Multiple micronutrient supplementation in HIV-infected children

A randomised trial among children aged 1-5 years in Uganda

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To my late sister, Rosette Ndeezi

She passed away in a tragic road accident. Your care, love and support for my children and continuous encouragement will always be remembered. May your soul rest in eternal peace.

Contributors

This thesis is a result of a joint collaboration between Makerere University and the University of Bergen for the joint PhD degree programme.

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The Centre for International Health, University of Bergen provided supervision of the candidate through Professor Thorkild Tylleskär.

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Acronyms and abbreviations

AIDS	Acquired Immune Deficiency Syndrome
ARI	Acute Respiratory Infections
ART	Antiretroviral therapy
ARV	Antiretroviral
CAI	Child Advocacy International
CD4	Cluster Differentiation 4
CDC	Centers for Disease Control and Prevention
CRF	Case Report Form
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
EAR	Estimated average requirement
ELISA	Enzyme-linked immunosorbent assay
HAART	Highly Active Antiretroviral Therapy
HAZ	Height-for-Age z score
HIV	Human Immunodeficiency Virus
ICP-AES	Inductively coupled atomic emission spectrophotometry
IL-2	Interleukin-2
IQ	Intelligence quotient
JCRC	Joint Clinical Research Centre
MMS	Multiple Micronutrient Supplementation
MOH	Ministry of Health
NGO	Non-governmental organisation
NK cell	Natural killer cell
PCR	Polymerase chain reaction
PI	Principal Investigator
PIDC	Paediatric Infectious Disease Clinic
PEPFAR	President's Emergency Plan for AIDS Relief
PMTCT	Prevention of Mother-to-Child Transmission
RDA	Recommended Dietary Allowance
RNA	Ribonucleic acid
SPSS	Statistical package for social sciences
UNAIDS	United Nations Joint Program on HIV/AIDS
UNICEF	United Nations Children's Fund
WAZ	Weight-for-Age z score
WHO	World Health Organization
WHZ	Weight-for-Height z score

Original papers

This thesis is based on the following 4 papers, referred to in the text by Roman numerals.

I. Ndeezi G, Tylleskär T, Ndugwa CM, Tumwine JK. Effect of multiple micronutrient supplementation on survival of HIV-infected children in Uganda: a randomized, controlled trial. *J Int AIDS Soc* 2010;**13**:18.

II. Ndeezi G, Tylleskär T, Ndugwa CM, Tumwine JK. Multiple micronutrient supplementation does not reduce diarrhoea morbidity in Ugandan HIV infected children; a randomised controlled trial. *Submitted*

III. Ndeezi G, Tumwine JK, Bolann BJ, Ndugwa CM, Tylleskär T. Zinc status in HIV-infected Ugandan children aged 1-5 years: a cross-sectional baseline survey. *BMC Pediatr* 2010;**10**:68.

IV. Ndeezi G, Tumwine JK, Ndugwa CM, Bolann BJ, Tylleskär T. Multiple micronutrient supplementation improves vitamin B_{12} and folate concentrations of HIV-infected children in Uganda: a randomized controlled trial. *Nutrition Journal* 2011;**10**:56

Abstract

Micronutrient deficiencies are common in children living in low-income countries, more so in malnourished and HIV-infected children. The routinely practiced interventions, such as vitamin A supplementation and other micronutrients in recommended dietary allowances (RDA), may not be sufficient to correct all the micronutrient deficiencies. Gaps still exist in determining the optimal composition, dosing and duration of supplementation. Highly active antiretroviral therapy tends to improve micronutrient status of HIV-infected persons, but most probably not back to normal concentrations, especially in areas where daily food intake is not micronutrient-rich.

Aims
The study aimed at determining the effect of multiple micronutrient supplementation on mortality, growth, diarrhoea and micronutrient concentrations of Ugandan HIV-infected children aged 1-5 years at paediatric HIV clinics.

Methods
Using a randomised controlled design, 847 confirmed HIV-infected children were enrolled, stratified into the highly active antiretroviral (HAART) and HAART naïve groups, and assigned to either an intervention supplement or standard of care comparative arm. The intervention consisted of 10 multivitamins and 4 minerals (vitamins A, B_1, B_2, Niacin, B_6, B_{12}, folate, C, D and E, plus minerals selenium, zinc, copper and Iodine) in 2 RDA doses, whereas the comparative supplement contained 6 multivitamins in 1-RDA as the 'standard of care' at 7 paediatric HIV clinics in Uganda. At enrolment, current and previous history of illness, anthropometric measurements and a detailed systemic examination was done. The trial supplement was administered orally, once daily for 6 months. Compliance was measured by weighing the remaining supplement at each monthly visit. The participants attended the study clinics on scheduled visits monthly for 6 months, at 9 and 12 months, and for treatment whenever the child was sick. All study clinics had in-patient facilities where very sick children could be hospitalised. Blood was drawn for a complete blood count, CD4+ cell count, C-reactive protein (CRP) and micronutrient assays, at baseline and 6 months visit. Study outcomes were measured at 12 months for mortality, 6 months for diarrhoea morbidity and the effect of supplementation on vitamin B_{12} and folate concentrations at 6months. Zinc status was reported as part of the baseline survey. For all the papers, data analysis was by arm and stratum.

Results
Of the 847 children, 85 (10.0%) were on HAART whereas 762 (90.0%) were HAART-naïve. Overall, 426 (50.3%) children, 43 on HAART and 383 HAART-naïve received the intervention whereas 421(49.7%), 42 on HAART and 379 HAART-naïve were treated with the comparative 'standard of care' supplement.

The mortality rate in the participants was 6.3 % at one year of follow-up. Mortality from all causes was 25/426 (5.9%) with intervention and 28/421 (6.7%) in the comparative arm. There was no difference between arms; the risk ratio was 0.9 (95% CI; 0.5-1.5) using the Kaplan-Meier survival analysis. As expected, mortality was lower in the HAART stratum,

2/85 (2.4%). Mean survival time was similar in both groups. Generally, weight-for-height and weight-for-age improved except height-for-age *z* scores, and there was no difference between the 2 arms.

There was no difference in the incidence and prevalence of diarrhoea in the 2 groups. The incidence of diarrhoea was 3.8 (95% CI; 3.4-4.3) in the intervention and 3.5 (95% CI; 3.1-4.0) in the comparative arm per child-year. The rate ratio was 1.1(95%CI; 0.9-1.3). In the HAART stratum, the incidence of diarrhoea was 1.7 (95%CI; (1.0-2.7) in the intervention and 1.5 (95%CI; 0.9-2.6) in the comparative arm. Although these children had fewer episodes of diarrhoea, there was no difference between the 2 arms. The rate ratio was 1.1 (95%CI; 0.5-2.3).

More than half the children had low zinc concentrations. Of the 247 children analysed for zinc status, 134 (54.3%) had zinc concentrations of < 10 μmol/L; 121/203 (59.6%) in the HAART naïve, and 13/44 (29.5%) in the HAAR-treated children, Odds ratio (OR) 3.5 (95%CI; 1.7-7.1).

At 6 months of supplementation, the children receiving the intervention had higher serum concentrations of vitamin B_{12} and folate compared to those who received the 'standard of care' supplement. In the intervention group, the median concentration (IQR) of vitamin B_{12} at 6 months was 401.5 (264.3-518.8) pmol/L compared to the baseline of 285.5 (216.5-371.8) pmol/L, $p<0.001$. The median (IQR) folate concentrations also increased from 17.3 (13.5-26.6) nmol/L to 27.7 (21.1-33.4) nmol/L, $p<0.001$. Of the 214 children, 60 (28.0%) had low vitamin B_{12} (<221picomoles per litre) concentrations at baseline compared to 42/214 (19.6%) at 6 months. Sixty two children (29.0%) had low folate (<13.4 nanomoles per litre) concentrations at baseline compared to 44/214 (20.6%) at 6 months. There was minimal reduction in the prorpotion of children with low vitamin B_{12} and folate concentrations.

There was a general increase in haemoglobin with no differences between the 2 groups, nor was there any significant change in CD4+ cell count. The supplement was well tolerated with no adverse effects.

Conclusion

A supplement of 2 RDAs of 14 micronutrients given to HIV-infected children for 6 months did not reduce mortality or diarrhoea morbidity, but improved vitamin B_{12} and folate concentrations compared to the comparative 'standard of care' arm. The supplement was well tolerated with no adverse effects. More than half of the children were zinc deficient, whereas one-third was vitamin B_{12} or folate deficiency. Routine supplementation with 2RDAs of multiple micronutrients to HIV-infected children in Uganda is recommended to reduce the magnitude of micronutrient deficiencies. Further studies to determine the impact of prolonged supplementation, inclusion of iron (our supplement did not contain iron) and supplementation in a larger group of HAART treated children are also recommended.

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Introduction

Vitamins and minerals are commonly termed 'micronutrients', which are distinguished from 'macronutrients' that are the energy-yielding compounds (proteins, carbohydrates and fat) in our diet. The topic of micronutrients is extremely broad and could be subject for a textbook-sized presentation. The scope of this thesis was a randomised clinical trial of supplementation of micronutrients to HIV-infected children in Uganda. In this introduction, I will try to bring the reader sufficiently up to date to understand the rationale for the study, but obviously there are a lot of things about micronutrients that have intentionally been left out to limit the size of this thesis. I hope the reader will find the selection adequate.

Micronutrients – vitamins and minerals

Micronutrients often constitute important building blocks in enzymes and other vital cellular structures, and they cannot be substituted by any other micronutrient or macronutrient; they are known as 'essential nutrients'. The micronutrients - vitamins and trace elements - cannot be synthesised by the body in sufficient amounts to maintain normal metabolism, growth and health. Therefore adequate intake depends on their availability in the diet or other external sources. For any micronutrient, an individual's requirements depends on a variety of factors, such as age, gender, physical activity, health status, physiological states (e.g. pregnancy) and the efficiency with which a person absorbs and metabolises micronutrients. Growing individuals, such as children and people with increased physiological demands (pregnant women), have a relatively greater need of vitamins and trace elements compared to others. For sick individuals, potential losses must also be factored in.

The average normal requirements for an individual can be estimated using average requirements (EARs) of a population. Every nutrient has a distribution of requirement described by a median/mean and standard deviation. The EAR is the intake level of a nutrient at which the needs of 50% of the population in that age-group and gender are met. In order to cover for the requirements of the whole population, another measurement - the Recommended Daily Allowance (RDA)- is needed. RDA is defined as the daily dietary intake level of a nutrient considered sufficient by the Food and Nutrition Board, National Academy of Sciences, Institute of Medicine, Washington to meet the requirements of nearly all (97-98%) the healthy individuals in each life-stage and gender group.[1] Mathematically, it is calculated based on the EAR in the following way. If the standard deviation (SD) of the EAR is available and the requirement for the nutrient is symmetrically distributed, the RDA is set at 2 SDs above the EAR:

$$\mathbf{RDA = EAR + 2\ SD\ (EAR)}$$

This level of intake statistically represents 97.5 percent of the requirements of the population (Figure 1). For some nutrients, data on the variability in requirements may be insufficient or unavailable to calculate a standard deviation, in which case a coefficient of variation (CV) for the EAR of 10% is assumed. Twice that amount added to the EAR is equivalent to the RDA. The resulting equation for the RDA is:

$$\mathbf{RDA = 1.2 \times EAR}$$

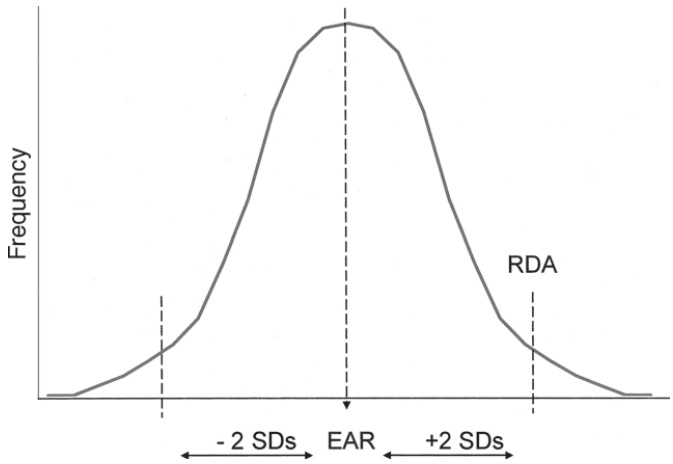

Figure 1. Distribution of requirements for a single nutrient in population for a single gender and a specific age group. The distribution is commonly a Gaussian curve. Estimated Average Requirement (EAR) for this group and how the Recommended Dietary Allowance (RDA) is derived by adding 2 standard deviations (SDs). Source: Tarasuk, 2006.

To illustrate how RDA varies with gender, age group and physiological states, the RDA for vitamin C are shown in Table 1. It is easy to see that the relative need is higher for growing individuals; a baby of 5 kg needs 50 mg of vitamin C compared to 90 mg in an adult who > 10 times heavier.

Micronutrients and biochemical functions

A number of micronutrients act as co-enzymes for many metabolic processes in the body. The co-enzymes help our bodies to utilise the macronutrients (carbohydrates, proteins and fats) by breaking them down for energy production. [2, 3] The majority of the B vitamins are mainly involved in energy metabolism, lipid and nucleic acids synthesis.[4] Vitamin B6 is a coenzyme in many enzyme reactions, particularly those responsible for amino-acid metabolism and transport. In addition, some of the micronutrients are involved in nucleic acid synthesis and formation of new cells. For instance, folate is required for synthesis of new red blood and gastrointestinal cells, and works with vitamin B_{12}. Vitamin C plays a role in collagen formation and iron absorption, and has multiple other functions as a co-enzyme or co-factor.[5, 6] In the metabolic processes the micronutrients co-operate, for instance, selenium promotes activity of vitamin E to prevent lipid oxidation and copper promotes iron metabolism. Some of the functions and symptoms of deficiency states of selected micronutrients are summarised in Table 2.

Magnitude of micronutrient deficiencies in children

Micronutrient deficiency has been coined 'hidden hunger', the reason being that, although it is not a gut-feeling, it may have devastating effects on an individual's health and general wellbeing. It is also wide spread – affecting more than 2 billion people globally – especially those living in low-income countries. It arises from lack of micronutrient-rich foods such as vegetables, fruits, animal products and fortified foods. Children and pregnant women with a relatively greater need of vitamins and trace elements are most affected by this scarcity,

making them more vulnerable to micronutrient deficiencies. The risk of micronutrient deficiencies is further increased in those with severe acute malnutrition, chronic infections and human immunodeficiency virus (HIV) infection. These conditions singly or together lead to reduced immunity and an increased risk of infectious diseases and mortality.[7]

Table 1. Recommended dietary allowance (RDA) for vitamin C according to gender age and physiological status.

Life stage	Age	Males (mg/day)	Females (mg/day)
Infants	0-6 months	40	40
Infants	7-12 months	50	50
Children	1-3 years	15	15
Children	4-8 years	25	25
Children	9-13 years	45	45
Adolescents	14-18 years	75	65
Adults	19 years & older	90	75
Smokers	19 years & older	125	110
Pregnancy	18 years & younger	-	80
Pregnancy	19 years & older	-	85
Breastfeeding	18 years & younger	-	115
Breastfeeding	19 years & older	-	120

Source: Micronutrient Information Center, Linus Pauling Institute, Oregon State University, USA

In order to understand how micronutrient deficiency leads to reduced immunity and recurrent infections, the following section summarises how the immune system works. It also deals with how HIV-infection and micronutrient deficiencies affect the immune system.

Table 2. The main functions and deficiency states of selected micronutrients

Micronutrient name	Main function	Main deficiency symptoms
Retinol (Vitamin A)	Maintain healthy surface linings of the eyes, the respiratory, urinary, and intestinal tracts, the skin and mucous membranes; night vision; bone development; antioxidant	Bitot's spots, xerophthalmia, corneal ulcers, scarring of the cornea and blindness; impaired dark adaptation. High incidence of respiratory illnesses and diarrhoea; increased mortality
Thiamin (Vitamin B_1)	Carbohydrate metabolism, coenzyme for synthesis of nucleic acids	Peripheral neuropathy, diminished sensation and weakness in the extremities. Muscle pain and tenderness, seizures (severe deficiency). Signs of congestive heart failure
Riboflavin (Vitamin B_2)	Metabolism of fats, carbohydrates and proteins. Protection from reactive oxygen species	Sore throat, stomatitis, cheilosis, inflammation and redness of the tongue, and seborrhoeic dermatitis. Normochromic normocytic anaemia
Niacin (Vitamin B_3)	Metabolism of carbohydrates, fats and proteins and synthesis of fatty acids and cholesterol	Dermatitis, diarrhoea, bright red tongue, vomiting, and diarrhoea, apathy and fatigue
Pyridoxine (Vitamin B_6)	Coenzymes that catalyse gluconeogenesis, synthesis of neurotransmitters, heme, nucleic acids, conversion of homocysteine to cysteine, production of lymphocytes and IL-2	Peripheral neuropathy, irritability, confusion, seizures in severe deficiency. Inflammation of the tongue, sores or ulcers of the mouth, and angular stomatitis. Anaemia.
Folic acid (Vitamin B_9)	Metabolism of nucleic acids and amino acids, synthesis of DNA, RNA, conversion of homocysteine to methionine	Megaloblastic anaemia and symptoms of anaemia (fatigue, weakness, and shortness of breath)

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Micronutrient name	Main function	Main deficiency symptoms
Cobalamin (Vitamin B_{12})	Amino acid metabolism, methylation of a number of sites in DNA and RNA, production of energy from fats and proteins, hemoglobin synthesis.	Megaloblastic anaemia, numbness and tingling of the extremities, difficulty walking, mood changes, tongue soreness, appetite loss, and constipation
Ascorbic acid (Vitamin C)	Synthesis of collagen, synthesis of carnitine which is essential for transport of fat into mitochondria for production of energy, metabolism of cholesterol to bile acids. Antioxidant, enhance chemotaxis and phagocytosis	Bleeding and bruising easily, hair and tooth loss, and joint pain and swelling, fatigue
Cholecalciferol Vitamin D)	Absorption of calcium and hardening of bones. DNA synthesis and transcription of genes, cellular differentiation, modulates immune cells e.g. dendritic cells and macrophages	Rickets, muscle weakness and pain, frequent bacterial infections
Alpha-tocopherol (Vitamin E)	Antioxidant, maintain integrity of cell membranes, affect the expression and activities of enzymes in immune and inflammatory cells	Peripheral neuropathy, muscle weakness
Iodine (I^-)	Production of thyroid hormones, regulating metabolism and development, growth and development of the nervous system	Fetal death, still-birth, birth defects, mental retardation, lower IQ and learning disabilities, poorer school performance, growth retardation, enlarged thyroid gland
Selenium (Se^{2+})	Regulates thyroid hormone (conversion of T4 to T3), a number of selenium dependent enzymes are antioxidants, facilitate cell growth and viability, promotes activity of vitamin E to reduce lipid oxidation	Increased infections, cardiomyopathy in severe deficiency, increased oxidative stress may favour HIV viral replication
Zinc (Zn^{2+})	Growth and development, immune response, neurological function, and reproduction. Nearly 100 Zinc-dependent enzymes, stabilises the structure of a number of proteins. Antioxidant; stabilises structure and function of cell membranes, regulate gene expression, influences hormone release and nerve impulse transmission, apoptosis. Bioavailability of folate in diet increased by zinc dependent enzymes; metabolism of vitamin A	Delayed growth and development, dermatitis, diarrhoea, increased susceptibility to infections, impaired wound healing, diminished appetite, impaired taste sensation
Copper (Cu^{2+})	Cellular energy production, formation of collagen and elastin, transport and mobilisation of iron from stores, synthesis of neurotransmitters, formation of myelin, formation of melanin, formation of antioxidant superoxide dismutase, regulate gene transcription, necessary for iron metabolism	Anaemia unresponsive to iron therapy, reduced neutrophil count, loss of pigmentation, neurological symptoms, and impaired growth
Iron (Fe^{2+})	Oxygen transport and storage, electron transport and energy metabolism, antioxidant and beneficial pro-oxidant functions, oxygen sensing, DNA synthesis	Microcytic-hypochromic anemia, brittle and spoon-shaped nails, sores at the corners of the mouth, taste bud atrophy, a sore tongue, pica (consumption of non-food items), impaired intellectual development and immune function.

Source of information: http://lpi.oregonstate.edu/infocenter/minerals:html and http://lpi.oregonstate.edu/infocenter/vitamin:html

The immune system

The immune system is a complex network of cells, tissues, proteins and organs that work together to defend the body from infectious micro-organisms and toxins. Our bodies are constantly exposed to germs or micro-organisms that cause diseases, however, the immune system continuously protect us from these invaders. In case they get in the body, the immune system tracks them down and eliminates them. There are 3 types of immunity – innate, adaptive and passive.

Innate immunity comprises of the skin and mucous membranes, phagocytic cells, and physiological barriers, such as the acidity/alkalinity (pH) of body fluids and temperature. Adaptive (acquired) immunity develops when one becomes exposed to different illnesses. Passive immunity is acquired from the mother and lost in infancy.

Most of the cells of the immune system responsible for the acquired immunity are white blood cells, the more important being lymphocytes, neutrophils and macrophages. We will focus on lymphocytes, which originate from stem cells in the bone marrow. One group migrates to the thymus for maturation, called T-lymphocytes or T-cells, while those maturing in the bone marrow are called B-lymphocytes or B-cells. Lymphocytes enter the bloodstream and become lodged in tissues and organs, such as the lymph nodes, spleen and tonsils.

One subgroup of T-cells are called CD4 cells because they express a glycoprotein called CD4 (cluster of differentiation 4) on the surface. CD4 cells coordinate the overall immune system and are the 'generals' of the immune army. The CD4 receptor helps the T-cell to recognise antigen fragments. In addition to destroying an antigen or infected cells, T-cells communicate or signal other cells, e.g. phagocytes, to destroy the invaders. T-cells can also react by secreting chemicals (cytokines and chemokines) that activate the surrounding immune cells.

Following exposure to antigens, B-cells produce antibodies. These are specific proteins (immunoglobulins) that lock onto the antigen and tag the antigen for destruction. After binding to the antigen, antibodies initiate the complement system, a group of specialised proteins that helps to remove antigen-antibody complexes. These antibodies remain in the body in case the antigen is re-introduced. This B-cell-dependent part of the immune system is most efficient in eliminating bacteria. For other microorganisms - viruses, fungi and special bacteria such as mycobacterium - T-cell mediated immunity is mostly involved. Other white cells e.g. macrophages and neutrophils, circulating in blood, survey the body and engulf antigens they encounter. They destroy the antigen by making toxic molecules, such as reactive oxygen intermediate molecules.

Another type of T-lymphocyte that exhibits the innate ability to detect and attack an intruder on its own exists, i.e. the natural killer (NK) cell. NK cells produce a substance called interferon that prevents viruses from replicating. They also release poisonous chemicals, e.g. nitric oxide, that can destroy the antigens from within.

HIV and the immune system

When the HIV enters the body, it attaches to CD4 cells, the commander of the other white blood cells in immunological processes. The virus multiplies within the cell and eventually lyses it. The new virions that are released infect other CD4 cells, eventually reducing the number of CD4 cells present. The immune system tries to fight infections by producing antibodies, but they are ineffective without CD4 to organise and regulate their function.

Assessment of the immune competence of HIV-infected subjects relies on measuring their CD4 cell count. For an adult, antiretroviral treatment is usually initiated when the CD4-count has dropped to or below 350 cells/µl. In children, it is more common to use CD4%, which is the proportion of CD4 cells in the total lymphocyte count. A value > 30% is considered normal, and a child with a percent of < 20% is immune deficient, severely so below 15%.

Micronutrients and the immune system

Cells of the immune system have a high replication rate, which means that they have a high demand for building blocks for new cells. Macronutrient and/or micronutrient malnutrition therefore rapidly affects both the antigen-specific and non-specific components of the immune system to cause a general 'down-tuning' of the system, changes that are in fact similar to immunodeficiency induced by HIV-infection. Micronutrient deficiencies are associated with lymphoid atrophy, reduced T-cell function and alterations in mucosal and other barrier surfaces.[8] It is difficult to assign individual micronutrients to specific functions in the immune system because their functions are interrelated and complement each other. For example, vitamins A, C, B, D, and E support the production of white blood cells, cytokines and antibodies, whereas some of these and other micronutrients strengthen the natural barriers and are strong antioxidants. [5, 9-14] During acute infections, there is increased utilisation of micronutrients to enhance the activity of the immune system or destroy free radicals. This results in the reduction of the blood concentrations of these micronutrients. Although the reduced concentrations may be due to redistribution, it is more probable that this loss has to be compensated by increased intake.

Effect of HIV-infection on micronutrient status and vice-versa

HIV attacks and directly destroys CD4 cells in the immune system, resulting in a decline of CD4 cells over several years until it reaches a critical point when infections of different kinds appear. This in turn affects the nutritional status by reducing dietary intake, impairing nutrient absorption and increasing nutrient utilisation[15] (Figure 2). Reduced dietary intake may be due to loss of appetite, and oral and oesophageal sores that affect the individual's desire and ability to eat.[16] Many of the opportunistic gut infections, such as *microsporidia, cryptosporidia, Giardia* destroy the absorptive surface of the intestines, leading to malabsorption and increased loss of micronutrients from the gut lumen.[17] Diarrhoea *per se,* without significant destruction of the absorptive surface, leads to nutrient loss. HIV itself may cause epithelial damage to the intestinal walls causing malabsorption.[18] Sometimes macronutrient malabsorption is accompanied by micronutrient malabsorption. For example, fat malabsorption affects the absorption of fat-soluble vitamins (A, D and E).[19] HIV-infection is associated with an increased basal metabolic rate, increased energy expenditure and protein catabolism.[20] Micronutrients are required to maintain all these processes along with increased immunological demands.

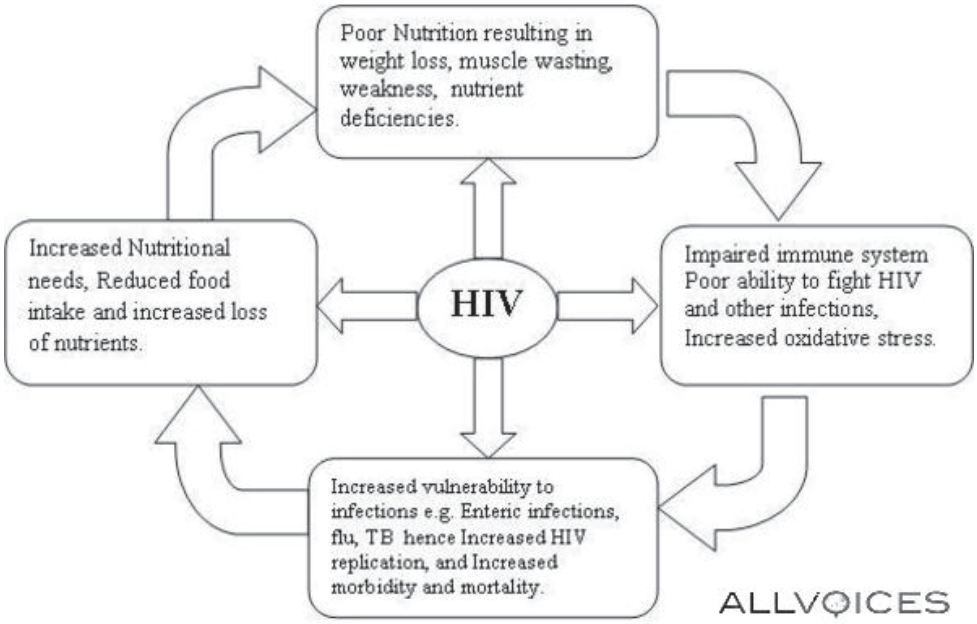

Figure 2. 'The vicious cycle of malnutrition and HIV'. Source: http://www.allvoices.com/contributed-news/6435380-vicious-cycle-of-malnutrition-and-hiv

Frequent or opportunistic infections are associated with increased oxidative stress and utilisation of antioxidant micronutrients, such as vitamins A, C, E, zinc, copper and selenium, resulting in reduced serum concentrations of these micronutrients. [21] The role of micronutrients in HIV-infection is mediated through the immune system, antioxidant status or possibly their antiviral effects.

Single or multiple micronutrient supplements?

In order to address micronutrient deficiencies, a plethora of micronutrient interventions have been explored. We have tried to review the literature on randomised micronutrient supplementation studies from 1980 to 2005 excluding those on adults, pregnant women, neonates, genetic disorders, cognitive function and food fortification. A few of the studies are summarised in Table 3. Not all studies from the same region reporting similar results have been included. Also studies using spreads or sprinkles to deliver the micronutrients have not been included in the summary table. Studies that were reported after 2005 have been included in the discussion. While most of the earlier studies concentrated on vitamin A, the majority of more recent studies addressed zinc supplementation. They tended to vary in their characteristics; some were community studies that included 'healthy' children, whereas others were hospital-based and followed participants in the communities. Most of

the studies were in children aged 6-59 months, although some included younger infants and school-age children. Most vitamin A and zinc community studies involved very large numbers of children. There were very few studies on other micronutrients or multiple micronutrients. For those who added multivitamins or other micronutrients, they were disease-targeted for treatment of diarrhoea [22-24] and given for a short period. The dosing schedule for vitamin A was almost consistent in most of the studies, while dosing schedules for zinc and the other micronutrients were not. Across these studies, the duration of supplementation varied from a few days to 24 months. Based on the different case scenarios and the settings, the outcomes were bound to vary. The main outcomes reported were growth, morbidity and mortality. In the majority of zinc studies, supplementation or adjunct therapy resulted in a significant reduction in diarrhoea morbidity, and vitamin A supplementation trials significantly reduced morbidity and mortality (from all causes).

The literature for the HIV-infected children is scant. The few studies that had been conducted before 2005 are summarised in Table 4, which excludes studies reporting the effect of maternal supplementation on infant and child outcomes. These studies were characterised by small numbers and almost all of them were concerned with vitamin A. Their findings were similar to those reported in other children - reduction in mortality from all causes with vitamin A and diarrhoea morbidity with zinc.

Table 3. Previous micronutrient supplementation studies in children, 1994 to 2005.

Authors	Country	Participant's age	Number	Intervention and dosage	Duration of follow-up	Findings
Benn CS, 2005[25]	Guinea-Bissau	6 months – 5 years	4983	Vitamin A: recommended dose or half dose, single dose	9 months	Mortality lower in children who took half dose
Brooks WA, 2005[26]	Bangladesh	2 – 12 months	1665	Zinc 70mg once/weekly for 12 months	12 months	Reduce mortality and pneumonia
Penny ME, 2004[27]	Peru	6 – 35 months with persistent diarrhoea	246	Zinc OR zinc +multiple micro at 1-2RDAs OR Placebo	6 months	Higher serum zinc, less diarrhoea and respiratory illness in zinc alone group
Brooks WA, 2004[28]	Bangladesh	2 – 23 months with severe pneumonia	270	Zinc or placebo	During hospitalisation	Reduced duration of severe pneumonia
Bhandari N, 2002[29]	Dakshinpuri, New Delhi	6 – 30 months	2482	Zinc gluconate	4 months	Reduced incidence of diarrhoea
Baqui AH, 2002[30]	Bangladesh	3 – 59 months	8070	Zinc, cluster randomisation	2 years	Shorter duration of diarrhoea, reduced incidence, reduced ARIs and admissions
Rahman MM, 2002[31]	Bangladesh	12 – 35 months	653	Zinc OR Vit A OR zinc + vit A OR placebo for 14 days	6 months	No effect on weight and length

Authors	Country	Participant's age	Number	Intervention and dosage	Duration of follow-up	Findings
Muller O, 2001[32]	Burkina Faso	6 – 31 months	709	Zinc 6 days/week for 6 months or placebo	6 months	No effect on malaria episodes, reduced diarrhoea
Varandas L 2001[33]	Mozambique	6 – 72 mo with severe malaria	570	Vitamin A OR placebo	6 weeks	Slight reduction in malaria mortality, not significant
Umeta M, 2000[34]	Ethiopia	6 – 12 months	2000	Zinc 10mg 6 days/ week for 6 months or placebo	6 months	Zinc increased growth in both stunted and non-stunted children, reduced illness due to diarrhoea, cough and fever
Shankar AH, 1999[35]	Papua New Guinea	6 – 60 months	274	Zinc 6 days/week for 46 weeks months		No effect on malaria
Roy SK, 1999[36]	Bangladesh	3 – 24 months with acute diarrhoea	65	Zinc with multivitamins versus zinc-free multivitamin	2 months	Fewer episodes of diarrhoea and respiratory illness, improved growth
Penny ME, 1999[22]	Peru	6 – 36 months	412	Zinc OR zinc +multiple micro at 1-2RDAs OR Placebo	2 weeks	Zn reduced duration of diarrhoea episodes
Sempértegui F, 1999[37]	Ecuador	6 – 36 months	400	Vitamin A or placebo, weekly low dose vitamin A	10 months	No impact on diarrhoea, reduced ALRT infections in underweight children
Rivera JA, 1998[38]	Guatemala	6 – 9 months	89	Zinc or placebo	7 months	Improved linear growth in those who were stunted
Semba RD, 1997[39]	Indonesia	9 months	394	Vit A / placebo single dose with measles vaccine	6 months	No difference in sero-conversion
Benn CS, 1997[40]	Guinea-Bissau	9 months	397	Vit A single dose with measles vaccine	9 months	Vit A had no effect on antibody response
Barreto ML, 1994[41]	Brazil	6 – 48 months	1240	Vitamin A, 3 cycles OR Placebo	12 months	Reduced incidence of diarrhoea, no effect on incidence of ARI
Bhandari N, 1994[42]	India	12 – 60 months	900	Vitamin A 200000 IU versus placebo	3 months	Reduced incidence of measles and not diarrhoea and not ARI

In all the trials, including HIV-infected and uninfected, the results were not consistent, even when studies were from the same region. For instance, zinc supplementation reduced

respiratory illness in some studies but not in others. The effect was observed in some cases who were deficient in the supplemented micronutrient.

The lack of effect of some of the interventions on some key child survival indicators, such as growth, may be evidence of the need to consider giving multiple micronutrients rather than single micronutrient supplementation. It has been argued that if you provide one vitamin or mineral to a person that is deficient in several, this intervention may not have any beneficial effect because of the overall deficiency.

Table 4. Previous micronutrient intervention studies in HIV-infected children.

