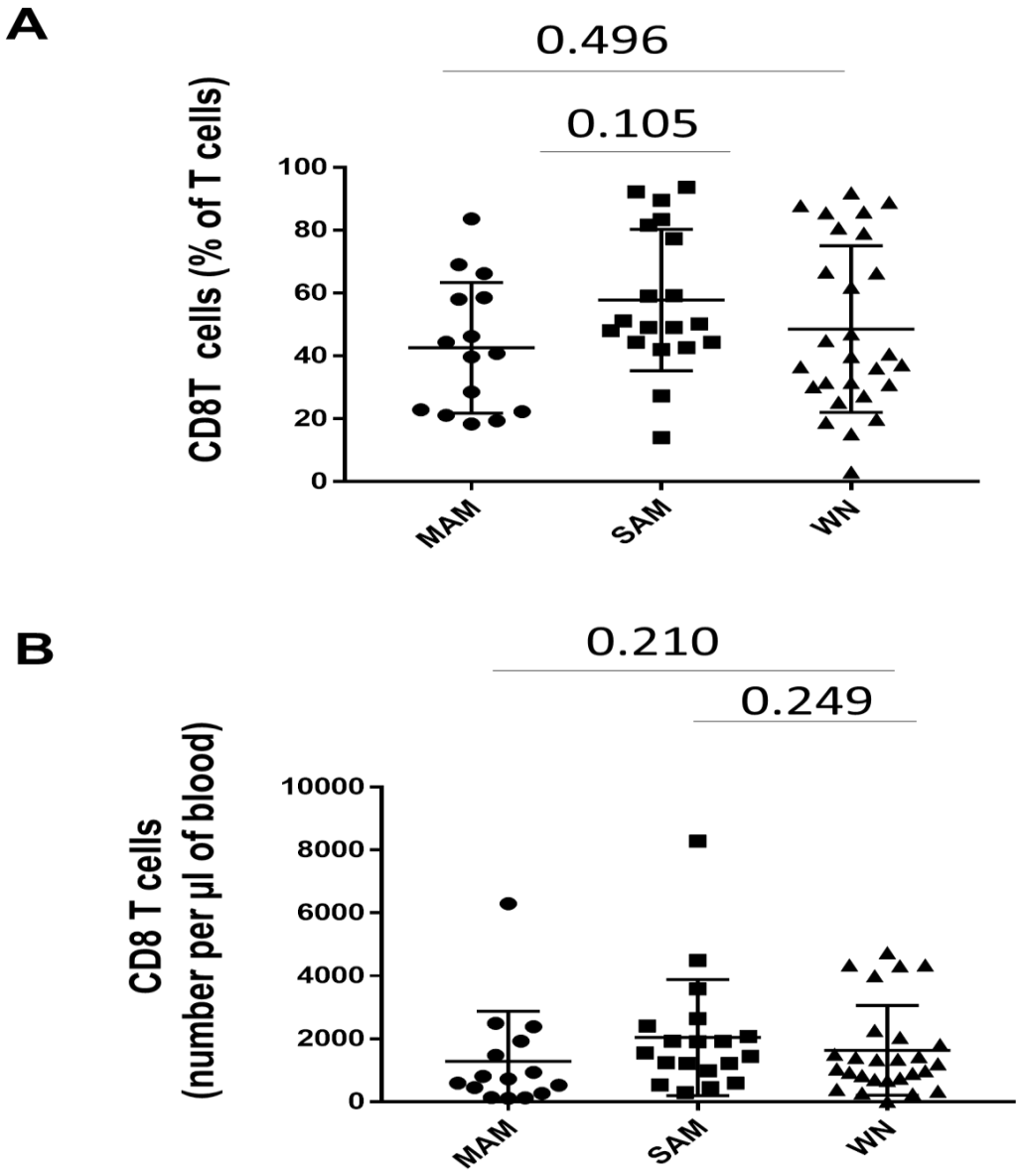


Figure 46: Effect of nutritional status on circulating CD8 T cell frequencies and numbers in ART-naïve children with HIV-infection.

*PBMC were isolated from 61 ART-naïve patients with HIV infection, of whom 27 were well-nourished (WN), 15 were moderately acutely malnourished (MAM) and 19 were severely acutely malnourished (SAM). Cells were stained with monoclonal antibodies specific for CD3, CD4 and CD8 and analysed by flow cytometry. CD8 T cells were defined as lymphocytes that were positive for CD8 and CD3 and negative for CD4. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD8 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

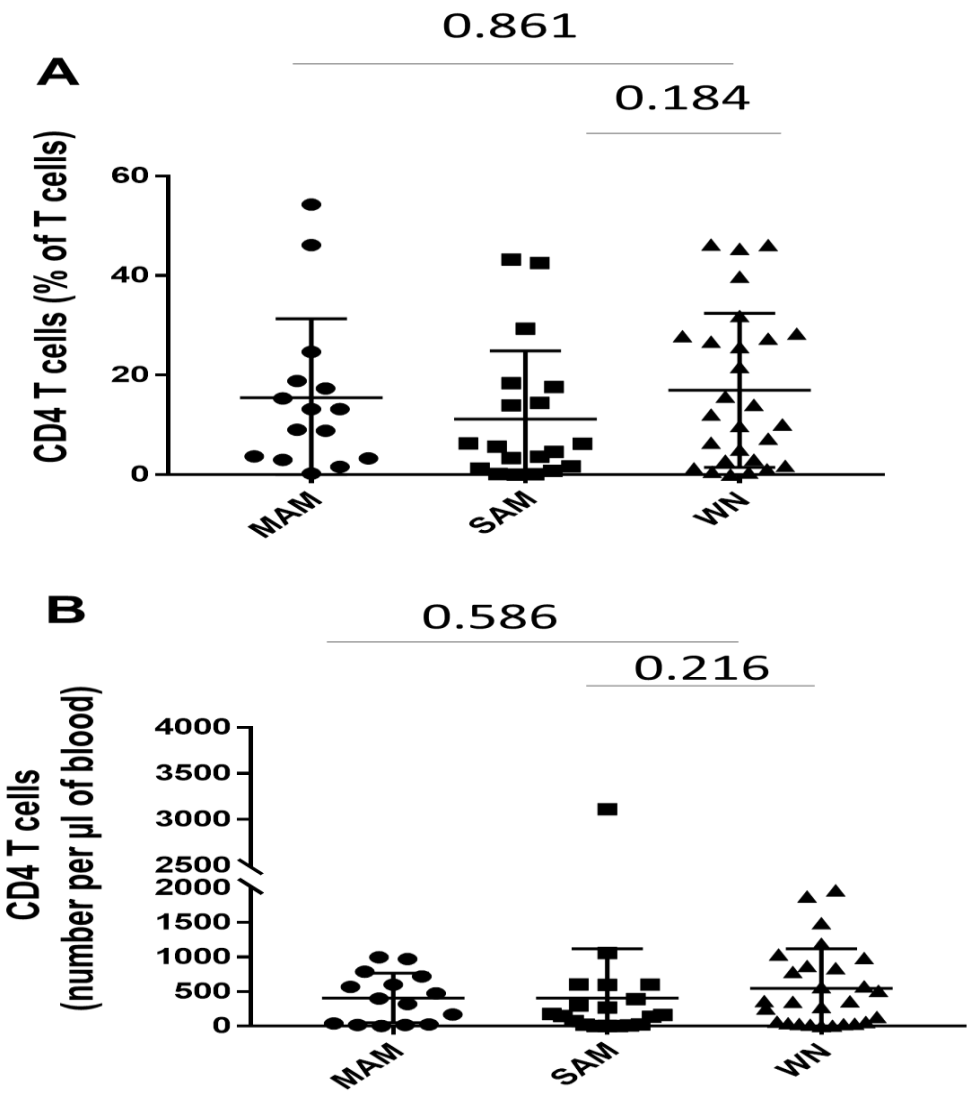


Figure 47: Effect of nutritional status on circulating CD4 T cell frequencies and numbers in ART-naïve children with HIV-infection.

*PBMC were isolated from 61 ART-naïve patients with HIV infection, of whom 27 were well-nourished (WN), 15 were moderately acutely malnourished (MAM) and 19 were severely acutely malnourished (SAM). Cells were stained with monoclonal antibodies specific for CD3, CD4 and CD8 and analysed by flow cytometry. CD4 T cells were defined as lymphocytes that were positive for CD4 and CD3 and negative for CD8. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD4 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

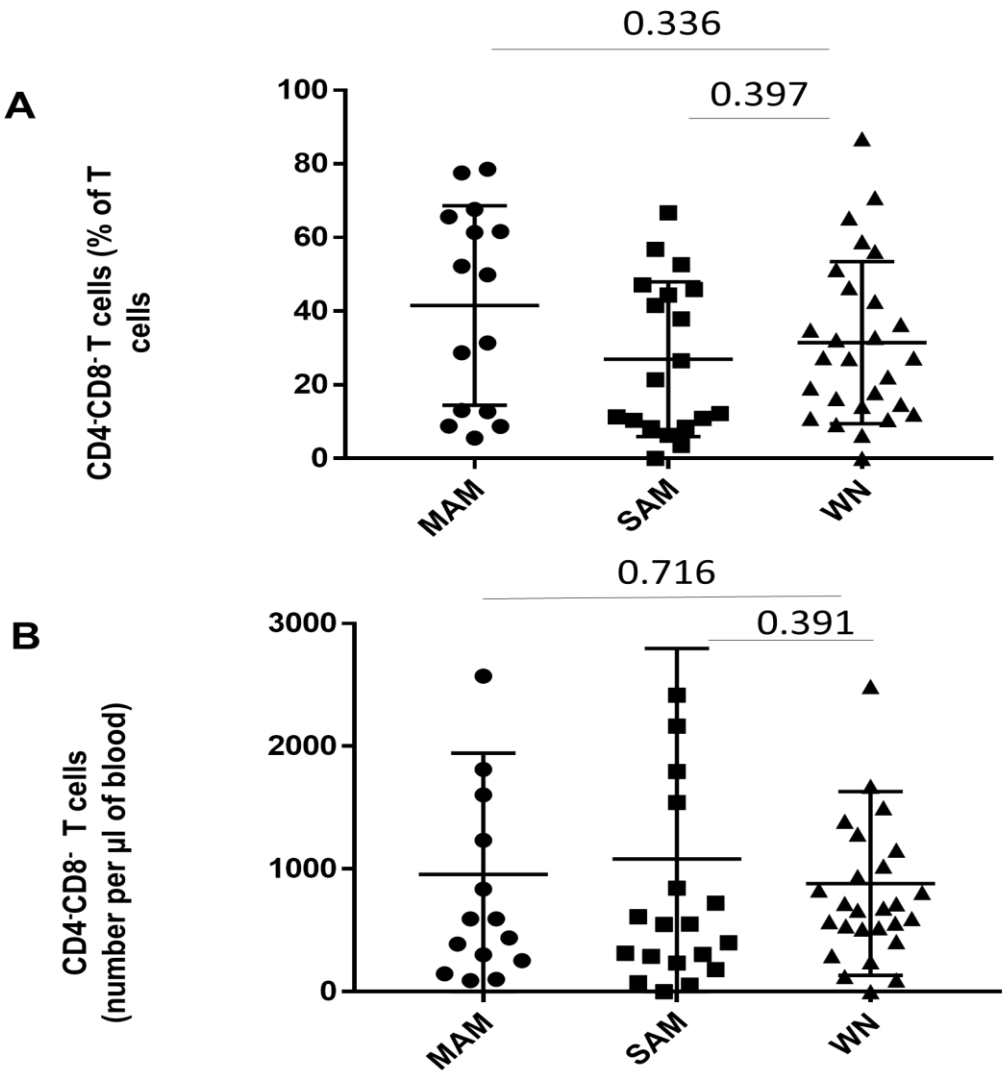


Figure 48: Effect of nutritional status on circulating CD4-CD8- T cell frequencies and numbers in ART-naïve children with HIV-infection.
*PBMC were isolated from 61 ART-naïve patients with HIV infection, of whom 27 were well-nourished (WN), 15 were moderately acutely malnourished (MAM) and 19 were severely acutely malnourished (SAM). Cells were stained with monoclonal antibodies specific for CD3, CD4 and CD8 and analysed by flow cytometry. CD4-CD8- T cells were defined as lymphocytes that were positive for CD3 and negative for CD4 and CD8. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD4-CD8- T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

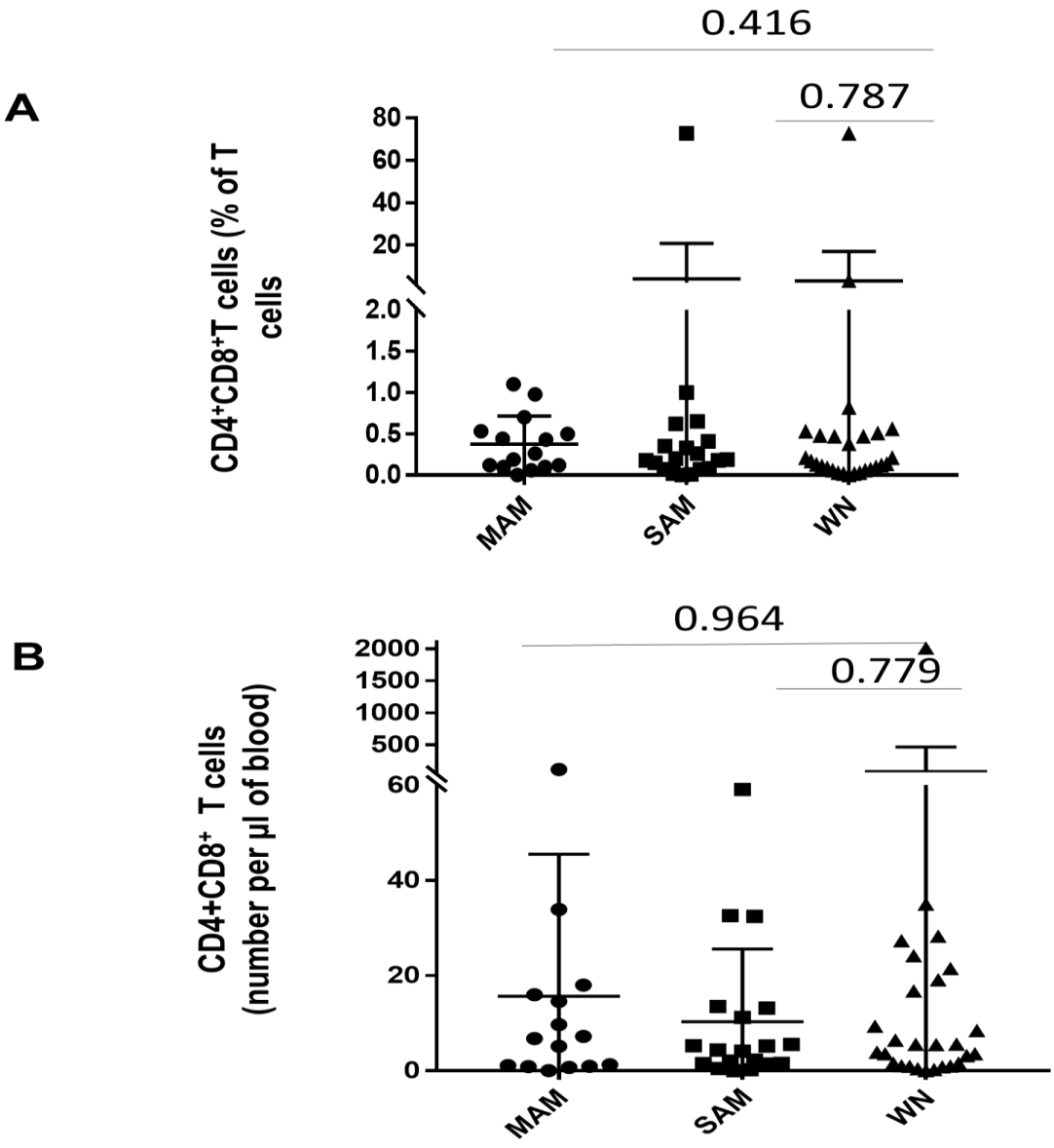


Figure 49: Effect of nutritional status on circulating CD4⁺CD8⁺ T cell frequencies and numbers in ART-naïve children with HIV-infection.
*PBMC were isolated from 61 ART-naïve patients with HIV infection, of whom 27 were well-nourished (WN), 15 were moderately acutely malnourished (MAM) and 19 were severely acutely malnourished (SAM). Cells were stained with monoclonal antibodies specific for CD3, CD4 and CD8 and analysed by flow cytometry. CD4⁺CD8⁺ T cells were defined as lymphocytes that were positive for CD3, CD4 and CD8. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD4⁺CD8⁺ T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

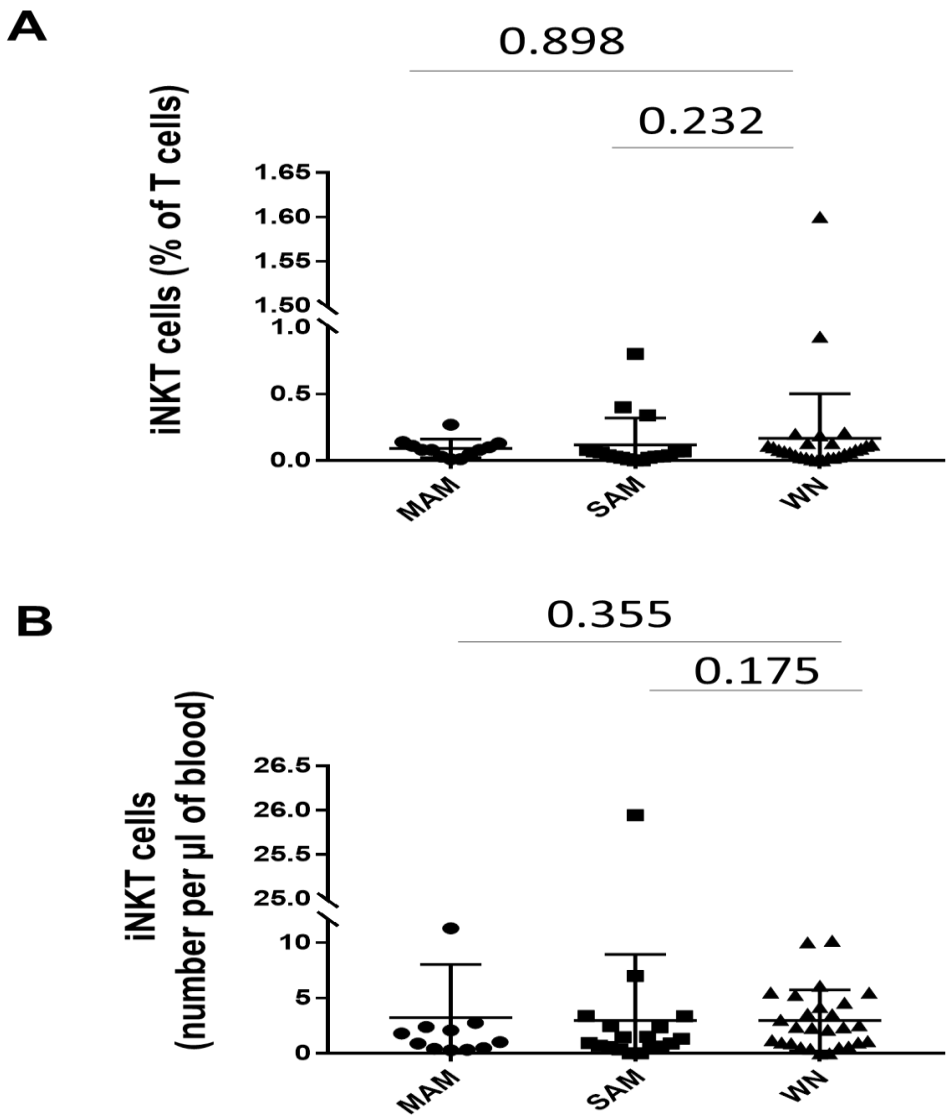


Figure 50: Effect of nutritional status on circulating iNK T cell frequencies and numbers in ART-naïve children with HIV-infection.
*PBMC were isolated from 61 ART-naïve patients with HIV infection, of whom 27 were well-nourished (WN), 15 were moderately acutely malnourished (MAM) and 19 were severely acutely malnourished (SAM). Cells were stained with monoclonal antibodies specific for CD3 and Vα24Jα18 and analysed by flow cytometry. iNK T cells were defined as lymphocytes that were positive for Vα24Jα18 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of iNK T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

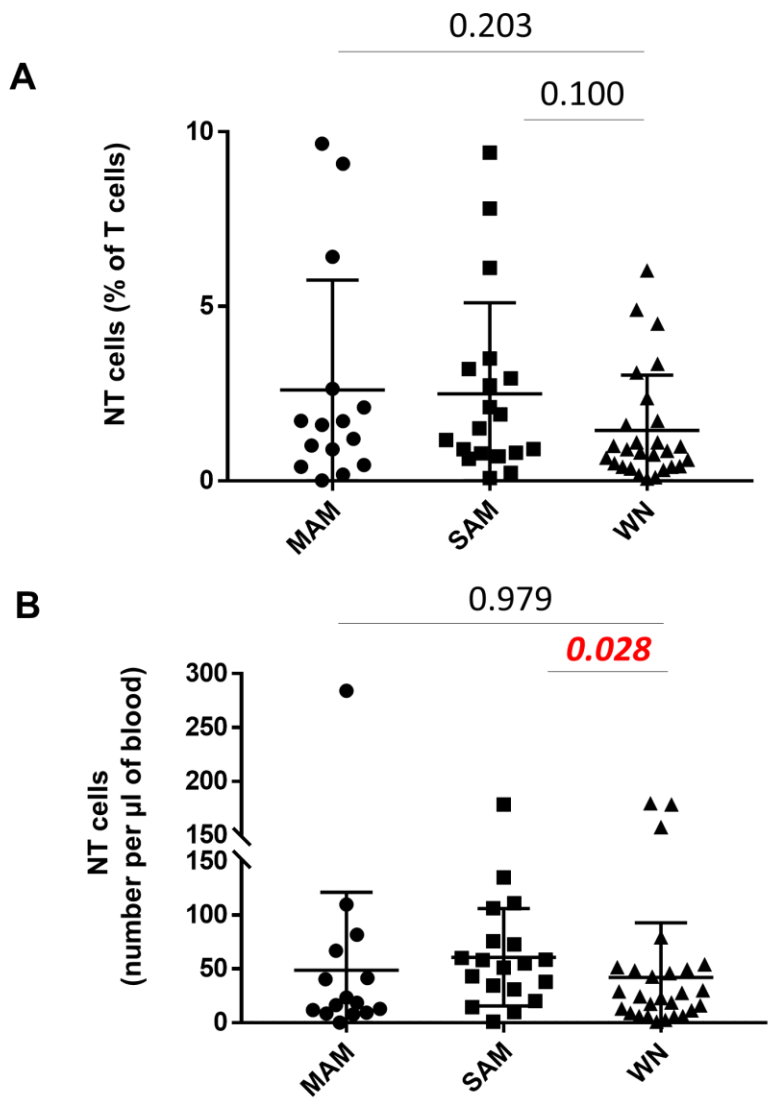


Figure 51: Effect of nutritional status on circulating NT cell frequencies and numbers in ART-naïve children with HIV-infection.

*PBMC were isolated from 61 ART-naïve patients with HIV infection, of whom 27 were well-nourished (WN), 15 were moderately acutely malnourished (MAM) and 19 were severely acutely malnourished (SAM). Cells were stained with monoclonal antibodies specific for CD3 and CD56 and analysed by flow cytometry. NT cells were defined as lymphocytes that were positive for both CD3 and CD56. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of NT cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

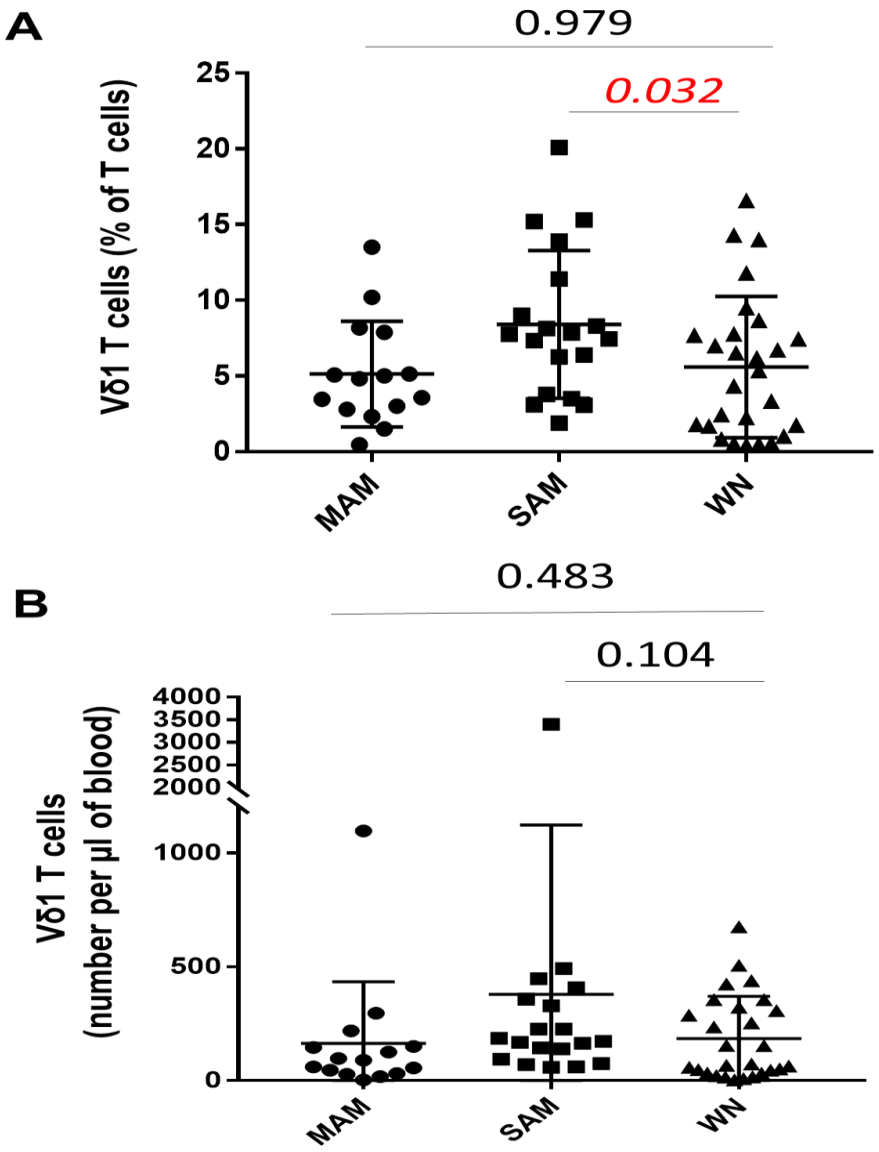


Figure 52: Effect of nutritional status on circulating Vδ1 T cell frequencies and numbers in ART-naïve children with HIV-infection.
*PBMC were isolated from 61 ART-naïve patients with HIV infection, of whom 27 were well-nourished (WN), 15 were moderately acutely malnourished (MAM) and 19 were severely acutely malnourished (SAM). Cells were stained with monoclonal antibodies specific for CD3 and Vδ1 and analysed by flow cytometry. Vδ1 T cells were defined as lymphocytes that were positive for Vδ1 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ1 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

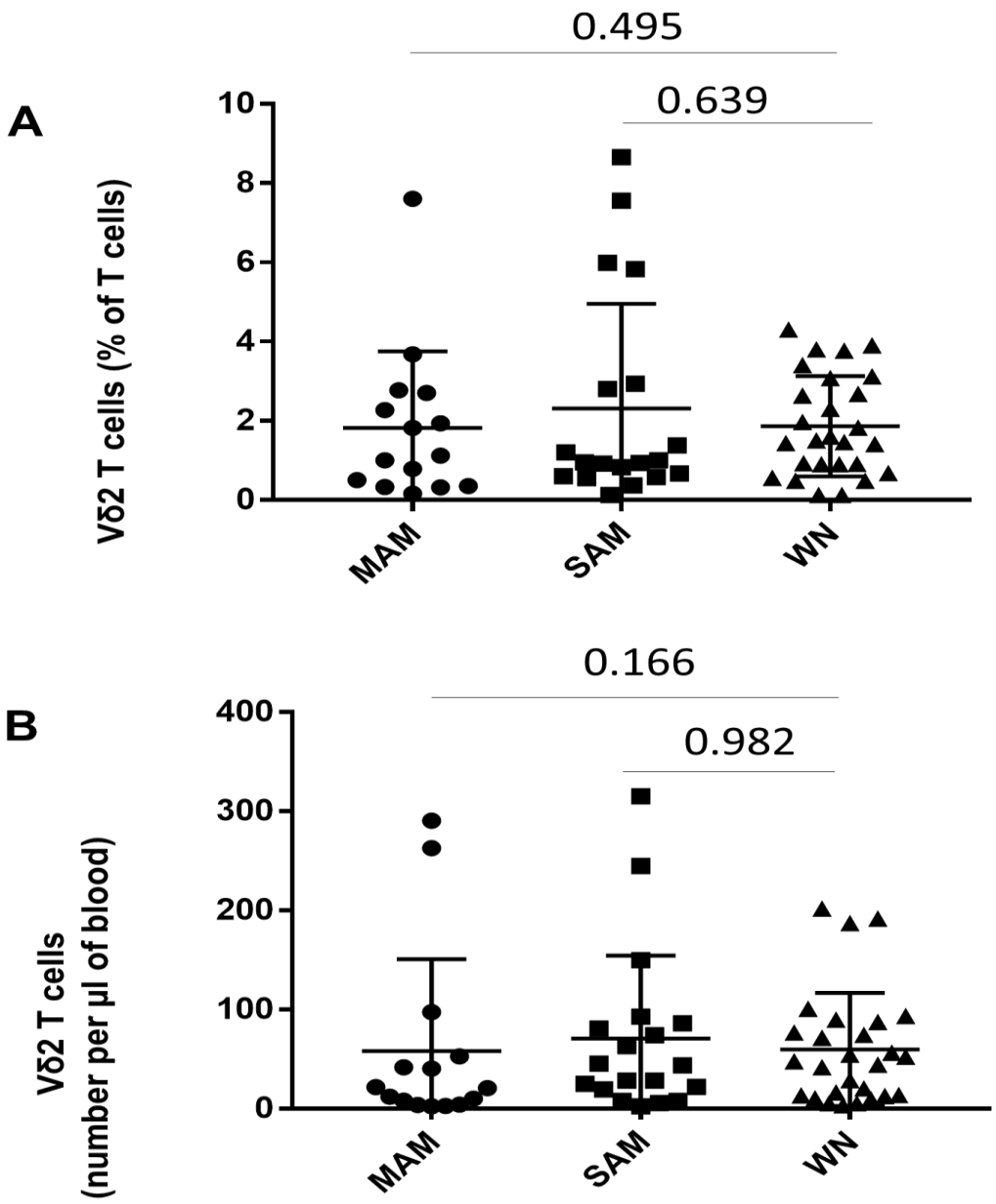


Figure 53: Effect of nutritional status on circulating Vδ2 T cell frequencies and numbers in ART-naïve children with HIV-infection.
*PBMC were isolated from 61 ART-naïve patients with HIV infection, of whom 27 were well-nourished (WN), 15 were moderately acutely malnourished (MAM) and 19 were severely acutely malnourished (SAM). Cells were stained with monoclonal antibodies specific for CD3 and Vδ2 and analysed by flow cytometry. Vδ2 T cells were defined as lymphocytes that were positive for Vδ2 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ2 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

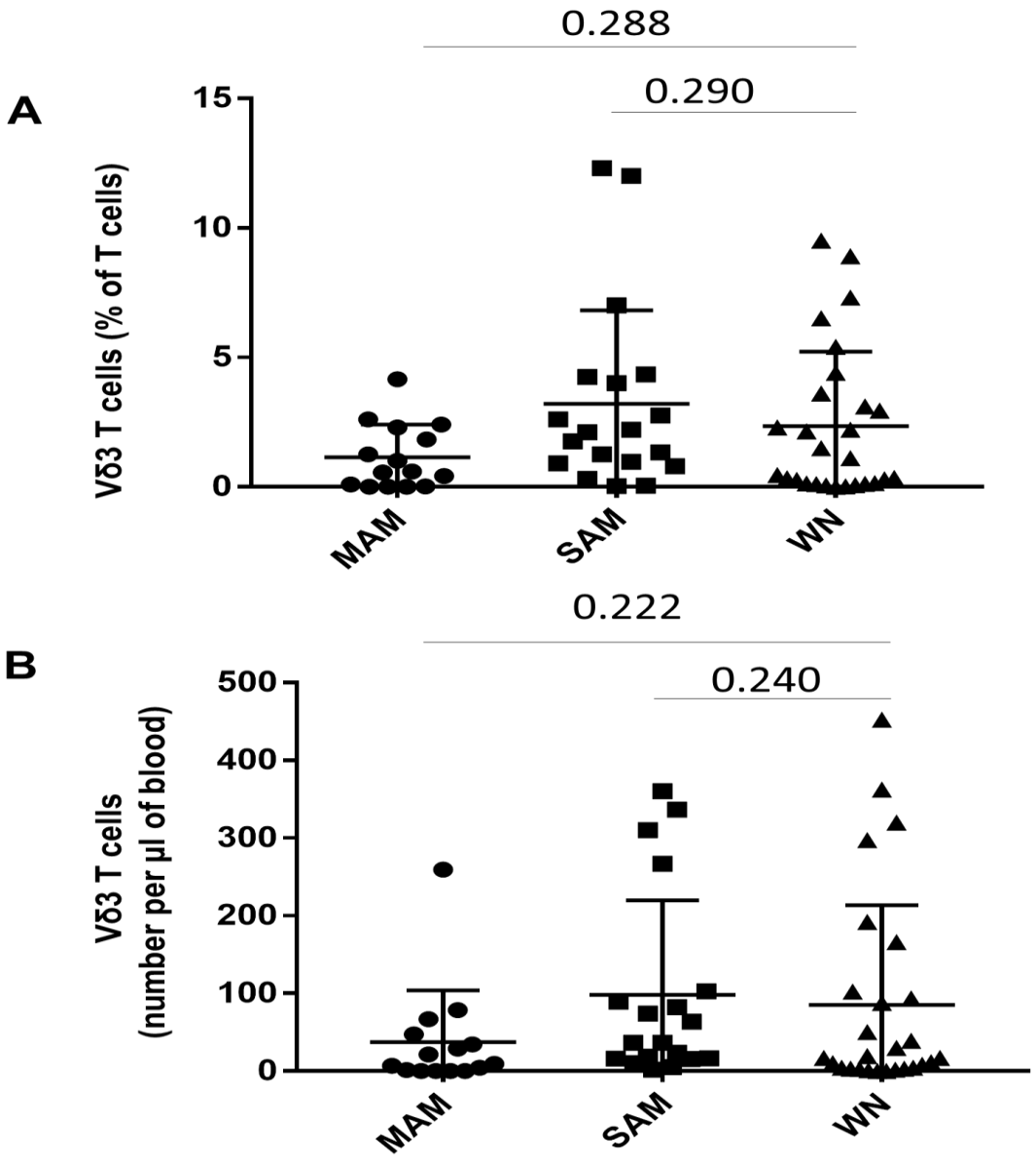


Figure 54: Effect of nutritional status on circulating Vδ3 T cell frequencies and numbers in ART-naïve children with HIV-infection.
*PBMC were isolated from 61 ART-naïve patients with HIV infection, of whom 27 were well-nourished (WN), 15 were moderately acutely malnourished (MAM) and 19 were severely acutely malnourished (SAM). Cells were stained with monoclonal antibodies specific for CD3 and Vδ3 and analysed by flow cytometry. Vδ3 T cells were defined as lymphocytes that were positive for Vδ3 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ3 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

Table 43. Summary results on effects of nutritional status on lymphocyte subset frequencies and numbers among the MAM and WN ART naïve HIV infected children at baseline.

Cells	MAM cell frequencies	WN cell frequencies	P-value	MAM cells/µL	WN cells/µL	P-value
T cells	**51.2**	**62.25**	**0.078**	**1,832**	**2,980**	**0.122**
B cells	**17**	**11.6**	**0.166**	**420**	**588**	**0.876**
NK cells	**4.99**	**3.25**	**0.051**	**231.5**	**139.3**	**0.405**
$CD8^+$ T cell	**40.8**	**39.8**	**0.496**	**727.5**	**1,216**	**0.210**
$CD4^+$ T cell	**13.2**	**12.1**	**0.861**	**398.3**	**358**	**0.586**
$CD4^-CD8^-$ T cell	**49.9**	**27.3**	**0.336**	**593.3**	**681.6**	**0.716**
$CD4^+CD8^+$T cell	**0.26**	**0.14**	**0.416**	**6.8**	**5.5**	**0.964**
iNK T cell	**0.08**	**0.08**	**0.898**	**0.44**	**0.92**	**0.355**
NT cell	**1.6**	**0.86**	**0.208**	**18.5**	**24.4**	**0.979**
Vδ1 T cell	**4.82**	**5.36**	**0.979**	**89.57**	**73.01**	**0.483**
Vδ2 T cell	**1.12**	**1.5**	**0.495**	**20.93**	**47.68**	**0.166**
Vδ3 T cell	**0.59**	**1.1**	**0.288**	**9.45**	**16.88**	**0.222**

Table 44. Summary results on effects of nutritional status on lymphocyte subset frequencies and numbers among the SAM and WN ART naïve HIV infected children at baseline.

Cells	SAM cell frequencies	WN cell frequencies	P-value	SAM cells/µL	WN cells/µL	P-value
T cells	**62.6**	**62.25**	**0.795**	**2,368**	**2,980**	**0.912**
B cells	**11.8**	**11.6**	**0.165**	**371.3**	**588.3**	**0.657**
NK cells	**4.66**	**3.25**	**0.136**	**208.3**	**139.3**	**0.249**
$CD8^+$ T cell	**50.2**	**39.8**	**0.105**	**1,550**	**1,216**	**0.249**
$CD4^+$ T cell	**5.61**	**12.1**	**0.184**	**162**	**358**	**0.2216**
$CD4^-CD8^-$ T cell	**21.4**	**27.3**	**0.397**	**547.5**	**681.6**	**0.391**
$CD4^+CD8^+$T cell	**0.19**	**0.14**	**0.787**	**4.33**	**5.5**	**0.779**
iNK T cell	**0.04**	**0.08**	**0.232**	**1.14**	**2.36**	**0.175**
NT cell	**1.5**	**0.86**	**0.100**	**54.87**	**24.43**	**0.028***
Vδ1 T cell	**7.76**	**5.36**	**0.032****	**172.5**	**73.01**	**0.104**
Vδ2 T cell	**0.95**	**1.5**	**0.639**	**43.93**	**47.68**	**0.982**
Vδ3 T cell	**2.1**	**1.1**	**0.290**	**36.37**	**16.88**	**0.240**

*Indicates that the median absolute count of NT cells was higher in SAM than WN, ART-N HIV infected children

** Indicates that the median absolute count of Vδ1 T cells was higher in SAM than WN, ART-N HIV infected children

Objective 3: Effects of ART on lymphocyte subset frequencies and numbers in malnourished children at baseline.

The effect of ART on circulating lymphocyte cell frequencies and numbers in malnourished children was assessed by isolating PBMC from 34 ART-N malnourished patients and 40 ART–E malnourished patients with HIV infection. We compared the subsets of each cell of interest by ART status at baseline. Cells were stained with monoclonal antibodies specific for CD3, CD4, CD8, CD19, CD56 and the Vα24Jα18, Vδ1, Vδ2 and Vδ3 TCR chains (Table 12), frequencies and absolute numbers of lymphocyte subsets were analysed by flow cytometry. Absolute numbers were calculated from full blood counts taken at the time of blood collection (Table 18 and 26).

The median frequencies of T cells were significantly higher among the ART-E in comparison to the ART-N (p-value=0.01) implying that the presence of ART played a role in repopulation of T cells. A similar trend was observed for absolute cell counts but this was not significant (p-value=0.918, Figure 55). B cell results, (Figure 56), were similar in the 2 groups. Figure 57 showed that NK absolute cell counts were lower in the ART-E children in comparison to the ART-N (p-value=0.031) children and the NK cell frequencies shows a similar trend but this difference was not significant (p-value=0.074). $CD4^+$ T cell absolute counts and percentages of total T cells, were significantly increased among the ART-E malnourished children in comparison to the ART-N malnourished children (p-value=<0.0002 and <0.0001 respectively, Figure 59). ART is known to restore the depletion of $CD4^+$ T cells if one takes treatment as instructed. The $CD8^+$ T cell frequencies and absolute count demonstrated a reduction in children who were ART-E but this was not significant (Figure 58). The median of $CD4^-CD8^-$ T cell frequencies and numbers were significantly higher in the ART-N than the ART-E children, (p-value=0.006 and p-value=0.029, respectively, Figure 60) while the $CD4^+CD8^+$ T cells were similar, (Figure 61). The subsets of iNK T and NT cells in the Figures 62-63 were increased in ART-E in comparison to those who were ART-N. Both the Vδ1 T cell frequencies and numbers were higher in the ART-N than the ART-E children (p-value=0.015 and p-value=0.023 respectively) illustrating that V δ1 T cells are expanded in HIV and ART decreases them towards the normal levels, (Figure 64). The V δ2 T subsets in figure 65 were similar. The

frequencies and absolute counts of V δ3 T cells were higher in the ART-N children than those who were ART-E but this trend was not significant, (Figure 66).

The findings of this study demonstrate that ART affects lymphocyte subsets in malnourished HIV infected children. These results are summarised in Table 45 and 46.

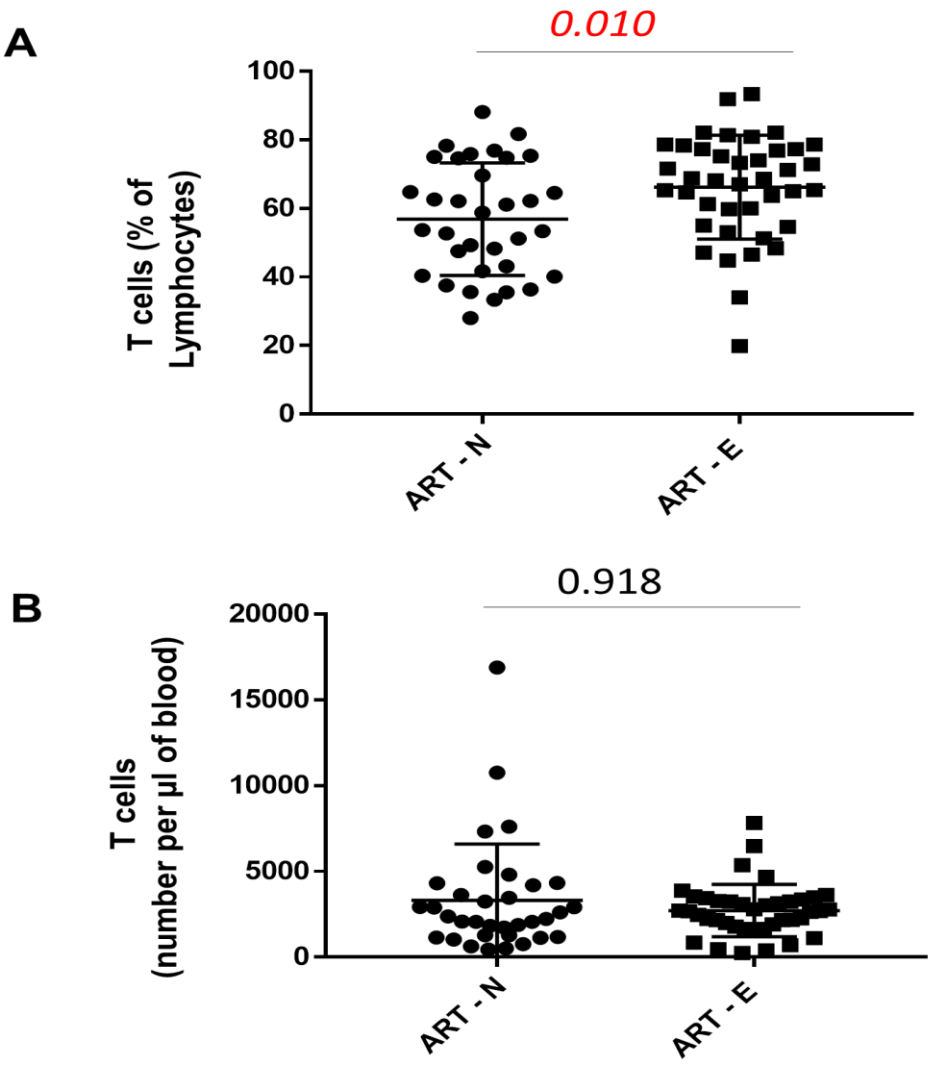


Figure 55: Effect of ART on circulating T cell frequencies and numbers in malnourished HIV infected children.

*PBMC were isolated from 34 ART-naïve malnourished patients and 40 ART – experienced malnourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD3 and analysed by flow cytometry. T cells were defined as lymphocytes that were positive for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

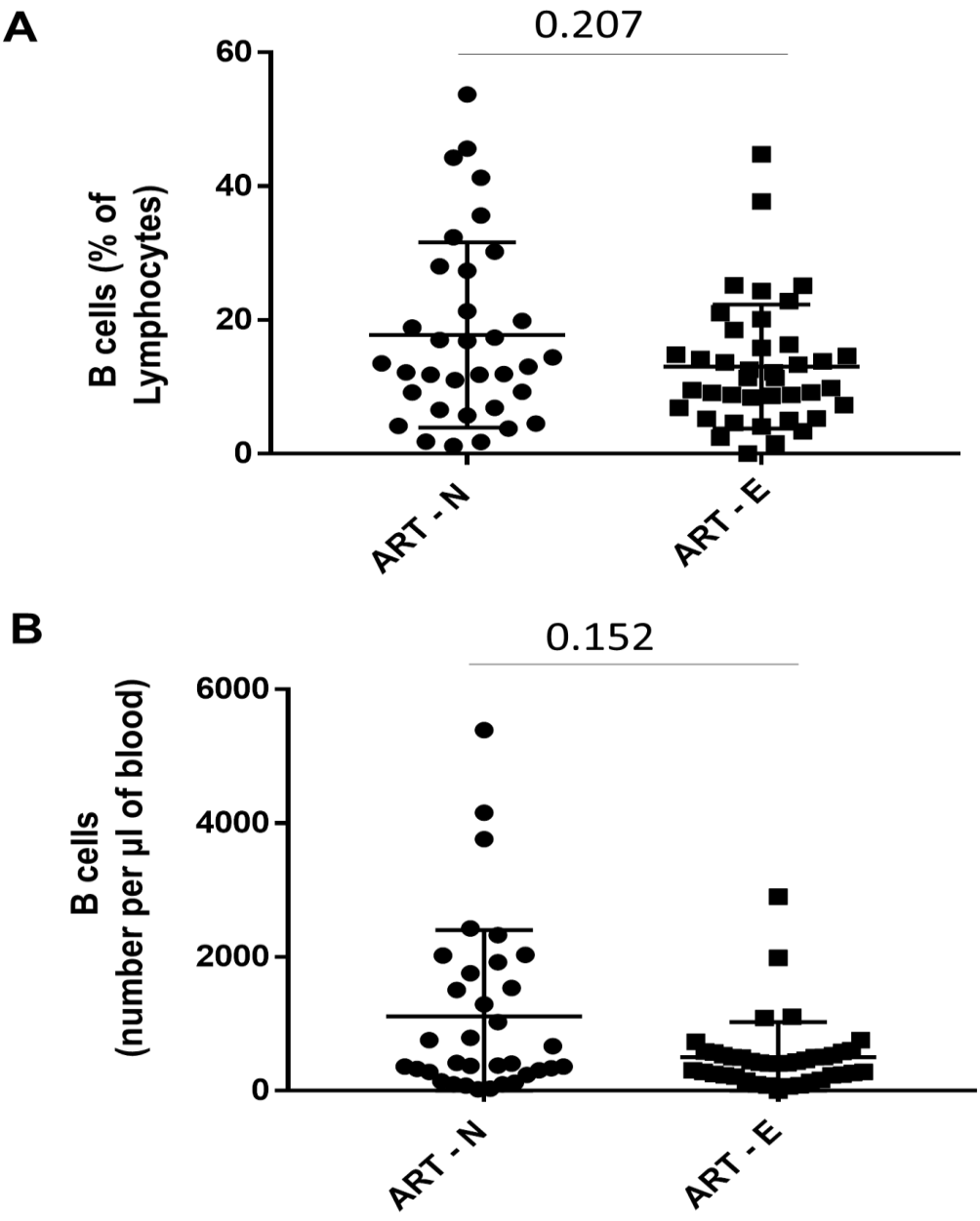


Figure 56: Effect of ART on circulating B cell frequencies and numbers in malnourished HIV infected children.

*PBMC were isolated from 34 ART-naïve malnourished patients and 40 ART – experienced malnourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD3 and CD19 and analysed by flow cytometry. B cells were defined as lymphocytes that were positive for CD19 and negative for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of B cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

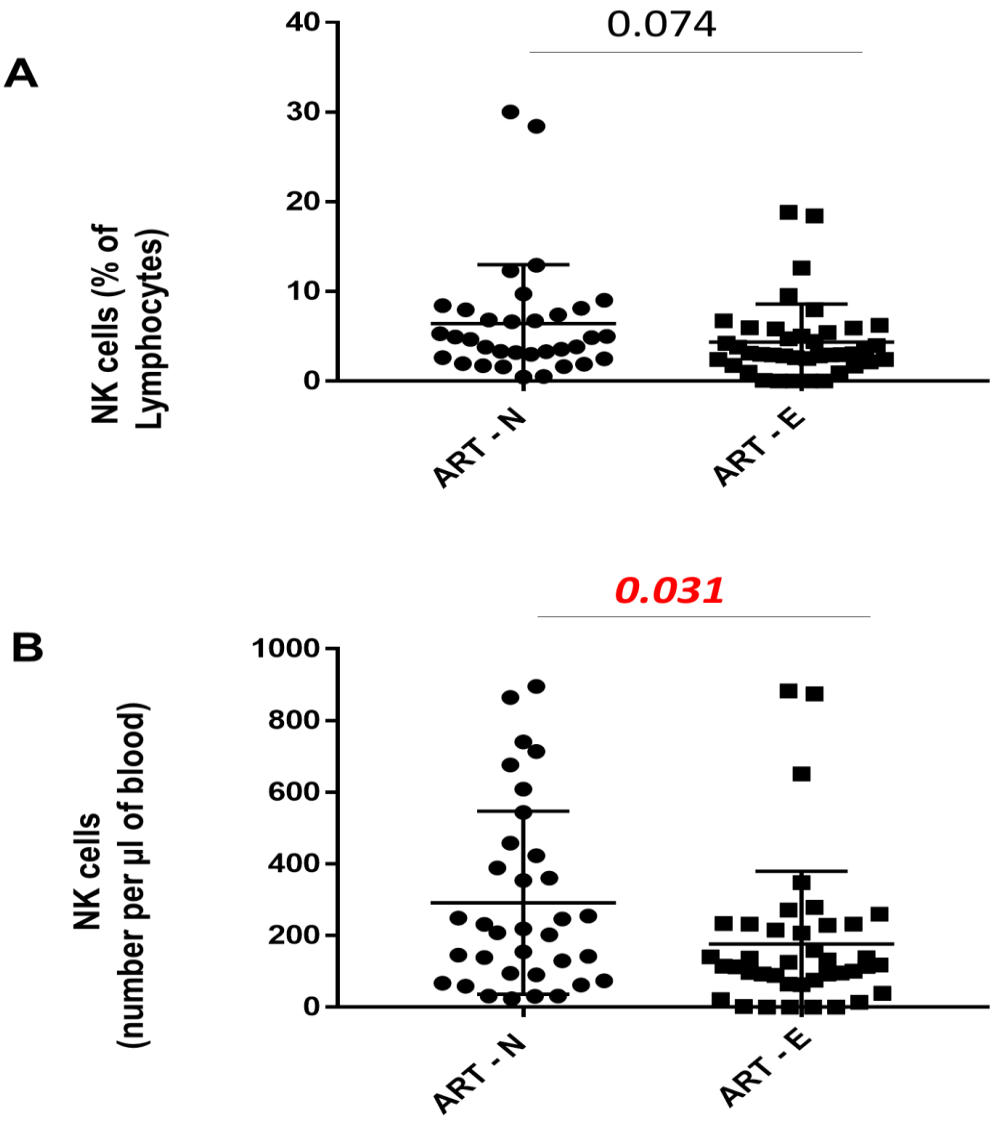


Figure 57: Effect of ART on circulating NK cell frequencies and numbers in malnourished HIV infected children.

