HIV-INFECTION IN SUB-SAHARAN AFRICA
From Quantity to Quality of Care

Minke Huibers

HIV-infection in sub-Saharan Africa; *from quantity to quality of care*

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Faculteit der Geneeskunde

Let us do research;
to improve care for those who need a better future.

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Part III HIV-infection in sub-Saharan Africa; Summary, considerations, implications and overall conclusion

Chapter 1

General introduction and outline of the thesis

Human Immunodeficiency Virus (HIV)

In the early 80s a mysterious and deadly disease was reported in the United States of America (1). Men who had sex with men suffered from various and ultimately lethal infections that appeared to be caused by an impaired immune system. Other at-risk groups that were later described included intravenous drug users, donor blood recipients and, lastly, heterosexuals. The disease was named Acquired Immune Deficiency Syndrome (AIDS), due to the clinical presence of a malfunctioning immune system. In 1984, Human Immunodeficiency Virus (HIV) was identified as the virus responsible for AIDS (1). HIV is a retrovirus that disrupts the host immune system by targeting and reducing the CD4-expressing white blood cells that regulate the immune response. Once infected, the host is unable to eliminate HIV, leading to progressive HIV infection, depletion of CD4 cells, immune deficiency and severe (opportunistic) infections from otherwise non-hazardous pathogens, ultimately leading to death.

HIV| The global burden

Since its recognition, the number of HIV infections has increased rapidly and the HIV epidemic has become one of the biggest challenges facing global health (1). So far, over 70 million people worldwide have been infected. Sub-Saharan Africa is the continent with the highest burden of HIV, where AIDS is the number one cause of death (2). Overall, in 2017 an estimated 36.7 million people were living with HIV worldwide, of which 1.8 million were children. Almost 90% of these children live in sub-Saharan Africa (2, 3).

HIV| Anti-Retroviral Treatment

Initially, successful treatments for HIV/AIDS involved drugs that had been developed to treat cancer (4). Azidothymidine, the first anti-retroviral treatment (ART), became available in 1987, and is a Nucleoside Reverse Transcriptase Inhibitor (NRTI). It directly inhibits reverse transcriptase, an enzyme that the HIV-virus uses to transcribe viral RNA into DNA, a crucial step in the replication of the virus. Initially Azidothymidine was given as a single agent, and markedly improved survival of HIV-infected patients. Unfortunately resistance to these treatments developed rapidly, a pattern that repeated each time new NRTI drugs were launched (5). Furthermore,

new drug classes, such as non-Nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs) were discovered. It was found that when drugs were given in combinations, the problem of resistance development was partly overcome. The first combination therapies were known as Highly Active Retroviral Therapy (HAART) and later as combined ART (cART). Currently, cART is called ART and includes a combination treatment of three types of ART from two or more different drug classes. Because NNRTIs and PIs act at different steps of the HIV life cycle (figure 1), a combination of these drugs maximally suppresses the replication of the virus.

In the last decade more classes of ART were discovered including intergrase inhibitors, fusion inhibitors and CCR5 antagonists. Currently these newer medications are not regularly available in sub-Saharan Africa. Despite these rapid developments and some interesting case reports, HIV can still not be cured as ART only suppresses HIV replication.

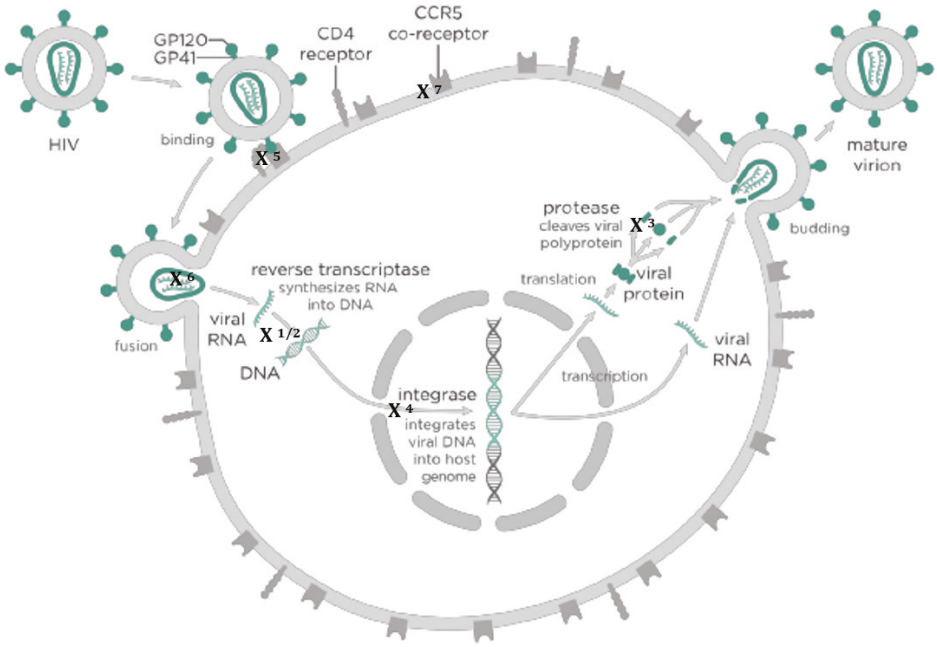

Figure 1: The life cycle of the Human Immunodeficiency Virus.
The different mechanisms of antiretroviral medication: $X^{1/2}$ (non) nucleoside reverse transcriptase inhibitors ((N)NRTI), X^{3} protease inhibitors, x^{4} intergrase inhibitors, X^{5} entry inhibitors including CCR5 antagonists (x^{5*}), x^{6}fusion inhibitors. *Source: Wikipedia*.

HIV| The public health approach

Despite the huge success implementing ART in the developed world at the end of the 20th century, the vast majority of HIV-infected people lived in low and middle-income countries (LMIC) and did not have access to the treatments. By the end of 2001, of the estimated 40 million people who were infected with HIV, only 1 million had access to adequate ART (6). Furthermore, HIV treatment guidelines emerging in the developed world required therapeutic and advanced laboratory resources that were not available in LMIC. A public health approach to make ART accessible and available in LMIC was introduced in 2002 (6). It included guaranteed supplies of drugs, minimal laboratory monitoring and standardized and simplified treatment protocols using a decentralized approach, with tasks distributed to all levels of health-workers.

Despite initial concerns about this public health approach, access to ART increased and life expectancy for HIV-infected patients in LMIC improved drastically. In 2014, a decade after the introduction of widespread ART to LMIC, two major programs were developed in addition to optimize the treatment of HIV worldwide. The Joint United Nations Program on HIV/AIDS (UNAIDS) launched "90-90-90" with the aim that by 2020, 90% of all people living with HIV would know their status, 90% of all people with diagnosed HIV infection would receive sustained ART and 90% of all people taking ART would have suppressed viral loads. Mathematical models showed that if these goals were met, HIV transmission could be stopped and AIDS could be eliminated as a public health threat by 2030 (7). In 2016 the World Health Organization introduced the "treat all" policy, which implemented a second major public health change in the global approach to HIV treatment. The "treat all" policy aimed to start ART in all HIV-infected persons regardless of their CD4 count (8). This recommendation was based upon strong evidence that early initiation of ART resulted in better clinical outcomes for people living with HIV, as compared to those whose treatment was delayed (8), further simplifying the decision to start ART. By mid-2018, 84% of LMIC adopted the treat all policy. By the end of 2020, given current commitments, 92% of all LMIC will have adopted the World Health Organization's " treat all" policy (9).

Despite some early scepticism both concepts proved to be feasible and HIV has changed from a lethal to a treatable disease in the eyes of healthcare professionals in LMIC (9). The number of people receiving ART increased from 1

million in 2001 to 21.7 million in December 2017, representing 59% of HIV-infected patients worldwide (10) .

Challenges

Challenges| HIV resistance

The World Health Organization recently reported a 4-25% prevalence rate of NNRTI resistance among adults retained on ART while rates of 47%-90% were found in unsuppressed HIV-infected patients (14). Urgent action is needed to restrict the increase of HIV drug resistance in the coming decade(s) in order to prolong the current success of ART in LMIC.

HIV creates, as part of its replication, billions of viruses each day, which also generates variations in the genetic structure (mutations) of these new viruses on daily basis. When these mutations occur during replication, whilst being exposed to a particular antiretroviral treatment, drug resistance may develop. The mutated virus may co-exist with the initial virus type (wild type) and may even become the dominant virus type. This may lead to disease progression in the host despite ART and can even infect other sexual partners or children born to HIV-infected mothers. This process is defined as transmitted drug resistance (11).

It is important to recognise factors influencing the development of HIV drug resistance. These factors are commonly subdivided into patient specific and programmatic factors. Patient factors include pre-treatment drug resistance and low adherence to ART (12-14). For children and adolescents, irregular clinical follow-up and HIV-associated stigma can especially compromise compliance. Programmatic issues that result in sub-optimal delivery of HIV care are a realistic threat in LMICs. Factors such as stock-outs of ART and limited human resources have direct and indirect negative impacts on the quality of treatment. HIV drug resistance is a growing global challenge.

Challenges| HIV in children

Over the past decade, the treatment of HIV-infected children has focused on the prevention of mother-to-child transmission (PMTCT) programmes, which has drastically reduced HIV transmission in LMIC (15) and facilitated the early diagnosis

of children who are infected despite PMTCT. Early detection and ART treatment in the first 24 months of life are of importance as they have improved life expectancy to approximately 91% (16). Despite these successes, the long-term outcome of those infected, as an increasing number of children live with HIV in LMIC, remains poor. Suboptimal viral suppression and treatment failure occurs in the first 12 months on ART in as many as one third of children (17-21). Compared to adults infected with HIV, the percentage of children harbouring resistant viral strains of HIV is very high.

The higher prevalence of resistance amongst children, as compared to adults may be explained by several factors. Firstly, children who are infected despite PMTCT have an increased risk of acquiring a drug resistant strain of HIV (19). Secondly, adherence to clinic visits and compliance with treatment may be poor since young children are fully dependent on their caregivers, who are commonly infected themselves or may even have died. Thirdly, paediatric treatment options and paediatric drug formulations are limited. This reduces the choice of alternative treatments in those who experience HIV drug toxicity or resistance (13, 17, 22). Lastly, significant changes in body weight due to malnutrition and catch-up growth during ART, as well normal physiological changes in childhood, cause constant changes in pharmacokinetics, which can result in suboptimal dosing of ART causing treatment failure and an increased risk of developing acquired drug resistant mutations (14).

Comorbidities

With the increased availability of ART in LMIC the focus of treatment is shifting from lifesaving to improving quality of life by treatment of comorbidities that negatively affect it. Common problems that occur in HIV-infected persons, especially in LMICs, include malnutrition, chronic diarrhoea and anaemia, amongst others.

Comorbidities| Diarrhoea

Acute diarrhoea is a major cause of death in LMIC, especially in young children if occurring in combination with malnutrition and/or HIV (23). Among children in sub-Saharan Africa, diarrhoea is the second leading cause of death (24). Diarrhoea is defined as the passage of three or more loose/liquid stools per day, or more frequent passage than normal for the individual. In the context of HIV it may more commonly

present as a chronic condition, defined as loose stools lasting for at least 4 weeks (25). In children infected with HIV several opportunistic pathogens can cause chronic diarrhoea, resulting in worsening malnutrition and increased risk of death (26).

The most common causes of diarrhoea in HIV-infected children are opportunistic infections with bacteria, viruses, protozoa, parasites and fungi. Secondary factors include food intolerance, side effects of HIV-related medication, HIV-enteropathy and nutrition deficiencies (25). Co-trimoxazole prophylaxis, prescribed as part of PCP infection prevention, has also been shown to reduce diarrheal illness in HIV–infected patients, possibly by treating bacterial gastrointestinal super-infections, prompting the World Health Organization to recommend co-trimoxazole prophylaxis for HIV-infected patients worldwide (27). Prevalence data on protozoal infections in patients with chronic diarrhoea range widely from 1-75%, and differ in demographics, seasonal variance and diagnostic methods (28, 29). Prevalence rates are higher when tested with sensitive Polymerase Chain Reaction (PCR) techniques in comparison to traditional microscopy (30, 31). However, PCR techniques are not commonly available in resource-limited settings and therefore reliable data from these areas are scarce (32). Interestingly, the prevalence of intestinal protozoa in HIV-infected adults after the introduction of ART has had a documented decreased both in industrialized nations and resource-limited settings (32-35). However, prospective data on children receiving ART treatment is lacking. Longitudinal data is needed in order to clarify which opportunistic infection may resolve through immune reconstitution due to ART alone and which infections might require targeted therapy (32).

Comorbidities| Anaemia

Anaemia is the most common haematological disorder among HIV-infected patients worldwide with an estimated prevalence of 60-95% (36). The World Health Organization defines anaemia as haemoglobin levels below 110-120 g/l depending on age. Severe anaemia is defined as a haemoglobin levels below 70 g/l (37). In sub-Saharan Africa, 60% of HIV-infected adults are anaemic and 22% are severely anaemic (38, 39). Moreover, in these settings, HIV-associated anaemia and severe anaemia are both independently associated with an increased one-year mortality of 8% and 55% respectively (40, 41). Anaemia can present with a wide range of clinical symptoms including fatigue, palpitations, shortness of breath and dizziness.

Fatigue is the most important symptom of anaemia and is strongly associated with a reduction in quality of life (42).

HIV-associated anaemia is typically multifactorial. Aetiologies include direct effect of HIV by apoptosis of bone marrow progenitors, micronutrient deficiencies due to malnutrition, infections, neoplastic disease and side effects of anti-retroviral treatment, such as with Zidovudine and Co-trimoxazole (44-48). Consequently, even in the presence of malaria parasites, the most common cause of severe anaemia in sub-Saharan Africa, additional or alternative causes should be considered (49). Micronutrient deficiencies associated with anaemia in HIV-infected adults include shortages of iron, folate or vitamin B12. Although iron deficiency is common in sub-Saharan Africa and likely contributes to HIV-associated moderate anaemia, its role in the development of severe anaemia is not fully understood. Most of the comprehensive studies suggesting that the role of iron is not as important in HIV-infected patients when compared with non-HIV-infected patients have been performed in children (50, 51). The data on adults is limited and comprehensive clinical data are needed to support the development of evidence-based guidelines for the prevention and treatment of severe anaemia in HIV-infected adults in this setting (52).

This thesis

This thesis| Research setting

The research presented in this thesis is based on clinical studies performed in Malawi and Uganda (figure 2).

This thesis| Research setting| Malawi

Malawi is a densely populated country located in south-east Africa with over 18 million inhabitants, of which half are under the age of 15 (Figure 3). Malawi is one of the poorest countries in the world with 50.7% of the population living in poverty and 25% in extreme poverty (53). HIV is highly prevalent with over one million people infected, of which 10% are below 15 years of age. HIV prevalence among 15-49 year-olds declined from a peak of 16.7% in 1999 to 10.6% in 2016 (54). Since 2005,

Figure 2. Study sites in this thesis; Malawi & Uganda (*).
This map was created with mapchart.net.

HIV treatment has been offered free of charge through the Malawi National AIDS program. In 2018 an estimated 69% of the HIV-infected patients were on ART (55).

The studies presented in this thesis were performed in Queen Elizabeth Central Hospital in Blantyre in collaboration with the College of Medicine Malawi, MLW (Malawi-Liverpool-Wellcome Trust) laboratories, Blantyre and the Global Child Health Group, Emma Children's Hospital Amsterdam University Medical Centres. The Queen Elizabeth Central Hospital is an academic referral hospital that serves Blantyre district, a (semi-) urban area in the Southern region of Malawi.

This thesis| **Research setting| Uganda**

Uganda is located in Eastern Africa with a total population of 40 million (2017) of which 55% are below 18 years of age (Figure 2). According to the World Bank's 2016 poverty assessment, poverty in Uganda between 2006 and 2013 declined rapidly. The population living below the national poverty line declined from 31.1% in 2006

to 19.7% in 2013 (56). In 2016 the total number of people with HIV in Uganda was 1.3 million, which is 6% of the population, reduced from 7.3% in 2011 (57).

The Ugandan study presented in this thesis formed part of the Pan African Studies to Evaluate Resistance (PASER). The PharmAccess Foundation and the Amsterdam Institute for Global Health and Development coordinated this program. PASER was established in 2006 as a multi-country capacity building and research program in collaboration with the World Health Organization Global HIV Drug Resistance Network (HIVResNet), for the assessment and prevention of HIV drug resistance in sub-Saharan Africa (58). The PASER program on African adults has been supplemented with similar paediatric studies: Monitoring Antiretroviral Resistance in Children (MARCH). The aim of MARCH was to strengthen the capacity of HIV drug resistance monitoring in children and to optimize care and treatment guidelines for paediatric ART programs in sub-Saharan Africa (19). The MARCH cohort was a prospective, observational cohort study of HIV-infected children under 12 years of age. In 2010 over 300 children were included in three sites in Uganda (19, 59).

This thesis| Outline of this thesis

This thesis focuses on various aspects of care of HIV-infected children and adults living in Sub-Sahara Africa and aims to gain further insights that will improve HIV treatment and optimize long-term survival in vulnerable patients where resources are limited.

Part I of the thesis focuses on the treatment of HIV-infected children in sub-Sahara Africa, in particular when treatment should be started and how to monitor treatment in a low resource setting. In **Chapter 2** we assess alternative diagnostic strategies to identify which HIV-infected Malawian children should qualify for ART, a decision based on regular testing (CD4 and viral load testing). **Chapter 3 and 4** present treatment outcomes, including viral failure and the development of drug-resistant mutations, in two cohorts of HIV-infected children in Malawi and Uganda respectively. In addition, in **Chapter 4** we focus on treatment failure and the differences between treatment failure during short (< 24 months) and long-term (24-48 months) ART in a cohort of Ugandan children.

Part II of the thesis evaluates common comorbidities of HIV in children and adults living in sub-Saharan Africa. In **Chapter 5** we describe the prevalence and clinical relevance of old and new intestinal protozoa in HIV-infected children

in Malawi using a multiplex real-time PCR platform. In **Chapter 6** we describe prevalence of potential aetiologies and outcome in HIV-infected adults presenting with severe anaemia (Haemoglobin (Hb) ≤ 70 g/l) in Malawi. Finally, in **Chapter 7**, we describe the importance of iron deficiency in this population as well as how to diagnose iron deficiency in this specific patient population.

Part III of the thesis is by **Chapter 8 and 9** used for an overall summary, considerations, implications and overall conclusion.

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Part I

HIV-infection in sub-Saharan Africa; treatment and monitoring

Chapter 2

An evaluation of alternative markers to guide initiation of anti-retroviral therapy in HIV-infected children in settings where CD4 assays are not available

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Abstract

Background: In settings where CD4-testing is not available, alternative markers to start paediatric anti-retroviral therapy (ART) could be used. A comprehensive evaluation of these markers has not been performed.

Methods: Prospective cross-sectional study of HIV-infected Malawian children not eligible for ART based on clinical criteria. Associations between CD4 and alternative markers (haemoglobin, total lymphocyte count (TLC), serum albumin, thrombocytes and growth parameters) were analysed and accuracy of existing and new cut-offs were evaluated.

Results: 417 children were enrolled. In 261 children aged 5 years or older, 155 (59%) qualified to start ART using CD4. In this group, only TLC was associated with CD4 ($p<0.001$). Sensitivity for TLC was 21% (95%-CI: 15-29%), using WHO cut-offs. Improved cut-offs increased sensitivity to 73% (95%-CI: 65-80%); specificity 62% (95%-CI: 52-72%).

Conclusion: Clinical staging alone is an unreliable strategy to start ART in children. TLC is the only alternative marker for CD4, cut-offs need to be revised though.

Background

HIV (human immunodeficiency virus) is a major cause of childhood morbidity and mortality in sub-Saharan Africa, where 90% of all HIV-infected children live (1, 2). Malawi is one of the most heavily affected countries with an estimated one million infected persons, including more than 120.000 children (2, 3). In the absence of anti-retroviral therapy (ART), the median survival of vertically infected African children is only 2 years (4). Increased access to ART has radically improved survival of these HIV-infected children (4, 5). Until 2013, the World Health Organization (WHO) recommended to commence ART in children two years of age or older based on an algorithm, using CD4 percentages or counts (6, 7). Since 2013 the WHO has recently modified their guidelines again and now recommend to start ART in all children less than five years regardless of their CD4 count (8). Therefore, only HIV-infected children of five years or older now require a CD4 count as part of the management algorithm.

However there are still settings where CD4 testing is still not available, or cost are too high, in which case the WHO clinical classification system i.e. 'clinical staging', occasionally combined with a total lymphocyte count (TLC), is used (7). Clinical staging may have major limitations because most clinical conditions used in the classification system are difficult, if not impossible, to diagnose especially in the same resource-limited settings where CD4 testing is not readily available (9). Further, the correlation of clinical staging with CD4 counts is poor, resulting in the misclassification of severely immune compromised children with low CD4 counts as clinical stage 1 or 2 (7). As a consequence, in resource-limited settings a considerable number of HIV-infected children that require ART are not enrolled in the government programmes and do not receive potentially life-saving ART.

These risks may be reduced by the introduction of alternative markers to identify adequate CD4. A well-known alternative marker used by the WHO is the TLC(7). Studies in resource-limited settings have found only a moderate correlation and a low sensitivity of TLC as a surrogate marker for CD4 (4, 10-12). Other markers that have been suggested in combination as surrogates for CD4 count include haemoglobin, platelet count, serum albumin and growth parameters (1,13-17). It remains unclear which marker or combination of markers best predicts CD4 values in African children because they have not previously been evaluated together in one

study population in a single study. We have therefore evaluated TLC, haemoglobin (Hb), platelet count, serum albumin and growth parameters as correlates of CD4 values.

Methods

Methods| Study population

A cross-sectional study of HIV-infected children was conducted in the Paediatric ART clinic of Queen Elizabeth Central Hospital, Blantyre, Malawi, from 2008-2010. On initial presentation to the clinic, all children underwent a full clinical evaluation including clinical staging. ART was commenced in those that were eligible according to the recommendations of the Malawi national guidelines. For purposes of this study, following written informed consent we enrolled HIV-infected children aged between 18 months and 18 years that were not yet eligible to start ART based on clinical staging according to the Malawi national guidelines which had been adapted from the WHO 2006 guidelines (7). This included children with stage 1, 2 or stage 3a disease (pulmonary tuberculosis, HIV-related chronic lung disease or weight for age < 2SD). Children who had received a blood transfusion in the previous month or children with a known cause of anaemia (e.g. sickle cell disease) were excluded.

Methods| Laboratory

HIV antibody testing (Abbott Determine HIV-1/2 Test, Uni-Gold HIV test) and counselling had been conducted prior to attending the HIV clinic. At enrolment for this study, blood was taken for full blood count (Beckman Coulter HMX, Beckman coulter, CA USA), CD4 count (flow cytometry, Becton Dickinson, CA USA), C-reactive protein and albumin (Modular P800 and Modular Analytics E170 systems, on a Roche 9000).

Methods| Definitions

Table 1 lists the WHO definitions applied in this study for anaemia, malnutrition and age-related indicators to start ART (4, 6, 7, 18-20). Young children are less than five years of age; older children are aged 5-18 years.

Methods| Statistical analysis

Data were entered and analysed using SPSS version 19.0 for MAC (IBM®). Baseline variables were analysed with Chi-square for dichotomous and categorical frequencies and with non-paired T-test or ANOVA for continuous variables. Univariate analysis and multi-variant linear regression analysis were used for the analysis of associations between CD4 count and CD4 percentage with potential predictors (haemoglobin, TLC, platelet count, albumin and growth parameters). CD4 cut-offs are based on the WHO 2013 guidelines(8). Predictors were included in a backward multi-variant model if a trend towards a univariate association was observed ($p<0.1$). The analysis was performed separately for children of less than 5 years and those aged 5 years and older (17). To optimize sensitivity and specificity, data were visualised by linear curves whereby potential new cut-offs were defined. All p-values presented are two-tailed and a significance level of <0.05 was used.

Methods| Ethical approval

The study was approved by the Research Ethics Committee of the College of Medicine, University of Malawi. The purpose of the study was explained to the guardians of each patient in Chichewa and written informed consent was obtained before inclusion into the study.

Malnutrition(20)	Definition
▪ Wasting	Weight-for height z score (WHZ)[1]/Body mass index z scores (BMIZ) <-2 SD[2]
▪ Severe wasting	Weight-for height z score (WHZ)[1]/Body mass index z scores (BMIZ) <-3SD[2]
▪ Stunting	Height-for-age z scores (HAZ)[1] <-2SD
▪ Severe stunting	Height-for-age z scores (HAZ)[1] <-3SD
▪ Underweight	Weight-for-age z scores (WAZ)[1] <-2SD
▪ Severe underweight	Weight-for-age z scores (WAZ)[1] <-3SD
Anaemia(18)	
▪ Age 18 months-59 months	Hb <11.0 g/dl
▪ Age 5-11.9 years	Hb <11.5 g/dl
▪ Boys age 12-15 years	Hb <12.0 g/dl
▪ Girls age > 12 years	Hb <12.0 g/dl
▪ Boys age > 15 years	Hb <13.0 g/dl
Indicators to start ART treatment	
CD4	
▪ Age 18-59 months (2008)	CD4 percentage <25% or CD4 count <750 cells/ mm^3
▪ Age 18-59 months (2013)	All
▪ Age >59 months (2008)	CD4 count <350 cells/ mm^3
▪ Age >59 months (2013)	CD4 count <500 cells/ mm^3
TLC(21)	
▪ Age 18-59 months	TLC <3000 cells/mm^3
▪ Age 36-59 months	TLC <2500 cells/mm^3
▪ Age 60-96 months	TLC <2000 cells/mm^3
▪ Age > 96 months	TLC <1200 cells/mm^3

Table 1. Definitions of malnutrition and anaemia and indicators for starting ART as used in this study.

[1]For children <5 years of age. [2] For children >5 years of age.

Results

In total, 417 children were enrolled in the study of which 261 (61%) were aged 5 years or older. Stage 1, 2 and 3a disease were diagnosed in 50%, 24% and 26% of children, respectively (Table 2). As defined by the selection criteria, none of the children qualified to start ART based on clinical staging at the time of enrolment.

After reviewing the CD4 results an additional 155 (59%) of 261 older children and all 156 young children would have qualified to start ART according to the WHO 2013 recommendations.

Results| Alternative markers and clinical staging groups

Admission variables and potential predictors stratified by age were compared between the three clinical stages; outcome is displayed in table 2. Children with clinical stage 1 disease had a significantly higher mean CD4 count/percentage compared with children with stage 2 and 3a disease combined (p≤0.03). Children with stage 3a disease were more commonly anaemic and had lower albumin levels compared with children with stage 1 and 2 combined (p=0.01 and p=0.01 respectively). No differences were found for TLC and platelets count between children in different staging groups.

Results| Alternative markers and CD4 results

Linear regression analysis among older children, identified univariate associations for CD4 count with TLC (beta=0.5 p <0.001) and BMI Z-score (BMIZ) (beta=0.1, p=0.05). Only the association with TLC remained significant in the multi-variant model (beta=0.5, p <0.001). In young children, univariate associations were found between CD4 percentage and TLC (beta=-0.2, p=0.003), height-for-age Z score (HAZ) (beta=0.2, p=0.004) and albumin (beta=0.2, p=0.016). In the multi-variant analysis, HAZ and TLC remained significantly associated with CD4 percentage (beta=0.2, p=0.006 and beta=-0.2, p=0.005 respectively). Linear regression outcome is displayed in table 3.

Results| Accuracy of alternative markers

The TLC would have correctly predicted ART eligibility on the basis of low CD4 results in 60 of 298 (20%) children if the WHO TLC cut-off were used, table 4. Sensitivity was similar in young and older children; 18,9%(95% CI: 13,0-26,2) for young children versus 21,3%(95% CI: 15,1-28,8) for older children, table 5. New TLC cut-off values were explored to improve the sensitivity of TLC in the older children. Optimal cut-offs were in general higher than WHO cut-offs: 3000 versus 2000 cells/mm^3 for the 59-96 months age group and 2500 versus 1200 cells/mm^3 for children

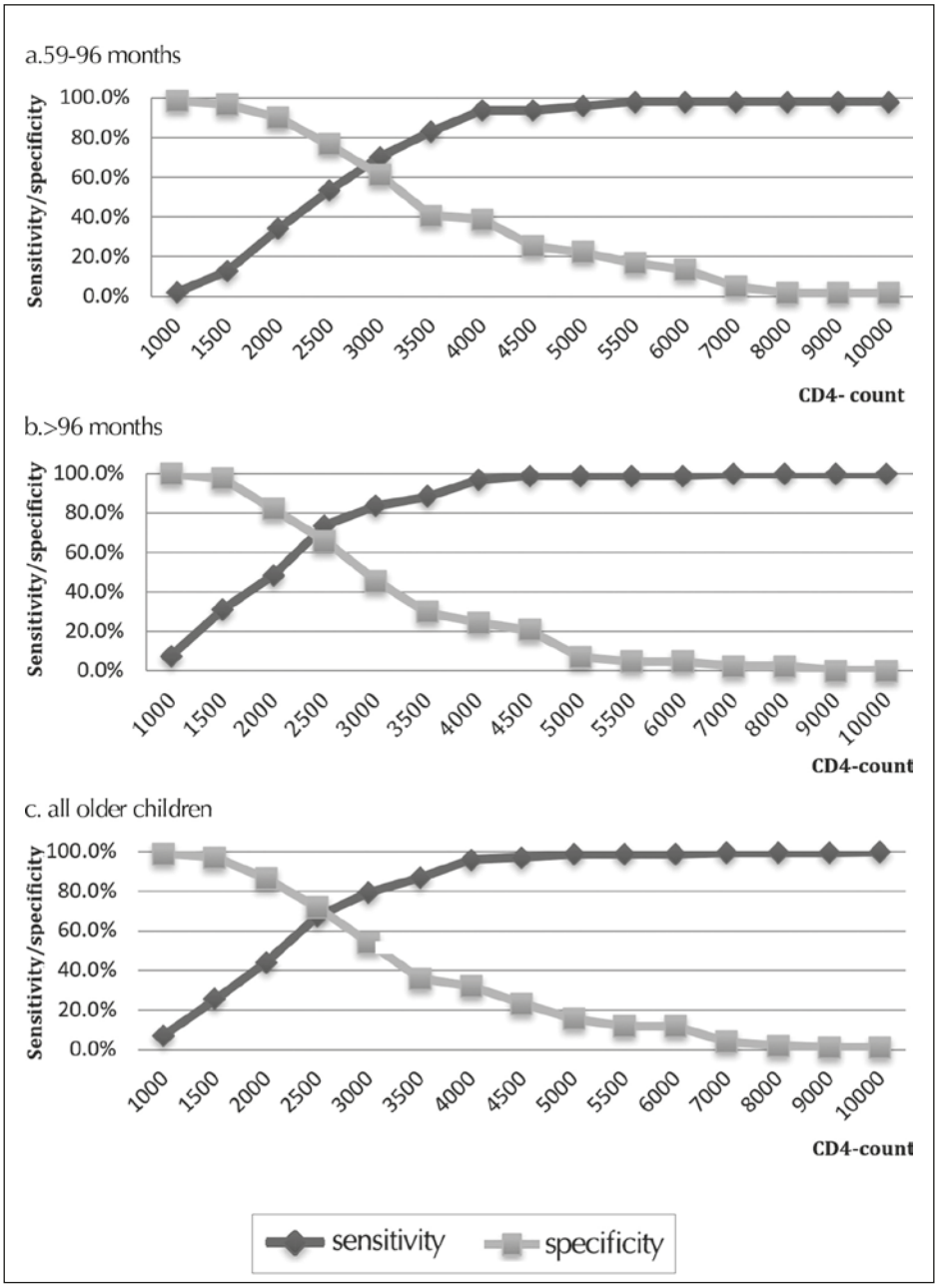

Figure 1. Optimal sensitivity and specificity for various total lymphocytes count cut offs by age group.

a) 59-96 months, new cut-off 3000 cells/m b) above 96 months, new cut-off 2500 cells/m. c) all older children, new cut-off 2500 cells/m.

aged 96 months or older, figure 1. Applying these new cut-offs increased the overall sensitivity for older children to 72,7% (95%CI: 64,8-79,6%), table 5.