Author	Country	Participants	Number	Intervention & dosage	Follow-up	Outcome
Semba RD, 2005[43]	Uganda	HIV-infected children at 15 months of age	181	Vit A / placebo supplementation quarterly	21 months	Reduced all cause of mortality by 46%
Bobat R, 2005[44]	South Africa	HIV-infected children 6 to 60 months	96 HIV-infected children	Zinc or placebo	6 months	Did not increase viral load, reduced diarrhoea morbidity
Villamor E, 2002[45]	Tanzania	6 – 60 months with pneumonia	687 (malaria 115, HIV 47)	Vitamin A or placebo	1 year	Improved linear growth in stunted children and weight gain in infants with malaria and HIV
Hanekom WA, 2000[46]	North America	Children receiving influenza vaccine	59	Vit A / placebo Single dose	2 weeks	Decreased HIV viral load
Fawzi WW, 1999[47]	Tanzania	Children admitted with severe pneumonia 6 months – 5 years	687 (58 HIV-infected children)	Vit A / placebo quarterly	24 months	Halved all cause mortality, reduced risk of severe watery diarrhoea. All cause mortality reduced by 63% in 58 HIV-infected children
Coutsoudis A, 1995[48]	South Africa	Infants born to 118 HIV-infected women	28 HIV-infected infants	Vit A / placebo supplementation quarterly from 1 month	15 months	Reduced all cause mortality by a third, no effect on mortality in a sub group of HIV-infected children but halved episodes of diarrhoea

Hence, there has therefore been a move towards use of multiple micronutrient supplements. However, the dilemma of how many micronutrients should be given and the doses to a deficient population remains controversial among many researchers and clinicians.

UNICEF, United Nations University (UNU), World Health Organization (WHO) and other partners held a consultative workshop in 1998 to discuss and propose a formulation for a multi-micronutrient supplement to be used in low-income countries.[49, 50] The supplement, called UNIMAP, for UN Multiple Micronutrient Preparation, contained 15 micronutrients (vitamin A, D, E, B_1, B_2, B_6, B_{12}, C, Niacin, Folic Acid, and Fe, Zn, Cu, I, and Se) at approximately the RDAs for pregnant women. The selection of the micronutrients was

based on previous reports on nutritional deficiencies, consequences of deficiency states to the mother and infant and known biological interactions between the different micronutrients.[51-53] Second, the dose, toxicity of some micronutrients, the cost, size of the pill and possible adverse effects were considered. The physiological doses were based on the US and Canadian recommendations [54] as being the most recent and best documented at that time.

The use of this UNIMAP supplement was to be piloted in several countries until subsequent research could substantiate its safety, efficacy and effectiveness. From a programme point of view, it was more efficient to combine several micronutrients into one supplement and consider other vulnerable groups, such as children. Some of the earlier studies showed no impact of the multi-micronutrient supplement on birth weight and infant mortality, except for one which showed a slight increase in birth weight. [55-58] A trial using 2RDAs showed a significant increase in birth weight.[59]

By 2003/4 when we designed this trial, there were no published studies on the effects of prolonged use of multiple micronutrient supplementation as the main intervention in young children. We therefore decided to conduct such a study using similar micronutrients suggested by UNICEF and partners, but we made one important change in excluding iron. The reason was that some studies had indicated that iron-supplementation could be harmful in HIV-infected persons. In HIV-infected adults living in developed countries, high iron stores, such as ferritin (after adjusting for acute infections) have been associated with increased mortality and rapid progression of HIV.[60-62] This was also supported by in vitro studies which showed that iron chelation was associated with reduced HIV replication.[63]

One or several RDAs of each micronutrient?

How much of each micronutrient should be provided to HIV-infected children? The recommended dietary allowance ideally is supposed to meet the nutrient requirement of 97.5% of healthy individuals in a life-stage and gender group in a population.[64] It was questionable if this would be adequate in HIV-infected children, the majority of whom had frequent illnesses associated with poor appetite, reduced intake and increased losses. In addition, the common diet in Uganda is not micronutrient-dense and most children do not routinely consume animal products, vegetables and fruits.[65] We therefore concluded that we should give more than one RDA based on an anticipated deficiency and increased needs, but how much more? We considered 1) earlier studies that had reported using larger doses of micronutrients[66, 67], and 2) the upper tolerable levels of intake and potential adverse effects for each of the micronutrients. Upper tolerable level (UL) defines the highest level of consumption where the risk of adverse effects or toxicity is zero (Fig 3).[1, 68]

Some vitamins e.g. vitamin A, may be harmful in larger doses. Furthermore, our supplement was to be given over extended periods of time (6 months). After consideration of these different factors, we decided to give 2RDA of each of the micronutrients, having no real data to support the use of different multiples of the RDA.

Furthermore, we decided to use the RDA of a 4-year old category, the group with the higher requirement, to warrant the highest probability that every child's requirements were met. We ensured that the doses of the individual micronutrients were not above the tolerable

upper levels at 2RDA doses. We also noticed that there were minor variations between the 1-3 and 4-8 age categories, and some doses were similar, such as iodine and vitamin D.

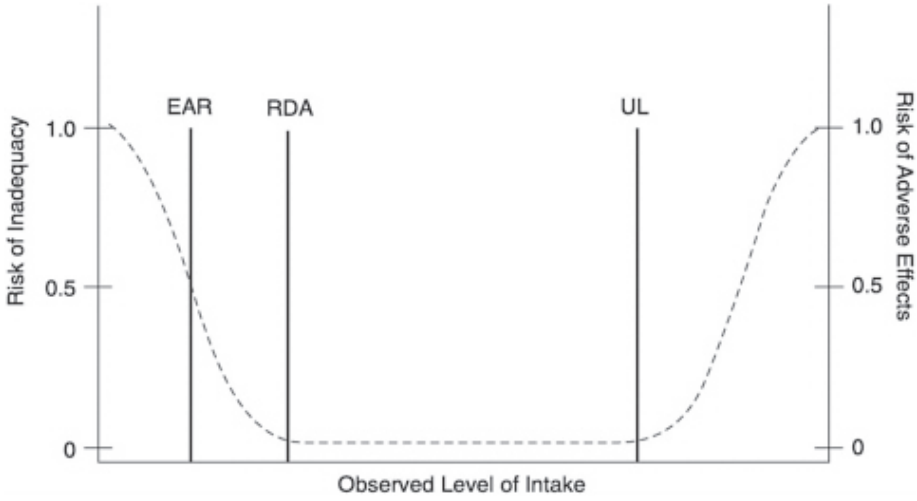

Figure 3. Theoretical curve of risk of adverse effects in relation to the level of intake of a particular nutrient. Above the upper tolerable level of intake (UL) there is a risk of adverse effects. Source: Renwich (2006)

Uganda

The context under which the study was conducted is described below.

Uganda is a landlocked country in East Africa lying on the equator. Lake Victoria covers a substantial portion of the southern part of the country. The climate is tropical which means a warm climate throughout the year with 2 rainy seasons in most of the country that remains permanently green throughout the year. Agriculture is the main economic activity relying on a few crops: maize, sweet potatoes, plantain, maize, sorghum, millet and pulses; vegetables and fruits tend to be seasonal. Livestock, poultry and fish also exist, but animal foods are rarely consumed on a regular basis. The northern part of the country is semi-arid and sometimes experiences prolonged drought, leading to crop failure and food insecurity.

The population of Uganda

Uganda has 32.7 million inhabitants and is one of the fastest growing populations[69] in the world at a rate of 3.3% (2009) compared to the world's average of 1.2%. Half the population is < 15 years of age. An estimated 20% of the population live in the urban areas, the main being the capital, Kampala. Uganda is generally a poor country with almost a third of the population living on less than one United States dollar per day.[70] The country is divided into 4 main regions (Central, Western, Eastern, and Northern) along the main ethnic groups, and into administrative units of 50,000 to 500,000 people called districts, of which there are currently 111 (i.e. in 2011).

The healthcare system of Uganda

The health system has 4 levels of health centres, district hospitals, regional and national referral hospitals. On top of the referral pyramid is the national referral level with 2 hospitals: the Mulago hospital for general care and Butabika hospital for psychiatric care, both located in the capital. The next level comprises of 13 regional referral hospitals, 3-4 in each of the main regions. At this level, the staffing ideally includes a paediatrician, a surgeon, a physician and a gynaecologist. This level is followed by the district hospitals which supervise the lower health levels: health centres IV (mini-hospitals), III (inpatient care), II (only outpatient care). The lowest level is 'Health Centre I'; it has no physical structures but is a village health team comprised of selected members of the community. Healthcare seeking does not follow this hierarchical order. Patients can present to the regional or national referral hospital for care without referral. Similar to most low-income countries, the reality is understaffing, frequent stock-outs and lack of adequate diagnostics. Frequent shortages in electricity and black-outs are also common, but the higher levels are usually better off. The Ministry of Health is mainly responsible for setting policies, standards and guidelines, soliciting for funding and supervision.

The private sector contributes 50% of healthcare delivery in Uganda. This includes the private-for-profit and the private not-for-profit, the latter mainly consisting of the faith-based organisations. Uganda uses a multi-sectorial approach to the control of AIDS (MACA) whose principle is that persons individually or collectively have a responsibility to fight the epidemic. The approach emphasizes the involvement of private and public actors, individuals and groups from highest political level to the grassroots.[71]

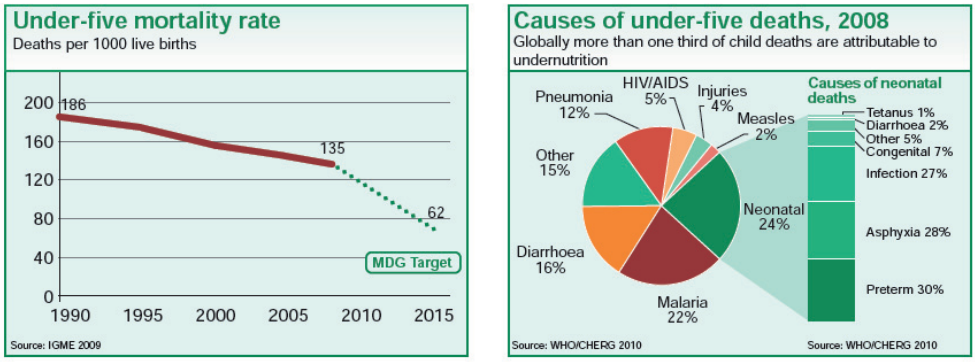

Figure 4. Under 5 mortality in Uganda: progress from 1990 to 2008 towards MDG 4 and main causes of under-5 deaths. Source: Countdown 2015.
http://www.who.int/child_adolescent_health/documents/9789241599573/en/index.html

Health indicators of Uganda

Uganda is making progress towards the millennium development goal number 4 of lowering the under-5 mortality by two-thirds between 1990 to 2015; however, this is not at the required pace (Figure 4).

Common causes of death in Uganda
Most of the common causes of death in children, as seen in Figure 4 are preventable, such as malaria, diarrhoea, pneumonia, HIV, malnutrition and neonatal causes. As in many low-income countries, malnutrition significantly contributes to morbidity and mortality (Figure 4 and Table 5).

Nutritional interventions in Uganda
Children aged 6 to 59 months receive a dose of vitamin A as routine supplementation. The coverage has been improving using the national child health days twice a year. Apart from this, there are no large-scale programs to prevent child malnutrition. Those in place are donor-initiated projects and most probably have a limited life-time. The proportion of children with diarrhoea who receive zinc as part of treatment is unknown. The choice of treatment given depends on how knowledgeable the health worker is. Sometimes multivitamin and rarely mineral supplementation is given as treatment to sick children at the discretion of the health worker.

HIV care services for children in Uganda
By 2005 Uganda was beginning to scale up paediatric HIV services following the "3 by 5" initiative, launched by UNAIDS and WHO in 2003; this aimed at improving access to antiretroviral therapy in low- and middle-income countries.[72] The major components of the HIV treatment package at that time included HIV testing and counselling services, treatment of common and opportunistic infections, nutritional counselling and routine multivitamin supplementation, with very few and severely ill children accessing anti-retroviral therapy. Most of the children had frequent visits to the clinics because of recurrent illness related to their poor immunological and nutritional state.

Table 5. Anthropometric and nutritional status of Ugandan children under 5 years of age.

Category	Prevalence (%)
Wasting	6
Stunting	38
Underweight	16
Vitamin A deficiency (<0.7 mmol/L)	20
All anaemia combined (Hb <11g/dl)	73
- Mild (Hb 10.0-10.9 g/dl)	22
- Moderate (Hb 7.0-9.9 g/dl)	43
- Severe (Hb <7.0 g/dl)	7
Zinc deficiency	20-69%

Rationale for the study of this thesis

There still is insufficient knowledge and evidence to guide appropriate micronutrient supplementation, especially in the most vulnerable groups. We know the diets for most Ugandan children comprise mainly of carbohydrate foods and plant proteins, which are unlikely to contain enough micronutrients for growth and cater for illness. And there is evidence to show that malnourished or HIV-infected persons have higher micronutrient requirements. We also know that more than one in two of the Ugandan HIV-infected children are malnourished,[73, 74] but how much more is required compared to the healthy population remains unclear.

Research-wise, previous micronutrient supplementation studies have largely focused on single or few micronutrient interventions probably because it is easier to attribute an outcome to a single micronutrient than many.

Implementation-wise, vitamin A supplementation is routinely given to all children under 5 years of age and an addition of multivitamins is given to HIV-infected children in Uganda. WHO recommends use of micronutrients in one RDA for HIV-infected persons, but it is not known whether the multivitamin supplements given at the HIV clinics and the doses are adequate to meet the requirements of these children

Highly active anti-retroviral therapy (HAART) is associated with a sustained increase in growth in HIV-infected children.[75] It is also known that HAART leads to improvement in micronutrient status[76] but it still may not correct all the deficiencies.

Theoretically, providing several micronutrients on a regular basis would improve child health; however, there is little evidence to support or refute this. In reality the best option would be to provide a supplement that would address all the micronutrient needs of the individual.

In order to try and fill in the knowledge gaps above, we hypothesised that providing 2RDAs of multiple micronutrients would reduce mortality (all causes), improve growth and reduce the frequency of illness compared to the 'standard of care' multivitamins. In the process, we also assessed the micronutrient status of the children by measuring some of the micronutrients in the blood.

Aims of the thesis

The overall aim of this thesis has been to assess whether a multiple micronutrient supplement could improve survival and weight gain, and also reduce morbidity of HIV-infected children living in Uganda compared to the 'standard of care multivitamin supplement'.

Primary objective

1. To assess whether multiple micronutrient supplementation could decrease mortality in Ugandan HIV-infected children aged 1-5 years (paper I)

Secondary objectives

2. To assess the effect of multiple micronutrient supplementation on incidence and prevalence of diarrhoea among HIV-infected children aged 1-5 years (paper II)
3. To assess the magnitude of zinc deficiency among HIV-infected children aged 1-5 years (paper III)
4. To assess the effect of multiple micronutrient supplementation on serum concentrations of vitamin B_{12} and folate among HIV-infected children aged 1-5 years (paper IV)

Subjects and methods

This thesis presents data from a randomised clinical trial of a multiple micronutrient supplement containing 14 micronutrients to HIV-infected children attending paediatric HIV clinics at 7 sites in Uganda. As it mainly describes a randomised clinical trial (RCT), we have followed the Consolidated Standards of Reporting Trials (CONSORT) checklist in the presentation of the methodology. The CONSORT group is one of the many initiatives to standardise reporting of scientific studies, and has produced the CONSORT Statement, which is an evidence-based minimum set of recommendations for reporting RCTs.[77]

Study sites

The studies were conducted in 7 sites (Figure 4). Three sites were located in the capital city (Kampala), while the other 4 sites were regional referral hospitals. The main site was Mulago hospital, the national referral hospital, situated in Kampala. Mulago has the oldest and biggest paediatric HIV clinic. The other sites in the capital city were Mildmay Centre, which is a non-governmental organisation based in the United Kingdom, and Nsambya Hospital, which is a faith-based private hospital. The 4 sites outside the capital city were Mbale hospital in the east, Lira in the north, Mbarara in the southwest and Masaka hospital in the south of the central region.

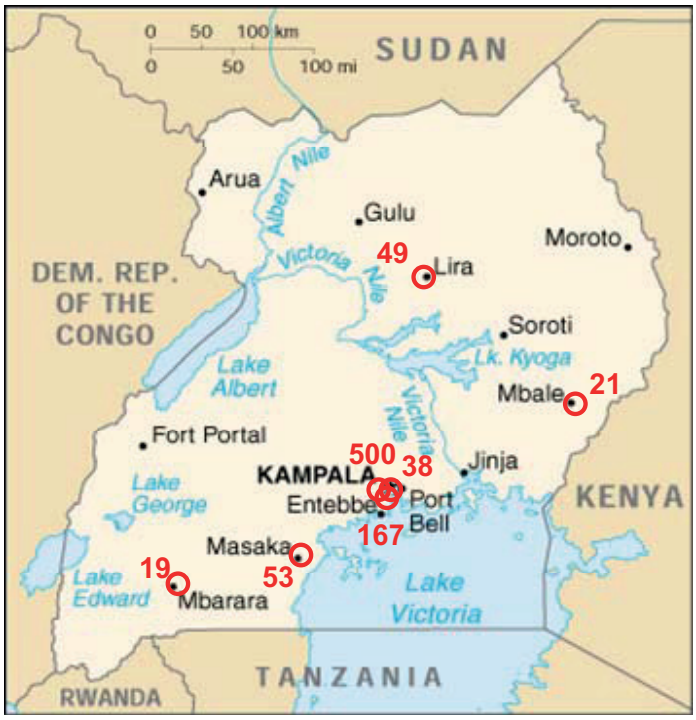

Figure 5. Map of Uganda showing the 7 study sites with numbers from each site.

The number of participants at the different sites varied since the paediatric HIV clinics were at different levels of development. Most of the regional sites were in their initial stages of setting up paediatric HIV clinics and had small numbers of patients registered in care. Most of the participants were from Mulago hospital, followed by the Mildmay Centre. All the study sites had in-patient wards where the children could be hospitalised if they became very ill.

Design

The study was a randomised controlled trial conducted between 2005 and 2008 at 7 paediatric HIV clinics in Uganda. Participants were allocated to the intervention or comparative 'standard of care' supplement in a ratio of 1:1. The participants were stratified into those receiving highly active anti-retroviral treatment (HAART), 10% and those not yet on HAART, 90% of the participants.

Papers I and II compared the outcomes in the intervention and comparative arm. In paper III a cross-sectional design was used to report baseline zinc status, whereas paper IV assessed the effect of the intervention on micronutrient concentrations.

Participants

Children aged 1-5 at the study clinics were screened for eligibility. The study profile shows the number of children screened, included, excluded and the number of children at different end-points (Figure 6).

Eligibility criteria

Inclusion criteria
HIV-infected children

- Aged 1-5 years
- HIV status previously confirmed by 2 ELISAs for children ≥ 18 months, and DNA PCR for those <18 months
- Unlikely to change residence during the course of the study
- Residing within 15 kilometres from the study clinics

Exclusion criteria

- Enrolled in other studies
- Mother or caretaker declined consent
- Unlikely to return to the clinic for regular follow-up, based on their previous record of non-adherence to clinic appointments

Clinical procedures
All eligible children had a detailed medical history followed by physical examination by one of the study doctors. Measurements included anthropometry, temperature, respiratory rate, and a systemic examination.

Follow-up

Children were followed monthly (routine visit) for the first 6 months during supplementation, and at 9 and 12 months (Figure 7). All clinical measurements made at baseline were repeated at monthly visits, except the laboratory tests were done at 0 and 6 months. Mothers/ caretakers were encouraged to attend a regular clinic managed by the study teams whenever the child was ill (inter-current visits). Transport was refunded to every mother/ caretaker attending a scheduled visit. Children who did not turn up for the scheduled visits were traced initially by telephone for those who had telephone contacts or by home visits on the 7th day.

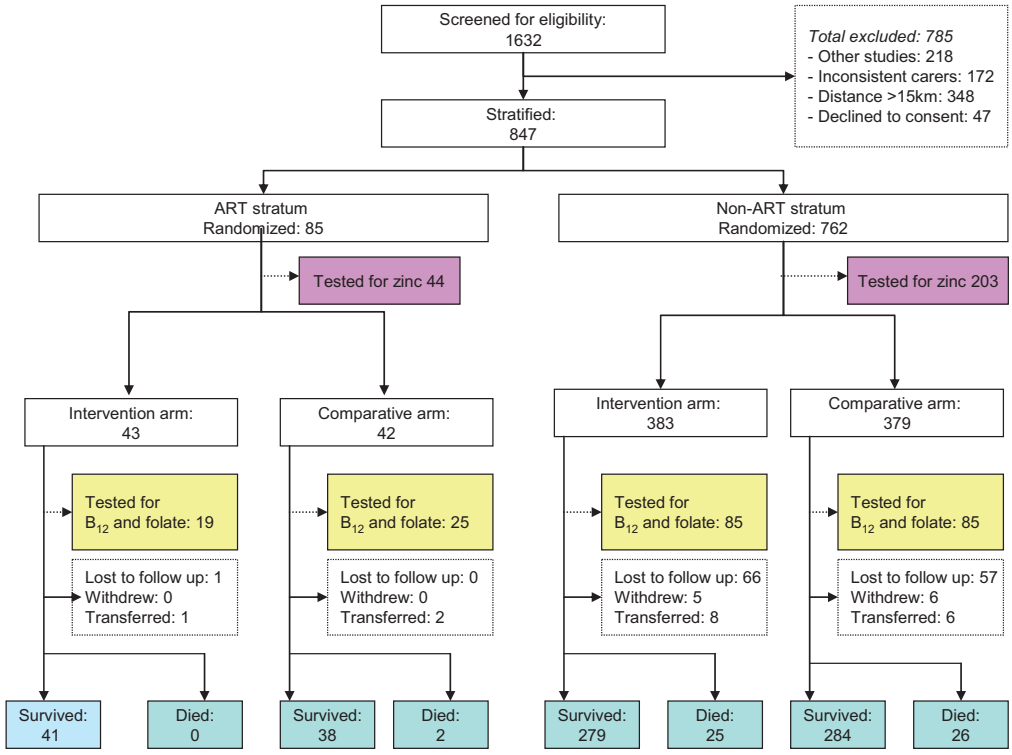

Figure 6. Trial profile of the study. Analysis in Papers I and II is based on the blue boxes, paper III on the purple boxes and paper IV on the yellow boxes.

Laboratory procedures

Blood samples (5-7 ml) were collected twice, at enrolment and 6 months (Figure 7) by venepuncture from the cubital fossa or dorsum of the hand using sterile techniques. A 2 ml sample was placed in a Beckton Dickinson Vacutainer® EDTA tube (purple-top) and 3-5 ml in a trace-element-free Vacutainer® (blue-top) tube. The EDTA samples were analyzed for a complete blood count using a Coulter counter (AcT 5Diff instrument Beckman Coulter), and CD4 cell count using a FACScan instrument and MultiSET software (Beckton Dickinson). Haematological analysis was performed by the Centre for Disease Control

(CDC) laboratory in Entebbe, Uganda, for the Kampala sites and at the Joint Clinical Research Centre (JCRC) laboratories at the rural sites.

Table 6. Study sites and number of participants recruited per site.

Study site	Location	Management	Total bed capacity	Number of HIV-infected children screened	Number enrolled
Mulago Hospital	Central (Kampala)	Government/ public and free	1500	1050	500
Mildmay Centre	Central (Kampala)	NGO/free services for children	Mainly outpatient services/ 30 beds for children	232	167
St Francis Hospital (Nsambya)	Central (Kampala)	Private faith-based not-for-profit	361	86	38
Masaka Regional Referral Hospital	South of central region (Masaka town)	Government/ public and free	330	78	53
Lira regional Referral Hospital	North (Lira town)	Government/ public and free	254	68	49
Mbale Regional Referral Hospital	East (Mbale town)	Government/ public and free	400	63	21
Mbarara Regional Referral Hospital	Southwest (Mbarara town)	Government/ public and free	300	55	19

The samples collected in trace element-free tubes were allowed to clot, centrifuged at 2000g, and the serum transferred to trace element-free cryo-tubes, kept and transferred in cool boxes within 3 hours to a -20°C refrigerator until they were shipped to the Clinical Chemistry Laboratory of Haukeland University Hospital, in Bergen, Norway, for biochemical analysis.

For trace element analysis, the samples were digested using a microwave oven, nitric acid and concentrated hydrogen peroxide,[78] and analysed using Inductively Coupled Atomic Emission Spectrophotometry (ICP-AES). Vitamin B_{12} was measured using Modular E (Roche) automatic analyzer by an electrochemiluminescence immunoassay, and folate on

Modular E (Roche) automatic analyzer using a competitive protein-binding assay. A coefficient of variation of 5% was used for the zinc, vitamin B_{12} and folate concentrations. Qualitative C-reactive protein (CRP) was measured at the study site before sample storage, using the latex immunoassay on one drop of serum (Human Gesellschaft fur Biochemica und Diagnostica mbH, Germany).

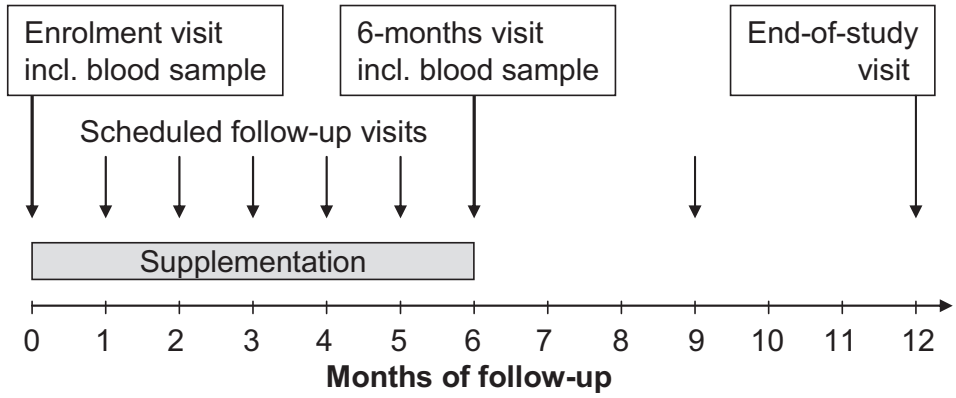

Figure 7. Plan of supplementation, follow-up and scheduled procedures at each visit.

Cut-off points for micronutrient concentrations

Because no Ugandan child reference values for zinc, vitamin B_{12} and folate concentrations have been established, we used cut-off values established by other researches. Low zinc was defined as a serum concentration of < 10.0 µmol/L.[79, 80] Vitamin B_{12} concentrations were considered low if < 221picomoles per litre (pmol/L), and folate concentrations were considered low if < 13.4 nanomoles per litre (nmol/L).[81-83] C-reactive protein (CRP) was positive if there was distinct agglutination on the latex immunoassay strip, which was consistent with a CRP of ≥ 6 mg/L. Anaemia was defined as a haemoglobin < 11 g/dl, using the WHO classification of anaemia in children aged 6 months to 5 years.[84]

Intervention

The trial supplement was a mixture of 10 vitamins and 4 trace elements in 2RDA doses (Table 7). The comparative supplement contained 6 vitamins that was 'standard of care' in 1RDA doses. The rationale for this composition has been previously provided.

The intervention was in form of a powder reconstituted with 10-20 ml of milk or water into a solution before being given to the child. The first dose was given at the clinic under guidance of the health worker, but subsequent doses were administered from home by the mother. Compliance was measured by weighing the remaining supplement when the children and mother returned for the monthly routine follow-up visits. The container held 140 g and the child was expected to consume 120 g. The daily dose was 4 g equivalent to a levelled scoop supplied by the manufacturer. The additional doses were to cater for spillage or in case the mother was not able to return on the exact day of follow-up. Supplements were given daily for 6 months (Figure 7).

Administration of the intervention
At the baseline visit, the study nurse demonstrated to the mother/caretaker how to measure a dose using a levelled scoop. Ten to 20 ml of milk or water was used to prepare a solution from the micronutrient powder. The mother prepared and gave the first dose under observation. Subsequent doses were administered at home.

Table 7. The formulation for the intervention supplements.

Micronutrient	Multivitamins 'Standard of care' 1RDA	Multiple micronutrients 2 RDA
Vitamin A (mcg)	400	800
VitaminB_1 (mg)	0.6	1.2
Vitamin B_2 (mg)	0.6	1.2
Niacin (mg)	8	16
Vitamin B_6 (mg)	-	1.2
Vitamin B_{12} (mcg)	-	2.4
Folate (mcg)	-	400
Vitamin C (mg)	25	50
Vitamin D (IU)	200	400
Vitamin E (mg)	-	14
Selenium (mcg)	-	60
Zinc (mg)	-	10
Copper (mcg)	-	800
Iodine (mcg)	-	180
Iron (mg)	-	-

Figure 8. Containers with the trial supplement.

Measuring compliance
At the monthly visit, mothers/caretakers came with the previous container of the supplement. The remaining amount was measured using a light-weight weighing scale to

ascertain how much supplement had been used. The actual amount of supplement taken against the expected was regarded as the compliance measure. Mothers answered a questionnaire on whether any doses had been missed and the reasons for non-compliance.

Outcomes

The study outcomes are summarised in Table 8, including information on when and how they were assessed.

Primary outcome: mortality (Paper I)
The primary outcome of the study was mortality. Participants were followed monthly on routine visits up to 6 months during supplementation, then at 9 and 12 months when the study ended. In addition to the routine visits mothers came to the study clinics for inter-current visits whenever the child was ill to be treated by the study doctor. If they required hospitalisation, they were admitted to the same hospital. Information about a death was communicated verbally to the study team or from hospital records when a child died in hospital. Those who were alive were censored at 12 months and those lost to follow-up immediately after the last visit.

Table 8. Outcomes of the study.

	Sample size	How assessed	When measured	Outcome
Effect of multiple micronutrient supplementation on survival (Paper I)	847	Participants followed up for 12 months, deaths recorded	At 12 months	Mortality
Effect of multiple micronutrient supplementation on diarrhoea (Paper II)	800 for incidence; 613 for prevalence	Episodes of diarrhoea counted on routine visits and 2 weeks before. Point prevalence at 6 months	At 6 months	Diarrhoea incidence and prevalence
Baseline zinc status (Paper III)	247	Measured serum zinc by ICP-AES	At baseline	Prevalence of zinc deficiency
Effect of multiple micronutrient supplementation on vitamin B_{12} and folate concentrations (Paper IV)	214	Measured serum vitamin B_{12} and folate	At baseline and 6 months	Vitamin B_{12} and folate concentrations

Secondary outcome: incidence and prevalence of diarrhoea (Paper II)
Episodes of diarrhoea occurring during supplementation, from the time of enrolment to 6 months were recorded. The time for assessing this outcome was limited to 2 weeks of observation for every routine visit, since we could not conduct surveillance visits in the homes. Episodes therefore included children who had diarrhoea at the routine visits, episodes occurring within the past 2 weeks, and any inter-current visits due to diarrhoea.

Episodes per person-time (weeks) of observation were calculated. For prevalence, the proportion of children presenting with diarrhoea at 6 months was determined.

Outcome 3: the proportion of children with a zinc concentration of <10.0 µmol/L (Paper III)
Zinc concentration was measured on serum samples collected at enrolment and reported in a sub-sample of 247 children. The proportion of children with serum zinc <10.0 µmol/L was regarded as zinc deficiency.

Outcome 4: Vitamin B_1 and folate concentrations pre- and post-supplementation (Paper IV)
Vitamin B_{12} and folate concentrations were measured both at baseline and at 6 months in a sub sample of 214 children. Vitamin B_{12} concentration was considered low if <221 pmol/L and folate <13.4 nmol/L.

Sample size

The estimated sample size of 411 children in each arm was based on data from 2 studies. The first assumption was a mortality rate in the comparative arm of 24% in one year, based on the mortality rate in a study conducted in Mulago Hospital, Kampala, before HAART was available to HIV-infected children in Uganda.[73] The second assumption – that all-cause mortality would be reduced to 14.4% (a 40% reduction) was based on a study of Tanzanian children aged 6 months to 5 years where supplementation with vitamin A was associated with a 49% reduction in overall mortality and a 63% reduction among HIV-infected children.[47] The 40% reduction level was used as we anticipated improved general care of HIV-infected children with time. Finally, we used a precision of 5%, and 95% confidence interval. The power estimate was 90% and we assumed a 10% loss to follow-up. The calculated sample size was 373 children plus 38 for 10% attrition rate (Total 411).

Table 9. Basis for the sample size calculation.

	Proportion (%)	Sample size
Mortality in Ugandan HIV-infected children pre-HAART (Berhane et al 1997)	24	
Assumptions:		
Mortality in study	14.4	373
Effect size	40	
Power	90	
Precision	5	
Loss to follow-up	10	38
Total sample size in each arm		411
Supporting evidence for mortality reduction		
Vitamin A in Tanzania children (Fawzi 1999) – All cases	49	
Vitamin A in Tanzania children (Fawzi 1999) – HIV-infected children)	63	

Randomisation

The participants were randomly assigned to either twice the recommended dietary allowance of 14 multiple micronutrients or the 6 multivitamin 'standard of care' supplement. To ensure equal distribution, randomisation was done by an independent researcher in permuted blocks of 4 to 20, using the Stata software and sent to the manufactures (NUTRISET) in France.

Allocation concealment or masking

The supplements were packed in sequentially numbered identical containers (Figure 8). Each participant number had 6 containers to be used in 6 months. The 2 strata were identified as S1 (HAART stratum) or S2 (non-HAART stratum) followed by a serial number. Following screening, an eligible participant underwent all the study procedures after which the next number of the supplement was issued by the study nurse. The intervention and standard of care supplements were both similar in colour, consistency, and odour, and were packaged in identical containers. The study staff, mothers/caretakers and investigators were all masked to treatment assignment. Serial interviews with the study staff indicated that they could never tell the difference between the two. The investigators had no access to the randomisation code until completion of the study.

Quality control

Each site had a minimum staffing of 3 members; a doctor, nurse/HIV counsellor and a laboratory person. At Mulago Hospital and Mildmay Centre, there were more doctors and an extra nurse. At each of the sites one of the investigators trained the staff and one paediatrician on the protocol and study procedures for 3 days before commencement of the study. Data collection materials were piloted and refined. At the Kampala sites where there were 2 doctors, they checked each others work so that mistakes could be corrected there and then. The principal investigator checked the questionnaires at the end of the day except on the days when she travelled to the regional sites. A paediatrician was in charge of supervising the junior doctors for the regional sites, and the PI supervised once in 2 weeks. The laboratories for sample analysis had been chosen because of their expertise and renowned experience. They had external quality control programmes in place.

Data management

Semi-structured case report forms (CRFs) were used to collect information at baseline, monthly visits, and inter-current visits, as well as at 6, 9 and 12 months. Information captured on the CRFs included physical findings, illnesses/admissions during the study period, medications given and laboratory investigations. An adverse event form was filled if one occurred. One of the investigators or site supervisors checked the forms daily for completeness. Any missing data or corrections were addressed. The CRFs were collected, cross-checked, placed in individual participant folders and kept by one of the investigators at a central office.