*PBMC were isolated from 34 ART-naïve malnourished patients and 40 ART – experienced malnourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD3 and analysed by flow cytometry. NK cells were defined as lymphocytes that were positive for CD56 and negative for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of NK cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

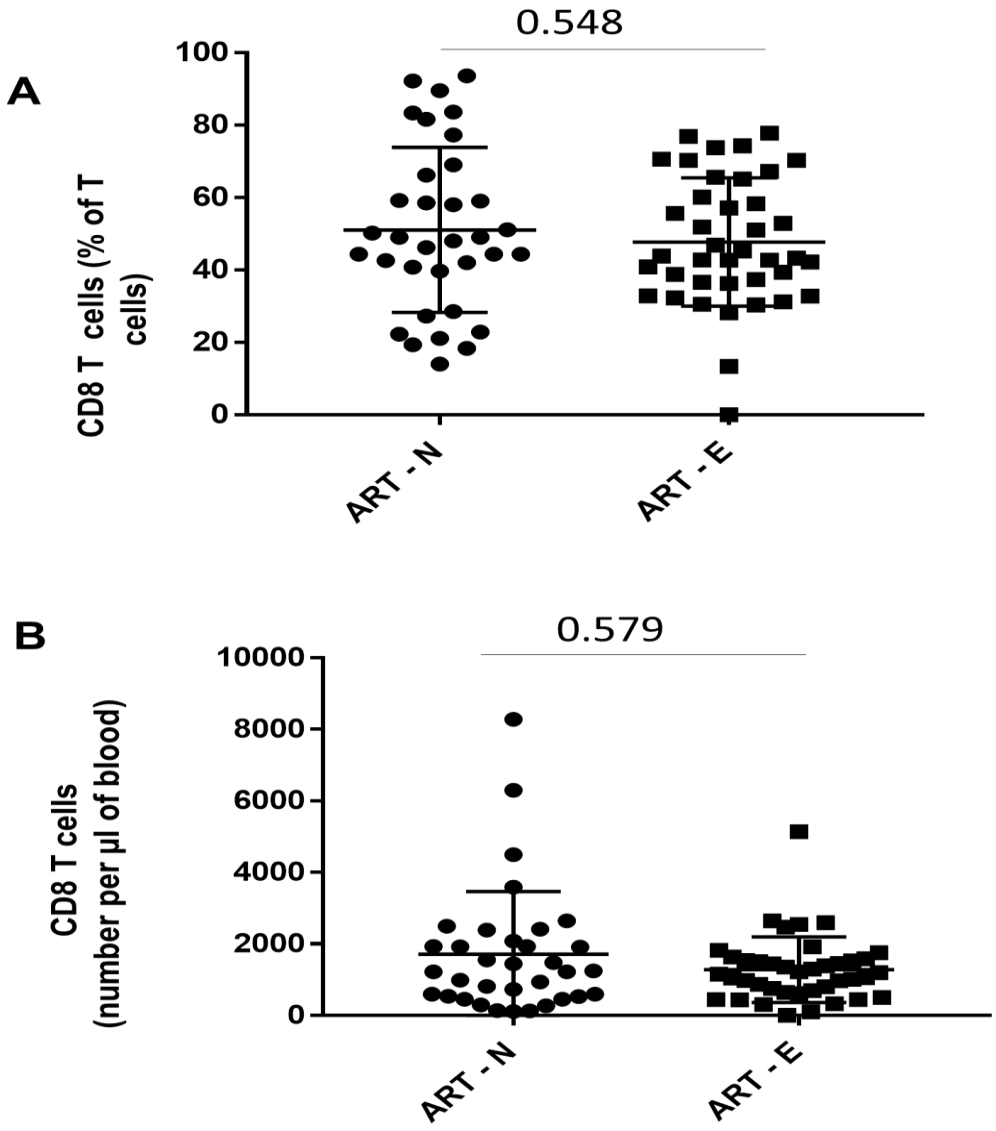


Figure 58: Effect of ART on circulating CD8⁺ T cell frequencies and numbers in malnourished HIV infected children.

*PBMC were isolated from 34 ART-naïve malnourished patients and 40 ART – experienced malnourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD4, CD8 and CD3 and analysed by flow cytometry. CD8 T cells were defined as lymphocytes that were positive for CD8 and CD3 and negative for CD4. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD8 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

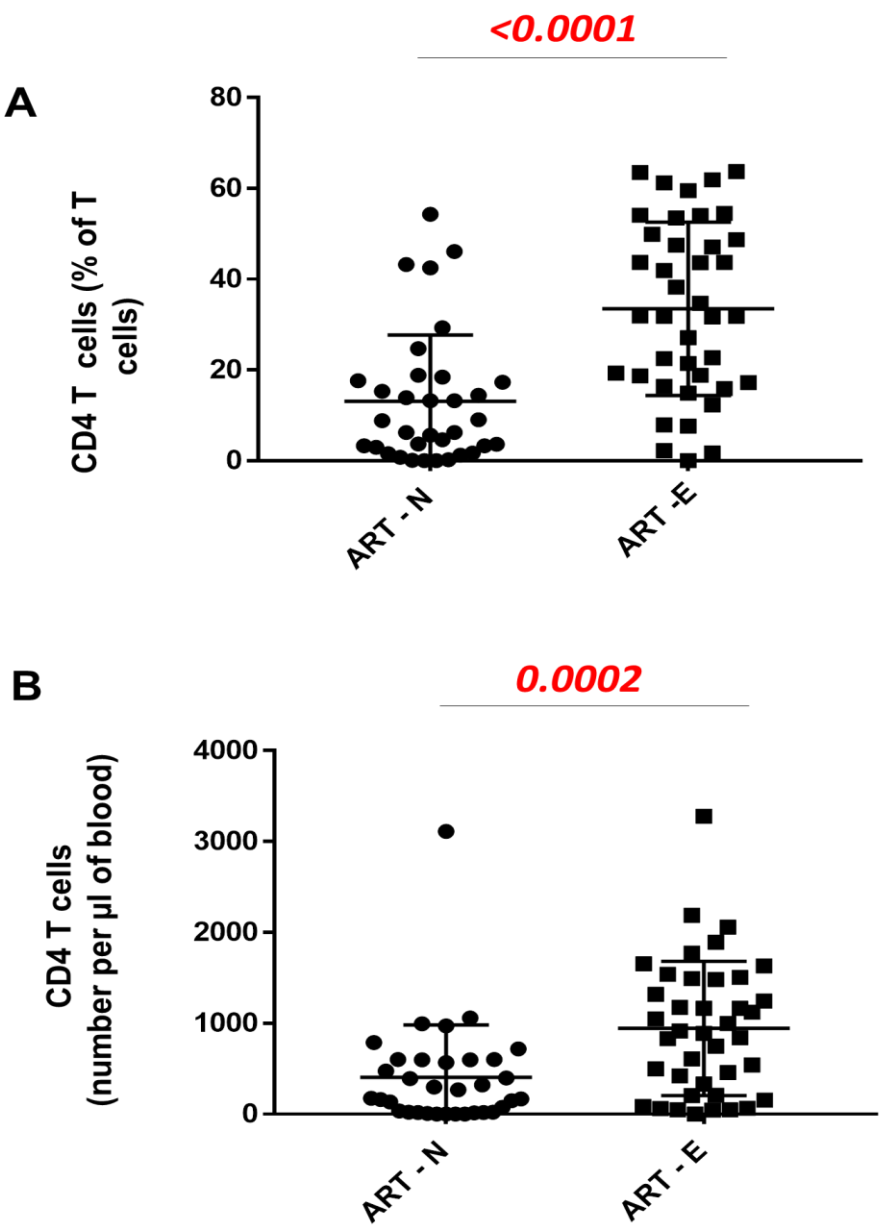


Figure 59: Effect of ART on circulating CD4⁺ T cell frequencies and numbers in numbers in malnourished HIV infected children.

*PBMC were isolated from 34 ART-naïve malnourished patients and 40 ART – experienced malnourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD4, CD8 and CD3 and analysed by flow cytometry. CD4 T cells were defined as lymphocytes that were positive for CD4 and CD3 and negative for CD8. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD4 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

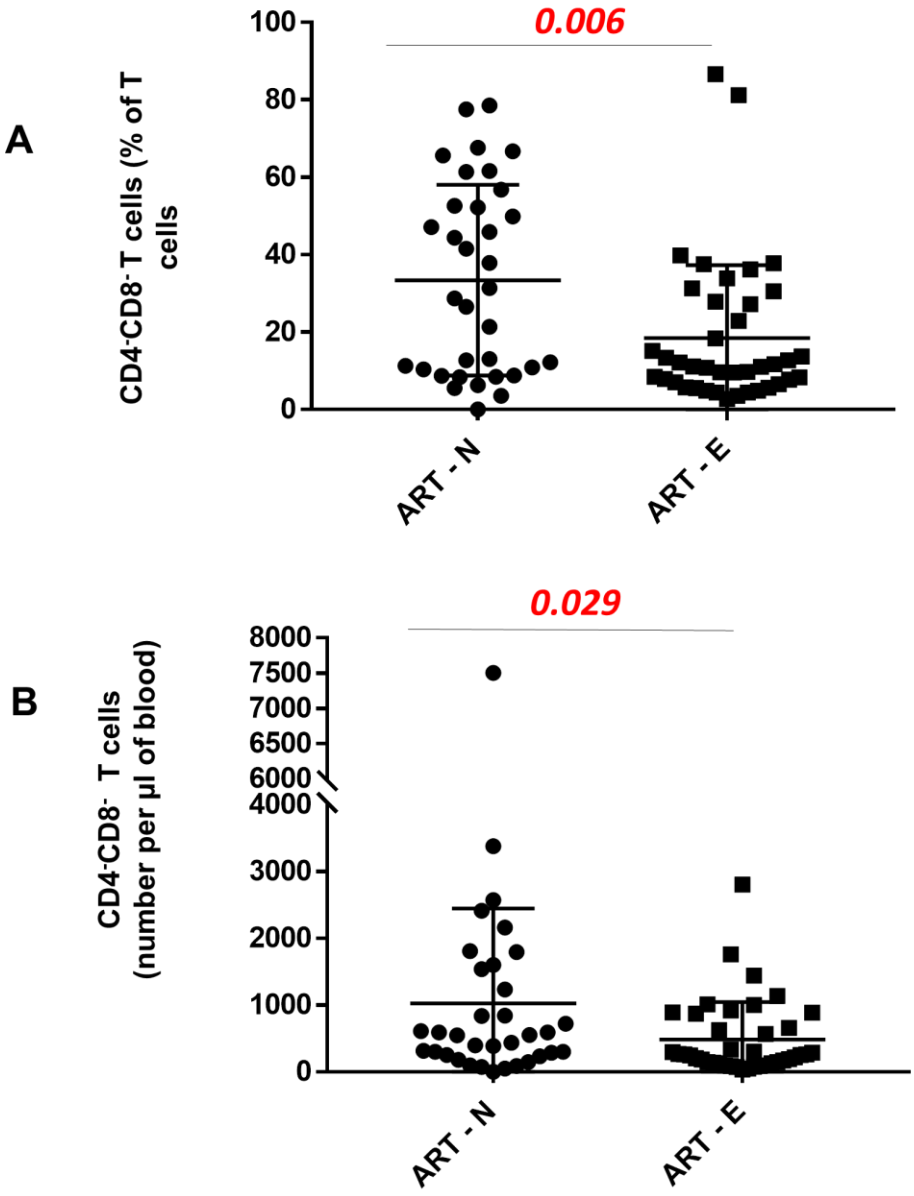


Figure 60: Effect of ART on circulating CD4-CD8- T cell frequencies and numbers in numbers in malnourished HIV infected children.

*PBMC were isolated from 34 ART-naïve malnourished patients and 40 ART – experienced malnourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD4, CD8 and CD3 and analysed by flow cytometry. CD4-CD8- T cells were defined as lymphocytes that were positive for CD3 and negative for CD4 and CD8. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD4-CD8 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

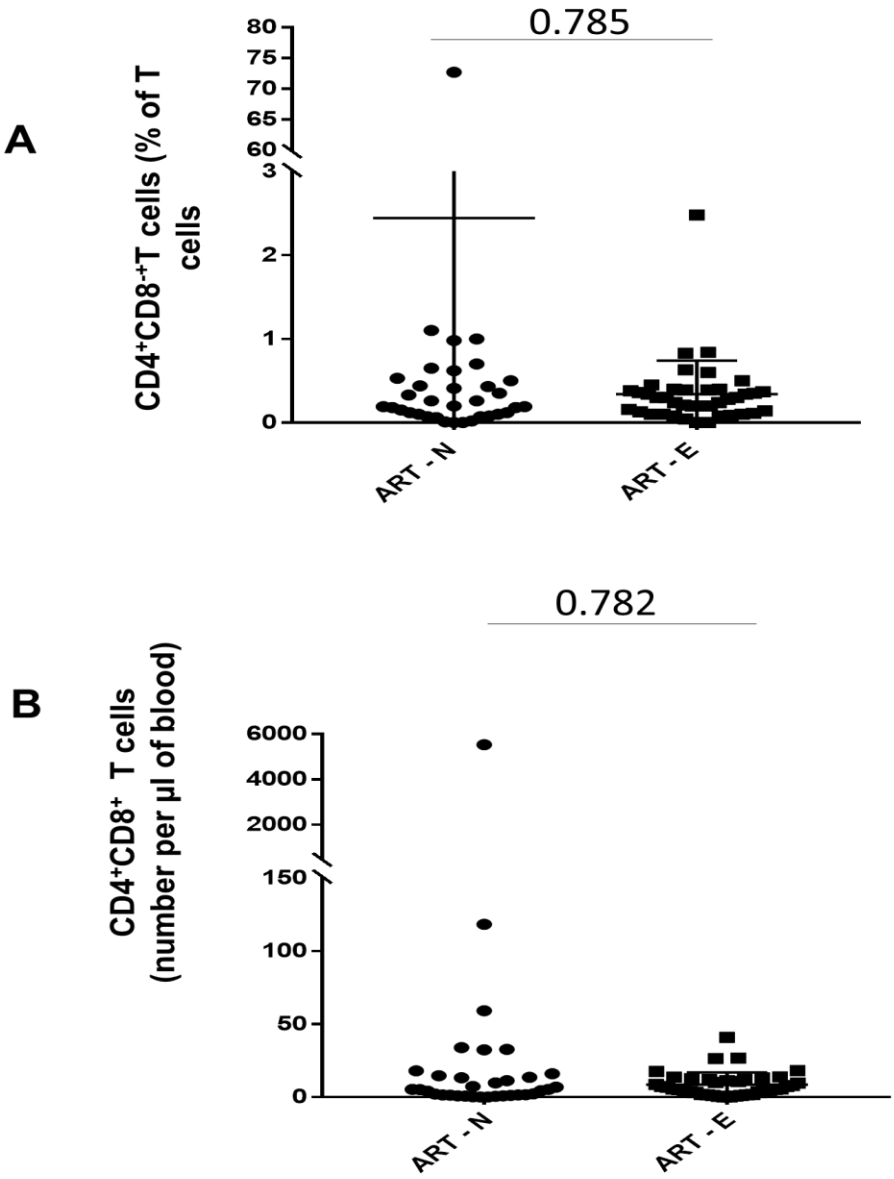


Figure 61: Effect of ART on circulating CD4⁺CD8⁺ T cell frequencies and numbers in malnourished HIV infected children.

*PBMC were isolated from 34 ART-naïve malnourished patients and 40 ART – experienced malnourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD4, CD8 and CD3 and analysed by flow cytometry. CD4⁺CD8⁺ T cells were defined as lymphocytes that were positive for CD3, CD4 and CD8. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD⁺CD8⁺ T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

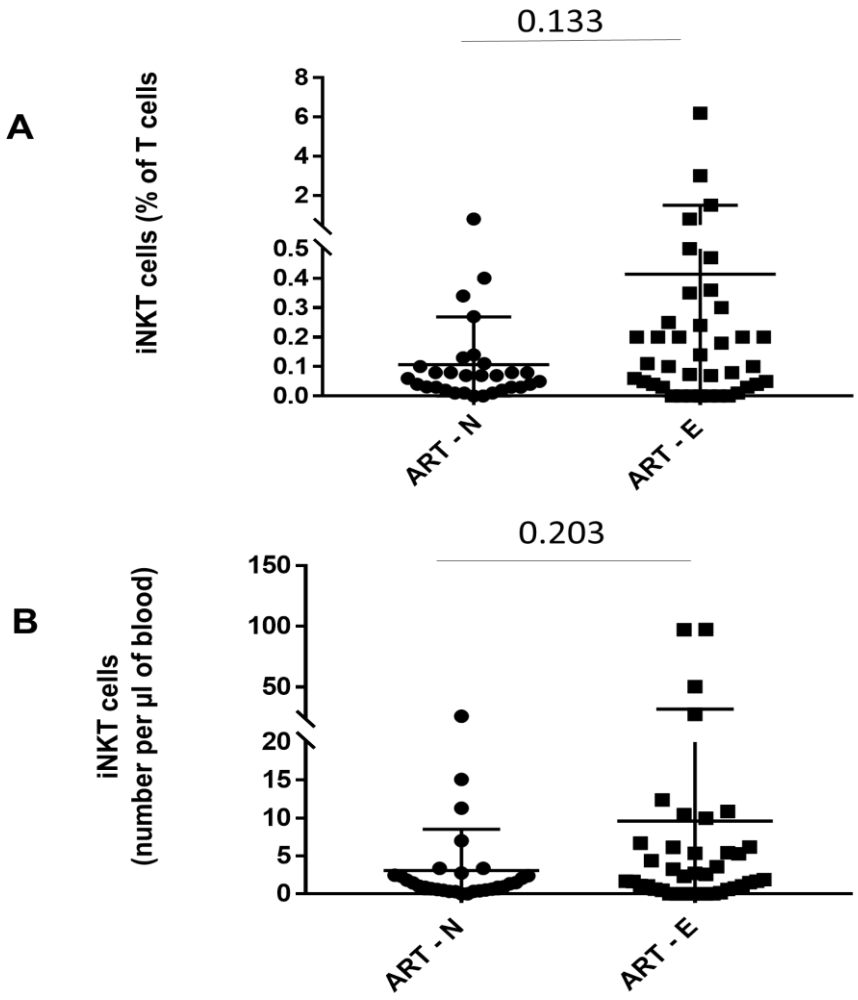


Figure 62: Effect of ART on circulating iNK T cell frequencies and numbers in malnourished HIV infected children.

*PBMC were isolated from 34 ART-naïve malnourished patients and 40 ART – experienced malnourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for Vα24Jα18 and CD3 and analysed by flow cytometry. iNK T cells were defined as lymphocytes that were positive for Vα24Jα18 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of iNK T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

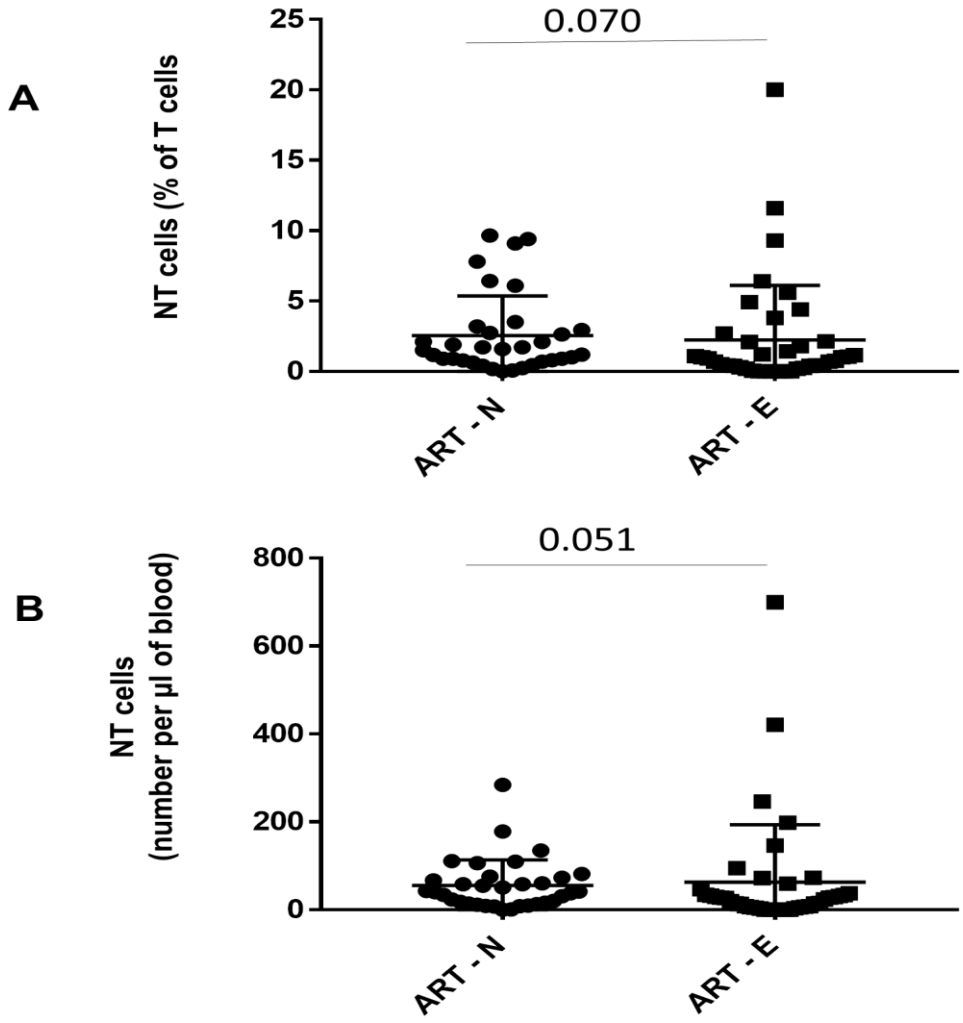


Figure 63: Effect of ART on circulating NT cell frequencies and numbers in malnourished HIV infected children.

*PBMC were isolated from 34 ART-naïve malnourished patients and 40 ART – experienced malnourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD56 and CD3 and analysed by flow cytometry. NT cells were defined as lymphocytes that were positive for CD3 and CD56. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of NT cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

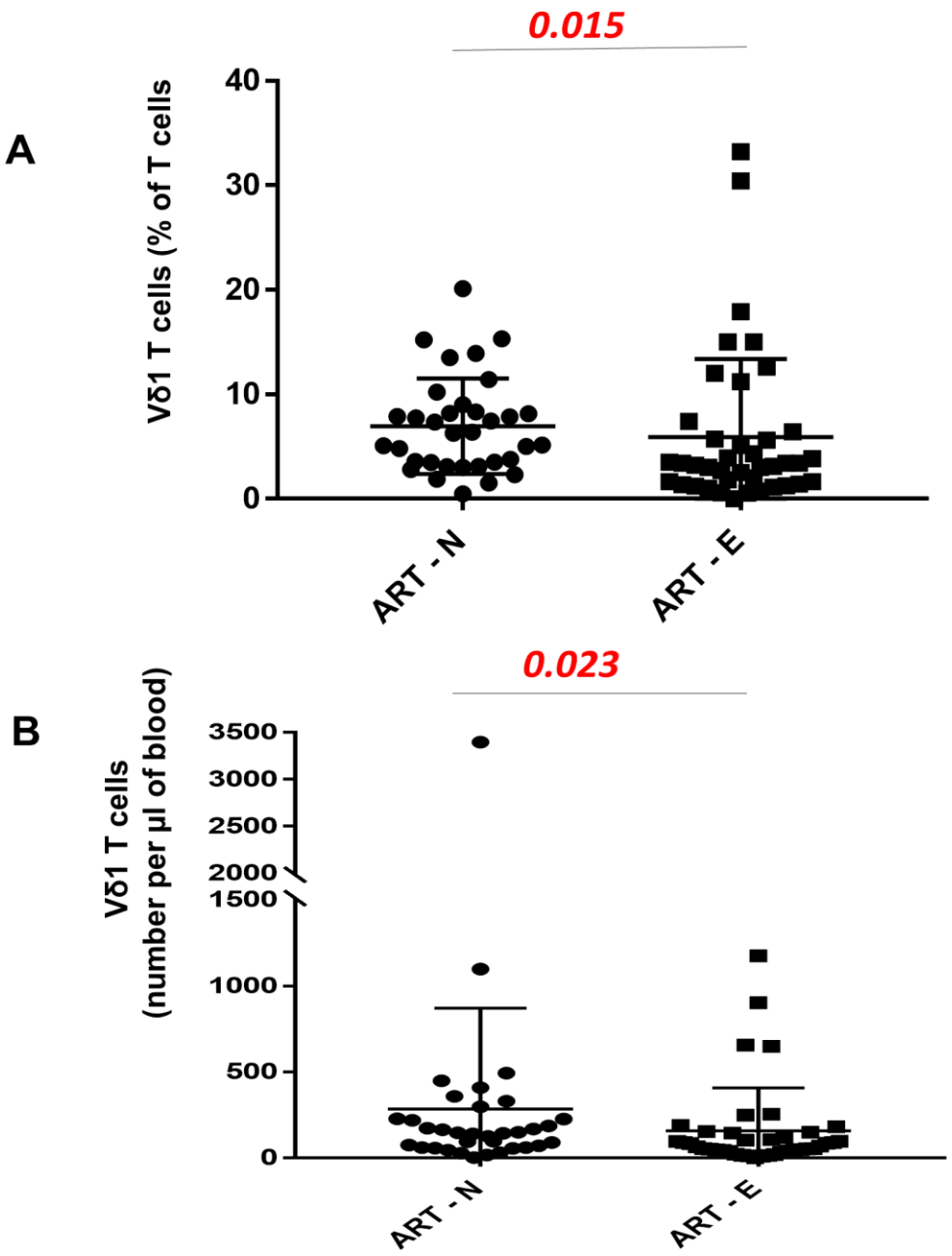


Figure 64: ***Effect of ART on circulating Vδ1 T cell frequencies and numbers in malnourished HIV infected children.***

*PBMC were isolated from 34 ART-naïve malnourished patients and 40 ART – experienced malnourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for Vδ1 and CD3 and analysed by flow cytometry. Vδ1 T cells were defined as lymphocytes that were positive for Vδ1 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ1 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

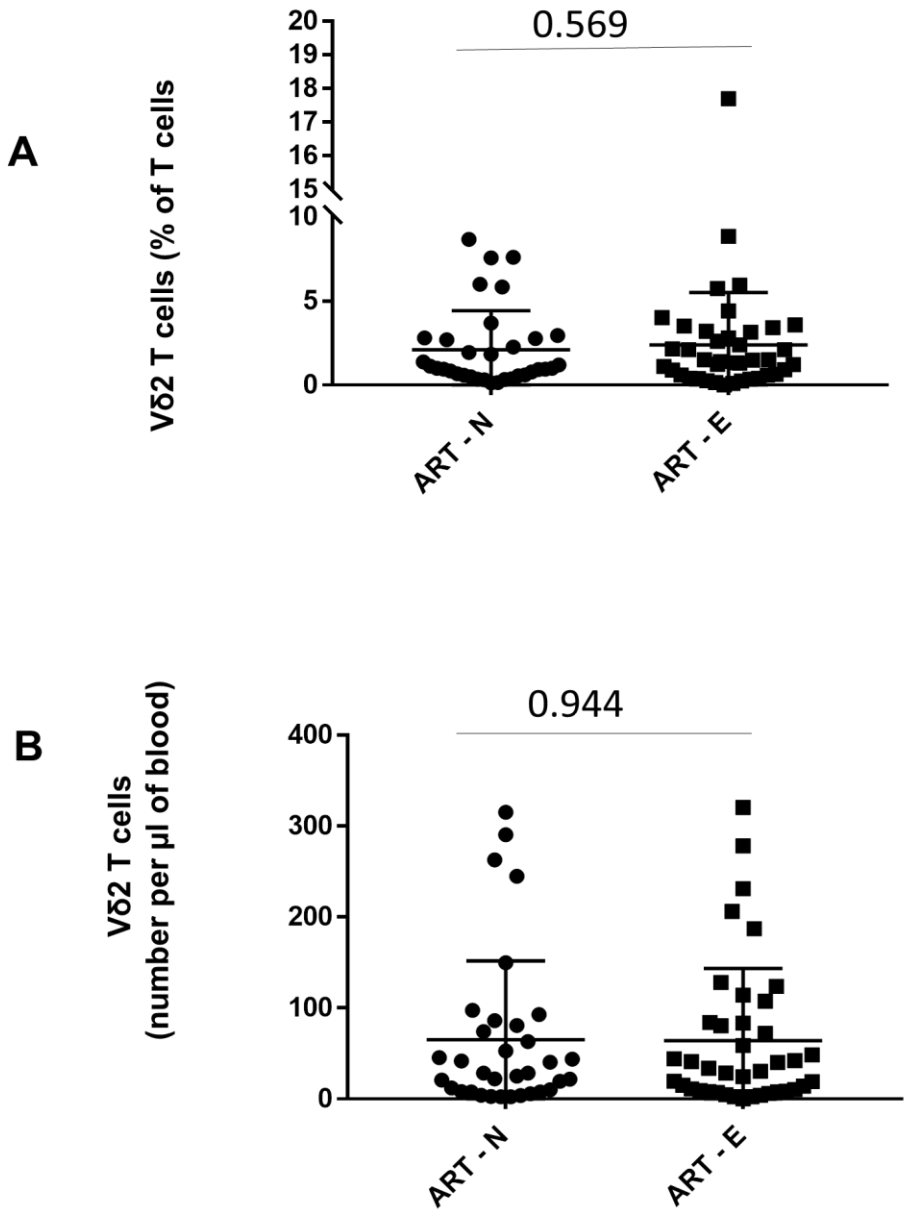


Figure 65: Effect of ART on circulating Vδ2 T cell frequencies and numbers in malnourished HIV infected children.

*PBMC were isolated from 34 ART-naïve malnourished patients and 40 ART – experienced malnourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for Vδ2 and CD3 and analysed by flow cytometry. Vδ2 T cells were defined as lymphocytes that were positive for Vδ2 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ2 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

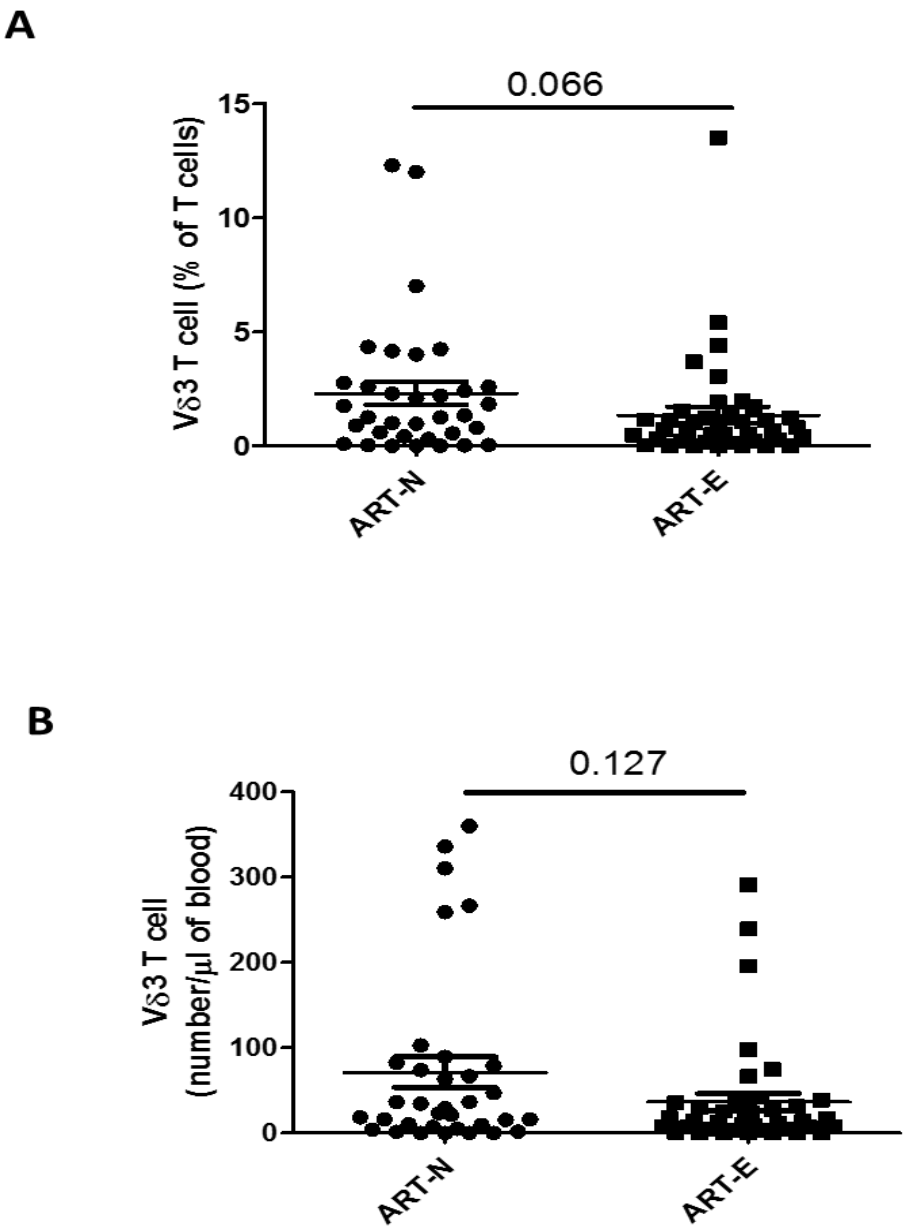


Figure 66: Effect of ART on circulating Vδ3 T cell frequencies and numbers in malnourished HIV infected children.

*PBMC were isolated from 34 ART-naïve malnourished patients and 40 ART – experienced malnourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for Vδ3 and CD3 and analysed by flow cytometry. Vδ3 T cells were defined as lymphocytes that were positive for Vδ3 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ3 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

Table 45. Summary results on effects of ART on lymphocyte subset frequencies in malnourished HIV infected children at baseline.

Cells	Median frequencies of ART-N	Median frequencies of ART-E	P-value
T cells	**56.25**	**68.4**	**0.010***
B cells	**13.25**	**11.3**	**0.207**
NK cells	**4.76**	**3.02**	**0.074**
$CD8^+$ T cell	**48.5**	**43.6**	**0.548**
$CD4^+$ T cell	**7.5**	**31.9**	**<0.0001*****
$CD4^-CD8^-$ T cell	**30.1**	**11.1**	**0.006********
$CD4^+CD8^+$ T cell	**0.2**	**0.29**	**0.785**
iNK T cell	**0.07**	**0.1**	**0.133**
NT cell	**1.55**	**0.84**	**0.070**
Vδ1 T cell	**6.33**	**3.31**	**0.015*********
Vδ2 T cell	**1**	**1.45**	**0.569**
Vδ3 T cell	**1.3**	**0.69**	**0.066**

*Indicates the median frequencies of T cells was higher in ART-E then ART-N, HIV infected children

*** Indicates the median frequencies of $CD4^+$ T cells were lower in ART-N than ART-E, HIV infected children

**** Indicates the median frequencies of DN were higher in ART-N than ART-E, HIV infected children

***** Indicates the median frequencies of Vδ1 T cells were higher in ART-N than ART-E, HIV infected children

Table 46. Summary results on effects of ART on lymphocyte subset absolute cell counts in malnourished HIV infected children at baseline.

Cells	Median absolute cell counts of ART-N	Median absolute cell counts of ART-E	P-values
T cells	**1,246**	**1,806**	**0.918**
B cells	**413.5**	**417.9**	**0.152**
NK cells	**213.7**	**116.6**	**0.031****
$CD8^+$ T cell	**1233**	**1176**	**0.579**
$CD4^+$ T cell	**221.1**	**901.6**	**0.0002*****
$CD4^-CD8^-$ T cell	**549.8**	**257.2**	**0.029******
$CD4^+CD8^+$ T cell	**5.2**	**6.2**	**0.782**
iNK T cell	**1.2**	**2.1**	**0.203**
NT cell	**40.92**	**25.64**	**0.051**
Vδ1 T cell	**144.1**	**76.36**	**0.023*******
Vδ2 T cell	**28.42**	**32.25**	**0.944**
Vδ3 T cell	**26.19**	**14.1**	**0.127**

** Indicates the median absolute count of NK cells was higher in ART-N than ART-E, HIV infected children

*** Indicates the median absolute count of $CD4^+$ T cells were lower in ART-N than ART-E, HIV infected children

**** Indicates the median absolute count of DN were higher in ART-N than ART-E, HIV infected children

***** Indicates the median absolute count of Vδ1 T cells were higher in ART-N than ART-E, HIV infected children

Objective 4: Effects of ART on lymphocyte subset frequencies and numbers in well-nourished HIV infected children at baseline.

The effects of ART on lymphocyte subset frequencies and numbers (in well-nourished children at baseline) was assessed by isolating PBMC from 27 ART-N, WN patients and 43 ART–E WN patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD3, CD4, CD8, CD19, CD56 and the Vα24Jα18, Vδ1, Vδ2 and Vδ3 TCR chains (Table 12). Frequencies and absolute numbers of lymphocyte subsets were analysed by flow cytometry. Absolute numbers were calculated from full blood counts taken at the time of blood collection (Table 19 and 26).

The median T cell frequencies, as percentages of lymphocytes, were higher in the ART-E than the ART-N children (p-value=0.033) though the absolute counts were higher in the ART-N compared to the ART-E children (p-value=0.045, Figure 67). The median absolute count of B cells was higher in the ART-N than ART-E children (p-value=0.015) while the median frequencies followed a similar pattern but were not significant (p-value=0.531, Figure 68). The frequencies and absolute cell counts of NK cells and $CD8^+$ T cells were similar in both groups; (Figure 69-70), although a non-significant decrease in $CD8^+$ T cells was observed. There was an increased trend observed in both the $CD4^+$ T cells absolute counts and frequencies, (Figure 71), in the ART-E than the ART-N children (p-value=0.0003 and 0.053 respectively). Both the median $CD4^-CD8^-$ T cell frequencies and numbers in WN children were increased (Figure 72) in the ART-N compared to the ART-E children (p-value=0.0002 and p-value<0.0001 respectively). The frequencies and absolute cell counts of $CD4^+CD8^+$ T cells, iNK T and NT cells were similar, although a non-significant increase in iNK T cells was observed among the ART-E children (Figure 73-75). Frequencies and absolute cell counts of γδ T cells were all increased in the ART-N children compared to the ART-E children, but this was not significant, (Figure 76-78). These results show that ART affects lymphocyte frequencies and numbers in children with HIV. These results are summarised in Table 47 and 48.

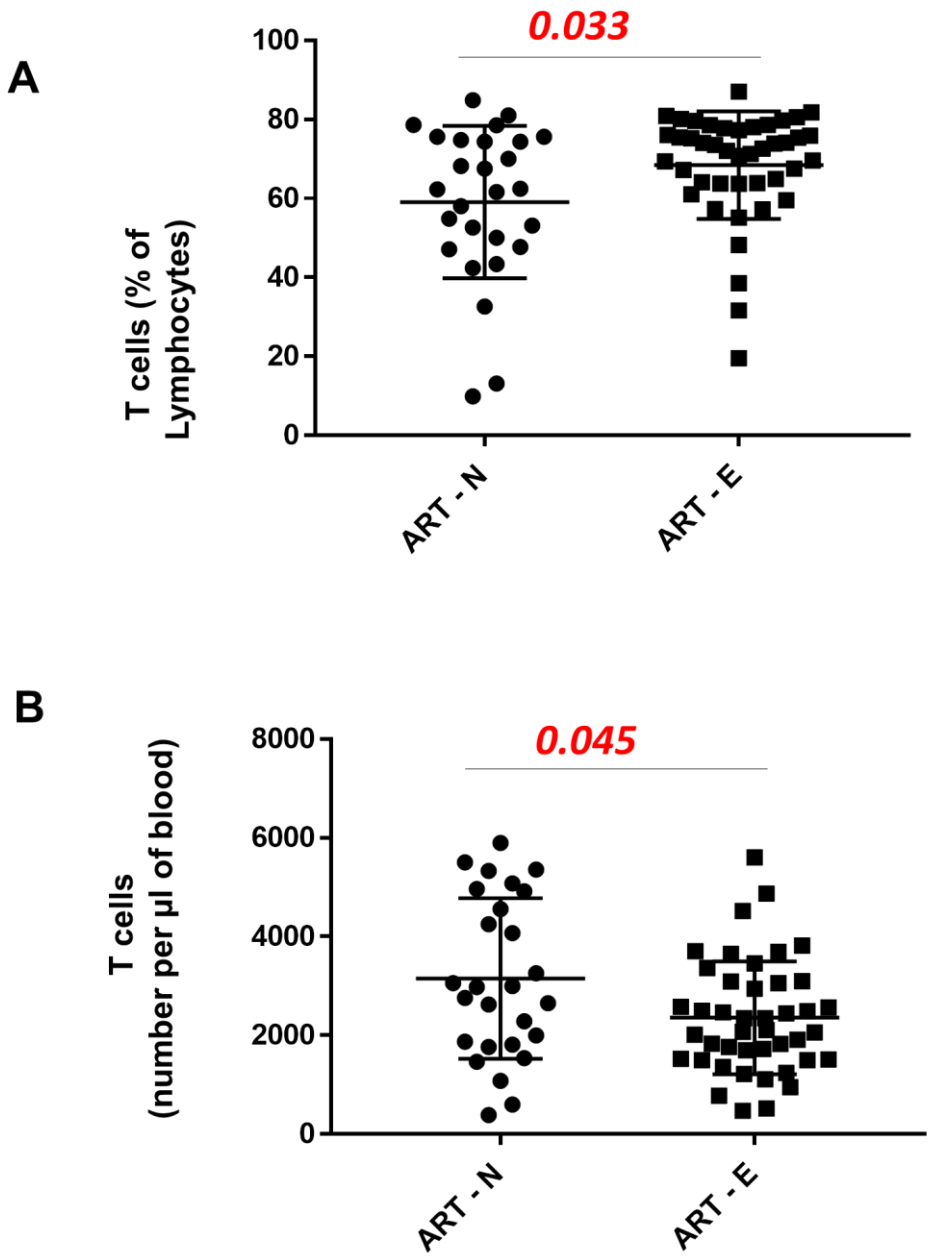


Figure 67: Effect of ART on circulating T cell frequencies and numbers in well-nourished children.

*PBMC were isolated from 27 ART-naïve well-nourished patients and 43 ART – experienced well-nourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD3 and analysed by flow cytometry. T cells were defined as lymphocytes that were positive for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

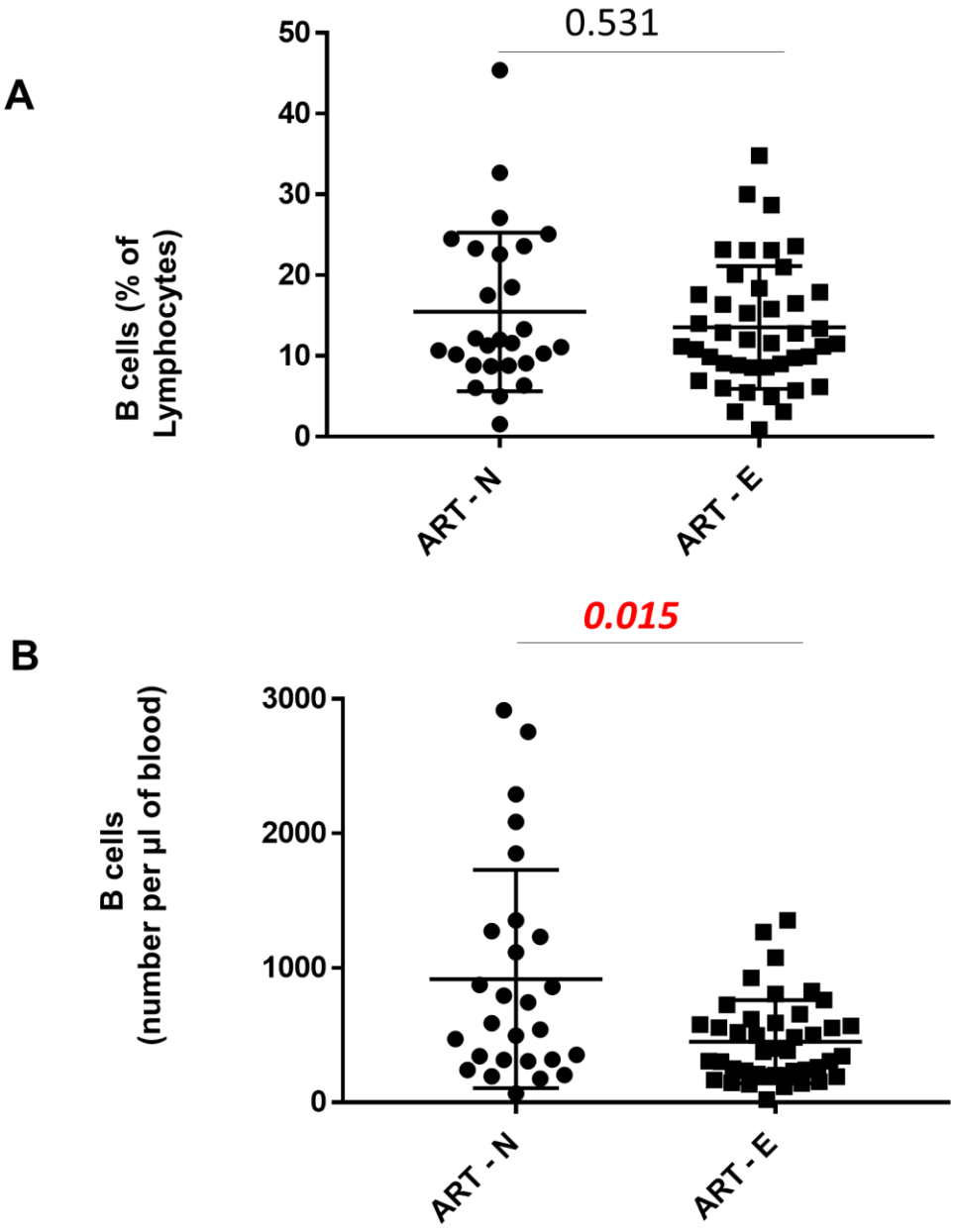


Figure 68: Effect of ART on circulating B cell frequencies and numbers in well-nourished children.

*PBMC were isolated from 27 ART-naïve well-nourished patients and 43 ART – experienced well-nourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD19 and CD3 and analysed by flow cytometry. B cells were defined as lymphocytes that were positive for CD19 and negative for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of B cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

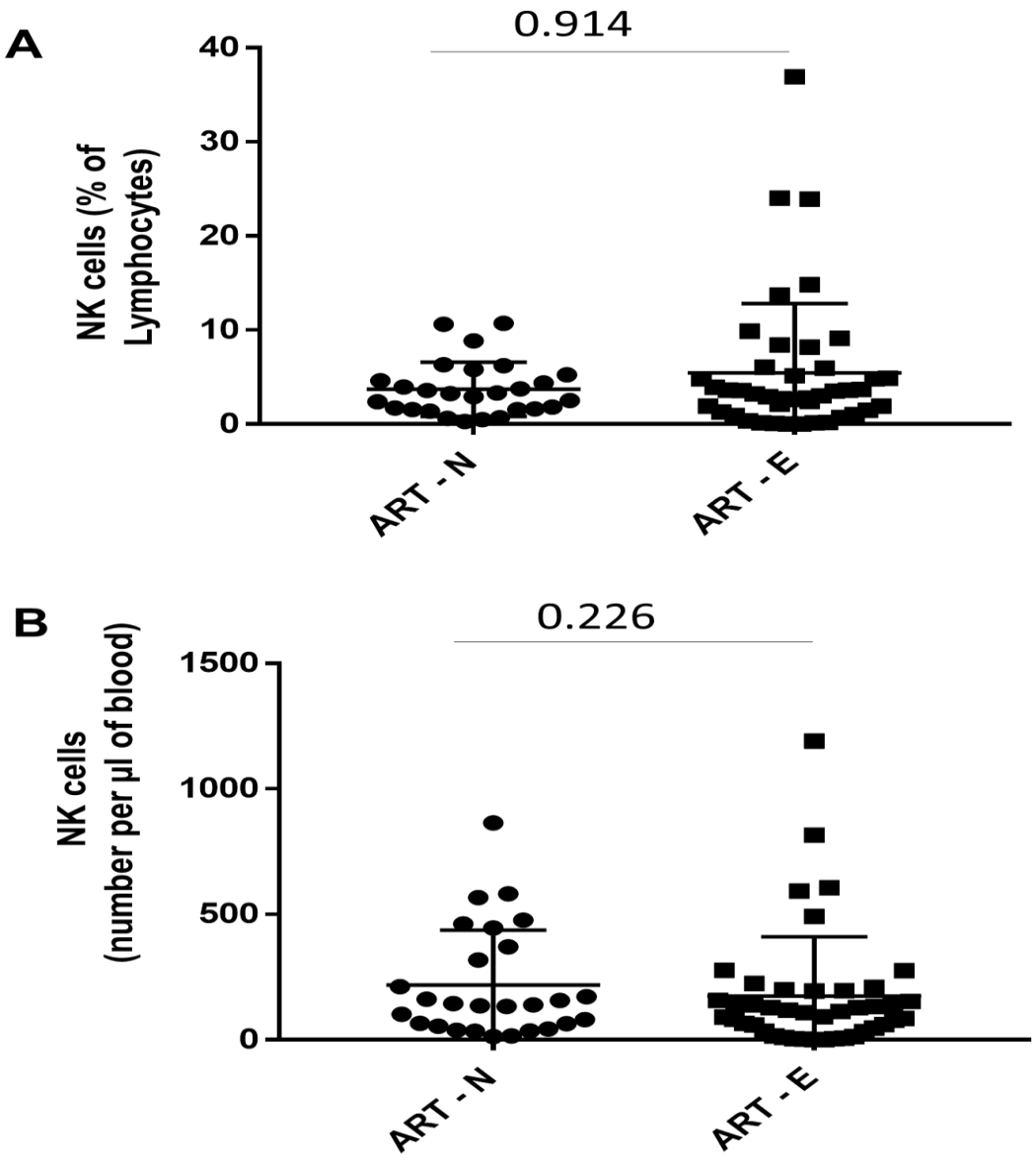


Figure 69: Effect of ART on circulating NK cell frequencies and numbers in well-nourished children.

*PBMC were isolated from 27 ART-naïve well-nourished patients and 43 ART – experienced well-nourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD56 and CD3 and analysed by flow cytometry. NK cells were defined as lymphocytes that were positive for CD56 and negative for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of NK cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

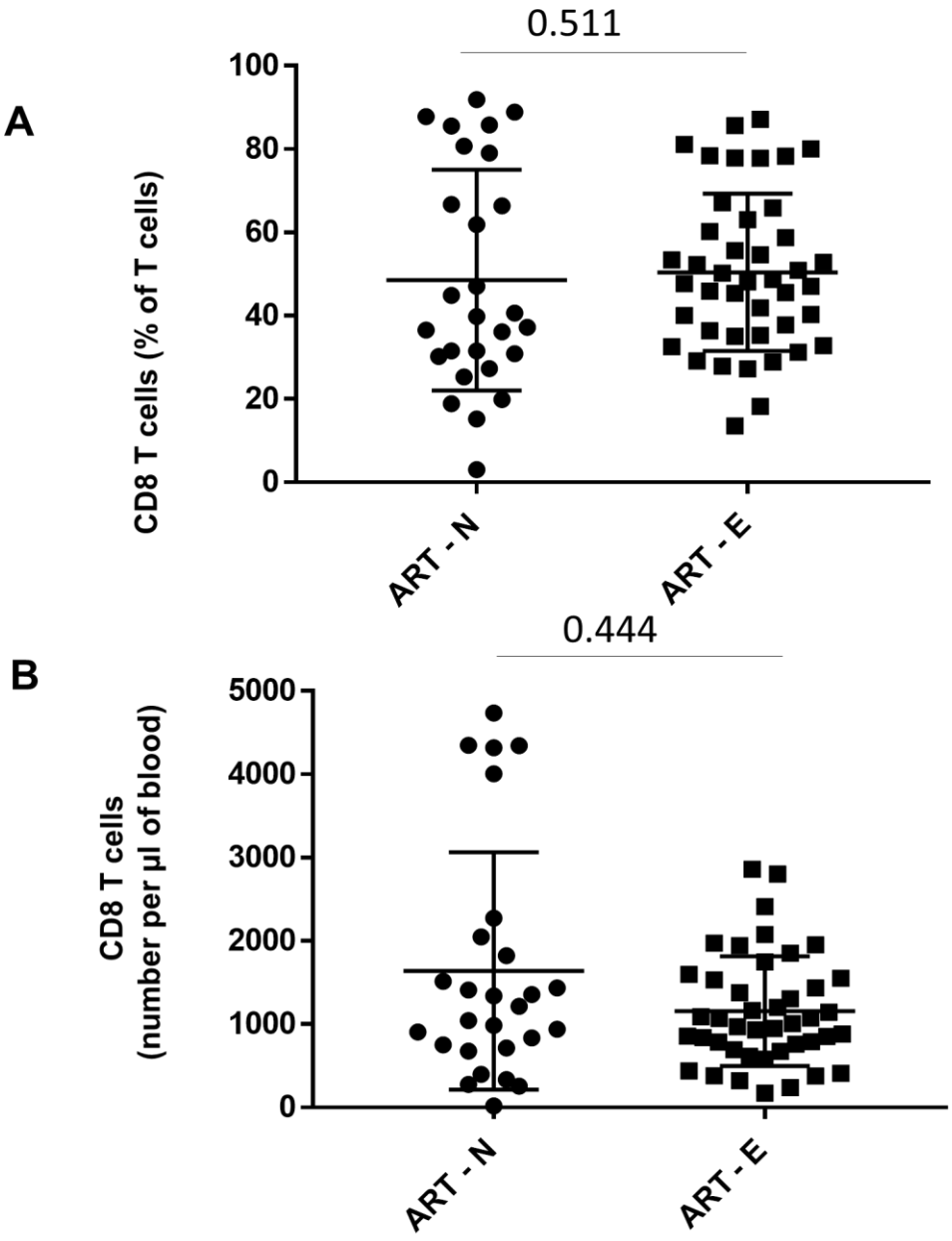


Figure 70: Effect of ART on circulating CD8 T cell frequencies and numbers in well-nourished children.

*PBMC were isolated from 27 ART-naïve well-nourished patients and 43 ART – experienced well-nourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD4, CD8 and CD3 and analysed by flow cytometry. CD8 T cells were defined as lymphocytes that were positive for CD8 and CD3 and negative for CD4. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD8 cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

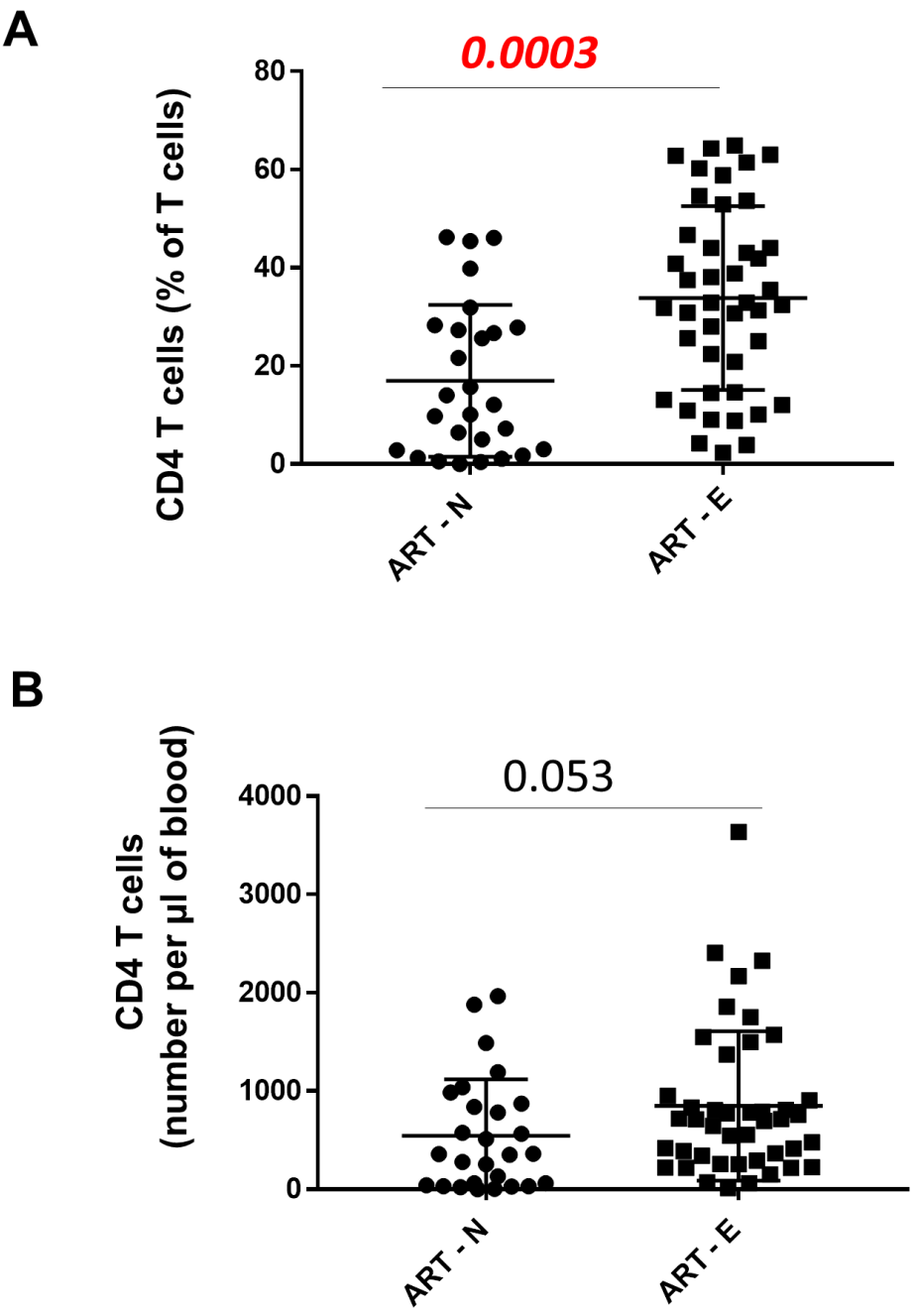


Figure 71: Effect of ART on circulating CD4⁺ T cell frequencies and numbers in well-nourished children.