Discussion

This is the first study to simultaneously evaluate all previously identified markers that are potential alternatives to CD4 values in children not eligible to start ART based on clinical staging alone. There are a number of important findings. First, if the WHO 2013 guidelines are applied, the majority (59%) of older children that did not qualify for ART on the basis of clinical staging alone were in fact eligible for ART on the basis of CD4 results. Second, a low TLC was associated with a low CD4-count in older children but sensitivity could be markedly improved by adopting cut-offs that are different to those currently recommended by WHO guidelines. Thirdly, we did not identify a reliable alternative predictor of CD4 percentage for the young children, which is consistent with previous studies that have recognised that clinical staging is a poor indicator of CD4 percentage in young children (11, 22, 23).

Since the study was designed several important changes took place: ART is given to all young children irrespective of CD4 values and CD4 testing has become more easily available (8). Still CD4 testing remains costly and (reliable) results are not always available in sub-Saharan African clinics. Therefor alternative markers for CD4 counts are still relevant in children aged 5 years or older. Using the 2013 criteria, 59% of the older children eligible for ART in our study would not have been identified using clinical criteria alone. We aimed to optimise the cut-off values if using TLC to improve sensitivity. Our findings are consistent with those from a previous study of Kenyan children that suggested that TLC cut-off values should be increased (24). That study evaluated the cut-off against WHO 2006 guidelines and suggested that 3000 cells/mm^{3}would be an optimal cut-off for older children. Our findings suggest that the optimal TLC cut-off for older children would be 2500 cells/mm^3.

The original WHO recommendations for using TLC published in 2006 were based on the correlation between TLC and a decline in CD4 count over time in children in USA and Europe (25, 26). Several studies have shown that TLC is a good predictor of mortality but correlates poorly with CD4 count (12, 23, 27, 28).

Furthermore, TLC varies between different populations as well as between individual (25, 26). Findings in African children may differ from counterparts in the USA and Europe for a number of reasons. Variations between environmental, nutritional and genetic factors are likely to have an impact (29, 30). Secondly, the recommended cut-offs were derived from cohort studies of HIV infected children irrespective of their clinical staging. They were not designed to detect children with low CD4 results that had no clinical signs of advanced HIV disease and yet this is the population for which TLC is currently being used. Although raising the cut-off will lower the threshold to put children on ART, we think there is a need to reconsider the TLC cut-offs values as per WHO guidelines to commence ART (7).

Low serum albumin was associated with clinical staging and CD4 values in the univariate analysis, but not in the multi-variant model. This association has not previously been studied in paediatric HIV populations. Our findings are consistent with studies in HIV-infected adults that reported that an association of serum albumin with HIV disease progression (14, 31). Low albumin levels can be an acute phase response (16, 32, 33), or caused by malnutrition or chronic disease (31, 34, 35). The fact that low albumin was not retained in a multi-variant model including markers for malnutrition suggests that this marker has limited added value over anthropometry alone.

Anaemia is a predictor of disease progression among HIV-infected children and adults (25, 36, 37). Although we found a difference in haemoglobin between the WHO staging groups, we were unable to find an association between low haemoglobin and CD4 values. Our results are consistent with the few studies conducted in African HIV-infected children showing that although anaemia may be a useful criterion to assess disease severity, defining cut-offs to guide the start of ART appears impossible(25, 37). Like anaemia, thrombocytopenia has been associated with disease progression and death in HIV-infected children in western countries (27, 28, 32, 38, 39). However, we did not find associations between platelet count and CD4 sub-groups or clinical stage. In our setting thrombocytes may have been influenced by co-infections such as malaria(29).

Conclusion

In summary, the majority of children that would not be eligible for ART on clinical staging had CD4 count eligible for ART according to current WHO guidelines. For older HIV-infected children in the African setting, the TLC cut-off when used as an alternative marker to CD4 count, when CD4 is not readily available, for decisions about commencing ART may need to be increased.

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		Age Group		WHO staging		
		18-59 months	>59 months	WHO stage I	WHO stage II	WHO stage III
Characteristics						
Number (%)		156 (37%)	261 (62.6%)	209 (50.1%)	101 (24.2%)	107(25.7%)
Sex (%)	Male	77 (49%)	113 (43.2%)	94 (45.0%)	45 (44.6%)	51(47.7%)
Age months, Median (IQR)		35.1 (25-47)	103.1 (76.1-132.8)	71.0 (45.6-109.5)	88.0 (54.7-121.8)	61.0(34.2-103.9)
Nutritional status						
BMI, Mean (SD)		15.9(1.7) ^1	15.3(1.6)	15.7(1.5) ^1	16.1(2.1)	14.8(1.5)
Underweight	Moderate	39(25%)^1	121(46%)	67 (32%)^1	40(40%)	53(50%)
Number (%)	Severe	19(12%)	24(9%)	8(4%)	7(7%)	28(26%)
Stunting	Moderate	115(74%)^1	146(56%)	110(53%)^1	58(57%)	93(87%)
Number (%)	Severe	68(44%)	68(26%)	45(22%)	25(25%)	66(62%)
Wasting	Moderate	5 (3%)^5	23 (9%)	10 (2%)^1	5 (1%)	13 (3%)
Number (%)	Severe	2 (1%)	7 (3%)	1 (0.25%)	0	8 (2%)
Laboratory results						
CD4 count, Mean (SD)		823.4(514.4) ^1	497.2(354.5)	674.9(474.4) ^3	531.2(358.0)	593.7(465.1)
CD4 percentage, Mean (SD)		18.3(10.3)	18.4(11.5)	19.8(11.2) ^4	16.3(9.7)	17.5(11.8)
Anaemia (%)		120(779%)^3	171(66%)	136(65%)^2	68(67%)	87(81%)
Haemoglobin (g/l), Mean (SD)		9.8(1.8) ^1	10.7(1.8)	10.6(1.9) ^1	10.6(1.6)	9.7(1.9)
Total lymphocytes count (x10*3/l), Mean (SD)		4.7(2.5) ^1	2.8(1.5)	3.5(2.1)	3.4(1.9)	3.8 (2.4)
Platelet count (x10*3/l), Mean (SD)		339(175) ^2	298(134)	314(143)	292(131)	333(183)
Albumin (g/l), Mean (SD)		33.8(6.0)	34.4(5.7)	35.2(5.8) ^1	34.7(4.9)	31.8(6.0)

Table 2: Baseline data

Abbreviations: IQR; Interquartile range, SD: standard deviation of mean.^ Significant difference between children aged under five and older than five years of age or significant difference between WHO staging groups (^1 p<0.001 ^2p=0.01, ^3=p=0.02 ^4p=0.03 ^5p=0.04). Definitions: [1,] Definitions by WHO of malnutrition and anaemia and indicators for starting ART as used in this study see table 1.

	Univariate				Multivariate			
	Beta	**Young children CD4 percentage (p-value)**	**Beta**	**Older children CD4 count (p-value)**	**Beta**	**Young children CD4 percentage (p-value)**	**Beta**	**Older children CD4 count (p-value)**
Anthropometry								
Body mass index z-score	-0.063	0.438	0.122	0.053				
Weight for height z-score	-0.020	0.805		**				
Height for age z-score	0.234	0.004	0.100	0.114	0.216	0.006		
Weight for age z-score	0.158	0.051	0.034	0.665				
Laboratory predictors								
Haemoglobin	0.041	0.611	0.049	0.437				
Total lymphocytes count	-0.237	0.003	0.534	<0.001	-0.219	0.005	0.534	<0.001
Platelets count	-0.004	0.964	0.901	0.368				
Albumin	0.200	0.016	0.106	0.098				

Table 3: Outcome univariate and multivariate analysis for predictive value of alternative makers for CD4 (beta and p-value).

(**Only available for age < 5yrs)

Start ART		WHO CD4 2006 (%)	WHO CD4 2013 (%)	WHO-TLC *(%)
All (n=417)		225 (54.0%)	311 (74,5%)	110 (24.4%)
Young children	All (n=156)	128 (86.5%)	**	28 (18.9%)
	18-35 months (n=78)	62 (79.5%)	**	7 (9.0%)
	36-59 months (n=78)	66 (84.6%)	**	21 (26.9%)
Older children	All (n=261)	97 (37.2%)	155 (59,4%)	82 (31.4%)
	60-96 months (n=109)	26 (23.4%)	48 (44,0%)	16 (20.6%)
	>96 months (n=152)	71 (47.3%)	107 (70,4%)	22 (11.0%)

Table 4. Total of children starting ART based on different guidelines.

Start ART based on WHO cut-offs CD4 count (WHO guideline 2013)(8) and total lymphocyte count based on study result, figure 3. *For some children total lymphocyte count was not performed; 18-35 months6 children, 36-59 months 2 children, 60-96 months. 2 children and >96 months 6 children. ** not measurable because 100% of these children do start ART based one age (<5 years) (WHO guideline 2013)(8).

		WHO-TLC cut-offs (%)		TLC New cut-offs -study (%)*	
		Sensitivity (95% CI)	Specificity (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)
Young children	All (n=156)	18,9% (13,0-26,2%)	**	**	**
	18-35 (n=78)	9,7% (4,0-19,6%)	**	**	**
	36-59 (n=78)	22,6% (18,0-39,1%)	**	**	**
Older children	All (n=261)	21,3% (15,1-28,8%)	94,2% (87,7-97,8%)	72,7% (64,8-79,6%)	62,1% (52,0-71,5%)
	60-96 (n=109)	34,0% (20,9-49,3%)	89,8% (79,2-96,2%)	70,2% (55,1-82,7%)	61,0% (47,4-73,5%)
	>96 ((n=152)	15,5% (9,2-24,0%)	100 % (91,8-100%)	73,8 % (64,2-82,0%)	65,9% (50,1-79,5%)

Table 5. Sensitivity and specificity for start ART based on different guidelines.

Sensitivity and specificity for start ART based on total of children starting ART (WHO guideline 2013) (8) versus WHO TLC cut-offs and TLC cut-offs based on study results figure 3. New cut-offs include; all older children; 2500 cells/m, 59-96 months; 3000 cells/m, above 96 months; 2500 cells/m.

*For some children total lymphocyte count was not performed; 18-35 months 6 children, 36-59 months 2 children, 60-96 months 2 children and >96 months 6 children. **. Not measurable because 100% of thesechildren do start ART based one age (<5 years) (WHO guideline 2013). [8]

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Chapter 3

High prevalence of virological failure and HIV-drug mutations in a first-line cohort of Malawian children

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Abstract

Background: Drug resistance mutations (DRMs) increasingly jeopardize paediatric HIV programs in sub-Saharan Africa. As individual monitoring of DRMs and viral loads has limited availability, population data on DRMs are essential to determine first-line susceptibility. Paediatric data from sub-Saharan Africa are scarce and unavailable for Malawi.

Objectives: To determine the prevalence of virological failure (VF) and DRMs among ART-naive HIV-infected Malawian children during the first year of first-line ART.

Methods: In a prospective cohort of HIV-infected Malawian children, on first-line treatment, children were followed monthly; blood was collected for viral load testing (6 and 12months) and genotypic resistance testing (12months). VF was defined as at least one viral load .1000 copies/mL or death after 6months of ART. DRMs were identified and susceptibility to NRTIs and NNRTIs was scored using the Stanford algorithm and by calculating genotypic susceptibility scores (GSSs).

Results: VF occurred in 66% (23/35) of the children during 12months of follow-up. DRMs were detected in 44% (15/34); all had NNRTI resistance and 12% (4/34) had dual-class NNRTI/NRTI resistance. Reduced susceptibility (DRMs and GSS 3) was seen in 41% (14/34) to their current first-line regimen. High-level resistance was most common for Nevirapine (26% (9/34)).

Conclusions: In this first report on VF and DRMs in children on first-line ART in Malawi, the rates of VF and DRMs were alarmingly high. Paediatric HIV programs in sub-Saharan Africa should emphasize programmatic evaluation of VF and include detection of DRMs to adjust and design adequate first- and second-line regimens and prevent widespread resistance in children.

Background

The development of drug resistance mutations (DRM's) among HIV-infected children in sub-Saharan African is increasing which jeopardizes the outcomes of paediatric HIV programs. Regular monitoring of viral load (VL) and sequencing data to detect DRM's is limited or not available for individual patients. Therefor monitoring first-line susceptibility and design of appropriate first and second line strategies should be based on population data on DRM's prevalence to prevent further accumulation of mutations in those who will require treatment for the rest of their lives (1). The data on DRM's in children on ART in sub-Saharan Africa is very scare (2). Although Malawi is heavily affected as 10% of the population is HIV-infected, data among children consisting DRM are limited and no data on DRM's in Malawian children during ART is available (3,4).

Objectives

This study was conducted to document the prevalence of virological failure (VF) and DRM's in HIV-infected Malawian children during the first year of first-line ART.

Methods

This study is a sub-study of a larger prospective cohort of HIV-infected children commenced on first-line ART in the paediatric ART-clinic of Queen Elizabeth Central Hospital, Blantyre, Malawi. The study includes ART-naïve HIV-infected children, aged 18 months-18 years, who were eligible to commence ART according to the Malawi National ART program guidelines (2008-2010). All children were started on a first-line regimen consisting of Stavudine Lamivudine and Nevirapine. Children were eligible for this sub-study if one or more samples were available for VL testing at either 6 or 12 months from recruitment. Virological outcome was evaluated by VF and viral suppression. VF was defined as either two consecutive detectable VL>1000 copies/mL taken at least 6 months after treatment initiation, or a VL>1000 copies/mL at the last available measurement, or death after at least 6 months of treatment (5). Viral suppression was defined as VL<1000 copies/ml. Severely immune compromised was defined according to the WHO criteria; age<59 months;

CD4% <10% or CD4 count<200 cells/mm^3, age>59 months: CD4 count<100 cells/mm^3 (6). At enrolment socio demographic, TB status, antrometry were taken and HIV diagnosis was confirmed (Abbott Determine HIV-1/2 Test). Venous blood sample were collected, 0, 6, 12 months for CD4 count (flow cytometry, Becton Dickinson, CA USA). VL testing was done at 6 and 12 months (Roche Amplicor; Roche, Basel, Switzerland; detection level 400 copies/ml). To evaluate DRM's after one year of ART genotypic resistance testing was performed, using dried blood spots collected at 12 months, which were available for 34 of 35 children. Outcome was not available during the time of study and therefore clinical regimens were not adjusted regarding this study outcome. Dried blood spots consisted of 50 µL of whole blood spotted on five circles of a Whatman Protein-Saveron 903-card filter paper (7). Elution of the nucleic acids was conducted and cDNA synthesis was performed using the Invitrogen Superscript one-step RT-PCR System with Platinum Taq High Fidelity. DNA and cDNA were subjected to a nested PCR amplifying a conserved region in the HIV-1 polymerase gene, followed by direct sequencing (7,8). Major DRM's were identified for NRTIs (Abacavir, Zidovudine, Stavudine, Didanosine, Embtricitabine, Lamivudine and Tenovir Disoproxil fumarate) and NNRTIs (Efavirenz, Etravirine, Nevirapine and Rilpivirine). Susceptibility to the prescribed regimen was determined by genotypic sensitivity score (GSS) using the Stanford University HIV drug resistance database, version 8.3 (9). Reduced susceptibility to the prescribed regimen was defined as GSS<3 (3 fully susceptible drugs).

Methods| Ethics

The study was approved by the Research Ethics Committee of the College of Medicine, University of Malawi and informant consent of the guardians was obtained.

Results

A total of 35 children with a mean age of 7.1 (SD 4.6) years were included. The baseline characteristics are shown in table 1. At 6 and 12 months respectively, 1/35(3.1%) and 2/35(6.3%) of the children were severe immune compromised. For 35 children a VL sample was available either at 6 months (n=24) or 12 months (n=23), including 12 children who had samples taken at both time points, table 2.

VF occurred in 23/35(65.7%) of the children during the 12 months of follow-up, table 2. Viral suppression was achieved in 12/24(50.0%) and 3/23(13.0%) at 6 and 12 months respectively.

Baseline Characteristics		**Kids with VL results (n=35) (n/N(%), mean**
General (total)		
Sex	male	17/35 (48.6%)
Age group	< 5 years	13/35 (37.1%)
Mother or child had received drugs for Prevention of	Yes	1/35 (2.8%)
Mother to Child Transmission (PMTCT) of HIV	No	17/35 (48.5%)
	Unknown	17/35 (48.5%)
Clinical suspect TB- Infection baseline[*1]		3/30(10.0%)
Immune status and Viral load		
WHO-staging	I/II	15/35(42.9%)
	III/IV	20/35(57.1%)
CD4 % (median, IQR)		17.5 (IQR1.1-28.5)
CD4 count (median, IQR) >		266.5 (IQR 19.0-654.0)
Severe immune compromised [*2]		11/31 (35.5%)
Viral load (log)[*3] (mean/SD)		5.4 (2.8-6.2)
Anaemia [*4]		20/33(60.6%)
Anthropometry		
Underweight[*5]		6/33 (18.2%)

Table 1. Baseline characteristics.

Abbreviations: PMTCT= prevention mother to child transmission. [*1] Clinical Tuberculosis (TB) infections was defined when children were on TB medication at enrolment or the clinical diagnose at enrolment based on X-ray was made. * Severe immune compromised: Age < 59 months a CD4% < 10% or a CD4 count <200 cells/mm3, age > 59 months a CD4 count < 100 cells/mm3 (5). [*3] Total Lymphocyte Counts (TLC): Age 18-59 months TLC < 3000 cells/mm^3, age 36-59 months TLC <2500 cells/mm^3, age 60-96 months TLC <2000 cells/mm^3 and age > 96 months TLC <1200 cells/mm^3 (5) [*4]. Viral load was measured 22 children. [*4] Haemoglobin level (Hb); Age 18 months-59 months and Hb< 11.0 g/dl, age 5-11.9 years and Hb< 11.5 g/dl, boys aged 12-15 year and Hb< 12.0g/dl, girls aged > 12 years Hb<12.0 g/dl and boys aged >15 years and Hb <13.0 g/dl (5). Underweight Body mass index z-scores (BMIZ) < -2 SD for all ages was used.

Genotypic resistance testing was available for 34/35 (97.1%) children; one patient did not have a dried blood spot available. DRM's were detected in

15/34(44.1%) children, with a range of 1-3 major mutations. All 15 children with a DRM had a mutations associated with a NNRTI resistance including 4/34(26.7%) with dual resistance (NNRTI and NRTI). Present DRM's are shown in figure 1a and table 2. At the end of follow up a total of 14/34(41.2%) children had resistance (DRM's and GSS< 3) against at least one drug of their first-line regimen. Drug susceptibility is shown in figure 1b. High-level resistance (GSS=1) for NRTI's and NNRTI's was seen for lamivudine in 4/34(11.8%) and for Nevirapine in 9/34(26.5%) children.

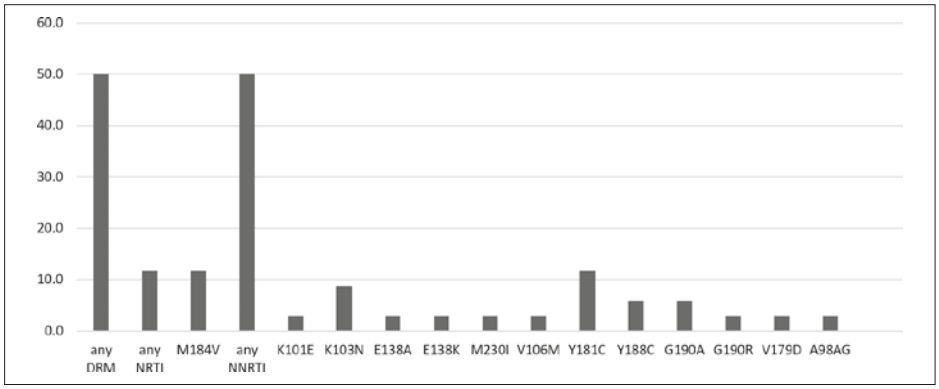

Figure 1a. Percentage of children with major HIV Drug Resistance Mutations (DRM's) found at 12 months of follow-up in this cohort.

Abbreviations: DRM: Drug Resistance Mutation, NRTI: Nucleoside reverse Transcriptase Inhibitors, NNRTI: Non- Nucleoside reverse Transcriptase Inhibitors. (9)

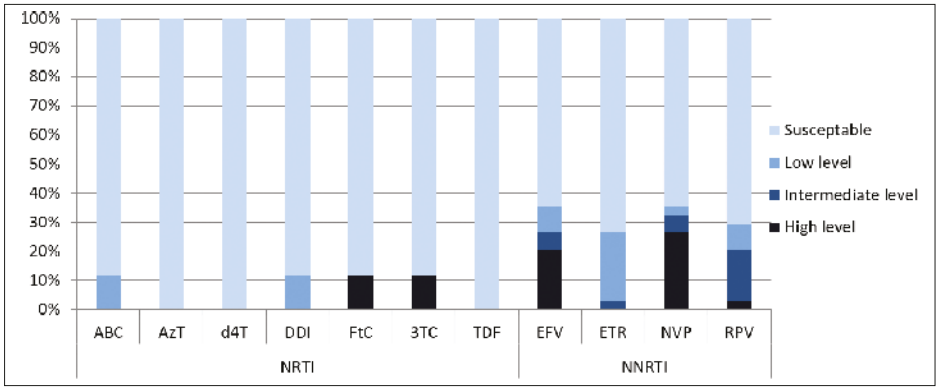

Figure 1b. Precentage of children with low (GSS0.5/0.75), intermediate (GSS0.25), high level resistance (GSS 0) or none reduced susceptible (GSS1).

Abbreviations: Nucleoside reverse Transcriptase Inhibitors (NRTI): Abacavir (ABC), Zidovudine (AZT), Stavudine (D4T), Didanosine (d4T), Embtricitabine (FTC), Lamivudine (3TC), Tenofovir (TDF). Non- Nucleoside reverse Transcriptase Inhibitors (NNRTI): Efavirenz (EFV), Etravirine (ETR), Nevirapine (NVP), Rilpivirine (RPV)

Discussion

This is the first report on VF and DRM's among children on first-line ART in Malawi and we identified that an alarming two thirds of ART-naïve children showed VF in the first year of treatment. As well nearly half of them showed NNRTI-mutation and more than a quarter had dual class resistance at the end of year one.

The high prevalence of VF (65.7%) and the low viral suppression rates of 50% and 13% at six and twelve months, are worse than previous reports from other sub-Saharan African countries. Previous reports show VF rates up to 50% and suppression rates of 74% (95%CI 70.2-78.2) during 12-24 months of ART (10-13). No data on HIV infected children in Malawi is available. The only comparable data from Malawi is a cohort of adolescents, which reports a suppression rate of 70% after 12-15 months of ART (12). Several potential causes may explain the poor outcome of our cohort. VF in children may be caused by pre-treatment DRM's and poor adherence (14-19). Pre-treatment data was not available for our cohort (48.5% unknown), however a study among Malawian children (age<15 years) on baseline of ART does report a 15% prevalence of pre-treatment DRM's (NNRTI: 14%, NRTI: 5%, dual class resistance: 4%) (3). The 15% prevalence of DRM's is similar to the 16%

reported in Ugandan and Nigerian children (16,20). The existence of pre-treatment DRM's is therefore an unlikely the only explanation of the poor performance of our Malawian cohort.

The pattern of DRM's found is comparable to previous reports on children and adults in sub-Saharan Africa (11, 12,15,19,21-24). Like our study Ugandan and Nigerian children most commonly showed NNRTI mutations (44%) and dual class resistance was present in 27%(15,19). This is not surprising as NNRTI resistance develops rapidly due to failed Prevention mother to child transmission (PMTCT) (2,12). As NVP is part of the PMTCT regimen in Malawi, and we found high level resistance was most common against Nevirapine this likely applies to our Malawian cohort. We did not find any TAM's (thymidine analogue mutations) among the tested children. This outcome is surprising, as children were exposed to ART for 12 months and TAM's mostly occur after several months of ART treatment. The combination of a high percentage of VF, but no TAM's after 12 months of treatment may suggest a very poor adherence. To address the high rates of DRM's and VF, the WHO introduced several key actions, which include a Protease Inhibitor (PI) based antiretroviral therapy regimen for all HIV-infected infants (<3years) in resource limiting settings (1). PI regimes are a promising next step among Malawian HIV-infected children as it will address the high rates of NNRTI resistance pre-treatment among these infants, however several logistic and financial barriers still delay implementation (2). Although resistance is less likely to develop in PI regimens programmatic monitoring of DRM's remains important to prevent a new resistance problem to occur in Malawi and other countries (1,15). Also the NNRTI drug mutations were the most common (44%) DRM's in our cohort as well and dual class resistance.

Presenting study has several shortcomings; first of all it was relatively small and lacked pre-treatment resistance and adherence data. Adherence was monitored by pill count and self-report, but documentation about clinician's suspicion of poor adherence was not sufficiently standardized to include these concerns to be evaluated in this study. Secondly, we applied the WHO definition for VF, which restricts the diagnosis of failure to only those with a detectable load or those who died after 6 months of ART (5). A total of 5 children died during the first 6 months of ART and were therefore excluded from this sub-study. The definition of VF contains two consecutive detectable VL>1000 copies/mL taken at least 6 months after treatment initiation, however if not available only one VL>1000 copies/mL at the

last available measurement will do. Because 13/23 (56.5%) VF diagnosis was based on one viral load sample above 1000 copies/ml outcome could be theoretically overrated. Baseline characteristics of the 35 included children, did not differ from the 70 excluded children, with missing viral load samples (data not displayed). Additionally, the study was performed in 2010 and local guidelines to start ART were used. Current WHO guidelines start ART early in the course containing the "treat all" policy of HIV disease regardless of CD4 count, which meant that the studied cohort was more severely immune compromised in comparison to current cohorts of children starting on ART (2).

Despite these flaws, our findings are important and alarming moreover as we known that ART resistance has gradually increased over the last years and treatment options did not change over the last years in Malawi (2,4,6). When DRM data is not available during ART, which was the case in our study and still the current practice in a lot of clinics in Malawi, children continue treatment on a partially active ART regimen, which increases the risk of further acquiring other DRM's (16). To improve future outcome of paediatric HIV programs, implementation of PI-based regimens and closer programmatic evaluation of viral loads and DRM's are essential. Both actions should deserve priority to prevent widespread resistance and further reduction of already limited treatment options in HIV-infected children in sub-Saharan Africa.

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Patient	Age (years)	VL6 (log)	VL12 (log)	VF	Drug Resistance Mutations
1	8		2,20	0	A98G
2	11	5,05	5,05	1	M184V, Y181C
3	5	2,61	4,42	1	
4	8	2,43		0	K103R
5	12	2,84		0	
6	2		3,12	1	
7	10	5,38		1	
8	5		4,03	1	G190R
9	12	4,18	4,10	1	Y181C, Y188C
10	5		2,42	0	
11	12		2,24	0	K103N
12	5	4,45	4,37	1	M184V, Y188C
13	2	3,67	3,21	1	M184V, K130N
14	1	2,65		0	
15	4		3,37	1	
16	9	2,44		0	
17	13	2,75		0	
18	12	3,53	3,35	1	
19	8	4,03	4,39	1	Y181C
20	3		4,93	1	
21	18	2,33	3,47	1	
22	13	4,06	5,96	1	*
23	2		3,40	1	K103N
24	10	5,26		1	
25	9	4,44	4,77	1	
26	3	2,30		0	
27	5		6,18	1	M230I
28	6		3,54	1	
29	2	2,68		0	V179VD
30	2	2,65		0	E138A
31	1	3,43		1	E138K, Y181C
32	14	3,02	5,20	1	M184V, G190A, A98AG
33	13	2,26	4,86	1	K101E, K103N, V106VM

Patient	Age (years)	VL6 (log)	VL12 (log)	VF	Drug Resistance Mutations
34	3	2,32		0	
35	2		4,35	1	G190A

Table 2. Viral Load (VL) (log) for months 6 (VL6) and 12 (VL12) and Genotypic resistance testing outcome tested at month 12.

* No Genotypic resistance testing available. Abbreviations: Virological Failure (VF)

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Chapter 4

Long term virological outcomes, failure and acquired resistance in a large cohort of Ugandan children

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Abstract

Objectives: Evaluate long-term virological failure (VF) and drug resistance among HIV-infected Ugandan children on first-line ART.