The data was entered at regular intervals and stored in Epidata version 3.1. at the Kampala office. The data-base was only accessible to the PI and the data entry person. In order to

minimise errors inbuilt consistency checks and restrictions were used on some of the variables. The data was transported into SPSS (version 15.0), frequencies run, and inconsistencies and outliers checked. The data was further cleaned with the assistance of case report notes kept at the central office. De-linking of the patients names was done before statistical analysis.

Statistical analysis
Statistical analysis was performed using SPSS version 15.0 (Table 10). Categorical characteristics were summarised into proportions while continuous variables were analysed using means and standard deviations. Baseline characteristics were compared in the 2 treatment groups using proportions, and differences were tested with the Chi-square or Fisher's exact test. Sub-group analysis was based on whether the children belonged to the HAART or HAART-naïve stratum.

In Paper I, Kaplan-Meier curves and the log-rank test were used to compare survival in the 2 arms. For Paper II, the incidence of diarrhoea was determined using rates, and rate ratios were used to compare differences in the 2 arms. In Paper III, frequencies were run and clinical characteristics were compared among zinc deficient (zinc <10.0 μmol/L) and non-deficient children using Fisher's exact test and odds ratios. In Paper IV, medians and their inter-quartile ranges were used to summarise the data. The median concentrations were compared using the Wilcoxon signed rank test.

Table 10. Statistical analysis for the papers.

	Design	Sample size	Statistical analysis
Paper I	Randomised controlled design	847 children	Kaplan Meier survival curves, Cox regression
Paper II	Randomised controlled design	800 for incidence; 613 for prevalence at 6 months	Rates and rate ratios, descriptive analysis, binary logistic regression, Fisher's exact test for comparisons
Paper III	Cross-sectional	247 children	Cross tabulations, Fisher's exact test, binary logistic regression
Paper IV	Randomised controlled design	214 pairs	Wilcoxon signed rank test, logistic regression

Ethical considerations

Permission to carry out the study was granted by Makerere University School of Medicine Research and Ethics Committee, the boards of management at the hospitals, the Uganda National Council for Science and Technology, and the Western Regional Ethics and Research Committee of Norway. Informed written consent was obtained from caretakers/mothers before their children could participate in the study. Counselling on adherence to treatment and anti-retroviral therapy was offered to all the parents/caretakers and their children. Whenever a child was eligible and ready to start anti-retroviral drugs, they were allowed to do so but remained in their pre-determined treatment arm.

Results

The results are presented in the 4 papers attached at the end of the recommendations. In this section, we have tried to summarise them for the reader.

Characteristics of the participants

The participants were 1-5 years of age, their mean age (Standard deviation, SD) being 33.4 (13.6) months. The majority were over 2 years of age. Boys and girls were equally represented. The baseline characteristics were similar in the intervention and comparative arm (Table 11).

Table 11. Baseline characteristics of HIV-infected children aged 1-5 years at paediatric HIV clinics in Uganda.

Baseline Characteristics	ART stratum (N=85)			Non-ART stratum (N=762)		
	Intervention; n/N (%)	Comparative arm; n/N (%)	p-value	Intervention; n/N (%)	Comparative arm; n/N (%)	p-value
Age <24 months	1/43 (2.3)	3/42 (7.1)	0.36	123/383 (32.1)	122/379 (32.2)	1.00
Sex : Male	20/43 (46.5)	23/42 (54.8)	0.52	201/383 (52.5)	182/379 (48.0)	0.25
Site: Kampala	40/43 (93.0)	40/42 (95.2)	1.00	313/383 (81.7)	311/379 (82.1)	0.93
Carer: Mother	24/43 (55.8)	21/42 (50.0)	0.67	278/383 (72.6)	278/379 (73.4)	0.87
Diarrhoea at enrolment	1/43 (2.3)	2/42 (4.8)	0.62	42/383 (11.0)	51/379 (13.5)	0.32
Persistent diarr-hoea at enrolm.	0/43 (0.0)	0/42 (0.0)	-	11/383 (26.8)	11/379 (23.4)	0.81
Diarrhoea in past 2 wks	0/43 (0.0)	0/42 (0.0)	-	13/383 (3.4)	14/379 (3.7)	0.85
Fever at enrolment	9/43 (20.9)	8/42 (19.0)	1.00	76/383 (19.8)	81/379 (21.4)	0.65
Cough at enrolment	27/43 (62.8)	26/42 (61.9)	1.00	220/383 (57.4)	219/379 (57.8)	0.94
Cotrimoxazole prophylaxis	43/43(100.0)	42/42(100.0)	-	340/383 (88.8)	334/379 (88.1)	0.82
Routine multivitamins	18/43 (41.9)	13/42 (31.0)	0.37	241/383 (62.9)	219/379 (57.8)	0.16
Vitamin A in past 6 months	20/43 (46.5)	16/42 (38.1)	0.51	183/383 (47.8)	175/379 (46.2)	0.66
WHO stage III or IV	-	-	-	124/383 (33.4)	114/379 (30.1)	0.53
CD4+ <25%	17/39 (43.6)	17/36 (47.2)	0.81	211/325 (64.9)	215/328 (65.5)	0.87
WHZ <-2	2/41 (4.9)	3/42 (7.1)	1.00	53/366(14.5)	50/367 (13.6)	0.75
HAZ <-2	22/40 (55.0)	23/41 (56.1)	1.00	190/358(53.1)	197/359 (54.9)	0.65
WAZ <-2	5/40 (12.5)	6/41 (14.6)	1.00	110/364 (30.2)	125/363 (34.4)	0.24
*CRP >6mg/L	15/31 (48.4)	11/30 (36.7)	0.44	122/260 (46.9)	119/247 (48.2)	0.79
*Zinc <10 µmol/L	5/31 (16.1)	14/29 (48.3)	0.01	86/143 (60.1)	84/133 (63.2)	0.62

Effect of multiple micronutrient supplementation on mortality

The overall mortality at 12 months of follow-up was 53/847 (6.3%). In the intervention arm 25/426 (5.9%) and in the comparative arm 28/421 (6.7%) children died. There was no difference between arms; the risk ratio was 0.9 (95% CI; 0.5-1.5) using the Kaplan-Meier survival analysis (Figure 9). However, those on HAART were less likely to die. In the HAART stratum, two out of the 85 (2.4%) children died compared with 51/762 (6.7%) in the non-HAART stratum. The numbers in the HAART stratum were too few to draw comparisons. In the non-HAART stratum, similar proportions of children in the intervention and comparative arm died, 25/383 (6.5%) in one arm and 26/379 (6.9%) in the other. The risk ratio was 1.0 (95% CI; 0.6-1.6). The mean survival time was similar in the 2 groups of children.

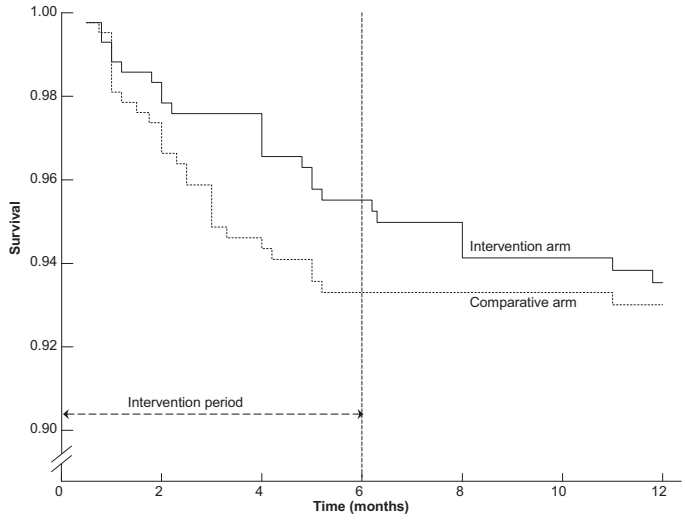

Figure 9. Survival in the two arms using Kaplan-Meier curves.

Presence of fever, hospitalization at enrolment visit, WHO Stage 3 or 4, and being underweight independently predicted early mortality. The common causes of death were pneumonia (accounting for 20/53, 37.7 %), acute febrile illness/malaria (11/53, 20.8%) and diarrhoea (6/53, 11.3%).

When the anthropometric measurements and the CD4+ cell count at 12 months were compared, no difference was found between the 2 groups.

Adverse effects related to the supplement were reported in 16 children (1.9%) and these included vomiting in 12 and diarrhoea in 4 children. A third of the 12 children vomited within 30 minutes of administering the supplement. There were equal numbers in each arm.

Effect of multiple micronutrient supplementation on diarrhoea morbidity

Of the 847 children randomised, 800 children contributed data on the incidence of diarrhoea (Table 12). Forty-seven children (25 in the intervention and 22 in the comparative arm) dropped out before their first routine follow-up visit. Of the 710 children who completed 6 months of follow-up, 613 were assessed for prevalence of diarrhoea at the 6 month visit. There were 516 episodes of diarrhoea in 7,336 person weeks, 270/3706 in the MMS and 246/3630 in the MV group. The incidence rate was 3.8 (95% CI; 3.4-4.3) in the MMS and 3.5 (95% CI; 3.1- 4.0) in the MV group per child year. The rate ratio was 1.1 (0.9-1.3), meaning that there was no difference in the incidence of diarrhoea. In the HAART stratum the incidence of diarrhoea was low but not different between the intervention and comparative arm.

Table 12. Incidence of diarrhoea in the intervention arm (MMS) compared to the comparative arm (MV).

Group	Arm	Number of participants with at least one follow-up	Episodes of diarrhoea	Obs. period (weeks)	Rate per child/year (95% CI)	Rate ratio (95% CI)	P value
Overall	MMS	401	270	3706	3.8 (3.4 – 4.3)	1.1 (0.9 – 1.3)	0.43
	MV	399	246	3630	3.5 (3.1 – 4.0)		
Non-HAART stratum	MMS	359	255	3246	4.0 (3.6 – 4.6)	1.1 (0.9 – 1.2)	0.44
	MV	358	233	3190	3.8 (3.4 – 4.3)		
HAART stratum	MMS	42	15	460	1.7 (1.0 – 2.7)	1.1 (0.5 – 2.3)	0.94
	MV	41	13	440	1.5 (0.9 – 2.6)		

The prevalence of diarrhoea at the 6 month visit was also similar in the 2 groups. Further analysis of the factors that were likely to predict diarrhoea revealed that age was the only significant factor.

Zinc status of HIV-infected children aged 1-5 years

Serum zinc concentration was assessed at baseline for a sub-sample of 247 children (Paper III). Of these, 134 (54.3%) had a serum zinc concentration of <10μmol/L, which we considered low. The mean (SD) serum zinc concentration was 10.0 (2.9) μmol/L, with a range of 5.6-29.5 μmol/L. There was no linear relationship between age, weight-for-height *z*-score, haemoglobin and zinc status. The mean serum zinc among children on HAART was 12.2 (SD 4.1) compared to 9.6 (SD 2.5) among those not receiving HAART, a statistically significant difference (OR 2.6; 95%CI; 1.6-3.5; Table 13).

Table 13. Serum zinc concentrations in the HAART and HAART naïve children

	Zinc <10 μmol/L	Zinc ≥10 μmol/L	p-value
On HAART (n=44)	13 (29.5%)	31 (70.5%)	<0.001
HAART naïve (n=203)	121 (59.6%)	82 (40.4%)	

Significant independent predictors of low zinc were: being HAART naïve and reported fever. The characteristics likely to be associated with low zinc concentration are presented in Table 14.

Table 14. Factors associated with low serum zinc concentration in 247 HIV-infected Ugandan children aged 1-5 years.

	Low zinc <10µmol/L n/N (%)	Unadjusted OR (95%CI)	Adjusted OR (95%CI)
On HAART	13/ 44 (29.5)	3.5 (1.7 – 7.1)	3.7 (1.8 – 7.7)
No HAART	121/203 (59.6)		
Reported fever	30/ 44 (68.2)	2.0 (1.0 – 4.1)	2.2 (1.1 – 4.6)
No fever	104/203 (51.2)		
WHO stage 3 or 4	42/ 62 (67.7)	2.1 (1.2 – 3.9)	1.5 (0.8 – 2.9)
WHO stage 1 or 2	92/185 (49.7)		
WAZ score <-2	38/ 56 (67.9)	2.1 (1.1 – 3.9)	1.2 (0.6 – 2.6)
WAZ score ≥-2	96/191 (50.3)		
CRP <6 mg/L	72/147 (49.0)	1.7 (1.0 – 2.9)	1.6 (0.9 – 2.7)
CRP ≥6 mg/L	62/100 (62.0)		

Effect of multiple micronutrient supplementation on vitamin B_{12} and folate concentrations

We compared the serum concentrations of vitamin B_{12} and folate at baseline and at 6 months in a sub-sample of 214 children. There were almost equal numbers in both the intervention and comparative arm. At baseline, 60/214 children (28.0%) had a low vitamin B_{12} concentration of <221.0 pmol/L and 62/214 (29.0%) had a low concentration of folate of <13.4 nmol/L. At 6 months, the children in the intervention arm had an increase in the concentrations of both vitamin B_{12} and folate, whereas the 'standard of care' arm showed almost no change with time. The mean difference in concentration of vitamin B_{12} was 106.5 (182.7) and that of folate was 7.8 (13.7) in the intervention arm, with minimal change in the comparative arm (Table 15, Figure 10).

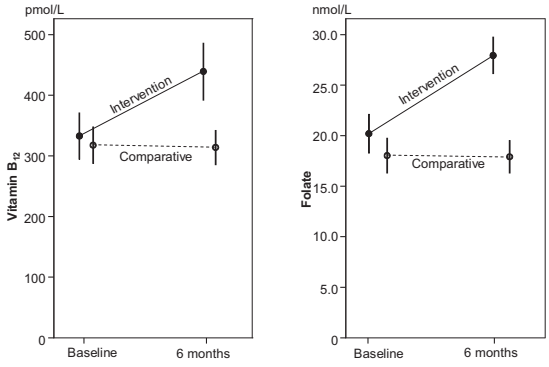

Figure 10. Mean serum concentrations of vitamin B_{12} and folate at baseline and at 6 months of supplementation.

The differences between the intervention and comparative arm were maintained in the 2 strata. Vitamin B_{12} status was not associated with baseline characteristics, whereas folate status was likely to be lower in the male and children of <24 months of age.

Table 15. Biochemical and haematological measurements at baseline and at 6 months of supplementation by intervention group.

	Multiple micronutrient supplementation group (n=104)		**Comparative 'standard of care' multivitamins group (n=110)**	
Measurement	**Median (IQR)**	**p-value**	**Median (IQR)**	**p-value**
Vitamin B_{12} (pmol/L)				
- Baseline	285.5 (216.5 – 371.8)	<0.001	280.0 (211.5 – 386.3)	0.78
- 6 months	401.5 (264.3 – 518.8)		288.5 (198.8 –391.0)	
- Change	90.5 (-0.8 – 203.5)		10.0 (-73.8 – 83.8)	
Folate (nmol/L)				
- Baseline	17.3 (13.5 – 26.6)	<0.001	15.7 (11.9 – 22.1)	0.44
- 6 months	27.7 (21.1 – 33.4)		16.5 (11.7 – 22.1)	
- Change	8.0 (-0.3 – 17.1)		-0.6 (-3.5 – 5.8)	
Haemoglobin (g/dl)				
- Baseline	10.0 (8.7 – 11.2)	0.04	9.8 (8.8 – 11.2)	<0.001
- 6 months	10.9 (9.4 – 11.7)		10.6 (9.6 – 11.7)	
- Change	0.3 (-0.4 – 0.9)		0.6 (-0.2 – 1.4)	
CD4+ count (cells/µL)				
- Baseline	1201 (822 – 1556)	0.16	1033 (728 – 1406)	0.52
- 6 months	1039 (725 – 1358)		1043 (704 – 1484)	
- Change	-137 (-348 – 254)		35 (-278 – 352)	

Wilcoxon Signed Ranks test was used to measure the difference between baseline and 6 months.

Discussion

This clinical trial of 2RDA multiple micronutrient supplementation in HIV-infected children demonstrated that this supplement was well tolerated by all subjects. However, there was no reduction in mortality compared to the 'standard of care' (of multivitamin supplement) at one year of follow-up, 6 months post-supplementation. The intervention neither improved growth nor reduced diarrhoea morbidity. Biochemical measurements showed that over half of the children were zinc deficient and close to one-third had low concentrations of vitamin B_{12} and folate. The deficiencies decreased following 6 months of supplementation but one-fifth of the children remained with low concentrations of vitamin B_{12} and folate. Children who had received the intervention were more likely to have higher serum concentrations of micronutrients.

Implications of the major findings

The main findings of this study and the potential explanations are summarised in Table 16.

Table 16. Main findings and their potential explanations.

Main findings	Potential explanations
No reduction in mortality No difference in growth	• Too short a supplementation period • Too low dose, higher dosing required • No placebo, comparative arm also had some impact • Too small difference in doses between intervention and comparative supplement • Too many other interventions in HIV
No reduction in diarrhoea morbidity	• Too short a supplementation period • Excluded peak age for diarrhoea morbidity, therefore fewer episodes • Other interventions
Low micronutrient concentrations in blood	• Micronutrient deficiency • Redistribution of zinc
Improved micronutrient status following supplementation	• Response to supplementation • The standard formulation did not contain folic acid and vitamin B_{12} • Evidence of deficiency

2RDAs of 14 micronutrients is safe

The study supplement with 14 micronutrients in the amount of 2RDAs per day was well tolerated. Diarrhoea and vomiting were reported by mothers or caretakers in ~2 in 100 children. The occurrence of gastrointestinal symptoms was variable in duration with a few episodes of vomiting within 30 minutes of administering the supplement. There were equal numbers in each arm indicating that these symptoms were unlikely to be due to the intervention supplement. Sometimes the children were taking multiple drugs and it may not be easy to attribute gastrointestinal symptoms to the intervention.

The risk of harm due to dietary supplements normally depends on the safe intake range. At the lower end of intake, the risk of compromised health increases due to deficiency and at higher doses the risk of compromised health due to toxicity increases (Figure 3). The safe intake range tends to vary with each micronutrient and may be narrow for some of them. From a safety point of view, 3 groups of nutrients can be identified, water-soluble vitamins, fat-soluble vitamins and minerals.

For the water-soluble vitamins, it is generally believed that higher doses are well tolerated since they are easily excreted. For example, vitamin C can be given in mega-doses without fear of toxicity other than osmotic diarrhoea. The margin is comparable or greater for most of the water-soluble vitamins.[85]

The fat-soluble vitamins tend to have a narrow safe intake range, such as vitamin A.[86] Single nutrient studies show that vitamin A accumulates in fatty tissues and the liver; toxicity occurs when the amount of vitamin A exceeds the capacity of retinol-binding protein to bind it. Excess vitamin A binds instead to lipoproteins, causing changes in biological membranes. Excess vitamin A in the liver may result in elevated liver cell enzymes as an indicator of potential liver damage.

The mineral supplements present a more varied picture. For example, selenium has a narrow range of safe intake such that using it in a more soluble form may increase uptake and the risk of toxicity. Our supplement also contained zinc whose adverse effects have been reported on in several studies. Although vomiting was reported in a few participants, there was no difference in the 2 groups. Previous studies reported that vomiting was twice as likely to occur in zinc-treated children, with no serious adverse events.[87] The human body tends to regulate the absorption and retention of zinc such that toxic levels do not accumulate in the body.[88] Zinc toxicity occurs following consumption of multiples of RDA; other minerals such as calcium and iron can be given in higher doses to most people without toxicity.

Our supplement contained only 2 RDAs and within the safety margin for all the micronutrients included. Other studies that have used multiple micronutrient supplements in the HIV-infected population have reported no adverse events.[89, 90]

No effect on mortality

Multiple micronutrient supplementation was not associated with reduced mortality compared to the 'standard of care' supplement. Were there any signs or tendencies that the intervention arm did better than the comparative arm? With a very generous and kind interpretation of the Kaplan-Meier curve, it could be argued that there is a tendency for the survival curves to diverge during the first 6 months with supplements, and a convergence could be observed after cessation (Figure 9). Would these curves have been different if the supplementation had been continued for a longer time, and with a larger dose difference between the arms? We can only speculate on this, but the actual data does not support any conclusion about a difference between the arms. We are also unlikely to ever get a better answer to this question because HAART has now become the standard of care for HIV-infected children. As expected, mortality was lower in children receiving HAART. Whether HIV-infected children on HAART require an MMS supplement is discussed below.

The care of HIV-infected children has improved with time as more knowledge is gathered about the disease. For example improved diagnosis and management of opportunistic infections improves the quality of life and reduces mortality.[91] Although these factors could have been equally shared in the 2 arms, they reduced the overall mortality thus reducing the power of the study.

Generally large studies are required for studying effects on mortality, as it is a relatively rare event. In the planning of this study we were facing very high mortality rates in this particular sub-population of HIV-infected children and we had indications that multiple micronutrients could dramatically improve their survival. At the time of calculating the sample size, an intervention effect size of 40% was used, anticipating a higher mortality than actually found. Fortunately, the overall mortality was much lower, only 6.3% compared to the anticipated 14.4%. The assumption for reduction of mortality was based on a small sample of HIV-infected children in Tanzania[47] in the absence of any large micronutrient supplementation studies reported in Africa at the time. The mortality in paediatric HIV was also much higher[73, 92, 93] than what we currently see. Although we factored in the impact of general improved care in the sample size, nevertheless, the study was underpowered for the mortality outcome, a limitation that has been acknowledged.

No effect on diarrhoea morbidity

The incidence of diarrhoea was 3-4 episodes per year in the non-HAART stratum, which is a quite common incidence among children in Africa.[94] Our study population was HIV-infected in whom we would thus expect a higher incidence. But the incidence was not elevated, and there was no difference between the intervention and comparative arm. Are there any explanatory factors?

It is possible that prophylaxis with cotrimoxazole could have reduced diarrhoea morbidity in the study population. A study of HIV-infected adults in Uganda showed a significant reduction in diarrhoea morbidity following cotrimoxazole prophylaxis,[95] although its impact on prevention of diarrhoea in HIV-infected children had been inconclusive in other studies.[96]

The different causes of diarrhoea could have influenced the results if not equally distributed in the 2 arms. The intervention could have had an impact on severity and duration of diarrhoea, factors not explored in our study.

Both the intervention and comparative supplements contained vitamin A, whose impact could have been similar on mucosal immunity and diarrhoea morbidity. Vitamin A supplementation reduces the severity of pathogen-induced diarrhoea, evidenced by reduced faecal concentrations of the monocyte chemoattractant protein-1, indicating that vitamin A has an anti-inflammatory effect in the gut.[97] However, the effect of vitamin A on mucosal immune response may be modified by the type of pathogen, resulting in no detectable difference in patients with different pathogenic infections.[98]

The intervention contained zinc, which reduces diarrhoea episodes and severity in HIV-infected, non-infected children and adults.[44, 99, 100] The effect could have been more

pronounced in those who were zinc deficient as shown in one of the adult studies [100]. It is therefore surprising that a zinc-containing supplement had no effect on diarrhoea incidence. The only plausible explanation is that the Uganda diet contains high concentrations of phytates from dietary plant material, including cereals, corn and rice; these chelate zinc and inhibit its absorption,[101] thereby minimising the difference between the 2 arms.

Some opportunistic gut infections are associated with damage to the intestinal villi[102] and this damage may not be repaired by micronutrients. Alternatively, the supplementation dose and period may not have been enough to facilitate complete repair.

Another intervention that may have influenced diarrhoea morbidity was the promotion at the time of the study of use of point-of-use chlorination and storage of water using safe water systems.[103] Some of the study participants received 'water guard' for chlorination of water and safe water storage containers. Unfortunately, no data was collected on this intervention.

Does this mean that this supplement has no real influence on diarrhoeal morbidity or did we fail to detect a true relation? It could be argued that the supplementation time was not long enough to create an effect on diarrhoea morbidity. Further, it could be argued that we did not include the peak age for diarrhoea (6 to 12 months) and subsequently fewer episodes were registered. However, the lack of effect is likely to be a reflection of the truth, since we have not identified any strong biasing factors and there was no tendency of difference between the arms.

The non-effect on diarrhoeal morbidity compares well with other recent multiple micronutrient supplementation studies among children in other low income countries that gave the supplement for 6 months.[104, 105]

Low serum micronutrient concentration – is it always equal to deficiency?
Ideally a measure of body stores of a micronutrient should determine its amount in the body. In most cases we use blood concentrations as proxy indicators of micronutrient status supposing that the blood concentration truly reflects body stores.

We know that during acute infections, serum concentrations of some micronutrients change, for example vitamin A and zinc tend to reduce whereas serum ferritin increases.[106, 107] If a sample is taken in such a situation and the micronutrient concentration is low, it is unclear whether this decrease is a real deficiency or the result of some redistribution. This becomes particularly difficult to evaluate when there is no follow-up measurement. In order to attribute a change to inflammation, another test that measures acute phase proteins is recommended. An example is CRP which was measured in our study. Indeed, zinc concentrations were related to fever or a positive CRP. Though not very reliable, serum zinc is the best measure of zinc status that is available to date.

For some micronutrients, their deficiency states can be reliably measured through their metabolic functions. For example, vitamin B_{12} and folate deficiency are associated with increased concentrations of methylmalonic acid and homocysteine.[108] Although we did not measure these metabolites, some studies in low-income countries indicated that low concentrations of folate and vitamin B_{12} were commonly associated with elevated

methylmalonic acid and homocysteine, indicating true deficiency.[82] Therefore the low concentrations in our study could actually imply true deficiency.

In a study of HIV-infected children in New York, all the participants had normal or elevated vitamin B_{12} and folate status,[109] despite not receiving multivitamins. There are definitely major differences in the diet of children in the United States of America compared to Ugandan children. Our study included younger children, the majority of whom were symptomatic and not on HAART compared to the New York study of older and HAART treated children.

Improved micronutrient status after supplementation

The increase in vitamin B_{12} and folate at 6 months implies that this was probably a true deficiency that responded to supplementation. Our study indirectly shows that the Ugandan children do not get adequate levels of vitamin B_{12} and folate in the diet or in the form of supplements. The 'standard of care' supplement did not contain vitamin B_{12} or folate. The fact that some children did not show normalisation of vitamin B_{12} and folate concentrations following supplementation with 2RDAs of multiple micronutrients (containing vitamin B_{12} and folate) could indicate that they require higher doses of at least some of the micronutrients or perhaps a longer duration of supplementation.

The children who did not show normalisation of their blood micronutrient concentrations are probably those who were more deficient or had multiple deficiencies. At the same time, they could have been the ones that benefited more than others. This argument is supported by previous reports that have shown that supplementation is more likely to be beneficial to micronutrient deficient children.[110-112]

Table 17. Main findings and their implications.

Main findings	Implications
2RDA well tolerated	• 2RDA safe
No reduction in mortality No difference in growth	• MMS in 2 RDA for 6 months does not reduce mortality • MMS in 2 RDA for 6 months does not improve growth • The comparative arm also had some effect and decreased the difference
No reduction in incidence of diarrhoea	• MMS in 2 RDA does not reduce diarrhoea incidence
Low micronutrient concentrations in blood Improved micronutrient status following supplementation Not all children normalised their blood concentrations of analysed micronutrients	• A high prevalence of micronutrient deficiencies • Possibly redistribution of zinc in infections • MMS in 2 RDA raises vitamin B_{12} and folate concentrations • Possible increase of the other micronutrients • An indirect evidence of deficiency • MMS in 2 RDA for 6 months not enough to raise concentrations to normal levels

We found no association between CD4+ cell count with vitamin B_{12} or folate concentrations. This was rather surprising, since adult studies have shown that low vitamin B_{12} is associated with low CD4+ lymphocyte count and faster HIV disease progression.[113-115] Low folate concentrations have not been previously associated with HIV disease progression. [115] There have been no similar studies in HIV-infected children residing in low-income countries to make comparisons. We have no clear explanation for the lack of association in this study.

Generally there was an increase in haemoglobin, with no significant differences in both arms. This is probably due to the fact that all participants increased in age during the trial. Although the supplement did not contain iron, it could potentially have had an impact on iron absorption (from the diet) or metabolism and other micronutrients involved in haemopoiesis.

Should HIV-infected children receive MMS?
Our findings demonstrate that the HIV-infected children were micronutrient deficient and not in only one micronutrient but probably several. It is also clear that the supplementation improved the concentrations of some of these micronutrients. On the other hand, our supplement neither reduced mortality nor diarrhoeal morbidity, so what should we recommend?

Based on general physiological knowledge that it is healthier for a subject to be replete in micronutrients than deficient, and on the finding that a large group of these children had low micronutrient concentrations and improved with supplementation, we recommend all Ugandan HIV-infected children to receive a multiple micronutrient supplement, even if it has no proven effect on mortality or morbidity. We also recommend that the multiple micronutrient supplement should be at least 2 RDAs.

Which composition of MMS should it be?
Because we need a considerable number of micronutrients and their assessment is often difficult, it is a good strategy to include as many as possible in a supplement.

Whether iron should be included remains contentious. In malaria endemic areas it was previously argued that iron supplementation increases malaria parasitaemia and severity.[116] Subsequent studies failed to show increased malaria morbidity.[117, 118] With adequate malaria diagnosis and treatment iron supplementation is associated with increased iron status and haemoglobin.

Previous studies in HIV-infected patients indicated that iron could be associated with increased HIV replication, more rapid disease progression and poor outcomes.[61, 62] With or without HIV infection iron is utilised by most pathogens and influences their virulence.[119] Most of the available literature is based on adult studies, not on HIV-infected children living in malaria-endemic areas. In Cochrane's 2009 review, there were no randomised trials of iron supplementation in HIV-infected children[120] and we have not found any to-date.

The arguments in support of including iron in the supplement are: 1) HIV and iron deficiency appear in the same regions of the world, 2) many children with anaemia are

treated with iron, irrespective of their HIV status, without any reported serious adverse effects, 3) several studies from malaria endemic areas indicate that iron supplementation during malaria illness does not increase parasitaemia or cause severe anaemia, and 4) iron supplementation in malaria endemic areas has been associated with improved haemoglobin and growth.[118]

Recommendation for HAART treated versus HAART naïve children?
Although the HAART children were better off, a significant number had low micronutrient concentrations. Since the diets for most children in Uganda are not micronutrient dense, we would recommend the same supplement and similar dosing for children receiving HAART. This proposal is supported by other studies; for example, a multiple micronutrient supplement administered to HIV-infected adults on HAART for at least 3 months improved their CD4 cell count within 12 weeks of follow-up compared to a placebo.[121] Other studies of one or two micronutrients resulted in improved antioxidant defence.[122]

How should the supplementation be administered?
Mothers were very comfortable with administering the powder form of the supplement. They equated it with giving powdered milk. However, a few mothers had problems administering the supplement for example, when having to travel with the child. In order to produce a relatively stable multiple micronutrient supplement that is easy to administer and store, we would recommend use of dispersible tables, preferably in blister packs.

Methodological considerations

A randomised controlled trial is a strong research design in studies of cause and effect associations. Any difference between the participants is supposed to be equally distributed between the arms and therefore the risk of bias is minimal; for instance, it is unlikely that one of the groups is richer or better educated than the other. In our study, randomisation took part in all the participating clinics which eliminates the risk of bias from the different sites.

Despite having used a 'gold standard' design, there are some issues that could have influenced the outcomes. The first is the intervention: we relied on the mothers for supervision and administration of the supplement. The best option would have been directly observed therapy, which was not possible in this study. We assessed compliance, which was similar in both arms, indicating that any effect would be equally distributed.

Second, we also relied on the mothers reporting one of the main outcomes: diarrhoea. We could also have missed diarrhoea episodes that occurred outside the clinic setting and were never reported, since we were unable to use home visits for ethical reasons. A record of morbidity would have been more complete had regular community visits been possible. This effect was minimised by encouraging mothers to return to the clinic on any day of the week whenever the child was sick. In addition, we restricted our analysis to 2 weeks of observation when we could fairly rely on the mother's recall for diarrhoea.

Third, micronutrient assays were only performed for children residing in Kampala city, and therefore it was not possible to assess differences with other sites. We could generally

conclude that our micronutrient results depict the micronutrient status of the children in Kampala.

Fourth, this thesis does not report any data on dietary habits of the study children, and therefore unable to tell whether this could have had an impact on the outcome. But again, with randomisation, it is unlikely to have any large hidden confounding element in the dietary habits.

Fifth, adult studies have shown that the time of blood collection and feeding influence micronutrient concentrations especially zinc.[123] However, it is unfair to starve children for purposes of blood collection. In addition, we were unable to conduct absorption studies to examine the impact of malabsorption on baseline micronutrient and post-supplementation status.

Sixth, due to the restrictions in blood volumes, we were unable to conduct other tests that may have confirmed vitamin B_{12} deficiency, such as methyl- malonic acid or homocysteine, and confirmed folate deficiency by red-cell folic acid status. Low micronutrient status remains a useful indicator in guiding detailed investigations for specific micronutrient deficiencies.

Seventh, the loss to follow-up was high, but similar between the 2 arms, and not very different from what other follow-up studies of HIV-infected subjects have reported.[124] The loss to follow-up in the rural sites was higher than in the capital city. We enrolled children who were living in internally displaced camps in the northern region; however, some of them relocated to their distant homes while still in the study and were therefore lost to follow-up. Those lost to follow-up were likely to be more sick, but with almost similar numbers in the two arms, and therefore less likely to influence the comparisons.

Strengths of this thesis

Our study had a larger sample size compared to other micronutrient supplementation studies in HIV-infected children. The study employed a 'gold standard' design with successful randomisation and blinding/masking. There are no published studies that had measured micronutrient concentrations or published the impact of micronutrient supplementation in Ugandan HIV-infected children.

Generalisability

The routinely available supplement for HIV-infected children in Uganda is mainly based on a few multivitamins. Our studies show that a significant proportion of the HIV-infected children have low concentrations of zinc, vitamin B_{12} and folate and that supplementation results in increased concentrations. These findings would apply to other HIV-infected children living in low-income countries in similar settings. The poor nutritional state, the micronutrient status and other baseline characteristics seemed similar to other African countries. The lack of effect on diarrhoea morbidity and mortality could also apply to similar settings, which is supported by the fact that other recent studies using multiple micronutrients in Africa, with supplementation for 6 months did not show an effect on child morbidity.[104, 105]

Conclusions and recommendations

Twice the recommended dietary allowance of 14 micronutrients compared with 1 RDA 6 multivitamins given as the 'standard of care' for 6 months did not significantly alter mortality, diarrhoea morbidity, but improved vitamin B_{12} and folate concentrations. The HAART-treated children had higher zinc concentrations and were less likely to die. A third to a half of the children had low serum concentrations of zinc, vitamin B_{12} and folate, and probably other micronutrients were deficient.