*PBMC were isolated from 27 ART-naïve well-nourished patients and 43 ART – experienced well-nourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD4, CD8 and CD3 and analysed by flow cytometry. CD4⁺ T cells were defined as lymphocytes that were positive for CD4 and CD3 and negative for CD8. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD4 cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

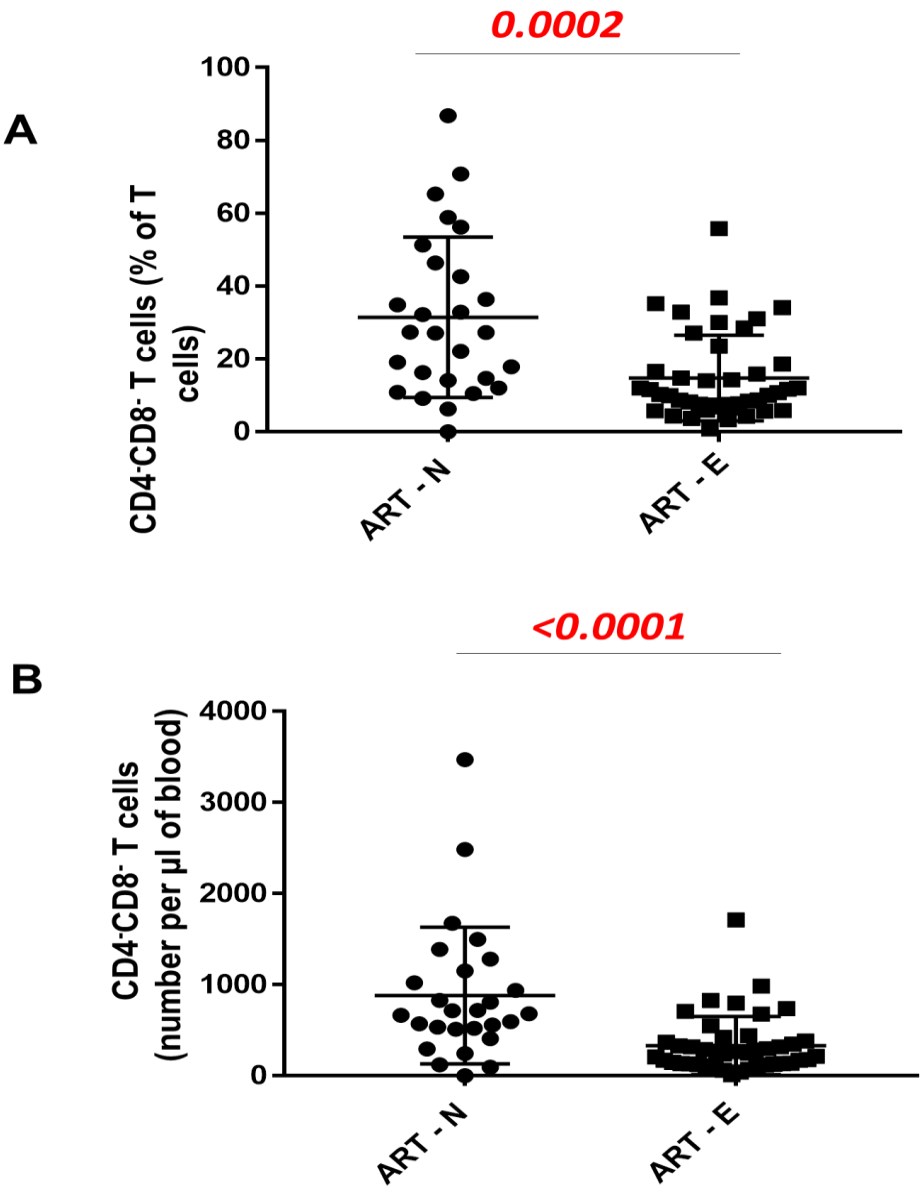


Figure 72: Effect of ART on circulating CD4-CD8- T cell frequencies and numbers in well-nourished children.

*PBMC were isolated from 27 ART-naïve well-nourished patients and 43 ART–experienced well-nourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD4, CD8 and CD3 and analysed by flow cytometry. CD4-CD8- T cells were defined as T cells lymphocytes that were positive for CD3 and negative for CD4 and CD8. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD4-CD8- T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

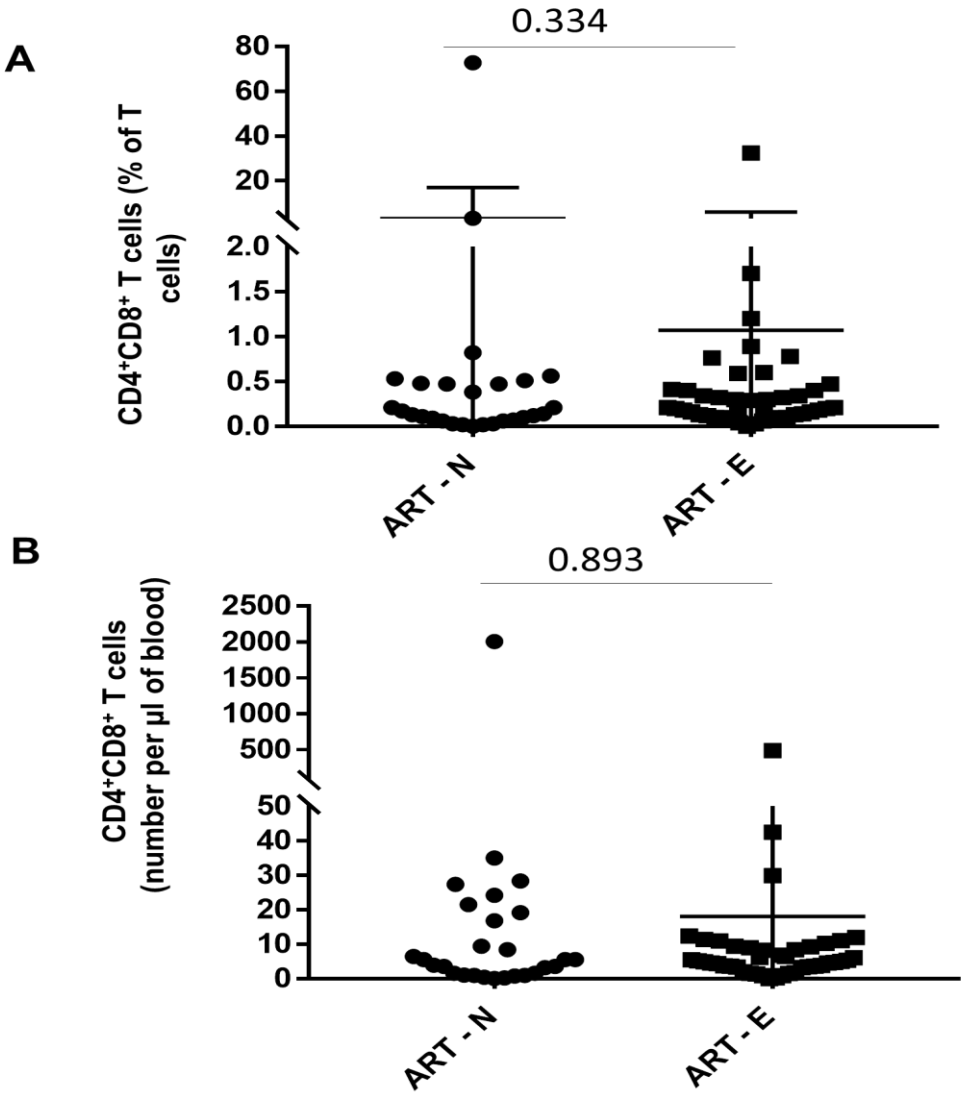


Figure 73: Effect of ART on circulating CD4⁺CD8⁺ T cell frequencies well-nourished children.

*PBMC were isolated from 27 ART-naïve well-nourished patients and 43 ART – experienced well-nourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD4, CD8 and CD3 and analysed by flow cytometry. CD4⁺CD8⁺ T cells were defined as T cells lymphocytes that were positive for CD3, CD4 and CD8. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD4⁺CD8⁺ T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

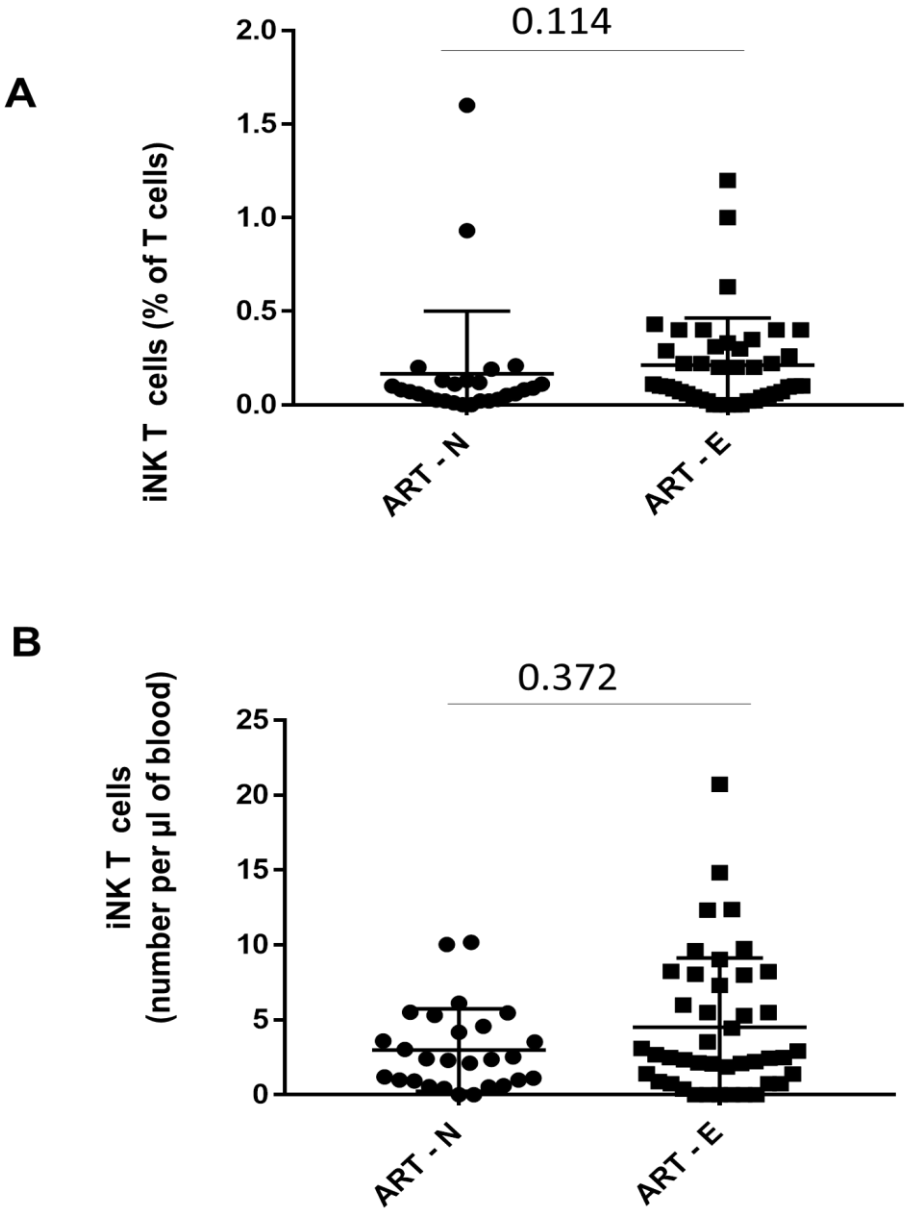


Figure 74: Effect of ART on circulating iNK T cell frequencies and numbers well-nourished children.

*PBMC were isolated from 27 ART-naïve well-nourished patients and 43 ART – experienced well-nourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for Vα24Jα18 and CD3 and analysed by flow cytometry. iNK T cells were defined as lymphocytes that were positive for Vα24Jα18 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of iNK T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

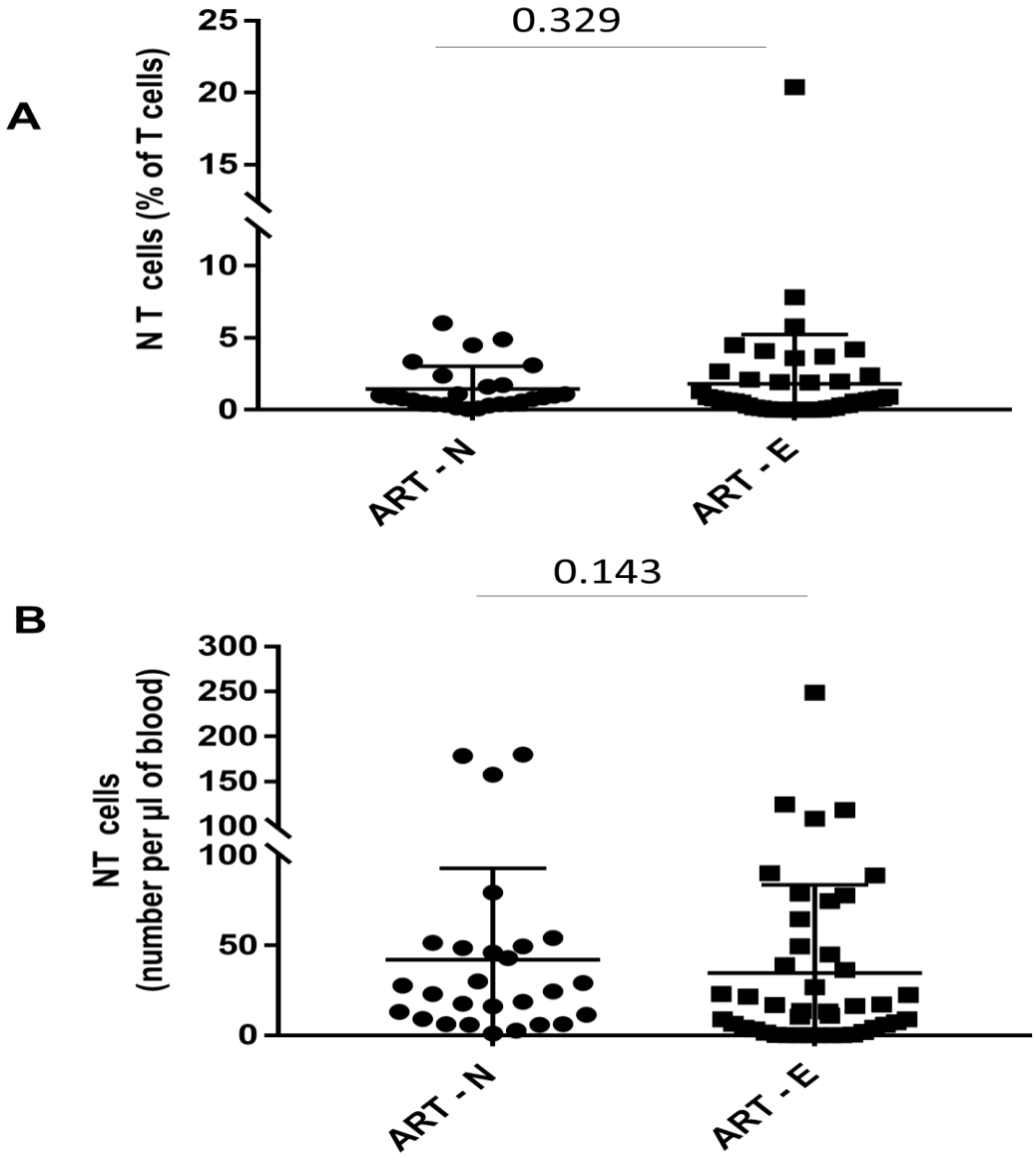


Figure 75: Effect of ART on circulating NT cell frequencies and numbers well-nourished children.

*PBMC were isolated from 27 ART-naïve well-nourished patients and 43 ART – experienced well-nourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for CD56 and CD3 and analysed by flow cytometry. NT cells were defined as lymphocytes that were positive for CD3 and CD56. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of NT cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

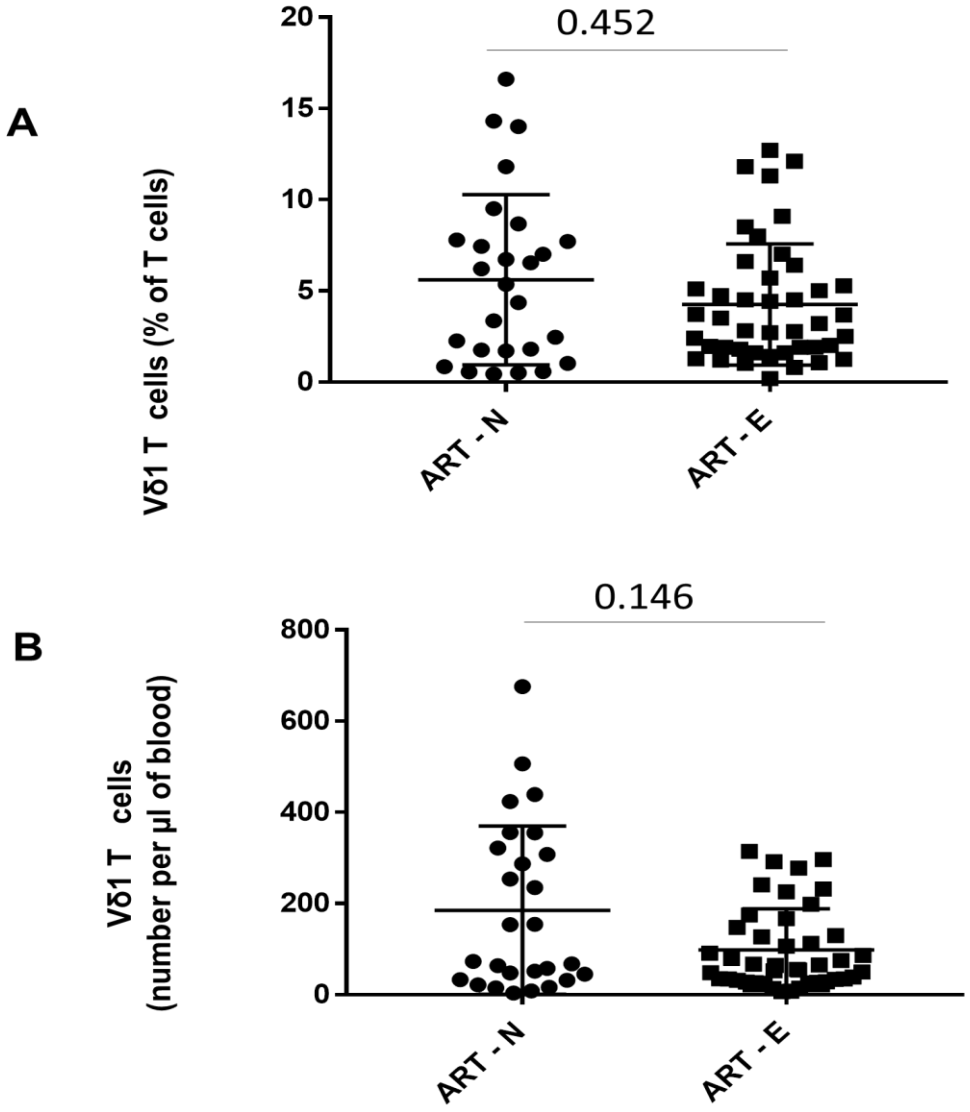


Figure 76: Effect of ART on circulating Vδ1 T cell frequencies and numbers in well-nourished children.

*PBMC were isolated from 27 ART-naïve well-nourished patients and 43 ART – experienced well-nourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for Vδ1 and CD3 and analysed by flow cytometry. Vδ1 cells were defined as lymphocytes that were positive for Vδ1 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ1 cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

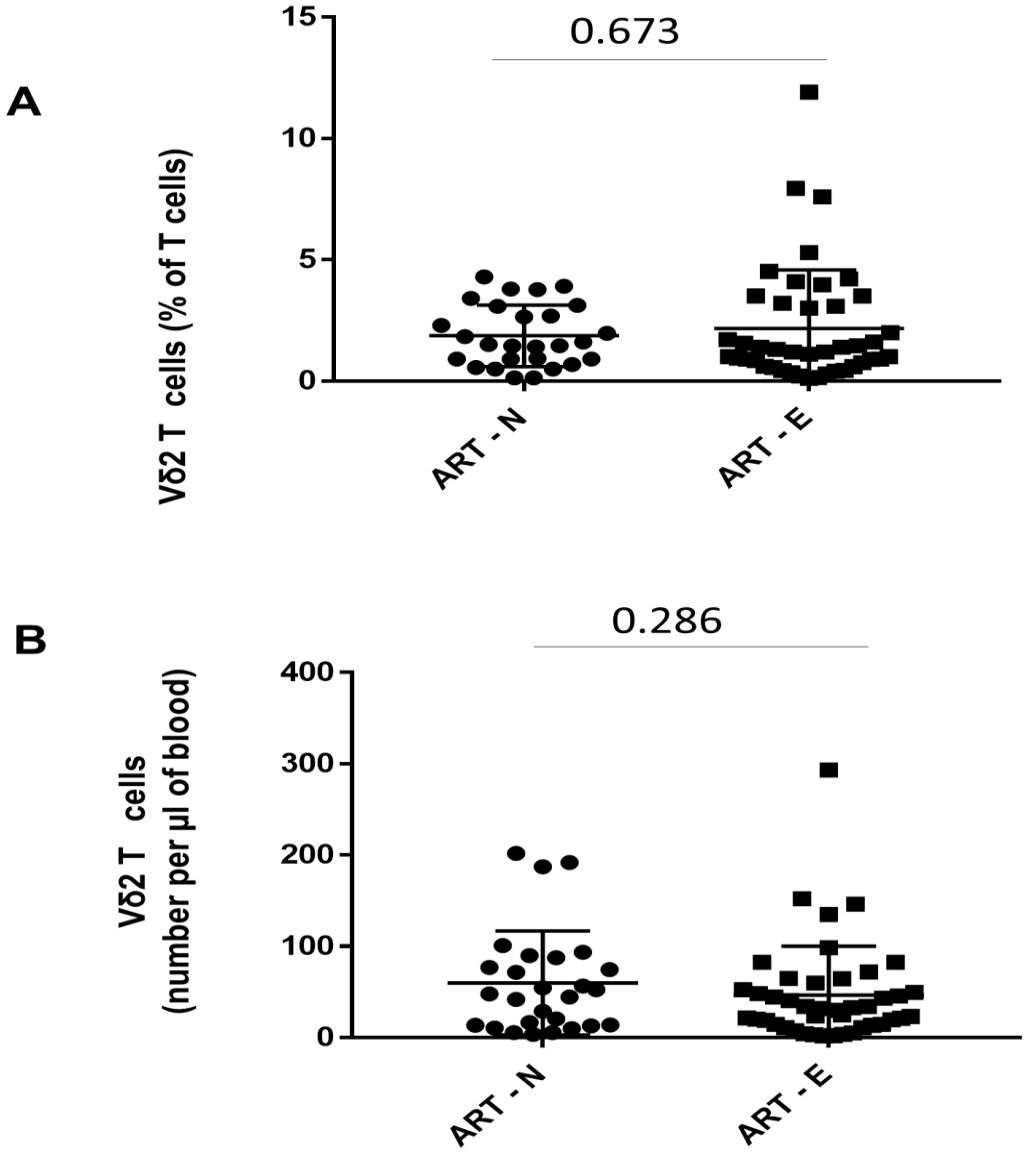


Figure 77: Effect of ART on circulating Vδ2 T cell frequencies and numbers in well-nourished children.

*PBMC were isolated from 27 ART-naïve well-nourished patients and 43 ART – experienced well-nourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for Vδ2 and CD3 and analysed by flow cytometry. Vδ2 cells were defined as lymphocytes that were positive for Vδ2 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ2 cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

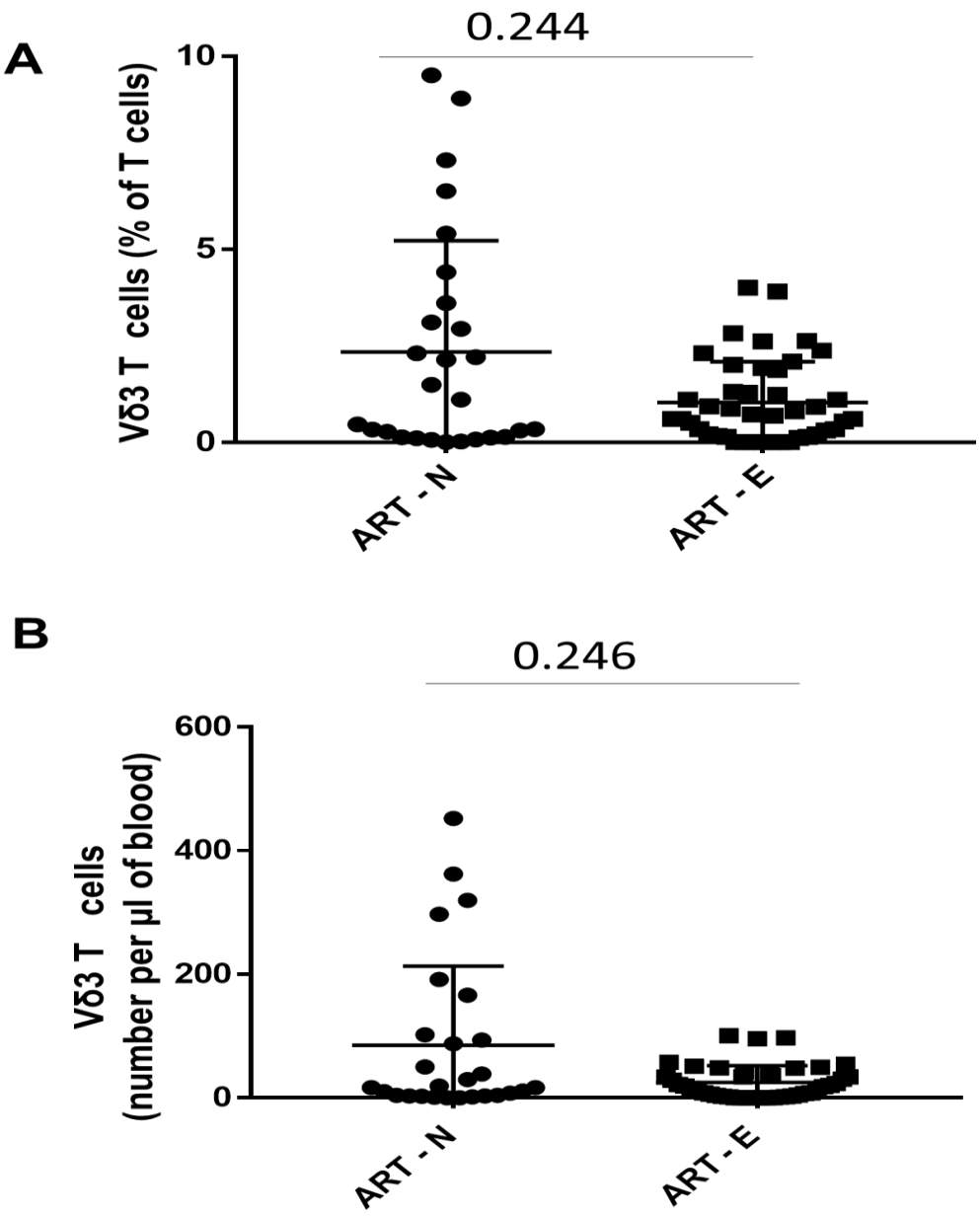


Figure 78: Effect of ART on circulating Vδ3 T cell frequencies and numbers in well-nourished children.

*PBMC were isolated from 27 ART-naïve well-nourished patients and 43 ART – experienced well-nourished patients with HIV infection. Cells were stained with monoclonal antibodies specific for Vδ3 and CD3 and analysed by flow cytometry. Vδ3 cells were defined as lymphocytes that were positive for Vδ3 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ3 cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

Table 47. Summary results on effects of ART on lymphocyte subset frequencies in well-nourished HIV infected children at baseline.

Cells	Median frequencies of ART-N	Median frequencies of ART-E	P-value
T cells	**62.25**	**72.65**	**0.033***
B cells	**11.6**	**11.6**	**0.531**
NK cells	**3.25**	**3.2**	**0.914**
$CD8^+$ T cell	**39.8**	**48.1**	**0.511**
$CD4^+$ T cell	**12.1**	**32.9**	**0.0003*****
$CD4^-CD8^-$ T cell	**27.3**	**10.3**	**0.0002********
$CD4^+CD8^+$ T cell	**0.14**	**0.21**	**0.334**
iNK T cell	**0.08**	**0.1**	**0.114**
NT cell	**0.86**	**068**	**0.329**
Vδ1 T cell	**5.36**	**3.2**	**0.452**
Vδ2 T cell	**1.5**	**1.2**	**0.673**
Vδ3 T cell	**1.1**	**0.69**	**0.244**

*Indicates the median frequency of T cells was higher in ART-E then ART-N, HIV infected children
***Indicates the median frequency of $CD4^+$ T cells were lower in ART-N than ART-E, HIV infected children
**** Indicates the median frequency of DN were higher in ART-N than ART-E, HIV infected children

Table 48. Summary results on effects of ART on lymphocyte subset numbers in well-nourished HIV infected children at baseline.

Cells	Median absolute counts of ART-N	Median absolute counts of ART-E	P value
T cells	**2980**	**2103**	**0.045***
B cells	**588.3**	**375.9**	**0.015****
NK cells	**3.25**	**3.2**	**0.226**
$CD8^+$ T cell	**1216**	**1007**	**0.444**
$CD4^+$ T cell	**358**	**711.4**	**0.053**
$CD4^-CD8^-$ T cell	**681.6**	**249**	**<0.0001****
$CD4^+CD8^+$ T cell	**5.5**	**4.96**	**0.893**
iNK T cell	**2.36**	**2.5**	**0.372**
NT cell	**24.43**	**13.58**	**0.143**
Vδ1 T cell	**73.01**	**64.31**	**0.146**
Vδ2 T cell	**47.68**	**31.74**	**0.286**
Vδ3 T cell	**16.88**	**18.45**	**0.246**

* Indicates median absolute count of T cells was higher in ART-N than ART-E, HIV infected children
**Indicates the median absolute count of B cells were higher in ART-N than ART-E, HIV infected children
***Indicates the median absolute count of $CD4^+$ T cells were lower in ART-N than ART-E, HIV infected children
**** Indicates the median absolute count of DN were higher in ART-N than ART-E, HIV infected children

Objective 5: Effects of RUTF supplementation on lymphocyte subset frequencies and numbers in ART-experienced malnourished children.

The effects of RUTF supplementation on lymphocyte subset frequencies and numbers in ART-E malnourished children was assessed by isolating PBMC from 40 ART-experienced malnourished children with HIV infection at baseline and 23 of these patients after 12 weeks of RUTF supplementation. By studying a cohort of ART-E malnourished children at baseline and 12 weeks, we were able to investigate the effect of RUFT alone. Cells were stained with monoclonal antibodies specific for CD3, CD4, CD8, CD19, CD56 and the Vα24Jα18, Vδ1, Vδ2 and Vδ3 TCR chains (Table12), frequencies and absolute numbers of lymphocyte subsets were analysed by flow cytometry. Absolute numbers were calculated from full blood counts taken at the time of blood collection (Table 30).

The frequencies and absolute counts of total T cells were similar at baseline and after 12 weeks of RUTF supplementation, (Figure 79). We observed an increase in B cell frequencies and absolute counts at 12 weeks (Figure 80). The absolute B cell increase was nearly statistically significant with a p-value=0.06 while the frequency change was not significant (p-value=0.272). The absolute counts and frequencies of NK cells, increased at 12 weeks and were statistically significant (p-value=0.01 and p-value=0.026 respectively, Figure 81).

As percentages of total T cells, frequencies and absolute $CD8^+$ T cell counts decreased at 12 weeks and the changes were statistically significant (p-value=0.026 and p-value=0.01 respectively, Figure 82). The $CD4^+$ T cell frequencies were surprisingly higher at baseline than 12 weeks (p-value=0.094) while the absolute counts were higher at 12 weeks (p-value=0.066, Figure 83). The absolute counts and frequencies of $CD4^-CD8^-$ T cells were higher at 12 weeks, (Figure 84), and statistically significant (p-value<0.0001 and p-value<0.0001 respectively). We observed an increased trend in frequencies and absolute counts of $CD4^+CD8^+$ T cells at 12 weeks although this difference as not significant, (Figure 85).

Of the 3 subsets of γδT cells measured, only Vδ2 T cell frequencies were significantly altered in patient groups and surprisingly increased at 12 weeks, (p-value=0.11 and p-value=0.033 respectively, Figure 88–Figure 90). These results show that nutritional

supplementation affects lymphocyte subsets numbers in malnourished HIV children. These results are summarised in Table 49 and 50.

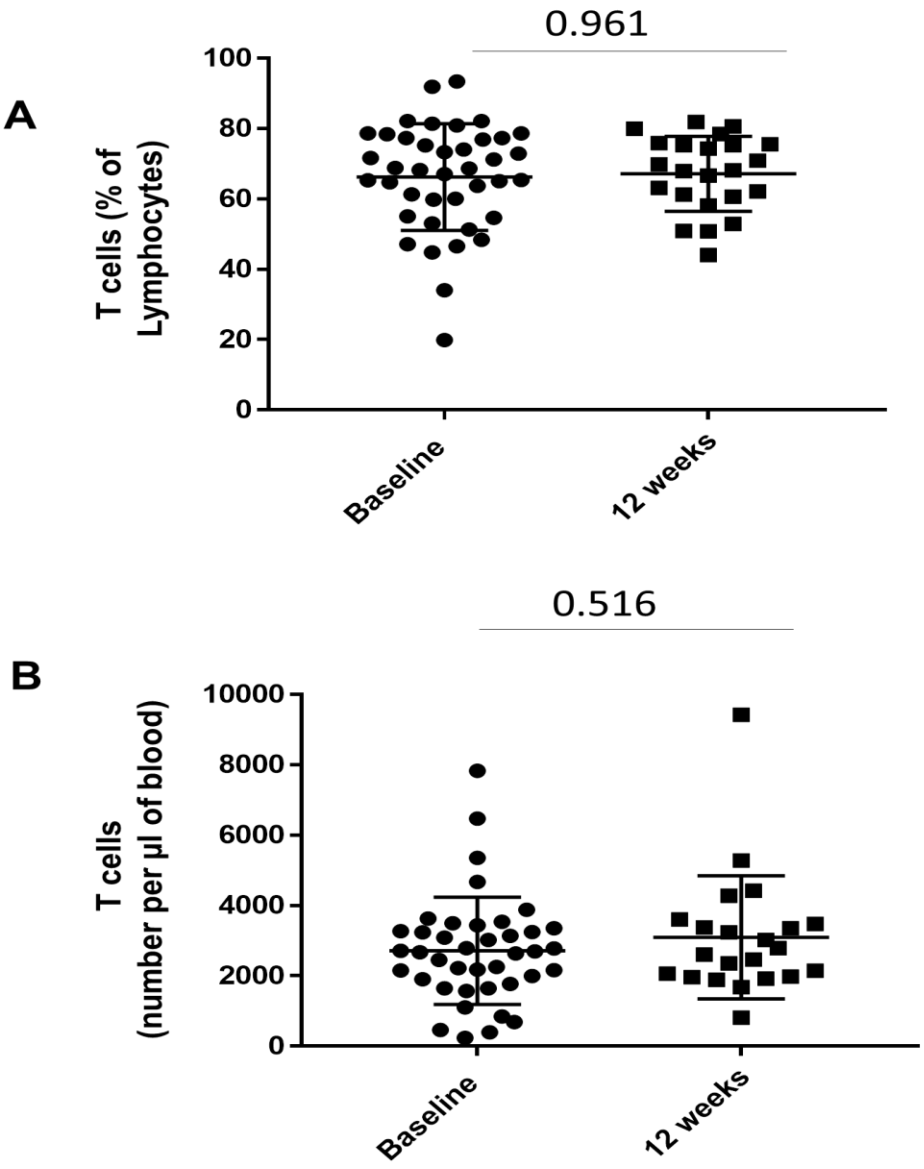


Figure 79: Effect of RUTF supplementation on circulating T cell frequencies and numbers in malnourished ART experienced children with HIV infection.

*PBMC were isolated from 40 ART-experienced malnourished children with HIV infection at baseline and 23 of these patients after 12 weeks of RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD3 and analysed by flow cytometry. T cells were defined as lymphocytes that were positive for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

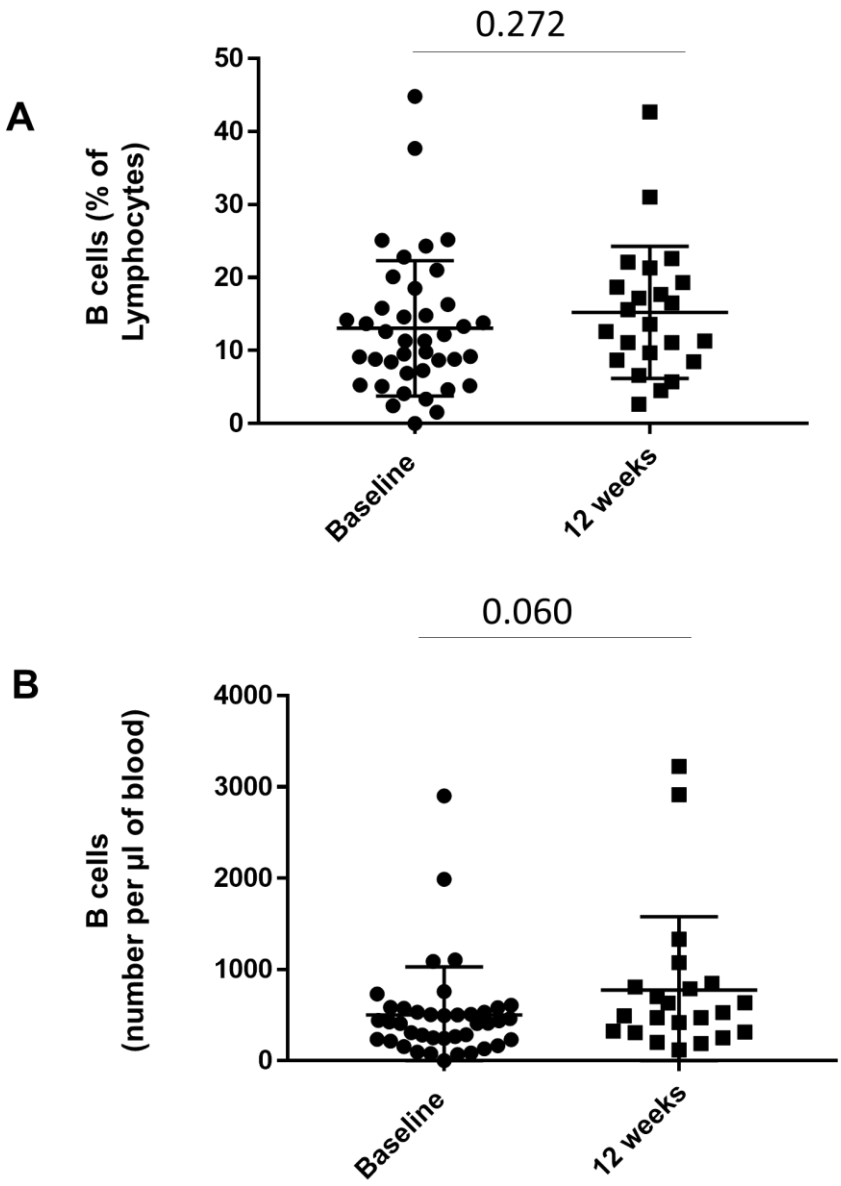


Figure 80: Effect of RUTF supplementation on circulating B cell frequencies and numbers in malnourished ART experienced children with HIV infection.

*PBMC were isolated from 40 ART experienced malnourished children with HIV infection at baseline and 23 of these patients after 12 weeks of RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD19 and analysed by flow cytometry. T cells were defined as lymphocytes that were positive for CD19 and negative for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of B cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

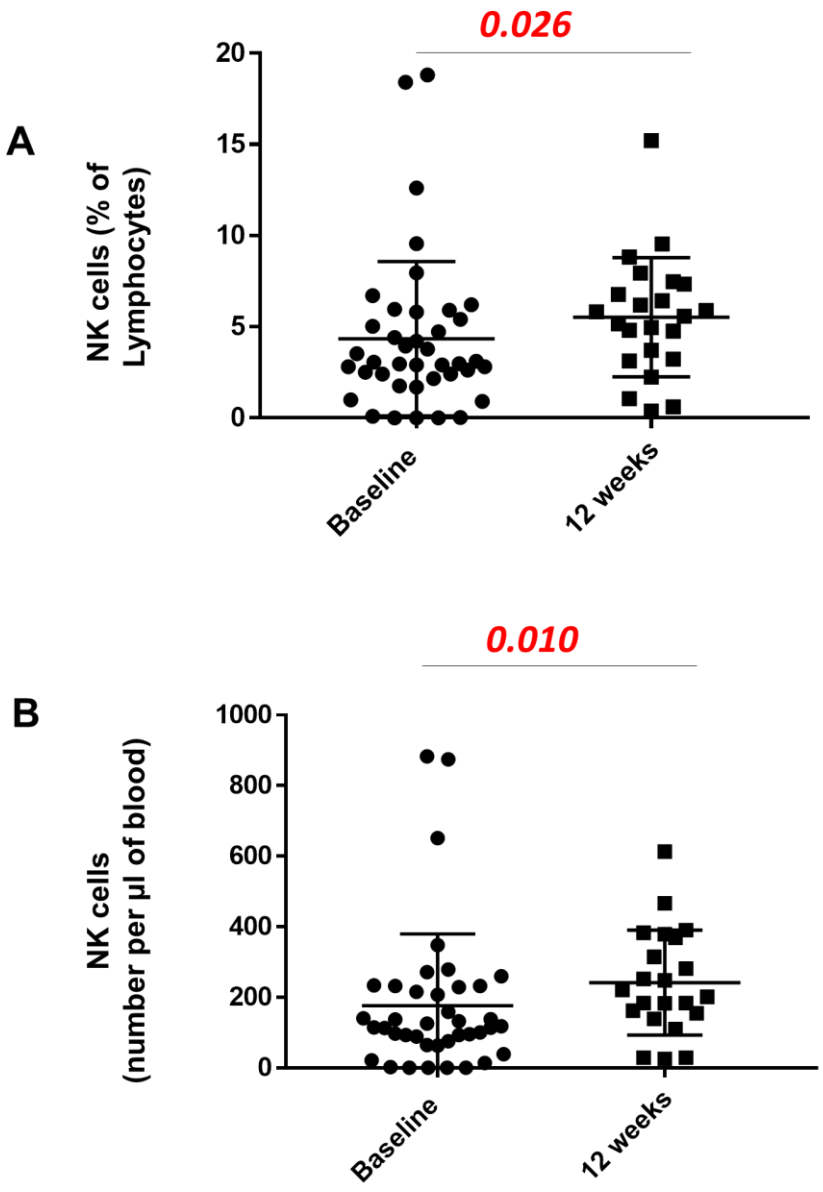


Figure 81: Effect of RUTF supplementation on circulating NK cell frequencies and numbers in malnourished ART experienced children with HIV infection.

*PBMC were isolated from 40 ART experienced malnourished children with HIV infection at baseline and 23 of these patients after 12 weeks of RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD56 and CD3 and analysed by flow cytometry. NK cells were defined as lymphocytes that were positive for CD56 and negative for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD56 cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

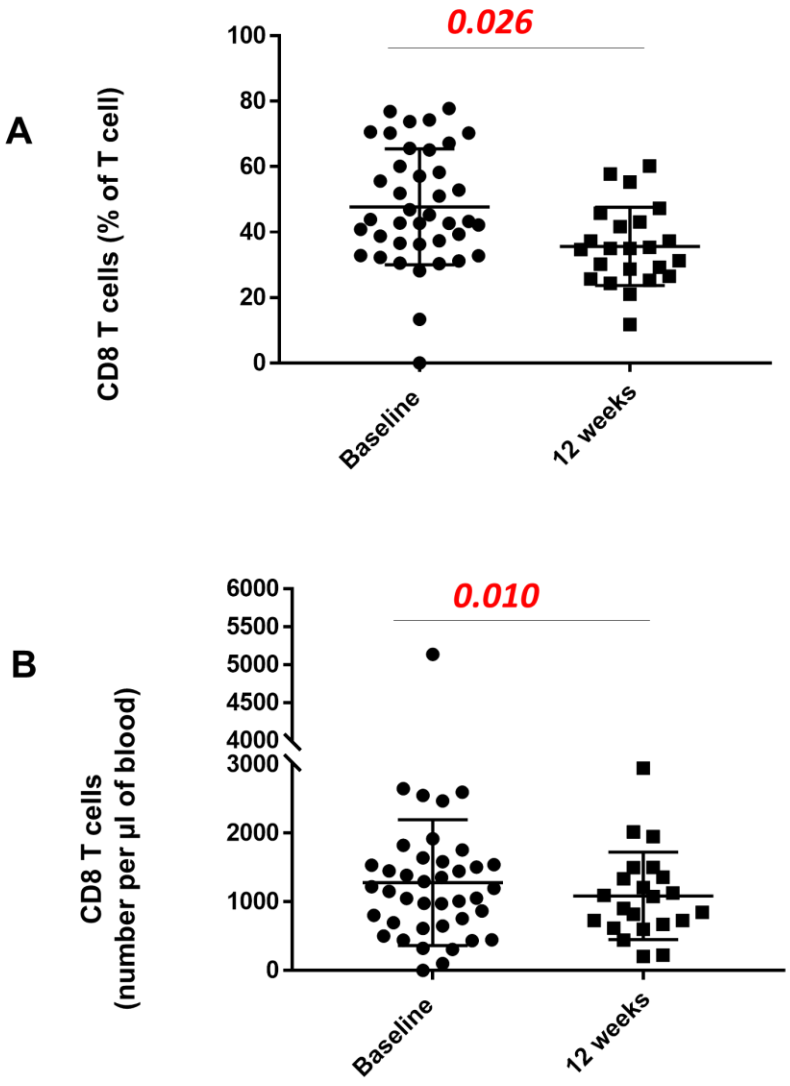


Figure 82: Effect of RUTF supplementation on circulating CD8 T cell frequencies and numbers in malnourished ART experienced children with HIV infection.

*PBMC were isolated from 40 ART experienced malnourished children with HIV infection at baseline and 23 of these patients after 12 weeks of RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD4, CD8 and CD3 and analysed by flow cytometry. CD8 cells were defined as lymphocytes that were positive for CD8 and CD3 and negative for CD4. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD8 cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

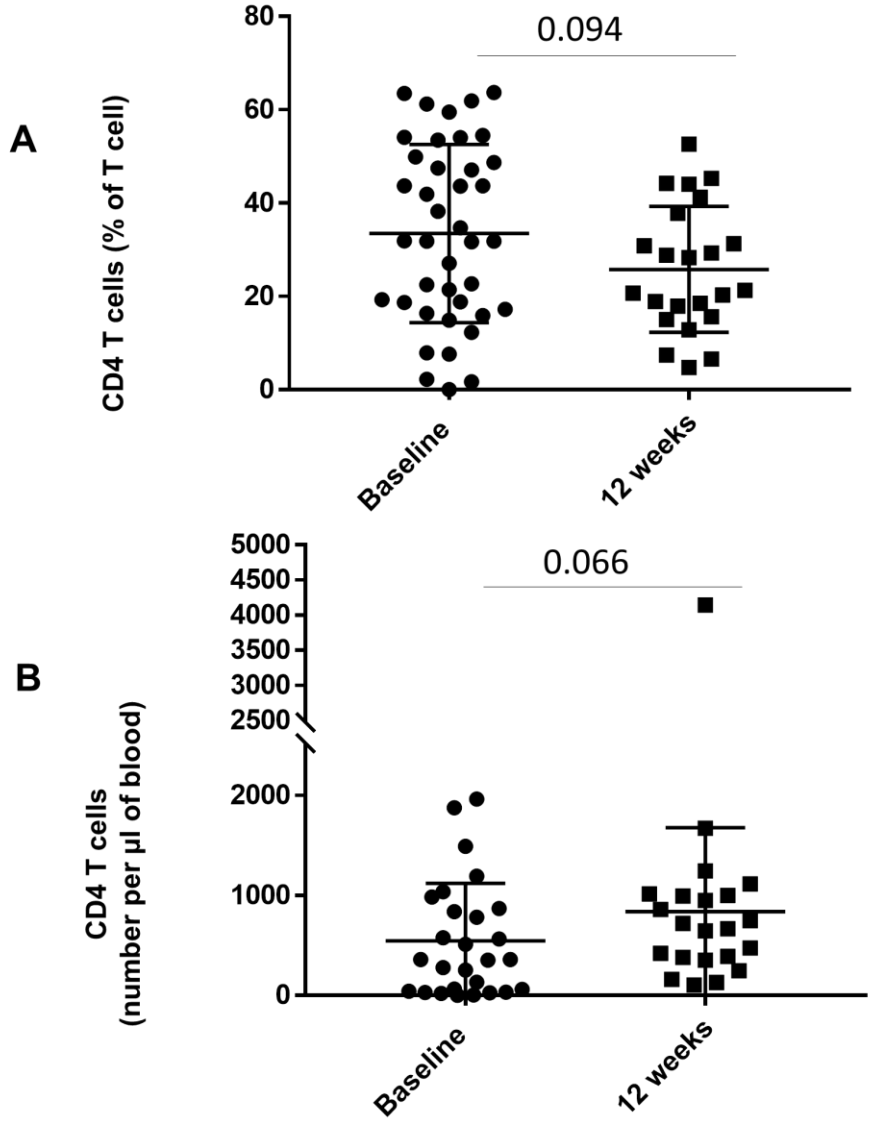


Figure 83: Effect of RUTF supplementation on circulating CD4 T cell frequencies and numbers in malnourished ART experienced children with HIV infection.
*PBMC were isolated from 40 ART experienced malnourished children with HIV infection at baseline and 23 of these patients after 12 weeks of RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD4, CD8 and CD3 and analysed by flow cytometry. CD4 cells were defined as lymphocytes that were positive for CD4 and CD3 and negative for CD8. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD4 cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

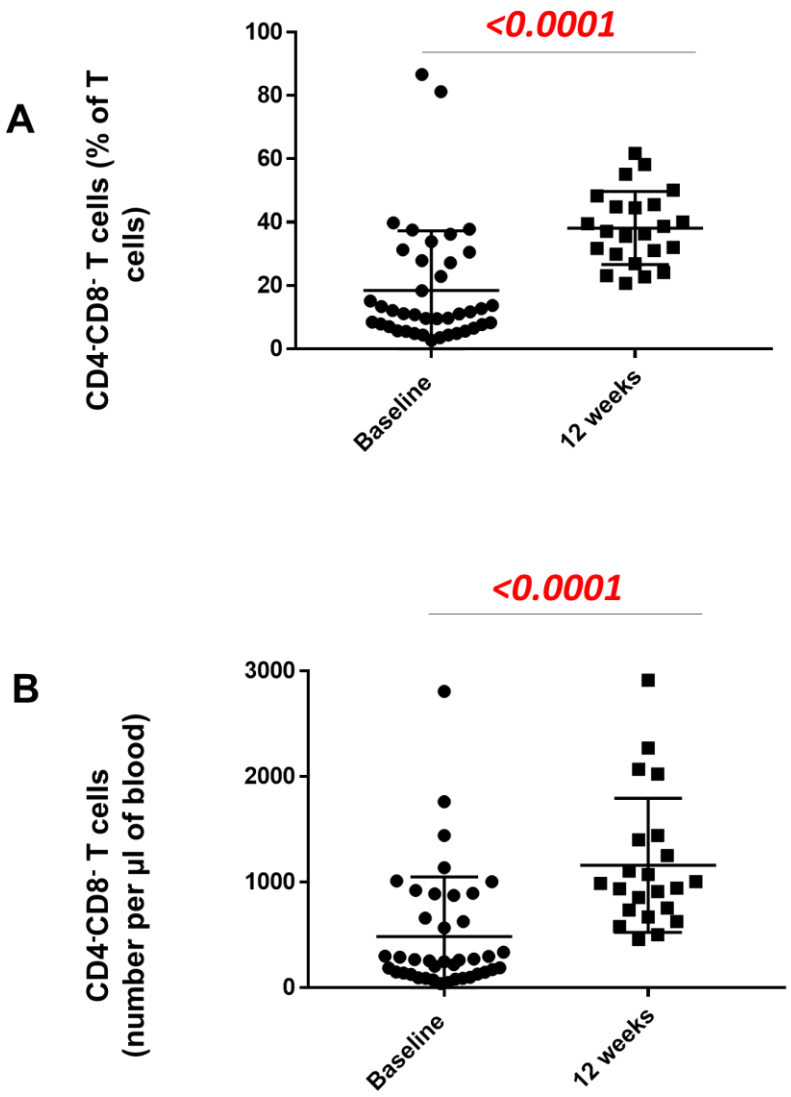


Figure 84: Effect of RUTF supplementation on circulating CD4-CD8- T cell frequencies and numbers in malnourished ART experienced children with HIV infection.