Methods: In a multi-centre prospective cohort study viral load (VL) and Drug Resistance Mutation (DRM) were investigated at start and 6-monthly intervals in children (age≤12 years). VF (two consecutive VLs>1,000 copies/ml or death after six months of ART) was defined as early VF (0-24 months of ART), or late VF (25-48 months of ART). An active regimen was defined partially active if a GSS-score was<3.

Results: From 2010-2011, 316 children were enrolled. Viral suppression was achieved in 75.8%, 71.5%, 72.6% and 69.2% at 12, 24, 36, 48 months. VF occurred in 111/286 (38.8 %), of which 67,6% was early and 32.4% late VF. Early VF was associated with a partially active regimen at baseline (OR: 6.0, 95%-CI: 1.9-18.5), poor adherence (OR: 3.1, 95%-CI: 1.3-7.4) and immunodeficiency (OR: 3.3, 95%-CI: 1.1-10.2). Late VF was associated with age>3 years (OR: 2.5, 95%-CI: 1.0-6.6) and WHO-stage 3/4 (OR: 4.2, 95%-CI: 1.4-13.4). Acquired DRMs were detected in 27.0% before 24 months, versus 14.4% after 24 months (p<0.001). 92.2% of the children with early VF, versus 56.2% with late VF had a partially active regimen (p<0.001).

Conclusion: VF rates were high, occurred predominantly in the first 24 months and appeared to increase again in year four. Risk factors and patterns of early VF/DRMs were different from late VF/DRMs. Virological control may improve by close monitoring and prompt switching to second line in the first 24 months. Late VF may be prevented by early start of ART.

Background

Since 2010 programs for treatment and prevention of Human Immunodeficiency Virus (HIV) infection in sub-Saharan Africa have scaled up rapidly (1). This has drastically changed the prognosis of children living with HIV, as survival rates, after 24 months of ART, have increased to 91% (2,3).

Although survival rates have improved, the rates of paediatric treatment failure are still unacceptably high (4). It has been estimated that 30% of the HIV-infected children in sub-Saharan Africa experience virological failure (VF) within the first 24 months of ART (3-6). Several factors that may contribute to the high failure rate have been identified including: high prevalence of pre-treatment drug resistance; suboptimal treatment monitoring due to the unavailability of viral load (VL) testing; limited availability of paediatric drug formulations and, finally, poor adherence among children and adolescents (4, 5).

The high failure rates and the lifelong need of ART stress the need for long-term and careful monitoring of this vulnerable group of HIV-infected children on ART. Only few studies have reported long-term treatment (≥ 24 months) outcomes of HIV-infected African children on first-line ART however (4-7). Studies on African adults reported a continuous increase of VF after 48 months of follow-up (8). There is limited data on 24-48 months and no published data on children on first line ART in sub-Saharan Africa.

The lack of long-term outcome negatively affects development and adjustment of ART protocols in sub-Saharan African children. For example we assume that risk factors for development of VF are the same for a child just started on ART and one that is on treatment for some years, whilst these may be very different due to virological, physiological and psychological changes over time. With the lack of studies beyond 24 months we have no data on this however.

In order to evaluate the longer-term effect of current first-line strategies and contribute to the development of second-line regimens for children, it is important to understand the development of virological failure (VF) over time, its determinants and the rate of resistance emergence in children. Therefore we assessed the long-term outcomes of a prospective cohort of Ugandan children including VF, acquired Drug resistance Mutations (DRM) and the predicted susceptibility of first-line ART regimen. In addition we compared the outcomes and determinant for VF and

acquired DRM in children experiencing an 'early VF' (<24 months after ART start) compared with a 'late VF' (25-48 months after ART start) (6).

Methods

The Monitoring Antiretroviral Resistance in Children (MARCH) study is a multicentre prospective observational cohort study of HIV-1-infected children who received HIV treatment and care at three Joint Clinical Research Centre (JCRC) Regional Centre's of Excellence (RCEs) based in Kampala, Mbale and Fort Portal, Uganda. The study methods and the results of baseline and the 24 months analysis have been published elsewhere (6, 9). In brief we enrolled HIV-infected children aged ≤12 years from January 2010 to August 2011. For this analysis, participants starting on first-line ART were included and followed up for 48 months. Children were started on ART based on 2006 World Health Organisation (WHO) treatment guidelines (10) though by August 2010 the clinics had adopted the revised 2010 treatment guidelines (11). Following this revision, all children <24 months of age were eligible for ART irrespective of their clinical stage or CD4 cell counts. A combination of two NRTI and one NNRTI, Efavirenz (EFV) or Nevirapine (NVP) were the preferred combinations of choice and recommended by the Ugandan guidelines (12). PI-based regimens (LPV/r-based) were prescribed for young children exposed to PMTCT, if the drugs were available. EFV was only given to children >3 years.

Sociodemographic and clinical data were collected at enrolment and at subsequent 3-monthly follow-up visits and aggregated in a web-based database. At baseline and every 6 months thereafter, viral load (VL) testing was done as well as genotypic resistance testing on specimens with VL >1000 copies/ml. Virological Failure (VF) was defined according to the WHO as two consecutive detectable VL >1000 copies/mL taken at least 6 months after treatment initiation(11). VF was classified as early or late if it was first diagnosed between 0-24 and 25-48 months after ART initiation, respectively. A second episode of VF was not analysed in this study. A VL >1000 copies/mL at the last available measurement, or death after at least 6 months of treatment, was also considered as failure. Viral suppression was defined as VL< 1000 copies/ml and defined per single visit. Major drug resistance mutations were identified based on the 2017 IAS mutation list (13). Susceptibility

to the prescribed regimen was determined by calculating the genotypic sensitivity score (GSS) using the Stanford algorithm (version 7.0)(14). Reduced susceptibility to the prescribed regimen was defined as a GSS <3, corresponding to less than 3 fully susceptible drugs. Acquired DRMs were defined as a new DRM following initiation of ART for both children with or without pre-treatment HIV drug resistance (PDR) and was measured when VL>1000 copies/ml was detected. Early acquired DRMs were defined as DRMs acquired at <24 months after treatment initiation whilst late acquired DRMs were defined as DRMs acquired between 25-48 months after ART start. All HIV-1 pol sequences in this study have been deposited in GenBank under the following accession numbers: MF357928–MF358296.

Methods| Definitions

Adherence to ART was assessed through pill count over the previous 30 days before a clinic visit and was based on caregiver's report. Adherence over time was calculated as the mean of these adherence reports, and was categorized as being suboptimal (≤95% adherence) or optimal (>95% adherence). Underweight was defined as Body Mass Index (BMI) Z-score for age (BAZ), below –2 standard deviation (SD)(15). Anaemia was defined as haemoglobin (Hb) <11.0 g/dl for children aged 0.5-4.99 years, Hb <11,5 g/dl for children aged 5.0-11.99 years, Hb< 12 g/dL 12.00-14.99 years, Hb<12 g/dL in girls >15 years and Hb<13 g/dL in boys age>15 years (16). Immune deficiency was defined by immune status based on WHO criteria and include CD4% <25 and ≥ 10 < 59 months or CD4 count <500 cells/mm3 and ≥ 100 cells/mm3 >59 months(10).

Methods| Ethics

The ethics committees of JCRC (approval reference 30 October 2009) and the Uganda National Council of Science and Technology (approval reference HS 721), and the Academic Medical Centre of the University of Amsterdam in the Netherlands approved the study protocol before commencement of the study (approval reference 09.17.1626).

Methods| Statistical analysis

Statistical analyses were performed using the statistical software package STATA version 12 (STATA Corp. LP, Texas, TX, USA). Baseline characteristics are presented

as proportions or medians with IQR. Group comparisons for categorical data were performed using the χ^2 test or Fisher's exact test, and for continuous data using the *t*-test or the Wilcoxon rank-sum test. Exact McNemar's Chi^2 was used to test difference in viral suppression and VF over time. Logistic regression was performed to model the association with explanatory variables and the presence of *early* versus *late* VF and of *early* versus *late* acquired DRM as well for explanatory variables and the presence of acquired DRM. Explanatory variables considered in the analysis were age, sex, WHO clinical stage at study entry, activity of baseline regimen (baseline drug resistance against active regimen, GSS<3), viral load at study initiation, adherence, previous use of NNRTI, exposure to PMTCT drugs and immunodeficiency at baseline. Explanatory variables associated with the outcome variables ($p < 0.10$) in the univariable analysis were included in the multivariable model in a stepwise approach. Biological plausible interactions were examined. Results were expressed as odds ratios (OR) with 95% confidence intervals (CI) and *p*-values are two-sided, with $p < 0.05$ regarded as statistically significant.

Results

A total of 316 children aged less than 12 years were started on first-line ART between January 2010 and August 2011 and were enrolled in the study cohort. Median age at baseline was 4.8 years (IQR 2.0-8.7). Baseline characteristics of the children are summarized in table 1 and have been published before (6). Of all participants 91.1% were initiated on a NNRTI-based regimen, 4.7% on a triple NRTI-based regimen and 4.1% on a PI-based regimen. Genotyping results at baseline were available for 85.4% of the children. At baseline one or more DRMs were detected in 16.7% and 7.8% had a predicted reduced susceptibility to at least one drug of their first line regimen (GSS<3), table 1. At 48 months of follow-up, 72.2% children were still in care and on first-line treatment, 5,4% were switched to second-line treatment, and 4,7% died. Outcome is shown in figure 1. Eleven of the 15 deaths (73.3 %) occurred in the first year of follow-up.

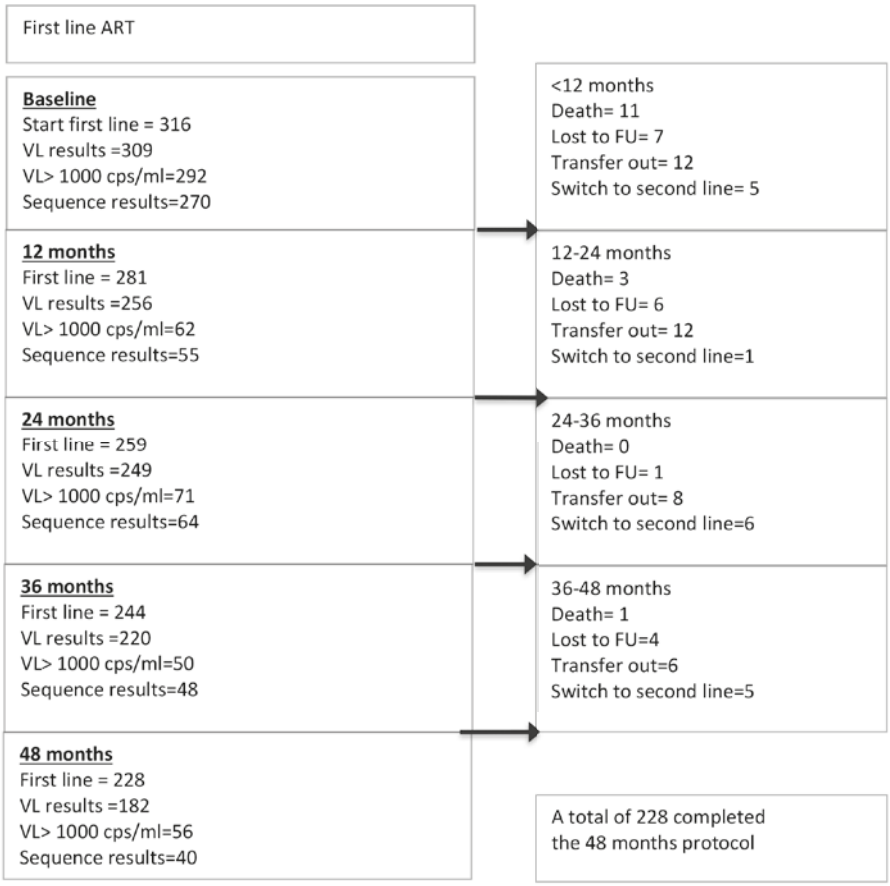

Figure 1. Follow-up of children within the MARCH study.
Abbreviations: Viral load (VL), loss to Follow-Up (loss to FU).

Results| Viral Suppression & failure

Viral suppression was achieved among 75.8%, 71.5%, 72.6%, and 69.2% of the children at 12, 24, 36, 48 months respectively (figure 2). Overall virological failure (VF) was detected in 38.8 % (111/286) of the children and occurred as early in 67.6% and late VF in respectively 32.4%, figure 2. Early VF was associated with a partially active regimen at baseline (GSS< 3) (OR: 6.0, 95%-CI 1.9-18.5), poor adherence (OR: 3.1, 95%-CI: 1.3-7.4,) and immunodeficiency at baseline (OR: 3.3 95%-CI: 1.1-10.2), table 2. Late VF was associated with an age above 3 years at ART start (OR: 2.5, 95%-CI: 1.0-6.6), and having WHO-stage 3/4 at ART start (OR: 4.2, 95%-CI: 1.4-13.4), table 2.

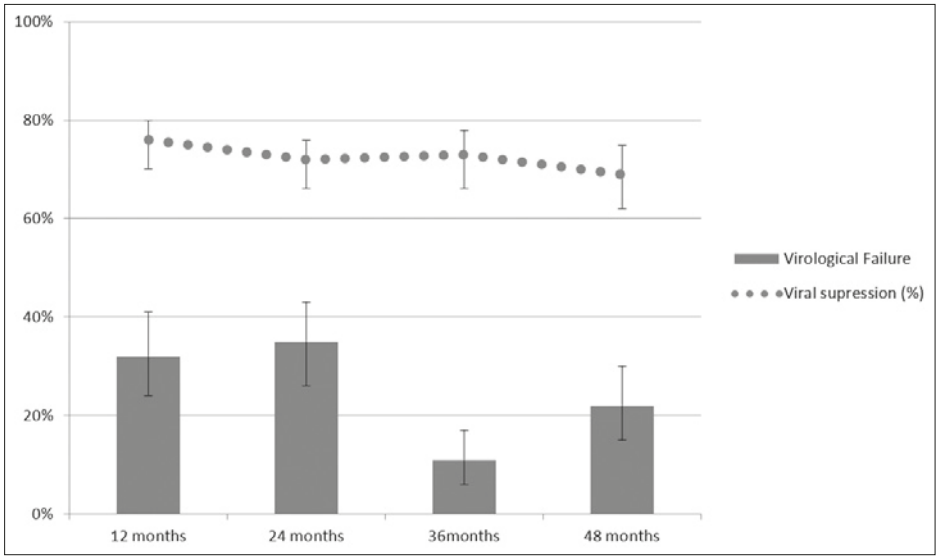

Figure 2. Viral Suppression and Virological Failure over 48 months.
Viral Suppression was defined according to the WHO as two consecutive detectable VL >1000 copies/mL taken at least 6 months after treatment initiation(11). Viral suppression was achieved among 75.8% (194/256), 71.5% (178/249), 72.6% (162/223) and 69.2%(126/182) at 12, 24, 36, 48 months respectively, the decrease in viral suppression between 36-48 months (*) is significant (McNemar's Chi2), p-value 0.005. Virological Failure (VF) was defined according to the WHO as two consecutive detectable VL >1000 copies/mL taken at least 6 months after treatment initiation(11). * *The increase in VF between 36-48 months (McNemar's Chi2), p= 0.05.

Results| Drug Resistance Mutations

During the entire study period 30% (81/270) of the children acquired one or more DRMs. Acquired DRMs more commonly occurred in the first 24 months (0-24 months) as compared to the period between 25-48 months (respectively 27.0%(73/270) and 14.4% (39/270); p< 0.001). A total of 11.5% children acquired one or more new DRMs in both time periods. DRMs detected included NNRTI mutations (96.3%; 78/81), NRTI mutations (92.6%; 75/81) and dual class mutations (NNRTI &NRTI 88.9%; 72/81). The most common NNRTI mutation was K103N, observed in 49.4% (40/81) of the children with an acquired DRM. A total of 27.4% (20/73) of the children acquired the K103N mutation in the first 24 months of treatment and 2.8% (1/39) after 24 months (p=0.036). M184V was the most common NRTI mutation in 88.8% (72/81) of the children. A total of 89.0% (65/73) of the children acquired

the M184V mutation in the first 24 months and 17.9% (7/39) after 24 months (p< 0.001). K65R mutation was only detected in the first 24 months of treatment in 2/81 children (2.5%, figure 3a).

To identify the children at risk for developing acquired DRMs within the first 24 months or after 24 months of first line ART treatment initiation, we explored potential determinants for early (<24 months) versus late (25-48 months) acquired DRMs. Early acquired DRMs was associated with the presence of pre-treatment DRMs leading to reduced activity of the first-line ART regimen (OR: 2.7, 95%-CI: 1.0-7.5) and a higher VL at ART start (OR: 2.6, 95%-CI: 1.2-2.9). No associations were identified for emergence of late acquired DRMs, table 3.

Results| Predicted drug susceptibility

We evaluated the association between drug susceptibility with early versus late VF. A total of 92.2% (59/64) of the children with early VF were treated with a partially active regimen at time of failure (GSS< 3), compared to 56.2% (18/32) of the children with late VF (p<0.001). High-level resistance (GSS=0) was most common for NVP in 67/74 (90.5%) and 20/35 (57.1%) (p<0.001) of the children with early and late VF, figure 3b. Among the 13 children receiving PI-based ART, only 2 children experienced VF one respectively 'early' and one 'late'; among those, no DRMs associated with PI resistance was detected.

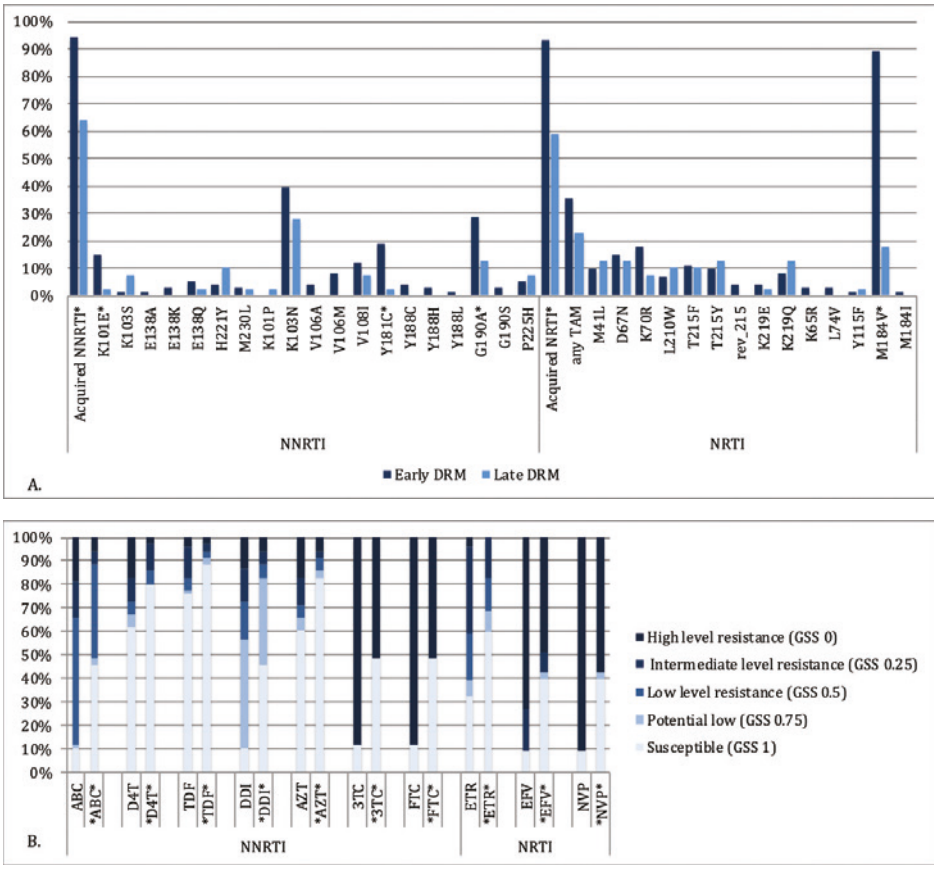

Figure 3. Specified acquired drug resistance mutations (DRM) & predicted drug susceptibility score.

A) Percentage of specified acquired DRM during the first 24 months (Early DRM; total number of children with acquired DRM =73) and after 24 months (late DRM; total number of children with acquired DRM =39) of treatment. * A significant difference between the 0-24 and 25-48 months of treatment ($p< 0.05$.); Acquired NNRTI p-value < 0.0001, K101E p-value =0.036, Y181C p-value =0.010, G190A p-value =0.044, NRTI p-value < 0.0001, M184V p-value < 0.0001.

B) GSS score for children with early virological failure (0-24 months) and late virological failure (25-48 months)(14). Outcome for late virological failure is indicated between asterisks with *xx*. Predicted susceptibility (genotype sensitivity score) was calculated using the Stanford algorithm version 7.0 (13). For participants with multiple genotypes, the most conservative susceptibility is plotted (i.e. the highest level of resistance). Abbreviations: Abbreviations: Drug Resistance Mutations (DRM), Non-Nucleoside reverse Transcriptase Inhibitors (NNRTI), Nucleoside reverse Transcriptase Inhibitors (NRTI), Abacavir (ABC), Stavudine (D4T), Tenovir (TDF), Didanosine (DDI), Zidovudine (AZT), Lamivudine (3TC), Embtricitabine (FTC), Etravirine (ETR), Efavirenz (EFV), Nevirapine (NVP).

Discussion

This is one of the first comprehensive studies evaluating long-term outcomes (48 months) of first line ART in ART-naïve African children. In this cohort of Ugandan children on first-line ART we identified that VF and acquired DRMs were common as they occurred in third of children during 48 months of follow-up. Although most VF occurred in the first 24 months a worrisome second decrease of viral suppression was seen at 48 months. Risk factors for early and late VF were different as early VF was associated with a partly active regimen at baseline and poor adherence and late VF was with advanced HIV disease stage and age ≥3 years at start of ART. Also the pattern of acquired DRMs was different over time as they occurred more common in the first 24 months of treatment, were more common and more likely to cause high level resistance as compared to late DRMs.

VF was detected in 38.8 % of our cohort. Although VF occurred mostly in the first 24 months of follow up, still a third of all failures was noted after 24 months of treatment. Viral suppression was achieved among 75.8% after 12 months and remained stable during months 12-36. A second decrease toward 69.2% suppression was seen at 48 months of follow-up and coincided with an increase in VF. The general results of our study are comparable to other long-term outcomes of paediatric HIV cohorts in sub-Saharan Africa (24-48 months) as they showed viral suppression rates of 60-75% and VF rates of 25-40% (5, 6, 17-19). Our finding that most VF occurred in the first 24 months corroborates with other reports in children (5, 6, 17-19). The worrisome deterioration of viral suppression rates between 36 and 48 months in our cohort has not been reported in children. Data on African adults show a significant stable viral suppression rates over the first 36 months of ART but a decrease of viral suppression and respectively increase of VF towards a plateau-phase around 42 months up to 72 months at follow-up (8). Data beyond 48 months follow up in children is lacking and needed to explore if this worrisome drop in viral suppression rates after 48 months persists.

To reduce development of resistance in children and develop effective guidelines including preventive measures we need to identify and understand potential risk factors for VF during (long-term) ART. We hypothesised that risk factors may be different for those failing early as compared to late and indeed identified differences. Late VF was associated with baseline age above 3 years and

an advanced baseline WHO stage whilst early VF was associated with pre-treatment DRMs and poor adherence (6). Risk factors have been associated with early VF in previous studies, however we are the first to compare early and late failure. Outcome is important and will help to provide guides on monitoring children at risk over long-term treatment plans.

Pre-treatment drug resistance mutations, which in our study were associated with early VF, was previously reported to be associated with VF, and was further reported in this as alarmingly prevalent amongst African (43%) and rapidly increasing (20). Adherence, associated with early VF in this study, is a general risk factor for VF, its effect even more pronounced in children less than 3 years of age and adolescents (21, 22). Older age and more advanced disease were associated with late VF in our study, have been associated with VF in other studies (23), though not specifically late VF. Given the lack of other studies separating late and early failure we can only speculate on the differences in risk factors. A possible explanation may be that early VF is caused by more important risk factors such as pre-treatment resistance, which mask less strong factors in the earlier years. Alternatively the effect of risk factors may vary over time, such as age and the life events that may affect treatment adherence and response. Despite the fact that we cannot fully explain these differences it is important to describe them as it may help to prioritize specific children and focus resources, which are commonly limited in sub-Saharan African settings.

Our data on DRMs showed that 30% of the children acquired mutations during 48 months. Mutations were obtained twice as much in the first 24 months, compared to the second period of follow-up. This outcome is in line with a previous report among HIV-infected children on long-term ART (7). Of note was that nearly all children with early VF had an acquired DRM as compared to 56% of children with late VF. We compared both the risk factors for and the patterns of acquired DRMs between early and late acquired DRMs. The risk factors for Early DRMs were different from those of late DRMs. Early acquired DRMs were associated with pre-treatment DRMs and a higher VL at ART start, both of which have been reported before. [6] The development of late DRMs was not associated to any of the factors tested. NNRTI resistance mutations were the most common mutations, which is comparable to previous reports on DRMs in HIV-infected children in sub-Saharan Africa (24,25). Resistance mutations as K65R, which triggers resistance against TDF, ABC, d4T, ddI, and rarely 3TC mostly generated by use of TDF or D4T (26),

were only identified in the early VF group. This corroborates with previous studies reporting K65R was mainly acquired within the first 24 months of treatment (5,27).

The clinical implications of these findings support the importance of regular VL testing within the first 24 months as well as the current strategy of initiating children on PI-based regimens. Encouraging adherence and a prompt switch of treatment regimen in this first 24 months of treatment is supported by our data, preferable preceded by drug resistance testing. In addition we have presented data on late ART failure which underscores the current trends of starting all children, regardless of age and disease staging, on early ART (11).

The major strength of our study is the comprehensive analysis of long-term outcomes of first line ART in HIV-infected children in sub-Saharan Africa, these are rare in the published literature. The Besides virological outcome, drug resistance mutations over time we tried to identify children at risk for failure after 24 months of treatment, which approach is unique. In addition our study has some potential limitations. Firstly, only major drug mutations were tested, so we may have missed minor mutations, contributing to resistance. However, since major drug mutations have an important role in susceptibility of the ART regimes and therefore acute treatment failure, we think our approach is of value. Secondly, the effect of drug class (NNRTI, PI, or triple NRTI) on VF or acquired drug resistance could not be investigated, because the numbers of children who were not on NNRTI-based regimen were too small. Thirdly, we possibly underestimated the prevalence of pre-treatment drug resistance, due to the fact that drug resistance mutations might be archived in older children and the median age in our cohort was relatively high with 4.7 years. Archived mutations could therefore be mistaken as acquired DRM. Fourthly, we may have overestimated the number of children failing at time point 48 months, as the definition of VF contained two viral loads >1000 copies/mL or a VL >1000 copies/mL on the last visit (11). Especially in children, who are known to have single episodes of detectable VL's, not necessarily meaning that a child is failing or developing resistance, this may have overestimated the amount of VF. Our worry that there may be a worsening of viral control is valid despite this possible bias as the number of viral suppression (not affected by this definition), decreased at 48 months. At last, adherence to ART was assessed through pill count over the previous 30 days before a clinic visit, which implicates that adherence was not measured in the first two months after the clinic visit. Adherence can therefore be underestimated in this

study. More long-term research is needed to evaluate our findings and concerns of deterioration in virological outcome after 48 months of treatment.

Conclusion

We conclude from one of the first very comprehensive studies evaluating long-term outcomes on paediatric ART African children that Virological Failure and acquired DRMs were common as they occurred in third of children during 48 months of follow-up. Although most VF occurred in the first 24 months a worrisome second decrease of viral suppression was seen at 48 months. Risk factors for early and late VF and DRMs were different. Although we cannot fully explain these findings it is important to describe them as it may help to prioritize and focus resources, which are commonly limited in sub-Saharan African settings. Clinical implication could include a) closer monitoring of children in the first 24 months of treatment and prompt switches to second line treatment and b) improved adherence support. In absence of pre-treatment drug resistance testing and individualised first line therapy the current strategy of starting children on a more robust second line (containing Protease inhibitors) should receive priority. The data on late failure underline the current trend to start all children (early) on ART (11). Future evaluation should prove the effect of these strategies.

Acknowledgments

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Baseline first line ART		
	n/N	**%**
Sex (male)	158/316	50.0
Age (median,IQR) yrs	4.77 (1.96-8.69)	
≥3 years	204/316	64.6
Previous antiretroviral treatment exposure (PMTCT) [1]		
Yes	16/316	5.1
No	264/316	83.5
Unknown	36/316	11.4
HIV subtype		
A	152/286	53.1
D	84/286	29.4
C/G	10/286	3.5
CRF	23/286	8.0
URF	16/286	5.6
Regimen[2]		
NNRTI-based	288/316	91.1
Triple NRTI	15/316	4.7
PI-based	13/316	4.1
Regimen, specified[3]		
AZT+3TC+ (EFV or NVP)	181/316	57.3
D4T+3TC+ (EFV or NVP)	99/316	31.3
ABC+3TC+(EFV or NVP)	8/316	2.5
ABC+3TC +(AZT or D4T)	15/316	4.7
3TC+LPV/r + (AZT or D4T)	13/316	4.1
Study site		
Kampala	90/316	28.5
Fort portal	118/316	37.3
Mbale	108/316	34.2
WHO stage[4]		
1	24/316	7.6
2	64/316	20.3
3	159/316	50.3
4	69/316	21.8

	n/N	%
CD4 category baseline[5]		
Normal	77/316	24.4
Diminished	167/316	52.8
Immune deficient	52/296	17.6
CD4 count/ul in children≥ 5 years (Median, IQR)	347 (210-689)	
CD4% in children< 5 years (median, IQR)	16.0 (10.9-21.4)	
Viral load (VL) at baseline		
<1000 cps/ml	17/309	5.5
1000-10,000 cps/ml	28/309	9.1
10,000-100,000 cps/ml	97/309	31.4
>100,000 cps/ml	167/309	54.0
Log VL baseline (median, IQR)	5.1 (4.5-5.6)	
Pre-treatment drug resistance mutations (PDR) and regimen activity[6]		
No PDR	225/316	71.2
PDR+GSS≥3	23/316	7.3
PDR+GSS<3 (Partially active regimen)	21/316	6.6
Unknown	47/316	14.9
Nutritional status at baseline		
BMI for age (BAZ) (median, IQR) [7]	-0,29 (-1.15 - 0.47)	
Underweight (BAZ<-2)	44/312	14.1
Haemoglobin (g/dL median, IQR)	11.0 (9.7-12.1)	
Anemia[8]	157/285	55.1

Table 1. Baseline characteristics.