Recommendation for practice
We recommend that all HIV-infected children in Uganda be supplemented with 2RDAs of multiple micronutrients based on the following reasons:

1) Our study has shown that Ugandan HIV-infected children are micronutrient deficient, not only in one micronutrient, but probably several.
2) Supplementation with 2RDAs improved concentrations of some of the analysed micronutrients, whereas the standard of care had no effect.
3) The Uganda diet for most children is carbohydrate and plant protein based and not micronutrient dense.
4) Most of the Uganda cereal foods and grains are locally processed and not fortified.
5) HIV-infected children are more likely to have increased micronutrient requirements because of increased morbidity and demands due to growth.

Recommendation for future research
Future studies in similar settings should consider:

1) Assessing the efficacy of different dosing schemes in HIV-infected children (various dosing schedules and types of micronutrients to include in a supplement)
2) Assessing the efficacy of prolonged supplementation.
3) Assess efficacy of an iron containing regimen of micronutrients.
4) Consider micronutrient intake at doses for risk reduction of disease, not only for the healthy population.
5) Assess efficacy in HAART-treated children.

More evidence is required regarding the best dosage schedules since RDAs are calculated for a healthy population.

Recommendation for policy
Currently the WHO recommends one RDA of multiple micronutrients daily to HIV-infected people. Our study has provided some evidence that 2RDA of multiple micronutrients is safe, and improves vitamin B_{12} and folate concentrations in Ugandan HIV-infected children, whereas 1RDA of a few multivitamins had no effect. A change of policy from 1RDA to 2RDA is recommended.

References

1. Dietary Reference Intakes. Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, Food and Nutrition BoardWashington, DC: *National Academies Press;* 1997.
2. Depeint F, Bruce WR, Shangari N, Mehta R, O'Brien PJ. Mitochondrial function and toxicity: role of the B vitamin family on mitochondrial energy metabolism. *Chem Biol Interact.* 2006;**163**(1-2):94-112.
3. Thompson J. Vitamins, minerals and supplements. 7: Minerals. *Community Pract.* 2007;**80**(8):38-40.
4. Singhal N, Austin J. A clinical review of micronutrients in HIV infection. *J Int Assoc Physicians AIDS Care (Chic Ill)*. 2002;**1**(2):63-75.
5. Maggini S, Wintergerst ES, Beveridge S, Hornig DH. Selected vitamins and trace elements support immune function by strengthening epithelial barriers and cellular and humoral immune responses. *Br J Nutr.* 2007;**98** Suppl 1:S29-35.
6. Mandl J, Szarka A, Banhegyi G. Vitamin C: update on physiology and pharmacology. *Br J Pharmacol.* 2009;**157**(7):1097-110.
7. Black RE, Morris SS, Bryce J. Where and why are 10 million children dying every year? *Lancet.* 2003;**361**(9376):2226-34.
8. Bhaskaram P. Micronutrient malnutrition, infection, and immunity: an overview. *Nutr Rev.* 2002;**60**(5 Pt 2):S40-5.
9. Bhatnagar S, Natchu UC. Zinc in child health and disease. *Indian J Pediatr.* 2004;**71**(11):991-5.
10. Chandra RK. Nutrition and the immune system: an introduction. *Am J Clin Nutr*. 1997;**66**(2):460S-3S.
11. Wintergerst ES, Maggini S, Hornig DH. Contribution of selected vitamins and trace elements to immune function. *Ann Nutr Metab*. 2007;**51**(4):301-23.
12. Semba RD. Vitamin A and human immunodeficiency virus infection. *Proc Nutr Soc.* 1997;**56**(1B):459-69.
13. Villamor E, Fawzi WW. Effects of vitamin a supplementation on immune responses and correlation with clinical outcomes. *Clin Microbiol Rev*. 2005;**18**(3):446-64.
14. Moriguchi S, Muraga M. Vitamin E and immunity. *Vitam Horm.* 2000;**59**:305-36.
15. Beisel WR. Nutrition in pediatric HIV infection: setting the research agenda. Nutrition and immune function: overview. *J Nutr*. 1996;**126**(10 Suppl):2611S-5S.
16. Suttajit M. Advances in nutrition support for quality of life in HIV+/AIDS. *Asia Pac J Clin Nutr.* 2007;**16** Suppl 1:318-22.
17. Lambl BB, Federman M, Pleskow D, Wanke CA. Malabsorption and wasting in AIDS patients with microsporidia and pathogen-negative diarrhea. *AIDS.* 1996;**10**(7):739-44.
18. Intestinal malabsorption of HIV-infected children: relationship to diarrhoea, failure to thrive, enteric micro-organisms and immune impairment. The Italian Paediatric Intestinal/HIV Study Group. *AIDS.* 1993;*7*(11):1435-40.
19. Semba RD, Tang AM. Micronutrients and the pathogenesis of human immunodeficiency virus infection. *Br J Nutr.* 1999;**81**(3):181-9.

20. Henderson RA, Talusan K, Hutton N, Yolken RH, Caballero B. Whole body protein turnover in children with human immunodeficiency virus (HIV) infection. *Nutrition.* 1999;**15**(3):189-94.
21. Srinivas A, Dias BF. Antioxidants in HIV positive children. *Indian J Pediatr*. 2008;**75**(4):347-50.
22. Penny ME, Peerson JM, Marin RM, Duran A, Lanata CF, Lonnerdal B, et al. Randomized, community-based trial of the effect of zinc supplementation, with and without other micronutrients, on the duration of persistent childhood diarrhea in Lima, Peru. *J Pediatr.* 1999;**135**(2 Pt 1):208-17.
23. Roy SK, Tomkins AM, Akramuzzaman SM, Behrens RH, Haider R, Mahalanabis D, et al. Randomised controlled trial of zinc supplementation in malnourished Bangladeshi children with acute diarrhoea. *Arch Dis Child.* 1997;**77**(3):196-200.
24. Roy SK, Tomkins AM, Haider R, Behren RH, Akramuzzaman SM, Mahalanabis D, et al. Impact of zinc supplementation on subsequent growth and morbidity in Bangladeshi children with acute diarrhoea. *Eur J Clin Nutr.* 1999;**53**(7):529-34.
25. Benn CS, Martins C, Rodrigues A, Jensen H, Lisse IM, Aaby P. Randomised study of effect of different doses of vitamin A on childhood morbidity and mortality. *BMJ*. 2005;**331**(7530):1428-32.
26. Brooks WA, Santosham M, Naheed A, Goswami D, Wahed MA, Diener-West M, et al. Effect of weekly zinc supplements on incidence of pneumonia and diarrhoea in children younger than 2 years in an urban, low-income population in Bangladesh: randomised controlled trial. *Lancet.* 2005;**366**(9490):999-1004.
27. Penny ME, Marin RM, Duran A, Peerson JM, Lanata CF, Lönnerdal B, et al. Randomized controlled trial of the effect of daily supplementation with zinc or multiple micronutrients on the morbidity, growth, and micronutrient status of young Peruvian children. *Am J Clin Nutr.* 2004;**79**(3):457-65.
28. Brooks WA, Yunus M, Santosham M, Wahed MA, Nahar K, Yeasmin S, et al. Zinc for severe pneumonia in very young children: double-blind placebo-controlled trial. *Lancet.* 2004;**363**(9422):1683-8.
29. Bhandari N, Bahl R, Taneja S, Strand T, Mølbak K, Ulvik RJ, et al. Substantial reduction in severe diarrheal morbidity by daily zinc supplementation in young north Indian children. *Pediatrics.* 2002;**109**(6):e86.
30. Baqui AH, Black RE, El Arifeen S, Yunus M, Chakraborty J, Ahmed S, et al. Effect of zinc supplementation started during diarrhoea on morbidity and mortality in Bangladeshi children: community randomised trial. *BMJ.* 2002 ;**325**(7372):1059.
31. Rahman MM, Tofail F, Wahed MA, Fuchs GJ, Baqui AH, Alvarez JO. Short-term supplementation with zinc and vitamin A has no significant effect on the growth of undernourished Bangladeshi children. *Am J Clin Nutr.* 2002;**75**(1):87-91.
32. Müller O, Becher H, van Zweeden AB, Ye Y, Diallo DA, Konate AT, et al. Effect of zinc supplementation on malaria and other causes of morbidity in west African children: randomised double blind placebo controlled trial. *BMJ.* 2001;**322**(7302):1567.
33. Varandas L, Julien M, Gomes A, Rodrigues P, Van Lerberghe W, Malveiro F, et al. A randomised, double-blind, placebo-controlled clinical trial of vitamin A in severe malaria in hospitalised Mozambican children. *Ann Trop Paediatr.* 2001;**21**(3):211-22.

34. Umeta M, West CE, Haidar J, Deurenberg P, Hautvast JG. Zinc supplementation and stunted infants in Ethiopia: a randomised controlled trial. *Lancet.* 2000;**355**(9220):2021-6.
35. Shankar AH, Genton B, Semba RD, Baisor M, Paino J, Tamja S, et al. Effect of vitamin A supplementation on morbidity due to Plasmodium falciparum in young children in Papua New Guinea: a randomised trial. *Lancet.* 1999;**354**(9174):203-9.
36. Roy SK, Tomkins AM, Haider R, Behren RH, Akramuzzaman SM, Mahalanabis D, et al. Impact of zinc supplementation on subsequent growth and morbidity in Bangladeshi children with acute diarrhoea. *Eur J Clin Nutr* 1999;**53**(7):529-34.
37. Sempértegui F, Estrella B, Camaniero V, Betancourt V, Izurieta R, Ortiz W, et al. The beneficial effects of weekly low-dose vitamin A supplementation on acute lower respiratory infections and diarrhea in Ecuadorian children. *Pediatrics.* 1999;*104*(1):e1.
38. Rivera JA, Ruel MT, Santizo MC, Lonnerdal B, Brown KH. Zinc supplementation improves the growth of stunted rural Guatemalan infants. *J Nutr.* 1998;**128**(3):556-62.
39. Semba RD, Akib A, Beeler J, Munasir Z, Permaesih D, Muherdiyantiningsih, et al. Effect of vitamin A supplementation on measles vaccination in nine-month-old infants. *Public Health.* 1997;**111**(4):245-7.
40. Benn CS, Aaby P, Balé C, Olsen J, Michaelsen KF, George E, et al. Randomised trial of effect of vitamin A supplementation on antibody response to measles vaccine in Guinea-Bissau, west Africa. *Lancet.* 1997;**350**(9071):101-5.
41. Barreto ML, Santos LM, Assis AM, Araújo MP, Farenzena GG, Santos PA, et al. Effect of vitamin A supplementation on diarrhoea and acute lower-respiratory-tract infections in young children in Brazil. *Lancet.* 1994;**344**(8917):228-31.
42. Bhandari N, Bhan MK, Sazawal S. Impact of massive dose of vitamin A given to preschool children with acute diarrhoea on subsequent respiratory and diarrhoeal morbidity. *BMJ.* 1994;**309**(6966):1404-7.
43. Semba RD, Ndugwa C, Perry RT, Clark TD, Jackson JB, Melikian G, et al. Effect of periodic vitamin A supplementation on mortality and morbidity of human immunodeficiency virus-infected children in Uganda: A controlled clinical trial. *Nutrition.* 2005;**21**(1):25-31.
44. Bobat R, Coovadia H, Stephen C, Naidoo KL, McKerrow N, Black RE, et al. Safety and efficacy of zinc supplementation for children with HIV-1 infection in South Africa: a randomised double-blind placebo-controlled trial. *Lancet.* 2005;**366**(9500):1862-7.
45. Villamor E, Mbise R, Spiegelman D, Hertzmark E, Fataki M, Peterson KE, et al. Vitamin A supplements ameliorate the adverse effect of HIV-1, malaria, and diarrheal infections on child growth. *Pediatrics.* 2002;**109**(1):E6.
46. Hanekom WA, Yogev R, Heald LM, Edwards KM, Hussey GD, Chadwick EG. Effect of vitamin A therapy on serologic responses and viral load changes after influenza vaccination in children infected with the human immunodeficiency virus. *J Pediatr.* 2000;**136**(4):550-2.
47. Fawzi WW, Mbise RL, Hertzmark E, Fataki MR, Herrera MG, Ndossi G, et al. A randomized trial of vitamin A supplements in relation to mortality among human immunodeficiency virus-infected and uninfected children in Tanzania. *Pediatr Infect Dis J.* 1999;**18**(2):127-33.

48. Coutsoudis A, Bobat RA, Coovadia HM, Kuhn L, Tsai WY, Stein ZA. The effects of vitamin A supplementation on the morbidity of children born to HIV-infected women. *Am J Public Health.* 1995;**85**(8 Pt 1):1076-81.
49. Multi–micronutrient supplements for pregnant women from developing countries. Summary of a workshop report. http://fex.ennonline.net/9/multi.aspx.
50. UNICEF. Composition of a multi-micronutrient supplement to be used in pilot programmes among pregnant women in developing countries: *proceedings of a joint UNICEF/UNU/WHO workshop,* July 1999. New York; 2000.
51. Suharno D, West CE, Muhilal, Karyadi D, Hautvast JG. Supplementation with vitamin A and iron for nutritional anaemia in pregnant women in West Java, Indonesia. *Lancet.* 1993;**342**(8883):1325-8.
52. Underwood BA, Arthur P. The contribution of vitamin A to public health. *FASEB J.* 1996;**10**(9):1040-8.
53. Hercberg S, Galan P, Preziosi P, Alfarez MJ, Vazquez C. The potential role of antioxidant vitamins in preventing cardiovascular diseases and cancers. *Nutrition.* 1998;**14**(6):513-20.
54. Recommended Dietary Allowances. *Food and Nutrition Board, National Research Council.* Washington, DC 1989.
55. Christian P, Shrestha J, LeClerq SC, Khatry SK, Jiang T, Wagner T, et al. Supplementation with micronutrients in addition to iron and folic acid does not further improve the hematologic status of pregnant women in rural Nepal. *J Nutr.* 2003;**133**(11):3492-8.
56. Christian P, West KP, Khatry SK, Leclerq SC, Pradhan EK, Katz J, et al. Effects of maternal micronutrient supplementation on fetal loss and infant mortality: a cluster-randomized trial in Nepal. *Am J Clin Nutr.* 2003;**78**(6):1194-202.
57. Ramakrishnan U, Gonzalez-Cossio T, Neufeld LM, Rivera J, Martorell R. Multiple micronutrient supplementation during pregnancy does not lead to greater infant birth size than does iron-only supplementation: a randomized controlled trial in a semirural community in Mexico. *Am J Clin Nutr.* 2003 ;**77**(3):720-5.
58. Friis H, Gomo E, Nyazema N, Ndhlovu P, Krarup H, Kaestel P, et al. Effect of multimicronutrient supplementation on gestational length and birth size: a randomized, placebo-controlled, double-blind effectiveness trial in Zimbabwe. *Am J Clin Nutr.* 2004;**80**(1):178-84.
59. Kaestel P, Michaelsen KF, Aaby P, Friis H. Effects of prenatal multimicronutrient supplements on birth weight and perinatal mortality: a randomised, controlled trial in Guinea-Bissau. *Eur J Clin Nutr.* 2005;**59**(9):1081-9.
60. Weinberg GA. Iron overload as a mechanism for the lowered survival in AIDS patients receiving dapsone-iron protoxalate for secondary prophylaxis of Pneumocystis carinii pneumonia. *J Infect Dis.* 1996;**174**(1):241-2.
61. Gordeuk VR, Delanghe JR, Langlois MR, Boelaert JR. Iron status and the outcome of HIV infection: an overview. *J Clin Virol.* 2001;**20**(3):111-5.
62. Semba RD, Shah N, Strathdee SA, Vlahov D. High prevalence of iron deficiency and anemia among female injection drug users with and without HIV infection. *J Acquir Immune Defic Syndr.* 2002;**29**(2):142-4.
63. Georgiou NA, van der Bruggen T, Oudshoorn M, Nottet HS, Marx JJ, van Asbeck BS. Inhibition of human immunodeficiency virus type 1 replication in human

mononuclear blood cells by the iron chelators deferoxamine, deferiprone, and bleomycin. *J Infect Dis.* 2000;**181**(2):484-90.
64. Micronutrient Information Center. [cited May 2004]; *http://lpi.oregonstate.edu/infocenter/*
65. Wamani H, Astrom AN, Peterson S, Tylleskar T, Tumwine JK. Infant and young child feeding in western Uganda: knowledge, practices and socio-economic correlates. *J Trop Pediatr.* 2005;**51**(6):356-61.
66. Tang AM, Graham NM, Saah AJ. Effects of micronutrient intake on survival in human immunodeficiency virus type 1 infection. *Am J Epidemiol.* 1996;**143**(12):1244-56.
67. USAID. HIV/AIDS and Nutrition. A review of the literature and recommendations for nutrirional care and support in sub-Saharan Africa; 2000.
68. Renwick AG. Toxicology of micronutrients: adverse effects and uncertainty. *J Nutr*. 2006;**136**:493S-501S.
69. Uganda on Track to Have World's Highest Population Growth. *http://www.worldwatch.org/node/4525*
70. Uganda population secretariat. *http://www.popsec.org/*
71. Government of Uganda. *Ministry of Health, Health sector strategic plan III 2010/11-2014/15.*
72. The '3 by 5' initiative. Treat 3 million people with HIV/AIDS by the year 2005. *www.who.int/3by5/en/*
73. Berhane R, Bagenda D, Marum L, Aceng E, Ndugwa C, Bosch RJ, et al. Growth failure as a prognostic indicator of mortality in pediatric HIV infection. *Pediatrics.* 1997;**100**(1):E7.
74. Kabue MM, Kekitiinwa A, Maganda A, Risser JM, Chan W, Kline MW. Growth in HIV-infected children receiving antiretroviral therapy at a pediatric infectious diseases clinic in Uganda. *AIDS Patient Care STDS.* 2008;**22**(3):245-51.
75. Verweel G, van Rossum AM, Hartwig NG, Wolfs TF, Scherpbier HJ, de Groot R. Treatment with highly active antiretroviral therapy in human immunodeficiency virus type 1-infected children is associated with a sustained effect on growth. *Pediatrics.* 2002;**109**(2):E25.
76. Rousseau MC, Molines C, Moreau J, Delmont J. Influence of highly active antiretroviral therapy on micronutrient profiles in HIV-infected patients. *Ann Nutr Metab.* 2000;**44**(5-6):212-6.
77. The CONSORT statement. *http://www.consort-statement.org/consort-statement/.*
78. Rahil-Khazen R, Henriksen H, Bolann BJ, Ulvik RJ. Validation of inductively coupled plasma atomic emission spectrometry technique (ICP-AES) for multi-element analysis of trace elements in human serum. *Scand J Clin Lab Invest.* 2000;**60**(8):677-86.
79. Hotz C, Peerson JM, Brown KH. Suggested lower cutoffs of serum zinc concentrations for assessing zinc status: reanalysis of the second National Health and Nutrition Examination Survey data (1976-1980). *Am J Clin Nutr.* 2003;**78**(4):756-64.
80. Brown KH, Rivera JA, Bhutta Z, Gibson RS, King JC, Lonnerdal B, et al. International Zinc Nutrition Consultative Group (IZiNCG) technical document #1. Assessment of the risk of zinc deficiency in populations and options for its control. *Food Nutr Bull.* 2004;**25**(1 Suppl 2):S99-203.

81. Carmel R, Green R, Rosenblatt DS, Watkins D. Update on cobalamin, folate, and homocysteine. Hematology Am Soc Hematol Educ Program. 2003:62-81.
82. Taneja S, Bhandari N, Strand TA, Sommerfelt H, Refsum H, Ueland PM, et al. Cobalamin and folate status in infants and young children in a low-to-middle income country in India. *Am J Clin Nutr.* 2007;**86**(5):1302-9.
83. Rogers LM, Boy E, Miller JW, Green R, Sabel JC, Allen LH. High prevalence of cobalamin deficiency in Guatemalan schoolchildren: associations with low plasma holotranscobalamin II and elevated serum methylmalonic acid and plasma homocysteine concentrations. *Am J Clin Nutr.* 2003;**77**(2):433-40.
84. WHO. Worldwide prevalence of anaemia 1993-2005. *WHO, Geneva, Switzerland;* 2008.
85. Hathcock JN, Azzi A, Blumberg J, Bray T, Dickinson A, Frei B, et al. Vitamins E and C are safe across a broad range of intakes. *Am J Clin Nutr.* 2005;**81**(4):736-45.
86. Mulholland AC, Benford JD. What is known about the safety of multivitamin-multimineral supplements for the generally healthy population? Theoretical basis for harm. *Am J Clin Nutr.* 2007;**85**(suppl):318S–22S
87. Lazzerini M, Ronfani L. Oral zinc for treating diarrhoea. *Cochrane Database Syst Rev.* 2008;(3):CD005436.
88. Valle BL, Falchuk KH. The biochemical basis of zinc physiology. *Physiol Rev.* 1993;**73**(1):79-118.
89. Fawzi WW, Msamanga GI, Wei R, Spiegelman D, Antelman G, Villamor E, et al. Effect of providing vitamin supplements to human immunodeficiency virus-infected, lactating mothers on the child's morbidity and CD4+ cell counts. *Clin Infect Dis.* 2003;**36**(8):1053-62.
90. Jiamton S, Pepin J, Suttent R, Filteau S, Mahakkanukrauh B, Hanshaoworakul W, et al. A randomized trial of the impact of multiple micronutrient supplementation on mortality among HIV-infected individuals living in Bangkok. *AIDS.* 2003;**17**(17):2461-9.
91. Mofenson LM, Brady TM, Danner PS, Dominguez LK, Hazra R, Handelsman E, et al. Guidelines for the prevention and treatment of opportunistic infections among HIV-exposed and HIV-infected children. *CDC, National Institutes of Health, Atlanta*; 2009 Aug.
92. Brahmbhatt H, Kigozi G, Wabwire-Mangen F, Serwadda D, Lutalo T, Nalugoda F, et al. Mortality in HIV-infected and uninfected children of HIV-infected and uninfected mothers in rural Uganda. *J Acquir Immune Defic Syndr.* 2006;**41**(4):504-8.
93. Little K, Thorne C, Luo C, Bunders M, Ngongo N, McDermott P, et al. Disease progression in children with vertically-acquired HIV infection in sub-Saharan Africa: reviewing the need for HIV treatment. *Curr HIV Res.* 2007 ;**5**(2):139-53.
94. Kosek M, Bern C, Guerrant RL. The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. *Bull World Health Organ.* 2003;**81**(3):197-204.
95. Mermin J, Lule J, Ekwaru JP, Malamba S, Downing R, Ransom R, et al. Effect of co-trimoxazole prophylaxis on morbidity, mortality, CD4-cell count, and viral load in HIV infection in rural Uganda. *Lancet.* 2004;**364**(9443):1428-34.
96. Grimwade K, Swingler GH. Cotrimoxazole prophylaxis for opportunistic infections in children with HIV infection. *Cochrane Database Syst Rev.* 2006(1):CD003508.

97. Long KZ, Santos JI, Estrada Garcia T, Haas M, Firestone M, Bhagwat J, et al. Vitamin A supplementation reduces the monocyte chemoattractant protein-1 intestinal immune response of Mexican children. *J Nutr.* 2006;**136**(10):2600-5.
98. Long KZ, Estrada-Garcia T, Rosado JL, Ignacio Santos J, Haas M, Firestone M, et al. The effect of vitamin A supplementation on the intestinal immune response in Mexican children is modified by pathogen infections and diarrhea. *J Nutr.* 2006;**136**(5):1365-70.
99. Humphreys EH, Smith NA, Azman H, McLeod D, Rutherford GW. Prevention of diarrhoea in children with HIV infection or exposure to maternal HIV infection. *Cochrane Database Syst Rev.* 2010;6:CD008563.
100. Baum MK, Lai S, Sales S, Page JB, Campa AM. Randomised, controlled clinical trial of zinc supplementation to prevent immunological failure in HIV-infected adults. *Clin Infect Dis.* 2010;**50**(12):1653-60.
101. Gibson RS, Bailey KB, Gibbs M, Ferguson EL. A review of phytate, iron, zinc, and calcium concentrations in plant-based complementary foods used in low-income countries and implications for bioavailability. *Food Nutr Bull.* 2010 ;**31**(2):S134-46.
102. Tumwine JK, Kekitiinwa A, Bakeera-Kitaka S, Ndeezi G, Downing R, Feng X, et al. Cryptosporidiosis and microsporidiosis in Ugandan children with persistent diarrhoea with and without concurrent infection with the human immunodeficiency virus. *Am J Trop Med Hyg.* 2005;**73**(5):921-5.
103. Lule JR, Mermin J, Ekwaru JP, Malamba S, Downing R, Ransom R, et al. Effect of home-based water chlorination and safe storage on diarrhea among persons with human immunodeficiency virus in Uganda. *Am J Trop Med Hyg.* 2005;**73**(5):926-33.
104. Untoro J, Karyadi E, Wibowo L, Erhardt MW, Gross R. Multiple micronutrient supplements improve micronutrient status and anemia but not growth and morbidity of Indonesian infants: a randomized, double-blind, placebo-controlled trial. *J Nutr.* 2005;**135**(3):639S-45S.
105. Luabeya KK, Mpontshane N, Mackay M, Ward H, Elson I, Chhagan M, et al. Zinc or multiple micronutrient supplementation to reduce diarrhea and respiratory disease in South African children: a randomized controlled trial. *PLoS One.* 2007;**2**(6):e541.
106. Filteau SM, Tomkins AM. Micronutrients and tropical infections. *Trans R Soc Trop Med Hyg.* 1994;**88**(1):1-3, 26.
107. Wieringa FT, Dijkhuizen MA, West CE, Northrop-Clewes CA, Muhilal. Estimation of the effect of the acute phase response on indicators of micronutrient status in Indonesian infants. *J Nutr.* 2002;**132**(10):3061-6.
108. Allen RH, Stabler SP, Savage DG, Lindenbaum J. Metabolic abnormalities in cobalamin (vitamin B_{12}) and folate deficiency. *FASEB J.* 1993;**7**(14):1344-53.
109. Malik ZA, Abadi J, Sansary J, Rosenberg M. Elevated levels of vitamin B_{12} and folate in vertically infected children with HIV-1. *AIDS.* 2009;**23**(3):403-7.
110. Fawzi WW, Mbise R, Spiegelman D, Fataki M, Hertzmark E, Ndossi G. Vitamin A supplements and diarrheal and respiratory tract infections among children in Dar es Salaam, Tanzania. *J Pediatr.* 2000;**137**(5):660-7.
111. Baqui AH, Zaman K, Persson LA, El Arifeen S, Yunus M, Begum N, et al. Simultaneous weekly supplementation of iron and zinc is associated with lower morbidity due to diarrhea and acute lower respiratory infection in Bangladeshi infants. *J Nutr.* 2003;**133**(12):4150-7.

112. Ruel MT, Rivera JA, Santizo MC, Lonnerdal B, Brown KH. Impact of zinc supplementation on morbidity from diarrhea and respiratory infections among rural Guatemalan children. *Pediatrics.* 1997;**99**(6):808-13.
113. Baum MK, Shor-Posner G, Lu Y, Rosner B, Sauberlich HE, Fletcher MA, et al. Micronutrients and HIV-1 disease progression. *AIDS.* 1995;**9**(9):1051-6.
114. Remacha AF, Cadafalch J. Cobalamin deficiency in patients infected with the human immunodeficiency virus. *Semin Hematol.* 1999;**36**(1):75-87.
115. Tang AM, Graham NM, Chandra RK, Saah AJ. Low serum vitamin B-12 concentrations are associated with faster human immunodeficiency virus type 1 (HIV-1) disease progression. *J Nutr.* 1997;**127**(2):345-51.
116. Smith AW, Hendrickse RG, Harrison C, Hayes RJ, Greenwood BM. The effects on malaria of treatment of iron-deficiency anaemia with oral iron in gambian children. *Ann Trop Paediatr.* 1989;**9**(1):17-23.
117. Mebrahtu T, Stoltzfus RJ, Chwaya HM, Jape JK, Savioli L, Miontresor A, et al. Low-dose daily iron supplementation for 12 months does not increase the prevalence of malarial infection or density of parasites in young Zanzibari children. *J Nutr.* 2004;**124**(11):3037-41.
118. Ojukwu JU, Okebe JU, Yahav D, Paul M. Oral iron supplementation for preventing or treating anaemia among children in malaria-endemic areas. *Cochrane Database Syst Rev.* 2009;(3):CD006589.
119. Friis H. Micronutrients and HIV infection. *CRC Press;* Boca Raton, Florida, USA: 2002.
120. Adetifa I, Okomo U. Iron supplementation for reducing morbidity and mortality in children with HIV. *Cochrane Database Syst Rev.* 2009(1):CD006736.
121. Kaiser JD, Campa AM, Ondercin JP, Leoung GS, Pless RF, Baum MK. Micronutrient Supplementation Increases CD4 Count in HIV-Infected Individuals on Highly Active Antiretroviral Therapy: A Prospective,Double-Blinded, Placebo-Controlled Trial. *J Acquir Immune Defic Syndr.* 2006;**42**(5):523-28.
122. Jaruga P, Jaruga B, Gackowski D, Olczak A, Halota W, Pawlowska M, et al. Supplementation with antioxidant vitamins prevents oxidative modification of DNA in lymphocytes of HIV-infected patients. *Free Radic Biol Med.* 2002;**32**(5):414-20.
123. Hambidge K M, Goodall M J, Stall C, Pritts J. Post-prandial and daily changes in plasma zinc. *J Trace Elem Electrolytes Health Dis*.1989;**3**(1):55-7.
124. Irlam JH, Visser ME, Rollins N, Siegfried N. Micronutrient supplementation in children and adults with HIV infection. *Cochrane Database Syst Rev.* 2005:CD003650.

Errata

- Page 19, table 3, last row:

was: 200 000

corrected to: 200 000 IU (International Units)

- Page 23, line 8:

was: 'Health Centre I'; it has physical structures

corrected to: 'Health Centre I'; it has no physical structures

- Page 33, 3rd paragraph, last sentence:

Was: Those who were alive or lost to follow-up were censored at 12 months.

Corrected to: Those who were alive were censored at 12 months and those lost to follow-up immediately after the last visit.

Papers

I. Ndeezi G, Tylleskär T, Ndugwa CM, Tumwine JK. Effect of multiple micronutrient supplementation on survival of HIV-infected children in Uganda: a randomized, controlled trial. *J Int AIDS Soc* 2010;**13**:18. (An errata list appears after the paper)

II. Ndeezi G, Tylleskär T, Ndugwa CM, Tumwine JK. Multiple micronutrient supplementation does not reduce diarrhoea morbidity in Ugandan HIV infected children; a randomised controlled trial. *Submitted*

III. Ndeezi G, Tumwine JK, Bolann BJ, Ndugwa CM, Tylleskär T. Zinc status in HIV-infected Ugandan children aged 1-5 years: a cross-sectional baseline survey. *BMC Pediatr* 2010;**10**:68.

IV. Ndeezi G, Tumwine JK, Ndugwa CM, Bolann BJ, Tylleskär T. Multiple micronutrient supplementation improves vitamin B_{12} and folate concentrations of HIV-infected children in Uganda: a randomized controlled trial. *Nutrition Journal* 2011;**10**:56

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Ndeezi *et al. Journal of the International AIDS Society* 2010, **13**:18
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RESEARCH Open Access

Effect of multiple micronutrient supplementation on survival of HIV-infected children in Uganda: a randomized, controlled trial

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Abstract

Background: Micronutrient deficiencies compromise the survival of HIV-infected children in low-income countries. We assessed the effect of multiple micronutrient supplementation on the mortality of HIV-infected children in Uganda.

Methods: In a randomized, controlled trial, 847 children aged one to five years and attending HIV clinics in Uganda were stratified by antiretroviral therapy (ART, n = 85 versus no ART, n = 762). The children were randomized to six months of either: twice the recommended dietary allowance of 14 micronutrients as the intervention arm (vitamins A, B_1, B_2, niacin, B_6, B_{12}, C, D and E, folate, zinc, copper, iodine and selenium); or the standard recommended dietary allowance of six multivitamins (vitamins A, D_2, B_1, B_2, C and niacin) as a comparative "standard-of-care" arm. Mortality was analyzed at 12 months of follow up using Kaplan Meier curves and the log rank test.

Results: Mortality at 12 months was 25 out of 426 (5.9%) children in the intervention arm and 28 out of 421 (6.7%) in the comparative arms: risk ratio 0.9 (95% CI 0.5 - 1.5). Two out of 85 (2.4%) children in the ART stratum died compared with 51 out of 762 (6.7%) in the non-ART stratum. Of those who died in the non-ART stratum, 25 of 383 (6.5%) were in the intervention arm and 26 of 379 (6.9%) in the comparative arm; risk ratio 1.0 (95% CI 0.6 - 1.6). There was no significant difference in survival at 12 months (p = 0.64, log rank test). In addition, there was no significant difference in mean weight-for-height at 12 months; 0.70 ± 1.43 (95% CI 0.52 - 0.88) for the intervention versus 0.59 ± 1.15 (95% CI 0.45 - 0.75) in the comparative arm. The mean CD4 cell count; 1024 ± 592 (95% CI 942 - 1107) versus 1060 ± 553 (95% CI 985 - 1136) was also similar between the two groups.

Conclusions: Twice the recommended dietary allowance of 14 micronutrients compared with a standard recommended dietary allowance of six multivitamins for six months was well tolerated, but it did not significantly alter mortality, growth or CD4 counts. Future intervention studies should carefully consider: (1) the composition and dosing of the supplements; and (2) the power needed to detect a difference between arms.

Trial Registration: ClinicalTrials.gov Identifier: NCT00122941

Background

Mortality in HIV-infected children living in low-income countries is still high compared with high-income countries [1,2]. Malnutrition in children under five years of age is highly prevalent and both macro and micronutrient deficiencies are likely to co-exist [3-6], especially among HIV-infected children.

Micronutrients are important for maintaining optimal functioning of the individual's immune response. Selenium and vitamin E are involved in the maintenance of the oxidant defence system, while zinc and vitamin A play a significant role in maintaining cellular integrity [7]. Vitamin B_{12} is important in the formation of proteins and proper functioning of a large number of enzymes and the immune system [8]. Deficiency of the important components of the endogenous anti-oxidant defence system leads to accumulation of oxidative stress, including oxidative damage [9]. Vitamin A and zinc deficiencies are asso-

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ciated with increased susceptibility to infections, increased severity of illness and mortality [10,11].