*PBMC were isolated from 40 ART experienced malnourished children with HIV infection at baseline and 23 of these patients after 12 weeks of RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD4, CD8 and CD3 and analysed by flow cytometry. CD4-CD8- T cells were defined as lymphocytes that were negative for CD4 and CD8 and positive for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD4-CD8- cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

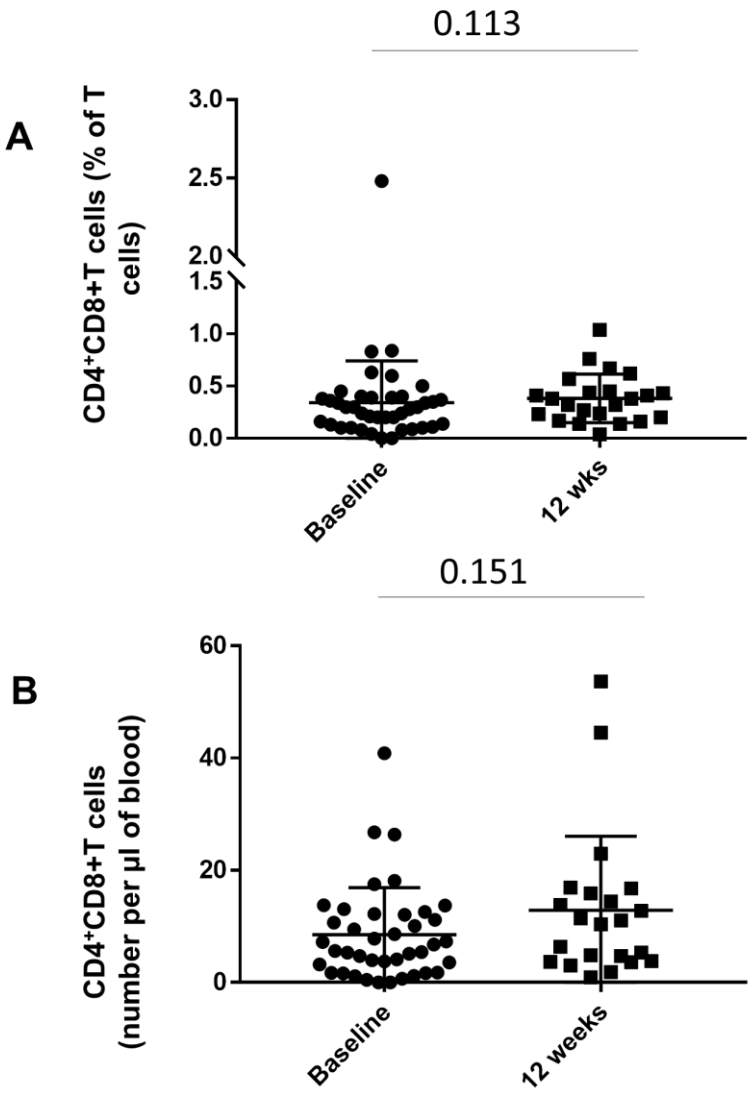


Figure 85: Effect of RUTF supplementation on circulating $CD4^+CD8^+$ T cell frequencies and numbers in malnourished ART experienced children with HIV infection.

*PBMC were isolated from 40 ART experienced malnourished children with HIV infection at baseline and 23 of these patients after 12 weeks of RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD4, CD8 and CD3 and analysed by flow cytometry. $CD4^+CD8^+$ cells were defined as lymphocytes that were positive for CD4, CD8 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of $CD4^+CD8^+$ cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

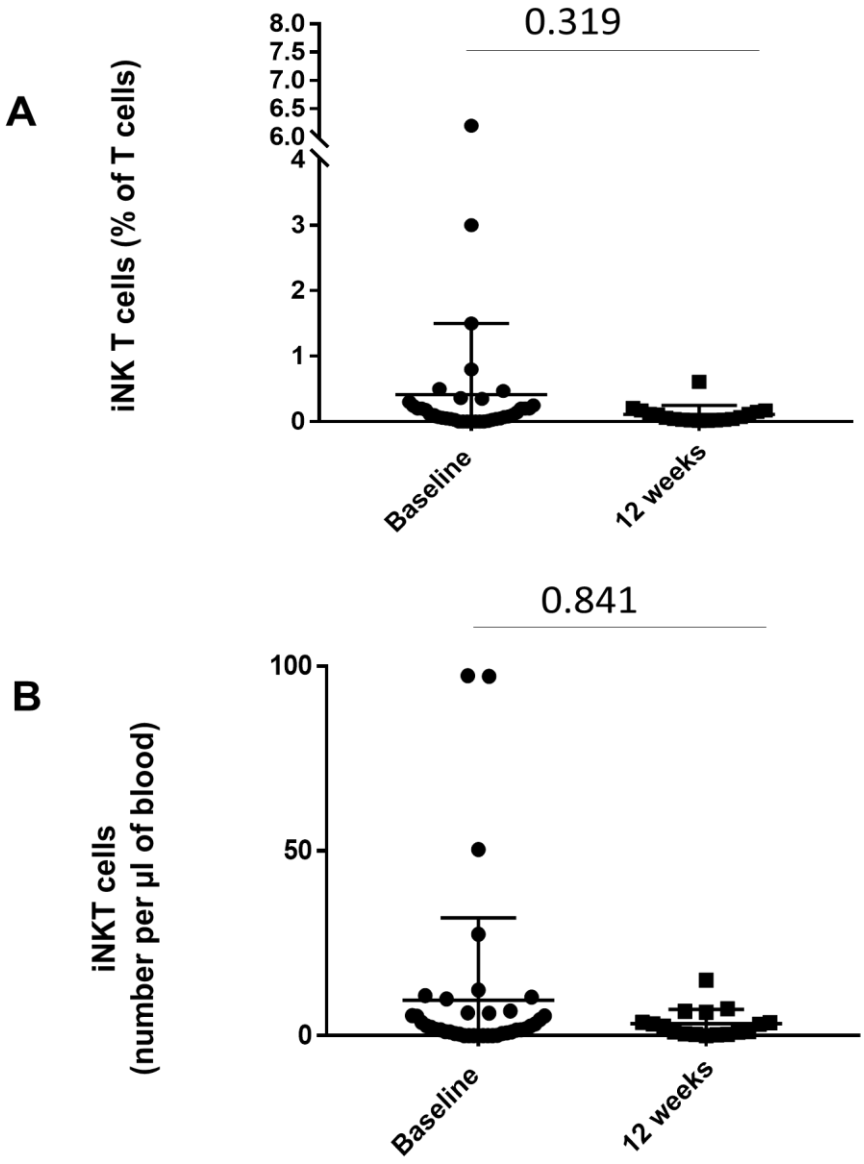


Figure 86: Effect of RUTF supplementation on circulating iNK T cell frequencies and numbers in malnourished ART experienced children with HIV infection.
*PBMC were isolated from 40 ART experienced malnourished children with HIV infection at baseline and 23 of these patients after 12 weeks of RUTF supplementation. Cells were stained with monoclonal antibodies specific for Vα24Jα18 and CD3 and analysed by flow cytometry. iNK T cells were defined as lymphocytes that were positive for Vα24Jα18 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of iNK T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

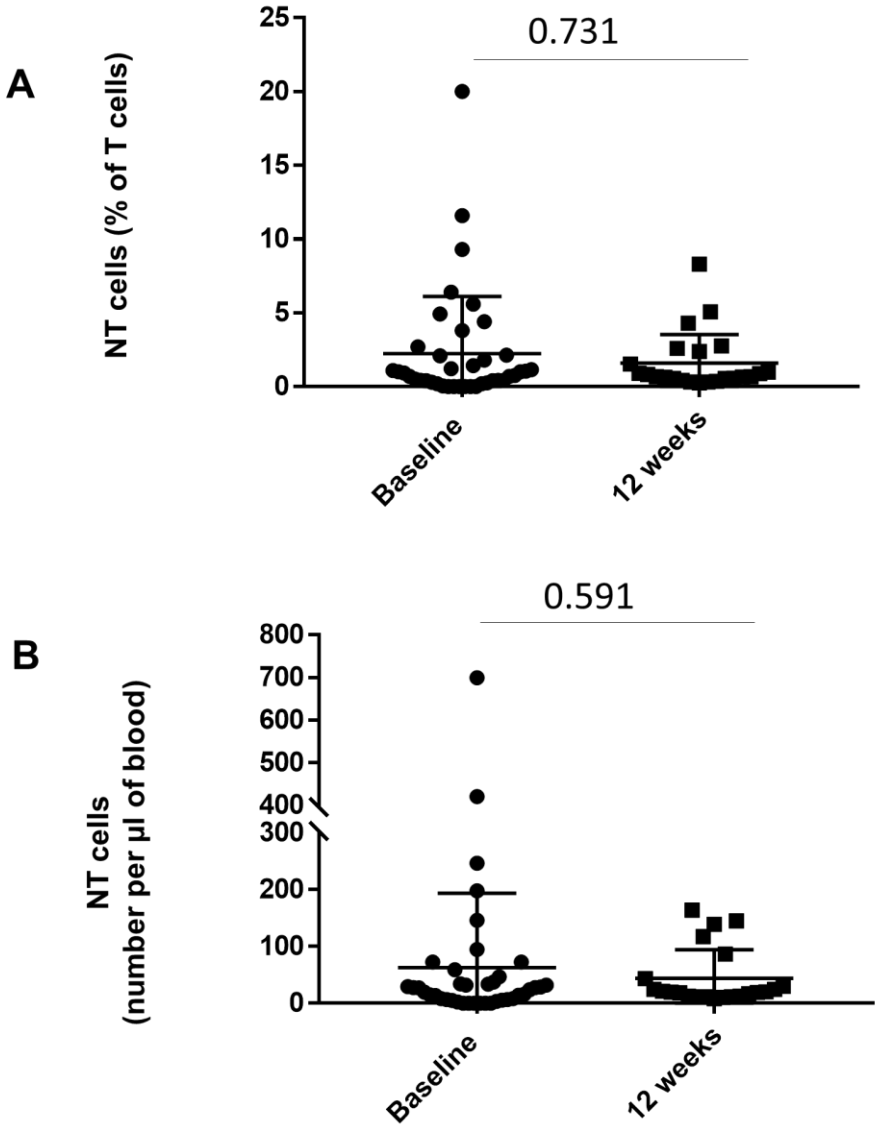


Figure 87: Effect of RUTF supplementation on circulating NT cell frequencies and numbers in malnourished ART experienced children with HIV infection.
*PBMC were isolated from 40 ART experienced malnourished children with HIV infection at baseline and 23 of these patients after 12 weeks of RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD56 and CD3 and analysed by flow cytometry. NT cells were defined as lymphocytes that were positive for CD3 and CD56. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of NT cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

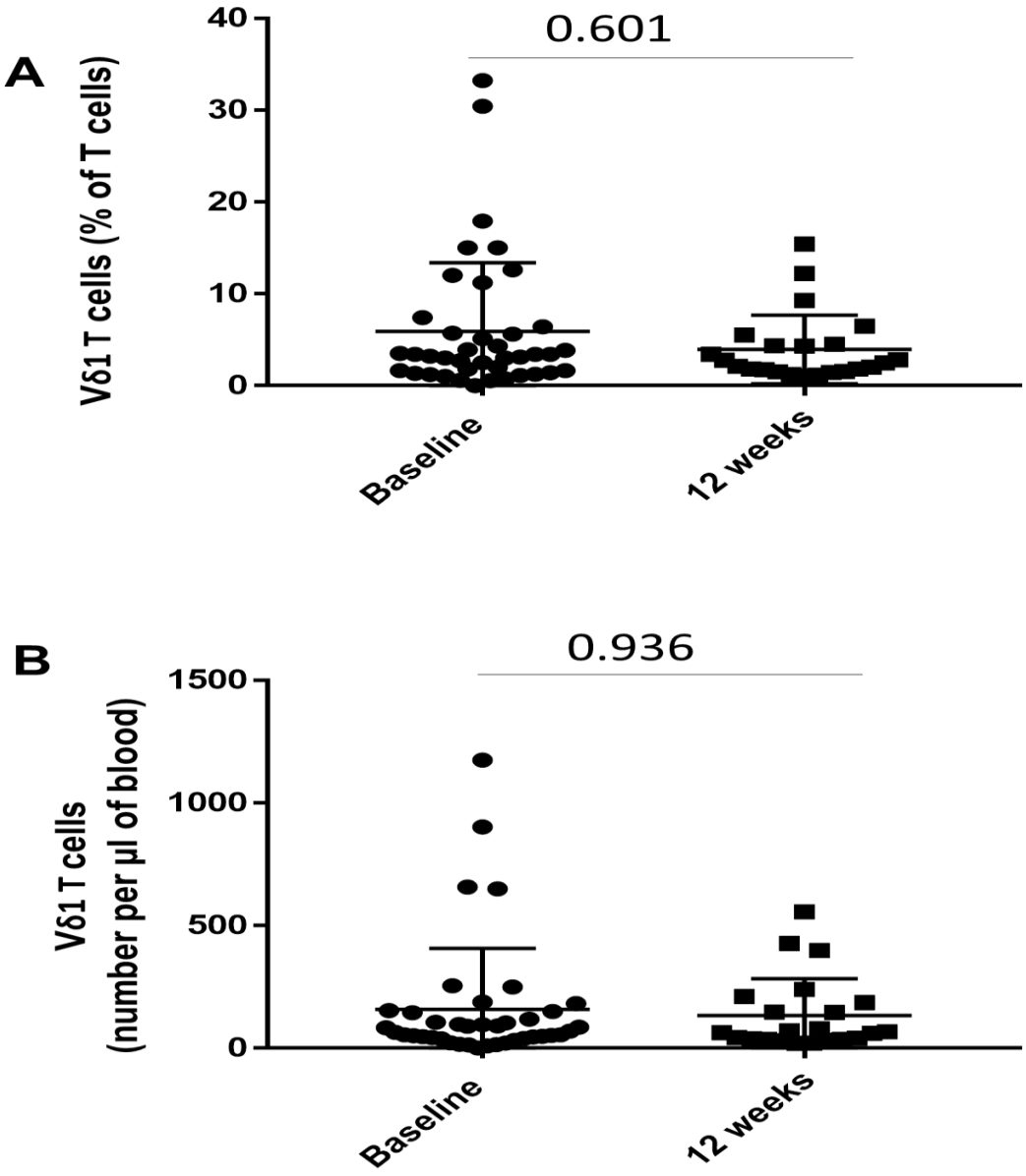


Figure 88: Effect of RUTF supplementation on circulating Vδ1 T cell frequencies and numbers in malnourished ART experienced children with HIV infection.

*PBMC were isolated from 40 ART experienced malnourished children with HIV infection at baseline and 23 of these patients after 12 weeks of RUTF supplementation. Cells were stained with monoclonal antibodies specific for Vδ1 and CD3 and analysed by flow cytometry. Vδ1 T cells were defined as lymphocytes that were positive for Vδ1 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ1 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

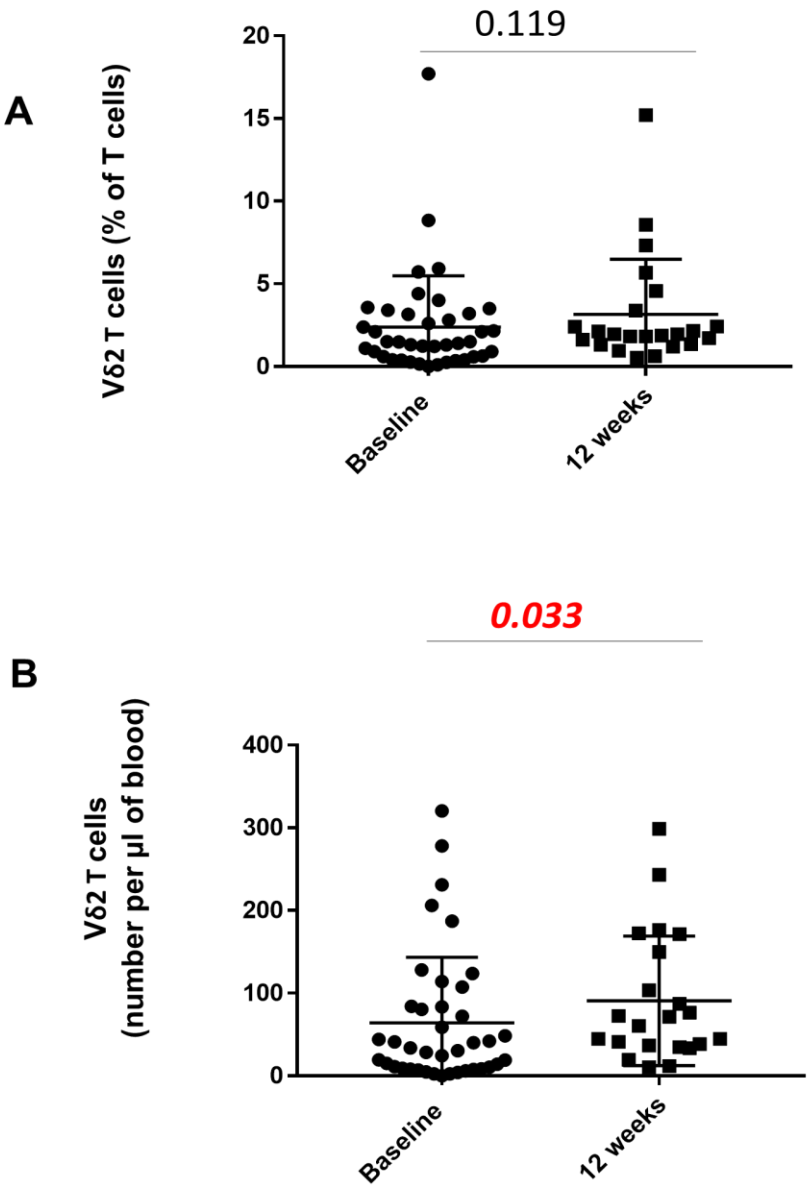


Figure 89: Effect of RUTF supplementation on circulating Vδ2 T cell frequencies and numbers in malnourished ART experienced children with HIV infection.

*PBMC were isolated from 40 ART experienced malnourished children with HIV infection at baseline and 23 of these patients after 12 weeks of RUTF supplementation. Cells were stained with monoclonal antibodies specific for Vδ2 and CD3 and analysed by flow cytometry. Vδ2 T cells were defined as lymphocytes that were positive for Vδ2 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ2 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

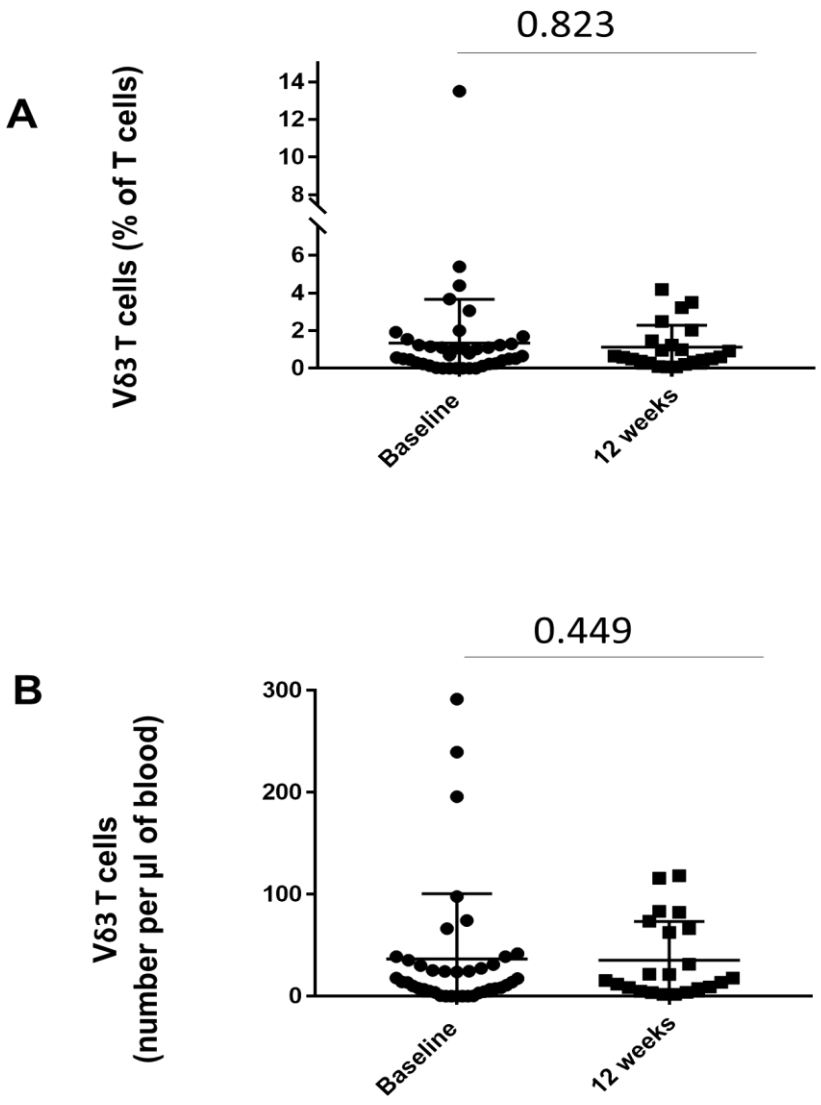


Figure 90: Effect of RUTF supplementation on circulating Vδ3 T cell frequencies and numbers in malnourished ART experienced children with HIV infection.

*PBMC were isolated from 40 ART experienced malnourished children with HIV infection at baseline and 23 of these patients after 12 weeks of RUTF supplementation. Cells were stained with monoclonal antibodies specific for Vδ3 and CD3 and analysed by flow cytometry. Vδ3 T cells were defined as lymphocytes that were positive for Vδ3 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ3 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

Table 49. Summary results on effects of RUTF on lymphocyte subset frequencies in malnourished ART-E HIV infected children.

Cells	Median frequencies at baseline	Median frequencies at 12 weeks	P-value
T cells	**68.4**	**68.1**	**0.961**
B cells	**11.3**	**13.6**	**0.272**
NK cells	**3.02**	**5.58**	**0.026***
$CD8^+$ T cell	**43.6**	**35.0**	**0.026****
$CD4^+$ T cell	**31.85**	**21.3**	**0.094**
$CD4^-CD8^-$ T cell	**11.05**	**37.1**	**<0.0001*****
$CD4^+CD8^+$ T cell	**0.29**	**0.38**	**0.113**
iNK T cell	**0.1**	**0.07**	**0.319**
NT cell	**0.84**	**0.67**	**0.731**
Vδ1 T cell	**3.31**	**2.47**	**0.601**
Vδ2 T cell	**1.45**	**1.95**	**0.119**
Vδ3 T cell	**0.69**	**0.62**	**0.823**

* Indicates the frequencies of NK cells were higher after 12 weeks of RUTF supplementation

** Indicates the frequencies of $CD8^+$ T cells were lower after 12 weeks of RUTF supplementation

***Indicates the frequencies of $CD4^-CD8^-$ T cell were higher after RUTF supplementation

Table 50. Summary results on effects of RUTF on lymphocyte subset absolute numbers in malnourished ART-E HIV infected children.

Cells	Median absolute cell counts at baseline	Median absolute cell counts at 12 weeks	P-value
T cells	**2684**	**2696**	**0.516**
B cells	**417.9**	**509.7**	**0.060**
NK cells	**116.6**	**210.9**	**0.010***
$CD8^+$ T cell	**660.3**	**658.0**	**0.010****
$CD4^+$ T cell	**901.6**	**693.8**	**0.066**
$CD4^-CD8^-$ T cell	**257.2**	**964.7**	**<0.0001*****
$CD4^+CD8^+$ T cell	**6.19**	**10.72**	**0.151**
iNK T cell	**2.10**	**2.65**	**0.841**
NT cell	**25.64**	**20.18**	**0.591**
Vδ1 T cell	**76.36**	**64.92**	**0.936**
Vδ2 T cell	**32.25**	**66.06**	**0.033******
Vδ3 T cell	**14.06**	**16.55**	**0.449**

* Indicates the absolute counts of NK cells were higher after 12 weeks of RUTF supplementation

** Indicates the absolute counts of $CD8^+$ T cells were lower after 12 weeks of RUTF supplementation

***Indicates the absolute counts of $CD4^-CD8^-$ T cell were higher after RUTF supplementation

**** Indicates the absolute count of Vδ2 T cells were higher after 12 weeks of RUTF supplementation

Objective 6: Changes in lymphocyte subset frequencies and numbers in ART-experienced well-nourished children at 12 weeks.

The changes of lymphocyte subset frequencies and numbers in ART-E, WN children was assessed by isolating PBMC from 43 ART-E,WN children with HIV infection at baseline and 22 of these patients after 12 weeks of follow up with no RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD3, CD4, CD8, CD19, CD56 and the Vα24Jα18, Vδ1, Vδ2 and Vδ3 TCR chains (Table 12), frequencies and absolute numbers of lymphocyte subsets were analysed by flow cytometry. Absolute numbers were calculated from full blood counts taken at the time of blood collection (Table 19 and 30). This was done to establish if there would be a change in lymphocyte subsets in children who are stable on ART, considered to have no nutrition challenge and basically doing well according to HIV programs.

The T and NK cell frequencies and absolute counts were similar though the absolute numbers of both were slightly raised at 12 weeks but this change was not significant, (Figure 91 and Figure 93). However, the frequencies and absolute counts of B cells were raised at 12 weeks significantly with p-value=0.022 and p-value=0.01 respectively, (Figure 92).

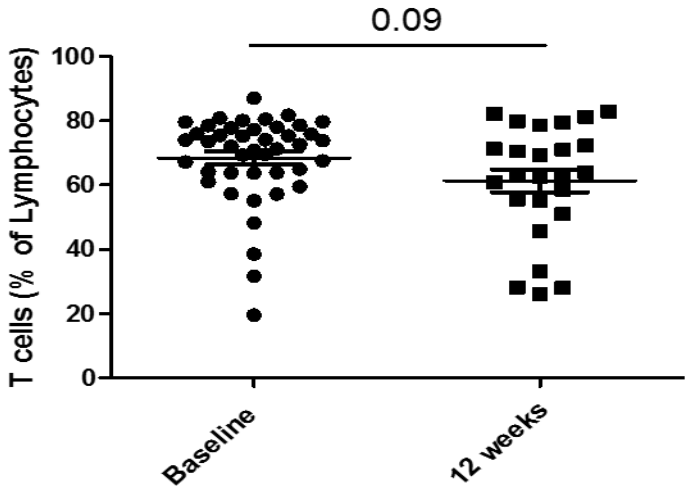
We observed lower $CD8^+$ T cell frequencies and absolute counts at 12 weeks with p-value<0.0001 and p-value=0.04 respectively, (Figure 94). The $CD4^+$ T cell frequencies and absolute counts were lower at 12 weeks this was significant, with p-value=0.05 and p-value<0.0001 respectively, (Figure 95). The $CD4^-CD8^-$ T cell frequencies and numbers were all higher at 12 weeks and were statistically significant with a p-value<0.0001, (Figure 96). Our results of $CD4^+CD8^+$T cell frequencies and numbers were higher at baseline than at 12 weeks, (p-value=0.223 and p-value=0.217, respectively, Figure 97).

The iNK T cell frequencies and absolute counts were not significantly different at both time points, (Figure 98), the NT cell frequencies and counts were higher at 12 weeks than at baseline, (Figure 99).

Among the WN ART-E children Vδ1 T and Vδ2 T cell frequencies and absolute counts were altered at 12 weeks, with Vδ1 T cells being decreased while Vδ2 T cell were increased though thte change was not significant. Vδ3 T cell were also found to decrease by 12 weeks

follow up, Figure 100 - Figure 102). These results show that in the absence of nutritional supplementation ART affects lymphocyte numbers in children with HIV. These results are summarised in Table 51 and 52.

A



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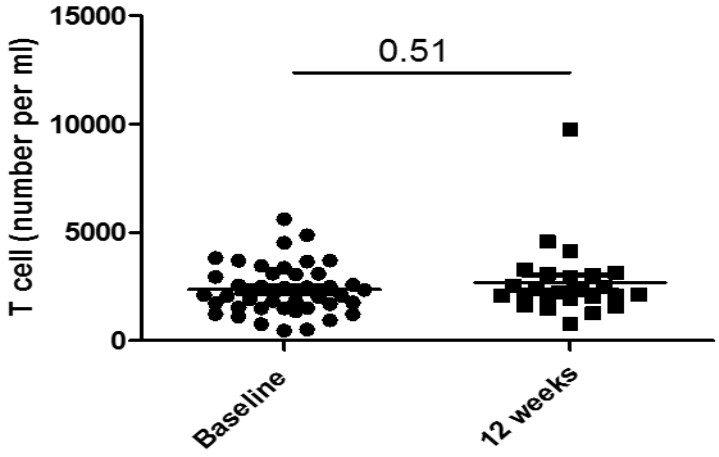

Figure 91: Changes in circulating T cell frequencies and numbers in well-nourished ART-experienced children over 12 weeks without RUTF supplementation.

*PBMC were isolated from 43 ART-experienced well-nourished children with HIV infection at baseline and 25 of these patients after 12 weeks of follow up. These patients received nutritional counselling but no RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD3 and analysed by flow cytometry. T cells were defined as lymphocytes that were positive for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

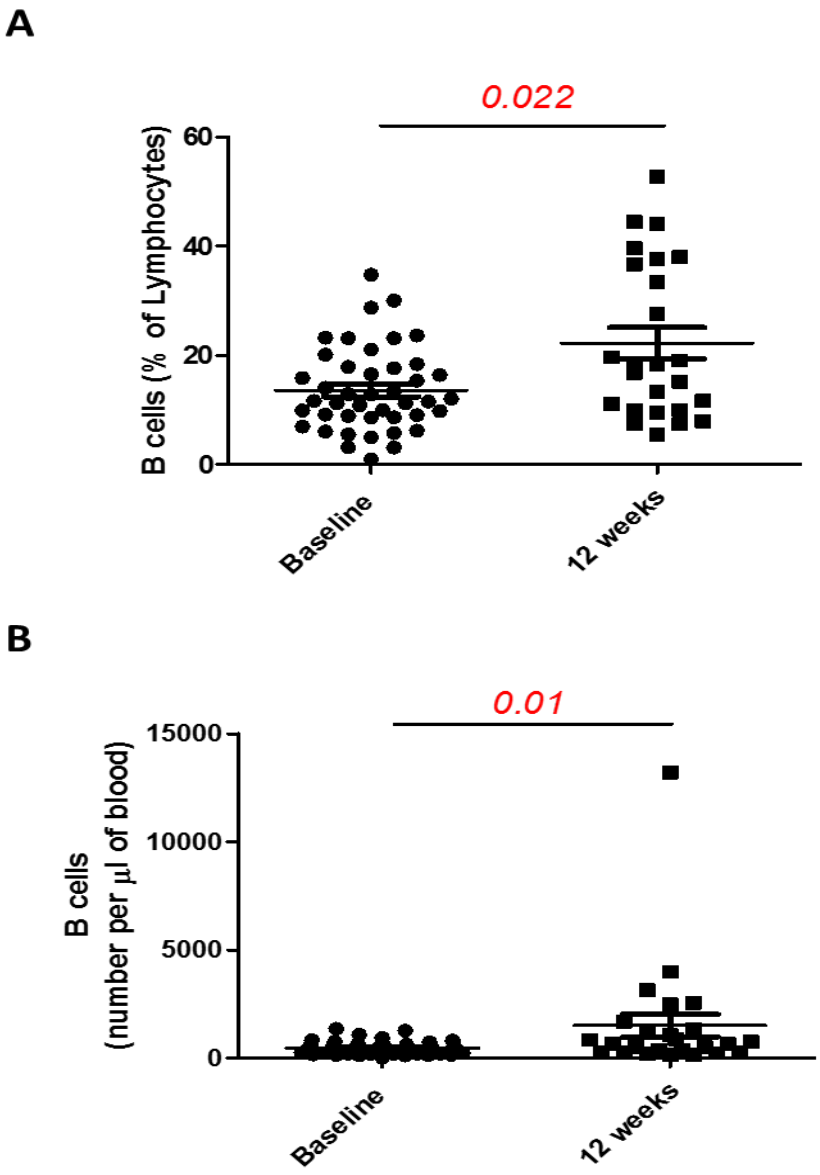

Figure 92: Changes in circulating B cell frequencies and numbers in well-nourished ART-experienced children over 12 weeks without RUTF supplementation.
*PBMC were isolated from 43 ART-experienced well-nourished children with HIV infection at baseline and 25 of these patients after 12 weeks of follow up. These patients received nutritional counselling but no RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD19 and CD3 and analysed by flow cytometry. B cells were defined as lymphocytes that were positive for CD19 and negative for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of B cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

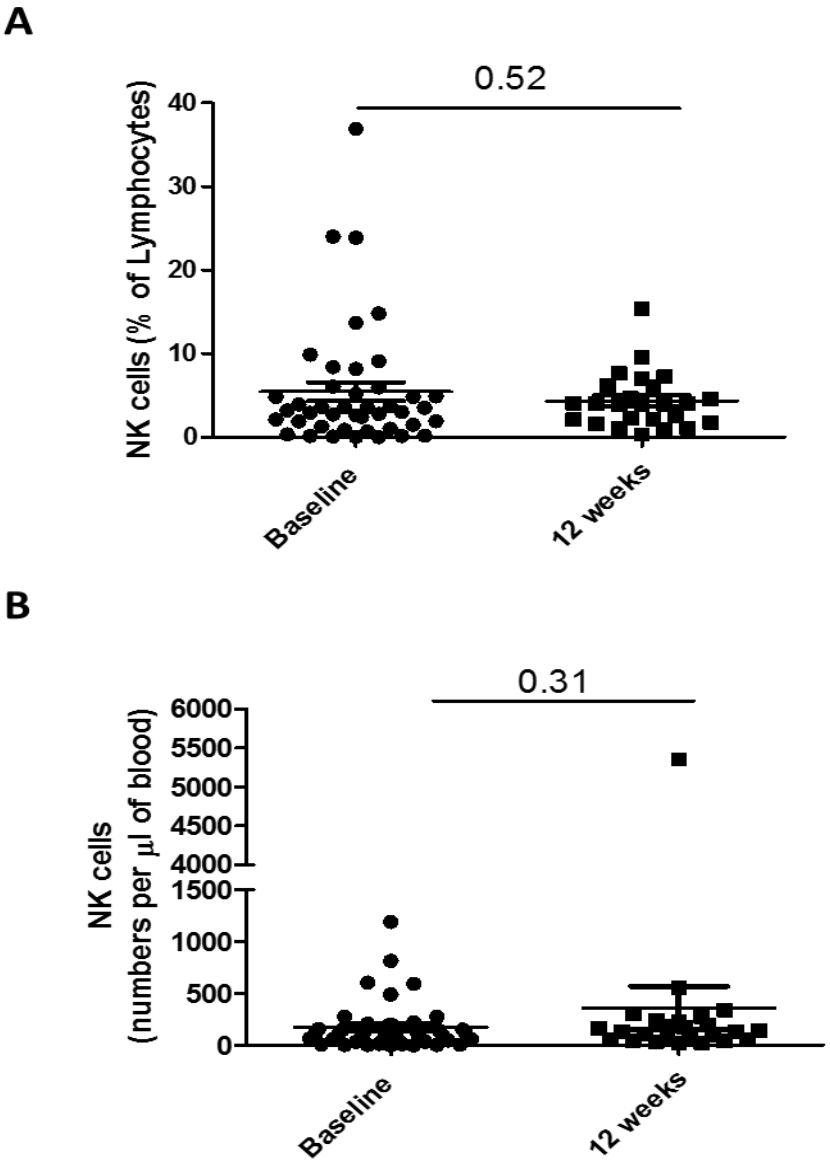

Figure 93: Changes in circulating NK cell frequencies and numbers in well-nourished ART-experienced children over 12 weeks without RUTF supplementation.

*PBMC were isolated from 43 ART-experienced well-nourished children with HIV infection at baseline and 25 of these patients after 12 weeks of follow up. These patients received nutritional counselling but no RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD56 and CD3 and analysed by flow cytometry. NK cells were defined as lymphocytes that were positive for CD56 and negative for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of NK cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

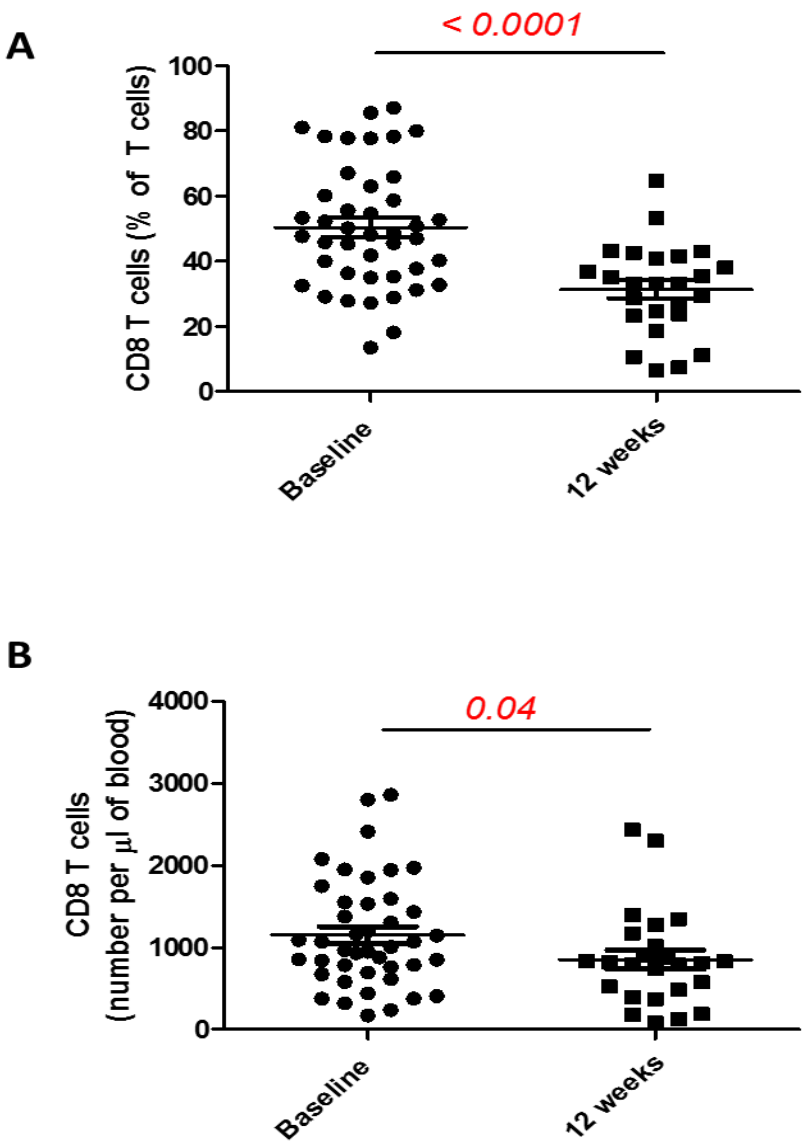

Figure 94: Changes in circulating CD8 T cell frequencies and numbers in well-nourished ART-experienced children over 12 weeks without RUTF supplementation.

*PBMC were isolated from 43 ART-experienced well-nourished children with HIV infection at baseline and 25 of these patients after 12 weeks of follow up. These patients received nutritional counselling but no RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD4, CD8 and CD3 and analysed by flow cytometry. CD8 cells were defined as lymphocytes that were positive for CD8 and CD3 and negative for CD4. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD8 cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

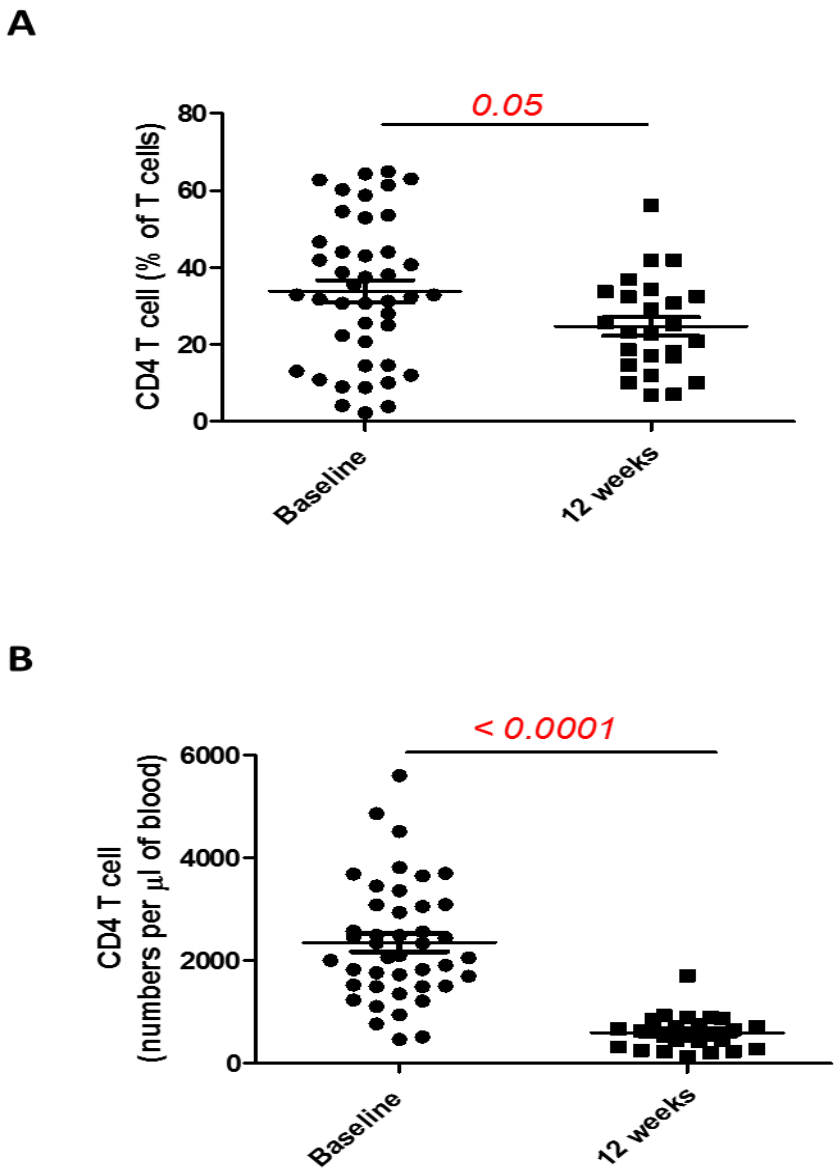

Figure 95: Changes in circulating CD4⁺ T cell frequencies and numbers in well-nourished ART-experienced children over 12 weeks without RUTF supplementation.

*PBMC were isolated from 43 ART-experienced well-nourished children with HIV infection at baseline and 25 of these patients after 12 weeks of follow up. These patients received nutritional counselling but no RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD4, CD8 and CD3 and analysed by flow cytometry. CD4 cells were defined as lymphocytes that were positive for CD4 and CD3 and negative for CD8. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD4 cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

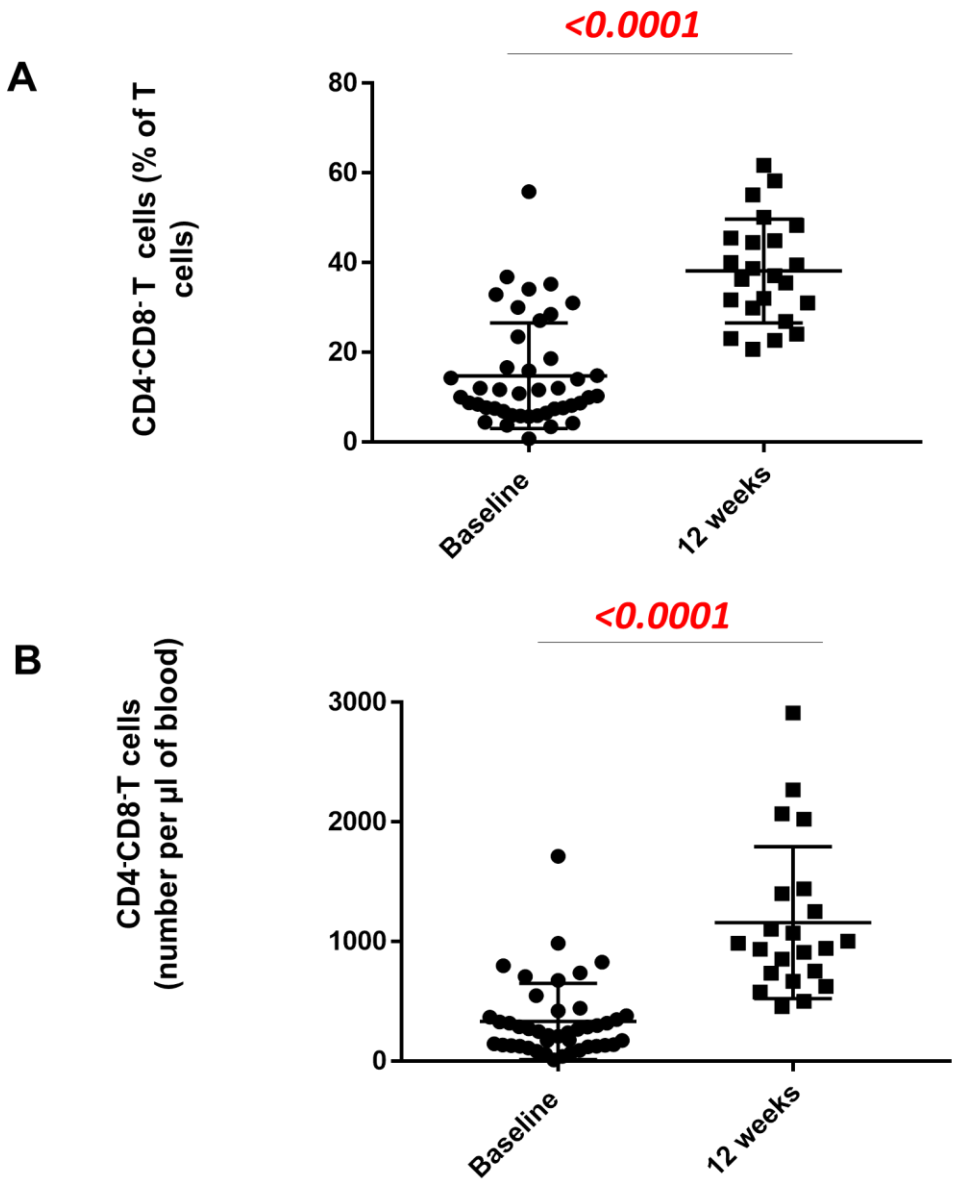

Figure 96: Changes in circulating CD4$^-$CD8$^-$ T cell frequencies and numbers in well-nourished ART-experienced children over 12 weeks without RUTF supplementation. *PBMC were isolated from 43 ART-experienced well-nourished children with HIV infection at baseline and 25 of these patients after 12 weeks of follow up. These patients received nutritional counselling but no RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD4, CD8 and CD3 and analysed by flow cytometry. CD4$^-$CD8$^-$ T cells were defined as lymphocytes that were negative for CD4 and CD8 and positive for CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD4$^-$CD8$^-$ cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

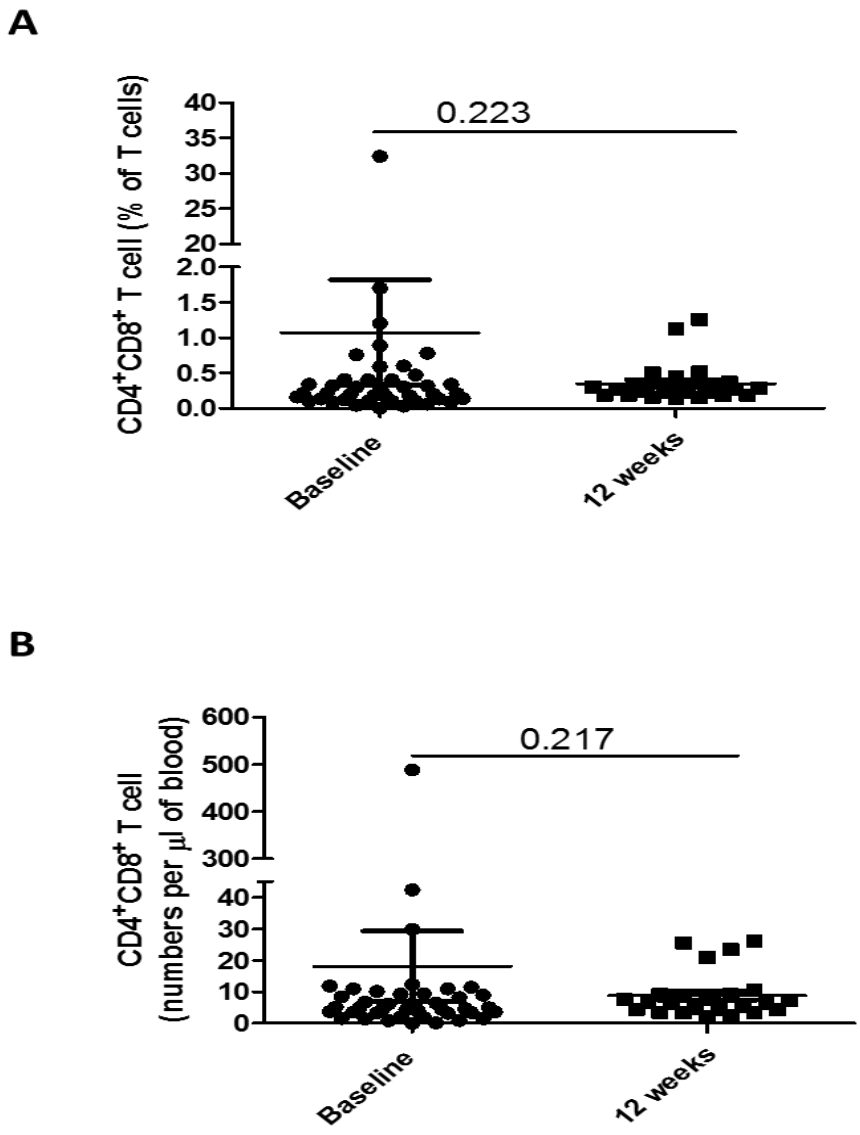

Figure 97: Changes in circulating CD4⁺CD8⁺ T cell frequencies and numbers in well-nourished ART-experienced children over 12 weeks without RUTF supplementation.

*PBMC were isolated from 43 ART-experienced well-nourished children with HIV infection at baseline and 25 of these patients after 12 weeks of follow up. These patients received nutritional counselling but no RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD4, CD8 and CD3 and analysed by flow cytometry. CD4⁺CD8⁺ T cells were defined as lymphocytes that were positive for CD4, CD8 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of CD4⁺CD8⁺ cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

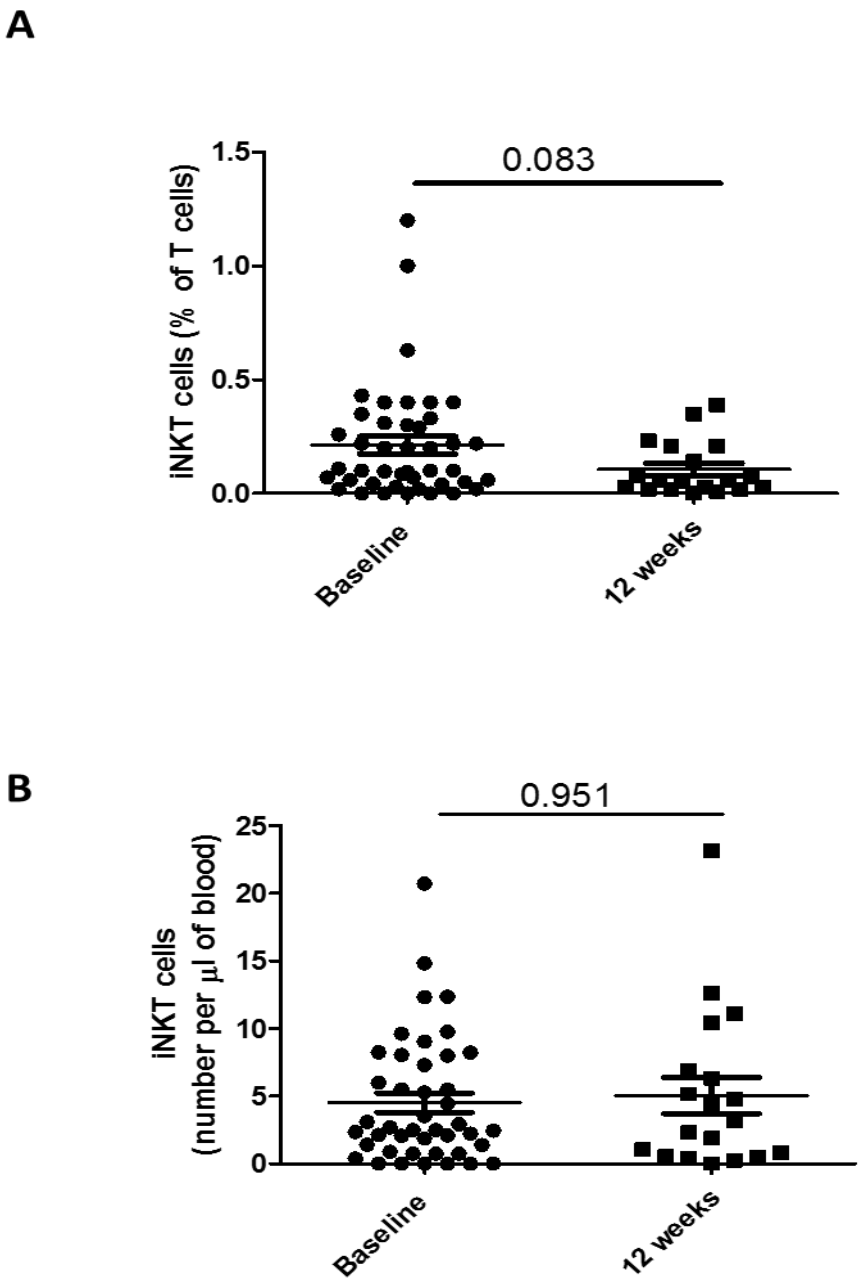

Figure 98: Changes in circulating iNK T cell frequencies and numbers in well-nourished ART-experienced children over 12 weeks without RUTF supplementation.