[1]PMTCT: prevention of mother-to-child. [2] Non- Nucloside reverse Transcriptase Inhibitors (NNRTI), Nucloside reverse Transcriptase Inhibitors (NRTI), Protease inhibitor (PI).[3] Abacavir (ABC), Zidovudine (AZT), Stavudine (D4T), Lamivudine (3TC), Efavirenz (EFV), Nevirapine (NVP), Lopinavir/Ritonavir (*LPV/r*) [4]World health Organisation (WHO) definition of clinical staging (27).[5] Immune deficient; CD4% <25 and ≥ 10 by age < 59 months or CD4 count <500 cells/mm3 and ≥ 100 cells/mm3 by age >59 months. Immunodeficiency; CD4% < 10% by age < 59 months or CD4 count <100 cells/mm3 by age > 59 months(10). [6] HIV Drug Mutation Resistance (DRM) transmission(13) [7]Body Mass index (BMI) Z-score for age (15)[8]Anemia; Haemoglobin (Hb) <11.0 g/dl by age 0.5-4.99 years, Hb <11,5 g/dl by 5.0-11.99 years, Hb< 12by age 12.00-14.99 years, Hb<12 by woman age >15 years and Hb<13 by men age>15 years (16).

		EARLY viral failure (<24 months); N=75/284 (26.4%)						LATE viral failure (25-48 months); N=36/283 (12.7%)					
		Univariate				Multivariate		Univariate				Multivariate	
		VF (n/N)	non-VF (n/N)	Odds ratio	95%-CI:	Odds ratio	95%-CI:	VF (n/N)	non-VF (n/N)	Odds ratio	95%-CI:	Odds ratio	95%-CI:
Age group At baseline	≥ 3 years	43/75	149/209	0.5	0.3-0.9	0.7	0.3-1.7	30/36	160/247	2.7	1.1-6.8	2.5	1.0-6.6
Sex	Male	44/75	99/209	0.6	0.37-1.1	0.7	0.3-1.4	17/36	125/247	1.5	0.6-2.3	1.1	0.5-2.2
WHO stage At baseline	WHO 3/4	54/75	148/209	1.1	0.59-1.9			31/36	170/247	2.8	1.1-7.5	4.2	1.4-13.4
Activity baseline regimen[1]	Partially active	14/64	7/177	6.8	2.6-17.8	6.0	1.9-18.5	2/32	19/208	0.9	0.8-1.1		
Baseline viral load (log) (Median, IQR)		5.32 (4.8-5.8)	4.9 (4.3-5.4)	1.8	1.30-2.5	1.4	0.9-2.3	4.9 (4.6-5.5)	5.11 (4.4-5.6)	1.0	0.8-1.4		
Adherence	≤ 95%	28/75	56/209	1.6	0.93-2.8	3.1	1.3-7.4	16/36	69/247	2.1	1.0-4.2	1.9	0.8-4.3
Type of NNRTI regimen	Nevirapine	51/75	106/209	I		I		18/36	138/247	I			
	Efavirenz	17/75	85/209	0.4	0.22-0.77	0.4	0.1-1.1	17/36	85/247	1.5	0.8-3.1		
	Others	7/75	18/209	0.8	0.32-2.0	0.6	0.2-2.1	1/36	24/247	0.3	0.04-2.5		
PMTCT-exposed		4/66	11/186	0.97	0.3-3.2			1/34	14/217	0.79	0.3-1.9		

		EARLY viral failure (<24 months); N=75/284 (26.4%)						LATE viral failure (25-48 months); N=36/283 (12.7%)					
		Univariate				Multivariate		Univariate				Multivariate	
		VF (n/N)	non-VF (n/N)	Odds ratio	95%-CI:	Odds ratio	95%-CI:	VF (n/N)	non-VF (n/N)	Odds ratio	95%-CI:	Odds ratio	95%-CI:
Immune status baseline													
	Normal[2]	9/67	65/200	I		I		10/33	64/233	I			
	Diminshed[3]	38/67	111/200	2.5	1.1-5.4	1.6	0.6-4.0	18/33	132/233	0.9	0.4-2.0		
	Immunodeficient[4]	20/67	24/200	6.0	2.4-15.0	3.3	1.1-10.3	5/33	37/233	0.9	0.3-2.7		
Underweight baseline	BMI for age (BAZ) <-2 SD	17/73	23/209	1.9	0.9-4.0	0.8	0.3-1.9	5/35	32/245	1.1	0.4-3.1		

Table 2. Factors associated with viral failure (VF) among children during 48 month of ART.

Multivariate outcome is corrected for gender, age and study site. Explanatory variables associated with the outcome variables ($p < 0.10$) in the univariable analysis were included in the multivariable model in a stepwise approach. Abbreviations: NNRTI: non-nucleoside reverse transcriptase; PMTCT: prevention of mother-to-child transmission; OR: odds ratio; VF: Virological Failure; VL: Viral Load; 95%-CI: 95% confidence [1.]Based on calculation of genotypic sensitivity score (GSS). Fully active no DRM and/or GSS>3. Partially active DRM and GSS<1; [2.] Age<5 and CD4%≥25% or age≥5 and CD4count>500; [3]. Age <5 years and CD4% <25% or age ≥5 years and CD4 count<500; [4.] Age<5 years and CD45<10% or age ≥5 years and CD4 count<100.

		Early acquired DRM N=73/270 (27.0%)						Late acquired DRM N=39/270(14.4%)					
		Univariate				Multivariate		Univariate				Multivariate	
		ADRM (n/N)	non-ADRM (n/N)	Odds ratio	95% CI:	Odds ratio	95% CI:	ADRM (n/N)	non-ADRM (n/N)	Odds ratio	95% CI:	Odds ratio	95% CI:
Age group	≥3 years	49/73	128/197	1.3	0.8-2.3	0.6	0.3-1.4	24/39	110/231	2.7	1.1-6.3	2.4	0.97-5.9
Sex	Male	45/73	92/197	0.6	0.3-0.9	0.6	0.3-1.2	14/39	119/231	0.5	0.3-1.1	0.5	0.2-1.0
WHO stage	WHO3/4	56/73	133/197	1.6	0.9-2.9			35/39	154/231	4.4	1.5-12.8	2.2	0.7-7.4
Baseline activity regimen	Partially active	13/73	12/196	3.3	1.4-7.7	2.7	1.0-7.5	5/39.	20/230	1.5	0.5-4.4		
Baseline viral load (log) (Median, IQR)		5.4 (4.8-5.9)	5.11 (4.6-5.5)	1.7	1.2-2.4	1.8	1.2-2.9	5.4 (4.8-5.7)	5.2 (4.6-5.6)	1.3	0.9-2.0		
Adherence	< 95% adherence	27/73	54/197	1.6	0.9-2.7			16/39	65/231	1.8	0.9-3.6	1.2	0.7-3.7
Type of NNRTI Regimen	Nevirapine	46/73	95/197	I		I		21/39	120/231	I			
	Efavirenz	20/73	80/197	0.5	0.3-0.9	0.6	0.2-1.6	17/39	83/231	1.17	0.6-2.4		
	Others	7/73	22/197	0.7	0.3-1.6	0.7	0.3-2.4	1/39	28/231	0.20	0.03-1.6		
PMTCT	Yes	2/73	12/197	2.4	0.5-11.1			0/36	14/204				

		Early acquired DRM N=73/270 (27.0%)						Late acquired DRM N=39/270(14.4%)					
		Univariate				Multivariate		Univariate				Multivariate	
		ADRM (n/N)	non-ADRM (n/N)	Odds ratio	95% CI:	Odds ratio	95% CI:	ADRM (n/N)	non-ADRM (n/N)	Odds ratio	95% CI:	Odds ratio	95% CI:
Immune status	Normal[2]	12/69	52/186.	I		I		8/37	56/218	I			
	Diminshed[3]	38/69	107/186	1.5	0.7-3.2	0.4	0.5-2.7	22/37	123/218	1.3	0.5-3.0		
	Immunodeficient[4]	19/69	27/186	3.0	1.3-7.2	1.2	0.7-5.1	7/37	39/218	1.3	0.4-3.8		
Underweight	BMI for age (BAZ) ≤-2 SD	14/71	21/195	2.0	0.97-4.3	1.6	0.7-3.8	7/38	28/228	1.6	0.6-4.0		

Table 3. Factors associated with acquired drug mutations (DRM) during 48 months.

Outcome is divided by acquired DRM during the first 24 months (Early DRM; total number of children with acquired DRM =73) and after 24 months (late DRM; total number of children with acquired DRM =39) of treatment. Explanatory variables associated with the outcome variables ($p<0.10$) in the univariable analysis were included in the multivariable model in a stepwise approach. Multivariate outcome was corrected for age and study site. [1.] Based on calculation of genotypic sensitivity score (GSS). Fully active no DRM and/or GSS>3. Partially active DRM and GSS<1; [2.] Age<5 and CD4% ≥25% or age≥5 and CD4count>500; [3.] Age <5 years and CD4% <25% or age ≥5 years and CD4 count<500; [4.] Age<5 years and CD45<10% or age ≥5 years and CD4 count<100.[5] Virological Failure (VF); 2 consecutive VLs >1000 cps/ml or 2 VLs>1000 cps/ml with one. Two or three missing measurements in between or VL>1000 on last measurement with one other VL sample during 48 months & dead after minimal 6 months of ART. Abbreviations: NNRTI: non-nucleoside reverse transcriptase; PMTCT: prevention of mother-to-child transmission; OR: odds ratio; VF: Virological Failure; VL: Viral Load; 95%-CI: 95% confidence interval.

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Part II

HIV-infection in sub-Saharan Africa; co-morbidities

Chapter 5

Multiplex real-time PCR detection of intestinal protozoa in HIV-infected children in Malawi, Enterocytozoon bieneusi is common and associated with gastrointestinal complaints and may delay BMI (nutritional status) recovery

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Abstract

Background: Intestinal protozoa are common opportunistic infections in HIV patients. Longitudinal studies on either the clinical relevance, or the effects of immune reconstitution by anti-retroviral therapy on intestinal protozoan infections are children is lacking however. This study investigates prevalence and clinical relevance of intestinal protozoa in HIV-infected Malawian children prior to and during their first year of ART.

Methods: Stool samples collected at enrolment and during follow-up were tested for non-opportunistic (*Giardia lamblia, Dientamoeba fragilis, Entamoeba histolytica*) and opportunistic protozoa (*Entroctytoon bieneusi, Encephalitozoon spp., Cryptosporidium spp and Cystoisospora belli*) using multiplex real-time Polymerase Chain Reaction (PCR). Associations between infections and clinical symptoms were evaluated using univariate methods.

Results: Non-opportunistic and opportunistic protozoa were detected in 40% (14/35) and 46% (16/35) of children at baseline respectively. *E. bieneusi* was the most prevalent protozoa (37%, 13/35) and associated with gastrointestinal complaints (43% in positive (10/13) versus 18% (4/22) in *E. bieneusi*-negative children, p=0.001). Body Mass Index (BMI) recovery during 12 months of ART was more commonly delayed in *E. bieneusi*-positive (+0.29+SD 0.83) than *E. bieneusi*-negative children (+1.03+SD 1.25; p=0.05). *E. bieneusi* was not detected after 12 months of ART.

Conclusion*: E. bieneusi* was the most prevalent opportunistic intestinal protozoa, present in over a third of study participants prior to initiation of ART. Although all children cleared *E. bieneusi* after 12 months of ART, *E. bieneusi* was associated with gastro-intestinal complaints and may delay BMI recovery. Trials to assess effect of treatment of *E. bieneusi* on nutritional status should be considered in HIV-infected African children.

Background

Intestinal protozoal infections, caused either by opportunistic or non-opportunistic species, can complicate HIV infection in adults and children. Protozoal infections can cause chronic diarrhoea and thereby malabsorption leading to malnutrition and dehydration, contributing to the high rates of morbidity and mortality observed in resource limited settings (1-3). Prevalence data on protozoal infections in patients with chronic diarrhoea range widely between 1-75%, which can be explained by differences in demographics, seasonal variance and diagnostic methods (4, 5). Prevalence rates are higher in those studies employing modern, more sensitive, Polymerase Chain Reaction (PCR) techniques (6, 7). However PCR techniques are not commonly available in resource limited settings and therefore reliable data from these areas are scarce.

Treatment of HIV infection has greatly improved over the last decade due to the massive scale up of ART availability. In general, ART reduces HIV viral load, permits immune reconstitution and reduces the risk of new opportunistic infections (8). Reduction of the prevalence of intestinal protozoa in HIV-infected adults after introduction of ART has been documented both in industrialized nations and resource limited settings (9-11). However, prospective data on children receiving ART treatment is lacking. Longitudinal data is needed in order to clarify which opportunistic infection may resolve through immune reconstitution due to ART alone and which infections might require more targeted therapy.

Therefore this prospective cohort study was conducted to document the prevalence of opportunistic and non-opportunistic intestinal protozoa in HIV-infected children in Malawi, during the first year of first line ART, using multiplex real-time PCR techniques. Additionally, the study investigated the dynamics and clinical aspects of the most common infections in order to evaluate their contribution to clinical symptoms and morbidity in Malawian children.

Methods

Methods| Study population

The study cohort included ART-naïve HIV-infected children initiating ART at the Queen Elizabeth Central Hospital, Blantyre, Malawi. Children aged 18 months to 18 years were enrolled prior to initiating ART, if they met ART initiation criteria outlined in the Malawi National ART program guidelines (2010-2012)(12, 13). Local guidelines, applicable at the time of the study have been described in detail elsewhere (14). In short, ART was initiated in children based on WHO staging as well as immune status. Clinical criteria included: ≥ 18 months of age with WHO clinical stage 3 and severe immune suppression was based on the WHO guideline at the time of the study: for children aged < 59 months by CD4%< 25% and/or CD4 <750 cells/mm and children aged > 59 months CD4 < 350 cells/mm (12).

Methods| Data collection

Following recruitment and informed consent, a standardised history was collected, including socio-economic data and gastro-intestinal symptoms. A physical examination was performed including collection of anthropometry. Children were monitored via data collection evaluating symptoms and anthropometry throughout the first 12 months of ART. Blood and stool samples were collected at 0, 6 and 12 months. This intestinal protozoa study was nested within a larger ART follow-up cohort study. Stool samples were stored and determined at a later point in time. All physicians were unaware of the PCR results.

Methods| Laboratory

HIV infection was confirmed using two HIV antibody tests (Abbott Determine HIV-1/2 Test and Uni-Gold HIV test). Full blood count (Beckman Coulter HMX, Beckman coulter, CA USA) and CD4 count (flow cytometry, Becton Dickinson, CA USA) were analysed at the MLW Clinical Research Programme laboratory. Aliquots were stored for batch analysis of HIV-1 viral load (Roche Amplicor; Roche, Basel, Switzerland). Stool samples were stored at -20 degrees Celsius within 24 hours after sampling and subsequently shipped to LUMC, Leiden, The Netherlands. DNA isolation, amplification and detection were performed at LUMC as described elsewhere with some modifications, in particular the combinations of targets (15-17). One

multiplex real-time PCR was used for the detection of DNA of the non-opportunistic protozoa *G. lamblia, D. fragilis* and *E. histolytica* (15) and another multiplex real-time PCR targeted opportunistic protozoa including microsporidia *E. bieneusi and Encephalitozoon spp.* (i.e. *E. intestinalis*)(18) with *Cryptosporidium spp(16) and C. belli* (17). Appropriate positive and negative controls where included in each assay. The PCR output consisted of a cycle threshold (Ct-) value representing the amplification cycle in which the level of fluorescent signal exceeded the background fluorescence. Intensity of infection was categorized for each target into low, moderate and high DNA levels based on Ct-value, respectively: higher than 35, between 35 and 30 and lower than 30, while negative PCR results were recoded as Ct=50 (19).

Methods| Definitions

Gastrointestinal symptoms were actively asked for at each planned clinic visit and included abdominal pain, diarrhoea or vomiting. Anaemia was classified using age and gender specific haemoglobin (Hb) cut offs: children aged 18 months-59 months Hb< 11.0 g/dl, children 5.0-11.9 years < 11.5 g/dl, boys 12-15 year Hb< 12.0 g/dl, girls > 12 years Hb 12.0 g/dl; boys >15 years: Hb <13.0 g/dl (20). Severe immune suppression was defined using the WHO definitions according to age (20): 18- 59 months CD4% < 10% or CD4 count < 200 cells/mm3; > 59 months: CD4 count < 100 cells/mm3(21). Anthropometric data, based on weight, length and age related Z-score were calculated based on WHO Multicentre Growth Reference Study Group (22, 23). Primary outcome was BMI for age z-score (BMIZ) as this parameter was applicable to children of all ages whilst weight for age (WAZ) only applies to children < 10 years and height for age (HAZ) is a late marker for growth recovery (>6-12 months). Malnutrition was defined as a body mass index z-scores (BMIZ) < -2 SD (24).

Methods| Statistical analysis

Data were double entered, cleaned and analysed using SPSS version 19.0. The study was primarily powered to detect prevalence of protozoa at baseline and their change over time. Secondly univariate analysis assessed risk factors for protozoal infection using chi square test or Fisher exact test for categorical variables and independent sample t-test for continuous variables. All p-values presented are two-tailed and a significance level of < 0.05 was used.

Methods| Ethical considerations

The purpose of the study was explained to the guardians of each patient in Chichewa and written informed consent was obtained before inclusion into the study from the parents or guardians and assent from the children, if applicable. The study protocol was approved by the Research Ethics Committee of the College of Medicine, University of Malawi.

Results

A total of 35 children were included with a mean age of 7.9 (SD 4.2) years. Baseline characteristics are shown in table 1. At baseline, severe immune suppression was prevalent among 30% (10/33). Prevalence of severe immune suppression did not differ significantly between the different age groups: 26% (5/19) in children <10 years, versus 36% (5/14) if > 10 years; p=0.56. Fifteen percent (5/34) of children were malnourished (BMIZ< -2 SD) at baseline, which dropped to 3% (1/33) after 12 months on ART. Gastrointestinal complaints occurred in 40% (14/35) of children during the first 6 months of follow-up, but these complaints were not associated to immunosuppression however (p=0.411). Stool samples were available for 35 children at baseline, 27 (77%) children at 6 months and 26 (74%) children at 12 months of follow-up.

Results | Non-opportunistic protozoa at baseline

Non-opportunistic protozoa were detected in 40% (14/35) of the children at baseline (table 2). *G. lamblia* was the most common protozoa and detected in 26% (9/35) of the samples. *D. fragilis* was prevalent in 17% (6/35) of children whilst *E. histolytica* was not detected. Three of nine children (33%) with *G. lamblia* infection at baseline had gastrointestinal complaints. Gastrointestinal complaints among children with *G. lamblia* were not more common than among children without *G. lamblia* (42% (11/26), p=0.22). At baseline, BMI for age z-scores (BMIZ) were -0.60 (SD 1.0) for *G. lamblia* positive children versus a mean of -0.55 (SD 1.4) for negative children, p=0.92. Also *D. fragilis* infections at baseline were not associated with gastrointestinal complaints or a significant difference in BMIZ scores. Respectively 50% (3/6) of the *D. fragilis* positive children showed gastrointestinal complaints

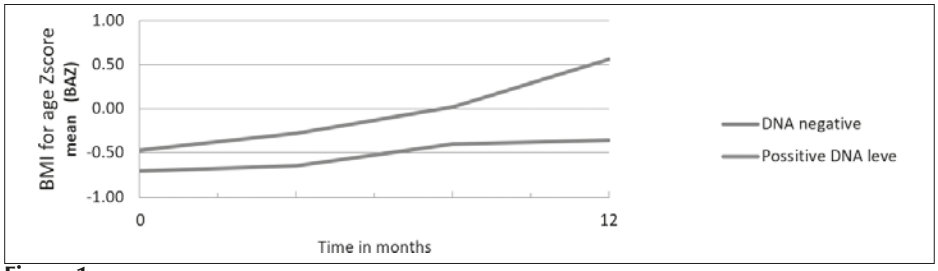

Figure 1a.

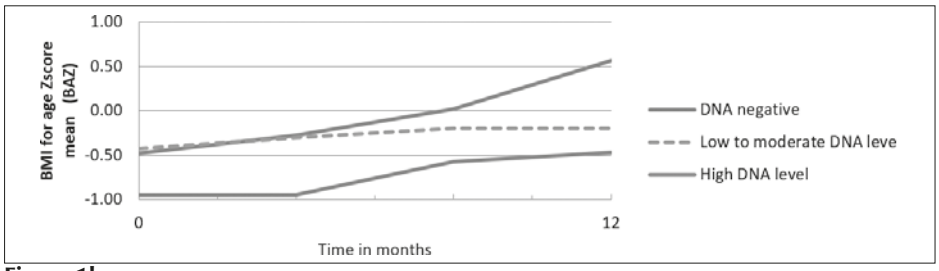

Figure 1b.

Figure 1. BMI for age-z scores (BMIZ) of children with and without detectable *E. Bieneusi* DNA levels during 12 months of ART.

BMI for age-z scores calculated based on WHO Multicentre Growth Reference Study Group (20, 21). **1a)** After 12 months follow-up the BMIZ remained lower in children with *E. bieneusi* in comparison to those without *E. bieneusi* infection; BMIZ -0.36 versus BMIZ +0.56; p=0.05. **1b)** After 12 months follow-up the BMIZ remained lower in children with *E. bieneusi* high DNA level in comparison to those with moderate to low and a negative *E. bieneusi* DNA level: BMIZ -0.47, -0.20 versus +0.56 for high, low to moderate and negative *E. bieneusi* DNA levels at baseline; p=0.25. Detected DNA level based on Ct-value: Low to moderate DNA level: Ct 30-50 and high DNA level: Ct<30.

versus 38% (11/29) among *D. fragilis* negative children, p=0.30. BMIZ at baseline was +0.56 (SD 0.9) versus +0.55 (SD 1.4) in *D. fragilis* positive and negative children respectively, p=0.99.

Results | Opportunistic protozoa at baseline

Opportunistic protozoa were detected in 46% (16/35) of children at baseline. *E. bieneusi* was the most common opportunistic protozoa, present in 37% of samples (13/35). Gastrointestinal complaints were more common among children with *E. bieneusi* at baseline than those without infection (43% (10/13) versus 18% (4/22), p=0.001 (table 3). Children with and without *E. bieneusi* had a similar BMIZ at

baseline; respectively BMIZ -0.6 (SD 1.0) versus BMIZ. -0.6 (SD 1.4), p=0.92. The clinical relevance of and *E. bieneusi* infection is described in detail below. *Cryptosporidium spp* was seen in 11% (4/35) of children, whilst *Encephalitozoon spp* (i.e. *E. intestinalis*) and *Cystoisospora belli* were not detected at baseline. *Cryptosporidium spp* infections at baseline were not associated with gastrointestinal complaints (42% (13/31) versus 25% (1/4) for *Cryptosporidium spp* positive and negative children respectively, p=0.42). No difference in mean BMIZ at baseline was seen: -0.16 (SD 0.4) versus -0.57 (SD 1.3), p=0.56 for *Cryptosporidium spp* positive and negative children respectively.

Results | Effect of 12 months ART on intestinal protozoa

The prevalence of non-opportunistic protozoa and opportunistic protozoa infections over time are described in table 2. Non-opportunistic protozoa were present in 43% (15/35) of the children at baseline and 42% (11/26) after 12 months of ART. The prevalence of *G. lamblia* dropped from 26% (9/35) to 11% (3/26), however new infections also occurred, table 2. Prevalence of *D. fragilis* increased from 17% (6/35) towards 31% (8/26) following ART initiation. All opportunistic protozoa, *including E. bieneusi*, were cleared after 12 months of ART.

Results | E. bieneusi

To identify children at risk for *E. bieneusi* and to assess the clinical importance of the infection, potential risk factors and symptoms were compared between children with and without *E. bieneusi*, table 3. *E. bieneusi* was more common in children older than 10 years of age when compared to younger children (57% (8/14) vs 24% (5/21) respectively, p=0.05). Children with *E bieneusi,* when compared to children without, did not suffer from more severe immune suppression at baseline (38.5% (5/13) versus 25.0% (5/15), p=0.46). However children with and without *E. bieneusi* had a similar BMIZ at baseline, significant differences were seen after 12 months of ART. Children with an *E. bieneusi* infection showed a trend towards lower BMIZ when compared to those without; BMIZ -0.36 (SD 0.97) versus BMIZ +0.56 (SD 1.39), p=0.052 (figure 1a). BMIZ recovery after 12 months was +0.29 (SD 0.83) versus +1.03 (SD 1.25) (p=0.050) for *E. bieneusi* infection versus non-infected children. Children with high levels of *E. bieneusi* DNA in their stool showed a non-significant trend towards a more delayed BMI recovery, figure 1b. BMIZ at 12 months of follow-up was -0.47

(SD 1.03), -0.20 (SD 0.97) versus +0.56 (SD 1.39) for high, low to moderate and negative baseline *E. bieneusi* DNA levels, respectively (p=0.25).

Discussion

This study presents important new PCR prevalence data evaluating non-opportunistic and opportunistic intestinal protozoa infections in HIV-infected African children receiving ART. *E. bieneusi,* an opportunistic infection, was the most prevalent intestinal protozoa. Despite being a rather unknown opportunistic infection amongst paediatric clinicians, *E bieneusi* was not only common but might be clinically relevant, as it was associated with both gastro-intestinal complaints and possible reduced BMI recovery. Although all children cleared *E. bieneusi* by 12 months of ART and gastro-intestinal complaints diminished, the BMI recovered poorly in those with an *E. bieneusi* infection.

Thirty seven percent of the ART naïve HIV-infected children were infected with *E. bieneusi* in this cohort. The reported prevalence of *E. bieneusi* in the literature shows wide ranges (0.8-79%) of *E. bieneusi* prevalence. This may be explained by differences in diagnostic methods, demographics and study populations, including ages, presence of clinical symptoms, other underlying conditions and severity of the immune suppression (25-27). Two other studies which used PCR-based assays in HIV-infected African children reported prevalence rates ranging from 20% - in a general population of HIV-infected children in South Africa to 79% - in children with HIV and diarrhoea in Uganda of *E. bieneusi* infection(5, 27). In the current study, all children cleared *E. bieneusi* and other opportunistic protozoa following immune recovery after 12 months of ART. This is the first paediatric cohort study evaluating non-opportunistic and opportunistic intestinal protozoa infections including *E. bieneusi* during ART treatment. However the data collected in children is corroborating with data in adults showing clearance of the *E. bieneusi* infection after receiving several months of ART (9, 10). The finding that children clear opportunistic protozoa are especially reassuring as children are considered to be more vulnerable to *E. bieneusi* as it is more common in children than adults (25).

Gastrointestinal complaints are commonly reported in microsporidian infections such as *E. bieneusi* (28, 29). In the current study 43% of the children

with a PCR-detected *E. bieneusi* infection described gastrointestinal complaints. The prevalence of gastrointestinal symptoms corroborates data in HIV-infected adults where *E. bieneusi* infection has been associated with diarrhoea and vomiting (28, 29). More importantly this cohort demonstrated that *E. bieneusi* is associated with a delayed BMI recovery, which persisted despite protozoal clearance and reduction of gastrointestinal complaints. Other, cross-sectional studies have also reported an association between malnutrition and lower rates of weight gain *E. bieneusi* infected HIV-patients (28, 30, 31). Malnutrition and lower rates of weight gain are usually attributed to on-going diarrhoea and impaired absorption of micronutrients due to mucosal damage as well as malabsorption caused by direct replication of protozoa in the epithelium of small intestine(29). In the presenting study, the gastrointestinal complaints resolved within 6 months and immune status improved during 12 months of ART treatment, while a poor nutritional state, signified by delayed BMI recovery, persisted. Secondly there was no association between potential confounders such as immune suppression at baseline, (table 3), neither tuberculosis infection at enrolment, nor ART failure at 12 months and BMI recovery in this population (data not shown). Further studies are needed to assess if the effect on BMI is secondary to a direct effect of E. bieneusi on the mucosa or if more factors are involved in the delayed BMI recovery.

Like other studies, a large part of patients with an *E. bieneusi* infection detected by PCR are asymptomatic (5, 25). However other studies have not assessed if the lower protozoal load may explain why some do not have symptoms (5, 25). Low detectable DNA levels may, for instance, reflect spore shedding rather than actual infection (6, 31-33). *E. bieneusi* spores are detected in stool specimens by microscopy for 9-33 days, while stool specimens evaluated by PCR are positive for 3-40 days longer (25). PCR results should be therefore be interpreted with some care, especially if low DNA levels are detected. Despite this we did identify a trend towards a poorer BMI recovery among children with high *E. bieneusi* DNA levels in this relatively small study.

None of the children received treatment following their PCR results in this study. Given our finding of a significant association between E. bieneusi and a poor BMI-recovery and clinical symptoms, the differences found were small and both may have multiple causes. Therefore the effect of therapy for E bieneusi on these outcomes should be tested in a placebo-controlled trial. Effective treatment for

E. bieneusi is complex, however. Albendazol has shown conflicting results in the treatment of different E. bieneusi subtype infections (34, 35). Fumagillin, a newer agent was shown to be effective in adults and was approved in 2005, but severe adverse effects limit its use and availability (35, 36). If these restrictions also apply to children is unclear as paediatric data are very limited despite showing good effectiveness and no side effects (36). TPN 470, the fumagillin analogue, may be a potentially safer alternative though it again lacks paediatric testing (2, 34, 35). Given the serious reduced BMI recovery in these HIV-infected children, phase 2 and 3 trials should be considered.