Studies in HIV-infected children have demonstrated that multiple, large doses of vitamin A reduces diarrhoea episodes, increases CD4 count and reduces all-cause mortality [12-14]. Zinc supplementation in children whose HIV status was not known in Asia and Latin America reduced the incidence, duration and severity of diarrhoea and pneumonia episodes [15]. In a study of efficacy and safety of zinc, mortality was lower in the zinc-supplemented group [16]. Most of these studies were single micronutrient interventions, yet deficiencies are less likely to exist singly: hence the efforts to provide multiple micronutrients as opposed to single nutrient supplements in both children and adults studies [17-21].

To achieve normal plasma levels of micronutrients, HIV-infected adults required multiples of the recommended dietary allowance (RDA) compared with HIV-negative men. Those consuming adequate recommended intake had a relatively high prevalence of deficiencies compared with uninfected adults with similar intake [22]. Multivitamin supplementation using multiples of the RDA resulted in reduction of progression to Stage 4 disease and mortality in pregnant and lactating women in Tanzania [13].

Supplementation with multiple micronutrients had no effect on mortality in one study of adults with HIV [23], while it reduced mortality in another trial that used multiples of RDAs in patients with CD4 counts of less than 200/mm^3 [18]. Hitherto, there are no published studies that have examined the effect of multiple micronutrient supplementation on mortality of HIV-infected children.

The objective of this study was to assess the efficacy of a supplement containing twice the RDA (2 RDA) of 14 multiple micronutrients on mortality of HIV-infected children in Uganda, and to document any adverse effects associated with this dosing of multiple micronutrients. We hypothesized that daily administration of twice the recommended dietary allowance of multiple micronutrients to HIV-infected children aged one to five years for six months would reduce all-cause mortality from 24% to 14.4% in one year.

Methods

Study design, site and population

This was a randomized, controlled, double-blind, hospital-based trial of a supplement containing twice the RDA (2 RDA) of 14 micronutrients (minerals and vitamins) versus a formula of six multivitamins at the standard RDA (1 RDA) dose administered to HIV-infected children for a period of six months and followed up to 12 months. The trial was conducted between June 2005 and June 2008 in Uganda at: the Paediatric HIV clinics of the national referral hospital (Mulago); two private, not-for-profit centres (Nsambya Hospital and Mildmay Centre) in Kampala, the capital city; and four regional hospitals (Mbale, Mbarara, Masaka, Lira).

At each site, a paediatrician was in charge of the study and worked with a nurse and laboratory technician, who had undergone training on study procedures. The principal investigator initiated the study at all the sites and supervised data collection once a week at the Kampala (central) sites and once every four weeks at the regional hospitals (rural sites). Similar operating procedures were followed.

The principal investigator or another paediatrician enrolled children aged one to five years whose mothers or caretakers gave informed written consent to participate and who had attended the clinic at least once. The mothers or caretakers also had to adhere to a regular study follow-up schedule for one year. Their HIV status had earlier been confirmed by either an antibody test or DNA-PCR if younger than 18 months of age. These children were stratified into two groups: those receiving antiretroviral drugs (ARVs) and those not yet started on antiretroviral therapy (ART). Children enrolled in other studies, those residing more than 15 kilometres from the clinic and those whose parents or caretakers were anticipating moving from the study area were excluded.

The study was approved by Makerere University College of Health Sciences Research and Ethics Committee, the participating hospitals, the Uganda National Council for Science and Technology and the Regional Committee for Medical Research Ethics, Western Norway. Counselling for initiation of ART and adherence was offered to all the participants, while ongoing adherence counselling was provided to those who were already receiving ART. Initiation of ART, treatment for concurrent illnesses and prophylaxis was offered according to the World Health Organization (WHO) and national paediatric HIV management guidelines.

Micronutrient supplements

The trial supplements were manufactured in the form of a white powder, packaged in plastic containers and serially labelled according to strata (S1 or S2). The intervention supplement consisted of twice the recommended dietary allowance (2 RDA) of vitamins A, B_1, B_2, niacin, B_6, B_{12}, C, D and E, folate, zinc, copper, iodine and selenium; the comparative "standard-of-care" supplement consisted of the RDA (1 RDA) of vitamins A, C, D, B_1, B_2 and niacin (Table 1).

The comparative "standard-of-care" supplement was designed to be similar to the regular multivitamin tablet supplied as the standard of care at paediatric HIV clinics in Uganda. Upon administration to the child, both supplements were dissolved in milk or water. The nurse demonstrated how to prepare a dose and allowed the mother

Table 1: The formulation of intervention supplement and the comparative "standard-of-care" supplement used in the supplementation trial of HIV-infected children, Uganda

Micronutrient	Intervention arm 2 RDA	Comparative arm "standard-of-care" 1 RDA
Vitamin A (mcg)	800	400
VitaminB1 (mg)	1.2	0.6
Vitamin B2 (mg)	1.2	0.6
Niacin (mg)	1.6	0.8
Vitamin B6 (mg)	1.2	-
Vitamin B12 (mcg)	2.4	-
Vitamin C (mg)	50	25
Vitamin D (IU)	400	200
Vitamin E (mg)	14	-
Folate (mcg)	400	-
Selenium (mcg)	60	-
Zinc (mg)	10	-
Copper (mcg)	800	-
Iodine (mcg)	180	-

to prepare and administer the first dose in the clinic. Each participant received 140g (one container) per month, which was equivalent to 35 doses.

Randomization and blinding

The eligible participants were randomized to either the intervention or "standard-of-care" in two strata. A WHO officer in Geneva, who was not part of the study team, generated the randomization code in permuted blocks of 4 to 20 using the Stata software. The list was sent to NUTRISET (France), which manufactured the trial supplements and packaged them in serially labeled identical containers. The consistency of the powder, colour and smell were similar. All investigators, staff and parents or caretakers were blinded to treatment assignment. The randomization code was made available to the investigators upon completion of data collection.

Clinical and laboratory assessment

Mothers or caretakers were interviewed about the children's previous medical, nutritional history and presenting symptoms. The participants underwent a detailed physical examination, including anthropometry and WHO staging for paediatric HIV/AIDS. Weight was taken using a scale (uniscale 01-410-15) to the nearest 0.1kg, and height was taken to the nearest 0.1 centimetre using a portable infant-child length-height measuring board (Shorr productions, Olney, Maryland, USA). A plastic tape was used to measure mid-arm circumference to the neatest 0.1cm. HIV/AIDS clinical disease staging was decided using clinical signs against the WHO classification for paediatric HIV [24].

Two millilitres (ml) of blood was collected in a 5ml EDTA vacutainer tube (Becton Dickinson, Franklin Lakes, N.J.) by venipuncture from the cubital fossa or dorsum of the hand, and was analyzed for a complete blood count (Act 5Diff instrument Beckman Coulter) and CD4 cell count (FACScan instrument and MultiSET software Beckton Dickinson). C-reactive protein (CRP) was analyzed using a qualitative method (Human Gesellschaft fur Biochemica und Diagnostica mbH, Germany). Agglutination indicated a C-reactive protein of more than 6mg/L and this was reported as a positive CRP. An additional 3-5ml blood sample was collected and serum was analyzed for zinc and other trace elements using inductively coupled atomic emission spectrometry (ICP-AES) [25].

Participants were followed at the clinics monthly for the first six months, and at nine and 12 months. A record of illness, anthropometry, physical examination findings, investigations and treatment was kept for each visit. Parents or caretakers were requested to report to the clinic whenever the child got sick, was admitted to hospital or died. Those admitted were followed until discharge, and if they died, information was recorded on a mortality and adverse event form. Information on missed doses of the supplement was recorded on each monthly visit for six months.

Compliance was assessed by measuring the remaining supplement using a light-weight weighing scale (Philips HR 2389/B 9.OV/DC). Each study participant was expected to take 4g of the supplement per day (one scoop) and this was equivalent to 120g in 30 days. A pro-

portion of the amount of supplement taken against the expected was used as a measure of compliance. Overall compliance was assessed at the end of six months, whereby the average compliance was derived by adding the compliance rates on all the six scheduled visits.

After six months, no study supplements were given, but the children received the regular multivitamin supplements from the clinic as the standard of care. Follow up to 12 months was to ascertain whether there was any sustained effect of the intervention. Children who were not brought for scheduled visits were traced by telephone or physically by the health visitor. Those who missed more than two scheduled visits and could not be traced were declared lost.

Outcomes

Information on mortality was collected from verbal reports by the parents or caretakers or from hospital records. Those who died at home were reported by telephone or through tracing. Side effects attributed to the supplement were assessed and recorded at the monthly visits. A serious adverse event (SAE) form was completed if an adverse event had occurred. Conditions that resulted in hospitalization, required medical intervention to prevent a serious outcome, or were life threatening or fatal were regarded and reported as SAEs. The paediatrician decided on the relationship to the intervention using a set of conditions or known side effects of micronutrients. All SAEs warranted stoppage of the trial supplement when closely related to the intervention.

Statistical issues

The estimated sample size of 411 children in each arm was based on data from two studies. The first assumption was a mortality rate in the comparative arm of 24% in one year, based on the mortality rate in a study conducted in Mulago Hospital, Kampala, before highly active antiretroviral therapy (HAART) was available to HIV-infected children [26]. The mortality among HIV-infected children occurring between the ages of 12 and 25 months was close to 24%. We assumed a similar mortality in the whole age span of one to five years.

The second assumption - that all-cause mortality would be reduced to 14.4% (a 40% reduction) - was based on a study in Tanzanian children aged six months to five years where supplementation with vitamin A was associated with a 49% reduction in overall mortality and 63% among HIV-infected children [13]. We decided to use the 40% reduction level as we anticipated improved general care of HIV-infected children over time. Finally, we used a precision of 5%, and 95% confidence interval. The power estimate was 90% with an assumption of 10% loss to follow up.

Weight-for-height (WHZ), height-for-age (HAZ) and weight-for-age (WAZ) z-scores were computed using the WHO International Growth References [27]. Sub-group analysis was based on whether the children were receiving ART or not.

Statistical analysis was performed using SPSS version 15.0. Baseline characteristics were compared in the two treatment groups using proportions, and differences were tested with the Chi-square or Fisher's Exact test. To determine the association between patients' characteristics and mortality, logistic regression was used. Risk ratios and 95% confidence intervals were used to test the strength of association. Comparisons of treatment efficacy were analyzed on intention-to-treat basis in the two arms. Kaplan Meier curves and the log rank test were used to compare survival in the two arms.

Results

A total of 1632 children aged 12 to 59 months attending paediatric HIV clinics at the study sites were screened for eligibility (Figure 1). Out of the 847 children enrolled, 704 (83.1%) were from the study sites in Kampala (the capital city); the rest were from the rural sites.

Baseline characteristics of participants

Almost equal proportions of children were assigned to the two arms in both strata. More than half of the children (470/847, 56%) were aged less than 36 months. Table 2 shows the baseline characteristics and these were comparable in the two groups.

Follow up

All the study participants took 85% or more of the study supplements. Adverse effects were reported in 16 children (1.9%) and these included vomiting in 12 children and diarrhoea in four children. Of the 12 children who vomited, 6/426 (1.4%) were in the intervention arm and 6/421(1.4%) in the comparative arm. These symptoms were minor and did not warrant stopping the supplement. There were no other adverse effects attributed to the intervention.

By 12 months, 124 (14.6%) children were lost to follow up: 67/426 (15.7%) in the intervention arm and 57/421(13.5%) in the comparative arm. Most of the participants lost to follow up (90/124; 72.5%) were from the central region (Kampala sites). However, the proportion of loss to follow up was higher in the regional than the central sites (23.8% vs. 12.8%; $p < 0.01$). Children who were not receiving cotrimoxazole routinely (22.7% vs. 13.7%; $p = 0.04$), CRP positive (13.5% vs. 9.0%; $p = 0.00$) and those who were underweight (19.9% vs. 12.1%; $p = 0.01$) were also more likely to be lost to follow up. The other characteristics were similar to those who completed the study.

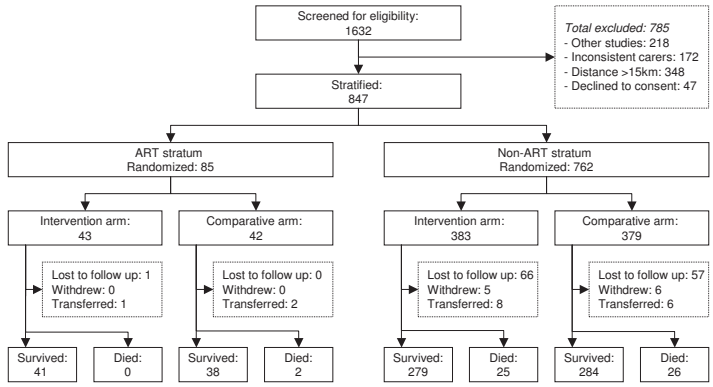

Figure 1 Trial profile.

Mortality

The overall mortality at 12 months of follow up was 53/847 (6.3%). In the intervention arm, the mortality was 25/426 (5.9%) and in the comparative arm 28/421 (6.7%). The difference between the arms was not statistically significant; risk ratio 0.9 (95% CI 0.5 - 1.5). In the ART stratum, two out of the 85 (2.4%) children died compared with 51/762 (6.7%) in the non-ART stratum. Of those who died in the non-ART stratum, 25/383 (6.5%) were in

Table 2: Baseline characteristics of the 847 Ugandan HIV-infected children aged 1-5 years enrolled in the micronutrient supplementation trial by ART stratum

Characteristics	ART stratum (n = 85)			Non-ART stratum (n = 762)		
	Intervention arm n = 43 (%)	Comparative arm n = 42 (%)	p-value	Intervention arm n = 383 (%)	Comparative arm n = 379 (%)	p-value
Age <36 months	8 (8.6)	16 (38.1)	0.06	219 (57.2)	227 (59.9)	0.46
Male	20 (46.5)	23 (54.8)	0.52	201 (52.5)	182 (48.0)	0.25
Caretaker: mother	24 (55.8)	21 (50.0)	0.67	278 (72.6)	278 (73.4)	0.87
Study site: Kampala	40 (93.0)	40 (95.2)	1.00	313 (81.7)	311 (82.1)	0.93
Fever	9 (20.9)	8 (19.0)	1.00	76 (19.8)	81 (21.4)	0.65
Diarrhoea	1 (2.3)	2 (4.8)	0.62	42 (11.0)	51 (13.5)	0.32
Cough	27 (62.8)	26 (61.9)	1.00	220 (57.4)	219 (57.8)	0.94
Persistent diarrhoea	0 (0.0)	0 (0.0)	-	11 (26.8)	11 (23.4)	0.81
Cotrimoxazole prophylaxis	43 (100.0)	42 (100.0)	-	340 (88.8)	334 (88.1)	0.82
Routine multivitamins	18 (41.9)	13 (31.0)	0.37	241 (62.9)	219 (57.8)	0.16
Vitamin A in past 6 months	20 (46.5)	16 (38.1)	0.51	183 (47.8)	175 (46.2)	0.66
WHO Stage 3 or 4	-	-	-	124 (33.4)	114 (30.1)	0.53
WHZ less than -2 z score	2 (4.6)	3 (7.1)	1.00	53 (13.8)	50 (13.2)	0.75
HAZ less than -2 z score	22 (51.2)	23 (54.7)	1.00	190 (49.6)	197 (51.9)	0.65
WAZ less than -2 z score	5 (11.6)	6 (14.3)	1.00	110 (8.7)	125 (32.9)	0.24
Current hospitalization	1 (0.02)	3 (7.1)	0.36	27 (7.0)	16 (4.2)	0.12
Ever hospitalized	25 (58.1)	27 (64.3)	0.66	226 (59.0)	200 (52.8)	0.09
CRP positive (n = 565)	15 (24.6)	11 (18.0)	0.65	122 (24.1)	119 (23.5)	0.70
CD4% <20 (n = 720)	10 (27.0)	8 (24.2)	1.00	124 (38.4)	136 (41.6)	0.42
Zinc <10 μmol/L (n = 336)	5 (26.3)	14 (73.7)	0.01	86 (50.6)	84 (49.4)	0.62

the intervention arm and 26/379 (6.9%) in the comparative arm; risk ratio 1.0 (95% CI 0.6 - 1.6).

Figure 2 shows the Kaplan Meier probability of survival by arm. There was no significant difference in survival at 12 months of follow up (log rank statistic 0.22, 1df, p-value 0.64). The mean survival time was 10.6 months (95% CI 10.3 - 10.9) in the intervention arm and 10.7 (95% CI 10.4 - 11.0) in the comparative arm. The mean survival time was 11.6 months in the ART (95% CI 11.3 - 12.0) stratum and 10.5 (95% CI 10.3 - 10.8) in the non-ART stratum.

On bivariate analysis for baseline characteristics, presence of fever, diarrhoea, cough, hospitalization at enrolment, low anthropometric indices below minus 2 z-scores, and WHO Stage 3 or 4 disease were associated with a shorter time of survival (Table 3). Those who were taking cotrimoxazole routinely within one month before the study had better survival. Other baseline characteristics and routine multivitamin supplementation were not significantly associated with mortality. At multivariate analysis, presence of fever, hospitalization at enrolment visit, WHO Stage 3 or 4, and being underweight independently predicted early mortality.

The most common cause of death was pneumonia accounting for 20/53 (37.7%)of the deaths: eight of the 20 children died of severe pneumonia, six of severe acute malnutrition with severe pneumonia, three of pnemocystis jiroveci pneumonia and a further three died of measles with severe pneumonia. Acute febrile illness and malaria accounted for 11/53 (20.8%) of the deaths, including eigth deaths due to acute febrile illness/malaria, two to cerebral malaria and one child died of malaria with severe anaemia.

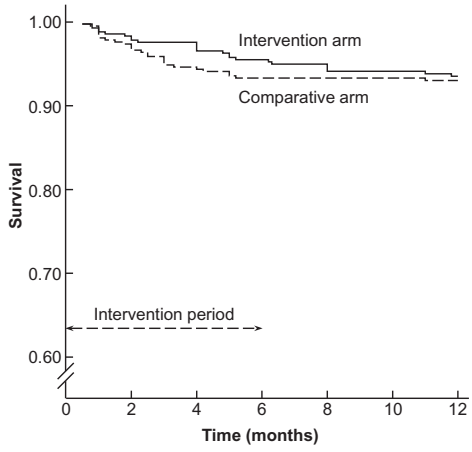

Figure 2 Kaplan-Meier survival curves of the two arms of the supplementation trial in HIV-infected children at 12 months of follow up.

Other causes of death were acute diarrhoea with dehydration (6/53; 11.3%), persistent diarrhoea with dehydration (3/53; 5.7%), measles with other complications (3/53; 5.7%), severe acute malnutrition with tuberculosis (2/53; 3.8%), pyogenic meningitis (2/53; 3.8%), cryptococcal meningitis (1/53; 1.9%), and Kaposi's sarcoma (1/53; 1.9%). In 4/53 (7.5%) children, the cause of death could not be established. Seven children died outside the hospital, including the four whose cause of death could not be ascertained.

Effect of multiple micronutrient supplementation on anthropometry and CD4 cell count

Multiple micronutrient supplementation had no effect on anthropometry as shown in table 4. CD4 cell count was available for 399 surviving children at one year of follow up. Of 195 children who had received twice the RDA of multiple micronutrients, 55 (28.2%) had CD4 cell counts <20% compared with 46/204 (22.5%) who received the standard of care. This difference was not significant (OR 0.74; 95% CI 0.74 - 1.17), implying that the intervention did not have an impact on CD4 count.

Discussion

Twice the recommended dietary allowance of 14 micronutrients given to HIV-infected children daily for six months showed no significant difference in all-cause mortality at 12 months of follow up compared with the "standard-of-care" of the RDA of six multivitamins. This lack of effect may be real. However, it might also be due to other factors.

The first possible reason for "no effect" is that we did not provide a true placebo since multivitamin supplementation was the standard of care in the paediatric HIV clinics in Uganda.

The second reason is the supplement composition and the dosage. In this study, we included 14 of the micronutrients judged to be vital and these were provided in the 2 RDA arm. This was done to minimize the risk of toxicity. But perhaps the individual vitamins and minerals should have been dosed higher, at least for those whose therapeutic window was wide [28]. In our supplement, we did not include iron, based on earlier studies that suggested that iron was potentially detrimental in HIV patients [29].

The third issue is the duration of supplementation and follow up, which have been variable in several studies. Although not significant, there is a divergence of the survival curves during the first six months with supplements and a convergence during follow up without supplementation. Would a longer supplementation time in a larger cohort have demonstrated an effect?

Table 3: Factors associated with mortality in HIV-infected children aged 1-5 years enrolled in the micronutrient supplementation trial in Uganda

Baseline characteristic	Unadjusted hazard ratio (95%CI)	p-value	Adjusted hazard ratio (95%CI)	p-value
Fever	3.7 (2.1 - 6.3)	<0.001	2.1 (1.1 - 3.9)	0.02
Diarrhoea	2.0 (1.0 - 3.9)	0.05	1.4 (0.6 - 2.9)	0.42
Cough	2.1 (1.1 - 3.8)	0.02	1.8 (0.9 - 3.5)	0.08
Hospitalization (current)	6.9 (3.6 - 13)	<0.001	2.6 (1.2 - 5.7)	0.02
Routine cotrimoxazole	0.4 (0.2 - 0.8)	0.01	0.4 (0.2 - 0.9)	0.03
Vitamin A in past 6 months	1.4 (0.8 - 2.4)	0.02	1.3 (0.7 - 2.3)	0.39
WHZ score <-2	3.3 (1.8 - 6.0)	<0.001	0.8 (0.4 - 1.7)	0.62
WAZ score <-2	5.4 (3.0 - 9.8)	<0.001	2.6 (1.2 - 5.8)	0.02
WHO Stage 3 or 4	5.1 (2.9 - 9.0)	<0.001	2.9 (1.5 - 5.6)	<0.01

The fourth issue is that the study was designed at a time when the mortality due to HIV was high and few children had an opportunity to receive ART [30]. The mortality figures in these clinics improved continuously over time due to improved care and access to antiretroviral therapy. This led to a lower mortality in the study than anticipated at the design stage.

As previously reported in sub-Saharan Africa, the mortality was lower in children who were already receiving ART at the time of enrolment compared with those who were not. This was comparable to mortality reported in children receiving HAART by other researchers in sub-Saharan Africa [31]. The factors associated with mortality in the regression model included low weight for age, advanced HIV disease (WHO Stage 3 and 4) and hospitalization at enrolment. These findings are not surprising since other studies have reported that malnutrition and symptomatic HIV disease are associated with increased mortality [13,14,32].

There was no difference in the impact of 2 RDA multiple micronutrients or the standard of care on anthropometric measurements and CD4 cell count. This lack of difference could be due to the same factors discussed here.

There were no major adverse events observed from our study, and this was similar to what other micronutrient supplementation studies have reported [16,19,33].

The loss to follow up was higher than anticipated and this could have influenced the study outcome. The children lost to follow up were more likely to be underweight than those who completed the study. Similarly, there were a higher proportion of CRP-positive children among those lost to follow up than among those who completed the study. The implications of these two findings are not clear, but indicate that those lost to follow up were more ill than those who completed the study [34].

At one of the regional sites, we included children who were living in internally displaced camps in northern Uganda (Lira), and some of these relocated to distant areas while still in the study. However, the overall loss to follow up was comparable to what other micronutrient supplementation studies in the HIV population have reported [35].

Conclusions

Twice the recommended dietary allowance of 14 micronutrients compared with 1 RDA of six multivitamins given as the "standard of care" for six months was well tolerated with no serious adverse events reported, but did

Table 4: The effect of multiple micronutrient supplementation on anthropometry and CD4 cell count in HIV-infected children aged 1-5 years

Measurement at 12 months	Intervention arm 2 RDA of 14 micronutrients		Comparative arm 1 RDA of 6 multivitamins "standard-of-care"		
	Mean (SD)	95% CI	Mean (SD)	95% CI	p value
Weight-for-height (WHZ)	0.70 (1.43)	0.52 to 0.88	0.59 (1.15)	0.45 to 0.75	0.39
Height-for-age (HAZ)	-2.17 (1.60)	-2.37 to -1.95	-2.42 (1.50)	-2.61 to -2.23	0.08
Weight-for-age (WAZ)	-0.78 (1.30)	-0.96 to -0.62	-0.97 (1.03)	-1.11 to -0.84	0.07
CD4 count	1024 (592)	942 to 1107	1060 (553)	985 to 1136	0.53

not significantly alter mortality, growth or CD4 counts in HIV-infected children aged one to five years. Patients on HAART had a considerably lower mortality compared to those without. Future intervention studies should carefully consider: (1) the composition and dosing of the supplements; and (2) the power needed to detect a difference between arms.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
GN, TT and JKT participated in the conception, design and implementation of the study, statistical analysis, interpretation and drafting of the manuscript. CMN participated in design and implementation of the study. All authors read and approved the final manuscript.

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References

1. Brahmbhatt H, Kigozi G, Wabwire-Mangen F, Serwadda D, Lutalo T, Nalugoda F, Sewankambo N, Kiduggavu M, Wawer M, Gray R: **Mortality in HIV-infected and uninfected children of HIV-infected and uninfected mothers in rural Uganda.** *J Acquir Immune Defic Syndr* 2006, **41:**504-508.
2. Villamor E, Misegades L, Fataki MR, Mbise RL, Fawzi WW: **Child mortality in relation to HIV infection, nutritional status, and socio-economic background.** *Int J Epidemiol* 2005, **34:**61-68.
3. Bhutta ZA: **Micronutrient needs of malnourished children.** *Curr Opin Clin Nutr Metab Care* 2008, **11:**309-314.
4. Eley BS, Sive AA, Abelse L, Kossew G, Cooper M, Hussey GD: **Growth and micronutrient disturbances in stable, HIV-infected children in Cape Town.** *Ann Trop Paediatr* 2002, **22:**19-23.
5. Faruque AS, Ahmed AM, Ahmed T, Islam MM, Hossain MI, Roy SK, Alam N, Kabir I, Sack DA: **Nutrition: basis for healthy children and mothers in Bangladesh.** *J Health Popul Nutr* 2008, **26:**325-339.
6. Lartey A: **Maternal and child nutrition in Sub-Saharan Africa: challenges and interventions.** *Proc Nutr Soc* 2008, **67:**105-108.
7. Semba RD, Tang AM: **Micronutrients and the pathogenesis of human immunodeficiency virus infection.** *Br J Nutr* 1999, **81:**181-189.
8. Tamura J, Kubota K, Murakami H, Sawamura M, Matsushima T, Tamura T, Saitoh T, Kurabayshi H, Naruse T: **Immunomodulation by vitamin B12: augmentation of CD8+ T lymphocytes and natural killer (NK) cell activity in vitamin B12-deficient patients by methyl-B12 treatment.** *Clin Exp Immunol* 1999, **116:**28-32.
9. Kupka R, Msamanga GI, Spiegelman D, Rifai N, Hunter DJ, Fawzi WW: **Selenium levels in relation to morbidity and mortality among children born to HIV-infected mothers.** *Eur J Clin Nutr* 2005, **59:**1250-1258.
10. Fischer Walker CL, Black RE: **Micronutrients and diarrheal disease.** *Clin Infect Dis* 2007, **45(Suppl 1):**S73-77.
11. Villamor E, Fawzi WW: **Vitamin A supplementation: implications for morbidity and mortality in children.** *J Infect Dis* 2000, **182(Suppl 1):**S122-133.
12. Coutsoudis A, Bobat RA, Coovadia HM, Kuhn L, Tsai WY, Stein ZA: **The effects of vitamin A supplementation on the morbidity of children born to HIV-infected women.** *Am J Public Health* 1995, **85:**1076-1081.
13. Fawzi WW, Mbise RL, Hertzmark E, Fataki MR, Herrera MG, Ndossi G, Spiegelman D: **A randomized trial of vitamin A supplements in relation to mortality among human immunodeficiency virus-infected and uninfected children in Tanzania.** *Pediatr Infect Dis J* 1999, **18:**127-133.
14. Semba RD, Ndugwa C, Perry RT, Clark TD, Jackson JB, Melikian G, Tielsch J, Mmiro F: **Effect of periodic vitamin A supplementation on mortality and morbidity of human immunodeficiency virus-infected children in Uganda: A controlled clinical trial.** *Nutrition* 2005, **21:**25-31.
15. Bhutta ZA, Nizami SQ, Isani Z: **Zinc supplementation in malnourished children with persistent diarrhea in Pakistan.** *Pediatrics* 1999, **103:**e42.
16. Bobat R, Coovadia H, Stephen C, Naidoo KL, McKerrow N, Black RE, Moss WJ: **Safety and efficacy of zinc supplementation for children with HIV-1 infection in South Africa: a randomised double-blind placebo-controlled trial.** *Lancet* 2005, **366:**1862-1867.
17. Fawzi WW, Msamanga GI, Spiegelman D, Wei R, Kapiga S, Villamor E, Mwakagile D, Mugusi F, Hertzmark E, Essex M, Hunter DJ: **A randomized trial of multivitamin supplements and HIV disease progression and mortality.** *N Engl J Med* 2004, **351:**23-32.
18. Jiamton S, Pepin J, Suttent R, Filteau S, Mahakkanukrauh B, Hanshaoworakul W, Chaisilwattana P, Suthipinittharm P, Shetty P, Jaffar S: **A randomized trial of the impact of multiple micronutrient supplementation on mortality among HIV-infected individuals living in Bangkok.** *AIDS* 2003, **17:**2461-2469.
19. Luabeya KK, Mpontshane N, Mackay M, Ward H, Elson I, Chhagan M, Tomkins A, Van den Broeck J, Bennish ML: **Zinc or multiple micronutrient supplementation to reduce diarrhea and respiratory disease in South African children: a randomized controlled trial.** *PLoS One* 2007, **2:**e541.
20. Semba RD, Kumwenda J, Zijlstra E, Ricks MO, van Lettow M, Whalen C, Clark TD, Jorgensen L, Kohler J, Kumwenda N, Taha TE, Harries AD: **Micronutrient supplements and mortality of HIV-infected adults with pulmonary TB: a controlled clinical trial.** *Int J Tuberc Lung Dis* 2007, **11:**854-859.
21. UNICEF, The Micronutrient Initiative: **Vitamin & mineral deficiency. A global progress report.** *Volume 2010.* New York: UNICEF and Micronutrient Initiative; 2004.
22. Baum MK, Shor-Posner G: **Nutritional requirements in HIV-1 infection.** *AIDS* 1994, **8:**715.
23. Kelly P, Katubulushi M, Todd J, Banda R, Yambayamba V, Fwoloshi M, Zulu I, Kafwembe E, Yavwa F, Sanderson IR, Tomkins A: **Micronutrient supplementation has limited effects on intestinal infectious disease and mortality in a Zambian population of mixed HIV status: a cluster randomized trial.** *Am J Clin Nutr* 2008, **88:**1010-1017.
24. WHO: **Case definitions of HIV for surveillance and revised clinical staging and immunological classification of HIV-related disease in adults and children.** Geneva, Switzerland: WHO; 2006.
25. Rahil-Khazen R, Henriksen H, Bolann BJ, Ulvik RJ: **Validation of inductively coupled plasma atomic emission spectrometry technique (ICP-AES) for multi-element analysis of trace elements in human serum.** *Scand J Clin Lab Invest* 2000, **60:**677-686.
26. Berhane R, Bagenda D, Marum L, Aceng E, Ndugwa C, Bosch RJ, Olness K: **Growth failure as a prognostic indicator of mortality in pediatric HIV infection.** *Pediatrics* 1997, **100:**E7.
27. WHO: **WHO Anthro 2005.** Geneva: WHO; 2005.
28. Renwick AG: **Toxicology of micronutrients: adverse effects and uncertainty.** *J Nutr* 2006, **136:**493S-501S.
29. McDermid JM, Jaye A, van der Loeff Schim MF, Todd J, Bates C, Austin S, Jeffries D, Awasana AA, Whittlex AA, Prentice A: **Elevated iron status strongly predicts mortality in West African adults with HIV infection.** *J Acquir Immune Defic Syndr* 2007, **46:**498-507.
30. Amolo Okero F, Aceng E, Madraa E, Namagala E, Serutoke J: **Scaling up antiretroviral therapy: experience in Uganda.** Geneva: WHO; 2003.
31. Sutcliffe CG, van Dijk JH, Bolton C, Persaud D, Moss WJ: **Effectiveness of antiretroviral therapy among HIV-infected children in sub-Saharan Africa.** *Lancet Infect Dis* 2008, **8:**477-489.
32. Walker AS, Mulenga V, Sinyinza F, Lishimpi K, Nunn A, Chintu C, Gibb DM: **Determinants of survival without antiretroviral therapy after infancy in HIV-1-infected Zambian children in the CHAP Trial.** *J Acquir Immune Defic Syndr* 2006, **42:**637-645.

33. Ndagije F, Baribwira C, Coulter JB: **Micronutrients and T-cell subsets: a comparison between HIV-infected and uninfected, severely malnourished Rwandan children.** *Ann Trop Paediatr* 2007, **27:**269-275.
34. Drain PK, Kupka R, Msamanga GI, Urassa W, Mugusi F, Fawzi WW: **C-reactive protein independently predicts HIV-related outcomes among women and children in a resource-poor setting.** *AIDS* 2007, **21:**2067-2075.
35. Irlam JH, Visser ME, Rollins N, Siegfried N: **Micronutrient supplementation in children and adults with HIV infection.** *Cochrane Database Syst Rev* 2005:CD003650.

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Errata in Paper I

Table 1. Errata in the text and Table 1.

Paper	Location	Reads	Should be
Paper I	Table 1, Niacin	Intervention 1.6	16
	Table 1, Niacin	Comparative arm 0.8	8.0
	Results section, line 3	704	705

Table 2. Errata in table 2.

Paper	Location	ART stratum			
Paper 1		Intervention arm reads, n (%)	Intervention arm, should be n (%)	Comparative arm, reads	Comparative arm, should be n (%)
Table 2	Age < 36 months	8 (8.6)	8 (18.6)		
	WHZ less than -2	2 (4.6)	2/41 (4.9)		
	HAZ less than -2	22 (51.2)	22/40 (55.0)	23 (54.7)	23/41 (56.1)
	WAZ less than -2	5 (11.6)	5/40 (12.5)	6 (14.3)	6/41 (14.6)
	CRP positive	15 (24.6)	15/31 (48.4)	11 (18.0)	11/30 (36.7)
	Zinc < 10µ mol/L	5 (26.3)	5/31 (16.1)	14 (73.7)	14/29 (48.3)
	Location	**Non-ART stratum**			
	WHZ less than -2	53 (13.8)	53/366(14.5)	50 (13.2)	50/367 (13.6)
	HAZ less than -2	190 (49.6)	190/358(53.1)	197 (51.9)	197/359 (54.9)
	WAZ less than -2	110 (8.7)	110/364 (30.2)	125 (32.9)	125/363 (34.4)
	CRP positive	122 (24.1)	122/260 (46.9)	119 (23.5)	119/247 (48.2)
	Zinc < 10µ mol/L	86 (50.6)	86/143 (60.1)	84 (49.4)	84/133 (63.2)

II

Multiple micronutrient supplementation does not reduce diarrhoea morbidity in Ugandan HIV-infected children: a randomised controlled trial

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Key words: Multiple micronutrients; Diarrhoea; HIV-infected; Children

Short title: micronutrients do not reduce diarrhoea

Word count: 2998

Figures:1

Tables: 4

Abstract

Objectives

To determine the effect of multiple micronutrient supplementation on incidence and prevalence of diarrhoea in Ugandan HIV infected children aged 1-5 years.