*PBMC were isolated from 43 ART-experienced well-nourished children with HIV infection at baseline and 25 of these patients after 12 weeks of follow up. These patients received nutritional counselling but no RUTF supplementation. Cells were stained with monoclonal antibodies specific for Vα24Jα18 and CD3 and analysed by flow cytometry. iNK T cells were defined as lymphocytes that were positive for CD3 Vα24Jα18. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of iNK T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

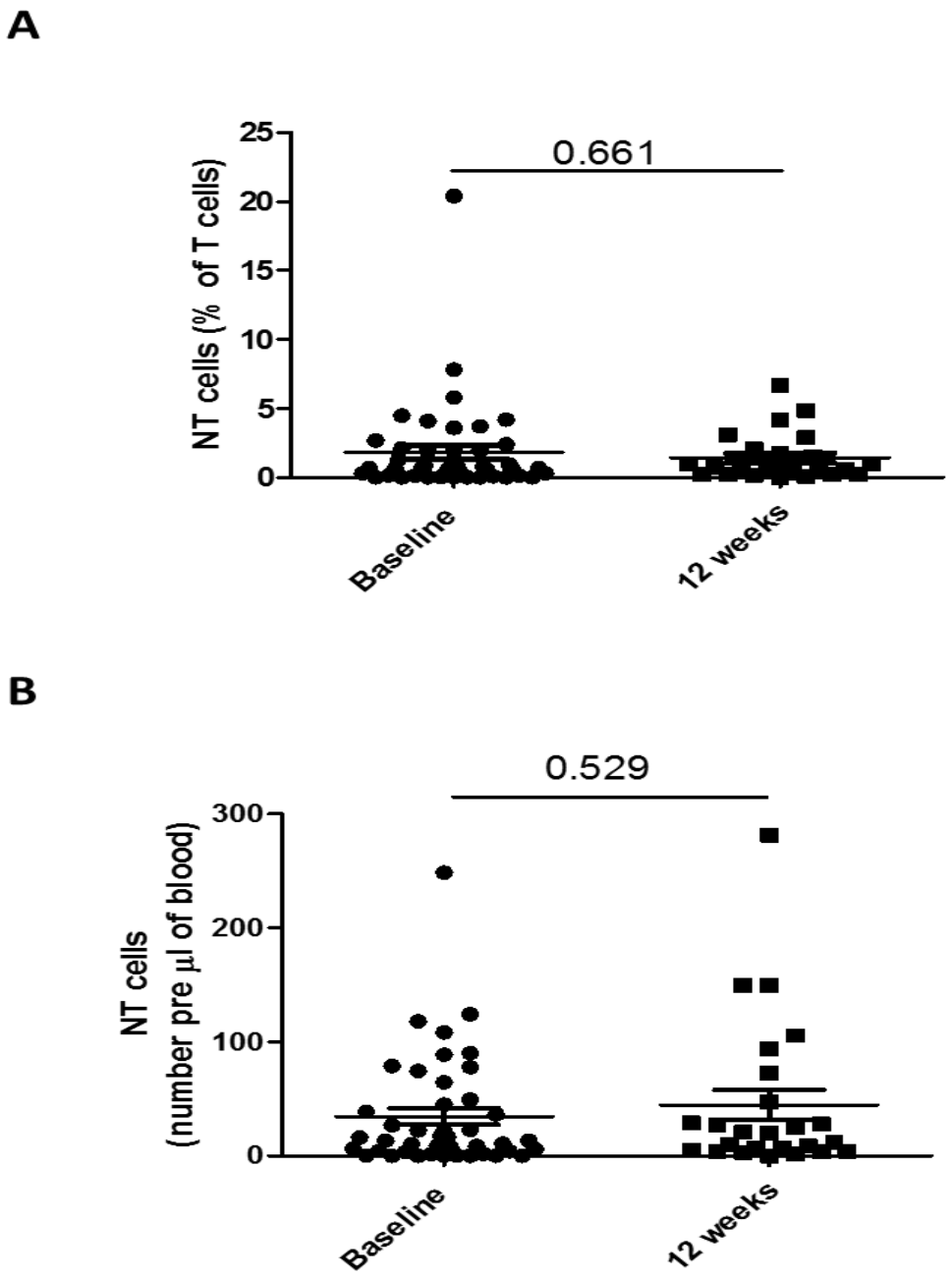

Figure 99: Changes in circulating NT cell frequencies and numbers in well-nourished ART-experienced children over 12 weeks without RUTF supplementation. *PBMC were isolated from 43 ART-experienced well-nourished children with HIV infection at baseline and 25 of these patients after 12 weeks of follow up. These patients received nutritional counselling but no RUTF supplementation. Cells were stained with monoclonal antibodies specific for CD56 and CD3 and analysed by flow cytometry. NT cells were defined as lymphocytes that were positive for CD3 and CD56. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of NT cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

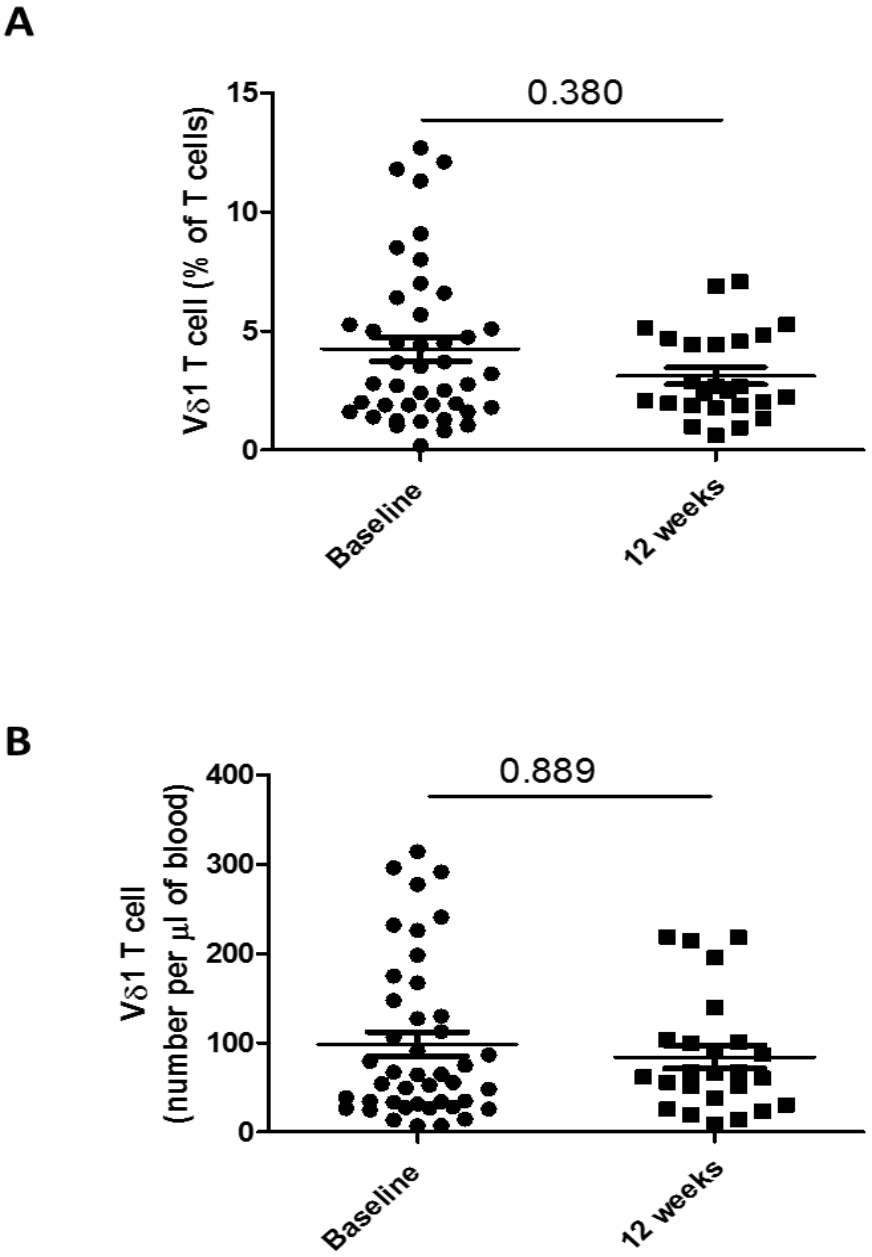

Figure 100: Changes in circulating Vδ1 cell frequencies and numbers in well-nourished ART-experienced children over 12 weeks without RUTF supplementation.

*PBMC were isolated from 43 ART-experienced well-nourished children with HIV infection at baseline and 25 of these patients after 12 weeks of follow up. These patients received nutritional counselling but no RUTF supplementation. Cells were stained with monoclonal antibodies specific for Vδ1 and CD3 and analysed by flow cytometry. Vδ1 cells were defined as lymphocytes that were positive for Vδ1 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ1 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

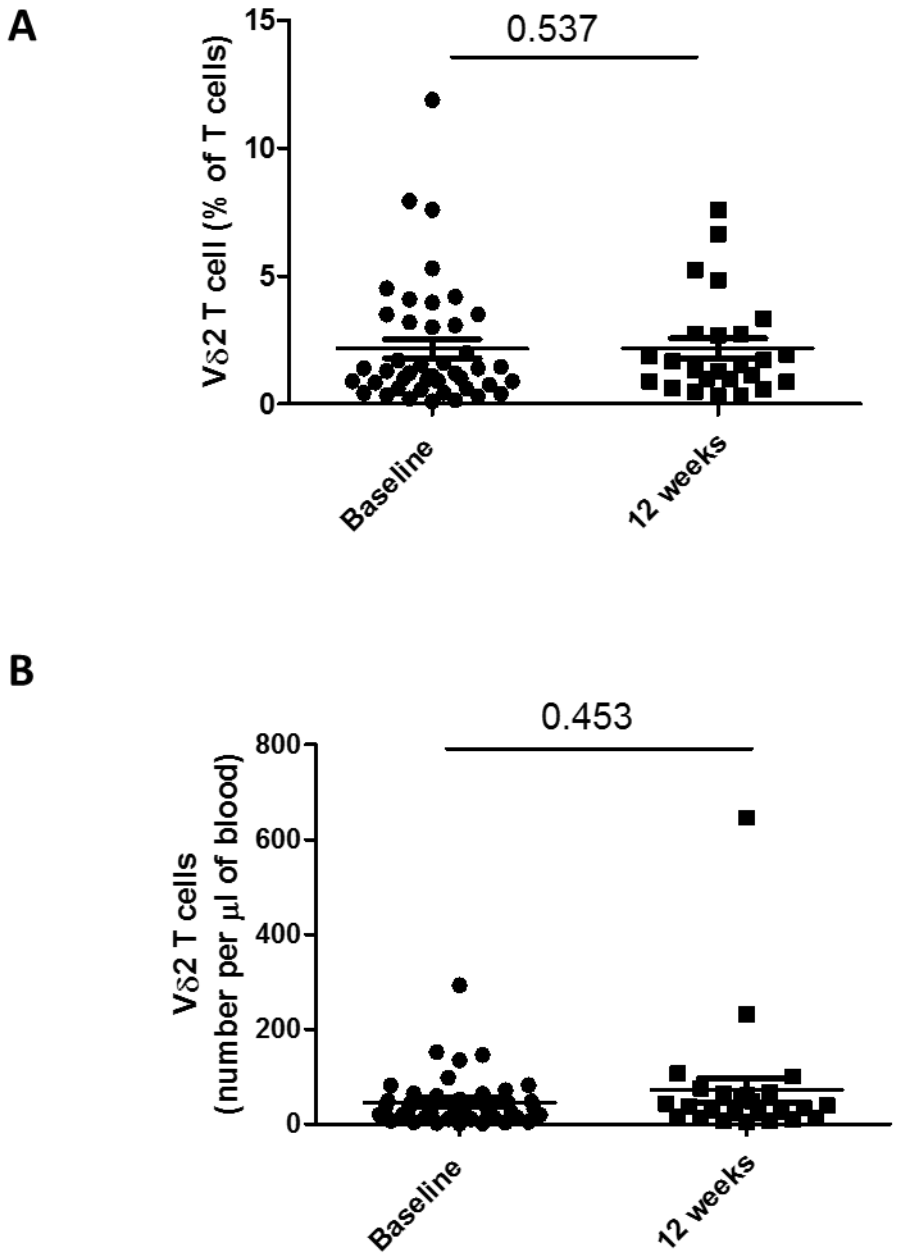

Figure 101: Changes in circulating Vδ2 cell frequencies and numbers in well-nourished ART-experienced children over 12 weeks without RUTF supplementation.

*PBMC were isolated from 43 ART-experienced well-nourished children with HIV infection at baseline and 25 of these patients after 12 weeks of follow up. These patients received nutritional counselling but no RUTF supplementation. Cells were stained with monoclonal antibodies specific for Vδ2 and CD3 and analysed by flow cytometry. Vδ2 cells were defined as lymphocytes that were positive for Vδ2 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ2 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

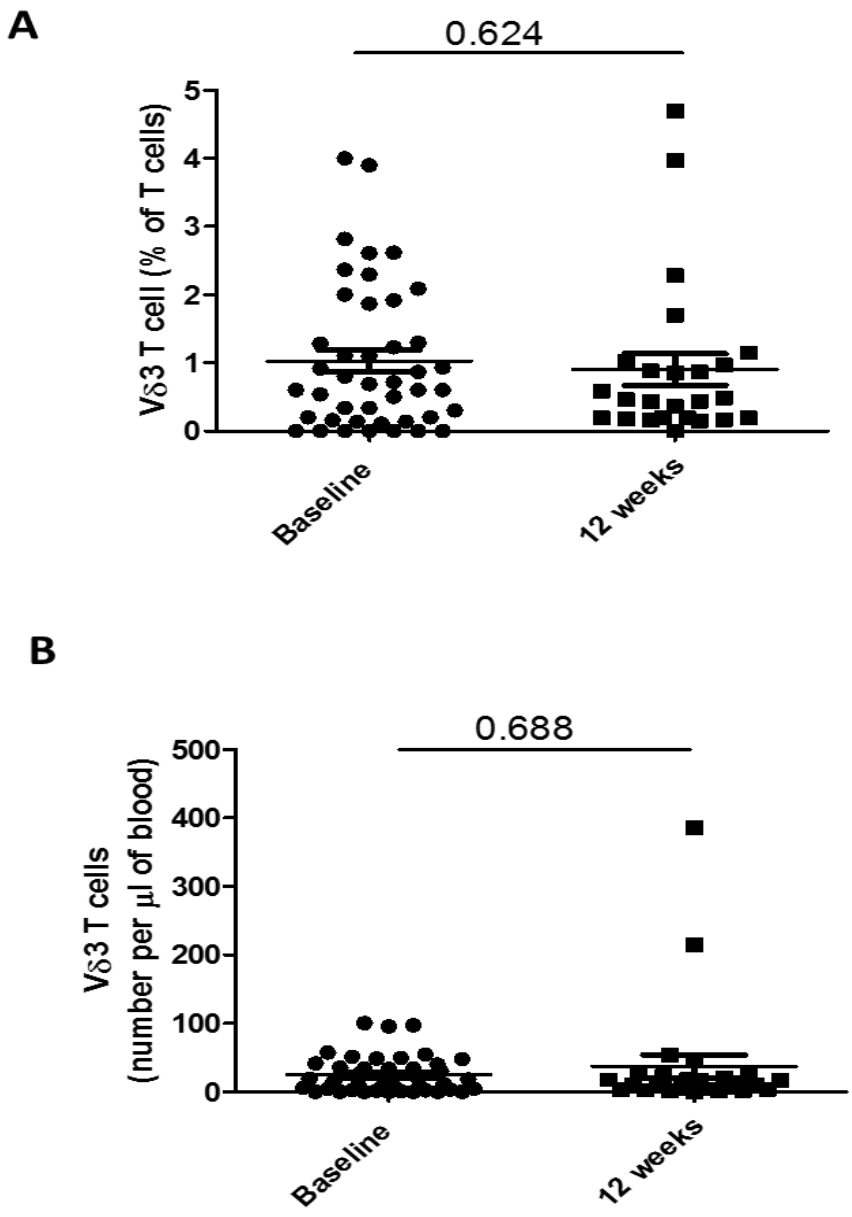

Figure 102: Changes in circulating Vδ3 cell frequencies and numbers in well-nourished ART-experienced children over 12 weeks without RUTF supplementation.

*PBMC were isolated from 43 ART-experienced well-nourished children with HIV infection at baseline and 25 of these patients after 12 weeks of follow up. These patients received nutritional counselling but no RUTF supplementation. Cells were stained with monoclonal antibodies specific for Vδ3 and CD3 and analysed by flow cytometry. Vδ3 cells were defined as lymphocytes that were positive for Vδ3 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ3 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

Table 51. Summary results on lymphocyte subset frequencies in ART-E well-nourished HIV infected children after 12 week follow up.

Cells	Median frequencies at baseline	Median frequencies at 12 weeks	P-value
T cells	**72.65**	**63.1**	**0.09**
B cells	**11.6**	**18.3**	**0.022***
NK cells	**3.2**	**3.95**	**0.52**
$CD8^+$ T cell	**48.1**	**33.1**	**<0.0001****
$CD4^+$ T cell	**32.9**	**23.2**	**0.05*****
$CD4^-CD8^-$ T cell	**10.3**	**42.2**	**<0.0001******
$CD4^+CD8^+$ T cell	**0.21**	**0.27**	**0.223**
iNK T cell	**0.1**	**0.06**	**0.083**
NT cell	**0.68**	**0.7**	**0.661**
Vδ1 T cell	**3.2**	**2.45**	**0.380**
Vδ2 T cell	**1.2**	**1.55**	**0.537**
Vδ3 T cell	**0.69**	**0.46**	**0.624**

* Indicates the frequencies of B cells were higher after 12 weeks of follow up
**Indicates the frequencies of CD8+ T cells were lower after 12 weeks of follow up
*** Indicates the median frequencies of $CD4^+$T cells were lower after 12 weeks of follow up
****Indicates the median frequencies of $CD4^-CD8^-$ T cell were higher after 12 weeks of follow up

Table 52. Summary results on lymphocyte subset numbers in ART-E well-nourished HIV infected children after 12 week follow up.

* Indicates the absolute counts of B cells were higher after 12 weeks of follow up

Cells	Median absolute counts at baseline	Median absolute counts at 12 weeks	P-value
T cells	**2103**	**2244**	**0.51**
B cells	**375.9**	**651.7**	**0.01***
NK cells	**113.6**	**134.8**	**0.31**
$CD8^+$ T cell	**1007**	**814.8**	**0.04****
$CD4^+$ T cell	**1508**	**303.6**	**<0.0001*****
$CD4^-CD8^-$ T cell	**249**	**1017**	**<0.0001********
$CD4^+CD8^+$ T cell	**4.96**	**6.64**	**0.217**
iNK T cell	**2.5**	**3.14**	**0.951**
NT cell	**13.58**	**20.21**	**0.529**
Vδ1 T cell	**64.31**	**65.31**	**0.889**
Vδ2 T cell	**31.74**	**38.14**	**0.453**
Vδ3 T cell	**18.45**	**10.91**	**0.688**

** Indicates the absolute counts of $CD8^+$ T cells were lower after 12 weeks of follow up

***indicated the absolute counts of CD4+ T cells were lower after 12 weeks of follow up

****Indicates the absolute counts of $CD4^-CD8^-$ T cell were higher after 12 weeks of follow up

Objective 7: Comparison of Vδ1, Vδ2 and Vδ3 T cell frequencies and numbers in immunological and virological responders and non-responders.

The sub classification of immunology into innate and adaptive immunity is being challenged [351]. There is growing evidence of innate-like T lymphocytes that express innate receptors to respond rapidly to stress despite expressing TCRs that are a hallmark of adaptive immunity. Innate T cells are a heterogeneous group of αβ and γδ T cells that respond rapidly upon activation. These innate T cells also share a non MHC class I or II restriction requirement for antigen recognition. Of the three major populations within the innate T cell group recognized, namely, invariant NK T cells, mucosal associated invariant T cells, and gamma delta T cells, we selected gamma delta T cells to further study their frequencies and numbers among immunological and virological responders in children with perinatally acquired HIV infection as they have been found to compensate for $CD4^+$ T cell functions in HIV disease progression. These cells recognize foreign/self-lipid presented by non-classical MHC molecules, such as CD1d, MR1, and CD1a. They are activated during the early stages of bacterial infection and act as a bridge between the innate and adaptive immune systems [352]. To compare Vδ1, Vδ2 and Vδ3 T cell frequencies and numbers in immunological and virological responders and non-responders, PBMC were isolated from 42 ART-E malnourished patients with HIV infection, of whom 4 were immunological non responders (INR) with $CD4^+$ T cell counts <200, 4 were intermediate responders (IMR) with $CD4^+$ T cell counts between 200-500 and 34 were adequate responders (AR) with $CD4^+$ T count >500 at baseline time point. At 12 week time point there was no child with an absolute $CD4^+$ T cell count less than 500, however, using $CD4^+$ T cell percentages there were children noted to have inadequate $CD4^+$ T cell response. Cells were stained with monoclonal antibodies specific for CD3, Vδ1, Vδ2 and Vδ3 and analysed by flow cytometry. Absolute numbers were calculated from full blood counts taken at the time of blood collection.

Vδ1 T cell frequencies were highest among children with INR and lowest in those with AR to ART illustrating that Vδ1 T cell numbers reduce in the presence of ART. This comparison was statistically significant with a p-value=0.046. The comparisons of absolute cell counts between children with IMR and AR was not significant with a p-value=0.7, (Figure 103).

Vδ2 T cell numbers were highest among the children with AR and lowest in those that had INR (p-value=0.006). The comparisons of Vδ2 T cell frequencies in the different groups were not statistically significant, (Figure 104).

Circulating Vδ3 T cell frequencies and numbers in ART-experienced children with HIV-infection classified according to their immunological responses did not yield any statistical significance, but the frequencies higher in those who had INR, (Figure 105).

Regarding virological responses, the PBMC were isolated from 85 ART-E patients with HIV infection, of whom 56 were virological non-responder (NR) with a viral load ≥20 viral copies /ml and 29 were adequate responders (AR) with a viral load < 20 viral copies/ml at 12 week follow up time point. Cells were stained with monoclonal antibodies specific for CD3, Vδ1, Vδ2 and Vδ3 and analysed by flow cytometry. Absolute numbers were calculated from full blood counts taken at the time of blood collection. The analysis showed that among the NR the circulating Vδ1 T cell frequencies and numbers were higher than those found in the responders. This was statistically significant (p-value=0.019 and 0.043, (Figure 106). Comparisons of circulating Vδ2 and Vδ3 T cell frequencies and numbers in ART-E children with HIV infection classified according to their virologic responses did not yield any statistical difference, however there was a modest increase of Vδ2 and Vδ3 T cells in the responders (Figure 107 and Figure 108).

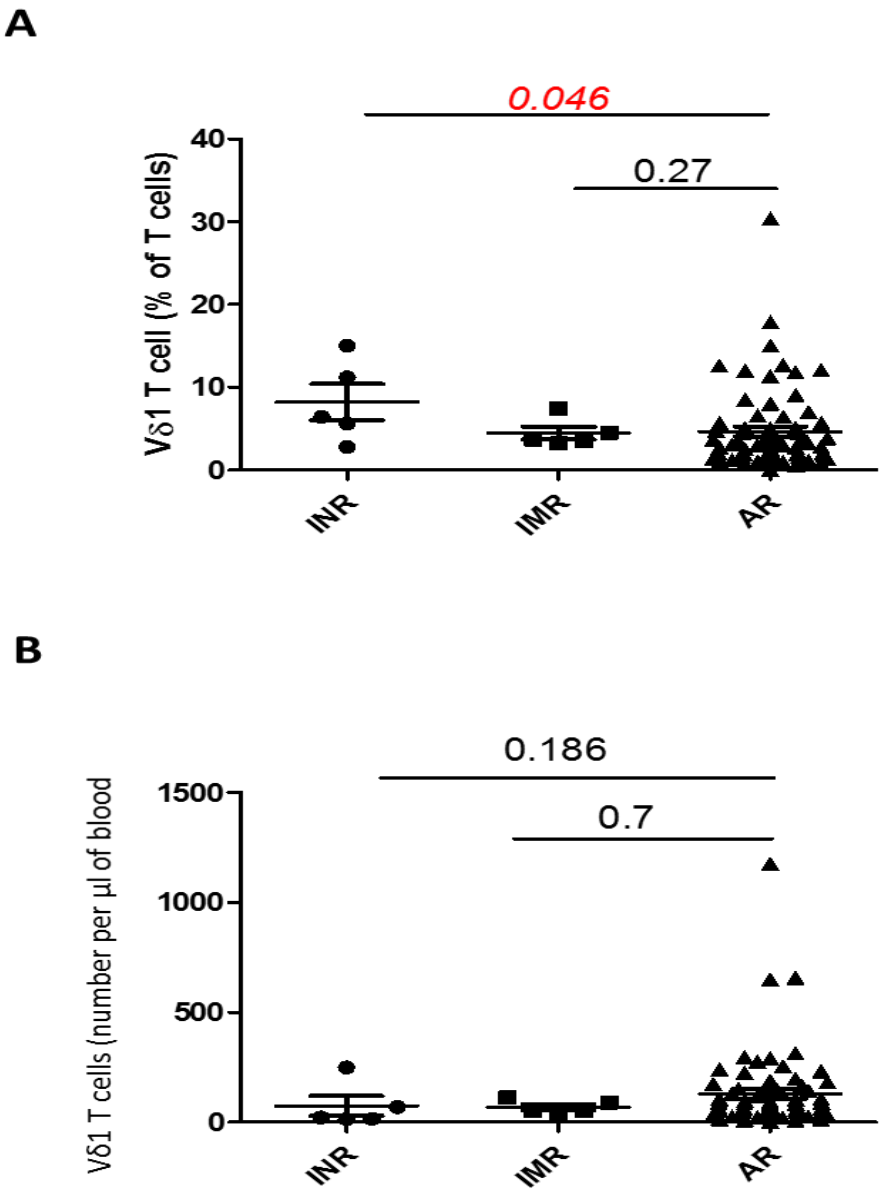

Figure 103: Circulating Vδ1 T cell frequencies and numbers in ART-experienced children with HIV-infection divided according to their immunological responses.

*PBMC were isolated from 42 ART-experienced malnourished patients with HIV infection, of whom 4 were immunological non responders (INR) with CD4 counts <200, 4 were intermediate responders (IMR) with CD4+ counts between 200-500 and 34 were adequate responders (AR) with CD4 count >500. Cells were stained with monoclonal antibodies specific for CD3 and Vδ1 and analysed by flow cytometry. Vδ1 T cells were defined as lymphocytes that were positive for Vδ1 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ1 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

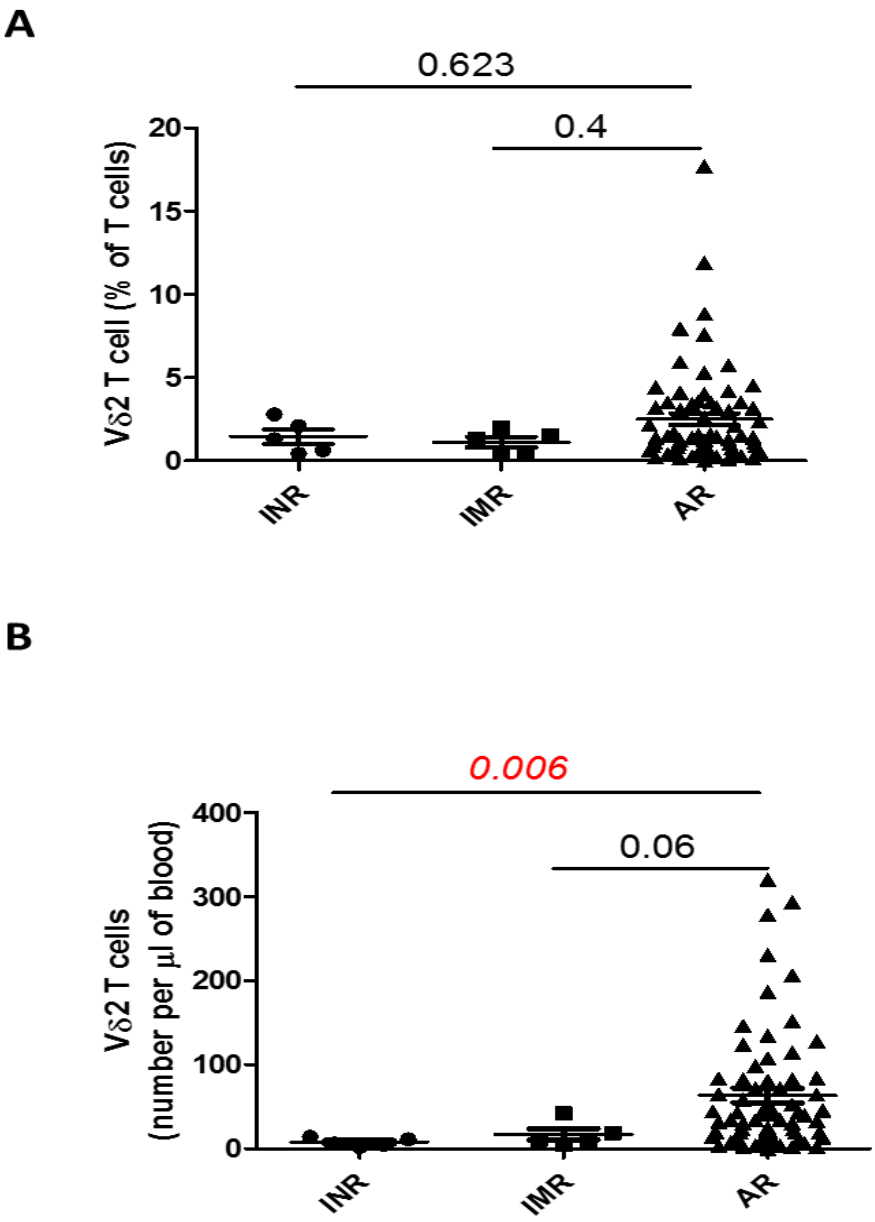

Figure 104: Circulating Vδ2 T cell frequencies and numbers in ART-experienced children with HIV-infection divided according to their immunological responses.

*PBMC were isolated from 42 ART-experienced malnourished patients with HIV infection, of whom 4 were immunological non responders (INR) with CD4 counts <200, 4 were intermediate responders (IMR) with CD4+ counts between 200-500 and 34 were adequate responders (AR) with CD4 count >500. Cells were stained with monoclonal antibodies specific for CD3 and Vδ2 and analysed by flow cytometry. Vδ2 T cells were defined as lymphocytes that were positive for Vδ2 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ2 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

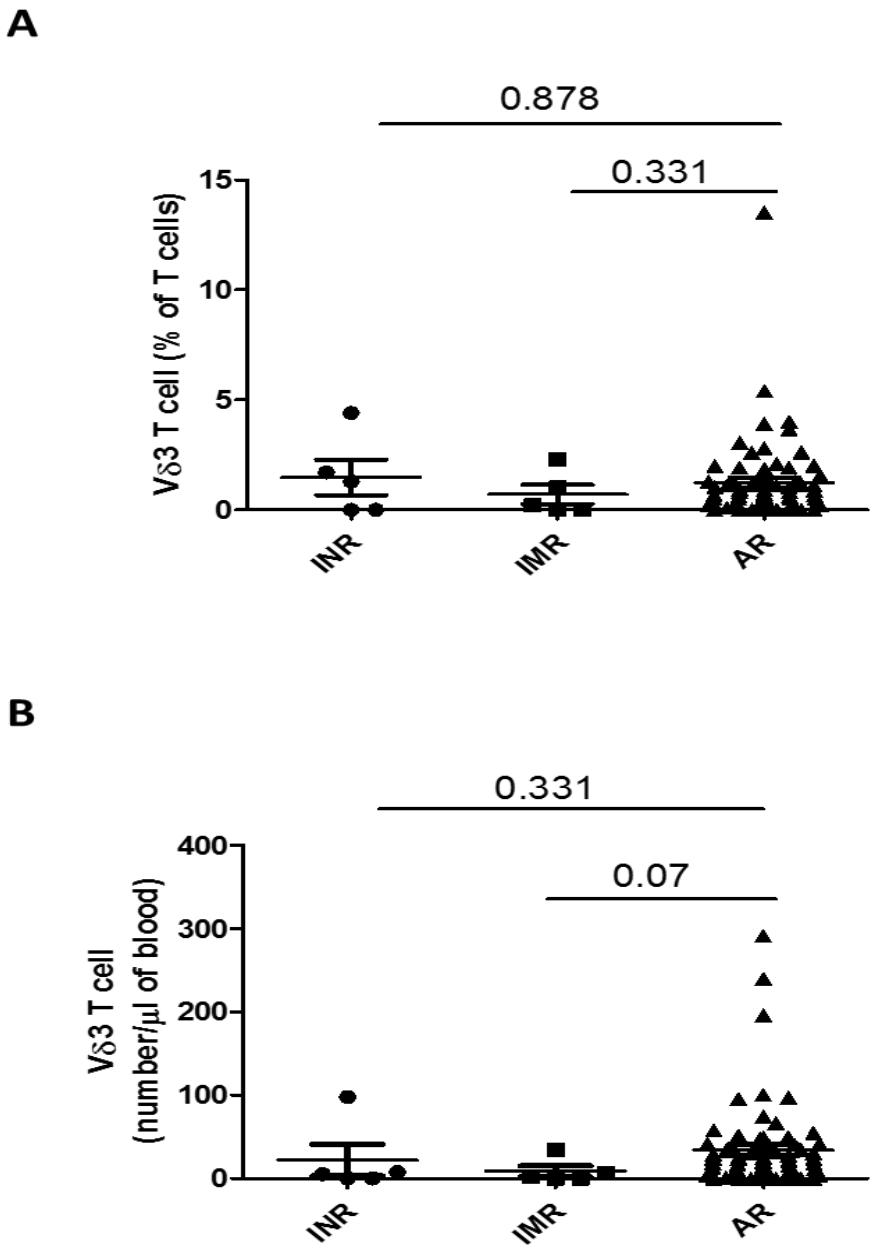

Figure 105: Circulating Vδ3 T cell frequencies and numbers in ART-experienced children with HIV-infection divided according to their immunological responses.
*PBMC were isolated from 42 ART-experienced malnourished patients with HIV infection, of whom 4 were immunological non responders (INR) with CD4 counts <200, 4 were intermediate responders (IMR) with CD4+ counts between 200-500 and 34 were adequate responders (AR) with CD4 count >500. Cells were stained with monoclonal antibodies specific for CD3 and Vδ3 and analysed by flow cytometry. Vδ3 T cells were defined as lymphocytes that were positive for Vδ3 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ3 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

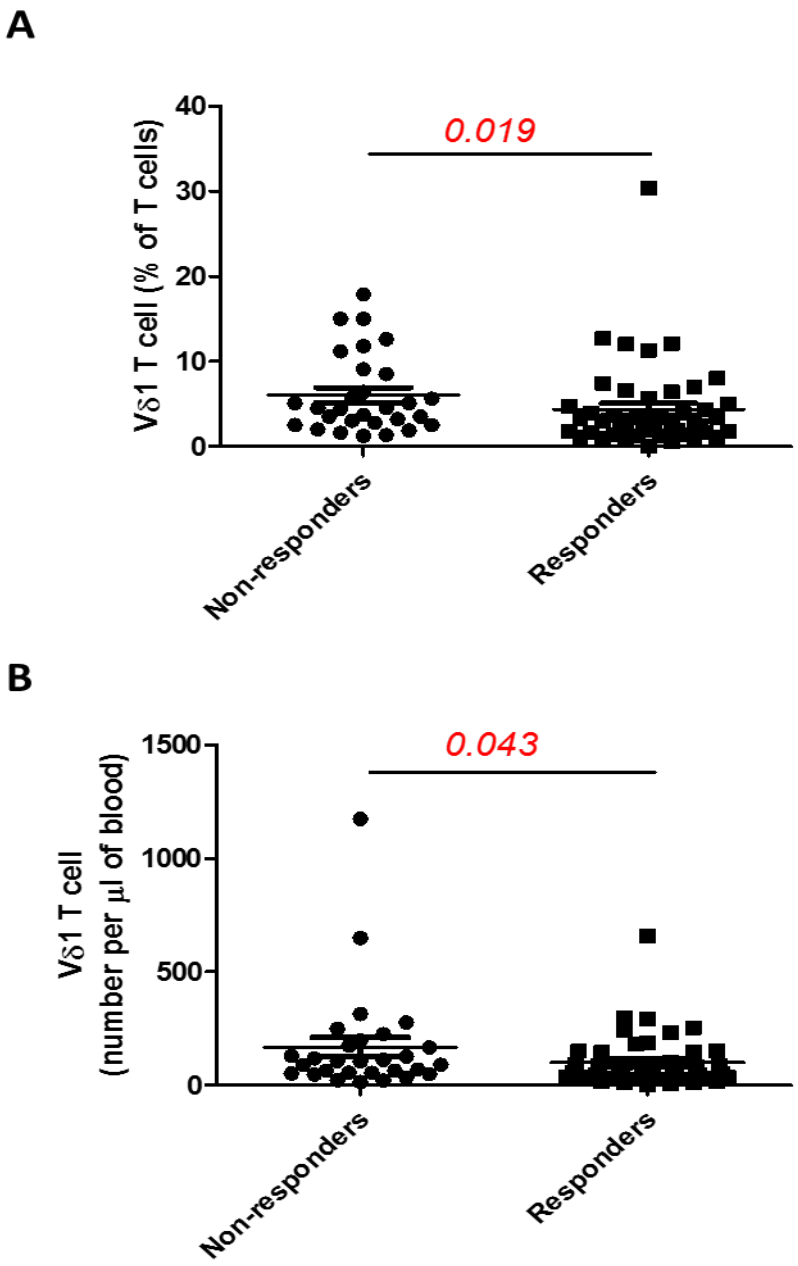

Figure 106: Circulating Vδ1 T cell frequencies and numbers in ART-experienced children with HIV-infection divided according to their virological responses.
*PBMC were isolated from 85 ART-experienced patients with HIV infection, of whom 56 had detectable viral loads ≥20 copies/ml and 29 patients had no detectable viral load copies. Cells were stained with monoclonal antibodies specific for CD3 and Vδ1 and analysed by flow cytometry. Vδ1 T cells were defined as lymphocytes that were positive for Vδ1 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ1 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

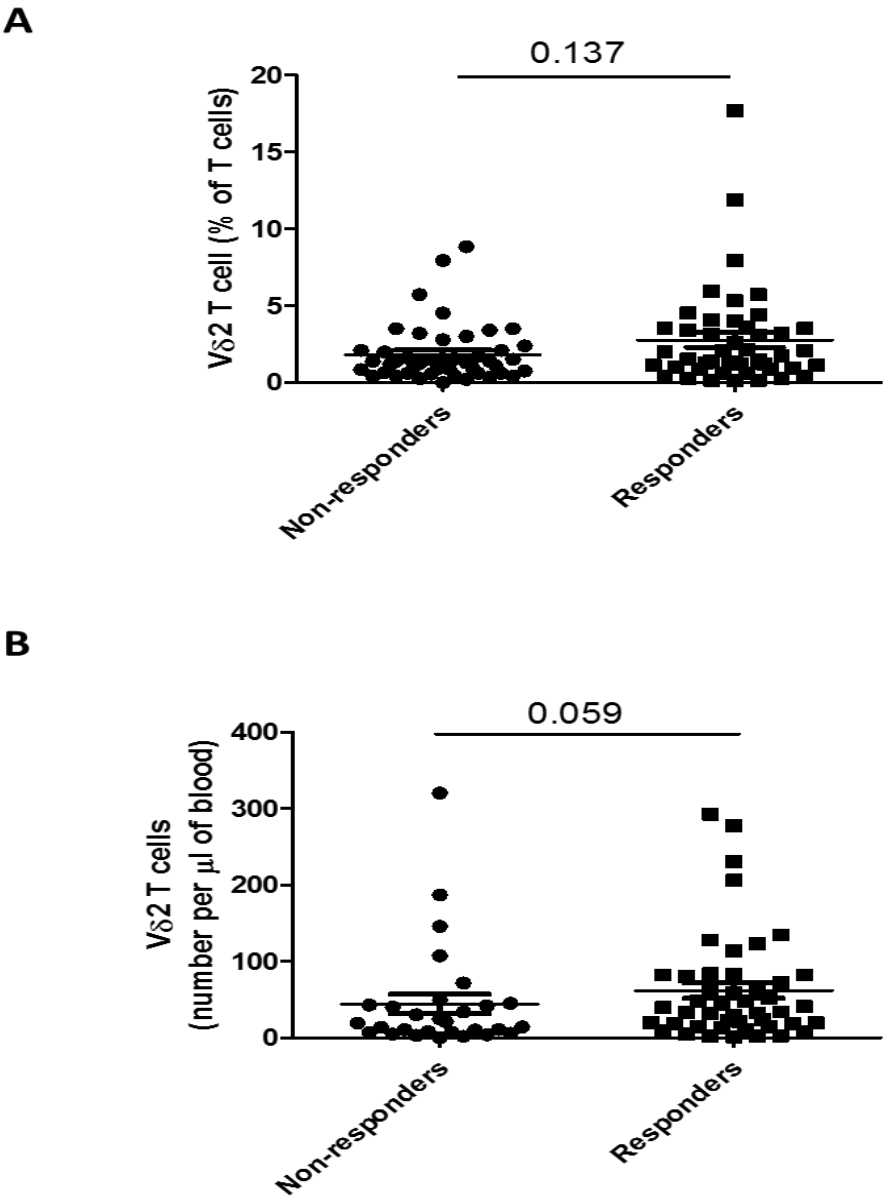

Figure 107: Circulating Vδ2 T cell frequencies and numbers in ART-experienced children with HIV-infection divided according to their virological responses.

*PBMC were isolated from 85 ART-experienced patients with HIV infection, of whom 56 had detectable viral loads ≥20 copies/ml and 29 patients had no detectable viral load copies. Cells were stained with monoclonal antibodies specific for CD3 and Vδ2 and analysed by flow cytometry. Vδ2 T cells were defined as lymphocytes that were positive for Vδ2 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ2 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

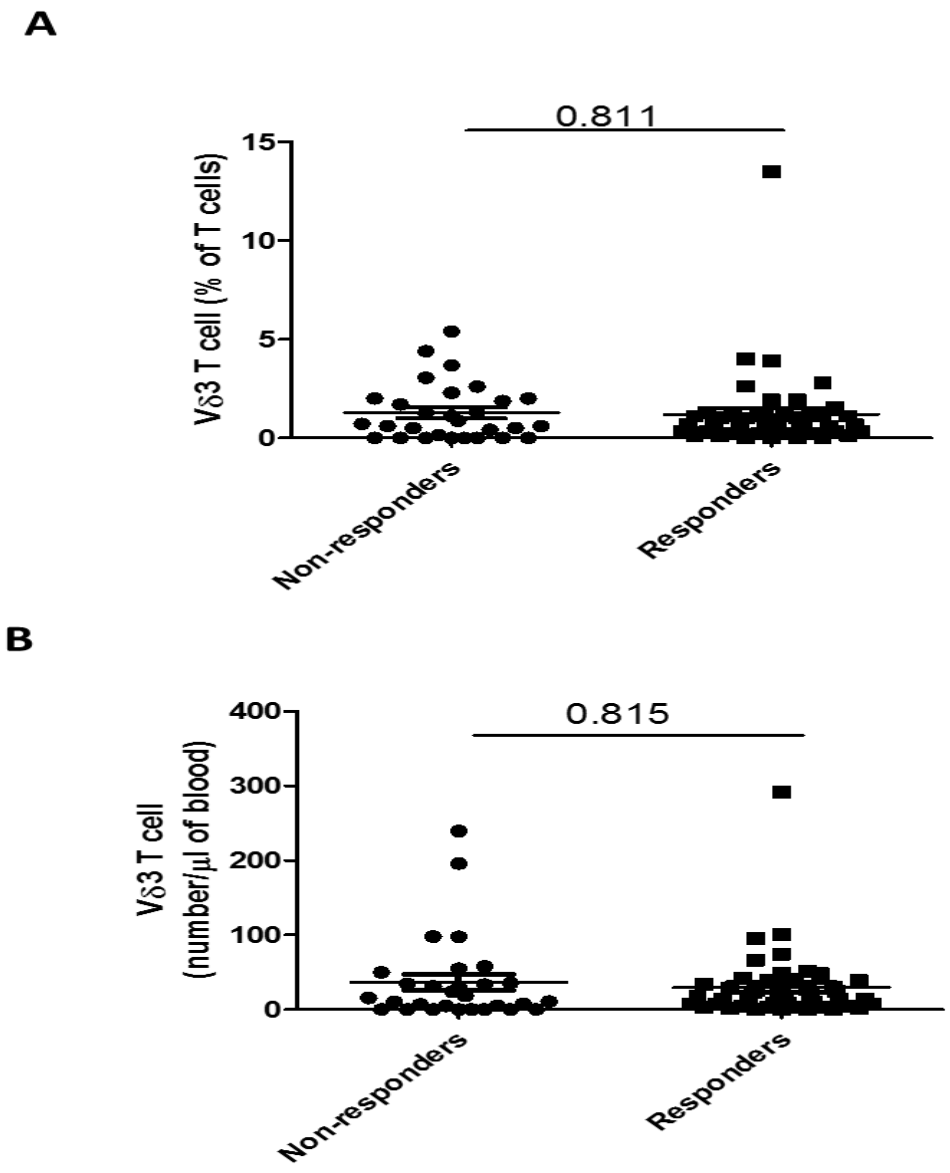

Figure 108: Circulating Vδ3 T cell frequencies and numbers in ART-experienced children with HIV-infection divided according to their virological responses.

*PBMC were isolated from 85 ART-experienced malnourished patients with HIV infection, of whom 56 had detectable viral loads ≥20 copies/ml and 29 patients had no detectable viral load copies. Cells were stained with monoclonal antibodies specific for CD3 and Vδ3 and analysed by flow cytometry. Vδ3 T cells were defined as lymphocytes that were positive for Vδ3 and CD3. Absolute numbers were calculated from full blood counts taken at the time of blood collection. Scatter plot show frequencies (**A**) and absolute numbers (**B**) of Vδ3 T cells present. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values.*

The summary findings on the comparison of Vδ1, Vδ2 and Vδ3 T cell frequencies and numbers in immunological and virological responders and non-responders are in tables 53-56.

Table 53. Comparison of Vδ1, 2, 3 T cell frequencies among immunological responders and non-responders

Cell	Median frequencies of INR	Median frequencies of AR	**P-value**
Vδ1	**6.4**	**3.0**	**0.046***
Vδ2	**1.3**	**1.4**	**0.623**
Vδ3	**1.3**	**0.72**	**0.878**

* Indicates the frequency of Vδ1 T cells were higher at baseline in INR than AR

Table 54. Comparison of Vδ1, 2, 3 T cell numbers among immunological responders and non-responders

Cell	**Median numbers of INR**	**Median numbers of AR**	**P-value**
Vδ1	**22.2**	**71.1**	**0.186**
Vδ2	**6.0**	**40.39**	**0.006***
Vδ3	**5.15**	**19.8**	**0.179**

* Indicates the absolute counts of Vδ2 T cells were lower at baseline

Table 55. Comparison of Vδ1, 2, 3 T cell frequencies among virological responders and non-responders

Cell	**Median frequencies of NR**	**Median frequencies of responders**	**P-value**
Vδ1	**4.5**	**2.89**	**0.019***
Vδ2	**1.27**	**1.61**	**0.137**
Vδ3	**0.72**	**0.7**	**0.811**

* Indicates the frequency of Vδ1 T cells were higher after 12 weeks of follow up in the virological NR than responders

Table 56. Comparison of Vδ1, 2, 3 T cell frequencies among virological responders and non-responders

Cell	**Median absolute numbers of NR**	**Median numbers of responders**	**P-value**
Vδ1	**106.2**	**54.11**	**0.043***
Vδ2	**19.35**	**37.17**	**0.059**
Vδ3	**15.44**	**17.66**	**0.815**

* Indicates the absolute counts of Vδ1 T cells were higher after 12 weeks of follow up in the virological NR than responders

Discussion

Several studies have been carried out on the effect of HIV and malnutrition on $CD4^+$ T cell and $CD8^+$ T cell subsets [5, 108]. However few studies have investigated the effects of HIV on other lymphocyte subsets such as $CD4^-CD8^-$, $CD4^+CD8^+$, iNK T, NT, Vδ1, Vδ2 and Vδ3 T cells, B and NK cells. Yet, for the immune system to function adequately and in totality, necessitates the proper functioning of all cells of the immune system. Furthermore, the effect of malnutrition is still understudied in the context of HIV and there are no studies to date on the effect of nutritional supplementation using RUTF on T, B, NK, $CD8^+$, $CD4^+$, $CD4^-CD8^-$, $CD4^+CD8^+$, iNK T, NT, Vδ1, Vδ2 and Vδ3 cells in children infected with HIV. Our main objectives were to find out the effects of HIV infection, ART, nutritional status and nutritional supplementation on lymphocyte subset frequencies and numbers among HIV infected children and to compare the γδ T cell subtypes frequencies and numbers among immunological and virological responders and non-responders. In view of our primary study objectives we did not explore gender effects on the immune system. None the less several studies have demonstrated that from the beginning of a life sex has a major impact on outcome of infectious diseases where the overall, morbidity and mortality rates are higher in the male sex right through life [353]. During childhood, studies have shown an increased risk of susceptibility and severity of infectious diseases for males; conversely, females reportedly mount stronger humoral and cellular immune responses to infection or antigenic stimulation than do males. The impact of sex hormones on the T-helper 1/T-helper 2 cytokine balance has been proposed to explain the higher severity of most infectious diseases in males [354]. In the current study we recognised the gender distribution and contribution in the demographic characteristics as presented in chapter 3 of this thesis. However our main aim was not to study gender effects on the immune system as that would need a different analysis plan that would be guided by gender associated drivers and take us to another path which the study was not designed to address.

Effects of HIV and nutritional status on lymphocyte subset frequencies and numbers

Malnutrition and HIV infection cause dysregulation of the immune system that may manifest with depletion or expansion of affected cell populations and their functions

[261]. Many studies have now clearly demonstrated that infection with HIV influences immune cell numbers and functions. Depletion of $CD4^+$ and Vδ2 T cells as a consequence of HIV infection in humans results in inversion of the CD4:CD8 and Vδ1:Vδ2 ratios [355-357]. Less well understood, however, are the effects of malnutrition and nutritional supplementation on immune cell repertoire. In addition, several studies have demonstrated that undernutrition is associated with immunosuppression, which leads to both increased susceptibility to infection and metabolic derangements [225]. Here, we describe the effects of HIV infection and nutritional status on lymphocyte subsets in HIV infected children. Understanding how lymphocyte subsets in HIV infected children with inadequate and abundant nutrients may enhance our ability to target immune cell metabolism and alter immunity in both malnutrition and HIV.

To study the effects of HIV and nutritional status on lymphocyte subsets in HIV infected children we studied the ART-N cohort of HIV infected children to eliminate the effects of ART in comparison with HIV negative healthy children. Previous studies have shown that T cells are depleted in HIV especially in advanced disease and based on those findings in the past, WHO recommended their use in treatment monitoring in the absence of $CD4^+$ T cell monitoring or were used in combination with $CD4^+$ T cell monitoring [358]. Additionally, during the early HIV programs, total lymphocyte counts were used as predictors of absolute $CD4^+$ T cell counts and percentages in HIV infected people and as surrogate markers for treatment monitoring [359]. The hallmark of AIDs in HIV disease is wasting previously known as the "Slim disease", [258] and having a total lymphocyte count, between 1- 2×10^9/L significantly predicated immunosuppression as measured by a $CD4^+$ T cell count less than 0.20×10^9/L in HIV-infected people [359]. In the current study we demonstrated low T cell counts in HIV infected ART-N children which was consistent with previous studies that used total lymphocyte counts for HIV disease monitoring. Interestingly, the SAM group had the highest levels of T cells while MAM were the lowest. This paradoxical presentation may be a result of active co-infections among those who had SAM leading to high T cell numbers or could possibly be due to a high number of exhausted T cells. However when the ART-N malnourished children were compared with HIV-NC the malnourished HIV infected children had T cell frequencies dramatically depleted. Previous studies have shown that in states of severe malnutrition T cell survival, proliferation, and

inflammatory cytokine production are all decreased. The altered T cell function and metabolism described in malnutrition has so far been associated with altered adipokine levels, most particularly decreased leptin [360]. Indeed, circulating leptin levels have been found to be low in malnutrition, and leptin has been shown to be a key link between nutrition and immunity [360]. Another study went further to demonstrate that low leptin levels were linked with higher risk of mortality in severely malnourished children and even higher in those who were HIV co-infected, however, those with higher leptin levels had a better treatment outcome [361]. Another research group reported that leptin played a key role in regulating energy intake and expenditure, appetite and hunger, metabolism and behavior and through its direct action on the brain regulated food intake and body weight, and also played a central role in the immune system [140]. In addition low leptin levels were found to compromise the ability to fight off infections such as HIV [361].

Most studies on B cells in HIV were carried out in adults; those performed in children are limited in number and have mainly investigated HIV antibody and vaccine response without reporting on quantification of B cells. A study on HIV infected adults and HIV negative controls on quantifying HIV specific IgA and IgG antibodies in mucosal surfaces found that, these antibodies were either absent or present at low levels and total IgA in comparison to IgG antibodies were low in the HIV infected adults [297]. In a similar study in HIV infected children, B cell function was assessed by quantification of IgG, IgG1 and IgG3 plasma levels and were all found to be significantly decreased compared to the HIV-negative controls [362]. Earlier studies in ART-N children, assessed IgA, IgG, and IgM serum as surrogate markers of HIV infection by assessing the presence of hypergammaglobulinemia [363]. Contrary to our study we measured B cell frequencies and numbers and found them to be comparable in the children who had SAM and WN, however, they were increased in MAM children. This may be due to less co-infection burden in the MAM children as compared to the SAM children whose status of malnutrition was less severe than those with SAM. The possible reason of lower B cell numbers and frequencies among the WN group may probably be due to a large percentage of children having detectable viral loads though anthropometrically are WN in comparison to the children who had MAM. Generally the children who were ART-N had lower B cell percentages than HIV-NC and some studies have shown that B cells may be severely

damaged during HIV infection with loss of memory B cells and decline of serological memory especially in those with chronic HIV with reduced B cell function [364]. Since we did not perform functional tests our results are not comparable to most other studies.