Besides *E. bieneusi, G. lamblia* was a common non-opportunistic pathogen. Prevalence of *G. lamblia* worldwide can differ enormously due to diagnostic test, population, demographics, season and ages (37, 38). Our reported PCR detected prevalence of 26% is similar to a previously reported prevalence of 30% among severely malnourished children in Blantyre, Malawi (39). Immune status recovery has a questionable effect on infection while the prevalence of *G. lamblia* dropped from 26% towards 11% during 12 months of ART, new infections also occurred. *G. lamblia* infection can present with gastro-intestinal complaints, but carriers may also be asymptomatic (37, 40). In this study, gastrointestinal complaints and malnutrition could not be linked to *G. lamblia* infection. As we detected mainly moderate and low DNA loads (78%), we may have identified a large proportion of asymptomatic carriers. As *G. lamblia* is not an opportunistic infection, it is unlikely that prevalence will be reduced by restored immunity due to ART.

Cryptosporidium spp occurred in this study but infections resolved after immune recovery during ART treatment and DNA levels were only high in two children. Earlier PCR-based studies in Malawi (Blantyre) among pre-school children with diarrhoea and an unknown HIV status showed a comparable prevalence of *Cryptosporidium spp* of 5-9%(41). Cryptosporidiosis is commonly associated with symptoms of diarrhoea, however in this study gastrointestinal complaints were not associated with infection (42). This effect may be explained by the relatively low to moderate infection prevalence of all *Cryptosporidium spp* infection at baseline as compared to other pathogens. Considering the low prevalence and the lack of an effective treatment in children, routine testing will likely not benefit children in an outpatient setting (43). Combined infections were not common among the presenting cohort. Clinical symptoms are therefore not related and influenced by co-infections.

This study had several limitations. Firstly, this study used local guidelines to start ART in Malawi in 2010, which meant that the studied cohort was severely immune compromised in comparison to current cohorts of children starting ART. Current WHO guidelines suggest start ART early in the course of HIV disease(21). Current HIV-infected populations may therefore have lower protozoal prevalence rates. Given the high prevalence in our study and the fact that HIV diagnosis still is often delayed in African settings we believe our findings are still important. Secondly, the number of children recruited is small and follow-up was stopped at 12 months; therefore associations may have been missed or suggested that required a larger sample size or prolonged follow-up. Still this study is the first paediatric cohort from Africa using PCR techniques with 12 months of follow-up during ART. Thirdly, the use of PCR may have overestimated the prevalence of pathogens over time as PCR also detects spores, which are not active pathogens (6, 31-33). However, we assessed the effect of DNA load and were able to show a trend towards a more delayed BMI recovery in children with a higher load. To confirm this effect more research is needed to investigate the duration of diminished growth after 24 months and the eventual effect of (repeated presumptive) treatment on growth over time. Lastly, we have not performed of *E. bieneusi* subtype analysis, this information could be of use as clinical presentation is influenced by genotype and therefore may vary in different geographic regions (44, 45).

Conclusion

This prospective cohort reports on the prevalence of intestinal protozoa in HIV-infected children in Malawi, during the first year of first line ART using multiplex real-time PCR techniques. *E. bieneusi* is a very common pathogen at start of ART and clinically important as it was associated with gastrointestinal complains and may be associated with prolonged reduced growth, a predictor of poor prognosis. Future studies should focus on trials to assess treatment options for *E. bieneusi* to improve symptoms and poor nutrition status.

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		Baseline	6 months	12 months
Sex	Boys	17/35(49%)		
Age	Years (mean/SD)	7.9 years (±4.2)		
	> 10 years	14/35 (40%)		
Toilet use	Private flushing	3/34 (9%)		
	Pit private	18/34 (53%)		
	Communal pit	13/34 (37%)		
Water source	Communal tap	28/34 (82%)		
	Borehole	5/34 (15%)		
	Others	1/34 (3%)		
Symptoms				
Gastrointestinal symptoms [*1]		14/35 (40%)[*2]	1/35 (3%) [*3]	
Diarrhoea		7/35 (20%) [*2]	0/35 (0%) [*3]	
Vomiting		8/35 (23%) [*2]	0/35 (0%) [*3]	
Immune status				
WHO classification	I	16/35 (46%)		
	II	7/35 (20%)		
	III	12/35 (34%)		
	IV	0/35 (0%)		
Severe immune suppression [*4]		10/33 (30%)	2/30 (7%)	2/33 (6%)
Haematology				
Anaemia[*5]		23/34 (68%)	6/33 (18%)	11/33 (33%)
Anthropometry				
BMI for age Z-score (BMIZ) (mean (SD)		-0.6 (1.3)	-0.1 (1.4)	+0.2 (1.3)

Table 1. Patient characteristics at baseline and during first 12 months on ART.

Abbreviations: SD= standard deviation. [*1] Gastrointestinal-symptoms include; abdominal pain or diarrhoea or vomiting. [*2] Complaints during 0-6 months follow-up *[3] complaints during 6-12 months follow-up [*4] Severe immune suppression: age.< 59 months: CD4% < 10% or a CD4 count < 200 cells/mm3(20); age > 59 months: CD4 count < 100 cells/mm3 (21). [*5] Anaemia: 18 -59 months: haemoglobin level (Hb) < 11.0 g/dl, 5.0-11.9 years Hb < 11.5 g/dl, boys 12-15 years: Hb< 12.0 g/dl, girls > 12 years: Hb <12.0 g/dl; boys >15 years: Hb <13.0 g/dl.

		Baseline (n=35)	6 months (n=27)	12 months (n=26)
Non-opportunistic protozoa				
G. lamblia	Total positive	9 (26%)	4 (15%)[*1]	3 (12%)[*2]
	High DNA level	2/9 (22%)	2/4 (50%)	1/3 (33%)
	Moderate DNA level	5/9 (56%)	0/4 (0%)	1/3 (33%)
	Low DNA level	2/9 (22%)	2/4 (50%)	1/3 (33%)
D. fragilis	Total positive	6 (17%)	6 (22%)[*3]	8 (31%)[*4]
	High DNA level	4/6 (67%)	2/6 (33%)	1/8 (13 %)
	Moderate DNA level	2/6 (33%)	2/6 (33%)	5/8 (63%)
	Low DNA level	0/6 (0%)	2/6 (33%)	3/8 (34%)
Opportunistic protozoa				
E. bieneusi	Total positive	13 (37%)	6 (22%)*[5]	0 (0%)
	High DNA level	7/13 (54%)	3/6 (50%)	
	Moderate DNA level	3/13 (23%)	2/6 (33%)	
	Low DNA level	3/13 (23%)	1/6 (17%)	
Cryptosporidium spp	Total positive	4 (11%)	3 (11%)[*6]	0 (0%)
	High DNA level	0/4 (0%)	1/3 (33%)	
	Moderate DNA level	0/4 (0%)	1/3 (33%)	
	Low DNA level	4/4 (100%)	1/3 (33%)	
C. belli	Total positive	0 (0%)	2(7%)[*7]	0 (0%)
	High DNA level		0/2 (0%)	
	Moderate DNA level		1/2 (50%)	
	Low DNA level		1/2 (50%)	

Table 2. Multiplex real-time PCR results of protozoal infections in HIV-infected children during the first year of ART.

E. histolytica and *Encephalitozoon spp* (i.e. *E. intestinalis*) were not detected. Combined *E. bieneusi-Cryptosporidium* infection at baseline was seen in 3%; 1/35 and E. *bieneusi* combined with *G. lamblia* infection at baseline was seen among 11%; 4/35. Detected DNA level based on Ct-value: Total positive: Ct<50, low DNA level: Ct 35-50, moderate DNA level: Ct30-35 and high DNA level: Ct<30. *[1] no new infections [*2] 2 new infections, [*3] 6 new infection [*4] 7 new infections, [*5] 4 new infections; [*6] 3 new infections, *[7] all new infections.

		E. bieneusi **Negative DNA level**	***E. bieneusi*** **Positive DNA level**	**p-value**	**Odds ratio**	**95% CI**
Sex	Boys	9/22 (41%)	8/13 (62%)	0.31	0.4	0.1-1.8
Age	Years (mean (±SD)	6.85 (±3.6)	9.8 (±4.5)	0.04	1.2	1.0-1.5
Symptoms						
Gastrointestinal symptoms[*1]	0-6 months[*2]	4/22 (18%)	10/13(43%)	0.001 [*4]	15.0	2.8-80.9
Diarrhoea	0-6 months[*2]	1/22 (5%)	6/13 (46%)	0.003 [*4]	18.0	1.8-176.6
Vomiting	0-6 months[*2]	1/22 (5%)	7/13 (54%)	0.001 [*4]	24.5	2.5-240.3
Immune status						
WHO	I	10/22 (46%)	6/13 (46%)	0.85	-	-
	II	5/22 (23%)	2/13 (15%)			
	III	7/22 (32%)	5/13(39%)			
	IV	0	0/39 (0%)			
Severe immune suppression [*3]	Baseline	5/20 (25%)	5/13 (39%)	0.41	1.9	0.4-8.5

Table 3. Factors associated at baseline and over 12 months follow-up with E. bieneusi infection at baseline.

Abbreviation: SD= standard deviation. [*1] Gastrointestinal symptoms include; abdominal pain or diarrhoea or vomiting. [*2] Complaints during 0-6 months follow-up [*3] Severe immune suppression: age.< 59 months: CD4% < 10% or a CD4 count < 200 cells/mm3(20); age > 59 months: CD4 count < 100 cells/mm3 (21).[*4] p-signifant < 0.05. Toilet use and water availability did not differ significant between children with and without E. bieneusi infection (data not shown).

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Chapter 6

Severe anaemia complicating HIV in Malawi; multiple co-existing aetiologies are associated with high mortality

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Submitted

Abstract

Background: Severe anaemia is a major cause of morbidity and mortality in HIV-infected adults living in resource-limited countries. Comprehensive data on the aetiology is lacking and needed to improve outcomes.

Methods: HIV-infected adults with severe (haemoglobin ≤70g/l) or very severe anaemia (haemoglobin ≤50 g/l) were recruited at Queen Elizabeth Central Hospital, Blantyre, Malawi. Fifteen potential causes of severe anaemia of anaemia and associations with anaemia severity and mortality were explored.

Results: 199 patients were enrolled: 42.2% had very severe anaemia and 45.7% were on ART. Over two potential causes for anaemia were present in 94% of the patients; including iron deficiency (55.3%), underweight (BMI<20: 49.7%), TB-infection (41.2%) and unsuppressed HIV-infection (viral load >1000 copies/ml) (73.9%). EBV/CMV co-infection (16.5%) was associated with very severe anaemia (OR 2.8 95% CI 1.1-6.9). Overall mortality was high (53%; 100/199) with a median time to death of 17.5 days. Death was associated with folate deficiency (HR 2.2; 95% CI 1.2-3.8) and end stage renal disease (HR 3.2; 95% CI 1.6-6.2).

Conclusion: Mortality among severely anaemic HIV-infected adults is strikingly high. Clinicians must be aware of the urgent need for a multifactorial approach, including starting or optimising HIV treatment; considering TB treatment, nutritional support and attention to potential renal impairment.

Introduction

Anaemia is recognized as the most common haematological complication of Human Immunodeficiency Virus (HIV) infection worldwide (1, 2). The World Health Organization (WHO) defines anaemia as a haemoglobin level below 110-120 g/l. In sub-Saharan Africa, 60% of HIV-infected adults are anaemic and 22% are severely anaemic (3, 4). Anaemia is independently associated with an increased one-year mortality in HIV infection of 8%, which rises to 55% in those with severe anaemia (5, 6).

To prevent and treat severe anaemia in HIV-infected patients, a comprehensive understanding of the aetiology and pathophysiology is essential. Severe anaemia in HIV infection has been associated with micronutrient deficiencies, infections (viral, bacterial and parasitic), medication induced (zidovudine and co-trimoxazole) and neoplastic diseases (7-11). Only a few studies have comprehensively studied the multifactorial aetiology and pathophysiology of HIV-associated severe anaemia in sub-Saharan Africa despite the high burden of HIV infection in this region (2). Commonly, studies only report on the association between HIV infection and a single cause of severe anaemia, for example iron deficiency, without considering the multiple causes of severe anaemia that may impact on an HIV-infected patient (12, 13). As a consequence, evidence to inform preventive or treatment guidelines for severe anaemia in HIV-infected patients in sub-Saharan Africa is scarce. In practice, severe anaemia management in HIV-infected patients is often still based on the same strategies used for non-HIV infected patients, including iron supplementation, malaria treatment and de-worming (2, 14). This strategy may be ineffective as the causes of HIV-associated severe anaemia may be different, and even harmful considering that (blind) iron supplementation may exacerbate infections and thus may deteriorate the patients condition (15, 16).

Better knowledge about the aetiology of severe anaemia is essential in order to develop evidence-based protocols and ultimately improve outcomes for HIV-infected adults with severe anaemia in sub-Saharan Africa. To address this knowledge gap, we performed a comprehensive observational cohort study to explore the prevalence of potential aetiologies of HIV-associated severe anaemia in Malawian in-patients and studied associations between these and the severity of the anaemia and patient outcomes.

Methods

An observational cohort study of HIV-infected patients with severe anaemia (haemoglobin ≤70 g/l) admitted to the Queen Elizabeth Central Hospital (QECH), Blantyre, Malawi between February 2010 and March 2011 was performed. All HIV-infected patients above 18 years of age with severe anaemia admitted to the general medical ward were approached and enrolled in the study if they provided informed consent. A case record form including a detailed medical history and physical examination was completed for each enrolled patient. On admission a venous blood sample was collected and a chest X-ray was performed. The patients were managed according to the hospital protocols, which included a blood transfusion if required, treatment of correctable conditions including anti-malarial medication and antibiotics. Antiretroviral treatment (ART) was provided according to the national Malawi guidelines, which stipulated that ARTs should only be initiated by specialist outpatient clinicians, so patients were not initiated on in-patient wards. At the time of the study first line ART was a combination of Stavudine, Lamivudine and Nevirapine and second line treatment was a combination of Zidovudine, Lamivudine, Tenofovir and Lopinavir/Ritonavir (17). ART was only prescribed for WHO stage 3 and 4 disease, or WHO stage 1 and 2 disease with CD4 count < 350 x 10^9/l (18). Co-trimoxazole was prescribed routinely to all HIV-infected patients on ART as Pneumocystis Jirovecii prophylaxis. For this study patients were followed up in the dedicated ART clinic after discharge. A community research nurse followed those who failed to attend their appointments up at home. Follow-up was done for a maximum of 365 days after enrolment when they attended the ART clinic for routine appointments or, if they failed to attend, by a home visit from a study nurse.

Methods| Laboratory assays

All samples were analysed within 24 hours of collection or stored at -80^{0}C for further analysis. On enrolment haemoglobin concentrations were measured on the ward using the HemoCue B-Haemoglobin analyser (HemoCue, Ängelholm, Sweden) to screen for eligibility. For patients enrolled in the study, the haemoglobin and red cell indices (MCV, MCH were determined and MCHC) using an automated analyser (Beckman Coulter, Durban, South Africa). CD4-cell counts were assessed using BD FACS Count (BD Biosciences, San Jose, CA, USA). Transferrin, iron, ferritin,

folate and vitamin B12 were analysed on Modular P800 and Monular Analytic E170 systems (Roche Diagnostics, Switzerland). Soluble transferrin receptor (sTfR) levels were measured using ELISA (Ramco Laboratories, TX, USA). Analyses for serum creatinine was by Beckman Coulter CX5 (ADVIA 2400 Siemens Healthcare Diagnostics). Renal function was measured by a estimating of Glomerular Filtration Rate (eGFR) using simplified Modification of Diet in Renal Disease (MDRD)-Study formula and the GFR was classified by Chronic Kidney Disease classification (19-21). For all tests the manufacturers' reference ranges were used; internationally accepted cut-offs were used to define deficiencies (22). Thick blood films were prepared and stained for malaria microscopy. Malaria was defined as the presence of *Plasmodium falciparum* asexual parasites in the blood films. HIV infection was confirmed using two point-of-care antibody tests (Unigold® and Determine®). A venous blood sample was inoculated into BACTEC Myco/F-Lytic culture vials and incubated in a BACTEC 9050 automated culture system (Becton Dickinson) for 56 days. Sub-culturing blood and sputum, susceptibility testing and isolate identification were performed by standard techniques(23). Possible contaminants were recorded as absence of pathogens. Sputum cultures were examined for mycobacteria using Ziehl–Nielsen staining. Whole-blood isolates were assessed for Epstein–Barr virus and cytomegalovirus infection by semi-quantitative PCR and for parvovirus B19 by real-time PCR (24). All chest X-rays were reviewed by a radiologist for signs of pulmonary tuberculosis (TB). `When TB was suspected, standardized treatment was started according to the local protocols.

Methods| Bone marrow

If the patient's clinical condition allowed and they provided consent, a bone marrow aspirate and trephine biopsy was performed. All bone marrow samples were taken from the posterior iliac crest. Samples of the aspirates were spread onto slides and trephine biopsies were fixed, decalcified and embedded in paraffin wax (25, 26). Bone marrow samples were sent to the Haematopathology Referral Centre at the Royal Liverpool University Hospital, Liverpool UK, for analysis. Sections of the trephine blocks were stained with haematoxylin and eosin and Giemsa, and also for iron with Perls stain, and for reticulin (25). All slides were examined for using a predefined format and diagnoses were allocated to the categories lymphoproliferative disease, myeloproliferative disease, myelodysplastic syndrome

(MDS) and TB (27-29). The need for additional histochemical (e.g. Ziehl-Neelsen) or immunohistological (e.g. CD3, CD20) staining was determined according to the local protocol in Liverpool depending on the preliminary morphological findings.

Methods| Definitions

The following potential factors involved in the aetiology of severe anaemia were defined and evaluated; 1) Unsuppressed HIV-infection; viral load ≥1000 copies/ml. 2) TB: one or more of the following were present: a) positive sputum culture, b) chest X-ray with signs of pulmonary TB and/or c) on going TB treatment at time of enrolment d) clinical diagnosis by local doctor including unknown generalized lymphadenopathy and/or night sweats of > 30 days and of unknown origin e) caseating granulomata in the bone marrow trephine. 3) Malaria: presence of malaria parasites in a thick blood film. 4) Parvovirus B19: viral load of >1000 copies/ml. 5) Cytomegalovirus (CMV); load of >100 copies/ml. 6) Epstein-Barr virus (EBV); viral load >100 copies/ml. 7) Bacteraemia; a blood culture growing a potential pathogen. 8) Underweight (BMI ≤18.5). 9) Serum folate deficiency (≤3 ng/l). 10) Vitamin B12 deficiency (≤180 pg/ml). 11). Iron deficiency was defined as MCV≤ 83 fl (3, 22, 30). 12) Zidovudine usage. 13) Co-trimoxazole usage. 14) Bone marrow disorders; lympho-proliferative disease, myeloid-proliferative disease or MDS. 15) Renal impairment: a GFR which either indicated impaired (GFR 15–59 ml/min/1.73 m^2) or End Stage (GFR ≤15 ml/min/1.73 m^2) Renal Disease (21, 31).

Methods| Statistics

Baseline characteristics and prevalence of potential risk factors are presented as proportions or medians with IQR. Logistic regression was performed to model the association between anaemia and potential factors involved severity of anaemia, Results are expressed as OR with 95% CI and p-values. Variables associated with the outcome variables ($P \leq 0.10$) in the univariate analysis were included in the multivariate model in a stepwise approach. Kaplan Meier survival curves were used to assess cumulative mortality. Significant differences were investigated with a Log Rank test. Uni- and multivariate analyses were done using logistic regression and Cox regression to describe predictors of mortality. P values of ≤ 0.05 were regarded as statistically significant. All reported P values were two-sided. The data were analysed using Stata (version 12) (STATA Corp. LP, Texas, TX, USA).

Methods| Ethics

The Research Ethics Committee of the College of Medicine, University of Malawi (P.09.09.824) and the Research Ethics Committee of Liverpool School of Tropical Medicine (research protocol 09.64) approved the study. The purpose of the study was explained to the patients in the local language Chichewa and written informed consent was obtained before inclusion in the study.

Results

In total, 199 patients were included in the study: 64.8% were female. The median age was 32 years (IQR 27-61 years). The median haemoglobin was 53 g/l (IQR 4.2-6.3) and 84 (42%) patients had very severe anaemia (hb≤50 g/l). A total of 91 (45.7%) patients were on ART at enrolment including 79.1% on first line ART. During the study period, an additional 41 (21%) patients started on ART treatment. Moreover 67.1% of the patients were immune suppressed with a CD4 count ≤ 200 cells/mm. Baseline characteristics of the patients are shown in table1. The prevalence of factors that were associated with severe anaemia, and factors that were co-existing in individual patients, are shown in table 2. Overall, patients had a mean of 3 (range 1-8) co-existing aetiologies of severe anaemia (figure 1).

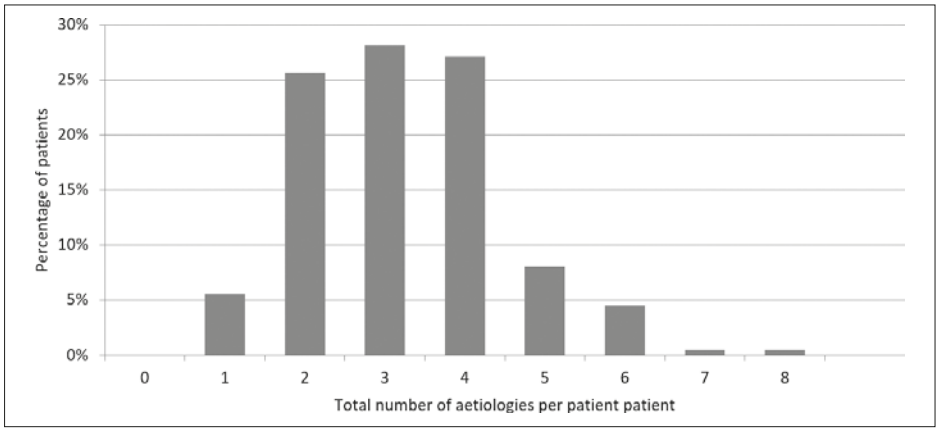

Figure 1. Total number aetiologies for severe anaemia co-existing in each patient (n=199). Mean is 3 factors (SD 1.3), range 1-8.

	Overall
Age, years (median IQR)	32 (IQR 27-61)
Sex (female)	129/199 (64.8%)
Haematology	
Haemoglobin (Hb) (median, IQR) g/l	53 (IQR 42-63)
Severe anaemia (Hb 51-70 g/l)	115/199 (57.8%)
Very severe anaemia (Hb ≤ 50g/l)	84/199 (42.2%)
Pancytopenia[1]	42 /184 (21.2%)
Mortality	
Overall mortality (365 days)	101/199 (50.8%)
Early mortality (60 days)	81/101 (80.2%)
Days until death (median, IQR)	17.5 (6-55)
HIV-disease and treatment	
CD4 (median, IQR)	175 (IQR 55-825)
CD4 ≤200 cells/mm^3	104/155 (67.1%)
Viral load ≥1000 copies/ml	136/184 (73.9%)
ART at enrolment	91/199 (45.7%)
First-line ART	72/199 (36.2%)
Second-line ART	11/199 (5.5%)
Non-specified ART	8/199 (4%)
Blood transfusions and supplemental treatment	
Blood transfusion at admission	47/199 (23.6%)
Folate supplementation at admission	57/199 (28.6%)
Iron supplementation at admission	81/199 (40.7%)
Vitamin B12 supplementation at admission	0/199 (-)

Table 1. Baseline characteristics of HIV-infected patients with severe anaemia at enrolment into study.

[1] Pancytopenia is defined as thrombocytopenia (≤150x10^9/l) and leukopenia (≤4x10^9/l) and severe anaemia (≤ 70 g/l) [22]. Abbreviations: Hb: Haemoglobin, ART: antiretroviral therapy

An unsuppressed HIV-infection was seen among 73.9% patients. TB was the second most common infection occurring in 82 (41.2%) of the patients. In 19 (23%) of these patients TB was diagnosed on their chest X-ray. Granulomata were seen in the bone marrow trephine in 15 (18%) patients of all the 82 patients that had a diagnosis of TB. 11 (13%) patients were on TB treatment at enrolment. 69/170 (40.5%) patients had evidence of current EBV infection and 57/170 (33.5%) had evidence of current CMV infection. Co-infection with CMV and EBV was found in

of 28/170 (16.5%) of the patients. Bacteraemia was diagnosed on a positive blood culture in 26 (13.1%); the most common pathogens found were E. Coli (12 patients; 42.9% and non-Typhoid Salmonella (5 patients; 19.9%). 74/148 (49.7%) patients were underweight and iron deficiency occurred in 61/180 (33.9%) of the patients. Bone marrow sampling was performed in 73 patients. Of these, 28 (38.4%) had morphological abnormalities with MDS being the most common abnormality (20 patients; 27.4%) (table 2). Renal impairment was diagnosed in 36/185 patients (19.5%) and 12 of these patients (33%) had end stage renal disease.

Comparing the different risk factors for very severe anaemia (Hb<50g/L) as compared to severe anaemia, EBV/CMV co-infection (OR 2.8 95% CI 1.1-6.9) was the only factor associated with very severe anaemia (table 2).

During the one-year follow-up period, 101 study patients (50.8%) died. The median time to death was 17.5 days (IQR 6-55) and 81 (80.2%) of these deaths occurred within 60 days of admission (figure 2). Folate deficiency and end stage renal disease were associated with mortality with Hazard Ratio 2.0 (95% CI 1.2-3.6) and Hazard Ratio 3.0 (95% CI 1.5-5.9) respectively (figure 3). Neither very severe anaemia (haemoglobin ≤50 g/l), nor the haemoglobin levels were associated with mortality in the study patients (Hazard Ratio 0.9, 95%CI 0.6-1.4 and Hazard Ratio 1.01, 95% CI 0.9-1.2 respectively).

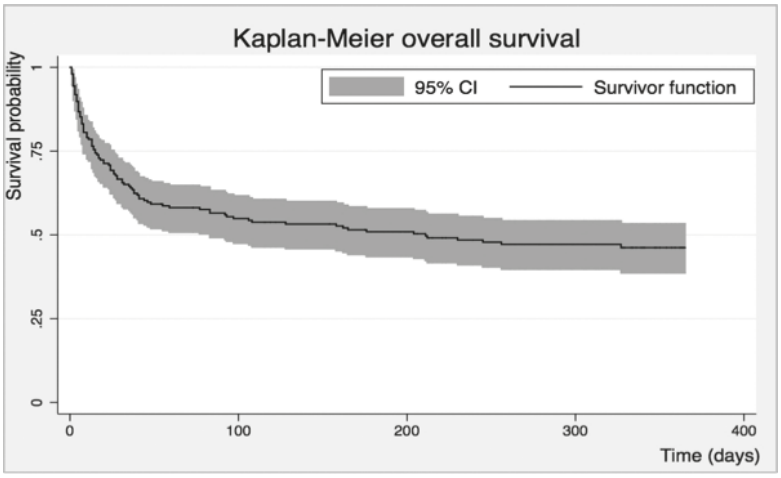

Figure 2. Kaplan Meyer curve for survival probability over time (days) for adult Malawian patients with HIV infection and severe anaemia during 365 days follow-up.
Abbreviation: 95% confidence interval (95%CI).

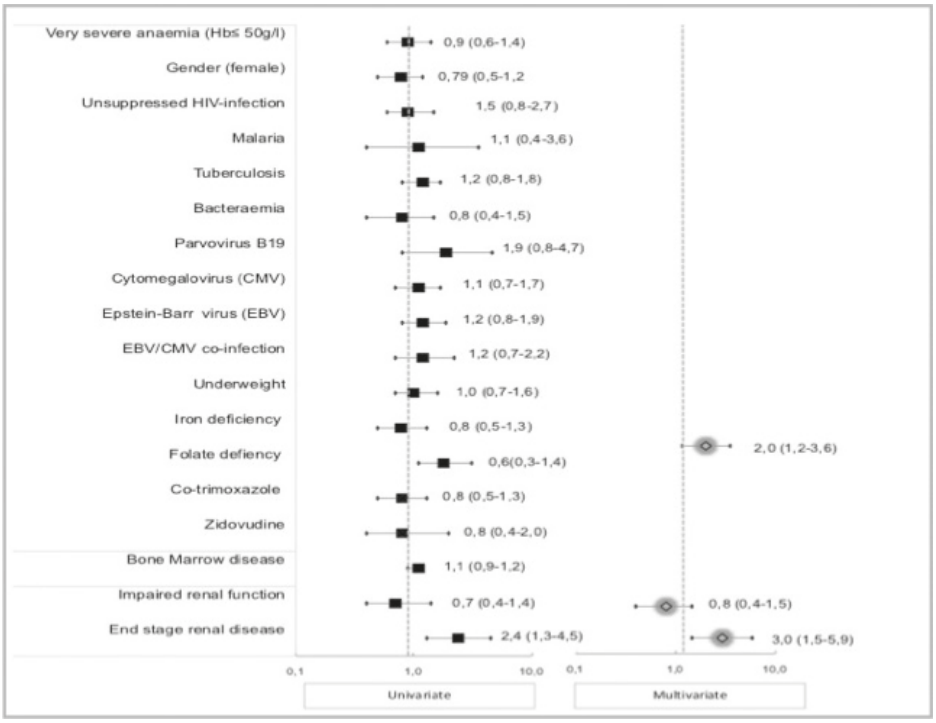

Figure 3. Risk factors for 365-day mortality in HIV-infected patients with severe anaemia. Univariate and multivariate Cox regression outcome. Folate deficiency (≤3 ng/l); HR 2.0 95% CI 1.2-3.6 and end stage renal disease (GFR ≤15); HR 3.0 5% CI 1.5-5.9, were associated with overall mortality. Abbreviations: Hb: Haemoglobin, GFR; Glomerular filtration rate, VL Viral Load

Discussion

In this study we described the prevalence of several potential aetiologies for severe and very severe anaemia in HIV-infected Malawian adults. Patients had a mean of three co-existing aetiologies potentially contributing to their anaemia, the most common one being unsuppressed HIV. Mortality in the study patients was extremely high, as 51% of the patients died within one year and most died within 60 days of admission. Severe anaemia in HIV-infected patients in a resource limited setting, such as Malawi, should therefore be treated as a multi-causal critical condition with high mortality.