Methods

We enrolled 847 HIV-infected Ugandan children in a trial of a supplement containing 14 micronutrients (MMS) given in twice the recommended dietary allowances (RDA) versus a 6 multivitamin (MV) supplement given in one RDA as the 'standard of care'. The participants were stratified into the highly active antiretroviral therapy (HAART) group of 85/847 (10.0%) and the non-HAART of 762/847 (90%) participants. The supplements were given daily for 6 months. Episodes of diarrhoea assessed at routine visits, sick visits and those reported within 2 weeks prior to the routine visit were counted against weeks of observation for each participant. Diarrhoea incidence per child was calculated as the number of episodes per child year. Rate ratios were used to compare person-time rates in the two groups.

Results

The incidence of diarrhoea was 3.8 (95% CI; 3.4 – 4.3) in the MMS and 3.5 (95% CI; 3.1 – 4.0) in the MV group per child year. The rate ratio was 1.1 (0.9 – 1.3), similar in both strata, except that HAART treated children had a lower incidence rate of diarrhoea. The prevalence of diarrhoea at 6 months was also similar in the two groups.

Conclusion

A 14 multiple micronutrient supplement given in 2RDA doses compared to a 6 multivitamin 'standard of care' supplement given in 1RDA dose did not reduce the incidence nor the prevalence of diarrhoea in HIV-infected children aged 1-5 years.

Trial registration: NCT00122941 (http://clinicaltrials.gov)

Background

HIV-infected children living in low-income countries have an increased risk of morbidity and mortality from common childhood diseases.[1-4] In addition, they tend to suffer from recurrent episodes of diarrhoea and have worse outcomes compared to HIV-uninfected children.[5-7]

Infectious morbidity is further compounded by the high prevalence of both macronutrient and micronutrient malnutrition[8] that tends to co-exist with the disease or occurs as a result of repeated infections. Micronutrient deficiencies are associated with reduced immunity[9-11] whereas infections aggravate micronutrient deficiencies by interfering with utilization, increasing losses, in addition to reduced nutrient intake.[12]

Several studies have shown that micronutrient interventions reduce childhood morbidity in low-income countries. Vitamin A supplementation reduces severity of infectious disease episodes in young children[13, 14] while zinc reduces incidence and duration of diarrhoea and respiratory infections.[15, 16] For many years vitamin A supplementation has been routinely practiced in children under 5 years of age whereas zinc has become standard treatment for diarrhoea since 2005[17] in children living in low-income countries.

Whereas HIV treatment programmes recommend use of micronutrients in the standard recommended dietary allowances, few studies have evaluated their efficacy in HIV-infected children. We hypothesised that children supplemented with twice the recommended dietary allowance (2RDA) of 14 multiple micronutrients (MMS) would get fewer episodes of diarrhoea compared to those receiving one RDA of 6 multivitamins (MV) as the 'standard of care' in a

period of 6 months. The effect of multiple micronutrient supplementation on diarrhoea morbidity was a secondary outcome of this trial. The primary outcome was the effect of multiple micronutrient supplementation on survival and the findings have been reported.[18]

Methods

Design

In a randomised trial conducted between 2005 and 2008, the participants were assigned to a 14 multiple micronutrient supplement (MMS) of 2RDA doses of the individual components or the 'standard of care' 6 multivitamin supplement in a 1:1 ratio.

Participants

We enrolled HIV-infected children aged 1-5 years whose HIV status had been confirmed and were coming for follow up at the HIV care clinics in seven hospitals. We only included those who were not enrolled in other studies, residing in a radius of 15 kilometres from the facility and whose mothers were willing to adhere to the supplement and the study follow up schedule. All consecutive patients attending the clinics were offered enrolment. In summary, 847 HIV-infected children aged 1-5 years were enrolled in the trial. Eighty five children belonged to the highly active anti-retroviral therapy (HAART) stratum and 762 to the non-HAART stratum. In the HAART stratum, 43 children were assigned to MMS and 42 the MV group. Of the 762 children in the non-HAART stratum, 383 received MMS and 379 were treated with MV (figure 1). The baseline characteristics of the participants are presented in table 1. This study was health facility based intentionally since most mothers refused home visits because of the stigma that was associated with HIV at that time.

Study sites

The study was conducted at 3 sites in Kampala; the capital city of Uganda and 4 regional hospitals situated in the South-western, Central, Eastern and Northern parts of the country. Each site had a designated paediatric HIV clinic though at different stages of development. Most of the

clinics at the regional hospitals were new and had few patients whereas the sites in the capital city were well established. The national referral hospital had the largest clinic with over 5000 HIV-infected children.

Ethical considerations

The study was approved by Makerere University College of Health Sciences Research and Ethics Committee, the Uganda National Council for Science and technology and the Regional Committee for Medical Research Ethics, Western Norway. Written informed consent was obtained from the parents or caretakers of the participants.

Trial supplements

The multiple micronutrient supplement consisted vitamin A, B_1, B_2, B_3, B_6, B_{12}, folate, C, D, E and trace elements; selenium, zinc, copper and iodine in 2RDAs. The multivitamin supplement contained vitamin A, B_1, B_2, B_3, C and D in 1 RDA doses as shown in table 2. The formula for MMS was based on two times the recommended dietary allowance for a 4 year old age group while the MV was based on one RDA dose for a similar age group.[19] We used the UNICEF recommended multiple micronutrient supplement for pregnant women and other vulnerable groups but we excluded iron since previous studies had indicated that iron could be harmful in HIV-infected persons.[20-22] There were no studies in children that had used a similar supplement by the time we conceptualised this study. We anticipated that HIV-infected children would have increased micronutrient requirements based on their nutritional state and increased infectious morbidity. There were two age bands in the RDA tables where our participants would fall; 1-3 and 4-8 years of age. We noted that there were minor variations in dosages of some micronutrients while others like iodine and vitamin D did not vary. We decided to use the group

with the higher requirement, to warrant the highest probability that every child's requirements are met. In addition it was easier to administer a uniform intervention. The multivitamin formula was similar to the 'standard of care' multivitamins given to HIV-infected children attending health care clinics in Uganda. Whereas the standard of care multivitamin was dispensed in tablet form the study supplements were manufactured as a powder. We chose to use a powder because it would be easier to administer in a reconstituted solution and we also considered the stability of the compound (upon the manufacturer's advice) since it contained many supplements. A daily dose was 4 g, measured by a scoop provided by the manufacturer. This was mixed with 10 – 20 ml of milk or water and administered orally.

Outcomes

Every child was assessed for diarrhoea at baseline, monthly (scheduled study clinic visits) and during inter-current/sick visits up to six months. The caretakers (mostly the mothers) were interviewed about any episodes of diarrhoea occurring within two weeks before the clinic visit. They were also asked whether the child had an episode of diarrhoea lasting for 14 days (2 weeks) or more in-between the clinic visits. Presence of diarrhoea was defined as passage of 3 or more loose/fluid stools in the previous 24 hours. Episodes which had persisted from the previous sick or routine visit were not counted again. If there was a diarrhoea-free interval of 3 or more days the illness was regarded as a new episode.

The diarrhoea incidence was calculated as follows:

1) We counted all new episodes of diarrhoea recorded from enrolment to 6 months
2) Each recall covered 2 out of 4 weeks since the last visit. This was set as the period of observation

3) If an inter-current visit occurred the period of observation increased and possibly the episode count
4) Total observation time per child was calculated by adding each individual's period of observation
5) Incidence rates were calculated by dividing the episodes of diarrhoea in each group with the total observation time in weeks
6) The rate ratio was calculated like this: incidence rate of MMS group divided by the incidence rate of MV group.

The prevalence of diarrhoea was determined by dividing the number of children who had diarrhoea at the 6 month's visit with the total number of participants who had information on diarrhoea at the 6 month's visit. The proportion of those who had diarrhoea in the MMS was compared with the proportion in the MV group using a two by two table. The Fisher's exact test was used to test for significance of the differences.

Sample size

The sample size was based on the primary outcome of survival for which 411 participants were required in each arm. The assumptions for the sample size calculation are indicated in a previous publication.[18]

Randomization

The eligible participants were randomized in a 1:1 ratio in permuted blocks of 4 to 20 to one of the study supplements. The randomisation code was generated using the Stata software by an independent researcher and sent to the manufactures (NUTRISET) in France. The supplements

were packaged in white plastic containers and labelled sequentially by the manufacturers. The participants were consecutively enrolled by the study doctor (at each of the sites there was at doctor responsible for the study) and the supplements were dispensed by the study nurse in serial order.

Masking

The intervention and standard of care supplement were both similar in colour, consistency, odour and packaged in identical containers. The study staff, mothers/ caretakers and investigators were all masked to treatment assignment. Serial interviews with the study staff indicated that they could never tell the difference between the two. The investigators had no access to the randomization code until completion of the study.

Compliance and adverse events

Compliance was assessed by weighing the remaining supplement using a light-weight weighing scale (Philips HR 2389/B 9.OV/DC) at each routine visit. Information about missed doses in the past 2 weeks was also collected. Each study participant took 4 g of the supplement per day (one scoop) and this was equivalent to 120 g in 30 days. The proportion of the amount of supplement taken against the expected was used as a measure of compliance. The overall compliance was derived by adding the monthly measurements divided by the 6 visits. And adverse effects that were attributed to the supplement by the mother or the doctor were recorded.

Quality control

Overall there were 10 doctors involved in the study. All of them were trained on the study protocol, the operating procedures and how to assess and manage HIV-infected children. A

majority of them were routinely involved in the care of sick children or HIV-infected children. Measurements such as anthropometry were done according to the protocol and standard operating procedures. At each site the doctor captured information on different forms (baseline, 6 months, monthly and inter-current visit).

Statistical analysis

Diarrhoea incidence was calculated as the number of episodes per child year. Rate ratios were used to compare person-time rates in the two groups using OpenEpi-Epidemiologic Calculators.[23] The prevalence of diarrhoea at 6 months and hospitalisations in the two groups were compared using the Fishers exact test. All tests were two-sided and considered significant if the p-value was <0.05. Children whose weight-for-height z-scores were less than -2 standard deviations below the mean were considered wasted, height-for-age z-scores less than -2 standard deviations were stunted, and less than -2 standard deviations weight-for-age z-scores underweight. Determinants for diarrhoea were evaluated by multiple regression in a stepwise fashion.

Results

Out of 847 HIV-infected children randomised to a 2RDA of 14 multiple micronutrients or 6 multivitamin supplement as 'standard of care' at paediatric HIV clinics in Uganda diarrhoea incidence was measured for 800 children, excluding 47 (25 in MMS and 22 in MV) who dropped out before their first routine follow up visit. Of the 710 children who completed 6 months of follow up, 613 were assessed for prevalence of diarrhoea at the 6 months visit (figure 1).

Baseline characteristics

Baseline characteristics were similar in the MMS and MV groups, (table 1). The majority of the participants [705/847 (83.2%)] were from the capital city.

Diarrhoea morbidity

There were 516 episodes of diarrhoea in 7336 person weeks; 270/3706 in the MMS and 246/3630 in the MV group. The incidence rate was 3.8 (95% CI; 3.4 – 4.3) in the MMS and 3.5 (95% CI; 3.1 – 4.0) in the MV group per child year. The rate ratio was 1.1 (0.9 – 1.3). Similarly, there were no differences in the HAART and non-HAART stratum except for the low incidence rate of diarrhoea in the HAART treated children (table 3). The overall incidence of diarrhoea was equivalent to 3.7 episodes per child per year; 4.0 and 3.8 in the non-HAART group, 1.7 and 1.5 in the HAART stratum.

The prevalence of diarrhoea at the 6 months visit was 53/613 (8.6%); 30/310 (9.7%) in the MMS and 23/303 (7.6%) in the MV group. This was not a statistically significant difference ($p > 0.99$). The baseline prevalence was 96/847 (11.3%); 43/426 (10.1%) in the MMS and 53/421 (12.6%) the MV group. The 6 months point prevalence was also not different from baseline ($p=0.36$).

Out of 115 hospitalisations, 24 were due to diarrhoea (20.9%); 11 in MMS and 13 in MV arm. There were no hospitalisations due to diarrhoea in the HAART stratum. Persistent diarrhoea was uncommon neither at baseline (22 cases) nor at 6 months (4 cases).

Age was the only baseline characteristic that was associated with diarrhoea [AOR 2.2 (95% CI: 1.2 – 4.4)]. Non use of HAART and poor nutritional indices were associated with diarrhoea at bivariate but not at multivariate analysis. Previous routine multivitamin supplementation was not associated with diarrhoea (table 4).

Compliance and adverse events

All participants took 85% or more of the study supplements. Adverse effects were reported in 16 children (1.9%); vomiting in 12 children and diarrhoea in four children. There were equal numbers in each arm; 8/426 (1.9%) in the MMS and 8/421(1.9%) in the MV group. These symptoms were minor and the supplement was not stopped.

Discussion

In this study, we found no difference in diarrhoea episodes in Ugandan HIV-infected children aged 1-5 years following supplementation with a 14 multivitamin and mineral supplement (MMS) compared to the 'standard of care' 6 multivitamin (MV) supplement. The incidence rate and the prevalence of diarrhoea were similar in the two groups. The incidence of diarrhoea was lower in the HAART treated stratum but not significantly different between the two intervention groups. Hospitalisations due to diarrhoea were also similar. Overall the incidence of diarrhoea was slightly higher than the estimated average of 3.2 episodes per child per year among children under five years of age living in low-income countries[24] but consistent with another almost similar study that included HIV-infected children.[25] Our study did not include the most at risk age group (6-12 months) on purpose because infant diagnosis of HIV was not readily available and there were very few infants attending the HIV clinics.

One possible explanation for the lack of effect is that the doses of the various components of the supplements may have been insufficient to meet the demands of these HIV-infected children. The nutritional needs for HIV-infected children remain largely unknown to date. Based on the serum zinc concentrations in this cohort of HIV-infected children (more than 50% had low zinc concentrations) it is possible that other micronutrient concentrations were low. The possibility of severe and multiple micronutrient deficiencies are further supported by other studies from the region. Vitamin A deficiency occurred in 83% of infants born to HIV-infected mothers in Tanzania.[26] In South Africa more than one in two HIV-infected children had two or more micronutrient deficiencies.[27] Some authors have suggested that intake of micronutrients in multiples of recommended dietary allowance may be required to achieve the normal micronutrient concentrations of HIV-infected persons.[28]

It is also possible that those who were more deficient or had multiple deficiencies could have benefited more that the others. This is supported by previous research that has shown that vitamin A supplementation in Tanzania children less than 5 years of age reduced acute diarrhoea in wasted children and signs of pneumonia in HIV-infected but not uninfected children[13]. Zinc supplementation studies have also shown that the effect on diarrhoea was more pronounced among children who were undernourished.[29, 30] It is also possible that the micronutrients could be more beneficial at reducing severity of illness but not episodes. In our study hospitalisation was anticipated to be a measure of severe illness. The lack of effect on this outcome could be due to the small number of hospitalisations. Mothers were allowed to return to the clinic whenever the child was sick on any day of the 5 days of the week except weekends. This may have facilitated early care seeking and reduced the frequency of severe illness and subsequently hospitalisations.

The lack of effect on morbidity by micronutrient supplementation in children has been reported by other researchers. In a study of Indonesian infants whose HIV status was not known supplementation with 15 multiple micronutrients in daily adequate intake amounts had no effect on days of illness due to diarrhoea, respiratory infection or fever during 6 months of supplementation[31] In a trial of 6-24 months old children with a stratum of 32 HIV-infected children (out of 373), a supplement containing 14 multiple micronutrients given in recommended dietary intake did not reduce diarrhoea morbidity, prevalence of respiratory symptoms or pneumonia.[32] In a Zambian study of infants whose HIV status was not known, there was no difference in the prevalence and causes of hospital referrals following addition of multiple micronutrients to a locally available food.[33]

Furthermore, the supplementation period may not have been long enough to create an impact. Micronutrient supplementation studies in adults have indicated that a significant proportion of HIV-infected patients may remain deficient despite supplementation.[34, 35] To create an effect on morbidity micronutrients may need to be given for a longer period and possibly in higher doses.

We are unable to make valid conclusions about the HAART stratum because of the small numbers. Children in this stratum were less sick at baseline and could have better micronutrient status as demonstrated by their higher zinc status.[36]

For ethical reasons, this was a facility-based study which could have missed some episodes of diarrhoea that occurred unreported. This may limit our ability to make comparisons with other studies that used weekly or bi-weekly visits. However, we also limited the time of observation to reasonable recall periods and any information bias would be equally distributed in the two groups.

Another reason for a non-effect is of course the fact that we used a multivitamin supplement in the comparative arm and not true placebo. But this remains speculations.

Conclusion

A 14 multiple micronutrient supplement given in 2RDA doses compared to a 6 multivitamin 'standard of care' supplement given in 1RDA did not reduce the incidence and prevalence of diarrhoea or diarrhoea related hospitalisations in HIV-infected children aged 1-5 years.

Competing interests

We declare that we have no conflict of interest.

Authors' contribution

GN participated in the conception, design and implementation of the study, statistical analysis, interpretation and writing the manuscript. TT and JKT participated in the conception and design of the study, implementation, statistical analysis, interpretation and writing the manuscript. CMN was involved in the design and supervised data collection. All authors read and approved the final manuscript.

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References

1. Bobat R, Moodley D, Coutsoudis A, Coovadia H, Gouws E. The early natural history of vertically transmitted HIV-1 infection in African children from Durban, South Africa. Ann Trop Paediatr. 1998 Sep;18(3):187-96.
2. Laufer MK, van Oosterhout JJ, Perez MA, Kanyanganlika J, Taylor TE, Plowe CV, et al. Observational cohort study of HIV-infected African children. Pediatr Infect Dis J. 2006 Jul;25(7):623-7.
3. Spira R, Lepage P, Msellati P, Van De Perre P, Leroy V, Simonon A, et al. Natural history of human immunodeficiency virus type 1 infection in children: a five-year prospective study in Rwanda. Mother-to-Child HIV-1 Transmission Study Group. Pediatrics. 1999 Nov;104(5):e56.
4. Taha TE, Graham SM, Kumwenda NI, Broadhead RL, Hoover DR, Markakis D, et al. Morbidity among human immunodeficiency virus-1-infected and -uninfected African children. Pediatrics. 2000 Dec;106(6):E77.
5. Kotloff KL, Johnson JP, Nair P, Hickman D, Lippincott P, Wilson PD, et al. Diarrheal morbidity during the first 2 years of life among HIV-infected infants. JAMA. 1994 Feb 9;271(6):448-52.
6. Pavia AT, Long EG, Ryder RW, Nsa W, Puhr ND, Wells JG, et al. Diarrhea among African children born to human immunodeficiency virus 1-infected mothers: clinical, microbiologic and epidemiologic features. Pediatr Infect Dis J. 1992 Dec;11(12):996-1003.
7. Thea DM, St Louis ME, Atido U, Kanjinga K, Kembo B, Matondo M, et al. A prospective study of diarrhea and HIV-1 infection among 429 Zairian infants. N Engl J Med. 1993 Dec 2;329(23):1696-702.
8. Singhal N, Austin J. A clinical review of micronutrients in HIV infection. J Int Assoc Physicians AIDS Care (Chic Ill). 2002 Spring;1(2):63-75.
9. Calder PC, Jackson AA. Undernutrition, infection and immune function. Nutr Res Rev. 2000 Jun;13(1):3-29.
10. Field CJ, Johnson IR, Schley PD. Nutrients and their role in host resistance to infection. J Leukoc Biol. 2002 Jan;71(1):16-32.
11. Scrimshaw NS, SanGiovanni JP. Synergism of nutrition, infection, and immunity: an overview. Am J Clin Nutr. 1997 Aug;66(2):464S-77S.
12. Bhaskaram P. Micronutrient malnutrition, infection, and immunity: an overview. Nutr Rev. 2002 May;60(5 Pt 2):S40-5.
13. Fawzi WW, Mbise R, Spiegelman D, Fataki M, Hertzmark E, Ndossi G. Vitamin A supplements and diarrheal and respiratory tract infections among children in Dar es Salaam, Tanzania. J Pediatr. 2000 Nov;137(5):660-7.
14. Hussey GD, Klein M. A randomized, controlled trial of vitamin A in children with severe measles. N Engl J Med. 1990 Jul 19;323(3):160-4.
15. Bhutta ZA, Bird SM, Black RE, Brown KH, Gardner JM, Hidayat A, et al. Therapeutic effects of oral zinc in acute and persistent diarrhea in children in developing countries: pooled analysis of randomized controlled trials. Am J Clin Nutr. 2000 Dec;72(6):1516-22.
16. Brooks WA, Santosham M, Naheed A, Goswami D, Wahed MA, Diener-West M, et al. Effect of weekly zinc supplements on incidence of pneumonia and diarrhoea in children younger than 2 years in an urban, low-income population in Bangladesh: randomised controlled trial. Lancet. 2005 Sep 17-23;366(9490):999-1004.

17. WHO. World Health Organization, United Nations Children's Fund, US Agency for International Development, Johns Hopkins Bloomberg School of Public Health. Implementing the new recommendations on the clinical management of diarrhoea: guidelines for policy makers and programme managers. Geneva:World Health Organization; 2006.
18. Ndeezi G, Tylleskar T, Ndugwa CM, Tumwine JK. Effect of multiple micronutrient supplementation on survival of HIV-infected children in Uganda: a randomized, controlled trial. J Int AIDS Soc. 2010;13:18.
19. Micronutrient Information Center. [cited May 2004]; Available from: http://lpi.oregonstate.edu/infocenter/
20. Friis H, Gomo E, Nyazema N, Ndhlovu P, Krarup H, Madsen PH, et al. Iron, haptoglobin phenotype, and HIV-1 viral load: a cross-sectional study among pregnant Zimbabwean women. J Acquir Immune Defic Syndr. 2003 May 1;33(1):74-81.
21. Gordeuk VR, Delanghe JR, Langlois MR, Boelaert JR. Iron status and the outcome of HIV infection: an overview. J Clin Virol. 2001 Feb;20(3):111-5.
22. Weinberg GA. Iron overload as a mechanism for the lowered survival in AIDS patients receiving dapsone-iron protoxalate for secondary prophylaxis of Pneumocystis carinii pneumonia. J Infect Dis. 1996 Jul;174(1):241-2.
23. Open Source Epidemiologic Statistics for Public Health version 2.3.1 http://www.openepi.com/OE2.3/Menu/OpenEpiMenu.htm
24. Bryce J, Boschi-Pinto C, Shibuya K, Black RE. WHO estimates of the causes of death in children. Lancet. 2005 Mar 26-Apr 1;365(9465):1147-52.
25. Chhagan MK, Van den Broeck J, Luabeya KK, Mpontshane N, Tucker KL, Bennish ML. Effect of micronutrient supplementation on diarrhoeal disease among stunted children in rural South Africa. Eur J Clin Nutr. 2009 Jul;63(7):850-7.
26. Chatterjee A, Bosch RJ, Hunter DJ, Manji K, Msamanga GI, Fawzi WW. Vitamin A and vitamin B-12 concentrations in relation to mortality and morbidity among children born to HIV-infected women. J Trop Pediatr. 2010 Feb;56(1):27-35.
27. Eley BS, Sive AA, Abelse L, Kossew G, Cooper M, Hussey GD. Growth and micronutrient disturbances in stable, HIV-infected children in Cape Town. Ann Trop Paediatr. 2002 Mar;22(1):19-23.
28. Baum M, Cassetti L, Bonvehi P, Shor-Posner G, Lu Y, Sauberlich H. Inadequate dietary intake and altered nutrition status in early HIV-1 infection. Nutrition. 1994 Jan-Feb;10(1):16-20.
29. Baqui AH, Zaman K, Persson LA, El Arifeen S, Yunus M, Begum N, et al. Simultaneous weekly supplementation of iron and zinc is associated with lower morbidity due to diarrhea and acute lower respiratory infection in Bangladeshi infants. J Nutr. 2003 Dec;133(12):4150-7.
30. Ruel MT, Rivera JA, Santizo MC, Lonnerdal B, Brown KH. Impact of zinc supplementation on morbidity from diarrhea and respiratory infections among rural Guatemalan children. Pediatrics. 1997 Jun;99(6):808-13.
31. Untoro J, Karyadi E, Wibowo L, Erhardt MW, Gross R. Multiple micronutrient supplements improve micronutrient status and anemia but not growth and morbidity of Indonesian infants: a randomized, double-blind, placebo-controlled trial. J Nutr. 2005 Mar;135(3):639S-45S.

32. Luabeya KK, Mpontshane N, Mackay M, Ward H, Elson I, Chhagan M, et al. Zinc or multiple micronutrient supplementation to reduce diarrhea and respiratory disease in South African children: a randomized controlled trial. PLoS One. 2007;2(6):e541.
33. Micronutrient fortification to improve growth and health of maternally HIV-unexposed and exposed Zambian infants: a randomised controlled trial. PLoS One.5(6):e11165.
34. Bogden JD, Baker H, Frank O, Perez G, Kemp F, Bruening K, et al. Micronutrient status and human immunodeficiency virus (HIV) infection. Ann N Y Acad Sci. 1990;587:189-95.
35. Skurnick JH, Bogden JD, Baker H, Kemp FW, Sheffet A, Quattrone G, et al. Micronutrient profiles in HIV-1-infected heterosexual adults. J Acquir Immune Defic Syndr Hum Retrovirol. 1996 May 1;12(1):75-83.
36. Ndeezi G, Tumwine JK, Bolann BJ, Ndugwa CM, Tylleskar T. Zinc status in HIV infected Ugandan children aged 1-5 years: a cross sectional baseline survey. BMC Pediatr. 2010 Sep 21;10(1):68.

Table 1. Baseline characteristics of HIV infected children aged 1-5years at paediatric HIV clinics in Uganda

Baseline Characteristics	ART stratum (n=85)			Non-ART stratum (n=762)		
	MMS n=43 (%)	MV n=42 (%)	P value	MMS n=383 (%)	MV n=379 (%)	P value
Age < 24 months	1 (2.3)	3 (7.1)	0.36	123 (32.1)	122 (32.2)	1.00
Sex : Male	20 (46.5)	23 (54.8)	0.52	201(52.5)	182 (48.0)	0.25
Site: Kampala	40 (93.0)	40 (95.2)	1.00	313 (81.7)	311 (82.1)	0.93
Carer: Mother	24 (55.8)	21 (50.0)	0.67	278 (72.6)	278 (73.4)	0.87
Diarrhoea	1 (2.3)	2 (4.8)	0.62	42 (11.0)	51 (13.5)	0.32
Persistent diarrhoea	0 (0.0)	0 (0.0)	-	11 (26.8)	11 (23.4)	0.81
Diarrhoea in past 2 weeks	0 (0.0)	0 (0.0)	-	13 (3.4)	14 (3.7)	0.85
Fever	9 (20.9)	8 (19.0)	1.00	76 (19.8)	81 (21.4)	0.65
Cough	27 (62.8)	26 (61.9)	1.00	220 (57.4)	219 (57.8)	0.94
Cotrimoxazole prophylaxis	43 (100.0)	42 (100.0)	-	340 (88.8)	334 (88.1)	0.82
Routine multivitamins	18 (41.9)	13 (31.0)	0.37	241 (62.9)	219 (57.8)	0.16
Vitamin A in past 6 months	20 (46.5)	16 (38.1)	0.51	183 (47.8)	175 (46.2)	0.66
WHO stage III or IV	-	-	-	124 (33.4)	114 (30.1)	0.53
*CD4+ < 25%	14 (38.9)	14 (42.4)	0.81	203 (64.0)	209 (64.7)	0.87
*WHZ less than -2	2 (4.6)	3 (7.1)	1.00	53(13.8)	50 (13.2)	0.75
*HAZ less than -2	22 (51.2)	23 (54.7)	1.00	190(49.6)	197 (51.9)	0.65
*WAZ less than -2	5 (11.6)	6 (14.3)	1.00	110 (28.7)	125 (32.9)	0.24
*CRP >6mg/L	15 (48.4)	11 (36.7)	0.44	122 (46.9)	119 (48.2)	0.79
*Zinc < 10 µmol/L	5 (16.1)	14 (48.3)	0.01	86 (60.1)	84 (63.2)	0.62

*These measurements were less than 847.
CD4+ cell percentages were available for 709 children; 353 in MMS and 356 in MV arm
CRP results were available for 568 children; 291 in MMS and 277 in MV arm
WHZ was available for 816 children; 407 in MMS and 409 in MV arm
WAZ was available for 809 children; 404 in each arm
HAZ was available for 798 children; 398 in MSS and 400 in MV arm.
Zinc results were available for 336 children; 174 in MMS and 162 in MV arm.

Table 2. The formulation for the intervention supplements

Micronutrient	Multivitamins 'Standard of care' 1RDA	Multiple micronutrients 2 RDA
Vitamin A (mcg)	400	800
VitaminB1 (mg)	0.6	1.2
Vitamin B2 (mg)	0.6	1.2
Niacin (mg)	8	16
Vitamin B6 (mg)	-	1.2
Vitamin B12 (mcg)	-	2.4
Vitamin C (mg)	25	50
Vitamin D (IU)	200	400
Vitamin E (mg)	-	14
Folate (mcg)	-	400
Selenium (mcg)	-	60
Zinc (mg)	-	10
Copper (mcg)	-	800
Iodine (mcg)	-	180
Iron (mg)	-	-

Table 3. Incidence of diarrhoea in the multiple micronutrient supplementation compared to the 'standard of care' multivitamin group

Group	Intervention	Number of participants with at least one follow up observation	Episodes of diarrhoea	Observation Period in weeks	Rate per child/year (95% CI)	Rate ratio (95% CI)	P value
Overall	MMS	401	270	3706	3.8 (3.4 – 4.3)	1.1 (0.9 – 1.3)	0.43
	MV	399	246	3630	3.5 (3.1 – 4.0)		
Non-HAART stratum	MMS	359	255	3246	4.0 (3.6 – 4.6)	1.1 (0.9 – 1.2)	0.44
	MV	358	233	3190	3.8 (3.4 – 4.3)		
HAART stratum	MMS	42	15	460	1.7 (1.0 – 2.7)	1.1 (0.5 – 2.3)	0.94
	MV	41	13	440	1.5 (0.9 – 2.6)		

MMS = Multiple micronutrient supplementation arm
MV = Multivitamin 'standard of care' arm

Table 4. Determinants of diarrhoea in HIV infected children supplemented with 2RDA multiple micronutrients or the 'standard of care' multivitamins

Variable	Number in group	Had an episode of diarrhoea: n, (%)	P-value	Unadjusted OR (95% CI)	Adjusted OR (95% CI)
Intervention					
MMS	426	111 (26.1)	0.52	0.9 (0.7 – 1.2)	
MV	421	101 (24.0)			
ART stratum					
HAART	85	13 (15.3)	0.03	0.6 (0.3 – 0.9)	
No-HAART	762	199 (26.1)			
Age					
< 24 months	249	86 (34.5)	<0.001	1.6 (1.3 – 2.1)	2.2 (1.2 – 4.4)
≥ 24 months	598	126 (21.1)			
Sex					
Male	426	114 (26.8)	0.26	1.2 (0.9 – 1.4)	
Female	421	98 (23.3)			
Routine MVs					
Yes	491	135 (27.5)	0.05	1.3 (1.0 – 1.6)	
No	356	77 (21.6)			
Vit A in past 6mo					
Yes	394	96 (24.4)	0.69	0.9 (0.7 – 1.2)	
No	453	116 (25.6)			
CD4+ %					
< 25%	440	127 (28.9)	0.60	1.1 (0.8 – 1.4)	
≥ 25%	269	72 (26.8)			
WHZ					
< -2 *z* score	108	30 (27.8)	0.47	1.1 (0.8 – 1.6)	
≥ -2 *z* score	708	174 (24.6)			
WAZ					
< -2	246	75 (30.5)	0.04	1.3 (1.1 – 1.6)	
≥ -2	562	133 (23.7)			
HAZ					
< -2	432	113 (26.3)	0.68	1.1 (0.8 – 1.3)	
≥ -2	366	91 (24.9)			
WHO staging					
I and II	598	141 (23.6)	0.14	0.8 (0.6 – 1.1)	
III and IV	249	71 (28.5)			
Zinc status					
< 10 µmol/L	189	53 (28.0)	0.53	1.1 (0.8 – 1.6)	
≥ 10 µmol/L	147	36 (24.5)			
C reactive protein					
< 6mg/L	267	86 (32.2)	0.07	1.3 (0.9 – 1.7)	
≥ 6mg/	301	76 (25.2)			

CTX = Cotrimoxazole
MMS = Multiple micronutrient supplementation
MV = Multivitamin
HAZ = Height-for-age *z* score
WAZ = weight-for age *z* score
WHZ = weight-for-height *z* score

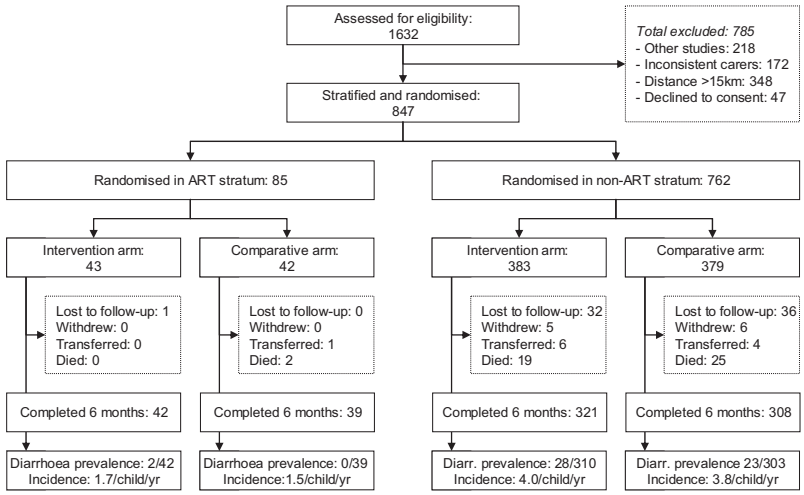

Figure 1. Trial profile.