In the setting of HIV infection, NK cells have been shown to play a vital role in viral protection and control, since they have a unique role in cytotoxicity of viral infected cells [365]. Deficiencies in NK cells have been associated with increased susceptibility to viral infection [365, 366]. Our study showed that NK cells were reduced in children who were ART-N, but were raised in the children who had SAM. SAM children on this study had several co-infections and some of these may have been viral in nature. Infections with viruses typically induce similar patterns of immune responses where in the acute phase of viral invasion there is usually an early induction of type 1 interferons secreted by dendritic cells, increased expression of IL-15, and a proliferation of NK cells. Subsequently, large quantities of Th1 type 1 cytokines (IFN-γ, TNF-α, and several chemokines) are released, which drive a strong Th1, followed by a rapid proliferation of T cells as seen in this study. This host response to infection aims at clearance of the viral infection or a reduction in viral replication in persistent viral infections [367]. Alternatively, the depletion of NK cells in children with HIV infection in this study may have been as a result of increased susceptibility to latent viral infections as they were severely immunocompromised leading to the reactivation of latent viruses such as CMV, HSV. This could have resulted in a transient increase in NK cell numbers due to their much needed cytotoxic ability towards viruses thus the increased activity in children who had SAM. This has also been demonstrated in patients with AIDS who are usually cachectic, by having increased risk of developing infection related cancers like KS. Furthermore in our study NK cells may have been increased in those with SAM in comparison to those who were WN since SAM is an AIDs defining illness or a condition according to WHO-HIV clinical staging and this has been shown to correlate with a state of severe immunosuppression. Severe immunosuppression in HIV is a conducive environment for developing or having an increased risk to OIs as already mentioned and malignancies (both HIV related and unrelated). Since NK cells play a pivotal role in immune response against HIV infection by destruction of infected cells and contribute to adequate and strong adaptive immune responses, by acting on dendritic, T, B, and epithelial cells, it is therefore not surprising

that they are increased when high states of viral replication occur. In our study we did not perform functional tests unfortunately, however, it is safe to hypothesise that increased NK cell activity may be a reflection of functional activity that may result in an increased state of cytotoxic capacity, IFN-γ and chemokines production. This has been found to be associated with resistance to HIV infection and delayed AIDS progression, demonstrating the importance of these cells in the antiviral response most especially in those who are malnourished. There are recent findings of showing a subpopulation of NK cells with adaptive functions that are associated with lower HIV viremia and HIV infection control [368]. Currently there is no data that shows that increased NK cells in HIV predispose to cancer. There is increased risk of developing cancer in HIV and is mainly related to severe immunosuppression that progressively develops in the absence of treatment or delayed initiation of treatment leading to the persistence of detectable viral load. Progressively the immune system's ability to control cancer cells from surviving is lost just like the increased risk of infection that becomes greater with increased immunosuppression occurs.

Malnutrition in HIV infection is mainly the marasmus type and $CD4^+$ T cell depletion is one of the immunological manifestations found to commonly occur in marasmus in the presence of HIV [5, 101]. The findings in the Ugandan study further suggested that those with Kwashakori type of malnutrition needed to have an appreciable level of immunocompetence and thus had higher $CD4^+$ T cells [5]. One of the unique observations of this study is that malnutrition affects $CD4^+$ T cells in ART-N children and this is evidenced by dramatic depletion among the SAM group with reciprocal expansion of $CD8^+$ T cells. Several observational studies strongly suggest that $CD8^+$ T cells play a crucial role in the control of HIV infection [369]. However, HIV specific $CD8^+$ T cell immunity alone is not sufficient to explain the large heterogeneity observed in the clinical and immunological manifestation of HIV disease [370]. Furthermore, comparing the malnourished ART-N children to HIV-NC showed that $CD4^+$ T cell depletion was profound. Using the WHO clinical staging of HIV, [371], moderate to severe forms of malnutrition are AIDs defining conditions due to the association to profound immunocompetence, but it is still not clear if the $CD4^+$ T cell depletion is merely due to disease progression resulting in malnutrition or malnutrition causing the HIV progression however both do aggravate the

immunological status as evidenced by presence of profound depletion of $CD4^+$ T cells in malnutrition with HIV.

The frequencies and functional activities of iNK T cells have not been well understood in HIV or malnutrition. However studies have shown that iNK T cells are important determinants for the progression of autoimmune disorders, tumors, and viral infections [372, 373]. Our study showed the iNK T cells were depleted in the children who were ART-N in comparison with those who were HIV-NC and there was no difference between the groups according to nutritional status. HIV infection depletes iNK T cells this may explain why HIV infected people are more predisposed to developing cancers and multiple infections as iNK T cells are necessary in cancer cell killing, dendritic cell activation in infection exposure and viral killing. There are limited studies of NT cells in HIV and none existent in malnourished HIV infected children. NT cell levels and functions were reduced in *M. tuberculosis* infected patients and these deficiencies were found to reflect the presence of active TB [374]. Our study showed similar findings of depletion of NT cells in HIV infection in children who were ART-N compared to HIV-NC and NT cells were more depleted in those with malnutrition than HIV-NC. However in our study we did not investigate for their relationship to TB but since they are dramatically depleted in malnutrition and some of the study patients had TB it is possible that they may be depleted in the presence of TB or their depletion increases risk of expressing TB disease. NT cells have been found to have antitumor effects and capacity to reprogram their cytolytic activity and cytokine production and therefore may be potential targets for immunotherapy in HIV [311]. Therefore deficiency of NT cells may increase the risk of OI and cancer occurrence in HIV. γδ T cells comprise of 3 groups and can be $CD4^+$, $CD8^+$, DN or DP [375]. Vδ1 T cells are limited in the peripheral circulation with majority being in the tissues or mucosa, recognise self, microbial antigens and stress induced molecules through the MHC unrestricted manner. Recent work showed that they were elevated in HIV most especially in those who are co-infected with *Candida albicans* [337, 376]. The current study showed similar findings that Vδ1 T cells were expanded in ART-N children in comparison to those who were HIV-NC and were strikingly elevated in those with malnutrition. Considering that malnutrition is an AIDs defining condition and children with late stage disease usually present with oral candidiasis, oropharyngeal or systemic

candidiasis, this may explain the dramatic expansion of Vδ1 T cells in this study. Surprisingly, contrary to several studies where Vδ2 T cells have been shown to be depleted in HIV, Vδ2 T cells in this study were expanded in HIV infected children in comparison to those who were HIV-NC but their expansion was less than that seen with the Vδ1 T cells thus preserving the inversion of the Vδ1:Vδ2 ratio. Vδ2 T cells may express low or no CD4 receptor, however, despite this, infection of these cells has been reported [377]. It has been demonstrated that the upregulation of the CD4 receptor on Vδ2 T cells renders them to become HIV targets *in vitro* therefore HIV-induced immune activation may allow infection of γδ T cells *in vivo*. The novel observation in this study is the expansion of Vδ3 T cells in the ART-N children in comparison to those who were HIV-NC and even greater expansion in those with SAM. This raise may be driven by the heavy co-infection burden on a background of chronic HIV infection per se. A previous study showed that Vδ3 T cells were found to be enriched in liver and in the peripheral circulation of patients with chronic viral infections and leukemias [378].

In summary nutritional status affects lymphocyte subsets however functional studies need to be done in order to appreciate the impact of the dysfunctional cells beyond the deficiencies in phenotypes. We have shown that though HIV profoundly affects lymphocyte subset, malnutrition plays a major role in worsening these effects. A profound depletion of T cells, NK cells, CD4:CD8 inversion, depletion of iNK T may depolarize the Th1 response, NT cells and the expansion of ɣδ T cells was noted in peripheral circulation of malnourished ART-N children. These findings demonstrate a sustained aberrant innate lymphocyte profile in HIV infected, ART-N children. This deregulates the immune responses in HIV and potentially account for the increased incidence of co-morbidities and treatment response beyond $CD4^+$ T cell recovery. This may need adjuvant immunotherapy to potentiate the effects of treatment of HIV by ART.

Effects of ART on lymphocyte subset frequencies and numbers among children.

To study the effect of ART on lymphocyte subsets in malnourished HIV infected children we compared the lymphocyte subsets of interest by ART status at baseline among the malnourished children and WN children. The ART-E cohort had been receiving ART for a

median time of 42 months when enrolling in the study and the ART-N cohort was initiating on ART at enrolment.

The hallmark of HIV infection is the progressive depletion of $CD4^+$ T cells with increased risk of OIs and death in those who develop the AIDS in the absence of ART. Commencing ART can achieve suppression of viral replication with a reciprocal increase in $CD4^+$ T-cell numbers in the majority of patients [379], resulting in dramatic decreases in morbidity and HIV/AIDS-related mortality [245], due to a reduced infection risk. ART may initially be related to IRIS, wherein patients experience paradoxical deterioration, despite efficient control of HIV viral replication and no apparent drug toxicity [242]. Alternatively, immune restoration on chronic ART use may also be incomplete. Relatively few individuals will achieve normal levels of $CD4^+$ T cells. Up to 20% of patients may experience immunologic non-response despite HIV virologic suppression, with limited $CD4^+$ T cell increase or none at all [380], however, the commonest reason is poor adherence to ART. Most importantly, those with recovery of near normal $CD4^+$ T cell counts may maintain chronic immune activation that has been linked to an increased risk of non-AIDS related morbidity and mortality, making it clear that the detrimental effects of HIV infection go beyond $CD4^+$ T cell depletion and recovery [381]. The current study demonstrates similar findings with other studies where total T cells and $CD4^+$ T cell numbers increased with reciprocal decline of $CD8^+$ T cells on chronic use of ART irrespective of nutritional status. In a study investigating B cells in acute and chronic HIV infection before and after reduction of HIV plasma viremia by ART in peripheral blood B cell counts were found to be significantly lower in both early and chronic HIV infected individuals compared with uninfected controls. B cell numbers in both groups increased significantly after ART introduction and the impact was better in those who initiated ART early [382]. In contrast, our study found that B cell numbers were lower in children who were ART-E compared to those who were ART-N. However, they were also lower in all ART-N children in comparison to HIV-NC. This implies that HIV infection affects B cell numbers and despite ART initiation, B cell numbers may not be restored to normal levels.

NK cells were lower among the ART-E compared to the ART-N irrespective of nutritional status. Though this has not been previously reported, it has been noted that after cord blood transplant these cells are the first innate lymphocyte cells to repopulate. Therefore

considering HIV results in profound immunosuppression with good ART adherence, achievement of early viral suppression may result in NK cells following a similar pattern as revealed in cord blood transplant, however our study participants who were ART-E [383], were also not well suppressed virologically. On the contrary, it is possible that though ART leads to the recovery of cells like $CD4^+$ T cells and T cells it may have untoward effects on NK cells but this has not been previously reported.

Though there are limited studies describing the response of $CD4^+CD8^+$ and $CD4^-CD8^-$ T cells to ART, the few that have been done demonstrated similar results to our study where there is an inverse response in these cells: $CD4^+CD8^+$ T cells were higher while $CD4^-CD8^-$ T cells were lower in those who were ART-E compared to ART-N. Persistent population of $CD4^+CD8^+$ T cells in ART-N have been shown in limited elite HIV controllers and are thought to be due to having an enhanced antiviral activity [384, 385] though some studies did not observe this [386]. Recent evidence has shown rare Gag+ cells that persist during the use of ART that are $CD4^-CD8^-$ T cells. These are thought to be a component of the HIV reservoir [308] and may explain the persistence of these cells after prolonged treatment.

In a study where iNK T cells were stimulated using α-GalCer to investigate the cytokine production (IFN-γ, TNF-α, IL-2, IL-4, and IL-21) and CD107a expression in HIV infected long-term non progressors, progressors, before and after ART in comparison with HIV negative participants, revealed that functional iNK T cells secreting cytokines were associated with non-progressive HIV infection but not with ART. They also demonstrated failure of restoration of the iNK T functionality after ART initiation [387]. Though, we did not perform functional studies, our results show that iNK T cell frequencies and numbers did not return to normal levels among the ART-E cohort. There is need for further studies since iNK T cells are important in the activation and regulation of immune responses. In addition iNK T cells being innate-like T cells that respond rapidly to a broad range of effector functions upon recognition of glycolipid antigens presented by CD1d and HIV infection carries Nef- and Vpu-dependent mechanisms that interfere with CD1d surface expression, indirectly suggests that iNK T cells have a crucial role they play in control of HIV infection. This role is actively targeted and affected by Nef- and Vpu-dependent viral immune evasion mechanisms early on in HIV [388].

Unlike classical T cells, NT cells possess both innate and adaptive immune functions and thus display both T and NK cells functions. They can express both MHC restricted and MHC-unrestricted cytotoxicity and secrete several cytokines. Their activation is crucial due to their ability to serve as an early source of regulatory cytokines and degranulation-related killing function [311, 389, 390]. NT cell frequencies and numbers were lower in the ART-E children compared to the ART-N children probably indicating the deleterious effects of ART on achieving complete immune restoration or the presence of ART results in their indiscriminate destruction affecting their double role in the innate and adaptive immune system if restored early on in the disease may prevent the deleterious effects of HIV/AIDs on the immune system by immunotherapy.

In perinatally acquired HIV infection, γδ T cell responses to HIV infection are complicated and not well-elucidated. Current evidence, though limited is from horizontally acquired HIV infection [357, 391]. Previous reports have indicated increased levels of Vδ1 T cells and decreased levels and function of Vδ2 T cells in chronically HIV-infected patients [391, 392]. Furthermore, changes in the Vδ2 T cell population correlated positively with $CD4^+$ T cell counts and negatively with viral loads [391]. The impact of ART on Vδ2 T cell recovery in HIV patients is controversial and inconclusive. A report in 2004 indicated partial recovery of the Vδ2 T cell population following long-term HIV-suppressive therapy [393]. However, a subsequent study demonstrated that ART failed to restore the Vδ2 repertoire in HIV-infected men [342]. The γδ T cells were affected by ART, with Vδ1 T and Vδ3 T cells being reduced while Vδ2 T cells were similar in the groups.

In summary, ART partially normalises the skewed lymphocyte subsets induced by HIV infection, however some perturbations persist probably due to persistent circulating viral load and late ART initiation with immune activation persisting in this cohort. Understanding the effects of HIV on unique lymphocyte subsets dysfunction and restoration or depletion following ART may provide important insights into the mechanisms of HIV pathogenesis and provide the opportunity for adjuvant immunotherapy.

Effects of RUTF supplementation on lymphocyte subset frequencies and numbers

The effects of RUTF supplementation on lymphocyte subset frequencies and numbers were assessed by studying a cohort of ART-E malnourished children at baseline and 12 weeks to minimize the effect of ART. The change in lymphocyte subset frequencies and numbers in ART-E WN children were also evaluated.

Standard nutritional rehabilitation has mainly targeted those who had SAM. It included the empirical use of antibiotic therapy; however with the introduction of RUTF in the management cascade of malnutrition from homes, this method has the added potential in reducing the use of antibiotics which was previously the routine. Some studies in primary malnutrition demonstrate that participants were treated with nutritional therapy alone, though in many studies we cannot be certain that antibiotics were not given. Thus compromising the comparisons we needed to make due to the heterogeneity of management of malnutrition. Studies confirm that the initial finding of thymolymphatic atrophy resolves with nutritional rehabilitation [394], and in parallel, T lymphocyte function as examined by cell proliferation and the TST improves [395, 396]. However, we need to recognise, that repeated TST will improve responses through the process of 'vaccination like reaction' alone. In addition, described defects of the innate immune system, such as complement levels [397], neutrophil microbicidal activity [394, 398], secretory capacity for IgA transport into secretions [399], and monocyte production of cytokines IL1β, TNFα, and IL6 [400, 401], have been found to improve on nutritional rehabilitation. Malnutrition affects the absorption of food nutrients resulting in a malabsorptive syndrome and HIV affects the gut, further worsening nutrient reabsorption. Studies in HIV infected adults have shown response to parental and enteral nutritional rehabilitation leading to increased body weight and fat mass which was similar to the results we reported on anthropometric parameter improvement [402, 403]. A study in children providing nutritional rehabilitation by the enteral route resulted in dramatic $CD4^+$ T cell count increase whereas those who received total parental feeding had no change in $CD4^+$ T cell numbers [404]. In our study we showed that T and B cell numbers increased marginally at 12 weeks while NK cells increased drastically. Though there are no previous studies showing the effect of nutritional supplementation on immune cell

restoration in HIV/AIDs in children, NK cells have been reported to play a crucial role in early immune reconstitution after receiving human cord blood transplant because they are the first lymphocyte subset to recover [383]. In our study there was an even more robust response in those who were WN demonstrable by the increase of T, B and NK cells. Probably in order to have a better immunological response in terms of increased numbers the period of providing RUTF should have been longer to allow for the intestinal repair in turn increased nutrient absorption. We propose that if RUTF was given in those who were WN immunological repose would be even better and could have better immunological value in those who are WN and to prevent malnutrition. There was no effect on inversed pattern of $CD4^+$ and $CD8^+$ T cells, similarly the DP and DN were still increased in those who were malnourished and DP were reduced. This may be due to the presence of ongoing infections like TB, malaria, candidiasis etc. These infections needed to first be treated and also provide longer period of nutritional supplementation to tease out the RUTF effects immunologically.

Efficient immune reconstitution requires the innate immune system to recover promptly for the acquired immune system to be able to reconstitute also. Depletion of iNK T cells did not improve after RUTF supplementation in our study. This pattern may be explained by similar findings from a study where numbers of iNK T cells were significantly decreased in TB patients and in HIV infected individuals who were ART-N or had detectable viremia despite receiving ART [405]. Our study participants especially those who were malnourished had a significant burden of OIs and these OIs such as TB depress iNK T cell and probably further mask the responses that may have been achieved through nutritional rehabilitation. There are limited studies on NT cells in HIV and nutritional rehabilitation. There are no studies to date describing the response to nutritional supplementation by γδ T cells. Vδ1 were decreased in the malnourished and Vδ2 T cell were interestingly increased in both the malnourished and WN. The effect of ART on Vδ1 T cells has shown to result into a decline trend to the pattern seen in HIV negative people. Whereas Vδ2 T cell recovery in HIV infected patients is controversial and inconclusive, an earlier study indicated partial recovery of the Vδ2 T cell population following long-term ART [393]. However, a subsequent study demonstrated that ART failed to normalize the Vδ2 T cells though there was an improved increase [342]. Our study describes for the first time the

effect of nutritional supplementation on γδ T cells where Vδ2 T cell frequencies and counts were significantly increased at 12 weeks, while Vδ1 T cell frequencies and counts were reduced which is similar to what is seen in the presence of ART. The Vδ2 T cells were raised at 12 weeks probably because the viral load was reducing and this is an indication of the cells tending to achieve the normal trends with reciprocal decrease of Vδ1 T cells. Vδ2 T cells in HIV infected persons are invaded by the HIV virus as it tries to evade the immune responses. These are destroyed by cells like Vδ1 T cells as they expand during viral replication and are known to kill off viral infected cells such as the Vδ2 T cells. However the Vδ2 T cells in HIV may increase due to the presence of other infections such as fungal infections like candida. Lastly in our study, Vδ1 T cell frequencies and counts may have reduced as the children were on ART as already explained above. Vδ1 and 2 T cells have been found to be activated by lipids, stress molecules and non-peptide antigens and could explain the expansion of Vδ2 T cells in this study as RUTF is lipid based. After stimulation of Vδ2 T cells they have a series of functions that may be set in motion and these include increased production of cytokines, chemokines and lytic enzymes through its effector arm or may result in cytolytic and non-cytolytic antiviral activity, they may also induce dendritic cell maturation, support B cell activities and presentation of antigens to $CD4^+$ T cells and $CD8^+$ T cells [406]. This increase may have contributed to the increase seen in the B cell numbers and counts after nutritional rehabilitation. This is the first study on Vδ3 T cells and we demonstrated a decline in those who were malnourished after nutritional supplementation but increased in those who were WN. This may be explained by the higher HIV burden in those who are WN and ART-E. On the other hand, enteral nutritional rehabilitation seems to contribute to the repair of the gut leading to increased nutrient absorption. This may result in the minimal change of the lymphocyte subsets as discussed, and additionally could be because one may need a longer period of taking the nutritional supplement for profound immune responses to be visible.

Comparison of Vδ1, Vδ2 and Vδ3 T cell frequencies and numbers in immunological and virological responders and non-responders.

Malnutrition has been shown to be associated with rapid $CD4^+$ T cell decline with increased rate of OIs. Malnutrition affects the absorption of nutrients resulting in a malabsorption syndrome through the extensive destruction of the intestinal villi. HIV

infection has been shown to cause gut destruction resulting an enteropathy which are characterised by changes in mucosal structure that have been found to be similar to lesions in coeliac disease. Though severe villous atrophy is rarely seen in HIV infected patients; in combination with malnutrition this may occur [407]. We could hypothesise that malnutrition affects immunological and viral response of innate T cells as a result of reduced ART absorption. Considering that now all PLWHIV/AIDs are eligible to initiate ART to improve their survival through suppression of HIV replication, one of the major challenges of HIV care will be to restore normal immune responses beyond focusing on only $CD4^+$ T cells. Suboptimal immune response is currently limited to defining the phenomenon based on $CD4^+$ T cell responses to ART and they have been associated with substantial increases in the risk of AIDS-related and non-AIDS-related mortality and morbidity [408-410]. Unfortunately, a significant proportion of HIV infected individuals are not able to achieve a normal $CD4^+$ T cell count or prolonged viral suppression despite receiving ART. A study revealed that nearly 25% of patients who started ART with a $CD4^+$ T cell count of <200 cells/μL did not achieve a $CD4^+$ T cell count of >500 cells/μL even after 7-10 years of treatment or longer [411]. We therefore focused on describing the trends of immunological response and viral response using γδ T cells as innate cells are the first line of defense and thus were interested in investigating their restoration on ART as they may be used in developing immune-adjuvants to potentiate the effects of ART.

The HIV infected children who were INR had profoundly high Vδ1 T cells compared with the AR. In addition those found to be virologically NR had astronomically high Vδ1 T cells in comparison to the viral responders. Increased trend of Vδ1 T cells in HIV has been shown in limited studies to express an increased cytolytic activity in the presences of infections like Candida [337, 341], HIV and CMV and also kill HIV infected cells [328-330, 338] and may account for the sustained increase of Vδ1 T cells in the INR. Interestingly, Vδ2 T cell numbers were high in the INR compared to those with AR and the Vδ2 levels were similar among the viral responders and none responders. This is contrary to what other studies have shown in response to ART [331]. This may be due to the presence of ongoing infections like TB and being an innate cell will respond to various infectious agents rapidly and probably remain elevated in the presence of an active pathogen that stimulates them [357]. In a recent study, Vδ3 T cells have been shown to induce

maturation of DC that elaborate cytokines that is CD1d-dependent. They also play a crucial role in B cell differentiation into plasma-secreting cells or memory cells, Ig class switching and antibody [412]. Vδ3 T cells had a downward trend with AR and viral response. There are limited studies reported on Vδ3 T cells behavior except for being expanded in HIV infected patients and chronic viral infections like hepatitis C patients and leukemias [413]. Our findings suggest that Vδ3 T cells may be a novel biomarker of disease severity or treatment response. We have shown that Vδ3 T cells are increased in HIV infection, malnutrition and more marked in those with SAM. It is therefore not surprising that among INR and viral NR they are greatly expanded too.

Conclusion

The prevention of infection predominantly relies on adequate barrier function and robust innate immunity which have been shown to be breached in HIV infection. Whereas clearance of an already established infection requires either a successful humoral response e.g. in malaria or a successful cell-mediated immune response e.g in TB, and the presence of occult or transient antigenemia may have accounted for the minimal response to nutritional supplementation. High OI burden therefore may have masked the immune responses necessitating to have had them treated first. The presence of malnutrition may have further masked the immune responses expected and we probably needed a longer period of nutritional supplementation. Nutritional supplementation may restore the lymphocyte subsets however this may have more value in those who are WN as we observed improvement in the absence of RUTF. The efficacy of nutritional supplementation may be better enhanced if provided before the development of AIDs defining conditions to potentiate robust immune responses and restoration. Lastly, substantial T cell subset recovery in malnutrition may be that the immune system is identifying RUTF as an antigen thus needs a longer time to develop immune tolerance to RUTF in order to then benefit from the nutritional supplementation using RUTF.

CHAPTER SIX: Pharmacological outcome results

Introduction

Pharmacokinetics (PK) is the study of the time course of drug absorption, distribution, metabolism and excretion. For the purposes of this study we measured the drug concentrations of the NNRTIs in plasma and these were NVP and EFV. The reason we studied the plasma concentrations of NVP and EFV is that they form the backbone of the first line ARV regimens and are the most commonly used ARV regimens in the Pediatric HIV programs. In addition their PK is understudied in the malnourished HIV infected children an important risk group for late presentation of HIV/AIDS yet we continue to provide and use these drugs in similar dosages as those given to the well-nourished HIV infected children. There is also no evidence currently when used concurrently with RUTF which is the standard of therapeutic food supplementation of uncomplicated acute malnutrition.

Non-nucleoside reverse transcriptase inhibitors (NNRTIs)

NNRTI are antiviral agents that bind non-competitively to HIV reverse transcriptase, as a result, blocking it and downstream prevents HIV viral replication. HIV reverse transcriptase, is an HIV enzyme that converts the HIV RNA into DNA through a process called reverse transcription [414]. NNRTIs comprise the commonest ART used in Uganda: NVP and EFV while the less frequently used ones are delavirdine, etravirine and rilpivirine. They are taken orally and have several different formulations however; the commonest formulations used in the study sites were syrups and tablets. The drugs may be dispensed as single drugs or in fixed dose combinations. They are well absorbed in the GIT and are transported across the GIT epithelium by several transport systems. NVP is absorbed in the jejunum, ileum, ascending colon and descending colon. The rate of absorption decreases from the jejunum right down to the descending colon [415]. In plasma they are transported by albumin or α-glycolipids or as free drug. They are generally metabolized by the liver using the cytochrome P450 enzyme system and excreted by the kidneys. They are also renowned for rapidly leading to virus-drug resistance development. The main mutations in HIV-1 are based on the emergence of the K103N and Y181C mutations [414]. K103N is a nonpolymorphic mutation selected in patients who may have been previously exposed to or receiving NVP and EFV. K103N

mutation reduces NVP and EFV susceptibility by about 50 and 20-fold, respectively. Other studies report that Y188C as uncommon nonpolymorphic mutation selected by NVP and EFV. However when it occurs, confers high-level resistance to NVP of greater than 50-fold reduced susceptibility and EFV 20-fold reduced susceptibility [416]. A systematic review across all pediatric HIV studies on viral resistance demonstrated that K103N and M184V were the most commonly detected mutations found in reverse transcriptase (39.8% and 76.6%, respectively) [417]. Another systematic review demonstrated that the commonest mutations were M184V (53%), K103N (31%) and K65R (7%) [418]. HIV drug resistance is currently defined as having mutation sequences classified as low, intermediate or high-level resistance according to the Stanford HIV data base algorithm. With respect to NNRTI resistance is defined as resistance to NVP or EFV. Substantial evidence from studies show that viral resistance is highest with NNRTs (61%), NRTI (55% and PIs (6%) [419].

Drug metabolism and malnutrition

Most PK studies are carried out in healthy adult populations, thus marginalizing pediatric populations. Thus, there is a paucity of crucial data on PK levels of ARVs in malnourished children yet they are a recognized risk group of delayed access to ART. Clinicians still benchmark on evidence accrued from studies done in well-nourished older children and adults and extrapolate to malnourished children. The knowledge gap still exists of lack of evidence in optimum timing of providing ART in malnourished HIV infected children especially with severe presentation of acute malnutrition necessitating supportive interventions in order to survive. In addition optimal dosing for ARVs in malnutrition has not been established and neither has the effect of nutritional supplementation on ARV PK been done.

NVP forms the backbone of the first line ART for both adults and children in LRS and this includes the malnourished children as well. NVP is taken orally and has several available formulations like syrups, tablets, may be as a single drug or in combination of other ARV drugs. Over 90% of NVP is absorbed across the GIT transport system. It is metabolized by CYP450 system in the liver by CYP 3A4 and 2B6 enzymes and excreted in the liver.

NVP has the property of auto-induction where it induces the production of CYP3A4 leading to its metabolism and thus decreased plasma half-life. Auto-induction is usually complete

by 28 days after initiation on an NVP based regimen resulting in a steady state plasma NVP trough concentration of 4.7 mg/ml (range: 3.6-6.4 mg/dl). It interacts with other drugs like rifampicin, ketoconazole leading to suboptimal levels of NVP in children receiving anti-TB drugs or anti-fungals [212, 420].

The majority of HIV malnourished children are treated for TB co-infection especially in the 1st 6 months after diagnosis of HIV. This implies that malnourished HIV infected children will have to be initiated or switched from NVP to an EFV based regimen. In addition, TB diagnosis poses a diagnostic challenge in pediatrics, moreso in malnutrition with HIV. Failure to thrive is one of the clinical signs used in making a TB diagnosis and is a cardinal symptom of progressive HIV. Therefore HIV malnourished children have a high probability of being treated with anti TB drugs. The diagnosis of TB depends on clinical signs and symptoms due to its paucibacillary characteristic in pediatrics that makes microbiological diagnosis difficult. This is further complicated by difficulty in collecting diagnostic samples in children. EFV exhibits pharmacokinetic variability causing varied clinical response with a narrow therapeutic range. Plasma concentration levels greater than 4 μg/ml are associated with more CNS toxicity including sleep disorders, hallucinations, insomnia and dizziness [183]. EFV concentrations below 1 μg, which are suboptimal for treatment of HIV, are associated with increased rate of virological failure. Inter-patient variability results from variability in hepatic metabolism and the extent of P-glycoprotein (Pg-P, a multi-drug resistant transport protein) mediated movement across plasma membranes [421]. EFV absorption is increased by fat-containing meals, potentially increasing plasma concentrations of the drug by 50%, of which 99.75% is bound to albumin. Hypothetically, in the current study we expected higher concentrations of EFV in the children who were supplemented with RUTF due to the high fat content in RUTF. EFV metabolism primarily is via CYP3A4, and 2B6; undergoes auto-induction (20-40%) during first two weeks of therapy whose major metabolite is inactive [422]. EFV metabolism has been shown to be majorly dependent on CYP2B6 activity; which exhibits variable levels of expression that is associated with genetic polymorphism. However, more recent studies have shown that EFV is extensively metabolized by intestinal and hepatic CYP3A4 enzyme and EFV is also known as to be a potent CYP3A4 modulator coupled with having drug-drug interactions. However there is paucity of data regarding CYP3A4 activity in HIV infected

malnourished patients most especially children [422]. The lack of knowledge in addition to the inter-individual and intra-individual variability in metabolism of EFV poses a challenge to the optimisation of drug levels available to clear the HIV infection.

A common polymorphism of CYP2B6 is a single nucleotide polymorphism, with a base change at position 516 from G to T (CYP2B6–516G > T), which is associated with reduced EFV metabolism resulting in elevated plasma EFV concentrations. It was recently found out that CYP2B6*6 accounts for about 50% of inter-individual variability of EFV PK as the main predictor of EFV plasma concentration in Ugandan HIV infected adults on an EFV based regimen [187]. This is not as extensively studied in children; however Saitoh et al reported the median oral clearance rate of EFV was significantly lower in children with the CYP2B6–516T/T genotype than in children with either the G/T or the G/G genotype [188] whereas expression of the CYP2B6*6-516GG genotype in children was associated with 50-70% of suboptimal EFV concentration of <1μg/ml [189]. Physiological changes in growth and development, immature enzyme systems and clearance mechanisms greatly affect drug pharmacogenetics in children. Recent studies done in children have found out that recommended dosing guidelines do not necessarily achieve optimal EFV concentrations [190]. According to WHO viral load monitoring guidelines, PLWHIV/AIDs undergo viral load testing once a year, if the viral load is found to be greater than 1000 copies/mL one undergoes intensified adherence counselling, after 6 weeks a repeat viral load test is done and if still above 1000 viral copies/mL, genotypic resistance testing is done [419].

Aims of the present study

This chapter will present the pharmacological results of the study. The main pharmacological objectives were:

1. To determine the impact of nutrition status on PKs of EFV and NVP
2. To determine the effects of nutritional supplementation on PKs of EFV and NVP
3. To determine the magnitude of treatment failure

Results

EFV and NVP plasma PK results at baseline and after nutritional supplementation

The average age of the children whose samples were analysed for drug concentration levels on the study was 7.3 years (SD±3.2) with male: female ratio of 1:1.2. Of the 120 plasma samples analysed, 48/120 (40%) children were receiving an EFV based regimen and 72/120 (60%) were on a NVP based regimen. Of the 48 EFV samples analysed, 23/48 (47.9%) and 25/48 (52.1%) were at baseline and 12 weeks respectively, of whom 7/23 (30.4%) were malnourished at baseline and 12/25 (48.0%) were previously malnourished among the 12 week samples. The EFV and NVP were reported to have been taken orally between 8.00 pm and 10.00 pm before the children went to sleep the previous evening. They were sampled between 8 am - 11 am the next morning before taking NVP dose for those who were on a NVP based regimen. We are therefore reporting mid-dose concentrations for EFV and trough plasma NVP concentration estimated at steady-state among the ART-E HIV infected children at baseline and at 12 week follow-up.

At baseline, 2/23 (8.7%), 11/23 (47.8%), 4/23 (17.4%) had sub-therapeutic, therapeutic and supra-therapeutic EFV concentrations, respectively and 8/23 (34.8%) had undetectable levels. At 12 weeks 10/25 (40%), 5/25 (20%), 10/25 (40%) had sub-therapeutic, therapeutic and supra-therapeutic EFV concentration respectively, (Figure 111 A), and 10/25 (40%) had no detectable plasma levels of EFV. Overall the plasma EFV concentrations were higher at baseline compared to 12 weeks. This difference was not statistically significant, (Figure 109). Comparing those who were malnourished at baseline and after receiving RUTF supplementation, the EFV concentration was slightly raised after RUTF supplementation. Whereas, in comparing the malnourished and WN children at baseline, EFV plasma concentration levels were higher compared to the WN with median concentration being 8.1 mg/l (IQR: 0-12.4) vs 5.4 mg/l (IQR: 0-11.5) but not significant. After RUTF supplementation the median EFV plasma concentration levels among those malnourished at baseline were higher in the malnourished compared to the WN and were not statistically significant. These data show that children with sub-therapeutic and supra-therapeutic levels of plasma EFV concentration were higher than those who had therapeutic levels at 12 weeks.

A total of 45/72 (62.5%) NVP samples and 27/72 (37.5%) at baseline and 12 weeks respectively were analysed, of whom 22/45 (48.9%) were malnourished at baseline and 12/27 (44.4%) were previously malnourished but received RUTF by 12 weeks. Overall, NVP plasma concentration was higher at baseline than 12 weeks and the difference was statistically significant (p-value=0.012), (Figure 110). At baseline 10/45 (43.5%), 11/45 (47.8%), 4/45 (17.4%) had sub-therapeutic, therapeutic and supra-therapeutic NVP concentrations respectively, (Figure 111.B). At 12 weeks 10/27 (40%), 5/27 (20%), 10/27 (40%) had sub-therapeutic, therapeutic and supra-therapeutic NVP concentrations respectively. At baseline, NVP concentration levels were lower among the malnourished compared to the WN with median concentration levels of 3.51 µg/ml (IQR: 1.10-7.41) and 6.83 µg/ml (IQR: 4.06-9.65) (p-value=0.036), respectively. After RUTF supplementation the median NVP plasma concentration levels among those malnourished was 3.51 µg/ml (IQR: 1.10-7.41) and 4.09 µg/ml (IQR: 1.00-6.30) at 12 weeks; (p-value=0.8), respectively.

The median length of being on ART among the HIV infected children was 17 (IQR: 1-48) months and 81/84 (96.4%) had a self- reported adherence score to ART of >95% at 12 weeks. We used a self-reporting to assess for ART adherence in this study. Though the adherence cards were present and regularly accessed from the Ministry of Health by the study sites they were no longer being used to assess adherence as it was noted on several occasions the carers would complete the cards while waiting in the appointment queues. Thus we used self-reporting knowing its limitations too.

In summary, overall, EFV plasma concentration was higher at baseline than at 12 weeks. Among the malnourished after nutritional supplementation they exhibited higher EFV levels at 12 weeks, when compared to WN at baseline and 12 weeks the malnourished children still had higher EFV plasma levels. NVP plasma concentrations were significantly higher at baseline before children received RUTF supplementation. When comparing the malnourished children before and after RUTF supplementation the NVP concentrations modestly increased after nutritional supplementation. When we compared the malnourished and WN at baseline the NVP levels were significantly lower among the malnourished. After 12 weeks of nutritional supplementation those previously malnourished at baseline had a higher NVP concentration in comparison to the WN.

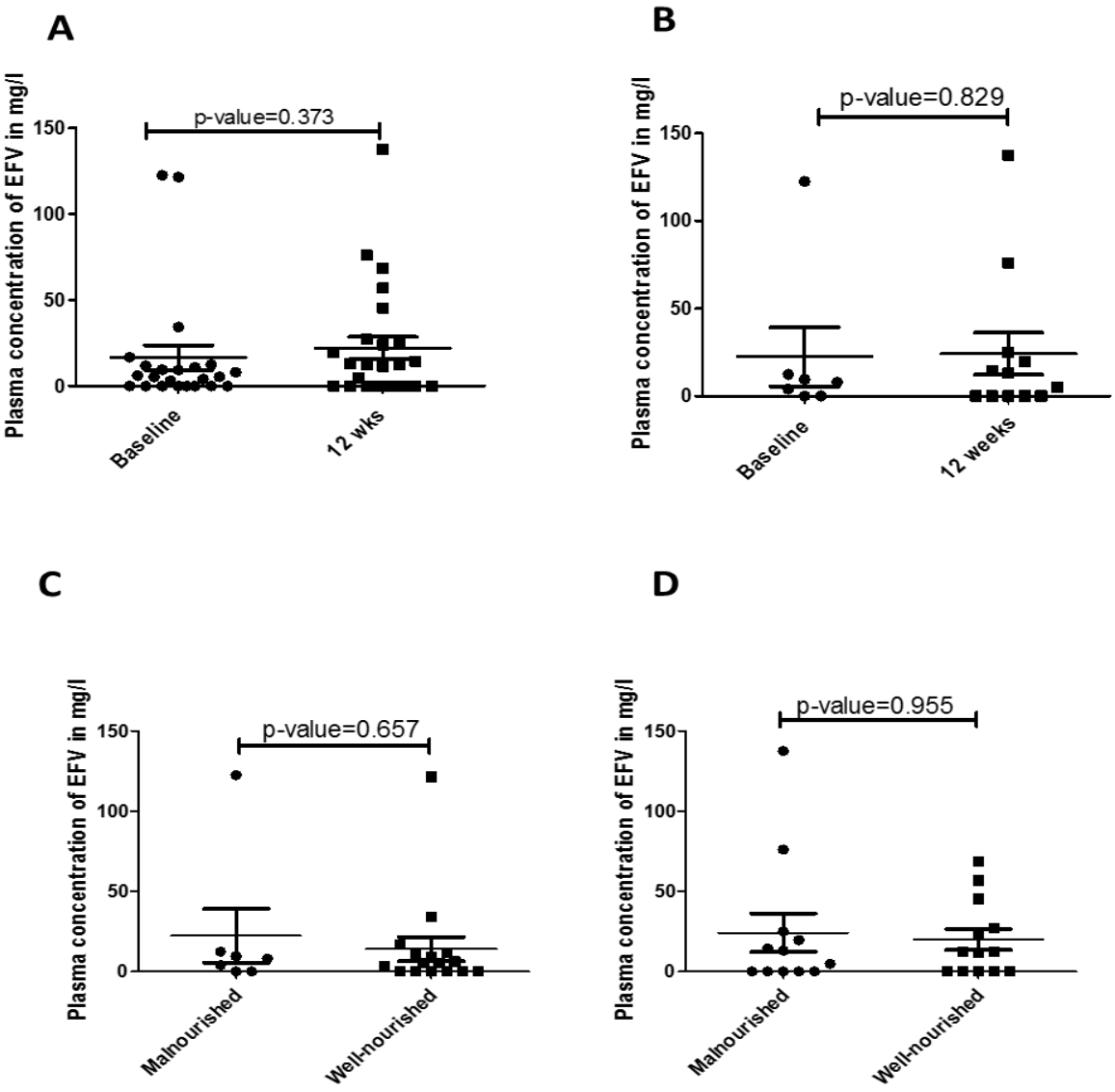

Figure 109: Changes in plasma concentration of Efavirenz (EFV) in ART-experienced children at baseline and 12 weeks follow up.

*Plasma samples were isolated from 23 ART-experienced children with HIV infection at baseline and 25 patients after 12 weeks of follow up. These patients received nutritional counselling +/- RUTF supplementation. Scatter plot show plasma concentrations of EFV comparing (**A**) EFV in ART-E children at baseline and 12 weeks, (**B**) malnourished children at baseline and at 12 weeks ART-E children, **(C)** ART-E malnourished and WN children on EFV at baseline and **(D)** malnourished and WN ART-E children at 12 weeks. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values where those in red are statistically significant.*

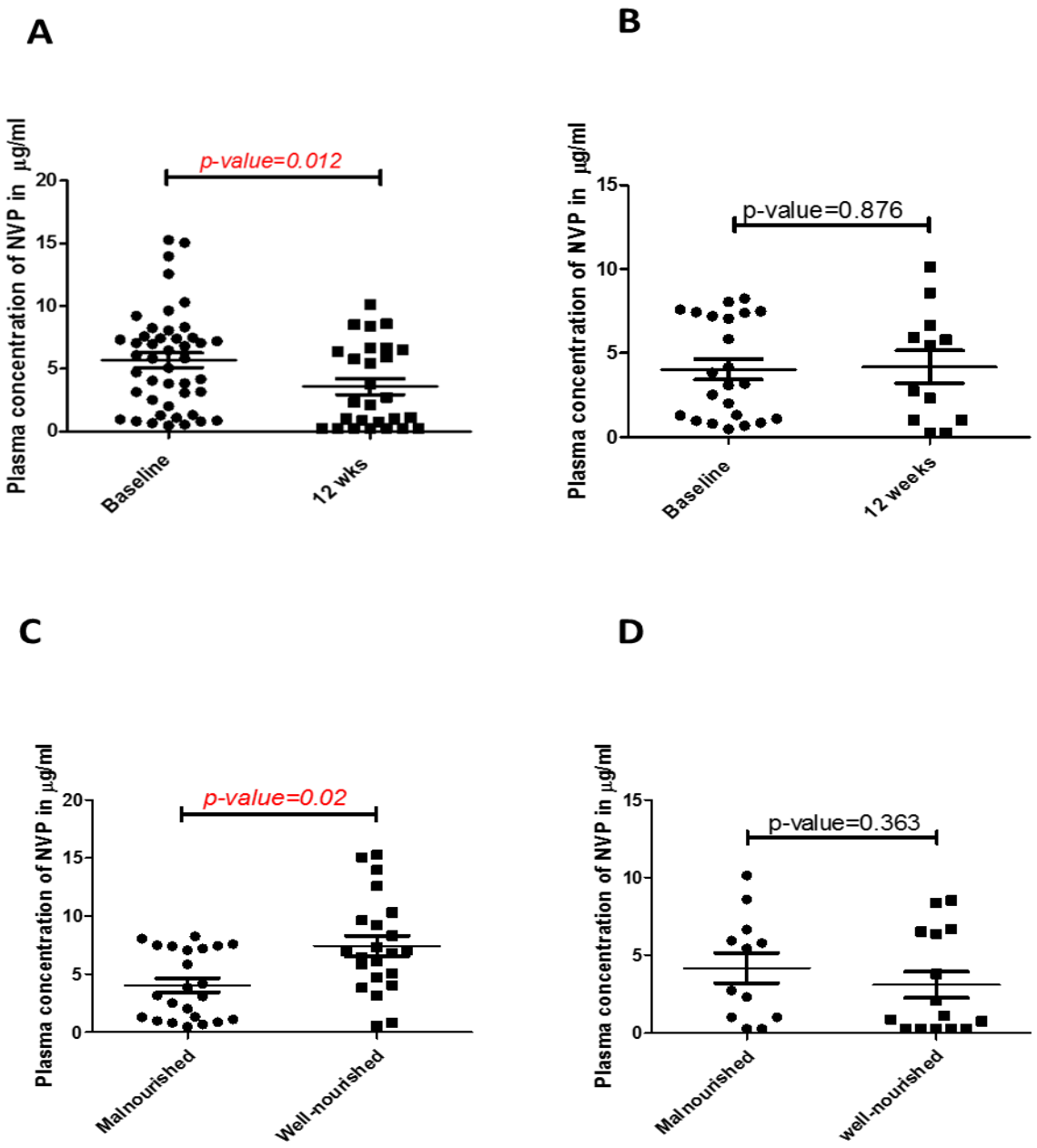

Figure 110: Changes in plasma concentration of Nevirapine (NVP) in ART-experienced children at baseline and 12 weeks follow up.

*Plasma samples were isolated from 45 ART-experienced children with HIV infection at baseline and 27 patients after 12 weeks of follow up. These patients received nutritional counselling +/- RUTF supplementation. Scatter plot show plasma concentrations of NVP comparing (**A**) NVP in ART-E children at baseline and 12 weeks, (**B**) malnourished children at baseline and at 12 weeks ART-E children, **(C)** ART-E malnourished and WN children on NVP at baseline and **(D)** malnourished and WN ART-E children at 12 weeks. Data were compared using the Mann Whitney U statistical test. Numbers in the graphs indicate P values where those in red are statistically significant.*

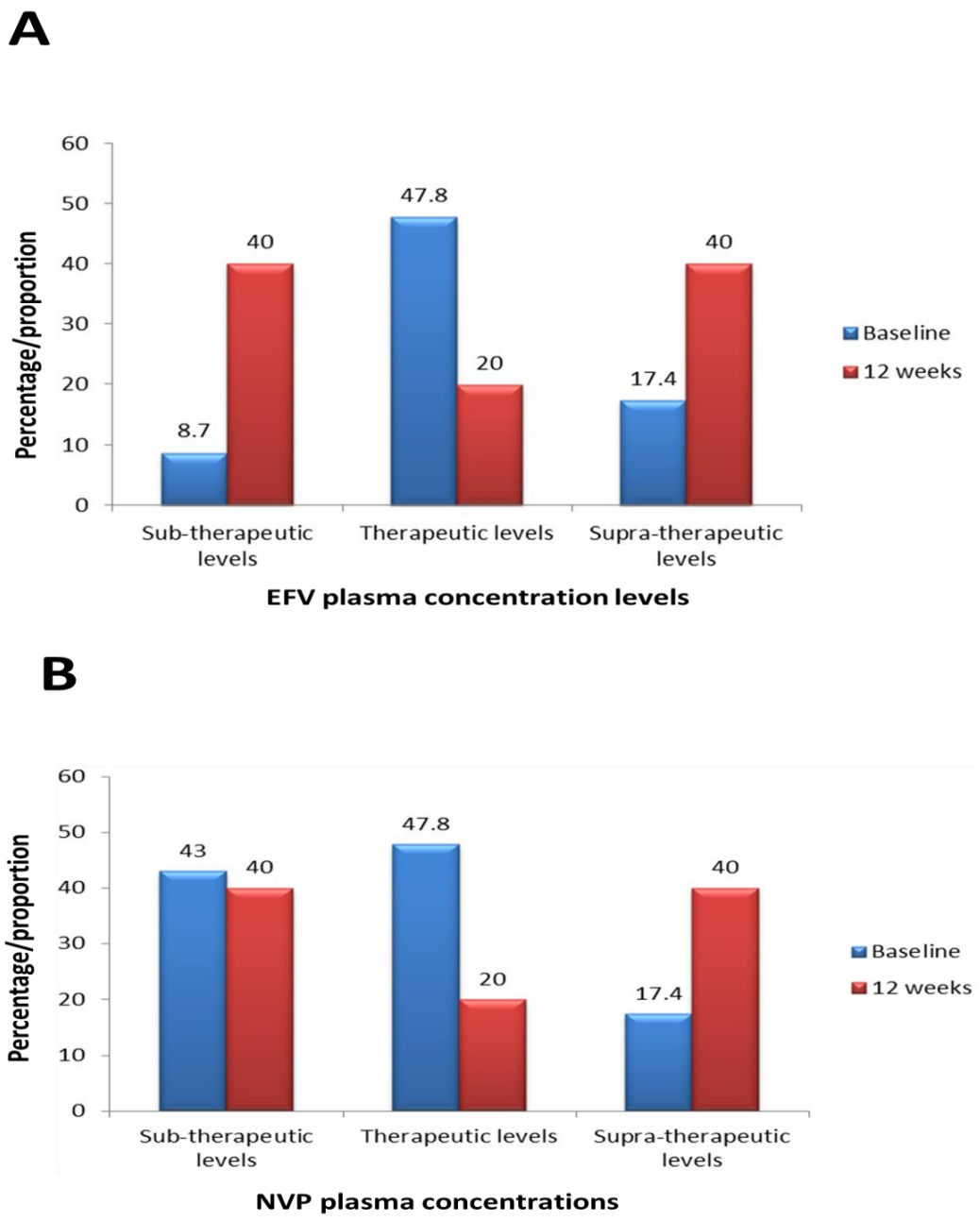

Figure 111: Effect of RUTF supplementation on plasma concentration of EVF and NVP in ART-experienced HIV infected children.

This is indicating sub-therapeutic levels of EFV < 1 mg/l and NVP <3µg/mL, therapeutic levels of EFV 1-4 mg/l and NVP 3-8 µg/mL and supra-therapeutic levels of EFV > 4 mg/l and NVP > 8 µg/mL. ***A,*** *EFV and* ***B,*** *NVP drug concentration levels. The blue bars denote the NNRTI plasma concentrations at baseline while the red bars denote the NNRTI plasma concentrations at 12 weeks.*

At baseline, of 90 ART-E HIV infected children studied, median time on ART was 42 months (IQR: 15-66 months), median $CD4^+$ T cell percentage, $CD4^+$ T cell count, and viral loads were 32% (IQR: 25-39%), 1,064 (IQR: 686-1,379) and 114 cps/mL (IQR: 19-17,139) respectively. Overall, 54.1% had immune reconstitution. Receiving PIs compared to NNRTIs based regimens conferred better virologic suppression (80% versus 20%; p-value=0.03). There was no difference in immunologic and virologic suppression amongst WN and malnourished ART-E children. Using WHO classification 61.7% had virologic suppression and 38.27% were not having viral suppression. Using the Cobas AmpliPrep/Cobas Taqman 48 machine (CAPCTH) detectable cut offs, 27.16% had undetectable viral load < 20 cps/ml and 72.84% had detectable viral load ≥ 20 cps/ml, (Figure 112).

The commonest HIV resistant polymorphisms on genotype testing among the study HIV infected children included: NRTI resistance polymorphisms: M184V, M41L, K70KR, D67N, K219Q and NNRTI resistance polymorphisms that includes K103V, Y181C and V108IV. Other RT polymorphisms included T200A, Q207A and K122E. Each child had more than 3 mutations in the 16 children who had viral genotyping done. Overall 16 children had their ART regimen switched during the study period as a result of virological failure, 4/16 who had viral failure had their viral load less than 1000 viral copies per ml.

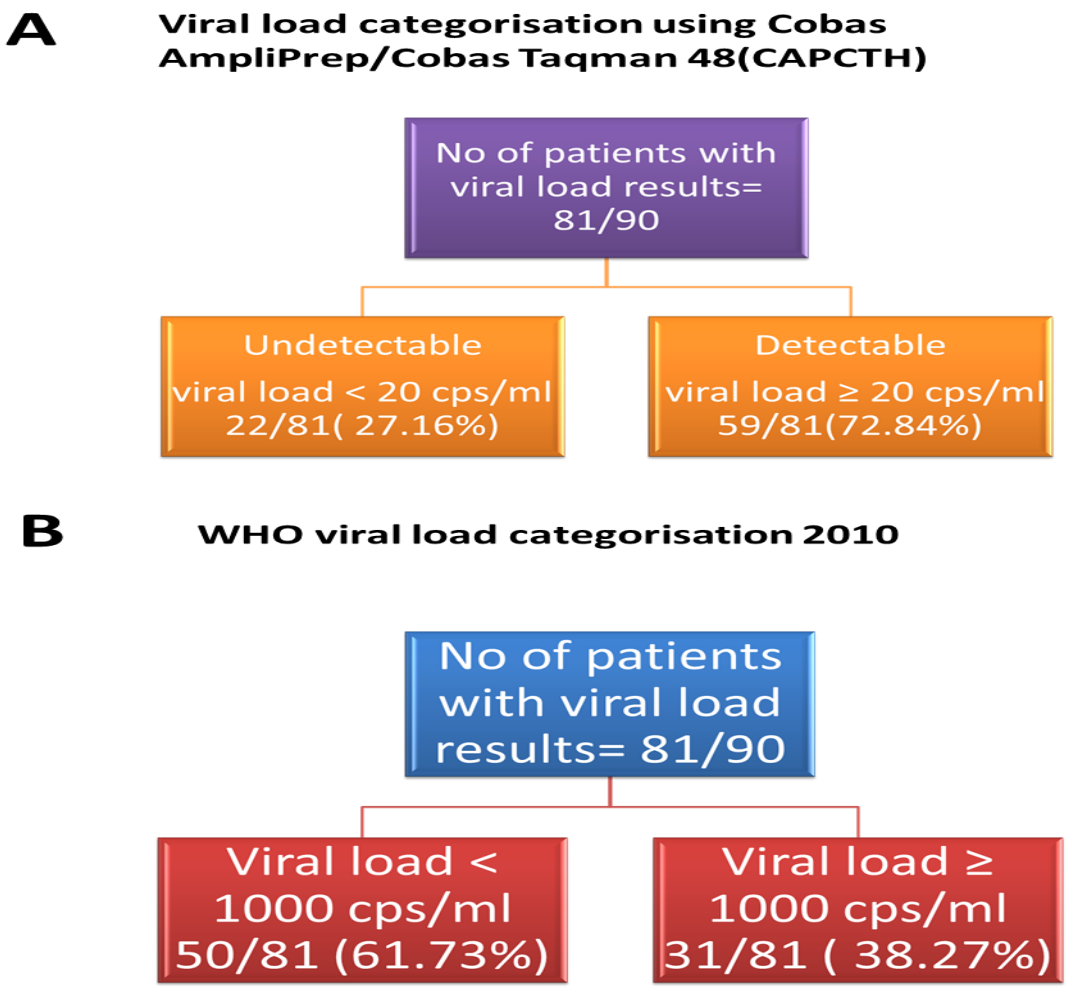

Figure 112: Viral load categorisation among the ART-experienced (ART-E) HIV infected children.