	Overall (n=199)	Severe anaemia N=115/199 (57.8%)	Very severe anaemia N=84/199 (42.2%)	Univariate			Multivariate		
				Odds	95% -CI	P-value	Odds	95% -CI	P-value
Sex (female)	129 (64.8%)	74/115 (65.2%)	55/84 (65.4%)	1.1	0.6-1.9	0.869	-	-	-
HIV									
CD4 ≤200 cells/mm^3	104/155 (67.1%)	62/87 (71.3%)	42/68 (61.8%)	1.04	0.6-1.8	0.889	-	-	-
Viral load ≥1000 copies/ml	136/184 (73.9%)	81/104 (77.9%)	55/80 (68.8%)	0.6	0.3-1.2	0.163	0.7	0.3-1.5	0.306
On ART at enrolment	91/199 (42.7%)	52/115 (40.9%)	39/84 (45.2%)	1.1	0.6-1.8	0.865	-	-	-
Infection									
Malaria	6/167 (1%)	3/100 (3.0%)	3/67 (4.5%)	1.5	0.3-7.8	0.617	-	-	-
Tuberculosis	82/199 (41.2%)	53/115 (46.0%)	29/84 (34.5%)	0.7	0.6-0.99	0.043	0.6	0.1-2.8	0.507
Bacteraemia[1]	26/199 (13.1%)	17/115 (61.7%)	9/82 (11.0%)	0.7	0.3-1.6	0.402	-	-	-
Parvovirus B19	7/170 (4.2%)	5/99 (5.0%)	2/71 (2.8%)	0.5	0.1-2.9	0.476	-		
Cytomegalovirus (CMV)	57/170 (33.5%)	28/99 (28.3%)	29/71 (40.8%)	1.8	0.9-3.3	0.088	-	-	
Epstein-Barr virus (EBV)	69/170 (40.6%)	40/99 (40.4%)	29/71 (40.8%)	1.0	0.6-1.9	0.954	-	-	-
EBV/CMV co-infection	28/170 (16.5%)	12/99 (12.1%)	16/71 (22.5%)	2.1	0.9-4.5	0.075	2.8	1.1-6.9	0.032
Malnutrition									
Underweight	74/148 (49.7%)	40/81 (49.3%)	34/68 (50.0%)	1.0	0.5-2.0	0.940	-	-	-
Vitamin B12 deficiency	2/194 (1.0%)	1/113 (0.8%)	1/81 (1.2%)	1.4	0.09-22.7	0.813	-	-	-
Iron deficiency	61/180 (33.9%)	38/104 (36.5%)	23/76 (30.3%)	0.8	0.40-1.42	0.380	-	-	-
Folate deficiency	23/194 (11.9%)	13/113 (11.5%)	10/84 (11.9%)	1.1	0.5-2.6	0.858	-	-	-
Medication									
Co-trimoxazole	163/199 (81.9%)	99/115 (81.6%)	64/84 (76.2%)	0.5	0.3-2.3	0.076	0.5	0.2-1.2	0.099
Zidovudine	13/199 (6.5%)	5/50 (10.0%)	8/40 (20.0%)	2.3	0.7-7.5	0.187	-	-	-
Renal function									
Normal (GFR >60)	149/185(80.5%)	90/105 (85.7%)	59/80 (73.8%)	I			I		
Impaired (GFR 15-60)	24/185 (13.0%)	12/105 (11.4%)	12/80 (15.0%)	1.5	0.6-3.6	0.339	1.0	0.7-5.0	0.176
End stage (GFR ≤15)	12/185 (6.5%)	3/105 (2.9%)	9/80 (11.3%)	4.6	1.2-17.6	0.027	3.0	0.7-13.1	0.140
Bone marrow									
Bone marrow disease	28/71 (39.4%)	19/42 (45.2%)	9/29 (31.0%)	0.5	0.2-1.5	0.231	-	-	
Aetiology									
Co-existing aetiologies per patient (mean, SD)	3.3 (1.3)	3.3 (1.2)	3.2 (1.4)	0.8	0.4-1.8	0.605	-	-	-

Table 2. Distribution and multivariate analysis of co-existing factors associated with severe and very severe anaemia (Hb≤ 50 g/l) in HIV-infected adults in Malawi.

[1] A total of 28-blood cultures were positive, the most common organisms were E. coli (42.9%; 12/28) and non-Typhoid Salmonella (17.9 %; 5/28).
Explanatory variables associated with the outcome variables (P>0.10) in the univariable analysis were excluded in the multivariable model in a stepwise approach (-). Abbreviation: GFR; Glomerular filtration rate.

Anaemia is an independent predictor of mortality and disease progression in HIV-infected patients and mortality increases with decreasing haemoglobin concentration (6, 32). Previous studies reported an estimated one-year mortality of 3.7% in HIV-infected patients without anaemia and up to 30-55% in severe anaemia in resource limited settings (5, 6). Our study results are consistent with these outcomes. Additionally we found that a mean of three contemporaneous aetiologies potentially contributed to severe anaemia in HIV-infected patients. This is important as it highlights the multi-causality of severe anaemia in this population. Clinicians should therefore not just focus on a single cause and single treatment for severe anaemia in HIV-infected patients. Different treatment protocols from those used traditionally to treat anaemia (e.g. iron and folate) will be needed to address these multiple co-existing aetiologies to enhance improvement of mortality amongst these patients. Also, the seriousness of severe anaemia in HIV-infected patients needs to be better recognized. Currently severe anaemia in HIV-infected patients is classified as a stage 3 clinical condition (33). However due to the high mortality found in our, and previous, studies, we recommend re-classifying severe anaemia as a marker of HIV stage 4 disease.

Unsuppressed HIV virus was present in 79% of the patients in our study population. HIV may cause anaemia directly through an inhibitory effect of the HIV-virus on the erythropoietin progenitor cells in the bone marrow, or indirectly through opportunistic infections causing anaemia (34). Although this potential direct/indirect risk factor was very common it was not associated with the severity of the anaemia or mortality in our population, explored in our multivariate analysis. This is in contrast with a large cross-sectional study in Tanzania showed that the risk of developing severe anaemia in HIV-infected patients increased two to three times among patients with advanced HIV disease (3). We therefor postulate that controlling HIV infection by starting or switching ART treatment should therefore be considered as the most important and urgent step in treatment protocols for severely anaemic HIV-infected patients. After our study had been completed guidelines for initiating ART changed. At the time of the study the trigger for starting ART was based on a patient's CD4 count, whereas it is currently recommended that ART should be started early in the course of HIV disease (17, 33). It will be important for the impact of this policy change on HIV-related anaemia, and its consequences, to be evaluated. Irrespective of the policy change for initiating ART, the findings of our study remain

very relevant because many HIV-infected patients in resource-limited settings present late in the course of their disease or are unable to access reliable supplies of ART, and are therefore likely to continue to have high levels of life-threatening anaemia.

TB has previously been associated with anaemia in HIV-infected patients (36). In our study patients TB was a common co-infection (41%). This prevalence is comparable to previous reports on TB prevalence (43%) among severely anaemic HIV-infected patients in Africa (8, 14). The pathophysiology of TB-associated anaemia in HIV-infected patients remains unclear. Bone marrow invasion by TB organisms or altered iron metabolism, as a side effect of tuberculostatic drugs have been described (36). Only 13% of patients were on TB treatment at enrolment. TB medication itself was not associated with the severity of anaemia (data not shown). Given that we and others have found TB in nearly half of the HIV-infected patients with severe anaemia, TB screening and rapid initiation of treatment should therefore be a high priority in the management of these patients, especially in resource limiting settings where there is a high TB prevalence.

Viral infections such as EBV, CMV and parvovirus B19 have been associated with anaemia in HIV-infected patients (35, 37). In our study EBV and CMV were common and present in 40% and 35% of patients respectively, whilst parvovirus B19 was less common (4%). Although parvovirus B19 is pathophysiological linked to mild anaemia, the role in the development severe anaemia in HIV infected patients has not been clearly established (38). Data on co-infections are very limited in African patients (39-41) and our study is the first to describe the association between co-existing CMV and EBV infections and very severe anaemia (haemoglobin ≤50 g/l). Possible explanations for this association include direct viral inhibition of erythropoiesis (39, 42, 43). The majority of our patients with co-infection had advanced HIV disease (88.5%) but the association between EBV and CMV co-infection and very severe anaemia remained significant even after correction for advanced HIV disease. As CMV is a treatable infection it will be important to determine the effect of ART on CMV infection and severe anaemia, as ART can reduce CMV infection by improving immune status (44).

Malaria is unsurprisingly associated with severe anaemia in HIV-infected patients in sub-Saharan Africa (2, 14). However, the contribution of malaria to anaemia in our study population was small however, as only 6 patients (3.5%) had malaria parasites on presentation. This is in line with data from other studies that

show that the role of malaria in causing anaemia, especially in HIV patients in sub-Saharan Africa (2, 14), is limited and likely to have been overestimated (40, 45).

Malnutrition was common in our population as half of the patients' had a BMI below 18.5m^2 and deficiencies of iron and folate were diagnosed in 33.9% and 12% of the patients respectively. The prevalence of iron deficiency in our study is higher than in previous studies on HIV-infected patients in sub-Saharan Africa populations that report prevalence rates of iron deficiency of 18%-25% of anaemic HIV-infected patients (46, 47). Interestingly, adults in sub-Saharan Africa with severe anaemia but without HIV infection, have a higher prevalence of iron deficiency (59%) (45). Data from children with severe anaemia in Malawi also showed a much higher overall prevalence of iron deficiency (47%) than the adults in our study (40). Studies presenting this outcome are all published before 2010, in this time period ART was less available and all included HIV-infected patients were ART naïve and sever immune suppression was highly present. In this group of HIV-infected patients with better immune systems the aetiology of severely anaemic may be more similar to the aetiology of non-HIV infected patients. It can be expected that the prevalence of condition such as iron deficiency is also more alike the HIV-uninfected groups (40). In addition to finding a high prevalence of iron deficiency, ours is the first study to document on a possible association between folate deficiency and increased mortality in severely anaemic HIV-infected patients. There are a limited number of studies describing micronutrient supplementation, including folate, for anaemic HIV-infected adults, but overall there appears to be little significant effect of supplementation on reducing morbidity and mortality (37, 48). Macronutrient support using, for example, fortified wheat flower, has had beneficial effects on anaemia reduction and micronutrient levels in populations in sub-Saharan Africa, but has never been tested in the context of severe anaemia in HIV-infected patients (49, 50). More research is therefore needed to evaluate the effectiveness of both macro- and micro- nutritional support in severely anaemic HIV-infected adults.

HIV-infected adults have an increased risk of neoplastic bone marrow diseases, which can often cause anaemia (13). However, prevalence data for such conditions among HIV-infected patients with anaemia, especially in resource limiting settings, are scarce. Only three of our study patients had confirmed bone marrow malignancies. In contrast MDS was common occurring in 27% of our patients (27, 51).

Renal impairment was a frequent finding among our study patients and has been linked to HIV disease progression, anaemia and poor outcomes in both wealthy and resource limited settings (52-55). Evaluation of renal function is an important component of severe anaemia treatment protocols (30, 53) since it may affect the choice of ART (52, 56). It may also have implications for clinical management, for example by introducing measures to prevent further deterioration or considering potential benefits of erythropoietin(53).

Our study has several limitations. We purposely did not explore factors potentially associated with anaemia that previous studies had shown were uncommon in Malawi, such as haemoglobinopathies and parasitic infections (40, 57). We also did not include an HIV-infected population without severe anaemia against which to compare the prevalence of anaemia aetiologies and clinical outcomes. Only a sub-population of our patients gave consent for bone marrow sampling. Although these were an unselected group of patients it is possible that this may have introduced a hidden bias, for example by excluding the patients who were particularly unwell. Nevertheless, our findings are very valuable since bone marrow data from HIV-infected African patients is very scarce.

Conclusion

Our study has demonstrated that severe anaemia in HIV-infected adults in Malawi is associated with multiple co-existing aetiologies and has a strikingly high mortality rate. Severe anaemia in HIV-infected patients is therefore a critical indicator of mortality and requires urgent and multiple interventions. Particularly important is the initiation of ART, the management of infections such as TB and CMV, and optimisation of renal function. Intervention studies are needed to properly define the role (and safety) of iron and folate supplementation, as well as to develop and evaluate guidelines, which are feasible and effective in resource-limited settings to help clinicians manage these patients more effectively.

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Chapter 7

Hepcidin and conventional markers to diagnose iron deficiency in severely anaemic HIV-infected patients in Malawi

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Submitted

Abstract

Introduction: Iron deficiency is a treatable cause of severe anaemia in low-and-middle-income-countries (LMIC). Diagnosing it remains challenging as peripheral blood markers poorly reflect bone-marrow iron deficiency (BM-ID), especially in the context of HIV-infection.

Methods: Severe anaemic (haemoglobin ≤70g/l) HIV-infected adults were recruited at Queen Elizabeth Central Hospital, Blantyre, Malawi. BM-ID was evaluated. Accuracy of blood markers including hepcidin alongside mean corpuscular volume, mean cellular haemoglobin concentration, serum iron, serum ferritin, soluble transferrin receptor (sTfR), sTfR -index, sTfR –ratio to detect BM-ID was valued by ROC area under the curve (AUC^{ROC}).

Results: Seventy-three patients were enrolled and 35 (48.0%) had BM-ID. Hepcidin and MCV performed best; AUC^{ROC} of 0.593 and 0.545. Other markers performed poorly (ROC<0.5). The AUC^{ROC} of hepcidin in males was 0.767 (sensitivity 80%, specificity 78%) and in women 0.490 (sensitivity 60%, specificity 61%).

Conclusion: BM-ID deficiency was common in severely anaemic HIV-infected patients and is an important and potential treatable contributor to severe anaemia. Hepcidin was the best, though still suboptimal, marker of BM-ID. Hepcidin, which is directly linked to iron absorption, is a very promising marker to guide curative iron supplementation policies in severely anaemic HIV-infected patients.

Introduction

Anaemia affects approximately a third of the world's population and substantially reduces the disability- adjusted life years worldwide (1). Iron deficiency contributes to development of anaemia and is diagnosed in more than half of all anaemic persons (2). Consequently, iron supplements remain the backbone of prevention and treatment protocols for anaemia.

Anaemia has an extensive list of potential causes. In sub-Saharan Africa, where this condition is most common, its aetiology is even more complex and in these setting aetiologies commonly co-incide requiring a multifactorial approach (3, 4). HIV may be the cause of anaemia by its direct effect on BM cells, but can also increase the range of aetiological factors to encompass opportunistic viral, bacterial and parasitic infections, drugs such as Zidovudine and co-trimoxazole, micronutrient deficiencies and neoplastic diseases (5, 6).

The exact role of iron deficiency, one of the few potentially preventable and treatable causes of anaemia, remains unclear due to its diagnostic challenges in HIV-infected patients in low resource settings (3, 4, 7). Peripheral blood markers, including erythrocyte indices, serum iron, ferritin, and soluble transferrin receptor (sTfR), have been evaluated but their accuracy is often negatively affected by inflammatory states and renal and liver conditions, which are common in both the African and HIV-infected populations (8-11). Previous studies therefore concluded that the uses of peripheral blood markers, such as ferritin, are not reliable without correction for inflammation (12). The evaluation of iron in the bone marrow is considered the 'gold standard' to diagnose iron deficiency, but bone marrow sampling is invasive and requires skilled staff for sampling and interpretation, which is challenging in low resource settings. Moreover, for large-scale use, a reliable peripheral blood marker is needed to replace bone-marrow biopsy to predict bone marrow iron deficiency (BM-ID).

Hepcidin is a relatively new marker, which regulates iron absorption from the gastrointestinal tract and iron release from stores, both of which are important pathways controlling the availability of iron for incorporation in the erythrocyte precursors (13). Increases of iron plasma levels stimulate the production of hepcidin, which blocks further iron absorption from the gastrointestinal tract and iron release

from storage. However, hepcidin is also an acute phase protein and serum levels increases during infections (14).

We investigated the prevalence of BM-ID in HIV-infected Malawian adult patients with severe anaemia. We further evaluated the accuracy of peripheral blood markers, as well of hepcidin to identify BM-ID in this population.

Methods

From February 2010 to March 2011, all adults admitted to the Department of Internal Medicine of the Queen Elizabeth Central Hospital (QECH), Blantyre, Malawi with a diagnosis of severe anaemia and HIV infection were approached for informed consent and study enrolment. This study is a sub-study of the larger observational cohort study (n=199) concerning severely anaemic (haemoglobin ≤ 70 g/l) HIV-infected patients. Bone marrow sampling was performed if the patient consented and the patient was clinically stable. Of the 199 included patients, 73 BM (37%) samples were included in this sub-study as the BM sampling was performed and the quality of the sample taken was appropriate.

Methods| Laboratory assays and blood markers

Haemoglobin concentration was measured on admission using the HemoCue B-Haemoglobin analyser (HemoCue, Ängelholm, Sweden) to screen patients for eligibility. After informed consent a venous blood sample was collected and bone marrow sample taken from the iliac crest. All blood samples were analysed within 24 hours of collection or stored at -80^{0}C. Haemoglobin and red cell indices (MCV, MCH and MCHC) were determined using an automated haematology analyser (Beckman Coulter, Durban, South Africa). CD4-cell counts were assessed using BD FACS Count (BD Biosciences, San Jose, CA, USA). Transferrin, iron, ferritin, folate and vitamin B12 were analysed on Modular P800 and Monular Analytic E170 systems (Roche Diagnostics, Switzerland). Soluble transferrin receptor (sTfR) levels were measured using ELISA (Ramco Laboratories, TX, USA). Commonly used ratios to define iron deficiency were calculated including the sTfR-index: sTfR (mg/L) divided by log ferritin (ug/L); and the 'sTfR ratio': sTfR (mg/L) x1000/ ferritin (ug/L) level (15). International accepted cut-offs were applied. For sTfR we used 2.75 mg/l and 3.6

mg/l and for the sTfR-index: 1.8 and 2.2, 2.8 respectively, as no international cut-offs have been defined, these represented the most resent consensus (16).

Serum hepcidin-25 measurements were performed in December 2012/ January 2013 (Testing lab: Hepcidinanalysis.com, Nijmegen, The Netherlands) by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (WCX-TOF MS) using synthetic hepcidin-24 as internal standard (17-19). Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ ionisation TOF MS platform (Bruker Daltonics, Bremen, Germany). Hepcidin concentrations are expressed as nanogram per mililitre (ng/ml). The lower limit of detection of this method was 0.5 ng/ml

Methods| BM-ID

Aspirate samples were spread onto slides and trephine biopsies were fixed, decalcified and embedded in paraffin wax (20, 21). Bone marrow samples were sent to the Haematology- Pathology Referral Centre at the Royal Liverpool University Hospital, Liverpool UK, for analysis. Sections of the trephine blocks were stained with Perls' Prussian Blue stain to detect iron stores(21). Intracellular iron in bone marrow trephine blocks was graded using the Stuart-Smith scale, which classifies the iron content of bone marrow into six grades (0–6). For bone marrow smears, iron was graded using Gale's grading (0-4). Iron deficiency was defined as no visible or severe reduced iron particles in a few reticulum cells under high power magnification; grade 0-1 on both scales (22, 23).

Methods| Infections

HIV infection was confirmed using two point-of-care antibody tests (Unigold® and Determine®). Different types and severity of on-going infections were evaluated. Including; HIV: CD4 counts ≤200 cells/mm^3 and/or viral load >1000 copies/ml. Malaria: presence of malaria parasites in a thick blood film assessed by light microscopy. Tuberculosis (TB) defined as one or more of the following: a) positive sputum culture; b) chest X-ray with signs of pulmonary tuberculosis and/ or; c) on-going TB treatment at time of enrolment; d) clinical diagnosis based on generalized lymphadenopathy and/or night sweats > 30 days with unknown origin; e) caseating granulomata in the bone marrow trephine. Bacteraemia was defined as blood cultures growing potential pathogen including streptococcus, enterococcus

and micrococcus species, non-Typhoid Salmonella and Klebsiella pneumonia. Furthermore, viral infections including Parvo-B19, Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) were evaluated by PCR and defined as positive by viral load >100 copies/ml.

Methods| Ethics

The Research Ethics Committee of the College of Medicine, University of Malawi (P.09.09.824) and the Research Ethics Committee of Liverpool School of Tropical Medicine (research protocol 09.64) approved the study. The purpose of the study was explained to the patients in the local language (Chichewa), and written informed consent was obtained before inclusion into the study.

Methods| Statistics

The data were analysed using Stata (version 12) (STATA Corp. LP, Texas, TX, USA). Baseline characteristics were compared between BM-ID and non-deficient patients using Chi-square test (dichotomous data) or t-test (continuous) or Pearson Chi-square test (continuous not normally distributed). Confounding was enhanced to evaluate hepcidin concentrations, gender, HIV disease progression, the use of ART as baseline, and TB infection (Pearson Chi-square test). The p-values reported are two-sided, and a level of $p<0.05$ was interpreted as significant.

The accuracy of the different peripheral blood markers, including hepcidin, to discriminate BM-ID were evaluated by receiver operating characteristics curves (ROC)(24). Corresponding areas under the curve (AUC^{ROC}) were created. AUC^{ROC} measures the two-dimensional area underneath the ROC curve and provides a summative measure of performance across all possible classification thresholds (25). The AUC^{ROC} <0.70 is weighed as low diagnostic; AUC^{ROC} of 0.70–0.90 as moderate diagnostic and a AUC^{ROC} ≥0.90, high diagnostic accuracy (26). Sensitivity and specificity were calculated for predefined internationally accepted cut-offs (8, 15, 16, 27). For hepcidin the best cut-off value for diagnosing BM-ID were determined using ROC-curve analyses with the Youden index (maximum (sensitivity + specificity – 1))(25). As gender differences are known for hepcidin (30) hepcidin outcome was hence evaluated by gender.

Results

Of the 73 HIV-infected adults in our sub-study, a total of 45 (61.6%) had severe anaemia (Hb 50-<70g/dL) and 28 (38.4 %) had very severe anaemia (Hb<50g/dL). The mean patient age was 33.7 (SD 8.7) years, and 43 (58.9%) patients were female. A CD4 count ≤ 200 cells/mm^3 was seen in 31/56 (55.4%) and a viral load >1000 copies/ml was present in 57/76 (75.0%) of patients. A total of 34/73 (46.6%) patients had been started on anti-retroviral ART treatment, of which most were on first line treatment at time of the study (Efavirenz, Lamivudine, Tenofovir). The most common infections in this population were tuberculosis (39/73; 53.4%) and EBV (30/45; 66.7%). All baseline characteristics are shown in table 1.

Results| BMI-ID and blood markers

BM-ID was seen among 35 (48.0%) of the patients, table 1. The performances of the peripheral blood markers to diagnose BM-ID are displayed in table 2. All markers displayed low diagnostic accuracy (AUC^{ROC}< 0.7). MCV had the highest AUC^{ROC} value of the common peripheral blood markers (0.545), the sensitivity and specificity using the common cut off of 83fL were 42% and respectively 67%. The use of hepcidin to detect BM-ID resulted in an AUC^{ROC} 0.593. We stratified the analysis for hepcidin according to gender; the AUC^{ROC} for men and women was 0.767 and 0.490 respectively. The optimal hepcidin concentration for the detection of BM-ID was ≤7 ng/ml; sensitivity 67% & specificity 67%. In males the optimum cut off was ≤6 ng/ml (sensitivity 80%; specificity 78%); whilst for women this was ≤7 ng/ml (sensitivity 60%; specificity 61%, figure 1). The hepcidin concentration did not differ significantly by gender (p=0.831), HIV disease progression (p=0.819), the use of ART at enrolment (p=0.616), and TB infection (p=0.590) in a univariate analysis.

Characteristic	Overall	Non BM-ID	BM-ID	P-value
BM-ID	**35/73 (48.0%)**	**38/73 (52.1%)**	**35/73 (48.0%)**	
Age, years (mean, SD)	33.7 (8.7)	32.7 (8.6)	34.7 (8.9)	0.331
Gender (female) (%)	43/73 (58.9%)	19/38 (50.0%)	24/35 (68.6%)	0.107
Haematology & iron markers				
Very severe anaemia (Hb≤ 50g/l) (%)	28/73 (38.4%)	13/38 (34.2%)	15/35 (42.9%)	0.448
Haemoglobin (Hb)(g/l), (median, IQR)	56.0 (43.0-63.0)	58.5 (45.0-64.0)	54.0 (36.0-63.0)	0.136
MCV (fl), (median, IQR)	85.8 (79.4-98.1)	87.3 (79.6-99.0)	83.5 (79.1-94.7)	0.534
MCH (pg/cells), (mean, SD)	29.0 (5.9)	23.6 (6.1)	26.3 (5.5)	0.084
Serum iron (umol/l), (median, IQR)	5.1 (3.3-11.1)	4.7 (3.0-7.9)	5.6 (3.8-22.2)	0.053
Ferritin (ug/dL), (median, IQR)	87.2 (49.6-100.0)	87.1 (50.1-97.1)	87.9 (36.0-100.0)	0.488
sTfR receptor (mg/l), (median, IQR)	2.9 (1.6-3.7)	2.8 (1.7-3.7)	3.0 (1.2-3.9)	0.934
sTfR index (median, IQR)	1.6 (0.8-2.2)	1.5 (0.9-2.1)	1.6 (0.6-2.6)	0.901
sTfR Ratio (median, IQR)	35.1 (17.5-69.3)	33.2 (21.3-61.9)	36.6 (11.5-79.6)	0.747
Hepcidin (ng/ml) (median, IQR)	7.3 (3.3-13.3)	9.2 (4.9-13.2)	5.1 (3.1-13.7)	0.196
HIV diseaseand treatment				
ART at enrolment (%)	34/73 (46.6%)	20/38 (52.6%)	14/35 (40.0%)	0.280
CD4 count ≤ 200 cells/mm^3	31/56 (55.4%)	15/28 (53.6%)	14/25(56.0%)	0.859
Viral load >1000 copies/ml	57/76 (75.0%)	25/38 (65.8%)	30/35 (85.7%)	0.048

Characteristic	Overall	Non BM-ID	BM-ID	P-value
Infection(s)				
Bacteraemia [3]	12/73 (16.4%)	6/38 (15.8%)	6/35 (17.1%)	0.876
Malaria[4]	3/63 (4.7%)	2/32 (6.3%)	1/31 (3.2%)	0.573
Tuberculosis[5]	39/73 (53.4%)	20/38 (52.6%)	19/35 (54.3%)	0.877
Epstein-Barr virus [6]	30/45 (66.7%)	19/26 (73.1%)	11/19 (57.9%)	0.286
Cytomegalo virus [6]	18/54 (33.3%)	11/28 (39.3%)	7/26 (26.9%)	0.336
Parvo-B19 virus[6]	1/59 (1.7%)	0/27 (-)	1/32 (3.1%)	0.230
Nutritional status				
Underweight (BMI < 18.5) (%)	22/49 (44.9%)	8/24 (33.3%)	14/25 (56.0%)	0.111

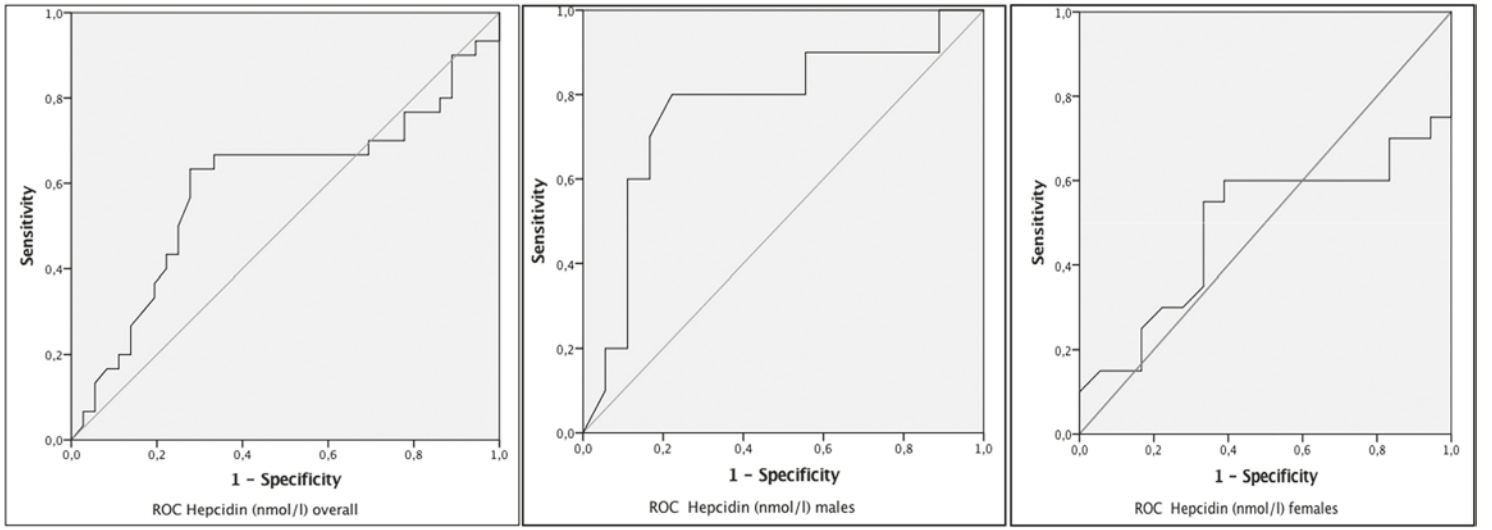
Table 1. Baseline characteristics in this population of severely anaemic HIV patients stratified according to bone marrow iron deficiency (BM-ID).

Abbreviations: ART: antiretroviral therapy. BMI: Body mass index. TB: Tuberculosis. [1] First line ART include combination of Stavudine (d4T), Lamivudine (3Tc) and Nevirapine (NVP) (28). [2] Advanced HIV disease including a CD4 count ≤ 200 cells/mm^3 and/or viral load > 1000 copies/ml. [3]Bacteraemia; a blood culture with clean growing potential pathogen including streptococcus (41.7%; 5/12), enterococcus (16.7%;2/12) and non-Typhoid Salmonella (16.7%;2/12). [4]Malaria: presence of malaria parasites on a thick blood film. [5]Tuberculosis (TB): one or more of the following present: a) positive sputum culture, b) chest X-ray with signs of pulmonary tuberculosis and/or c) on-going TB treatment at time of enrolment d) clinical diagnosis by local doctor including unknown generalized lymphadenopathy and/or night sweats > 30 days with unknown origin e) caseating granulomata in the bone marrow trephine. [6] Epstein-Barr, cytomegalo- and parvo-B19 virus infection are diagnosed by a virus load of 1000 copies/ml. Abbreviations: MCV; mean cellular volume, MCH; mean corpuscular haemoglobin, s-TfR: Soluble transferrin receptor, TfR-index (sTfR(mg/L) /Log ferritin(ug/L)), TfR Ratio (sTrR(mg/L))x1000/ferritin(ug/L)).