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RESEARCH ARTICLE Open Access

Zinc status in HIV infected Ugandan children aged 1-5 years: a cross sectional baseline survey

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Abstract

Background: Low concentrations of serum zinc have been reported in HIV infected adults and are associated with disease progression and an increased risk of death. Few studies have been conducted in HIV infected children in Africa. We determined serum zinc levels and factors associated with zinc deficiency in HIV infected Ugandan children.

Methods: We measured the baseline zinc status of 247 children aged 1-5 years enrolled in a randomised trial for multiple micronutrient supplementation at paediatric HIV clinics in Uganda (http://ClinicalTrials.gov NCT00122941). Zinc status was determined using inductively coupled atomic emission spectrophotometry (ICP-AES). Clinical and laboratory characteristics were compared among zinc deficient (zinc <10.0 μmol/L) and non deficient children. Logistic regression was used to determine predictors of low serum zinc.

Results: Of the 247 children, 134 (54.3%) had low serum zinc (< 10.0 μmol/L). Of the 44 children on highly active antiretroviral therapy (HAART), 13 (29.5%) had low zinc compared to 121/203 (59.6%) who were not on HAART. Overall, independent predictors of low zinc were fever (OR 2.2; 95%CI 1.1 - 4.6) and not taking HAART (OR 3.7; 95% CI 1.8 - 7.6).

Conclusion: Almost two thirds of HAART naïve and a third of HAART treated HIV infected children were zinc deficient. Increased access to HAART among HIV infected children living in Uganda might reduce the prevalence of zinc deficiency.

Background

Zinc deficiency is wide spread in low-income countries and is responsible for 4% of childhood deaths and 1% of the burden of disease in Africa, Latin America and Asia [1]. Populations in sub-Saharan Africa and South East Asia have the greatest risk of zinc deficiency because of inadequate zinc intake in about one third of the population [2].

Zinc is a component of various metallo-enzymes, proteins and cell membranes; and plays an important role in immune regulation. Zinc deficiency increases susceptibility to oxidative stress and impaired cell membrane function [3,4]. In HIV infected adults, low serum zinc has been associated with more advanced HIV disease and increased mortality [5-7].

Whereas some studies of HIV infected children in high-income countries have indicated that micronutrient deficiencies are uncommon [8], the reverse is true in low-income countries. The zinc status of HIV infected children in Uganda has not been reported. In this paper we report the magnitude of zinc deficiency and associated factors in a group of HIV infected children in Uganda.

Methods

Study sites

This study was part of a baseline assessment of children enrolled in a multiple micronutrient supplementation trial carried out between June 2005 and June 2008. This paper presents data from 3 of 7 clinics involved in the study, namely the paediatric HIV clinics at the national referral hospital (Mulago), Mildmay Centre and Nsambya hospital. These centres had laboratory facilities for blood sampling and freezing before transportation for analysis at a distant laboratory. Mulago is the national

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referral hospital; Mildmay Centre and Nsambya are private, not-for-profit hospitals. All the three are situated in the capital city, Kampala. The Mulago Hospital Paediatric HIV Clinic is the largest in the country and cares for more than 8000 patients. The Mildmay Centre, Uganda, is an HIV/AIDS referral and training institution, 12 kilometres South of Kampala city centre and cares for about 1500 HIV infected children, while Nsambya hospital cares for an equal number of HIV infected children.

Design and subjects

We here report the baseline zinc levels of children aged 1-5 years who enrolled in the multiple micronutrient supplementation (MMS) study (ClinicalTrials.gov Identifier: NCT00122941).

The MMS study was a randomised trial of a 14 vitamins and minerals versus a six multivitamin supplement as 'standard-of-care' for 6 months, among 847 HIV infected children, with a highly active anti-retroviral therapy (HAART) strata comprising 10% of the study participants. The study enrolled HIV infected children who had at least attended the clinic once and were coming for follow-up. Those who were enrolled in other studies were excluded.

Children in the HAART strata had already been started on anti-retroviral therapy (ART) at the study clinics before enrolment into the study. The 2006 World Health Organization (WHO) guidelines for initiating anti-retroviral therapy in children had been used, in addition to a 'social criteria' that was conductive for initiating ART. Children aged 1 - 3 years were initiated on ART if their CD4+ T cell count was below 20% or if they had WHO stage 3 or 4 disease. Those above 3 years of age were initiated on ART if their CD4+ T cell count was < 15% or if they had WHO stage 3 or 4 disease [9]. The social criteria meant that the child had a consistent caretaker who was adherent to previous clinic appointments, attended at least 2 counseling sessions on adherence to ART and had consented for initiation of therapy.

Due to cost, zinc was analysed at baseline only for children who had sufficient serum collected on both samplings (baseline and at 6-month follow-up). Of the 847 children who participated in the multiple micronutrient supplementation study, 705 were enrolled at the 3 sites where zinc analysis was possible. Two hundred and forty seven children of the 705 (35.0%) had complete clinical data and laboratory analyses at baseline to be included in this paper (figure 1). Laboratory data was declared incomplete as long as a participant did not have one or more of the tests. Out of the 458 exclusions, 261 children had haematology and CRP results but no micronutrient test (zinc inclusive) result, 64 had other micronutrient tests done except zinc, 89 had baseline zinc results but no second sample and 44 had missing samples. Overall insufficient samples contributed 414/458 exclusions while 44 were due to missing samples.

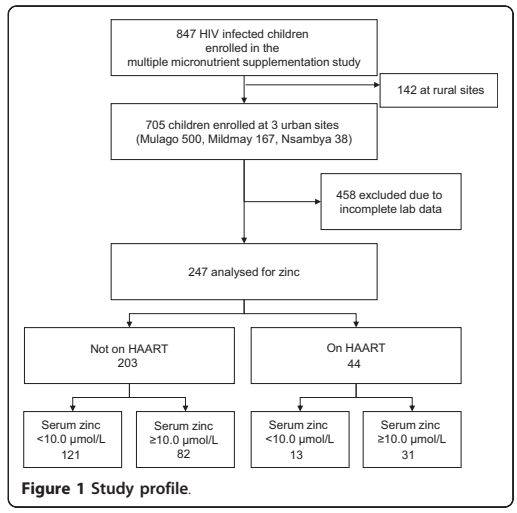

Figure 1 Study profile.

Ethical issues

Written informed consent from the mothers/caretakers was obtained in English or Luganda, one of the commonly spoken local languages in the study area. Permission to conduct the study was granted by Makerere University College of Health Sciences, the Mildmay Centre, Nsambya and Mulago hospitals Research and Ethics Committees. Permission was also granted by the Uganda National Council for Science and Technology and the Regional Committee for Medical and Health Research Ethics, Western Norway ("REK Vest"). Counselling for initiation of HAART and adherence was offered to those who were eligible to start antiretroviral therapy. Treatment for illness and prophylaxis was offered according to the WHO and national paediatric HIV management guidelines.

Procedures

History and physical examination

Mothers/caretakers were interviewed about the child's medical history, nutritional history and symptoms. A detailed physical examination was conducted by one of the study doctors. Weight was taken using a standardized uniscale 01-410-15 to the nearest 0.1 kg and height was taken using the Shorr portable infant/child length/height (Shorr productions, Olney, Maryland, USA) measuring board to the nearest 0.1 centimetre. HIV/AIDS clinical disease staging was determined using

the World Health Organisation (WHO) classification for paediatric HIV/AIDS [10].

Laboratory procedures

Three to 5 millilitres (ml) of blood was collected in a 5 ml trace element free vacutainer tube (Becton Dickinson, Franklin Lakes, N.J) by venepuncture from the cubital fossa or dorsum of the hand irrespective of when the last meal was eaten. The sample was centrifuged at 2000 g within one hour of collection. Serum was transferred into trace element-free cryo-tubes, and transported within 3 hours to a minus 20°C refrigerator until shipment. Zinc was analysed using Inductively Coupled Atomic Emission Spectrophotometry (ICP-AES) [11] at the Clinical Chemistry Laboratory, Haukeland University Hospital, Bergen, Norway. The samples were digested using a microwave oven, nitric acid and concentrated hydrogen peroxide, as described by Rahil-Khazen et al [12]. The analytical coefficient of variation was about 3 %. An additional 2 ml sample was collected in an EDTA containing vacutainer tube and analysed for haemoglobin, white cell count and CD4+ T cell count within 12 hours of collection. A complete blood count was performed using the Act 5Diff instrument (Beckman Coulter) and CD4 count was done using a FACScan instrument and MultiSET software (Beckman Dickinson). Qualitative C-reactive protein (CRP) was measured using the latex immunoassay on one drop of serum (Human Gesellschaft fur Biochemica und Diagnostica mbH, Germany). Distinct agglutination indicated a C-reactive protein (CRP) content ≥ 6 mg/L. This rapid procedure was used in order to feedback the result to the attending paediatrician/doctor.

Low zinc was defined as a serum concentration below 10.0 μmol/L and an elevated CRP was defined as ≥6 mg/L. We used the WHO definition for anaemia where by children aged 6 months to 5 years are considered to be anaemic if their haemoglobin levels are below 11 g/dl [13]. We did not adjust the haemoglobin levels for ethnicity or altitude.

Statistical issues

Data were analysed using SPSS version 15. Categorical characteristics were summarised into proportions while continuous variables were analysed using means and standard deviations. Demographic, anthropometry and clinical characteristics were compared among zinc deficient (zinc less than 10.0 μmol/L) and non deficient children using Fisher's exact test and odds ratios. All factors with a p-value of less than 0.20 by bivariate analysis were retained in the multiple logistic regression model to determine factors independently associated with low zinc. Weight for height (WHZ), height for age (HAZ) and weight for age (WAZ), z-scores were calculated using the WHO Anthro software and reference population [14] to assess anthropometric status.

Results

Of the 247 children analysed for serum zinc, 134 (54.3%) had low zinc < 10μmol/L. Their mean (SD) age was 33.4 (13.8) with a range of 12.0 to 65.5 months. The male to female ratio was approximately 1:1.

Clinical and haematological findings

Generally the children were unhealthy. More than half (141/247, 57.1%) had cough, which was the commonest symptom, followed by skin rash in about half (121/247, 49.0%) and fever in a fifth (94/247, 17.8%) of the children. More than half of the children (108/247, 53.0%) were stunted, almost a quarter were underweight (53/247, 21.5%) and a tenth was wasted. Twenty five percent (62/247) had advanced HIV disease with WHO stage 3 or 4. Absolute CD4+ T cell count ranged from 46 - 3769 with a mean (SD) of 1129(615). One third of the children (85/247) were severely immune-compromised with CD4+ T cell counts of less than 20%. Qualitative C-reactive protein was elevated in 100 out of 247 (40.5%) children. The mean haemoglobin (SD) was 9.9 (1.6) g/dl with a range of 4.6 - 13.7 g/dl. Two thirds of the children (170/247, 68.8%) were anaemic with haemoglobin of less than 11 g/dl.

There were no differences in sex, age, anthropometry, morbidity and CD4+ T cell count among those tested for zinc versus those who were not tested.

Characteristics of children on HAART

The children receiving HAART were older with a mean (SD) age of 42.3 (10.7) compared to the non-HAART group with 31.4 (13.7) months. Other descriptive characteristics were similar in HAART treated and HAART naïve children as shown in table 1.

The proportion of HAART children who were tested for zinc (44/85, 55.0%) was significantly higher (p = 0.00) than the non-HAART (203/847, 32.5%) children analyzed for zinc. The mean serum zinc among children on HAART was 12.2 (SD 4.1) compared to 9.6 (SD 2.5) among those not receiving HAART, a statistically significant difference (OR 2.6; 95%CI 1.6 - 3.5). The mean duration of HAART was 10.2 months for the 13 zinc deficient and 9.5 months in children who were not zinc deficient, with a range of one to 21 months. Of the 44 children, 33 (75%) had received ART for 12 or more months.

Serum zinc and factors associated with low zinc concentrations

The mean (SD) serum zinc concentration was 10.0 (2.9) μmol/L with a range of 5.6 - 29.5 μmol/L. There was no linear relationship between age, WHZ and haemoglobin versus zinc status as shown in Figure 2, 3 and 4. In

Table 1 Characteristics of HIV infected children aged 1-5 years by HAART status at paediatric HIV clinics in Uganda

Variable (mean, SD)	HAART (n = 44)	No HAART (n = 203)	Mean difference (95% CI)
Age in months	42.3 (10.7)	31.4 (13.7)	10.9 (6.6 - 15.2)
Weight for height z score	0.7 (1.4)	-0.2 (1.4)	0.9 (0.4 - 1.4)
Weight for age z score	-0.7 (1.1)	-1.3 (1.3)	0.6 (0.2 - 1.0)
Height for age z score	-2.1 (1.4)	-2.1 (1.6)	0.0 (-0.5 - 0.5)
Haemoglobin (g/dl)	10.9 (1.2)	9.8 (1.6)	1.1 (0.6 - 1.6)
Absolute CD4+ cell count (cells/μ L	1132 (664)	1128 (607)	6 (-198 - 206)
Serum zinc (μmol/L)	12.2 (4.1)	9.6 (2.5)	2.6 (1.6 - 3.5)

addition there was no significant difference in mean age, anthropometry, haemoglobin and CD4+ cell count among children with low serum zinc (< 10μmol/L) compared to those with normal zinc levels (table 2).

Factors associated with low serum zinc concentrations at bivariate analysis were: being HAART naïve, reported fever, underweight, WHO stage 3 or 4 disease and elevated CRP (table 3). The mean (SD) serum zinc among CRP negative children was 10.2 (2.5) compared to 9.8 (3.6) in CRP positive children. This difference was not statistically significant.

In the multivariate model the only significant independent predictors of low zinc were: being HAART naïve and reported fever. Of the 44 children on HAART, 13 (29.5%) had low serum zinc compared to 121 of 203 (59.6%) in the non-HAART group. This difference was statistically significant [Adjusted OR 3.7 (95% CI 1.8-7.7)]. When fever was excluded from the model elevated CRP was a significant predictor of low zinc, illustrating that fever and CRP are closely related.

Discussion

The prevalence of zinc deficiency in this group of HIV infected children aged 1-5 years attending paediatric HIV clinics in Uganda was high (54.3%). This was higher than what was reported in a previous study of children aged 6-36 months with persistent diarrhoea but of undetermined HIV status at Mulago hospital, Uganda [15]. While we used a cut off of 10 μmol/L to indicate zinc deficiency, the previous study had used 4.73 μmol/L assuming a normal serum zinc level of 8.99 (SD 2.13) μmol/L measured from a control group of healthy children. What was regarded as normal is below the mean (SD) serum zinc of 10.0 (2.9) reported in our study. An earlier community study in Kampala had found a serum zinc range of 8.4 - 20.9μmol/L [mean(SD) 10.1(3.2)] among children aged 4 -14 years [16]. Based on these two previous studies, it is possible that the zinc status in our study is similar to zinc levels in the general population of children in a similar age group in Uganda. The prevalence was twice as high in children who had not yet started HAART compared to those receiving HAART. This was not influenced by the duration on HAART. This implies that HAART may protect against zinc deficiency but will not completely eliminate it.

Compared to other studies of HIV infected children in Africa, the prevalence of zinc deficiency was higher than

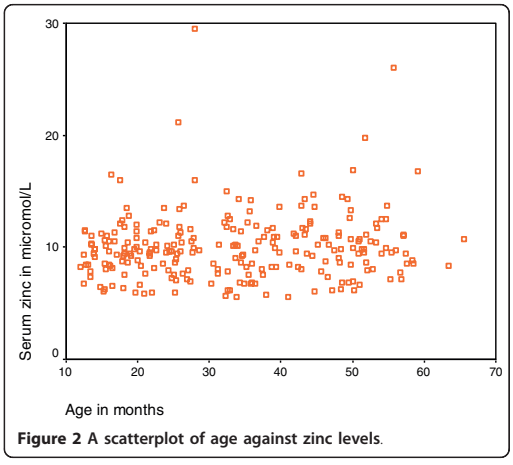

Figure 2 A scatterplot of age against zinc levels.

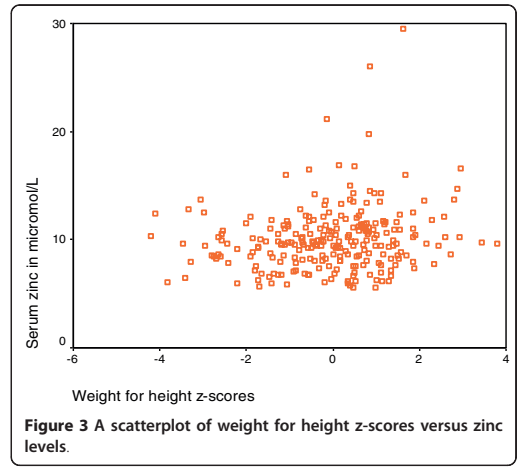

Figure 3 A scatterplot of weight for height z-scores versus zinc levels.

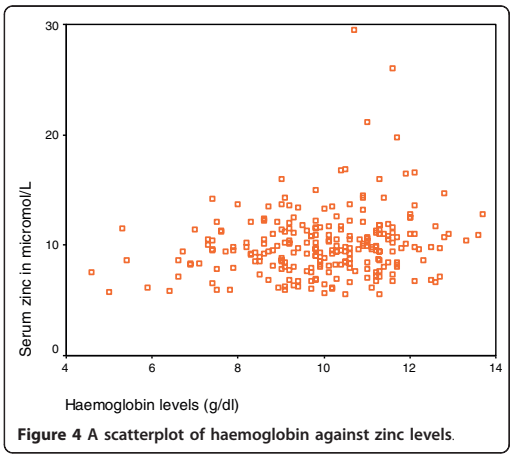

Figure 4 A scatterplot of haemoglobin against zinc levels.

what was reported in South Africa and Rwanda [17,18]. Our study had a larger sample size and older children compared to the two studies which enrolled children from 2 months of age.

We did not find any association between age and zinc status in our study. All the children enrolled in this study were above one year of age, very few of whom were still breastfeeding. Other studies have shown that micronutrient deficiencies are less likely to occur below 24 months of age [17] and that breastfeeding is protective [19] against low serum zinc. Further more, there was no significant difference in mean zinc concentrations between girls and boys. Similar findings were reported from children whose HIV status was not known in low-income families in California [20].

Surprisingly, there was no significant association between zinc status and diarrhoea in the current study, possibly because there were very few children with diarrhoea. Studies in other developing countries have shown an association between low zinc and diarrhoea [21]. As expected, the presence of fever was significantly associated with low zinc levels. Fever is an indicator of infection and this may be associated with increased acute phase proteins and consequently reduced serum zinc [22]. Other common illnesses such as cough and skin rash were not associated with low zinc either.

There was a weak association between underweight and zinc status although a previous Uganda study of children with persistent diarrhoea [15] and a South Africa study of HIV infected children [17] did not find any association between zinc and nutritional status. Some authors have shown that severely malnourished children have a higher anti-oxidant activity and this is associated with low serum zinc [19]. Although anaemic children were more likely to have low zinc, the association was not significant. Other studies in zinc deficient low-income countries such as India and Cambodia have reported a close association between zinc deficiency and anaemia [19,23]. The lack of association in our study could not be explained.

Advanced HIV disease (WHO stage 3 and 4) was associated with low serum zinc but probably confounded by other factors. Advanced HIV disease is more likely to be associated with recurrent acute infections and an elevated acute phase response interpreted as low zinc. Our findings are similar to a South African study where the prevalence of zinc deficiency increased with HIV disease staging [6]. CD4+ T cell count had no association with zinc status in the current study. This finding is similar to what was reported in studies of HIV infected adults at Tufts university and Medical Centre in the nutrition for health living cohort [24] and in South Africa [6].

Children who were on HAART were less likely to be zinc deficient. This is further supported by a study in adults that showed that patients receiving HAART have better micronutrient indices including zinc than those not yet on HAART [25]. However, two other studies indicated that zinc deficiency remains highly prevalent in HIV infected adults on HAART [7,24].

We acknowledge that infant, child feeding practices and maternal HIV status influence the diet and mode of feeding [26], and may subsequently affect zinc intake. Adult studies have shown that the time of blood

Table 2 Characteristics of HIV infected children aged 1-5 years by zinc status at paediatric HIV clinics in Uganda

Variable (mean, SD)	Serum zinc < 10 μmol/L	95% CI	Serum zinc = 10 μmol/L	95% CI
Age in months	32.2 (14.0)	29.8 to 34.6	34.7 (13.6)	32.2 to 37.2
Weight for height z score	-0.2 (1.4)	-0.5 to 0.0	0.1 (1.5)	-0.2 to 0.4
Weight for age z score	-1.4 (1.3)	-1.6 to -1.2	-1.0 (-1.3)	-1.2 to -0.7
Height for age z score	-2.3 (1.4)	-2.5 to -2.0	-1.9 (1.8)	-2.3 to -1.6
Haemoglobin (g/dl)	9.7 (1.7)	9.5 to 10.0	10.2 (1.5)	9.7 to 10.5)
Absolute CD4+ cell count (cells/μ L)	1056 (613)	950 to 1162	1214 (610)	1099 to 1330
*On HAART (n,%)	13 (29.5)		31 (70.5)	

*Children on HAART are expressed as a percentage (%).

Table 3 Factors associated with low zinc in 247 HIV infected Ugandan children aged 1-5 years

Variable	No. of children	Low zinc < 10μmol/L (n; %)	Unadjusted OR (95%CI)	p-value	Adjusted OR (95%CI)
Age < 36 months	146	86 (58.2)	1.5 (0.9 - 2.5)	0.153	1.0 (0.6 - 1.8)
Age ≥ 36 months	101	49 (48.5)			
Male	128	75 (58.6)	1.4 (0.9 - 2.4)	0.162	0.7 (0.4 - 1.1)
Female	119	59 (49.6)			
On HAART	44	13 (29.5)	3.5 (1.7 - 7.1)	0.000	3.7 (1.8 - 7.7)
No HAART	203	121(59.6)			
Reported fever	44	30 (68.2)	2.0 (1.0 - 4.1)	0.046	2.2 (1.1 - 4.6)
No fever	203	104 (51.2)			
Current diarrhoea	20	15 (75.0)	2.7 (1.0 - 7.7)	0.062	2.2 (0.8 - 6.7)
No Diarrhoea	227	119 (52.4)			
Cough present	141	81 (57.4)	1.4 (0.8 - 2.2)	0.249	
No cough	106	53 (50.0)			
WHO stage 3 or 4	62	42 (67.7)	2.1 (1.2 - 3.9)	0.018	1.5 (0.8 - 2.9)
WHO stage 1 or 2	185	92 (49.7)			
WHZ score < -2	24	15 (62.5)	1.5 (0.6 - 3.5)	0.519	
WHZ score ≥ -2	223	119 (53.4)			
WAZ score < -2	56	38 (67.9)	2.1 (1.1 - 3.9)	0.022	1.2 (0.6 - 2.6)
WAZ score ≥ -2	191	96 (50.3)			
HAZ score < -2	136	80 (58.8)	1.5 (0.9 - 2.5)	0.124	1.3 (0.8 - 2.3)
HAZ score ≥ -2	111	54 (48.6)			
CRP < 6 mg/L	147	72 (49.0)	1.7 (1.0 - 2.9)	0.051	1.6 (0.9 - 2.7)
CRP ≥ 6 mg/L	100	62 (62.0)			
Hb < 11 g/dl	170	98 (57.6)	1.6 (0.9 - 2.7)	0.130	1.1 (0.6 - 2.0)
Hb ≥ 11 g/dl	77	36 (46.8)			
CD4 < 20%	85	52 (61.2)	1.5 (0.9 - 2.5)	0.139	1.1 (0.6 - 1.9)
CD4 ≥ 20%	162	82 (50.6)			

collection and feeding influence zinc levels [27], factors not controlled for in this study. The exclusions though numerous, were not systematic and therefore less likely to influence the results of our study. Low zinc levels were associated with fever or CRP implying that infection and acute phase proteins may have influenced the zinc status of the study children. Other researchers have previously confirmed that serum zinc is affected by the serum protein level and any acute-phase reactions [28]. Although the concentration of serum zinc gives limited information on the total zinc content in the body, there is no better way of determining zinc status that has been established [29]. The generalisability of this study is limited to the HIV infected children since we did not have a control group of HIV un-infected children.

Coupled with the already existing poor nutritional and immunological status, low zinc status in Ugandan HIV infected children is likely to remain a significant contributor to increased morbidity especially among those not yet receiving HAART.

Conclusion

While almost two thirds of untreated HIV infected children were zinc deficient, zinc deficiency occurred in only a third of those on HAART. Increased access to HAART among HIV infected children living in Uganda might reduce the prevalence of zinc deficiency in this population.

Acknowledgements

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Authors' contributions

GN, TT and JKT participated in the conception, design and implementation of the study, statistical analysis, interpretation and drafting of the manuscript. CMN participated in study design and drafting of the manuscript. BJB analysed the serum samples for zinc and participated in drafting the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interest.

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References

1. Fischer Walker CL, Ezzati M, Black RE: **Global and regional child mortality and burden of disease attributable to zinc deficiency.** *Eur J Clin Nutr* 2009, **63(5)**:591-597.
2. Brown KH, Rivera JA, Bhutta Z, Gibson RS, King JC, Lonnerdal B, Ruel MT, Sandtrom B, Wasantwisut E, Hotz C: **International Zinc Nutrition Consultative Group (IZiNCG) technical document #1. Assessment of the risk of zinc deficiency in populations and options for its control.** *Food Nutr Bull* 2004, **25(1 Suppl 2)**:S99-203.
3. Bhaskaram P: **Micronutrient malnutrition, infection, and immunity: an overview.** *Nutr Rev* 2002, **60(5 Pt 2)**:S40-45.
4. O'Dell BL: **Role of zinc in plasma membrane function.** *J Nutr* 2000, **130(5S Suppl)**:1432S-1436S.
5. Baum M, Campa A, Lai S, Lai H, Page J: **Zinc status in human immunodeficiency virus type 1 infection and illicit drug use.** *Clin Infect Dis* 2003, **37(Suppl 2)**:S117-123.
6. Visser ME, Maartens G, Kossew G, Hussey GD: **Plasma vitamin A and zinc levels in HIV-infected adults in Cape Town, South Africa.** *Br J Nutr* 2003, **89(4)**:475-482.
7. Wellinghausen N, Kern WV, Jochle W, Kern P: **Zinc serum level in human immunodeficiency virus-infected patients in relation to immunological status.** *Biol Trace Elem Res* 2000, **73(2)**:139-149.
8. Henderson RA, Talusan K, Hutton N, Yolken RH, Caballero B: **Serum and plasma markers of nutritional status in children infected with the human immunodeficiency virus.** *J Am Diet Assoc* 1997, **97(12)**:1377-1381.
9. WHO: **Antiretroviral Therapy for HIV Infection in Infants and Children in Resource-Limited Settings: Towards Universal Access.** Geneva, Switzerland 2006.
10. WHO: **Case definitions of HIV for surveillance and revised clinical staging and immunological classification of HIV-related disease in adults and children.** Geneva, Switzerland 2006.
11. Bolann B, Rahil-Khazen R, Henriksen H, Isrenn R, Ulvik R: **Evaluation of methods for trace-element determination with emphasis on their usability in the clinical routine laboratory.** *Scand J Clin Lab Invest* 2007, **67(4)**:353-366.
12. Rahil-Khazen R, Henriksen H, Bolann BJ, Ulvik RJ: **Validation of inductively coupled plasma atomic emission spectrometry technique (ICP-AES) for multi-element analysis of trace elements in human serum.** *Scand J Clin Lab Invest* 2000, **60(8)**:677-686.
13. WHO: **Worldwide prevalence of anaemia 1993-2005.** Geneva, Switzerland 2008.
14. WHO: **WHO Anthro 2005.** Geneva, Switzerland 2005.
15. Bitarakwate E, Mworozi E, Kekitiinwa A: **Serum zinc status of children with persistent diarrhoea admitted to the diarrhoea management unit of Mulago Hospital, Uganda.** *Afr Health Sci* 2003, **3(2)**:54-60.
16. Bimenya GS, Lutalo-Bosa AJ, Nzaro E: **Serum zinc levels in normal children (HbAA) and sickle cell children (HbSS) in and around Kampala.** *East Afr Med J* 1980, **57(12)**:825-827.
17. Eley BS, Sive AA, Abelse L, Kossew G, Cooper M, Hussey GD: **Growth and micronutrient disturbances in stable, HIV-infected children in Cape Town.** *Ann Trop Paediatr* 2002, **22(1)**:19-23.
18. Ndagije F, Baribwira C, Coulter JB: **Micronutrients and T-cell subsets: a comparison between HIV-infected and uninfected, severely malnourished Rwandan children.** *Ann Trop Paediatr* 2007, **27(4)**:269-275.
19. Thakur S, Gupta N, Kakkar P: **Serum copper and zinc concentrations and their relation to superoxide dismutase in severe malnutrition.** *Eur J Pediatr* 2004, **163(12)**:742-744.
20. Schneider JM, Fujii ML, Lamp CL, Lonnerdal B, Zidenberg-Cherr S: **The prevalence of low serum zinc and copper levels and dietary habits associated with serum zinc and copper in 12- to 36-month-old children from low-income families at risk for iron deficiency.** *J Am Diet Assoc* 2007, **107(11)**:1924-1929.
21. Bhandari N, Bahl R, Hambidge KM, Bhan MK: **Increased diarrhoeal and respiratory morbidity in association with zinc deficiency–a preliminary report.** *Acta Paediatr* 1996, **85(2)**:148-150.
22. Duggan C, MacLeod WB, Krebs NF, Westcott JL, Fawzi WW, Premji ZG, Mwanakasale V, Simon JL, Yeboah-Antwi K, Hamer DH: **Plasma zinc concentrations are depressed during the acute phase response in children with falciparum malaria.** *J Nutr* 2005, **135(4)**:802-807.
23. Anderson V, Jack S, Monchy D, Hem N, Hok P, Bailey K, Gibson R: **Co-existing micronutrient deficiencies among stunted Cambodian infants and toddlers.** *Asia Pac J Clin Nutr* 2008, **17(1)**:72-79.
24. Jones CY, Tang AM, Forrester JE, Huang J, Hendricks KM, Knox TA, Spiegelman D, Semba RD, Woods MN: **Micronutrient levels and HIV disease status in HIV-infected patients on highly active antiretroviral therapy in the Nutrition for Healthy Living cohort.** *J Acquir Immune Defic Syndr* 2006, **43(4)**:475-482.
25. Rousseau MC, Molines C, Moreau J, Delmont J: **Influence of highly active antiretroviral therapy on micronutrient profiles in HIV-infected patients.** *Ann Nutr Metab* 2000, **44(5-6)**:212-216.
26. Fadnes LT, Engebretsen IM, Wamani H, Semiyaga NB, Tylleskar T, Tumwine JK: **Infant feeding among HIV-positive mothers and the general population mothers: comparison of two cross-sectional surveys in Eastern Uganda.** *BMC Public Health* 2009, **9**:124.
27. Hambidge KM, Goodall MJ, Stall C, Pritts J: **Post-prandial and daily changes in plasma zinc.** *J Trace Elem Electrolytes Health Dis* 1989, **3(1)**:55-57.
28. Wieringa FT, Dijkhuizen MA, West CE, Northrop-Clewes CA, Muhilal : **Estimation of the effect of the acute phase response on indicators of micronutrient status in Indonesian infants.** *J Nutr* 2002, **132(10)**:3061-3066.
29. Thompson RP: **Assessment of zinc status.** *Proc Nutr Soc* 1991, **50(1)**:19-28.

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Multiple micronutrient supplementation improves vitamin B_{12} and folate concentrations of HIV infected children in Uganda: a randomized controlled trial

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Abstract

Background: The effect of multiple micronutrient supplementation on vitamin B_{12} and folate has hither to not been reported in African HIV infected children. This paper describes vitamin B_{12} and folate status of Ugandan HIV infected children aged 1-5 years and reports the effect of multiple micronutrient supplementation on serum vitamin B_{12} and folate concentrations.

Methods: Of 847 children who participated in a multiple micronutrient supplementation trial, 214 were assessed for vitamin B_{12} and folate concentrations pre and post supplementation. One hundred and four children were randomised to two times the recommended dietary allowance (RDA) of a 14 multiple micronutrient supplement (MMS) and 114 to a 'standard of care' supplement of 6 multivitamins (MV). Serum vitamin B_{12} was measured by an electrochemiluminescence immunoassay and folate by a competitive protein-binding assay using Modular E (Roche) automatic analyzer. Vitamin B_{12} concentrations were considered low if less than 221picomoles per litre (pmol/L) and folate if < 13.4 nanomoles per litre (nmol/L). The Wilcoxon Signed Ranks test was used to measure the difference between pre and post supplementation concentrations.

Results: Vitamin B_{12} was low in 60/214 (28%) and folate in 62/214 (29.0%) children. In the MMS group, the median concentration (IQR) of vitamin B_{12} at 6 months was 401.5 (264.3 - 518.8) pmol/L compared to the baseline of 285.5 (216.5 - 371.8) pmol/L, $p < 0.001$. The median (IQR) folate concentrations increased from 17.3 (13.5 - 26.6) nmol/L to 27.7 (21.1 - 33.4) nmol/L, $p < 0.001$. In the 'standard of care' MV supplemented group, the median concentration (IQR) of vitamin B_{12} at 6 months was 288.5 (198.8 - 391.0) pmol/L compared to the baseline of 280.0 (211.5 - 386.3) pmol/L while the median (IQR) folate concentrations at 6 months were 16.5 (11.7 - 22.1) nmol/L compared to 15.7 (11.9 - 22.1) nmol/L at baseline. There was a significant difference in the MMS group in both vitamin B_{12} and folate concentrations but no difference in the MV group.

Conclusions: Almost a third of the HIV infected Ugandan children aged 1-5 years had low serum concentrations of vitamin B_{12} and folate. Multiple micronutrient supplementation compared to the 'standard of care' supplement of 6 multivitamins improved the vitamin B_{12} and folate status of HIV infected children in Uganda.

Trial registration: http://ClinicalTrials.govNCT00122941)

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Background

Vitamin B_{12} and folate deficiency are relatively common in low income countries compared to high income countries, particularly in communities where the diet is predominantly vegetarian [1-4], where major sources of vitamin B_{12} such as meat, fish, poultry, milk and fortified breakfast cereals [5] are consumed in small amounts or are not readily available. Dietary sources of folate, on the other hand, are more prevalent as they include green leafy vegetables, fruits and dried beans and peas [6]. During infancy and childhood folate and vitamin B_{12} deficiency have been associated with failure to thrive, reduced physical activity and cognitive function and megaloblastic anaemia [7,8]. However many of the symptoms are non-specific and may result from a variety of medical conditions.