Using WHO classification 61.7% had virologic suppression and 38.27% were not having viral suppression. Using the Cobas AmpliPrep/Cobas Taqman 48 machine (CAPCTH) 27.16% had undetectable viral load < 20 cps/ml and 72.84% a detectable viral load ≥ 20 cps/ml. This is indicating the different methods used to categorise virologic failure among the ART-E children. ***A,*** *CAPCTH viral load categorisation 2010,* ***B,*** *WHO viral load cateaorisation 2010*

Discussion

Introduction

The pharmacokinetics (PK) of ARVs in malnourished children and those receiving nutritional supplementation in the form of RUTF or any other therapeutic food supplement have not been well characterized. The determinants of PK of ARVs in children include and are not limited to developmental changes due to growth, effects of co-administered drugs like anti-TB drugs, presence of diseases like OIs, malnutrition and food insecurity [420, 423]. This may be aggravated by the characteristics of the carer of the child as children depend on carers back at home such as their level education, health literacy, nutrition status and availability. Food insecurity as a catalyst for the development of malnutrition has been found to enhance poor adherence to ART in LRS in both children and adults [424]. Food insecurity and inability to consume adequate food when one is ill are determinants of the variability in ARV exposure since the circumstances affect both the drug absorption and metabolism. Variability of ARV PK has serious implications for clinical outcomes with erratic exposure to ARVs there is a potential of increased emergence for virologic failure and/or drug resistance and this may contribute to malnutrition being associated with HIV progression and increased drug pressure where there are limited choices [425]. Previous studies investigating ARV PKs in malnourished HIV infected children are inconclusive. The limited studies available suggest that WHO dosing guidelines for ARV based on weight yield suboptimal levels of ARV in children, specifically for LPV and EFV [426, 427]. Additionally, earlier studies in SSA children reported more reduced concentrations of ARV [428-430]. It remains unknown why sub-Saharan HIV infected children receiving ART as recommended by WHO guidelines have been found to have suboptimal levels and whether malnutrition alters pediatric ARV levels, more information is needed. Ultimately our aim in this chapter was to determine the impact of nutrition status and nutritional supplementation on plasma concentration levels at 12 hours after dosing with EFV and NVP. The current knowledge of drug concentration levels of EFV and NVP in the context of malnutrition in HIV is limited and inconclusive. Findings from this study may contribute information about the treatment continuum of malnutrition and HIV infection and guide additional interventions in the management of this critically ill population.

The impact of nutrition status and nutritional supplementation on PKs of EFV and NVP

Efavirenz

In the current study, at baseline, a modest proportion of the children had therapeutic levels of EFV concentration, while the proportion of children with undetectable EFV and sub-therapeutic levels were unacceptably high at 34.8% and 17.4%, respectively. After 12 weeks follow up with the malnourished children receiving RUTF, the proportion of children with sub-therapeutic levels drastically increased from the baseline level with a paradoxical decrease in the proportion of children with therapeutic levels. There was also a drastic increase of children in the supra-therapeutic levels (40%) in addition the proportion of children who had sub-therapeutic levels had undetectable levels of EFV (40%) and this may have been as a result of non-adherence. Our findings, together with those of previous studies, indicate that many children dosed according to the current guidelines do not achieve adequate ARV drug levels in this case, EFV plasma concentration [430] despite reporting high levels of adherence. We report for the first time that EFV plasma concentration in the presence of RUTF is associated with either sub-therapeutic or supra-therapeutic levels. EFV absorption in the GIT is known to be increased by a fatty meal and usually patients will be advised to take the dose on an empty stomach: 1 hour before a meal or 2 hours after a meal and to ensure they take it around the same time daily. RUTF has high fat content and this has the potential of increasing the absorption of EFV. Knowing the challenges of taking medication and feeding in children, it is possible that the children who contributed to the increase in supra-therapeutic levels may have taken the RUTF with the EFV dose resulting to the potentiated absorption of EFV by consumption of RUTF. Consequently, in the children who developed supra-therapeutic levels may have been at a risk of developing toxicity to EFV and some may have developed the side effects. The undesirable effects of EFV may have been temporarily incapacitating most especially in those who are genetically slow metabolisers of EFV [176] prompting the non-adherence gradually to treatment. Therefore the surge in sub-therapeutic and high undetectable plasma levels in EFV seen in this study after nutritional supplementation could be a result of children experiencing undesirable effects of EFV in the early period of RUTF supplementation and possibly opting to stop taking the EFV in order to avert the undesirable effects and subsequently resulting in the low plasma concentrations or

undetectable EFV levels and the viral failure in this study population. Malnutrition may reduce albumin or α-acid glycoprotein concentrations transiently, though this was not demonstrated in this study, thereby increasing the free fraction of highly protein bound EFV thus enhancing drug metabolism and elimination and could be accounting for the high proportions of children seen with sub-therapeutic levels after nutritional supplementation [431]. In addition since EFV is highly protein-bound and therefore will have limited central nervous system penetration which is a sanctuary of HIV virus may explain the reason of poor viral suppression despite high levels of reported adherence [432].

Among the malnourished children, the EFV concentration levels at baseline were higher compared to the WN and though had a similar pattern after RUTF supplementation the median EFV levels among those malnourished were still higher than those among the WN. The reason for this pattern is unknown because in malnutrition where there is protein dysregulation, we would expect to have more EFV free drug that would rapidly undergo metabolism and eliminated leading to higher sub therapeutic levels in the malnourished than the WN and the reverse on nutritional supplementation.

Nevirapine

A study on the effect of malnutrition on the PK and virologic outcomes of ARVs in food insecure HIV infected children in rural Uganda documented a significant high proportion of malnourished children having sub-optimal levels of EVF and LPV but high NVP levels using dry blood spots [425]. These findings were similar to our findings where overall, NVP plasma concentrations were significantly higher at baseline before children received RUTF supplementation. However NVP levels were significantly lower among the malnourished compared to the WN at baseline meaning that malnutrition may be reducing the levels of NVP which were noted to increase after RUTF supplementation. Our study reports for the first time the effect of RUTF supplementation on NVP; we observed a significant reduction in plasma NVP levels and this phenomenon may be explained by metabolites of RUTF affecting the bio-availability of NVP. In contrast to other studies which have shown high NVP plasma levels in malnutrition, our study found the contrary in comparison to the WN may also be explained by diminished auto-induction of NVP metabolism due to malnutrition [433].

In our study, at baseline 43.5%, 47.8%, 17.4% had sub-therapeutic, therapeutic and supra-therapeutic NVP concentrations. While at 12 weeks 40%, 20%, 40% had sub-therapeutic, therapeutic and supra-therapeutic NVP concentrations respectively. Studies in young children aged below 9 years indicate that NVP metabolism has been found to be more rapid than that observed in children >8 years, and that younger children require higher doses of NVP to achieve therapeutic concentration. Sub-therapeutic NVP concentration during early therapy can result in slower viral clearance, increased risk of development of drug resistance and virological failure [434]. The children with HIV in Africa pharmacokinetics and adherence of simple antiretroviral regimens (CHAPAS) trial in children older than 2 years of age on NVP dose escalation reported sub-therapeutic NVP concentration in 12% of the children [435] while our study showed a higher proportion of children having sub-therapeutic levels while in the NVP steady-state phase in addition an even larger proportion of those with undetectable levels. Therefore having high sub-therapeutic levels in our study at baseline and at 12 weeks is a recipe for developing resistance to NVP which has future implications on the alternative choice of drug regimen.

The magnitude of treatment failure among the children who were ART-experienced

Virologic response to ART typically results in a substantial rise in $CD4^+$ T cell counts [371]. We investigated viral response in ART-E HIV infected children. We then categorized the viral loads according to the WHO classification and the cut offs of the AmpliPrep/Cobas Taqman 48 machine (CAPCTH) used at MRC laboratory in Uganda. A Tanzanian study on development of HIV drug resistance and therapeutic failure in children and adolescents in rural settings noted an emerging public health concern characterised by high virological failure of 25.4% of children in the pediatric HIV programs [436]. ART associated drug resistance mutations (DRM) were identified in 90%, with multiclass resistances in 79%. Suboptimal adherence was found to be highly associated with developing DRM [436]. A retrospective study done on the magnitude and predictors of ART treatment failure among HIV infected children in several hospitals in Ethiopia revealed the prevalence of ART failure in children to be 18.8% and majority (12.2%) of them were clinical failures compared to immunological failures [437]. However they used clinical and immunological parameters for defining failure to treatment that are known to have low sensitivity in

detecting virologic failure which was the reason of change of WHO guidelines to have a more sensitive and specific method. Thus the recent roll out of viral testing capability in HIV programs to monitor treatment [224]. The current study according to WHO classification of virological failure, 38.27% was not virologically suppressed thus suspected to have virologic failure. A study done on pretreatment HIV drug resistance results in virological failure and accumulation of additional resistance mutations in Ugandan children showed that one-third of children experienced virologic failure within 24 months of initiation of an NNRTI-based regimen which forms the backbone of first-line treatment of HIV. This emerging evidence from the current study suggests that majority of children are receiving a partially effective regimen on the HIV programs and is similar to the Ugandan study [438].

The CAPCTH machine used for viral testing on the study showed that 72.84% were not suppressed virologically which was significantly higher than 38.27% by current WHO viral load classification for viral load monitoring. The current study findings using the current WHO guidelines for viral monitoring provide the evidence for us to hypothesis that we are possibly missing the opportunity of identifying virological failure early enough so as to manage the challenge appropriately; waiting for PLWHIV/AIDs to have viral load greater than 1000 copies per ml may be deleterious and undermines the gains made. The children on this study whose viral loads were between 20-1000 viral copies could already have virologic failure but since the present guidelines dictate the use of a higher cut off may not be identified early enough. Additionally children may fail to have undetected viral load and stabilize with an intermediate viral status that drives a more robust immune activation resulting in negative cumulative effects. Therefore there is need to carry out further studies to elucidate viral failure in a timely way and develop interventions to reduce the effects of continued immune activation as they could be highly contributing to the development of non-communicable diseases. This may provide a multi-prong approach of prevention of NCDs in PLWHIV/AIDs. The majority (80%) of patients receiving an NNRTI regimen had both nucleoside reverse-transcriptase inhibitor (NRTI) and NNRTI resistance mutations. The commonest mutations reported in children receiving an NNRTI-based regimen were M184V and K103N and M184V on PI-based regimens with 30.1% had

one or more thymidine analogue mutation (TAM) and 6% had ≥3 TAMs. Only one child on a PI-based regimen harbored a major PI resistance mutation [239].

Conclusion

Though PK studies in children, especially the malnourished groups, are under-represented the current study shows the presence of altered ARV plasma concentrations in a cohort of Ugandan ART-experienced children. The undetectable plasma EFV concentrations were high at baseline and even higher at 12 week despite high reported adherence rates. Because low EFV concentrations are associated with the rapid emergence of NNRTI resistant mutations and treatment failure, the current recommended EFV doses should be re-evaluated, especially in LRS, where therapeutic drug monitoring is not readily available. After nutritional supplementation, a high proportion of children with supra-therapeutic and undetectable plasma levels of EFV were observed in our study implying the possible paradoxical effect of a high fat content supplement and this maybe a driver of developing resistance. NVP plasma concentrations were significantly higher at baseline before children received RUTF supplementation probably demonstrating that weak auto-induction process of NVP in malnutrition thus ending up with a higher set point of NVP in children who are malnourished and the interaction of NVP and RUTF metabolites after nutritional supplementation. Additionally NVP levels were may be lower among the malnourished compared to the WN at baseline meaning that malnutrition did affect drug bio-availability. Further studies to assess the mechanism of malnutrition on ARV PKs and clinical outcomes in children residing in resource-limited settings are warranted. There is an urgent need to establish routine drug monitoring in pediatric HIV programs to appreciate the origin of high virological failure rates seen in this study. Finally, further studies to elucidate viral failure among children with intermediate viral levels may lead to mitigating or reducing the effects of continued immune activation.

CHAPTER SEVEN: Qualitative study results

Introduction

Sub-standard nutrition undermines the health of children as a whole and puts them at an increased risk of morbidity and mortality especially in the context of HIV co-infection. One of the undesirable outcomes of poor nutrition is risk of impaired mental development of a child especially those under five years of age [439]and the impact on those above 5 years of age is poorly understood. Malnutrition arises when one's daily diet is unable to provide satisfactory calories and protein for growth and maintenance or maybe incapable of wholly harnessing the food consumed due to illness [440]. In developing countries, malnutrition accounts for over 300,000 deaths per year in children under five years and globally contributes indirectly to over a half of all the deaths in children [17]. According to the UDHS in 2010 the prevalence of HIV among the malnourished children was high ranging from 30-50% [441]. The commonest type of malnutrition in HIV was reported to be the non-edematous type compared to odematous malnutrition [18]. Mortality among the malnourished children who are HIV infected is elevated four fold in comparison with uninfected malnourished children regardless of nutritional rehabilitation. Therefore the initiation of ART is required to prevent HIV disease progression and associated mortality [442, 443]. With both global and national movements geared to increase access to ART for all eligible HIV infected patients, achieving EMTCT and 90-90-90 global goal is possible and therefore, there is need to describe the social attributes that influence RUTF and ART utilisation among children so that no one is left behind [228, 444]. There is paucity of information on knowledge, perceptions and attitudes in nutrition in HIV and most qualitative studies in nutrition have been done in the area of infant feeding [187].

In this study, knowledge was defined as awareness or familiarity or practical understanding about the nutrition interventions in HIV programs by the primary carer of HIV infected child. We defined perceptions as the way the primary carer of the HIV infected children understood and interpreted the nutrition information in HIV and how they applied it in the management of the children in their care.

Adherence to RUTF was taken to be consumption >80% of the RUTF prescribed for the 12 week period, while adherence to ART was referred to as any child who missed no more

than one dose per week and consumed more than 95% of the prescribed doses correctly at review every month.

A carer in this study was defined as the primary person who had consistently assumed responsibility for the housing, health, or safety of the child (individuals who administered the child's medication daily and bringing the child for clinic appointments) [445].

Aims of the present study

This chapter will present the qualitative study results. The main outcomes of interest were to explore and understand the knowledge, practices and attitudes of carers of HIV-infected children towards feeding malnourished children and the use of RUTF and ART. The specific objectives were:

1. To assess knowledge and perception of health and malnutrition.
2. To examine the malnutrition burden and attitudes in the community.
3. To describe the interventions/programmes in place for malnutrition.
4. To establish RUTF and ART perceptions and behaviour.
5. To obtain suggestions for the improvement of nutrition service delivery.

A purposeful sample of 160 adults was utilized to answer the research questions above. We had proposed to perform 12 FGDs with at least one per site (12 participants in each FGD) and 16 in-depth interviews (IDIs) at least 2 per site. We were able to carry out only one FGD per site, each comprising 10 women and we were not able to form a group for men as they were few in number. The FGD and IDIs included a target group (carers for HIV positive children and peer mothers). Regarding the IDIs, a total of 16 key informant interviews were done with 2 fathers and 14 mothers. We were able to carry out the qualitative study concurrently with the quantitative study.

Results

The age range of participants was 18-45 years. The study participants noted that poor sanitation, hygiene and feeding practices, lack of treatment (negligence and shortage of drugs), poor child care, stress and HIV/AIDS were the main causes of malnutrition in their

communities. HIV/AIDS was mentioned to cause low appetite, and oral sores with difficulty in swallowing, resulting in malnutrition. Having a malnourished child exposed both mother and child to stigmatisation and hindered a child's adherence to ART. Assessment and management of malnutrition were perceived as beneficial mainly to HIV infected children. RUTF was provided as part of the study to manage malnutrition and health talks about RUTF were given through peer mothers. However, most study participants whose children were not malnourished expressed limited knowledge and understanding of the use of RUTF. The carers who knew about RUTF, got it from the health facility when their children suffered malnutrition, they used it thrice daily. The key likes about RUTF were; being ready and easy to use in management of malnutrition, caused rapid weight gain, increased strength rapidly, early satiety, boasted immunity and improved the quality of life as children were then able to take ART as their appetite improved. The limited availability of RUTF and stigmatisation were major barriers to its use especially outside the study setting. However, most study participants expressed limited knowledge and understanding of RUTF and ART in the context of malnutrition.

Participants' perspectives and understanding of health and malnutrition

Perceptions about health

We explored participants' perceptions about health and found that they understood health in three dimensions; good health meaning no health complaint, active child living positively and good sanitation and hygiene. Below are the illustration quotes:

*"The person may have the HIV virus but as long as they are healthy since they do not fall sick often or frequently, medicine is taken in time, they are practicing good food nutrition, having enough sleep meaning you sleep at the right time and wake up at the right time". (***FGD, 28 years old, mother, Mildmay)**

"Just like they mentioned earlier the child feeds well, plays, sleeps well". **(FGD, 26 years old, mother, Kawala)**

"Some children get diarrhea, the child is underweight, the child is sickly, the child is unhappy and miserable, and the child is really demotivating". **(FGD, 29 years old, mother, Mwanamugimu)**

"Even growth may show the child is not healthy, when the child is maybe 6 months and it is supposed to sit you find that the stage when the child is supposed to walk instead the child crawls. The child's growth is slow and this may be because the child is unhealthy ". **(FGD, 33years old, mother, Kisenyi)**

"Sometimes the child refuses to take the breast milk, or if you give the child breast milk instead they just vomit back when they are unhealthy ". **(FGD, 24 years old, mother, Komamboga)**

"I understand that a child is in a good health state when he or she feeds well, plays with his mates and does everything well, that is how I know that a child is in good health". **(FGD, 34 years old, mother, Kitebi)**

"I cry when a healthy child is mentioned. I envy a healthy child, mine is not, and I imagine my child would be walking by now. She thinks of "omwana omugongobavu" when they mention a malnourished child. A malnourished child does not develop and achieve millstones in life" **(KII, 29 years old, peer mother Mildmay)**

Perceptions about Malnutrition

Many participants described malnutrition by its symptoms and signs. They mentioned that a person suffering from malnutrition has weight and age that don't tally, (underweight, bones are visible from outside), low appetite, poor hair texture (hair falls off, brown hair), child is not playful, may have a swollen abdomen, legs, body, constant diarrhea, stunted growth, bad health (sickly child), wrinkled/ scaly skin, yellowing of skin, red eyes, body weakness, choosy in food types. This was expressed by mothers in the following ways:

"Malnourishment means a child is not fed well, and the child looks skinny, does not look good at all; the hair is yellow, the hair tends to fall off, no cheeks, and the stomach swells up, sometimes the legs also swell up". **(FGD, 35 years old, mother, Mildmay)**

"A child with malnourishment is one who does not feed properly, whatever you give the child to eat, does not give them strength. That is for example eating unhealthy food, or not eating at all, the child looks miserable, pale and unhealthy". **(FGD, 36 years old, mother, Mildmay)**

"A malnourished child doesn't grow, all the time the child's growth is stunted, does not progress in growth. Someone could say maybe the child is 4 years old and yet when you look at them they are not that age, the house helps take the milk and starves the child sometimes". **(KII, 38 years old, peer mother Mildmay)**

"The child generally does not look good, does not enjoy him/herself, does not play, and does not behave like ordinary children. They even have stunted growth, they do not grow normally as normal and healthy children should grow. (**FGD, 19 years old, mother, Mildmay)**

"A healthy child should be seen to grow that is their weight should increase their height should increase. A healthy child is active and if they are already talking then you will know that they are healthy. A healthy child will complain if they have a problem.
Malnutrition is inadequacy of feeds or nutrients in a child or adult. Signs include abnormal hair, skin changes, loss of weight, decreased activity of children, swelling and they are sickly". **(KII, 28 years old, mother Mildmay)**

"Malnutrition is when the body is not growing well because of poor feeding. The weight and age do not tally, the child may look stunted, the hair is not healthy and legs are swollen. The common cause of death in children is malaria. Malnutrition can cause death because when the baby is malnourished he will fall sick all the time". **(KII, 26 years old, mother Mildmay)**

Frequently participants mentioned the causes of malnutrition and these included, poverty (lack of funds to buy nutritious food), poor sanitation and hygiene, poor feeding (lack balanced diet, poor weaning system), lack of treatment (negligence, lack of drugs), poor child care, stress, HIV/AIDS (causes low appetite, oral sores cause difficulty in swallowing, poor adherence to ART), low appetite of pregnant mother or child, limited plumpynut and poor pregnancy care hence low birth weight babies as expressed below.

"For some cases it is not about the earnings, because they may be earning a decent living but they don't know how to feed their children nutritiously. They think they will only drink soda, eat chips; eat meat only without giving them vegetables, fruits. They stick to the fancy food thinking it's healthy yet it's not. And the foods they despise are the most healthy ones for

instance food that does not have oils or fats like boiling green vegetables without frying it with cooking oil, giving them beans, groundnut paste, among others. But most people despise these healthy foods, yet these same foods are nutritious. They have the money but their children are unhealthy, they are many people with this kind of life style. They want to only feed on classy foods, biscuits, sweets, ice cream". **(FGD, 36 years old, mother, Mildmay)**

"HIV can cause malnutrition if people do not take food and drugs on time. HIV can cause malnutrition because the virus feeds on the sick person. Management of malnutrition is by giving children soya, mixed foods, mwana mugimu" **(KII, 26 years old, peer mother Kisenyi)**

"You cannot eat as an adult when the child fails to eat. Sometimes the child lacks appetite. Getting proper medicine to make them eat is not there. You can try and try, food is there but they do not want to eat". **(FGD, 25 years old, mother Kiswa)**

"Most kids who are malnourished if you see the parents who bring them, they are good looking, with a good hairstyle but when the child has kwashiorkor, and there are many mothers like that. I see them at EID they come good looking you ask them what's the problem with the kid, they say the kid doesn't drink efficiently but when the mother eats and drinks well." **(KII, 30 years old, peer mother Kiswa)**

"You can leave a kid with food under the care of the maid, when you return the maid tells you, the kid eats well yet it may not be so". **(FGD, 24 years old, mother, Kiswa)**

"Little earnings; hence no money to feed the child appropriately. You may start hearing people say, that child is malnourished, has a bad skin, yellow eyes, does not feed well, you obviously hear signs and symptoms of malnutrition from neighbours". **(FGD, 20 years old, mother, Mildmay)**

"When you neglect the child, not give them food, lock them in the house, you put them at risk of malnutrition. (**FGD, 26 years old, mother, Kitebi)**

"Malnutrition; is simply not eating nutritious food, the child does not have nutrients suitable for their growth and development ...[not audible]. The child gets worms in the stomach so the stomach

swells. Sometimes the kid has wounds in the throat and cannot swallow food easily". **(FGD, 28 years old, mother, Komamboga)**

*"Even when a person has money, they may not be able to buy nutritious food, sometimes couples may have misunderstandings and sometimes they fight at night instead of cooking food for the children, the children end up sleeping hun*gry". **(FGD, 27 years old, mother Komamboga)**

"Malnutrition may occur when one has just commenced ARVS as they may cause loss of appetite and illness". **(KII, 30 years old, mother Kiswa)**

"The reason these children are malnourished is because their parents do not know how to look after them, others are just careless, and others lack knowledge on how to care for babies. The malnourished are mostly young like those with 9 months and mostly boys". **(KII, 28 years old, carer- mother, Mildmay)**

Common diseases among children

The most common diseases are malaria, cough and flu. They mostly affect children. Causes include many mosquitoes in the area that bite the children, neighbours with cough and flu that transmit the infections to other children. Other diseases include the following:

common cold (commonly referred to as flu), chicken pox, measles, malaria, typhoid, mumps, ringworms, whooping cough, pneumonia, mental disability, diarrhea, tooth decay, headache, vomiting and stomach pain.

"Most of these diseases come as a result of poor hygiene and sanitation" **(FGD, 36 years old, mother, Mildmay)**

Effects of malnutrition in the community

Effects of malnutrition highlighted included**,** death**,** stunted growth**,** lowers child's immunity**,** kwashiorkor**,** malnourished children and their mothers get stigmatised**,** poor adherence to ART due to lack of food, pill burden as expressed by participants in the following ways;

"People in the community discriminate against those with malnutrition they think the child might be having HIV because if there is diarrhea, the child will lack appetite, also may vomit and lose weight". **(KII, 24 years old, mother, Mwanamugimu)**

*"I hate being embarrassed in front of people they talk saying the child of that woman is malnourished and people keep insulting the child". (***FGD, 21 years old, mother, Mildmay)**

"Every time your child is insulted you cannot feel good at all; sometimes you feel like abandoning the child. One time people abused me; they did not want to associate with me because of a malnourished child". **(FGD, 19 years old, mother, Mildmay**)

"Our children are hindered from going to good schools because of the condition of taking them to boarding section. We won't be able to monitor the children's feeding and adherence to medicine, and also the way the child is treated at school; and yet there is no way of explaining to the teachers because the child will be stigmatized, and this may affect their education yet our children have to study. We want our children to at least go to boarding schools, but when we think about it, the child won't take the medication due to fear of being seen by peers." **(FGD, 38 years old, mother, Kitebi)**

Management of Malnutrition

There are a few health programs in place for the management of childhood malnutrition in the community. The majority of respondents do not know where nutritional services are found in their area. The few available nutritional health services in the area are well or over utilized by the community. These are provided by the government freely. Nutritional health information is communicated to communities when they fall sick and go to the health center. As one mother expressed, *"we are advised to have small vegetable gardens like greens, onions so that you have fresh food instead of buying from the market yet the food stuffs have spent a week and the nutrients are not there anymore".* (**FGD, 31 years, mother, Mildmay)**

Participants described where malnourished children are taken for treatment and these included, government facilities such as Mwanamugimu- Mulago, health centre 3, KCCA

clinics), Rubaga, Nsambya Hospitals, local herbalists, some choose to stay home, nearby private clinics, church for prayers, NGOs like Baylor and Mildmay center as some mothers elaborated below.

"Some people can take their child to church instead, they can be misled. The nutrition information is communicated to us through the places where people mostly go to such as the churches, hospitals". **(FGD, mother 28 years, Kiswa)**

Despite the availability of conventional medicine to treat malnutrition some participants reported usage of herbal remedies as expressed by a career. "*They tell us to take some local herbs to increase the blood of the child".* **(FGD, 27 years old, mother, Kitebi)**

"There are some cases where you may take the child to a medical doctor yet the issue is not medical-related". **(FGD, 28 years old, mother, Mildmay)**

An ill child is taken to the nearest health Centre. Village Health Teams treat children under 5 some people treat themselves using herbal medicines and others go to traditional healers". **(KII, 26 years, mother Mildmay)**

"People with malnutrition in her community are treated by being given a balanced diet. They are also offered physiotherapy where their legs are stretched" **(KII, 28 years, mother Mildmay)**

Burden of malnutrition

Participants mentioned that the most affected by malnutrition problem were children those aged between 0-5 years, those under 9 months, women and adults especially the elderly as stated below:

"Children and women. Because if a child falls sick, it is the mothers that go an extra mile in taking care of them. Sometimes the father may tell you to go away and leave his house with your child; if you explain that the child is sick of malaria some men may say, "What do you want me to do". The men just seem less concerned with the children's health. It is the children and the women…" (**KII, 29 years, peer mother Mildmay)**

"Another challenge we face, especially for us with school going children, they cannot go to boarding school especially at a young age. You cannot go to boarding schools, we won't know what the child feeds on, and also explaining to the doctors that the child is in boarding, how will they give the child the medication, or how will they monitor the child. The teachers and fellow students themselves may also start to stigmatize the child once they find out the child is HIV+… is on medication." **(FGD, 40 years old, mother, Mildmay)**

This is what one participant said, "I have *not noticed childhood malnutrition in my area but I know the risk factors for malnutrition are; poverty, unavailability of food".* **(KII, 28 years, mother, Mwanamugimu)**

Experience with RUTF (Perceptions and behavior)

RUTF was provided as part of the study at health facilities to manage malnutrition and health talks about RUTF were given through peer mothers. However, most study participants whose children were not malnourished expressed limited knowledge and understanding of use of RUTF. The carers who knew about it, got it from the health facility when their children suffered malnutrition, they used it thrice daily. This is what participants had to say:

"*We have plumpynut at Mwana Mugimu; we use it most of the time. It tastes like groundnuts mixed with milk, some carers give it to the child takes it four times a day, some make them suck it."* **(FGD, 35 years, peer mother Mwanamugimu)**

"Personally I have used it before, I was looking so bad, I had stress they told me to go to Mwana Mugimu, and I used it. I think it's one of the fast moving actions in life. When they tell you to take two sachets in a day, you get strength instantly. It improves weight and appetite. At home children would eat some as snacks". **(KII, 30 years, peer mother Kiswa)**

"It is a type of feed they give to children who are malnourished as well as the malnourished adults; it is just like a groundnut, RUTF also brings about a difference; and the child gains more weight, and even the hair which used to fall off, you will start to see that the hair grows back in its normal shape", **(KII, 29 years, peer mother Mildmay)**

"I took my child to the hospital and the doctor analysed the situation and saw that my child was very malnourished, so they prescribed the RUTF for the child". **(FGD, 26 years old, mother, Kisenyi)**

Likes of RUTF

The key likes about RUTF were; being ready and easy to use in management of malnutrition, caused rapid weight gain, increased strength, early satiety, boasted immunity and improved quality of life as children were then able to take ART as their appetite improved.

"I first heard of RUTF from Mildmay about 3 years ago when this daughter was given RUTF for 2 months until she gained 10 kgs. I believe that RUTF is good for children, easily satisfies, improves appetite rapidly, and restores good health in terms of rapid gain of weight" **(KII, 30 years, mother, Mildmay)**

"RUTF boosts children's bodies because it has foods such in carbohydrates. She thinks it is easy to use RUTF at home because it is not sold, it is given free of charge. Her child's experience with using it has been a good one; the child likes it and takes it well". **(KII, 26years, mother, Mildmay)**

"RUTF helps the child improve immediately in about a week, appetite improved, RUTF is easy to use at home but the children may not like it because of the smell, taste as it irritates them". **(KII, 42years, mother Kawala)**

"I think that RUTF is used for management and prevention of malnutrition and it is easy to use at home. My daughter has received RUTUF twice and it was helpful in improving her appetite and weight gain". **(KII, 23 years, mother Mildmay)**

"RUTF is easy to use at home the child eats it without difficulty her child has received RUTF twice" **(KII, 25 years, mother Kawala)**

"It's only given to children who are malnourished with low kgs, it tastes like groundnut paste, and they tell you to add it in the child's porridge. The child gains weight very fast, because the

child's weight was down but within 3 weeks that they predict". **(FGD, 27 years old, mother, Mildmay)**

"It really helped the child because the child started doing what she couldn't do, she got more strength. The child, who used not to walk, started walking". **(FGD, 26 years old, mother, Komamboga)**

"The doctors also give us instructions on how to use those feeds because you cannot just do it your way. It is easy to use because you are supposed to use it 3 times a day; in the morning, afternoon and night. You put it in the food you have cooked". **(FGD, 28 years old, mother, Komamboga)**

Dislikes of RUTF

The limited availability RUTF was a major barrier to its use especially outside the study setting. Other dislikes include side effects, bad smell, referral challenges to access the RUTF; some are ignorant about its usage. Most study participants expressed limited knowledge and understanding of RUTF as mentioned below:

"I had rapid weight gain in a very short time. I was told when I gain 50 kg I stop because I started when I was 48 kg but in two weeks I had gained up to 52 kg. Upon taking you get palpitations and stop. It requires one to take plenty of water". **(FGD, 29 years old, mother, Kisenyi)**

"It is not easy to give RUTF at home as one needs where to be supplied with RUTF as it is not found on the market". **(FGD, 35 years old, mother, Mildmay)**

"In some areas there are no trained personnel to teach the people in the community about food nutrition and to also supply the RUTF feeds" **(FGD, 25 years, mother, Mwana Mugimu)**

Challenges to utilization of health care.

Challenges faced by participants when they are referred to nutritional health services include lack of money to pay for the services and lack of money for transport. Other

challenges in access to health care include the following: poor adherence to drugs**,** persistent infections**,** lack of drugs**,** rude health workers**,** and poor access to nutrition support. This is what participants had to say:

When you take a child to hospital to examine the problem, you might have recognized a high temperature, tried giving panadol in vain, upon reaching the hospital they say the child has an infection but no malaria. **(FGD, 27 years, mother Kiswa)**

"Children take a lot of pills, can't they reduce the number of pills taken; because the tablets are so many and yet they also take the pills at many different times in a day. It really stresses our children". **(FGD, 35years, mother Kitebi)**

"Sometimes you can go and find no drugs. They prescribe the drugs yet you lack funds to buy them, the government should inject more funds so that drugs are available". **(FGD, 22 years, mother, Kiswa)**

"They say the drugs are finished and even when it is available they sell it expensively and they send us to other clinics yet they get the medicine from the hospitals". **(FGD, 31years old, mother, Mildmay)**

"There are some care givers who are irresponsible and they contribute to poor adherence to medication by their children, but some of us may have a challenge of taking the medications so some of us explain and we even have proof from the child herself that she takes the medicine or not: But some doctors act so harsh and rude and yet we are the people who treasure the lives of our children the most". **(FGD, 37years old, mother, Mildmay)**

"The main challenges are unavailability of nutritional services in our area. There is no nutritional education. Provision of services at village level is not available at all".
(KII, 29 years, peer mother Mildmay)

Suggestions to improve nutrition service delivery in their communities

The study participants in the FDGs suggested several ways in which their community nutritional service delivery can be improved, Table 57.

"Hospitals should be put up in the village areas as well to reduce the challenge of long distance. I reside in Nateete, near the Bazadde Clinic. They always urge parents to go for trainings about the nutrition of children". **(FGD, 28 years old, mother, Mildmay)**

"People should take the responsibility and train others about proper nutrition of children. We are requesting that it is done in the places such as churches, mosques, even in schools". **(FGD, 22 years old, mother, Mildmay)**

"Our children need bursaries; our children are neglected a lot by their fathers because they think the children will die anytime soon". **(FGD, 30 years old, mother, Mildmay)**

"The village health teams should improve on what they do and should increase on their sensitization of what they do in villages and local communities". **(KII, 29 years, peer mother Mildmay)**

"Schools should be sensitized that even if a child is HIV+ it is not the end of the world. But what frightens me is that if I take my child to boarding school, they may not give him/her the medication. The child may also forget to take the pills. **(FGD, 39 years old, mother, Kitebi)**

"Get a separate day for children only, without any other category of patients to make the process faster for us so that we take the children home much quicker, since they are always shouting, crying. But then there are people who do not have children and the lines are very long, and the children become uncomfortable because of waiting too long". **(FGD, 23 years old, mother, Mildmay)**

"Would like people in her community to be educated on how to spend time with her children feeding them. "Nutritional messages can also be passed on through newspaper" **(KII, 29 years, mother Mildmay)**

"The recommendation would be to form small income generation groups, sensitisation, screening for malnutrition to start on early treatment and management .The people responsible for nutrition health service should do the work more frequently not once in 5 years". **(KII, 29 years, peer mother Mildmay)**

"The government should provide enough supplies and those that order for them should order in time. The RUTF could be put on open market at affordable price very sick children and those who have failed to respond are referred out of the area". **(KII, 26 years, peer mother Mildmay)**

"There is need to distribute food to those who are unable to support themselves in their community. There is need for greater sensitization of people in the community regarding nutrition". **(KII, 27 years, peer mother Mildmay)**

"The health workers should go down to the community and hold talks about nutrition because if a person does not fall sick then they will not get to know about the nutritional services that are available. When there is no one to teach you, then you will not know if your nutrition is good or bad". **(KII, 25 years, mother Mildmay)**

Table 57. The suggestions enumerated by the FGD participants to improve nutrition service delivery in their communities.

List of suggestions to improve nutrition services in their communities
1-Increase nutritional health tips via TV, radio, newspapers
2-Parents should be advised to give children nutritious foods
3- Pregnant women should be counselled about good nutrition
4- Provide separate day for child care to reduce waiting time
5- Establish nutrition health programs closer to the community
6- Educate more people about RUTF (benefits, usage and access)
7- Train nutrition staff on customer care
8- Government inject more funds, increase staff, avail more health facilities
9- Government provide enough supplies of RUTF
10-Avail of bursaries for formerly malnourished children
11- Train more village health team to avail nutritional information in the community
12-Encourage "peer to peer" nutritional advise to encourage personal responsibility
13-Mothers to locate IGA for self-reliance to support child nutrition
14- Distribute food to children who can't afford
15- Encourage nutrition education at schools, LCs, places of worship and all media places.

Discussion

Malnutrition is a major contributor to morbidity and mortality experienced in childhood; however, the precise benefits of nutritional supplementation are poorly understood in the context of HIV. The use of ART in treatment of HIV in PLWHIV/AIDs has greatly improved their quality of life and in addition improved their survival especially when initiated timely [446]. Increasingly, in developed countries, researchers and program planners have come to appreciate that understanding health seeking behavior and increasing community involvement are essential determinants for improving service delivery and optimizing the meagre services available in a more cost-effective model. This, however, is still limited in LRS as they tend to deliver a generic service package with limited input from the community they are targeting [447] and thus the lack of knowledge or understanding and information the health services available at a community level usually manifests among the intended users. A study done in rural Uganda on nutritional status of children living in a community with high HIV prevalence revealed that HIV infection had a direct effect in worsening children's nutritional status however it was a cross-sectional quantitative study design that did not delve into the carers perceptions and health needs or concerns of their children [448].

Knowledge and perception of health and malnutrition by primary carers

In our study we explored the perceptions of the primary carers in understanding what the health of their children pertained and we found that they had a similar perception and understanding of health in a pediatric context. They understood health in three dimensions where having good health was synonymous with having no health complaint, having an active child living positively and in an environment of good sanitation and hygiene indirectly indicated the child was healthy. However, there is profound paucity of studies in LRS on primary carer perceptions and knowledge relating to caring for their children more so in the HIV context. Currently, the majority of studies that have been done in assessing the knowledge and perceptions were among health care providers as opposed to primary carers. In a community study on understanding community perceptions of health and social needs in a rural Balinese village in Indonesia showed that the secondary consequences of unemployment and income reduction had a negative impact on population health resulting into children being involved in child labor at the village level

and this was not in an HIV context [449]. In our study, low economic status was mentioned as one of the barriers of achieving a healthy status among the children of our study participants. Furthermore, in the current study many participants described malnutrition by its symptoms and signs. They mentioned that a person suffering from malnutrition has weight and age that don't tally, (underweight, bones are visible from outside), low appetite, poor hair texture (hair falls off, brown hair), child is not playful, may have a swollen abdomen, legs, body, constant diarrhea, stunted growth, bad health (sickly child), wrinkled/ scaly skin, yellowing of skin, red eyes, body weakness, choosy in food types. The primary carers in our study demonstrated in-depth that they had a wealth of knowledge of the presentation of malnutrition. Health literacy as a discrete form of literacy, different from general literacy, has become increasingly important for social, economic and health transformation and development. However in LRS the opportunity of utilizing the health literacy of the people is still limited yet this would efficiently contribute to improvement of health service delivery. Improvement in the health literacy of a community would complement efforts to improve health services and disease control progressively as this would enhance and utilise community participation [450].

Malnutrition burden and attitudes in the community

Effects of malnutrition highlighted by the primary carers of the HIV infected children in this study included**,** death**,** stunted growth**,** lowers child's immunity**,** kwashiorkor**,** malnourished children and their mothers get stigmatised**,** poor adherence to ART due to lack of food, pill burden as expressed by participants. Maintaining optimal nutrition in HIV-infected children poses a significant challenge to carers as having a malnourished child may expose the possibility of existence of HIV in that family; this maybe a source of stigmatization. This may be further compounded by poor ART adherence in the presence of food insecurity. Malnourished children infected with HIV may experience numerous complications that compromise nutritional status as mentioned by the carers in this study. In addition the patient is at high risk for developing OIs that can dramatically affect any part of the body especially the lungs, central nervous system, gastrointestinal tract, and skin resulting in a critically sick child. Such infections apart from causing the person to be stigmatised for HIV are common causes of morbidity and mortality. Impaired nutritional status may further compromise the patient's immunocompetence especially with very low

measures of lean body mass occurring in those patients close to death in the absence of ART [451].

Participants mentioned that the most affected by the problem of malnutrition were children aged between 0-5 years, those under 9 months, women and adults, especially the elderly. A community study done in children less than 3 years on dietary and environmental risk factors involved in the etiology of early childhood malnutrition in Uganda indicated the risk factors for acute malnutrition were poor health, the use of unprotected water supplies, lack of charcoal as fuel, food insecurity and lack of personal hygiene which was similar to what was mentioned by the primary carers in the current study [452]. Similarly, the participants iterated the causes of malnutrition that included but not limited to poverty (lack of funds to buy nutritious food), poor sanitation and hygiene, poor feeding (lack balanced diet, poor weaning system), lack of treatment (negligence, lack of drugs), poor child care, stress, HIV/AIDS (causes low appetite, oral sores cause difficulty in swallowing, poor adherence to ART), low appetite of pregnant mother or child, limited plumpynut and poor pregnancy care hence low birth weight babies. According to the UDHS 2010 showed that malnutrition mainly affected infants and those under 5 years of age and commonest form was marasmus and mainly in HIV [453]. This implies that the primary carers did appreciate which ages in the community are most affected by malnutrition and in addition adult studies are now showing that people affected by HIV and co-infected with TB present with malnutrition.

Interventions/programs in place for management of malnutrition

There are a few health programs in place in the communities for the management of childhood malnutrition as mentioned by some of the respondents. Majority of respondents do not know where nutritional services are found in their area. The few available nutritional health services in the area are over utilized as they are few nutrition specific services in the community. These are mainly provided freely by the government, however, are overwhelmed and are still imbedded in the colonial health structure that has not expanded to incorporate the needs of the communities they serve. Inequalities of provision of health services and access to quality health care are still an underserved concern in Uganda even in the urban settings. A systematic review done in Uganda on

access to and utilisation of health services for the poor in Uganda showed that the poor and vulnerable experience a greater burden of disease, but have lower access to health services than the less poor. Nutritional services are not mentioned [454]. In the current study the primary carers iterated that nutritional health information is usually communicated in untimely manner to the communities when they have fallen sick or when they go to seek treatment at the health center or facility. Therefore community health education is limited in the information provided to the community or may not be timely or carry the appropriate and relevant information or may be delivered to a very ill patient when their main priority is the drive to survive the illness therefore may not appropriately concentrate to internalise the information provided.

RUTF and ART perceptions and behavior

RUTF was provided as part of the study at health facilities to manage malnutrition and health talks about RUTF were given through peer mothers. However, most study participants whose children were not malnourished expressed limited knowledge and understanding of the use of RUTF. The carers who knew about RUTF, accessed the information from the health facility when their own children suffered malnutrition. They also mentioned that when administering the RUTF they provided it thrice daily. A large study on a community-based management of child malnutrition in Zambia from 6 to 59 months old with MAM demonstrated good program performance with high recovery rates (86.1%) while receiving RUTF. They found that children who had a higher risk of mortality had SAM, HIV infection, very low WAZ-score and health problems at admission implying that the children were very ill and probably unable to take the RUTF orally [455]. The key likes about RUTF were; being ready and easy to use in management of malnutrition, caused rapid weight gain, increased strength rapidly, early satiety, boasted immunity and improved the quality of life as children were then able to take ART as their appetite improved. The organoleptic characteristics of RUTF were found to be acceptable effective in treating SAM among Vietnamese HIV patients [456]. In a Tanzanian study where RUTF was found to improve under-nutrition among ART-treated, HIV-positive children in Dar-es-Salaam documented evidence of its impact being closely linked to improved anthropometrics for HIV infected children. In the compiled evidence, RUTF increased average weight gain in HIV infected children, especially when given with ART [457].

Another study done in Malawi in Lilongwe showed that HIV-infected children gained more weight than children not infected with HIV although HIV infected children had longer hospitalizations and their rate of weight gain was slightly higher than the HIV negative children [458]. A systematic study on nutritional supplementation in HIV-Infected children in LRS also showed that RUTF were associated with improvement in selected anthropometrics [213]. The limited availability of RUTF was a major barrier to its use especially outside the study setting, other dislikes included side effects, bad smell, referral challenges to access the RUTF; some were ignorant about its usage and expressed limited knowledge and understanding of RUTF. According to findings on a study on acceptability of outpatient usage of RUTF in HIV-Infected Senegalese children and adolescents, RUTF acceptability was found to be satisfactory and more than half the children had successful weight recovery, although adherence to RUTF prescription was suboptimal but had positive organoleptic appreciation of RUTF [263]. There are still limited studies on knowledge and perceptions of RUTF among carers of children who are HIV infected yet health literacy or knowledge is a set of skills, including functional, interactive, numeracy that are needed by people to function effectively in utilisation of health care information. Therefore knowledge in health issues is a determinant of good health. Other common determinants of health include distance one needs to travel to health facility, perceived quality of care and availability of drugs as they are key to utilisation [450]. Another important key health determinant and easy to address with community participation is behavior component that is modified positively by having good knowledge of the subject matter.

Suggestions for the improvement of nutrition service delivery

In this current study the challenges faced by participants when they are referred to nutritional health services include lack of money to pay for the services and lack of money for transport. Other challenges in access to health care include the following: poor adherence to drugs**,** persistent infections**,** lack of drugs**,** rude health workers**,** and poor access to nutrition support. This study demonstrates that challenges faced by participants are similar to other findings in LRS and are contributed from both the service providers and the consumers [454].

Conclusion

The findings and recommendations from this qualitative study can be utilised by the community to develop educative media information to promote good nutrition change, ART adherence in management of malnutrition in the context of HIV. Behavior is modifiable and provides addressable factors for health promotion efforts. Lifestyle improvement and better use of formal preventive health services are among these factors. Although many health conditions are exacerbated or ameliorated through behavior there are no studies that prove the causation. Nevertheless, primary career behaviors have an overall impact on their children's health, and can affect that of others.

CHAPTER NINE: General discussion and recommendations

Introduction

This study was undertaken to describe the role of nutrition as a determinant of clinical, immunological and pharmacological outcome in HIV-infected children in Uganda. A total of 278 HIV infected children at the study sites were screened according to the UNCST guidelines, 156 fulfilled the study criteria and were enrolled into the study between January 2015 to December 2017. One hundred and twenty-two children were excluded from the study. This high exclusion rate ensured that the appropriate study population was selected. In addition screening human subjects for research purposes protected them from unnecessary investigations and increased their chance of survival if they were very sick [176].

SSA is home to the majority of children and adolescents living with HIV and the majority of the ART-N cohort in our study presented with advanced disease (WHO stage 3 or Stage 4 disease). Their median circulating $CD4^+$ T cell % was 15% of all T cells implying severe immunosuppression and is comparable to the reporting method of immunological profiling in the HIV programs. This is similar to what the other studies have shown where substantial proportions of children and adolescents initiate treatment in SSA with advanced disease (46.3–72.0) % [224] coupled with a multitude of comorbidities such as TB and malnutrition (33–54) % that tend to be associated with early mortality and poor clinical outcomes [5, 355].

Clinical discussion and recommendations

With numerous programs targeting HIV prevention like PMTCT, EMTCT, EID, the regional expansion of ART (TREAT), early initiation of ART, post exposure prophylaxis, pre-exposure prophylaxis, voluntary medical male circumcision but not limited to these, the pediatric HIV epidemic is evolving [214]. However children still present late with advanced AIDS. Therefore, in this study we excluded children who were very sick with complicated late disease presentation as they were not able to pass the appetite test which involved eating ¾ of the provided RUTF portion in the presence of the healthcare worker screening or enrolling the study participants. In 2014, it is estimated that 220,000 children were infected by HIV through PMTCT thus making HIV/AIDs a major cause of child

morbidity and mortality in SSA [228]. In the current study over a 12 week period the rate of hospitalization in this study cohort was 14.7%, mortality was 3.2% and only 56% returned for the follow up. Slightly less than a half of the study participants were unable to fulfill their 12 week follow up time point. The high proportion of children in the HIV programs lost to follow up or who attend in a haphazard manner threaten the integrity of the gains made in fighting HIV. Despite the significant HIV disease burden in paediatrics, adolescents, orphans and vulnerable children, children living with HIV have been found to fall through the gaps of poorly coordinated health systems and HIV programmes due to several factors. A Zimbabwean study showed that adolescents had higher rates of loss to follow up when compared to other age-groups [459]. A systematic review on outcomes of HIV infected patients lost to follow-up in African treatment programmes also showed that there was a huge burden of children not retained in care and only 73.4% were successfully traced. Of those, 34.2% had died and 23.9% had been transferred to other health centres. In addition, 13 studies reported several children who had experienced treatment interruption [460]. In our study we were unable to ascertain the causes of loss to follow up for all study participants, however, we postulate that some of the participants may have failed to keep appointments due to several reasons such as being unwell, or having no readily available carer to bring them for follow up. For those whom we were able to call up and interview, a few reported that death and changing address as the cause of failing to keep the appointments. However children who are not retained in care or irregularly attend need to be prioritized as they seem to be those who may tend to falter on treatment. However we also acknowledge that due to the narrow period set to follow-up the study participants we excluded patients who came back out of the designated time range by the study. We wanted to ensure we captured the immunological changes our main outcomes in a tight time period. We were trying to avoid a lot of varying changes as they would be difficult to interpret and contextualize.

In the absence of ART in children infected by HIV through MTCT, 50% of these children will die before their 2nd birthday and 75% before their 5th birthday [14]. Therefore there is need for early diagnosis and treatment to achieve the 90-90-90 target. The commonest OIs in this study cohort were mainly TB and candidiasis. These pathogens in the presence of HIV in an underdeveloped immune system in childhood present a management

challenge especially in malnutrition. The immune system of early childhood is characterized by a robust innate immune system but an underdeveloped adaptive immune system. This compromises immunity against pathogens such as HIV, candidiasis and TB. Consequently, OIs will tend to thrive and be rampant in such an environment and in the presence of ART have been recognised to reduce [237]. Since OIs in HIV lead to severe life threatening presentations that are often difficult to manage, their reduction by the introduction of ART has been reported to reduce the mortality [224]. A similar trend of reduced OI episodes, severity and mortality in children on ART was noted in the current study.

In malnutrition there is usually structural damage to the gut epithelial surface resulting in flattening of the villi in turn affecting the efficiency of nutrient absorption. In HIV infection there is also a unique HIV related enteropathy involving progressive structural damage of the intestinal villi and has been correlated multiple factors that include progressive depletion of $CD4^+$ cells in the gut mucosa, HIV viral replication resulting from infection of increased numbers of susceptible target cells thus maintaining the vicious cycle of destruction [407]. In the presence of malnutrition, gut destruction is thought to be compounded with malabsorptive consequences of nutrients consumed and altered microbiota. The children's epithelium is physiologically more permissive to antigens than that of adults especially in the prematurity. This is potentiated by the fact that approximately 1/3 of infants born to HIV infected mothers are premature, consequently with markers of microbial translocation elevated compared to adults with HIV and HIV negative children in the early life. Ideally exclusive breastfeeding protects the microbiota as the negative impact of an ever changing diet is minimized thus reducing mucosal inflammation and microbial translocation. Nonetheless, breast milk has been found to transmit HIV to children during lactation.

Despite numerous descriptions of the infectious and non-infectious causes of IRIS, the overall incidence remains largely unknown. This study provides the first documented burden of IRIS among malnourished children in Uganda where there were only 5 cases noted. The types of IRIS category seen in this study were only TB/IRIS. There was no re-feeding syndrome noted in this study.

Recommendations

Our study findings confirm that the introduction of ART has tremendously revolutionalised child survival of those infected with HIV in LRS evidenced by the marked reduction in the rates of OIs episodes, hospitalization and mortality transforming HIV into a manageable chronic illness. Additionally we have demonstrated that high immune reconstitution does occur despite late initiation of ART, however there are high rates of inadequate virologic suppression. Though other studies have shown that nutrition status to be associated with immunologic profile in our current study though there was a trend of association, this was not significant. Nutritional status was not associated with virologic suppression thus there is need for viral load guided ART switching since we are still utilizing clinical failure and immunological failure to guide ART switching.

Nutrition discussion and recommendations

The evidence base of the role of nutrition status and nutritional supplementation in HIV with malnutrition in children has been specifically derived from observational studies in HIV infected children, or extrapolated from those who are HIV negative; however is still limited [273]. Nonetheless, there has been crucial information generated over time, despite the ethical limitation to carrying out a randomised clinical study which would entail selective denial of the food or ART as an intervention in a population that needs these interventions. Adverse nutritional outcomes such as growth faltering and an array of metabolic derangements, are common in children infected with HIV and have been found to be major contributors to poor survival [254]. The association between HIV infection and low WAZ or growth faltering in HIV infected children has been reported in both resource rich and resource limited settings. In adults, the nutritional consequences of HIV were also among the first to be recognized as AIDs defining illnesses [215]. With the introduction of RUTF in the management of severe forms of malnutrition in a home setting, there is need to develop appropriate utilisation in the context of HIV. In our study though we demonstrated that there was improvement of anthropometric indices after nutritional supplementation children did not meet the targets set by WHO [288]. This may be as a result of non-adherence to RUTF or ART which are known to improve nutritional status. The non-adherence to RUTF may be because there are other people in the households who may share the nutritional supplement, as reported in other studies

assessing acceptability of RUTF in communities where it was introduced. Though the reported adherence to ART in the present study was high there was poor viral suppression in some children and evidence of undetectable plasma drug levels in our study. Primary carers quoted that having a child who was malnourished and using RUTF was a source of stigmatisation. Carers also reported that food insecurity was a source of poor adherence to ART. Other studies have shown that there was an improvement of anthropometric indices after nutritional supplementation despite inadequate adherence [259]. Using the 24 hour dietary recall method at baseline, we found that children had a high reported nutrient daily intake for each macronutrient assessed. This may imply that, although HIV infected children have a high nutrient intake, they may have absorption challenges as a result of malnutrition and HIV. Studies have shown that HIV infection and malnutrition negatively impact on each other [261]. HIV infection has also been shown to increase energy requirements and affects nutritional status through increase in resting energy expenditure, reduction in food intake, nutrient malabsorption and loss, and complex metabolic alterations that culminate in weight loss and wasting which is common in AIDS and younger people are more affected [349, 448]. In addition, the emergence of progressive lipodystrophy syndrome may affect the achievement of anthropometric targets. A study in HIV infected malnourished children in high resource settings found that enteral feeding increased $CD4^+$ T cell counts, whereas parental feeding had no effect. This allows us to hypothesise that nutritional supplementation in HIV with malnutrition may necessitate a treatment approach where exposure to therapeutic feeds is necessary in order to first restore the microbiota in the gut and prime the repair of the absorptive surface so as to efficiently benefit from nutritional supplementation. Therefore, there is a need to prolong the period of treating malnourished children with RUTF or even extend the use of RUTF to all HIV infected people on ART.