Potential markers	AUC^{ROC}	95%-CI	Cut-off	Sensitivity	95%-CI	Specificity	95%-CI
MCV (fl)[1]	0.545	0.404-0.685	≤83	42%	25.2-58.8%	67%	51.0-83.0%
MCH (pg/cells) [1]	0.365	0.230-0.499	≤27	52%	35.2-69.0%	29%	13.7-44.3%
Serum iron (mmol/l) [1]	0.368	0.239-0.498	≤ 10	60%	43.8-76.2%	18%	5.8-30.2%
Ferritin (mg/l) [1]	0.441	0.293-0.588	≤30	13%	1.0-25.0%	88%	76.7-99.3%
			≤70	30%	13.6-46.4%	66%	49.6-82.4%
sTfR receptor (mg/l)[3]	0.522	0.378-0.667	≥1.8	71%	55.0-87.0%	29%	13.7-44.3%
			≥2.8	58%	40.6-73.1%	56%	39.3-72.7%
			≥3.6	32%	15.6-48.2%	76%	61.6-99.4%
			≥8.0	3%	0-9.0%	100%	-
sTfR index [4]	0.523	0.375-0.672	≥1.8	47%	29.1-64.9%	67%	46.3-79.7%
			≥2.2	27%	11.1-42.9%	75%	60.0-90.0%
			≥2.8	20%	5.7-34.3%	81%	67.4-94.6%
			≥3.5	10%	0-20.7%	97%	75.1-86.9%
sTfR Ratio [4]	0.508	0.359-0.656	≥100	17%	0-23.4%	91%	71.1-90.9%

Table 2. Accuracy of peripheral blood markers to detect bone marrow iron deficiency (gold standard).

Abbreviations: AUC: area under curve of receiver operating characteristic (ROC), where 0.5 would be expected by chance and 1 denotes a perfect test. 95%-CI: 95% confidence interval. MCV; mean cellular volume, MCH; mean corpuscular haemoglobin, sTfR: Soluble transferrin receptor, sTfR-index (sTfR(mg/L) /Log ferritin(ug/L)), sTfR Ratio (sTrR(mg/L))x1000/ferritin(ug/L)).[1] (29) [2] (11) [3] (29) [4](15, 16)



Hepcidin	AUC^{ROC}	95%CI	Optimal hepcidin (nmol/L) cut-off for BMI-ID	Sensitivity	95%CI	Specificity	95%CI
Overall	0.593	0.447-0.739	7.0	67%	50.2-83.8	67%	51.6-82.4
Males	0.767	0.567-0.960	6.0	80%	55.2-100.0	78%	52.9-91.1
Females	0.490	0.298-0.682	7.0	60%	38.5-81.5	61%	38.5-83.5

Figure 1. Hepcidin (nmol/L) ROC curve by gender with optimal cut-off.

The best cut-off value for diagnosing BM-ID was determined by the Youden index (maximum (sensitivity+specificity –1)) in the ROC-curve (25).
Abbreviations: AUC^{ROC}: area under curve of receiver operating characteristic

Discussion

In this study on hepcidin and conventional markers to detect BM-ID in severely anaemic HIV-infected patients in Malawi, we found that BM-ID was present in almost half of our patients. In the first study evaluating hepcidin as a marker for BM-ID among severely anaemic HIV-infected adults in such a setting we found that hepcidin had the highest AUC^{ROC} 0.593 amongst all peripheral markers and therefore was the best marker to use for detecting BM-ID. MCV was found to be the best conventional peripheral blood marker for BM-ID. As the MCV is commonly provided as part of routine full blood counts this marker may be of some use in resource-limited settings.

BM-ID was highly prevalent among our population of HIV-infected and severely anaemic patients. The prevalence of BM-ID in this study is higher than previous reports on similar populations that were published in the pre-ART era when BM-ID was reported to be 18%-25% in severely anaemic HIV patients (8, 30). The increased prevalence of BM-ID may be explained by the effect of ART. Previous reports on HIV-associated anaemia before 2010 included patients who were mostly ART naïve and advanced HIV disease and/or severe immune suppression were common (8). Although VL above 1000 copies/ml (75%) and CD4 counts below 200 cells/ml were (55.4%) still common in the presenting population, median CD4 counts (325 cells/mm^3) were much higher than in the previous reports (median 67 cells/ml) (4, 8). Initiation of ART aims to stop HIV disease progression, promote immune reconstitution and reduce the risk of (opportunistic) diseases. This may also be reflected in our cohort, and has likely changed the aetiology of severe anaemia among HIV-infected patients as compared to older studies. In this group of HIV-infected patients with better immune systems the aetiology of severely anaemic may be more similar to the aetiology of non-HIV infected patients. The BM-ID prevalence of 48% is comparable to previous findings among HIV-uninfected African populations with severe anaemia, which supports this hypothesis (8). Our data need to be confirmed in the on-going 'treat all' era but they could have great impact on preventive and curative policies concerning iron supplementation in severely anaemic HIV-infected patients. Irrespective of the cause, the role of iron supplementation to prevent and treat severe anaemia appears to have gained importance.

Our results concerning the accuracy of peripheral blood markers to detect BM iron deficiency indicate that it is not easy to reliably detect those with deficient BM iron stores, which corroborates with previous studies (8, 9). Hepcidin, a specific hormone in metabolising iron, did perform slightly better than conventional markers, but remained far from good or even perfect. Additionally, hepcidin is a key player in the absorption of iron and thus may be used to not only select those needing iron but also may predict iron supplementation, response, safety and timing. Hepcidin as a possible marker for BM-ID, has not been evaluated before in this population of severely anaemic HIV-infected adults in Africa. It is not surprising that hepcidin remains from prefect as a marker for BM-ID as we know that hepcidin levels are also affected by inflammation which is highly present among HIV-infected patients, especially living in resource-limited settings (31-33). For example, when hepcidin levels are high, the absorption of dietary iron and release of macrophage iron to serum are blocked as protection, resulting in a relative hypoferremia and an increase iron into the macrophages, which is thought to be anti-infective. Consequently during malaria or TB infection or immune deficiency with low CD4 counts, hepcidin levels are increased (32, 33). Among children in Malawi, including children with HIV, our group previously reported low hepcidin levels (34). Further, hepcidin was suggested as a possible useful marker in guiding iron therapy in severely anaemic children, as low hepcidin levels were related toward a diminished expected up regulation of hepcidin by inflammation and iron deficiency due to an increase of erythropoietin in this population (34). Our data on hepcidin and (standardized) identified cut-offs are highly likely to be relevant as there is a need for a reliable marker to define BM-ID and to start iron supplementation among HIV-infected patients in resource limited settings such as Malawi. Intervention studies, using hepcidin as a marker, should be performed to assess feasibility and effect of such an intervention.

Worldwide hepcidin concentrations are measured by various methods, which differ considerably in absolute hepcidin concentrations (35). Recently, secondary hepcidin reference material, that has been value assigned by a primary reference material, has become available (36). Standardization in February 2019 of a similar hepcidin assay as we used in 2012/2013 for our study, resulted in (only) 5.4 % increase of hepcidin concentrations (C. Laarakker and D. Swinkels unpublished data) (37). For this specific patient population, our study thus provides a first and rough estimate for cut off point that are universally applicable by other assays that they are

standardized using this same reference material. However, for formal universal use of these cut-off points these values should be confirmed by studies that directly measure samples with a standardized hepcidin method. Additionally, hepcidin optimal cut-offs were more sensitive and lower for man in comparison to woman. A direct clarification for this effect is challenging. Age and gender differences in hepcidin concentrations are known. Within the mean age group of our study population, 30-35 years of age, (normal) hepcidin concentrations were reported to be higher on man than woman (36, 38). However reference data available is coming from Europe and no hepcidin reference levels are known for an African population. Therefore a direct comparison is challenging. At last an explanation(s) can be found in levels of infection or control of the HIV disease, which in our population was not different for woman and man (data not shown). Outcome should be formally confirmed with studies that directly measure with the standardized hepcidin method to enhance confirmed hepcidin cut-offs in this specific patient population.

Iron deficiency is treatable and preventable, however supplementation has been associated with an increased number of severe infections, including an increase in malaria, which, especially in an immune compromised population, may be dangerous (39, 40). Therefore a reliable diagnosis of iron deficiency is important, as supplementation will put the patient at risk. Our study underlined that the current used and known peripheral blood markers performed poorly. For several decades clinicians and researchers have evaluated peripheral markers to diagnose BM-ID in laboratory resource limiting settings like Malawi and reported poor performance which is commonly considered to be caused by inflammatory conditions which are common in African and especially in HIV infected patients (8, 9). Some of the markers tested, such as sTfR concentrations, alone or in combination with other markers, were designed to better reflect iron stores (41) irrespective of inflammation (42). These markers did not perform well in this study either. Potential explanations for the poor performances of sTfR in our population may be the lack of clear cut-offs, as suggested by other studies (8) and the fact that sTfR is also influenced by erythropoietin, which may play an (even more) important role in severe HIV-associated anaemia (32). The best performing conventional peripheral blood marker was MCV. Microcytosis is commonly used as a screening test for deficiency (27, 43); however, MCV was never found to be an accurate predictor of BM-ID (8, 9, 44). Although MCV did not have a high sensitivity or specificity and the AUC^{ROC} was

of low diagnostic value, it remains of some use in this population as it was the best available common marker and it is relatively easily available as part of automated full blood counts.

Our study has several shortcomings. Firstly, bone marrow testing was only performed in a subset of patients, which may have introduced a sampling bias. Reasons for not taking bone marrow included a severe clinical condition of the patient or patients not consenting to this aspect of the study. However, it is one of the largest studies with BM results to date. Secondly, our study was performed in 2010 when antiretroviral treatment (ART) was provided according to the national and hospital guidelines. Accordingly, ART could only be started in the outpatient ART clinic after discharge from hospital. Currently ART is started much earlier in the course of HIV infection so our study patients are likely to have had more advance disease than current patients. Nevertheless, this is the first study combining bone marrow data with a large set of peripheral blood markers, including hepcidin, in a group of HIV-infected severely anaemic African patients. We believe our study provides important information, which is valuable for clinicians who care for HIV-infected persons with severe anaemia in Malawi and other resource-limited settings.

Conclusion

Bone marrow iron deficiency was present in almost half of severely anaemic HIV-infected adults. This, substantial increase compared to data from the pre-ART era, underline the potential importance of preventive and therapeutic role of iron supplementation to reduce the problem of severe anaemia in HIV-infected patients. Detection and safe treatment of BM-ID is hampered by a lack of peripheral iron markers. Hepcidin was found to be the most accurate marker and could be used to guide and predict the effect of iron supplementation. Although hepcidin evaluations are not routinely available in settings such as Malawi, our study findings are important as hepcidin could guide and predict the effect of safe iron supplementation. This is important because of the potential risk of increased infection risk due to iron supplementation, its effect and safety should be evaluated in future intervention studies.

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Part III

HIV-infection in sub-Saharan Africa; Summary, considerations, implications and overall conclusion

Chapter 8

Summary

Summary

Until today the HIV-virus has infected over 70 million people worldwide (1) in one of the biggest global health challenges ever faced. In 2018 this included a total of 1.8 million children (age < 15 years) with almost ninety percent of these children living in sub-Saharan Africa (2,3). Over the last decade important steps have been taken worldwide to improve HIV care for adults and children, including in sub-Saharan Africa.

Now that ART has become more accessible (4-6), further progress in the fight against HIV will require the determination of health care providers and policymakers to further increase the quantity of those receiving treatment, alongside improvement in the quality of HIV care in sub-Saharan Africa. In addressing the HIV pandemic, the quality of HIV care will become increasingly important as resistance to HIV drugs is rising. This thesis focused on multiple aspects of the care of HIV-infected children and adults living in sub-Sahara Africa and aimed to gain further insight into optimal HIV treatment in order to improve long-term survival for these patients: **HIV-infection in sub-Saharan Africa; *from quantity to quality of care*.**

Summary | Treatment and monitoring

The first part (**Part 1**) of this thesis focused on HIV treatment, with special attention paid to initiation and monitoring. During the last decade, many aspects of HIV care have improved. Specifically, with widespread implementation of the World Health Organization's "treat all" recommendation in 2016, all HIV-infected patients receive ART regardless of age or CD4 counts. Previously, as CD4 counts were regularly not available, clinical criteria were used to start ART. As a result, patients were identified late. The results of our prospective cross-sectional study on HIV-infected Malawian children, who were not eligible for ART based on clinical criteria as reported in **Chapter 2,** indicated that there are no adequate clinical markers to replace CD4 counts. This negative finding has provided support for the current "treat all" approach. In **Chapter 3** and **Chapter 4** we described the short and long-term treatment outcomes for children on ART and focused on HIV drug-resistant mutations. **Chapter 3** reported data from a prospective cohort of HIV-infected Malawian children with high rates of HIV drug resistance and treatment failure after the first year of ART. These results emphasized the need for paediatric HIV treatment

programs to improve treatment follow-up that, even in low resource settings, should include regular viral load testing to allow adequate treatment switches and reduce the further development of resistance. **Chapter 4** presented long-term follow-up data (4 years) from a paediatric multicentre cohort of HIV-infected children on first-line ART in Uganda. Using this we reported a high prevalence of treatment failure and resistance to HIV drugs in one of the very few long-term datasets from Africa. Treatment failure occurred predominantly in the first 24 months. However, a second increase in treatment failure incidence occurred at year four. Children whose treatment had failed in the first 24 months had different risk factors, with greater occurrence of early treatment failure patterns and acquired HIV drug resistance mutations than children whose treatment failed after 24 months. These findings suggested that children with treatment failure in the first 24 months may benefit from repeated viral load monitoring and prompt switching to second line treatment, whilst treatment failure after 24 months may be prevented by earlier commencement of ART, which again supports the "treat all" recommendations of the World Health Organization.

Summary | HIV-infection in sub-Saharan Africa; co-morbidities

The second part of this thesis (**Part II**) focused on co-morbidities of HIV-infected children and adults in sub-Saharan Africa. Diarrhoea is an important cause of mortality in children under the age of five worldwide and highly common in HIV-infected children. In **Chapter 5,** using PCR techniques, we described the prevalence and clinical relevance of specific intestinal protozoa in HIV-infected Malawian children with diarrhoea before and during their first year of ART. Surprisingly, we found that a relatively unknown opportunistic pathogen, E. bieneusi, was the most prevalent opportunistic intestinal protozoa, and was present in over a third of study participants prior to the initiation of ART. Although all children were clear of E. bieneusi after 12 months of ART without specific treatment, E. bieneusi and its treatment may be of clinical importance as it was associated with initial gastro-intestinal complaints and a potentially delayed BMI recovery over 12 months of follow-up.

Severe anaemia is a major cause of morbidity in HIV-infected patients in LMIC and is associated with increased mortality. Previously, our research group reported on a large dataset involving children with severe anaemia in Malawi, including those

who were HIV-infected. However, comprehensive data on the aetiology of HIV-associated severe anaemia in adults especially on ART is lacking (7). In **Chapter 6** we reported the results of a comprehensive observational study that explored the mortality and potential aetiologies of HIV-associated severe anaemia (Hb≤70 g/L) in Malawian adults. In our population, mortality among severely anaemic HIV-infected adults was strikingly high (50%); suggesting that severe anaemia in HIV-infected adults must be investigated and treated urgently. In addition, all of the adults in the study averaged three potential aetiologies of (severe) anaemia, indicating that the aetiology of severe anaemia in this population is multifactorial, with several coinciding diseases occurring. This highlights the need for clinicians to be wary of the possibility that multiple causes of severe anaemia must be identified and treated.

Iron deficiency has always been regarded as a primary cause of (severe) anaemia in LMIC. In the search for the aetiology of severe anaemia in HIV-infected patients we identified in **Chapter 6** that iron deficiency may be an important contributor to the development of severe anaemia, as it was prevalent in 55% of our population. In a sub-study presented in **Chapter 7**, we evaluated and described the difficulties of diagnosing bone marrow iron deficiency using peripheral blood markers in this specific patient population. The study showed that these markers performed poorly in determining iron deficiency in this group of patients. Hepcidin, a specific hormone in metabolising iron, did perform slightly better than conventional markers and this might aid the decision as to whom iron supplements may be safely and effectively prescribed.

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Chapter 9

Considerations, implications and overall conclusion

Considerations and implications

This thesis outlines the different challenges faced in providing care for HIV-infected children and adults living in sub-Saharan Africa, and there are several clinical implications that emerge from these results.

Considerations and implications| HIV-treatment in sub-Saharan Africa

Due to the scaling-up of ART over the last decade, the number of people receiving ART has increased enormously from 1 million in 2001 to 21.7 million, representing 59% of HIV-infected patients worldwide, by December 2017 (1) . However, 90% of the USAID 90-90-90 goals for 2020 are still far from being attained. In addition, although survival rates for children affected with HIV have improved drastically, incidence of suboptimal viral suppression and HIV resistance among children are a concern and much more common than in HIV-infected adults living in LMIC. Viral suppression rates for children range from 60-75%, in contrast to 85% for adults (2, 3). Additionally, HIV drug resistance at the start of ART among PMTCT exposed HIV-infected children is 43%, in comparison to 10% for adults (2). This inequity is dangerous and the imbalance in funding between paediatric and adult HIV research is problematic and must be addressed (4, 5). If not, the next generation of HIV patients will be faced with increasing treatment failure and drug resistance.

Considerations and implications | Improved monitoring of treatment

Over the past decade, the emphasis of HIV programs in LMIC has shifted from quantity to quality of care. In addition to adherence support, viral load monitoring has become the backbone of high quality paediatric HIV care. Since 2016, the World Health Organisation has recommended routine viral load testing at 6 and 12 months after ART initiation, and thereafter every 12 months. Our data from Malawi and Uganda support these recommendations. The data presented in chapters 3 and 4 emphasize the need for viral load testing at regular intervals during the first 24 months of treatment, and at least yearly thereafter. Country guidelines should be adjusted. For instance, at the time of writing, according to the current Malawian Guideline of Clinical Management of HIV in children and adults, viral load testing is only done at 6 months, 2 years and every 2 years thereafter. Based on the data that we report from Malawi, we strongly recommend increasing the frequency of viral

load testing, to at least the World Health Organisation's recommended frequency, to improve treatment monitoring and to enhance the detection of early treatment failure and (future) resistance (6).

Considerations and implications | Improved antiretroviral treatment

HIV resistance rates in children are high and worrisome. Previously, we reported that pre-treatment drug resistance was around 17% among children in LMIC (7, 8). In this thesis we reported that the rate is now even higher, with a prevalence of acquired NNRTI and/or NRTI resistance of 30% after 48 months of treatment. Because of these resistance patterns, Protease-Inhibitor (PI)-based ART regimens, which are known to reduce early infant mortality and HIV disease progression in non-LMIC settings, are more important in the management of HIV-infected children (9). In line with this, the World Health Organisation now recommends PI-based first-line regimens worldwide, including in LMIC (10). PI-regimens, including one PI and two NRTI's, have been shown to be effective and robust for adolescents and adults in LMIC (11, 12). Long-term data comparing PI-based versus NNRTI-based regimens for children, especially in LMIC, are sparse, but expected to be good as PMTCT programs do not incorporate PI-based regimens and these regimens are robust (resistance is less likely to be developed after a missed doses) (11, 12). However the feasibility of large-scale implementation of PI-based regimens in LMIC settings remains challenging (13). For example, Lopinavir/Ritonavir (LPV/r; Kaletra) is the most commonly used PI in LMIC. LPV/r is supplied in tablet, capsule (pellet) and liquid form. Treatment with LPV/r has several disadvantages. Firstly, the tablet of LPV/r is 5 times as expensive as the broadly used Nevirapine (an NNRTI) (14). Secondly, liquid paediatric formulations would be most ideal from a dosing perspective as, for example, the concentration of widely available liquid formulations is such that a child would have to ingest a large volume in order to receive an adequate dose of the medication (28 mL per dose for a 10 kg child). A more concentrated form is expensive to make and storage of the medication requires a refrigerator. This leaves the LPV/r capsules as "the best" available option for children. Capsules are opened and the pellets inside are given to children, especially if they are under 3 years of age. However, it is difficult to give pellets to small children on a spoon. In addition, the bioavailability of these pellets is uncertain (13). In conclusion, though

the recommendation is appropriate, practicalities on the ground make meeting these recommendations challenging.

Because overall drug resistance in HIV is a major problem and second line treatments (PI-based regimens) for children are not optimal, new treatment options are urgently needed. Introduced in 2007, intregrase inhibitors are relatively new to the market and therefore research on effectiveness, especially in children in LMIC, is limited. However, intregrase inhibitors have several advantages including mild side effects, uncommon drug-to-drug interactions and a high genetic barrier, reducing incidence of drug resistance. The US Food and Drug Administration and European Medical Agency currently approve three intregrase inhibitors for children and adolescents who have HIV-1 infection. Raltegravir is approved for children aged 4 weeks to 18 years, while Dolutegravir and Elvitegravir, co-formulated with Cobicistat, Emtricitabine, and Tenofovir Alafenamide (E/C/FTC/TAF), are approved for children from 6 years of age and above 30 kg of weight (15). Studies of intergrase inhibitors in children and adolescents, with the exemption of Dolutegravir, are scarce and therefore additional studies investigating the safety and efficacy of these drugs in children are needed (15). Raltegravir is recommended as a second and third-line treatment in LMIC, but is rarely available in LMIC, including in Malawi and Uganda (6). Stakeholders are making efforts to improve availability and cost-effectiveness for Intregrase inhibitors, especially for Dolutegravir. For example, in Malawi, by 2019 administration of Dolutegravir will be commenced in boys above 30 kg (16, 17). Implementation of intergrase inhibitors as a treatment, especially in children in LMIC, will take time.

Finally, as the result of the successful scaling-up of ART, including PMTCT, over the last decade, fewer young children are getting infected with HIV. Increased prevention of mother-to-child transmission will ultimately reduce the need for paediatric antiretroviral drugs and thus limit the financial incentives for pharmaceutical companies to invest in paediatric drug formulations. The children who require ART will become increasingly difficult to treat over time. New trials that compare PI-based and Intergrase inhibitor-based regimes in children living in LMIC are therefore urgently needed, alongside efforts to increase drug availability for those most in need.

Considerations and implications | Comorbidity during HIV-infections

With increasing survival rates due to the availability of ART, advances in HIV care will focus more and more on the impact of comorbidities on survival and quality of life. Two major contributors to the comorbidity of HIV-infected patients in LMIC are diarrhoea and (severe) anaemia. Improving care at this level will become increasingly important.

Diarrhoea is a common and important comorbidity of HIV-infected patients, especially with regard to children, where diarrhoea is associated with a high mortality (18). In LMIC, the available investigations do not usually identify specific pathogens. PCR-techniques offer fast and reliable results for the detection gastrointestinal protozoa and can be helpful in the detection of causative pathogens of diarrhoea (19). Novel combined PCR panels are even more efficient since more pathogens can be detected with a single test. Identification of causative agents may lead to specific treatments that could improve acute and chronic diarrhoea and malnutrition, thus impacting morbidity and high mortality rates among children with HIV in LMIC (20, 21). Surprisingly, E. bieneusi was the most prevalent opportunistic intestinal protozoa in our study cohort. It might be clinically relevant, as it was associated with both gastro-intestinal complaints and possible reduced BMI recovery. New PCR results are interesting but additional clinical research is needed to follow-up long-term consequences and possible treatment options in these children.

Severe anaemia in HIV-infected patients in a resource-limited setting, such as Malawi, is a critical condition with high rates of mortality, where various factors may be simultaneously responsible (22). Guidelines for diagnosis and treatment of severe anaemia in HIV-infected adults or children are rarely available in sub-Saharan Africa, including in Uganda and Malawi (22, 23). Based on our results we recommend a more comprehensive approach to the diagnosis of severe anaemia that includes the evaluation of HIV treatment failure, exclusion of TB, identification of renal failure and adequate nutritional support. Iron deficiency has also been identified as a major cause of severe anaemia in this patient population. However, diagnosis of iron deficiency remains difficult in severely anaemic HIV patients in LMIC. Finally, in view of these challenges, we recommend the use of Hepcidin, a hormone involved in the metabolising of iron, for improvement on diagnostics. However more research is needed to confirm this recommendation. Addressing the diagnosis and treatment of severe anaemia among HIV-infected patients is vitally important. Improved diagnosis

and trials to compare multifactorial treatment protocols may yield impactful results for this vulnerable population.

Overall conclusion

Altogether, this thesis emphasizes the importance of the early initiation of ART, the need for more intensive viral load monitoring and awareness of supportive care among HIV-infected patients in sub-Saharan Africa. Towards more and, above all, improved HIV care in LMIC for both adults and children; ***"From quantity to quality of care"!***

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Addenda

NEDERLANDSE SAMENVATTING

Humaan Immunodeficiëntie Virus (HIV)

Begin jaren 80 werd vanuit de Verenigde Staten voor het eerst melding gemaakt van een mysterieuze en dodelijke ziekte (1). Aanvankelijk werd gedacht dat alleen homoseksuele mannen aan deze ziekte leden, maar later werden ook intraveneuze drugsgebruikers, ontvangers van donorbloed en heteroseksuelen beschreven, die stierven door dodelijke infecties als gevolg van een afgezwakt immuunsysteem. Vanwege dit falende immuunsysteem werd de ziekte "Acquired Immune Deficiency Syndrome" (AIDS) genoemd. In 1984 werd vervolgens het virus dat verantwoordelijk is voor AIDS ontdekt (1). Dit retrovirus genaamd "Humaan Immunodeficiëntie Virus" (HIV) infiltreert in het immuunsysteem van de gastheer en tast daarna specifieke witte bloedcellen (CD4) aan, waardoor het immuunsysteem verzwakt wordt. Een patiënt besmet met HIV is niet in staat het virus zelf te klaren, wat leidt tot een toename van de infectie, afname van aantal CD4-cellen en daardoor falend immuunsysteem met ernstige (opportunistische) infecties en uiteindelijk de dood tot gevolg. Sinds de ontdekking van HIV, nam het aantal infecties explosief toe en is de HIV-epidemie één van de grootste epidemieën die de wereld gekend heeft (1). In 2017 leefden wereldwijd naar schatting 36,7 miljoen mensen met HIV, van wie 1.8 miljoen kinderen. Bijna negentig procent van deze kinderen woont in sub-Sahara Afrika (2,3).

HIV| Behandeling

In de strijd tegen HIV/AIDS werden verschillende geneesmiddelen ontwikkeld. Azidothymidine was in 1987 het eerste medicijn dat beschikbaar werd. Azidothymidine remt het enzym reverse transcriptase, dat door het HIV-virus wordt gebruikt om viraal RNA te transcriberen naar DNA, een cruciale stap in de replicatie van het virus. De groep medicamenten met dit werkingsmechanisme behoren tot de Nucleoside Reverse Transcriptase-Remmers (NRTI). Aanvankelijk veroorzaakte de introductie van Azidothymidine een indrukwekkend afname van de sterftecijfers onder HIV-geïnfecteerde patiënten. Enkele jaren later verzwakte dit effect als gevolg van resistentie van het HIV virus voor Azidothymidine. Een patroon dat zich herhaalde na de introductie van nieuw ontwikkelde NRTI's (4). In

de opeenvolgende jaren werden ook andere soorten geneesmiddelen geproduceerd, zoals niet-nucleoside reverse transcriptase-remmers (NNRTI's) en proteaseremmers (PI's). Door verschillende medicatiesoorten met elkaar te combineren werd de resistentie voor HIV aanzienlijk verminderd. Deze combinatietherapie werd bekend onder de naam *"Highly Active Antiretroviral Therapy"* (HAART) en wordt tegenwoordig Anti-Retroviral Treatment (ART) genoemd. In de afgelopen jaren zijn er verschillende soorten ART beschikbaar gemaakt, waaronder intergrase remmers, fusieremmers en CCR5-antagonisten. Ondanks al deze ontwikkelingen kan ART momenteel alleen het HIV virus onderdrukken; volledige genezing is tot op de dag van vandaag niet mogelijk.

Ondanks het enorme succes van ART in de Westerse wereld had de overgrote meerderheid van HIV-geïnfecteerde patiënten wonend in landen met een laag of middelhoog inkomen (LMIC) geen toegang tot ART. Eind 2001 waren naar schatting 40 miljoen mensen besmet met HIV en voor slechts 1 miljoen van hen was behandeling beschikbaar (5). Bovendien werden de richtlijnen voor behandeling van HIV in het Westen ontwikkeld, daar waar medicatie, geavanceerde laboratoriumtechnologie en medisch personeel voldoende aanwezig zijn. Deze Westerse richtlijnen waren daardoor niet toepasbaar in LMIC, waar medicatie en ondersteunende zorg slechts heel beperkt mogelijk waren en nog steeds zijn. Om deze reden werd in 2002 door de World Health Organisatie (WHO) een nieuwe 'public health approach' geïntroduceerd met als doel om ART beter toegankelijk te maken voor patiënten in LMIC (5). Deze aanpak omvatte gestandaardiseerde en vereenvoudigde behandelprotocollen met een gegarandeerde levering van geneesmiddelen. Daarbij werd gebruik gemaakt van een decentralisatie van zorg, waarmee taken verdeeld werden over medisch personeel met verschillende scholingsniveaus en minimale monitoring van laboratoriumwaarden. Deze nieuwe benadering was zo succesvol dat de levensverwachting van HIV-geïnfecteerde patiënten, behandeld met ART, drastisch verbeterde.