A significant number of HIV-infected children in low-income countries remain at risk of increased morbidity due to immunodeficiency related to HIV infection in addition to micronutrient deficiencies. Low vitamin B_{12} status has been associated with poor immunological status and HIV disease progression in adults [9]. In follow up studies of HIV infected pregnant women micronutrient supplementation resulted in improved child growth, haematological indices and vitamin B_{12} levels [10].

Food supplementation and multiple micronutrient fortification of foods and beverages in children with unknown HIV status living in low income countries have shown improved haemoglobin levels and concentrations of deficient micronutrients [2,11-14].

We hypothesised that supplementation with two recommended dietary allowances (RDA) of 14 multiple micronutrients (MMS) would increase serum vitamin B_{12} and folate concentrations compared to a 1 RDA of 6 multivitamin (MV) 'standard of care' supplement. We here report the baseline vitamin B_{12} and folate status in Ugandan HIV infected children aged 1-5 years and the effect of multiple micronutrient supplementation on serum concentrations of vitamin B_{12} and folate.

Methods

Design

The study was a randomised controlled trial conducted between 2005 and 2008 at 7 paediatric HIV clinics in Uganda. Participants were allocated to the intervention or 'standard of care' supplement in a 1:1 ratio.

Participants

HIV infected children aged 1- 5 years presenting at the study clinics for follow up visits were eligible for the trial. Children who had enrolled in other studies or were unable to adhere to a regular follow up schedule and those whose mothers or guardians declined consent were excluded. There were no other micronutrient supplementation studies going on at the time so the exclusion for those who were participating in other studies was done to avoid participant fatigue and interference with procedures. Multivitamin supplementation was routinely practiced at the study clinics but this was not an exclusion criteria. Eligible participants were consecutively enrolled, assigned to the study intervention or 'standard of care' supplement and followed for one year.

The study sites have been previously described [15]. Three sites were located in the capital city of Uganda (Kampala) and these were Mulago (the national referral hospital), Mildmay Centre and Nsambya hospital. The other sites were situated in the regional hospitals in the east, south-west, central and north of the country. For logistical reasons, transport and storage, it was not possible to collect samples for biochemical tests from all the sites. Therefore blood samples for biochemical tests were only collected from the three Kampala sites. The samples were subsequently shipped to the clinical chemistry laboratory at Haukeland University Hospital, Bergen (Norway), where vitamin B_{12} and folate were analysed.

The trial enrolled 847 children at all the seven study sites. Out of 705 children from whom blood samples could be stored for micronutrient tests, 261 had no sufficient samples, 230 had other micronutrient tests done or had either baseline but no result at the second sampling. Because of multiple analyses 214 children had both baseline and 6 months results for vitamin B_{12} and folate concentrations. There were no significant differences in demographic and clinical characteristics such as age, sex, anthropometric measurements and other laboratory measurements like CD4 + cell count among those who had results for vitamin B_{12} and folate compared to those who did not.

The study was approved by the College of Health Sciences Research and Ethics Committee, Makerere University, Kampala, Uganda; the Uganda National Council for Science and Technology, and the Regional Committee for Medical Research Ethics, Western Norway.

Cotrimoxazole prophylaxis, initiation of ART, management of common illnesses and opportunistic infections was offered using the national guidelines for Paediatric HIV care.

Intervention

The trial supplements were manufactured in powder form and packaged by NUTRISET, France using a formula that was determined by the investigators based on twice the recommended dietary intake for a 4 year old category [6]. We decided to use 2 RDA based on the fact that many children were malnourished despite routine supplementation with multivitamins. Secondly some

previous studies of HIV infected adults had indicated that HIV infected persons may require multiples of RDA in order to achieve normal serum concentrations of micronutrients. Thirdly, looking at the tables of the nutritional requirements for children [6] we noted that there are 2 age bands where our participants belonged, 1-3 and 4-8 years of age. We noticed that there were minor variations in dosages of some micronutrients while others like iodine and vitamin D did not vary. We therefore decided to use the 4 year old category for our study. In addition it was easier to administer a uniform intervention. There were no similar studies in the region and there was no literature to guide us on the micronutrient status of Ugandan HIV infected children. Also there were no food composition tables based on the local foods so we could not estimate how much micronutrients children get from the usual diet. The MMS comprised of 800 mcg vitamin A, 1.2 mg vitamin B_1, 1.2 mg vitamin B_2, 16 mg niacin, 1.2 mg vitamin B_6, 2.4 mcg vitamin B_{12}, 50 mg vitamin C, 400 IU vitamin D, 14 mg vitamin E, 40 mcg folate, 60 mcg selenium, 10 mg zinc, 800 mcg copper and 180 mcg iodine. The multivitamin 'standard of care' supplement contained 400 mcg vitamin A, 0.6 mg vitamin B_1, 0.6 mg vitamin B_2, 8 mg niacin, 25 mg vitamin C and 200 IU vitamin D. The contents and formula for the MV supplement was based on the regular multivitamins supplied at the study clinics as routine care of HIV infected children. The daily dose was 4 g and this was equivalent to a levelled scoop supplied by the manufacturer. The powder was mixed with 10 to 20 ml of milk or water. The first dose of the supplement was administered at the study clinic following a demonstration and under observation by the study nurse. At the time of administering the first dose we counselled the mother on the importance of completing the dose and ensuring that the daily dose was given. Whenever the child vomited during or within 30 minutes of administering the dose a repeat dose was given. Mothers were given calendar charts and instructed to tick on the appropriate date whenever a dose was given. They would return to the clinic with the container/remaining supplement together with the calendar charts on routine follow up visits. The remaining amount of supplement was measured using a light weight scale and the level of compliance determined using the proportion of the supplement consumed against the expected. The supply for one month was 140 grams and the expected dose for 30 days was 120 grams. The remaining 20 grams was to cater for vomited doses or in case there was spillage. The mothers administered the subsequent doses from home and it was not possible to observe them. The supplements were given for a period of 6 months when the second sample of blood was drawn.

Outcomes

The outcome measures were serum vitamin B_{12} and folate concentrations pre and post supplementation and factors associated with low concentrations at baseline. These were secondary objectives of the trial. Survival was assessed as the primary objective whose findings have been previously reported [15].

Randomisation and blinding

The randomisation sequence was generated using the stata soft ware in variable blocks of 4 to 20. The supplements were manufactured and packaged in 140 g plastic containers which were sequentially numbered. RB generated the randomisation sequence at Geneva and sent it to the manufacturers in France. The randomisation code was kept at Geneva and by the manufacturers. The treatment assignment was revealed upon completion of the study.

Participants were enrolled by the principal investigator and the other study doctors. The trial nurse at the study sites dispensed the supplement in serial order. The colour, consistency and odour of the intervention and 'standard of care' supplement were similar. The principal investigator, study personnel including doctors and nurses and the caretakers/participants were all blinded to treatment assignment.

Clinical and laboratory measurements

At enrolment demographic information, history of the child's illness and findings on physical examination were recorded. Non fasting blood samples were drawn for CD4+ cell count and micronutrient analysis using procedures previously described in the same cohort [15]. Vitamin B_{12} was measured by electrochemiluminescence immunoassay, and folate by a competitive protein-binding assay on Modular E (Roche) automatic analyzer. The coefficient of variation for the assays was less than 5%. To determine cut off values for low vitamin B_{12} and folate, we considered what other researchers have used since there were no reference values for Ugandan children. Vitamin B_{12} concentrations were considered low if less than 221 picomoles per litre (pmol/L), combining both very low (<148 pmol/L) and marginal (148 - 221 pmol/L) status. Concentrations equal or > 221 pmol/L was considered to be normal. Folate concentrations were low if < 13.4 nanomoles per litre (nmol/L), similarly combining both very low (<6.8 nmol/L) and marginal (6.8 - 13.4 nmol/L) status. Concentrations ≥ 13.4 nmol/L were regarded as being normal [16,17].

Statistical analysis

Change in micronutrient concentrations of each participant was computed in SPSS as follows: concentration at

6 months minus concentration at baseline. Medians and their interquartile ranges were used for summarising the data. Because vitamin B_{12} and folate concentrations were not normally distributed the Wilcoxon Signed Ranks test was used to measure the difference between baseline and 6 months in each group. Differences in categorical variables were tested with the Pearson chi-square or Fischer's exact test. Differences were considered significant if a two-sided p-value was less than 0.05. Multiple regression analysis was performed to examine factors associated with low vitamin B_{12} or folate concentrations at baseline assessment.

Results

Baseline characteristics of participants

Of the 214 children with both baseline and 6 months results for vitamin B_{12} and folate, 104 (48.6%) received MMS while the rest received the 'standard of care' MV supplement. The distribution of children by arm and strata is presented in figure 1. Males and females were equally represented. The median age (IQR) was 33.2 months (19.6 - 44.4) in the MMS and 30.3 (19.8 - 45.5) in the MV group. Baseline characteristics are described in table 1. There were no significant differences between the two treatment groups.

Overall, 60 children (28.0%) had low vitamin B_{12} concentrations less than 221.0 pmol/L, and 62 (29.0%) had low folate concentrations less than 13.4 nmol/L. Among the children with low vitamin B_{12} status, 13 (6.0%) had very low and 47 (22.0%) marginal concentrations (148 - 221 pmol/L). Of the 62 children with low folate status, 3 (1.4%) had very low (<6.8 nmol/L) and 59 (27.6%) had marginal concentrations (6.8 - 13.4 nmol/L).

Comparisons between baseline and 6 months' vitamin B_{12} and folate concentrations

Baseline median serum vitamin B_{12} and folate concentrations were similar in the two groups. Following supplementation with multiple micronutrients (MMS) vitamin B_{12} concentrations increased from a median (IQR) of 285.5 (216.5 - 371.8) to 401.5 (264.3 - 518.8) pmol/L at 6 months. This difference was statistically significant ($p < 0.001$). Similarly folate concentrations increased from 17.3 (13.5 - 26.6) to 27.7 (21.1 - 33.4) nmol/L and this difference was also significant, $p < 0.001$ (Table 2). There was no significant difference in the MV 'standard of care' group.

Of the 44 children who were on HAART, 19 received MMS while 25 received MV. In this stratum the median (IQR) concentration of vitamin B_{12} in the MMS arm at baseline was 262.0 (215.0 - 342.0) compared to 453.0 (261.0 - 594.0) pmol/L at 6 months, $p = 0.002$. The median (IQR) folate concentrations increased from 18.6 (13.8 - 23.9) to 25.0 (21.3 - 32.9) nmol/L, $p = 0.040$. Although the numbers were small these were significant differences. There was no significant difference in the MV group. Median (IQR) baseline vitamin B_{12} concentrations were higher in the HAART stratum [306.5

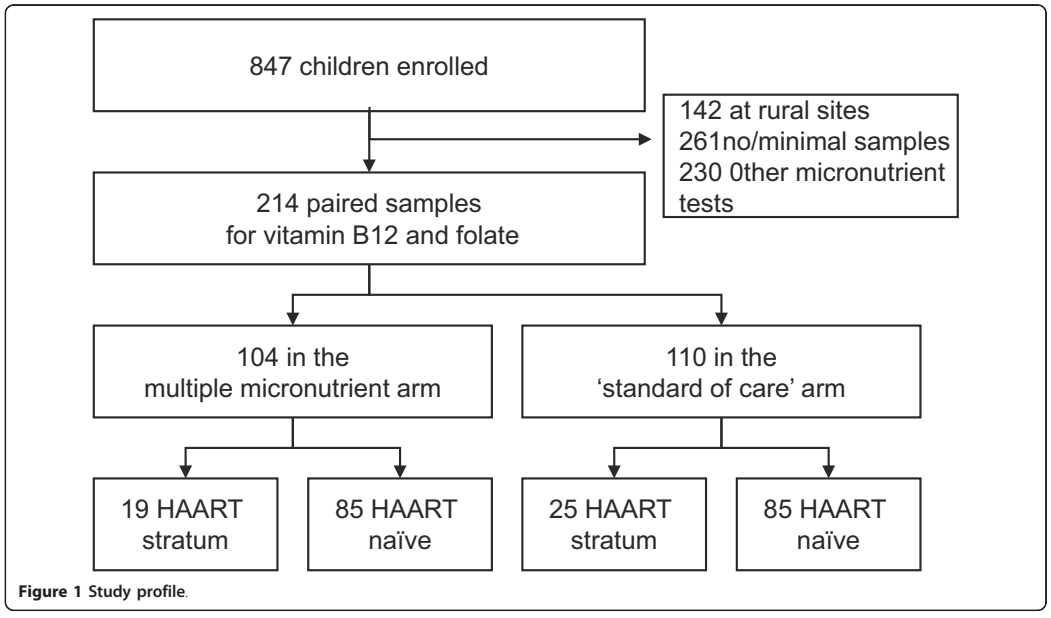

Figure 1 Study profile.

Table 1 Characteristics of 214 Ugandan HIV infected children analysed for vitamin B_{12} and folate by intervention group

	Number (N)	MMS n (%)	'Standard of care' MV n (%)	p-value
Sex: Male	100	49 (49.0)	51 (51.0)	>0.99
Age less than 24 months	70	33 (47.1)	37 (52.9)	0.77
Mother as carer	146	73 (50.0)	73 (50.0)	0.56
On HAART	44	19 (43.2)	25 (56.8)	0.50
Routine CTX	193	93 (48.2)	100 (51.8)	0.82
Routine MV	144	68 (47.2)	76 (52.8)	0.66
WHZ < -2	28	14 (50.0)	14 (50.0)	>0.99
HAZ < -2	113	56 (49.6)	57 (50.4)	0.89
WHO stage 3 or 4	56	26 (46.4)	30 (53.6)	0.76
CD4+ T cells < 25%	114	50 (43.9)	64 (56.1)	0.20
Elevated CRP (> 6 g/dl)	67	33 (49.3)	34 (50.7)	0.42
Low haemoglobin (< 11 g/dl)	127	61 (48.0)	66 (52.0)	0.89
Low vitamin B_{12} (< 221 pmol/L)	60	29 (48.3)	31 (51.7)	0.96
Low folate (< 13.4 nmol/L)	62	25 (40.3)	37 (59.7)	0.13

MMS = multiple micronutrient supplementation
HAART = Highly active anti-retroviral therapy
CTX = Cotrimoxazole
MV = 'standard of care' multivitamins
WHZ = Weight for height z-score
HAZ = Height for age z-score
WHO = World Health Organisation
CD4+ = Cluster of differentiation 4
CRP = C-reactive protein

(209.3 - 372.8) pmol/L] compared to the non-HAART stratum [280.0 (218.0 - 386.3) pmol/L], but not significantly so. Folate concentrations were almost the same [16.1 (13.5 - 22.0) nmol/L] in the HAART and [16.0 (12.1 - 24.9) nmol/L] non-HAART stratum. Thirteen out of 40 children (32.5%) who were on HAART had CD4+ cell percent < 25 compared to 100/159 (63.5%) who were HAART naïve, p = 0.001 (Fisher's exact test). Median (IQR) duration of anti-retroviral therapy was 9 (6.0 - 14.3) months.

Table 2 Biochemical and haematological measurements at baseline and at 6 months of supplementation by intervention group

	Multiple Micronutrient Supplementation group (n = 104)		Comparative 'standard of care' multivitamins group (n = 110)	
Measurement	**Median (IQR)**	**P-value**	**Median (IQR)**	**p-value**
Vitamin B_{12} (pmol/L)				
Baseline	285.5 (216.5 - 371.8)	<0.001	280.0 (211.5 - 386.3)	0.78
6 months	401.5 (264.3 - 518.8)		288.5 (198.8 - 391.0)	
Change	90.5 (-0.8 - 203.5)		10.0 (-73.8 - 83.8)	
Folate (nmol/L)				
Baseline	17.3 (13.5 - 26.6)	<0.001	15.7 (11.9 - 22.1)	0.44
6 months	27.7 (21.1 - 33.4)		16.5 (11.7 - 22.1)	
Change	8.0 (-0.3 - 17.1)		-0.6 (-3.5 - 5.8)	
Haemoglobin (g/dl)				
Baseline	10.0 (8.7 - 11.2)	0.04	9.8 (8.8 - 11.2)	<0.001
6 months	10.9 (9.4 - 11.7)		10.6 (9.6 - 11.7)	
Change	0.3 (-0.4 - 0.9)		0.6 (-0.2 - 1.4)	
CD4+ count (cells/μL)				
Baseline	1201 (822 - 1556)	0.16	1033 (728 - 1406)	0.52
6 months	1039 (725 - 1358)		1043 (704 - 1484)	
Change	-137 (-348 - 254)		35 (-278 - 352)	

Wilcoxon Signed Ranks test was used to measure the difference between baseline and 6 months.

Vitamin B_{12} and folate status at 6 months of supplementation

Overall, 42 (19.6%) children had low concentrations of vitamin B_{12} at 6 months, 9 (4.2%) with very low and 33 (15.4%) with marginal concentrations. Nine children (1.4%) were in the MMS and 33 (78.6%) in the MV group. This was a statistically significant difference. The odds ratio was 4.5 (95% CI; 2.0 - 10.0). Folate concentrations were low in 44/214 (20.6%) children at 6 months of follow up; almost all of them had marginal concentrations. Five children (11.4%) were supplemented with MMS and 39 (88.6%) MV; Odds ratio 10.8 (95% CI; 4.1 - 28.9). Low vitamin B_{12} and folate concentrations were more frequent in the MV supplemented group.

Other clinical and haematological findings

Eight of the 214 children had signs of neurological disease, 6 with delayed or loss of developmental milestones, 3 of whom had low or marginal vitamin B_{12} status. There was a significant increase in the haemoglobin status in both groups as shown in table 2. There was no significant change in the CD4 cell counts in either the MMS or MV group.

HAART strata and initiation of HAART during the study

Of the 44 children on HAART at enrolment, 15 (34.1%) and 10 (22.7%) had low vitamin B_{12} and low folate concentrations, respectively. The prevalence of low micronutrient status of these two was not significantly different between the HAART and non-HAART treated children. Of the 170 HAART naïve children, 21 started HAART during the study; 13 in MMS and 8 in MV group. This difference was not significant, p = 0.35. Seven of the 21 children who initiated HAART during the study had low vitamin B_{12} concentrations at enrolment.

Factors associated with low vitamin B_{12} or folate concentrations

Vitamin B_{12} status was not closely associated with most of the baseline characteristics (Table 3). However being male, age less than 24 months and a haemoglobin < 11 g/dl were associated with low folate concentrations. At multivariate analysis only age and the male sex remained significantly associated with low serum folate concentrations.

Discussion

This paper describes the vitamin B_{12} and folate concentrations of HIV-infected Ugandan children aged 1-5 years and the effect of 2RDA of 14 multiple micronutrients that contained vitamin B_{12} and folate versus the 'standard of care' multivitamins in 1RDA without vitamin B_{12} and folate. Almost a third of the children had low vitamin B_{12} and low folate concentrations at baseline. Very low serum concentrations were uncommon. MMS containing vitamin B_{12} and folate improved both vitamin B_{12} and folate concentrations compared to the 'standard of care' multivitamins.

Vitamin B_{12} and folate concentrations in our study are comparable or slightly lower than what has been reported in children living in other low-income countries [1,2,4,17]. Our findings are also comparable to what other studies in HIV infected adults reported before the HAART era. These studies showed that low vitamin B_{12} concentrations were relatively common with a prevalence ranging between 10 and 35% [18-22].

There are few studies that have examined vitamin B_{12} and folate in HIV infected children in Africa. The prevalence of low vitamin B_{12} concentrations in our study was much higher than the prevalence of 5% reported in South African HIV infected children [23]. Contrary to our findings a study of HIV infected children in New York showed elevated vitamin B_{12} and folate status [24]. Our study included younger children, the majority of whom were symptomatic and not on HAART compared to the New York study. In our study the lack of differences between the HAART and non-HAART stratum in baseline vitamin B_{12} or folate concentrations could be explained by the short duration of HAART compared to other studies.

Twice the recommended dietary allowance of multiple micronutrients improved vitamin B_{12} and folate status compared to the 'standard of care'. This is not surprising since the standard of care supplement did not contain vitamin B_{12} and folate. This implies that the standard of care multivitamin is not enough and many more micronutrients may be required to correct micronutrient deficiencies. A significant number of children still had low concentrations of vitamin B_{12} and folate at the end of 6 months, implying that the duration of supplementation needed to be extended.

Low vitamin B_{12} could be related to folate deficiency or to low vitamin B_{12} binding proteins. Although we did not find an association between low vitamin B_{12} and white blood cell count this does exclude the possibility that low vitamin B_{12} concentrations could be related to neutropenia.

We observed that there was an association between low folate concentrations and low haemoglobin which indicates that the anaemia could partly be attributed to low folate concentrations. In both treatment groups the haemoglobin improved compared to baseline levels. We could not conclusively attribute the anaemia to low folate concentrations since we did not measure red blood cell folate concentrations. Measuring both serum folate and Red cell folate would have yielded more diagnostic information of folate deficiency. However sub-

Table 3 Factors associated with low vitamin B_{12} and folate among HIV infected children in Uganda

Baseline characteristics	Number N	Low vitamin B_{12} status[a] n (%)	Un adjusted OR (95% CI)	Adjusted OR (95%CI)
Vitamin B_{12} status				
Age < 24 months	70	19 (27.1)	0.9 (0.6 - 1.5)	
Age ≥ 24 months	144	41 (28.5)		
Male	100	33 (33.0)	1.4 (0.9 - 2.1)	1.6 (0.8 - 3.1)
Female	114	27 (23.7)		
Weight for height *z* score < -2	28	7 (25.0)	0.9 (0.4 - 1.7)	
Weight for height *z* score ≥ -2	182	52 (28.6)		
Haemoglobin < 11 g/dl	127	37 (29.1)	1.1 (0.7 - 1.7)	
Haemoglobin ≥ 11 g/dl	87	23 (26.4)		
CD4+ < 25%	114	33 (28.9)	1.1 (0.6 - 2.1)	
CD4+ ≥ 25%	85	23 (27.1)		
On HAART	44	15 (34.1)	1.3 (0.8 - 2.1)	
HAART naïve	170	45 (26.5)		
Routine CTX prophylaxis	193	56 (29.0)	1.5 (0.6 - 3.8)	
No routine CTX prophylaxis	21	4 (19.0)		
Previous routine multivitamins	144	40 (27.8)	1.0 (0.6 - 1.5)	
No routine multivitamins	70	20 (28.6)		
Folate status		**Low folate**		
Age < 24 months	70	27 (38.6)	1.6 (1.1 - 2.4)	2.2 (1.1 - 4.5)
Age ≥ 24 months	144	35 (24.3)		
Male	100	36 (36.0)	1.6 (1.0 - 2.4)	2.1 (1.1 - 4.0)
Female	114	26 (22.8)		
Weight for height *z* score < -2	28	7 (25.0)	0.8 (0.4 - 1.7)	
Weight for height *z* score ≥ -2	182	54 (29.7)		
Haemoglobin < 11 g/dl	127	45 (35.4)	1.8 (1.1 - 2.9)	1.9 (0.9 - 4.6)
Haemoglobin ≥ 11 g/dl	87	17 (19.5)		
CD4+ < 25%	114	36 (31.6)	1.2 (0.7 - 1.9)	
CD4+ ≥ 25%	85	22 (25.9)		
On HAART	44	10 (22.7)	0.7 (0.4 - 1.3)	
HAART naïve	170	52 (30.6)		
Routine CTX prophylaxis	193	58 (30.1)	1.6 (0.6 - 3.9)	
No routine CTX prophylaxis	21	4 (19.0)		
Previous routine multivitamins	144	44 (30.6)	1.2 (0.7 - 1.9)	
No routine multivitamins	70	18 (25.7)		

[a]Low vitamin B_{12} < 221 pmol/L,
Low folate < 13.4 nmol/L

normal serum folate is a useful indicator of folate status that warrants reporting. In a landscape of multiple deficiencies, such as HIV-infected children in a low-income country, there is always the potential for other deficiencies to alter the response, for instance, iron deficiency (the supplement did not contain iron). Folate deficiency has been reported in almost one in two anaemic HIV infected patients [25]. Other authors have reported associations between anaemia, gender and folate deficiency in children whose HIV infection status was not known [26].

Neither MMS nor the 'standard of care' MV improved the immunological status of the study children. In fact there was a slight deterioration in the CD4+ cell counts among the HAART naïve MMS group. This was contrary to findings of a trial in HAART treated HIV infected adults living in the USA where multiple micronutrient supplementation was associated with improved CD4+ cell count compared to a placebo [27]. The lack of effect on CD4+ cell count in our study could be explained by the natural immunological deterioration or it may be apparent due to the small numbers in the HAART group. It is also possible that the supplementation was not long enough to show an impact on CD4+ cell count since one in five children still had low vitamin B_{12} and folate concentrations at 6 months of follow up.

In our setting it is possible that low concentrations of vitamin B_{12} and folate may be related to consumption of marginal or low levels of vitamin B_{12} and folate since twice the recommended dietary intakes increased serum concentrations as opposed to the multivitamin supplement which did not contain vitamin B_{12} or folate. Some authors have shown that Vitamin B_{12} deficiency is rare in HIV infected persons consuming vitamin B_{12} well above the recommended nutrient intakes [28]. In Uganda one third of children aged between 6 and 24 months are likely to be getting diary products in their diet and very few are likely to be getting other animal source foods [29].

We are not certain whether our findings are similar to children in the general population since we had no control group of HIV uninfected children. A study of both HIV-infected and exposed uninfected children in Brazil showed no differences in micronutrient status [30]. However another study indicated that HIV infected children had significantly lower folate levels than the reference children while vitamin B_{12} was similar [31].

We were unable to measure serum homocysteine and methyl malonic acid concentrations which are more reliable indicators of vitamin B_{12} deficiency because of the limited amount of blood that we could draw from the children as we had multiple micronutrients to test for. We also did not conduct absorption studies to examine the impact of malabsorption on baseline micronutrient and post-supplementation status. This paper does not describe the dietary habits of the study children and whether they had an impact on the outcome.

Conclusion

Low vitamin B_{12} and folate concentrations are common in Ugandan HIV infected children aged 1-5 years. Twice the recommended dietary allowance of 14 multiple micronutrients as opposed to the 6 multivitamin 'standard of care' supplement improved the vitamin B_{12} and folate status of HIV-infected children in Uganda.

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Authors' contributions

GN participated in the conception, design and implementation of the study, statistical analysis, interpretation and writing of the manuscript. JKT participated in the conception, design and implementation of the study, statistical analysis, interpretation and drafting of the manuscript. CMN participated in the design and implementation of the study. BJB participated in the design, supervised the laboratory work and drafting of the manuscript. TT participated in the conception, design and implementation of the study, statistical analysis, interpretation and drafting of the manuscript. All the authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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References

1. Allen LH, Rosado JL, Casterline JE, Martinez H, Lopez P, Munoz E, Black AK: **Vitamin B-12 deficiency and malabsorption are highly prevalent in rural Mexican communities.** *Am J Clin Nutr* 1995, **62(5)**:1013-1019.
2. Siekmann JH, Allen LH, Bwibo NO, Demment MW, Murphy SP, Neumann CG: **Kenyan school children have multiple micronutrient deficiencies, but increased plasma vitamin B-12 is the only detectable micronutrient response to meat or milk supplementation.** *J Nutr* 2003, **133(11 Suppl 2)**:3972S-3980S.
3. García-Casal MN, Osorio C, Landaeta M, Leets I, Matus P, Fazzino F, Marcos E: **High prevalence of folic acid and vitamin B12 deficiencies in infants, children, adolescents and pregnant women in Venezuela.** *Eur J Clin Nutr* 2005, **59(9)**:1064-1070.
4. Taneja S, Bhandari N, Strand TA, Sommerfelt H, Refsum H, Ueland PM, Schneede J, Bahl R, Bhan MK: **Cobalamin and folate status in infants and young children in a low-to-middle income country in India.** *Am J Clin Nutr* 2007, **86(5)**:1302-1309.
5. Subar AF, Krebs-Smith SM, Cook A, Kahle LL: **Dietary sources of nutrients among US children, 1989-1991.** *Pediatrics* 1998, **102(4 Pt 1)**:913-923.
6. **Micronutrient Information Center.** [http://lpi.oregonstate.edu/infocenter/].
7. Monsen AL, Refsum H, Markestad T, Ueland PM: **Cobalamin status and its biochemical markers methylmalonic acid and homocysteine in different age groups from 4 days to 19 years.** *Clin Chem* 2003, **49(12)**:2067-2075.
8. Zengin E, Sarper N, Caki Kiliç S: **Clinical manifestations of infants with nutritional vitamin B deficiency due to maternal dietary deficiency.** *Acta Paediatr* 2009, **98(1)**:98-102.
9. Baum MK, Shor-Posner G, Lu Y, Rosner B, Sauberlich HE, Fletcher MA, Szapocznik J, Eisdorfer C, Buring JE, Hennekens CH: **Micronutrients and HIV-1 disease progression.** *AIDS* 1995, **9(9)**:1051-1056.
10. Baylin A, Villamor E, Rifai N, Msamanga G, Fawzi WW: **Effect of vitamin supplementation to HIV-infected pregnant women on the micronutrient status of their infants.** *Eur J Clin Nutr* 2005, **59(8)**:960-968.
11. Ash DM, Tatala SR, Frongillo EA Jr, Ndossi GD, Latham MC: **Randomized efficacy trial of a micronutrient-fortified beverage in primary school children in Tanzania.** *Am J Clin Nutr* 2003, **77(4)**:891-898.
12. Osei AK, Rosenberg IH, Houser RF, Bulusu S, Mathews M, Hamer DH: **Community-level micronutrient fortification of school lunch meals improved vitamin A, folate, and iron status of schoolchildren in Himalayan villages of India.** *J Nutr* **140(6)**:1146-1154.
13. Varma JL, Das S, Sankar R, Mannar MG, Levinson FJ, Hamer DH: **Community-level micronutrient fortification of a food supplement in India: a controlled trial in preschool children aged 36-66 mo.** *Am J Clin Nutr* 2007, **85(4)**:1127-1133.
14. Chatterjee A, Bosch RJ, Hunter DJ, Manji K, Msamanga GI, Fawzi WW: **Vitamin A and vitamin B-12 concentrations in relation to mortality and morbidity among children born to HIV-infected women.** *J Trop Pediatr* 2010, **56(1)**:27-35.
15. Ndeezi G, Tumwine JK, Bolann BJ, Ndugwa CM, Tylleskar T: **Zinc status in HIV infected Ugandan children aged 1-5 years: a cross sectional baseline survey.** *BMC Pediatr* 2010, **10(1)**:68.

16. Carmel R, Green R, Rosenblatt DS, Watkins D: **Update on cobalamin, folate, and homocysteine.** *Hematology Am Soc Hematol Educ Program* 2003, 62-81.
17. Rogers LM, Boy E, Miller JW, Green R, Sabel JC, Allen LH: **High prevalence of cobalamin deficiency in Guatemalan schoolchildren: associations with low plasma holotranscobalamin II and elevated serum methylmalonic acid and plasma homocysteine concentrations.** *Am J Clin Nutr* 2003, **77(2)**:433-440.
18. Harriman GR, Smith PD, Horne MK, Fox CH, Koenig S, Lack EE, Lane HC, Fauci AS: **Vitamin B12 malabsorption in patients with acquired immunodeficiency syndrome.** *Arch Intern Med* 1989, **149(9)**:2039-2041.
19. Rule SA, Hooker M, Costello C, Luck W, Hoffbrand AV: **Serum vitamin B12 and transcobalamin levels in early HIV disease.** *Am J Hematol* 1994, **47(3)**:167-171.
20. Remacha AF, Cadafalch J: **Cobalamin deficiency in patients infected with the human immunodeficiency virus.** *Semin Hematol* 1999, **36(1)**:75-87.
21. Tang AM, Graham NM, Chandra RK, Saah AJ: **Low serum vitamin B-12 concentrations are associated with faster human immunodeficiency virus type 1 (HIV-1) disease progression.** *J Nutr* 1997, **127(2)**:345-351.
22. Hepburn MJ, Dyal K, Runser LA, Barfield RL, Hepburn LM, Fraser SL: **Low serum vitamin B12 levels in an outpatient HIV-infected population.** *Int J STD AIDS* 2004, **15(2)**:127-133.
23. Eley BS, Sive AA, Abelse L, Kossew G, Cooper M, Hussey GD: **Growth and micronutrient disturbances in stable, HIV-infected children in Cape Town.** *Ann Trop Paediatr* 2002, **22(1)**:19-23.
24. Malik ZA, Abadi J, Sansary J, Rosenberg M: **Elevated levels of vitamin B12 and folate in vertically infected children with HIV-1.** *AIDS* 2009, **23(3)**:403-407.
25. Castro L, Goldani LZ: **Iron, folate and vitamin B12 parameters in HIV -infected patients with anaemia in southern Brazil.** *Trop Doct* 2009, **39(2)**:83-85.
26. Pasricha SR, Black J, Muthayya S, Shet A, Bhat V, Nagaraj S, Prashanth NS, Sudarshan H, Biggs BA, Shet AS: **Determinants of anemia among young children in rural India.** *Pediatrics* 2010, **126(1)**:e140-149.
27. Kaiser JD, Campa AM, Ondercin JP, Leoung GS, Pless RF, Baum MK: **Micronutrient Supplementation Increases CD4 Count in HIV-Infected Individuals on Highly Active Antiretroviral Therapy: A Prospective, Double-Blinded, Placebo-Controlled Trial.** *J Acquir Immune Defic Syndr* 2006, **42(5)**:523-528.
28. Dowling S, Lambe J, Mulcahy F: **Vitamin B12 and folate status in human immunodeficiency virus infection.** *Eur J Clin Nutr* 1993, **47(11)**:803-807.
29. Wamani H, Astrom AN, Peterson S, Tylleskar T, Tumwine JK: **Infant and young child feeding in western Uganda: knowledge, practices and socio-economic correlates.** *J Trop Pediatr* 2005, **51(6)**:356-361.
30. Monteiro JP, Freimanis-Hance L, Faria LB, Mussi-Pinhata MM, Korelitz J, Vannucchi H, Queiroz W, Succi RC, Hazra R: **Both human immunodeficiency virus-infected and human immunodeficiency virus-exposed, uninfected children living in Brazil, Argentina, and Mexico have similar rates of low concentrations of retinol, beta-carotene, and vitamin E.** *Nutr Res* 2009, **29(10)**:716-722.
31. Vilaseca MA, Sierra C, Colomé C, Artuch R, Valls C, Muñoz-Almagro C, Vilches MA, Fortuny C: **Hyperhomocysteinaemia and folate deficiency in human immunodeficiency virus-infected children.** *Eur J Clin Invest* 2001, **31(11)**:992-998.

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