Children who were anaemic in this study were found to have lower nutrient intake than those who did not have anaemia. Anaemia may cause loss of appetite, increase oxidative stress lead to lethargy and in ability to feed. Anaemia is prevalent in HIV infected children [275], therefore, interventions to prevent its occurrence may result in improved nutrient utilisation and quality of life. Importantly this is the first study to demonstrate that low fibre intake was associated with being severely immunosuppressed. It is well established

that fibre in diet improves intestinal movements, found to be protective towards developing fat deposition in the arteries [280]. High-fibre diet may be found to be useful in accelerating gut repair in HIV malnourished people and thus increased enhancement of nutrient absorption. Further investigation is warranted to determine the impact of dietary intake of specific nutrients on HIV progression and chronic complication risk in children who are HIV infected receiving ART so that better diets specific to HIV nutritional challenges can be designed and implemented.

Our findings show persistently high levels of inflammatory markers such as CRP and LDH and this may indicate the presence of ongoing OIs [285]. CRP is an acute phase reactant and has been reported to increase in patients with HIV disease progression and is further compounded by the high microbial translocation and immune activation in the absence of ART. In some patients, ART has had toxic effects on various organs and the inflammatory response may contribute to increased activities of LDH in HIV infected individuals. The increased levels in this study may also be due to the body's response to presence of RUTF metabolites [235, 286]. In this study, the ART-N malnourished children had lower serum lipid levels than the WN children, but these normalized probably by the combined effect of ART and nutritional supplementation for 12 weeks. ART-E children had significantly raised serum lipid levels in comparison to ART-N children, however, upon initiation of ART among the ART-N children similar trends of serum lipid levels were rapidly attained as the ART-E cohort indicating that ART profoundly affects lipid metabolism and thus there is need to establish robust monitoring for early detection of possible non-communicable disease such as cardiovascular disease. Previous studies have documented similar findings [284].

Recommendations

Nutritional supplementation improves anthropometry as observed in several studies however the majority did not attain the recommended targets. This may be due to several factors such as suboptimal adherence to RUTF and ART as shown in this study; though lipodystrophy may be one of the causes of failing to attain the WHO targets. The trend of lipid profile is similar to that demonstrated in previous studies revealing that in the presence of wide use of ART there is need for monitoring of lipid profiles to prevent or timely identify possible emergency of non-communicable diseases. Inflammatory markers

declined towards normal levels after 12 weeks of nutritional supplementation however did not attain normal levels. More studies are needed to assess the contribution of RUTF metabolites in contributing to the high levels of inflammatory marker and also the effect of a high fiber diet in modification of diets specific for HIV infected patients. Therefore there is urgent need to develop more sensitive bio-markers for monitoring response to treatment when using therapeutic food supplementation.

Immunology discussion and recommendations

HIV is a lymphotrophic virus that affects both the innate and acquired immune system. This HIV virus affects several lymphocyte subsets other than just the $CD4^+$ T cell subsets resulting to clinical picture of AIDs [461]. The hallmark of AIDS is a progressive depletion of $CD4^+$ T cell repertoire and impairment of cellular immunity leading to susceptibility to OIs.

γδ T cells include $CD4^+$ and $CD8^+$ T cells and are thought to be involved in HIV disease progression. They are comprised of 3 main subsets Vδ1, 2 and 3 and they produce large amounts of cytokines from the Th1 pathway when activated such as IFNγ [462]. Vδ2 T cells are known to recognize phosphorylated non-peptide antigens, not presented by the MHC, and as such they are a link between the innate and acquired immunity [462]. In HIV, several studies have demonstrated an inverse relationship between the Vδ1 and the Vδ2 T cells characterised by an over expansion of the Vδ1 T cell subset as the Vδ2 T cell subset is depleted early in the disease and this worsens in the absence of ART [158, 331, 333, 338, 393, 463, 464]. In our study we report a similar pattern where the Vδ1 T cell population is markedly expanded in the ART-N compared to HIV negative children with profound depletion of the Vδ2 T cell. This phenomenon was most dramatic in the SAM, followed by the MAM and WN ART naïve children. Vδ1 T cells are thought to mediate antiviral activity and assist in the translocation of bacteria across the gut [465, 466]. On initiation of ART there is a reverse of the phenomenon however normal levels are not achieved as seen in other studies [393]. Our study, also demonstrates for the first time, that among the virological non-responders Vδ1 T cells are markedly expanded and Vδ2 T cells are profoundly depleted. When Vδ2 T cells are co-cultured with HIV infected lymphocytes, studies have shown that they become stimulated to produce IFγ and TNFα *in vitro* indicating that Vδ2 T cell count and activity directly correlate with CD4+ T cell count and

inversely to viral load early in the disease. The relationship between CD4+ and Vδ2 T cells count depletion with increased viral loads may be explained by the direct target of HIV infection thus apoptosis of infected cells resulting in reduced numbers, or indirectly by the continued antigen stimulation by the HIV and OIs. Thus the combined effect of the two pathways possibly surmount into the depletion of Vδ2 T cells in HIV.

Recommendations

Vδ1 T cells and $CD56^+$ T cells are unconventional populations of T cells with innate immune functions, which are predominantly found in the intestine and liver. The expansions of these cells in the circulations of SAM children with HIV may be due to their migration to the periphery in response to malnutrition and may contribute to the immunodeficiency associated with malnutrition.

ART leads to sustained expansion of $CD4^+$ T cells in both malnourished and WN with HIV infection and may cause depletions of NK and B cells in malnourished and WN children, respectively. A striking decrease in the numbers and frequencies of DN cells in ART-E patients, suggests that ART may impair populations of innate T cells, which frequently display DN phenotypes. Analysis of individual innate T cell populations indicated that Vδ1 T cells, known to expand in patients with HIV, were depleted from the circulation of ART-experienced malnourished, but not WN, but that other innate T cell populations were found at similar numbers and frequencies in all groups. Thus, ART can efficiently promote recovery of the $CD4^+$ T cell repertoire in malnourished and WN but may have adverse effects on innate T cell populations. Therefore, γδ T cell numbers can be boosted as done in cancer patients by immunotherapy.

Similar changes in circulating lymphocyte numbers over a 12-week period among ART experienced HIV-infected children were seen in both malnourished children who received RUTF supplementation and well-nourished children who did not receive RUTF. These results indicate that effects of RUTF supplementation on lymphocyte numbers are negligible compared to the effects of ART.

Vδ1 T cells are expanded in patients with HIV infection since they produce IFN-γ and IL-17 which are lost in HIV infections and their numbers decrease in patients with good immunological and virological responses to ART. In contrast, Vδ2 T cells are depleted in

HIV infection and they remain decreased in patient with a virological non-response, suggesting that they may become infected and killed by HIV.

Indeed due to the vital role NK cells play in immune response to HIV infection NK cells would be good candidates for developing immunotherapy to HIV. Due to recent findings of a subpopulation of NK cells with adaptive immune responses and associated with lower HIV viremia and control of infection. These evidences, suggest that NK cells can be a viable alternative for new treatment and possibly vaccination strategies to overcome limitations of current vaccination and treatment approaches for this virus [368]. Regarding the development of an HIV cure the current strategy is based on a combination of strategies which involve the reactivation of the HIV virus from latency and then improvement of immune effector cell responses to eliminate infected cells. The γδ T cells are a unique subset of effector T cells that can traffic to tissues, and selectively target cancer or virally infected cells without requiring MHC presentation. A study done on γδ T cells especially the Vδ1 T cells derived from from HIV-infected virologically suppressed donors were expanded with bisphosphonate pamidronate. They demonstrated the ability to target and clear autologous HIV reservoirs upon latency reversal. This provided evidence that they can potentially be used as immunotherapeutic agents in HIV eradication strategies [467].

Pharmacology discussion and recommendations

Routine evaluation of plasma concentrations of ARV drugs is not generally recommended in the management of children with HIV infection and HIV viral monitoring has recently been recommended in HIV programs and is slowly being rolled out in LRS [468]. Undetectable and sub-therapeutic levels of drugs were unacceptably frequent in patients given ART in the present study and even higher after nutrition supplementation. The proportion of children with undetectable and sub-therapeutic levels was higher for those who were on EFV than NVP drug combination. Currently in the pediatric HIV programs the commonest ART regimen used is an NNRTI based [212, 371]. Most children continue to be on the 1st choice regimen dictated upon by the treatment guidelines [212] which is an NNRTI based regimen and studies have shown that dosing guidelines can result in suboptimal concentrations [428]. The rate of switching from 1st line to second line is slow as it involves complex processes leading to delay in switching despite studies now showing the existence of high levels of sub-therapeutic levels of ARVs in children [430].

There is need of introducing therapeutic drug monitoring of ARVs in LRS in order to maintain and potentiate the goals made by availing ART for all and also as a determinant of achieving the 90-90-90 targets of viral suppression [228]. The high levels of undetectable drug levels of NVP and EFV further escalated after RUTF supplementation in this study may be explained by unbecoming effects of the drugs leading to patients opting to stop taking the drugs despite reporting high adherence levels to ART.

The goal of TDM in the setting of ART is to optimize treatment responses, tolerability, and minimize drug-associated toxicity. TDM may be useful in clinical management with drugs that have a known exposure-response relationship and a relatively narrow therapeutic window of desirable concentrations, as such EFV. The therapeutic window of EFV has been described as a range of concentrations that are associated with the greatest likelihood of achieving the desired therapeutic response while reducing the frequency of drug-associated adverse reactions in clinical investigations [429]. EFV and NVP have plasma trough concentrations associated with viral efficacy and drug levels associated with toxicity, thus TDM may have benefits in optimizing ART in pediatrics [434]. However, most TDM targets have been established in adult studies with a few of the ARVs having had target concentrations validated in pediatric studies.

According to WHO classification of virological failure, 38.27% of the children in the present study were not virologically suppressed and were suspected to have virologic failure. Using the CAPCTH machine used for viral testing 72.84% of the children were not suppressed virologically and this was significantly higher than 38.27% using current WHO definitions of viral failure and resistance. There is a growing problem of treatment failure that threatens to undermine the efforts gains made in fighting the HIV epidemic in LRS despite the successful roll out of ART for all PLWHIV/AIDs. Pediatric cases of ART failure rates ranged from 19.3% to over 32% [469]. Most studies have been retrospective in design and a retrospective cohort study done in Uganda and Mozambique also found that ART failure rate was 29% [470] and other retrospective cohort conducted in Jimma University hospital reported 11.5% immunologic treatment failure [469]. There are various challenges described in different studies; the sociodemographic factors like age, baseline clinical factors like baseline CD4 count and WHO clinical stage, drug–drug

interactions, drug side effects, drug toxicity, or inadequate adherence to treatment are some of the factors associated with treatment failure [469, 471, 472].

Recommendations

Though PK studies in children, especially the in malnourished groups are under-represented, the current study shows the presence of sub-therapeutic ARV plasma concentrations in a cohort of Ugandan ART-experienced children. Despite the average length of being on ART being 42 months there is high level of undetectable NVP and EFV at the 12 week time point indicating poor adherence despite high reported adherence rates. There is need to introduce TDM in routine pediatric HIV programs because low EFV and NVP concentrations are associated with the rapid emergence of NNRTI resistant mutations and treatment failure, the current recommended doses should be re-evaluated, especially in RLS, where therapeutic drug monitoring is not readily available. There is an urgent need to establish routine drug monitoring in pediatric HIV programs to appreciate the origin of high virological failure rates seen in this study. Further studies to elucidate viral failure among children with intermediate viral levels may lead to mitigating or reducing the effects of continued immune activation. Prompt ART switching to potentiate benefits of pediatric ART is needed irrespective of nutritional status.

Knowledge, attitude and perceptions findings discussion and recommendations

Health literacy may be defined as the degree to which the decision maker has the capacity in acquisition, processing, understanding and synthesising basic health information and services needed to make appropriate health decisions when the need arises. This is dependent on several factors which mainly stem from the individual characteristics and systemic factors [450]. Some of the factors include education level which has been shown to improve understanding greatly and be able to communicate effectively. Community-based Therapeutic Care is the management of severe malnutrition through community mobilisation, supplementary feeding, outpatient therapeutic feeding and in-patient care for those with medical complications [473]. Most of the primary carers in this study had knowledge of what a healthy child should be and described signs and symptoms of an unhealthy child, and could list the characteristics of the state of being healthy. However they expressed limited knowledge of nutritional supplementation when using RUTF

especially on how it is administered to a malnourished child and where to access it from. Their knowledge was limited in the area of co-administering RUTF and ART. There are limited studies elsewhere assessing knowledge on utilization of RUTF among carers.

We also demonstrated that the carers who demonstrated substantial knowledge regarding the management of malnutrition were those whose children or children under their care were previously admitted in a health facility for malnutrition. They mainly had information of hospital based management of malnutrition and had limited knowledge of community based management of malnutrition in a home setting. Their main concern though was that they expressed that though services where present they were still limited and mainly available at Mwanamujimu Nutritional Rehabilitation unit at the National Referral Hospital which for many was inconvenient.

Primary carers also noted that one of the challenges they were faced with was having a malnourished child in the community and receiving RUTF as both enhanced social stigma. The presence of malnutrition and utilisation of RUTF was associated with being HIV positive. This may have been because RUTF was initially provided in HIV settings and had identifiable or distinct packaging. This hindered people seeking nutritional support. A similar study in HIV infected adults in Ethiopia utilising RUTF as a supplement showed that the intention to use RUTF was also high. However, patients experienced remarkable challenges associated with their use of RUTF. Apart from having side effects; they felt to a greater extent stigmatised and discriminated. They also reported having problems during RUTF handling and transportation. RUTF misuse was practiced frequently and in variety of forms [474]. Another study done in Kenya on consumption of RUTF showed some challenging realities of RUTF use in adults similar to our study like poor adherence and sharing with other household members. However they noted that diet boredom was also a challenge which was not noted in our study. Most patients reported that they felt stigmatised for consuming RUTF more so than for HIV drugs, which is similar to our study except that in our study we did not compare the stigmatisation [475]. Another study in adults showed that half of RUTF users indicated they could not comply with the full prescription due to the taste of the product, diet boredom and clinical conditions associated with HIV (esophageal thrush, lack of appetite, nausea and vomiting). Sharing RUTF rations with other household members was also common, mainly due to poverty

and household food insecurity [476]. Another study done in Malawi on acceptability of RUTF among PLWH also showed that patients experienced gastrointestinal symptoms like nausea, diarrhea, vomiting and dyspepsia [477].

On the contrary, carers attributed the challenge of food insecurity to poor adherence to ART as they expressed the challenges of taking their drugs when they had no food to take as it was difficult to handle the drugs due to the unsavory effects of the drugs. Several studies have demonstrated that adherence to ART is critical in achieving viral suppression in order to mitigate the spread of HIV and improve the quality of patients infected with HIV [478]. This in turn reduces the HIV related morbidity and mortality. Food insecurity is emerging as an important barrier to adherence to care and treatment for PLHIV which was similar to what primary carers in our study reported [479].

The participants also reported limited involvement at a community level in provision of their inputs in decision making on choices of health services to be availed in the community. They informed the researches that the felt left out in issues of decision making regarding their own or children's health. Primary carers noted that the government needed to do more in terms of providing quality nutritional services as the services available were mainly at the national referral hospital, inaccessible and with limited resources as the services were riddled with repeated or prolonged shortages of medical supplies like drugs and RUTF.

Carers who had previous experience of using RUTF noted that it was good for their children as they improved tremendously in a short time, and it was easy to use and to consume alongside other foods. This was also documented in a Malawain study where more than half of malnourished, HIV infected children ART-N were shown to have benefited from home-based nutritional rehabilitation with RUTF and it was associated with more rapid weight gain and a higher likelihood of reaching 100% WHZ [480].

Recommendations

The use of RUTF was widely associated with HIV in this study and since RUTF has unique identifiable packaging there is need to find innovative ways of packaging or dispensing as to avert the stigma attached to its use so that utilization is highly optimized. There is need to strengthen nutritional and ART education among carers of HIV malnourished children.

General conclusions

Implications for clinical practice

There is high unmet need for effective and timely initiation of ART in pediatric programs, as children continue to access care late, often with AIDS defining illness, and with high rates of loss-to-follow once in care. Immune reconstitution was marginal with high virologic failure and high incidence of suboptimal and undetectable drug levels despite reported high ART adherence. There is need for the introduction of therapeutic drug monitoring of ARVs in the pediatric HIV programs in LRS as this can reduce the delay in switching and therefore promote prompt ART switching to potentiate benefits of ART needs to be prioritized irrespective of nutritional status.

Implications for further research

Though ART leads to sustained expansion of $CD4^+$ T cells in both malnourished and WN children with HIV infection, it may cause depletions of other cells and complete immune recovery is not achieved. ART may have adverse effects on innate T cell populations. Vδ1 T cells are expanded in HIV infection and reduced among immunological and virological responders. Immunotherapies using innate T cells may potentiate ART effect to attain complete immune recovery. However there is need for further studies in this area of immunotherapy in HIV.

Regarding nutritional supplementation there is need to provide nutritional supplementation for a longer period and in those who are well nourished in order to appreciate the interaction of RUTF with ART and immune responses. However a proof of concept needs to be done.

Proposed Future plans

We are currently preparing to work on the global metabolomics profiling on the remaining samples which were ethically cleared for this purpose. Based on previous work done we have comprehensively studied the metabolomics of a cohort of young Rwandan children suffering from severe and mild malaria, paired with community healthy controls in equal sized groups. The focus of these investigations was to thoroughly map and understand the acute phase response in malaria and understand the metabolic responses in the human host. We have identified a number of metabolic determinants for severe malaria and a

method of staging the patients metabolically. We have also generated a way to understand the role of eicosanoids and other small pro- and anti-inflammatory molecules in these patients. Furthermore, since the lipidome generated the most divergent metabolites in these groups we proceeded to understand the perturbations of the lipid pathways in the malaria infected patients. Lastly, we have used a proteomics array method to validate and also expand upon a set of protein biomarkers for malaria staging. We would like to repeat this in children with malnutrition with HIV and come up with better prognostic biomarkers based on metabolomics profiling. In addition we would like to map out if there is a unique metabolomic foot print by children who are malnourished, poor immunological and virological responders in comparison to those who are HIV unexposed and nutritionally healthy.

Policy implications

There is need to strengthen nutritional and ART education among carers of HIV malnourished children and destigmatize the use of RUTF. Lastly for success of pediatric HIV programs especially the nutritional component there is need to promote community involvement so that the end users can be involved in making relevant decisions on how services are streamlined to meet their needs.

List of completed manuscripts or in preparation

1. Clinical outcomes of HIV-infected children in Pediatric HIV programs in resource-constrained-settings: Implications of achieving HIV viral suppression. ***Judy Orikiiriza,*** Ben Kikaire, Jane Nakawesi, Allen Mukhwana, Mubiru Frank, Christine Mugasha, Dorothy Turitwenka, Andrew Kambugu, Mohammed Lamorde, Johan Normark, Jane Achan, Victor Musiime, Fiona Lithander, Ann Mullen, Nazarius M. Tumwesigye, Derek G. Doherty, Schlech Walters, Martina Hennessey, Grace Ndeezi (In draft form).

2. Caregivers' knowledge, perceptions and experience of using Ready to use therapeutic food among HIV infected children: A case study of Uganda. ***Judy Orikiiriza ,*** Allen Muhwana, Adelline Twimukye, Ben Kikaire, Jane Nakawesi, Christine Mugasha, Dorothy Turitwenka, Rachel Kansiime, Andrew Kambugu, Mohammed Lamorde, Johan Normark, Jane Achan, Victor Musiime, Grace Ndeezi, Fiona Lithander, Ann Mullen, Nazarius M.

Tumwesigye, Martina Hennessey, Derek G. Doherty, Joseph Rujumba, Charles Hilliard (In draft form).

3. Effects of HIV infection, nutritional status, ART and nutritional supplementation on lymphocyte subset frequencies and numbers in HIV-infected children. ***Judy Orikiiriza,*** Ben Kikaire, Jane Nakawesi, Dorothy Turitwenka, Nabatanzi Rose, Christine Mugasha, Andrew Kambugu, Mohammed Lamorde, Frank Mubiru, Johan Normark, Jane Achan, Victor Musiime, Fiona Lithander, Ann Mullen, Ken Scott, Paul Spiers , Martina Hennessey, Derek G. Doherty (In draft form).

4. Effect of Ready-to-use therapeutic food on efavirenz and nevirapine plasma levels in Malnourished HIV-infected Children in Uganda. ***Judy Orikiiriza,*** Ben Kikaire, Kuteesa Bisaso, Sandra Naluyima, Jane Nakawesi, Dorothy Turitwenka, Andrew Kambugu, Mohammed Lamorde, Frank Mubiru, Johan Normark, Jane Achan, Grace Ndeezi, Victor Musiime, Derek G. Doherty, Fiona Lithander, Anne Mullen, Jackson Mukonzo, Martina Hennessy (In draft form).

5. The continuum of Pediatric HIV care and treatment: Implications of immunologic and virologic response among ART experienced children in Uganda. ***Orikiiriza. J,*** Nakawesi. J, Kikaire. B, Turitwenka. D, Mubiru. F, Nabatanzi R, Mugasha. CG, Johan Normark, Doherty DG. (In draft form).

6. γδ T cells in immunological and virological responders to antiretroviral therapy. ***Judy Orikiiriza,*** Ben Kikaire, Jane Nakawesi, Dorothy Turitwenka, Nabatanzi Rose, Christine Mugasha, Andrew Kambugu, Mohammed Lamorde, Frank Mubiru, Johan Normark, Jane Achan, Victor Musiime, Fiona Lithander, Ann Mullen, Ken Scott, Paul Spiers , Martina Hennessey, Derek G. Doherty (In draft form).

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Appendix 1: Information sheet- English version

Study title 'To determine the role of nutrition as a determinant of immune function and pharmacological outcome for HIV infected malnourished children in Uganda'

Introduction

Dear Parent or legal guardian,

You and your child have been invited to take part in a research project concerning HIV and nutrition. Before joining the project in question, you need to read this information form, because it contains important information to assist you in deciding whether or not to participate. We urge you to ask as many questions as you wish in order to make sure that you understand the procedures for the study as well as the risks and benefits involved. If you have a question about this document that has not been sufficiently answered or explained, do not hesitate to ask one of the research team members for more information. Your participation in this trial is voluntary. You may choose not to have your child participate in this study. Choosing to participate or not will not affect either your own or your child's future treatment in any way. You and your child will still have all the benefits that would otherwise be available for you.

Why are we doing this study?

We are attempting to explore how nutritional supplementation will influence the effects of antiretroviral therapy (ART) in children. Your child will be asked to take a nutritional supplement daily for 12 weeks during ART treatment incase they are malnourished. We will then want to see how the effects of the nutritional supplementation on your childs ability to metabolise the HIV drugs and immune system and after 6 and 12 weeks.

How many participants will participate in this study?

75 malnourished children and 75 well-nourished children.

Plumpy-nut and Midazolam

All malnourished participants will receive plumpy nut which is a nutrition supplement that has been approved by WHO for use among malnourished people. It is a paste and rich in many nutrients required for the body. For the well-nourished they will not be required to take this supplement.

What will happen during the research?

Our attempts to studying this are based on a 15-30 millilitre blood sample that will be drawn during the initial visit and 12 weeks. A urine sample will be taken as well at 0 and 12 weeks. These samples will be used in part for diagnostic purposes and we are requesting that they may also be used for this research.

Your child will in addition to this be examined in detail regarding nutritional assessment which will entail weight, height and mid upper arm circumference measurement. The analyses will in no way interfere with the medical treatment of your child instead, it will ensure quality healthcare for your child. Parts of the samples will be saved and stored and at a later stage transported to the Republic of Ireland for further analysis. The samples will be screened for Hepatitis B and C, and other worms, amoebiasis, pneumoccocci and tuberculosis.

Storage of specimen and genetic testing

The stored samples will be made anonymous during the analysis in the Republic of Ireland, only the researcher in Uganda will have access to the results. You will have to acknowledge if you want to be informed of the results of the analysis or not by the researcher, and leave a way for him or her to contact you. In case you change your mind in either way during or after the study, you may inform the researcher, and he or she will comply with your wishes. We will also perform some genetic studies in order to examine which drug is most beneficial to your child.

What will the study pay for?

Before taking part in this study, we will do an interview and medical evaluation in order to find out if your child qualifies for our study. Should your child qualify for the study and you agree to participate, we will pay for any tests or assessment costs that are not a standard part of diagnostics, but are the direct result of participating in the study.

Carer qualitative interview

In addition we will also be seeking to better understand feeding practices of children in your community and your experience in managing your child during the 12 weeks of the study. Therefore we will also be requesting your participation either in a focus group discussion or an in-depth interview. The focus group discussions will comprise groups of 8-12 people members where the researcher will ask questions for the group to discuss in the field of HIV and nutrition for 30-40 min. The researcher will request to audio record the discussions however if you decline we could perform recording by a note-taker only. The written script will be available to you in the language the focus group discussion was carried out.

Who will be able to see your medical information?

All documents that identify your child will be held in the strictest confidence and will not be released to the public. The research team will only use your personal information and child's information to perform the study. This information may include your child's name, your name, address, medical history, and data from your child's medical consultations. However, this information will not be entered into the study data sent to the researchers. You and your child will be identified by a code number in each report for publication produced based on this study. In order to ensure that the study data collected about you and your child are correct and truly relate to both of you, you can request to cross check. Members of the Ethics Committee will have access to the research data but they cannot link it to you in anyway. Nevertheless these persons will be required to keep this information confidential. By signing this document, you are permitting this access. The researcher may use the data from the study, which are sent to them, for the following purposes:

- To see if the intervention is working properly
- To compare the results with previously published data
- You have the right to demand that the researcher allow you to view the personal data collected on you and your child and make any corrections to it, which may be necessary.

What are the benefits of being in this study?

All participants will benefit from nutritional counselling and assessment which will improve on their dietary habits and in turn improve their health status. They will also benefit from the routine tests that will be offered regularly by the study and will inform their medical care. The study has made provisions to pay for the expense of traveling to a centre and other expenses related to participating in the study. Genetic testing will help in the correct choice of ART regimen.

What will happen at the end of the study?

At the end of the study, we will be sharing what we have learnt with the participants and with the community through the Ministry of Health. We will do this by meeting first with the participants and then with the larger community. A written report will also be given to the participants upon request, which they can share with their families. We will also publish the results in order that other interested people may learn from our research.

Who can I talk to about this study?

If you have any questions you may ask them now or later, even after the study has started. If you wish to ask questions later, you may contact any of the following:

1. Dr. Judy Orikiiriza

Infectious Diseases Institute,

Makerere College of Health Sciences

Makerere University

Email address: orikiirij@tcd.ie

Telephone: +256712962843

2. Dr. Andrew Kambugu

Infectious Diseases Institute

Makerere College of Health Sciences

Makerere University

Email address: akambugu@idi.co.ug

Telephone: +256772507636

4. The Chairperson of the School of Public Health Higher Degree and ethics committee
   Makerere College of Health Sciences
   Dr. Susan Kiwanuka
   Telephone: +256718060387; Office line: 0312291397
   Address: P.O. Box 7072 Kampala
   Email address skiwanuka@nusph.ac.ug

The study has been approved by the Uganda National Council of Science and Technology (UNCST) through the higher degree Institutional review board of School of Public Health Makerere College of Health Sciences and Trinity College Dublin Research Board. These are committees from the different collaborating institutes whose task is to make sure that research participants are protected from harm and thus ensuring that we comply with medical ethics standards. Additionally, the trial will be conducted in accordance with the Helsinki Declaration and the Guide on Best Clinical Practices and Good Laboratory Practices.

Appendix 2: Consent form: English version

Study title 'To determine the role of nutrition as a determinant of immune function and pharmacological outcome for HIV infected malnourished children in Uganda'
QUESTIONNAIRE STUDY CODE……
I, hereby, fully consent for my child and myself to participate in this study on the **"Nourish work package 1 study on the role of nutrition as a determinant of Immune function and treatment outcome for HIV infected children in Uganda'** I agree to be interviewed concerning the health of my child and my own health under this study. I understand that I will not be compensated for my time as a result of my child or myself participating in this study. I have been fully informed about the purposes of the interviews and discussions that will be conducted. I also understand that I may withdraw at any time with no consequences whatsoever to my child's or my health. I agree that on condition of anonymity, the information obtained from these assessments shall be used for developing better programs, educational and research purposes only. The results of this study will assist in availing information to be used in management of HIV and malnutrition co-morbidities unique to these disorders. Upon request, I shall have access to a summary of the research finding at the completion of this study. I am also aware that I can contact Dr. Judy Orikiiriza on Telephone +256712962843, Dr Andrew Kambugu +256772507636 and Dr Suzanne Kiwanuka +256772496136 in Uganda for any further clarifications or questions in the specific sites.

Address Telephone Number Email address	I want to be informed about the findings of these findings.

My signature below, is my acknowledgement that:
- I have read the information sheet and consent form for this study (or the contents of the information sheet and consent form for this study have been read to me), and I have understood and consented for my child and myself to be involved in the study;
- I had the opportunity to ask questions and the answers were satisfactory;
- I took time to discuss this information with others and to decide to take part or not;
- I will receive a dated and signed copy of the consent form;
- I agree for my child to take part in this study;
- I agree to take part in this study and
- I have decided upon if I want to be informed about the outcomes of your study.

Subject	**Person obtaining consent**	**Researcher /Interviewer**
Name (BLOCK LETTERS), signature/ thumbprint	**Name (BLOCK LETTERS), signature/ thumb print**	**Name (BLOCK LETTERS), signature**
Date:	**Date:**	**Date:**

Appendix 3: Assent form for children aged 8-12 years-English version

Study title 'To determine the role of nutrition as a determinant of immune function and pharmacological outcome for HIV infected malnourished children in Uganda'

Clinic ID no……Study no……

Introduction: Dr Orikiiriza Judy of the Department of Research Infectious diseases, is conducting a study on nutrition and HIV in children in Kampala. Permission for me to carry out this study has been obtained from relevant authorities, and your parent /guardian has accepted you to participate in this study. I am kindly requesting you to allow participating in this important study.

Purpose of the study: A research is a way of finding out new information about something. We are doing a research to find out some factors that can cause some children on drugs treating HIV and nutritional supplementation in the malnourished children improve. We are asking children who are receiving treatment from Kampala to be part of the study. When you accept to be part of the study, we shall request you to respond to some questions concerning your health and family. A doctor will then examine and also remove some blood from you 15mls (about 3 tea spoonfulls) using a needle so that we can carry out some tests.

Risks and Benefits: During the process of removing blood, you will feel some pain as the needle enters your body but this will not last long and after the needle is out you will not feel pain again. We shall also give you a small injection on one of the arms to access if your immunity is improving. The results from the investigations done will assist us in the management of your illness.

Patient's rights: Participation in the study is voluntary. You are free to decline to participate, or withdraw from the study at any time and this will not affect your management in any way.

You are free to ask any questions now or if you have any questions later, you can call or ask your parent to call Dr Judy Orikiiriza on 0712962843.

Statement of assent

I have fully understood the purpose and nature of this study and also understand that my participation is voluntary and no consequences will result if I refuse to participate or withdraw from the study.

…….. …… ……..

Patient's name Name, signature/finger print of Patient Date

Authorized representative. Dr Judy Orikiiriza on 0712962843

Appendix 4. Screening form for study participants

Study title 'To determine the role of nutrition as a determinant of immune function and pharmacological outcome for HIV infected malnourished children in Uganda'

Patient initials:……Age in years……Sex 1. Male 2. Female [ ]

Site of recruitment:

1. TASO 2. KCC clinics 3. Mwanamugimu Unit 4. Other

Tick the appropriate

a. Inclusion criteria:

1. Projects 1 and 2 Inclusion criteria:

a. Project I: HIV-infected children aged 6 months to 12 years, including WN, MAM and SAM patients initiating on ART within 2 weeks, whose carer is aged ≥18 years and has provided informed consent.

b. Project II: MAM HIV-infected children aged 6 months to 12 years stabilised on ART for at least 2 weeks and initiating on RUTF, whose carer is aged ≥18 years and has provided informed consent.

Exclusion criteria:

1. Previous enrolment in a nutritional therapeutic program in the last 3 months
2. Children involved in an on-going nutrition study
3. Children with clinically suspected or confirmed malignancy
4. Children exhibiting any specific food intolerance
5. Children who are vomiting profusely (over 3 times daily)
6. Children living outside 50 km radius from IDI clinics in Kampala.
7. Children whose carers do not want to disclose their home address.
8. Children whose cause of malnutrition is compounded by congenital malformations, chromosomal disorders, metabolic disorders, congenital immune disorders, cerebral palsy
9. Children with a severe disability limiting the possibility of investigations
10. Children who plans to leave the catchment area in the next 6 months

Project 3 Inclusion criteria:

Carers of children participating in Project I & II who have consented.

Appendix 5: Baseline research questionnaire

Study title 'To determine the role of nutrition as a determinant of immune function and pharmacological outcome for HIV infected malnourished children in Uganda'

Patients Clinic IPNo……..

Patient's Initials:……..

Study Code no……..

Date of Review……

Is your child on ART? 1. Yes 2. No.

If yes for how long have you been on ART?.....days/months/years

Type if HAART regimen if receiving ART ……..

Date and Time of last dosing of ART:……

A. Socio-Demographic details.

a) Age……months/years

b) i) Date of birth…/…../…… Birth weight:……..Kg

c) Sex 1. Male 2. Female [ ]

d) Religion……

e) Tribe……..

f) Home address……..

g) Telephone number (1)……..

h) Telephone number(2)……

i) Site of recruitment:

1. Mild may center
2. TASO
3. KCC clinics. Specify……..
4. Mwanamugimu Unit
5. Other

j) Next to kin (circle the appropriate): 1. Father 2. Mother 3. Auntie 4. Uncle 5. Sister 6. Brother 7. Grandfather/mother 8. Good Samaritan 9. Adopted parents 10. Other……..

k) Is mother alive? 1. Yes 2. No 3. Don't know [ ] If ' 3 specify……..

l) Is the mother aware of her HIV status? 1. Yes 2. No [ ]

m) If yes is/was the mother HIV positive? 1. Yes 2. No [ ]

n) If the father aware of his HIV status 1. Yes 2. No [ ]

o) Is father HIV positive? 1. Yes 2. No [ ]

p) Weight of mother in Kgs [ ] Height in ms [ ] MUAC in cm [ ]

q) Is the mother on HAART? 1. Yes 2. No [ ] if no why?.....

r) For how long has the mother been on HAART? [ ] days, months, years

s) Mothers CD4 count……..in the last 6 months Don't know [ ] Didn't do it [ ] Results were lost [ ]

t) Viral load in the last 1 year…….. Present [ ] Absent [ ] if present specify……..

u) If mother is alive what is her education status? Specify……

v) What is the mothers occupational status? Specify……..

w) What is the fathers occupational status? Specify……..

x) Specify the marital status of the mother……

y) Mothers monthly income. Specify in UGS……

z) Family income in UGS……

B. Past Medical history

i) Previous treatment of Tuberculosis. Yes 2. No [ ]

ii) How many times has your child been treated for TB

a. Duration……

ii) History of contact with adult with PTB. Specify contact if any……

1. Yes 2. No [ ]

iii) Has your child suffered from persistent diarrhea for duration of more than 2 weeks in the past 6 months? 1. Yes 2. No

iv) Previous blood transfusion in the last 3 months if yes; product given……

1. Yes 2. No [ ] Episodes……

v) Previous admissions in the last 3 months. Specify admission indication if any……

1. Yes 2. No [ ]

vi) Has the child had a previous episode of malnutrition? 1. Yes 2. No

vii) If yes how many times in the last 2 years and when? ……

C. Immunisation history

i) Is your child immunized up to date/completed? 1. Yes 2. No [ ]

ii) If no why is the child not immunized?

a. Child was unwell

b. Mother was unwell and was unable to take the child

c. Lack of transport

d. HCW said it wasn't appropriate to vaccinate the child. Specify ……..

e. Other reason for not vaccinating the child specify……..

iii) If yes mention the vaccinations received and when the child received them.

iv) Presence of vaccination card and appropriately filled. 1. Yes 2. No [ ]

v) If no specify the gaps……

vi) Is it corresponding with what the caretaker reported in section C (i) and (iii) above? 1. Yes 2. No [ ]

D. Symptoms of present illness and duration at time of interview.

i) Presence of cough at interview

1. Yes 2. No [ ] Duration……days/months/years.

ii) Fever at interview

1. Yes 2. No [ ] Duration……days/months/years.

iii) Difficulty in breathing at interview

1. Yes 2. No [ ] Duration……days/months/years.

iv) Grunting respiration at interview

1. Yes 2. No [ ] Duration……days.

v) Convulsions at interview

1. Yes 2. No [ ] Duration……days.

vi) Presence of oral sores at interview

1. Yes 2. No [ ] Duration……days.

vii) Neck or axillary swelling present

1. Yes 2. No [ ] Duration……days/months/years.

viii) Skin lesions when present

1. Yes 2. No [ ] Duration…….days/months/years.

ix) Presence of Abscess(es)

1. Yes 2. No [ ] Duration……..days

x) Neck pain present

1. Yes 2. No [ ] Duration…….days.

xi) Presence of painful swallowing

1. Yes 2. No [ ] Duration…….days.

E. General examinations

i) Temperature……..ºC

ii) Pallor

1. None 2. Mild 3. Moderate. 4. Severe

iii) Jaundice

1. Yes 2. No [ ]

iv) Presence of dehydration

1. No 2. Some 3. Severe

v) Cyanosis present

1. Yes 2. No [ ] If yes specify……

vi) Lymphadenopathy present

1. Yes 2. No [ ] Specify……..

vii) Oral lesions present.

1. Yes 2. No [ ] Specify type……

viii) Finger clubbing present

1. Yes 2. No [ ] Specify the grade……..

ix) Dental carries

1. Yes 2.No [ ] Specify……..

x) Anthropometry

Weight……..kg

Length/height……cm

Mid upper arm circumference……..cm

Head circumference……cm

Chest circumference……..cm

Waist circumference……..cm

Comment on anthropometry……..

xi) Body composition:

a. Biceps skin fold thickness……..mm

b. Triceps skin fold thickness……..mm

c. Gluteal skin fold thickness……..mm

F. Respiratory system examination

i) Respiratory rate……..breaths/min

ii) Signs of respiratory distress

1. Yes 2. No [ ] Specify

i) chest indrawing 1. Yes 2.No [ ]

ii) Intercoastal reccesion . 1.Yes 2.No [ ]

iii) Flaring of ala nasai . 1.Yes 2.No [ ]

iv) Tachypnea 1. Yes 2.No [ ]

v) Subcoastal recessions. 1.Yes 2.No [ ]

iii) Crepitations present

1. Yes 2. No [ ]

iv) Bronchial breathing

1. Yes 2. No [ ]

G. Cardiovascular examination

i) Pulse rate……..beats/min. B.P…….mmHg

ii) Heart sounds

1. Normal 2. Abnormal [ ]

H. Central nervous system

i) Loss of Consciousness

1. Yes 2. No [ ] If yes, Specify……..

ii) Behavior pattern

1. Normal 2. Abnormal [ ]

iii) Craniopathies

1. Yes 2. No [ ] If yes, specify type of craniopathy……

iv) Muscle power

1. Normal 2. Abnormal [ ] If yes, Specify if its upper or lower limb and grade……

v) Muscle Tone

1. Normal 2. Increased 3. Decreased [ ]

vi) Deep tendon reflexes

1. Normal 2. Increased 3. Reduced [ ]

vii) Presence of clonus

1. Yes 2. No [ ] If yes, Grade the clonus……..

viii) Abnormal movements

1. Yes 2. No [ ] If yes, specify……

ix) Planter reflex

1. Normal 2. Abnormal [ ]

x) Meningeal signs

1. Present 2. Absent [ ]

I. Abdominal examination

i) Abdominal distension

1. Yes 2. No [ ]

ii) Hepatomegaly

1. Yes 2. No [ ]

a. Tenderness of Liver

1. Yes 2. No [ ]

iii) Splenomegaly

1. Yes 2. No [ ]

a. Tenderness of the spleen

1. Yes 2. No [ ]

J. Skin examination

i) Rashes

1. Yes 2. No [ ]

a. Type of skin lesions

i. Kaposis sarcoma 1. Yes 2. No [ ]

ii. Generalised marculo parpular rash 1. Yes 2. No [ ]

iii. Verruca planus 1. Yes 2. No [ ]

iv. Teania capitis 1. Yes 2. No [ ]

K. WHO staging at initiation of HAART.

1. Stage I 2. Stage II 3. Stage III 4. Stage VI [ ]

L. Radiological investigations during clinic visit

ix) CXR appearances

1. Date……

2. Findings……...

x) Abdominal ultra sound findings

1. Date……

2. Findings……...

xi) PPD test

1. <5mm 2. 5-10mm 3. 10-15mm 4. >15mm. [ ]

xii) Diagnosis……..(where applicable)

Appendix 6: Follow up questionnaire and adherence assessment form

Study title 'To determine the role of nutrition as a determinant of immune function and pharmacological outcome for HIV infected malnourished children in Uganda'

Patients Clinic IPNo……..

Patient's Initials……..

Study Code no……..

Date of Review……..
Duration on HAART……..days

Type if HAART regimen ……..

Date and Time of last dosing of ART:……..

A. Socio-Demographic details.

a) Age……..

b) Sex 1. Male 2. Female [ ]

c) Follow up site:

1. TASO
2. Mildmay center
3. KCC clinics
4. Mwanamugimu Unit
5. Other

d) Scheduled visit 1. Yes 2. No [ ] if no specify……..

e) Next of kin (circle the appropriate): 1. Father 2. Mother 3. Auntie 4. Uncle 5. Sister 6. Brother 7. Grandfather/mother 8. Good Samaritan 9. Adopted parents 10. Other……..

f) WHO staging at follow up of HAART. 1. Stage I 2. Stage II 3. Stage III 4. Stage VI [ ]

B. Symptoms of present illness and duration at time of follow up.

i) Presence of cough at interview

1. Yes 2. No [ ] Duration…….days/months/years.

ii) Fever at interview

1. Yes 2. No [ ] Duration…….days/months/years.

iii) Difficulty in breathing at interview

1. Yes 2. No [ ] Duration…….days/months/years.

iv) Grunting respiration at interview

1. Yes 2. No [ ] Duration…….days.

v) Convulsions at interview

1. Yes 2. No [ ] Duration…….days.

vi) Presence of oral sores at interview

1. Yes 2. No [ ] Duration…….days.

vii) Presence of vomiting

1. Yes 2. No [ ] Duration…….days.

viii) a) Presence of diarrhea

1. Yes 2. No [ ] Duration…….days.

b) If yes it is bloody diarrhea? 1. Yes 2. No [ ]

ix) Neck or axillary swelling present

1. Yes 2. No [ ] Duration…….days/months/years.

x) Skin lesions when present

1. Yes 2. No [ ] Duration…….days/months/years.

xi) Presence of Abscess(es)

1. Yes 2. No [ ] Duration……..days

xii) Neck pain present

1. Yes 2. No [ ] Duration…….days.

xiii) Presence of painful swallowing. If yes specify location……

1. Yes 2. No [ ] Duration…….days.

C. General examinations

i) Temperature……..ºC

ii) Pallor

1. None 2. Mild 3. Moderate. 4. Severe

iii) Jaundice

1. Yes 2. No [ ]

iv) Presence of dehydration

1. No 2. Some 3. Severe

v) Cyanosis present

1. Yes 2. No [ ] If yes specify……

vi) Lymphadenopathy present

1. Yes 2. No [ ] Specify……..

vii) Oral lesions present.

1. Yes 2. No [ ] Specify type……

viii) Dental carries

1. Yes 2.No [ ] Specify……..

ix) Anthropometry

Weight……..kg

Length/height……cm

Mid upper arm circumference……cm

Head circumference……cm

Chest circumference……..cm

Waist circumference……..cm

Comment on anthropometry……..

x) Body composition:

a. Biceps skin fold thickness……..mm
b. Triceps skin fold thickness……..mm
c. Gluteal skin fold thickness……..mm

xi) WHO staging at initiation of HAART. 1. Stage I 2. Stage II 3. Stage III 4. Stage VI [ ]

ADHERENCE ASSESSMENT.

a) RUTF

a. Has your child consumed the RUTF as prescribed? 1. Yes 2. No
b. If No why has your child unable to consume the prescribed amounts?
c. Specify……
d. Count the number of sachets: 1. >80% -100% adherent 2. 50-80% adherent 3. <50% adherent

b) ART

a. Has your child taken the ART as prescribed? 1. Yes 2. No [ ]
b. If no specify the reason
c. In the last 7 days how many times has your child missed to take any of the drugs?
d. What caused them not to take the medication?
   i. Forgot 1. Yes 2. No [ ]
   ii. Run out of medication 1. Yes 2. No [ ]

iii. Carer travelled 1. Yes 2. No [ ]
iv. Child travelled 1. Yes 2. No [ ]
v. Taste and smell of medication 1. Yes 2. No [ ]
vi. Side effects 1. Yes 2. No [ ]
vii. Lost the medicines 1. Yes 2. No [ ]
viii. Pills very many 1. Yes 2. No [ ]
ix. Dosing instructions not understood 1. Yes 2. No [ ]
x. Different primary carers 1. Yes 2. No [ ]
xi. Illness of child 1. Yes 2. No [ ]
xii. Illness in the family 1. Yes 2. No [ ]
xiii. Inability to identify the medications 1. Yes 2. No [ ]
xiv. Financial constraints 1. Yes 2. No [ ]
xv. No food 1. Yes 2. No [ ]

xvi. Other……
…….

e. Have you carried the adherence card? 1. Yes 2. No [ ]

f. If no why did the carer not come with their adherence card?
Specify……

g. Has the carer ticked the adherence card appropriately? 1. Yes 2. No [ ]

h. Has the carer carried the pill packs? 1. Yes 2. No [ ]

i. Why didn't the carer carry the pill packs?
Specify……

j. Document the name and number of pills prescribed to the patient on the last visit in the table below in k.

k. Do pill counts and record the names and number of pills returned in the format below in k.

Type of drug on prescription	Drug 1		Drug 2		Drug 3	
Number of pills on prescription						
Number of pills on visit						
Tabs per dose eg 1 or 2 tabs	AM	PM	AM	PM	AM	PM
Pill count balance						

l. Grade the adherence 1. >95% 2. 90-95% 3. >85%-<90% 4. <85% [ ]

m. Is the child disclosed to? 1. Yes 2. No [ ]

n. If above 8 years of age why aren't they disclosed to?

Specify……
…

o. Are siblings tested? 1. Yes 2. No 3. I don't know [ ]

p. Is prevention education provided to the carer today? 1. Yes 2. No [ ] tick where appropriate.

i. ABC 1. Yes 2. No [ ]
ii. Family planning 1. Yes 2. No [ ]
iii. Disclosure 1. Yes 2. No [ ]
iv. STD prevention 1. Yes 2. No [ ]
v. Adherence to medications 1. Yes 2. No [ ]
vi. Positive living 1. Yes 2. No [ ]
vii. PMTCT information 1. Yes 2. No [ ]
viii. Use of safe water 1. Yes 2. No [ ]
ix. VCT child blood tests partner testing information 1. Yes 2. No [ ]
x. Prevention of drug substance abuse 1. Yes 2. No [ ]
xi. Nutrition counselling 1. Yes 2. No [ ]

xii. Other……..

Appendix 7: Household questionnaire

Study title 'To determine the role of nutrition as a determinant of immune function and pharmacological outcome for HIV infected malnourished children in Uganda'

Instruction: Circle the appropriate score

A1 Child study code			
A2 sex of respondent		1- Male 2- Female	
A3 Education of Primary carer		1- P1-P7 2- S1-S4 3- S5-S6 4- Diploma and above 5- None	
A4 Marital Status of Primary carer		1- Married 2- Single 3- Widow 4- Widower	
A5 Location	District	Sub-county:	Parish
A6 Position held by respondent in the community			
B: Number of people in the household		Specify:	
B1: Number of Children in household		Specify:	
B2: Number of school going children		Specify:	
B3: Sex and age of the children		Specify:	
C: Socio-economic status			
C1: Does the primary carer belong to any community development scheme?		Yes [ ] No [ ]	
C2: What community scheme does the primary carer belong to?		Specify:	
D: Revenue generation **D1: What are the sources of revenue for the family?**		Specify:	
D2: What is the total revenue of your family during the last financial year?		Specify:	
E1: What is the most commonly eaten food?		Specify:	
E2: Where do you usually get your food from?		Specify:	

Appendix 8: 24 hour dietary recall questionnaire

Study title 'To determine the role of nutrition as a determinant of immune function and pharmacological outcome for HIV infected malnourished children in Uganda'

This part of the interview is to enable us to find out what your child has eaten the previous day. This includes all that your child has eaten including drinks, snacks, sauces, spices, and salad dressings will need to be recalled.

	Column 1		**Column 2**		**Column 3**	**Column 4**	**Code use only**	
Quick List of Food Items	A. Time	A. Occasion	A. Food/Drink and additions	B. Description of food/drink and ingredient	How much of this (FOOD) did you actually (eat/drink)?	Where did you obtain the (FOOD)?	Food code	Amount
1.								
2.								
3.								
4.								
5.								
6.								
7.								
8.								
9.								
10.								
11.								
12.								
13.								
14.								
15.								
16.								
17.								
18.								
19.								
20.								
21.								
22.								
23.								
24.								
25.								

Occasion: 1. Breakfast 2. Break tea 3. Lunch 4. Dinner 5. Late night meal 6. Fruit 7. Food and/or beverage break, snack, local drink/alcohol or other beverage 8. Other (specify):_____

Source of food: 1. Homemade 2. Restaurant/cafeteria/fast food shop/deli 3. Food stall/hawker 4. Supermarket/Food store 5. Workplace tuck shop 6. Day care 7. Friend/relative's home 8. Party/BBQ/banquet/special event 9. Other (specify):_____

9. Was the amount of food that your child ate yesterday about usual, less than usual, or more than usual?
(1) Usual- ***Go to 10*** *(2) Less than usual-* ***Go to 9a*** *(3) More than usual -****Go to 9b***

9a. What is the main reason the amount your child ate yesterday was less than usual?

(1) Sickness

(2) Short of money

(3) Traveling

(4) At a social function, special meal or on a special day

(5) On vacation

(6) Too busy

(7) Not hungry

(8) At school

(9) Fasting period

(10) Bored with no apparent reason

(11) Stressed

(12) Other reason:_____

9 b. What is the main reason the amount your child ate yesterday was more than usual?

(1) Traveling

(2) At a social function, special meal, or on a special day

(3) On vacation or day off

(4) Very hungry

(5) Bored or stressed

(6) Some other reason: _____

10. How would you describe your childs current dietary habit? [Show card]

(1) No special diet, my child eats almost everything

(2) Vegetarian

(3) Special diet:_____

Appendix 9: In depth interview guide for qualitative study

Appendix 9: Key informant guide for the qualitative study

Study title 'To determine the role of nutrition as a determinant of immune function and pharmacological outcome for HIV infected malnourished children in Uganda'

Name of study site: _____

Type of Group: _____

Number of participants: _____

Venue: _____

Length of Discussion: _____

Date: _____

Moderator: _____

Note-taker: _____

My name is _____ and my colleague is _____. We are working with the Nutritional and treatment outcome: Development of a Ugandan-Irish HIV/Nutrition Research Cluster (NOURISH) in a study being conducted on DETERMINING THE ROLE OF NUTRITION AS A DETERMINANT OF IMMUNE FUNCTION AND PHARMACOLOGICAL OUTCOME FOR HIV INFECTED MALNOURISHED CHILDREN ON ART IN UGANDA. Your community has been chosen in the country for this project. We are holding discussions with you on issues related to nutritional health services and use of ready to use therapeutic foo (RUTF) in your community, your experiences with using RUTF, availability and access, major concerns about its use and how to address them. Your participation in this discussion is very valuable to the study. All information will be used without mentioning your names and will be held in confidence within the research team and among its collaborators. The benefit of participating in this study is that you will help us to understand the community here and help in the development of nutritional health programmes that can better meet your needs.

We would like to ask for your permission to audio record the discussion so that we can capture all the ideas expressed.

We expect this discussion to last approximately 30-40 minutes.

INTRODUCTIONS.

Participants to sign a consent sheet showing informed acceptance to participate in the discussions

1. Perceptions of health and malnutrition

- [ICE BREAKER]. When you hear the word health, what comes to your mind? PROBE for elements or aspects of health. ASK: How can you recognize a "healthy person"?
- Please describe what people in this community regard as the most important elements of health and health services. PROBE for satisfaction with essential health services.
- If somebody falls ill in this community, where does he/she go for treatment? PROBE for use or non-use of different opportunities including traditional healers/medicine and health facilities.
- When you hear the word malnutrition, what comes to your mind? PROBE for elements or aspects of malnutrition. ASK: How can you recognize a "malnourished person"?

2. Malnutrition burden in the area

- What are the most common diseases in this area? What are the main factors for the mentioned diseases? (Probe on the causes for the various diseases)
- Who are the most affected? Probe for adult males, females, young males, females, children, why do you think .....are the most affected?
- PROBE for presence of childhood malnutrition

3. Interventions/programmes in place for malnutrition in children

- What health programmes are in place for management of childhood malnutrition in your community?
- Who provides these nutrition service deliveries in this region? Are they able to reach everyone? Probe for access
- Which organizations/companies are providing childhood nutritional health services in this community (inter-sectoral)?
- What type of support are the partners providing?
- How are community members involved in health service delivery? How has the community responded to these efforts? PROBE
- As members of this community, what would you say are the challenges experienced most by health service providers in this community?
- How best do you think these challenges can be addressed?

4. Community utilization of RUTF and nutritional health services

- What is your view regarding the community's utilization of the available nutritional health services in place in this area? PROBE for children.

- What are the constraints/challenges facing the community members in terms of utilizing the services? What opportunities exist? PROBE for challenges encountered in use of RUTF.
- How best do you think the health service UTILIZATION challenges that you have mentioned can best be addressed?
- For what nutritional services are children referred outside of this area?
- What challenges do the community members face when they are referred for nutritional health services?
- How best do you think these challenges can be addressed?
- How is nutritional health information communicated to communities in this area? PROBE for use of RUTF in children
- What can be done to improve how nutritional information is communicated in this community?

5. RUTF perceptions and behaviour

- Which age group is most affected with HIV in this community? Probe for adult men, adult women, young men, young women, children, etc.
- What puts young males at risk for HIV? PROBE for young females, adult male, adult females, truck drivers.
- How do people here treat someone who is infected with HIV?

6. Conclusion

- What, in your view have we not discussed concerning the nutrition health of this community, services and information available that we have not mentioned but you believe is important for us to know?
- Do you have any other recommendation you would like to make concerning nutrition health services availability in this community? PROBE for information needs.