In 2014, bijna een decennium na de introductie van de public health approach, werden twee nieuwe omvangrijke behandelprogramma's ontwikkeld met als doel om wereldwijd de behandeling van HIV te optimaliseren. Het eerste programma genaamd "90-90-90", is een initiatief gelanceerd Verenigde Naties inzake HIV / AIDS (UNAIDS) (6). De intentie van "90-90-90" is dat in 2020; 90% van de mensen met HIV hun status kent, dat 90% van de positief geteste patiënten

medicamenteuze behandeling krijgt en dat bij 90% van de behandelde patiënten het HIV virus onderdrukt is. Dit met het ultieme doel dat in 2030 de HIV-verspreiding is gestopt waardoor AIDS niet langer een bedreiging voor de volksgezondheid vormt (6). De tweede belangrijke nieuwe benadering van de behandeling van HIV is het WHO advies om daadwerkelijk alle HIV-geïnfecteerden te behandelen met ART, ongeacht hun leeftijd of het aantal CD4-cellen (7). Deze aanbeveling is gebaseerd op uitgebreid wetenschappelijk onderzoek dat heeft aangetoond dat het vroegtijdig starten van ART resulteert in betere klinische uitkomsten vergeleken met patiënten die later starten met behandeling. In 2018 werd in 84% van de LMIC dit beleid gevolgd en verwacht wordt dat tegen het einde van 2020 dit percentage tot 92% is toegenomen (8). Als gevolg van al deze inspanningen steeg het aantal onder behandeling zijnde HIV patiënten van 1 miljoen in 2001 naar 21.7 miljoen in december 2017, wat 59% van de met HIV-geïnfecteerde patiënten wereldwijd vertegenwoordigt (9).

HIV| Resistentie

HIV is een snel replicerend virus dat miljarden nieuwe virus per dag creëert. In dit replicatieproces kan het virus muteren en varianten van de oorspronkelijke genetische structuur vormen. Wanneer deze mutaties gevormd worden ten tijde van ART gebruik, ontstaat naast het initiële virustype een nieuw virus, dat minder onderdrukt wordt door de medicatie en daarmee leidt tot falen van de behandeling. Dit nieuwe virustype kan zich onder de behandeling met (suboptimale) ART blijven repliceren en wordt de meest dominante virus variant in het lichaam van de geïnfecteerde patiënt (3).

Resistentie tegen HIV-geneesmiddelen kan worden veroorzaakt door verschillende factoren die te maken hebben met het soort geneesmiddel, de patiënt zelf of het behandelprogramma. Zo is bijvoorbeeld therapietrouw een belangrijke patiënt gerelateerde factor waarbij door suboptimale medicatiespiegels de kans op resistentie hoger wordt (10-12). Therapietrouw kan nadelig worden beïnvloed door bijvoorbeeld HIV-geassocieerd stigma en onregelmatige klinische follow-up. Deze factoren spelen met name een rol bij kinderen en adolescenten. Daarnaast zijn omgevingsfactoren zoals stock-outs van ART en beperkte aanwezigheid van medisch personeel om voorlichting of behandeling te geven een realistische bedreiging voor een optimale uitvoering van HIV-programma's in LMIC.

HIV-resistentie is in toenemende mate een bedreiging voor HIV-geïnfecteerde patiënten. Een recent rapport (2017) van de WHO toonde een NNRTI-resistentie prevalentie met een spreiding van 4-25%. Met name kinderen blootgesteld aan ART door "Prevention of Mother to Child Treatment" (PMTCT) hebben een hoger risico op resistentie. Bij patiënten met het falen van behandeling en hoge concentratie van virus in het bloed liepen de percentages zelfs op tot 47%-90% (13). Het is essentieel om aandacht te hebben voor HIV-resistentie om het huidige succes van ART in LMIC niet te verliezen.

HIV| Kinderen

In het laatste decennium hebben HIV-programma's zich toegespitst op de preventie van moeder-op-kind transmissie (PMTCT/ Prevention of Mother to Child Transmission), waardoor de HIV-overdracht van moeder naar het kind in LMIC drastisch is verminderd (14). Tevens werden kinderen die ondanks PMTCT geïnfecteerd zijn geraakt eerder gediagnosticeerd en behandeld, waardoor de levensverwachting van kinderen enorm verbeterd is en er tot op heden een overleving van 91% in de eerste 24 maanden van behandeling is bereikt (15). Echter, op de lange termijn zijn de resultaten van behandeling van HIV-geïnfecteerde kinderen in LMIC nog steeds suboptimaal. In een eerdere publicatie van onze groep lieten wij zien dat bij een derde van HIV-geïnfecteerde kinderen in Uganda, ondanks behandeling, er onvoldoende onderdrukking van het virus was en zij zelfs faalden op de behandeling 24 maanden na de start van de behandeling (16-19). Dit aantal is veel hoger dan bij volwassenen en heeft verschillende oorzaken. Allereerst hebben kinderen die aan PMTCT zijn blootgesteld een verhoogd risico op het ontwikkelen van resistentie, dit omdat ze al prenataal in aanraking zijn geweest met medicatie (18). Daarnaast zijn jonge kinderen volledig afhankelijk van hun verzorgers, die vaak zelf zijn geïnfecteerd of zelfs zijn overleden, wat een negatief effect kan hebben op de therapietrouw. Vooral bij adolescenten is therapietrouw een uitdaging vanwege de angst voor stigmatisering naast al de normale puberleeftijd gerelateerde problemen met therapietrouw (12). Tot slot is de aanwezigheid van kindvriendelijke medicatie, zoals aangepaste doseringen, in LMIC vaak schaars waardoor het nemen van medicatie vaak betekent dat er een grote hoeveelheid pillen ingenomen moet worden (15, 20). Tevens vereist de enorme verandering in lichaamsgewicht als gevolg van ondervoeding en inhaalgroei tijdens ART, evenals

normale fysiologische veranderingen in de kindertijd, een frequente dosisaanpassing van medicatie (11, 12, 15). Al deze factoren zorgen voor suboptimale dosering van ART en kan de behandeling doen falen (12).

HIV| Co-morbiditeiten

Met de toegenomen beschikbaarheid van ART in LMIC verschuift de focus van levensreddende behandeling naar optimale kwaliteit van leven. Veelvoorkomende problemen die optreden bij HIV-infecties, met name in LMIC, zijn ondervoeding, diarree en bloedarmoede.

Acute diarree is bij zowel kinderen als volwassenen in LMIC een belangrijke doodsoorzaak, met name in combinatie met HIV-infectie (21). Diarree wordt gedefinieerd als het passeren van vloeibare ontlasting drie of meer keer per dag. Men spreekt van chronische diarree, wanneer dit ontlastingspatroon tenminste 4 weken aanhoudt (22). Kinderen met HIV lijden vaak aan chronische diarree, wat ondervoeding veroorzaakt en uiteindelijke verhoogd risico op overlijden geeft (23). De meest voorkomende oorzaken van diarree bij HIV-geïnfecteerde kinderen zijn opportunistische infecties met bacteriën, virussen, protozoa, parasieten of schimmels. Daarnaast kan diarree een bijwerking van ART zijn of een gevolg van HIV-enteropathie of ondervoeding (22). Prevalentiegegevens over protozoa infecties bij HIV-patiënten met chronische diarree variëren sterk (1-75%) en verschillen per samenstelling van de bevolking, seizoen en diagnostische tests (24,25). De prevalentiepercentages zijn bijvoorbeeld hoger bij testen waarbij meer gevoelige technieken om polymerase kettingreactie (PCR) te bepalen gebruikt worden, dan bij testen met traditionele microscopie (26,27). PCR-technieken zijn echter over het algemeen niet beschikbaar in LMIC en daarom zijn betrouwbare gegevens uit deze gebieden schaars. Afname van de prevalentie van intestinale protozoa bij volwassen HIV-patiënten na introductie van ART is beschreven in zowel Westerse landen als LMIC (28, 29). Gegevens van HIV-geïnfecteerde kinderen ontbreken, maar zijn voor een adequate behandeling van diarree essentieel.

Bloedarmoede is de meest voorkomende hematologische aandoening bij HIV-geïnfecteerde patiënten wereldwijd met een geschatte prevalentie van 60-95% (30). De WHO definieert bloedarmoede als hemoglobinewaarden onder de 110-120 g/l, afhankelijk van de leeftijd, en ernstige anemie als een hemoglobinegehalte onder de 70 g/l (31). In sub-Sahara Afrika heeft 60% van de HIV-geïnfecteerde

volwassenen bloedarmoede (32,33). De oorzaak van HIV-geassocieerde anemie wordt vaak multifactorieel genoemd. De etiologie van bloedarmoede omvat een tekort aan micronutriënten, infecties inclusief het HIV-virus, neoplastische ziekten en bijwerkingen van geneesmiddelen zoals Zidovudine en Cotrimoxazol (34-38). Voorbeelden van een tekort aan micronutriënten zijn een tekort aan ijzer, foliumzuur en vitamine B12. IJzergebrek komt vaak voor in sub-Sahara Afrika en is geassocieerd met matige tot ernstige anemie. De rol van ijzergebrek, vergelijking tot andere oorzaken van ernstige bloedarmoede bij HIV-geïnfecteerden patiënten is niet geheel duidelijk (39, 40). Malaria wordt in Afrika nog steeds als een van de meest voorkomende oorzaken van anemie gezien. Echter, studies bij kinderen met een ernstige anemie tonen aan dat zelfs bij de aanwezigheid van malariaparasieten naar alternatieve oorzaken van ernstige bloedarmoede moet worden gezocht (35). De meesten van deze studies zijn bij kinderen en niet bij volwassenen uitgevoerd. Studies onder HIV-geïnfecteerde volwassenen behandeld met ART, waarbij mogelijke oorzaken en gevolgen van HIV-geassocieerde (ernstige) anemie worden geëvalueerd zijn schaars. Hierdoor zijn evidence-based preventieve of therapeutische richtlijnen voor HIV-geïnfecteerde volwassenen met ernstige bloedarmoede in deze setting sporadisch beschikbaar (34,41).

Dit proefschrift

Dit proefschrift richt zich op verschillende aspecten van de zorg voor HIV-geïnfecteerde kinderen en volwassenen in sub-Sahara Afrika en heeft als doel bij te dragen aan verbetering van HIV-behandeling om zo de overlevingskansen voor deze patiënten te optimaliseren in LMIC. **Hoofdstuk 1** geeft een overzicht van de toenemende kennis die in de loop der jaren over HIV is verkregen.

Het eerste deel **(Part I)** van dit proefschrift richt zich op de behandeling van HIV met speciale aandacht voor de vraag wie en wanneer behandeld moet worden en hoe het effect van deze behandeling te evalueren. De afgelopen tien jaar is er wereldwijd veel verbeterd in de behandeling van HIV. Een belangrijke verbetering is de vereenvoudiging van de behandelrichtlijnen en de *"treat all"* aanbeveling van de WHO in 2016, waarbij ART gestart wordt bij alle kinderen ongeacht hun leeftijd of het aantal CD4 cellen. Het bepalen van het aantal CD4 cellen is in landen met

een laag of middeninkomen (LMIC) vaak onbetaalbaar en daardoor niet beschikbaar. In deze landen werd gebruik gemaakt van klinische criteria met als nadeel dat kinderen vaak (te) laat in hun ziekteproces geïdentificeerd werden. In **Hoofdstuk 2** evalueren we in een prospectieve cross-sectionele studie verschillende alternatieve markers bij HIV-geïnfecteerde kinderen in Malawi. Deze kinderen kwamen eerder op basis van klinische criteria niet in aanmerking voor ART. Uit deze studie kunnen we concluderen dat het hanteren van alleen klinische criteria een onbetrouwbare strategie is om ART te starten en dat er geen adequate markers zijn om CD4-aantallen te vervangen. Een bevinding die de huidige WHO "treat all" benadering ondersteunt. In **Hoofdstuk 3** en **Hoofdstuk 4** beschrijven we het effect van HIV-resistentie bij de behandeling van kinderen met ART op zowel de korte (<24 maanden) als de lange termijn (>24 maanden). Het prospectieve cohort, beschreven in **Hoofdstuk 3**, rapporteert gegevens over het falen van de behandeling en resistentie tegen HIV-medicatie in Malawi bij HIV-geïnfecteerde kinderen gedurende eerste jaar na het starten van ART. De hoge mate van resistentie tegen HIV-medicatie en falen van de behandeling benadrukt de noodzaak om de follow-up van pediatrische HIV-behandelingsprogramma's te verbeteren. **Hoofdstuk 4** presenteert de resultaten van een lange-termijn follow-up studie onder HIV-geïnfecteerde kinderen in Oeganda. Alle kinderen ontvingen eerstelijns ART. In een van de weinige lange-termijn datasets die gedaan zijn in Afrika, melden we een hoge prevalentie van therapie falen en resistentie tegen HIV-geneesmiddelen 4 jaar na start van behandeling. Hoewel het falen van behandeling voornamelijk in de eerste 24 maanden van de behandeling optrad, werd een zorgwekkende tweede piek in het falen van behandeling gezien in het vierde jaar na start van ART. Kinderen die in de eerste 24 maanden faalden, hadden andere risicofactoren in vergelijking met de kinderen die na de eerste 24 maanden van behandeling faalden. Uit deze studie concluderen we dat kinderen met therapie falen in de eerste 24 maanden van behandeling baat kunnen hebben bij frequente monitoring van de concentratie virusdeeltjes in hun bloed en indien nodig snel moeten overschakelen naar tweedelijns behandeling. Het falen van behandeling bij kinderen na 24 maanden behandeling, kan mogelijk worden voorkomen door vroegtijdige start van ART, wat opnieuw de 'treat all' aanbevelingen van de WHO onderstreept.

Het tweede deel van dit proefschrift (**Part II**) richt zich op co-morbiditeiten van HIV-geïnfecteerde kinderen en volwassenen in sub-Sahara Afrika. Diarree is

wereldwijd een van de belangrijkste oorzaken van sterfte bij kinderen onder de vijf jaar en HIV-geïnfecteerde kinderen hebben hier regelmatig last van. In **Hoofdstuk 5** richten we ons op chronische diarree. Met behulp van moderne PCR-technieken onderzoeken we de prevalentie en klinische relevantie van intestinale protozoa bij HIV-geïnfecteerde kinderen in Malawi vóór en tijdens hun eerste ART-jaar. Verrassend vonden we dat E. bieneusi de meest voorkomende opportunistische intestinale protozoa infectie is voorafgaand aan de start van ART. Een E. bieneusi infectie werd gevonden bij meer dan een derde van de patiënten. Door het verbeteren van de immuunstatus en zonder directe behandeling van E. bieneusi werd na 12 maanden behandeling met ART bij geen van de kinderen E. bieneusi. meer aangetoond. Echter de behandeling van E. bieneusi kan klinisch van belang zijn, omdat de aanwezigheid van deze infectie bij start van ART werd geassocieerd met gastro-intestinale klachten en mogelijk een vertraagd herstel van ondergewicht gedurende de 12 maanden van follow-up.

Een andere belangrijke co-morbiditeit onder HIV-geïnfecteerde patiënten in sub-Sahara-Afrika is (ernstige) bloedarmoede. Studies onder kinderen met ernstige anemie in sub-Sahara-Afrika, inclusief HIV-geïnfecteerde kinderen, toonden aan dat de oorzaak van ernstige anemie vaak multifactorieel is (35). Echter uitgebreide gegevens over de verschillende gecombineerde oorzaken van HIV-geassocieerde ernstige bloedarmoede bij volwassenen zijn nauwelijks beschikbaar. In **Hoofdstuk 6** rapporteren we de resultaten van een observationele studie bij HIV geïnfecteerde volwassen naar de mortaliteit en de etiologie van HIV-geassocieerde ernstige bloedarmoede (Hb≤70 g / L) in Malawi. Sterfte onder HIV-geïnfecteerde patiënten met ernstige anemie was opvallend hoog, namelijk 50% van onze studiepopulatie. Alle onderzochte volwassenen hadden gemiddeld ten minste drie mogelijke oorzaken van ernstige anemie. Gezien de hoge mortaliteit moet ernstige anemie bij een met HIV-geïnfecteerde volwassene zeer serieus genomen worden, waarbij een multifactoriële aanpak noodzakelijk is. In de zoektocht naar de etiologie van (ernstige) bloedarmoede wordt ijzergebrek vaak gezien als een van de oorzaken. Uit onze resultaten van de studie beschreven in **Hoofdstuk 6** blijkt inderdaad ijzergebrek een van de mogelijke aanwezige oorzaken van ernstige anemie bij volwassen patiënten met HIV-infectie. In een sub-studie, gepresenteerd in **Hoofdstuk 7**, evalueren we de problemen van het diagnosticeren van ijzertekort met perifere bloedmarkers in vergelijking met de gouden standaard van beenmergdeficiëntie in deze specifieke

patiëntenpopulatie. Deze studie laat zien hoe lastig het is om ijzergebrek te bepalen in deze specifieke groep van patiënten. Mogelijk dat Hepcidine, een hormoon betrokken bij ijzermetabolisme, in deze patiëntenpopulatie een rol kan spelen in de diagnose en effectief suppletie van ijzersupplementen noodzakelijk is.

Klinische implicaties en toekomstperspectieven

Dit proefschrift bespreekt de verschillende uitdagingen in de zorg voor HIV-geïnfecteerde kinderen en volwassenen die leven in sub-Sahara Afrika en deze studieresultaten kunnen verschillende klinische consequenties hebben.

HIV-behandeling in sub-Sahara Afrika

Als gevolg van de betere beschikbaarheid van ART in het afgelopen decennium is het aantal mensen dat met ART behandeld wordt enorm toegenomen, van 1 miljoen in 2001 tot bijna 22 miljoen in december 2017, een aantal dat 59% van de met HIV-geïnfecteerde patiënten wereldwijd vertegenwoordigt (3). Ondanks deze verbetering in de toegang tot therapie is het USAID '90-90-90' streven dat in 2020 90% van de HIV-geïnfecteerde patiënten onder behandeling moet staan nog ver weg. Door de betere beschikbaarheid van ART nam het overlevingspercentage van kinderen met HIV drastisch toe. Echter de percentages onder kinderen blijven achter in vergelijking met de successen die bij HIV-geïnfecteerde volwassenen in LMIC geboekt zijn. Zo wordt bijvoorbeeld virale suppressie gerapporteerd bij 60-75% van de kinderen als gevolg van behandeling met ART, dit in tegenstelling tot de 85% van de volwassenen in deze landen (12,42). Eveneens is de prevalentie van HIV resistentie onder volwassen voordat ze starten met ART circa 10%, terwijl er bij HIV-geïnfecteerde kinderen, blootgesteld aan PMTCT, een prevalentie van 43% bekend is. Als dit verschil niet aangepakt wordt, zullen we te maken krijgen met steeds meer kinderen die falen onder ART behandeling en toenemende percentages van HIV-resistentie. Verbeterde kwaliteit van zorg in pediatrische HIV-programma's voor LMIC is daarom noodzakelijk en onderstaande suggesties kunnen daar aan bijdragen.

Verbetering van monitoren tijdens behandeling

In het afgelopen decennium is de focus van HIV-programma's in LMIC langzaam van kwantiteit naar kwaliteit van zorg verschoven. Naast bevorderen van therapietrouw is monitoring van virusconcentratie in het bloed de ruggengraat van kwalitatief betere pediatrische HIV-zorg. Vanaf 2016 adviseert de WHO om 6 en 12 maanden na initiatie van ART routinematig de virusconcentratie in het bloed te testen en vervolgens elke 12 maanden tijdens de verdere behandeling. Onze gegevens uit Malawi en Oeganda ondersteunen deze aanbeveling. De gepresenteerde resultaten in hoofdstuk 3 en 4 onderstrepen de noodzaak van het regelmatig testen van virus in het bloed gedurende de eerste 24 maanden en tenminste jaarlijks na 24 maanden. Tot december 2018 werd het testen van virusconcentratie in het bloed van HIV-geïnfecteerde kinderen en volwassen alleen gedaan op 6 maanden, 2 jaar en 4 jaar na aanvang van de behandeling en vervolgens om de 2 jaar. Per januari 2019 is dit advies aangepast en wordt het aanbevolen na de eerste 6 maanden de virusconcentratie in het bloed jaarlijks te evalueren. Op basis van onze huidige uitkomsten kunnen we deze verandering van harte aanbevelen. Met het verhogen van de testfrequentie voor virusconcentratie in het bloed kan falen van behandeling worden erkent en behandeling worden verbeterd dan wel aangepast, dit om mogelijke HIV-resistentie vorming en langdurig falen van behandeling te voorkomen. Om de doelen van het USAID 90-90-90 programma te bereiken zal 90% van alle mensen die ART gebruikt ook in 90% van de gevallen viraal onderdrukt moeten zijn. Dit kan alleen maar gerealiseerd worden als de kwaliteit van de zorg aanzienlijk verbetert door onder andere de concentratie van virus in het bloed regelmatig te testen en testmogelijkheden beter beschikbaar te maken. Zo kan bij therapie falen eerder overgegaan worden op andere behandelopties.

Verbetering van antiretrovirale behandeling

HIV-resistentie bij kinderen is hoog en zorgwekkend. Naast de beschreven percentages van 45% voor behandeling van ART bij kinderen blootgesteld aan PMTCT, meldden wij eerder dat 17% van de kinderen in de eerste 2 jaar van behandeling resistentie ontwikkelt (18). In dit proefschrift hebben we aangetoond dat na 4 jaar behandelen, dit aantal nog hoger is, met een prevalentie van verworven NNRTI en NRTI-resistentie van 30% (17,18). Vanwege deze hoge resistentie worden alternatieve ART-regimes op basis van PI's en intergrase remmers belangrijker.

De WHO onderstreept dit advies op basis van studies waarin is aangetoond dat behandeling met PI de vroege kindersterfte met 76% en de progressie van HIV-ziekte met 75% kan verminderen bij kinderen ouder dan een jaar (10,11). Voor adolescenten en volwassenen is aangetoond dat PI-regimes in combinatie met NRTI-basis effectief zijn in LMIC (8,43). Grootschalige implementatie van op PI-gebaseerde behandelregimes in LMIC zijn echter een uitdaging (44). Bijvoorbeeld Lopinavir/Ritonavir (LPV /r; Kaletra) is een de meest gebruikte PI in LMIC en wordt geleverd in tabletten, capsules (met daarin pellets) en in drankvorm. Behandeling met LPV/r heeft verschillende nadelen, ten eerste is een tablet van LPV/r vijfmaal zo duur als bijvoorbeeld het doorgaans gebruikte Nevirapine (45). Ten tweede zouden vloeibare toedieningsvormen het meest ideaal zijn vanuit het doseringsperspectief, maar er moet met de huidige concentraties een grote hoeveelheid vloeistof worden ingenomen voor een adequate behandeling (voor een kind met een gewicht van 10 kg is dit 28 ml per dag). Een meer geconcentreerde vorm betekent een meer kostbare productie en daarnaast is voor het bewaren van deze medicatie is een koelkast noodzakelijk. Daarom is een LPV / r capsule “de beste” beschikbare optie voor kinderen, capsules worden geopend en de pellets aan de kinderen gegeven. Praktisch gezien is het moeilijk om pellets op een lepel aan kleine kinderen te geven en tevens is het onduidelijk of de farmacologische beschikbaarheid beïnvloed wordt, wanneer de medicatie op deze manier toegediend wordt (44).

Aangezien de resistentie tegen HIV een groot probleem is en de tweedelijnsbehandeling voor kinderen met op PI-gebaseerde regimes een uitdaging blijkt, zijn nieuwe behandelopties vereist. De in 2017 geïntroduceerde intregrase remmers zijn relatief nieuw op de markt en daarmee is onderzoek naar de effectiviteit, vooral bij kinderen in LMIC, beperkt. In Malawi is recent, in januari 2019, de intregrase remmer Dolutagravir beschikbaar gekomen voor jongens zwaarder dan 30 kg en voor vrouwen boven de vruchtbare leeftijd (> 40 jaar). Verschillende belanghebbenden spannen zich in voor betere beschikbaarheid en kosteneffectiviteit, maar directe resultaten voor kinderen in LMIC zullen nog even op zich laten wachten (46,47). Het succes van betere beschikbaarheid van ART, inclusief afname van de wereldwijde overdracht van moeder op kind, heeft ertoe geleid dat minder kinderen op jonge leeftijd met HIV besmet raken. Verhoogde preventie van overdracht van moeder op kind zal uiteindelijk de behoefte aan antiretrovirale geneesmiddelen bij kinderen verminderen en dus zal de financiële prikkel voor

farmaceutische bedrijven om te investeren in pediatrische medicijnformules afnemen. De behandeling van kinderen die ART nodig hebben zal in de loop van de tijd steeds complexer worden, omdat zij vaak prenataal al blootgesteld zijn geweest aan ART en als gevolg daarvan meer resistentie hebben ontwikkeld. Nieuwe onderzoeken die behandeling met op PI- en intergrase gebaseerde regimes vergelijken bij kinderen in LMIC zijn broodnodig, naast inspanningen om de beschikbaarheid van deze geneesmiddelen voor de meest behoeftigen te vergroten.

Co-morbiditeit tijdens HIV-infecties in sub-Sahara Afrika

Met de toenemende overlevingskansen door de verbeterde beschikbaarheid van ART, zal de HIV-zorg in LMIC zich de komende decennia meer en meer op de kwaliteit van het leven gaan richten. Twee belangrijke co-morbiditeiten onder HIV-geïnfecteerde patiënten in LMIC zijn diarree en (ernstige) anemie. Als we de kwaliteit van zorg voor HIV-geïnfecteerde patiënten in LMIC willen verbeteren zal zorg gericht op deze twee co-morbiditeiten van belang zijn.

Diarree is veelvoorkomend onder HIV-geïnfecteerde patiënten en is met name bij kinderen geassocieerd met een hoge mortaliteit (21). Nieuwe PCR-technieken bieden snelle en betrouwbare resultaten voor de detectie van gastro-intestinale protozoa die diarree kunnen veroorzaken (48). Verrassend genoeg was E. bieneusi de meest voorkomende opportunistische infectie in ons studiecohort. Deze uitkomst kan klinisch relevant zijn, omdat dragerschap voor start van ART werd geassocieerd met zowel gastro-intestinale klachten en verminderd herstel van ondervoeding gedurende het jaar van follow-up. Aanvullend klinisch onderzoek naar dit mogelijke pathogeen is nodig om de gevolgen op lange termijn en mogelijke behandelingsopties bij deze kinderen in kaart te brengen.

Ernstige bloedarmoede bij HIV-geïnfecteerde patiënten in LMIC is geassocieerd met een hoge mortaliteit en gebleken is dat verschillende factoren verantwoordelijk zijn voor het ontstaan hiervan (34). Richtlijnen voor diagnose en behandeling van ernstige bloedarmoede bij HIV-geïnfecteerde volwassenen of kinderen zijn zelden beschikbaar in sub-Sahara Afrika, zo ook bij onze studiepopulaties in Oeganda en Malawi (34,49). Op basis van onze resultaten willen we een gestructureerde aanpak van de analyse en behandeling van ernstige bloedarmoede aanbevelen, waarbij rekening wordt gehouden met de effectiviteit van HIV-behandeling, het uitsluiten van tuberculose en nierfalen en het optimaliseren van de voedingsondersteuning.

Ook ijzergebrek is geïdentificeerd als een belangrijke oorzaak van ernstige anemie in deze patiëntenpopulatie. Echter diagnostiek naar ijzergebrek blijft tot op de dag van vandaag lastig bij ernstige anemische HIV patiënten in LMIC. Ondanks deze uitdagingen geven wij tot slot een aanbeveling om deze diagnostiek te verbeteren door het gebruik van het Hepcidine, een hormoon dat betrokken is bij ijzermetabolisme. Gezien de hoge mortaliteit van HIV-geïnfecteerde patiënten met ernstige bloedarmoede heeft deze specifieke patiëntenpopulatie recht op meer aandacht en verbeterde zorg met adequate behandelprotocollen.

Conclusie

Al met al benadrukt dit proefschrift het belang van vroege initiatie van ART, de behoefte aan intensievere monitoring van virusconcentratie in het bloed en aandacht voor verschillende co-morbiditeiten onder HIV-patiënten in sub-Sahara Afrika. Laten we streven naar niet alleen meer toegankelijke, maar met name verbeterde HIV-zorg in LMIC, voor zowel volwassenen als kinderen; "***From quantity to quality of care***".

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LIST OF PUBLICATIONS

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Minke Huibers was born on January 21th, 1984, in Ermelo. After completing secondary school at the Christelijk College Groevenbeek in Ermelo she started studying medicine at the Free University of Amsterdam (Vumc) in 2002. During her studies, Minke was an active member of the International Federation of Medical Students' Associations (IFMSA). Throughout her medical studies she did two clinical rotations in tropical medicine including the St. John of God hospital, Duayaw Nkwanta, Ghana in 2005 and the Mulanje Mission Hospital in Malawi in 2009 respectively. A research internship in 2007 was done at the Radboud University Nijmegen in collaboration with Kilimanjaro medical centre (KCMC) Moshi, Tanzania on drug resistance exposure in HIV infected mothers and their children.

After obtaining on her medical degree, Minke started working as junior doctor (ANIOS) in paediatrics in respectively Westfriesgasthuis, Hoorn and OLVG-West Amsterdam. In 2011 she started a research project on predictors of low CD4 counts on HIV infected children in Malawi, supervised by dr. Job Calis and prof. Michael Boele van Hensbroek. After 6 months of research, Minke started her training as resident in paediatrics at the University of Amsterdam, which she finished in December 2016. During her training she worked for 12 months as a resident in training (AIOS) in the General paediatrics St. Elisabeth Hospital Willemstad, Curacao. As well she did a clinical rotation in infectious disease; including tropical infectious diseases and a rotation in paediatric cardiology in Emma children's Hospital (EKZ), AMC, Amsterdam.

After her graduation, Minke continued her PhD on HIV, working on different aspects of HIV among adults and children in Malawi and Uganda Global Child Health Group in collaboration with the Amsterdam Institute on Global Health and Diseases (AIGHD) and collaborated with the Liverpool School of tropical medicine and Hygiene, in order to complete her PhD research. Minke is currently working as a paediatrician in Lilongwe, Malawi. She is employed by Baylor College of medicine, Texas Children Houston, USA, Haematology Oncology Paediatrics Excellence (HOPE), were she provides care for children with cancer in resource limiting settings. Minke Huibers is married (2008) to Anton van Veenhuisen and has two children; Pien Sijgje van Veenhuisen (2015) and Teun Pieter van Veenhuisen (2017).